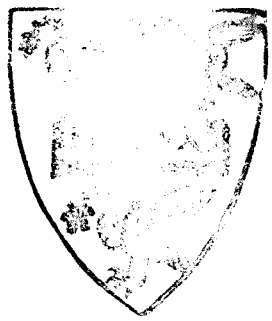
NOVEL SYNAPTIC MECHANISMS OF THE CEREBELLUM



"Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by Andrew Mollison Batchelor"

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ABSTRACT

The cerebellar cortex contains only five cell types and these are organised in a regular anatomical fashion. The excitatory synapses all seem to use glutamate as the transmitter substance whereas the inhibitory ones use GABA. The cerebellar cortex is therefore an ideal area of the brain in which to study the actions of these major transmittters.

Special biplanar slices preserving the three dimensional nature of the cerebellar cortex were used to study synaptic transmission in the mossy fibre and the parallel fibre pathways using a grease gap recording technique. The advantages of this technique over conventional electrophysiological recordings are particularly suited to studying second messenger functions. In addition, some intracellular recordings with simultaneous microfluorometric measurement of intracellular calcium were performed.

Synaptic communication between the major input to the cerebellar cortex, the mossy fibres, and the granule cells is known to be mediated by both the NMDA and the AMPA/kainate glutamate receptor types. A colleague and myself found evidence that the nitric oxide donor, nitroprusside, blocked the function of NMDA receptors and that this was not in fact due to nitric oxide but to a redox effect. In addition, I found some circumstantial evidence that there may be an AMPA receptor modulated by Mg^{2+}. I found no evidence that the obligatory co-agonist at NMDA receptors, glycine, was modulated in a controlled fashion as a co-transmitter.

At the glutamatergic synapses made between the axons of the granule cells, the parallel fibres, with the sole output of the cerebellar cortex, the Purkinje cells, there are no functional NMDA receptors. The inhibition at this synapse is mediated by the stellate and basket cells via $GABA_A$ receptors. I found evidence for direct activation of postsynaptic $GABA_B$ receptors as well as presynaptic ones modulating transmitter release. Glutamate also acts at second messenger linked receptors, the metabotropic glutamate receptors (mGluR). Activation of mGluR's on Purkinje cells by the selective agonist 1S,3R-1-amino-1,3-cyclopentanedicarboxylic acid causes an inward current with a concomitant increase in intracellular Ca^{2+}, which was mainly located in the soma of Purkinje cells from rats aged 14-21 days. Using the grease-gap technique I found evidence that mGluR's could be stimulated synaptically by parallel fibres.

The use of the grease-gap technique has allowed recording of second messenger mediated effects from the synapses of the cerebellar cortex which could otherwise have been overlooked using conventional electrophysiological techniques. The involvement of second messenger linked receptors adds another layer of computational complexity to the cerebellar cortex.

OUTLINE

CONTENTS

List of Abbreviations

βARK	β-adrenoreceptor receptor kinase
γ-DGG	γ-D-glutamylglycine
1S,3R-ACPD	active enantiamer of *t*-ACPD
5-HT_3	5-hydroxytryptamine
A1	adenosine type-1
AC	adenylate cyclase
ACh	acetylcholine
AChR	acetylcholine receptor
AHP	afterhyperpolarisation
AMPA	α-amino-3-hydroxy-5-methyl-5-isoxazolepropionate
AP3	L-2-amino-3-phosphopropionate
AP4	L-2-amino-4-phosphonobutyrate
AP5	D-2-amino-5-phosphonopentanoate
APB	see AP4
ATP	adenosine trisphosphate
CaM-KII	Ca^{2+}/calmomulin-dependent protein kinase II
cAMP	cyclicAMP
CCT	cuneocerebellar tract
cGMP	cyclic guanosine monophosphate
CN PDE	cyclicnucleotide phosphodiesterase
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CREB	cAMP response element binding protein
D-AP5	D-2-amino-5-phosphonopentanoate
DAA	D-α-aminoadipate
DAG	diacylglycerol
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DSCT	dorsal spinocerebellar tract aka Flechsig's or Clarke's tract
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)

EAA	excitatory amino acids
EDRF	endothelium derived relaxing factor
EGF	epidermal growth factor
epsp	excitatory postsynaptic potential
ER	endoplasmic reticulum
g-protein	GTP-binding proteins
G_{α}-GTP	alpha subunit of g-protein complexed with GTP
$G_{\beta\text{-}\gamma}$	beta-gamma complex of g-protein
GABA	γ-amino-butyric acid
$GABA_A$	GABA receptor - subtype A
$GABA_B$	GABA subtype B
GAD	L-glutamic acid decarboxylase
GAMS	γ-glutamylaminomethylsulphonate
GC	guanylate cyclase
GDEE	glutamate diethyl ester
GDP	guanine diphosphate
$GluR_{1\text{-}4}$	glutamate receptor subunits type 1 to 4 (aka $GluR_{A,B,C\ \&\ D}$; AMPA family)
$GluR_{5,6\ \&\ 7}$	glutamate receptor subunits type 5,6 and 7 (kainate family)
G_o	subfamily of g-proteins
G_t	subfamily of g-proteins known as transducins
GTP	guanine trisphosphate
Hb	haemoglobin
HPLC	high performance liquid chromotography
IBMX	isobutylmethylxanthine
$InsP_3$	inositol-1,4,5-trisphosphate
$InsP_3R$	inositol-1,4,5-trisphosphate receptor
IP_3	inositol-1,4,5-trisphosphate
KA1 & KA2	kainate subunits 1 and 2 (kainate family)
LOC	ligand operated channels
LRN	lateral reticular nucleus
LTP	long term potentiation

metHb	methaemoglobin
mGluR	metabotropic glutamate receptor
$mGluR_{1-7}$	metabotropic glutamate receptors types 1 to 7
mRNA	messenger RNA
NA	Noradrenaline
nAChR	nicotinic acetylcholine receptor
NBQX	2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline
NMDA	N-methyl-D-aspartate
NMDAR1	NMDA receptor, type 1
NMDAR2	NMDA receptor, type 2
NO	Nitric oxide
non-NMDA	glutamate receptors not belonging to the NMDA, kainate and AMPA class
NOS	nitric oxide synthase
NPH	nucleus prepositus hypoglossi
NRTP	nucleus reticularis tegmenti pontis
PDE-1	Type-1 phophodiesterases
PDGF	platelet derived growth factor
PI	phosphoinositides
PKA	protein kinase A
PKC	protein kinase-C
PKG	cGMP-dependent protein kinase
PLC	phospholipase-C
PRN	paramedian reticular nucleus
PtdIns-PLC	phospholipase-C isozymes
PtdIns(4)P	phosphatidylinositol-4-phosphate
PtdIns(4,5)P2	phosphatidylinositol-4,5-bisphosphate
Qp	quisqualate receptor linked to phosphoinositide hydrolysis (now called mGluR)
RNA	ribonucleic acid
RyanR	ryanodine receptor
SIN-1	derivative of molsidomine

SMOC	second messenger operated channels
SNAP	S-nitrosoacetylpenicillamine
SNOG	S-nitrosoglutathione
SNP	sodium nitroprusside
t-ACPD	trans-1-amino-1,3-cyclopentanedicarboxylic acid
TM	transmembrane
VIP	vasoactive intestinal peptide
VOC	voltage operated channels
VSCT	ventral spinocerebellar tract aka Gower's tract
$[K^+]_o$	extracellular concentration of potassium ions

SECTION I - INTRODUCTION

A. THE CEREBELLUM

1. ANATOMY

a. Gross Anatomy.

The cerebellum occupies approximately 10% of the brain volume yet contains more than half the neurones of the total brain, in man. It comprises of a convoluted sheet of cortex, which is superficially divided by two deep fissures running in the saggital plane into the vermis and the two hemispheres. In addition, there are nodes attached at the lateral extremes of the hemispheres called the flocculi. The sheet is folded to form folia which are evident as shallow grooves in the transverse direction. Each of these folia is identified with a roman numeral following the system of Larsell.

b. Cellular Architecture.

One of the most striking features of the cerebellar cortex is the simplicity of its neuronal organisation. The grey matter is less than 1 mm thick and contains three distinct layers, the molecular layer, the Purkinje cell layer and the granule cell layer. The main input to the cerebellar cortex is the mossy fibres which arise from the pons and brainstem area. These synapse onto granule cells in a tight cluster referred to as a glomeruli. The granule cells in turn send an axon through the Purkinje cell layer into the molecular layer where they bifurcate forming parallel fibres. The parallel fibres synapse onto the Purkinje cell dendrites, the Purkinje cells innervate the deep cerebellar nuclei. The parallel fibres also synapse onto two sets of inhibitory interneurones; the Golgi cells which feed back to the mossy fibre - granule cell synapses; and the basket/stellate cells which feedforward on to the

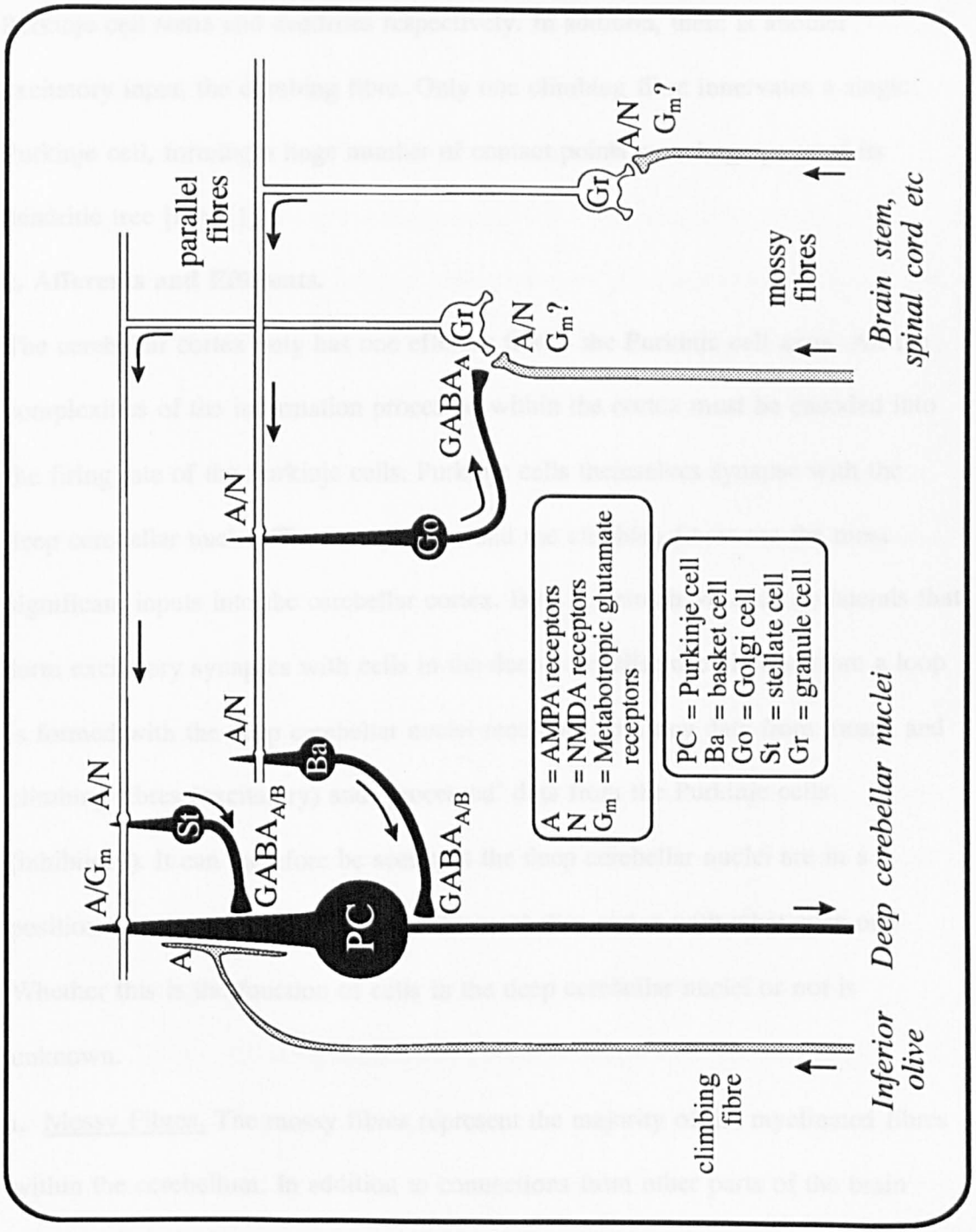


Figure I(1): Synaptic connections and receptors in the cerebellar cortex. Inhibitory (GABAergic) neurones are black, excitatory inputs are grey and the excitatory interneurones are white. Proposed location of amino acid receptors is also included.

