EPILEPTIFORM ACTIVITY IN ISOLATED CORTEX AND HIPPOCAMPAL PREPARATIONS, AND ITS MODULATION BY PURINERGIC COMPOUNDS

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Summary

1. This dissertation addresses both the physical requirements (in terms of the minimal mass of tissue needed) for the generation and propagation of epileptiform activity, and the purinergic modulation of this epileptiform activity in the cerebral cortex. Studies on the minimal mass were performed in rat somatosensory cortex *in vivo*, using subpial transsections based on a recently developed neurosurgical approach to the treatment of drug-resistant epilepsy (Morrell *et al.*, 1989: "Multiple subpial transection: a new approach to the surgical treatment of focal epilepsy"), in conjunction with the iontophoretic application of the convulsant penicillin. For a detailed analysis of the structural requirements for epileptiform activity and the rapid application and wash-out of drugs of known concentration, a novel *in vitro* model of the isolated neocortical column was developed which allowed the manipulation of radial intracortical pathways via pressure ejection of drugs at various cortical depths, and the isolation of specific layers by subsectioning the tissue. It therefore provided a unique way of studying the intrinsic pathways of individual cortical layers. The epileptiform potentials in rat and mouse neocortical cylinders were recorded with standard extracellular recording techniques in an attempt to elucidate some of the aspects of the ongoing debate on whether the so-called 'cortical columns' are hard-wired information processing units, or functional groups of co-active neurones whose configuration varies with the task performed.

2. After preliminary findings in rat neocortex *in vivo* had indicated that the minimal mass, *i.e.* the smallest block of cortical brain tissue able to generate epileptiform spiking, was small enough to be investigated *in vitro*, work on the subsequently developed *in vitro* model of the cortical column clearly showed that the minimal diameter was below that of the postulated columns defined by the so-called 'barrel' structures found in layer IV of the rat somatosensory cortex. Furthermore, blocks of brain tissue (containing an estimated 1500 neurones) which excluded layer IV displayed epileptiform activity which was indistinguishable from that observed in preparations containing all neocortical layers, or indeed from records obtained by other groups in chronically isolated neocortex *in situ*. It is concluded that the minimal

mass problem is, to a certain extent, merely a question of the degree of connectivity; from the results of the present study, no horizontal boundaries corresponding to the postulated 'cortical modules' were found to exist with respect to the generation of paroxysmal discharges. Similarly, the importance of layer IV in the generation of epileptiform discharges appears to pertain only to the specialised case of discharges induced by GABA receptor blockade, as exclusion of this layer did not affect the ability of the tissue to display the spikes/afterdischarges commonly observed in intact cortical slices *in vitro*, and in cortical subpial isolations *in vivo*.

3. In Part Two, the purinergic modulation of this epileptiform activity was studied in mouse neocortical cylinders and in rat hippocampal slices. The inhibitory effects of adenosine (a metabolite of nucleotide hydrolysis which may act as an 'endogenous anticonvulsant') on low-magnesium ACSF-induced epileptiform activity are described, followed by an analysis of the factors contributing to the proconvulsant effects caused by selective adenosine A1 receptor blockade *in vitro*. The main compound used was 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX), a highly selective A1 antagonist which in previous *in vitro* studies had been reported to induce persistent epileptiform activity by an unknown mechanism after transient application (Alzheimer *et al.*, 1989: "Transient and selective blockade of adenosine A1 receptors by DPCPX causes sustained epileptiform activity in hippocampal CA3 neurons of guinea pigs"); these prolonged epileptogenic effects are not usually observed after the application of less selective adenosine antagonists.

4. Despite the marked proconvulsant effects reported for DPCPX *in vitro*, the drug does not induce seizures when administered *in vivo*. A similar effect was observed in the present study *in vitro*; on the basis of these findings, it is suggested that the main physiological role of adenosine in the brain is to exert an *activity-dependent* negative feedback control which limits the effects of calcium influx through voltage-activated Ca^{++} channels, rather than the 'inhibitory purinergic tone' proposed by some authors.

5. Based on the physico-chemical properties of DPCPX, and on a comparison with the effects of less lipophilic alkylxanthine adenosine antagonists, it is proposed that the affinity of these compounds for the adenosine A1 receptor is governed to a large extent by the degree to which they accumulate in the lipid bilayer of cell membranes. Theoretical considerations, and the fact that DPCPX could be displaced by theophylline, indicate that an accumulation within the lipid bilayer, and subsequent binding to the A1 receptor *via* the plasma membrane is the most likely route of access. The model also provides an explanation for the previously reported unusually rapid recovery of adenosine-mediated inhibition during DPCPX-mediated A1 blockade.

6. HPLC analysis of the superfusate of incubated hippocampal slices indicated a substantial release of endogenous adenosine. During prolonged periods of epileptiform activity in the presence of the highly selective DPCPX-mediated blockade of A1 receptors, the endogenously released adenosine may cause excitatory effects through the activation of A2 receptors. The recently described function of adenylyl cyclase as a coincidence detector which can integrate information from multiple signalling pathways suggests that A2-mediated activation of adenylyl cyclase is likely to be maximal under these conditions. Recent results obtained with genetic 'knockout' mutants or the i.c.v. administration of irreversible Gs protein activators suggest that the gene transcription and protein synthesis induced by increased cAMP levels may be involved in the pathophysiological changes which occur in the epileptic brain. This hypothesis is supported by clinical observations indicating that some methylxanthine-induced toxic effects (seizures) may not respond to anticonvulsant treatment, while at the same time showing a poor correlation with xanthine serum levels.

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ABBREVIATIONS USED

AC:	adenylyl cyclase
ACSF:	artificial cerebrospinal fluid
AD:	afterdischarge
ADA:	adenosine deaminase
ADP:	adenosine diphosphate
AED:	antiepileptic drug
AMP:	adenosine 5'-monophosphate
AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AOPCP:	α,β-methylene adenosine-5'-diphosphate
4-AP:	4-aminopyridine
APNEA:	N6-2-(4-amino-3-iodo-phenyl)ethyladenosine
APV:	aminophosphonovaleric acid
ATP:	adenosine triphosphate
BBB:	blood-brain barrier
CA1, (CA3):	subregion 1 (subregion 3) of the hippocampus
cAMP:	cyclic adenosine-3',5'-monophosphate (cyclic AMP)
CHA:	N6-cyclohexyladenosine
CHAPS:	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CNQX:	6-cyano-7-nitroquinoxaline-2,3-dione
CNS:	central nervous system
CPA:	N6-cyclopentyladenosine
CPT:	8-cyclopentyl-1,3-dimethylxanthine
CREB:	cAMP response element-binding protein
DRB:	5,6-dichloro-1-β-D-ribofuranosyl benzimidazole
DMSO:	dimethylsulphoxide
DPCPX:	1,3-dipropyl-8-cyclopentyl-xanthine
ECS:	electroconvulsive shock
EEG:	electroencephalogram
EPSP:	excitatory postsynaptic potential
GABA:	γ-amino-butyric acid

Gpp(NH)p:	5'-guanylylimidodiphosphate
GTP:	guanosine triphosphate
HPLC:	high performance liquid chromatography
5-HT:	serotonin
IIS:	interictal spike
IPSP:	inhibitory postsynaptic potential
IU:	international units
LTP:	long-term potentiation
MAP:	multiple action potential
NAD:	nicotinamide adenine dinucleotide
NBA:	nitrobenzylalanine
NECA:	5'-N-ethylcarboxamidoadenosine
NMDA:	N-methyl-D-aspartate
PDE:	phosphodiesterase
PIA:	N6-phenyl-isopropyladenosine
PKA:	protein kinase A
PKC:	protein kinase C
PMBSF:	posteromedial barrel subfield
PTZ:	pentylenetetrazole
SAH:	S-aminohippuric acid
SI:	primary somatosensory cortex
TEA:	tetraethylammonium
TMA:	tetramethylammonium
t-ACPD:	trans-1-aminocyclopentane-1,3-dicarboxylic acid
XAC:	xanthine amine congener

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1 INTRODUCTION TO PART ONE

Although the mechanisms by which specific manipulations of cellular excitability can lead to a control of seizures are well understood, the origin of spontaneous seizures and therefore the aetiology of epilepsy in man is still unclear, regardless of the fact that a number of predisposing factors have been found which include central nervous system (CNS) infections in early childhood, head trauma, and genetic factors (Annegers *et al.*, 1980; Annegers, 1994). The long list of potential causative factors currently under discussion is mirrored by the large number of animal models of epilepsy. Experimental seizures may for example be induced by the application of chemoconvulsants like GABA-antagonists or excitatory amino acids, acute or chronic electric stimulation, or the creation of chronic foci induced by alumina cream, iron compounds or cold lesions (Löscher and Schmidt, 1988). In fact, it is probably the ease with which seizures can be evoked experimentally which has been partly responsible for the popularity of epilepsy research, as evidenced by the steadily growing number of citations on epilepsy or seizures available in the computerised Medline database (Fisher, 1991).

However, the precise changes that lead to a human brain suffering from repeated epileptic seizures, i.e. the cellular and molecular basis of the development and lasting predisposition for seizures, are unknown. There has been discussion (e.g., Ayala *et al.*, 1973) as to whether the tendency of the vertebrate brain to suffer seizures can be explained in terms of changes in cellular properties (decrease in GABAergic transmission, abnormal channel properties or ionic buffering) or changes in the connections of otherwise healthy cells (the network theory). Arguments in favour of both models have been found, and the aetiologies of the various epileptic syndromes in Man are probably a mixture of both: a change in the cellular properties initiating seizures, and a network of neurones with a certain degree of connectivity to allow the expression of seizures. The principle that - independently of pathological changes at the cellular level - a network of given size and connectivity is needed to express a seizure has been linked to the idea of functional units (the cortical columns defined

below), leading to the concept that a certain 'minimal mass' is needed for the generation of seizure activity.

1.1 The modular organisation of the neocortex

The mammalian cerebral cortex contains about 10^9 nerve cells in a relatively amorphous sheet covering the phylogenetically more ancient regions of the brain. Based on variations in the relative thickness of the various layers and on detailed mapping studies, the cortex has been subdivided into more than 40 functionally distinct areas in the horizontal plane. Along the vertical axis, a six-layered pattern first introduced by the anatomist Brodmann (1909) has come to be accepted as the standard. The layers differ in their composition, functional properties and sets of connections. On Nissl-stained sections, they can be defined as follows (from pia to white matter):

- Layer I (the plexiform or molecular layer) contains very few cell bodies (mainly neuroglial cells, and some - mostly GABAergic - neurones), and the apical dendritic tufts of layer II, III and V pyramidal cells. It is the site of synaptic terminations from cortico-cortical projections, and occupies about 5% of the distance between pia and white matter
- Layer II (the external granular layer), which consists of small, tightly packed cell bodies, and Layer III (the external pyramidal layer), a thick layer containing large pyramidal cells. Layers II and III are not separated by a clear border on Nissl-stained sections; they occupy about 20% of the distance between pia and white matter
- Layer IV (the internal granular layer) is densely packed with the somata of small pyramidal and non-pyramidal cells. It receives most of the thalamocortical afferent input, and occupies about 10% of the cortical depth
- Layer V (the internal pyramidal layer) consists primarily of large, loosely dispersed pyramidal cell somata, and is often subdivided into superficial (Va) and deep (Vb) zones. Its thickness is about 20% of cortical depth.

- Layer VI (the polymorph layer) is composed of relatively tightly packed, small round cell somata that belong mostly to a modified form of pyramidal cells (thickness approximately 40%).

With the exception of laminae I and VI, the cortical layers contain predominantly projection neurones (such as pyramidal cells) with axons leaving the cortex and projecting to different parts of the brain including other areas of the neocortex. In addition to these principal neurones, intrinsic interneurones of variable morphology are found in all cortical layers. The granular layer (IV) represents the input relay station of the neocortex and is therefore most prominently developed in primary sensory areas. It consists mainly of intrinsic cortical interneurones (Miller, 1988).

Besides the horizontal structure characterised by a specific pattern of afferent and efferent fibre connections, vertical organisational features have been proposed: in sensory and other areas of the cerebral neocortex, afferent information from the thalamus is thought to be processed by vertically oriented neuronal assemblies, or columns (Mountcastle, 1979). These structural or functional modules, which are connected to each other by horizontal fibres (Gilbert, 1985), are considered to be the functional units of the neocortex, and the synaptic networks within a column have been assumed to provide the basis for the higher integrative and cognitive functions of the mammalian brain. The concept of a columnar organisation of the cortex is based on evidence obtained from developmental, electrophysiological and morphological studies.

1.1.1 *Ontogenesis*

Newly generated neurones in the developing cerebral cortex migrate outward from a proliferative zone near the cerebral ventricle (which is divided by glial septa into columns of precursor or stem cells termed proliferative units by Rakic [1974]), through a cell-sparse intermediate zone, and into the cortical plate below the pial surface of the brain (Luskin and Shatz, 1985). The postmitotic neurones migrate to

their prespecified areal and laminar position by following shafts of radial glial cells through the cellular lattice of the intermediate and subplate zones. These elongated non-neuronal cells stretch across the fetal cerebral wall from the beginning of corticogenesis and are the main reason for the radial organisation of the cortex. All postmitotic neurones generated in a single proliferative unit have been claimed to form a morphologically identifiable stack of neurones in the cortex variously called an "ontogenetic" or "embryonic" column (Rakic, 1978), in which earlier generated neurones occupy deeper positions and therefore those arriving later have to pass them to become situated more superficially (the 'inside out' gradient of neurogenesis). Recombinant retrovirus studies demonstrated that a single progenitor cell can produce more than one cell phenotype, most of which end up within the same radial ontogenetic column (Wetts and Fraser, 1988). This suggests that the evidence for a common origin of a group of neurones within a cortical columnar module is fairly strong, although there are exceptions to this point-to-point mapping between the ventricular zone and cortical plate, which suggests that areal specification occurs after neurogenesis: Walsh and Cepko (1992) reported that clonally related neurones in the cerebral cortex of the rat can become widely dispersed across functional areas of the neocortex. Further indications that radially oriented groups of neurones form a functional unit come from dye coupling experiments. Dye coupling, an indicator of electrotonic coupling via gap junctions, is extensive in immature rat neocortex and declines rapidly between birth and 10 days of age (Connors *et al.*, 1983); this corresponds to the critical period of development of the primary somatosensory cortex in rats.

Fluorescent calcium indicator labelling studies by Yuste *et al.* (1992) have revealed distinct domains of spontaneously co-active neurones in tangential slices of developing rat neocortex; the authors suggest that "this nonsynaptic cellular communication via gap junctions may influence subsequent synaptic rearrangements: if synapses are formed, retained or modified by activity-dependent mechanisms, selective synapse formation could occur among members of a domain, resulting in an adult column".

1.1.2 *Electrophysiology and morphology*

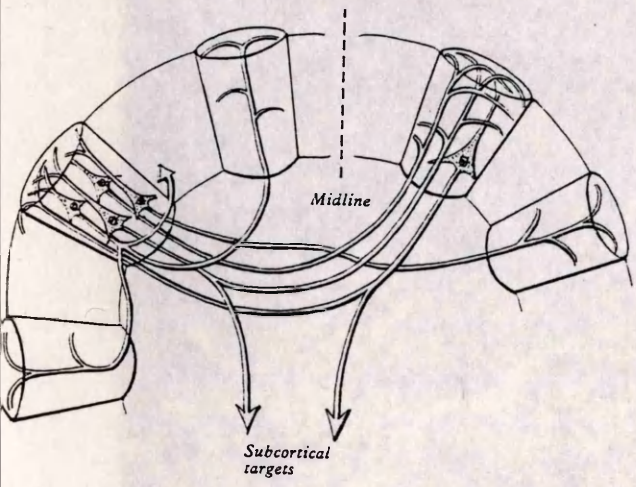
The first experimental evidence for columnar domains in mature neocortex came from electrophysiological data from primary sensory cortex (Mountcastle, 1957), where vertical microelectrode penetrations encountered neurones with similar receptive fields, whereas tangential penetrations revealed cells whose orientation preference shifted systematically. The findings confirmed the hypothesis advanced by Lorente de No (1938) that the elementary pattern of organisation in the cerebral cortex is a vertically oriented column of neurones. Vertically oriented functional columns have since been demonstrated in other cortical areas of various species (Mountcastle, 1979); in the visual cortex, neurones that respond preferentially to stimuli presented to one eye or stimuli having a particular orientation, wavelength, or direction of movement are grouped together in independently organised, vertically oriented multicellular arrays (Berman *et al.*, 1987, Hubel and Wiesel, 1962, LeVay *et al.*, 1987, Michael, 1978). Other studies have shown that, in the cat and monkey primary somatosensory cortices, rapidly adapting neurones which respond only at the onset and offset of skin indentation, and slowly adapting neurones which respond throughout the stimulus, are situated within separate slablike columns of cortex (Dykes *et al.*, 1980; Sur *et al.*, 1984). Columns of cells with similar receptive field properties have also been identified in the primary (Favorov *et al.*, 1987) and secondary (Alloway and Burton, 1985) somatosensory cortices of the cat. In the primary auditory cortex of the cat are columns of cells that respond to the same or to similar frequencies or to auditory stimuli from one or both ears (Abeles and Goldstein, 1970). Neurones within a single efferent column in motor cortex receive proprioceptive input from the same joints or muscles that are activated by cells in that column, as well as from skin that is superficial to the target muscles (Asanuma, 1975); the diameter of these cortical motor columns is about 1 mm in cross section. Columns for visual features of objects in monkey inferotemporal cortex having an average diameter of 400 µm were described by Fujita *et al.* (1992). Iterated modular units have also been demonstrated by histological means, leading to the definition of ocular dominance stripes in layer IV of the primary visual cortex, alternating bands of cortico-cortical connections related to monaural or binaural responses in the auditory

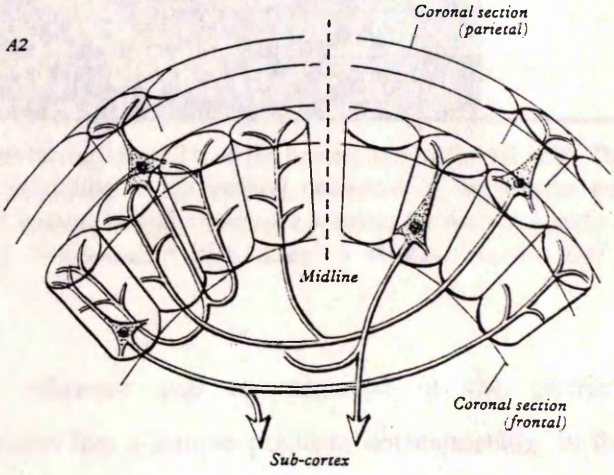
system of monkeys (Imig and Brugge, 1978), and so-called 'barrels' in the rodent primary somatosensory cortex.

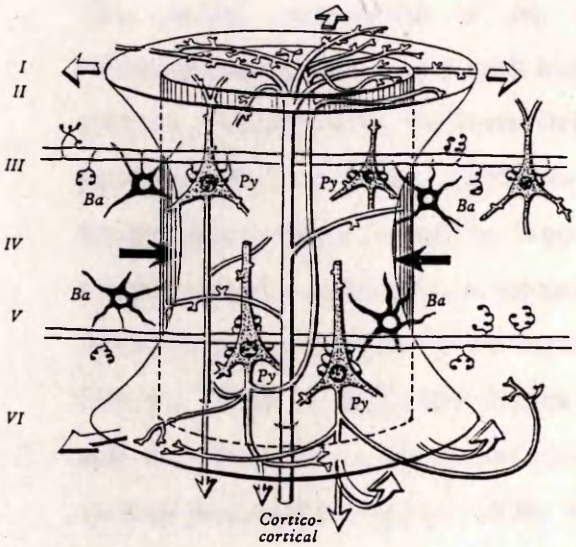
1.2 Barrels

In the rat, the somatosensory cortex (the region most frequently used in studies on the mechanisms of neocortical epileptogenesis) comprises the rostro-medial part of the parietotemporal cortex. It can be subdivided cytoarchitecturally into a primary region, thought to be the principal termination site of receptotopically organised specific thalamocortical afferent projections, and areas of secondary sensory cortex whose receptive fields are less specific, are usually not lateralised, and which receive projections from associated primary sensory cortex, commissural input from contralateral cortex, and parallel afferent input from the thalamus as well (Kolb, 1990). Primary somatosensory cortex (SI - referred to as sensorimotor cortex SmI by Woolsey) may be readily identified in Nissl-stained (Chapin and Lin, 1990) or cytochrome oxidase-stained (Land and Simons, 1985) tangential sections through layer IV by clearly defined aggregates of granule cells (see Fig. 1). These aggregations of layer IV neurones - called "barrels" - are related one-to-one to individual vibrissae on the contralateral mystacial pad (Woolsey and van der Loos, 1970; vibrissae are tactile organs consisting of a central hair in a mechanically isolated hair follicle receiving a segregated and substantial sensory innervation which are moved back and forth at frequencies of 7 Hz in rats and 15 Hz in mice during exploratory behaviour, and are arranged in species-specific, stereotyped, grid-like patterns in which each vibrissa has a unique position).

CORTICAL COLUMNAR MODULES







Williams,P., Warwick, R., Dyson, M. & Bannister , L. (1989) Gray's Anatomy, 37th edition. Churchill Livingstone, Edinburgh. p. 1053

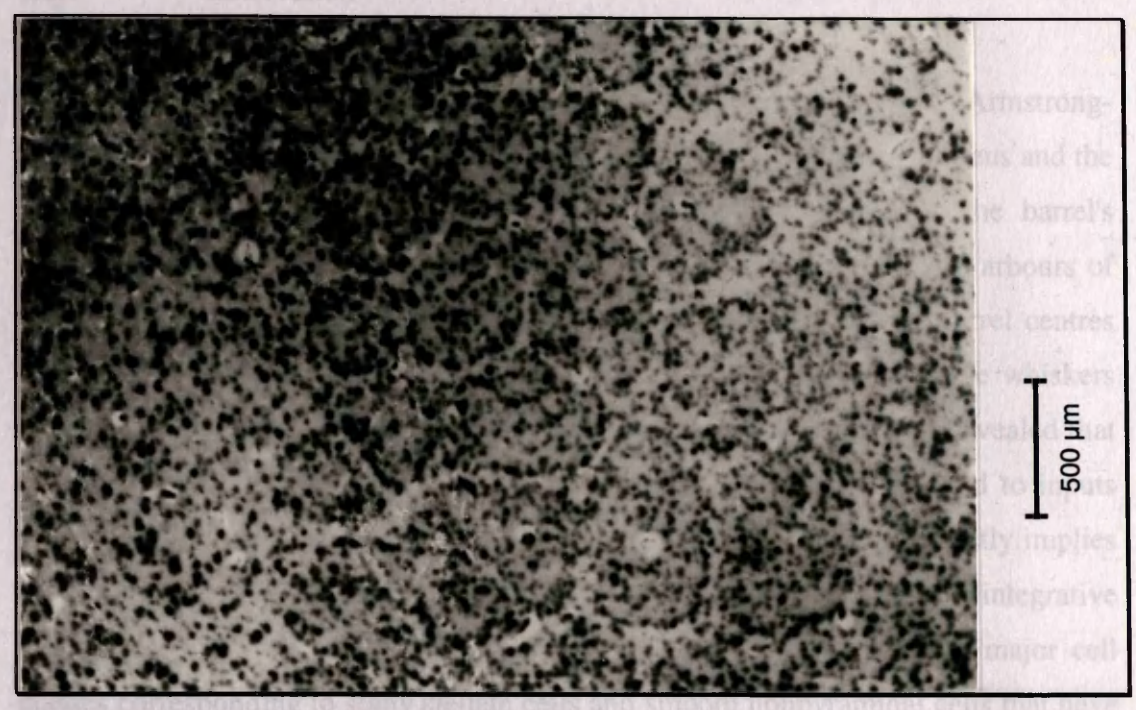


Figure 1: Thionine-stained tangential section through layer IV of the postero-medial barrel field. The tissue was flattened between glass slides according to the method described by Strominger and Woolsey (1987), fixed and stained in a 10 % neutral formalin / thionine solution for one week and cut at 20 µm. A schematic illustration of columnar modules is shown on the left.

This spatial organisation of the vibrissal pad is mirrored in the primary somatosensory cortex, where each barrel has a unique position corresponding to the vibrissa projecting to it; the barrel field as a whole is homeomorphic to the whisker pattern of the face. Numerous studies using a variety of approaches have supported the hypothesis first advanced by Woolsey and van der Loos (1970) that a barrel is a morphologically identifiable component of a functional cortical column that extends throughout the thickness of the cortical sheet (Armstrong-James, 1975; Land and Simons, 1985). In single unit studies this is evidenced by one whisker evoking the sole or maximally excitatory response from all drivable cells encountered within a vertical penetration (Simons, 1978). Further evidence for the central role played by the barrels in sensory information processing comes from the finding that markers for intense metabolic activity (mitochondrial enzyme staining, blood vessel density, and Na^+/K^+ ATPase activity) are all greatest in the barrels (Riddle *et al.*, 1993).

1.2.1 *Innervation*

Barrels receive their afferent sensory inputs via a trisynaptic pathway (Armstrong-James and Fox, 1987) reaching the cortex via the principal trigeminal nucleus and the thalamic ventral posteromedial nucleus; these projections constitute the barrel's predominant source of extrinsic inputs (Land *et al.,* 1986). The terminal arbours of thalamocortical axons in layer IV are confined almost exclusively to barrel centres (Bernardo and Woolsey, 1987). However, the pathway does not correlate whiskers and barrels in a simple one-to-one fashion, as more recent studies have revealed that most neurones projecting from the thalamus to the barrel cortex respond to inputs form a large number of whiskers (Simons and Carvell, 1989); this indirectly implies that the one-to-one response of individual barrels is based on an active integrative process rather than a restricted afferent input. The barrels contain two major cell classes corresponding to spiny stellate cells and smooth nonpyramidal cells that have been described in different cortical areas in a variety of species (Woolsey *et al.,* 1975); the total cell number of large barrels in the rat averages 2500 neurones. The dendritic fields of intrinsic barrel neurones are confined to the barrel, and their axons ramify extensively within it, where they come in close apposition to barrel neurones of both classes (Harris and Woolsey, 1983). Since thalamic contacts account for only 20 percent of all barrel synapses (White, 1989) and since locally projecting axons of non-barrel neurones largely avoid barrel centres, it appears that a majority of synaptic connections in a barrel arise from neurones within it. Thus, available evidence strongly indicates that a barrel is a densely interconnected network of layer IV neurones that receives its predominant afferent drive from a single external source (Kyriazi and Simons, 1993).

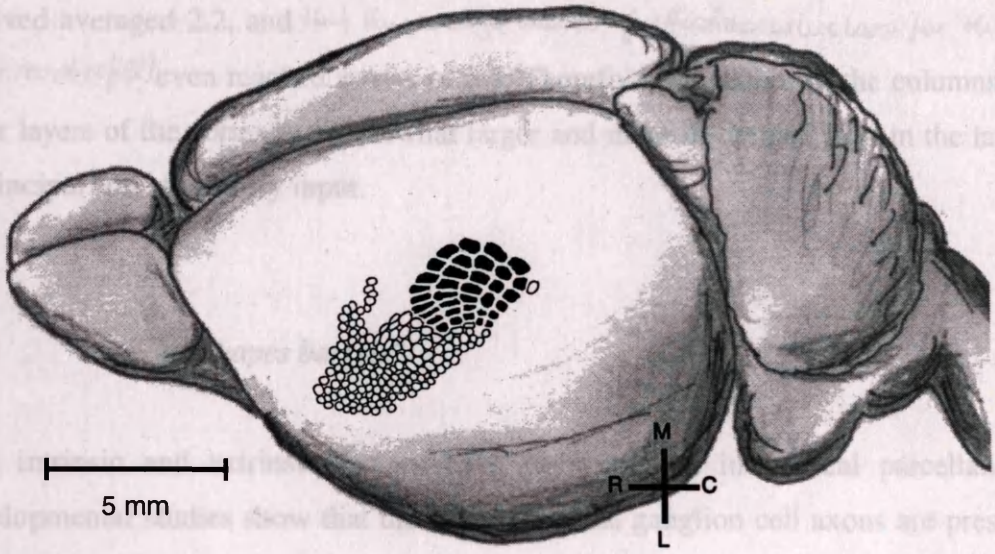


Figure 2: Location of the barrel field on the left hemisphere of a rat brain. The posteromedial barrel subfield (PMBSF; filled black circles) corresponds to the major whiskers on the mystacial pad. The drawing of the barrel field was taken from Woolsey and van der Loos (1970); the position was determined according to the definition of area SI by Schober (1986) and relative to prominent anatomical landmarks such as the middle cerebral artery

1.2.2 *Receptive fields*

The receptive fields in the SI barrel cortex are small in the layer IV barrels and distinctly larger outside of them (Swadlow, 1989); the functional data are consistent with anatomical descriptions of segregated afferent inputs from barreloids (the homeomorphic representation of the barrel field at the second relay station of the trigeminal pathway, at the level of the ventrobasal complex of the thalamus [Van der Loos, 1976]) to homologous cortical barrels (Land *et al.*, 1986) and of interconnections among neighbouring barrel columns by way of supra- and infragranular routes that largely avoid barrel centres (Bernardo *et al.*, 1990).

Specifically, the idea that progressively larger receptive fields are elaborated as intracortical processing of information proceeds from layer IV and deep layer III to more superficial layers and then to deep layers is supported by a comparative

electrophysiological and histochemical study (Ito, 1992) which showed that the mean dendritic field size of layer Vb pyramidal neurones in terms of the number of barrels involved averaged 2.2, and that the average number of effective whiskers for that cell (= receptive field) even reached a value of 5.8. Therefore, the extent of the columns in lower layers of the cortex are somewhat larger and more ill-defined than in the layer of principal somatosensory input.

1.2.3 *What shapes barrels?*

Both intrinsic and extrinsic factors have been implied in cortical parcellation. Developmental studies show that the first trigeminal ganglion cell axons are present within the brain stem on the eleventh day of gestation (E11) in the rat, whereas peripheral axons do not reach the mesenchyme of the developing whisker pad until day E13 (Scarisbrick and Jones, 1993); however, the subsequent superimposition of the pattern of whisker follicles on neural networks at successive relay stations of the CNS is generated in an activity-dependent manner, as injury of the whisker follicles in rat pups disrupts the formation of characteristic cytoarchitectonic and histochemical patterns within the vibrissal representation found in the ventrobasal complex of the thalamus and in the somatosensory cortex (Belford and Killackey, 1980). The sensory input relayed through the thalamic afferents appears to be a critical factor in determining later cytoarchitecture (Erzurumlu and Jhaveri, 1990; Schlaggar and O'Leary, 1991). That the barrel field formation is defined mainly by sensory input rather than glia is supported by an electrophysiological study (McCandlish *et al.*, 1993) of the development of the SI cortical barrel field map; although the development of the physiological map of the representation of the body surface follows the same lateral-to-medial gradient as morphological studies, the morphological map lagged behind the physiological map by about 48 hours. Evoked responses could be detected as early as 12 hr after birth in the rat, suggesting that thalamocortical afferents have reached the developing cortical plate and are functional before glial cells are first detected: this can be seen as further evidence for a purely functional origin of cortical pattern formation. Final evidence that barrels are not simply an extrapolation of predetermining ontogenetic columns as proposed by

Rakic comes from an analysis of virally tagged cortical clones which revealed no coincidence with barrel structures (Walsh and Cepko, 1992). In summary, the sharp boundaries observed in mature neocortex are likely to be due to activity-dependent mechanisms, being shaped both by spatial cues in the early phases of cortical development, and by extensive axonal remodelling and pruning which is thought to require neuronal activity.

1.2.4 *What is their function?*

As mentioned earlier, it is a widely held belief that information is processed within columns in the cortex (Mountcastle, 1957), but experimental evidence on how this might occur, or how information might be associated between columns, is sparse. However, the sequence of connections between layers has been found to be relatively uniform, and may provide an insight into how the cortex 'works'. Studies on the flow of excitation within the barrel cortex upon striking a single vibrissa (Armstrong-James *et al.,* 1992) reveal the following picture: layer IV is activated before any other layer but is closely followed by cells in layer Vb. The next group of cells to fire were found to lie directly above the barrel (layer III). Next, layer Va cells fired simultaneously below the main barrel and neighbouring barrels; at the same time, layer II cells became active. Therefore, information is first relayed within a column before relay to cells in adjacent columns takes place. (The septal regions above, below and between barrels are innervated via a different pathway relayed by the rostral zone of the thalamic posterior complex [Diamond *et al.,* 1992], which itself receives corticofugal inputs from the barrels [cortico-thalamo-cortical loop]). Why these cortical columns should be apparent at the macroscopic level is beyond the scope of this introduction. Woolsey and van der Loos (1970) speculated that "the size of the mouse cerebral cortex may approach a minimum; [..] neurones in layer IV in the mouse cerebral cortex are perhaps just barely enough for the cortical manipulation of sensory data" (p. 238), while Krubitzer *et al.* (1993) advanced the hypothesis that modules within a cortical field may represent the initial stage of the evolution of a new cortical field by a gradual aggregation and eventual segregation of functionally distinct neuronal groups.

1.2.5 *Are barrels identical with cortical columns?*

Cortical columns have been defined in various ways. It has been suggested recently (White and Peters, 1993) that barrels actually comprise a large number of cortical modules consisting of clusters of layer V apical dendrites with a centre-to-centre spacing of about 25 µm; the authors drew the conclusion that the pyramidal cell modules (which have been found throughout the cerebral cortex) are fundamental neuronal units, and that the various functional areas of the cortex in different species are organised according to a common, basic plan. However, evidence for a clear functional segregation of computational tasks into cell subgroups within the barrel hollow is sparse. Therefore, barrels are still regarded as functional units subserving a single computational task; reference to the idea that barrels correspond to columns is frequently found in recent publications. For example, Welker *et al.* (1993) state that "a barrel is regarded as layer IV's morphological substrate of a cortical column, i.e. the barrel column" (and see Ito, 1992).

1.3 The 'Minimal mass' problem in epilepsy research

Related to the question of whether there are defined cortical processing units shaped by a stringent (pre-existing) organisational principle forming the whole of the neocortical sheet, and which by definition should be able to perform a defined computational task, is whether these columns coincide with the minimal amount of cortical tissue involved in the generation and maintenance of epileptiform activity, the so-called 'minimal mass'.

1.3.1 *Historical background*

The first indication that epileptiform spiking could be evoked in a subregion of the neocortex came from an *in vivo* study by Kristiansen and Courtois (1949) who demonstrated that paroxysmal discharges could be evoked in a completely isolated portion of the suprasylvian gyrus in the cat in which the pial circulation was left

essentially intact. Echlin (1959) isolated "an area of cortex approximately 2 to 2.5 cm square and 1 cm in thickness by extensive subpial section along four sides and wide undercutting" in the frontal or parietal lobes of 90 Rhesus monkeys and was able to demonstrate an enhanced epileptiform response to topical eserine solution up to three and a half years later. Similar results were obtained by Sanders and Pinski (1967) in cortical isolations in the suprasylvian gyrus in the cat measuring 18-25 mm long, 3 mm wide and 4 mm deep.

The findings were extended to the human CNS with the observation that epileptiform activity could still be seen in isolated cerebral cortex in patients who had undergone functional hemispherectomy, suggesting that "intracortical connections allow sufficient neuronal synchrony to produce epileptiform discharges" (Pierre-Louis and Morrell, 1992).

1.3.2 *Multiple subpial transections*

The concept of a minimal mass needed for the generation of epileptic activity saw its first clinical application with the development of a novel surgical technique for the inactivation of foci in refractory partial epilepsy by Morrell and Hanbery (1969; see Fig. 3). It was based on a number of observations which indicated that whereas the columnar organisation of the cerebral cortex discussed above results in a vertical processing of information, the spread of epileptic activity to previously uninvolved brain regions occurs in a horizontal fashion; and furthermore, that the amount of cortical tissue needed to generate the seizure activity has a critical lower limit: Sperry *et al.* (1955) had demonstrated that surgical cross-hatching of the visual cortex did not impair visual perception in the cat, which suggested that most of the functionally important connections of a cortical territory were arranged in vertical columns and that, if this columnar organisation was preserved, the horizontal fibres could be sectioned without causing serious disability.

Other studies on the minimal mass *in vivo* indicated that a minimal contiguous volume of cortical tissue was necessary to sustain synchronous spiking. Duysens and

McLean (1974) reported that the reduction of a cobalt gelatine focus in monkey cortex to a 2 mm^2 area by four subpial vertical cuts abolished its epileptic activity. In 1977, Reichenthal and Hochermann had showed that after the topical application of penicillin to the exposed rat cortex *in vivo*, the division of the active epileptic focus by a subpial cut only resulted in active subfoci if the surface area exceeded 0.5 mm^2. Lueders *et al.* (1981) showed that stable penicillin foci generated by weak penicillin solutions had a minimal area of distribution of 12.5 mm^2 in the cat, as two foci separated by four millimetres on the same gyrus were consistently dependent and simultaneous, whereas foci separated by 6 mm were almost always independent. Cortical islands greater than 5 mm in width, or horizontal connections of greater than 5 mm, were known to be able to support paroxysmal discharges (Tharp, 1971). More recently, Silva-Barrat *et al.* (1992) showed that the neurones in rat somatosensory cortex responding to the discontinuation of GABA infusion with epileptiform bursting (the so-called "GABA withdrawal syndrome") were located within 0.7 to 1.2 mm from the infusion site. Morrell's own preliminary studies on small alumina cream foci in monkeys indicated that epileptiform potentials disappeared when the focus was transsected, and he therefore assumed that "the spontaneous generation of epileptiform potentials in intact [human] brain appears to require several millimetres of contiguous tissue" (Morrell and Hanbery, 1969).

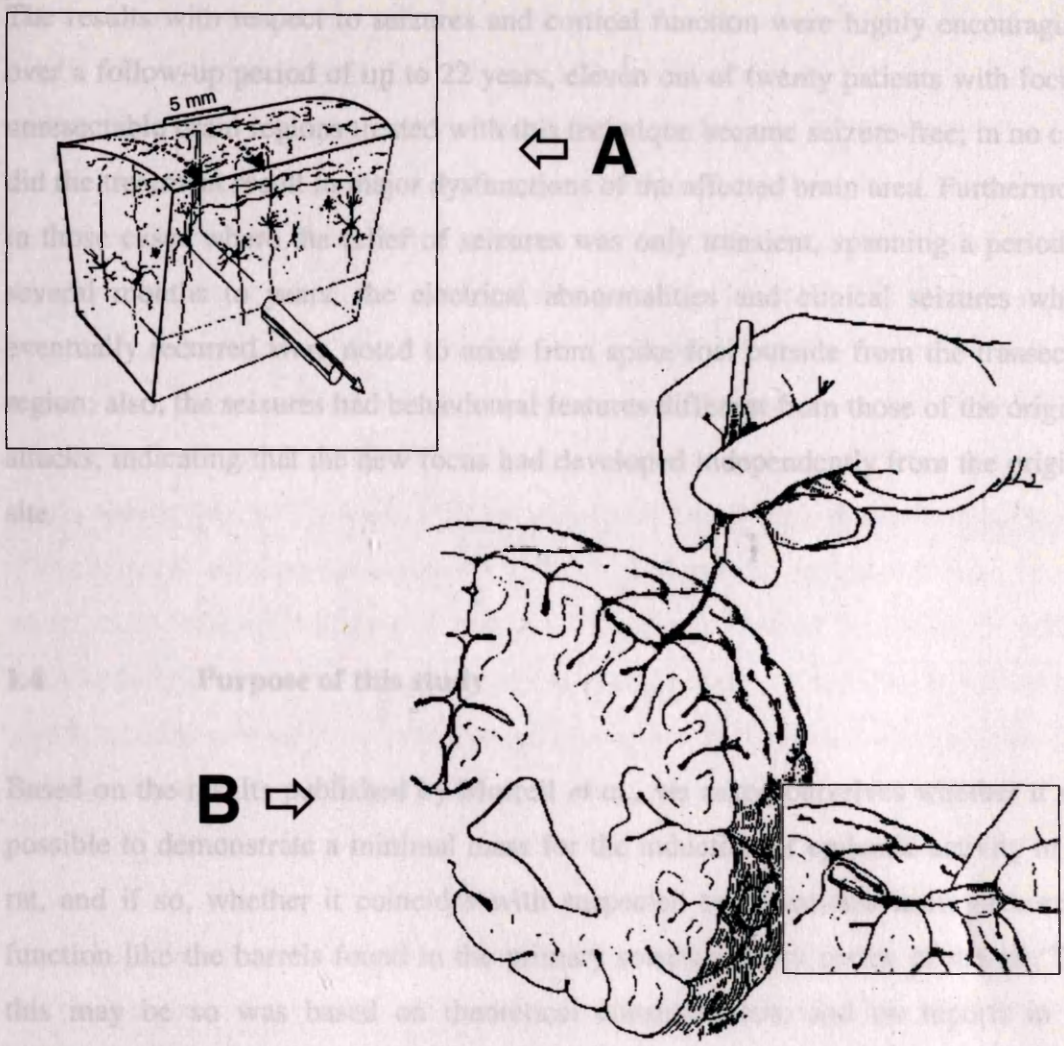


Figure 3: Multiple subpial transections, a method devised by F. Morrell *et al.* for the treatment of focal epilepsy: a small, right-angled hook is inserted underneath the pia and drawn through the gray matter (middle, and right inset). Further transections are performed at 5 mm intervals to include the entire area of electrical abnormality (adapted from Morrell *et al.*, 1989)

The surgical technique, which requires the severing of tangential intracortical fibres while preserving the vertical fibre connections of both incoming and outgoing nerve pathways and of the penetrating blood vessels (which also have a vertical orientation), was intended particularly to be used in those cases where the epileptogenic lesion lies in "unresectable" cortex; that is, those cerebral regions subserving speech, memory, and primary motor and sensory function. It consisted of a number of parallel incisions spanning the depth of the cortex which were performed by introducing a heavy steel wire with a right-angled hook through a small, pin-sized pial opening, pushing it through the grey matter and then gently drawing it back across the sulcus to sever all horizontal connections.

The results with respect to seizures and cortical function were highly encouraging: over a follow-up period of up to 22 years, eleven out of twenty patients with foci in unresectable brain regions treated with this technique became seizure-free; in no case did the treatment result in major dysfunctions of the affected brain area. Furthermore, in those cases where the relief of seizures was only transient, spanning a period of several months to years, the electrical abnormalities and clinical seizures which eventually recurred were noted to arise from spike foci outside from the transected region; also, the seizures had behavioural features different from those of the original attacks, indicating that the new focus had developed independently from the original site.

1.4 Purpose of this study

Based on the results published by Morrell *et al.*, we asked ourselves whether it was possible to demonstrate a minimal mass for the induction of epileptic activity in the rat, and if so, whether it coincides with suspected organisational units of cortical function like the barrels found in the primary somatosensory cortex of the rat. That this may be so was based on theoretical considerations, and on reports in the literature that small penicillin foci evoked *in vivo* resulted in a zone of columnar activation in sensory cortex (Collins, 1978) as measured by the [^{14}C]-deoxyglucose method in the rat, and that the propagation of epileptiform discharges in rat neocortical slices was spatially periodical in the somatosensory (but not the secondary visual) cortex, with the dominant spatial frequency averaging one millimetre (Chervin *et al.*, 1988). Also, Gabor *et al.* (1979) had reported that in cat visual cortex, the margin of a penicillin focus coincided with a shift in the preferred axis of movement of individual neurones, and stated that "the minimal epileptogenic aggregate is probably no smaller than the number of cells with the same preferred axis of movement and ocular dominance [...] physiologically defined neuronal populations (columns) may be the subunits of epileptogenesis".

In its original form, the aim of the research project was stated as follows (O. Holmes, 1990): "Several laboratories have shown that, both in vivo and in the tissue slice, epileptiform manifestations cannot be induced in a partially isolated island of neocortical tissue of less than a certain critical size. In this laboratory it has further been shown that the enhancement of somatosensory evoked potentials which heralds the onset of overt spontaneous spiking also requires a critical mass of neural tissue of the same dimensions. These observations indicate that the neuronal circuitry subserving epileptiform manifestations is much more spatially extensive than that subserving normal electrical activity such as the electroencephalogram and normal somatosensory evoked potentials. [The] objective is to specify the anatomical layout of the cortical circuitry necessary for epilepsy to occur. In the anaesthetised rodent, using acute focal epileptogenesis induced with agents such as penicillin, [it will be investigated] whether the minimal cortical mass subserving epilepsy is the cortical barrel, whether several barrels are needed or whether epilepsy depends on some other modular cortical organisation which is out of register with the barrel fields."

These questions are also relevant to the ongoing discussion as to whether epilepsy is due to changes in individual neurones (the "epileptic neurone" hypothesis) or whether the behaviour of an entire population has been altered (the "circuit" hypothesis). The approach chosen for the present study involved the induction of epileptiform (interictal) spiking in isolated rat somatosensory (SI) cortex both *in vivo* and *in vitro* with the help of chemical convulsants.

1.4.1 *Evoked potentials and interictal spikes*

Evoked potentials, which can be monitored in non-invasive surface electroencephalographic (EEG) recordings, are the sum of many thousands of synchronised postsynaptic potentials produced by parallel cortical dendritic processes extending normal to the pial surface in response to physiological stimuli, and reflect mainly the activity of pyramidal cells. They play an important role in CNS diagnostic routines in the clinical setting, and are the main tool for the mapping of

representational regions in the vertebrate cortex. In this study, they were used to locate the forepaw region within the primary somatosensory cortex, and to gauge the physiological health of the cortex. The field potentials recorded at depth reflect the sum of dipole activity in the surrounding cortex. In the anaesthetised rat these field potentials show slow, random oscillations due to remaining cortical activity, and also a certain degree of noise due to the mechanical movement imposed on the electrode tip by blood flow and respiration. When convulsant drugs are applied to the cortex, a general increase in activity can be observed which is then followed by the development of epileptic discharges; these represent the synchronous activity of a large number of neurones. The most regular in form and the most easily identifiable discharge is the spontaneous interictal spike.

Interictal spikes are abrupt, spontaneous changes in potential lasting about 70 ms which are frequently found between seizures in EEG recordings in human patients; they are electrically similar to seizures, but are self-limiting and brief enough not to interfere with normal behaviour, and often represent the most reliable indicator for an epileptic syndrome; furthermore, they are often localised in the brain region in which seizures originate in a given patient. They are, however, clearly pathological, as the mismatch between information content (which is inversely correlated to synchrony) and energy cost is similar to that of an epileptic fit. The events leading to an interictal EEG spike have been elucidated in studies combining *in vitro* electrophysiology and computer modelling of neuronal networks. Pyramidal neurones, which rarely fire action potentials in response to a single excitatory postsynaptic potential (EPSP), may recruit additional neurones through sequential activation under circumstances where GABAergic inhibition is reduced or excitatory transmission is facilitated. An increasing number of pyramidal cells then fire bursts of action potentials in response to a single synaptic input, until a critical fraction of cells in the population becomes simultaneously active; and then virtually all the remaining neurones become recruited simultaneously, leading to the interictal spike which can be detected in the EEG. This kind of paroxysmal activity requires recurrent innervation and is therefore not observed in structures lacking such connections, like the cerebellar cortex. In human epileptic foci, seizures have been speculated to be triggered by spontaneously spiking

'epileptic neurones' at the centre of the focus which occasionally recruit a critical mass of surrounding neurones (Shaw and Chadwick, 1987).

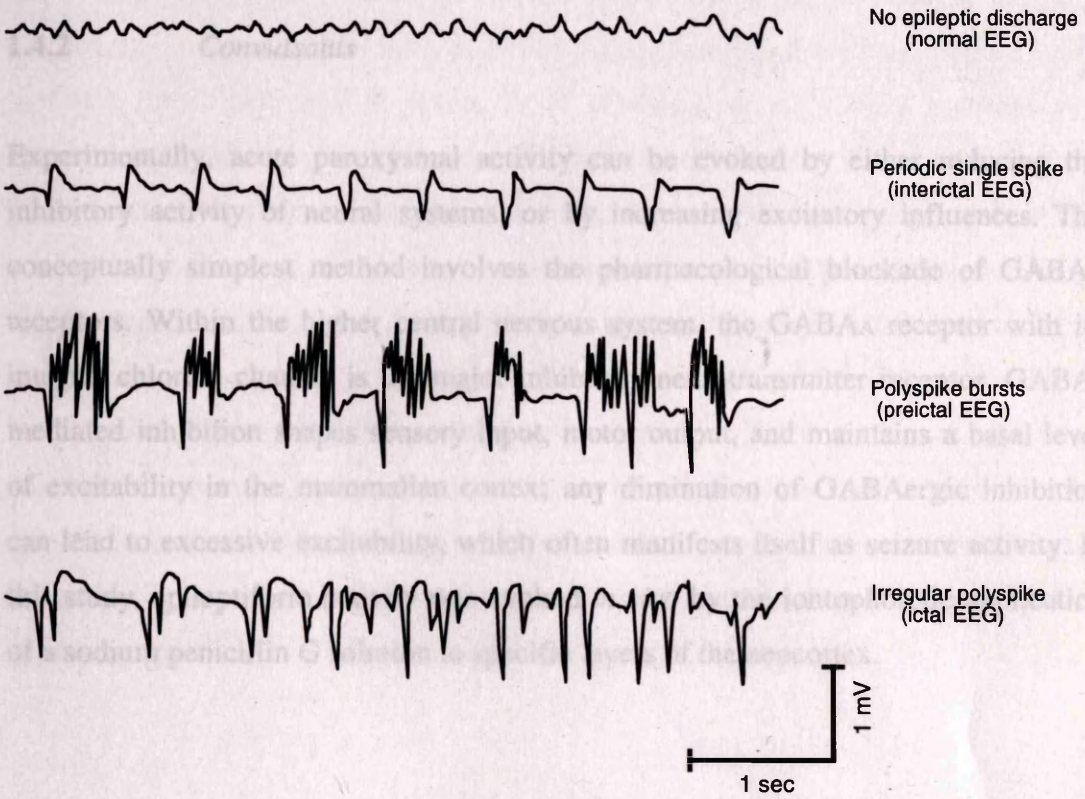


Figure 4: Discharge patterns observed during the transition from normal to ictal EEG in a quisqualate (30 µg i.c.v.)-treated, freely moving rat (modified from Kanai *et al.*, 1992)

The role played by interictal spikes within the epileptic syndrome is not yet fully understood: they could be either abortive seizures, or represent a necessary build-up to the seizure proper, or be a completely independent phenomenon associated with epileptic activity. Interictal spikes may be entirely lacking between clinical seizures, or only be present in significant numbers in the wake of a seizure; other patients have frequent interictal spikes, but few seizures (Fisher, 1991). However, most researchers assume that interictal spikes represent a minor form of epileptic seizure; this is supported by results from experimentally induced seizures in intact animals, where the transition to generalised tonic-clonic seizures after the administration of chemical convulsants is graded, leading from periodic single spikes over polyspike bursts (preictal EEG) to irregular polyspike patterns (ictal EEG; see Fig. 4). It can therefore

be assumed that interictal spikes provide a valid if simple system for investigating the basic mechanisms of epilepsy.

1.4.2 *Convulsants*

Experimentally, acute paroxysmal activity can be evoked by either reducing the inhibitory activity of neural systems, or by increasing excitatory influences. The conceptually simplest method involves the pharmacological blockade of $GABA_A$ receptors. Within the higher central nervous system, the $GABA_A$ receptor with its integral chloride channel is the major inhibitory neurotransmitter receptor. GABA-mediated inhibition shapes sensory input, motor output, and maintains a basal level of excitability in the mammalian cortex; any diminution of GABAergic inhibition can lead to excessive excitability, which often manifests itself as seizure activity. In this study, epileptiform activity was evoked *in vivo* by the iontophoretic application of a sodium penicillin G solution to specific layers of the neocortex.

In common with several other clinically used antibiotics (Grondahl and Langmoen, 1993), penicillin G is convulsant when applied to the central nervous system; it acts mainly by blocking GABAergic postsynaptic inhibition (Schwartzkroin and Prince, 1980), and the epileptogenic effect appears to be linked to the beta-lactam ring, as enzymatic cleavage of this ring results in loss of epileptogenic activity (Gutnick and Prince, 1971). It has been shown to reduce the mean open time of GABA-activated channels (Chow and Mathers, 1986) and is extensively used in models of absence seizures (the feline generalised penicillin epilepsy - Quesney and Gloor, 1978), and for evoking local epileptiform activity in the cortex. The focal intracortical application of convulsants has been routinely used in this laboratory and has led to the discovery that layer IV is the most sensitive to the convulsant action of penicillin (Holmes and Lockton, 1982), of other GABA-blocking agents, and possibly also of other, unrelated convulsants. Layer IV therefore appears to play a cardinal role in the generation of bicuculline/penicillin-induced abnormalities seen in *in vivo* and *in vitro* recordings and current source density analysis. Several research groups have focused

on these possible "generator zones", which have been identified in various regions within the brain, and which have revealed the important role not only of layer IV in the neocortex, but also of several limbic regions (area CA3 in the hippocampus and amygdala), and of the area tempestas, an epileptogenic trigger zone in the deep prepiriform cortex (Piredda and Gale, 1985). The presence of a unique population of 'intrinsic burst' neurones in layers IV-V (Connors *et al.*, 1982) provides one mechanism by which seizures may be initiated in the neocortex. Layer IV is also the target for most of the thalamic input (the main afferent system within the cortex), and it easy to envisage it as the most vulnerable region to the potentiating effect of GABA antagonists, as it can be regarded as the starting point for the cortical signal processing pathway. Consequently, the peak sensitivity of layer IV discovered by Holmes and Lockton (1982) was taken to indicate the site of generation of cortical epileptiform events (a conclusion based on the assumption that GABA receptor density is uniform throughout the cortex) and led to the assumption that barrels, which are a prominent feature of SI layer IV in the rat, might be the basic unit of epileptogenesis.

In the *in vitro* experiments, epileptiform spiking was induced by superfusing the tissue with magnesium-free artificial cerebrospinal fluid (ACSF). The physiological roles of magnesium ions in neuronal function include the stabilisation of neuronal membranes by screening membrane surface charges, the regulation of neurotransmitter release by the partial antagonism of presynaptic calcium ion entry, a voltage-sensitive block of the NMDA-receptor linked channel, and the involvement in a number of cellular metabolic processes such as the regulation of ATP and cyclic nucleotides which themselves affect neuronal excitability. It is therefore not surprising that nominally magnesium-free ACSF leads to a distinctly altered neuronal excitability, resulting in several types of epileptiform activity: spontaneous EPSP-like depolarisations, epileptiform bursts, spreading depression, and even full-blown electrographic seizures - hence the use of parenteral magnesium sulphate for the treatment of eclampsia (Lindheimer, 1993) and alcohol withdrawal seizures (it has to be noted, however, that its clinical use is limited by its failure to reach adequate levels in the brain: in a patient suffering from intractable status epilepticus treated

with magnesium sulphate, cerebrospinal fluid magnesium levels were only slightly above baseline, despite an increase in serum magnesium levels to 7.1 mM (Fisher *et al.*, 1988)).

2 METHODS

2.1 Experiments *in vivo*

For the *in vivo* experiments, a total of 46 male Sprague-Dawley Albino rats (weighing 200 - 300 g) bred in the animal house of the University were used. (The rat, being a lissencephalic animal, is particularly useful for *in vivo* studies involving the cerebral cortex.) Prior to an experiment, each animal was deeply anaesthetised using ethyl chloride vapour and a subsequent intraperitoneal injection of urethane (ethyl carbamate, 25% w/v in distilled water) 1.2 to 1.6 ml, equivalent to 2 g/kg of body weight. The depth of anaesthesia was arranged to be such that the animal just failed to exhibit a withdrawal reflex when the toes of a hind limb were pinched. A midline incision 3 cm long was performed in the skin of the throat, and the sternohyoid muscles were parted by blunt dissection to expose a section of the trachea; a tracheal cannula was then inserted and secured with thread to facilitate spontaneous respiration (some animals had a mild throat infection, which would have led to the accumulation of fluid in the trachea).

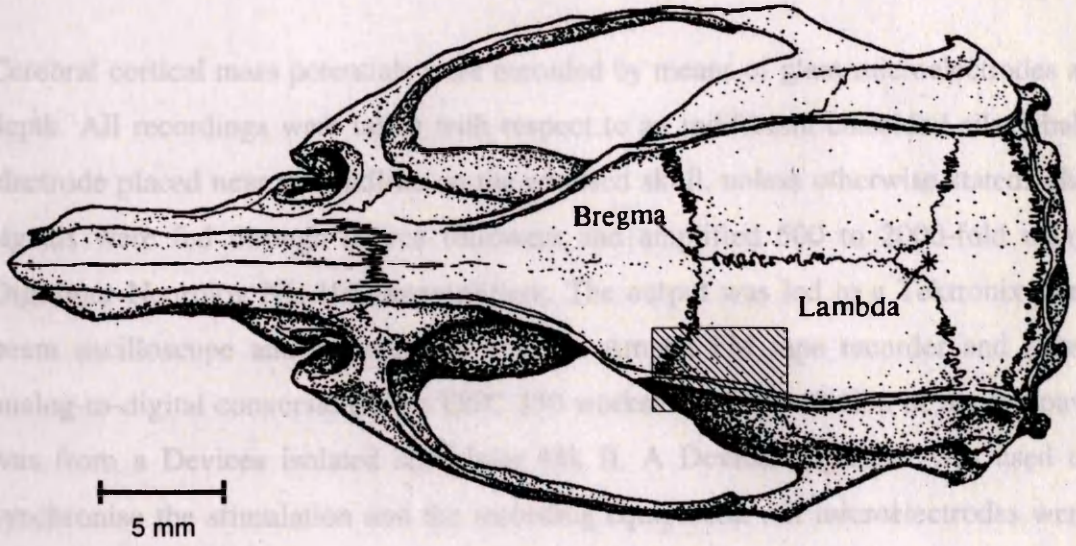


Figure 5: Rat skull; the shaded square indicates the area of bone removed prior to recording (illustration adapted from Paxinos and Watson, 1986)

The head of the animal was mounted in a stereotaxic frame and held at three points: two metal rods with conical ends were pushed one into each auditory meatus, and the snout was gripped with a screw clamp. The body temperature was kept at 37°C by a heating pad warmed from a water bath. The skull was exposed, and after the removal of part of the left temporal muscle to expose the parietal bone, a unilateral craniotomy (3 x 3 mm) was made using a Kaltenbach and Voigt dental drill to expose a region of the left hemisphere centred around the intersection of the coronal suture and the sagittal ridge, as this point lies almost directly over the forepaw area of the primary sensory cortex (see Fig. 5). Under a Zeiss binocular dissecting microscope to provide a 10- to 16-fold magnification, the dura mater was carefully resected by first piercing it with a fine surgical needle and then carefully cutting it back to expose the arachnoid; during the resection, the exposed neural tissue was covered with a thin film of saline to keep it moist. Once any bleeding had stopped, the exposed cortex was covered with liquid paraffin oil at 37°C to prevent it from drying out. Finally, stimulating electrodes consisting of a fine silver wire loop coated with electrode gel were connected to the second and fourth digit of the right forepaw; the stimulation consisted of rectangular voltage pulses of 0.1 msec duration and an amplitude of 10 to 90 V applied every 4 seconds.

Cerebral cortical mass potentials were recorded by means of glass microelectrodes at depth. All recordings were taken with respect to an indifferent chlorided silver ball electrode placed near the midline on the exposed skull, unless otherwise stated. The signals were fed through source followers and amplified 500 to 2000-fold using Digitimer Neurolog NL 103 preamplifiers. The output was led to a Tektronix dual beam oscilloscope and recorded on both an Ampex FM tape recorder and (after analog-to-digital conversion) on a DEC 350 workstation. Stimulation of the forepaw was from a Devices isolated stimulator Mk II. A Devices digitimer was used to synchronise the stimulation and the recording equipment. All microelectrodes were made from borosilicate glass capillaries (Kwik-Fil, Clark Electromedical Instruments) pulled on a two-stage laboratory-made vertical puller. Recording electrodes (tip size 1 μm, resistance around 2 MΩ) were backfilled with 4 molar sodium chloride solution. Iontophoresis was used to eject a controlled flow of

penicillin into a discrete area of the cortex. To this end, a separate glass micropipette with slightly larger tip diameter was used to avoid clogging of the electrode tip; a tip size of between 5 and 10 µm could be regularly achieved by breaking back the tip of a normal electrode under visual guidance on a 40x binocular microscope.

These electrodes were then backfilled with sodium penicillin G (500 mM) solution. After checking that the electrode resistance was below 5 MΩ, and after careful rinsing of the tip to remove penicillin solution adhering to the outside of the tip, the electrodes were ready for use. Because penicillin is a negatively charged ion, it is retained within the electrode by the application of a positive (holding or backing) current of +100 nA, and expelled by a negative current (-100 to -500 nA) once the electrode has been placed at the required cortical depth. After ejection, the iontophoretic current was reversed to reimpose the backing current, and the electrode was removed from the cortex to minimise passive leakage. The cortical depth of 0.7 mm for both the recording and iontophoresis electrodes was based on previous studies performed in this laboratory (Holmes and Lockton, 1982) indicating that layer IV is the most sensitive layer to the epileptogenic actions of penicillin and other GABA-blocking agents.

2.1.1 *Isolation procedure*

One hour after the cortex had been exposed, the recording electrode was placed on the surface of the cortex near the location of the forepaw area of the primary somatosensory cortex (estimated from anatomical landmarks) and advanced into the cortex to a depth of 700 µm until the correct position was found; this was confirmed by the presence of strong negative going evoked potentials triggered by the stimulating electrodes at a rate of 4 Hz. For the comparison of the enhancement of evoked potentials and of the generation of epileptiform spiking, the isolations were performed within the forepaw area; if the isolation was to be within the barrel field area, the final location of the isolation was more medial. As no evoked potentials

were recorded in the latter case, the correct placement of the isolation had to be controlled by histological means after the end of the experiment.

After a control period of up to 30 minutes, the recording electrode was withdrawn and a partial isolation of the area around the electrode track was performed. Round isolations were achieved by using a tungsten wire helix (see Fig. 6) with an electrolytically sharpened tip, while rectangular isolations were performed by using an L-shaped tungsten microelectrode. In both cases, the isolation was performed by carefully introducing the tip of the wire underneath the pia before pushing the cutting edge down to the required depth. If the isolation was performed properly, the resulting bleeding was minimal and of a transient nature; the recording electrode could then be reintroduced along the same track and to the same depth, and more transients were recorded until the amplitude of the evoked potentials had stabilised at or near the pre-isolation level. Then, a sodium penicillin G-filled iontophoretic electrode was lowered into the cortex as close as possible to the recording electrode, and a given amount of penicillin was expelled.

Ideally, the isolations should contain a single cortical unit, or module. The diameters of individual barrels in the primary sensory cortex of the adult rat range from around 200 µm for the smallest ones up to 500 - 600 µm for the large posteromedial barrel subfield (PMBSF) barrels, and from 150 µm (small barrels) to 350 µm (PMBSF barrels) in the mouse. If one accepts the hypothesis that barrels represent single cortical modules (see Introduction), the diameter of 'cortical units' should vary over a similar range throughout the primary somatosensory cortex, with a median value of 350 µm. As a starting value, a helix of tungsten wire with a diameter of 600 µm was therefore manufactured and used.

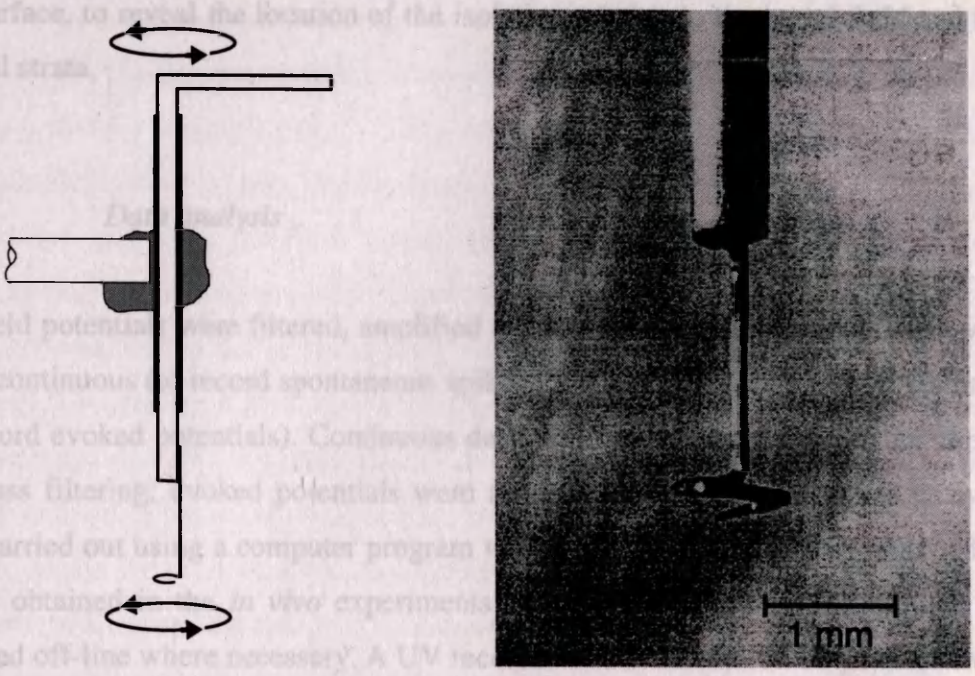


Figure 6: Isolator used for the round subpial partial isolations. Left: schematic drawing. A 24 gauge wire carrying the tungsten helix turns inside a 36 gauge hypodermic cannula glued to a supporting rod which is connected to the micromanipulator. Right: The tip of the tungsten wire (diameter 180 µm) was sharpened electrolytically in KNO_2 as described by Hubel (1957). The helix is glued to its supporting wire using dental wax to allow repositioning under heat.

2.1.2 *Histology*

At the end of each experiment, the brains were removed and fixed in 10% neutral formalin. To assess the depth of the isolation, coronal sections 20 µm in thickness were cut on a cryostat and thaw-mounted onto slides before embedding in Burr Aquamount (see Fig. 14). Alternatively, the entire brain was sectioned at the level of the isolation and photographed. In those experiments where the position of the isolation relative to the barrel field cortex had to be visualised, the brains were treated according to the method described by Strominger and Woolsey (1987): the cortex of the left cerebral hemisphere was gently divided from the underlying white matter with a moistened spatula and placed between two coated glass slides separated 1 mm by spacers. The flattened cortex was stained and fixed by immersion in 10% neutral formaline / thionine nuclear stain solution for three weeks, and wax

embedded. Subsequently, serial sections were cut horizontally, that is, parallel to the pial surface, to reveal the location of the isolation relative to the barrel field and the cortical strata.

2.1.3 *Data analysis*

The field potentials were filtered, amplified and digitised. Digital data sampling was either continuous (to record spontaneous spikes) or triggered by the forepaw stimulus (to record evoked potentials). Continuous data could then be processed using digital bandpass filtering: evoked potentials were averaged. Acquisition and data analysis were carried out using a computer program written by Dr. O. Holmes (CompA). The results obtained in the *in vivo* experiments were stored on magnetic tape and re-analysed off-line where necessary. A UV recorder was available for the production of permanent records of the EEG trace displayed on a dual beam oscilloscope.

2.2 Experiments *in vitro*

For the *in vitro* experiments described below and in Part Two of the study, a total of 90 male Wistar rats (weighing 160 - 260 g) bred in the animal house of the University, and 97 female BalbC mice (weighing 25 - 40 g) obtained from a commercial supplier were used. The animals were injected with urethane 2.5 g/kg (as 25% w/v in water) and decapitated by guillotine while in a state of heavy sedation or unconsciousness (previous studies had indicated that urethane appears to interfere minimally with the outcome of electrophysiological and pharmacological studies in the brain slice [Feldman and Felder, 1988]). The surface of the cortex was then rapidly exposed under a constant stream of cold, oxygenated artificial cerebrospinal fluid (ACSF; composition [in mM]: NaCl 120, KCl 1.1, $CaCl_2$ 2, $MgSO_4$ 1, KH_2PO_4 2.2, $NaHCO_3$ 26, glucose 10) by inserting a pair of forceps into the rostral end of a fine cut made along the right temporal ridge and then lifting the whole tectum and dura off in one movement.

At first, the cylinders were excised using a glass punch manufactured by drawing out a piece of glass microelectrode tubing containing a metal rod of given diameter (300 - 1000 µm) over a gas flame; this resulted in a glass punch with extremely fine walls and a sharp cutting edge. The resulting neocortical cylinders were electrophysiologically viable, but the method did not yield sufficiently standardised preparations and was therefore abandoned. All experiments described on the following pages were therefore performed in tissue preparations obtained as follows: After exposing the surface of the cortex, a slab of cortical tissue centred around the SI area of the left hemisphere was sliced off using a small razor blade. The slab was positioned upside down (i.e. with the white matter uppermost) on a McIlwain tissue chopper and cut at the required thickness, turned by 90° and cut again. Although the resulting tissue sections were blocks of rectangular shape, they will be referred to as 'cylinders' to illustrate the fact that they represent a model of the cortical column, in analogy to the term 'column', which has been used to describe functional units such as the ocular dominance stripes in the visual cortex (interestingly, the basic cylindrical module has an equivalent in computer modelled neuronal networks, where a cylindrical configuration of the hypothetical layers is often adopted in order to avoid edge discontinuities and wrap-around connections (Walker and Akers, 1988)). Positioning the slab upside down ensured that all cuts were perpendicular to the pial surface. The moistened filter paper with the cross-sectioned slab was then transferred to a petri dish containing oxygenated, cold (5°C) ACSF, and the cortical 'cylinders' were gently teased apart using two glass electrodes with firepolished rounded tips. Finally, the individual cylinders were transferred to a petri dish placed in an incubation chamber constantly bubbled with carbogen (95% O_2 / 5% CO_2) and left to recover at room temperature for at least one hour. Cooling of the incubation chamber to 5°C for the length of the incubation period in an effort to improve the recovery of the slices did not have the desired effect. If anything, the slices were less viable; this could be due to the inhibition of essential enzymatic reparation processes occurring after the severe perturbations inflicted by the excision. After a period of at least one hour, a single cylinder was then placed in a submersion-type recording chamber, pinned down with two blunt glass microelectrodes and superfused by gravity at a rate of 5 ml/min with ACSF saturated with 95% O_2 / 5% CO_2 and maintained at 29°C. The medium had the following composition [in mM]: NaCl 120,

KCl 1.1, $CaCl_2$ 2, $MgSO_4$ 1, KH_2PO_4 2.2, $NaHCO_3$ 26, glucose 10. Magnesium-free ACSF was prepared by omitting $MgSO_4$ from the normal ACSF. Glass microelectrodes were pulled on a single-stage puller, and backfilled with Ca^{2+}- and glucose-free ACSF. The tips of the microelectrodes were broken back under visual guidance to a tip diameter of around 2 µm; this resulted in electrode resistances of 2 - 5 MΩ.

For the localised application of Mg^{++} solutions, small amounts of magnesium sulphate solution (100 mM in distilled water) were injected at various cortical depths in mouse cortical cylinders (diameter 450 µm) displaying spontaneous spiking in low-magnesium ACSF. To this end, a micropipette (tip diameter 10 µm) filled with $MgSO_4$ solution was connected to a syringe driver via rigid small-diameter tubing, and brief (2 - 5 seconds) pressure pulses were applied after the micropipette had been positioned at the required depth. The pipette was then removed from the bath to minimise passive leakage. The duration of the pulse was chosen to be such that a single droplet of fluid could be observed to form at the tip of the micropipette when the pipette was outside the bath.

Data were recorded both as continuous chart recorder tracings at slow speed for the assessment of spiking frequencies, and as print-outs of a Gould digital oscilloscope to record single events at a higher resolution. For illustration purposes, chart recordings were scanned using a Hewlett Packard scanner with a resolution of 600 x 600 dpi and re-traced in a graphics program (CorelDraw, version 3.0) to allow the inclusion into graphs independently of the originally used chart speed and amplification factor. It should be noted that the chart record is of a semiquantitative nature: each upstroke represents one burst discharge, but the deflections (which usually had an amplitude of 0.1 to 1 mV) do not accurately reflect the burst amplitude due to the low-pass filtering effect of the chart recorder; therefore, only the records obtained on the digital oscilloscope are voltage-calibrated.

In order to illustrate the correlation between the barrel field structure in layer IV and the cuts made to obtain the tissue cylinders, a slab of cortex from the left hemisphere of a rat brain was cross-cut at 450 µm as shown in Fig. 7, fixed in 10% formaline solution and then placed in liquid gelatine (5%) for 30 minutes before the addition of two drops of the hardening agent glutaraldehyde (25%); this method was found to be the best way of embedding which ensured that the spatial relationship between the isolated tissue segments was retained. The tissue was then covered in gelatine (15%) to which more glutaraldehyde had been added, and left to cool down to room temperature. Subsequently, the preparation was sectioned at 45 µm on a cryostat, and the sections were transferred to phosphate buffer (0.1 M), placed on microscope slides and left to dry. After staining the sections by the Nissl method (rinse in aqua dest. for 5 minutes, sodium acetate 0.1 M (pH = 5) for 5 minutes, staining with cresyl violet (in sodium acetate, pH = 4.2) for 3 to 5 minutes, and a further rinse with aqua dest.), the sections were dehydrated in an alcohol series (70% for 20 seconds, 90% for 20 seconds, 96% for 1 minute, 100% for two minutes (repeated twice), and xylol for 10 minutes (repeated 3 times)), embedded in DePeX Entellan, and covered.

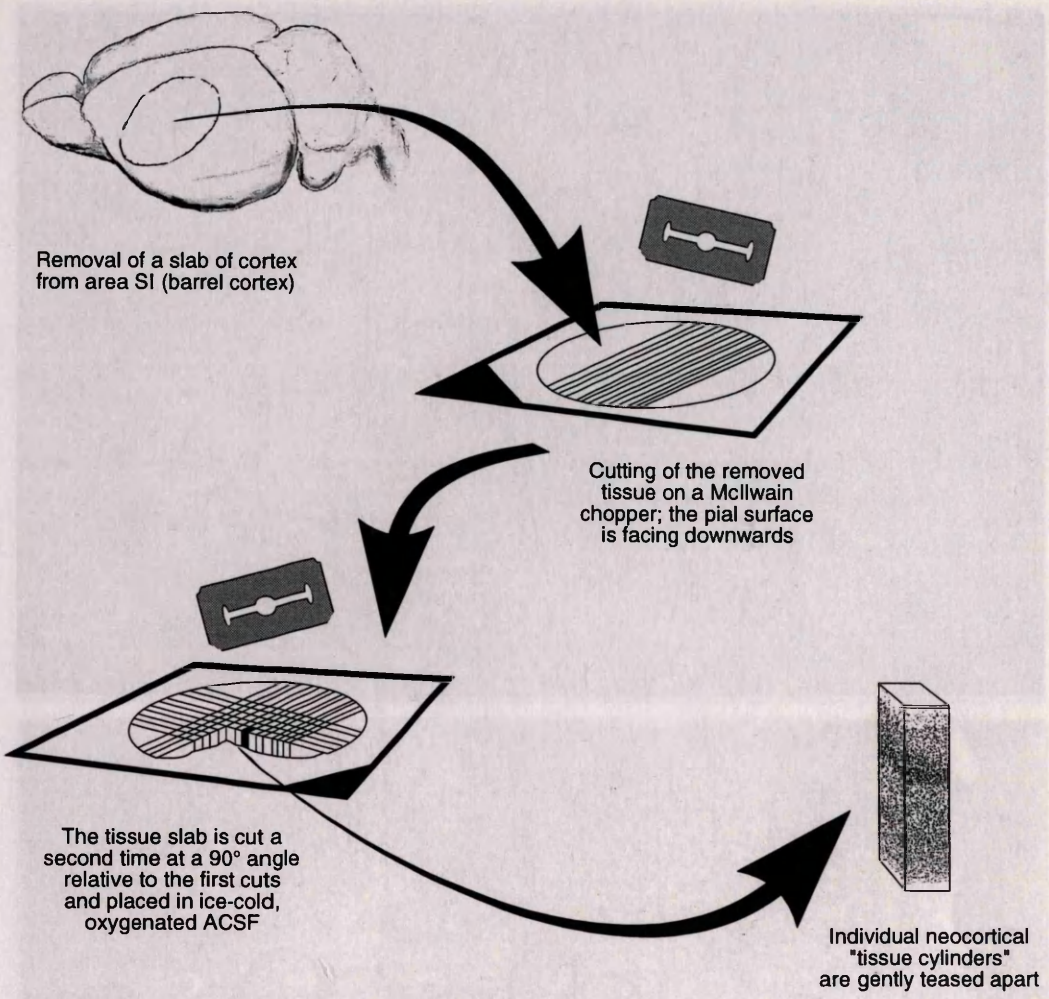


Figure 7: Cortical cylinders were prepared by removing a slab of primary somatosensory cortex which was placed on moistened filter paper with the pial side facing downwards to ensure that cuts were vertical to the pial surface. The slab was then sliced on a MacIlwain tissue chopper at the required thickness, turned by 90 degrees and cut again. After transfer to a petri dish filled with ice-cold, oxygenated ACSF, the individual tissue sections were gently teased apart using fire-polished glass micropipettes, and transferred to a holding chamber.

The tissue sections were photographed on a Nikon Biphot (Japan) microscope equipped with Nikon Plan 20x and 10x objectives, using Agfa XR 100 film.

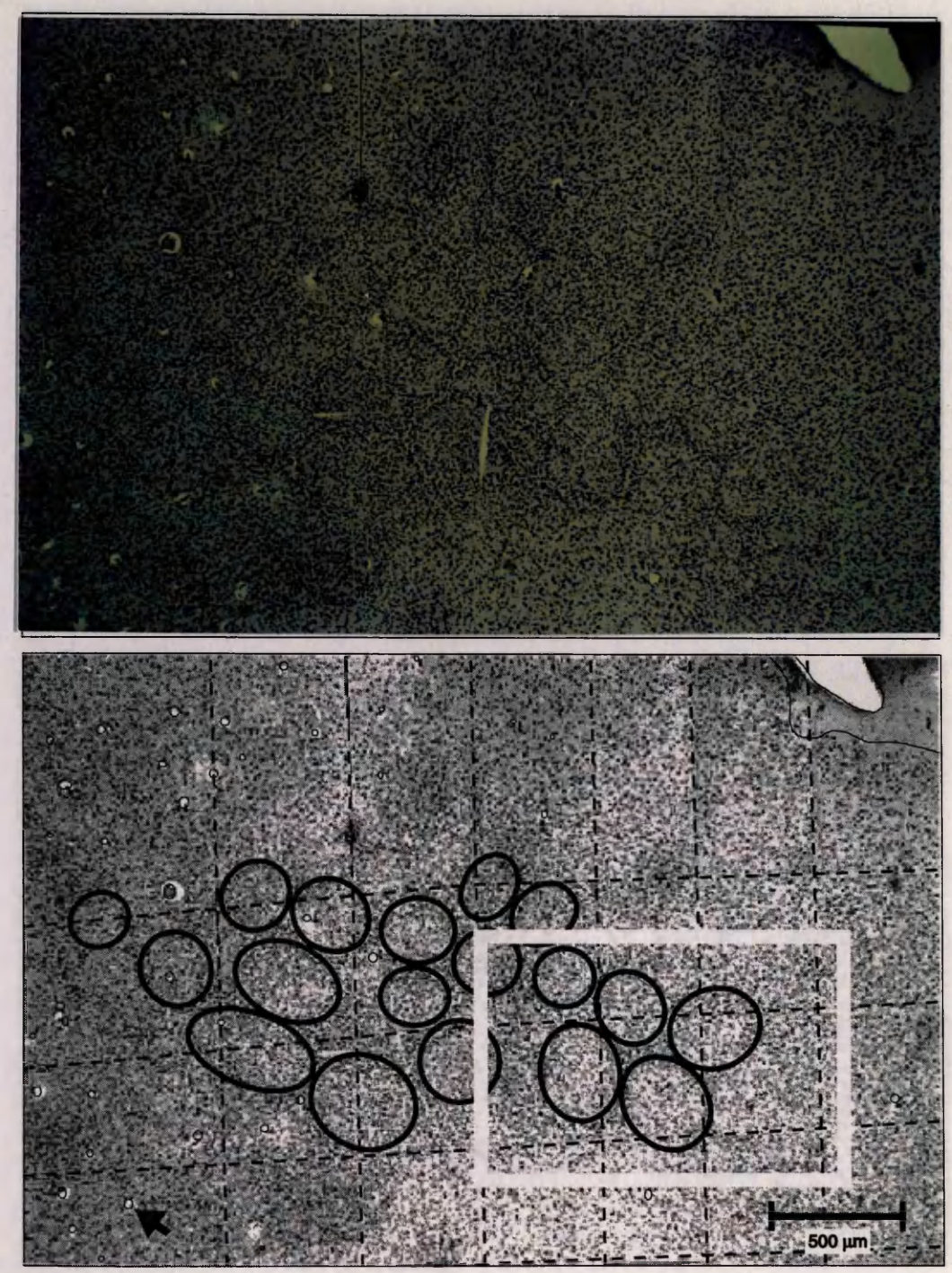


Figure 8: Tangential Nissl-stained cryostat section through layer IV of primary somatosensory cortex which had been cross-sectioned (dashed lines; cf. Fig. 7) on a Campden Vibroslice at 450 µm, embedded in gelatine to preserve the spatial orientation of the cylinders, stained with cresyl violet, and sectioned at 45 µm (Histology: Gaby Klausa). The approximate position of several barrels is indicated by thick black circles; the area indicated by the white rectangle is expanded in Fig. 9. The small circles (arrow) indicate small arterioles.

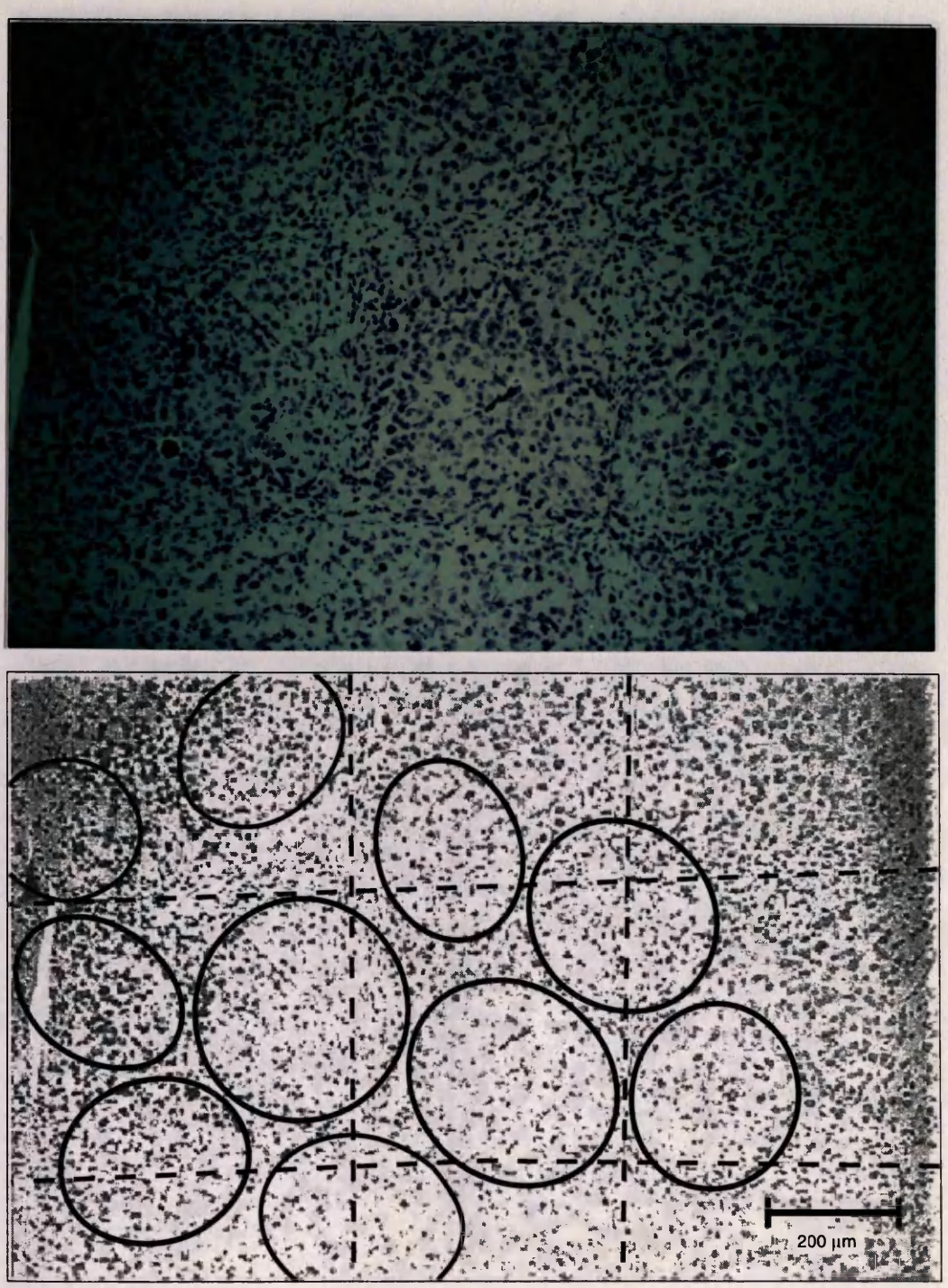


Figure 9: Detail of Figure 8 at higher magnification. Only 2 out of 10 barrels are entirely within the boundaries of the tissue sections demarcated by a zone of shrunk cell bodies approximately 50 µm wide (cf. Fig. 10).

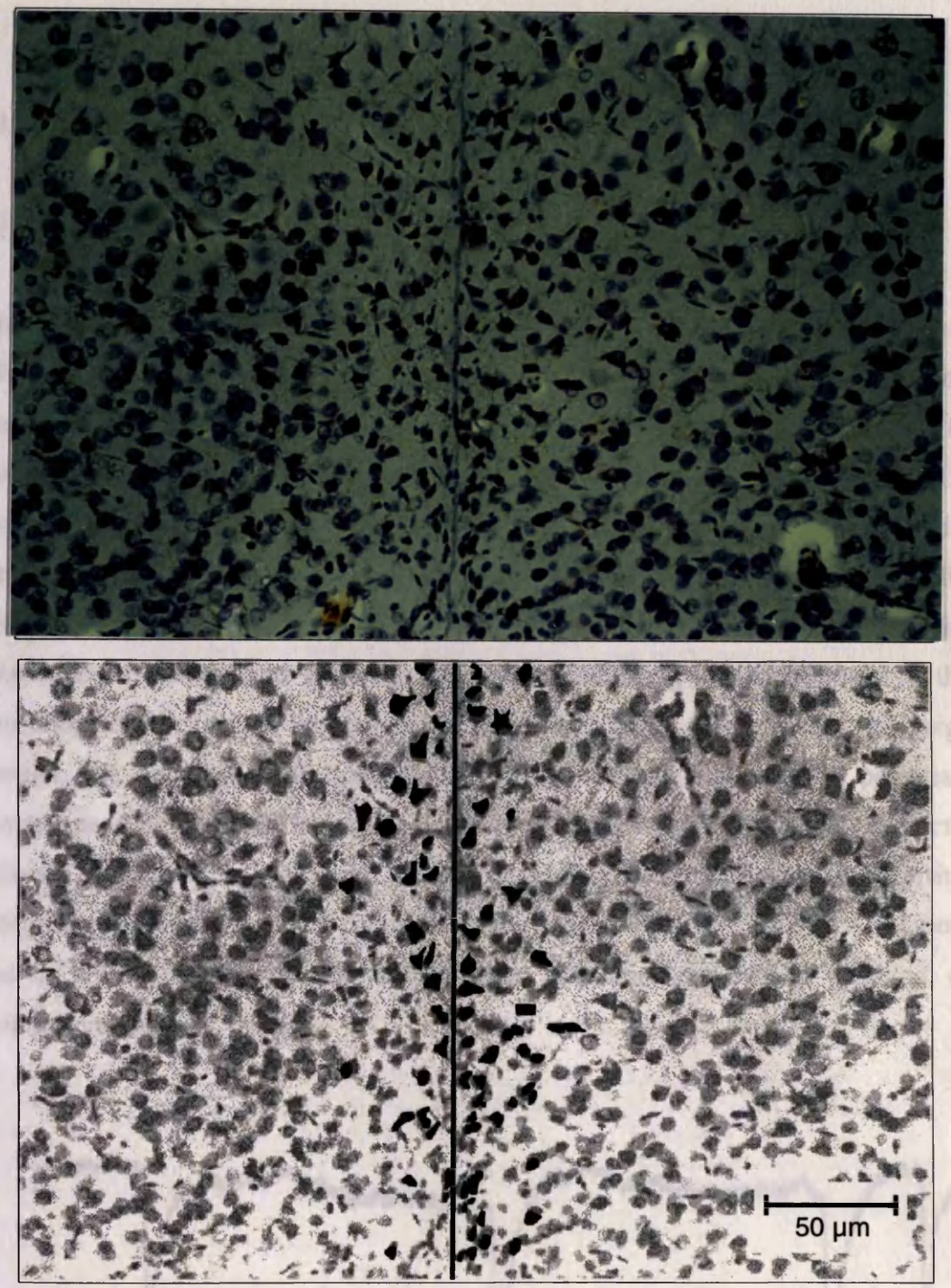


Figure 10: Zone of damage along a cut perpendicular to the pial surface. The extent of the damaged area (recognisable by collapsed pyramidal cell somata) varied from cut to cut, but averaged 50 µm on either side. In the schematic drawing, damaged cell somata are indicated by filled shapes.

3 RESULTS

3.1 Results *in vivo*

3.1.1 *Epileptiform activity: Spontaneous spikes and evoked potentials*

Once the cortex had been exposed, finding the region targeted by the forepaw afferents was usually straightforward. The microelectrode penetrations produced no major damage, and it was usually sufficient to record from three or four locations to find a site presenting with large amplitude evoked potentials (as shown in Fig. 13, these were near maximal at the recording depth of 700 µm, which was chosen for its reported high sensitivity to GABAergic blockade). After the application of penicillin (either by topical application to the pial surface, or by iontophoresis at depth), the amplitude of the evoked potentials increased, indicating a disinhibition of the physiological circuitry. If the amount of penicillin applied to the cortex was large enough, the appearance of high voltage, sharp negative epileptiform potentials characterised by a greater latency and massively increased amplitude (the 'interictal spikes') could then be observed. Evoked potentials and spontaneously occurring interictal spikes were the main parameters used to assess the viability of the isolation, and its ability to support either an enhanced somatosensory evoked potential, or epileptiform activity, or both.

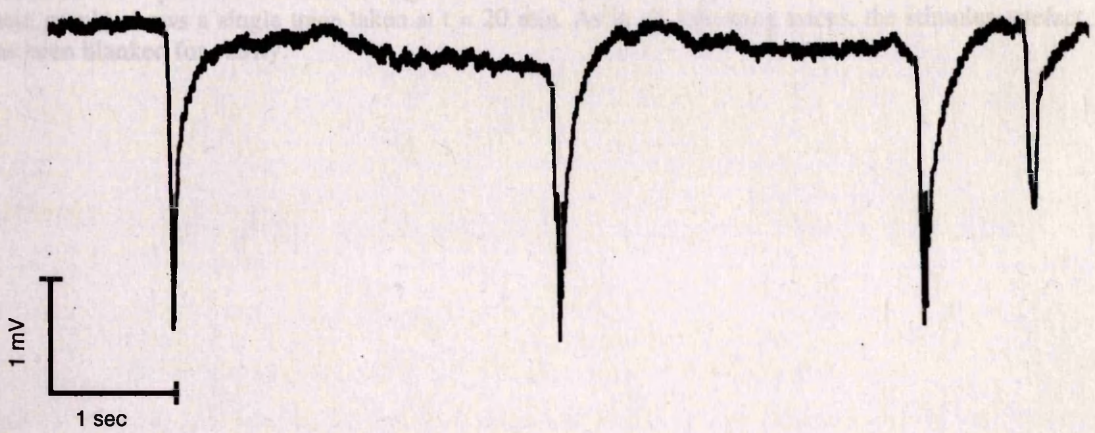


Figure 11: Large amplitude interictal spikes recorded from undamaged cortex after the application of a dilute penicillin solution to the exposed pial surface

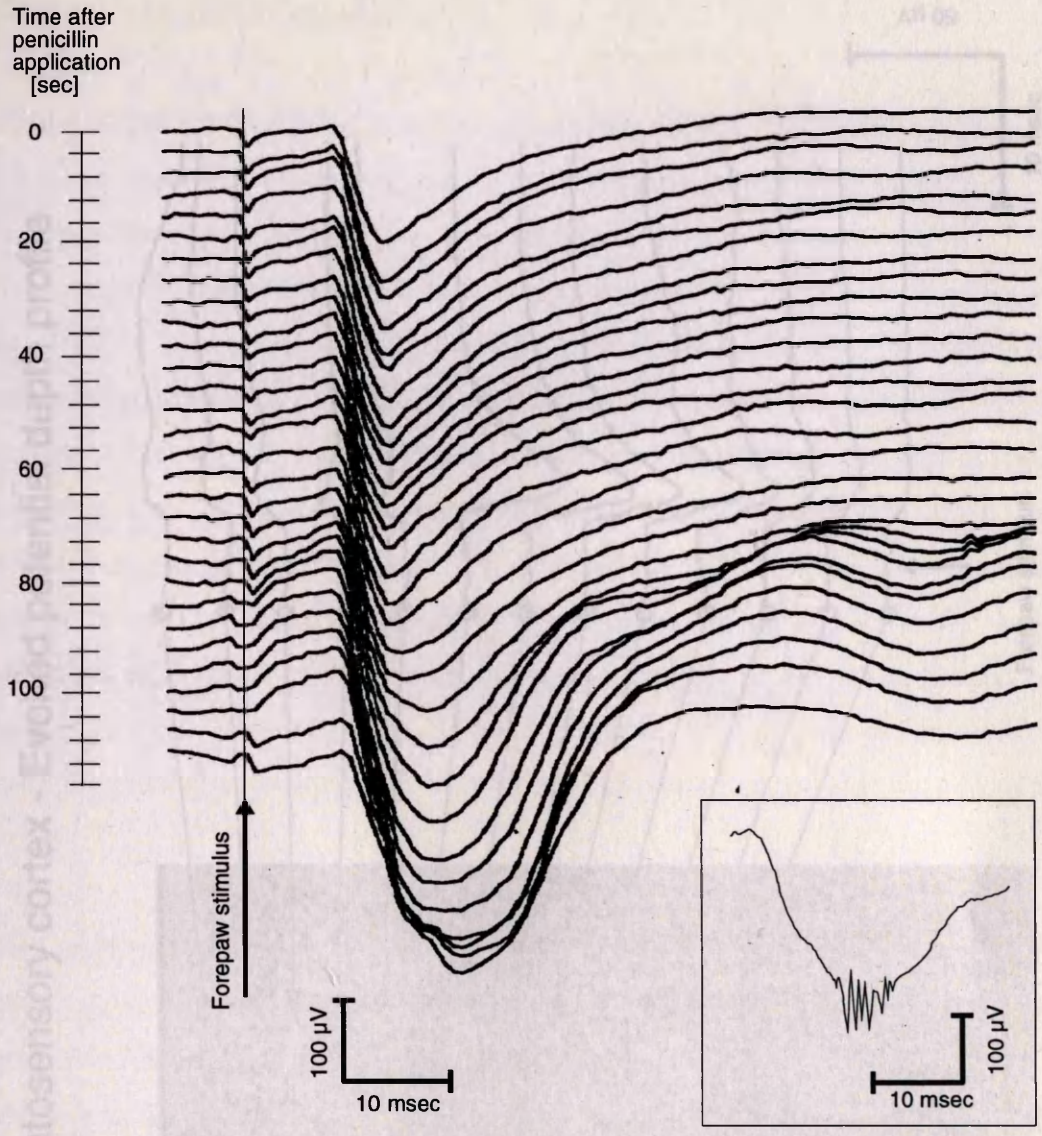


Figure 12: Effects of topically applied penicillin solution on SI evoked potentials, recorded at a depth of 700 µm: each trace represents the average of 10 forepaw potentials, evoked at a rate of 15/min. The record covers a period of 2 min, starting at the time of penicillin application. The inset (scale same as main graph) shows a single trace taken at t = 20 min. As in all following traces, the stimulus artefact has been blanked for clarity.

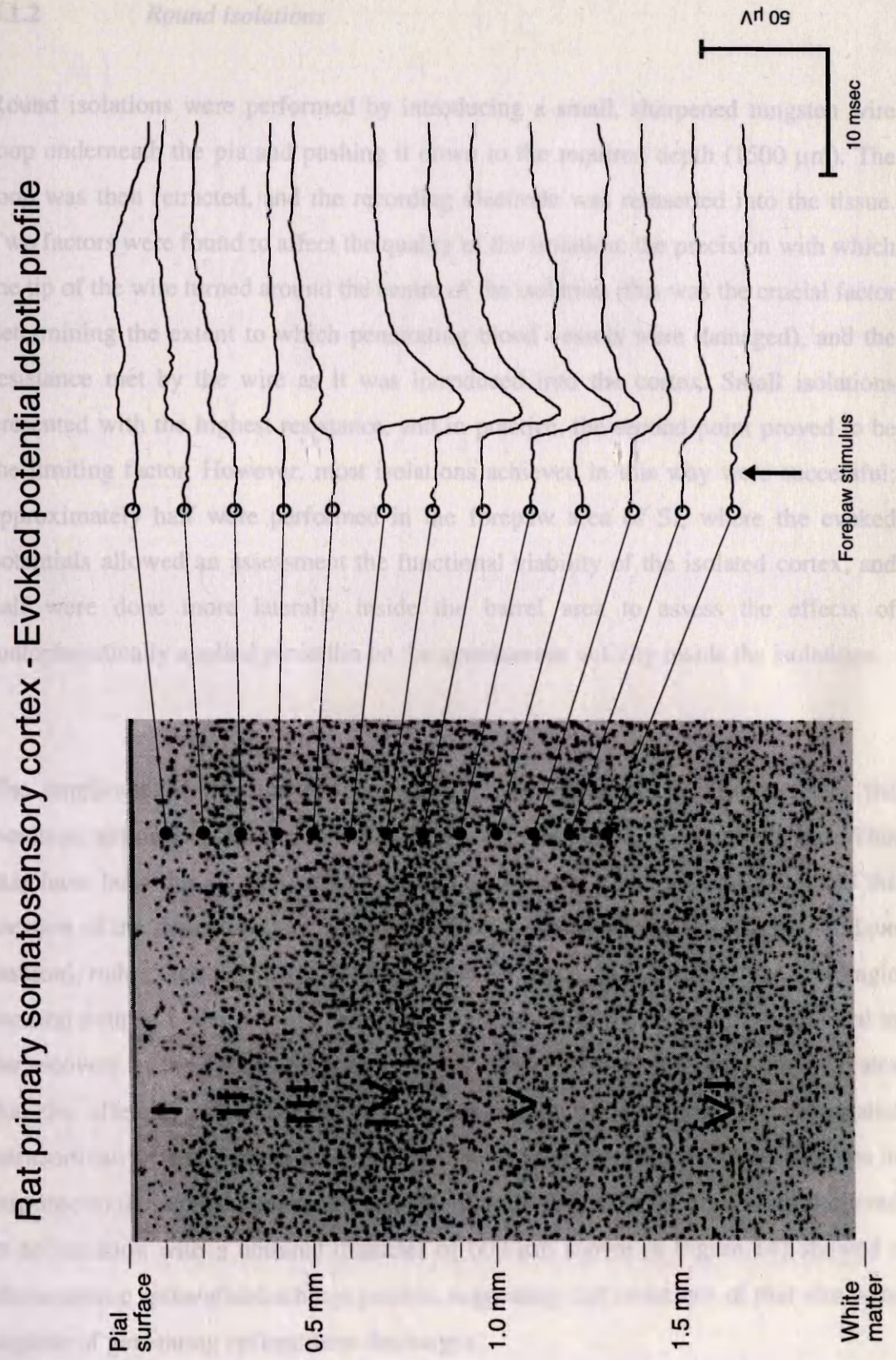


Figure 13: 100 µm interval evoked field potential profile in the forepaw area of rat primary somatosensory cortex, correlated to a photomicrograph of a nuclear-stained cross-section through SI cortex. The evoked negativities are strongest in the middle layers; no evoked potentials were seen in deep layers.

3.1.2 *Round isolations*

Round isolations were performed by introducing a small, sharpened tungsten wire loop underneath the pia and pushing it down to the required depth (1500 µm). The loop was then retracted, and the recording electrode was reinserted into the tissue. Two factors were found to affect the quality of the isolation: the precision with which the tip of the wire turned around the centre of the isolation (this was the crucial factor determining the extent to which penetrating blood vessels were damaged), and the resistance met by the wire as it was introduced into the cortex. Small isolations presented with the highest resistance, and in practice, the second point proved to be the limiting factor. However, most isolations achieved in this way were successful; approximately half were performed in the forepaw area of SI, where the evoked potentials allowed an assessment the functional viability of the isolated cortex, and half were done more laterally inside the barrel area to assess the effects of iontophoretically applied penicillin on the spontaneous activity inside the isolations.

The amplitude of the evoked potentials recovered within 30 minutes after the isolation, although in some cases to a level which was below the control value. This may have been due to the fact that the isolation was not centred exactly around the location of the thalamocortical afferents (which enter the cortex in a slightly oblique fashion), rather than to irreversible damage to the tissue, as the application of a single incision with an L-shaped wire (see below) through the forepaw area often resulted in the recovery of the evoked potentials on one side of the incision only; this indicates that the afferent input to discrete locations within the cortex and is propagated intracortically via horizontal connections. The spiking observed in these isolations in response to the topical application of penicillin (cf. for example the spiking observed in an isolation with a nominal diameter of 600 µm shown in Figure 14) showed a characteristic spike/afterdischarge pattern, suggesting that isolations of that size were capable of generating epileptiform discharges.

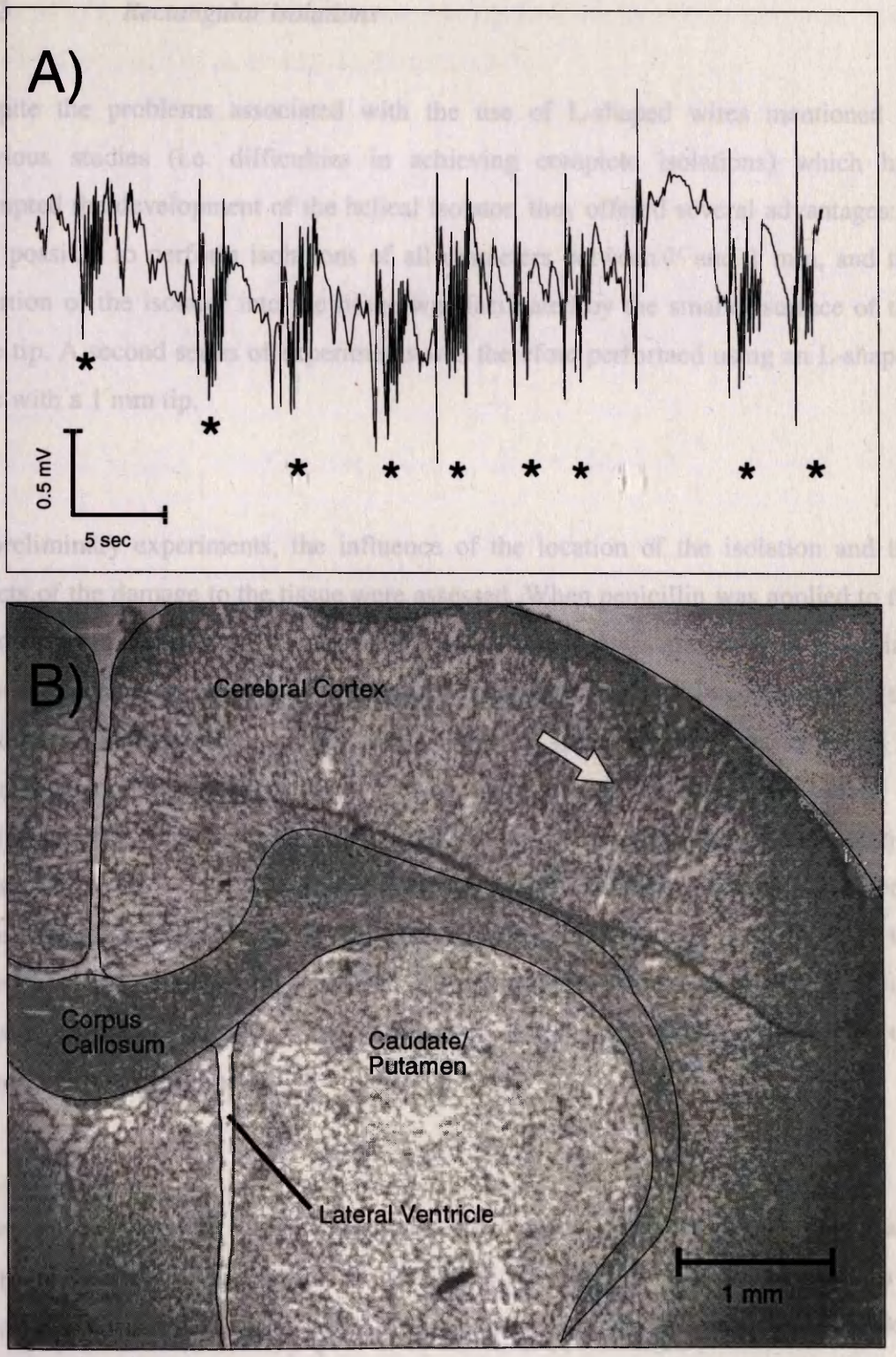


Figure 14: Spike/afterdischarge complexes recorded inside a round isolation (diameter 600 µm) after topical application of penicillin solution (**A**), and coronal cryostat section (30 µm) of a different rat brain at the level of a round isolation, after fixation in 10% neutral formalin solution (**B**): two parallel incisions (indicated by the arrow) reaching from underneath the pia down to the white matter can be clearly seen. Embedding medium: Burr Aquamount.

3.1.3 *Rectangular isolations*

Despite the problems associated with the use of L-shaped wires mentioned in previous studies (i.e. difficulties in achieving complete isolations) which had prompted the development of the helical isolator, they offered several advantages: it was possible to perform isolations of all diameters between 0.5 and 1 mm, and the insertion of the isolator into the tissue was facilitated by the smaller surface of the wire tip. A second series of experiments was therefore performed using an L-shaped wire with a 1 mm tip.

In preliminary experiments, the influence of the location of the isolation and the effects of the damage to the tissue were assessed. When penicillin was applied to the middle of an incision of 1 mm length and 1 mm depth, the evoked potentials recovered within 40 minutes on one side of the cut only; penicillin application to the same location led to strong enhancement within 20 seconds, which abated within 30 minutes. Spontaneous spikes also occurred, even though the depression caused by cutting had been rather severe. As suspected after the round isolations, the isolation clearly had to be around the target zone of the specific forepaw afferents if one were to use the evoked potentials as a means of assessing functional viability and the strength of the response to penicillin; but the incision itself had no effect on the spiking; therefore damage to the surrounding tissue is of minor relevance to the outcome of the experiment.

To achieve a series of subpial isolations of decreasing diameter in one animal and within one cortical area, nested isolations were made which resulted in isolations of three different sizes (see Fig. 15). In the experiment shown overleaf, the evoked potentials in two of the smallest incisions (= quadrants) recovered quite readily, especially in quadrant II, where the amplitude and short latency suggest a close proximity to the forepaw region whereas they appeared blunt in the other two, indicating a deafferentiation as described above. Enhancement of evoked potentials after the application of penicillin occurred in all but the smallest isolation. This

appeared to indicate that the lower limit was reached which, at 0.25 mm^2, was quite close in size to that of an average cortical barrel.

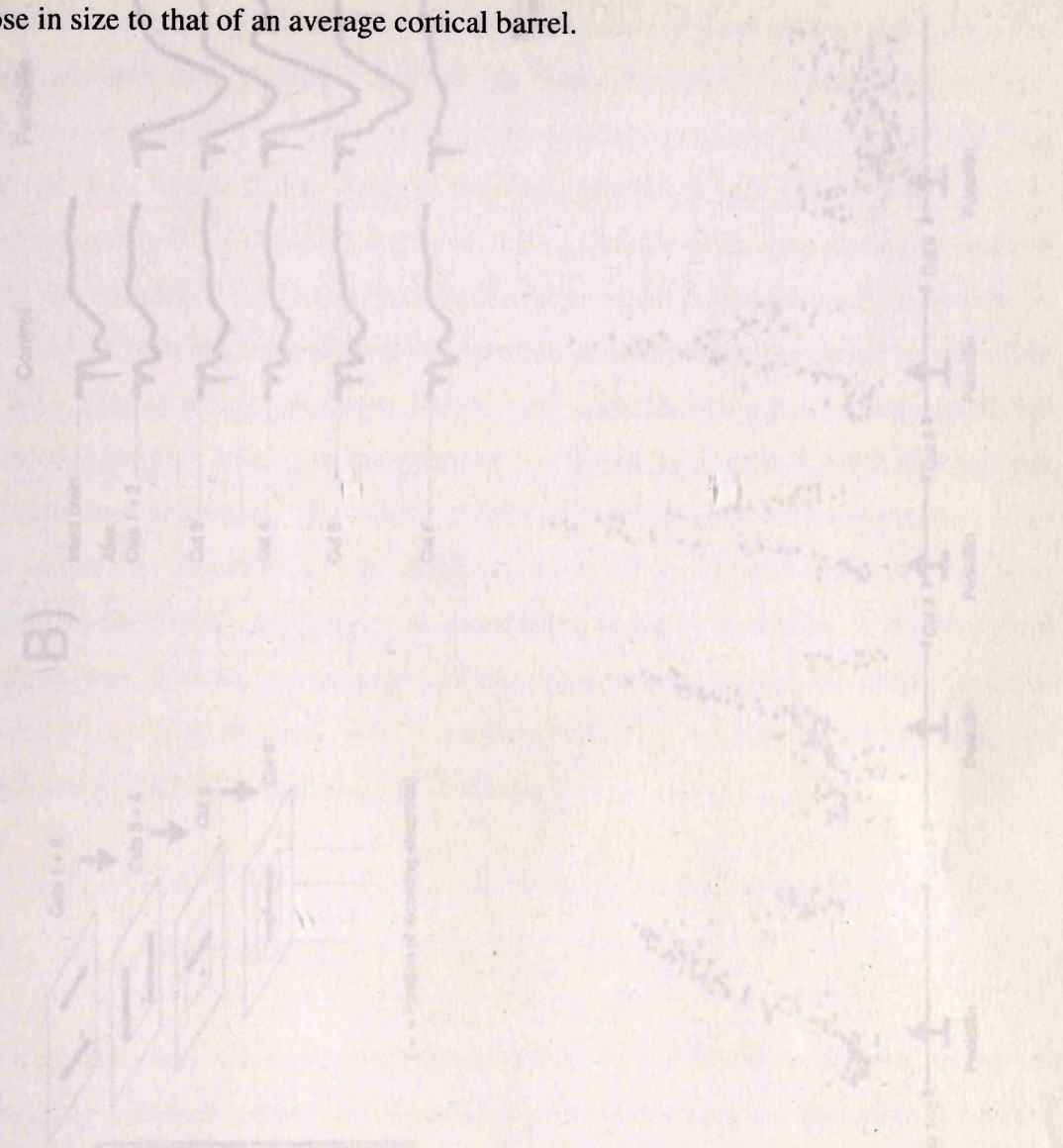


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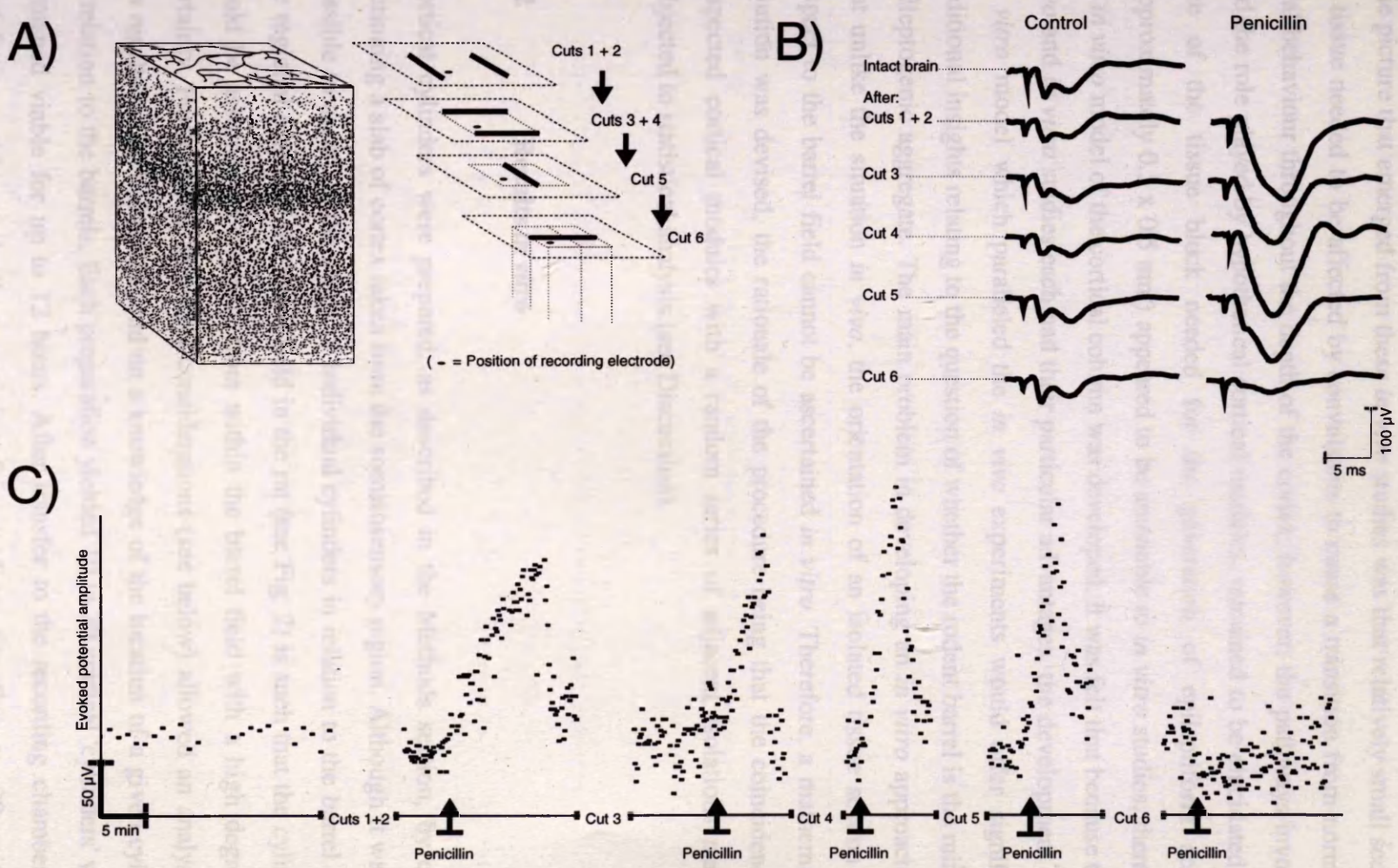
Figure 15: Effects of iontophoretically applied penicillin solution on somatosensory evoked potentials inside a progressively smaller subpial isolation (rat neocortex). The uppermost trace represents a control evoked potential; the following 5 pairs of traces represent evoked potentials recorded at the site marked by a circle in the corresponding schematic drawing of the isolation (length of each cut: 1 mm) before (top) and after (bottom) the iontophoretic application of penicillin. Each trace is the average of 5 recordings. The amplitude of the control evoked potentials remains constant, but the penicillin-induced enhancement is absent in the smallest isolation (size: 0.5 x 0.5 mm)


A)
Cuts 1 + 2
Cuts 3 + 4
Cut 5
Cut 6
(- = Position of recording electrode)
B)
Control
Penicillin
Intact brain
After:
Cuts 1 + 2
Cut 3
Cut 4
Cut 5
Cut 6
100 µV
5 ms
C)
Evoked potential amplitude
50 µV
5 min
Cuts 1+2
Cut 3
Cut 4
Cut 5
Cut 6
Penicillin
Penicillin
Penicillin
Penicillin
Penicillin

(Figure 15)

The picture that emerged from these *in vivo* studies was that relatively small sections of tissue needed to be affected by convulsants to cause a transition from normal to ictal behaviour throughout the depth of the cortex; however, the pathways involved, and the role played by hypothetical cortical modules, remained to be elucidated. The size of the tissue block needed for the generation of epileptiform activity (approximately 0.5 x 0.5 mm) appeared to be amenable to *in vitro* studies; therefore, an *in vitro* model of the cortical column was developed. It was felt that because the *in vivo* and *in vitro* studies each had their particular advantages, the development of an *in vitro* model which paralleled the *in vivo* experiments would offer significant additional insights relating to the question of whether the rodent barrel is the minimal epileptogenic aggregate. The main problem in developing an *in vitro* approach was that unlike the situation *in vivo*, the orientation of an isolated tissue section with respect to the barrel field cannot be ascertained *in vitro*. Therefore, a mathematical solution was devised, the rationale of the procedure being that the coincidence of suspected cortical modules with a random series of adjacent isolations may be subjected to statistical analysis (see Discussion).

3.2 Results *in vitro*

Cortical cylinders were prepared, as described in the Methods section, by cross-sectioning a slab of cortex taken from the somatosensory region. Although it was not possible to ascertain the origin of individual cylinders in relation to the barrel field, the regional extent of the barrel field in the rat (see Fig. 2) is such that the cylinders could be assumed to originate from within the barrel field with a high degree of certainty. Furthermore, statistical considerations (see below) allowed an analysis of the results which did not depend on a knowledge of the location of a given cylinder in relation to the barrels. Each preparation yielded 10 to 20 cortical 'cylinders' which remained viable for up to 12 hours. After transfer to the recording chamber and superfusion with nominally magnesium-free medium for five to 20 minutes, spontaneous epileptiform discharges were routinely observed. The discharges consisted of a large initial spike followed by gradually developing, recurring clusters of afterdischarges separated by short silent intervals of increasing duration which

lasted up to 20 seconds. In some instances, the discharges of several individual neurones could be discriminated (multiple action potential recordings). These recordings were usually only obtained in layer III; the main repetitive discharges could be recorded at all cortical depths.

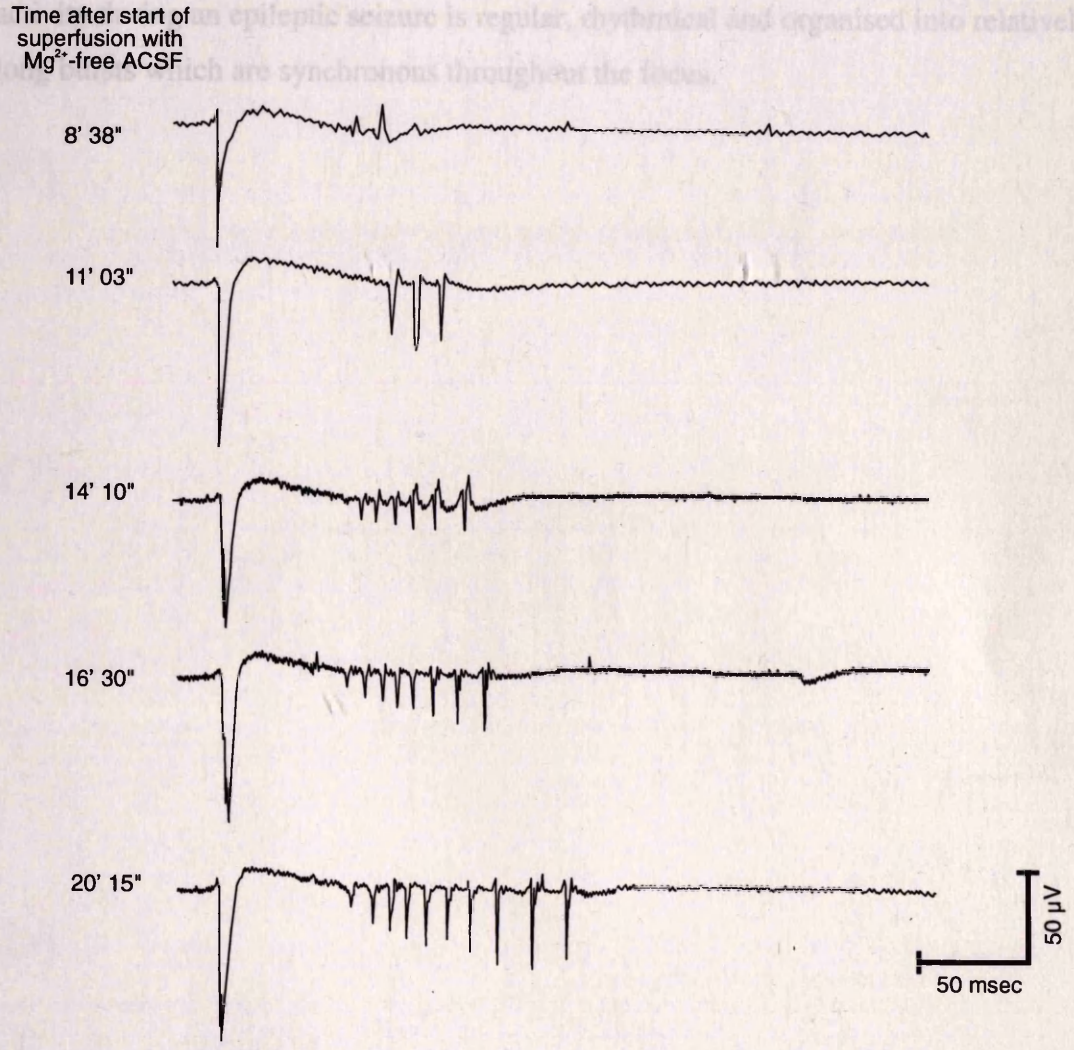


Figure 16: Gradual development of afterdischarges in a spontaneously spiking rat neocortical cylinder (diameter 500 µm). Perfusion with magnesium-free ACSF started at time = 0'00". Traces were bandpass-filtered to remove low frequency components.

In Fig. 17, details of the activity in spontaneously spiking tissue cylinders are shown. Small, nonsynchronous action potentials and EPSPs which increase in number over time and summate temporally can be seen to form clusters that appear as irregular depolarisations. These depolarisations progressively become larger in amplitude and

higher in frequency, finally giving rise to an epileptiform burst, consisting of a large population burst followed by a series of smaller bursts (afterdischarges) riding on a prolonged depolarisation wave, which invades the entire cylinder. The electrographic pattern mirrors the situation in epileptic foci *in vivo*: while the discharging focus is characterised by sporadic, random discharges during the interictal period, the seizure activity during an epileptic seizure is regular, rhythmical and organised into relatively long bursts which are synchronous throughout the focus.

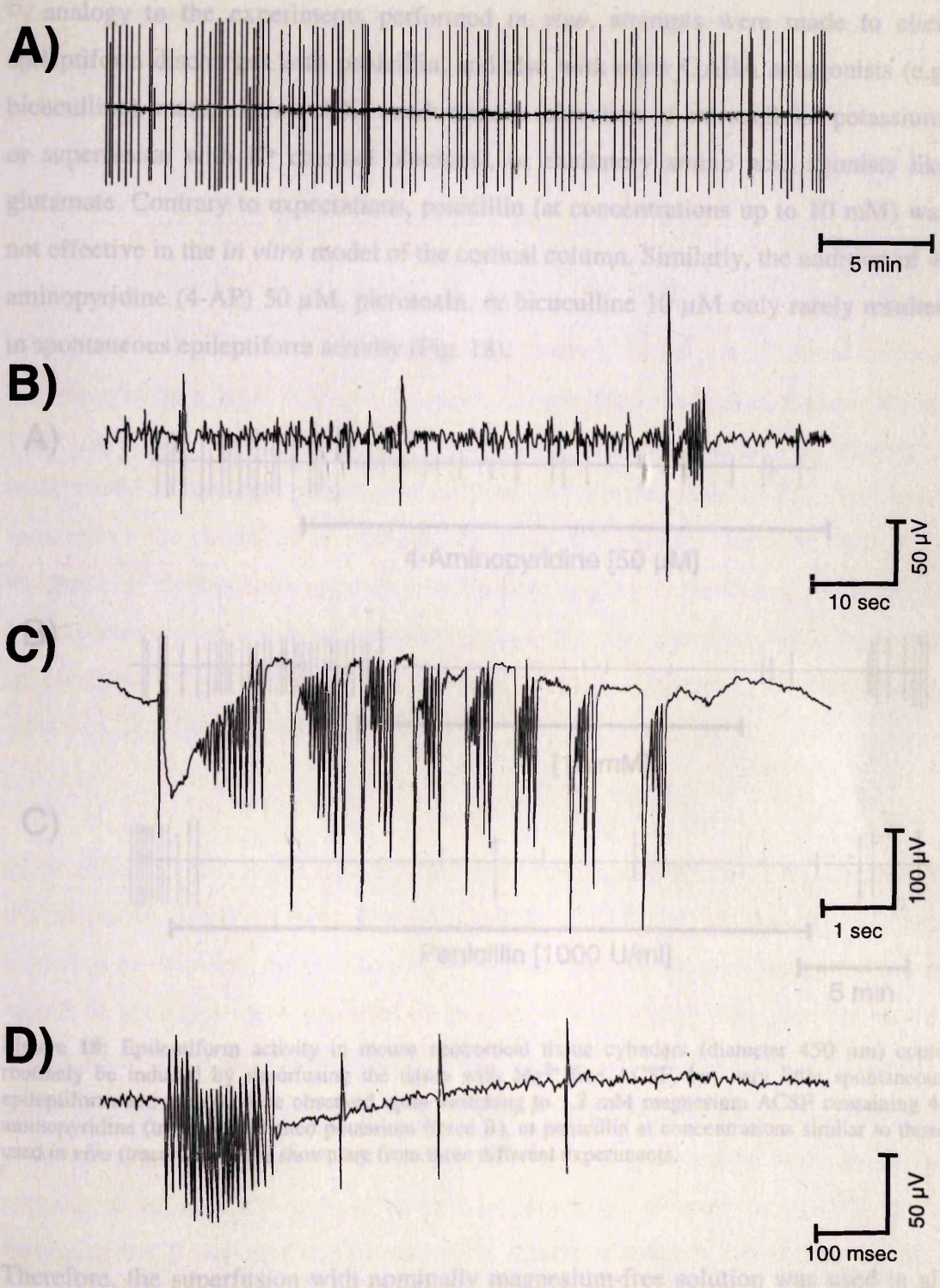


Figure 17: Epileptiform activity in mouse neocortical tissue cylinders (diameter 450 µm) in Mg^{2+}-free ACSF. Traces **A** and **B** are (low-pass filtered) chart recordings, while traces **C** and **D** are plots from a digital oscilloscope. In some instances, the high level of spontaneous background activity which precedes the main spike could be recorded (= multiple action potential recording, **B**); small interictal spikes can be seen to occur with increasing frequency before a large spike/afterdischarge complex. Only the latter (**C**) can be called 'epileptic', as it represents a recurrent, synchronised discharge involving the entire substrate. Trace **D** shows an action potential burst fired by a single neurone during an afterdischarge.

By analogy to the experiments performed *in vivo*, attempts were made to elicit epileptiform discharges with penicillin, and also with other GABA antagonists (e.g. bicuculline), manipulation of K^+ conductances (elevation of extracellular potassium, or superfusion with K^+ channel blockers), or excitatory amino acid agonists like glutamate. Contrary to expectations, penicillin (at concentrations up to 10 mM) was not effective in the *in vitro* model of the cortical column. Similarly, the addition of 4-aminopyridine (4-AP) 50 µM, picrotoxin, or bicuculline 10 µM only rarely resulted in spontaneous epileptiform activity (Fig. 18).

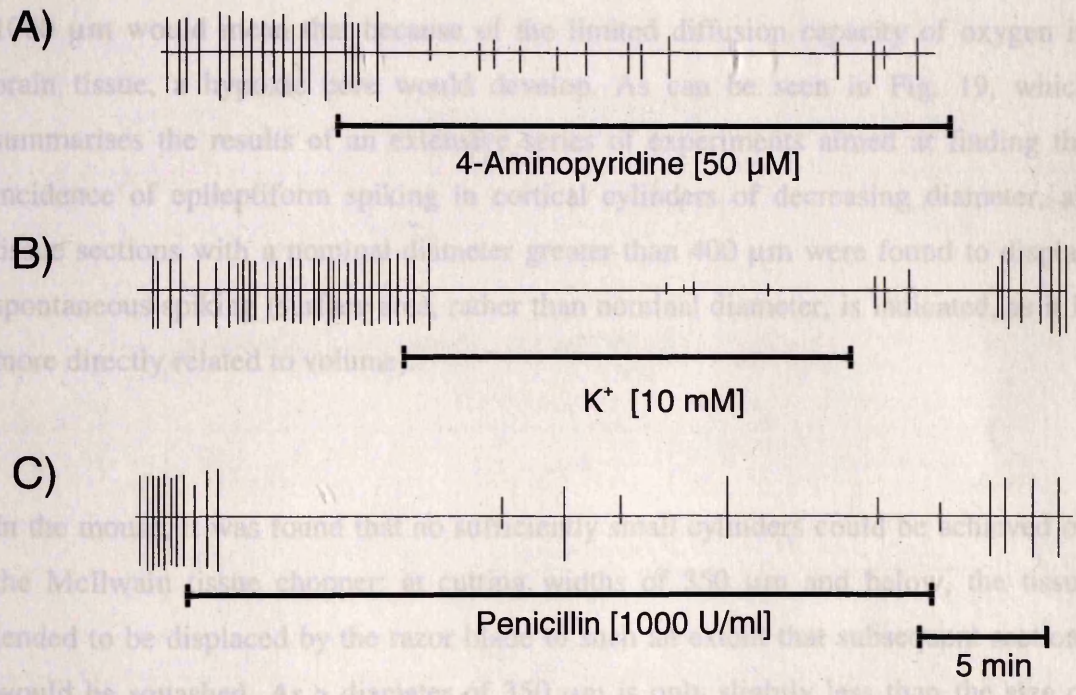


Figure 18: Epileptiform activity in mouse neocortical tissue cylinders (diameter 450 µm) could routinely be induced by superfusing the tissue with Mg^{2+}-free ACSF, but very little spontaneous epileptiform activity could be observed upon switching to 1.2 mM magnesium ACSF containing 4-aminopyridine (trace A), elevated potassium (trace B), or penicillin at concentrations similar to those used *in vivo* (trace C). Traces shown are from three different experiments.

Therefore, the superfusion with nominally magnesium-free solution was used in all subsequent experiments to achieve a rapid and reliable induction of interictal spiking mediated by NMDA receptor activation. The spiking could be completely abolished by aminophosphonovaleric acid (APV) 50 µM, with half-maximal suppression occurring at a concentration of 1 µM. The $GABA_B$ agonist baclofen inhibited the

epileptiform activity induced with Mg^{++}-free solution with an approximate IC_{50} of 70 nM (not shown)

3.2.1 *Smallest cylinders*

With practice, it was found that it was possible to reproducibly obtain cortical cylinders which covered a large range of diameters. In the rat, nominal cylinder diameters ranging from 1000 µm down to 350 µm were studied; diameters beyond 1000 µm would mean that because of the limited diffusion capacity of oxygen in brain tissue, a hypoxic core would develop. As can be seen in Fig. 19, which summarises the results of an extensive series of experiments aimed at finding the incidence of epileptiform spiking in cortical cylinders of decreasing diameter, all tissue sections with a nominal diameter greater than 400 µm were found to display spontaneous spiking (surface area, rather than nominal diameter, is indicated, as it is more directly related to volume).

In the mouse, it was found that no sufficiently small cylinders could be achieved on the McIlwain tissue chopper: at cutting widths of 350 µm and below, the tissue tended to be displaced by the razor blade to such an extent that subsequent sections would be squashed. As a diameter of 350 µm is only slightly less than the size of large barrels in the mouse (Woolsey and van der Loos, 1970), a congruence of barrels and the minimal mass could not be excluded with this method. Therefore, the presence of generator zones within these cylinders was investigated in an attempt to confirm or exclude the layer IV barrels as generators of epileptic activity in the vertical plane. However, a comparison of the results obtained in the rat (Fig. 19; little or no paroxysmal activity is seen in small cylinders) with tissue of similar proportions which yielded robust spiking in the mouse (Fig. 20), already appeared to indicate that the degree of connectivity, rather than the absolute cell number, is the determining factor in the ability of a tissue segment to generate paroxysmal discharges (Chesi, 1992).

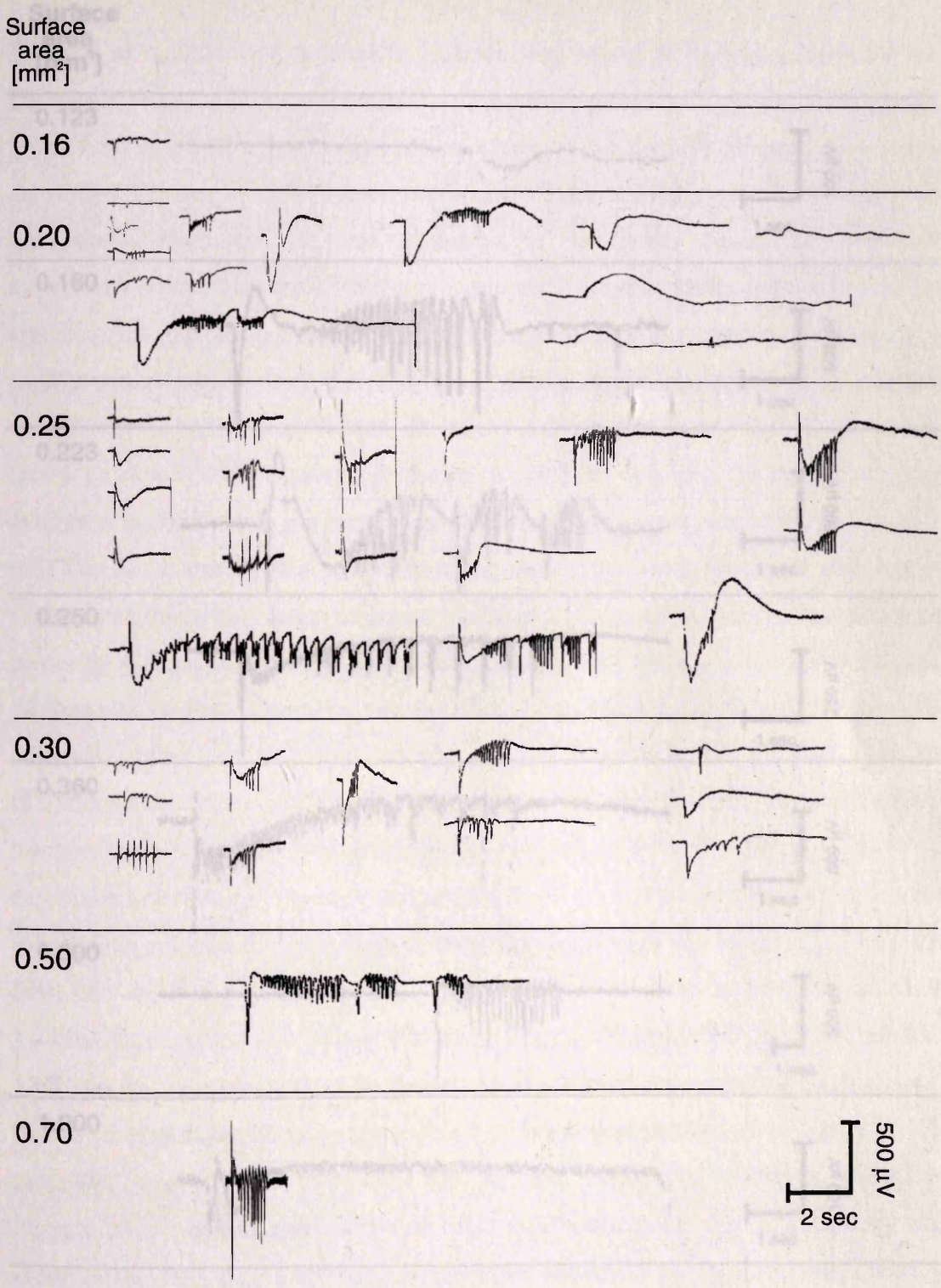


Figure 19: Representative records from sets of rat cortical cylinders of increasing diameter which displayed spontaneous spiking in magnesium-free ACSF; for each of the three smallest sizes, a total of 20 cylinders from the left SI cortex of one rat were used. Traces for larger diameters are included for illustration purposes. The smallest diameter for which spiking could still be regularly observed was 400 µm (= surface area of 0.20 mm²)

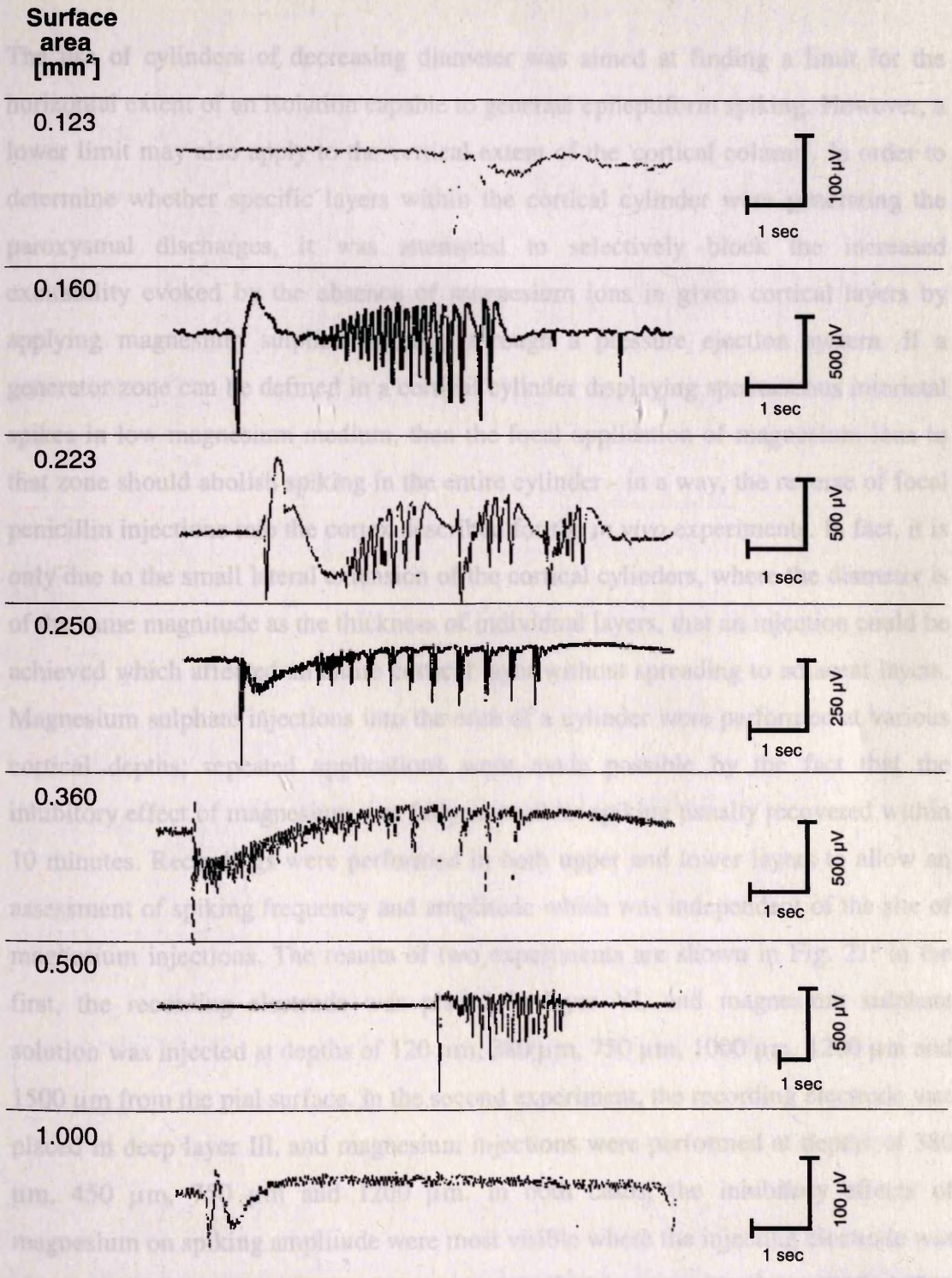


Figure 20: Representative records of epileptiform spiking observed in mouse cortical cylinders of increasing diameter incubated in magnesium-free ACSF. The traces are shown for illustration purposes; spiking could even be observed in the smallest technically feasible cylinders (surface area 0.123 mm²).

3.2.2 *Origin of spiking: magnesium injections*

The use of cylinders of decreasing diameter was aimed at finding a limit for the horizontal extent of an isolation capable to generate epileptiform spiking. However, a lower limit may also apply to the vertical extent of the 'cortical column'. In order to determine whether specific layers within the cortical cylinder were generating the paroxysmal discharges, it was attempted to selectively block the increased excitability evoked by the absence of magnesium ions in given cortical layers by applying magnesium sulphate solution through a pressure ejection system. If a generator zone can be defined in a cortical cylinder displaying spontaneous interictal spikes in low-magnesium medium, then the focal application of magnesium ions to that zone should abolish spiking in the entire cylinder - in a way, the reverse of focal penicillin injections into the cortex described for the *in vivo* experiments. In fact, it is only due to the small lateral extension of the cortical cylinders, where the diameter is of the same magnitude as the thickness of individual layers, that an injection could be achieved which affected an entire cortical layer without spreading to adjacent layers. Magnesium sulphate injections into the core of a cylinder were performed at various cortical depths; repeated applications were made possible by the fact that the inhibitory effect of magnesium was fully reversible: spiking usually recovered within 10 minutes. Recordings were performed in both upper and lower layers to allow an assessment of spiking frequency and amplitude which was independent of the site of magnesium injections. The results of two experiments are shown in Fig. 21: in the first, the recording electrode was placed in layer VI, and magnesium sulphate solution was injected at depths of 120 µm, 380 µm, 750 µm, 1000 µm, 1200 µm and 1500 µm from the pial surface. In the second experiment, the recording electrode was placed in deep layer III, and magnesium injections were performed at depths of 380 µm, 450 µm, 750 µm and 1200 µm. In both cases, the inhibitory effects of magnesium on spiking amplitude were most visible where the injecting electrode was close to the recording electrode, as expected. However, no location was found to suppress spiking frequency in a preferential fashion; this is in contradiction to a postulated generator zone in layers II - IV. Several other experiments using different strengths of magnesium sulphate solutions, injection times and recording sites were performed, with essentially identical results to those illustrated below.