Purkinje cell soma and dendrites respectively. In addition, there is another excitatory input, the climbing fibre. Only one climbing fibre innervates a single Purkinje cell, forming a huge number of contact points over large parts of its dendritic tree [Fig.I(1)].

c. Afferents and Efferents.

The cerebellar cortex only has one efferent fibre - the Purkinje cell axon. All the complexities of the information processed within the cortex must be encoded into the firing rate of the Purkinje cells. Purkinje cells themselves synapse with the deep cerebellar nuclei. The mossy fibres and the climbing fibres are the most significant inputs into the cerebellar cortex. Both systems have axon collaterals that form excitatory synapses with cells in the deep cerebellar nuclei. Therefore a loop is formed with the deep cerebellar nuclei receiving both raw data from mossy and climbing fibres (excitatory) and 'processed' data from the Purkinje cells (inhibitory). It can therefore be seen that the deep cerebellar nuclei are in a position to compare data going in to the cerebellar cortex with what goes out. Whether this is the function of cells in the deep cerebellar nuclei or not is unknown.

i. Mossy Fibres. The mossy fibres represent the majority of the myelinated fibres within the cerebellum. In addition to connections from other parts of the brain (extracerebellar) some mossy fibres arise from neurones within the deep cerebellar nuclei (nucleocortical). If the cerebellar peduncles (through which all extracerebellar mossy fibres run) are cut, about 5% of mossy fibre terminals remain (Hamori, Mezey & Szentagothai, 1981). Indicating that this projection does indeed exist but probably has a less important role. The remaining 95% can be assembled

into three groups: somatosensory inputs from the spinal cord; vestibular inputs and descending inputs from the cerebral cortex which connect via the pontine nuclei.

Spinocerebellar connections. Groups of neurones within the spinal cord relay information from proprioceptors, exteroceptors and enteroceptors via the spinocerebellar tracts. There are two main pathways that directly connect with the cerebellum the dorsal and the ventral spinocerebellar tracts. There are several other groups of spinocerebellar mossy fibres that are indirectly connected. The DSCT (dorsal spinocerebellar tract aka Flechsig's or Clarke's tract) is composed of fast, 30 - 100 ms^{-1}, myelinated axons emanating from the nucleus dorsalis of the dorsal horn of spinal segments T_1-L_4. These neurones are activated by proprioceptors (Ia muscle spindle fibres, Ib Golgi tendon organ afferents and also afferents of the Ruffini endings of joint capsules) and exteroceptors (fast and slow adapting mechanoreceptors) located in the hindlimb and the trunk areas. The axons ascend the spinal cord and enter the cerebellum via the restiform body. The VSCT (ventral spinocerebellar tract aka Gower's tract) also contains fast (90 ms^{-1}) myelinated axons emanating from the ventrolateral grey matter of lumber spinal cord segments. These cells are stimulated by Ib Golgi tendon organ fibres, Ia muscle spindle and receive inputs from descending motor pathways. It is thought that this pathway may monitor the activity of the interneurones lying in the spinal segments (Lundberg, 1971). The *cuneocerebellar tract* (CCT) conveys axons from the cuneate and external cuneate nuclei to the cerebellum. These nuclei relay information from the forelimbs via the ascending spinal fibres of the dorsal funiculus. The neurones in the external cuneate mainly receive proprioceptive inputs whereas the neurones in the cuneate itself mainly receive exteroceptor

inputs. In addition to the spinal inputs the CCT receives information from cranial nerves VIII (acoustic), IX (glossopharyngeal) and X (vagus). There is also evidence that there is a direct autonomic input from cranial nerves IX and X to the cerebellum (Sobusiak, Zimny & Matlosz, 1971). Several other areas associated with autonomic function seem to project to the cerebellum. Including the nucleus of the solitary tract, the dorsal motor vagal nucleus and the parabrachial nucleus.

Vestibular inputs. The VIIIth cranial nerve (acoustic) contains two classes of information: auditory from mechanoreceptors of the organ of Corti and vestibular from the semicircular canals and otolith organs. There is some evidence of auditory information being forwarded by the cochlear nucleus and also via the pons. In addition, there is a wealth of evidence pointing towards a role for the cerebellum in processing information from the organs of balance. There are three semicircular canals in each side of the head. This allows detection of angular acceleration in three-dimensional space. This is achieved by a viscous fluid contained within the canals which, when displaced by movements of the head, causes motion of mechano-sensitive hair cells. The otolith organs, namely the utricle and the saccule, have similar cells which signal changes in the inclination of the head relative to the direction of gravitational pull. These organs, along with visual cues and information from pressure sensitive receptors in the feet and joints, allow the brain to keep the body balanced, at rest, and much more demandingly, during movement as well. The vestibulocerebellar afferents are collaterals of the primary vestibular afferents which project to the vestibular nucleus in the medulla, they enter the cerebellum as mossy fibres, primarily innervating the flocculus. There are also secondary projections from the vestibular nucleus which innervate most of the

cerebellar cortex. The otolithic afferents seem to innervate the flocculus and other areas. *Pontine and reticular mossy fibres.* These mossy fibres are somewhat different from the two groups discussed above. They do not deal with inputs from sensory systems but instead come from the 'higher' brain areas. There are four areas of the reticular formation that project to the cerebellum: the lateral reticular nucleus (LRN); the nucleus reticularis tegmenti pontis (NRTP); the paramedian reticular nucleus (PRN) and the perihypoglossal nuclei. The LRN lies in the medulla lateral to the inferior olive. It receives afferents from both spinal and supraspinal areas. The former is thought to be the most important. The information processed is thought to be the product of the activities of spinal motor centres. The NRTP is located dorsal to the pontine nuclei. Inputs arise mainly from supraspinal areas including the cerebral cortex and the deep cerebellar nuclei. Interestingly, there is also a visual input from some pretectal areas, this projects mainly to the flocullus. The PRN and perihypoglossal nuclei receive diverse inputs from mainly supraspinal areas, a specific function is not known for any of these areas except for the nucleus prepositus hypoglossi (NPH) which has been intensely studied. This nucleus receives inputs from three areas: vestibular nuclei; various parts of the reticular formation and from the oculomotor and accessory oculomotor nuclei. Collaterals of cerebellar efferents also project to the oculomotor nucleus (Yingcharoen & Rinvik, 1982). Thus the NPH is thought to play a critical role in the coordination of eye movements. The pontine nuclei are the largest source of mossy fibres in the rat and other higher vertebrates. They are a heterogenious group of nuclei which have been divided into six subnuclei: nucleus dorsolateralis; nucleus lateralis; nucleus medialis, nucleus paramedianus, nucleus peduncularis and

nucleus ventralis (Brodal and Jahnsen, 1946). All the efferent fibres project to most parts of the cerebellum, mainly contralaterally, via the brachium pontis. The most important input to the pontine nuclei is the descending one from layer V pyramidal cells in the cerebral cortex. The most prominent connections come from the motor (area 4), somatosensory (areas 1, 2 & 3) and parietal association (areas 5 & 7) cortices. There are also significant connections from the visual cortex (areas 17 & 18) and to a lesser extent the auditory cortex. A number of the corticopontine projections seem to arise from collaterals of corticospinal neurones. In addition to the cortical inputs, there are some tectal inputs: the dorsolateral pontine nucleus receives inputs from the superior colliculus and the ipsilateral dorsolateral pontine nucleus from the inferior colliculus. The pontine nuclei also receive inputs from the lateral geniculate nucleus, deep cerebellar nucleus and ascending spinal fibres (not rats).

ii. The climbing fibres. The climbing fibres are the other main source of afferent fibres to the cerebellar cortex. They all stem form the inferior olivary nuclei and project to the contralateral side where they synapse in a highly specialised manner with the Purkinje cells. On route the climbing fibres send collaterals to the deep cerebellar nuclei. The inferior olive receives inputs from many different areas of the nervous system. Spino-olivary pathways: ventral; dorsal; dorsolateral and lateral, these communicate activities in cutaneous and proprioceptive sensors (Oscarsson, 1973), but, interestingly, they only seem to respond to external stimuli and not to planned motor actions (Gellman, Gibson & Houk, 1983). Several areas of the medulla project to the inferior olive, the trigeminal (containing information from sensory receptors on the face of the animal and in particular the vibrissae of

rats) and vestibular nuclei and miscellaneous parts of the reticular formation. The inferior olivary nuclei receive visual information from three domains, the superior colliculus, pretectal nuclei and the nucleus of the accessory optic tract. The visual information is used for at least two purposes, assisting the vestibular apparatus in maintaining balance and as an integral part of ocular reflexes.

2. FUNCTION

a. **Comparative and evolutionary aspects.**

The cerebellum is part of the hindbrain termed the metencephalon. It is relatively well developed in birds and mammals. By examining comparative aspects of the cerebellum we can construct a history of the evolution of the cerebellum as a part of the brain. The cerebellum has been studied intensively in turtle, frog, alligator, birds, mouse, rat and cat as well as human. Amazingly from such a varied group the cerebellum is one of the best conserved features of the brain. The same basic cellular architecture is there and the channels and receptors are remarkably similar. In the turtle for example, which is thought of as a living fossil since its body-form has not changed in millions of years, the cerebellar cortex is very similar to rat. The main difference is that it does not have basket cells only stellate cells. Apart from this the Purkinje cells behave much like rat ones (Midtgaard & Hounsgaard, 1989). The cerebellum is thought to have evolved from the lateral line organ in fish. This organ has to process a huge amount of information about water moving past the lateral line and give useful information about the speed and orientation of the fish. The human cerebellum also receives a lot of information about the interaction of the animal with the outside world. Vestibular information is crucial for orientation, as is information from the joints and muscles.

b. **Pathology.**

Most of the information about how damage to the cerebellum affects behaviour in man came from studying patients injured during the First World War (Holmes, 1939). He found three main effects: *hypotonia* - the patients were unable to react properly to their limbs being moved. During rapid movements they tend to be unable to predict the end of a movement and tend to overshoot; *cerebellar ataxia* - a whole catalogue of inabilities during voluntary movements, errors in the speed and distance to be moved and interestingly, sometimes failure to put together the components of a complex movement in terms of timing; *intention tremor* - a strange type of tremor where the finer the control needed the worse the tremor gets. There is often disorders of articulating speech due to damage to the vermis. Most cerebellar damage is compensated for very well which makes it very hard to dissociate the problems caused by the trauma rather than the selective damage.

c. **Function.**

The cerebellum seems to play a part in the processing of many different kinds of information. Perhaps it is not useful to think of one function for the cerebellum but a whole variety which by virtue of the homogeneity of the cerebellar wiring must need similar kinds of characteristics, particularly the amount of parallelism and the speed of processing. There are three broad groups of 'functions'. *Vestibular* - the inputs from the semicircular canals of the ear seem to be a major function of the most lateral portions of the hemispheres. The outputs from this area are concerned with posture and orientation. *Motor execution* - the control of movements. *Planning and timing* - complex movements can be achieved better with practice and concentration, the cerebellum is thought to play a special role in hand - eye

coordination.

B. SYNAPTIC TRANSMISSION

1. NEURONES ARE EXCITABLE

An animal collects information from the outside world via sensory receptors: the eyes, ears, nose and touch receptors, for example. The animal then processes this information and acts appropriately, usually with some movement. Between the sensory receptors and the muscle cells lies the central nervous system. The central nervous system comprises of a complex arrangement of neurones and their support cells. The neurones collectively bestow all the intricacies of an animal's action and behaviour - reaching an apogee in the sophisticated workings of the human mind. A neurone's most prominent characteristic is its ability to fire action potentials. Action potentials are a property of the permeability of the cell's membrane combined with the electrochemical gradients maintained across it. There is a large inward chemical gradient of Na^+ ions across the cell membrane, normally the cell is relatively impermeable to these ions but when the cell is depolarised (made internally more positive) special Na^+ channels are induced to open, this causes further depolarisation which opens more of these voltage-sensitive Na^+ channels and so on. The Na^+ channels are preprogrammed to open for a short time and this along with other mechanisms allows rapid repolarisation of the membrane. The activity at one point in the membrane spreads to neighbouring regions of the cell membrane to depolarise them above the threshold for Na^+ channel opening. The final result is a very short lived polarity shift (approximately 1 msec) across the membrane which travels away from the starting point. These are known as action potentials or 'spikes'.

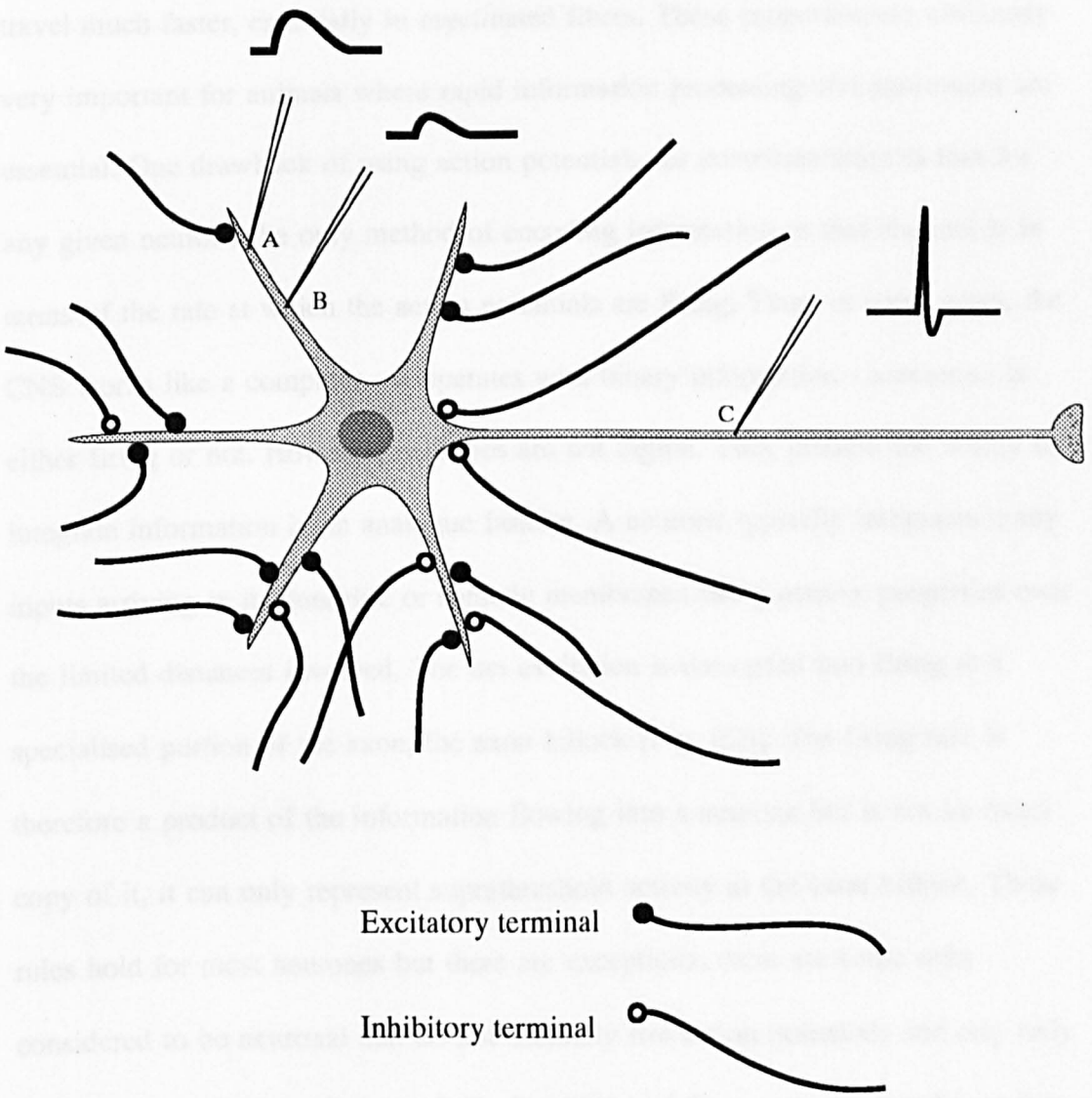


Figure I(2): Dendritic integration of information in a schematic neurone. The dendrites of the neurone are contacted by many excitatory ( ) and inhibitory ( ) terminals. The voltage response is measured at different points with intracellular electrodes A, B and C. The excitatory postsynaptic potential (epsp) recorded by A is larger than that recorded by B due to the dendrite being a leaky conductor. The sum of all the epsp's and inhibitory postsynaptic potentials (ipsp's) decides whether the neurone should fire an action potential or not. The resultant action potential is recorded by electrode C.

2. INFORMATION PROCESSING IN AN INDIVIDUAL NEURONE

Action potentials allow conduction of information over distances much greater than permitted by the passive electrical properties of a neurone. Action potentials also travel much faster, especially in myelinated fibres. These properties are obviously very important for animals where rapid information processing and movement are essential. One drawback of using action potentials for communication is that for any given neurone the only method of encoding information in that channel is in terms of the rate at which the action potentials are firing. Thus, in some ways, the CNS works like a computer - it operates with binary information - a neurone is either firing or not. However, neurones are not digital. They possess the ability to integrate information in an analogue fashion. A neurone typically integrates many inputs arriving in its dendritic or somatic membranes using passive properties over the limited distances involved. The net excitation is converted into firing at a specialised portion of the axon, the axon hillock [Fig. I(2)]. The firing rate is therefore a product of the information flowing into a neurone but is not an exact copy of it, it can only represent suprathreshold activity at the axon hillock. These rules hold for most neurones but there are exceptions: there are some cells considered to be neuronal that do not normally fire action potentials and rely only on passive conduction (Roberts & Bush, 1981) and there are cells that fire spikes in the dendrites as well as in the axon.

3. SYNAPTIC TRANSMISSION - CHEMICAL MAINLY.

Neurones contact each other at a site called a synapse. Morphologically a synapse is distinguishable by certain features. When viewed using an electron microscope, the most noticeable features are that at certain points the cell membranes of two

cells approach each other very closely, about 20 nm, called the synaptic cleft, and here the edges of the cell cytoplasm are dark - the post and presynaptic densities - and in addition there are many small round vesicles usually accompanied by numerous mitochondria, on the presynaptic side of the synapse. When an action potential reaches the specialised region called the axon terminal (synaptic bouton) or a swelling of the axon called a varicosity, the action potential is translated back into a passively conducted potential and acts on voltage- sensitive Ca^{2+}-channels, these open briefly, which in turn sets off a sequence of events which ends in a vesicle containing transmitter substance fusing with the membrane and spilling its contents out into the synaptic cleft (Kelly, 1993). The released transmitter diffuses to the recipient cells where it binds to specific postsynaptic receptor molecules situated on the plasma membrane. For fast and high frequency firing the transmitter should only be capable of acting for a very short period. The concentration of transmitter quickly returns to very low levels due to two factors: one, the transmitter diffuses very quickly away from the synaptic cleft into the extrasynaptic space and secondly there are highly specialised mechanisms for transporting the substance back into the cells that released it or into surrrounding glial cells or alternatively enzymatically cleaving the substance into less reactive products which are then taken up.

4. EXCITATORY TRANSMISSION

Excitatory transmission can be defined as a synaptic input that increases the chance of the recipient cell firing. Generally this means that excitatory receptors result in depolarisation of the cell towards the threshold for action potential generation by allowing influx of cations Na^+ and Ca^{2+} or stopping the efflux of K^+ (it is also

theoretically possible that closing of, say, Cl^- channels could increase the probability of firing to a given input by increasing the input resistance of the cell not by depolarising it). For example the excitatory effects of motorneurones is mediated through nicotinic AChR's. It was initially thought that all chemical transmission was excitatory.

5. INHIBITORY TRANSMISSION.

When Cajal drew his beautiful neuronal circuits he had no concept of the idea that there could be inhibitory connections between neurones, when he drew the direction of flow of information he assumed that it was all excitatory. At around the same time, Sherrington was sure that the relaxation of muscles antagonistic to a contracted muscle was due to inhibitory innervation of the former. It was not properly realised until much later that this inhibition was due to the action of inhibitory interneurones. Inhibition can be defined as the input of a cell that decreases the likelihood of that cell firing, all other things being equal. Inhibitory pathways can act directly onto the postsynaptic membrane and release an inhibitory transmitter, most commonly the amino acids glycine or GABA (γ-amino-butyric acid). These usually act on receptors that increase the conductivity of the membrane to Cl^-, this has two effects, it tends to hyperpolarise the cell towards the Cl^- reversal potential and the high conductance of these channels causes a decrease in the membrane resistance so that incoming excitatory inpulses are dissipated and are less efficacious. In addition, inhibitory synapses can be situated on the axon hillock of the neurone where they have a very powerful effect. Besides opening Cl^- channels inhibitory synapses are thought to employ other mechanisms, opening of K^+ channels or closing of Ca^{2+} channels.

6. CHANNELS AND RECEPTORS

a. Channels.