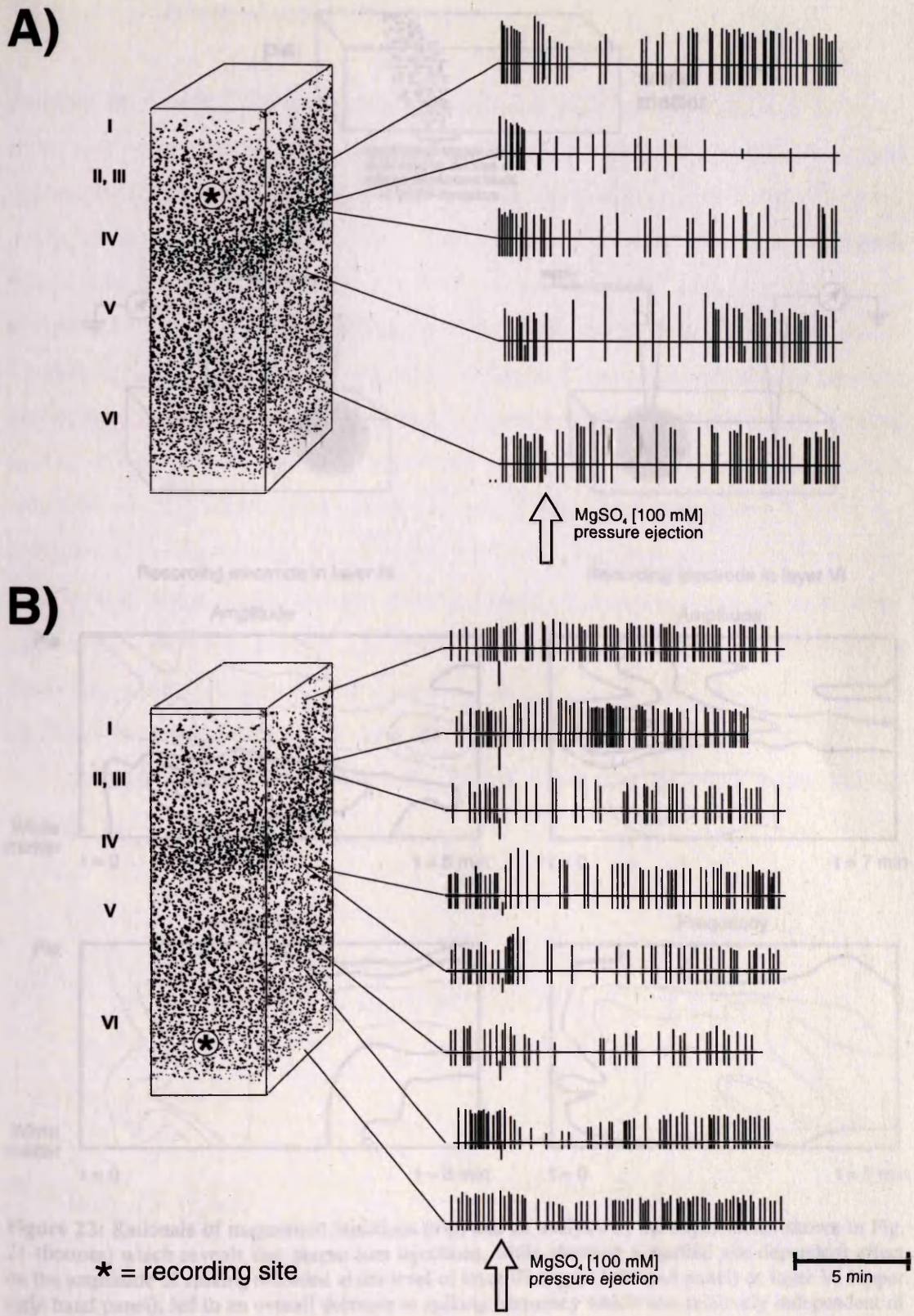


Figure 21: Pressure ejection of magnesium sulphate solution into different layers in two mouse neocortical cylinders. The recording electrode was placed in either layer III (A) or VI (B), and injections were made into layers III, IV, V and VI (A) and into I, III, V, VI and white matter (B) at the time indicated by the arrow.

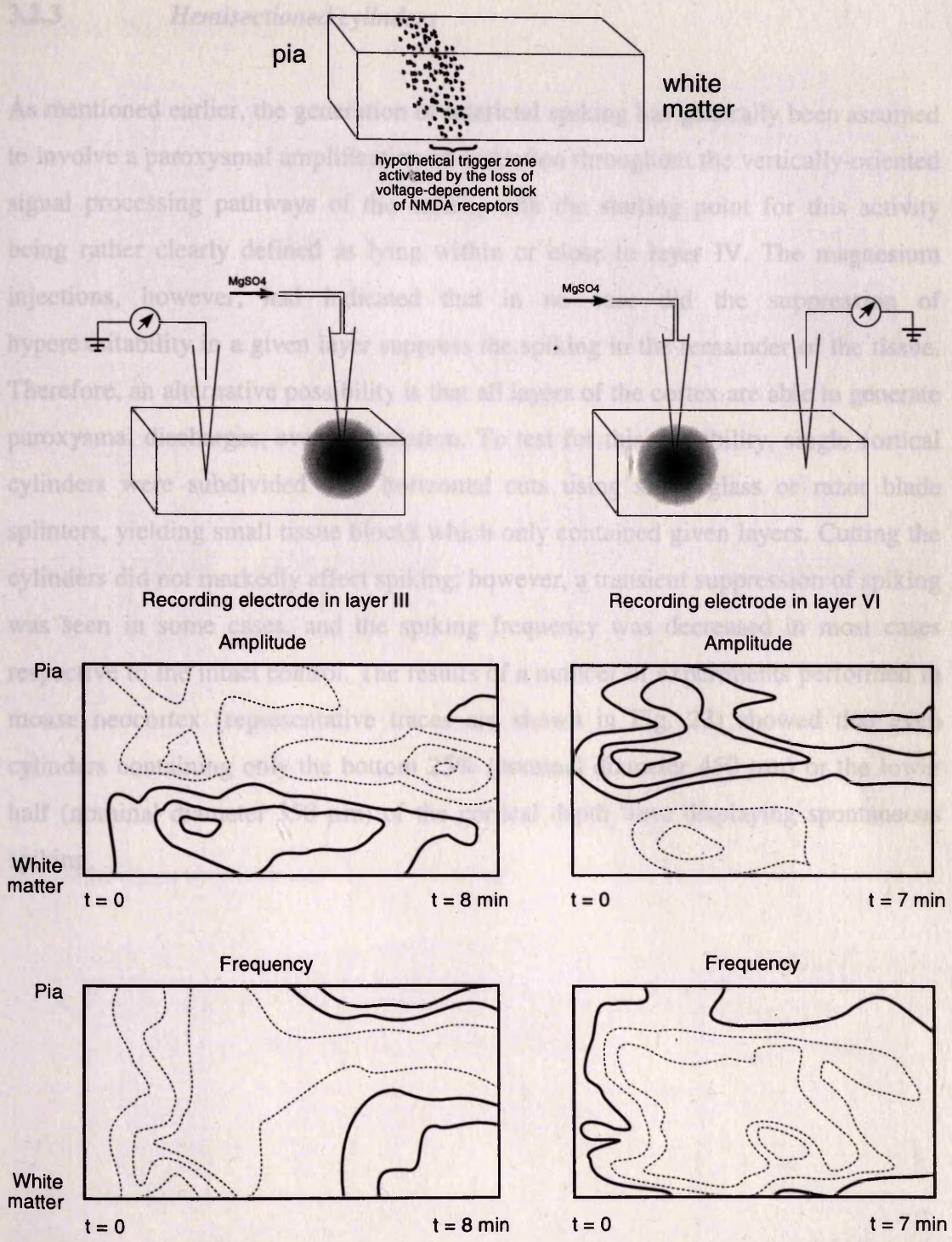
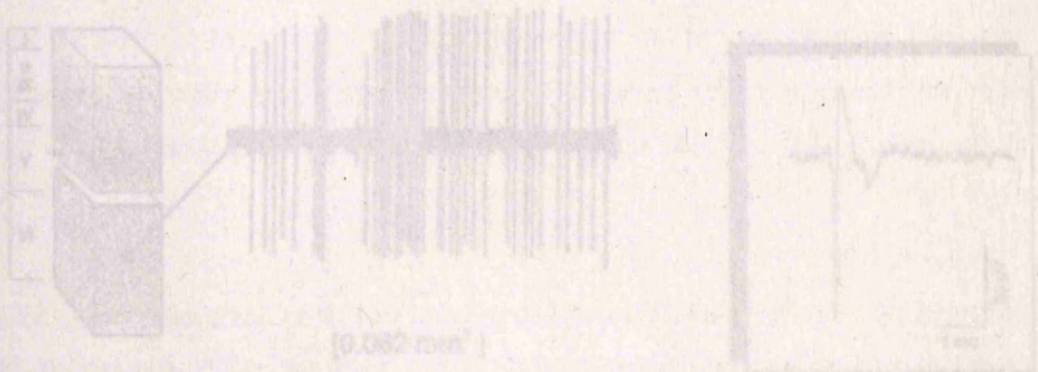


Figure 22: Rationale of magnesium injections (top) and an analysis of the experiments shown in Fig. 21 (bottom) which reveals that magnesium injections, while showing a marked site-dependent effect on the amplitude of spiking recorded at the level of layer III (upper left hand panel) or layer VI (upper right hand panel), led to an overall decrease in spiking frequency which was relatively independent of the site of injection, suggesting that the spiking observed in magnesium-free ACSF does not originate in a discrete 'trigger zone'. Dashed lines represent a decrease relative to control, while solid lines mark an increase relative to control; the distance between two lines corresponds to a change of 20%.

3.2.3 *Hemisectioned cylinders*

As mentioned earlier, the generation of interictal spiking has generally been assumed to involve a paroxysmal amplification of excitation throughout the vertically-oriented signal processing pathways of the cortex, with the starting point for this activity being rather clearly defined as lying within or close to layer IV. The magnesium injections, however, had indicated that in no case did the suppression of hyperexcitability in a given layer suppress the spiking in the remainder of the tissue. Therefore, an alternative possibility is that all layers of the cortex are able to generate paroxysmal discharges, even in isolation. To test for this possibility, single cortical cylinders were subdivided with horizontal cuts using small glass or razor blade splinters, yielding small tissue blocks which only contained given layers. Cutting the cylinders did not markedly affect spiking; however, a transient suppression of spiking was seen in some cases, and the spiking frequency was decreased in most cases respective to the intact control. The results of a number of experiments performed in mouse neocortex (representative traces are shown in Fig. 23) showed that even cylinders containing only the bottom 25% (nominal diameter 450 µm) or the lower half (nominal diameter 350 µm) of the cortical depth were displaying spontaneous spiking.



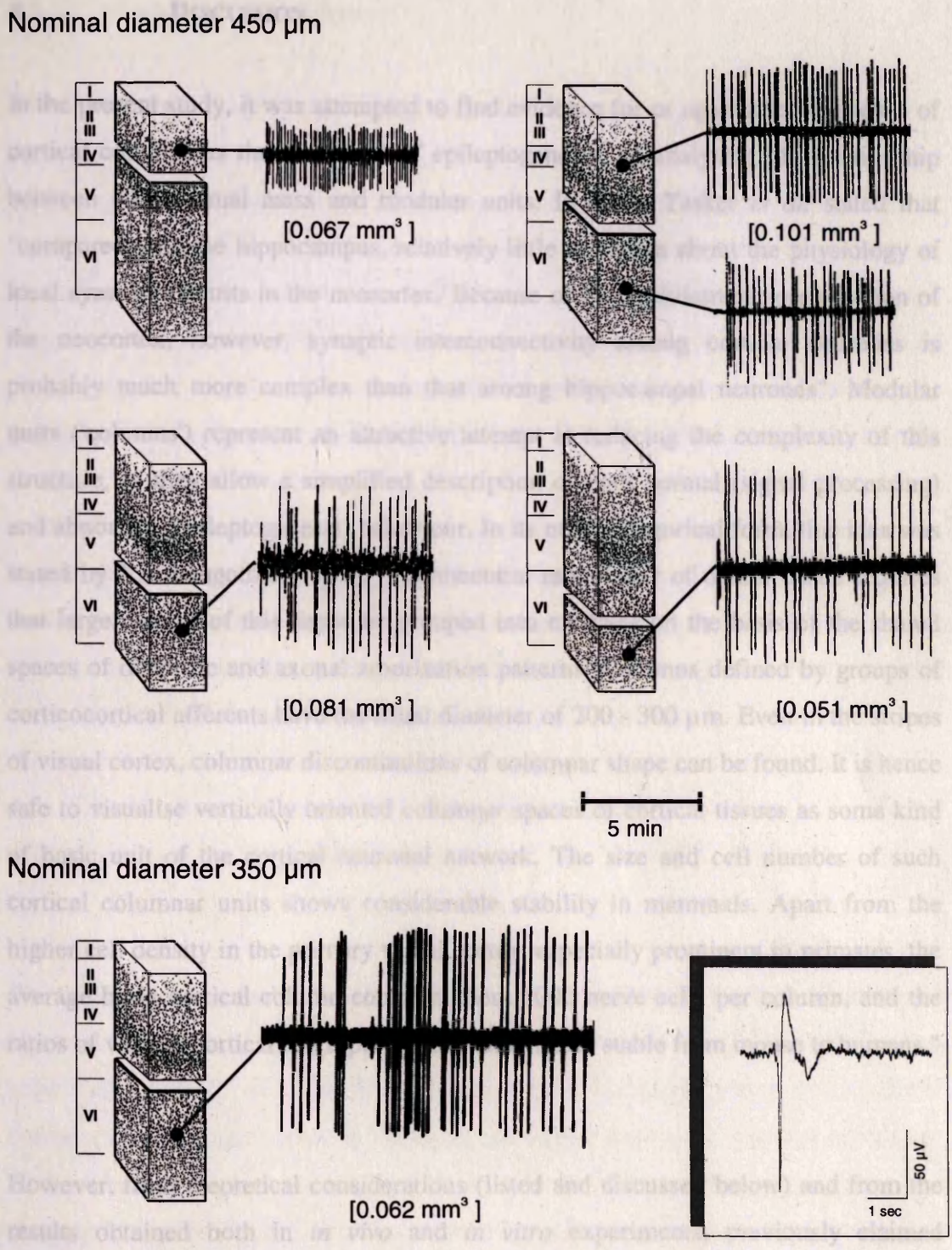


Figure 23: Recordings from five representative experiments using hemisectioned mouse neocortical 'cylinders' (nominal diameters 450 or 350 µm): spontaneous spiking could be observed even in preparations containing only either layers I - III (top left) or the lower half of layer VI (middle right), indicating that layer IV or an intact vertical information processing pathway is not needed for the generation of interictal spikes. The approximate volume of the segments in which spikes were recorded is indicated in square brackets. The inset (lower right) expands the discharges shown in the adjacent recording: a secondary burst resembling an afterdischarge can be seen.

4 DISCUSSION

In the present study, it was attempted to find evidence for or against the existence of cortical columns as the basic unit of epileptogenesis, by analysing the relationship between the minimal mass and modular units. In 1992, Tasker *et al.* stated that "compared with the hippocampus, relatively little is known about the physiology of local synaptic circuits in the neocortex. Because of the multilaminar organisation of the neocortex, however, synaptic interconnectivity among cortical neurones is probably much more complex than that among hippocampal neurones". Modular units ('columns') represent an attractive attempt at reducing the complexity of this structure, as they allow a simplified description of both normal (signal processing) and abnormal (epileptogenesis) behaviour. In its most categorical form, this idea was stated by J Szentagothai (1987): "Architectural modularity of neural tissue requires that large chunks of this tissue be grouped into modules on the basis of the shared spaces of dendritic and axonal arborization patterns. Columns defined by groups of corticocortical afferents have the usual diameter of 200 - 300 µm. Even in the stripes of visual cortex, columnar discontinuities of columnar shape can be found. It is hence safe to visualise vertically oriented columnar spaces of cortical tissues as some kind of basic unit of the cortical neuronal network. The size and cell number of such cortical columnar units shows considerable stability in mammals. Apart from the higher cell density in the primary visual cortex, especially prominent in primates, the average basic cortical column contains about 5000 nerve cells per column, and the ratios of various cortical cell types remain remarkably stable from mouse to humans."

However, from theoretical considerations (listed and discussed below) and from the results obtained both in *in vivo* and *in vitro* experiments, previously claimed requirements for the generation of epileptiform discharges (i.e. unidirectional, polysynaptic pathways initiating in layer IV, and a minimal mass coinciding with an area of cortex occupied by a single barrel) do not appear to be essential.

4.1 *In vivo* isolations

The first series of subpial isolations was performed using a helical, sharpened wire developed for this purpose. The main reason for using a helical wire for the subpial isolation of a small island of cortical tissue was that it would keep the damage caused by the isolation to a minimum, as the insertion of the wire loop (which had an electrolytically sharpened tip) was made through a single hole in the pia: because the helix had been centred with high accuracy, the turning underneath the pia occurred along a single track. Also, the resulting isolation would be complete, regular, and of a precisely defined diameter, unlike the isolations performed with L-shaped wire hooks (see below), which often left gaps at the corners of the rectangular isolation, where the diameter of the isolation varied between experiments, and where the introduction had to be performed four times, through two holes in the pia. It was felt that the circular and complete isolation was the best possible way of isolating tissue corresponding in size and shape to the somatosensory barrels, without causing too much damage to the arachnoid. That the isolation had to be complete for the experiment to be valid is indicated by the results of a study by Boakes *et al.* (1988), who found that as little as 20% of the cortical depth needed to be intact for epileptiform activity to spread past the incision.

The resulting isolations were shown to support the spike/afterdischarge patterns of electrographic activity typically seen in comparable isolations of larger size (as first suggested by Echlin *et al.* in 1952, the spikes/afterdischarges observed in isolated cortex may be caused in part by the de-afferentiation due to the isolation procedure: similar electroencephalographic patterns were noted adjacent to a brain tumour in a patient). However, it was found that the method could not be used for larger series of experiments, as the results were not easily reproducible. The main technical difficulty with the helical isolator lay in the high mechanical resistance of the tissue: a slow insertion of the helix using the micromanipulator drive resulted in a depression of the cortex by more than half a millimetre before the pressure was high enough for the helix to cut through the tissue. Therefore, the insertion had to be performed by giving a sharp blow to the supporting structure. As a result, although the isolation would be

complete and of a known diameter, the insertion depth could not be guided, and the blow to the isolator often resulted in a skewed track, which meant that when the helix was retracted, it was along a different track, resulting in a higher degree of damage. Also, the manufacturing of the wire loop was technically demanding and time-consuming; therefore, the range of diameters for the subpial isolations was limited, and a series of isolations of graded diameter to determine the minimal diameter for the induction of epileptiform spiking would have been extremely difficult to perform.

The subsequently used rectangular isolations showed that isolations with a diameter of 500 µm approached the limit for the generation of interictal spikes, as penicillin-induced enhancement of somatosensory evoked potentials was no longer present after an isolation measuring 0.5 x 1.0 mm was subdivided into two smaller cortical isolations measuring 0.5 x 0.5 mm (Fig. 15). However, the use of the L-shaped isolator frequently resulted in incomplete isolations still connected to the surrounding tissue by tissue bridges. At the same time, other groups had perfected the *in vivo* penicillin model to overcome the inherent limitations of the current approach. In fact, exact assessments of the amount of tissue involved in the generation of interictal spiking after the focal application of penicillin are hampered by the inability to measure the radial diffusion velocity of the drug molecules, although several models have been developed to this end (e.g. Holmes and Lockton, 1982). At the same time, the borders of the cuts constituting the partial isolation do not represent a diffusion barrier. Therefore, it becomes increasingly difficult to achieve high localised penicillin concentrations within an ever smaller isolation, even if the iontophoretic current is increased substantially, as the radially diffusing penicillin will always affect the surrounding tissue as well. Both limitations were elegantly overcome by Lehmenkühler *et al.* (1991), who applied penicillin to the motor cortex of anaesthetised rats via a pressure ejection system and used co-applied tetramethylammonium chloride as a marker substance (TMA^+ is a quaternary ammonium compound which cannot cross the cell membrane, and which can therefore be used as a marker for the extracellular space). Using this approach, they found that spikes could be elicited if penicillin concentrations of 1-2 mM were achieved in a tissue sphere with a diameter of 300 µm. No differential sensitivity

profile was reported, but they remarked that no spikes could be induced below a cortical depth of 1300 µm, i.e. below middle to deep layer V; this is in extension to the findings by Chatt and Ebersole (1984) that injections of radioactively labelled penicillin shown to be limited to either of the non-granular layers (III or V) did not induce epileptogenesis.

4.2 *In vitro* columns (cortical cylinders)

The experiments described in the first part of the study had been performed *in vivo*, as the architecture and normal function of neural tissue is best preserved in model systems based on the intact brain. The neocortex was used both because it was the most amenable structure for surgical manipulations, and because its highly complex yet repetitive architecture represents a major challenge to the understanding of the organisation of the brain. The main object was to find the minimal mass required for the initiation and expression of 'epileptogenesis', that is, of paroxysmal discharges ('spikes'), and to evaluate whether this minimal mass coincides in any way with the barrels found in layer IV of the rodent somatosensory cortex. The results from the *in vivo* studies had indicated that the minimal mass of cortical tissue needed for the generation of epileptiform discharges was not only small enough to be investigated *in vitro* (i.e. with a diameter below 500 µm), but also possibly smaller than a single cortical column, i.e. only a fraction of the cortical module accessible *in vivo*. Therefore, an *in vitro* model of the cortical column was developed (referred to as 'cortical cylinder') which had to fulfil the following requirements: it had to be similar in shape to the *in vivo* isolation, and the paroxysmal activity observed *in vitro* had to be comparable to that recorded *in vivo*.

The cortical cylinders developed as an *in vitro* model for the study for the mechanisms involved in the generation of epileptiform spiking are similar in size and shape to the cortical column postulated by several workers, that is, cylindrical and perpendicular to the pial surface; the cylindrical shape had also the advantage of allowing easier access to oxygen and drugs. In fact, the tissue pO_2 generally

decreases at the core of the tissue as the brain slice increases in thickness; it has been reported (Fujii *et al.*, 1982) that the maximum section thickness for viable slices is 430-600 µm. Tissue cylinders permit a better perfusion of the tissue with oxygen and drugs, thus allowing larger tissue diameters to be achieved. Also, pyramidal cell-dense laminae (II/III and V-VI) appear much more translucent than layers containing fewer somata and a higher percentage of myelinated fibres in epi-illuminated sections. Accordingly, transverse cuts could be performed under a binocular microscope, and the locations of both superficial and deep laminae accurately targeted by virtue of their well-defined cytoarchitectural characteristics. Similar to the experiments *in vivo*, it was first attempted to obtain round isolations by retrieving the tissue sections with a glass punch; because individual sections could be obtained from an otherwise intact brain, this would have allowed to perform histological sections of the cortex after the experiment to determine the position of the isolation relative to the barrel field. However, the method was found to be too unreliable for extended studies, because the tissue sections were of poor quality (possibly because the cortex could not be cooled down sufficiently *in situ*, or because the sections were squashed during the insertion of the punch). Therefore, rectangular sections cut on a conventional tissue chopper were used for most experiments.

Contrary to expectations, penicillin could not be used to generate spikes/afterdischarges in the *in vitro* model, although it displayed proconvulsant effects when spiking had been induced by other means, e.g. by superfusing the cylinders with (nominally) magnesium-free ACSF (the concentration of magnesium ions in 'magnesium-free' ACSF prepared with standard reagents is below 1 µM). This is in accordance with studies in human temporal lobe neocortical slices maintained *in vitro* (Hwa *et al.*, 1991), where the $GABA_A$ blocker bicuculline (50 µM) failed to evoke spontaneously occurring epileptiform activity (which could however be elicited by single-shock stimuli) in over 60 slices examined, and in rat neocortical slices, where superfusion with penicillin (3.4 mM) did not result in depolarisation shifts "in contrast to the *in vivo* focus" (Gutnick *et al.*, 1982). Similarly, Mattia *et al.* (1993) observed that bath application of 4-aminopyridine (100 µM) only induced spontaneous epileptiform potentials in 6% of rat neocortical slices. On the other

hand, several other studies, e.g. by Aram *et al.* (1989) report the reliable induction of spontaneous epileptiform bursts with bicuculline 5 - 100 µM, picrotoxin (another $GABA_A$ receptor antagonist) 50 - 100 µM, 4-aminopyridine (4-AP) 20 - 100 µM, tetraethylammonium (TEA) 2 - 4 µM, and carbachol 10 - 100 µM. The induction of spontaneous epileptiform activity is even more easily achieved in immature neocortex, suggesting that the developing brain has a propensity for generating paroxysmal discharges: Lee and Hablitz (1991) elicited prolonged ictal discharges in neocortical slices from young (8 - 15 days of age) animals superfused with 50 µM picrotoxin. This contrasts with the reliable induction of spiking by penicillin observed in adult rats (this study) *in vivo*. A possible explanation for the failure of penicillin to induce spontaneous spiking in the *in vitro* cortical cylinder would be that GABA antagonists may depend on intrinsic cortical activity for the induction of paroxysmal discharges, i.e. that GABA blockade amplifies existent activity, rather than being involved in the generation of paroxysmal discharges. In the usually quiescent *in vitro* preparation, this basal activity (that is, the input *via* thalamic afferents) is lacking, and must be provided experimentally by an electrical or chemical stimulus (Connors, 1984). Therefore, the superfusion with magnesium-free ACSF was chosen as the epileptogenic stimulus, and the assumption of the equivalence of the epileptiform activity recorded in the *in vivo* and *in vitro* models of the isolated cortical column was based on the similarity of the spike/afterdischarge patterns.

Afterdischarges are more complex than the single synchronised population burst ('interictal spike') that is the predominant feature of the *in vivo* epileptiform activity which was evoked by the topical application of penicillin to the intact cortex, but clearly similar to the activity recorded in the isolated cortex *in vivo* both in this study (Fig. 14) and in other studies reported in the literature (e.g. the activity in chronically isolated cat neocortex after the topical application of eserine which was reported by Kristiansen and Courtois in 1949). At the cellular level, they consist of a series of synchronised bursts at about 65 to 85 ms intervals (Miles *et al.*, 1984), where the first burst is longer than later (secondary) bursts, as the latter occur when the cell membrane is shunted by intrinsic synaptic conductances and consequently requires

more current for their activation; the sequence is terminated by a prolonged after-hyperpolarization. Their substrates are the recurrent excitatory synapses which terminate on the dendritic regions of pyramidal cells which generate repetitive calcium spikes in response to the sustained depolarisation provided for example by NMDA receptor activation in the low-magnesium model. The resulting dendritic calcium potentials are thought to initiate somatic bursts which propagate axonally to re-activate non-NMDA receptors at recurrent synapses with a timing that helps to maintain afterdischarge synchrony (Traub *et al.*, 1993).

After it had been shown that the cortical cylinders obtained by cross-sectioning a slab of somatosensory cortex displayed regular, spontaneously recurring spikes/afterdischarges very similar to those previously recorded *in vivo*, a series of progressively smaller cylinders was made until a diameter was reached at which spontaneous spiking could no longer be observed. Theoretical considerations (Fig. 24) show that when a group of adjacent segments is isolated from an array of contiguous modular units, the probability of any one of the segments containing at least one intact module increases gradually from p = 0 to p = 1 as the diameter of the isolation increases from ***d*** (the mean distance between the centres of the modules) to 2.1547 * ***d***. This calculation is based on the assumption that the modules in question are densely packed, and that they are fixed in space; it does not depend on the shape of the isolation (round or square). It essentially indicates that not all isolations of a given diameter, which presumably are placed randomly with regard to compartmental boundaries, should display epileptiform activity if the diameter of the isolation is below 2.16 * ***d***, and that the probability of observing spikes should reach 0 for isolations of diameter ***d*** or less. The range of diameters over which spiking is only occasionally observed becomes even wider if the modules in question are of varying sizes or not arranged in a regular array, as is the case for the barrels of the somatosensory cortex; the range [***d;*** 2.16 * ***d***] therefore represents a conservative estimate.

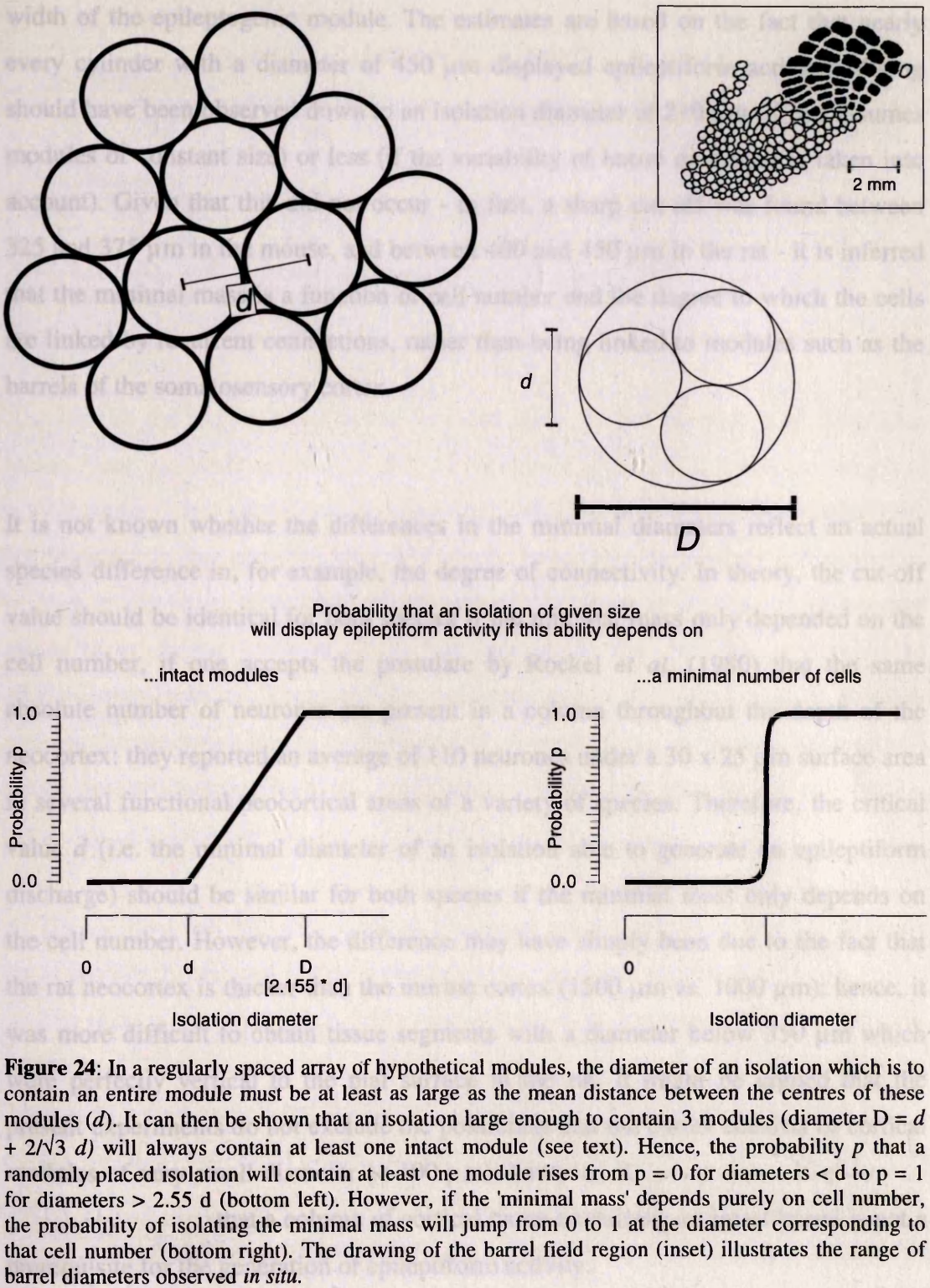


Figure 24: In a regularly spaced array of hypothetical modules, the diameter of an isolation which is to contain an entire module must be at least as large as the mean distance between the centres of these modules (*d*). It can then be shown that an isolation large enough to contain 3 modules (diameter D = *d* + $2/\sqrt{3}$ *d)* will always contain at least one intact module (see text). Hence, the probability *p* that a randomly placed isolation will contain at least one module rises from p = 0 for diameters < d to p = 1 for diameters > 2.55 d (bottom left). However, if the 'minimal mass' depends purely on cell number, the probability of isolating the minimal mass will jump from 0 to 1 at the diameter corresponding to that cell number (bottom right). The drawing of the barrel field region (inset) illustrates the range of barrel diameters observed *in situ.*

While the randomly cross-chopped tissue cylinders cannot provide direct evidence concerning the topology of the isolations within the barrel field cortex, the above calculations allow to place approximate lower and upper bounds on the average

width of the epileptogenic module. The estimates are based on the fact that nearly every cylinder with a diameter of 450 µm displayed epileptiform activity: spiking should have been observed down to an isolation diameter of 210 µm (if one assumes modules of constant size) or less (if the variability of barrel diameters is taken into account). Given that this did not occur - in fact, a sharp cut-off was found between 325 and 375 µm in the mouse, and between 400 and 450 µm in the rat - it is inferred that the minimal mass is a function of cell number and the degree to which the cells are linked by recurrent connections, rather than being linked to modules such as the barrels of the somatosensory cortex.

It is not known whether the differences in the minimal diameters reflect an actual species difference in, for example, the degree of connectivity. In theory, the cut-off value should be identical for both species if the minimal mass only depended on the cell number, if one accepts the postulate by Rockel *et al.* (1980) that the same absolute number of neurones are present in a column throughout the depth of the neocortex: they reported an average of 110 neurones under a 30 x 25 µm surface area in several functional neocortical areas of a variety of species. Therefore, the critical value ***d*** (i.e. the minimal diameter of an isolation able to generate an epileptiform discharge) should be similar for both species if the minimal mass only depends on the cell number. However, the difference may have simply been due to the fact that the rat neocortex is thicker than the murine cortex (1500 µm *vs.* 1000 µm): hence, it was more difficult to obtain tissue segments with a diameter below 350 µm which were perfectly vertical to the pial surface in the rat. It might be argued that the present experiments do not exclude the possibility that the cortex consists of cortical modules of very small diameter (< 100 µm); however, it appears highly likely that a column of cortical tissue containing an intact barrel is not a prerequisite for the generation of epileptiform activity.

		Layer I	Layers II, III	Layer IV	Layer V	Layer VI
Layer boundaries						
	Mouse	0-150 µm	150-350 µm	350-480 µm	480-650 µm	650-1000 µm
	Rat	0-100 µm	100-400 µm	400-600 µm	600-900 µm	900-1500 µm
Cell density ($x1000/mm^3$)						
Rat	Neurones	12	71	88	46	47
	GABA	6	18	23	15	10

Table 1: Average cortical thickness of rat and mouse barrel cortex. Data are taken from Ren *et al.* (1992) for the rat, and from Welker *et al.* (1993) for the mouse.

The idea of the rodent barrel as the minimal mass for the generation of discharges stems from the observation of a unit of functionally co-active neurones congruent with the anatomical barrel, and therefore obviously presumes that these modular units are invariant in size and over time. The results from the present study indicated that, at least as far as the generation of paroxysmal discharges is concerned, no modular parcellation exists; however, this may not hold true for the processing of physiological activity. Therefore, the definition of the functional role of modular units in the neocortex hinges on the question of whether the connections subserving a single computational task are of a fixed nature. For the representation of motor tasks, a number of hypotheses have been listed by Montgomery *et al.* (1992):

a) The cardinal cell hypothesis, which holds that behavioural functions are uniquely and consistently encoded in the discharge activity of single neurones;
b) The mass action hypothesis: behavioural function is represented not in individual neurones, but in the combined activities of groups of neurones; macroscopic states emerge as a continuum from the discrete activities of large groups of neurones
c) The 'parallel distributed processing' hypothesis favoured by the authors, which they define by analogy to parallel processing in computer science, where the solution to a computational problem is represented in the pattern of interconnections and not in the states of the processors themselves.

Similarly, physiological functions would be represented in the patterns of neuronal interactions and not in the activities of single neurones: the response to any input is distributed over a population of neurones, with each neurone contributing a small

amount to the final response. Thus, neurones may be multipotential with respect to their functional roles, and their connections may be viewed as physiological rather than strictly anatomical. Variable weighing of individual connections by inhibition of interneurones or presynaptic inhibition, as well as short-term enhancement of excitatory synapses may allow neuronal activation through specific anatomical linkages in one task and a different set of linkages in a different task. Corroborative experimental evidence for this hypothesis comes from demonstrations of neuronal multipotentiality in both invertebrate (Hooper and Moulins, 1989) and vertebrate (Grillner, 1991) nervous systems.

Also, the long-held belief that the cortical representation of the sensory periphery is 'hard wired' in adults has become less and less tenable; moreover, the potential for change is not restricted to the primary sensory cortical areas, but has also been described for motor areas: when bicuculline is infused into an identified movement representation such as the forelimb zone, subsequent intracortical microstimulation mapping reveals that stimulation in nearby sites that originally elicited vibrissa movements now gives rise to forelimb movements (Jacobs and Donoghue, 1991), leading to the assertion that "inhibitory circuits are critically placed to maintain or readjust the form of cortical motor representations". This is at variance with earlier results from cat visual cortex (Gabor *et al.*, 1979), which had indicated that physiologically defined neuronal populations remain intact in the presence of GABA antagonists (in this case, penicillin). The broadening of receptive fields after the application of picrotoxin (Batuev *et al.*, 1982) or bicuculline also points towards a crucial role of intracortical inhibition (e.g. the strong surround inhibition that characterises barrel neurone receptive fields - Kyriazi and Simons, 1991) in defining the shape of modular units; therefore, the use of GABA antagonists like penicillin is bound to interfere with investigations of cortical columns. Enhancement of excitatory amino acid transmission on the other hand should interfere less with the definition of columns by their inhibitory influences and give a more accurate picture of the true effects of intracortical boundaries on the generation of epileptiform activity. It has to be mentioned that the receptive fields (as the main categorising point of columns) in somatosensory cortex have also been shown to be affected by deep anaesthesia

(Armstrong-James and George, 1988) and by the attentive state of the animal (Hyvärinen *et al.*, 1980); urethane, the anaesthetic used in this study, has also been claimed by Simons *et al.* (1992) to dramatically alter cortical responses to peripheral stimulation, although interictal-like epileptiform events in kindled rats were reported to be relatively unaffected (Haberly and Sutula, 1992).

Finally, boundaries of cortical representation can also be subject to chronic changes. A striking example for re-wiring of the cortical representation comes from the work of Siucinska and Kossut (1994), who demonstrated that the common fur of mystacial pads after vibrissectomy performed in adult rats can be shown to shift its cortical representation to the site of the barrel field over a time course of several weeks; in intact rats, the fur representation is located outside the barrel field (Sharp *et al.*, 1988). In Man, a physiological correlate of the well-known remodelling of the sensory map which can occur after limb amputation (Ramachandran, 1994) was demonstrated by magnetic source imaging techniques (Flor *et al.*, 1995; a similar degree of cortical reorganization was recently demonstrated to occur outside the somatosensory cortex by Seitz *et al.* (1995)). In the somatosensory cortex of adult owl and squirrel monkeys, alterations of input due to nerve section or the repeated imposition of particular stimuli can lead to changes in individual map boundaries (Kaas *et al.*, 1983). These changes occur both acutely and chronically. Before and after the changes, the maps have receptive fields with sharp continuous borders no thicker than one or two cell diameters, despite the fact that the arbours of input axons from the thalamus extend over much wider distances (Landry and Deschenes, 1981). This constitutes a powerful argument against cell bodies as the defining principle of cortical units: if single neurones were units of selection during such alterations, one would not expect continuous sharp boundaries; therefore, module boundaries are determined by an organizatory principle with higher resolution. Whether this principle is based on the shaping influence of inhibitory connections or on more complex forms of computational interaction remains to be elucidated; but the demonstration of a high functional, spatial and temporal variability of the processing patterns found in the physiologically active neocortex suggest that the absence of a

spatially fixed 'modular' structure of the cortex found for the minimal epileptogenic aggregate in this study also extends to the functional level.

4.3 Layer IV is not essential for the generation of epileptiform activity

Next to the question of whether the minimally active epileptogenic combinate represents (or is coincidental with) a cortical column, this study had also aimed at investigating the requirements for the generation of epileptiform activity within a column, i.e. along the vertical extent of the tissue. Results from previous studies had indicated that certain layers within the cortex may be involved in the generation of epileptiform discharges: layer IV in the penicillin model (Holmes and Lockton, 1982) and in the *in vitro* application of glutamate and high potassium (Connors, 1984), and layer II in the strychnine model (Ebersole and Chatt, 1986); based on current source density analysis, Barth *et al.* (1990) postulated a trigger zone in the supragranular layers. The observations led to the hypothesis that a network of intrinsically bursting neurones located in middle (Chagnac-Amitai and Connors, 1989) or upper (Langdon and Sur, 1990) cortical layers initiate synchronous epileptiform activity. This gave rise to a theory on the generation of cortical epileptiform activity which assigns a critical role to the excitatory interneurones (spiny stellate cells - Lund, 1984) within the thalamocortical networks in layer IV of many primary sensory areas (Faingold and Fromm, 1992), and which envisages the initiation to be mediated by the recruitment of reciprocal, recurrent AMPA receptor-mediated excitation between neurones in this layer (Jones and Lambert, 1990). This would be followed by amplifying activation of pyramidal cells near the border of layers III/IV, subsequent hypersynchronised depolarisation of other supragranular pyramidal cells, and finally, activation of large pyramidal cells in the infragranular layer (Ebersole and Chatt, 1986). Obviously, the theory that the mechanisms involved in the generation of ictal activity require the same pathways as physiological activity, i.e. generator cells (intrinsic burst generators) at the starting point of a diverging polysynaptic pathway with feed-forward amplification starting from the layer receiving most of the thalamic input (IV) would have major implications for the explanation of the

mechanism involved in the generation of seizures. There have been reports that kainate injections into various layers of the neocortex *in vivo* did not result in a sensitivity profile similar to that observed with penicillin (Faingold and Fromm, 1992); however, these models also assumed that the columnar pathways (which have been modelled for the primary visual cortex by Patton *et al.* [1992] in a recent study) within the cortex needed to be intact for the epileptiform discharge to be sustained. Indeed, in a recent publication by Jones (1994), the suggestion that epileptiform activity elicited in the entorhinal cortex (the major source of afferent input to the hippocampus) by depletion of magnesium or $GABA_A$ receptor blockade is initiated in layers IV/V and that activity in other cortical layers is synaptically driven by these structures was re-iterated for a cortical region outside the somatosensory area.

It was therefore surprising to find that in sectioned cortical cylinders which contained only layers I to III, or the lower half of layer VI, were still able to generate and sustain spontaneous epileptiform activity, indicating that layer IV, which had clearly been shown to be necessary for the generation of spiking *in vivo* (Lockton and Holmes, 1980, Ebersole and Chatt, 1980), could be excluded from a cortical cylinder *in vitro*. This suggests that the pathways used in the generation of interictal spikes do not coincide with those involved in the normal processing of somatosensory information, on which the previously mentioned models had been based. In theory, the central role played by layer IV of primary somatosensory cortex (which is not only the main target for the extracortical input, but also possesses the highest density of GABA receptors and GABAergic interneurones [Chudler *et al.*, 1988]) *in vivo* may not be present *in vitro*, and under circumstances where increased glutamatergic activity, rather than decreased GABAergic inhibition, present the epileptogenic stimulus. However, this is not substantiated by the results of work performed by Connors (1984) on the laminar sensitivity profile of adult guinea pig somatosensory cortex to the focal application of convulsants *in vitro*. Here, the pressure ejection of bicuculline, high potassium or glutamate led to synchronised bursts which propagated through the whole slice; however, the threshold level of convulsant needed was found to vary systematically across laminae, with a sharp minimum always observed in the middle layers (either layer IV or upper layer V).

In summary, the findings of the present study indicate that the paroxysmal activity did not depend on intact cortical modules (columns) for either its generation or its propagation, as subsections excluding the layer IV barrel structure and containing only layer VI, or only layers I-III, were still able to generate population spikes. The results are in keeping with the assumption that the generation of paroxysmal activity is an inherent property of all neuronal tissue satisfying basic parameters of connectivity (recurrence and/or divergence and sufficient synaptic strength; the requirement for recurrent connections is demonstrated by the observation that the cerebellar cortex, which lacks a recurrent excitatory system, fails to develop interictal spikes in response to topically applied strychnine - Brookhart *et al.,* 1950). This view is supported by theoretical modelling (in their much-cited computer model of an unstable neuronal network, Traub and Wong (1983) showed that a small (100 cells) highly interconnected excitatory network can generate a paroxysmal discharge), and by the observation that hippocampal microcultures, where synaptic connections were present in almost all instances between any two neurones, had a 'minimal mass' of two cells (Segal and Furshpan, 1990; M. Segal reported a further refinement of this technique in 1994, where epileptiform activity was measured in a single hippocampal neurone possessing excitatory autapses). The question of what amount of CNS tissue is needed to produce this epileptiform activity was first addressed by Miles *et al.* (1984), who recorded from progressively smaller pieces of the CA3 region of the hippocampus. The smallest pieces that supported picrotoxin-induced epileptiform activity were estimated to contain about 2000 pyramidal neurones; the connectivity has been estimated at 1 - 5% (Miles and Wong, 1986).

The smallest viable tissue section displaying epileptiform spiking in the present study measured 450 x 450 x 250 µm (based on an average cortical thickness of the mouse of 1000 µm), giving a total volume of 0.051 mm^3. If one accepts (in analogy to the observations made by Prince and Tseng (1993) in subpial isolations of rat cortex) that the shrunken pyramidal neurones lining the cut in Fig. 10 demarcate the zone of damage caused by cutting, this zone appears to extend to a depth of 50 µm (see Fig. 10); therefore, the volume of tissue containing viable cells can be estimated to be around 0.032 mm^3. A volume of 0.032 mm^3 corresponds to a total cell number of

roughly 1200 neurones, according to the quantitative analysis published by Ren *et al.* (1992). The results from the present study therefore indicate that epileptiform spiking can arise from and be sustained by isolated portions of the rodent somatosensory neocortex containing less than 2000 pyramidal cells (to put it into perspective, it has been estimated that large barrels in layer IV of rat somatosensory cortex consist of an average of 2500 neurones). This also suggests that the degree of connectivity of pyramidal cells in deep layers of the neocortex is comparable to that observed in the hippocampus. It should be noted that the estimates of the cell numbers involved in paroxysmal activity assume that a hemisectioned cortical cylinder represents a physiological substrate for neuronal activity. However, some components such as the calcium-mediated dendritic electrogenesis implied in the recurrent afterdischarge activity regarded as 'true epileptiform activity' may no longer be generated in tissue sections lacking these apical dendrites. It could also be argued that sectioning of the apical dendrites would inflict serious damage to most pyramidal cells within the tissue, therefore distorting the response properties of the tissue; however, intracellular recordings by M. Gutnick (personal communication) during a study on active conductances in the dendritic tree have shown that those neurones that survive dendrotomy appear to be physiologically healthy. Also, Prince and Tseng (1993) reported that in contrast to results from axotomised spinal motoneurones, axotomised neurones from undercut cortex showed no signs of damage during intracellular recordings.

The results regarding the physiological substrate for the generation of epileptiform activity which were obtained in the present study also point to a methodological shortcoming inherent in many studies concerning the computational abilities of the neocortex: the working of the cortex has frequently been defined by its reaction to specific somatic afferent inputs. The activity defined by a given experimental approach only represent a snapshot of the cortical activity - it would be necessary to record from a given cell during all physiologically possible inputs to be able to say conclusively in which processes the cell in question takes part. This reductionist approach resulted in impressive and important discoveries on the primary targets of afferent inputs and their organisation, but is of necessity partially or wholly blind to

parallel or subsequent stages of information processing, or purely intracortical processes, and therefore to a (presumably) substantial fraction of computational power of the cortex. As we now know, thalamic axons contribute no more than about 20% of the excitatory synapses in layer IV, the main layer of termination of thalamic axons (White, 1989). Most of the remaining excitatory synapses are from intracortical sources (Douglas and Martin, 1991), and the results from the present study indicate that the intracortical network forms a substantial part of the substrate for neuronal computing.

In summary, although a columnar organisation in the sense that the sequence of afferent informational relay within the cortex often occurs in a strictly vertical fashion has been amply demonstrated with electrophysiological and anatomical means, the 'columnar' organisation of the cortex has to be qualified in two ways: it is columnar mainly with respect to the first stages of afferent sensory input, and the shape of the columns is determined by inhibitory mechanisms (which will vary over time) at least as much as by 'hard' synaptic wiring, implying that boundaries between columns can shift depending on the task performed by that region of the cortex. Both restrictions are an acknowledgement of the high degree of synaptic connectivity, which is also the basic requirement for the generation of paroxysmal discharges. In conclusion, both the results from the present study, which indicate that the excitatory connections within the cortex are sufficiently dense to allow the generation and maintenance of a paroxysmal discharge in tissue segments containing only a fraction of the vertical extent of the 'cortical column', and data from other studies on the influence of inhibitory connections point to a view of the cortex as a highly interconnected excitatory matrix shaped by inhibitory influences which can change over time, rather than a static array of hard-wired, parallel circuits.

4.4 Pertinence to human epilepsy

The surgical treatment of refractory epilepsy devised by Frank Morrell and co-workers was based mainly on evidence obtained in animal models of epilepsy

involving GABA blockade. However, it is becoming increasingly clear that GABAergic mechanisms play a more differentiated role in epileptic neural networks. For example, it has been suggested (Lytton and Sejnowski, 1991) that in absence epilepsy, a form of human epilepsy in which GABAergic inhibition remains intact throughout the seizure, it is the firing of inhibitory interneurones that may be the main mechanism of phase-locking. The limited range of axonal arborization of most inhibitory interneurones (generally < 1 mm) could limit the spatial extent of this mechanism of synchronisation to a single 'column', thereby providing a means of synchronising information processing without the risk of this synchrony spreading to adjacent cortical areas. This again postulates that cortical 'columns' (i.e. co-active units) are shaped by their inhibitory input, therefore complicating the search for a correlation between columns and penicillin-triggered epileptiform activity. What are the conclusions that can be drawn from the present study? The positive results obtained with the 'multiple subpial transection' technique were probably not due to the fact that the remaining brain tissue strips were below the absolute critical mass needed for the generation of epileptic activity. The procedure either prevented the spread of epileptic activity from a focus which was still active (Albowitz and Kuhnt (1995) have recently shown that the spread of epileptiform activity in neocortical slices is quite effectively blocked by cuts through the supragranular layers), or else - since no epileptiform activity was recorded from the transected region - the underlying disturbance was more subtle and disseminated in these patients than is the case with chemically induced epileptiform activity in the experimental setting, and probably required the synchronisation of abnormal activity over a large mass of contiguous brain tissue. The therapeutic success obtained by Morrell and co-workers is not in itself sufficient evidence for the existence of cortical modules responsible for the generation of seizures; rather, it illustrates the difference between ictogenesis (the generation of paroxysmal activity) and epilepsy (the tendency of a brain to suffer recurrent seizures): while the modification of almost any parameter of neuronal function, such as connectivity, ionic conductances, the extracellular environment, or synaptic divergence, can be used to induce epileptiform activity, little is known about the actual physiopathological changes which cause seizures in the patient.

The preparation developed in this study, the 'cortical cylinder', does not represent the 'basic modular unit' proposed by several workers, but it has several appealing features: it preserves the basic radial organisation of the cortex in the smallest possible preparation, and it appeared to be a suitable model for further pharmacological investigations into the generation of epileptiform discharges. It was therefore also used in some of the experiments presented in the second part of this study, which concerned itself with the pharmacological aspects of epileptogenesis, rather than its physiology, and which centred on the role played by adenosine in epilepsy: purinergic transmission has been the subject of active research ever since the first demonstration of adenosine's cardinal role as inhibitory neuromodulator, and adenosine agonists may even have therapeutic potential as novel antiepileptic drugs.

5 INTRODUCTION TO PART TWO

The epilepsies are characterised by the periodic occurrence of abnormal neuronal discharges which can present as one or more of a wide variety of seizure types which are classified according to their localisation, their onset, and the degree of the concomitant impairment or loss of consciousness. The existing antiepileptic drugs (AED) reflect this broad spectrum, with some drugs reliably controlling seizures of one type while being ineffective or even proconvulsant in others. Despite their multiple and often overlapping effects, antiepileptic drugs can be broadly grouped into two major classes: those which decrease excitation, and those which potentiate inhibitory mechanisms. Examples for the former would include activity-dependent sodium channel blockers such as phenytoin and valproate, and calcium channel blockers like ethosuximide. The latter include drugs which enhance GABAergic transmission; these include the benzodiazepines and barbiturates, and a group of four recently introduced agents, felbamate, gabapentin, lamotrigine and vigabatrin (Kälviäinen *et al.*, 1993). Despite the existence of these effective anticonvulsants which may satisfactorily control seizures in the majority of individuals with epilepsy, the number of intractable cases still exceeds the number of people suffering from brain tumours, multiple sclerosis, amyotrophic lateral sclerosis and many other 'unsolved' neurological illnesses (Kurtzke, 1982). The need for more effective, less toxic antiepileptic drugs is therefore as great as ever, but advances have not been as quick as expected. One problem, discussed in Part One, lies in the gap between our understanding of the mechanisms of ictogenesis (i.e. the precise cellular changes which occur during seizures) and epileptogenesis (i.e. the pathophysiological changes which predispose a patient to recurrent epileptic fits). Another difficulty lies in transforming advances in the laboratory into clinical therapies. Several promising developments, such as the NMDA antagonists, had to be halted due to toxicity, side-effects, pharmacodynamic problems (inability to cross the blood brain barrier, poor solubility) or other reasons; yet, the development of antiepileptic drugs based on novel mechanisms of action remains one of the most hopeful avenues of research. One example would be the use of the purinergic modulation of neuronal excitability. The following chapters report some studies on the "endogenous anticonvulsant" adenosine and discuss its antiepileptic potential.

5.1 Adenosine: a neuromodulatory purine

Since the first description by Sattin and Rall (1970) of the membrane-bound receptor-mediated effects of the endogenous purine adenosine on adenylyl cyclase in guinea pig cerebral cortex, a multitude of other effects mediated by the nucleoside have been described. Adenosine has been shown to inhibit lipolysis (Londos *et al.*, 1978), to affect cardiac rate and contractility, modulate myometrial and vascular smooth muscle tone, renal vasoconstriction and white blood cell function (Olsson and Pearson, 1990; Matias *et al.*, 1991), to cause vasodilation in coronary (Berne, 1980) and cerebral blood vessels, inhibit platelet aggregation (Haslam and Rosson, 1975), to exert potent bronchoconstriction (Driver *et al.*, 1993), and neuromodulatory actions at both peripheral and central sites in the nervous system (see Nicoll *et al.*, 1990; Stone, 1991; Stone and Simmons, 1991): accumulation of extracellular adenosine has been suggested to play a role in inhibiting the brainstem and basal forebrain cholinergic neurones implied in EEG arousal and wakefulness (Rainnie *et al.*, 1994).

5.1.1 *Origin*

Adenosine is a metabolic breakdown product of nucleotide (i.e., 5'-AMP and cAMP - Gereau and Conn, 1994) hydrolysis. Its tissue levels are governed mainly by the state of metabolic activity. The rate-limiting factor of intracellular adenosine production is the limited availability of AMP; as the cytosolic AMP concentration is an order of magnitude below the Km of 5'-nucleotidase, the catalytic rate is directly proportional to substrate concentration. Adenosine levels therefore rise concomitantly with any mismatch between energy supply and demand; as the initial concentration of ATP is approximately 50 times higher than that of AMP, a small percentage fall in ATP concentration can lead to a large proportional rise in AMP, which is then hydrolysed via cytosolic 5'-nucleotidases (Meghji, 1993). A second pathway, which involves the hydrolysis of S-adenosyl-L-homocysteine, is oxygen insensitive and is therefore not accelerated during hypoxia and ischemia (Lloyd *et al.*, 1988).

Adenosine is also formed extracellularly through the sequential hydrolysis of ATP by (almost exclusively glia-bound [Kreutzberg *et al.*, 1986]) ecto-5'-nucleotidases. ATP is stored and co-released with several excitatory transmitters, e.g. noradrenaline in postganglionic sympathetic neurones (Burnstock, 1986), acetylcholine, or glutamate at CNS terminals (Zimmermann, 1994a). Both ATP and ADP exert a feed-forward inhibition on the 5'-ectonucleotidase: this leads to a delayed production of adenosine from released ATP, which only reaches significant levels once the concentrations of ATP and ADP have fallen substantially (Meghji, 1993). Adenosine is then either catabolised by adenosine deaminase to the relatively inactive compound inosine, or transported back into the cell by a bi-directional nucleoside transporter (Geiger and Fyda, 1991) where it is inactivated either by adenosine deaminase or by adenosine kinase (see Fig. 25).

5.1.2 *Extracellular levels*

Unlike most other neurotransmitters known to date, intracellular adenosine is not released by exocytosis of synaptic vesicles, but by direct diffusion across the cell membrane. Adenosine crosses cell membranes by a combination of simple and facilitated diffusion, that is, it moves down a concentration gradient. Although the cytosolic free adenosine concentration was once assumed to lie within the low micromolar range (Prince and Stevens [1992] estimated the interstitial concentration to be about 10 μM), this probably represents an overestimate due to the invasive assay procedures; more recent estimates give a figure of around 80 nM under normoxic conditions, rising to around 2 μM during hypoxia. The interstitial concentration of adenosine of intracellular origin will vary over a similar range, although at lower absolute values, as three intracellular metabolic processes (deamination, rephosphorylation, and sequestration through conversion into S-adenosyl-L-homocysteine) will compete with the adenosine transport system. Under resting conditions, the larger fraction of extracellular adenosine originates from the hydrolysis of ATP. Unfortunately, the direct assessment of the extracellular concentration, which in the case of adenosine (which exerts its actions mainly via cell surface adenosine receptors) represents the physiologically active concentration, is

almost impossible to determine due to the many factors regulating the concentration in the interstitial compartment. Most estimates in the literature range from a few hundred nanomolar down to a median value well under 100 nM under resting conditions; similar levels (190 nM) have been found to be present in whole venous blood samples from human patients (Guieu *et al.*, 1994).

However, the extracellular adenosine concentration may become considerably elevated - either as a by-product of increased intracellular metabolic activity, or as the result of activity-dependent ATP release - during periods of ischemia, intense neuronal activity or physical brain injury. This response to factors that increase cellular metabolic demand relative to available metabolites leads to the enhancement of a voltage-dependent, non-GABAergic chloride conductance (Mager *et al.*, 1990), and a hyperpolarization of pyramidal cells via a G-protein mediated increase of a postsynaptic K^+ conductance (Thompson *et al.*, 1992, Pan *et al.*, 1994) shared by $GABA_B$ receptors and 5-HT1a receptors (McCormick and Williamson, 1989). A presynaptic reduction in excitatory (but not inhibitory - Lambert and Teyler, 1991) transmitter release (possibly via the inhibition of an axon terminal ω-conotoxin-sensitive Ca^{2+} current) has been described in the hippocampus (but cf. Ulrich and Huguenard, 1995). In addition, adenosine has several other non-neuronal effects, like vasodilation (Linden, 1991) and the increase of glucose uptake, that also serve to adapt to excessive metabolic demand, and has therefore been termed a "retaliatory metabolite" (Newby *et al.*, 1990) mediating a metabolic negative feedback.

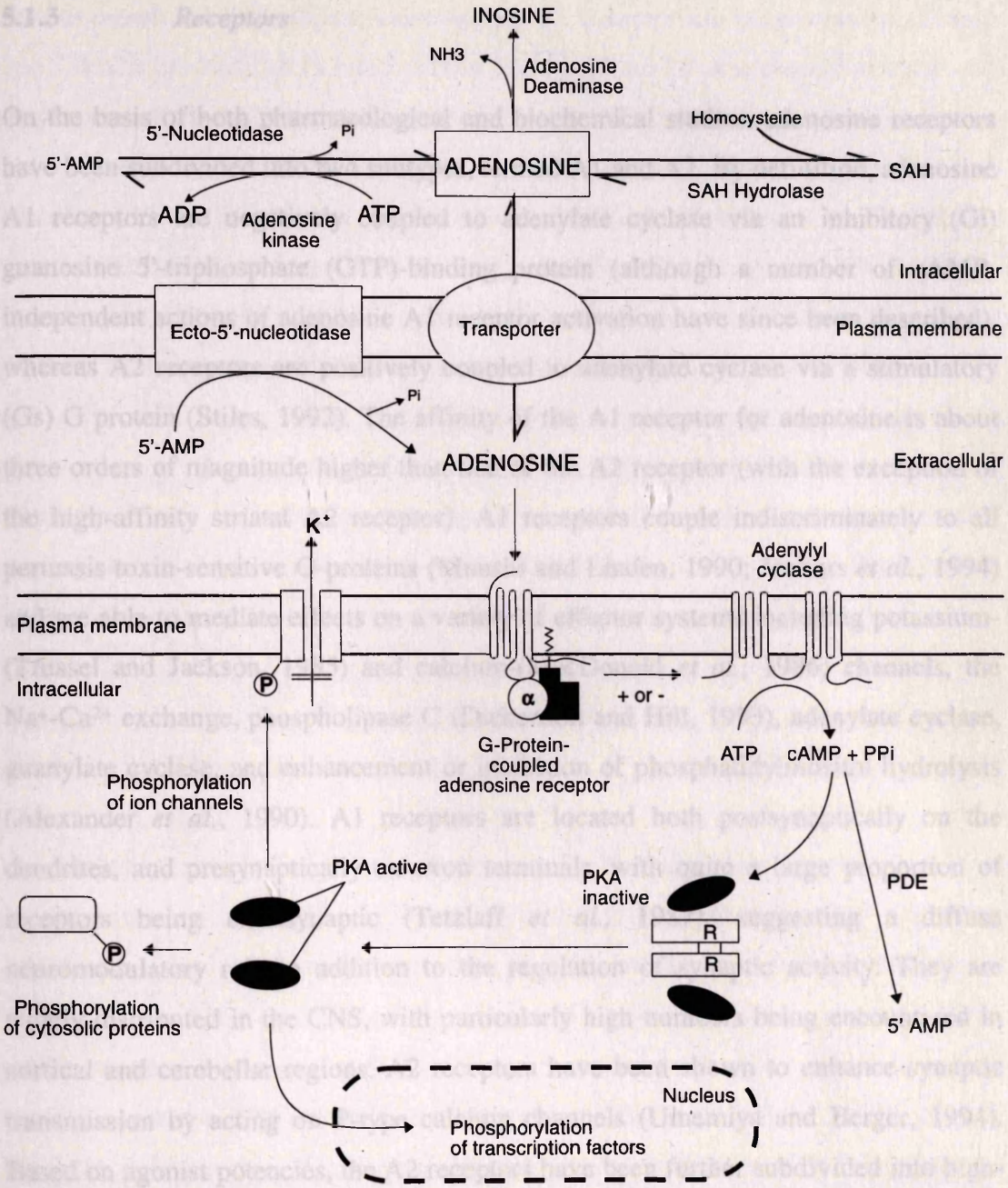


Figure 25: Adenosine: origin, metabolism and effector pathways (adapted from Murray *et al.*, 1993 [metabolism] and Zimmermann, 1994 [mechanism of action]). SAH, S-adenosyl-L-homocysteine; PKA, protein kinase A; PDE, Ca^{2+}/calmodulin-dependent phosphodiesterase. Adenosine is produced either intracellularly by one of the pathways indicated at the top, or extracellularly through the sequential hydrolysis of ATP, and mediates its effects through adenylyl-cyclase-linked G-protein-coupled receptors by either increasing (A2) or decreasing (A1) cAMP levels.

5.1.3 *Receptors*

On the basis of both pharmacological and biochemical studies, adenosine receptors have been subdivided into two subtypes, termed A1 and A2. By definition, adenosine A1 receptors are negatively coupled to adenylate cyclase via an inhibitory (Gi) guanosine 5'-triphosphate (GTP)-binding protein (although a number of cAMP-independent actions of adenosine A1 receptor activation have since been described), whereas A2 receptors are positively coupled to adenylate cyclase via a stimulatory (Gs) G protein (Stiles, 1992). The affinity of the A1 receptor for adenosine is about three orders of magnitude higher than that of the A2 receptor (with the exception of the high-affinity striatal A2 receptor). A1 receptors couple indiscriminately to all pertussis toxin-sensitive G-proteins (Munshi and Linden, 1990; Jockers *et al.*, 1994) and are able to mediate effects on a variety of effector systems including potassium-(Trussel and Jackson, 1985) and calcium-(MacDonald *et al.*, 1986) channels, the Na^{+}-Ca^{2+} exchange, phospholipase C (Dickenson and Hill, 1993), adenylate cyclase, guanylate cyclase, and enhancement or inhibition of phosphatidylinositol hydrolysis (Alexander *et al.*, 1990). A1 receptors are located both postsynaptically on the dendrites, and presynaptically on axon terminals, with quite a large proportion of receptors being extrasynaptic (Tetzlaff *et al.*, 1987), suggesting a diffuse neuromodulatory role in addition to the regulation of synaptic activity. They are widely distributed in the CNS, with particularly high numbers being encountered in cortical and cerebellar regions. A2 receptors have been shown to enhance synaptic transmission by acting on P-type calcium channels (Umemiya and Berger, 1994). Based on agonist potencies, the A2 receptors have been further subdivided into high-affinity (striatal A2a, localised to dopamine-rich regions of the brain) and low-affinity (peripheral A2b) receptors (Bruns *et al.*, 1986); both have been successfully cloned (Libert *et al.*, 1991; Maenhaut *et al.*, 1990), and there is evidence for two A2-like genes on the human genome.

The existence of an A3 receptor (Zhou *et al.*, 1992) which binds the ^{125}I-radiolabelled A1-selective agonist N6-2-(4-amino-3-iodophenyl)-ethyladenosine (APNEA), but not the xanthine antagonists DPCPX (1,3-dipropyl-8-cyclopentyl-

xanthine) and XAC (xanthine amine congener), has recently been reported (Fozard and Carruthers, 1993). It is more closely related to the A1 than the A2 receptor and displays marked species variability, with the rat A3 receptor amino acid sequence showing only 74% sequence homology with the receptors from the other species that have been cloned (sheep and human; Linden, 1994). Its importance lays in the fact that A3 receptors can trigger mast cell degranulation (Doyle *et al.*, 1994) and may therefore be involved in both the bronchoconstrictor action of adenosine, and the antiasthmatic properties of theophylline and related compounds.

Finally, a fourth adenosine binding site (the so-called P-site) located intracellularly on the catalytic unit of adenylate cyclase exists which inhibits cAMP formation at supraphysiological adenosine concentrations (Londos *et al.*, 1983).

5.1.4 *Binding mechanics*

Adenosine receptor affinity for agonists is strongly dependent on the state of G protein interaction. The A1 receptor exists in two affinity states for agonist binding, which correspond to the receptors coupled and uncoupled to a G-protein, respectively (Lohse *et al.*, 1984); agonist binding promotes the conversion from a high- to a low-affinity state (Casado *et al.*, 1991). A1 receptors couple largely indiscriminately to pertussis-sensitive GTP binding proteins (but cf. Fredholm *et al.*, 1989), and the coupling between the A1 receptor and the G-protein appears to be very tight: in solubilised receptors, 80% appear to be in the high-affinity form; addition of guanine nucleotides (GTP or its stable analogue 5'-guanylylimidodiphosphate (Gpp(NH)p)) partially shifts the agonist-specific high-affinity state of the receptor (reflecting the ternary complex between agonist, receptor and G-protein) into the low-affinity state (Ströher *et al.*, 1989), which represents the receptor dissociated from the G-protein. In slices, a large percentage of the receptors appears to be coupled to G-proteins, although the intracellular concentrations of GTP present in intact cells (around 1 mM - Fredholm and Jonzon, 1988) should suffice to convert a proportion of receptors to the low-affinity state. Estimates for the proportion of A1 receptors in the high-affinity

state range from 33% (Green and Stiles, 1986) in rat cortical slices to 81% in calf brain membranes (Garritsen *et al.*, 1990); in human hippocampal slices, binding of [^{3}H]PIA to the low affinity state could not be reliably detected in the absence of GTP (Ulas *et al.*, 1993). Following chronic caffeine treatment, a higher percentage of receptors is in the high-affinity form, but at the same time the ternary complex is more sensitive to GTP; this results in an increased efficiency of adenosine (Green and Stiles, 1986) either as a consequence of changes in the adenosine receptor or Gi protein quantity or functionality. More recent reports (Ramkumar *et al.*, 1988) suggest an increase in Gi proteins (evidenced by increased pertussis toxin-catalysed [^{32}P]NAD incorporation into the αi subunit).

The high adenosine occupancy of the A1 receptor has also led to continuing debate about the existence of two affinity states for antagonist binding. The increase in Bmax of DPCPX binding after the addition of Gpp(NH)p (Klotz *et al.*, 1990) had originally been taken to represent preferential binding of antagonists to the uncoupled state of the receptor. However, the effect may be due to the dissociation of endogenous adenosine from receptor sites (Parkinson and Fredholm, 1992); in these binding studies performed in rat striatal slices, endogenous adenosine was found to be continuously present despite the addition of adenosine deaminase (2 IU/ml). Mg^{2+} was found to decrease the dissociation rate of bound endogenous adenosine from A1 receptors, thus limiting the access of DPCPX to the binding site; this may also explain the apparent inhibitory effects of Mg^{2+} on antagonist binding (Reddington *et al.*, 1989a). Additional evidence for an indiscriminate binding of adenosine A1 antagonists to both states of the receptor came from studies showing that antagonists can bind to both coupled receptor-G protein complexes and small uncoupled receptors (Reddington *et al.*, 1989b). Furthermore, the concept that adenosine antagonists bind preferentially to uncoupled receptors (Leung *et al.*, 1990), implying "negative efficacy", that is the ability to trigger inverse agonist responses even in the absence of agonists (Klotz *et al.*, 1990), may be based on the erroneous assumption that membrane-bound and solubilised receptor preparations are devoid of endogenous adenosine. In fact, Schiemann *et al.* (1990) showed that the increase in Bmax for DPCPX observed in smooth muscle membrane preparations after the

addition of GTP analogues was due to residual adenosine trapped in inside-out vesicles (and therefore inaccessible to adenosine deaminase) in concentrations of up to 400 nM; and Prater *et al.* (1992) demonstrated the continued presence of a 'cryptic' adenosine pool in CHAPS-solubilised receptor preparations by radio-immuno assay which was inaccessible to degradation by adenosine deaminase, showing that a Mg^{2+}-dependent GTPγS-induced increase in antagonist binding to solubilised receptors is also due to the unmasking of binding sites occupied by contamination with residual adenosine. Despite these observations, reverse intrinsic activity of antagonists on G protein-coupled receptors continues to be a subject of speculation (Schütz and Freissmuth, 1992).

5.1.5 *Effects in vivo*

In the experimental animal, adenosine agonists possess psychomotor depressant effects. They have been shown to produce a marked decrease in locomotor activity in rodents (Heffner *et al.*, 1989), a decrease in schedule-controlled behaviour of rodents and monkeys, and catalepsy when given intracerebroventricularly at higher doses (Ferre *et al.*, 1991). Adenosine has also demonstrated anticonvulsant properties in audiogenic (Maitre *et al.*, 1974), chemically induced (Murray and Szot, 1985), and kindling models of epilepsy: adenosine, adenosine agonists and the adenosine uptake blocker papaverine reduce the severity and duration of amygdala-kindled seizures (Albertson *et al.*, 1983). These results suggest that endogenous adenosine is an important regulator of the spread and duration of seizures, and adenosine analogues may represent a novel and effective treatment for epilepsy (Chin, 1989).

Adenosine antagonists on the other hand, which include the dietary methylxanthines caffeine and theophylline, not only inhibit adenosine analogue psychomotor depressant effects, but, when administered alone, have psychomotor stimulant effects: they increase locomotion in mice (Seale *et al.*, 1986), and caffeine has been found clinically to increase anxiety in healthy volunteers (Loke *et al.*, 1985). These central effects of methylxanthines, as well as their multiple peripheral effects

(lipolysis in adipose tissue, increased renal blood flow, increased heart rate) have been proposed to be mainly due to antagonism of endogenous adenosine (Snyder, 1985), a suggestion based on the assumption that adenosine exerts a tonic inhibitory action referred to as "purinergic tone".

5.1.6 *Adenosine effects: A1 or A2 mediated?*

Originally, the central stimulant actions of methylxanthines were thought to be related exclusively to their A1 antagonist properties, as their activity correlated well with their capacity to compete with radioligand binding to A1 receptors; however, more recent data suggest that the A2 receptor subtype may also be responsible for these effects (Nikodijevic *et al.,* 1990), confirming the assumption of Spealman and Coffin (1986) that most of the behavioural effects of adenosine agonists (Phillis, 1990) and low-dosed antagonists are likely mediated by the A2 receptor, while highly selective A1 antagonist compounds like KFM 19 (a potential cognitive enhancer - Schingnitz *et al.,* 1991) and DPCPX (see below) exert relatively minor effects in the central nervous system.

In fact, the potencies of different methylxanthines to produce an increase in schedule-controlled behaviour in monkeys correlate better with their affinities for A2 receptors. Also, caffeine induces a c-fos expression in mouse brain which cannot be antagonised by an A1 adenosine analogue, but is blocked by a potent non-specific A2 adenosine analogue (Nakajima *et al.,* 1989). Additionally, a postsynaptic A2-induced decrease in agonist binding to dopamine D2 receptors may explain some of the stimulatory effects of low doses of caffeine (Fredholm *et al.,* 1993). In support of this theory, Ferré *et al.* (1993) have described a potent A2/dopamine D2 receptor interaction after the application of low-dosed xanthines, with A2 receptor activation inhibiting the effects of dopamine D2 receptor stimulation (although some doubts have now been raised as to the relevance of these effects). Interestingly, the effects of low doses of caffeine (10 mg/kg) in rats generalise not only to other adenosine antagonist xanthines, but also to dopamine stimulating agents (Mumford and

Holtzman, 1991) which are known to act mainly in striatal regions with a high density of A2 receptors. High-affinity A2a receptors are restricted mainly to the basal ganglia, although A2a mRNA has been shown to be expressed in the hippocampus and cerebral cortex as well (Cunha *et al.*, 1994). Therefore, and despite the lower affinity of A2 receptors, low doses of methylxanthines *in vivo* appear to act mainly via A2 receptors, and most of the behavioural effects of low-dosed adenosine agonists and antagonists can therefore be explained by actions at A2 receptors. The convulsant properties of adenosine antagonists on the other hand appear to be mediated by A1 receptor blockade.

5.2 Adenosine antagonists

The acute administration of methylxanthines at high doses can cause severe CNS side-effects: Caffeine can produce seizures in rats at high doses (200-400 mg/kg) (Chu, 1981), reduces the threshold for the initial (tonic) phase of electroconvulsive seizures at 100 mg/kg in mice (Chen *et al.*, 1968), and prolongs post-stimulation afterdischarges in the amygdaloid kindling model of partial seizures (50 mg/kg) (Albertson *et al.*, 1983); the acute intraperitoneal administration of caffeine 20-40 mg/kg was found to be associated with increased rates of seizure upon challenge with pentylenetetrazole (File *et al.*, 1988); and chronically ingested caffeine (20-100 mg/kg/day) increases the percentage of rats having convulsions in response to low-dosed PTZ (File *et al.*, 1988; Cutrufo *et al.*, 1992). While paraxanthine is its main metabolite, caffeine is also partly metabolised to theophylline (Tang-Liu *et al.*, 1983); in rats, a dose of 25 mg/kg i.p. can be estimated to correspond to a peak brain concentration of 60 µM. In the maximal electroshock seizure test in mice (a model for human grand mal seizures), aminophylline, but not 8-(p-sulfophenyl)-theophylline (a theophylline derivative unable to cross the blood-brain barrier) in the dose of 238 µmol/kg, impaired the protective action of several anticonvulsants, indicating a central site of action of methylxanthines (Borowicz *et al.*, 1993). Acute intraperitoneal administration of theophylline base up to 280 µM resulted in increased activity and slight toxic side-effects in rats (Thithapandha *et al.*, 1972). Oral doses of theophylline up to 100 mg/kg stimulated locomotor activity in rats,

although no wet dog shakes or other behaviours characteristic of limbic seizures could be observed in the absence of additional stimulation with subcutaneously administered kainic acid (Ault *et al.*, 1987). Similarly, aminophylline at 100 mg/kg i.p. did not affect motor activity significantly or alter the EEG in chronic amygdala-kindled rats (Handforth and Treiman, 1994). Acute intravenous infusions of theophylline and some of its derivatives at higher doses invariably lead to convulsions; the ED_{50} is around 175 mg/kg in the rat (Norenberg and Chu, 1977), and decreases with increasing phylogenetic brain complexity. In man, as in primates, side-effects are commonly, although not always, observed at concentrations above 20 µg/ml, and seizures are most often seen at theophylline serum levels of more than 50 µg/ml (Zwillich *et al.*, 1975). Theophylline-induced seizures have been reported in patients with no previous history of epilepsy and no gross brain pathology (Mori *et al.*, 1992); they are often initially focal or unilateral (Nakada *et al.*, 1983), and respond poorly to antiepileptic medication with intravenous diazepam, phenytoin and phenobarbital (Zwillich *et al.*, 1975); it is worth noting, however, that methylxanthines have also been deliberately used in an attempt to increase the therapeutic effect of electroconvulsive shock (ECS): Swartz and Lewis (1991) report that in patients undergoing electroconvulsive shock (ECS) treatment, premedication with theophylline significantly lengthened seizure duration without causing adverse effects. *In vitro*, theophylline-induced interictal spiking can be routinely achieved in the hippocampal slice (Ault and Wang, 1986).

Since the discovery that the methylxanthines caffeine and theophylline act partly via antagonism of endogenous adenosine at A1 receptors, a large number of highly selective synthetic analogues have been developed by introducing substitutions at the N-1, N-3, N-7 and C-8 positions of xanthines. General problems with some of these compounds are poor aqueous solubilities and variable activities as cyclic nucleotide phosphodiesterase inhibitors; the problems are avoided with polar compounds like BWA-1433U and 1,3-dipropyl-8-sulfophenylxanthine, which do not cross the cell membrane. Substitutions at the methylxanthine structure led to the development of compounds with increasing selectivity and potency, such as 8-phenyltheophylline (Collis *et al.*, 1985) or 8-cyclopentyltheophylline (CPT). The most potent and

selective A1 agonist resulted from the combination of a 1,3-dipropyl-substitution and an 8-cyclopentyl-substitution: 8-cyclopentyl-1,3-dipropylxanthine, or DPCPX (Bruns *et al.*, 1987). DPCPX binds to rat brain membrane A1 receptors with a Kd of 0.42 nM and possesses a 700-fold selectivity for the A1 over the A2 receptor (Lohse *et al.*, 1987).

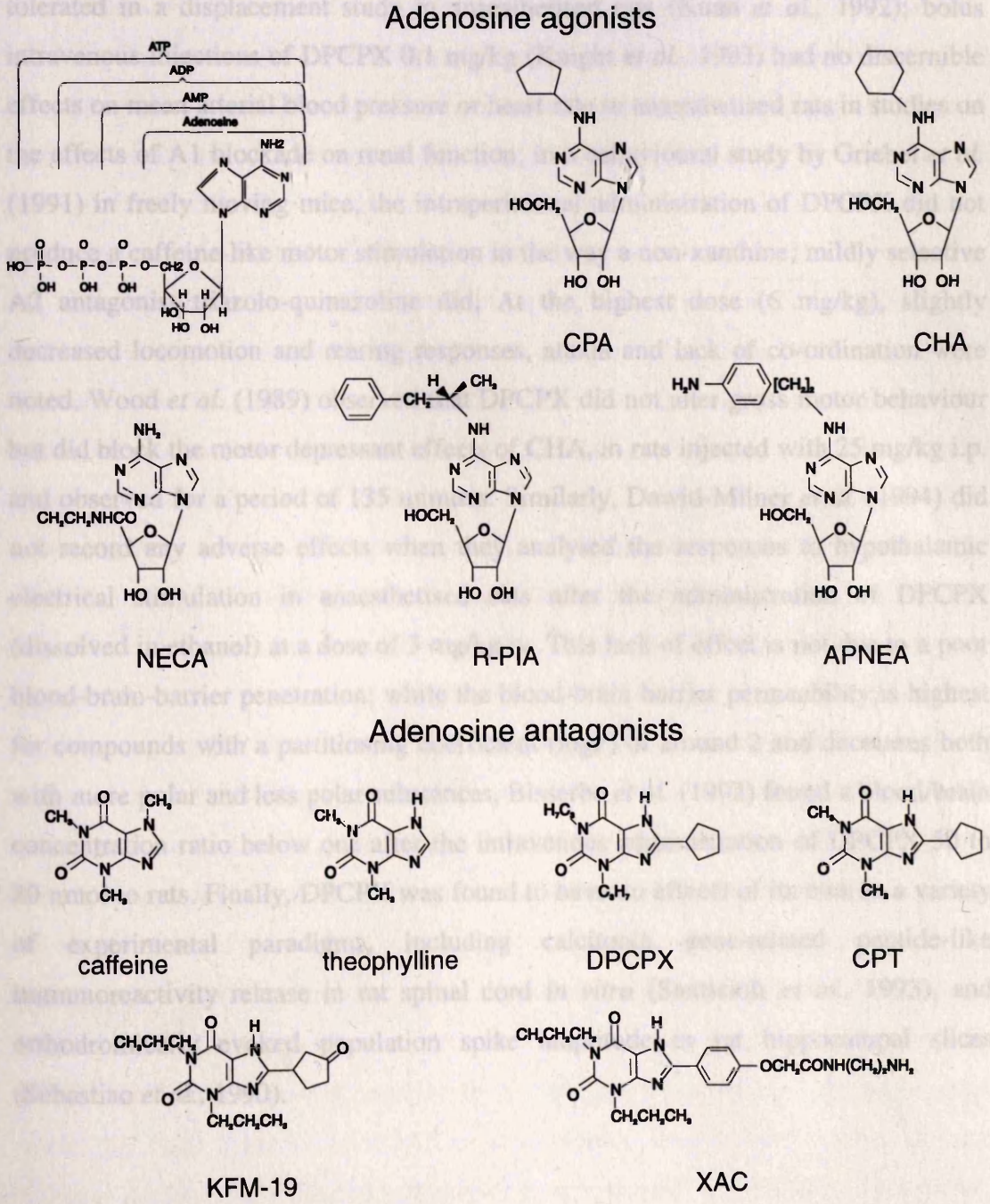


Figure 26: Structure of adenosine and adenosine agonists, and of alkylxanthine adenosine receptor antagonists used (or mentioned) in this study

However, despite its high affinity for the adenosine A1 receptor, DPCPX has few effects when administered in uninjured and unstimulated tissue: in organotypic rat hippocampal slice cultures, Thompson *et al.* (1992) reported that bath application of DPCPX 200 nM did not affect either the resting membrane potential or the amplitude of synaptic responses. It also concords with the results of numerous *in vivo* studies: the intravenous infusion of DPCPX 100 µg/kg/min over several hours was well tolerated in a displacement study in anaesthetised rats (Kuan *et al.,* 1992); bolus intravenous injections of DPCPX 0.1 mg/kg (Knight *et al.,* 1993) had no discernible effects on mean arterial blood pressure or heart rate in anaesthetised rats in studies on the effects of A1 blockade on renal function; in a behavioural study by Griebel *et al.* (1991) in freely moving mice, the intraperitoneal administration of DPCPX did not produce a caffeine-like motor stimulation in the way a non-xanthine, mildly selective A2 antagonist triazolo-quinazoline did. At the highest dose (6 mg/kg), slightly decreased locomotion and rearing responses, ataxia and lack of co-ordination were noted. Wood *et al.* (1989) observed that DPCPX did not alter gross motor behaviour but did block the motor depressant effects of CHA, in rats injected with 25 mg/kg i.p. and observed for a period of 135 minutes. Similarly, Dawid-Milner *et al.* (1994) did not record any adverse effects when they analysed the responses to hypothalamic electrical stimulation in anaesthetised cats after the administration of DPCPX (dissolved in ethanol) at a dose of 3 mg/kg iv. This lack of effect is not due to a poor blood-brain-barrier penetration; while the blood-brain barrier permeability is highest for compounds with a partitioning coefficient (logP) of around 2 and decreases both with more polar and less polar substances, Bisserbe *et al.* (1992) found a blood/brain concentration ratio below one after the intravenous administration of DPCPX 50 to 80 nmol to rats. Finally, DPCPX was found to have no effects of its own in a variety of experimental paradigms, including calcitonin gene-related peptide-like immunoreactivity release in rat spinal cord *in vitro* (Santicioli *et al.,* 1993), and orthodromically evoked population spike amplitude in rat hippocampal slices (Sebastiao *et al.,* 1990).

5.3 Object of study

The (mainly) inhibitory effects of adenosine on cell excitability and metabolism have stimulated the search for therapeutic indications of compounds which mimic its physiological effects. Research has concentrated mainly on cardioprotective (Engler, 1994) and neuroprotective (MacGregor *et al.*, 1993; Schubert and Kreutzberg, 1993) mechanisms with a view to limiting the tissue damage ('reperfusion injury') observed after myocardial or cerebral ischemia. Adenosine agonists have also been suggested to be potentially useful antiepileptic compounds, prompted partly by the marked inhibitory effects of adenosine on neuronal excitability, but mainly by the proconvulsant effects of compounds (such as the methylxanthines) known to be adenosine receptor antagonist. However, the mechanisms involved in the generation of methylxanthine-induced seizures are not fully understood. Originally, the discovery of highly selective antagonists such as the alkylxanthine DPCPX had led to the hope that the antagonism of endogenous adenosine may be unambiguously identified as the cause for methylxanthine-induced convulsions. However, results from several recent studies have rather confused the picture. In particular, the administration of relatively high doses of DPCPX has been shown to produce little more than slight behavioural disturbances in intact animals, while reliably inhibiting the effects of exogenous adenosine receptor agonists (see above). Also, several reports had indicated that transient blockade of A1 receptors *in vitro* resulted in persistent epileptiform activity: in 1989, Alzheimer *et al.* reported that "a transient suppression of an inhibitory purinergic tonus by DPCPX leads to sustained interictal-like epileptiform activity arising in area CA3 [in guinea pig hippocampal slices]. Once induced, the spontaneous burst discharges were apparently irreversible within the observation period, even after prolonged washout (2-3 hr) in normal solution. In contrast, the hyperpolarising action of exogenous adenosine, which was substantially reduced by DPCPX, recovered within 30-60 min of drug washout, indicating that DPCPX was not irreversibly bound to the A1 receptor"; and in 1993, the same group stated that "our data do not allow us to determine how and to what extent the different A1 receptor-mediated pathways [i.e. activation of a potassium conductance, decrease of a calcium conductance, and inhibition of adenylyl cyclase] contribute to the long-term effects observed after a transient removal of endogenous adenosinergic

inhibition. Further research will be required to explain the delicate relationship between adenosine tonus and neuronal excitability in terms of its molecular machinery". This poses some important questions relating to the presence or absence of the inhibitory purinergic tonus postulated by several groups, and to the role of adenosine in epilepsy. Therefore, the effects of DPCPX and two other structurally related xanthines on epileptiform activity were investigated in the *in vitro* mouse neocortical cylinder and the rat hippocampal slice in an effort to answer the following questions: What is the persistent effect of DPCPX due to? Is there a basal purinergic (inhibitory) tone? And what are the implications for the use of purinergic mechanisms in the treatment of epilepsy?

6 METHODS

Mouse neocortical cylinders were prepared as described in Chapter 2, usually with a nominal diameter of 450 µm. Hippocampal slices were prepared as follows: Male Wistar rats (180-240 g) were anaesthetised with intraperitoneally injected urethane (25% w/v, 5 ml/kg body weight) and decapitated. The brain was quickly removed, and the hippocampus was dissected free in ice cold, oxygenated ACSF (composition [in mM]: NaCl 120, KCl 1.1, $CaCl_2$ 2, $MgSO_4$ 1, KH_2PO_4 2.2, $NaHCO_3$ 26, glucose 10; prepared freshly each day). Slices (450 mm thick) were cut perpendicular to the long hippocampal axis on a McIlwain tissue chopper, placed on moistened filter paper in a Petri dish together with a small amount of ACSF, and transferred to an interface holding chamber constantly bubbled with carbogen (95% O_2 / 5% CO_2) to equilibrate. After a period of at least one hour, a single slice was then placed in a submersion-type recording chamber, pinned down with two blunt glass microelectrodes and superfused by gravity at a rate of 5 ml/min with ACSF saturated with 95% O_2 / 5% CO_2 and maintained at 29°C. Magnesium-free ACSF was prepared by omitting $MgSO_4$ from the normal ACSF, without adjusting for molarity. Glass microelectrodes were pulled on a single-stage puller, and backfilled with Ca^{2+}- and glucose-free ACSF. The tips of the microelectrodes were broken back under visual guidance to obtain a resistance of 2 to 5 MΩ. Extracellular field potentials were recorded from the stratum pyramidale in the CA3 subfield.

The following drugs were used: 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), cyclopentyltheophylline (CPT) (both RBI), theophylline, adenosine (both Sigma), adenosine deaminase (ADA; the enzyme which converts adenosine to inosine), and N6-cyclopentyladenosine (CPA, a synthetic adenosine analogue). DPCPX was dissolved in 10% dimethylsulfoxide (DMSO), while CPT was dissolved in NaOH 1 M; both were stored as 100 µM stock solutions at 4°C. Theophylline (dissolved in NaOH 0.5 M to a final concentration of 50 mM) and adenosine (dissolved in calcium-free ACSF to a final concentration of 10 mM) stock solutions were prepared freshly each day. Further dilutions were made with calcium-free medium. Equivalent quantities of the solvent had no effect. Different drugs were switched to the bath

perfusion system by means of three-way taps. At the flow rate of about 5 ml/min, about 15 seconds were required until the drug reached the bath.

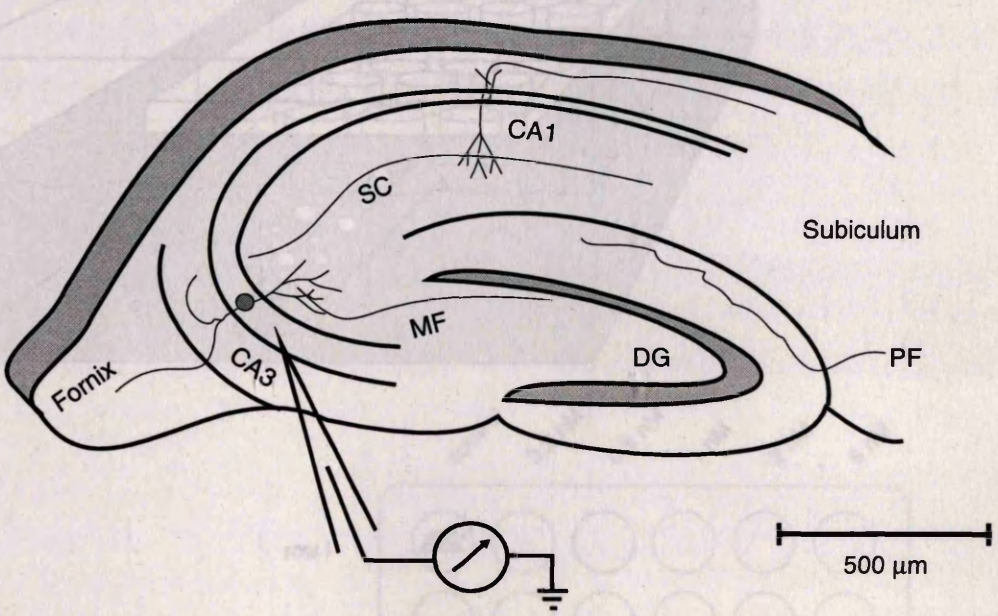


Figure 27: The rat hippocampal slice (schematic drawing): extracellular field potentials were recorded in the stratum pyramidale of the CA3 subfield, which is innervated by the mossy fibre (MF) pathway from the dentate gyrus (DG, innervated by a projection from the entorhinal cortex - PF [perforant pathway]) and projects to the CA1 subfield via the Schaffer collaterals (SC). The schematic is intentionally simplified and omits a number of important connections.

In some instances, slices were incubated in individual wells of a 24-well tissue culture plate containing 0.5 ml of oxygenated ACSF per well after the initial resting period of 60 minutes. The tissue culture plate was left to float in a holding chamber containing 300 ml of constantly bubbled ACSF. This resulted in a continuous gentle movement of the plate in a humidified O_2/CO_2 atmosphere. Where this was done to assess the amount of adenosine and its metabolites secreted by the slices, ACSF samples were then withdrawn from the wells at various time points during the incubation period, as described in the results section. For the incubation of individual slices with DPCPX, a further 500 µl of ACSF containing a defined amount of the drug were added to the wells after an incubation period of 30 minutes, and the slices were transferred to the recording chamber at the end of a further incubation period, which ranged from 15 minutes to one hour.

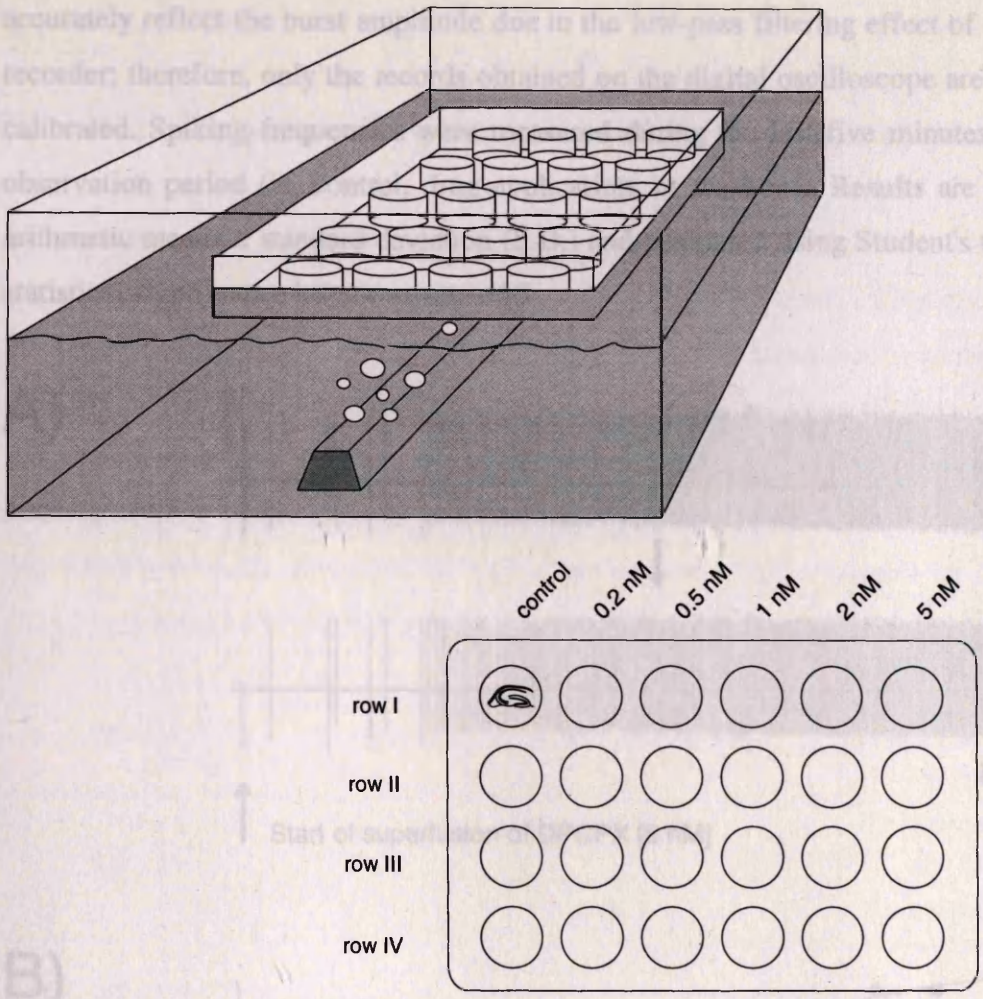


Figure 28: Method used for the incubation of up to 24 hippocampal slices in defined amounts of drug-containing ACSF. The slices are placed in individual wells containing 0.2 ml ACSF, to which a further 200 µl are added after a given equilibration period. The culture plate is floating on constantly bubbled ACSF inside a holding chamber; the continuous movement in moistened carbogen ensures optimal oxygenation.

Data were recorded both as continuous chart recorder tracings at slow speed for the assessment of spiking frequencies, and as print-outs of a Gould digital oscilloscope to record single events at a higher resolution. For illustration purposes, chart recordings were scanned using a Hewlett Packard scanner with a resolution of 600 x 600 dpi and re-traced in a graphics program (CorelDraw, version 3.0) to allow the inclusion into graphs independently of the originally used chart speed and amplification factor. Both the amplitude and the frequency are reproduced faithfully (see Figure 29 below), except where the frequency was so high as to prevent an adequate resolution: in these cases, the trace was included as the original bitmap. It should be noted that the chart record is of a semiquantitative nature: each upstroke represents one burst discharge, but the deflections (which usually had an amplitude of 0.1 to 1 mV) do not

accurately reflect the burst amplitude due to the low-pass filtering effect of the chart recorder; therefore, only the records obtained on the digital oscilloscope are voltage-calibrated. Spiking frequencies were measured during the last five minutes of each observation period (ie. control, drug application, or washout). Results are given as arithmetic means ± standard deviation (S.D.) and compared using Student's t test; the statistical significance level was set at 5%.

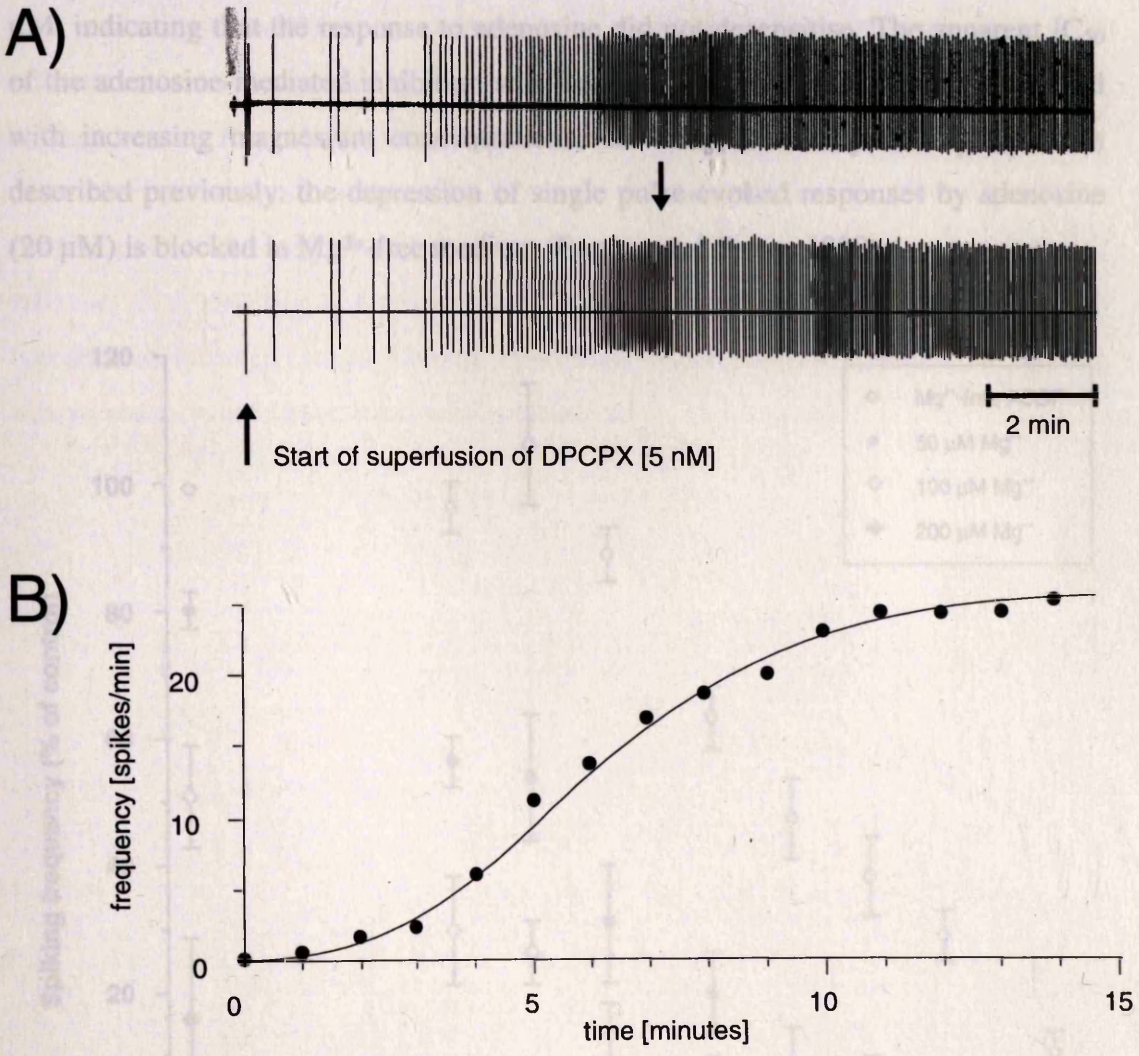


Figure 29: Equivalence of original and re-traced Y-t chart recordings. Shown are the results of an experiment testing the time needed for DPCPX (5 nM)-induced spiking in a rat hippocampal slice to reach a stable level. The drug was added to the superfusate at the point indicated by the arrow; the time to steady state is plotted below. The top trace is a bitmap image of the original recording, while the bottom trace represents the re-drawn copy. The illustrations of spiking frequencies used in the figures are re-traced, unless otherwise stated.

7 RESULTS

7.1 Adenosine effects in hippocampal and neocortical preparations

Adenosine rapidly and reversibly suppressed spontaneous spiking induced by perfusion with magnesium-free medium in mouse neocortical cylinders. Spiking frequency decreased to a similar extent upon consecutive exposures to adenosine 100 µM, indicating that the response to adenosine did not desensitise. The apparent IC_{50} of the adenosine-mediated inhibition was found to be 87.9 ± 1.3 µM, and decreased with increasing magnesium concentrations; this magnesium-dependency has been described previously: the depression of single pulse-evoked responses by adenosine (20 µM) is blocked in Mg^{2+}-free medium (Bartrup and Stone, 1988).

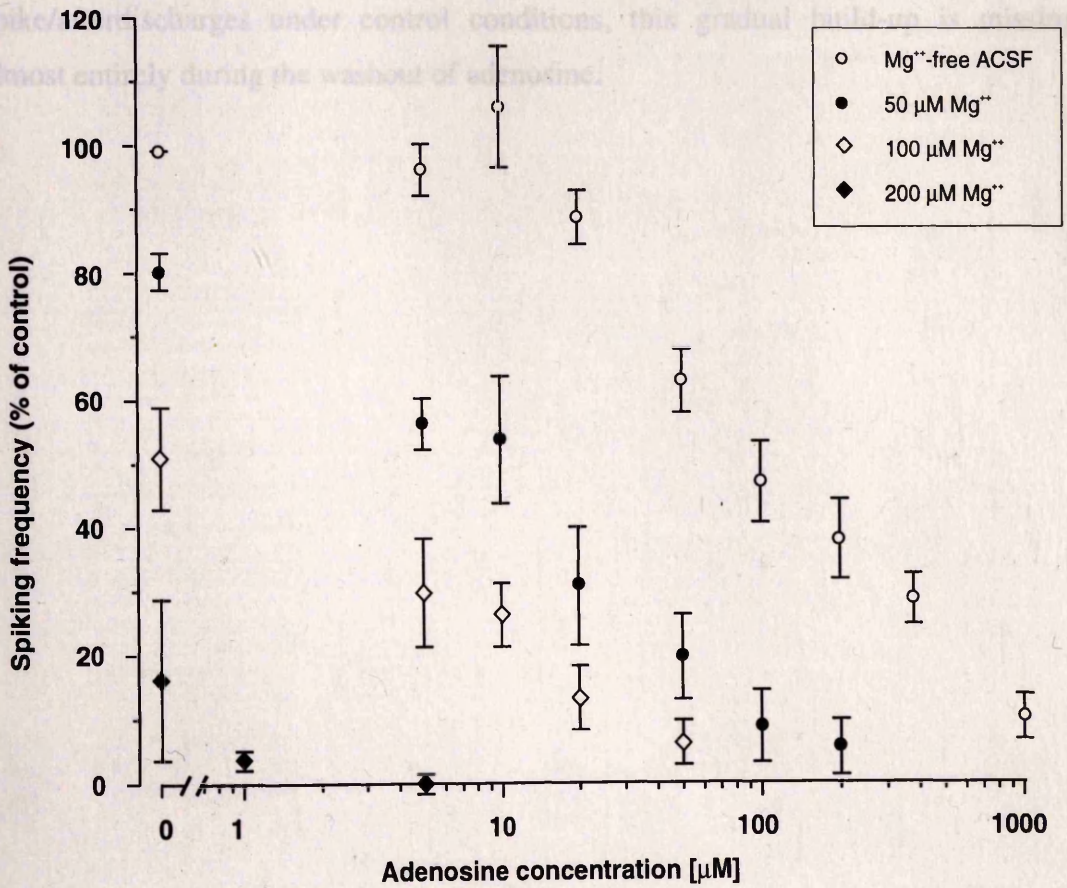


Figure 30: Adenosine effects on spontaneous spiking in neocortical cylinders superfused with ACSF containing 0, 50, 100 or 200 µM magnesium sulphate (n = 6 for each magnesium concentration). Adenosine displays a tendency towards enhancing effects at low doses (10 µM) in magnesium-free ACSF.

The fitting of a sigmoidal function to the data gave no satisfactory result, possibly due to the fact that the effects of adenosine are markedly affected by uptake and metabolism. The fit improved when a basal interstitial concentration of 2 μM was included to account for the increase in extracellular adenosine levels reported to occur during seizure-like activity. In some cases, perfusion with low doses of adenosine (10 μM) was observed to result in a facilitatory effect, which however failed to reach statistical significance (Fig. 30). A seemingly unrelated, but possibly very germane observation was that after the perfusion with adenosine, a transient rebound increase in the spiking frequency to above control levels was observed; this is illustrated in Fig. 31 (A). A record of the activity of several individual neurones located in proximity to the recording electrode (B) suggests that the rebound increase in epileptiform burst frequency is due to a reduction in the bursting threshold: whereas the continuous firing by individual cells only occasionally results in spike/afterdischarges under control conditions, this gradual build-up is missing almost entirely during the washout of adenosine.

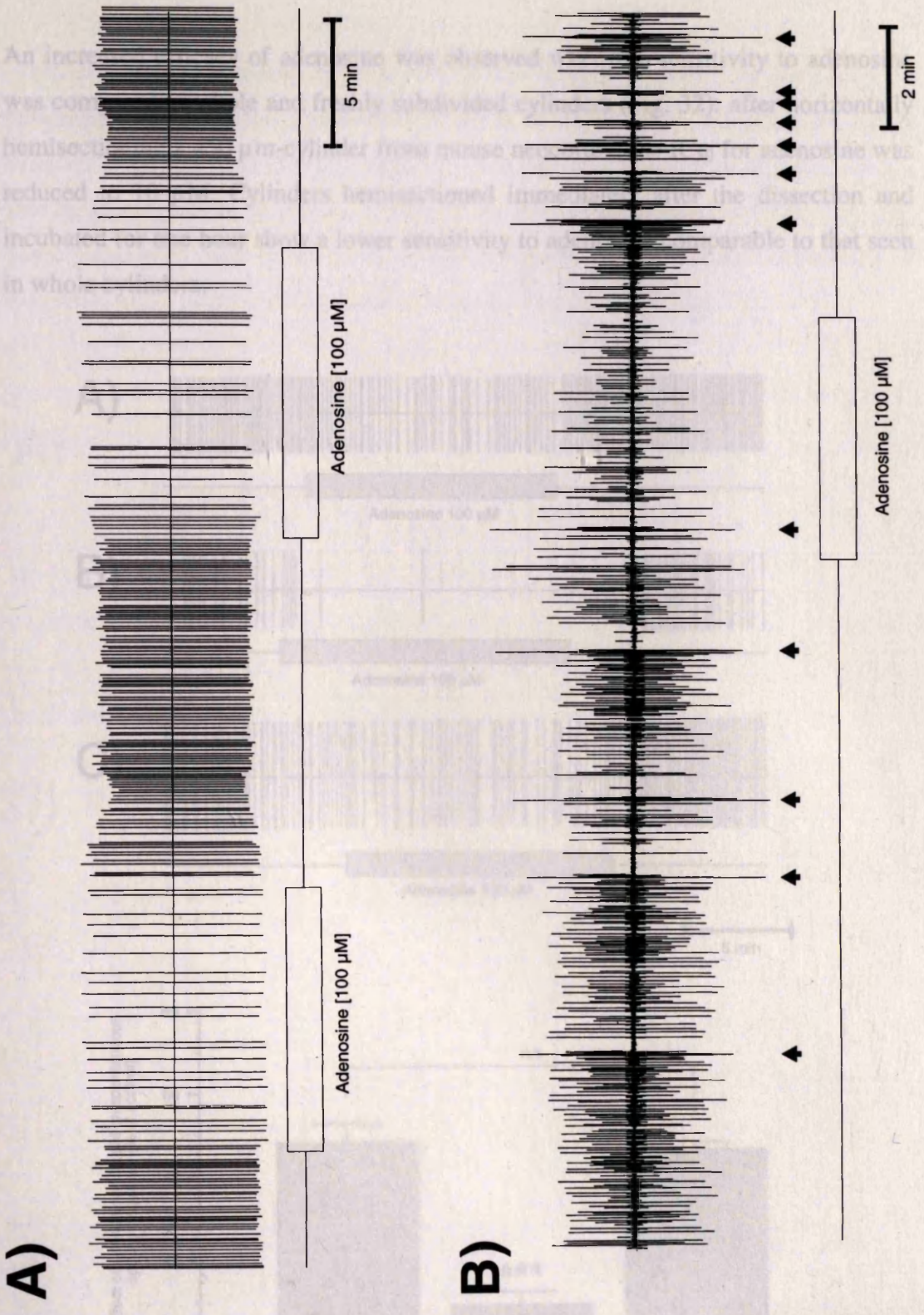


Figure 31: Response of a mouse neocortical cylinder (450 µm) to repeated applications of adenosine 100 µM in zero Mg^{2+} ACSF: During washout, a transient rebound increase in spiking frequency is observed (illustrated in a recording of the activity of several individual neurones in the vicinity of the electrode, below; the spike/afterdischarge bursts are indicated by arrows).

An increased efficacy of adenosine was observed when the sensitivity to adenosine was compared in whole and freshly subdivided cylinders (Fig. 32): after horizontally hemisectioning a 450 µm-cylinder from mouse neocortex, the IC_{50} for adenosine was reduced to 10 µM. Cylinders hemisectioned immediately after the dissection and incubated for one hour show a lower sensitivity to adenosine comparable to that seen in whole cylinders.

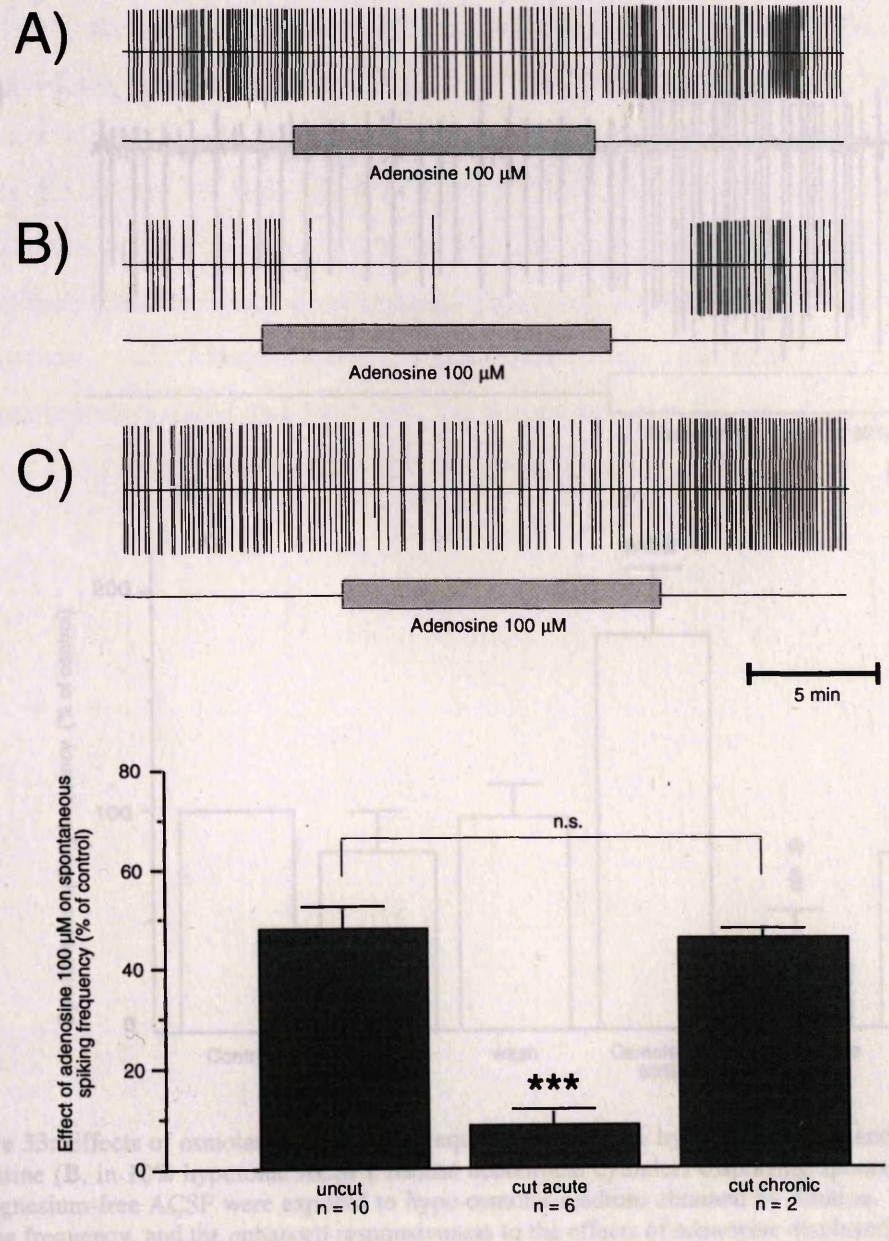


Figure 32: Acutely hemisectioned cylinders were markedly more sensitive to the superfusion with adenosine 100 µM. This sensitivity returned to control levels in hemisectioned cylinders incubated for several hours (**A**: intact cylinder; **B**: cut cylinder - 10 minutes after hemisection; **C**: cut cylinder - 150 minutes after hemisection). ***: $p < 0.001$ (unpaired t-test).

A similar marked increase in sensitivity to the inhibitory effects of adenosine on epileptiform activity was observed in a series of experiments using hypo-osmolar solution to achieve cell swelling similar to that reported to occur in damaged slices. The osmolarity of the ACSF was decreased by adding distilled water (10% v/v). The experiments with hypo-osmolar ACSF demonstrated both an increased excitability of the tissue, and a significantly increased response to adenosine (Fig. 33).

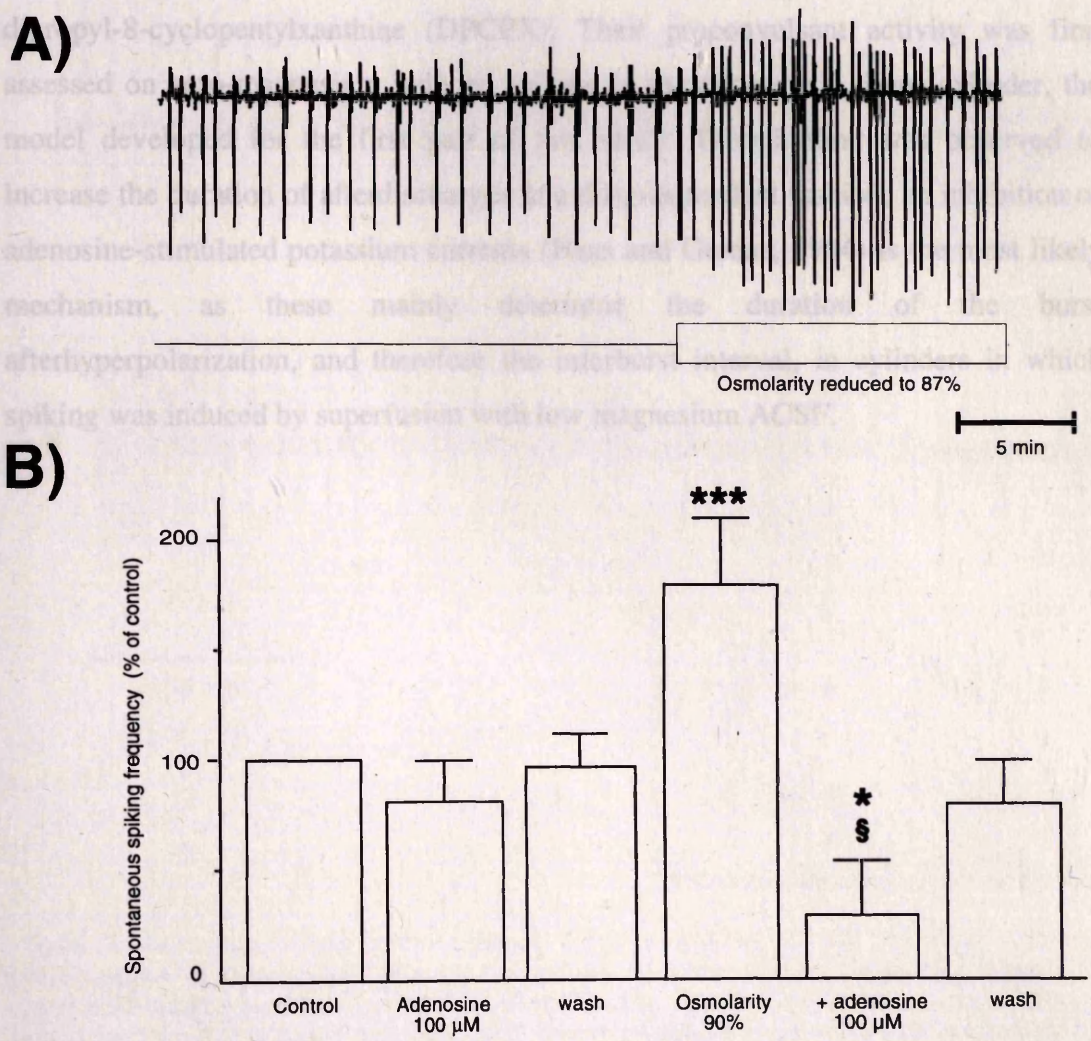


Figure 33: Effects of osmolarity on spiking frequency (**A**, in 13% hypotonic ACSF) and sensitivity to adenosine (**B**, in 10% hypotonic ACSF). Mouse neocortical cylinders displaying spontaneous spiking in magnesium-free ACSF were exposed to hypo-osmolar medium obtained by dilution. The increased spiking frequency, and the enhanced responsiveness to the effects of adenosine displayed in (**B**) are the results (mean ± SD) obtained in five experiments. * and ***: $p<0.05$ and $p<0.001$ vs. control; §: $p<0.05$ vs. first adenosine superfusion (two-sided t-test).

7.2 Methylxanthine effects in hippocampal and neocortical preparations

Both the naturally occurring methylxanthines caffeine and theophylline, and synthetic analogues with higher affinity for the adenosine A1 receptor have been shown to be potent proconvulsants in animal studies (see Introduction). The compounds used for the assessment of the role of purinergic neuromodulation in epileptiform activity included three alkylxanthines with different degrees of selectivity and affinity for the adenosine A1 receptor: theophylline, cyclopentyltheophylline (CPT) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). Their proconvulsant activity was first assessed on zero-magnesium induced spiking in the neocortical tissue cylinder, the model developed for the first part of this study. Theophylline was observed to increase the duration of afterdischarges in a dose-dependent fashion; an inhibition of adenosine-stimulated potassium currents (Haas and Greene, 1984) is the most likely mechanism, as these mainly determine the duration of the burst afterhyperpolarization, and therefore the interburst interval, in cylinders in which spiking was induced by superfusion with low magnesium ACSF.

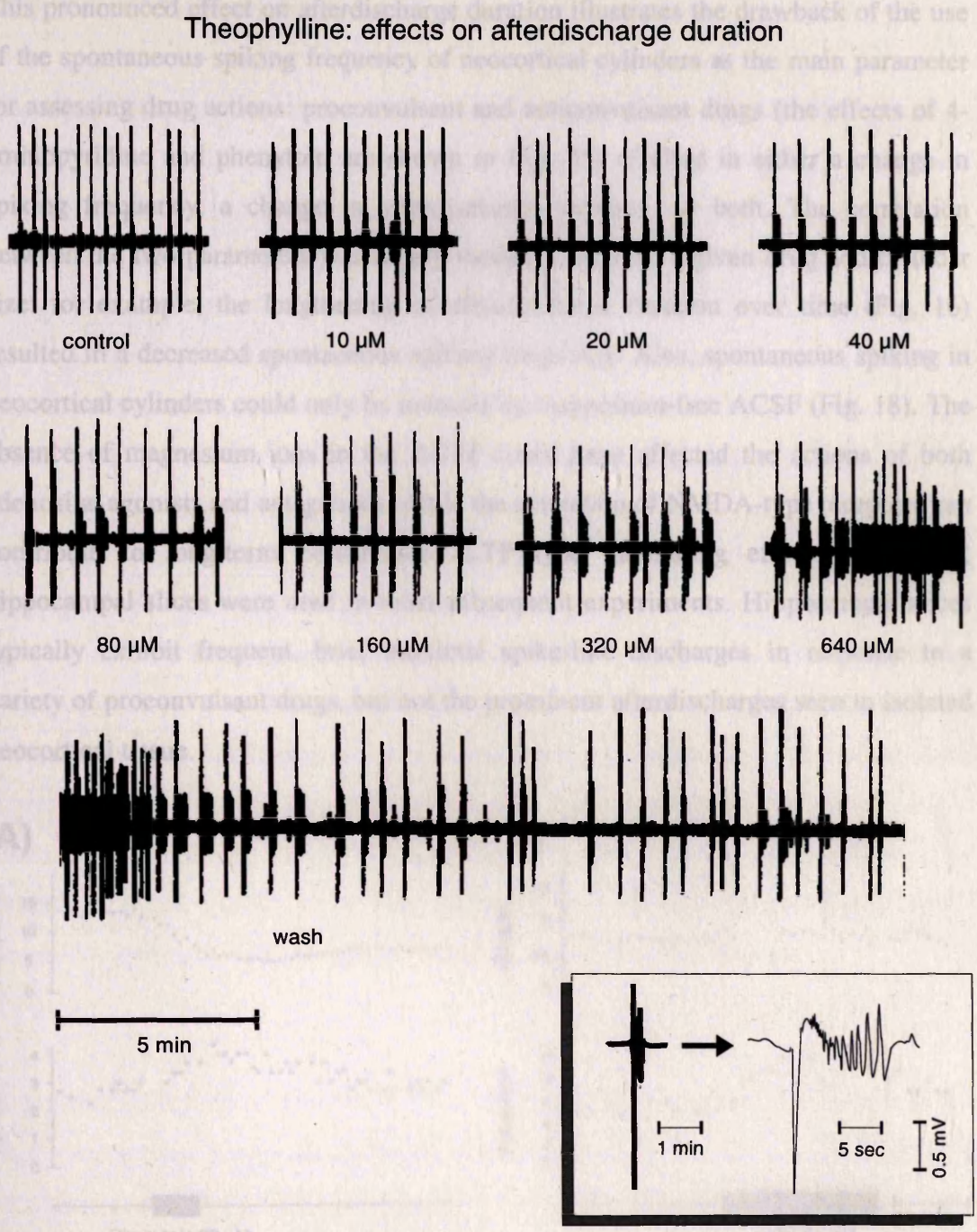


Figure 34: Rat cortical cylinder (nominal diameter 600 µm) incubated in 0 Mg^{++} ACSF and increasing concentrations of theophylline. Whereas the spiking frequency remains relatively constant, the duration of the afterdischarge (seen as high frequency/low amplitude bursts immediately following each interictal spike) is greatly prolonged, and becomes continuous at a theophylline concentration of 640 µM (compare with Fig. 17).

This pronounced effect on afterdischarge duration illustrates the drawback of the use of the spontaneous spiking frequency of neocortical cylinders as the main parameter for assessing drug actions: proconvulsant and anticonvulsant drugs (the effects of 4-aminopyridine and phenytoin are shown in Fig. 35) resulted in either a change in spiking frequency, a change in afterdischarge duration, or both. The correlation between the two parameters was highly variable, even for a given drug and cylinder size: for example, the lengthening of afterdischarge duration over time (Fig. 16) resulted in a decreased spontaneous spiking frequency. Also, spontaneous spiking in neocortical cylinders could only be induced by magnesium-free ACSF (Fig. 18). The absence of magnesium ions in the ACSF could have affected the actions of both adenosine agonists and antagonists, while the activation of NMDA-type receptors can contribute to long-term potentiation (LTP)-type facilitating effects. Therefore, hippocampal slices were used in most subsequent experiments. Hippocampal slices typically exhibit frequent, brief interictal spike-like discharges in response to a variety of proconvulsant drugs, but not the prominent afterdischarges seen in isolated neocortical tissue.

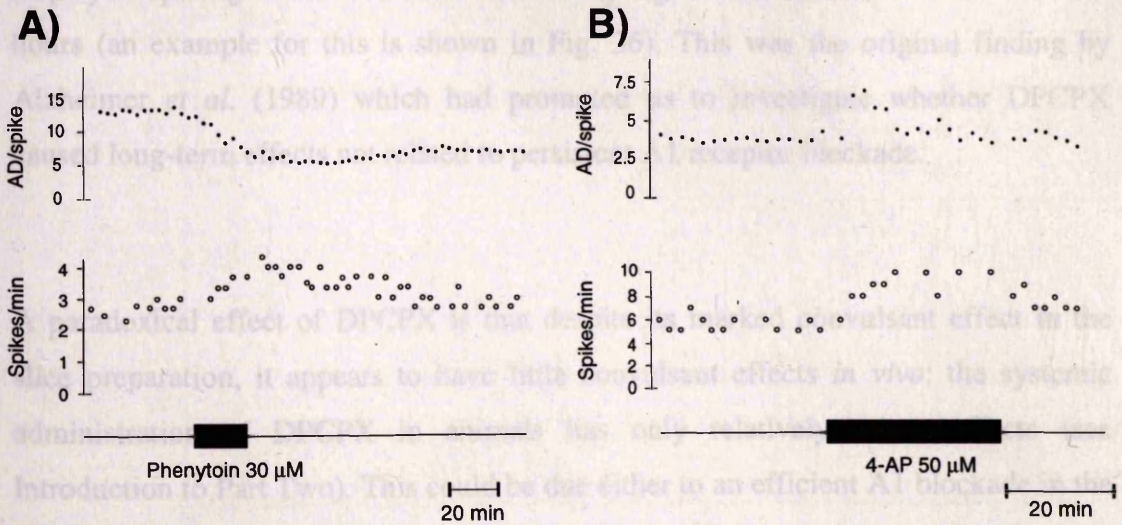


Figure 35: Variable effect of phenytoin and 4-AP on spiking frequency and afterdischarge (AD) duration (expressed as number of afterdischarges/burst). Preliminary experiments showed that neocortical cylinders, while being an adequate model for the sensorimotor 'column', could not be used for quantitative studies on the effects of drugs, as the parameters used (spontaneous spiking frequency, or number of afterdischarges) were correlated in a variable fashion. As can be seen, phenytoin at clinically relevant concentrations led to an increase in spiking and a concomitant decrease in the number of afterdischarges, while 4-AP led to a transient increase in the number of afterdischarges/spike, but a persistent increase in the spiking frequency.

7.2.1 *Time course of methylxanthine-mediated effects*

The main effect to be examined, the apparently persistent convulsant effect produced by DPCPX in the hippocampal slice, is illustrated in Fig. 36. All three methylxanthines tested in this study induced spontaneous spiking in normal medium containing 1.2 mM magnesium. Theophylline, a relatively water-soluble methylxanthine with a low affinity for the A1 receptor, induced spontaneous interictal spiking which reached stable levels within two to three minutes at concentrations of 50 μM; as this was approximately the time needed for the drug to reach the slice via the perfusion system at the flow rate used, the effect can be considered to occur almost immediately. The effect of theophylline was reversed within 20 minutes of washout (but see below). Cyclopentyltheophylline (CPT) demonstrated higher potency and a slower wash-out, taking approximately one hour to wash out. DPCPX induced spiking which did not abate even after a prolonged washout (6 hours). A monoexponential decay with a half-life (T½) of 80 minutes could be fitted to a subset of washout data; however, a sizeable proportion of slices displayed spiking which did not abate to any significant extent even after several hours (an example for this is shown in Fig. 36). This was the original finding by Alzheimer *et al.* (1989) which had prompted us to investigate whether DPCPX caused long-term effects not related to persistent A1 receptor blockade.

A paradoxical effect of DPCPX is that despite its marked convulsant effect in the slice preparation, it appears to have little convulsant effects *in vivo*: the systemic administration of DPCPX in animals has only relatively minor effects (see Introduction to Part Two). This could be due either to an efficient A1 blockade in the absence of a physiologically relevant purinergic tone, or to an inaccessibility of the A1 receptor for DPCPX under basal conditions.

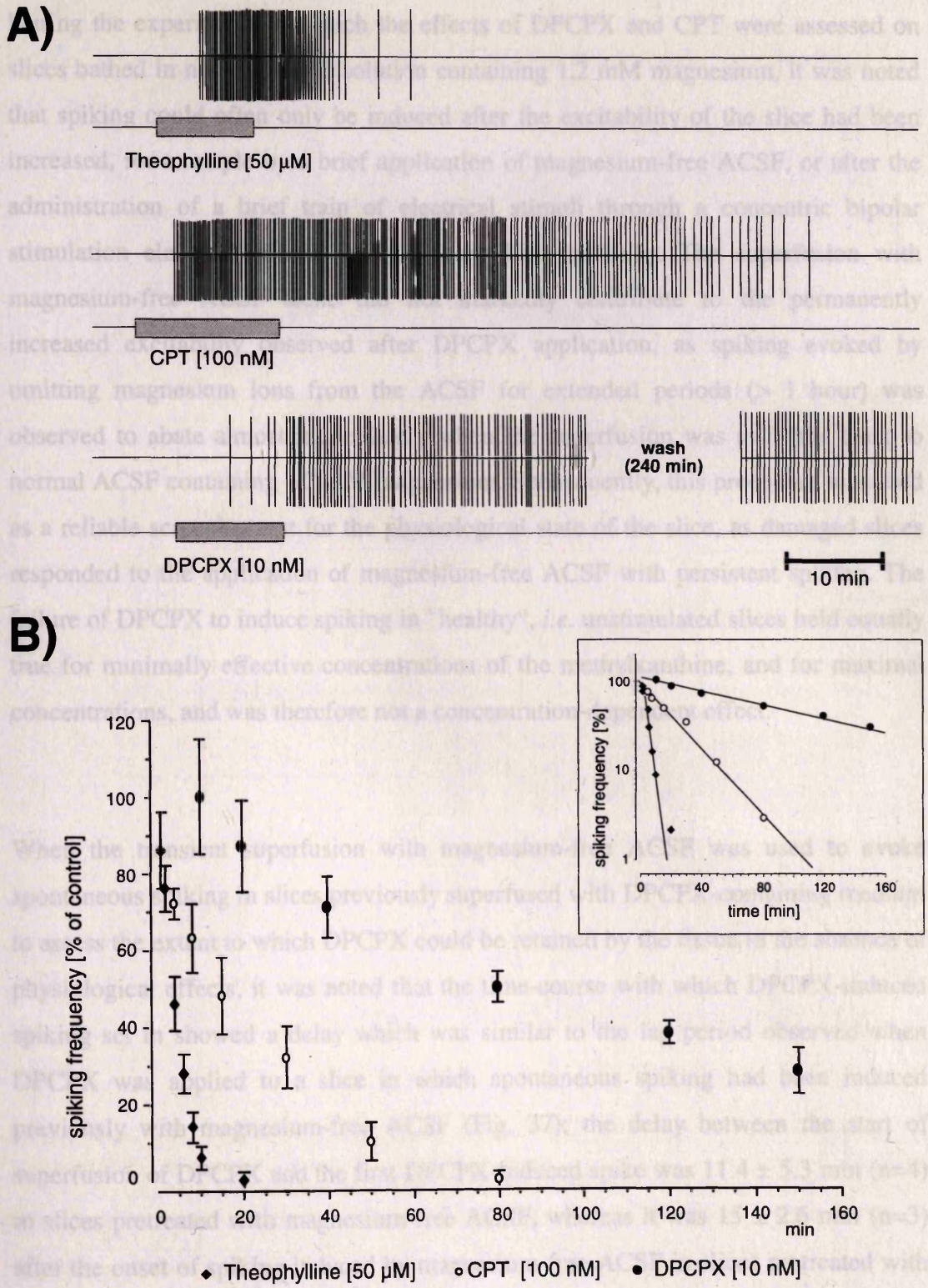


Figure 36: Time course of decay of spontaneous spiking frequency after the application of methylxanthines. Representative traces from three single experiments using theophylline, CPT or DPCPX (**A**), and averaged values (mean ± SD, n=5 per compound; **B**). Theophylline washed out within 20 minutes, while the proconvulsant effects of CPT abated within 80 minutes. DPCPX-induced spiking lasted for several hours.

During the experiments in which the effects of DPCPX and CPT were assessed on slices bathed in normal ACSF solution containing 1.2 mM magnesium, it was noted that spiking could often only be induced after the excitability of the slice had been increased, for example by a brief application of magnesium-free ACSF, or after the administration of a brief train of electrical stimuli through a concentric bipolar stimulation electrode placed in the mossy fibre pathway. The superfusion with magnesium-free ACSF alone did not markedly contribute to the permanently increased excitability observed after DPCPX application, as spiking evoked by omitting magnesium ions from the ACSF for extended periods (> 1 hour) was observed to abate almost immediately when the superfusion was switched back to normal ACSF containing 1.2 mM magnesium. Subsequently, this procedure was used as a reliable screening test for the physiological state of the slice, as damaged slices responded to the application of magnesium-free ACSF with persistent spiking. The failure of DPCPX to induce spiking in "healthy", *i.e.* unstimulated slices held equally true for minimally effective concentrations of the methylxanthine, and for maximal concentrations, and was therefore not a concentration-dependent effect.

When the transient superfusion with magnesium-free ACSF was used to evoke spontaneous spiking in slices previously superfused with DPCPX-containing medium to assess the extent to which DPCPX could be retained by the tissue in the absence of physiological effects, it was noted that the time-course with which DPCPX-induced spiking set in showed a delay which was similar to the lag period observed when DPCPX was applied to a slice in which spontaneous spiking had been induced previously with magnesium-free ACSF (Fig. 37); the delay between the start of superfusion of DPCPX and the first DPCPX-induced spike was 11.4 ± 5.3 min (n=4) in slices pretreated with magnesium-free ACSF, whereas it was 15 ± 2.6 min (n=3) after the onset of spiking induced by magnesium-free ACSF in slices pretreated with DPCPX.

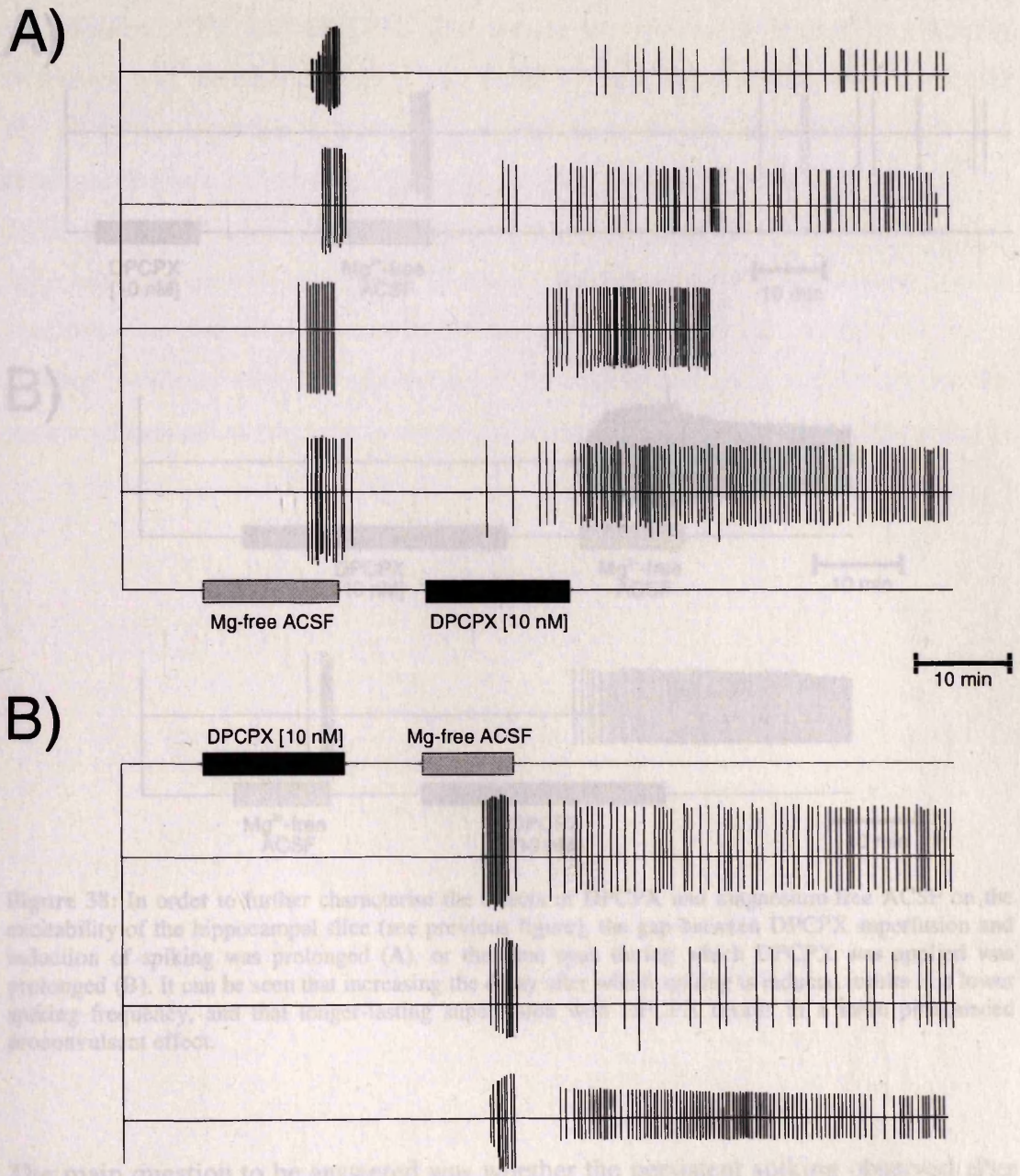


Figure 37: In slices pre-treated by superfusion with Mg^{2+}-free ACSF, DPCPX induced spontaneous spiking (results of 4 experiments shown in **A**); by contrast, naive slices did not respond to DPCPX with spontaneous spiking until superfused with Mg^{2+}-free ACSF (**B**, 3 experiments): in the latter case, DPCPX-induced spiking appeared with a characteristic lag period which could last several minutes.