A lipid bilayer is relatively impermeable to ions since ions depend on an aqueous environment for their stability. The crux of neuronal communications is the ability to selectively alter the permeability of the cell membrane to different ions. This is achieved by regulation of pore-forming proteins in the plasma membrane. When a channel opens ions can move through it if their electrochemical gradient allows. The pore of a channel can select between types of ion on the basis of charge and size. It is therefore possible to get channels that preferentially allow passage of Na^+, Ca^{2+}, K^+, Cl^- or combinations thereof. Channels are formed by the complex folding of protein molecules. As for any protein, the folding pattern depends on the repulsive and attractive forces between the amino acid side chains. A channel can exist in at least two isomeric forms, one that has an open ionophore and the other closed. Channels can be grouped into three types by virtue of what normally regulates the isomerisation between open and closed states [Fig.I(3)]. The first group are called ligand operated channels (LOC's), these are induced to open by an endogenous ligand binding to the extracellular surface of the channel protein. The second group are similar except the ligand is on the cytoplasmic side and these are referred to as second messenger operated channels (SMOC's). The third group has already been mentioned for their role in generating action potentials, the voltage operated channels (VOC's). These change their protein conformation when the potential difference across them is altered. Channel opening can be regulated by more than one of the above, in fact, this is more the rule than the exception. For example, some ligand gated channels are also voltage-sensitive, voltage changes

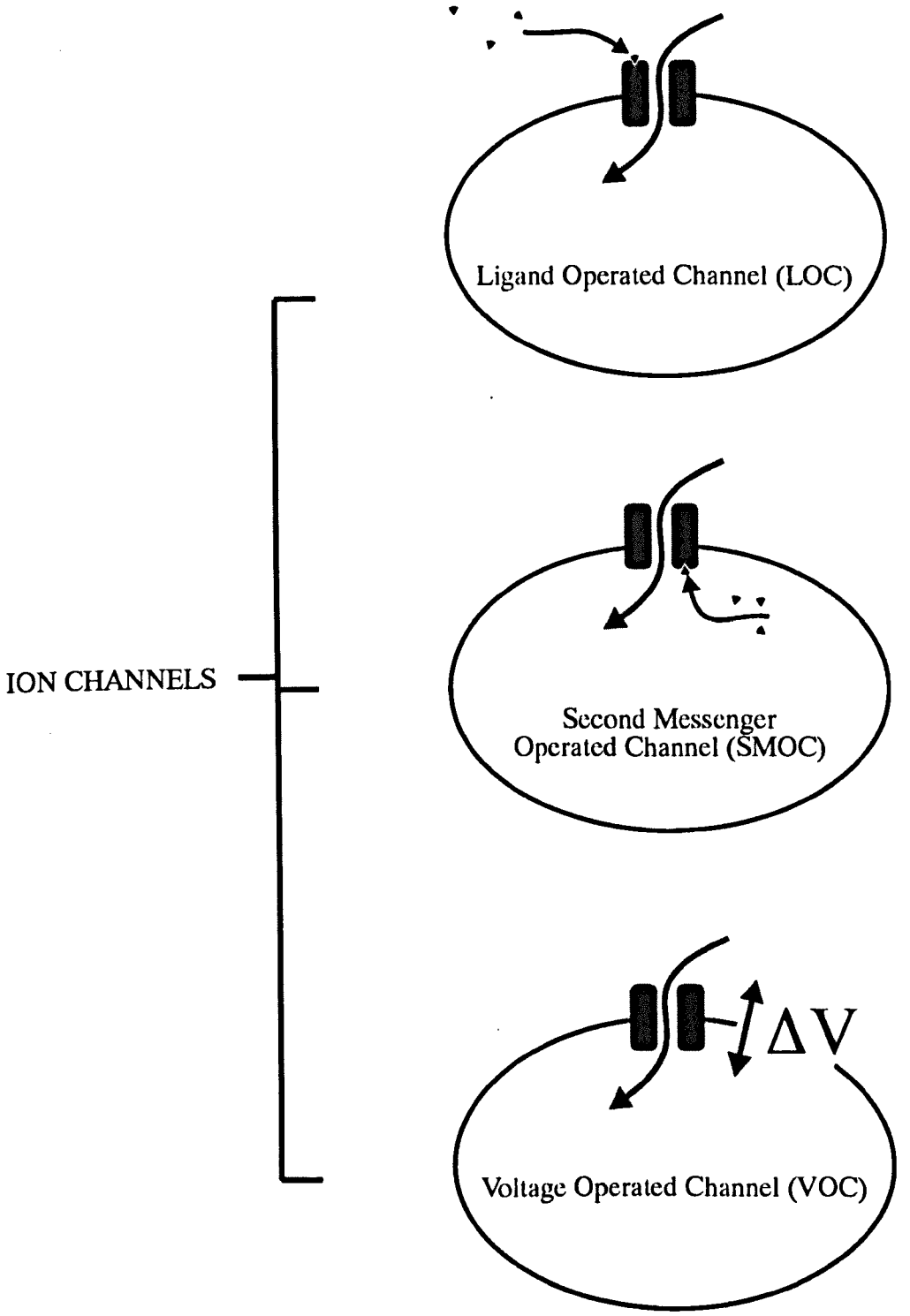


Figure I(3): Three different classes of membrane channels. The binding of an agonist or a second messenger regulates the transition between open and closed state of the channel for the top two types. The bottom type is opened when the electrical potential across it is changed.

alone cannot open the channel but they can modulate the average open time of the channel or its conductance. Most, if not all, channels can be phosphorylated by a kinase, activated by some second messenger pathway, which can alter the properties of the channel.

b. Receptors.

Receptors are protein molecules that have binding sites for a specific messenger molecule. Activation of the receptor leads to a conformational change in the protein resulting in a change in the functioning of the protein, which then directly or indirectly affects the cell. Receptors can be divided up in numerous ways [Fig.I(4)]. One distinction that is relatively easy to apply is whether the receptor is situated inside the cell or not. For an extracellular messenger to diffuse into the cell it must be lipid soluble. Most messenger molecules are not lipid soluble, the main exception being the steroid hormones. In the nervous system such intracellular receptors are probably most important during development and as a route for communication between the periphery and the central nervous system. They are generally best suited to slow transmission of information. One newly found messenger molecule that breaks all these rules is the gas nitric oxide. It is lipid soluble but due to its physicochemical properties is most suitable as a short range messenger. The intracellular receptor for nitric oxide seems to be the haemoprotein guanylyl cyclase. All other kinds of receptors fall into the category of cell surface receptors, these are by far the most common type used in the functioning of the CNS. We can divide cell surface receptors into three categories which reflects the signal transduction method used. The simplest are the ionotropic or channel-linked receptors. The messenger molecule binds to the extracellular domains of the

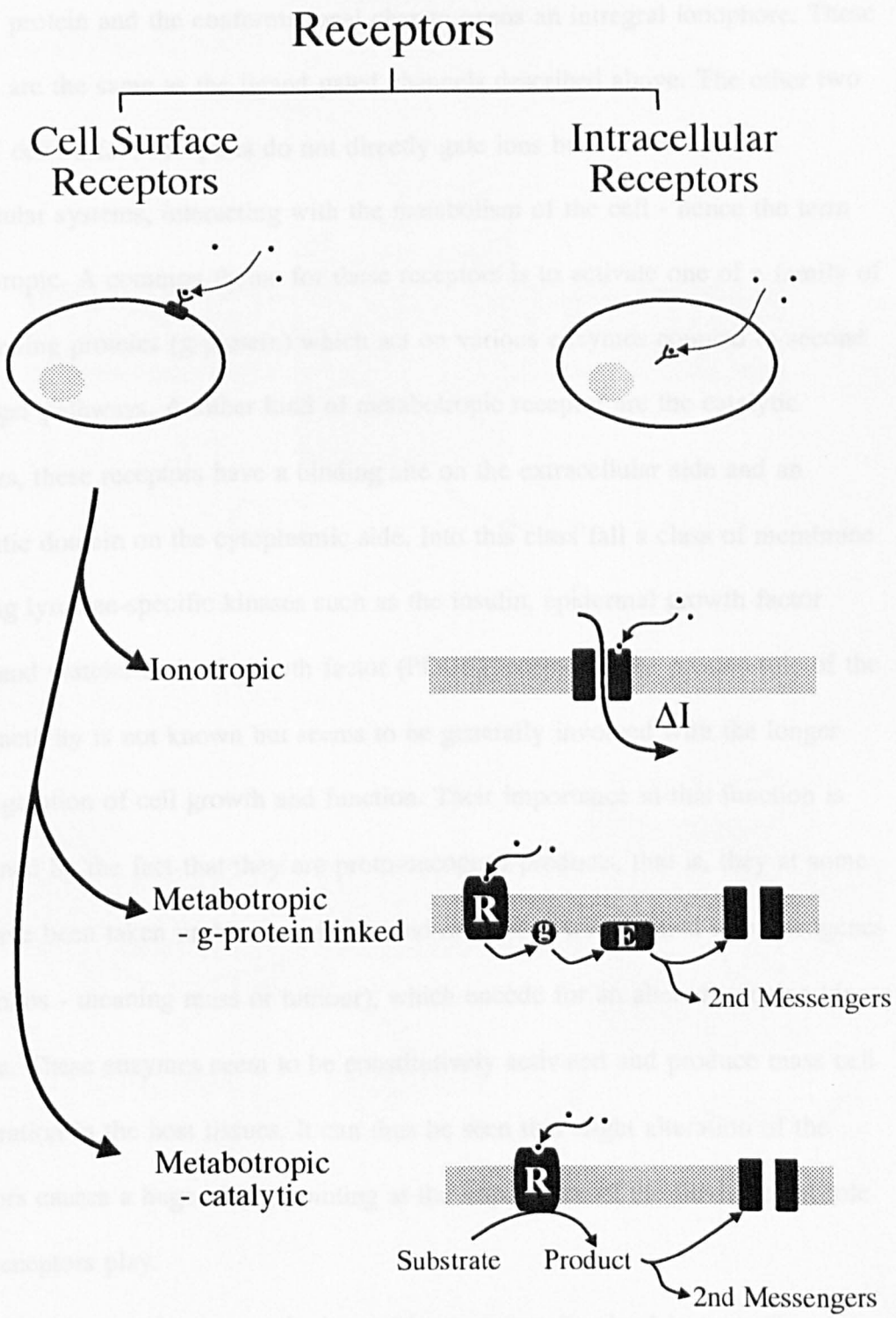
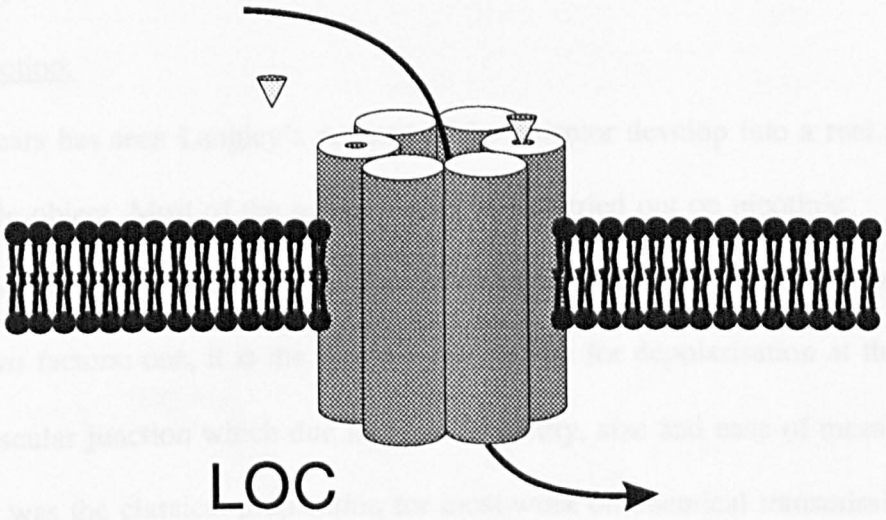