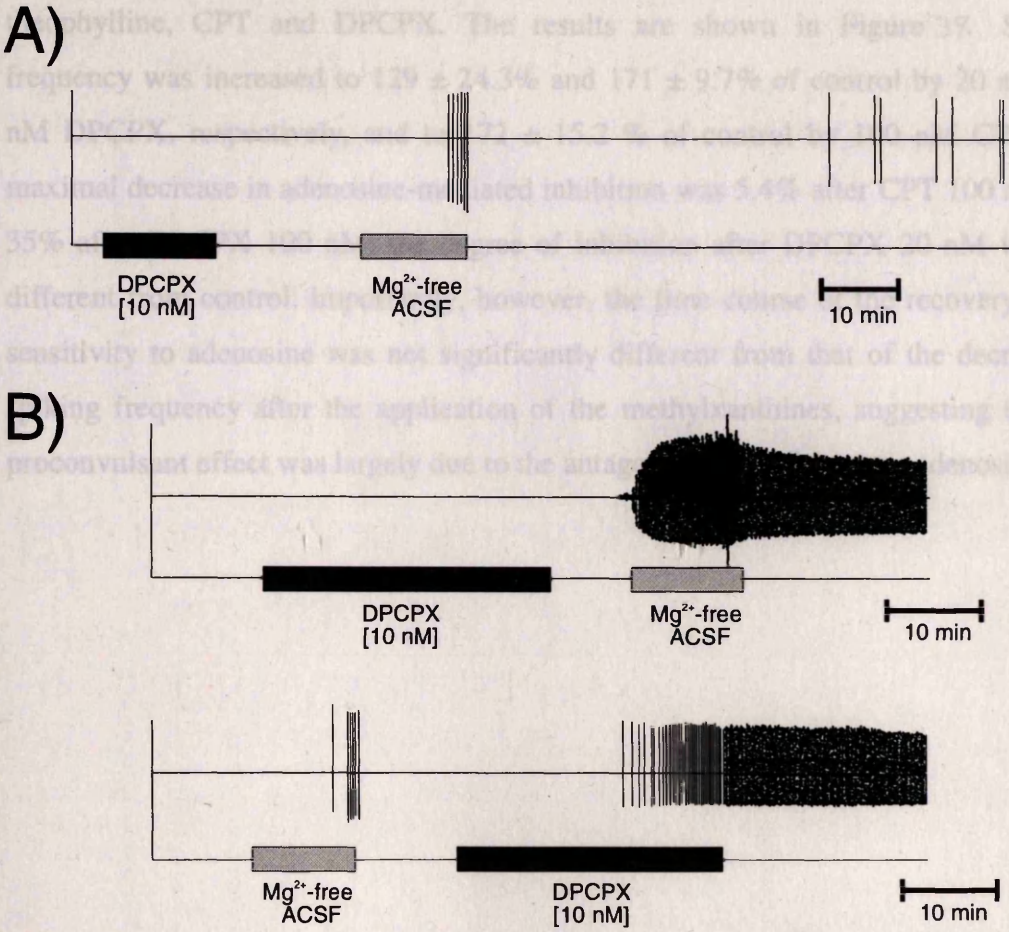
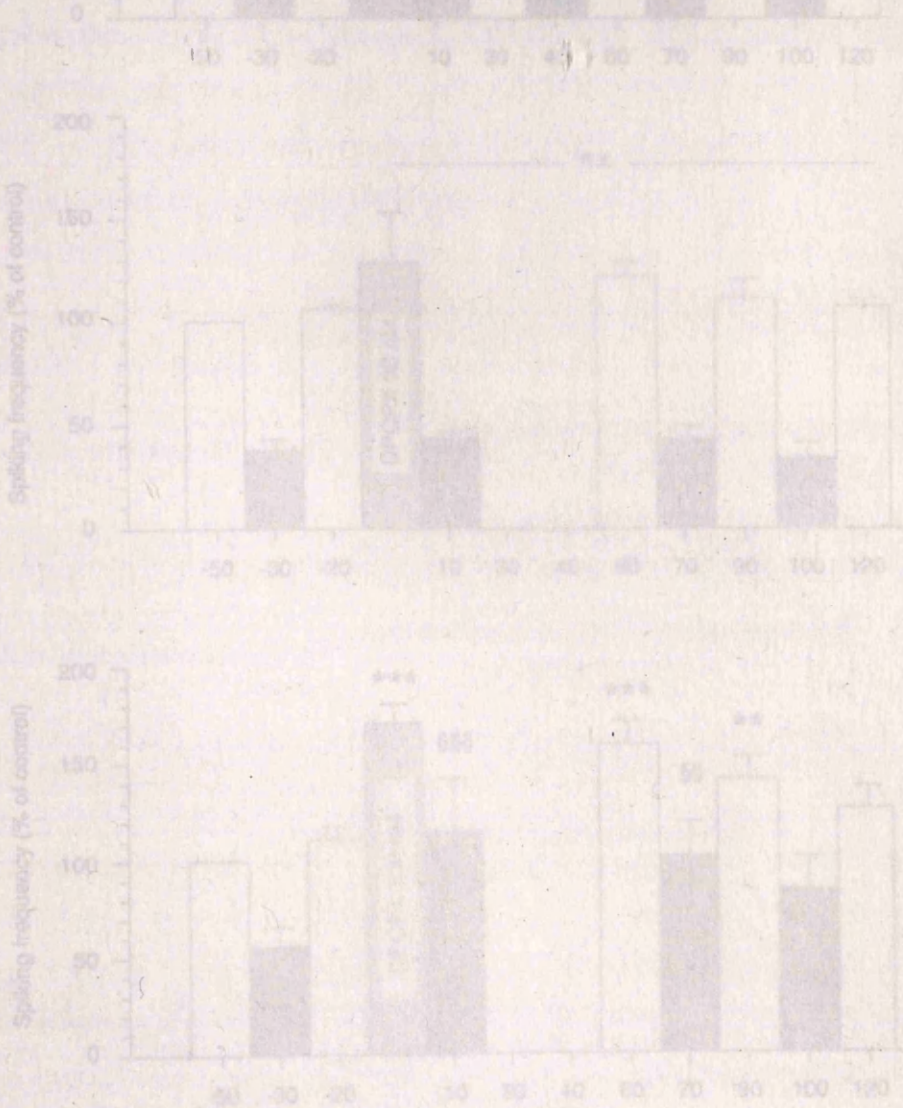


Figure 38: In order to further characterise the effects of DPCPX and magnesium-free ACSF on the excitability of the hippocampal slice (see previous figure), the gap between DPCPX superfusion and induction of spiking was prolonged (A), or the time span during which DPCPX was applied was prolonged (B). It can be seen that increasing the delay after which spiking is induced results in a lower spiking frequency, and that longer-lasting superfusion with DPCPX results in a more pronounced proconvulsant effect.

The main question to be answered was whether the persistent spiking observed after the application of DPCPX was due to adenosine receptor blockade. A series of experiments was devised where the sensitivity of spontaneously spiking neocortical cylinders to adenosine (100 µM) was assessed before and after a brief superfusion with DPCPX (20 or 100 nM) or CPT (100 nM). The reason for using the zero magnesium paradigm was that a control parameter for the potency of adenosine before the application of the methylxanthines was needed, as direct intracellular measurements of the adenosine-induced hyperpolarization were not possible. The effects of a brief (10 min) application of adenosine (100 µM) were assessed at 30 minute intervals before and after the application of each of the three antagonists, i.e.

theophylline, CPT and DPCPX. The results are shown in Figure 39. Spiking frequency was increased to 129 ± 24.3% and 171 ± 9.7% of control by 20 and 100 nM DPCPX, respectively, and to 172 ± 15.2 % of control by 100 nM CPT. The maximal decrease in adenosine-mediated inhibition was 5.4% after CPT 100 nM and 35% after DPCPX 100 nM; the degree of inhibition after DPCPX 20 nM was not different from control. Importantly, however, the time course of the recovery of the sensitivity to adenosine was not significantly different from that of the decrease in spiking frequency after the application of the methylxanthines, suggesting that the proconvulsant effect was largely due to the antagonism of endogenous adenosine.



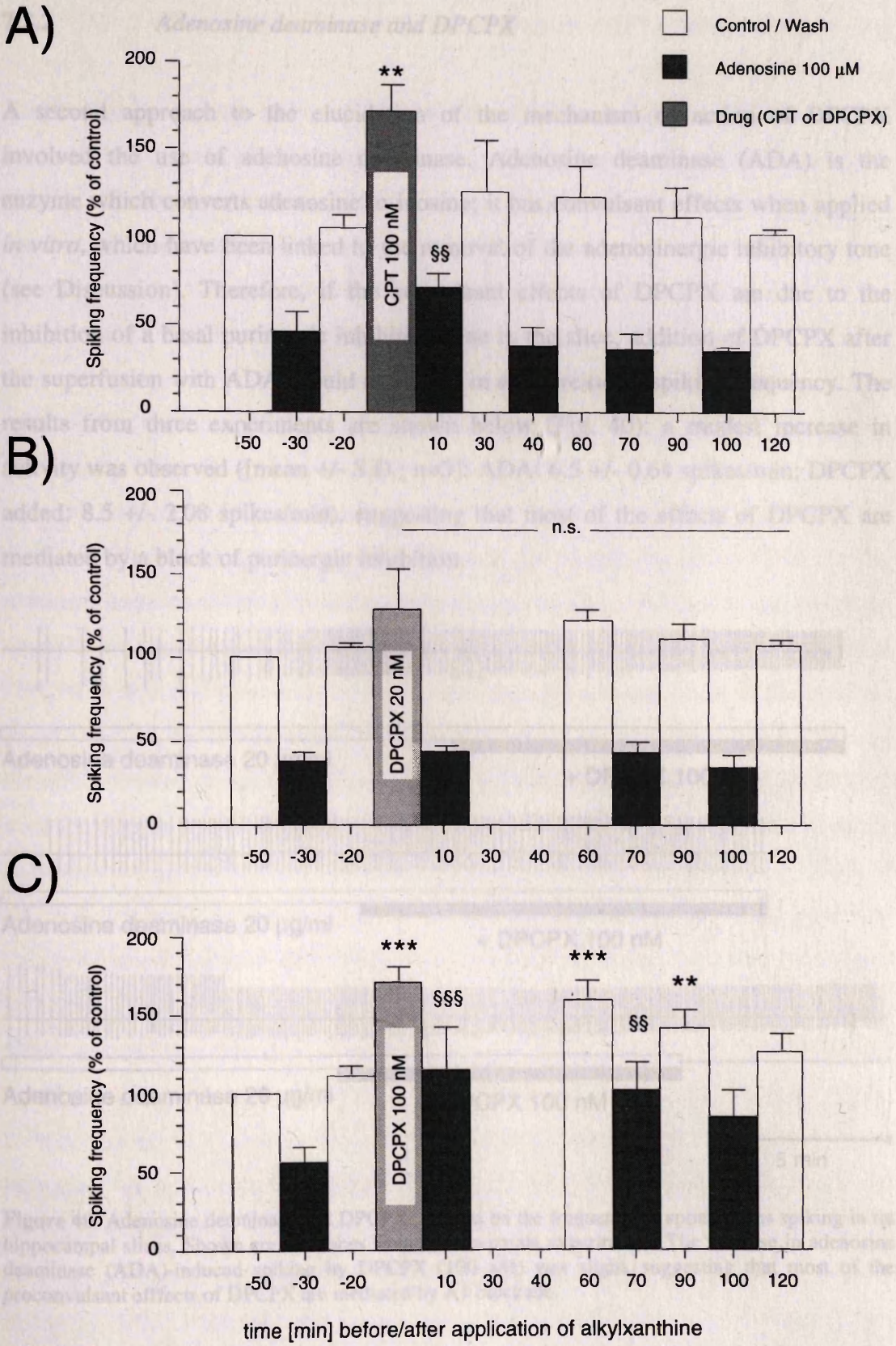


Figure 39: Inhibiton of zero Mg^{++}-induced spiking in rat hippocampal slices by adenosine before and after the application of CPT (100 nM) or DPCPX (20 nM or 100 nM) (n=5 for each graph). **=P<0.01; ***=P<0.001 vs. control; §§=P<0.01, §§§=P<0.001 vs. first superfusion with adenosine. (x axis: time in minutes before/after methylxanthine application).

7.2.2 *Adenosine deaminase and DPCPX*

A second approach to the elucidation of the mechanism of action of DPCPX involved the use of adenosine deaminase. Adenosine deaminase (ADA) is the enzyme which converts adenosine to inosine; it has convulsant effects when applied *in vitro*, which have been linked to the removal of the adenosinergic inhibitory tone (see Discussion). Therefore, if the convulsant effects of DPCPX are due to the inhibition of a basal purinergic inhibitory tone in the slice, addition of DPCPX after the superfusion with ADA should not result in an increase in spiking frequency. The results from three experiments are shown below (Fig. 40): a modest increase in activity was observed ([mean +/- S.D.; n=3]: ADA: 6.5 +/- 0.64 spikes/min; DPCPX added: 8.5 +/- 2.08 spikes/min), suggesting that most of the effects of DPCPX are mediated by a block of purinergic inhibition.

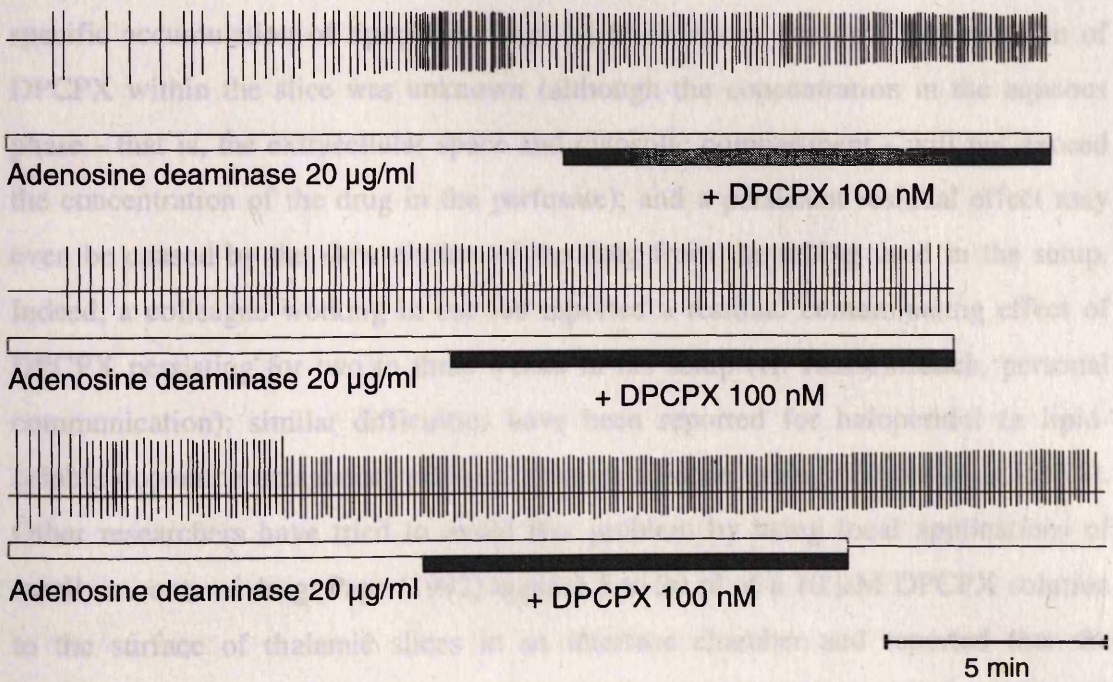


Figure 40: Adenosine deaminase and DPCPX: effects on the frequency of spontaneous spiking in rat hippocampal slices. Shown are the traces from three separate experiments. The increase in adenosine deaminase (ADA)-induced spiking by DPCPX (100 nM) was slight, suggesting that most of the proconvulsant efffects of DPCPX are mediated by A1 blockade.

7.2.3 *Adenosine and metabolite concentrations - HPLC assay*

The second question to be answered with respect to the actions of DPCPX was the nature of the persistent convulsant effect of DPCPX reported by Alzheimer *et al.* (1989) and also observed in the present study. Several possibilities had to be considered: The persistent effect could simply be due to a persistence of the drug at the receptor, or to a persistence of the drug in the experimental setup (i.e., an artefact), or to the induction of long-term, secondary changes in the tissue.

The results from the present experiments had indicated that the spiking observed after the addition of DPCPX to the perfusate was most likely exclusively due to the blockade of adenosine A1 receptors. However, the application of DPCPX via the perfusing solution introduced two uncontrollable variables: because of the avid non-specific accumulation of lipophilic drugs by brain slices, the end concentration of DPCPX within the slice was unknown (although the concentration in the aqueous phase - that is, the extracellular space and cytosolic compartment - will not exceed the concentration of the drug in the perfusate); and a persistent residual effect may even be caused by the slow elution of the drug from the tubing used in the setup. Indeed, a colleague working in our lab reported a residual contaminating effect of DPCPX persisting for two to three weeks in his setup (H. Hosseinzadeh, personal communication); similar difficulties have been reported for haloperidol (a lipid-soluble dopamine antagonist) perfused through silicone tubing (Starke *et al.*, 1978). Other researchers have tried to avoid this problem by using local applications of small amounts of drug: Pape (1992) applied 5 to 20 pl of a 10 µM DPCPX solution to the surface of thalamic slices in an interface chamber and reported that the responses to adenosine had almost completely recovered from block within 33 minutes. A final proof for the persistence of the drug in the tissue and for the degree of accumulation in the lipid phase could have been obtained by measuring the drug levels by HPLC. However, the system in use in the Department was a reverse phase liquid chromatography system and therefore unable to assay lipophilic compounds.

At first, it was attempted to minimise the problems introduced by the adsorption of DPCPX to tubing by using two separate perfusion systems for DPCPX-containing

ACSF and control medium. However, this still left the problem of not knowing the amount of drug bound by the tissue. To circumvent both problems (i.e. contamination of the setup, and accumulation of drug at non-specific binding sites in the tissue), a method was devised by which individual slices could be incubated in small amounts of ACSF for extended periods of time before assessing the excitability in the experimental setup. Because the accumulation of endogenous adenosine and other metabolites could have interfered with the binding of the drug (see Introduction to Part Two), it was also necessary to assess the amount of adenosine and its metabolites secreted by the slices over time. To this end, ACSF samples were taken from the wells and analysed by HPLC, as described below.

The amount of endogenous adenosine which accumulated in the incubation medium was an important factor for several reasons: firstly, it can give an indication of the state of the slices, as damaged or hyperexcitable slices are known to secrete increased amounts of adenosine; and second, endogenous adenosine could have interfered with the binding of purinergic compounds (cf. Schiemann *et al.*, 1990). To assess the amount of adenosine and adenosine metabolites secreted by the slices, ACSF samples (50 µl) were withdrawn from two wells (containing one slice from each hippocampus each in an initial volume of 450 µl) before, and at 0, 10, 30, 60 and 120 min after transfer of the slices. A third slice was incubated in parallel and assessed for electrophysiological viability after two hours. Purines were analysed by parallel high performance liquid chromatography (HPLC, Severn Analytical Solvent Delivery System SA6410B) and UV detection at 254 nm (Severn Analytical UV/vis Absorbance Detector SA6500). A Techsphere C18 3 µm microsphere column (10 cm x 4.6 mm) was used to separate adenosine, inosine, hypoxanthine and xanthine by isocratic reverse phase chromatography. The mobile phase was 0.01 M sodium phosphate (NaH_2PO_4) with 6% methanol (HPLC grade) pH 6.1 at a flow rate of 0.8 ml/min. The detection limit for adenosine was 0.5 pmol.

The calibration curves for adenosine, inosine and xanthine (range 0.05 - 20 µM) and for hypoxanthine (range 0.025 - 5 µM) were linear over the entire range. The concentrations were calculated from peak absorption heights according to the

formula x = (y-*c*)/*m*, with the constants *c* and *m* being determined graphically. The spread of the values (mean ± S.D.) is given below:

Adenosine	m = 3.269 +/- 0.024	c = -0.183 +/- 0.166
Inosine	m = 9.399 +/- 0.123	c = -0.639 +/- 0.429
Xanthine	m = 7.260 +/- 0.056	c = -0.030 +/- 0.197
Hypoxanthine	m = 12.414 +/- 0.195	c = -0.210 +/- 0.360

The population spike of the control slice (measured in the CA1 subfield after orthodromic stimulation) had an amplitude of 6 mV and remained stable over time, indicating good electrophysiological viability.

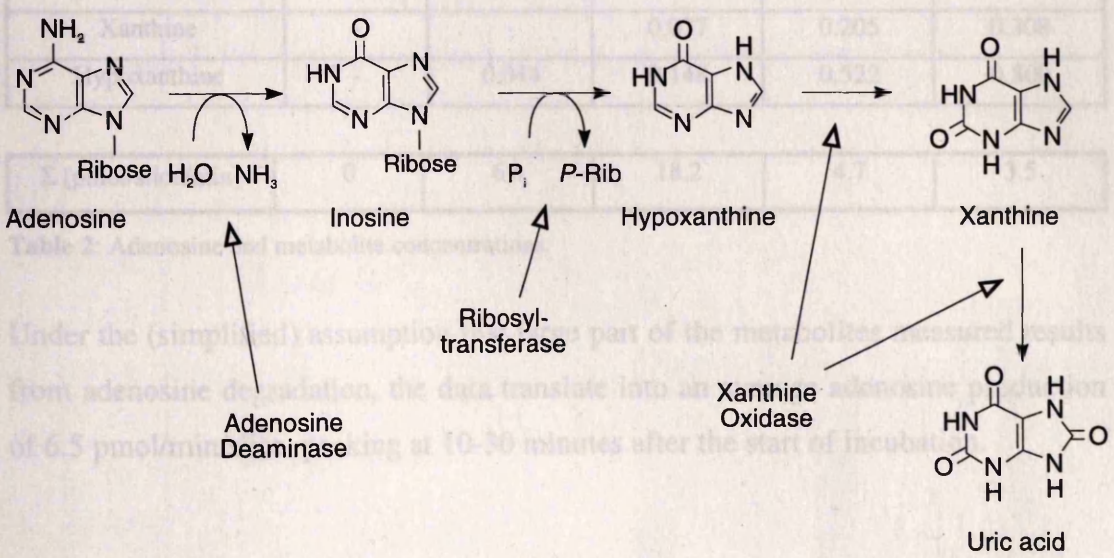


Figure 41: Principal intermediates and enzyme-catalysed transformations involved in adenosine metabolism.

The results from the two hippocampal slices incubated in parallel were as follows (in μM):

Slice A					
	0 min	10 min	30 min	60 min	120 min
Adenosine	-	-	0.065	0.097	0.129
Inosine	-	0.028	0.128	0.100	0.167
Xanthine	-	-	0.128	0.128	0.205
Hypoxanthine	-	0.070	0.435	0.383	0.644
Slice B					
	0 min	10 min	30 min	60 min	120 min
Adenosine	-	-	0.032	0.065	0.129
Inosine	-	-	0.056	0.122	0.200
Xanthine	-	-	0.077	0.205	0.308
Hypoxanthine	-	0.044	0.148	0.522	0.800
Σ [pmol/slice/min]	0	6.4	18.2	4.7	3.5

Table 2: Adenosine and metabolite concentrations

Under the (simplified) assumption that large part of the metabolites measured results from adenosine degradation, the data translate into an average adenosine production of 6.5 pmol/min/slice, peaking at 10-30 minutes after the start of incubation.

The results of the HPLC analysis suggest a continuous production of adenosine by the slice (indicated by the increasing levels of both adenosine and its metabolites over time), but at a relatively low level: the overall concentration of adenosine in the tissue culture wells over a two hour period remained well below 200 nM and therefore at levels similar to those reported to be present in brain tissue *in vivo*, although of course the measurement of adenosine concentration in the bulk medium may not truly reflect the local concentration present in the immediate vicinity of the adenosine receptors. The values compare with 0.5 μM adenosine found by Fowler (1993) after the incubation of rat hippocampal slices (thickness 400 μm) in a volume of 1.7 ml for one hour.

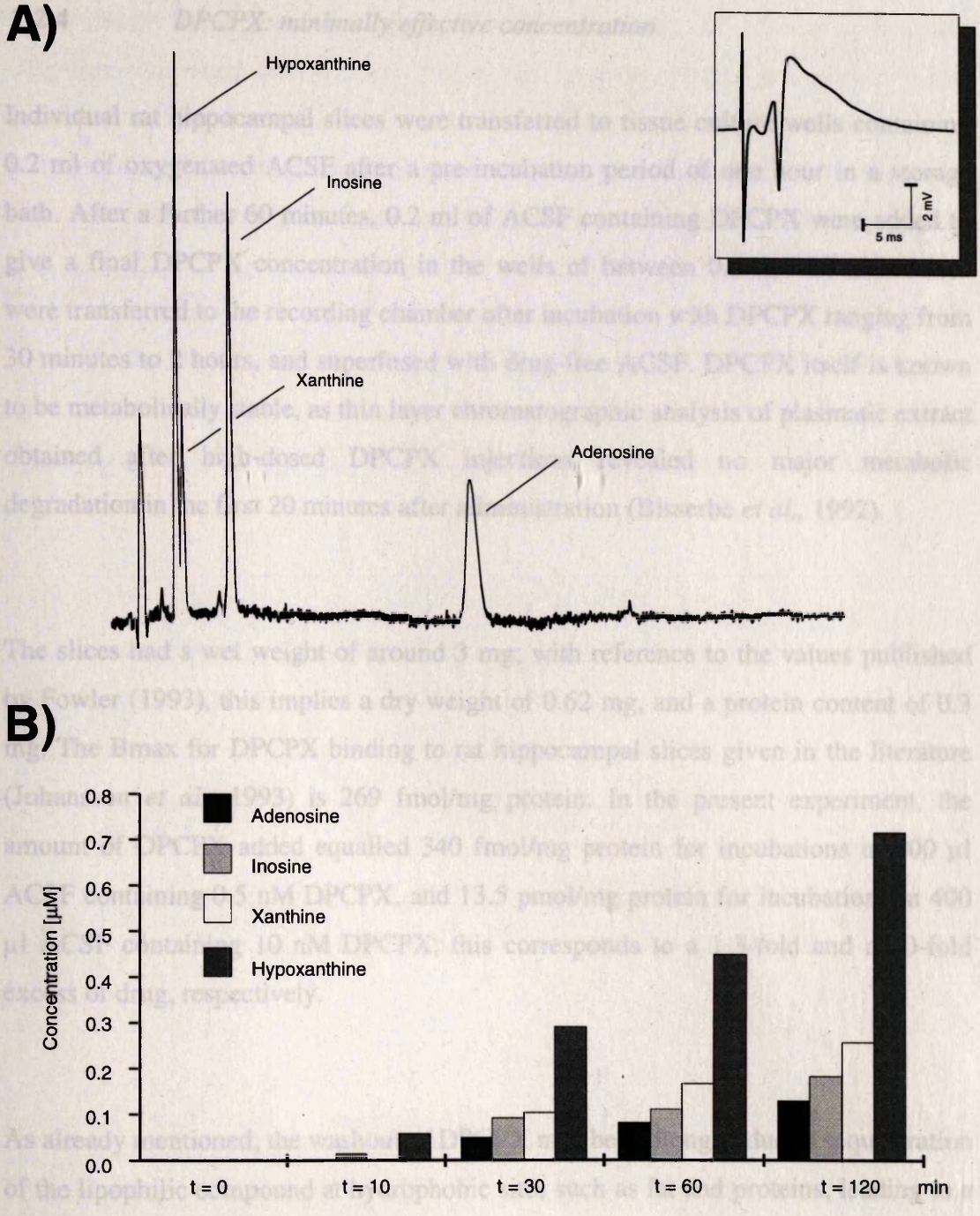


Figure 42: Adenosine and metabolite concentrations during incubation of hippocampal slices. Top: Elution of reference standards (1 µM each of hypoxanthine, xanthine, inosine and adenosine). Inset: Test for electrophysiological viability of a hippocampal slice from the same animal incubated in parallel. The population spike recorded in area CA1 after orthodromic stimulation remained stable over time. Bottom: Adenosine and metabolite concentrations secreted by hippocampal slices incubated in 400 µl ACSF over a 2 hour incubation period. Pooled results from two slices (one from each hemisphere)

7.2.4 *DPCPX: minimally effective concentration*

Individual rat hippocampal slices were transferred to tissue culture wells containing 0.2 ml of oxygenated ACSF after a pre-incubation period of one hour in a storage bath. After a further 60 minutes, 0.2 ml of ACSF containing DPCPX were added to give a final DPCPX concentration in the wells of between 0.2 and 10 nM. Slices were transferred to the recording chamber after incubation with DPCPX ranging from 30 minutes to 2 hours, and superfused with drug-free ACSF. DPCPX itself is known to be metabolically stable, as thin layer chromatographic analysis of plasmatic extract obtained after high-dosed DPCPX injections revealed no major metabolic degradation in the first 20 minutes after administration (Bisserbe *et al.,* 1992).

The slices had a wet weight of around 3 mg; with reference to the values published by Fowler (1993), this implies a dry weight of 0.62 mg, and a protein content of 0.3 mg. The Bmax for DPCPX binding to rat hippocampal slices given in the literature (Johansson *et al.,* 1993) is 269 fmol/mg protein. In the present experiment, the amount of DPCPX added equalled 340 fmol/mg protein for incubations in 200 µl ACSF containing 0.5 nM DPCPX, and 13.5 pmol/mg protein for incubations in 400 µl ACSF containing 10 nM DPCPX; this corresponds to a 1.3-fold and a 50-fold excess of drug, respectively.

As already mentioned, the washout of DPCPX may be prolonged due to sequestration of the lipophilic compound at hydrophobic sites such as fat and proteins, leading to a substantial accumulation (see Fig. 57; and cf. Lüllman *et al.,* 1979), and resulting in a 'depot action' of the perfused drug. The degree to which absorption to lipophilic sites may affect both the extent of receptor occupancy and the duration of the washout was analysed by incubating slices in varying, but limited amounts of DPCPX. However, the difference in response patterns of slices incubated in either 200 or 400 µl of ACSF containing either 2 or 5 nM DPCPX was not statistically significant. Therefore, the data from experiments using 200 µl of incubation medium were pooled with those using 400 µl to give the results shown in Fig. 43. It was obviously

not possible to define the degree of A1 blockade, as electrophysiological measurements were only started during the washout phase; however, a semi-quantitative dose-response curve could be constructed by classifying the spiking patterns observed in slices incubated in various concentrations of DPCPX, which indicated that concentrations as low as 500 pM had a proconvulsant effect.

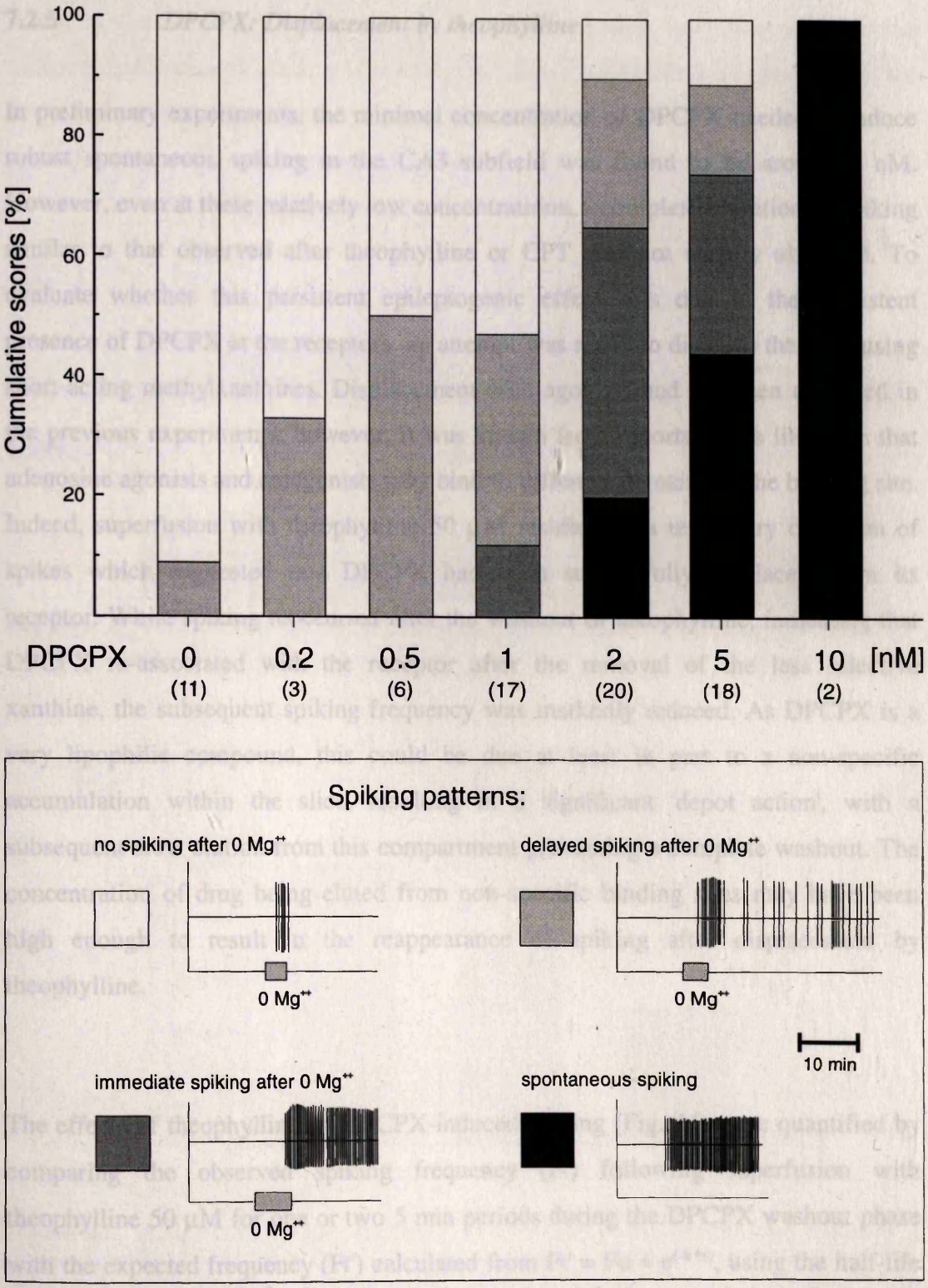


Figure 43: Incubation of rat hippocampal slices in small volumes of ACSF containing defined amounts of DPCPX (see text). The columns (top) illustrate the frequency of occurrence of the four spiking patterns (defined below): no DPCPX-induced spiking after the induction of zero-magnesium spikes; appearance of DPCPX-induced spikes after a lag period; DPCPX-induced spiking immediately after zero-magnesium-induced spiking; appearance of spontaneous spikes upon transfer of the slice to the recording chamber. The number of slices used at each concentration is given in brackets. Slices were from a total of 12 animals.

7.2.5 *DPCPX: Displacement by theophylline*

In preliminary experiments, the minimal concentration of DPCPX needed to induce robust spontaneous spiking in the CA3 subfield was found to be around 5 nM. However, even at these relatively low concentrations, a complete cessation of spiking similar to that observed after theophylline or CPT was not usually observed. To evaluate whether this persistent epileptogenic effect was due to the persistent presence of DPCPX at the receptors, an attempt was made to displace the drug using short-acting methylxanthines. Displacement with agonists had not been observed in the previous experiments; however, it was known from reports in the literature that adenosine agonists and antagonists may bind to different domains of the binding site. Indeed, superfusion with theophylline 50 µM resulted in a temporary cessation of spikes which suggested that DPCPX had been successfully displaced from its receptor. While spiking reoccurred after the washout of theophylline, indicating that DPCPX re-associated with the receptor after the removal of the less selective xanthine, the subsequent spiking frequency was markedly reduced. As DPCPX is a very lipophilic compound, this could be due at least in part to a non-specific accumulation within the slice, resulting in a significant 'depot action', with a subsequent slow elution from this compartment preventing a complete washout. The concentration of drug being eluted from non-specific binding sites may have been high enough to result in the reappearance of spiking after displacement by theophylline.

The effects of theophylline on DPCPX-induced spiking (Fig. 44) were quantified by comparing the observed spiking frequency (Ft) following superfusion with theophylline 50 µM for one or two 5 min periods during the DPCPX washout phase with the expected frequency (Ft') calculated from $Ft' = Fo * e^{(-k*t)}$, using the half-life of drug washout measured in 6 control slices briefly superfused with DPCPX 5-10 nM ($T_{1/2}$, 80 min) under identical conditions. As can be seen from Fig. 44, the observed decrease in spiking frequency is highly significant; this indicates a displacement of DPCPX from A1 receptors. It also suggests that most of the spiking observed during the prolonged washout is due to the persistence of DPCPX at the

receptors. Interestingly, no decrease in spiking frequency was observed after the transient application of adenosine or cyclopentyladenosine (CPA), suggesting that the effect was specific for adenosine antagonists. Similarly, a transient suppression of spiking using high magnesium concentrations (10 mM), or the excitatory acid antagonist kynurenate (1 µM) was without effect on the subsequent spiking frequency.

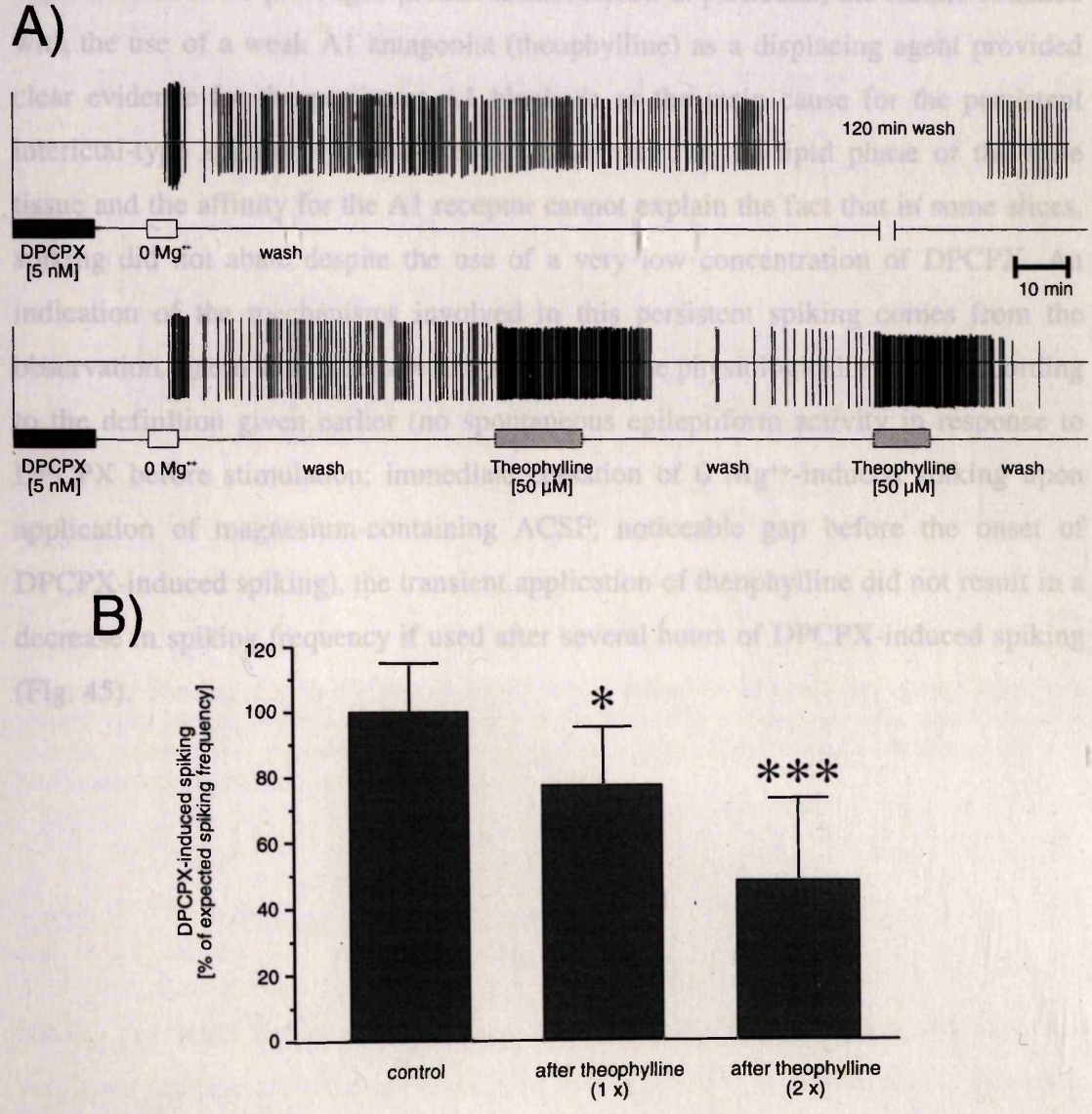


Figure 44: Theophylline 50 µM transiently inhibited DPCPX-induced spiking, indicating a displacement of DPCPX from its receptor (**A**). Re-association takes place from a reservoir within the tissue, probably the lipid phase; however, spiking frequency decreases below the value expected from washout data (Fig. 36): (**B**) illustrates the decrease induced by single (1x) and repeated (2x) brief applications of theophylline (50 µM) expressed as percentage of the expected value (n = 6; *: $p < 0.05$, ***: $p < 0.001$)

7.2.6 *Persistent effects of DPCPX*

The results obtained so far had suggested that of the various factors presumed to be involved in the 'persistent spiking' described in the literature for DPCPX, those associated with the high lipophilicity and the high affinity for the A1 receptor could explain most of the prolonged proconvulsant effect. In particular, the results obtained with the use of a weak A1 antagonist (theophylline) as a displacing agent provided clear evidence for the persistent A1 blockade as the main cause for the persistent interictal-type spiking. However, both the affinity for the lipid phase of the slice tissue and the affinity for the A1 receptor cannot explain the fact that in some slices, spiking did not abate despite the use of a very low concentration of DPCPX. An indication of the mechanisms involved in this persistent spiking comes from the observation that in some slices which appeared to be physiologically viable according to the definition given earlier (no spontaneous epileptiform activity in response to DPCPX before stimulation; immediate cessation of 0 Mg^{++}-induced spiking upon application of magnesium-containing ACSF; noticeable gap before the onset of DPCPX-induced spiking), the transient application of theophylline did not result in a decrease in spiking frequency if used after several hours of DPCPX-induced spiking (Fig. 45).

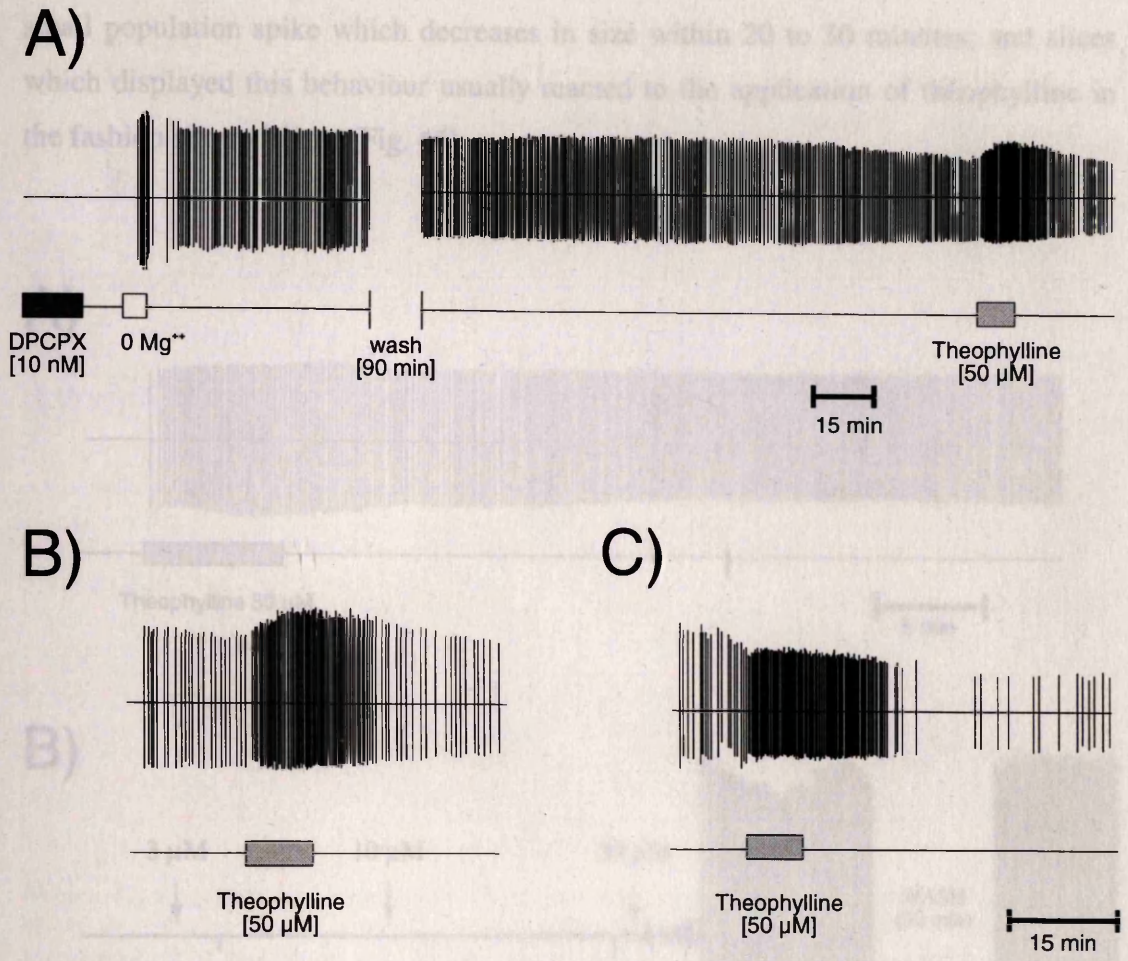


Figure 45: Theophylline 50 µM was no longer able to reduce DPCPX-induced spiking when used several hours into the experiment. The spiking pattern observed in a rat hippocampal slice is shown in (**A**); the last section is expanded in (**B**). For comparison, a trace obtained when theophylline was used 20 minutes after the induction of spiking is shown in (**C**).

7.2.7 *Persistent effects of theophylline*

Finally, a related observation is shown in Fig. 46. Ault *et al.* (1987) had described persistent spiking after the application of theophylline in a concentration similar to that used in the present experiments in the isolated rat hippocampus *in vitro*. They ascribed this phenomenon to a 'kindling' effect. However, it could be demonstrated in the present study that this persistent spiking only occurred after the application of theophylline when the slice had been damaged in some way during the preparation or the subsequent incubation. The state of the slice can be easily assessed by measuring the peak height of evoked population spikes in the CA3 subfield over time (not shown). By convention, damaged slices are characterised by an unusually large or

small population spike which decreases in size within 20 to 30 minutes; and slices which displayed this behaviour usually reacted to the application of theophylline in the fashion shown below (Fig. 46).

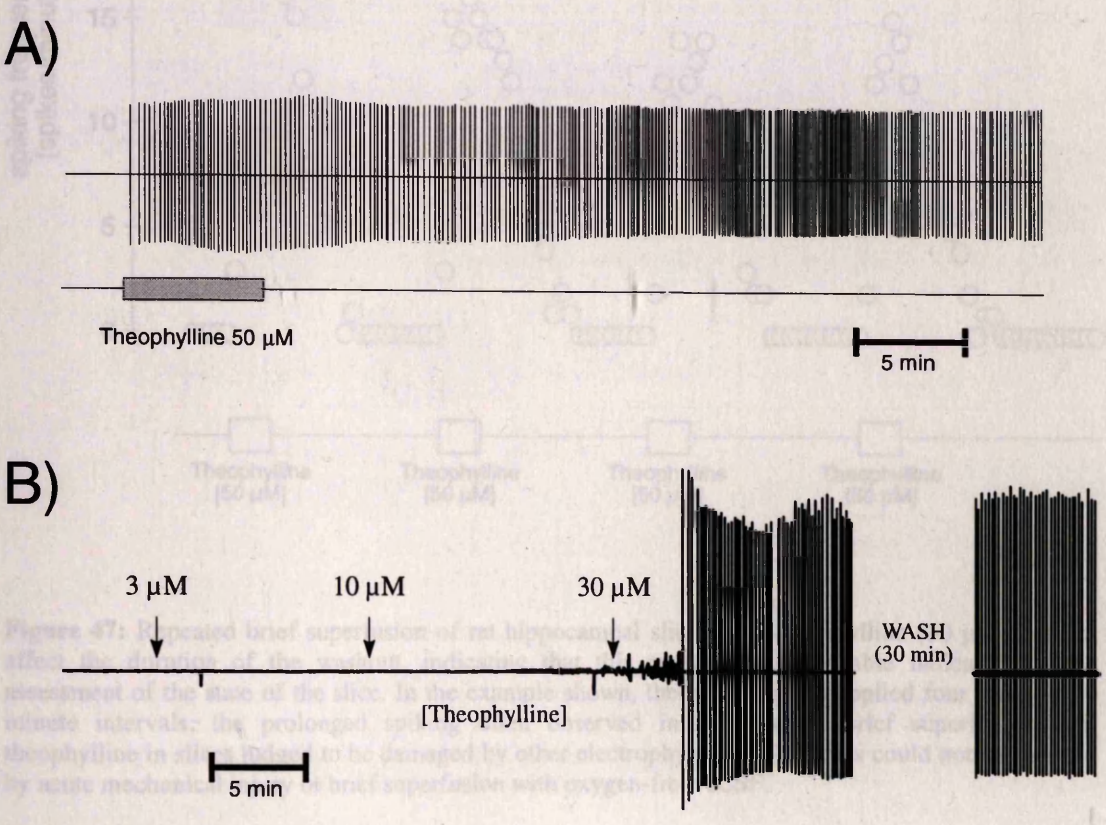


Figure 46: Persistence of theophylline-induced spiking in damaged hippocampal slices: persistent spiking in rat hippocampal slice induced by brief (5 min, indicated by scale bar) superfusion with theophylline 50 µM (**A**) and similar result in hippocampal slice superfused with theophylline 30 µM as reported by Ault *et al.* (1987) (**B**)

The exact mechanism of this damage remains to be elucidated; attempts to reproduce this damage by either mechanically squashing the tissue after the slice had been placed in the recording chamber, or by introducing a brief (10 minute) period of hypoxia, did not result in a different response to theophylline (Fig. 47). However, an indication of the mechanisms involved may come from an experiment in which a low concentration of adenosine (5 µM) was present for one hour during the washout of DPCPX 5 nM. Here, the application of theophylline after one hour resulted in a markedly prolonged proconvulsant effect (Fig. 48).

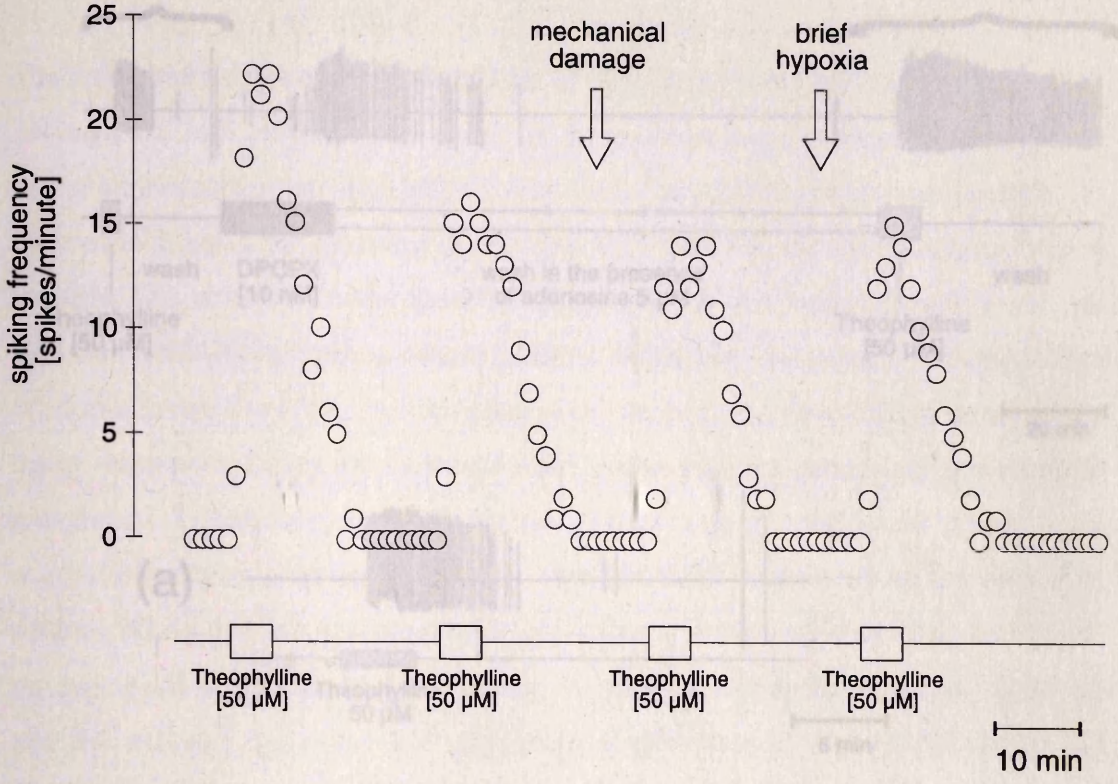


Figure 47: Repeated brief superfusion of rat hippocampal slices with theophylline (50 µM) did not affect the duration of the washout, indicating that this response is a reliable indicator for the assessment of the state of the slice. In the example shown, theophylline was applied four times at 30 minute intervals; the prolonged spiking often observed in response to brief superfusion with theophylline in slices judged to be damaged by other electrophysiological means could not be induced by acute mechanical injury or brief superfusion with oxygen-free ACSF.

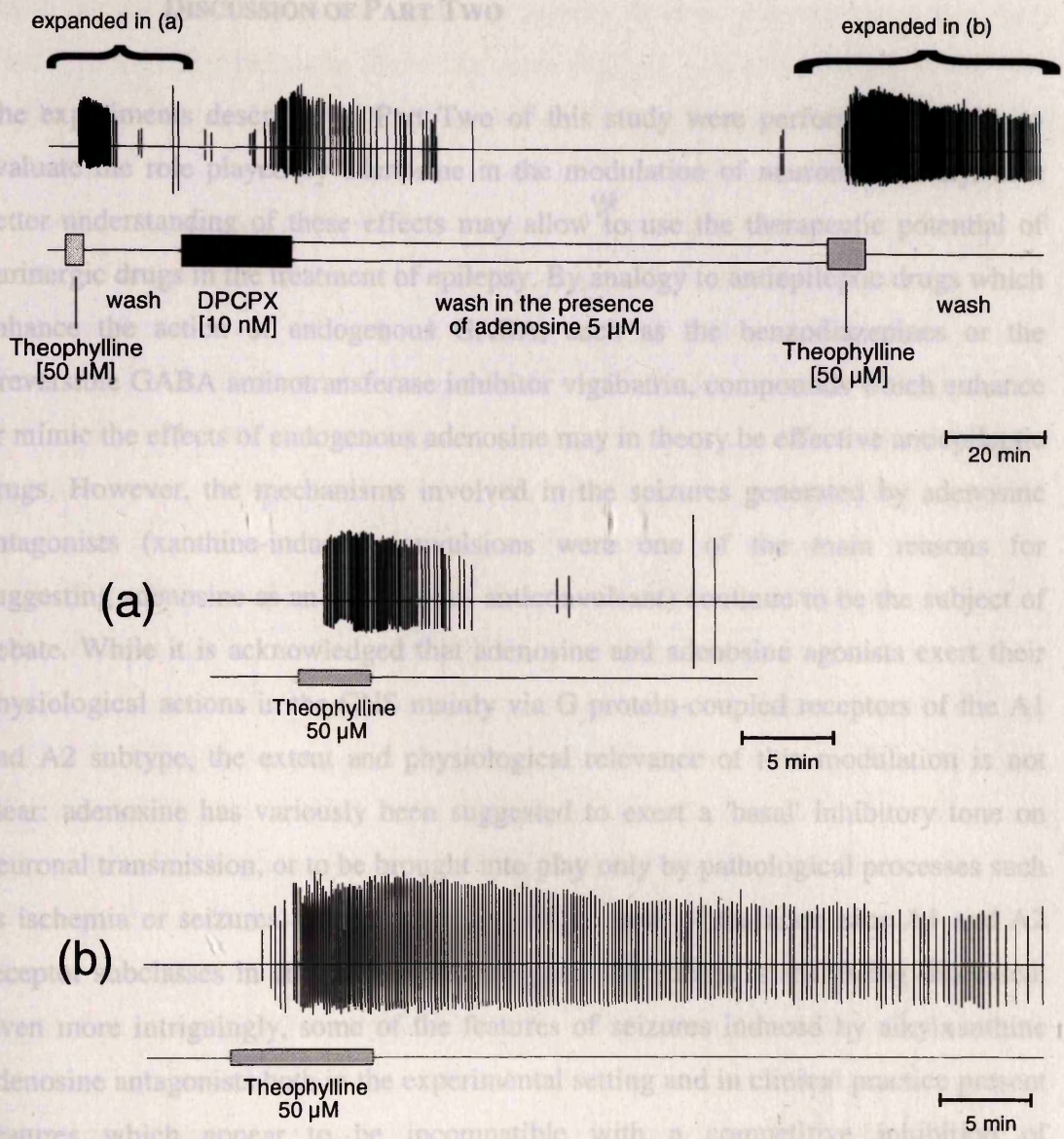


Figure 48: After an initial superfusion with theophylline to test the state of the slice (a), DPCPX (10 nM) was applied for 20 min, followed by a washout phase in the presence of a low concentration of adenosine. A second superfusion with theophylline (b) resulted in a significantly prolonged response.

8 DISCUSSION OF PART TWO

The experiments described in Part Two of this study were performed in order to evaluate the role played by adenosine in the modulation of neuronal activity, as a better understanding of these effects may allow us to use the therapeutic potential of purinergic drugs in the treatment of epilepsy. By analogy to antiepileptic drugs which enhance the action of endogenous GABA, such as the benzodiazepines or the irreversible GABA aminotransferase inhibitor vigabatrin, compounds which enhance or mimic the effects of endogenous adenosine may in theory be effective antiepileptic drugs. However, the mechanisms involved in the seizures generated by adenosine antagonists (xanthine-induced convulsions were one of the main reasons for suggesting adenosine as an endogenous anticonvulsant) continue to be the subject of debate. While it is acknowledged that adenosine and adenosine agonists exert their physiological actions in the CNS mainly via G protein-coupled receptors of the A1 and A2 subtype, the extent and physiological relevance of this modulation is not clear: adenosine has variously been suggested to exert a 'basal' inhibitory tone on neuronal transmission, or to be brought into play only by pathological processes such as ischemia or seizures. Also, the relative importance of the adenosine A1 and A2 receptor subclasses in effects mediated by purinergic drugs is still being discussed. Even more intriguingly, some of the features of seizures induced by alkylxanthine adenosine antagonists both in the experimental setting and in clinical practice present features which appear to be incompatible with a competitive inhibition of endogenous adenosine, suggesting either that the modulatory effect of adenosine can be downregulated under some circumstances, or that secondary effects are involved in the proconvulsant action of alkylxanthines. A better understanding of these effects is clearly cardinal to an assessment of adenosine's physiological role and therapeutic potential.

8.1 Effects of adenosine

The effects of adenosine on both the neocortical tissue cylinder and the hippocampal slice preparation were characterised first in order to be able to assess the extent to

which adenosine can modulate excitatory activity *in vitro*. It needs to be said that, similar to the attempts to use round isolations (both *in vivo* and *in vitro*) in the first part of this study, the originally planned experimental design (which was to be an extension of the method used by Aram *et al.*, 1989) proved not to be practical: it was found that the spontaneous spiking frequency of neocortical cylinders, which was to be used as the main parameter to test for the effects of various purinergic compounds on epileptiform activity, was too variable (see Fig. 35). In the original publication by Aram *et al.* (1989), both the frequency of burst discharges and the number of afterpotentials per burst were measured in spontaneously spiking neocortical slices superfused with magnesium-free ACSF: the differential sensitivity of both parameters to various antiepileptic drugs was used by the authors to elucidate the mechanisms contributing to spiking frequency and afterdischarge generation. However, this feature (that is, the differential response of two related parameters) was felt to be a disadvantage in the present study, as the temporal variability in the number of afterdischarges, and their unpredictable effect on spiking frequency imposed significant variability on the data (a similar effect was noted by Watts and Jefferys (1993): "longer bursts were followed by brief periods of inactivity before the re-emergence of short bursts"). Therefore, neocortical cylinders were only used in those instances where the modulation of epileptiform activity induced by magnesium-free ACSF was to be studied. All other experiments, that is, those involving spontaneous spiking induced by other means, were conducted in hippocampal slices. Hippocampal slices obtained from adult rats usually display only interictal-type activity, whereas both interictal and ictal activity can be evoked in hippocampal slices from young (12-18 days old) rats (Fueta and Avoli, 1993).

Three aspects of adenosine's action were studied: the extent to which adenosinergic modulation is influenced by the absence of magnesium ions, since magnesium-free ACSF was used to induce epileptiform spiking in both the cortex and the hippocampus; the response to adenosine under various experimental conditions, such as repeated application (to assess desensitisation), osmotic changes, or mechanical injury to the tissue; and the basal rate of adenosine production and metabolism.

8.1.1 *Role of magnesium ions*

Adenosine consistently inhibited the 0 Mg^{2+}-induced spiking observed in both neocortical cylinders and hippocampal slices in a dose-dependent fashion, although the data could not be described by a simple sigmoidal function, suggesting the involvement of additional factors such as a high basal adenosine concentration or saturable uptake sites; the concentration needed to evoke a half-maximal inhibition of spiking induced by magnesium-free ACSF (IC_{50}) was estimated at 88 µM (Fig. 30). It should be noted, however, that this represents an underestimate due to the avid uptake mechanisms for adenosine present in the tissue, which result in a decreased concentration in the extracellular space surrounding the receptor. The affinity constant of adenosine at A1 receptors has been inferred to be very close to that of 2-chloroadenosine (2-CA, a non-metabolised analogue of adenosine), *i.e.* 12.8 nM (Bruns *et al.*, 1986); an exact assessment of its potency may be possible in hippocampal monolayer slice cultures (Thompson *et al.*, 1992) and, to a lesser extent (due to the receptor antagonist activity inherent to most uptake blockers), by using dipyridamole or other uptake blocking agents. Interestingly, a tendency towards an enhancing effect of adenosine on epileptiform spiking was noticed at low concentrations (1 to 10 µM; Fig. 30), which however failed to reach statistical significance. It will be discussed in more detail in section 8.2.

When the magnesium concentration of the ACSF was gradually increased, an apparent increase in the potency of adenosine was observed (Fig. 30), in accordance with a study by Bartrup and Stone (1988) reporting that the inhibition mediated by adenosine may be reduced in magnesium-free solution; this suggested that the spiking observed in magnesium-free ACSF may be due in part to the loss of adenosinergic inhibition. A low concentration of magnesium ions may influence the actions of adenosine by several mechanisms. Mono- and divalent cations can modulate receptors by either affecting their conformational states or their coupling with second messenger systems. Nanomolar concentrations of magnesium ions (which are present even in nominally magnesium-free ACSF due to impurities of the salts used) are required for GTPase activity (Gilman, 1987). The magnesium

dependency of adenosine's effects in the hippocampus has been reported to be due to the enhancement of adenosine receptor agonist binding by divalent cations (Yeung *et al.*, 1985): magnesium ions are a necessary prerequisite for the induction of the high-affinity state of the A1 receptor coupled to a G-protein (Goodman *et al.*, 1982) and inhibit the coupling of GTP (and therefore the conversion of the receptor to the low-affinity state). Indeed, a reduction of the magnesium concentration to 0.5 mM decreased radio-iodinated hydroxyphenyl-isopropyladenosine binding to rat brain A1 receptors by 45% (Ströher *et al.*, 1989), and both Ca^{2+} and Mg^{2+} have been shown to be necessary for the maximal binding of cyclohexyladenosine in rat cortical synaptosomes (Traversa *et al.*, 1990), as well as to obtain the maximum binding of R-PIA to A1 receptors from rat cortical membranes (Yeung *et al.*, 1987). Parkinson and Fredholm (1992), while failing to find an effect on equilibrium binding constants, noted that magnesium had pronounced effects on the kinetics of both agonist and antagonist binding, reducing both association and dissociation rates. In electrophysiological studies, a reduction in the sensitivity to adenosine has been observed in magnesium-free medium in the rat hippocampus *in vitro* (Bartrup and Stone, 1988). The residual effects of adenosine in magnesium-free solution may be due to the depression of stimulus train-evoked Ca^{2+} entry (Schubert *et al.*, 1993). Alternatively, the reduced presynaptic sensitivity to adenosine in low magnesium medium has been suggested to be due mainly to higher amounts of Ca^{2+} ions entering the nerve terminals (Smith and Dunwiddie, 1993). Under these circumstances, the decreased efficacy of adenosine would reflect a nonspecific enhancement of synaptic transmission, rather than a direct inhibition of its binding to the receptor. It appears likely that both the reduction in adenosine's postsynaptic effects (the activation of potassium conductances) and an enhancement of activity-dependent presynaptic calcium entry play a role in the observed decrease in adenosine-mediated inhibition. The magnesium dependence of adenosine's effects was not the subject of the present study; however, it is relevant in so far as magnesium-free ACSF was used to induce paroxysmal spiking in some experiments performed to assess the mechanism of action of drugs acting at adenosine receptors. Magnesium ions may alter the binding of endogenous adenosine and therefore the percentage of available receptors; they also affect the gating / voltage-dependency of the NMDA receptor channel, and therefore the development of LTP-type effects. The

influences of these factors are discussed under the appropriate headings in the following chapters.

8.1.2 *Increased sensitivity to adenosine*

An increased efficacy of adenosine was observed when the sensitivity to adenosine was compared in whole and freshly subdivided cylinders (Fig. 32). That this is not due to permanent changes caused for example by the sectioning of the apical dendrites is shown by the finding that cylinders hemisectioned immediately after the dissection and incubated for one hour show a lower sensitivity to adenosine comparable to that seen in whole cylinders. The results from the differential sensitivity to adenosine in cylinders cut during the preparation and cylinders cut acutely prior to recording indicate that the heightened reactivity is a transient effect, which could be related to an increase of basal adenosine release in response to injury (Fig. 49); it confirms the role of adenosine as a protective metabolite.

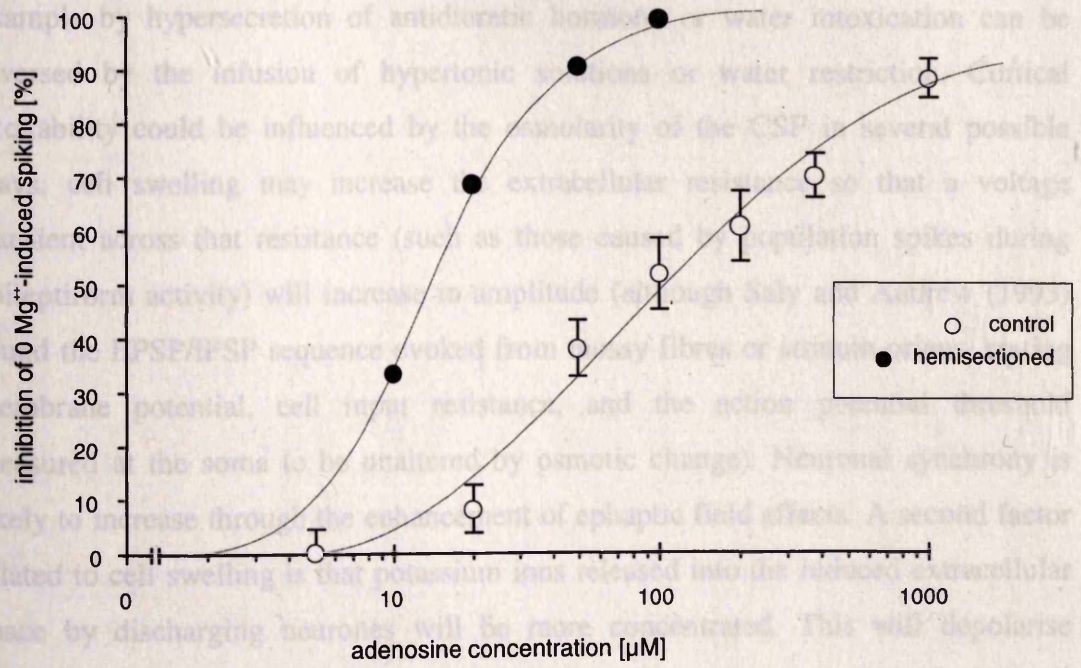


Figure 49: The sensitivity of 0 Mg^{++}-induced spiking in mouse neocortical cylinders to the inhibitory effects of adenosine was markedly increased following a horizontal cut through the middle cortical layers. The graph shows a representative adenosine dose-response curve for a hemisectioned mouse neocortical cylinder (nominal diameter 450 µm), and control values obtained in intact cylinders (cf. Fig. 32).

Alternatively, the enhanced sensitivity to exogenous adenosine observed in acutely hemisectioned cylinders may be related to a swelling of neuronal cell bodies due to injury qualitatively similar to that observed in hypo-osmotic solution (Andrew and Macvicar, 1994), and would imply that purinergic neuromodulators may be effective anticonvulsants in situations where brain oedema or swelling is present. The use of hypo-osmolar ACSF revealed an increased excitability of the tissue (Fig. 33) similar to that reported by Löchner *et al.* (1992) and Saly and Andrew (1993). ACSF compositions reported in the literature vary widely; a survey of all papers published in *Brain Research* in 1993 (103 publications in volumes 600 to 632; see Appendix A) which mention the composition of the artificial CSF solution used reveals that in the hypo-osmolar ACSF used in the present study, only the sodium and chloride ion concentrations are outside the normal range (defined as ± 2 SD), resulting in a significantly reduced osmolarity. Therefore, the increase in epileptiform activity is most probably an osmotic effect. Water retention or water intoxication has been reported to increase seizure frequency in known epileptics, and may even precipitate seizures in otherwise healthy individuals (Dawson *et al.*, 1961). Conversely, the hypo-osmotic symptoms (restlessness, muscle twitching, seizures) caused for example by hypersecretion of antidiuretic hormone or water intoxication can be reversed by the infusion of hypertonic solutions or water restriction. Cortical excitability could be influenced by the osmolarity of the CSF in several possible ways: cell swelling may increase the extracellular resistance so that a voltage transient across that resistance (such as those caused by population spikes during epileptiform activity) will increase in amplitude (although Saly and Andrew (1993) found the EPSP/IPSP sequence evoked from mossy fibres or stratum oriens, resting membrane potential, cell input resistance, and the action potential threshold measured at the soma to be unaltered by osmotic change). Neuronal synchrony is likely to increase through the enhancement of ephaptic field effects. A second factor related to cell swelling is that potassium ions released into the reduced extracellular space by discharging neurones will be more concentrated. This will depolarise surrounding neurones and glia more than if the cells were not swollen (Osehobo *et al.*, 1992), further increasing excitability. Heinemann *et al.* (1994) reported that epileptiform activity in the hippocampal slice is accompanied by a shrinkage of the extracellular space of between 10 and 20%, suggesting that increased excitability and

cell swelling are positively coupled. In the present study, the use of 10% hypo-osmolar ACSF was associated with a transient increase in spontaneous spiking frequency in mouse neocortical cylinders by an average of 77%, and a concomitant increase in the sensitivity to the anticonvulsant effects of exogenous adenosine. Kimelberg *et al.* (1990) have speculated that swelling may nonsynaptically release amino acids and other potential neuroactive substances, either from surrounding neurones or from glial cells; hyperosmotic media were found to inhibit voltage-dependent calcium influx and peptide release in Aplysia neurones (Löchner *et al.*, 1992). The results from the present study (Fig. 33) suggest that the activity of exogenous neuromodulators can be enhanced in hypo-osmotic medium; however, further research will be required to explain the exact mechanisms of the enhanced responsiveness to adenosine observed in acutely hemisectioned cortical cylinders or following acute overhydration.

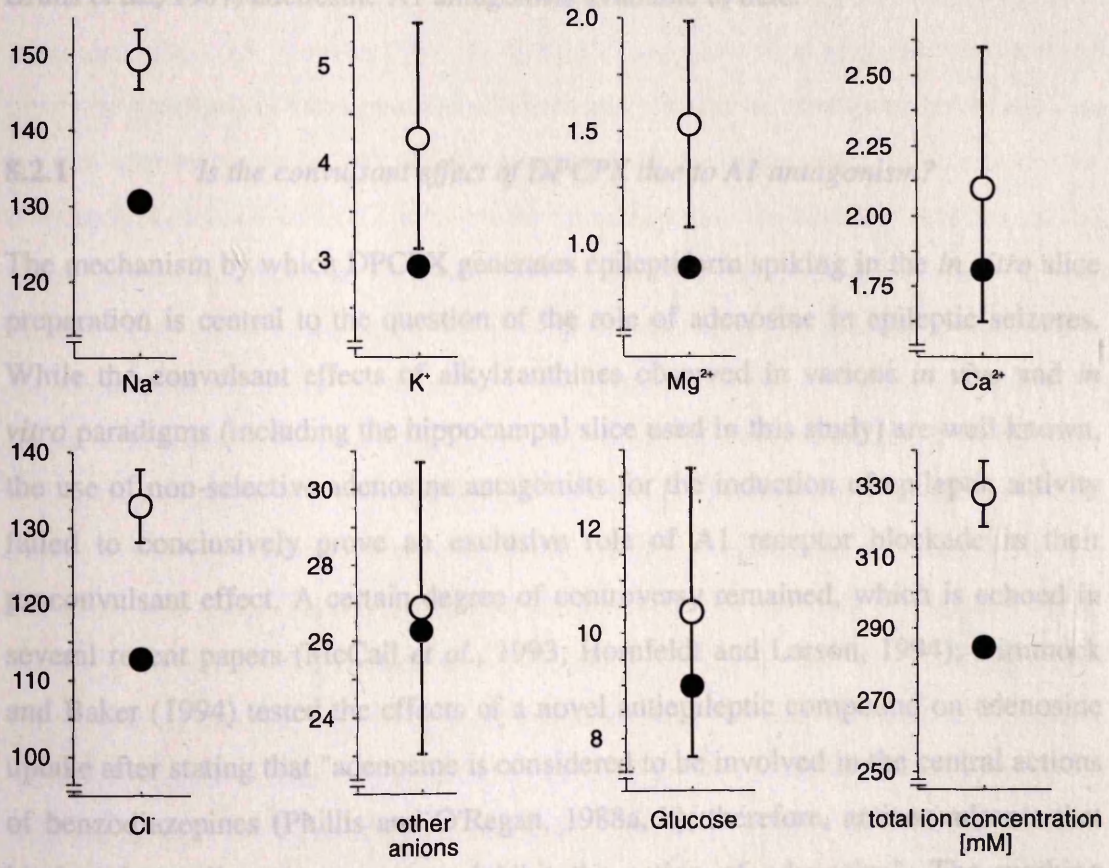


Figure 50: A comparison of the composition of the hypo-osmolar solution used in the present study (filled circles; see also Fig. 33) with ACSF compositions reported in the literature. The unfilled circles represent (in mM) the concentrations (mean ± SD) of ACSF compositions used for *in vitro* mammalian brain slice preparations which were published in the 1993 volumes of the journal **Brain Research** (see Appendix A).

8.2 Effects of alkylxanthines

An understanding of the mechanisms involved in the effects exerted by adenosine in the intact brain is of major importance for the assessment of a possible therapeutic role of adenosinergic compounds in the treatment of epilepsy. As stated earlier, the involvement of A1 receptor blockade has been implied in some models of *in vitro* epileptogenesis. In the present study, three chemically related antagonists at the adenosine A1 receptor were used in order to evaluate the physiological role played by adenosine in normal and 'epileptic' brain tissue: the methylxanthine theophylline (1,3-dimethylxanthine), a naturally occurring, non-selective adenosine antagonist and phosphodiesterase inhibitor, and the two synthetic alkylxanthine derivatives cyclopentyltheophylline (which exhibits 130-fold selectivity for the A1 over the A2 receptor - Bruns *et al.*, 1986), and DPCPX, one of the most selective (740-fold - Bruns *et al.*, 1987) adenosine A1 antagonists available to date.

8.2.1 *Is the convulsant effect of DPCPX due to A1 antagonism?*

The mechanism by which DPCPX generates epileptiform spiking in the *in vitro* slice preparation is central to the question of the role of adenosine in epileptic seizures. While the convulsant effects of alkylxanthines observed in various *in vivo* and *in vitro* paradigms (including the hippocampal slice used in this study) are well known, the use of non-selective adenosine antagonists for the induction of epileptic activity failed to conclusively prove an exclusive role of A1 receptor blockade in their proconvulsant effect. A certain degree of controversy remained, which is echoed in several recent papers (McCall *et al.*, 1993; Hornfeldt and Larson, 1994); Dimmock and Baker (1994) tested the effects of a novel antiepileptic compound on adenosine uptake after stating that "adenosine is considered to be involved in the central actions of benzodiazepines (Phillis and O'Regan, 1988a, b); therefore, anticonvulsants that bind at benzodiazepine receptors inhibit the action of adenosine". The working hypothesis on which the present study was based, namely that the proconvulsant effects of DPCPX cannot be caused by a mechanism other than A1 receptor blockade, was based on the exclusion of several alternative explanations for both the

generation and the persistence of the epileptiform activity: The effects of alkylxanthines on intracellular calcium mobilisation (caffeine modulates the ATP-dependent calcium uptake by binding to the endoplasmatic reticulum binding site for ryanodine [Sawyonok and Yaksh, 1993]) have only been observed at concentrations in the millimolar range. Similarly, an inhibition of phosphodiesterase by DPCPX (similar to that which has been postulated for caffeine and related compounds) is unlikely, since the maximal inhibition of human platelet cAMP phosphodiesterase at a concentration of 100 µM (which represents the limit of solubility for DPCPX) was only 31% (Lohse *et al.*, 1987). In addition, rolipram (a selective phosphodiesterase inhibitor) did not affect evoked field potentials in the hippocampal slice (Alzheimer *et al.*, 1993), and enprofylline (a phosphodiesterase inhibitor with low affinity for adenosine receptors) is far less epileptogenic than theophylline (Czuczwar *et al.*, 1987). Phosphodiesterase inhibitors are usually central depressants (Beer *et al.*, 1972), although recent reports (see for instance Chaviez-Noriega and Stevens, 1994) have described an excitatory response to bath-applied forskolin (an activator of adenylate cyclase) in rat hippocampal slices maintained *in vitro* (and see below). A further argument in favour of A1 receptors as the main site of action of the alkylxanthines used in this study comes from receptor distribution profiles of the main adenosine receptor subtypes: A1 receptors are highly enriched in the hippocampus (particularly in the CA1 subfield - Onodera and Kogure, 1988), while the corresponding A1 mRNA is particularly enriched in the stratum pyramidale (Johansson *et al.*, 1993), indicating that A1 receptors are predominantly located on intrinsic pyramidal cell terminals and dendrites. High-affinity A2a receptors are sparse in the hippocampus (Fink *et al.*, 1992), although Sebastiao and Ribeiro (1992) have reported marked and specific excitatory effects of a selective A2 agonist (CGS 21680, 3-10 nM) on field potentials in the rat hippocampal slice. The largely parallel timecourse of the decrease in spiking frequency and the recovery of the inhibitory effect of adenosine after the application of CPT or DPCPX (Fig. 39) also suggests that the effects of these alkylxanthines are due to the inhibition of endogenous adenosine, although the inhibition of adenosine's effects was slightly less marked than expected from previously published values (eg., Grover and Tyler (1993) reported that adenosine 100 µM depressed EPSP's by 51% in hippocampal slices, but only by 9% in the presence of DPCPX 200 nM). Finally, the effects of adenosine

deaminase and DPCPX were shown to be non-additive in several *in vitro* studies, excluding a novel, non-A1 mechanism of DPCPX, and substantiating the view that A1 receptor blockade by DPCPX is necessary for its proconvulsant effect. Adenosine deaminase is the enzyme which converts adenosine to inosine; it has convulsant effects when applied *in vitro*, which have been linked to the removal of the adenosinergic inhibitory tone; Alzheimer *et al.* (1993) failed to observe additive effects of adenosine deaminase and DPCPX which could have suggested an excitatory effects of DPCPX not attributable to interference with an inhibitory adenosinergic tone. Similarly, Feuerstein *et al.* (1988) reported that the effects of micromolar DPCPX and adenosine deaminase on 5-HT release were in the same order of magnitude. Adenosine deaminase and DPCPX displayed a trend towards an additive effect on spontaneous epileptiform activity in the present study (Fig. 40). The concentration of adenosine deaminase may have been submaximal, despite the fact that 3 units/ml of adenosine deaminase (one unit is defined as the amount needed to deaminate 1 µM of adenosine to inosine per minute at 25°C) have been shown to result in maximal effects on electrically evoked overflow of tritiated compounds in rabbit brain cortex slices (von Kügelen *et al.*, 1992). An alternative explanation will be presented in Section 8.2.4.

In summary, most of the evidence suggests that the proconvulsant effects of DPCPX cannot be attributed to a mechanism not linked to an antagonism of endogenous adenosine at central A1-type receptors. However, some aspects of DPCPX-induced epileptiform activity appear to be incompatible with the expected response extrapolated from results obtained with the less selective A1 antagonists theophylline and CPT. The main inconsistencies regard the inability of DPCPX to induce seizures *in vivo* (see below), and the remarkably prolonged proconvulsant effect of this compound *in vitro*, which appeared to be independent of a persistent blockade of adenosine receptors (Alzheimer *et al.*, 1989): "the spontaneous burst discharges in guinea pig hippocampal slices, once induced [by DPCPX], were apparently irreversible within the observation period, even after prolonged washout (2-3 hours) in normal solution; in contrast, the hyperpolarising action of exogenous adenosine,

which was substantially reduced by DPCPX, recovered within 30-60 min of drug washout, indicating that DPCPX was not irreversibly bound to the A1 receptor".

8.2.2 *Onset of DPCPX-induced spiking*

When the ability of adenosine antagonists to induce seizures *in vivo* is compared to their affinity at the A1 receptor, an interesting discrepancy is noted. Despite its exceptionally high affinity for the A1 receptor (Kd: 0.42 nM in rat brain membranes - Bruns *et al.*, 1987), there have so far been no descriptions in the literature of seizures triggered by the administration of DPCPX to animals *in vivo*: the intravenous infusion of DPCPX 100 µg/kg/min over several hours was well tolerated in a displacement study in anaesthetised rats (Kuan *et al.*, 1992); bolus intravenous injections of DPCPX 0.03 mg/kg (Knight *et al.*, 1991) or 0.1 mg/kg (Knight *et al.*, 1993) had no discernible effects on mean arterial blood pressure or heart rate in anaesthetised rats in studies on the effects of A1 blockade on renal function, while persistently preventing the bradycardia induced by exogenous adenosine. In a behavioural study by Griebel *et al.* (1991) in freely moving mice, the intraperitoneal administration of DPCPX did not produce a caffeine-like motor stimulation in the way a non-xanthine, mildly selective triazolo-quinazoline A2 antagonist did. At the highest dose (6 mg/kg), slightly decreased locomotion and rearing responses, ataxia and lack of co-ordination were noted. Similarly, Dawid-Milner *et al.* (1994) did not record any adverse effects when they analysed the responses to hypothalamic electrical stimulation in anaesthetised cats after the administration of DPCPX (dissolved in ethanol) at a dose of 3 mg/kg intravenously. This lack of effect is not due to a poor blood-brain-barrier penetration; after the intravenous administration of DPCPX 50 to 80 nmol to rats, Bisserbe *et al.* (1992) found a blood/brain concentration ratio below one, which is in line with current concepts of blood-brain-barrier permeability: Hansch *et al.* (1968) have suggested that a log P value of about 2 represents the ideal hydrophobic character for penetration into the CNS.

The results are mirrored by some studies performed in *in vitro* preparations. In organotypic rat hippocampal slice cultures, Thompson *et al.* (1992) reported that bath application of DPCPX at a concentration of 200 nM did not affect either the resting membrane potential or the amplitude of synaptic responses, and Wu and Saggau (1994) noted that DPCPX 200 nM did not affect resting calcium levels in hippocampal slices. DPCPX was also found to have no effects of its own in a variety of experimental paradigms, including calcitonin gene-related peptide-like immunoreactivity release in rat spinal cord *in vitro* (Santicioli *et al.,* 1993), and the orthodromically evoked population spike amplitude in rat hippocampal slices (Sebastiao *et al.,* 1990). These findings were confirmed in the present study: DPCPX at concentrations between 5 nM and 10 μM failed to induce epileptiform spiking in rat hippocampal slices. However, spiking was readily induced by DPCPX in slices which had been exposed to either brief tetanic stimulation or (in most cases) a transient superfusion with magnesium-free ACSF. Conversely, if the stimulus preceded the application of DPCPX (by up to 30 minutes), spiking was observed within fifteen minutes of the start of superfusion with DPCPX. Interestingly, the onset of DPCPX-induced spiking appeared to be qualitatively similar both when stimulation preceded the application of DPCPX, or when the inducing stimulus was given some time after the application of DPCPX. In particular, a characteristic gap was observed before the onset of spiking in both cases (Figs. 37 and 38). This may be due to either one of two (mutually exclusive) mechanisms: either the drug is prevented from accessing the A1 receptor under resting conditions and only binds to it after an increase in excitability, or else A1 receptors are fully blocked by DPCPX without affecting the excitability in the absence of increased synaptic activity; the latter would suggest a predominantly presynaptic site of action for the inhibitory effects of endogenous adenosine. The two alternatives are denoted by curves (A) and (B) in the illustration below (Fig. 51).

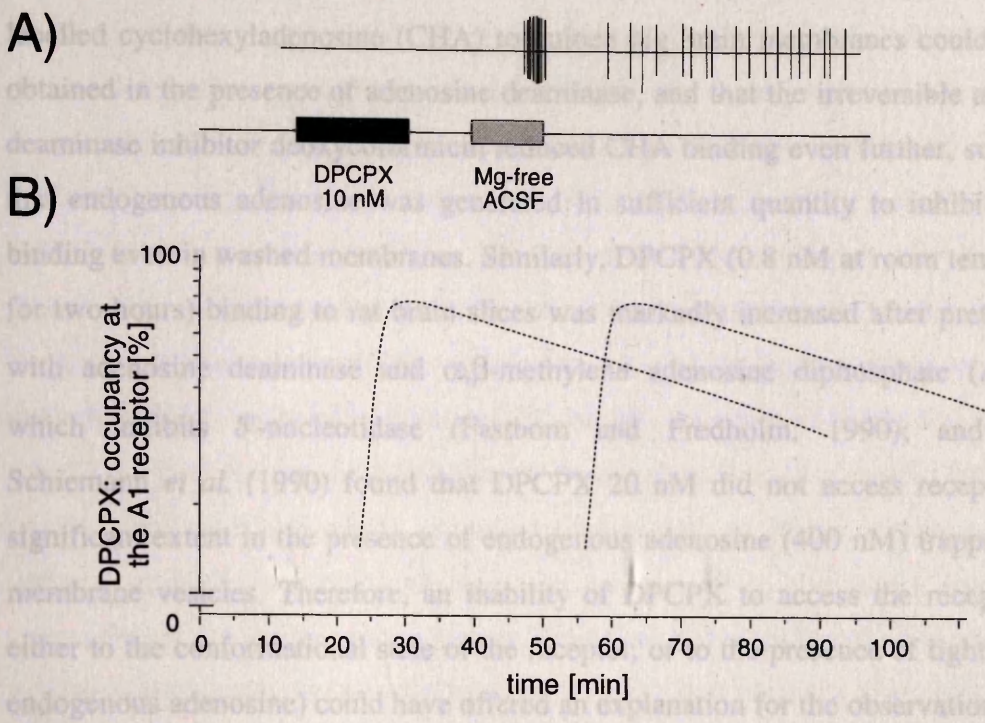


Figure 51: The gap observed before the onset of DPCPX-induced spiking suggests one of two mutually exclusive mechanisms for the onset of receptor occupancy: either the proconvulsant effect is directly related to A1 occupancy, or else adenosine A1 receptors can be blocked without affecting the excitability of the slice until spiking is induced by the superfusion with magnesium-free ACSF. The alternatives are illustrated by the curves shown in B.

An inability to access the receptor could either be due to an unfavourable conformational state (implying negative efficacy), or to the presence of tightly bound endogenous adenosine. The former has been suggested for many antagonists at calcium and sodium channels: lipid-soluble toxin binding to a strongly hydrophobic region such as at the boundary between membrane lipids and the membrane-crossing segments of the channel is known to be greatly enhanced by all treatments that promote or prolong opening of sodium channels, including modification with scorpion toxins and repetitive depolarisation. "Evidently the toxins are free to approach and leave their binding site from the lipid membrane only while the channel is open" (Hille, 1992).

Alternatively, the receptor may be occupied by endogenous adenosine. Adenosine binds with nanomolar affinity to the high-affinity state of the A1 receptor, and very tightly bound endogenous adenosine can prevent DPCPX binding to the receptor: as early as 1980, Bruns *et al.* described that satisfactory binding of radioactively

labelled cyclohexyladenosine (CHA) to guinea pig brain membranes could only be obtained in the presence of adenosine deaminase, and that the irreversible adenosine deaminase inhibitor deoxycoformicin reduced CHA binding even further, suggesting that endogenous adenosine was generated in sufficient quantity to inhibit agonist binding even in washed membranes. Similarly, DPCPX (0.8 nM at room temperature for two hours) binding to rat brain slices was markedly increased after pretreatment with adenosine deaminase and α,β-methylene adenosine diphosphate (AOPCP), which inhibits 5'-nucleotidase (Fastbom and Fredholm, 1990); and finally, Schiemann *et al.* (1990) found that DPCPX 20 nM did not access receptors to a significant extent in the presence of endogenous adenosine (400 nM) trapped inside membrane vesicles. Therefore, an inability of DPCPX to access the receptor (due either to the conformational state of the receptor, or to the presence of tightly bound endogenous adenosine) could have offered an explanation for the observation that the superfusion with magnesium-free ACSF resulted in the appearance of epileptiform activity, as the absence of magnesium ions has been shown to affect both the affinity of the A1 receptor for adenosine, and its conformational state (see above). However, GTP binding to the alpha subunit of the G protein, which is necessary for any G protein-mediated effects of adenosine, is known to lead to a decrease in the affinity of adenosine for the A1 receptor. As the functional effects of adenosine agonists are mediated by the low-affinity state of the receptor (Tawfik-Schlieper *et al.*, 1989), persistent high-affinity bound adenosine would imply the absence of a basal inhibitory purinergic activity; if one accepts the unlikely model of a constitutively active A1 receptor which is inaccessible to DPCPX, this fails to explain the marked effects of exogenous adenosine in all studies listed above, which furthermore could reliably be antagonised by DPCPX. Therefore, an inaccessible adenosine receptor can not be the sole reason for the lack of effect of adenosine antagonists *in vivo* and under certain circumstances (present study) *in vitro*.

Finally, the lag period observed before the appearance of DPCPX-induced spiking is probably not due to a slow association of the antagonist to the receptors, although the time scale was comparable to values reported in the literature (Gerwins *et al.*, 1990); the mechanism may even be independent of the alkylxanthine used. This is suggested

by the fact that a prolonged "rebound secondary afterdischarge" similar to that observed in slices preincubated with DPCPX has been observed *in vivo* after electrically induced seizures in rats pretreated with CPT (Dragunow and Robertson 1987; and see Fig. 52) or with caffeine (Burdette and Dyer, 1987). Also, Peters *et al.* (1984) describe a case of status epilepticus incurred in an elderly patient on theophylline medication after the administration of electroconvulsive therapy (ECT): the latency between the end of the induced seizure and the appearance of spontaneous convulsions was reported to be about 20 seconds. The serum level of theophylline was 15.6 mg/ml, suggesting that - because theophylline is approximately 60% protein bound - the free concentration was around 9.4 mg/ml, or 70 µM. Status epilepticus induced by ECT is exceedingly rare; the causal link to the serum theophylline concentration is therefore rather strong. Therefore, although the slow appearance of spiking under circumstances of A1 receptor blockade is clearly a reproducible effect previously described for seizures induced *in vivo* - and described for the first time for an *in vitro* paradigm in the present study -, it is not likely to be related to the slow association kinetics of the A1 antagonist DPCPX. Rather, it appears that this may be a general feature of alkylxanthine-induced seizures.

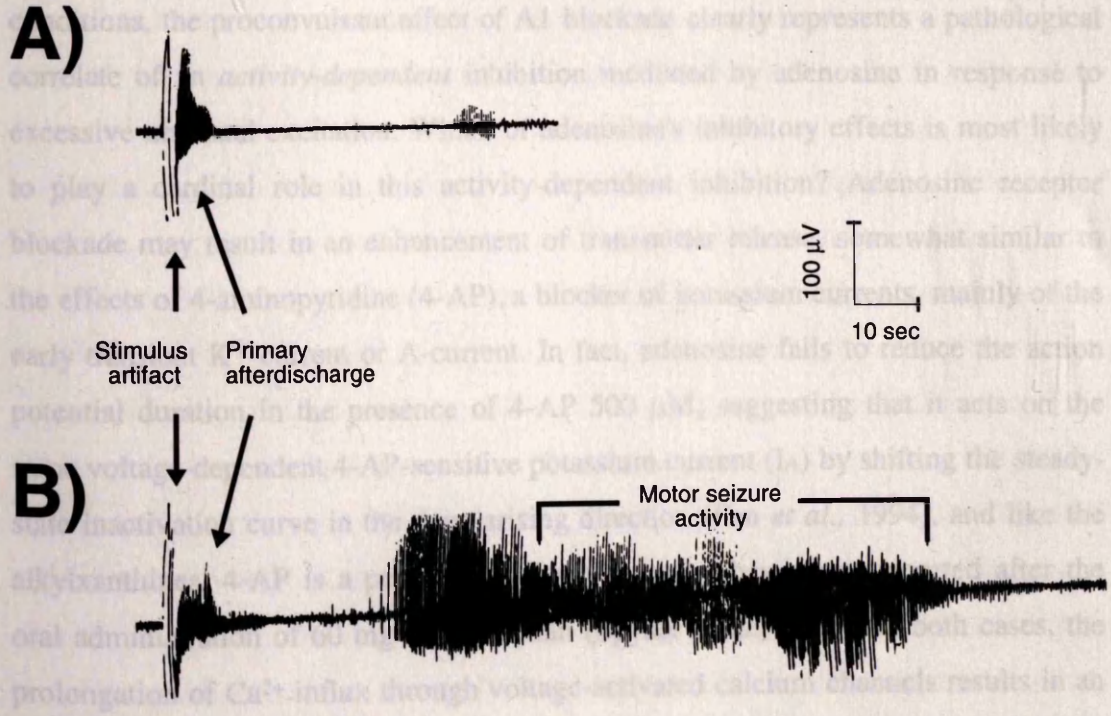


Figure 52: EEG records of vehicle(A)- and 8-cyclopentyl-1,3-dimethylxanthine(B)-treated rats *in vivo* (adapted from Dragunow and Robertson, 1987): after several tens of seconds, a secondary afterdischarge develops in methylxanthine-treated animals which converts from partial to generalised.

The most parsimonious explanation of the data reported both here and in the literature is that the postulated "basal purinergic tone" appears to be of minor functional importance. The concept of a purinergic tone was based in part on estimates of interstitial adenosine levels in the micromolar range. However, the basal concentration of adenosine may have been overestimated due to the invasive methods (such as microdialysis) used for its assessment. Thompson *et al.* (1992) have suggested that the large amount of tissue damage inherent in the preparation of slices, as well as the low rate of exchange between the bathing saline and the extracellular space in the centre of the slice, may lead to an aberrant increase in the extracellular adenosine concentration. Further evidence for the assumption that adenosine is only present at physiologically effective levels under pathophysiological conditions comes from the observation that the binding enhancer PD 81,723, which reduced abnormal hippocampal activity evoked in low-magnesium ACSF, had no effect on hippocampal field potentials under normal conditions (Janusz and Berman, 1993).

Since the antagonism of adenosine receptors has only marginal effects under resting conditions, the proconvulsant effect of A1 blockade clearly represents a pathological correlate of an *activity-dependent* inhibition mediated by adenosine in response to excessive neuronal excitation. Which of adenosine's inhibitory effects is most likely to play a cardinal role in this activity-dependent inhibition? Adenosine receptor blockade may result in an enhancement of transmitter release, somewhat similar to the effects of 4-aminopyridine (4-AP), a blocker of potassium currents, mainly of the early transient K^+ current or A-current. In fact, adenosine fails to reduce the action potential duration in the presence of 4-AP 500 μM, suggesting that it acts on the same voltage-dependent 4-AP-sensitive potassium current (I_A) by shifting the steady-state inactivation curve in the depolarising direction (Pan *et al.*, 1994), and like the alkylxanthines, 4-AP is a potent convulsant: seizures have been reported after the oral administration of 60 mg 4-AP in man (Spyker *et al.*, 1980). In both cases, the prolongation of Ca^{2+} influx through voltage-activated calcium channels results in an increased evoked transmitter release, while resting release remains relatively unaffected (Thesleff, 1980); the net effect would consist of an increase in excitability,

rather than an increase in excitation, although the physiologial relevance of these findings has been questioned (Hu, 1993). However, while a nonselective enhancement of transmitter release may be a powerful proconvulsant mechanism, it fails to explain the fact that spiking induced by magnesium-free solution permitted the induction of spiking by DPCPX up to 20 minutes later (although presumably not hours later, since the dissection procedure, which is also known to result in a transient hyperexcitable state of the tissue, did not affect the response to DPCPX). The superfusion with magnesium-free ACSF was in most cases discontinued as soon as epileptiform spiking occurred, resulting in a total of less than ten interictal-type spikes. Such a weak activation is unlikely to result in an increase in excitation strong enough to be influenced by a facilitation of synaptic release one quarter of an hour later. In fact, the superfusion of slices with magnesium-free ACSF for periods of up to one hour did not result in persistent spiking or other signs of persistently increased neuronal activity, although spiking was observed to be induced more rapidly during subsequent perfusions with magnesium-free ACSF, indicating that some subtle change had taken place. A possible mechanism is suggested by the differential effects of conventional (high potassium) and excitatory amino acid-induced depolariations on intracellular calcium levels: a persistent activation of postsynaptic voltage-dependent calcium channels has been described to occur in isolated CA1 neurones after the application of excitatory amino acids (Wadman and Connor, 1992) which led to a sustained calcium influx into the cell. The spread of a region of elevated intracellular calcium towards the soma could be arrested by application of a kinase inhibitor, and was dependent on the presence of extracellular calcium. Interestingly, the transient elevation of intracellular calcium observed after high potassium-evoked depolarisations, which could be repeated many times without apparent potentiation, was significantly enhanced after challenge with glutamate: "Applying a same K^+ challenge to the cell as was given before the EAA [excitatory amino acid] stimulation now caused a much larger Ca^{2+} change. [...] This high K^+-protocol could be repeated several times without further change to the response. We consistently observed a significant augmentation of the responses to K^+ depolarisation by EAA stimulus even when the persisting effects of the EAA stimulus itself, observed in normal saline, were vanishingly small. Repeated K^+ depolarisations, on the other hand, did not lead to a persistent response, ie. the Ca^{2+} levels returned to predepolarisation levels or

below. [...] The application of GABA facilitated the return to low calcium levels." (Wadman and Connor, 1992). These observations are remarkably similar to the effects described in the present study. If the main physiological effect of adenosine is to exert a negative feedback on the persistent activation of voltage-gated calcium channels by glutamate acting at NMDA receptors, these findings may allow to explain both the failure of DPCPX to induce seizures during physiological brain activity, and the observation that this became possible after the activation of NMDA receptors by superfusion with magnesium-free ACSF.

The alkylxanthine-induced seizures may therefore represent an activity-dependent disinhibition of an adenosine-mediated presynaptic inhibition of excitatory amino acid release. The low convulsant (i.e. seizure-*inducing*) activity of the selective A1 antagonist DPCPX, which contrasts markedly with the effects of less specific compounds such as caffeine and theophylline may be due to the fact that seizures induced by the latter may involve mechanisms other than A1 receptor blockade, i.e. phosphodiesterase (PDE) inhibition, intracellular calcium release or even an elevation of the interstitial pH. In that case, theophylline-induced seizures would represent a two-stage process - the induction of paroxysmal activity, *e.g.* through an inhibition of cAMP metabolism, and a prolongation of the seizure caused by the antagonism of adenosine-mediated inhibition. There is some evidence to support such an assumption: in an article on the EEG effects of theophylline medication, Shucard *et al.* (1985), while stating that "the relationship between theophylline dosage, serum levels and measures of CNS activity is not a simple one", presented data which suggest that the incidence of EEG abnormalities observed in patients treated with theophylline is markedly increased (25% *vs.* 13%) by the concomitant use of ephedrine, a non-selective beta-adrenergic compound.

The failure to induce seizures by A1 blockade may also offer an explanation for the surprisingly high alkylxanthine doses used in some previously published studies: after some reports (eg. Dodd *et al.*, 1986) had indicated that drugs such as carbamazepine may work by binding to adenosine receptors, Hornfeld and Larson

(1994) published a study in which they claimed that neither carbamazepine, nor cyclohexyladenosine or dipyridamole influenced theophylline-induced seizures at clinically relevant doses. However, theophylline was used at doses of up to 6.5 g/kg. Hoffman *et al.* (1993) assessed the effect of pretreating rats with various clinically used antiepileptic drugs prior to a constant rate infusion of theophylline base, and found that the theophylline concentration at the onset of the first major seizure was increased from 285 mg/kg in control animals to 368 and 359 mg/kg after pretreatment with diazepam and clonazepam, respectively, whereas phenytoin (8 mg/kg i.v.) and magnesium sulphate (300 mg/kg i.v.) were without effect. In the light of the present study, these theophylline doses would appear to have induced seizures by a non-specific mechanism, as the concentration used in the present study (50 μM) corresponds to an approximate dose of 20 mg/kg. Similarly, the DPCPX concentration (100 nM) used by Alzheimer *et al.* (1989) may have been sufficient for the induction of spiking and the antagonism of the hyperpolarising effects of adenosine 50 μM, but supramaximal with respect to the maintenance of epileptiform spiking (for which a minimal concentration of 1 to 5 nM was found to be sufficient in the present study - Fig. 43).

8.2.3 *Duration of action of DPCPX: Role of the lipid bilayer compartment*

As discussed in the previous section, the available evidence suggests that the spiking observed after the application of DPCPX is directly related to its blockade of adenosine A1 receptors, at least during the early phase of DPCPX-induced spiking: the main mode of action is likely to be an inhibition of the negative feedback exerted by endogenously released adenosine on increased calcium entry caused by glutamate receptor activation. Several studies have shown that endogenous extracellular adenosine levels rise in response to increased neuronal activity. For example, glutamate released at excitatory synapses can increase extracellular adenosine by acting at both NMDA- and non-NMDA receptors: NMDA-receptor activation results mainly in the release of nucleotides which are then hydrolysed to adenosine, while AMPA-receptor activation results mainly in the release of adenosine itself (Craig and White, 1993). Hoehn and White (1990) have suggested that low level NMDA

activation may already lead to full purine release. In the present study, a substantial amount of adenosine (estimated at 7 pmol/slice/minute) could be shown to be produced by hippocampal slices during a 2 hour incubation period (Fig. 42). Similarly, experimental ischemia, or the induction of experimental seizures in animals, can cause an increase in extracellular adenosine levels (Schrader *et al.*, 1980) which is potentiated by adenosine derived from the hydrolysis of activity-dependent ATP release. The time scale of the rise in extracellular adenosine concentrations is in the range of several tens of seconds, and may be explained by the feed-forward inhibition of 5'-ectonucleotidase by both ATP and ADP: this leads to a delayed production of adenosine from released ATP, which only reaches significant levels once the concentrations of ATP and ADP have fallen substantially (Meghji, 1993).

A transient block of the control mechanism which limits the extent of excitatory responses occurring after NMDA receptor activation should result in an equally transient increase in excitation. However, as noted by Alzheimer *et al.* (1989), the spiking induced by DPCPX (which was reported to persist for "the entire observation period", *i.e.* for several hours) appears to be irreversible, despite the fact that a rapid recovery of the inhibitory effects of exogenously applied adenosine (Fig. 53, below) suggested that the persistent effects of DPCPX were not due to a continuing blockade of postsynaptic A1 receptors.

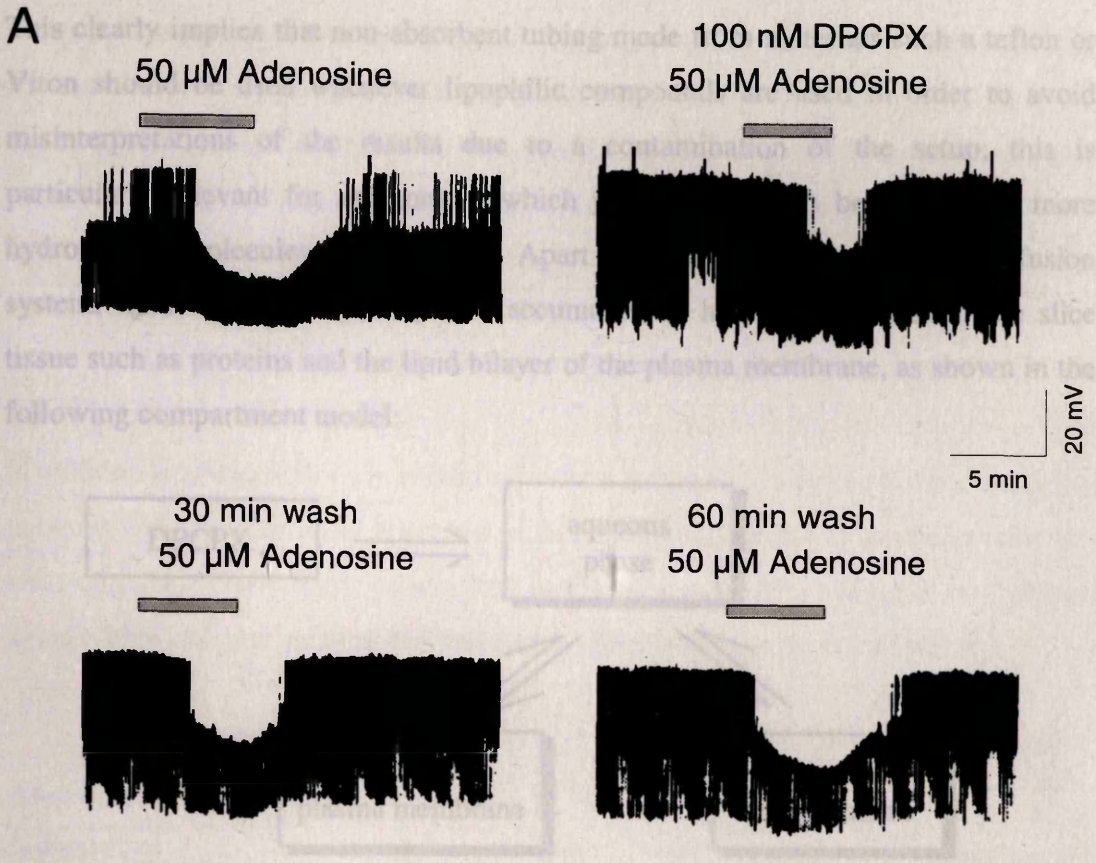
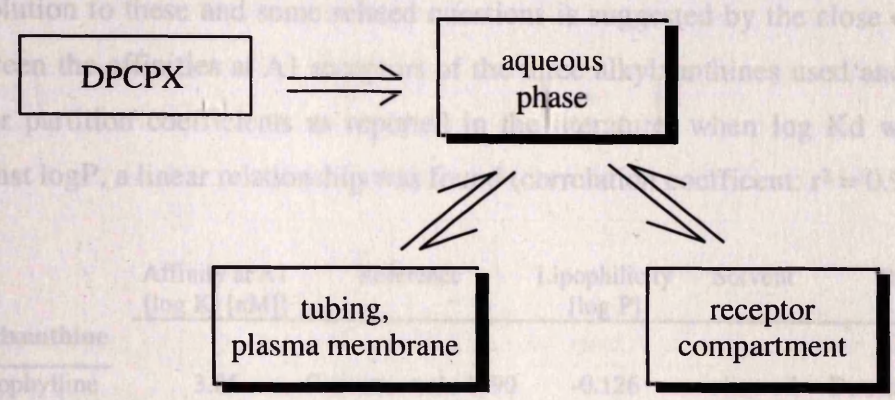


Figure 53: The hyperpolarizing response evoked by the application of adenosine 50 µM, which is transiently suppressed after the brief superfusion of guinea pig hippocampal slices with DPCPX 100 nM, recovers within 60 minutes of washout; the epileptiform activity persists for several hours (data from Alzheimer *et al.*, 1989). Intracellular recordings; downward deflections represent hyperpolarizing current injections.

A prolonged drug washout is not an unusual feature when highly lipophilic compounds are used; these may wash out slowly due to several reasons. Firstly, the drug may be absorbed to plastic and rubber components used in the experimental setup. For the experiments described in the present study, silicone tubing was used; and Starke *et al.* (1978) have conclusively shown in a study on the contrasting reports on the effects of dopamine autoreceptor stimulation that silicone tubing can absorb a substantial amount of lipophilic drugs, which are then slowly eluted upon washout: "After perfusion of silicone tubings with 3H-haloperidol [10 µM] and subsequent washing for 18 hours, the radioactivity in the effluent corresponded to a haloperidol concentration of 28 nM, showing that [...] in spite of careful and copious washing enough haloperidol was retained by, and eluted from, the tubings to interfere seriously with [...] the next experiment.". Indeed, a colleague (H. Hosseinzadeh) working in our laboratory reported a prolonged (> 2 weeks) effect of a single application of DPCPX on extracellular field recordings obtained in rat brain slices.

This clearly implies that non-absorbent tubing made from materials such a teflon or Viton should be used whenever lipophilic compounds are used in order to avoid misinterpretations of the results due to a contamination of the setup; this is particularly relevant for antagonists, which generally tend to be larger and more hydrophobic molecules than agonists. Apart from the absorption to the perfusion system, lipid soluble drugs may also accumulate at hydrophobic sites in the slice tissue such as proteins and the lipid bilayer of the plasma membrane, as shown in the following compartment model:



If access to the receptor is via the aqueous phase, then non-specifically bound drug in the lipid membrane can act as a depot from which leaching into the aqueous phase may occur. As absorption at non-specific binding sites is usually relatively rapid, the sequestration of drug molecules into the lipid phase should decrease the maximal receptor occupancy if the amount of drug delivered to the tissue is limited.

The offset rate is compounded by an additional first-order process, although washout takes place eventually. The introduction of a second compartment (the lipid bilayer) may also explain the delayed onset of action of DPCPX, which in some cases peaked well after the superfusion of the drug was discontinued, although both a contamination of the setup and the accumulation at nonspecific binding sites in the slice cannot result in drug concentrations at the receptor which are higher than the concentration originally found in the perfusing solution if the access to the receptor is via the aqueous phase. However, this model would imply features which are incompatible with the observed results: in particular, the prolonged washout from the

receptor compartment would still reflect the degree of A1 blockade, suggesting that the sensitivity to the inhibitory effects of adenosine should recover in parallel to the decay of the frequency of DPCPX-induced spiking; this was not found to be the case in the original publication by Alzheimer *et al.* (1989), although the results could not be conclusively reproduced in the present study (Fig. 39; see below for a discussion of this discrepancy).

A solution to these and some related questions is suggested by the close correlation between the affinities at A1 receptors of the three alkylxanthines used and their oil-water partition coefficients as reported in the literature: when log Kd was plotted against logP, a linear relationship was found (correlation coefficent: $r^2 = 0.99$).

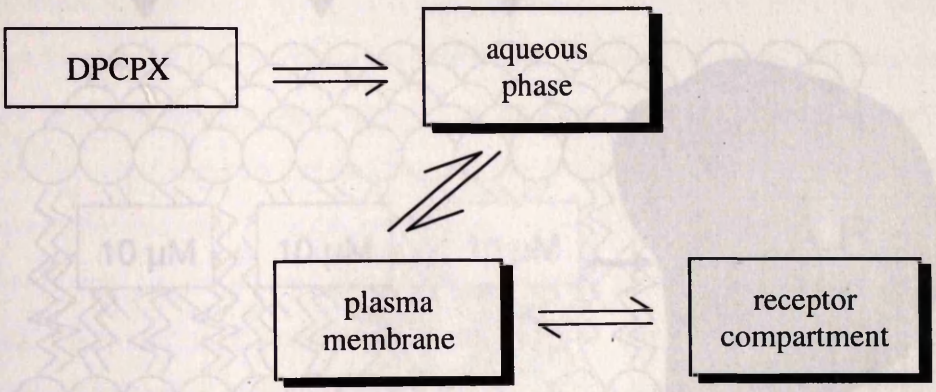
Alkylxanthine	Affinity at A1 [log Ki [nM]]	Reference	Lipophilicity [log P]	Solvent	Reference
Theophylline	3.85	Gerwins *et al.*, 1990	-0.126	olive oil	Donoso *et al.*, 1994
CPT	1.04	Bruns *et al.*, 1987	2	octanol	Bruns *et al.*, 1985
DPCPX	-0.25	Bruns *et al.*, 1987	3.6	octanol	Bruns *et al.*, 1987

Table 3: A comparison of the affinities of the three alkylxanthines used in this study and their lipophilicity (expressed as the logarithm of the partitioning coefficient P) reported in the literature revealed a significant correlation, suggesting that their apparent affinity for the A1 receptor may to a large extent be a function of their accumulation in the plasma membrane.

Despite the fact that the rate of washout depends on the amphiphilicity of the molecule rather than its partitioning coefficient, this suggested the possibility that the duration of action was governed mainly by the concentration in the lipid phase, and prompted the development of a model based on an access to the antagonist binding site *via* the lipid bilayer. Evidence to suggest that some drugs approach the receptor in two-dimensional space by first partitioning into the neuronal lipid membrane was first described by Kokubun and Reuter (1984) for nimodipine, a 1,4-dihydropyridine which blocks L-type voltage-sensitive calcium channels. This route of access is also highly likely with other lipophilic compounds such as DPCPX (L. Herbette, personal communication), as well as applying to adenosine itself, which is uncharged at physiological pH. Adenosine and its synthetic analogues probably cross the cell membrane to a similar extent as alkylxanthines; however, adenosine agonists and

antagonists may bind to different domains of the receptor. This is suggested by the observation that preincubation with adenosine agonists offered relatively little cross-protection against the loss of antagonist binding induced by diethyl pyrocarbonate (a histidine-specific reagent), and vice versa (Klotz *et al.*, 1988). Site-specific mutation of one of the histidine residues at the binding site decreased antagonist but not agonist binding (Olah *et al.*, 1992), and a threonine (Thr-277) residue was shown to be important for agonist, but not for antagonist binding (Townsend-Nicholson and Schofield, 1994). Modelling studies on drug-receptor interaction (Allende *et al.*, 1993) also indicate that alkylxanthines bind to a domain which is different from the agonist binding domain. This may turn out to be a general feature of agonist/antagonist binding; some authors claim that it may not even be valid to assume a single pharmacopore for agonist binding (Neve and Wiens, 1995).

The postulated route of access for alkylxanthine adenosine antagonists is summarised by the following model:



An approach to the receptor *via* the lipid bilayer has two major implications: firstly, the biologically active concentration at the antagonist binding site is likely to be in equilibrium with the plasma membrane concentration and therefore (depending on the partitioning coefficient of the drug) far higher than suggested by the concentration in the aqueous phase. The octanol:water partitioning coefficient (a commonly used measure of lipophilicity) may even represent an underestimate of the degree of drug concentration, as the chemical differences between biological lipid membranes and bulk solvent systems can make the latter unreliable predictors of drug concentration in the membrane: Herbette *et al.* (1989) observed a 7-fold higher partition coefficient

for nimodipine in biological membranes than would be expected from its octanol/water partition coefficient

Additionally, the assumption that the affinity constants based on the concentration in the aqueous phase are hybrid constants, consisting of the lipid partitioning coefficient P and an affinity constant at the antagonist binding site (denoted by K_{eff}) suggests that when the potentiating effect on receptor binding conveyed by the access via the lipid phase is taken into account, these 'true' affinity constants may be of comparable magnitude for a range of alkylxanthines as different as theophylline, CPT and DPCPX (Fig. 54, below).

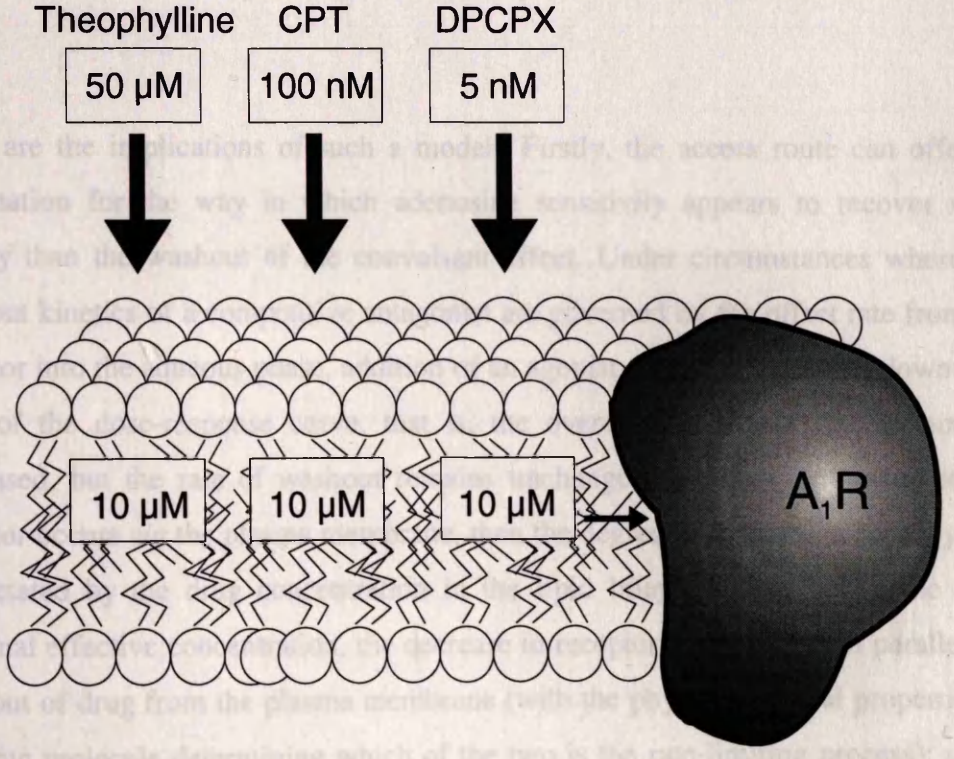


Figure 54: Aqueous concentrations of lipophilic drugs may not reflect their true affinity at the receptor if access to the binding site is via the lipid membrane; in that case, the biologically relevant concentration is the concentration in the lipid membrane, which can be deduced from the lipid partitioning coefficient P. In theory, DPCPX, CPT and theophylline may have broadly similar affinities at the adenosine A1 receptor.

The exact value of K_{eff} will be rather difficult to assess, as the true partitioning coefficient for biological membranes may be different from that obtained in solvents such as octanol or olive oil; furthermore, the collision probability for a drug molecule

in a two-dimensional plane is higher than in a three-dimensional (aqueous) space. However, it may offer an explanation for the finding that the affinity of alkylxanthines at adenosine A1 receptors is to some extent inversely correlated with their degree of phosphodiesterase inhibition. For example, enprofylline (3-propylxanthine), a potent phosphodiesterase inhibitor, has virtually no adenosine antagonist activity, whereas DPCPX has a relatively low affinity for PDE (maximal inhibition: 31% - Lohse *et al.*, 1987); caffeine and theophylline display intermediate values. An inverse correlation becomes plausible if access to the binding site for inhibitors of PDE is assumed to be *via* the aqueous phase: indeed, PDE are mainly found in the cytosolic fraction (Xiong *et al.*, 1995); and the membrane-anchoring N-terminal domain of cAMP PDE, which targets this PDE to membranes, can be removed without affecting its enzymatic activity (Lobban *et al.*, 1994).

What are the implications of such a model? Firstly, the access route can offer an explanation for the way in which adenosine sensitivity appears to recover more rapidly than the washout of the convulsant effect. Under circumstances where the washout kinetics of a competitive antagonist are governed by the offset rate from the receptor into the aqueous phase, addition of an agonist results in a parallel downward shift of the dose-response curve, that is, the overall duration of the washout is decreased, but the rate of washout remains unchanged. If, however, access to the receptor occurs *via* the plasma membrane, then the degree of receptor occupancy will be dictated by the drug concentration in the lipid bilayer. At or below the half-maximal effective concentration, the decrease in receptor occupancy will parallel the washout of drug from the plasma membrane (with the physicochemical properties of the drug molecule determining which of the two is the rate-limiting process); under these circumstances, the recovery of sensitivity of the tissue to the agonist will occur at a similar rate to the washout of the antagonist. In the presence of a large excess of antagonist molecules in the membrane (as is likely to occur with aqueous DPCPX concentrations above 50 nM; see Fig. 43), on the other hand, the same agonist concentration will have a proportionally smaller effect on the degree of receptor binding by the antagonist (and vice versa). The ability to recognise this non-linear agonist response hinges on the accuracy with which agonist-mediated changes can be

detected. Clearly, the lower the agonist concentration used, the more the timecourse of the recovery of agonist-mediated effects will parallel the decline in antagonist-induced effects, *ie.* the washout. Conversely, relatively high adenosine concentrations such as those used in Fig. 53 (50 μM) will result in an apparently biphasic recovery of agonist-mediated inhibition (Fig. 55, inset; and cf. the return of adenosine-induced S-H interval prolongation to 90% of its control value 35 minutes after the application of DPCPX to guinea pig atrioventricular nodes, as reported by Dennis *et al.*, 1992).

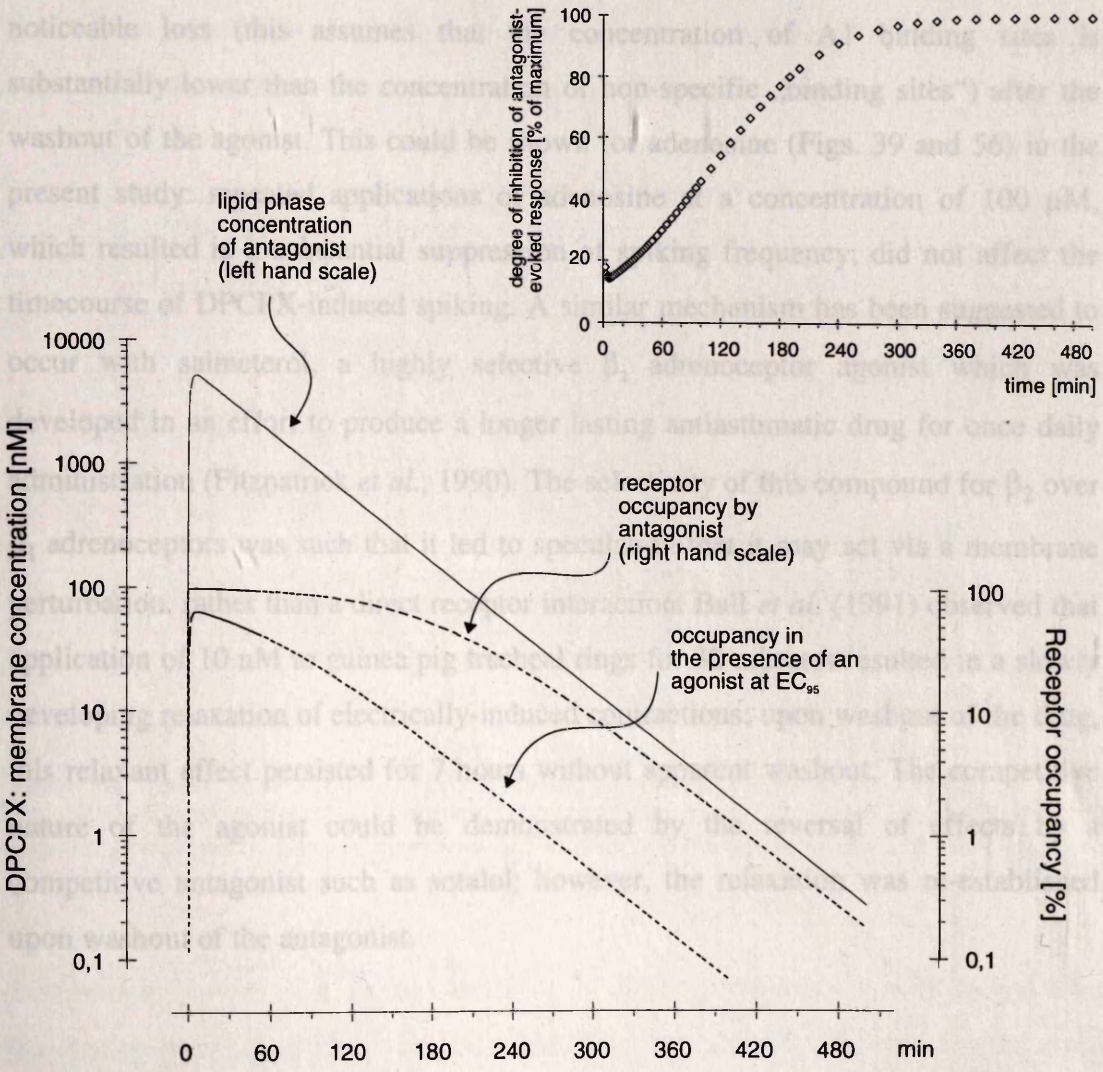


Figure 55: Theoretical calculations show that if drug access to the receptor is via the cell membrane, then the solubility of the drug in the aqueous phase can determine the apparent rate of offset at the receptor. If the fall in concentration in the lipid phase is monoexponential, then the receptor occupancy curves will be sigmoidal; addition of an agonist results in an apparent leftward shift of this curve. The curves are based on hypothetical values (k_a and k_b at the lipid compartment: 0.6 and 0.02; Kd of DPCPX: 5 nM; adenosine concentration: EC_{95}) and are used to illustrate how the sensitivity to the inhibitory effects of adenosine and the receptor occupancy may appear to follow different timecourses at antagonist concentrations above Kd.

Thus, to assume the presence of an excess drug concentration in the membrane during the initial phase of the washout of lipophilic drugs such as DPCPX allows to account for the unusual time course of the recovery of agonist-mediated effects reported previously (Alzheimer *et al.*, 1989).

Secondly, addition of high concentrations of agonist will displace the antagonist into the lipid phase, from where it is free to re-associate with the receptor without noticeable loss (this assumes that the concentration of A1 binding sites is substantially lower than the concentration of non-specific „binding sites") after the washout of the agonist. This could be shown for adenosine (Figs. 39 and 56) in the present study: repeated applications of adenosine at a concentration of 100 µM, which resulted in a substantial suppression of spiking frequency, did not affect the timecourse of DPCPX-induced spiking. A similar mechanism has been suggested to occur with salmeterol, a highly selective β_2 adrenoceptor agonist which was developed in an effort to produce a longer lasting antiasthmatic drug for once daily administration (Fitzpatrick *et al.*, 1990). The selectivity of this compound for β_2 over β_1 adrenoceptors was such that it led to speculation that it may act via a membrane perturbation, rather than a direct receptor interaction. Ball *et al.* (1991) observed that application of 10 nM to guinea pig tracheal rings for 30 minutes resulted in a slowly developing relaxation of electrically-induced contractions; upon washout of the drug, this relaxant effect persisted for 7 hours without apparent washout. The competitive nature of the agonist could be demonstrated by the reversal of effects by a competitive antagonist such as sotalol; however, the relaxation was re-established upon washout of the antagonist.

Similarly, a transient displacement of DPCPX molecules from A1 receptors into the surrounding lipid phase by adenosine would account for the observation that the rate of washout of DPCPX (taken to parallel the decrease in spiking frequency) was not increased by repeated brief applications of adenosine (Fig. 56, below). This could have been due to the avid uptake mechanisms which exist for the purine, which mean that sufficiently high concentrations may not be achieved at the receptor. The

removal of the agonist by uptake or hydrolysis could have been avoided by the use of synthetic analogues of adenosine such as 2-chloroadenosine or cyclopentyladenosine (CPA). Preliminary findings suggest that CPA does not result in a displacement of DPCPX in the present paradigm; however, the use of long-acting adenosine analogues does not allow to discriminate between a factual displacement of DPCPX and a persistent physiological antagonism. Alternatively, the fact that adenosine, while being able to prevent the binding of DPCPX in various experimental paradigms (see above), was unable to achieve a similar displacement may indicate that a large proportion of adenosine A1 receptors were converted to the low-affinity state by the antagonist.

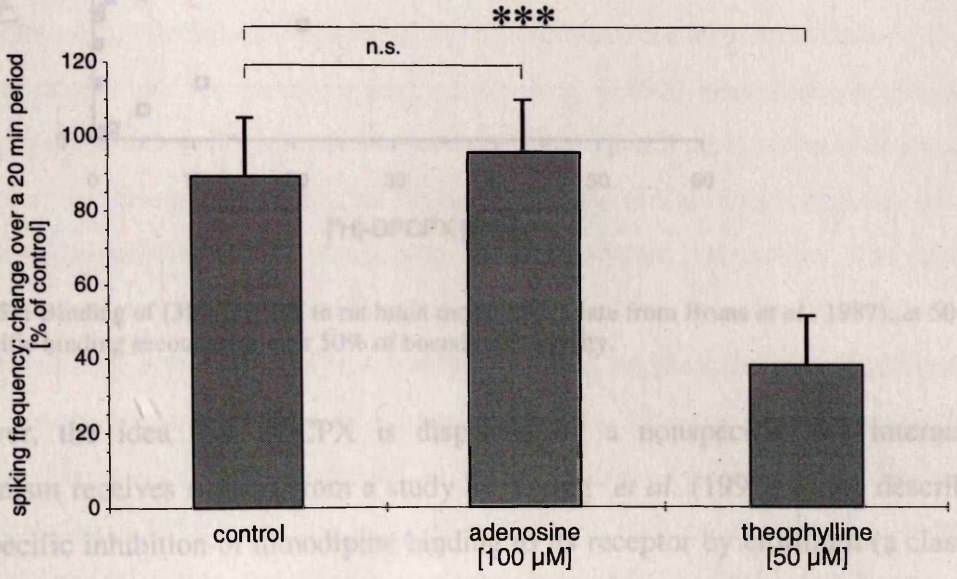


Figure 56: The decrease in DPCPX (10 nM)-induced spontaneous spiking over a 20 minute period was not significantly different in either untreated slices or after transient superfusion of adenosine (100 µM), but was markedly enhanced by transient superfusion of theophylline (50 µM). ***: $p < 0.001$

Intriguingly, however, a marked reduction in spiking frequency was observed after the transient application of theophylline (Figs. 44 and 56). It was known from previously published studies that even weak A1 antagonists like caffeine are able to displace DPCPX to a large extent from its receptor (Deckert *et al.*, 1993). If the hypothesis of a two-stage process of antagonist binding to the A1 receptor proposed in the previous section is accepted, then the finding that adenosine agonists and antagonists differentially affect the displacement of DPCPX from the tissue may imply that alkylxanthine antagonists compete for DPCPX binding at both specific

(receptor) and non-specific (membrane) binding sites. Traditionally, non-specific binding sites have been defined as the non-saturable compartment in receptor binding studies (see *e.g.* Fig. 57, below).

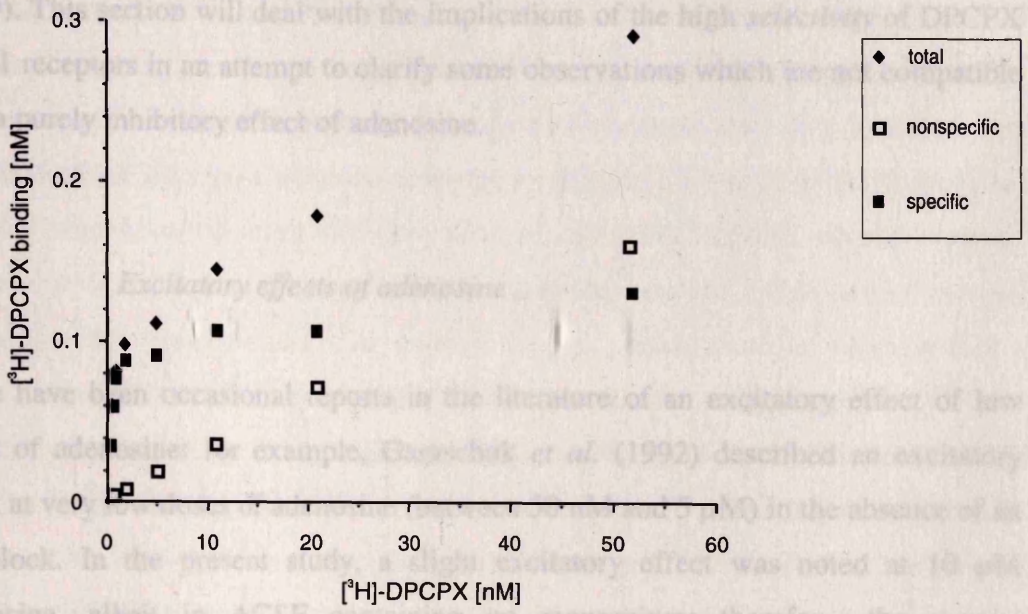


Figure 57: Binding of [3H]-DPCPX to rat brain membranes (data from Bruns *et al.*, 1987): at 50 nM, nonspecific binding accounts for over 50% of bound radioactivity.

However, the idea that DPCPX is displaced by a nonspecific site interaction mechanism receives support from a study by Young *et al.* (1992) which describe a non-specific inhibition of nimodipine binding to its receptor by clofilium (a class III antiarrhythmic) in cardiac sarcolemmal membranes. Their results suggest that by virtue of their membrane localisation, some amphiphilic drugs can inhibit dihydropyridine binding to their receptor site by drug-drug interactions within the membrane.

8.2.4 *Persistent effects of alkylxanthines*

The previous sections have described the extent to which the apparent high affinity of DPCPX for both the plasma membrane and the adenosine A1 receptor may affect its actions in nervous tissue. Following the observation that a transient application of an antagonist, but not of an agonist, results in a permanent displacement of DPCPX from its receptor, a model based on the accumulation of DPCPX in the lipid bilayer

and its subsequent association with the receptor was developed in which washout is determined by the lipophilicity of the compound as well as its affinity for A1 receptors. The model could be shown to explain the apparent rapid recovery of adenosine sensitivity during a prolonged washout described by Alzheimer *et al.* (1989). This section will deal with the implications of the high *selectivity* of DPCPX for A1 receptors in an attempt to clarify some observations which are not compatible with a purely inhibitory effect of adenosine.

Excitatory effects of adenosine

There have been occasional reports in the literature of an excitatory effect of low doses of adenosine: for example, Garaschuk *et al.* (1992) described an excitatory effect at very low doses of adenosine (between 50 nM and 5 μM) in the absence of an A1 block. In the present study, a slight excitatory effect was noted at 10 μM adenosine, albeit in ACSF containing no magnesium; therefore, the agonist concentrations are not directly comparable: adenosine binding to the A1 receptor may be preferentially impaired by a low concentration of magnesium ions. Snyder *et al.* (1981) reported that low doses of adenosine agonists enhanced locomotor activity, and Morgan *et al.* (1989) described a proconvulsant effect of intraperitoneal 2-chloroadenosine, N6-cyclohexyladenosine and NECA on subsequent xanthine amine congener-(XAC)-induced convulsions in mice. This fits in with the findings in the present study of a trend towards an enhanced spiking frequency with low doses (10 μM, Fig. 30) of adenosine, and the rebound phenomenon observed during the wash-out of adenosine 100 μM (Fig. 31; under control conditions, the interval between spike/afterdischarge complexes was characterised by a silent period followed by the gradual build-up of excitatory potentials (Fig. 17), whereas the period immediately following the application of high concentrations of adenosine was characterised by a markedly reduced interval between spike/afterdischarges). Both phenomena have also been described by others while this work was in progress: Nishimura *et al.* (1992) described that the population spike recorded in the granule cell layer of guinea pig hippocampal slices was depressed immediately after the application of adenosine (50 μM), but that after the removal of adenosine from the medium a rebound

phenomenon appeared which they termed 'post-inhibitory excitation'. As with other potentiation-type effects, a vast number of potential mechanisms have been discussed for this effect. Nishimura *et al.* (1992) suggested the involvement of a protein kinase C (PKC)-mediated effect, since the excitatory effects of adenosine were blocked in the presence of PKC inhibitors such as melittin and polymyxin B. The protein kinases C, a group of calcium-dependent protein phosphorylating enzymes of which at least seven isoforms have been identified (Nelsestuen and Bazzi, 1991), are activated either through Ca^{2+} ions entering for example through NMDA receptor-linked channels, or through the activation of receptors coupled to phosphatidyl inositol turnover, such as the metabotropic glutamate receptors. Phosphatidyl inositol hydrolysis by phospholipase A results in the production of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG); the former causes a release of Ca^{2+} ions from internal stores (Miyazaki *et al.*, 1992) and the latter is an endogenous PKC activator (Nishizuka, 1992). PKC activity rises gradually after transient ischemia (Onodera *et al.*, 1989), and may be associated with LTP. Phorbol esters, which stimulate PKC, have been shown to potentiate synaptic transmission in the hippocampus (Malenka *et al.*, 1986). Increases in membrane-associated PKC have also been observed in hippocampus- (Daigen *et al.*, 1991) and amygdala-kindled rats (Beldhuis *et al.*, 1993). PKC activation results in the formation of two membrane-associated states (Nelsestuen and Bazzi, 1991); one form needs co-factors, while the other forms an irreversible complex with the membrane and its activity does not depend on co-factors. Increased PKC activation caused by receptor activation, increased Ca[i], decreased protein phosphatase activity (Cohen *et al.*, 1990), or formation of PKC activators such as DAG or cis-fatty acids results in hyperexcitability of the brain (Kishimoto *et al.*, 1989; Murakami and Routtenberg, 1986). The PKC-induced hyperexcitability may be mediated through phosphorylation of the NMDA receptor. This has been shown to induce a change in the kinetics of Mg^{2+} binding to the NMDA receptor, enabling an activation of NMDA receptor channels at more negative membrane potential levels (Chen and Huang, 1992). Under these circumstances, Mg^{2+} is released from the receptor site at about resting membrane potential, and PKC activation reduces the indirect inhibitory effects of adenosine by reducing the voltage-dependent block of NMDA channels, resulting in a lower frequency being required for calcium influx (Ben-Ari *et al.*, 1992). The loss

of the voltage-dependent magnesium-block means that membrane-stabilising modulators like adenosine are less effective in preventing NMDA channel-mediated calcium influx. Finally, adenosine and A1 agonists, but not A2 agonists, attenuated the vasoconstrictor and negative inotropic effects of the phorbol ester PMA (a protein kinase C activator) (Lasley *et al.*, 1994).

Also, the fact that stable adenosine analogues (NECA, 2-CA, CHA) were able to evoke similar excitatory effects (Morgan *et al.*, 1989) indicates that the excitatory effects are indeed mediated by adenosine acting at adenosine receptors, and not an effect due to metabolic sequelae of adenosine release (adenosine receptor-dependent inhibition of metabolic pathways such as cGMP formation, or an effect of an adenosine metabolite), although the reported excitatory effects of both adenosine agonists (CHA, R-PIA, CGS-21680, NECA) and antagonists (8-CPT, DMPX, CGS-15943, theophylline) in guinea pig superior collicular slices (Ishikawa *et al.*, 1994) awaits independent confirmation. The excitatory actions of adenosine at the cellular level have been suggested to be due to its differential activity on calcium currents. After the observation by Kuroda (1990) of increased presynaptic calcium influx following the selective adenosine receptor activation in neuroblastoma cells, Sakurai and Okada (1992) described an excitatory response to adenosine in hippocampal synapses which resulted in an increased release of glutamate. Subsequently, Mogul *et al.* (1993) found that in acutely dissociated guinea pig hippocampal CA3 cells, blocking the A1 receptors with high concentrations of cyclopentyltheophylline (CPT, 500 μM) unmasked a potentiation of a calcium current by adenosine 50 μM which was insensitive to ω-conotoxin, but sensitive to ω-agatoxin-IVA, suggesting that adenosine activates a P-type calcium current. A similar calcium current potentiation was observed with N6-[2-(3,5-dimethyloxyphenyl)-2-(2-methylphenyl)ethyl)-adenosine (DPMA; 100 nM), a selective A2 agonist, but not with CGS 21680 1 μM, a selective A2a agonist. The results suggest that the excitatory effects of adenosine may be mediated via A2b receptors. This receptor subtype has a markedly lower affinity for adenosine compared to A1, and may therefore have been overlooked in functional studies on adenosinergic modulation. A mechanism involving P-type calcium channels could also help to explain the observation that pure adenosine

transport inhibitors like nitrobenzylthioinosine are markedly less potent anticonvulsants than dilazep (Murray *et al.*, 1993), which has a similar affinity for the adenosine transporter, but additional calcium channel antagonist activities (Nakagawa *et al.*, 1986).

DPCPX: a functional A2 agonist?

Conceivably, the excitatory effects of endogenously released adenosine acting at A2 receptors may be unmasked or enhanced by the selective blockade of A1 receptors by DPCPX, resulting in an action of DPCPX indistinguishable from that of an A2 agonist. This hypothesis assumes that the adenosine receptor block exerted by low DPCPX concentrations is selective for the A1 receptor. Whereas theophylline (Ki at A2b: 4800 nM) and caffeine (Ki at A2b: 48100 nM) (Bruns *et al.*, 1980) are relatively nonspecific, DPCPX is highly A1 specific, allowing endogenously released adenosine (see below) to act either at A2a receptors (both the expression of adenosine A2a receptor mRNA, and the presence of specific binding sites have recently been demonstrated in the hippocampus (CA1, CA3 and dentate gyrus) and cerebral cortex (Cunha *et al.*, 1994)) or at the ubiquitous low-affinity A2b receptor, whose main pharmacodynamic responses include cAMP generation (Bruns *et al.* (1986) give Ki values for adenosine of 12.8 nM, 37.0 nM and 750 µM at A1, A2a and A2b receptors, respectively).

On the basis of previously published results on excitatory effects of adenosine and adenosine agonists such as the observation by Garaschuk *et al.* (1992) of an adenosine-dependent enhancement of alkylxanthine-induced excitation in the rat hippocampus, theoretical considerations, and the results from the present study, a model for the mechanism by which DPCPX may cause persistent proconvulsant effects was developed. It incorporates the two characteristic features of the action of DPCPX (the prolonged persistence in the tissue due to its lipophilicity, and its selectivity), and is based on the following assumptions (see also Fig. 58):

(i) - the initial proconvulsant effect of DPCPX is an enhancement of excitatory activity via a block of A1-mediated inhibition of (presynaptic) transmitter release triggered by other means, and of (postsynaptic) NMDA receptor-mediated calcium currents

(ii) - this proconvulsant effect results in both a prolonged increase in extracellular adenosine levels (cf. Fig 42, and a large, protracted calcium influx into the cells

(iii) - the decreased DPCPX-induced spiking upon application of theophylline during the early phase is due to a displacement of DPCPX from A1 receptors and the cell membrane, resulting in an overall decrease in spiking frequency. (The failure of theophylline to achieve this effect during the late phase (Fig. 45) in some cases may therefore suggest that the spiking is mediated by an effect other than A1 blockade)

(iv) - when DPCPX is applied at low concentrations, the concentration of DPCPX at the receptors present during the prolonged washout is in the range of selective A1 blockade, i.e. A2 receptors are still accessible; this would not be the case with less selective adenosine antagonists

(vi) - under these circumstances (i.e. blocked A1 receptors, accessible A2 receptors, and elevated extracellular adenosine levels), DPCPX acts as a functional A2 agonist, resulting in increased cAMP levels which lead to further adenosine release

In this model, adenosine released during ictal activity may have excitatory effects at A2 receptors which are normally counterbalanced by its activity at A1 receptors (upper left inset). In the presence of low concentrations of DPCPX (Fig. 58, main panel), this excitatory effect may be unmasked due to the A1 selectivity, whereas theophylline and related alkylxanthines, which are relatively nonspecific adenosine receptor antagonists, block both effects (upper right inset).