Figure I(4): Different classes of receptor. Some ligands, notably the steroid hormones, can diffuse through the lipid membrane and act on intracellular receptors. Most ligands cannot penetrate the cell membrane and interact with cell surface receptors. This class can be further subdivided into ionotropic, receptor molecules that also have an integral ion channel, or metabotropic. Metabotropic receptors have been further subdivided here to distinguish two ways of coupling to intracellular enzymes - the catalytic type with built in metabolic enzyme activity and the type that uses a G-protein to communicate with enzyme systems.

receptor protein and the conformational change opens an intregral ionophore. These proteins are the same as the ligand gated channels described above. The other two types of cell surface receptors do not directly gate ions but instead activate intracellular systems, interacting with the metabolism of the cell - hence the term metabotropic. A common theme for these receptors is to activate one of a family of GTP-binding proteins (g-protein) which act on various enzymes coupled to second messenger pathways. Another kind of metabotropic receptor are the catalytic receptors, these receptors have a binding site on the extracellular side and an enzymatic domain on the cytoplasmic side. Into this class fall a class of membrane spanning tyrosine-specific kinases such as the insulin, epidermal growth factor (EGF) and platelet derived growth factor (PDGF) receptors. The precise role of the kinase activity is not known but seems to be generally involved with the longer term regulation of cell growth and function. Their importance in this function is underlined by the fact that they are proto-oncogene products, that is, they at some time have been taken up by retroviruses and have been transformed into oncogenes (Gk. onkos - meaning mass or tumour), which encode for an altered tyrosine kinase enzyme. These enzymes seem to be constitutively activated and produce mass cell proliferation in the host tissues. It can thus be seen that slight alteration of the receptors causes a huge effect, pointing at the importance of the fundamental role these receptors play.

The two main transduction methods used by receptors involved in synaptic transmission in the central nervous system are the ionotropic and the g-protein linked metabotropic cell surface receptors [Fig.I(5)]. From the following examples it will be seen that there are common themes to these transduction pathways.

a - Ionotropic Receptor



b - Metabotropic Receptor

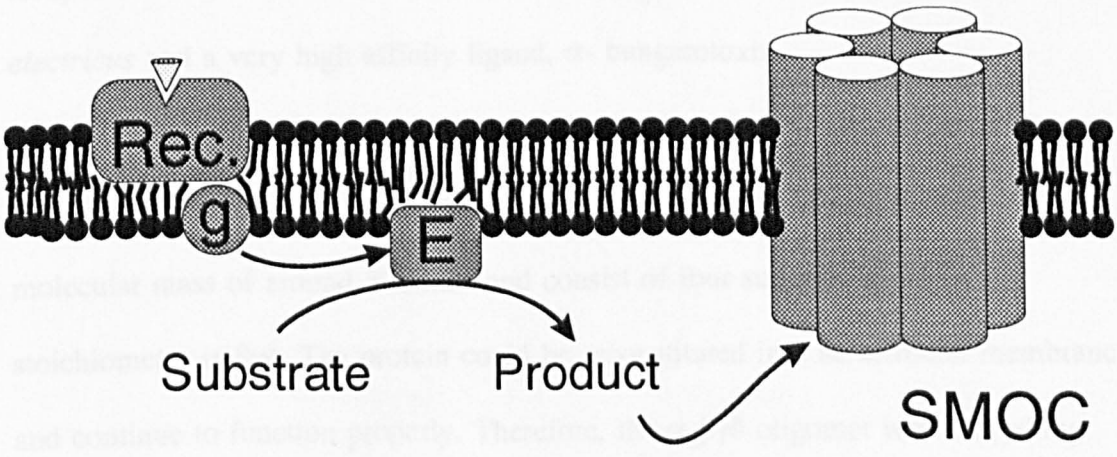


Figure I(5): The two main types of receptors involved in synaptic transmission. a. The ligand binds to the binding site of the ionotropic receptor which induces a conformational change which results in an ionophore opening through the protein. The receptor classification ionotropic is the same as the channel class LOC (Ligand Operated Channel). b. the ligand binds to a receptor molecule and a conformational change is communicated to the cytoplasmic side of the protein and activates a G-protein (g) which in turn activates particular second messenger producing enzymes (E). The second messengers can activate some channels (SMOC - Second Messenger Operated Channel) amongst many other things.

c. An Example of an Ionotropic Receptor - The Nicotinic Acetylcholine Receptor.

i. Introduction.

Ninety years has seen Langley's concept of the receptor develop into a real and observable object. Most of the primary work was carried out on nicotinic acetylcholine receptors (nAChR's). This receptor became the focus of intense study due to two factors: one, it is the receptor responsible for depolarisation at the neuromuscular junction which due to its accessibility, size and ease of measuring a response was the classical preparation for most work on chemical transmission. The second reason, particularly at the stage of purification, identification, sequencing and structural analyses, was that there was a particularly rich source of the receptors available from the electric ray, *Torpedo*, or the electric eel *Electrophorus electricus* and a very high affinity ligand, α- bungarotoxin.

The nAChR is constructed from subunits. Using the practical advantages above, an α-bungarotoxin- binding protein was purified and found to have a relative molecular mass of around 300 kDa and consist of four subunits in the stoichiometry $\alpha_2\beta\gamma\delta$. The protein could be reconstituted into an artificial membrane and continue to function properly. Therefore, the $\alpha_2\beta\gamma\delta$ oligomer was everything that was needed to show ACh binding, an ion channel and activation/inactivation kinetics similar to the normal AChR in vivo. Molecular biological techniques showed that the subunits had very similar amino-acid sequences and that injection of mRNA for the four subunits resulted in fully functioning receptors (Lindstrom, Schoepfer & Whiting, 1987). Analysis of the hydrophobicity of the amino acid sequence suggested that there were four portions that were likely to represent

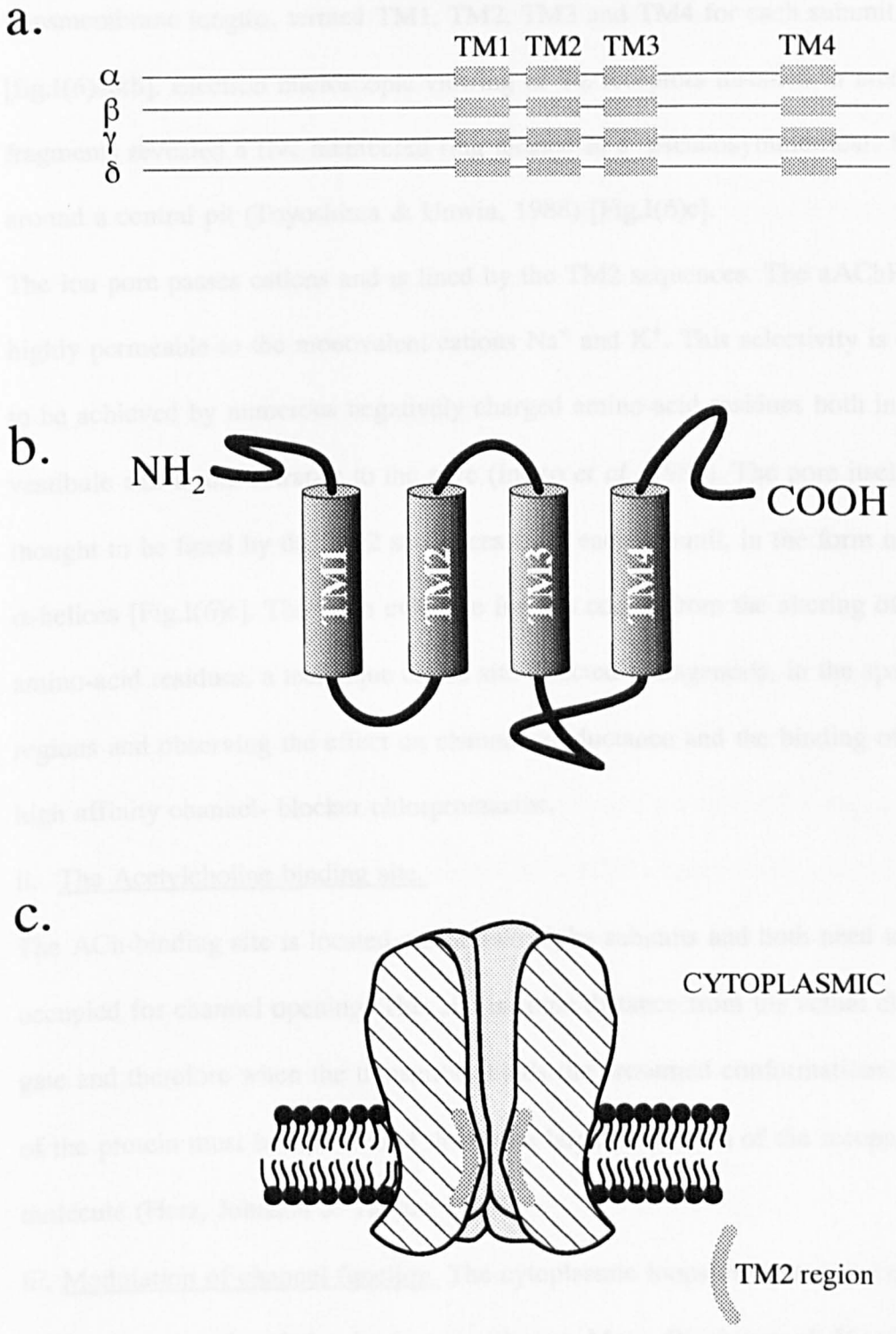


Figure I(6): Molecular nature of the nAChR. a.The subunits first cloned were α, β, γ and δ. The primary sequence of these subunits is very similar with 4 putative transmembrane domains in each (TM1-4). b. The secondary structure of one of the subunits, the TM domains are thought to be α-helices, two cytoplasmic loops and one extracellular loop and the COOH and NH_2 groups both being extracellular. c. The native nAChR molecule is thought to be constructed from five subunits, in this case, two α subunits and one each of β, γ and δ. Each subunit has four transmembrane domains and are thought to position their TM2 portions to form the actual pore (the shaded regions).

transmembrane lengths, termed TM1, TM2, TM3 and TM4 for each subunit [fig.I(6)a&b]. Electron microscopic viewing of the receptors installed in membrane fragments revealed a five membered ring located in a 'pseudosymmetrical' fashion around a central pit (Toyoshima & Unwin, 1988) [Fig.I(6)c].