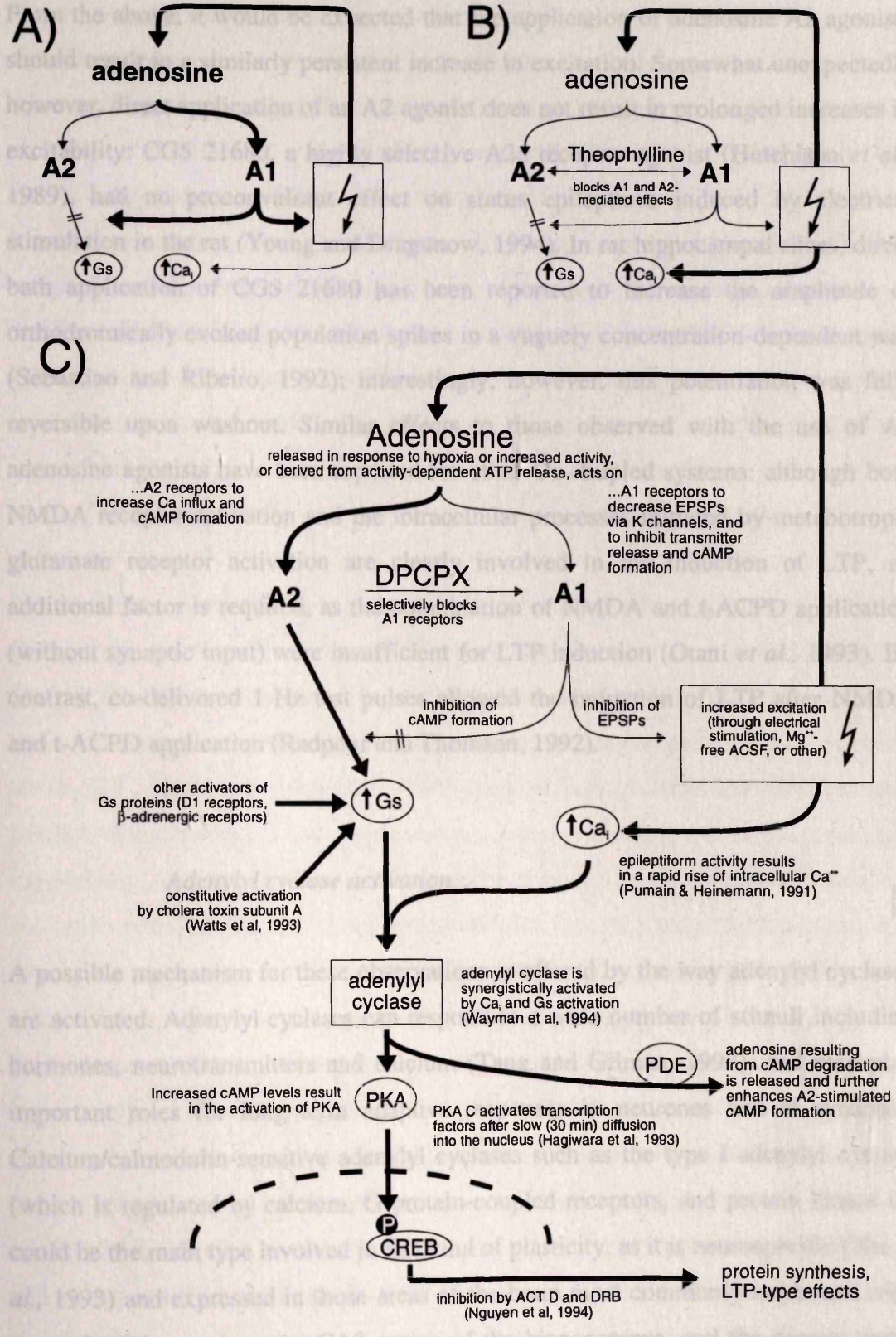


Figure 58: DPCPX may increase long-term excitability by acting as a functional A2 agonist. Shown are the role of adenosine as a 'retaliatory metabolite' (A), the way in which theophylline causes epileptiform activity by inhibiting the action of endogenous adenosine (B), and the proposed mechanism for the long-term effects of DPCPX (C). References are those referred to in the main text.

PKA C = Subunit C of protein kinase A

From the above, it would be expected that the application of adenosine A2 agonists should result in a similarly persistent increase in excitation. Somewhat unexpectedly however, direct application of an A2 agonist does not result in prolonged increases in excitability: CGS 21680, a highly selective A2a receptor agonist (Hutchison *et al.*, 1989), had no proconvulsant effect on status epilepticus induced by electrical stimulation in the rat (Young and Dragunow, 1994). In rat hippocampal slices, direct bath application of CGS 21680 has been reported to increase the amplitude of orthodromically evoked population spikes in a vaguely concentration-dependent way (Sebastiao and Ribeiro, 1992); interestingly, however, this potentiation was fully reversible upon washout. Similar effects to those observed with the use of A2 adenosine agonists have been reported for other Gs-coupled systems: although both NMDA receptor activation and the intracellular processes initiated by metabotropic glutamate receptor activation are clearly involved in the induction of LTP, an additional factor is required, as the combination of NMDA and t-ACPD application (without synaptic input) were insufficient for LTP induction (Otani *et al.*, 1993). By contrast, co-delivered 1 Hz test pulses allowed the induction of LTP after NMDA and t-ACPD application (Radpour and Thomson, 1992).

Adenylyl cyclase activation

A possible mechanism for these observations is offered by the way adenylyl cyclases are activated. Adenylyl cyclases can respond to a wide number of stimuli including hormones, neurotransmitters and calcium (Tang and Gilman, 1992), and may play important roles for long term adaptive responses in neurones and at synapses. Calcium/calmodulin-sensitive adenylyl cyclases such as the type I adenylyl cyclase (which is regulated by calcium, G protein-coupled receptors, and protein kinase C) could be the main type involved in this kind of plasticity, as it is neurospecific (Xia *et al.*, 1993) and expressed in those areas of the brain most commonly associated with neuroplasticity, such as the CA3 region of the hippocampus, and the dentate gyrus (Xia *et al.*, 1991). The involvement in LTP and memory is suggested by observations with type I adenylyl cyclase-deficient mice (Wu *et al.*, 1995) which showed markedly decreased LTP in response to tetanic stimulation, and an impaired performance in the

Morris water task. Interestingly, type I adenylyl cyclase (I-AC), which can be stimulated by activated Gs proteins in purified enzyme preparations, failed to be stimulated by the activation of either beta-adrenergic receptors or glucagon receptors in a study by Wayman *et al.* (1994). However, when intracellular calcium was elevated by adding the calcium ionophore A23187 or carbachol, I-AC became sensitive to isoprenaline or glucagon, suggesting the need for a synergistic stimulation of the enzyme by calcium and G protein-coupled receptors to achieve maximum cAMP signals. The effects were quite marked, with A23187 stimulating I-AC activity 3-fold, while the combination of A23187 and glucagon resulted in a 90-fold increase in cAMP levels. This property has led Bourne and Nicoll (1993) to suggest that adenylyl cyclases can function as 'coincidence detectors'.

Similar effects have also been described for other systems: Radpour and Thomson (1992) reported that the activation of metabotropic glutamate receptors following NMDA activation results in a potentiation which develops slowly over a period of tens of minutes, as compared to the few minutes that are required to fully establish stable LTP. Direct activation of NMDA receptors through treatment of slices with NMDA alone (either by bath application or by iontophoresis) does not produce a long-lasting potentiation of synaptic responses (Manabe *et al.*, 1992). Similarly, co-activation of metabotropic glutamate receptors and beta-adrenergic receptors causes a synergistic increase in cAMP formation in the rat hippocampus, which could not be achieved by isoproterenol or trans-ACPD alone (Gereau and Conn, 1994). The coactivation resulted in a long-lasting (>30 min) increase in cell excitability which appears to be due to the activation of cAMP-dependent protein kinase, since it was completely blocked by protein kinase inhibitors. The physiological effects of second messenger-activated kinases include depolarization and blockade of the Ca^{++} dependent K^+ current I_{AHP} responsible for the slow afterhyperpolarization (Madison and Nicoll, 1988), and the decrease of $GABA_A$-mediated chloride influx (Sigel and Baur, 1988); at the same time, the target G proteins of both A1 and $GABA_B$ receptors are inactivated by PKC and other second messenger-activated kinases (Houslay, 1991), possibly by disrupting the coupling of G proteins to calcium channels (Swartz, 1993).

As shown in Fig. 58, DPCPX may act in a similar fashion by causing both an activation of Gs proteins coupled to A2 receptors (via the activity-dependent increase in extracellular adenosine), and an increase in intracellular calcium (via the enhancement of synaptic activity through the inhibition of adenosine's inhibitory effects at A1 receptors); during interictal spiking, large and very rapid decreases in extracellular calcium have been observed, which clearly suggest a concomitant increase in the intracellular calcium concentration (e.g. Pumain and Heinemann, 1991). The particular significance for the present study lies in the fact that in the case of selective A1 blockade and subsequent A2 activation, this A2 activation is further enhanced by the release of adenosine resulting from the intracellular metabolic degradation of cAMP: low concentrations of DPCPX in the presence of increased synaptic activity may therefore represent a uniquely powerful stimulus for adenylyl cyclase activation. It is conceivable that this mechanism may account for an effect of DPCPX observed in the present study, which could not be explained by either an artefact (i.e., contamination of the setup) or by persistent A1 blockade due to accumulation in the lipid phase. As shown in Fig. 44, the application of theophylline during the early phase of DPCPX-induced spiking led to a transient suppression, and a subsequent significant reduction, of the epileptiform activity, indicating that the proconvulsant effect was related to the blockade of A1 receptors. However, in some slices, no such effect was observed (Fig. 45) when theophylline was applied several hours after the induction of spiking by DPCPX, indicating that the spiking during the late phase was independent of DPCPX blockade of the A1 receptor (the effect was apparently specific for DPCPX, as the application of theophylline or magnesium-free ACSF for over an hour did not result in persistent activity upon washout (data not shown)). If the development of a form of interictal spiking which could not be terminated by displacement with theophylline was indeed time-dependent, this may be an indication for the proposed second-messenger-dependent mechanism. Indeed, although cAMP levels have been shown to rise rapidly in response to receptor stimulation, the development of long-term changes in cellular activity is often a relatively slow process, requiring a prolonged or repeated stimulation of adenylyl cyclase. Nishimura *et al.* (1992) found that adenosine 100 nM evoked a lasting response when applied for 30 minutes, but not when applied for 10 minutes; Garaschuk *et al.* (1992) obtained a massive (4-fold) increase in excitatory

postsynaptic currents after the application of adenosine 10 μM in the presence of CPT 5 μM for 10 minutes which decreased only slowly over the next 40 minutes. The slow onset of cAMP-dependent responses has been suggested to be due to the fact that cAMP generated at synapses and the tips of neurites must diffuse back to the cell body of the neurone (Bacskai *et al.,* 1993). Alternatively, this slow onset may be due to the kinetics of cAMP-activated PKA translocation into the nucleus, as Hagiwara *et al.* (1993) have shown in cultured PC12 cells that the entry of the PKA C subunit into the nucleus occurs over a 30 minute period. C subunit entry into the nucleus is then followed by the activtion of transcriptional factors, as reviewed by Hunter (1995), pp. 229-230 (and see Fig. 58): "binding of cAMP to the inactive PKA holoenzyme, much of which is anchored in the perinuclear region of the cytoplasm through membrane-associated anchoring proteins, releases the active C subunit, which is then competent to phosphorylate its substrates [...]. A fraction of the C subunit population translocates into the nucleus, apparently through passive diffusion, where it phosphorylates its nuclear targets. PKA helps its own cause by activating an inhibitor of protein phosphatase 1, which is the main phosphatase acting on PKA substrates. [...] One of the major nuclear PKA substrates is the transcription factor CREB (= cAMP response element-binding protein), which binds to a palindromic sequence known as the cAMP-regulated enhancer in cAMP-inducible genes. A cAMP-induced gene expression is important in many cellular responses, including the establishment of long-term memory. The PKA C subunit phosphorylates a single serine residue of CREB, resulting in increased transactivating activity."

Protein synthesis has long been suspected to be involved in LTP-type effects: for example, Sargent Jones *et al.* (1992) have shown that the application of the protein synthesis inhibitors cycloheximide, anisomycin or puromycin block the maintenance (but not the induction) of epileptogenesis induced by electrical stimulation in rat hippocampal slices. The involvement of gene transcription in LTP-like mechanisms evoked by cAMP-dependent mechanisms was demonstrated by Nguyen *et al.* (1994) who showed that the long-term potentiation produced in hippocampal slices either by tetanic stimulation or by application of a synthetic cAMP analogue could be

prevented by the simultaneous application of the transcriptional inhibitors actinomycin D and dichloro-D-ribofuranosyl-benzimidazole. The finding of a critical time window in their study (application during the first 120 min following tetanic stimulation prevented the induction of LTP, whereas delayed application starting two hours after the first tetanus was without effect) is consistent with the recruitment of immediate response genes, many of which are expressed between 0.5 and 3 hours after LTP induction in the hippocampus (Abraham *et al.*, 1991). The implication that CREB is involved in LTP-type effects is greatly strengthened by results obtained with experiments on hippocampal slices from CREB deficient mutant mice, where LTP induced by tetanic stimulation decayed to baseline within 90 minutes (Bourtchuladze *et al.*, 1994), even though two other forms of synaptic plasticity (paired-pulse facilitation and post-tetanic potentiation) were normal. Remarkably, the same cAMP signal transduction pathway, including its nuclear components, seems to be required for memory-related functions in a wide variety of species, as long-term memory in the fruitfly *Drosophila* could also be blocked by disrupting CREB function (Yin *et al.*, 1994). Finally, it is of interest to note that in the work by Nishimura *et al.* (1992) describing a 'post-inhibitory excitation' after the application of adenosine which was mentioned earlier, reference is made to the fact that "when the periods of application of adenosine were more than 30 minutes, the population spike maintained a higher plateau level even after washing the slice for two hours. The rebound and facilitating 'post-inhibitory excitation' could be induced at all [adenosine] concentrations greater than 10 μM"; they conclude that "whether or not the post-inhibitory excitation and excitatory effect of adenosine at low doses are mediated by A1 or A2 receptors should be further investigated by using specific agonists and antagonists".

In summary, it is suggested that DPCPX may act to maximally stimulate neuronal adenylyl cyclase by virtue of its selective blockade of A1 receptors. Upon an increase in excitation, either through electrical stimulation or the removal of extracellular magnesium ions, this A1 blockade results in both a massive increase in excitation and a concomitant elevation of adenosine levels in the tissue. At low DPCPX concentrations, this endogenous neuromodulator may then be able to stimulate

adenylyl cyclase via A2 receptors. The synergistic effects of elevated calcium concentrations and Gs activation lead to maximal adenylyl cyclase activity (not noted after the direct application of A2a agonists, as shown by Phillis [1990] for CGS21680), which is further potentiated by the elevated extracellular adenosine levels resulting from cAMP hydrolysis. Elevated intracellular cAMP results in short term changes such as increased synapsin I phosphorylation at synaptic terminals, and in the transcription of genes involved in long-term changes in cellular excitability via a PKA-mediated activation of transcription factors at the level of the soma. The time-course of PKA activation, and the finding of a critical time window for the induction of persistent LTP (Nguyen *et al.,* 1994) suggest a possible mechanism by which DPCPX (a long-acting, selective A1 blocker) may activate a persistent increase in excitability.

Epilepsy models and increased cAMP levels

Cyclic AMP-mediated effects have previously been implied in excitability changes (*e.g.* the down-regulation of the fast desensitizing glycine current in hypothalamic neurons of the rat - Agopyan *et al.,* 1993), behavioural arousal, and synaptic plasticity underlying learning and memory. The fact that Gs-stimulated adenylyl cyclase needs additional factors (increased intracellular calcium) for maximal activation, and that the effects are in part mediated by a slow process involving protein transcription, may explain the difficulty in establishing unambiguously that modulation of neuronal excitability is mediated by protein phosphorylation. In fact, while phosphorylation-dependent modulation of ion channels from the brain have been demonstrated in lipid bilayers and expression systems (Reinhart *et al.,* 1991), it has been difficult to show that protein phosphorylation actually mediates transmitter effects on neuronal excitability in the central nervous system (Pedarzani and Storm, 1993), or even to unambiguously establish the role of cAMP in long-term increases in excitability: LTP-inducing tetanic stimulation does not result in a measurable increase in cAMP in hippocampal slices (Frey *et al.,* 1993), suggesting that any increases may be highly localized. However, recent advances in the development of specific protein kinase and phosphatase inhibitors, and the use of more physiological

models to include several aspects of neuronal activity involved in the generation of long-term changes in excitability, should provide detailed insights into the way these changes are mediated. The fact that these pathways exist in adult brain tissue also suggests an involvement in physiological brain function; this is further indication for the similarity between physiological changes in synaptic plasticity (*ie.* learning and memory; the underlying assumption being that memories are stored in nerve networks through changes in the strength of synapses) and abnormal changes leading to excessive activity and the ability to generate seizures.

Strong evidence for a role of G protein-mediated effects on cellular excitability stems from the animal models of epilepsy developed by JG Jefferys and his co-workers using the application of minute quantities of cholera or pertussis toxins to the brain. These models differ from commonly used models in that their main action is on G proteins and second messenger systems, rather than directly on synaptic transmission, as is the case with the acute application of penicillin to block synaptic inhibition (Part One of this study, *in vivo*), or the lowering of extracellular magnesium concentration to increase excitatory synaptic transmission (Part One, *in vitro*). However, they are functionally similar to the potent adenylyl cyclase activation postulated to be induced by DPCPX, as both cholera toxin (which permanently activates Gs proteins) and pertussis toxin (which acts by inhibiting Gi proteins) shift the balance towards an activation of second messenger-mediated pathways. Cholera toxin injections into various brain regions (Watts *et al.*, 1993; Williams *et al.*, 1993) cause long-lived epileptic foci which generate interictal discharges and brief epileptic seizures intermittently over many days; the A subunit of the toxin which, when introduced into the cell, stimulates adenylyl cyclase, is probably responsible for this effect. Cyclic AMP accumulation has been observed to occur; the prolonged duration of the epileptic syndrome most likely reflects the sustained elevation of cAMP due to the permanent ADP ribosylation of stimulatory G proteins (Gs). The permanent activation is terminated only by the turnover of the G proteins in the membrane. When analysed *in vitro*, the focus is found to extend around 2 mm around the injection track. The main difference found intracellularly is an impairment of potassium current-mediated accommodation of action potential discharges (Watts *et*

al., 1993): cAMP has been demonstrated to depress the slow afterhyperpolarisation in hippocampal neurones (Madison and Nicoll, 1986).

Similarly, an electrical stimulus given to rats pre-treated three days prior to stimulation with pertussis toxin (a compound which inactivates Gi proteins) resulted in status epilepticus, suggesting that impairment of G protein-linked receptors could be involved in the development of status epilepticus. The blockade of A1 receptor-activated potassium channels appeared not to be involved in this effect, as phaclofen (an antagonist at $GABA_B$ receptors coupled to the same subset of K^+ channels as A1 receptors) failed to induce status in this model (Young and Dragunow, 1994). This increase in excitability, or susceptibility to seizures, may be related to other frequently used epilepsy models: for example, elevated cAMP levels have been reported in some experimental models of epilepsy such as seizures induced by $FeCl_2$ injections into the sensorimotor cortex (Hattori, 1990).

Persistent effects of theophylline

As mentioned earlier, the response of hippocampal slices to brief superfusions with theophylline (50 µM) was used routinely to assess the state of the slice. It could be shown that slices judged to be electrophysiologically 'healthy' by other means invariably showed a rapid decline in spontaneous spiking during the washout of theophylline; spiking usually stopped completely within ten to 15 minutes. This did not change after repeated applications of theophylline (Fig. 47), even if slices had been mechanically injured or exposed to brief episodes of low oxygen. 'Damaged' slices on the other hand were characterised by persistent spiking. This long-lasting effect, which was similar to that described for DPCPX in hippocampal slices, and to the clinical observations of theophylline-induced seizures refractory to standard antiepileptic treatment (see below), was also noted by Ault *et al.* (1986, 1987), who reported persistent spontaneous bursting after the application of theophylline (30 µM), but assumed this to be a general property of theophylline: "this effect did not reverse during 15-20 min of washing the slices with theophylline-free medium,

suggesting that the slices were 'kindled' ", in analogy to the long-lasting or even permanent increases in CNS excitability which can be experimentally produced in previously healthy animals by repeated electrical stimulation (Goddard *et al.*, 1969). This results in progressive development of electro-encephalographic and behavioural epileptiform manifestations, such that, for example, a behavioural seizure is readily evoked by stimulation of previously kindled rats after a period of three months without stimulation; kindled brains retain a state of excitability to stimulation which may last for the entire life of the rat. The cellular and/or molecular mechanisms underlying establishment and particularly maintenance of the kindled state are still unclear (Beldhuis *et al.*, 1993), although some indication comes from the observation that protein synthesis inhibition with cycloheximide, anisomycin or puromycin blocks the maintenance, but not the induction of epileptogenesis in rat hippocampal slices by electrical stimulation (Sargent Jones *et al.*, 1992).

It is difficult to assess the reasons for the prolonged epileptiform spiking observed in response to theophylline, as it was found to be impossible to mimick the damage by experimental procedures. Cyclic AMP-mediated effects may be involved, since the application of a low concentration of adenosine (5 μM) for 60 minutes during the washout of DPCPX (10 nM) resulted in a massively prolonged duration of action of theophylline, suggesting that the combined administration of DPCPX and adenosine caused an increase in excitability similar to that observed in 'damaged' slices. However, this is rather tentative, since the persistent spiking observed after the administration of theophylline in damaged slices (Fig. 46) may be due to any of a number of possible mechanisms which increase the excitability of the slice. Several recent reports have focused on the role of NMDA channels in anoxia-dependent LTP. A second mechanism for increased excitability after damage is an alteration in the response of ATP-sensitive potassium channels. Ocana and Baeyens (1994) reported that antinociception produced by R-PIA in mice was inhibited by the K_{ATP} channel blockers gliquidone > glipizide > glibenclamide, increased by the K_{ATP} opener cromakalim, and not affected by the potassium channel blockers 4-aminopyridine and tetraethylammonium.

Independently of the exact mechanism resulting in this exaggerated response to theophylline, these experiments may be regarded as a useful test for uncovering a state of abnormal excitability: in this view, the persistent spiking reported by Ault *et al.* (1987) would not represent a 'kindling', i.e. causative, effect of theophylline, but the unmasking of a 'kindled' state of the brain tissue by theophylline. Similarly, the uncovering of an increased excitability by theophylline may be the reason for the idiosyncratic nature of theophylline-induced seizures in the clinical setting. Theophylline is widely used as bronchodilator despite its relatively narrow range of therapeutic plasma concentrations. One of the most serious manifestations of chronic theophylline toxicity is the occurrence of generalised seizures. These have frequently been reported to be refractory to standard treatment with anticonvulsant drugs (Yarnell and Chu, 1975; Zwillich *et al.*, 1975; Nakada *et al.*, 1983; Peters *et al.*, 1984), and often result in either permanent neurological damage or death. Although early reports suggested that these convulsive episodes only occurred in the high (toxic) dose range, recent reports indicate that seizures may occur within the therapeutic range or even below (Bahls *et al.*, 1991). The fact that theophylline-induced seizures are associated with a wide range of plasma theophylline concentrations indicates that the pharmacodynamics of theophylline neurotoxicity may depend on variables such as pathophysiological conditions and concomitant drug therapy, and many other variables that are as yet unrecognised: no causative lesions and no prior history of epilepsy were found in five out of 14 elderly patients who died after status epilepticus which occurred during theophylline therapy (Mori *et al.*, 1992; status epilepticus is a life-threatening condition characterized by unremitting seizure activity, which usually does not begin as an unlimited seizure, but rather builds up gradually from initially discrete seizures, suggesting a failure of endogenous seizure-terminating systems). Further evidence for the idiosyncratic nature of these seizures is the fact that the convulsions observed in the presence of methylxanthines do not invariably lead to an entry into status epilepticus, but instead may be self-limiting. This is observed both with ECS treatment (studies reporting the use of caffeine to prolong electroconvulsive shock treatment include Coffey *et al.*, 1990; Cantu and Korek, 1991; Francis and Fochtmann, 1994; Lurie and Coffey, 1990; and Rasmussen and Zorumski, 1993), and in accidental poisoning: in a recently reported case, Würl (1994) described an extremely brief, spontaneously subsiding

tonic-clonic seizure in a patient who had ingested 500 g of ground coffee beans, resulting in caffeine plasma levels of 29 µg/ml.

These clinical reports of a highly variable response to theophylline (ranging from a prolongation of induced seizures to life-threatening status epilepticus) and the results from the present study (see Fig. 46) suggest that alkylxanthines may contribute to paroxysmal activity in one of two (not mutually exclusive) ways: either by enhancing neuronal activity through the inhibition of adenosine-mediated effects on transmitter release and the postsynaptic membrane potential, or by unmasking an underlying brain dysfunction. Whether the latter is related to the postulated second messenger-induced changes triggered by the activation of A2 receptors as discussed in the previous section remains to be clarified.

Conclusion

The present study has attempted to elucidate some of the physical (connectivity of neurones) and biochemical (cellular excitability) requirements for the generation of epileptiform seizures and ultimately, for epilepsy. It could be shown that the cortical tissue dimensions required for epileptiform activity can be surprisingly small, and do not require the inclusion of either a specific cortical layer or of a presumptive 'basic cortical module' such as the barrels found in rodent primary somatosensory cortex. Also, the continuing debate on the role of third-messenger mediated effects in LTP-type mechanisms, and in particular of methylxanthine-mediated seizures ("caffeine lengthens the duration of convulsions [but] the mechanism of caffeine's effect is unknown" - McCall *et al.*, 1993), may be due to a combination of factors. Firstly, pharmacological blockade of the adenosine A1 receptor in intact brain tissue does not necessarily induce epileptiform activity. While the present studies do not allow to discriminate between an inaccessible A1 receptor and the lack of a physiologically relevant adenosinergic inhibitory tone as the reason for the inability of DPCPX to induce epileptiform spiking both *in vitro* (Chapter 7) and *in vivo* (see Chapter 5), they do suggest that the marked increase in interstitial adenosine levels under pathological circumstances plays an important role in counteracting glutamatergic/cholinergic

excitation. The concept of a basal 'purinergic tone' may have been based on overestimates of extracellular adenosine concentrations obtained by biochemical *ex vivo* techniques or invasive sampling procedures; this has recently been supported by an electrophysiological study (Dunwiddie and Diao, 1994). Alternatively (and based in part on the observation that the focal application of AOPCP results in severe seizure activity in the rat - Zhang *et al.*, 1993), the A1 receptor may be occupied by tightly bound endogenous adenosine, which displays nanomolar affinity towards the high-affinity state of the A1 receptor. This, however, leaves open the question as to which configuration of the agonist/receptor/G protein complex is involved in the effects mediated by adenosine. Secondly, while it is feasible that long-term blockade of A1 receptors may cause persistent changes in excitability by a mechanism outlined in the previous section, the main reason for the persistence of seizure-like activity following A1 receptor blockade (such as, for instance, status epilepticus during theophylline therapy; or the persistent spiking in hippocampal slices described by Ault *et al.*, 1987; or the 'late' component of DPCPX-induced spiking shown in Fig. 45) is probably the unmasking of an underlying neuronal dysfunction. Thirdly, it could be shown that the timecourse of DPCPX-induced epileptiform activity can be adequately explained by assuming that methylxanthines access their binding site *via* the plasma membrane. The resulting 'depot action' of the drug could be shown to account for both the prolonged effects of DPCPX, and for the apparent rapid recovery of agonist-mediated inhibition. Intriguingly, the differential effects of adenosine receptor agonists and antagonists on the washout of DPCPX may indicate that binding of alkylxanthines to the A1 receptor involves a two-stage process: anchoring of the molecule within the plasma membrane, and occupation of the binding site on the receptor. The involvement of both non-specific (membrane) and specific (receptor protein) binding components has recently been suggested for dihydropyridines (Young *et al.*, 1992); the present study may furnish the functional evidence for the proposed mechanism of binding.

The final chapter represents a brief discussion of the potential of purinergic compounds as antiepileptic drugs, and suggestions for further studies on the observations discussed in the previous sections.

9 THERAPEUTIC USE OF PURINERGIC COMPOUNDS

As changes in adenosinergic systems have been implied in the pathophysiology and biochemistry of conditions as diverse as anxiety and sleep disorders, psychosis, neurodegenerative pathology, cerebral ischemia and convulsions (Bisserbe *et al.*, 1992), adenosine or related compounds could potentially have a wide therapeutic scope. Furthermore, several adenosinergic drugs are approved for human use; adenosine for example has already been licensed for clinical use in several European countries: it is currently one of the most effective drugs for the treatment of supraventricular cardiac arrhythmias in juvenile patients (Clarke *et al.*, 1987).

As far as epilepsy is concerned, the depressant action exerted by adenosine upon central neurones may enable endogenous adenosine or a related compound to act as an antiepileptic agent. Several lines of evidence support this: first, brain levels of adenosine increase within seconds following the onset of experimental seizures (Schrader *et al.*, 1980). Second, adenosine, its analogues and adenosine uptake inhibitors are effective in decreasing epileptiform activity in both *in vivo* and *in vitro* models of epilepsy (Barraco *et al.*, 1984, Ault and Wang, 1986; but cf. the lack of effect of R-PIA on strychnine- and pentylenetetrazole-induced convulsions at doses which exhibited marked locomotor depressant effects (Snyder *et al.*, 1981)), while adenosine antagonists possess potent convulsant activity, as discussed in this study. Third, theophylline-induced increases in cyclohexyladenosine binding in the brain were found to parallel an increase in seizure threshold in rats (Szot *et al.*, 1987). Finally, while adenosine inhibits synaptic transmission at virtually all excitatory synapses in the hippocampus, it does not inhibit transmission at GABAergic synapses (Yoon and Rothman, 1991; Lambert and Teyler, 1991; but cf. O'Regan *et al.*, 1992). How likely is the development of novel antiepileptic drugs based on purinergic mechanisms?

Owing to the high density of peripheral receptor sites, it may not be feasible to use either adenosine (which shows poor blood-brain-barrier permeability [Berne *et al.*,

1974] and has an extremely short half-life) or adenosine agonists for chronic administration. Besides their peripheral side-effects such as hypotension, adenosine agonists also have severe effects on psychomotor performance and produce marked sedation (Ahlijanian and Takemori, 1986) and myasthenic effects; this makes them unsuitable for chronic (prophylactic) administration. Also, maintaining epileptic patients continuously on a prophylactic dose of an A1 agonist could only work if tolerance did not develop to the agent. However, there is evidence that desensitisation to the effects of A1 agonists occurs with prolonged exposure: chronic adenosine agonist administration was found to lead to relatively rapid desensitisation of the A1 receptor-adenylate cyclase system (Parsons and Stiles, 1987). The desensitisation may occur at either the A1 receptor itself or at the level of the G proteins. A1 receptor density has been found to be reduced after prolonged R-PIA exposure as revealed by receptor binding assays (Green *et al.*, 1990), and the percentage of A1 receptors in the high-affinity state was also reduced after the desensitisation with PIA (Ramkumar *et al.*, 1991). Because the high-affinity state is thought to occur when the receptor is attached to its G-protein, desensitisation could be the result of depletion of the G proteins or failure of the G proteins to couple properly to the receptor. Indeed, reduced levels of the three alpha subunits of Gi protein have been reported by Longabaugh *et al.* (1989) in the A1 receptors of rat adipocytes desensitised by R-PIA.

For these reasons, antiepileptic drug development has recently begun to focus on agents which potentiate the action of endogenously released adenosine and therefore may be a way of limiting seizure duration. These include substances such as soluflazine (Boissard and Gribkoff, 1993) and other adenosine uptake inhibitors, or blockers of enzymatic degradation: while phosphorylation of adenosine by the kinase appears to predominate under normal physiological conditions, adenosine deaminase contributes significantly to adenosine degradation under pathophysiological conditions such as ischemia or hypoxia (Zhang *et al.*, 1993); therefore, adenosine deaminase inhibitors may be one possible way of enhancing the protective effect of adenosine, particularly since re-uptake of adenosine generated from the activity-dependent co-release of ATP with glutamate is quantitatively more important than

the block of release via the bi-directional carrier. Recently, the adenosine binding enhancer PD 81,723, which is thought to allosterically modify the A1 receptor (Bruns and Fergus, 1990), has been reported to enhance adenosine's physiological activity in isolated guinea pig hearts (Kollias-Baker *et al.*, 1994). This has been suggested to be due to a stabilization of the receptor-G protein interaction in the presence of agonists, thus providing a site- and event-specific potentiation of the effects of endogenously released adenosine. Protective effects in the CNS remain to be demonstrated; as PD 81,723 may act by stabilizing the high-affinity (G-protein bound) state of the A1 receptor, the effects on adenosine binding may be more pronounced than the enhancement of its G protein-mediated effects.

Alternatively, upregulation of adenosine A1 receptors in response to chronic treatment with low-dosed A1 antagonists may exert a protective effect, as suggested by Rudolphi *et al.* (1989) and by Pagonopoulou (1993). Long-term administration of caffeine results in significant habituation to its behavioural effects, and plasma levels of caffeine 98 μM and theophylline 56 μM have been observed to be well tolerated in rats after long-term treatment (Johansson *et al.*, 1993). The exact mechanism is unknown, but has been proposed to be independent of mRNA upregulation and possibly involves a redistribution of adenosine receptors from the outer membrane into cytosolic vesicles. Whereas a desensitization of the A1-adenylate cyclase system is observed upon chronic exposure to R-PIA (Parsons and Stiles, 1987), Ramkumar *et al.* (1988) have reported a Gi upregulation in rat cerebral cortex (determined by pertussis toxin-mediated labelling) after chronic caffeine treatment. A variation in A1 receptor/G-protein coupling, reflected in a decreased potency of GTP in shifting high-affinity sites to low-affinity, has been observed after chronic treatment with theophylline (Fastbom and Fredholm, 1990). Intriguingly however, patients who chronically receive theophylline generally will have greater manifestations of toxicity at comparable toxic levels than those patients who have achieved that same level due to an acute exposure (Olson *et al.*, 1985); this may represent further evidence for the differential effects of methylxanthines on seizure initiation (by an adenosine A1 receptor-independent mechanism) and augmentation (through the blockade of A1 receptors).

Independently of the mechanisms involved, an assessment of the value of prophylactic treatment with agents which enhance the actions of endogenous adenosine by one of the mechanisms discussed above (adenosinergics, binding enhancers/uptake inhibitors, or A1 receptor upregulation) depends on whether adenosine is a tonically acting 'endogenous anticonvulsant', or whether its effects are confined to ongoing seizures, *i.e.* if adenosine was brought into play as an anticonvulsant only by the seizure itself (as suggested by During and Spencer, 1992). The results from the present study suggest that the latter may be the case. In line with these considerations is the fact that an inhibition of the 'purinergic tone' often results in significant increases in seizure duration without affecting the seizure threshold: McCall *et al.* (1993) found that caffeine (242 mg intravenously) did not change the convulsive threshold in 12 patients undergoing electroconvulsive therapy for major depression, while reliably lengthening seizure duration (and see Shapira *et al.*, 1987). Similar observations have been observed by a number of other groups: for example, Krahl *et al.* (1995) reported that afterdischarge threshold and behavioural seizure stage in amygdala-kindled rats were not affected by an adenosine agonist (metrifudil) which significantly reduced afterdischarge duration, and Albertson *et al.* (1983) noted that "despite large increases in afterdischarge durations seen with both threshold and suprathreshold amygdaloid stimulation [in kindled rats], no change in seizure threshold was noted after aminophylline [100 mg/kg i.p.]". The main implication from the present study would appear to be that an adenosinergic basal tone is indeed of minor importance under resting conditions, but that adenosine may be able to limit the sequelae of NMDA receptor activation. Clinically therefore, purinergic drugs would be expected to contribute mainly to the limitation of epileptiform activity, although there is evidence to suggest that adenosinergic mechanisms may only play a relatively minor role in this respect: White *et al.* (1993) have claimed that NMDA activation leads to a loss of adenosine efficiency because the requirements to overcome the magnesium-block decrease, leading to a decreased effect of hyperpolarising agents. Secondly, the fact that seizures occurring in the presence of high methylxanthine levels are self-limiting in most cases (*e.g.*, Würl, 1994) suggests that adenosine-mediated inhibition is not the main mechanism involved in the termination of epileptic convulsions. Also, the potential involvement of second-messenger evoked effects implied by recent reports of excitatory effects of

adenosine and the activation of adenylyl cyclases would suggest that while the A1-mediated inhibition of these mechanisms may be important for the normal functioning of the brain, a failure of this inhibition may induce irreversible changes, implying that adenosinergic compounds may only be of use within a narrow time window during the onset of paroxysmal activity. Indeed, epilepsy itself may be caused by mechanisms similar to those involved in the second-messenger mediated excitation described in this study. Therefore, a study of these mechanisms, rather than of the balance between inhibitory and excitatory synaptic transmission, may be of particular relevance to the understanding of human idiopathic epilepsy.

An entirely different approach to the therapeutic use of purinergic mechanisms may involve the potentially anticonvulsant effects of A1 blockade in absence seizures. One of the most disabling aspects of epilepsy is the unpredictability of individual seizures, despite the fact that numerous studies now show that seizure occurrence is not a random process. An examination of the behavioural and environmental settings in which seizures occur and of the associated cerebral physiology indicate that, at least for certain common types of epilepsy, clinical seizures occur at periods related to the sleep-wake cycle. In petit mal epilepsy, a remarkable suppression of seizure discharges during rapid-eye-movement (REM) sleep, and an augmentation of spike activity in these patients during non-REM sleep was reported

Rodin (1984). Adenosine acting at A1 receptors, and GABA acting at $GABA_B$ receptors converge in part on identical effector pathways (Thompson *et al.*, 1992). $GABA_B$-mediated de-inactivation of low-threshold calcium currents through late inhibitory postsynaptic potentials from reticular thalamic neurones has a marked effect on rhythmical spindle activity (Destexhe and Babloyantz, 1993) in thalamocortical neurones. A1 receptor-mediated decreases in the hyperpolarization-activated cation current I_H (Pape, 1992) via a decrease in intracellular cAMP increase the amplitude and duration of strong membrane hyperpolarisations, thereby also promoting spindle oscillations in the thalamus. A similar oscillation in thalamocortical afferents has been suggested to be the mechanism underlying absence seizures (Steriade, 1990). The involvement of adenosine in sleep and EEG arousal (Rainnie *et al.*, 1994) and the convergence of

GABAergic and adenosinergic mechanisms in those pathways involved in thalamocortical spindle generation would therefore suggest that in these patients an intriguing effect of A1 blockade, as well as the use of orally available $GABA_B$ antagonists (Olpe *et al.*, 1993), might be the suppression of petit mal-type fits, although a recent report (Ulrich and Huguenard, 1995) suggests that adenosine may negatively affect the amplitude rather than the frequency of thalamic oscillations. It may therefore be of interest to study the effects of adenosine A1 antagonists such as theophylline on the EEG of patients suffering from absence (petit mal) epilepsy, using methods similar to those described by Shucard *et al.* (1985).

Further studies

The following points are suggested for further studies:

(a) the involvement of A1 receptor blockade in the initiation of seizures was assumed to be minimal in the present study. Experimentally, this may be shown by assessing the seizure threshold to electrical stimulation in the presence of a high level of DPCPX *in vivo*. It is predicted that the seizure threshold should be unchanged in either the presence or absence of DPCPX, whereas the time to onset, and the duration, of the seizures would be markedly affected. *In vitro,* the use of a broad range of adenosine agonists and antagonists on hippocampal slices pre-incubated in defined amounts of DPCXP may be instrumental in corroborating the proposed model of a two-stage binding of DPCPX to the A1 receptor. Ideally, one would also like to assess the molecular location of various alkylxanthines within the lipid bilayer.

(b) the time course and extent of adenylyl cyclase activation by convergent stimuli such as the 'functional A2 activation' proposed for DPCPX may be studied biochemically, using for example the method described by Salomon (1991), whereby the adenine nucleotide pool of the cell is labelled with [3H]-adenine prior to stimulation. A2 activation could be stimulated by the concomitant application of an

A1-selective concentration of DPCPX (5 nM) and low concentrations of adenosine (5 μM), similar to the experiment shown in Fig. 48. The recent development of CREB-deficient mutants (Bourtchuladze *et al.*, 1994) may greatly facilitate the demonstration of a role for cAMP-induced changes in protein synthesis in the long-term effects of a synergistic activation of adenylyl cyclase by the functional A2 agonism of DPCPX during epileptiform activity. It may be of interest to study whether the proposed mechanism for the long-term excitatory effects of DPCPX described in this study also applies to other antagonistic transmitter systems, such as the D1/D2 dopamine receptor system: dopaminergic D1/D5 receptors have been reported to induce a protein synthesis-dependent late potentiation in the CA1 region of the hippocampus while this work was in progress (Huang and Kandel, 1995).

References

Abbracchio MP, Brambilla R, Rovati GE, Cattabeni F (1993): Prolonged agonist exposure induces imbalance of A1 and A2 receptor-mediated functions in rat brain slices. *Drug Dev Res* **28**, 364-368

Abeles M, Goldstein MH (1970): Functional architecture in cat primary auditory cortex - columnar organization and organization according to depth. *J Neurophysiol* **33**, 172-187

Abraham WC, Dragunow M, Tate WP (1991): The role of immediate early genes in the stabilization of long-term potentiation. *Mol Neurobiol* **5**, 297-314

Aden U, Lindstrom K, Bona E, Hagberg H, Fredholm BB (1994): Changes in adenosine receptors in the neonatal rat brain following hypoxic ischemia. *Brain Res Mol Brain Res* **23**, 354-358

Agopyan N, Tokutomi N, Akaike N (1993): Protein kinase A-mediated phosphorylation reduces only the fast desensitizing glycine current in acutely dissociated ventromedial hypothalamic neurons. *Neurosci* **56**, 605-615

Ahlijanian MK, Takemori AE (1986): Cross-tolerance studies between caffeine and (-)-N6-(phenylisopropyl)-adenosine (PIA) in mice. *Life Sci* **38**, 577-588

Albertson TE, Stark LG, Joy RM, Bowyer JF (1983): Aminophylline and kindled seizures. *Exp Neurol* **81**, 703-713

Albowitz B, Kuhnt U (1995): Epileptiform activity in the guinea-pig neocortical slice spreads preferentially along supragranular layers - recordings with voltage-sensitive dyes. *Eur J Neurosci* **7**, 1273-1284

Alexander SPH, Hill SJ, Kendall DA (1990): Is the adenosine receptor modulation of histamine-induced accumulation of inositol phosphates in cerebral cortical slices mediated by effects on calcium ion fluxes? *J Neurochem* **55**, 1138-1141

Allende G, Casado V, Mallol J, Franco R, Lluis C, Canela EI (1993): Role of histidine residues in agonist and antagonist binding sites of A1 adenosine receptor. *J Neurochem* **60**, 1525-1533

Alloway KD, Burton H (1985): Submodality and columnar organisation of the second somatic sensory cortex in cats. *Exp Brain Res* **61**, 128-140

Alzheimer C, Sutor B, ten Bruggencate G (1989): Transient and selective blockade of adenosine A1-receptors by DPCPX causes sustained epileptiform activity in hippocampal CA3 neurones of guinea pigs. *Neurosci Letts* **99**, 107-112

Alzheimer C, Sutor B, ten Bruggencate G (1993): Disinhibition of hippocampal CA3 neurones induced by suppression of an adenosine A1 receptor-mediated inhibitory tonus - pre- and postsynaptic components. *Neurosci* **57**, 565-575

Andrew RD, Macvicar BA (1994): Imaging cell volume changes and neuronal excitation in the hippocampal slice. *Neurosci* **62**, 371-383

Annegers JF, Grabow JD, Groover RV, Laws ER, Elveback LR, Kurland LT (1980): Seizures after head trauma: a population study. *Neurology* **30**, 683-689

Annegers JF (1994): Epidemiology and genetics of epilepsy. *Neurol Clin* **12**, 15-29

Aram JA, Lodge D (1988): Validation of a neocortical slice preparation for the study of epileptiform activity. *J Neurosci Methods* **23**, 211-224

Aram JA, Martin D, Tomczyk M, Zeman S, Millar J, Pohler G, Lodge D (1989): Neocortical epileptogenesis *in vitro*: studies with N-methyl-D-aspartate, PCP, sigma and dextromethorphan receptor ligands. *J Pharmacol Exp Therap* **248**, 320-328

Armstrong-James M (1975): The functional status and columnar organisation of single cells responding to cutaneous stimulation in neonatal rat somatosensory cortex SI. *J Physiol (Lond)* **246**, 501-538

Armstrong-James M, Fox K (1987): Spatiotemporal convergence and divergence in the rat SI barrel cortex. *J Comp Neurol* **263**, 265-281

Armstrong-James M, George MJ (1988): Influence of anaesthesia on spontaneous activity and receptive field size of single units in rat SmI neocortex. *Exp Neurol* **99**, 369-387

Armstrong-James M, Fox K, Das-Gupta A (1992): Flow of excitation within rat barrel cortex on striking a single vibrissa. *J Neurophysiol* **68**, 1345-1358

Asanuma H (1975): Recent developments in the study of the columnar arrangements of neurones within the motor cortex. *Physiol Rev* **55**, 143-156

Ault B, Joyner JL, Olney MA, Wang CM (1986): Endogenous adenosine modulates neuronal activity in hippocampal area CA3. *Br J Pharmacol* **88**, 461P

Ault B, Wang CM (1986): Adenosine inhibits epileptiform activity arising in hippocampal area CA3. *Br J Pharmacol* **87**, 695-703

Ault B, Olney MA, Joyner JL, Boyer CE, Notrica MA, Soroko FE, Wang CM (1987): Pro-convulsant actions of theophylline and caffeine in the hippocampus - implications for the management of temporal lobe epilepsy. *Brain Res* **426**, 93-102

Ayala GF, Dichter M, Gumnit RJ, Matsumoto H, Spencer WA (1973): Genesis of epileptic interictal spikes - new knowledge of cortical feedback systems suggests a neurophyiological explanation of brief paroxysms. *Brain Res* **52**, 1-17

Bacskai BJ, Hochner B, Mahaut-Smith M, Adams SR, Kaang BK, Kandel ER, Tsien RY (1993): Spatially resolved dynamics of cAMP and protein kinase A subunits in Aplysia sensory neurons. *Science* **260**, 222-226

Bahls FH, Ma KK, Bird TD (1991): Theophylline-associated seizures with "therapeutic" or low toxic serum concentrations: risk factors for serious outcomes in adults. *Neurology* **41**, 1309-1312

Ball DI, Brittain RT, Coleman RA, Denyer LH, Jack D, Johnson M, Lunts LHC, Nials AT, Sheldrick KE, Skidmore IF (1991): Salmeterol, a novel, long-acting β2-adrenoceptor agonist - characterization of pharmacological activity in vitro and in vivo. *Br J Pharmacol* **104**, 665-671

Barraco RA, Swanson TH, Phillis JW, Berman RF (1984): Anticonvulsant effects of adenosine analogs on amygdaloid-kindled seizures in rats. *Neurosci Letts* **46**, 317-322

Barth DS, Baumgartner C, Di S (1990): Laminar interactions in rat motor cortex during cyclical excitability changes of the penicillin focus. *Brain Res* **508**, 105-117

Bartrup JT, Stone TW (1988): Interactions of adenosine and magnesium on rat hippocampal slices. *Brain Res* **463**, 374-379

Batuev AS, Alexandrov AA, Scheynikov NA (1982): Picrotoxin action on the receptive fields of the cat sensorimotor cortex neurones. *J Neurosci Res* **7**, 49-55

Beer B, Chasin M, Clody DE, Voge JR, Horovitz ZP (1972): AMP phosphodiesterase in brain - effect on anxiety. *Science* **176**, 428-430

Beldhuis HJA, de Ruiter AJH, Maes FW, Suzuki T, Bohus B (1993): Long-term increase in protein kinase C-gamma and muscarinic acetylcholine receptor expression in the cerebral cortex of amygdala-kindled rats - a quantitative immunocytochemical study. *Neurosci* **55**, 965-973

Belford GR, Killackey HP (1980): The sensitive period in the development of the trigeminal system of the neonatal rat. *J Comp Neurol* **193**, 335-350

Ben-Ari Y, Gho M (1988): Long-lasting modification of the synaptic properties of rat CA3 hippocampal neurones induced by kainic acid. *J Physiol (Lond)* **404**, 365-384

Ben-Ari Y, Aniksztejn L, Bregestovski P (1992): Protein kinase C modulation of NMDA currents - an important link for LTP induction. *Trends Neurosci* **15**, 333-339

Berman N, Payne BR, Labar DR Murphy EH (1987): Organisation of orientation and direction selectivity in areas 17 and 18 of cat cerebral cortex. *J Neurophysiol* **58**, 676-699

Bernardo KL, Woolsey TA (1987): Axonal trajectories between mouse somatosensory thalamus and cortex. *J Comp Neurol* **258**, 542-564

Bernardo KL, McCasland JS, Woolsey TA, Strominger RN (1990): Local intra- and interlaminar connections in mouse barrel cortex. *J Comp Neurol* **291**, 231-255

Berne RM, Rubio R, Curnish RR (1974): Release of adenosine from ischemic brain, ist effects on cerebral vascular resistance and its incorporation into cerebral adenine nucleotides. *Circ Res* **35**, 262-271

Berne RM (1980): The role of adenosine in the regulation of coronary blood flow. *Circ Res* **47**, 807-813

Berne M, Winn HR, Rubio R (1982): In: Cerebral hypoxia in the pathogenesis of migraine (eds FC Rose, WK Amery), p.82-91. Pitman, London

Bisserbe JC, Pascal O, Deckert J, Maziere B (1992): Potential use of DPCPX as probe for *in vivo* localization of brain A1 adenosine receptors. *Brain Res* **599**, 6-12

Boakes RJ, Burns BD, Webb AC (1988): Transmission of burst responses through slices of rat cerebral cortex. *J Physiol (Lond)* **404**, 467-478

Böhm M, Brückner R, Meyer W, Nose M, Schmitz W, Scholz H, Starbatty J (1985): Evidence of adenosine receptor-mediated isoprenaline-antagonistic effects of the adenosine analogs PIA and NECA on force of contraction in guinea-pig atrial and ventricular cardiac preparations. *Naunyn-Schmiedeberg's Arch Pharmacol* **331**, 131-139

Boissard CG, Gribkoff VK (1993): The effects of the adenosine reuptake inhibitor soluflazine on synaptic potentials and population hypoxic depolarizations in area CA1 of rat hippocampus in vitro. *Neuropharmacol* **32**, 149-155

Borowicz K, Kozicka M, Kleinrok Z, Czuczwar SJ (1993): Influence of aminophylline and 8-(p-sulfophenyl)theophylline on the anticonvulsive action of diphenylhydantoin, phenobarbital, and valproate against MES-induced convulsions in mice. *J Neural Transm (Gen Sect)* **93**, 157-163

Bourne HR, Nicoll R (1993): Molecular machines integrate coincident synaptic signals. *Cell* **78**, 65-75

Bourtchuladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G, Silva AJ (1994): Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell* **79**, 59-68

Brodmann K (1909): Vergleichende Lokalisationslehre der Großhirnrinde in ihren Prinzipien dargestellt aufgrund des Zellenbaues. Barth, Leipzig.

Brookhart B, Moruzzi G, Snider R (1950): Spike discharges of single units within cerebellar cortex. *J Neurophysiol* **13**, 465-486

Bruns RF, Daly JW, Snyder SH (1980): Adenosine receptors in brain membranes: binding of N6-cyclohexyl[3H]adenosine and 1,3-diethyl-8-[3H]phenylxanthine. *Proc Natl Acad Sci USA* **77**, 5547-5551

Bruns RF, Lu GH, Pugsley TA (1986): Characterization of the A2 adenosine receptor labelled by [3H]NECA in rat striatal membranes. *Mol Pharmacol* **29**, 331-346

Bruns RF, Fergus JH, Badger EW, Bristol JA, Santay LA, Hartman JD, Hays SJ, Huang CC (1987): Binding of the A1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Arch Pharmacol* **335**, 59-63

Bruns RF, Fergus JH (1990): Allosteric enhancement of adenosine A1 receptor binding and function by 2-amino-3-benzoylthiophenes. *Mol Pharmacol* **38**, 939-949

Burdette LJ, Dyer RS (1987): Differential effects of caffeine, picrotoxin and pentlenetetrazol on hippocampal afterdischarge activity and wet dog shakes. *Exp Neurol* **96**, 381-392

Burnstock G (1986): The changing face of autonomic neurotransmission. Acta Physiol Scand **126**, 67

Cantu TG, Korek JS (1991): Caffeine in electroconvulsive therapy. *DICP* **25**, 1079-1080

Casado V, Mallol J, Canela EI, Lluis C, Franco R (1991): The binding of [3H]R-PIA to A1 adenosine receptors produces a conversion of the high to the low-affinity state. *FEBS Lett* **286**, 221-224

Chagnac-Amitai Y, Connors BW (1989): Synchronized excitation and inhibition driven by intrinsically bursting neurons in neocortex. *J Neurophysiol* **62**, 1149-1162

Chapin JK, Lin CS (1990): The somatic sensory cortex of the rat. In: The cerebral cortex of the rat (eds B Kolb and RC Tees) p. 341- 380. MIT Press, Cambridge MA

Chatt AB, Ebersole JS (1984): Identification of penicillin diffusion at the onset of epileptogenesis in the cat striate cortex following differential laminar microinjection. *Brain Res* **290**, 361-366

Chaviez-Noriega LE, Stevens CF (1994): Increased transmitter release at excitatory synapses produced by direct activation of adenylate cyclase in rat hippocampal slices. *J Neurosci* **14**, 310-317

Chen G, Ensor CR, Bohner B (1968): Studies on drug effects on electrically induced extensor seizures and clinical implications. *Arch Int Pharmacodyn* **172**, 183-218

Chen L, Huang LY (1992): Protein kinase C reduces Mg2+ block of NMDA-receptor channels as a mechanism of modulation. *Nature* **356**, 521-523

Chervin RD, Pierce PA, Connors BW (1988): Periodicity and directionality in the propagation of epileptiform discharges across neocortex. *J Neurophysiol* **60**, 1695-1713

Chesi AJR (1992): An *in vitro* approach to the 'minimal mass' problem in neocortical epilepsy. *Neurosci Letts* Suppl **42**, S39

Chin JH (1989): Adenosine receptors in brain: neuromodulation and role in epilepsy. *Ann Neurol* **26**, 695-698

Chow P, Mathers D (1986): Convulsant doses of penicillin shorten the lifetime of GABA-induced channel openings in cultured central neurones. *Br J Pharmacol* **88**, 541-547

Chu NS (1981): Caffeine- and aminophylline-induced seizures. *Epilepsia* **22**, 85-94

Chudler EH, Pretel S, Kenshalo DR (1988): Distribution of GAD-immunoreactive neurons in the first (SI) and second (SII) somatosensory cortex of the monkey. *Brain Res* **456**, 57-63

Clarke B, Till J, Rowland E, Ward DE, Barnes PJ, Shinebourne EA (1987): Rapid and safe termination of supraventricular tachycardia in children by adenosine. *Lancet* **1(8528)**, 299-301

Cohen P, Holmes CFB, Tsukitani Y (1990): Okadaic acid - a new probe for the study of cellular regulation. *Trends Biochem Sci* **15**, 98-102

Coffey CE, Figiel GS, Weiner RD, Saunders WB (1990): Caffeine augmentation of ECT. *Am J Psychiatry* **147**, 579-585

Collins RC (1978): Use of cortical circuits during focal penicillin seizures: an autoradiographic study with [14C]deoxyglucose. *Brain Res* **150**, 487-501

Collis MG, Palmer DB, Saville VL (1985): Comparison of the potency of 8-phenyltheophylline as an antagonist at A1 and A2 adenosine receptors in atria and aorta from the guinea pig. *J Pharm Pharmacol* **37**, 278-280

Colotta V, Cecchi L, Catarzi D, Melani F, Filacchioni G, Martini C, Tacchi P, Lucacchini A (1993): Novel adenosine receptor ligands: 1,3-disubstituted[1]benzopyrano[2,3-c]pyrazol-4-ones. Synthesis and structure-activity relationships. *Receptors Channels* **1**, 111-119

Connors BW, Gutnick MJ, Prince DA (1982): Electrophysiological properties of neocortical neurons *in vitro*. *J Neurophysiol* **48**, 1302-1320

Connors BW, Benardo LS, Prince DA (1983): Coupling between neurons of the developing rat neocortex. *J Neurosci* **3**, 773-782

Connors BW (1984): Initiation of synchronized neuronal bursting in neocortex. *Nature* **310**, 685-687

Craig CG, White TD (1993): NMDA and non-NMDA-evoked adenosine release from rat cortical slices: distinct purinergic sources and mechanisms of release. *J Neurochem* **60**, 1073-1080

Cunha RA, Johansson B, Vanderploeg I, Sebastiao AM, Ribeiro JA, Fredholm BB (1994): Evidence for functionally important adenosine A2a receptors in the rat hippocampus. *Brain Res* **649**, 208-216

Cutrufo C, Bortot L, Giachetti A, Manzini S (1992): Differential effects of various xanthines on pentylenetetrazole-induced seizures in rats - an EEG and behavioural study. *Eur J Pharmacol* **222**, 1-6

Czuczwar SJ, Kleinrok Z, Turski L, Turski WA (1987): Effect of aminophylline and enprofylline on the protective efficacy of common antiepileptic drugs against electroconvulsions. *Epilepsia* **28**, 383

Daigen A, Akiyama K, Otsuki S (1991): Long-lasting change in the membrane-associated protein kinase C activity in the hippocampal kindled rat. *Brain Res* **545**, 131-136

Dawid-Milner MS, Silva-Carvalho L, Goldsmith GR, Spyer KM (1994): A potential role of central A1 adenosine receptors in the responses to hypothalamic stimulation in the anaesthesized cat. *J Autonom Nerv Syst* **49**, 15-19

Dawson J, Anderson WM, Margerison JH (1961): Water, electrolytes and the EEG in a case of focal cortical epilepsy with diabetes insipidus on varying drug regimes. *Epilepsia* **2**, 144 - 160

Deckert J, Berger W, Kleopa K, Heckers S, Ransmayr G, Heinsen H, Beckmann H, Riederer P (1993): Adenosine A1 receptors in human hippocampus: inhibition of [3H]DPCPX binding by antagonist drugs. *Neurosci Letts* **150**, 191-194

Dennis D, Jabobson K, Belardinelli L (1992): Evidence of spare A1-adenosine receptors in guinea pig atrioventricular node. *Am J Physiol* **262**, H661-H671

Destexhe A, Babloyantz A (1993): A model of the inward current IH and its possible role in thalamocortical oscillations. *NeuroReport* **4**, 223-226

Diamond ME, Armstrong-James M, Budway MJ, Ebner FF (1992): Somatic sensory responses in the rostral sector of the posterior group and in the ventral posterior medial nucleus of the rat thalamus: dependence on the barrel field cortex. *J Comp Neurol* **319**, 66-84

Dickenson JM, Hill SJ (1993): Adenosine A1-receptor stimulated increases in intracellular calcium in the smooth muscle cell line, DDT1MF-2. *Br J Pharmacol* **108**, 85-92

Dimmock JR, Baker GB (1994): Anticonvulsant activities of 4-bromobenzaldehyde semicarbazone. *Epilepsia* **35**, 648-655

Dodd PR, Watson WEJ, Johnston GAR (1986): Adenosine receptors in post-mortem human cerebral cortex and the effect of carbamazepine. *Clin Exp Pharmacol Physiol* **13**, 711-722

Donoso P, O'Neill SC, Dilly KW, Negretti N, Eisner DA (1994): Comparison of the effects of caffeine and other methylxanthines on [Ca2+]i in rat ventricular myocytes. *Br J Pharmacol* **111**, 455-458

Douglas WW (1968): Stimulus-secretion coupling - the concept and clues from chromaffin and other cells. *Br J Pharmacol* **34**, 451-474

Douglas RJ, Martin KA (1991): Opening the grey box. *Trends Neurosci* **14**, 286-293

Doyle MP, Linden J, Duling BR (1994): Nucleoside-induced arteriolar constriction: a mast cell-dependent response. *Am J Physiol* **266**, H2042-H2050

Dragunow M, Robertson HA (1987): 8-Cyclopentyl-1,3-dimethylxanthine prolongs epileptic seizures in rats. *Brain Res* **417**, 377-379

Dragunow M, Goddard GV, Laverty R (1987): Proconvulsant effects of theophylline on hippocampal afterdischarges. *Exp Neurol* **96**, 732-735

Driver AG, Kukoly CA, Ali S, Mustafa SJ (1993): Adenosine in bronchoalveolar lavage fluid in asthma. *Am Rev Respir Dis* **148**, 91-97

Dunwiddie TV, Diao L (1994): Extracellular adenosine concentrations in hippocampal brain slices and the tonic inhibitory modulation of evoked excitatory responses. *J Pharmacol Exp Therap* **268**, 537-545

During J, Spencer DD (1992): Adenosine - a potential mediator of seizure arrest and postictal refractoriness. *Ann Neurol* **32**, 618-624

Duysens J, McLean DR (1974): The effect of subpial vertical cortical incisions on a cobalt gelatin focus. *Epilepsia* **1**, 579-591

Dykes RW, Rasmusson DD, Hoeltzell P (1980): Organization of primary somatosensory cortex in the cat. *J Neurophysiol* **43**, 1527-1546

Ebersole JS, Chatt AB (1980): The laminar susceptibility of cat visual cortex to penicillin-induced epileptogenesis. *Neurology* **30**, 355

Ebersole JS, Chatt AB (1986): Spread and arrest of seizures - the importance of layer 4 in laminar interactions during neocortical epileptogenesis. In: Advances in Neurology Vol 44 (eds AV Delgado-Escueta, JAA Ward, DM Woodbury, RJ Porter), p. 515-558. Raven, New York

Echlin FA, Arnett V, Zoll J (1952): Paroxysmal high-voltage discharges from isolated and partially isolated human and animal cerebral cortex. ???

Echlin FA (1959): The supersensitivity of chronically "isolated" cerebral cortex as a mechanism in focal epilepsy. *EEG Clin Neurophysiol* **11**, 697-722

Engler RL (1994): Harnessing nature's own cardiac defense mechanism with acadesine, an adenosine regulating agent: importance of the endothelium. *J Card Surg* **9**, 482-492

Erzurumlu RS, Jhaveri S (1990): Thalamic axons confer a blueprint of the sensory periphery onto the developing rat somatosensory cortex. *Dev Brain Res* **56**, 229-234

Faingold CL, Fromm GH (1992): Drugs for control of epilepsy, p.151. CRC Press, Florida

Fastbom J, Fredholm BB (1990): Effects of long-term theophylline treatment on adenosine A1-receptors in rat brain - autoradiographic evidence for increased receptor number and altered coupling to G-proteins. *Brain Res* **507**, 195-199

Favorov OV, Diamond ME, Whitsel BL (1987): Evidence for a mosaic representation of the body surface in area 3b of the somatic sensory cortex of cat. *Proc Natl Acad Sci USA* **84**, 6606-6610

Feldman PD, Felder RB (1988): Compatibility of urethane sedation with studies of adrenergic systems in the rat brain slice. *J Neurosci Methods* **24**, 189

Ferre S, Rubio A, Fuxe K (1991): Stimulation of adenosine A2 receptors induces catalepsy. *Neurosci Letts* **130**, 162-164

Ferre S, O'Connor WT, Fuxe U, Ungerstedt U (1993): The striopallidal neuron: a main locus for adenosine-dopamine interactions in the brain. *J Neurosci* **13**, 5402-5406

Feuerstein TJ, Bär KI, Lücking CH (1988): Activation of A1 adenosine receptors decreases the release of serotonin in the rabbit hippocampus, but not in the caudate nucleus. *Naunyn-Schmiedeberg's Arch Pharmacol* **338**, 664-670

File SE, Baldwin HA, Johnston AL, Wilks LJ (1988): Behavioural effects of acute and chronic administration of caffeine in the rat. *Pharmacol Biochem Behav* **30**, 809-815

Fink JS, Weaver DR, Rivkees SA, Peterfreund RA, Pollack AE, Adler EM, Reppert SM (1992): Molecular cloning of the rat A2 adenosine receptor - selective co-expression with D2 dopamine receptors in rat striatum. *Mol Brain Res* **14**, 186-195

Fisher RS, Kaplan PW, Krumholz A (1988): Failure of high-dose intravenous magnesium sulfate to control myoclonic status epilepticus. *Clin Neuropharmacol* **11**, 537-544

Fisher RS (1991): Neuronal damage and epilepsy: basic and clinical interface. *Epilepsy Res* **10**, 80-89

Fitzpatrick MF, Mackay T, Driver H, Douglas NJ (1990): Salmeterol in nocturnal asthma - a double blind, placebo controlled trial of a long acting inhaled beta 2 agonist. *BMJ* **301**, 1365-1368

Flor H, Elbert T, Knecht S, Wienbruch C, Pantev C, Birbaumer N, Larbig W, Taub E (1995): Phantom-limb pain as a correlate of cortical reorganization following arm amputation. *Nature* **375**, 482-484

Fowler JC (1993): Changes in extracellular adenosine levels and population spike amplitude during graded hypoxia in the rat hippocampal slice. *Naunyn-Schmiedeberg's Arch Pharmacol* **347**, 73-78

Fozard JR, Carruthers AM (1993): Adenosine A3 receptors mediate hypotension in the angiotensin II-supported circulation of the pithed rat. *Br J Pharmacol* **109**, 3-5

Frankenhäuser B, Hodgkin AL (1957): The action of calcium on the electrical properties of squid axons. *J Physiol (Lond)* **137**, 218-244

Francis A, Fochtmann L (1994): Caffeine augmentation of electroconvulsive seizures. *Psychopharmacol* **115**, 320-324

Fredholm BB, Jonzon B (1988): Adenosine receptors - agonists and antagonists. In: Role of adenosine in cerebral metabolism and blood flow (eds. V Stefanovich and I Okayayuz-Baklouti), p. 17-45. VSP BV, Zeist.

Fredholm BB, Proctor W, Van der Ploeg I, Dunwiddie TV (1989): *In vivo* pertussis toxin treatment attenuates some, but not all adenosine A1 effects in slices of rat hippocampus. *Eur J Pharmacol* **172**, 249-262

Fredholm BB, Johannson B, van der Ploeg I, Hu PS, Jin S (1993): Neuromodulatory roles of purines. *Drug Dev Res* **28**, 349-353

Freissmuth M, Schütz W, Linder ME (1991): Interactions of the bovine brain A1-adenosine receptor with recombinant G protein alpha-subunits. Selectivity for rGi alpha-3. *J Biol Chem.* **266**, 17778-17783

Freissmuth M, Selzer E, Schütz W (1991): Interactions of purified bovine brain A1-adenosine receptors with G proteins - reciprocal modulation of agonist and antagonist binding. *Biochem J* **275**, 651-656

Frey U, Huang Y, Kandel ER (1993): Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. *Science* **260**, 1661-1664

Fueta Y, Avoli M (1993): Tetraethylammonium-induced epileptiform activity in young and adult rat hippocampus. *Dev Brain Res* **72**, 51-58

Fujii T, Baumgartl H, Lubbers DW (1982): Limiting section thickness of guinea pig olfactory cortical slices studied from tissue pO2 values and electrical activities. *Pflügler's Arch Pharmacol* **393**, 83-87

Fujita I, Tanaka K, Ito M, Cheng K (1992): Columns for visual features of objects in monkey inferotemporal cortex. *Nature* **360**, 343-346

Gabor AJ, Scobey RP, Wehrli CJ (1979): Relationship of epileptogenicity to cortical organization. *J Neurophysiol* **42**, 1609-1625

Garaschuk O, Kovalchuk, Krishtal O (1992): Adenosine-dependent enhancement by methylxanthines of excitatory transmission in hippocampus of rats. *Neurosci Letts* **135**, 10-12

Garritsen A, Ijzerman AP, Beukers MW, Cragoe EJ, Soudijn W (1990): Interaction of amiloride and its analogues with adenosine A1 receptors in calf brain. *Biochem Pharmacol* **40**, 827-834

Geiger JD, Fyda DM (1991): Adenosine transport in nervous tissues. In: Adenosine in the Nervous System (ed TW Stone), p. 1-23. Academic Press, London

Gereau RW, Conn PJ (1994): Potentiation of cAMP responses by metabotropic glutamate receptors depresses synaptic transmission by a kinase-independent mechanism. *Neuron* **12**, 1121-1129

Gerwins P, Nordstedt C, Fredholm BB (1990): Characterization of adenosine A1 receptors in intact DD1 MF-2 smooth muscle cells. *Mol Pharmacol* **38**, 660-666

Gilbert CD (1985): Horizontal integration in the neocortex. *TINS* **8**, 160-165

Gilman AG (1987): G proteins - transducers of receptor-generated signals. *Annu Rev Biochem* **56**, 615-649

Goddard GV, McIntyre DC, Leech K (1969): A permanent change in brain function resulting from daily electrical stimulation. *Expl Neurol* **25**, 295-330

Goodman RR, Cooper MJ, Gavish M, Snyder SH (1982): Guanine nucleotide and cation regulation of the binding of [3H]cyclohexyladenosine and [3H]diethylphenylxanthine to adenosine A1 receptors in brain membranes. *Mol Pharmacol* **21**, 329-335

Green RM, Stiles GL (1986): Chronic caffeine ingestion sensitizes the A1 adenosine receptor-adenylate cyclase system in rat cerebral cortex. *J Clin Invest* **77**, 222-227

Green A, Johnson JL, Milligan G (1990): Down-regulation of Gi subtypes by prolonged incubation of adipocytes with an A1 adenosine receptor agonist. *J Biol Chem* **265**, 5206-5210

Griebel R, Saffroy-Spittler M, Misslin R, Remmy D, Vogel E, Bourguignon J (1991): Comparison of the behavioural effects of an adenosine A1/A2-receptor antagonist, CGS 15943A, and an A1-selective antagonist, DPCPX. *Psychopharmacol* **103**, 541-544

Grillner S (1991): Recombination of motor pattern generators. *Curr Opinion Biol* **1**, 231-233

Grondahl TO, Langmoen IA (1993): Epileptogenic effects of antibiotic drugs. *J Neurosurg* **78**, 938-943

Grover LM, Teyler TJ (1993): Role of adenosine in heterosynaptic, posttetanic depression in area CA1 of hippocampus. *Neurosci Lett* **154**, 39-42

Guieu R, Sampieri F, Bechis G, Rochat H (1994): Use of HPLC to measure circulating adenosine levels in migrainous patients. *Clin Chim Acta* **227**, 185-194

Gutnick MJ, Prince DA (1971): Penicillinase and the convulsant action of penicillin. *Neurology* **21**, 759-764

Gutnick MJ, Connors BW, Prince DA (1982): Mechanisms of neocortical epileptogenesis *in vitro*. *J Neurophysiol* **48**, 1321-1335

Haas HL, Greene RW (1984): Adenosine enhances afterhyperpolarization and accommodation in hippocampal pyramidal cells. *Pflügler's Arch* **402**, 244-247

Haberly LB, Sutula TP (1992): Neuronal processes that underlie expression of kindled epileptiform events in the piriform cortex in vivo. *J Neurosci* **12**, 2211-2224

Hagiwara M, Brindle P, Harootunian A, Armstrong R, Rivier J, Vale W, Tsien R, Montminy MR (1993): Coupling of hormonal stimulation and transcription via the cyclic AMP-responsive factor CREB is rate limited by nuclear entry of protein kinase A. *Mol Cell Biol* **13**, 4852-4859

Handforth A, Treiman DM (1994): Effect of an adenosine antagonist and an adenosine agonist on status entry and severity in a model of limbic status epilepticus. *Epilepsy Res* **18**, 29-42

Hansch C, Steward AR, Anderson SM, Bentley D (1968): ??? *J Med Chem* **11**, 1-16

Harris RM, Woolsey TA (1983): Computer-assisted analyses of barrel neuron axons and their putative synaptic contacts. *J Comp Neurol* **220**, 63-79

Haslam RJ, Rosson GM (1975): Effects of adenosine on levels of cAMP in human blood platelets in relation to adenosine incorporation and platelet aggregation. *Mol Pharmacol* **11**, 528-544

Hattori Y (1990): Regional differences in responsiveness of adenosine-sensitive cAMP-generating systems in chronic epileptic cerebral cortex of the rat. *J Neurochem* **55**, 1294-1303

Heffner TG, Wiley JN, Williams AE, Bruns RF, Coughenour LL, Downs DA (1989): Comparison of the behavioral effects of adenosine and dopamine antagonists in mice. *Psychopharmacol* **98**, 31-37

Heinemann U, Dreier J, Leschinger A, Stabel J, Draguhn A, Zhang C (1994): Effects of anticonvulsant drugs on hippocampal neurons. *Hippocampus* **4**, 291-296

Herbette LG, Vant Erve YMH, Rhodes DG (1989): Interaction of 1,4-dihydropyridine calcium channel antagonists with biological membranes - Lipid bilayer partitioning could occur before drug binding to receptors. *J Mol Cell Cardiol* **21**, 187-201

Hille B (1992): Ionic channels of excitable membranes. 2nd edition, p. 455. Sinauer, Mass.