The ion pore passes cations and is lined by the TM2 sequences. The nAChR is highly permeable to the monovalent cations Na^+ and K^+. This selectivity is thought to be achieved by numerous negatively charged amino-acid residues both in the vestibule and at the entrance to the pore (Imoto *et al.*, 1988). The pore itself is thought to be lined by the TM2 sequences from each subunit, in the form of α-helices [Fig.I(6)c]. The main evidence for this comes from the altering of single amino-acid residues, a technique called site-directed mutagenesis, in the specified regions and observing the effect on channel conductance and the binding of the high affinity channel- blocker chlorpromazine.

ii. <u>The Acetylcholine binding site.</u>

The ACh-binding site is located on the two alpha subunits and both need to be occupied for channel opening. This site is some distance from the actual channel gate and therefore when the transmitter binds the presumed conformational change of the protein must be transmitted through a large proportion of the receptor molecule (Herz, Johnson & Taylor, 1989).

iii. <u>Modulation of channel function.</u> The cytoplasmic loops express many consensus regions for phosphorylation by kinases (Swope, Moss, Blackstone & Huganir, 1992). In addition to phosphorylation, the function of nAChR's can be altered by having a different subunit composition. Most obvious is that the γ subunit only exists in foetal muscle whereas in the adult it is replaced by the ε subunit, the

functional consequence of this is that of a higher conductance channel in the adult (Mishina, Takai, Imoto, Noda, Takahashi, Numa, Methfessel & Sakmann, 1986). There are many more subunits of the nAChR found throughout the body, several α subunits and at least three β subunits have been found, these subunits can group together presumably in the same general form as the torpedo nAChR but are believed to express subtly different behaviour (Role, 1992).

iv. A superfamily of ionotropic receptors.

Not only do all the subunits of the nAChR have a large degree of homology with each other but as has come to light as more and more receptors are cloned is the fact that there is a large degree of homology between different channels. The members of the nAChR family are thought to be the $GABA_A$, glycine and $5\text{-}HT_3$. Apart from the obvious differences in terms of transmitter binding sites and ion selectivity these diverse channels seem to be structuraly very similar and are presumed to follow the general pattern of the nAChR. Functionally all of these receptors are responsible for very fast actions in the range of a few milliseconds to a few tens of milliseconds. However, there is another super-family of receptor emerging which are quite different - the second messenger linked or metabotropic receptors.

d. Metabotropic Receptors - The β_2 Adrenergic Receptor

The superfamily of metabotropic, g-protein or second-messenger linked receptors contains numerous receptor types with common themes (Dohlman, Caron & Lefkowitz, 1987). This family has genetic homology of 10-20% between the different groups e.g. between muscarinic and β-adrenergic receptors, but shows much higher homology in certain regions of the gene, especially the cytoplasmic

loops formed by the characteristic seven transmembrane domains [Fig.I(7)a]. The family contains the α and β-adrenoceptors, dopamine D1 to D5, adenosine A1, histamine H2, 5-HT_1 and 5-HT_2, muscarinic acetylcholine receptors and rhodopsin (and other opsins), amongst others. The β_2-adrenergic receptor is one of the most studied metabotropic receptors, and was one of the first to have is primary amino-acid sequence read (Dixon et al, 1986). Noradrenaline is the transmitter at most of the neuro-effector synapses of the sympathetic nervous system. On the basis of the relative potencies of adrenaline, noradrenaline and isoprenaline Ahlquist, in 1948, proposed that there were two different adrenergic receptors, which he called α and β adrenoceptors, as predicted by Langley in 1905. Ten years later a selective β-antagonist, dichlorophenylisoprenalinel, was found. Another ten years passed and in 1967 Lands *et al* proposed that β-receptors were composed of two types, β_1 and β_2, to account for inconsistencies in the effects in different tissues. The β_1 were located in the heart and in the intestinal smooth muscle whereas the β_2 were in the bronchial, vascular and uterine smooth muscle. This allowed selective manipulation of bronchial and cardiac receptors both of which are important sites for clinical intervention in angina pectoris and bronchoconstrictive disorders such as asthma. Molecular biological techniques have agreed generally with the pharmacological findings and in addition to finding genes for β_1 and β_2 there also seems to be a third type the β_3. The extracellular sites contain sites for N-linked glycosolation, asparagine-linked oligosaccharides and cysteine-cysteine disulphide bridges. The β_2-adrenergic receptor has on its cytoplasmic carboxyl terminal a serine and threonine rich sequence, this is believed to be the site for phosphorylation by the β-adrenergic receptor kinase (βARK). The βARK

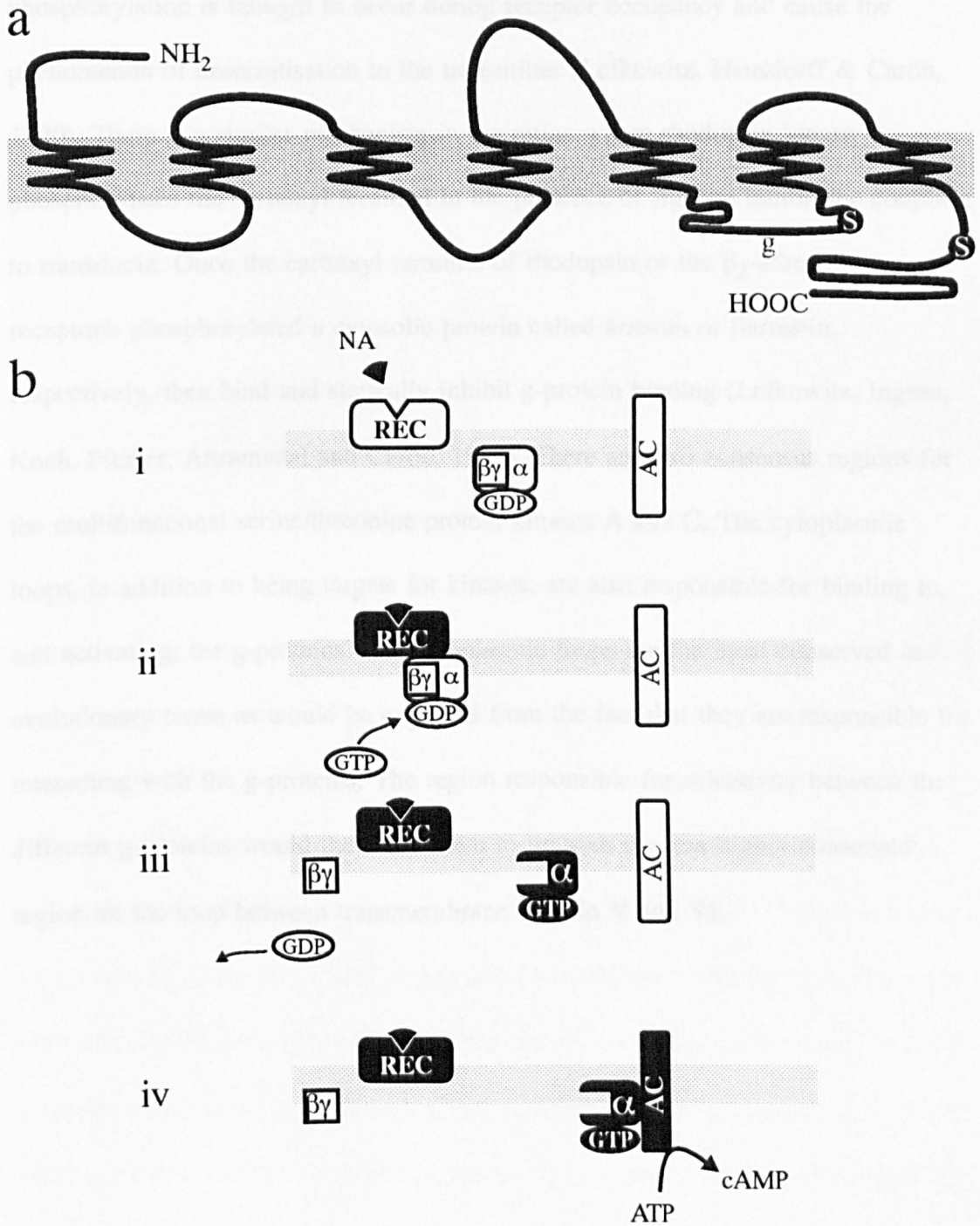


Figure I(7): The structure of the β2-adrenoreceptor and its interactions with G-proteins and adenylyl cyclase. a. The 7 transmembrane domains are typical of the G-protein linked receptor family. The 'g'designates the proposed G-protein binding area. The **S** are serines that can be phosphorylated. b. The mechanism from transmitter (noradrenaline; NA) to production of cAMP. The darker enzymes are activated. In (i) nothing is activated but when NA binds to the receptor (rec) in (ii) the receptor is activated which allows the inactive G-protein complex to bind. The GDP is exchanged for GTP and activates the G-protein (iii). The Gα-GTP complex dissociates from the βγ and moves through the membrane to activate adenylyl cyclase (AC) in (iv). Inactivation occurs as the GTP is hydrolysed to GDP and the G-protein reforms.

phosphorylation is thought to occur during receptor occupancy and cause the phenomenon of desensitisation to the transmitter (Lefkowitz, Hausdorff & Caron, 1990). There is a similar mechanism in the retina where rhodopsin kinase phosphorylates the carboxyl terminal in the presence of light to inhibit the coupling to transducin. Once the carboxyl terminal of rhodopsin or the β_2-adrenergic receptoris phosphorylated a cytosolic protein called arrestin or βarrestin, respectively, then bind and sterically inhibit g-protein binding (Lefkowitz, Ingese, Koch, Pitcher, Attramadal and Caron, 1992). There are also consensus regions for the multifunctional serine/threonine protein kinases A and C. The cytoplasmic loops, in addition to being targets for kinases, are also responsible for binding to, and activating, the g-proteins. The cytoplasmic loops are the most conserved in evolutionary terms as would be expected from the fact that they are responsible for interacting with the g-proteins. The region responsible for selectivity between the different g-proteins would therefore seem to lie with the less highly conserved region on the loop between transmembrane domain V and VI.

C. SECOND MESSENGERS.

1. G-PROTEINS COUPLE CELL SURFACE RECEPTORS TO INTRACELLULAR ENZYME SYSTEMS.

In the early 1970's it was noticed that hormonal regulation of adenylate cyclase was dependent on guanine trisphosphate (GTP). Indeed, activation of the cell-surface receptor switched on GTPase activity and this was essential for effective coupling to the adenylate cyclase. It is now known that when an agonist binds to the receptor the cytoplasmic face of the receptor can then bind a G-protein (more formally called a GTP-binding protein). A G-protein is a heterotrimer containing one each of a selection of α, β and γ subunits. When inactive it is a stable complex with guanine diphosphate (GDP) bound. When activated by binding to the receptor - agonist complex it swaps its GDP for GTP and splits into two components G_{α}-GTP and $G_{\beta\text{-}\gamma}$. The receptor is still activated and can repeat this for many G-protein heterotrimers. The GTP-G_{α} part is responsible for activating the effector enzyme, the GTP-G_{α} moiety is hydrolysed back to GDP-G_{α} and can then rebind to a $G_{\beta\text{-}\gamma}$. Thus one activated receptor can activate more than one G-protein and each G-protein can activate more than one effector enzyme, so overall there is scope for significant amplification of the signal (Gilman, 1987; Hepler & Gilman, 1992 and Hille, 1992). The β-adrenoceptor was one of the first G-protein mediated systems to be characterised. The agonist (endogenously it would be noradrenaline) binds into the agonist-binding site of the receptor and promotes a conformational change that is communicated throughout the molecule which then has the ability to bind the G-protein consisting of the $G_{\alpha s}$, a G_{β} and a G_{γ}. $G_{\alpha s}$ is the name given to the α subunits that are known to stimulate adenylyl cyclase. There are many G_{α}

subunits and they are divided into groups depending on known targets. Besides the $G_{\alpha s}$ there are ones that inhibit adenylyl cyclase $G_{\alpha i}$ and there is less well defined group that do no regulate adenylyl cyclase but stimulate other effector systems, G_o. The G-protein activated by rhodopsin is called transducin (or G_t) and activates a phosphodiesterase (Lagnado & Baylor, 1992). In the case of the β-adrenergic receptor the $G_{s\alpha}$-GTP is liberated from the receptor and moves in the membrane to activate adenylyl cyclase [Fig.I(7)c], thus causing a rise in the cytoplasmic cyclicAMP (cAMP) concentration. CyclicAMP activates a specific kinase, protein kinase A (PKA) which can phosphorylate many proteins in the cell. What has been described above is the mechanism of the β-adrenoceptor but there are many other G-protein linked receptors that use different second messenger pathways. The system that has been best characterised is the stimulation of phospholipase-C by a G-protein which leads to elevations of two second messengers inositol trisphosphate ($InsP_3$) and diacylglycerol (DAG) which play a role in controlling many cellular processes.

2. G-PROTEIN STIMULATED PLC LEADS TO THE BIFURCATING $InsP_3$ AND DAG PATHWAYS.

The plasma membrane primarily consists of phospholipids, with glycolipids, cholesterol and proteins making up the rest. The phospholipids are dominated by phosphoglycerides, which is glycerol esterified to the carboxyl groups of two fatty acid chains with the remaining carbon esterified to phosphoric acid. This is the simplest phosphoglyceride and is is called phosphatidate. This in turn can be esterified to one of several alcohols: serine, ethanolamine, choline, glycerol and inositol. In addition to phosphoglycerides there is another major phospholipid -

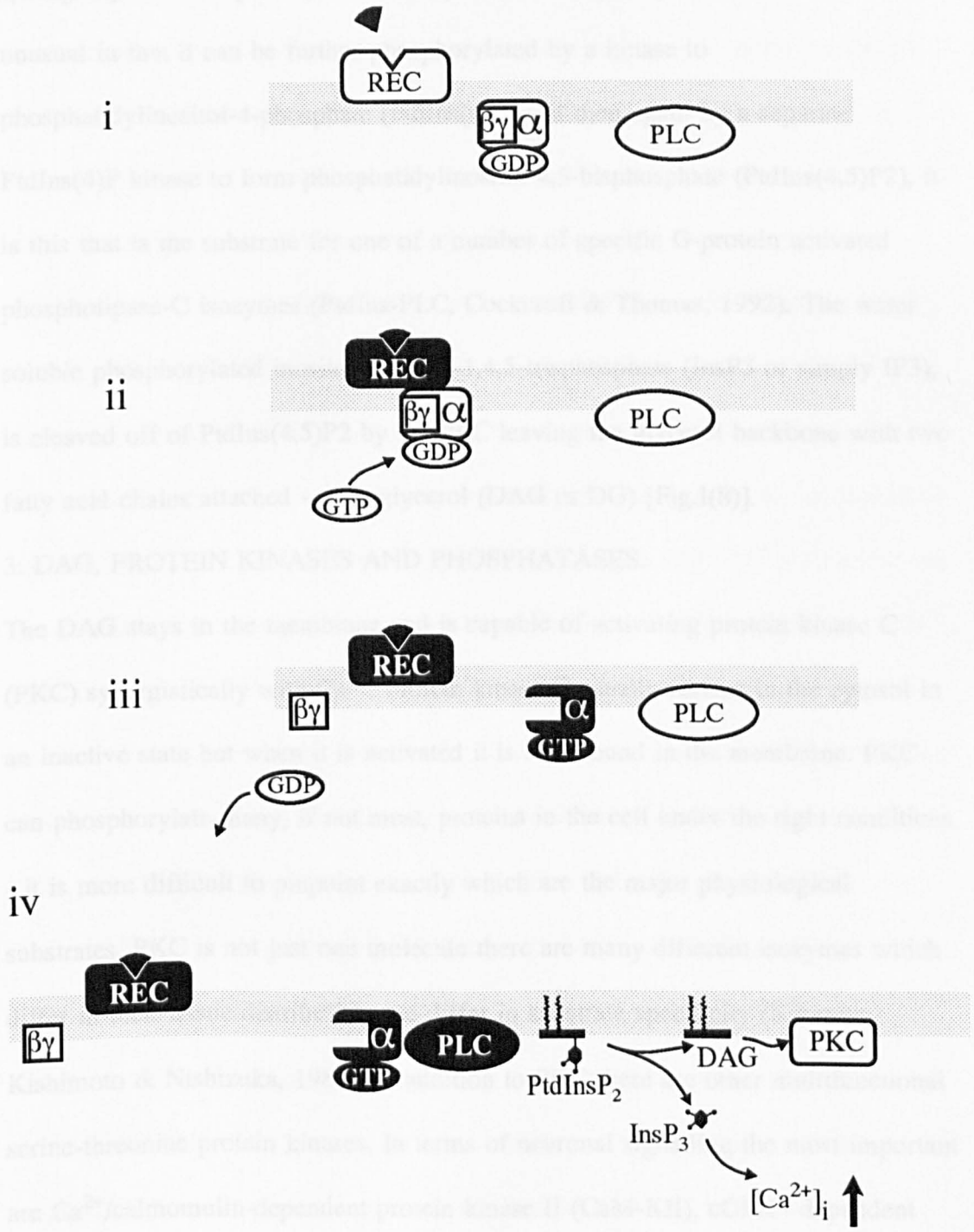


Figure I(8): G-protein stimulation of phospholipase-C (PLC) leads to two bifurcating messenger pathways. The stimulation of PLC via an activated G-protein is similar to the scheme in Fig.I(7). PLC hydrolyses the phosphatidylinositol(4,5)bisphosphate ($PtdInsP_2$) which leaves diacylglycerol (DAG) which diffuses through the membrane and stimulates protein kinase-C (PKC). The inositol-1,4,5-trisphosphate cleaved off is soluble and diffuses to specific $InsP_3$ receptors located on the endoplasmic reticulum and liberates Ca^{2+} from them.

sphingomyelin. Phosphatidylinositol is present in only very small amounts, it is unusual in that it can be further phosphorylated by a kinase to phosphatidylinositol-4-phosphate (PtdIns(4)P) and then again by a separate PtdIns(4)P kinase to form phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2), it is this that is the substrate for one of a number of specific G-protein activated phospholipase-C isozymes (PtdIns-PLC, Cockcroft & Thomas, 1992). The water soluble phosphorylated inositol, inositol-1,4,5-trisphosphate (InsP3 or simply IP3), is cleaved off of PtdIns(4,5)P2 by the PLC leaving the glycerol backbone with two fatty acid chains attached - diacyglycerol (DAG or DG) [Fig.I(8)].

3. DAG, PROTEIN KINASES AND PHOSPHATASES.

The DAG stays in the membrane and is capable of activating protein kinase C (PKC) synergistically with Ca^{2+}. Protein kinase C usually resides in the cytosol in an inactive state but when it is activated it is then found in the membrane. PKC can phosphorylate many, if not most, proteins in the cell under the right conditions - it is more difficult to pinpoint exactly which are the major physiological substrates. PKC is not just one molecule there are many different isozymes which differ in their tissue distribution and differ in substrate specificity (Kikkawa, Kishimoto & Nishizuka, 1989), in addition to PKC there are other multifunctional serine-threonine protein kinases. In terms of neuronal signalling the most important are Ca^{2+}/calmomulin-dependent protein kinase II (CaM-KII), cGMP- dependent protein kinase (PKG; Hofman *et al.*, 1992) and cAMP-dependent protein kinase (PKA) already mentioned above. The dephosphorylation of phosphoproteins is achieved by a group of phosphatases, these have heterogenious tissue distribution and are highly regulated (Cohen, 1989). The situation with the kinases and the

phosphatases is further complicated by the fact that the kinases and phosphatases can themselves act as substrates for phosphorylation/dephosphorylation reactions (Cohen, 1992). Proteins can be phosphorylated on tyrosine residues instead of serine/threonine, there is a whole different set of kinases and phosphatases for these reactions (Wagner, Mei & Huganir, 1991) including many receptor molecules which are themselves tyrosine kinases when activated (Pazin & Williams, 1992).

4. $InsP_3$ AND CALCIUM SIGNALLING.

G-protein activation of PLC results in the formation of $InsP_3$ which is known to release Ca^{2+} from an internal non-mitochondrial store likely to be the endoplasmic reticulum (ER) or a subset of that apparatus. This results in a rapid increase in the free calcium concentration of the cytoplasm, $[Ca^{2+}]_i$ and is often associated with secondary influx of Ca^{2+} from outside the cell. The $InsP_3$ binds to a specific receptor situated in the membrane of the ER. The receptor molecule had been long known as a marker for Purkinje cells known as P400 (Mikoshiba, Huchet and Changeux, 1979). This receptor has been purified and reconstituted (Ferris, Huganir, Supattapone & Snyder, 1989) and cloned (Furuichi, Yoshikawa, Miyawaki, Wada, Maeda and Mikoshiba, 1989) and found to be similar to the ryanodine receptor of muscle sarcoplasmic reticulum (Mignery, Sudhof, Takei and De Camilli, 1989). The receptor in its natural state is a homotetramer with one $InsP_3$ binding site per subunit with a positive cooperative effect *in vivo* but not when reconstituted. The difference is thought to be due to the lack of an associated Ca^{2+}- binding protein, calmedin, this is also thought to be resposible for the difference in Ca^{2+} modulation of $InsP_3$ binding (Ferris & Snyder, 1992). The ion channel is formed by the receptor proteins itself yet there is no similarity to any

voltage-gated channels or plasma membrane ionotropic receptors (Mikoshiba *et al.*, 1993). There are two types of $InsP_3R$, each with its own gene, $InsP_3R_1$ and $InsP_3R_2$. Further variants are generated by alternative splicing. The receptor binds $InsP_3$ with a K_m in the nM range but is capable of binding other inositol phosphates but only in the μM range. $InsP_3$ binding and function can be blocked by heparin or a monoclonal antibody (18A10) to the C-terminus of the type-1 $InsP_3R$ (Nakade, Maeda & Mikoshiba, 1991). There is strong evidence for a regulatory role of PKA. The $InsP_3$ signalling pathway is an evolutionary ancient mechanism as indicated by the fact that it is present in all vertebrates and many invertebrates and even plays an important role in the plant kingdom (Drobak, 1992). The ryanodine receptor (RyanR) is cloned and sequenced for both the cardiac and skeletal muscle types. In cardiac muscle Ca^{2+} entering the cell through dihydropyridine- sensitive Ca^{2+}-channels binds to RyanR and elicits Ca^{2+} release from internal stores. The skeletal muscle dihydropyridine molecule is a non-conducting voltage-sensor located in the plasma membrane which physically couples to the ryanodine receptors located on the sarcoplasmic reticulum and again induces release of internal Ca^{2+}. Both ryanodine receptor types are capable of Ca^{2+}-induced Ca^{2+} release and are stimulated by methylxanthines, in particular caffeine. Neurones also contain ryanodine binding sites and possess Ca^{2+}-induced Ca^{2+} release pools which are also caffeine sensitive (Henzi & MacDermott, 1992). Antibodies to the skeletal muscle RyanR cross react with the presumed neuronal RyanR (Ellisman *et al.*, 1990) and cross react with a large number of brain areas especially the hippocampus and cerebral cortex. The resting $[Ca^{2+}]_i$ is kept very low in neurones, in the region of 100 nM compared with a few mM outside the

cell. The electrochemical driving force is therefore very great. Other second messengers can be kept at a low concentration in the cell by specific catabolic enzymes, the same is not true for Ca^{2+}. Resting $[Ca^{2+}]_i$ is controlled by many processes. The Na^+/Ca^{2+} exchanger and Ca^{2+}-ATPase pumps are responsible for removing large quantities of Ca^{2+} out of the cell. The endoplasmic reticulum and mitochondria are equipped with Ca^{2+}-ATPase pumps with differing affinities and capacities which means that the release of internal Ca^{2+} is almost self-sufficient in that most of the Ca^{2+} is taken back up internally. There are also many Ca^{2+}-binding proteins which act to buffer the $[Ca^{2+}]_i$ and inhibit small fluctuations (Carafoli, 1987; Tsien & Tsien, 1990). Elevations of $[Ca^{2+}]_i$ can arise from influx or release from stores. It would seem that a cell could only have one target for raised $[Ca^{2+}]_i$. In reality cells contain many enzymes which are modified by Ca^{2+} over the range of concentrations that could occur *in vivo*. The Ca^{2+} signal can be segregated in terms of its spatial characteristics. Steep concentration gradients for Ca^{2+} are possible within very localised areas of the cell (Llinas, Sugimori & Silver, 1992). In addition the temporal characteristics are often varied to a huge degree. The interaction of Ca^{2+} with the other second messengers is complex and allows a few second messenger pathways to interact and produce a multitude of different signals.