Hoehn K, White TD (1990): NMDA, kainate and quisqualate release endogenous adenosine from rat cortical slices. *Neurosci* **39**, 441-450

Hoffman A, Pinto E, Gilhar D (1993): Effect of pretreatment with anticonvulsants on theophylline-induced seizures in the rat. *J Crit Care* **8**, 198-202

Holmes O, Lockton JW (1982): Penicillin epileptogenesis in the rat: diffusion and the differential laminar sensitivity of the cortex cerebri. *Brain Res* **231**, 131-141

Hooper SL, Moulins M (1989): Switching of a neuron from one network to another by sensory-induced changes in membrane properties. *Science* **244**, 1587-1589

Hornfeldt CS, Larson AA (1994): Adenosine receptors are not involved in theophylline-induced seizures. *J Toxicology - Clinical Toxicology* **32**, 257-265

Houslay MD (1991): Crosstalk - a pivotal role for protein kinase C in modulating relationships between signal transduction pathways. *Eur J Biochem* 195, 9-27

Hu PS (1993): On the usefulness of Fura-2 measurements of intrasynaptosomal calcium levles in rat cortical synaptosomes to study mechanisms of presynaptic function. *Acta Physiol Scand* **148**, 115-123

Huang Y, Kandel ER (1995): D1/D5 receptor agonists induce a protein synthesis-dependent late potentiation in the CA1 region of the hippocampus. *Proc Natl Acad Sci USA* **92**, 2446-2450

Hubel DH (1957): Tungsten microelectrode for recording from single units. *Science* **125**, 549-550

Hubel DH, Wiesel TN (1962): Receptive fields, binocular interaction and functional architecture in the cat's visual cortex. *J Physiol (Lond)* **160**, 106-154

Humphrey NK, Weiskrantz L (1967): Vision in monkeys after removal of the striate cortex. *Nature* **215**, 595-597

Hunter T (1995): Protein kinases and phosphatases - the Yin and Yang of protein phosphorylation and signalling. *Cell* **80**, 225-236

Hutchison AJ, Webb RL, Oei HH, Ghai GR, Zimmermann MB, Williams M (1989): CGS 21680, an A2 selective adenosine receptor agonist with preferential hypotensive activity. *J Pharmacol Exp Therap* **251**, 47-55

Hwa GGC, Avoli M, Oliver A, Villemure JG (1991): Bicuculline-induced epileptogenesis in the human neocortex maintained in vitro. *Exp Brain Res* **83**, 329-339

Hyvärinen J, Poranen A, Jokinen Y (1980): Influence of attentive behaviour on neuronal responses to vibration in primary somatosensory cortex of the monkey. *J Neurophysiol* **43**, 870-882

Imig TJ, Brugge JF (1978): Sources and terminations of callosal axons related to binaural and frequency maps in primary auditory cortex of cat. *J Comp Neurol* **182**, 637-660

Ishikawa A, Kurihara E, Okada Y (1994): Excitatory effects of adenosine receptor agonists and antagonists on neurotransmission in guinea pig superior collicular slices. *Neurosci Lett* **171**, 129-132

Ito M (1992): Simultaneous visualization of cortical barrels and horseradish peroxidase-injected layer 5b vibrissa neurones in the rat. *J Physiol (Lond)* **454**, 247-265

Jacobs KM, Donoghue JP (1991): Reshaping the cortical motor map by unmasking latent intracortical connections. *Science* **251**, 944-947

Jacobson KA, van Galen PJM, Ji X, Ramkumar V, Olah ME, Stiles GL (1993): Molecular characterization of A1 and A2a adenosine receptors. *Drug Dev Res* **28**, 226-231

Janusz CA, Berman RF (1993): The adenosine binding enhancer PD 81,723 inhibits epileptiform bursting in the hippocampal brain slice. *Brain Res* **619**, 131-136

Ji X, Stiles GL, van Galen PJM, Jacobson KA (1992): Characterization of human striatal A2a-adenosine receptors using radioligand binding and photoaffinity labelling. *J Receptor Res* **12**, 149-169

Jockers R, Linder ME, Hohenegger M, Nanoff C, Bertin B, Strosberg AD, Marullo S, Freissmuth M (1994): Species difference in the G protein selectivity of the human and bovine A1-adenosine receptor. *J Biol Chem* **51**, 32077-32084

Johansson B, Ahlberg S, van der Ploeg I, Brene S, Lindefors N, Persson H, Fredholm BB (1993): Effect of long-term caffeine treatment on A1 and A2 adenosine receptor binding and on mRNA levels in rat brain. *Naunyn-Schmiedeberg's Arch Pharmacol* **347**, 407-414

Jones RSG, Lambert JDC (1990): Synchronous discharges in the rat entorhinal cortex *in vitro*: site of initiation and the role of excitatory amino acid receptors. *Neurosci* **34**, 657-670

Jones RSG (1994): Synaptic and intrinsic properties of neurons of origin of the perforant path in layer II of the rat entorhinal cortex in vitro. *Hippocampus* **4**, 335-353

Kaas JH, Merzenich MM, Killackey HP (1983): The reorganization of somatosensory cortex following peripheral-nerve damage in adult and developing mammals. *Annu Rev Neurosci* **6**, 325-356

Kälviäinen R, Keränen T, Riekkinen PJ (1993): Place of new antiepileptic drugs in the treatment of epilepsy. *Drugs* **46**, 1009-1024

Kanai H, Ishida N, Nakajima T, Kato N (1992): An analogue of Joro spider toxin selectively suppresses hippocampal epileptic discharges induced by glutamate. *Brain Res* **581**, 161-164

Karnup SV (1992): Background firing activity in guinea-pig neocortex in vitro. *Neurosci* **48**, 915-924

Kimelberg HK, Goderie SK, Higman S, Pang S, Waniewski RA (1990): Swelling-induced release of glutamate, aspartate and taurine from astrocyte cultures. *J Neurosci* **10**, 1583-1591

Kishimoto AK, Mikawa K, Hashimoto K, Yasuda I, Tanaka S, Tominaga M, Kuroda T, Nishizuka Y (1989): Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain). *J Biol Chem* **264**, 4088-4092

Klotz KN, Lohse MJ, Schwabe U (1988): Chemical modification of A1 adenosine receptors in rat brain membranes. *J Biol Chem* **263**, 17522-17526

Klotz KN, Keil R, Zimmer FJ, Schwabe U (1990): Guanine nucleotide effects on [3H]DPCPX binding to membrane-bound and solubilized A1 adenosine receptors of rat brain. *J Neurochem* **54**, 1988-1994

Knight RJ, Collis MG, Yates MS, Bowmer CJ (1991): Amelioration of cisplatin-induced acute renal failure with 8-cyclopentyl-1,3-dipropylxanthine. *Br J Pharmacol* **104**, 1062-1068

Knight RJ, Bowmer CJ, Yates MS (1993): The diuretic action of DPCPX, a selective A1 adenosine receptor antagonist. *Br J Pharmacol* **109**, 271-277

Kokubun S, Reuter H (1984): Dihydropyridine derivatives prolong the open state of the Ca channels in cultured cardiac cells. *Proc Natl Acad Sci USA* **81**, 4824-4827

Kolb B (1990): Organization of the neocortex of the rat. In: The cerebral cortex of the rat (eds B Kolb and RC Tees) p. 21-34 and 459-472. MIT Press, Cambridge MA

Kollias-Baker C, Ruble J, Dennis D, Bruns RF, Linden J, Belardinelli L (1994): Allosteric enhancer PD 81,723 acts by novel mechanism to potentiate cardiac actions of adenosine. *Circ Res* **75**, 961-971

Krahl SE, Treas LM, Castle JD, Berman RF (1995): Attenuation of in vivo and in vitro seizure activity using the adenosine agonist metrifudil. *Drug Dev Res* **34**, 30-34

Kreutzberg GW, Heymann D, Reddington M (1986): 5'-nucleotidase in the nervous system. In *Cellular Biology of Ectoenzymes* (eds GW Kreutzberg, D Heymann and H Zimmermann), p. 147-164. Springer Verlag, Berlin.

Kristiansen K, Courtois G (1949): Rhythmic electrical activity from isolated cerebral cortex. *EEG Clin Neurophysiol* **1**, 265-272

Krubitzer LA, Calford MB, Schmid LM (1993): Connections of somatosensory cortex in megachiropteran bats: the evolution of cortical fields in mammals. *J Comp Neurol* **327**, 473-506

Kuan C, Herzer WA, Jackson EK (1992): An experimental paradigm for investigating the role of endogenous adenosine/A1 receptor interactions *in vivo*. *J Pharmacol Exp Therap* **263**, 657-662

Kuroda Y (1990): Activity-dependent release of ATP and adenosine derivatives can trigger molecular cascades for the memory process in the human brain. *Jpn J Pharmacol* **52**, 70

Kurtzke JF (1982): The current neurologic burden of illness and injury in the United States. *Neurology* **32**, 1207-1214

Kyriazi HT, Simons DJ (1991): Computer simulations of thalamo-cortical response transformations in the whisker/barrel system. *Neurosci Abstr* **17**, 1109

Kyriazi HT, Simons DJ (1993): Thalamocortical response transformations in simulated whisker barrels. *J Neurosci* **13**, 1601-1615

Lambert NA, Teyler TJ (1991): Adenosine selectively depresses excitatory synaptic transmission in area CA1 of the rat hippocampus. *Neurosci Letts* **122**, 50-52

Land PW, Simons DJ (1985): Cytochrome oxidase staining in the rat SmI barrel cortex. *J Comp Neurol* **238**, 225-235

Land PW, Simons DJ, Buffer SA (1986): Specificity of thalamocortical connections in the rat somatosensory system. *Soc Neurosci Abstr* **12**, 1434

Landry P, Deschenes M (1981): Intracortical arborizations and receptive fields of identified ventrobasal thalamocortical afferents to the primary somatic sensory cortex in the cat. *J Comp Neurol* **199**, 345-371

Langdon RB, Sur M (1990): Components of field potentials evoked by white matter stimulation in isolated slices of primary visual cortex - spatial distributions and synaptic order. *J Neurophysiol* **64**, 1484-1501

Lasley RD, Noble MA, Paulsen KL, Mentzer RM (1994): Adenosine attenuates phorbol ester-induced negative inotropic and vasoconstrictive effects in rat hearts. *Am J Physiol* **266**, H2159-H2166

Lee WL, Hablitz JJ (1991): Excitatory synaptic involvement in epileptiform bursting in the immature rat neocortex. *J Neurophysiol* **66**, 1894-1901

Lehmenkühler A, Nicholson C, Speckmann EJ (1991): Threshold extracellular concentration distribution of penicillin for the generation of epileptic foci measured by diffusion analysis. *Brain Res* **561**, 292-298

Lerer B, Stanley M, Altman H, Keegan M (1986): An animal model of electroconvulsive therapy-induced amnesia - possible neurochemical correlates. *Ann N Y Acad Sci* **462**, 91-98

Leung E, Jacobson KA, Green RD (1990): Analysis of agonist-antagonist interactions at A1 adenosine receptors. *Mol Pharmacol* **38**, 72-83

LeVay S, McConnell SK, Luskin MB (1987): Functional organization of primary visual cortex in the mink (Mustela vison), and a comparison with the cat. *J Comp Neurol* **257**, 422-441

Libert F, Schiffmann SN, Lefort A, Parmentier M, Gérard C, Dumont JE, Vanderhaeghen JJ, Vassart G (1991): The orphan receptor cDNA RDC7 encodes an A1 adenosine receptor. *EMBO J* **10**, 1677-1682

Linden J (1991): Structure and function of A1 adenosine receptors. *FASEB J* **5**, 2668-2676

Linden J (1994): Cloned adenosine A3 receptors - pharmacological properties, species differences and receptor functions. *Trends Pharmacol Sci* **15**, 298-306

Lindheimer MD (1993): Hypertension in pregnancy. *Hypertension* **22**, 127-137

Lloyd HGE, Deussen A, Wuppermann H, Schrader J (1988): The transmethylation pathway as a source for adenosine in the isolated guinea-pig heart. *Biochem J* **252**, 489-494

Lobban M, Shakur Y, Beattie J, Houslay MD (1994): Identification of two splice variant forms of type IVB cyclic AMP phosphodiesterase, DPD (rPDE-IVB1) and PDE-4 (rPDE-IVB2) in brain: selective localization in membrane and cytosolic compartments and differential expression in various brain regions. *Biochem J* **304**, 399-406

Löchner KJ, Knox RJ, Connor JA, Kaczmarek LK (1992): Hyperosmotic media inhibit voltage-dependent calcium influx and peptide release in Aplysia neurons. *J Membr Biol* **128**, 41-52

Lockton JW, Holmes O (1980): Site of the initiation of penicillin-induced epilepsy in the cortex cerebri of the rat. *Brain Res* **190**, 301-304

Lockton JW, Holmes O (1983): Penicillin epilepsy in the rat - the responses of different layers of the cortex cerebri. *Brain Res* **258**, 79-89

Lohse MJ, Lenschow V, Schwabe U (1984): Two affinity states of Ri adenosine receptors in brain membranes. *Mol Pharmacol* **26**, 1-9

Lohse MJ, Klotz KN, Lindenborn-Fotinos J, Reddington M, Schwabe U, Olsson RA (1987): DPCPX - a selective high affinity antagonist radioligand for A1 adenosine receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* **336**, 204-210

Loke WH, Hinrichs JV, Ghonheim MM (1985): Caffeine and diazepam: separate and combined effects on mood, memory and psychomotor performance. *Psychopharmacol* **87**, 344-350

Londos C, Cooper DMF, Schlegel W, Rodbell M (1978): Adenosine analogs inhibit adipocyte adenylate cyclase via a GTP-dependent process - basis for actions of adenosine and methylxanthines on cAMP production and lipolysis. *Proc Natl Acad Sci USA* **75**, 5362-5366

Londos C, Wolff J, Cooper DMF (1983): Adenosine receptors and adenylate cyclase interactions. In: Regulatory Function of Adenosine (eds RM Berne, TW Rall and R Rubio) p. 17-32. Nijhoff, Boston MA

Longabaugh JP, Didsbury J, Spiegel A, Stiles G (1989): Modification of the rat adipocyte A1 adenosine receptor-adenylate cyclase system during chronic exposure to an A1 adenosine receptor agonist - alterations in the quantity of Gs alpha and Gi alpha are not associated with changes in their mRNAs. *Mol Pharmacol* **36**, 681-688

Lorente de No R (1938): Cerebral cortex - architecture, intracortical connections, motor projections. In: The Physiology of the Nervous System, 3rd edition (ed JF Fulton), p. 291-325. Oxford University Press, London

Löscher W, Schmidt D (1988): Which animal models should be used in the search for new antiepileptic drugs? *Epilepsy Res* **2**, 145-181

Lueders H, Bustamante LA, Zablow L, Goldensohn ES (1981): The independence of closely spaced discrete experimental spike foci. *Neurology* **31**, 846-851

Lüllman H, Timmermans PBMWM, Ziegler A (1979): Accumulation of drugs by resting or beating cardiac tissue. *Eur J Pharmacol* **60**, 277-285

Lund JS (1984): Cellular components of the cerebral cortex. In: Cerebral Cortex Vol 1 (eds A Peters and EG Jones), p 255-308. Plenum Press, New York

Lurie SN, Coffey CE (1990): Caffeine-modified electroconvulsive therapy in depressed patients with medical illness. *J Clin Psychiatry* **51**, 154-157

Luskin MB, Shatz CJ (1985): Neurogenesis of the cat's primary visual cortex. *J Comp Neurol* **242**, 611-631

Lytton WW, Sejnowski TJ (1991): Simulations of cortical pyramidal neurons synchronized by inhibitory interneurons. *J Neurophysiol* **66**, 1059-1078

MacDonald RL, Skerritt JH, Werz MA (1986): Adenosine agonists reduce voltage-dependent calcium conductance of mouse sensory neurons in cell cultures. *J Physiol (Lond)* **370**, 75-90

MacGregor DG, Miller WJ, Stone TW (1993): Mediation of the neuroprotective action of R-phenylisopropyl-adenosine through a centrally located adenosine A1 receptor. *Br J Pharmacol* **110**, 470-476

Madison DV, Nicoll RA (1986): Cyclic adenosine-3',5'-monophosphate mediates ß-receptor actions of noradrenaline in rat hippocampal pyramidal cells. *J Physiol (Lond)* **372**, 245-259

Madison DV, Nicoll RA (1988): Norepinephrine decreases synaptic inhibition in the rat hippocampus. *Brain Res* **442**, 131-138

Maenhaut C, Van Sande J, Libert F, Abramowicz M, Parmentier M, Vanderhagen JJ, Dumont JE, Vassart G, Schiffmann S (1990): RDC8 codes for an adenosine A2 receptor with physiological constitutive activity. *Biochem Biophys Res Comm* **173**, 1169-1178

Mager R, Ferroni S, Schubert P (1990): Adenosine mediates a voltage-dependent chloride conductance in cultured hippocampal neurons. *Brain Res* **532**, 58-62

Maitre M, Ciesielski L, Lehmann A, Kempf E, Mandel P (1974): Protective effect of adenosine and nicotinamide against audiogenic seizures. *Biochem Pharmacol* **23**, 2807-2816

Malenka RC, Madison DV, Nicoll RA (1986): Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature* **321**, 175-177

Manabe T, Renner P, Nicholl RA (1992): Postsynaptic contribution to long-term potentiation revealed by the analysis of miniature synaptic currents. *Nature* **355**, 50-55

Matias A, Albino-Teixeira A, Polonia J, Azevedo I (1991): Long-term administration of DPSPX causes arterial hypertension. *Eur J Pharmacol* **193**, 101-104

Mattia D, Hwa GGC, Avoli M (1993): Epileptiform activity induced by 4-aminopyridine in guinea-pig and rat neocortices. *Neurosci Lett* **154**, 157-160

McCall WV, Reid S, Rosenquist P, Foreman A, Kiesow-Webb N (1993): A reappraisal of the role of caffeine in ECT. *Am J Psychiatry* **150**, 1543-1545

McCandlish CA, Li CX, Waters RS (1993): Early development of the SI cortical barrel field representation in neonatal rats follows a lateral-to-medial gradient: an electrophysiological study. *Exp Brain Res* **92**, 369-374

McCormick DA, Williamson A (1989): Convergence and divergence of neurotransmitter action in human cerebral cortex. *Proc Natl Acad Sci USA* **86**, 8098-8102

Meghji P (1993): Storage, release, uptake and inactivation of purines. *Drug Dev Res* **28**, 214-219

Michael CR (1978): Color vision mechanisms in monkey striate cortex: Simple cells with dual opponent-color receptive fields. *J Neurophysiol* **41**, 1233-1249

Miles R, Wong RKS, Traub RD (1984): Synchronized afterdischarges in the hippocampus - contribution of local synaptic interaction. *Neurosci* **12**, 1179-1189

Miles R, Wong RKS (1986): Excitatory synaptic interactions between CA3 neurons in the guinea pig hippocampus *in vitro*. *J Physiol (Lond)* **373**, 97-113

Miller MW (1988): Development of projection and local circuit neurons in neocortex. In: Cerebral cortex, Vol. 7: Development and Maturation of the Cerebral Cortex (eds: A Peters and EG Jones), pp. 133-175. Plenum Press, New York.

Miyazaki S, Yuzaki M, Nakada K, Shirakawa H, Nakanishi S, Nakade S, Mikoshiba K (1992): Block of Ca^{++} wave and Ca^{++} oscillation by antibody to the inositol-1,4,5-trisphosphate receptor in fertilized hamster eggs. *Science* **257**, 251-255

Mogul DJ, Adams ME, Fox AP (1993): Differential activation of adenosine receptors decreases N-type but potentiates P-type Ca^{2+} current in hippocampal CA3 neurones. *Neuron* **10**, 327-334

Montgomery EB, Clare MH, Sahrmann S, Buchholz SR, Hibbard LS, Landau WM (1992): Neuronal multipotentiality - evidence for network representation of physiological function. *Brain Res* **580**, 49-61

Morgan PF, Deckert J, Jacobson KA, Marangos PJ, Daly JW (1989): Potent convulsant actions of the adenosine receptor antagonist, xanthine amine congener (XAC). *Life Sci* **45**, 719-728

Mori H, Mizutani T, Yoshimura M, Yamanouchi H, Shimada H (1992): Unilateral brain damage after prolonged hemiconvulsions in the elderly associated with theophylline administration. *J Neurol Neurosurg Psychiatry* **55**, 466-469

Morrell F, Hanbery JW (1969): A new surgical technique for the treatment of focal cortical epilepsy. *EEG Clin Neurophysiol* **26**, 120

Morrell F, Whisler WW, Bleck TP (1989): Multiple subpial transection: a new approach to the surgical treatment of focal epilepsy. *J Neurosurg* **70**, 231-239

Mountcastle VB (1957): Modality and topographic properties of single neurons of cat's somatic sensory cortex. *J Neurophysiol* **20**, 408-434

Mountcastle VB (1979): An organizing principle for cerebral function: the unit module and the distributed system. In: The *Neurosci*ences (eds FO Schmitt and FG Worden) p. 21-42. MIT Press, Cambridge MA

Mumford GK, Holtzman SG (1991): Qualitative differences in the discriminative stimulus effects of low and high doses of caffeine in the rat. *J Pharmacol Exp Therap* **258**, 857-865

Munshi R, Linden J (1990): Interaction of purified bovine brain A1 adenosine receptors with guanine nucleotide binding proteins of human platelet membranes following reconstitution. *Mol Pharmacol* **38**, 170-179

Murakami K, Routtenberg A (1986): Protein kinase C activation by *cis*-fatty acid in the absence of Ca^{++} and phospholipids. *J Biol Chem* **261**, 15424-15429

Murray TF, Szot P (1985): A1 adenosine receptor mediated modulation of seizure susceptibility. In: Neurotransmitters in Epilepsy (eds PL Morselli, KG Lloyd, RG Fariello and J Engel Jr), p. 341-353. Raven Press, New York.

Murray TF, Zhang G, Franklin PH (1993): Manipulation of endogenous adenosine affects seizure susceptibility. *Drug Dev Res* **28**, 410-415

Nakada T, Kwee IL, Lerner AM, Remler MP (1983): Theophylline-induced seizures - clinical and pathophysiological aspects. *West J Med* **138**, 371-374.

Nakagawa Y, Gudenzi M, Mustafa SJ (1986): Calcium entry blocking activity of dilazep and other adenosine potentiating compounds in guinea pig atria. *Eur J Pharmacol* **122**, 51-58

Nakajima T, Daval JL, Morgan PF, Post RM, Marangos PJ (1989): Adenosinergic modulation of caffeine-induced c-fos mRNA expression in mouse brain. *Brain Res* **501**, 307-314

Nanoff C, Freissmuth M, Tuisl E, Schütz W (1990): P2-, but not P1-purinoceptors mediate formation of 1, 4, 5-inositol trisphosphate and its metabolites via a pertussis toxin-insensitive pathway in the rat renal cortex. *Br J Pharmacol* **100**, 63-68

Nelsestuen GL, Bazzi MD (1991): Activation and regulation of protein kinase-C enzymes. *J Bioenerget Biomembr* **23**, 43-61

Neve KA, Wiens BL (1995): Four ways of being an agonist - multiple sequence determinants of efficacy at D2 dopamine receptors. *Biochem Soc Transactions* **23**, 112-116

Newby AC, Worku Y, Meghji P, Skladanowski AC (1990): Adenosine: a retaliatory metabolite or not? *News Physiol Sci* **5**, 67-70

Nguyen PV, Abel T, Kandel EC (1994): Requirement of a critical period of transcription for induction of a late phase of LTP. *Science* **265**, 1104-1106

Nicoll RA, Malenka RC, Kauer JA (1990): Functional comparison of neurotransmitter receptor subtypes in the mammalian central nervous system. *Physiol Rev* **70**, 513-565

Nikodijevic O, Daly JW, Jacobson DA (1990): Characterization of the locomotor depression produced by an A2-selective adenosine agonist. *FEBS Lett* **261**, 67-70

Nishimura S, Okada Y, Amatsu M (1992): Post-inhibitory excitation of adenosine on neurotransmission in guinea pig hippocampal slices. *Neurosci Letts* **139**, 126-129

Nishizuka Y (1988): The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**, 661-665

Nishizuka Y (1992): Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 607-614

Norenberg MD, Chu NS (1977): Aminophylline-induced preictal alterations in cortical astrocytes. *Exp Neurol* **54**, 340-351

Ocana M, Baeyens JM (1994): Role of ATP-sensitive K+ channels in antinociception induced by R-PIA, an adenosine A1 receptor agonist. *Naunyn-Schmiedeberg's Arch Pharmacol* **350**, 57-62

Okada Y, Sakurai T, Mori M (1992): Excitatory effect of adenosine on neurotransmission is due to increase of transmitter release in the hippocampal slices. *Neurosci Letts* **142**, 233-236

Olah ME, Ren H, Ostrowski J, Jacobson KA, Stiles GL (1992): Cloning, expression, and characterization of the unique bovine A1-adenosine receptor - studies on the ligand binding site by site-directed mutagenesis. *J Biol Chem* **267**, 10764-10770

Olpe HR, Steinmann MW, Ferrat T, Pozza MF, Greiner K, Brugger F, Froestl W, Mickel SJ, Bittiger H (1993): The actions of orally active GABAb receptor antagonists on GABAergic transmission *in vivo* and *in vitro*. *Eur J Pharmacol* **233**, 179-186

Olsson RA, Pearson JD (1990): Cardiovascular purinoceptors. *Physiol Rev* **70**, 761-845

Olson KR, Benowitz NL, Woo OF (1985): Theophylline overdose - acute single ingestion versus chronic repeated overmedication. *Am J Emerg Med* **3**, 386-394

Onodera H, Kogure K (1988): Differential localization of adenosine A1 receptors in the rat hippocampus - a quantitative autoradiographic study. *Brain Res* **458**, 212-217

Onodera H, Araki T, Kogure K (1989): Protein kinase C activity in the rat hippocampus after forebrain ischemia - autoradiographic analysis by [3H]phorbol 12,13-dibutyrate. *Brain Res* **481**, 1-7

O'Regan MH, Simpson RE, Perkins LM, Phillis JW (1992): Adenosine receptor agonists inhibit the release of GABA from the ischemic rat cerebral cortex. *Brain Res* **582**, 22-26

Osehobo EP, Andrew RD (1992): Osmotic effects upon the theta rhythm, a natural brain oscillation in the hippocampal slice. *Exp Neurol* **124**, 192-199

Otani S, Ben-Ari Y, Roisin-Lallemand MP (1993): Metabotropic receptor stimulation coupled to weak tetanus leads to long-term potentiation and a rapid elevation of cytosolic protein kinase C activity. *Brain Res* **613**, 1-9

Pagonopoulou O, Angelatou F, Kostopoulos G (1993): Effect of pentylenetetrazol-induced seizures on A1 adenosine receptor regional density in the mouse brain - a quantitative autoradiographic study. *Neurosci* **56**, 711-716

Pan WJ, Osmanovic SS, Shefner SA (1994): Adenosine decreases action potential duration by modulation of A-current in rat locus coeruleus neurons. *J Neurosci* **14**, 1114-1122

Pape HC (1992): Adenosine promotes burst activity in guinea-pig geniculocortical neurones through two different ionic mechanisms. *J Physiol (Lond)* **447**, 729-753

Parkinson FE, Fredholm BB (1992): Magnesium-dependent enhancement of endogenous agonist binding to A1 adenosine receptors: a complicating factor in quantitative autoradiography. *J Neurochem* **58**, 941-950

Parsons WJ, Stiles GL (1987): Heterologous desensitisation of the inhibitory A1 adenosine receptor-adenylate cyclase system in rat adipocytes. *J Biol Chem* **262**, 841-847

Patton P, Thomas E, Wyatt RE (1992): A computational model of vertical signal propagation in the primary visual cortex. *Biol Cybern* **68**, 43-52

Paxinos G, Watson C (1986): The rat brain in stereotaxic coordinates. Academic Press, New York.

Pedarzani P, Storm JF (1993): PKA mediates the effects of monoamine transmitters on the K^+ current underlying the slow spike frequency adaptation in hippocampal neurons. *Neuron* **11**, 1023-1035

Peters SG, Wochos DN, Peterson GC (1984): Status epilepticus as a complication of concurrent electroconvulsive and theophylline therapy. *Mayo Clin Proc* **59**, 568-570.

Phillis JW, O'Regan MH (1988a): Benzodiazepine interaction with adenosine systems explains some anomalies in GABA hypothesis. *Trends Pharmacol Sci* **9**, 153-154

Phillis JW, O'Regan MH (1988b): The role of adenosine in the central actions of the benzodiazepines. *Prog Neuropsychopharmacol Biol Psychiatry* **12**, 389-404

Phillis JW (1990): The selective adenosine A2 receptor agonist, CGS 21680, is a potent depressant of cerebral cortical neuronal activity. *Brain Res* **509**, 328-330

Pierre-Louis SJ, Morrell F (1992): Epileptiform activity in chronically isolated cerebral cortex in humans. *EEG Clin Neurophysiol* **82**, 248-254

Piredda S, Gale K (1985): Evidence that the deep prepiriform cortex contains a crucial epileptogenic site. *Nature* **317**, 623-625

Prater MR, Taylor H, Munshi R, Linden J (1992): Indirect effect of guanine nucleotides on antagonist binding to adenosine receptors - occupation of cryptic binding sites by endogenous vesicular adenosine. *Mol Pharmacol* **42**, 765-772

Prince DA, Stevens CF (1992): Adenosine decreases neurotransmitter release at central synapses. *Proc Natl Acad Sci USA* **89**, 8568-8590

Prince DA, Tseng GF (1993): Epileptogenesis in chronically injured cortex - in vitro studies. *J Neurophysiol* **69**, 1276-1291

Pull I, McIlwain H (1972): Adenine derivatives as neurohumoral agents in the brain - the quantities liberated on excitation of superfused cerebral tissues. *Biochem J* **130**, 975-981

Pumain R, Heinemann U (1991): Changes in extracellular ions associated with epileptiform discharges. In: Generalized epilepsy - neurobiological approaches (eds M Avoli, P Gloor, G Kostopoulos and R Naquet), pp. 61-85. Birkhäuser, Boston.

Quesney LF, Gloor P (1978): Generalized penicillin epilepsy in the cat: correlation between electrophysiological data and distribution of 14C-penicillin in the brain. *Epilepsia* **19**, 35-45

Radpour S, Thomson AM (1992): Synaptic enhancement induced by NMDA and Qp receptors and presynaptic activity. *Neurosci Lett* **138**, 119-122

Rainnie DG, Grunze HCR, McCarley RW, Greene RW (1994): Adenosine inhibition of mesopontine cholinergic neurons - implications for EEG arousal. *Science* **263**, 689-692

Rakic P (1974): Neurons in Rhesus monkey visual cortex - systematic relation between time of origin and eventual disposition. *Science* **183**, 425-427

Rakic P (1978): Neuronal migration and contact guidance in the primate telencephalon. *Postgrad Med J* **54**, 25-40

Ramachandran VS (1994): Phantom limb pain, neglect syndromes, repressed memories, and Freudian psychology. *Int Rev Neurobiol* **57**, 291-333

Ramkumar V, Bumgarner JR, Jacobson KA, Stiles GL (1988): Multiple components of the A1 adenosine receptor-adenylate cyclase system are regulated in rat cerebral cortex by chronic caffeine ingestion. *J Clin Invest* **82**, 242-247

Ramkumar V, Olah ME, Jacobson KA, Stiles GL (1991): Distinct pathways of desensitization of A1- and A2-adenosine receptors in DDT1 MF-2 cells. *Mol Pharmacol* **40**, 639-647

Rasmussen KG, Zorumski CF (1993): Electroconvulsive therapy in patients taking theophylline. *J Clin Psychiatry* **54**, 427-431

Reddington M, Alexander SP, Hoppe E, Kreuzberg GW (1989a): Sensitivity to Mg^{2+} of ligand binding to A1 adenosine receptors in rat brain and adipocytes. In: Adenosine Receptors in the Nervous System (ed JA Ribeiro) p. 95-101. Taylor & Francis, London

Reddington M, Klotz KN, Lohse MJ, Hietel B (1989b): Radiation inactivation analysis of the A1 adenosine receptor of rat brain - decrease in radiation inactivation size in the presence of guanine nucleotide. *FEBS Lett* **252**, 125-128

Reichenthal E, Hochermann S (1977): The critical cortical area for development of penicillin-induced epilepsy. *EEG Clin Neurophysiol* **42**, 248-251

Reinhart PH, Chung S, Martin BL, Brautigan DL, Levitan IB (1991): Modulation of calcium-activated potassium channels from rat brain by protein kinase A and phosphatase A2. *J Neurosci* **11**, 1627-1635

Ren Q, Aika Y, Heizmann CW, Kosaka T (1992): Quantitative analysis of neurons and glial cells in the rat somatosensory cortex, with special reference to GABAergic neurons and parvalbumin-containing neurons. *Exp Brain Res* **92**, 1-14

Rhodes DG, Newton R, Butler R, Herbette L (1992): Equilibrium and kinetic studies of the interactions of salmeterol with membrane bilayers. *Mol Pharmacol* **42**, 596-602

Riddle DR, Gutierrez G, Zheng D, White LE, Richards A, Purves D (1993): Differential metabolic and electrical activity in the somatic sensory cortex of juvenile and adult rats. *J Neurosci* **13**, 4193-4213

Rockel AJ, Hiorns RW, Powell TPS (1980): The basic uniformity in structure of the neocortex. *Brain* **103**, 221-244

Rodin E (1984): Sleep deprivation and epileptological sleep implications. In: Epilepsy, sleep, and sleep deprivation (eds. R Degen and E Niedermeyer). Elsevier, Amsterdam, p. 293-301.

Rudolphi KA, Keil M, Fastbom J, Fredholm BB (1989): Ischemic damage in gerbil hippocampus is reduced following upregulation of adenosine (A1) receptors by caffeine treatment. *Neurosci Lett* **103**, 275-280

Sakurai T, Okada Y (1992): Excitatory effect of adenosine on neurotransmission and the release of glutamate from hippocampal slices of guinea pig. *J Physiol (Lond)* **446**, P380

Salomon Y (1991): Cellular responsiveness to hormones and neurotransmitters: conversion of [3H]adenine to [3H]cAMP in cell monolayers, cell suspensions, and tissue slices. *Methods Enzymol* **195**, 22-28

Saly V, Andrew RD (1993): CA3 neuron excitation and epileptiform discharge are sensitive to osmolarity. *J Neurophysiol* **69**, 2200-2208

Sanders HD, Pinski C (1967): Facilitation and spatial summation in neurons concerned with epileptiform afterdischarge in the isolated cerebral cortex of the cat. *Can J Physiol Pharmacol* **45**, 965-970

Santicioli P, Del Bianco E, Maggi CA (1993): Adenosine A1 receptors mediate the presynaptic inhibition of calcitonin gene-related peptide release by adenosine in the rat spinal cord. *Eur J Pharmacol* **231**, 139-142

Sargent Jones L, Grooms SY, Lapadula DM, Lewis DV (1992): Protein synthesis inhibition blocks maintenance but not induction of epileptogenesis in hippocampal slice. *Brain Res* **599**, 338-344

Sattin A, Rall TW (1970): The effect of adenosine and adenine nucleotides on the cAMP content of guinea pig cerebral cortex slices. *Mol Pharmacol* **6**, 13-23

Sawyonok J, Yaksh T (1993): Caffeine as an analgesic adjuvant - a review of pharmacology and mechanisms of action. *Pharmacol Rev* **45**, 43-85

Scarisbrick IA, Jones EG (1993): NCAM immunoreactivity during major developmental events in the rat maxillary nerve-whisker system. *Dev Brain Res* **71**, 121-135

Schiemann WP, Walther JM, Buxton ILO (1990): On the ability of endogenous adenosine to regulate purine nucleoside receptor binding of antagonists in smooth muscle cells. *J Pharmacol Exp Therap* **255**, 886-892

Schingnitz G, Küfner-Mühl U, Ensinger H, Lehr E (1991): A novel xanthine derivative for the treatment of cognitive deficits. *Biol Psychiatr* **29**, 694S

Schlaggar BL, O'Leary DDM (1991): Potential of visual cortex to develop an array of functional units unique to somatosensory cortex. *Science* **252**, 1556-1560

Schober W (1986): The rat cortex in stereotaxic coordinates. *J Hirnforschung* **27**, 121-143

Schrader J, Wahl M, Kuschinski W, Kreutzberg GN (1980): Increase of adenosine content in cerebral cortex of the cat during bicuculline-induced seizures. *Pflügler's Arch* **387**, 245-251

Schubert P, Kreutzberg GW (1993): Cerebral protection by adenosine. *Acta Neurochir Suppl Wien* **57**, 80-88

Schubert P, Keller F, Rudolphi K (1993): Depression of synaptic transmission and evoked NMDA Ca influx in hippocampal neurons by adenosine and its blockade by LTP or ischemia. *Drug Dev Res* **28**, 399-405

Schütz W, Stroher M, Freissmuth M, Valenta B, Singer EA (1991): Adenosine receptors mediate a pertussis toxin-insensitive prejunctional inhibition of noradrenaline release on a papillary muscle model. *Naunyn Schmiedeberg's Arch Pharmacol* **343**, 311-316

Schütz W, Freissmuth M (1992): Reverse intrinsic activity of antagonists on G protein-coupled receptors. *Trends Pharmacol Sci* **13**, 376-380

Schwartzkroin PA, Prince DA (1980): Changes in excitatory and inhibitory synaptic potentials leading to epileptogenic activity. *Brain Res* **183**, 61-76

Seale TW, Ablaq KA, Cao W, Parker KM, Rennert OM, Carney JM (1986): Inherent hyporesponsiveness to methylxanthine-induced behavioral changes associated with supersensitivity to NECA. *Pharmacol Biochem Behav* **25**, 1271-1277

Sebastiao AM, Stone TW, Ribeiro JA (1990): The inhibitory adenosine receptor at the neuromuscular junction and hippocampus of the rat - antagonism by 1,3,8-substituted xanthines. *Br J Pharmacol* **101**, 453-459

Sebastiao AM, Ribeiro JA (1992): Evidence for the presence of excitatory A2 adenosine receptors in the rat hippocampus. *Neurosci Letts* **138**, 41-44

Segal MM, Furshpan EJ (1990): Epileptiform activity in microcultures containing small numbers of hippocampal neurons. *J Neurophysiol* **64**, 1390-1399

Segal MM (1994): Endogenous bursts underlie seizurelike activity in solitary excitatory hippocampal neurons in microcultures. *J Neurophysiol* **72**, 1874-1884

Seitz RJ, Huang Y, Knorr U, Tellmann L, Herzog H, Freund HJ (1995): Large-scale plasticity of the human motor cortex. *NeuroReport* **6**, 742-744

Shannon MW, Maher T (1993): Anticonvulsant activity of Adenocard in experimental theophylline-induced seizures. *Vet Hum Toxicol* **35**, 338

Shapira B, Lerer B, Gilboa D, Drexler H, Kugelmass S, Calev A (1987): Facilitation of ECT by caffeine pretreatment. *Am J Psychiatry* **144**, 1199-1202

Sharp FR, Gonzales MF, Morgan CW, Morton MT, Sharp JW (1988): Common fur and mystacial vibrissae parallel sensory pathways - C14 2-deoxyglucose and WGA-HRP studies in the rat. *J Comp Neurol* **270**, 446-469

Shatz CJ (1992): Dividing up the neocortex. *Science* **258**, 237-238

Shaw MDM, Chadwick DW (1987): Epilepsy and neurosurgery. In: Northfield's Surgery of the Central Nervous System, 2nd edition (ed JD Miller), p. 598ff. Blackwell, Edinburgh.

Shucard DW, Spector SL, Euwer RL, Cummins KR, Shucard JL, Friedman A (1985): Central nervous effects of antiasthmatic medication - an EEG study. *Ann Allergy* **54**, 177-184

Sigel E, Baur R (1988): Activation of protein kinase C differentially modulates neuronal Na, Ca and GABAa channels. *Proc Natl Acad Sci USA* **85**, 6192-6196

Silva-Barrat C, Araneda S, Menini C, Champagnat J, Naquet R (1992): Burst generation in neocortical neurones after GABA withdrawal in the rat. *J Neurosci* **67**, 715-727

Simons DJ (1978): Response properties of vibrissa units in the rat SI somatosensory neocortex. *J Neurophysiol* **41**, 798-820

Simons DJ, Carvell GE (1989): Thalamocortical response transformation in the rat vibrissa/barrel system. *J Neurophysiol* **61**, 311-330

Simons DJ, Carvell GE, Hershey AE, Bryant DP (1992): Responses of barrel cortex neurons in awake rats and effects of urethane anaesthesia. *Exp Brain Res* **91**, 259-272

Siucinska E, Kossut M (1994): Plasticity of mystacial fur representation in SI cortex of adult vibrissectomized rats - a 2DG study. *Neuroreport* **5**, 1605-1608

Smith DA, Dunwiddie TV (1993): Effects of bivalent cations on adenosine sensitivity in the rat hippocampal slice. *Brain Res* **617**, 61-68

Snyder SH, Katims JJ, Annau Z, Bruns RF, Daly JF (1981): Adenosine receptors and behavioural actions of methylxanthines. *Proc Natl Acad Sci USA* **78**, 3260-3264

Snyder SH (1985): Adenosine as a neuromodulator. *Annu Rev Neurosci* **8**, 103-124

Spealman RD, Coffin VL (1986): Behavioral effects of adenosine analogs in squirrel monkeys: relation to adenosine A2 receptors. *Psychopharmacol* **90**, 419-421

Sperry RW, Miner N, Myers RE (1955): Visual pattern perception following subpial slicing and tantalum wire implantation into visual cortex. *J Comp Physiol Psychol* **48**, 50-58

Spyker DA, Lynch C, Shabanowitz J, Sinn JA (1980): Poisoning with 4-aminopyridine - report of three cases. *Clinical Toxicology* **16**, 487-497

Starke K, Reimann W, Zumstein A, Hertting G (1978): Effect of dopamine receptor agonists and antagonists on release of dopamine in the rabbit caudate nucleus *in vitro*. *Naunyn-Schmiedeberg's Arch Pharmacol* **305**, 27-36

Steriade M (1990): Spindling, incremental thalamocortical responses, and spike-wave epilepsy. In: Generalized Epilepsy -Neurobiological Approaches (eds M Avoli, P Gloor, P Kostopoulos and R Naquet), p. 161-180. Birkhäuser, Boston.

Stiles GL (1992): Adenosine receptors. *J Biol Chem* **267**, 6451-6454

Stone TW (1991): *Adenosine in the nervous system*. Academic Press, London.

Stone TW, Simmons HA (1991): *Purines: basic and clinical aspects.* Kluwer Press, Dordrecht.

Ströher M, Nanoff C, Schütz W (1989): Differences in the GTP-regulation of membrane-bound and solubilized adenosine A1-receptors. *Naunyn-Schmiedeberg's Arch Pharmacol* **340**, 87-92

Strominger RN, Woolsey TA (1987): Templates for locating the whisker area in fresh flattened mouse and rat cortex. *J Neurosci Methods* **22**, 113-118

Sur M, Wall JT, Kaas J (1984): Modular distribution of neurons with slowly adapting and rapidly adapting responses in area 3b of somatosensory cortex in monkeys. *J Neurophysiol* **51**, 724-743

Swadlow HA (1989): Efferent neurons and suspected interneurons in S-I vibrissa cortex of the awake rabbit: receptive fields and axonal properties. *J Neurophysiol* **62**, 288-308

Swartz CM, Lewis RK (1991): Theophylline reversal of electroconvulsive therapy (ECT) seizure inhibition. *Psychosomatics* **32**, 47-51

Swartz KJ (1993): Modulation of Ca^{2+} channels by protein kinase C in rat central and peripheral neurons - disruption of G protein-mediated inhibition. *Neuron* **11**, 305-320

Szentágothai J (1987): Modular architectonics of neuronal centers. In: Encyclopedia of Neuroscience (ed. G. Adelman). Birkhäuser, Basel, p. 74-77

Szot P, Sanders RC, Murray TF (1987): Theophylline-induced upregulation of A1-adenosine receptors associated with reduced sensitivity to convulsants. *Neuropharmacol*ogy **26**, 1173-1180

Takacs J, Saillour P, Imbert M, Bogner M, Hamori J (1992): Effect of dark rearing on the volume of visual cortex (areas 17 and 18) and number of visual cortical cells in young kittens. *J Neurosci* Res **32**, 449-459

Tang WJ, Gilman AG (1992): Adenylyl cyclases. *Cell* **70**, 869-872

Tang-Liu DDS, Williams RL, Riegelman S (1983): Disposition of caffeine and its metabolites in man. *J Pharmacol Exp Therap* **224**, 180-185

Tasker JG, Peacock WJ, Dudek FE (1992): Local synaptic circuits and epileptiform activity in slices of neocortex from children with intractable epilepsy. *J Neurophysiol* **67**, 496-507

Tawfik-Schlieper H, Klotz KN, Kreye VA, Schwabe U (1989): Characterization of the K+ channel-coupled adenosine receptor in guinea pig atria. *Naunyn-Schmiedeberg's Arch Pharmacol* **340**, 684-688

Tetzlaff W, Schubert P, Kreutzberg GW (1987): Synaptic and extrasynaptic localization of adenosine binding sites in the rat hippocampus. *Neurosci* **21**, 869-875

Tharp BR (1971): The penicillin focus: a study of field characteristics using cross-correlation analysis. *EEG Clin Neurophysiol* **31**, 45-55

Thesleff S (1980): Aminopyridines and synaptic transmission. *Neurosci* **5**, 1413-1419

Thithapandha A, Maling Harriet M, Gilette JR (1972): Effects of caffeine and theophylline on activity of rats in relation to brain xanthine concentrations. *Proc Soc Exp Biol Med* **139**, 582-586

Thompson SM, Haas HL, Gähwiler BH (1992): Comparison of the actions of adenosine at pre- and postsynaptic receptors in the rat hippocampus *in vitro*. *J Physiol (Lond)* **451**, 347-363

Townsend-Nicholson A, Schofield PR (1994): A threonine residue in the 7th transmembrane domain of the human A1 adenosine receptor mediates specific agonist binding. *J Biol Chem* **269**, 27370-27376

Traub RD, Wong RKS (1983): Synchronized burst discharge in the disinhibited hippocampal slice. II. Model of the cellular mechanism. *J Neurophysiol* **49**, 459-471

Traub RD, Miles R, Jefferys JGR (1993): Synaptic and intrinsic conductances shape picrotoxin-induced synchronized afterdischarges in the guinea-pig hippocampal slice. *J Physiol (Lond)* **461**, 525-547

Traversa U, Florio C, Rosati AM, Vertua R (1990): Differential effect of calcium and magnesium ions on [3H]cyclohexyladenosine binding to rat cerebral cortical particulate preparations. *Pharmacol (Life Sci Adv)* **9**, 233-242

Trussel LO, Jackson MB (1985): Adenosine-activated potassium conductance in cultured striatal neurons. *Proc Natl Acad Sci USA* **82**, 4857-4861

Ulas J, Brunner LC, Nguyen L, Cotman CW (1993): Reduced density of adenosine A1 receptors and preserved coupling of adenosine A1 receptors to G proteins in Alzheimer hippocampus: a quantitative autoradiographic study. *Neurosci* **52**, 843-854

Ulrich D, Huguenard JR (1995): Purinergic inhibition of GABA and glutamate release in the thalamus - implications for thalamic network activity. *Neuron* **15**, 909-918

Umemiya M, Berger AJ (1994); Activation of adenosine A1 and A2 receptors differentially modulates calcium channels and glycinergic synaptic transmission in rat brainstem. *Neuron* **13**, 1439-1446

van der Loos H (1976): Barreloids in mouse somatosensory thalamus. *Neurosci Lett* **2**, 1-6

Vistisen K, Poulsen HE, Loft S (1992): Foreign compound metabolism capacity in man measured from metabolites of dietary caffeine. *Carcinogenesis* **13**, 1561-1568

von Kügelgen I, Späth L, Starke K (1992): Adenosine but not an adenine nucleotide mediates tonic purinergic inhibition, as well as inhibition by glutamate, of noradrenaline release in rabbit brain cortex slices. *Naunyn-Schmiedeberg's Arch Pharmacol* **346**, 677-684

Wadman WJ, Connor JA (1992): Persisting modification of dendritic calcium influx by excitatory amino acid stimulation in isolated CA1 neurons. *Neurosci* **48**, 293-305

Walker MR, Akers LA (1988): A neuromorphic approach to adaptive digital circuitry. Proceedings of the 7th Annual International IEEE Conference on Computers and Communications, p. 19, March 16, 1988

Walsh C, Cepko CL (1992): Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. *Science* **255**, 434-440

Watts AE, Jefferys JGR (1993): Effects of carbamazepine and baclofen on 4-AP-induced epileptic activity in rat hippocampal slices. *Br J Pharmacol* **108**, 819-823

Watts AE, Whittington MA, Jefferys JGR (1993): Epileptic focus induced in rat by intrahippocampal cholera toxin: neuronal properties *in vitro*. *Neurosci* **55**, 45-56

Wayman GA, Impey S, Wu Z, Kindsvogel W, Prichard L, Storm DR (1994): Synergistic activation of the type I adenylyl cyclase by Ca^{2+} and Gs-coupled receptors in vivo. *J Biol Chem* **269**, 25400-25405

Welker E, Armstrong-James M, Van der Loos H, Kraftsik R (1993): The mode of activation of a barrel column - response properties of single units in the somatosensory cortex of the mouse upon whisker deflection. *Eur J Neurosci* **5**, 691-712

Wetts R, Fraser SE (1988): Multipotent precursors can give rise to all major cell types of the frog retina. *Science* **239**, 1142-1145

White EL (1978): Identified neurons in mouse SmI cortex which are postsynaptic to thalamocortical axon terminals: a combined Golgi, electron microscopic and degeneration study. *J Comp Neurol* **181**, 627-661

White EL (1989): Cortical circuits: synaptic organization of the cerebral cortex - Structure, function and theory, p.194. Birkhäuser, Boston.

White EL, Peters A (1993): Cortical modules in the posteromedial barrel subfield (SmI) of the mouse. *J Comp Neurol* **334**, 86-96

White TD, Craig CD, Hoehn K (1993): Extracellular adenosine, formed during low-level NMDA receptor activation, provides an inhibitory threshold against further NMDA receptor-mediated neurotransmission in the cortex. *Drug Dev Res* **28**, 406-409

Williams SF, Colling SB, Whittington MA, Jefferys JG (1993): Epileptic focus induced by intrahippocampal cholera toxin in rat - time course and properties in vivo and in vitro. *Epilepsy Res* **16**, 137-140

Wood PL, Kim HS, Boyar WC, Hutchison A (1989): Inhibition of nigrostriatal release of dopamine in the rat by adenosine receptor agonists - A1 receptor mediation. *Neuropharmacol* **28**, 21-25

Woolsey TA, van der Loos H (1970): The structural organization of layer IV in the somatosensory region (SI) of mouse cerebral cortex. *Brain Res* **17**, 205-242

Woolsey TA, Dierker ML, Wann DF (1975): Mouse SmI cortex: qualitative and quantitative classification of Golgi-impregnated barrel neurons. *Proc Natl Acad Sci USA* **72**, 2165-2169

Woolsey TA, Wann JR (1976): Areal changes in mouse cortical barrels following vibrissal damage at different postnatal ages. *J Comp Neurol* **170**, 53-66

Wu LG, Saggau P (1994): Adenosine inhibits evoked synaptic transmission primarily by reducing presynaptic calcium influx in area CA1 of hippocampus. *Neuron* **12**, 1139-1148

Wu Z, Thomas SA, Villacres EC, Xia Z, Simmons ML, Chavkin C, Palmiter RD, Storm DR (1995): Altered behaviour and long-term potentiation in type I adenylyl cyclase mutant mice. *Proc Natl Acad Sci USA* **92**, 220-224

Würl P (1994): Lebensgefährliche Coffeinintoxikation unter Verwendung von Kaffee als Rauschmittel. *Wien Klin Wochenschr* **106**, 359-361

Xia Z, Refsdal CD, Merchant KM, Dorsa DM, Storm DR (1991): Distribution of mRNA for the calmodulin-sensitive adenylate cyclase in rat brain - expression in areas associated with learning and memory. *Neuron* **6**, 431-443

Xia Z, Choi EJ, Wang F, Blazynski C, Storm DR (1993): Type I calmodulin-sensitive adenylyl cyclase is neural specific. *J Neurochem* **60**, 305-311

Xiong Y, Westhead EW, Slakey LL (1995): Role of phosphodiesterase isoenzymes in regulating intracellular cyclic AMP in adenosine-stimualted smooth muscle cells. *Biochem J* **305**, 627-633

Yarnell PR, Chu NS (1975): Focal seizures and aminophylline. *Neurology* **25**, 819-822

Yeung SMH, Fossom LH, Gill DL, Cooper DMF (1985): Magnesium ion exerts a central role in the regulation of inhibitory adenosine receptors. *Biochem J* **229**, 91-100

Yeung SMH, Perez-Reyes E, Cooper DMF (1987): Hydrodynamic properties of adenosine Ri receptors solubilized from rat cerebral cortical membranes. *Biochem J* **248**, 635-642

Yin JCP, Wallach JS, Del Vecchio M, Wilder EL, Zhou H, Quinn WG, Tully T (1994): Induction of a dominant negative CREB transgene specifically blocks long-term memory in Drosophila. *Cell* **79**, 49-58

Yoon KW, Rothman SM (1991): Adenosine inhibits excitatory but not inhibitory synaptic transmission in the hippocampus. *J Neurosci* **11**, 1375-1380

Young HS, Skita V, Mason RP, Herbette LG (1992): Molecular basis for the inhibition of 1,4-dihydropyridine calcium channel drugs binding to their receptors by a nonspecific site interaction mechanism. *Biophys J* **61**, 1244-1255

Young D, Dragunow M (1994): Status epilepticus may be caused by loss of adenosine anticonvulsant mechanisms. *Neurosci* **58**, 245-261

Yuste R, Peinado A, Katz LC (1992): Neuronal domains in developing neocortex. *Science* **257**, 665-669

Zhang G, Franklin PH, Murray TF (1993): Manipulation of endogeous adenosine in the rat prepiriform cortex modulates seizure susceptibility. *J Pharmacol Exp Therap* **246**, 1415-1424

Zheng D, Purves D (1995): Effects of increased neuronal activity on brain growth. *Proc Natl Acad Sci USA* **92**, 1802-1806

Zhou QY, Li C, Olah ME, Johnson RA, Stiles GL, Civelli O (1992): Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. *Proc Natl Acad Sci USA* **89**, 7432-7436

Zimmermann H (1994): Synaptic transmission - Cellular and molecular basis. Thieme, Stuttgart

Zimmermann H (1994a): Signalling via ATP in the nervous system. *TINS* **17**, 420-426

Zwillich CW, Sutton FD, Neff TA, Cohn WM, Matthay RA, Weinberger MM (1975): Theophylline-induced seizures in adults - correlation with serum concentrations. *Ann Int Med* **82**, 784-787

Appendix A

ACSF composition given in 103 research articles describing an in vitro mammalian brain slice preparation published in "Brain Research" in 1993

Author	Source	Slice preparation	Ion concentration [mM]									
[First author]	[Vol: pp]	[Species, region]	Na	K	Mg	Ca	Cl	HCO3	SO4	PO4	Glu	total
Alberi et al.	624: 326-30	Rat brainstem	150.0	6.25	1.0	2.0	144.0	15	1.0	1.25	10	330.50
Albowitz et al.	631: 329-33	Guinea pig cortex	151.3	5.00	2.0	2.0	133.0	26	2.0	1.25	10	332.50
Ambros-Ingerson et al.	620: 237-44	Rat hippocampus	150.0	4.25	2.5	3.4	133.8	26	2.5	1.25	10	335.70
Ballanyi et al.	607: 99-107	Rat brainstem	150.3	6.00	2.2	2.0	133.0	26	2.2	1.30	10	333.00
Behbehani et al.	612: 56-60	Rat periaqueductal gray	150.0	6.24	1.3	2.5	134.0	26	1.3	1.24	10	332.58
Bellinger et al.	628: 227-34	Rat hippocampus	155.3	3.50	1.5	2.0	137.5	24	1.5	1.25	10	336.50
Benardo	607: 81-8	Guinea pig hippocampus	150.0	5.00	1.6	2.0	136.2	26			10	330.80
Benardo	627: 314-24	Rat cortex	150.0	5.00	1.6	2.0	136.2	26			10	330.80
Bernath et al.	632: 232-8	Rat striatum	152.3	5.00	1.5	2.0	137.0	26		1.25	10	335.00
Berry et al.	628: 99-104	Rat cortex	150.0	6.25	2.0	2.0	147.0	16		1.25	10	334.50
Bittar et al.	620: 181-8	Rat hippocampus	150.0	4.25	1.5	2.5	135.0	26		1.25	10	332.50
Bostwick et al.	629: 79-87	Rat hippocampus	144.5	4.50	1.2	1.3	125.4	25	1.2	1.20	11.5	316.80
Caudle	613: 247-50	Rat hippocampus	149.6	6.10	2.4	2.5	133.9	25.6	2.4	1.20	10	333.70
Chang et al.	603: 302-8	Rat hippocampus	144.0	5.00	1.6	2.5	127.0	26	1.6	1.00	10	318.70
Chieng et al.	626: 136-42	Rat brainstem	152.2	2.50	1.2	2.5	135.9	25		1.20	11	331.50
Cohen et al.	601: 80-7	Rat hippocampus	146.2	3.30	0.9	1.3	125.9	25	0.9	1.23	10	314.76
Colbert et al.	606: 87-91	Rat hippocampus	153.0	3.10	1.5	2.0	133.0	26	1.5	1.10	10	331.20
Coulter et al.	631: 137-42	Mouse thalamus/cortex	157.3	3.00	2.0	2.0	141.0	26		1.25	10	342.50
Cristofol et al.	606: 237-43	Rat hippocampus	144.2	4.70	1.2	1.3	125.3	25	1.2	1.20	10	314.10
Czeh et al.	632: 195-208	Rat hippocampus	154.0	5.50	1.2	1.2	140.3	24			10	336.20
Davidson et al.	615: 229-39	Rat striatum	149.0	3.25	2.0	2.0	130.0	25	2.0	1.25	11	325.50
Dawe et al.	625: 63-74	Rat hippocampus	151.3	4.00	1.5	2.5	133.0	26	1.5	1.25	10	331.00
Erwin et al.	629: 59-66	Mouse brain	142.8	5.88	1.2	1.2	125.1	24.8	1.2	1.18	10	313.32
Fan et al.	632: 225-31	Rat hippocampus	146.2	3.00	1.2	2.6	128.2	25	1.2	1.20	5	313.60

Ferchmin et al.	601: 95-102	Rat hippocampus	151.3	3.30	2.0	2.0	136.3	25		1.25	10	331.10
Ferraro et al.	629: 103-8	Human neocortex	143.0	5.60	1.2	2.4	127.2	25	1.2	1.20	10	316.80
Fortin et al.	630: 125-35	Rat brainstem	150.0	6.30	2.2	2.0	133.0	26	2.2	1.30	10	333.00
Gellman et al.	600: 63-73	Rat cortex	153.3	5.00	2.0	2.0	135.0	26	2.0	1.25	10	336.50
Goshima et al.	617: 167-70	Rat striatum	143.4	5.93	1.2	2.5	128.2	25	1.2	1.19	11.1	319.68
Guitart et al.	611: 7-17	Rat nucleus accumbens	152.3	5.00	2.0	2.0	139.0	25		1.25	10	336.50
Haddad et al.	625: 261-8	Rat brainstem	152.3	3.10	1.3	2.4	132.9	26	1.3	1.25	10	330.50
Haj-Dahmane et al.	614: 270-8	Rat Brainstem	146.2	3.50	1.3	2.0	130.1	25		1.20	11	320.30
Hedberg et al.	632: 239-48	Rat cortex	153.3	5.00	2.0	2.0	139.0	26		1.25	10	338.50
Hesen et al.	627: 159-67	Rat hippocampus	150.3	3.50	1.5	2.0	131.5	25	1.5	1.25	10	326.50
Hirai et al.	629: 23-30	Rat superior colliculus	151.0	4.24	1.3	2.0	132.0	26	1.3	1.24	10	329.08
IIngram et al.	602: 325-30	Rat BedNStriaTerm	150.0	4.50	1.2	1.0	129.3	26	1.2	1.25	10	324.40
Janusz et al.	619: 131-6	Rat hippocampus	147.2	5.00	1.3	2.4	129.8	26	1.3	1.24	10	324.28
Jing et al.	604: 251-9	Rat hippocampus	154.0	3.50	1.2	1.2	138.3	24			10	332.20
Kahle et al.	609: 201-10	Rat hippocampus	153.0	3.25	2.0	2.0	133.0	26	2.0	1.25	10	332.50
Karst et al.	612: 172-9	Rat hippocampus	150.3	3.50	1.5	2.0	131.5	25	1.5	1.25	10	326.50
King et al.	619: 120-30	Rat NuclTractSolit	150.0	6.25	1.3	2.5	134.0	26	1.3	1.25	25	347.60
Kleschevnikov et al.	611: 295-9	Rat hippocampus	150.0	4.25	1.3	2.4	131.8	26	1.3	1.25	10	328.30
Kral et al.	612: 278-88	Rat cortex	151.3	3.00	1.8	1.6	130.2	26	1.8	1.25	10	326.90
Lapchak et al.	613: 239-46	Rat cortex	145.0	5.80	1.2	2.4	129.4	25	1.2	1.20	9.9	321.10
Larson et al.	600: 97-102	Rat hippocampus	150.0	4.25	2.5	3.4	133.8	26	2.5	1.25	10	335.70
Lee et al.	609: 313-5	Gerbil hippocampus	149.7	4.55	2.4	2.0	131.3	25.7	2.4	1.25	10	329.30
Lewis et al.	610: 267-75	Rat DRG	153.0	3.10	1.3	2.0	132.9	26	1.3	1.20	10	330.80
Lin et al.	603: 117-20	Rat hippocampus	143.2	4.70	1.2	2.5	129.1	25		1.20	11	317.90
Livingston et al.	621: 97-105	Guinea pig brainstem	157.3	3.00	2.0	2.0	141.0	26		1.25	10	342.50
Maeda et al.	619: 324-30	Guinea pig hippocampus	150.0	3.24	3.0	3.0	132.0	26	3.0	1.24	10	331.48
Magnuson et al.	628: 317-20	Rat pons	143.0	3.00	0.8	2.5	126.0	24	0.8	1.00	10	311.12
Mason	600: 174-8	Rat hippocampus	150.0	3.60	1.0	2.5	131.3	26	1.0	1.30	10	326.70
Matsumura et al.	626: 343-6	Rat NTractSolit	139.0	3.00	1.0	2.0	122.0	25		1.00	11	304.00
McIntyre et al.	624: 268-76	Rat amygdala	150.0	5.00	2.0	2.0	133.0	26	2.0		10	330.00
Meijer et al.	603: 284-8	Hamster SuprXNucl	148.3	3.00	1.3	1.5	128.0	25	1.3	1.25	10	319.60

Merlin et al.	627: 330-40	Rat hippocampus	150.0	5.00	1.2	1.7	134.8	26			10	328.70
Mitani et al.	601: 103-10	Gerbil hippocampus	151.3	5.00	2.0	2.0	133.0	26	2.0	1.25	10	332.50
Miyamoto et al.	605: 287-92	Guinea pig Sup Coll	151.0	4.24	1.3	2.0	132.0	26	1.3	1.24	10	329.08
Morrisett et al.	600: 235-42	Rat hippocampus	146.2	3.30	1.2	1.9	127.1	25	1.2	1.23	10	317.16
Nolan et al.	630: 101-14	Rat medulla	143.0	5.40	0.4	1.8	125.0	26	0.4	1.00	10	312.98
Otani et al.	613: 1-9	Rat hippocampus	152.2	3.50	1.3	2.0	136.1	25		1.20	10	331.30
Pai et al.	621: 215-21	Mouse brain	149.0	6.20	1.3	1.3	131.6	25	1.3	1.20	10	326.90
Palij et al.	607: 134-40	Rat BedNStriaTerm	149.0	3.25	2.0	2.0	130.0	25	2.0	1.25	11	325.50
Papas et al.	607: 54-60	Rat hippocampus	152.2	3.50	1.3	2.0	136.1	25		1.20	5	326.30
Penning et al.	620: 301-4	Guinea pig hippocampus	147.3	3.10	1.5	2.0	130.1	26		1.30	10	321.30
Potier et al.	616: 236-41	Rat hippocampus	146.2	2.50	1.3	2.5	126.5	26.2	1.3	1.00	11	318.50
Ragozzino et al.	628: 115-20	Rat hippocampus	152.3	2.50	1.0	2.0	133.5	26		1.25	25	343.50
Roberts	620: 251-8	Rat hippocampus	153.3	3.50	2.0	2.0	133.5	26	2.0	1.25	10	333.50
Rothberg et al.	631: 227-34	Rat hippocampus	151.5	4.50	1.2	1.9	132.7	25.9	1.2	1.20	10	330.10
Saito	612: 9-15	Rat cortex	145.0	6.00	1.2	2.6	152.4	5		1.20	10	333.40
Sakai et al.	613: 326-30	Rat hippocampus	144.1	4.50	1.3	2.5	126.5	26.2	1.3	0.92	11	318.34
Schiess et al.	604: 283-97	Rat amygdala	143.2	4.70	1.2	2.5	129.1	25		1.20	11.5	318.40
Schreurs et al.	631: 235-40	Rabbit Cerebellum	152.4	3.00	1.2	2.1	132.2	26	1.2	1.40	10	329.50
Schurr et al.	601: 317-20	Rat hippocampus	150.0	5.00	2.0	3.0	135.0	23	2.0	3.00	10	333.00
Schurr et al.	614: 10-4	Rat hippocampus	150.0	5.00	2.0	3.0	135.0	23	2.0	3.00	10	333.00
Seutin et al.	630: 341	Rat ventral tegmental area	145.2	2.50	1.2	2.4	135.7	18		1.20	11	317.20
Shafer et al.	629: 133-40	Rat cortex/hippocampus	142.8	5.88	1.2	0.8	124.2	24.8	1.2	1.18	10	311.97
Shahi et al.	627: 261-6	Rat hippocampus	150.0	4.25	1.0	3.0	135.0	26		1.25	10	332.50
Shibata et al.	603: 248-54	Rat hippocampus	152.6	4.20	1.3	1.5	136.2	22.4	1.3	1.20	10	330.70
Shibata et al.	606: 259-66	Rat SuprXNucl	151.4	5.40	1.3	1.5	136.2	22.4	1.3	1.20	10	330.70
Shibata et al.	615: 95-100	Rat SuprXNucl	151.4	5.40	1.3	1.5	136.2	22.4	1.3	1.20	10	340.70
Smith et al.	608: 259-65	Rat hippocampus	150.0	5.00	1.0	2.5	135.0	26		1.00	10	330.50
Somjen et al.	631: 118	Rat hippocampus	152.3	2.50	2.0	2.0	135.5	26		1.25	10	331.50
Stittsworth et al.	630: 21-7	Rat hippocampus	150.4	6.00	1.5	2.0	133.0	26.4	1.5	1.00	10	341.80
Sugita et al.	612: 151-5	Rat amygdalayla	153.2	2.50	1.3	2.4	135.9	26		1.20	10	332.50
Tang et al.	614: 125-30	Rat hypothalamus	151.1	5.00	1.3	2.4	133.7	26	1.3	1.20	10	332.00

Tauboll et al.	623: 329-33	Rat hippocampus	151.0	5.00	2.0	2.0	133.0	26	2.0	1.00	10	332.00
Ting et al.	610: 16-23	Rat hippocampus	151.3	4.00	1.5	2.5	133.0	26	1.5	1.25	5	326.00
Tolchard et al.	609: 21-8	Rat NuclTRactSolit	150.0	4.50	2.4	1.0	129.3	26	2.4	1.25	10	326.80
Torocsik et al.	612: 306-12	Rat striatum	151.5	5.60	0.9	1.4	146.2	14.3		1.20	5.5	326.60
Villani et al.	606: 304-8	Rat hippocampus	149.0	2.40	1.1	2.0	132.6	25			10	322.10
Wang et al.	616: 144-53	Rat NuclTRactSolit	150.0	6.25	1.3	2.5	134.0	26	1.3	1.25	25	347.60
Wang et al.	627: 299-306	Rat ventral tegmental area	149.7	3.75	2.4	2.5	131.5	25.7	2.4	1.25	10	329.20
Watson et al.	601: 129-35	Rat hippocampus	150.4	4.50	1.5	2.0	131.5	26.4	1.5	1.00	10	328.80
Waxham et al.	609: 1-8	Rat hippocampus	146.2	2.50	1.3	2.5	126.5	26.2	1.3	1.00	11	319.20
Waxham et al.	609: 1-8	Rat hippocampus	152.0	3.00	2.0	2.0	135.0	26		2.00	10	342.00
Womble et al.	621: 87-96	Rat amygdala	151.2	3.50	1.5	3.0	133.5	26.2	1.5	1.00	11	332.40
Xie et al.	604: 173-9	Guinea pig hippocampus	147.8	3.10	2.0	2.0	131.1	26		1.80	10	323.80
Xu et al.	624: 162-70	Rat hippocampus	146.2	3.30	1.2	2.5	128.3	25	1.2	1.23	10	318.96
Xue et al.	626: 272-7	Rat hippocampus	150.0	6.25	2.0	2.4	137.8	26		1.25	10	335.70
Yamada et al.	624: 336-8	Rat hippocampus	129.4	3.20	2.0	2.0	133.2	3.4	2.0		10	285.20
Yu et al.	602: 191-9	Hamster Suprachiasm Nucl	149.5	4.50	2.0	2.5	132.3	25.5	2.0	1.20	10	329.50
Zidichouski et al.	611: 313-21	Rat brainstem	152.2	2.50	1.2	2.4	135.7	25		1.20	11	331.20

	Na	K	Mg	Ca	Cl	HCO	SO4	PO4	Glu	total
Arithmetic mean:	149.32	4.28	1.53	2.11	132.97	24.67	1.564	1.254	10.4	327.97
Standard deviation:	3.884	1.164	0.456	0.489	4.832	3.56	0.486	0.29	2.75	9.227
(n = 103)										