5. CYCLIC-AMP, CYCLIC-GMP AND NITRIC OXIDE.

a. CyclicAMP

CyclicAMP is formed by adenylate cyclase from adenosine trisphosphate (ATP). Adenylate cyclase is activated by metabotopic receptors via G_s and inactivated via G_i. There is also a Ca^{2+}/calmodulin dependent adenylate cyclase in brain and this

has been found in brain areas associated with learning (Xia, Refsdal, Merchant, Dorsa & Storm, 1991). As mentioned above cAMP activates a multifunctional serine/threonine protein kinase called protein kinase A (PKA). This can phosphorylate many proteins within the cell. One of its most interesting and powerful systems is the regulation of gene transcription. The active subunit of PKA, the C-subunit, is transported into the nucleus where it phosphorylates CREB (cAMP response element binding protein) this allows it to bind to certain recognition sequences with the aid of other proteins and transcribe specific genes such as the genes for VIP, somatostatin and proenkephalin. The response time of the CREB system is very fast with times of less than 30 mins from stimulation to new protein (Montminy, Gonzalez & Yamamoto, 1990). CyclicAMP also plays a pivotal role in the transduction of olfactory signals. Odourous ligands bind to G-protein coupled receptors on olfactory sensory neurones and induce a rise in [cAMP] which is thought to directly gate channels. The channels have recently been cloned and are related to the cGMP- gated channels in visual transduction (Buck & Axel, 1991).

b. Phosphodiesterases.

The breakdown of cAMP is essential if it is to act as a signalling molecule. CyclicAMP is only known to be catablised by one route and that is via a cyclicnucleotide phosphodiesterase (CN PDE) to 5'- AMP. The level of cyclicnucleotide in the cell at any given moment is a function of the cyclase and phophodiesterase activity. There are several different types of CN PDE and their functions are inseperable from the actions of cGMP, which will be discussed further below. Phosphodiesterases have been grouped into five main types taking

into account their substrate preferences, their regulation and their genetic resemblance (Beavo, 1990). Type-1 phophodiesterases, PDE-I, is activated by Ca^{2+}/calmodulin and prefers cGMP. PDE-II is equally eager to breakdown cGMP and cAMP but is activated by cGMP. PDE-III prefers cAMP and is switched off by cGMP but this isozyme does not seem to occur in the brain. PDE-IV almost exclusively hydrolyses cAMP. PDE-V is selective for cGMP and can be activated by light in the retina and probably by cGMP itself elsewhere. The pharmacology of all these PDE's is very indistinct. There are some fairly selective inhibitors for example rolipram and zaprinast for PDE-III and PDE-V respectively (Thompson, 1991). Methylxanthines such as caffeine and isobutylmethylxanthine (IBMX) are non-selective inhibitors of the the above PDE's and have proved useful in elucidating the function this far. Another novel Ca^{2+}-stimulated PDE was overlooked because it was not sensitive to IBMX. This, I suppose, should be called PDE-VI. It had long been thought that guanylyl cyclase (GC) the enzyme responsible for producing cGMP was inhibited by Ca^{2+}. When GC was isolated it was found that Ca^{2+} no longer inhibited the ability of GC to raise cGMP levels. It was found that there was a missing protein - a Ca^{2+}-dependent PDE (Mayer, Klatt, Böhme & Schmidt, 1992).

c. CyclicGMP.

Guanylyl cyclase converts GTP into cGMP. In parallel to cAMP, many actions of cGMP are thought to occur through a specific serine/threonine protein kinase, protein kinase-G (PKG; Hofman *et al.*, 1992). The actual substrates for phosphorylation are not clear for any system in which PKG is involved, but in smooth muscle it seems that it reduces $[Ca^{2+}]_i$, it is not known whether this is due

to enhanced transport out of the cell, into the internal stores or blockade of further Ca^{2+} release. In the brain the cerebellar Purkinje cells are by far the richest source of GC, of the Type-Iα. Here the substrate of interest is thought to be an inhibitor of a phosphatase. CyclicGMP can also act in two other ways, one, it can stimulate the PDE-II which would then result in reduction of cAMP levels (inhibition of PDE-III is not thought to be present in brain) or, like cAMP, it can directly gate ion channels (see also Doerner & Alger, 1988). Visual transduction relies on a light activated phosphodiesterase reducing the cGMP levels and allowing a tonically activated cGMP-dependent channel to close.

d. Nitric oxide.

Nitric oxide (NO) is a free radical gas which has a very high diffusion coefficient and is chemically very reactive. It was first found to have a transmitter function when the elusive endothelium derived relaxing factor (EDRF) was discovered to be NO (Palmer, Ferrige and Moncada, 1987). The first indication that NO was present in neuronal tissue was that isolated cerebellar granule cells, when stimlated with NMDA, released a factor that relaxed adjacent smooth muscle (Garthwaite, Charles & Chess-Williams, 1988). This was later identified as being mediated by NO released from the cells (Garthwaite, Garthwaite, Palmer & Moncada, 1989). The enzyme nitric oxide synthase (NOS) was subsequently isolated (Bredt & Snyder, 1990) and then cloned (Bredt *et al.*, 1991). Neuronal NOS is a haemoprotein which is activated by Ca^{2+}/calmodulin and converts arginine into citrulline and NO (Mayer, 1993). In the brain as in blood vessels NO stimulates guanylate cyclase and elevates cGMP levels. The mapping of brain areas containing NOS (as shown by NADPH-diaphorase histochemistry) and those containing NO elevated cGMP

levels has shown that both are widespread but with subtle differences in location. This suggests that perhaps guanylate cyclase is the major target for NO in the brain (E. Southam, pers. comm.). NO can also have actions that are independent of cGMP - nitrosylation of proteins is thought to occur. There is evidence that NO plays a role in gene expression (Peunova & Enikolopov, 1993). There is also a debate over whether NO is responsible for some forms of excitotoxic cell death (Dawson, Dawson, Bartley, Uhl & Snyder, 1993). There is no doubting the ability of NO to kill cells as shown by its role in the armoury of the macrophage but whether the levels of NO produced by neurones is high enough to be toxic *in vivo* following an ischaemic or anoxic insult is still not resolved (Dawson, Kusumoto, Graham, McCulloch & Macrae, 1992). Nitric oxide has some particular properties which have tempted theoretical analysis of its neuronal functions (Gally, Montague, Reeke & Edelman, 1990). Firm evidence of the functions of NO in the central nervous system is still lacking. A role in long term potentiation (LTP) in the hippocampus is still not resolved although there is little doubt that under specific circumstances it does play a role, the physiological relevance has not been pinned down (Schuman & Madison, 1993).

6. SLOW SYNAPTIC EVENTS.

The examples of inhibitory and excitatory transmission discusssed in previous sections are mediated through ionotropic receptors and are responsible for short time course events in the range of a couple of milliseconds to tens of milliseconds. Direct point-to-point synaptic transmission can also follow a much slower timecourse, even via the same transmitter substances. This is mediated by the complex interweaving second messenger cascades described above. Ionotropic

receptors can directly gate Ca^{2+} into the cell. This can have a multitude of consequences via Ca^{2+}/calmodulin activated enzymes PKC, NOS, CaMK-II, etc. It can also directly activate Ca^{2+}-activated K^+ or Cl^- conductances which usually cause hyperpolarisation of the cell and are responsible for afterhyperpolarisations (AHP's; Lancaster, Nicoll & Perkel, 1991). AHP's are responsible for controlling the cells ability to fire sustained periods of high frequency action potentials. Ca^{2+} influx into the presynaptic terminal accumulates during moderate to high frequency firing and can bring about paired pulse facilitation, so that the next action potential that invades the synaptic terminal encounter an already raised Ca^{2+} level which it adds to by opening voltage-activated Ca^{2+}-conductances therefore causing more transmitter to be released. Second messengers also have the ability to close tonically open channels (usually K^+), this has never been found for ionotropic receptors but is a viable possibility. The classic example of this is the m-current. ACh activates muscarinic receptors which through an indistinct second messenger pathway act on special K^+ channels, the M-channels, and closes them (Adams, Brown & Constanti, 1982). This raises the excitabilty of the cell and is observed as the suppression of the ability of the cell to accommodate during repetitive firing. Noradrenaline (NA) can function in a similar fashion. In the hippocampus NA acts on β-adrenoceptors to elevate cAMP which closes a Ca^{2+}-activated K^+-conductance, it too stops spike accommodation (Nicoll, Madison and Lancaster, 1987). Longer term actions of second messengers can be mediated through phosphorylation of receptors (Swope, Moss, Blackstone & Huganir, 1992) or control of gene transcription which has the potential to change the synapse in a permanent fashion.

7. PRESYNAPTIC RECEPTORS.

All of the above modifications by second messengers occur in the postsynaptic cell but, second messengers can affect the release of transmitter from the terminals as well. Neurotransmitter receptors on the synaptic terminals when activated by the same transmitter that is released from the terminal (autoreceptors) or by a different transmitter from nearby terminals (presynaptic heteroceptors) can result in an increase or decrease in transmitter release. Presynaptic inhibition is a common mechanism whereby transmitter released from the terminal, in addition to diffusing to the postsynaptic membrane, feeds back onto autoreceptors on the terminal and reduces subsequent release. This is responsible for and studied by using paired-pulse depression of the postsynaptic response (Starke, 1981). Presynaptic receptors are quite often identical to receptors functioning in postsynaptic roles. They are thought to mediate their effects by inhibiting subsequent Ca^{2+} influx through voltage-sensitive Ca^{2+}-channels or via increasing K^{+}-conductances (Thompson, Capogna & Scanziani, 1993; Huston, Scott & Dolphin, 1990).

D. AMINO ACIDS AS NEUROTRANSMITTERS.

1. INTRODUCTION.

Quantitatively amino acids are the most common chemical transmitters in the vertebrate nervous system. Several amino acids have been proposed as candidates, these have been divided into excitatory amino acids (EAA), glutamate, aspartate, cysteic acid and homocysteic acid - which depolarise neurones, and inhibitory amino acids, GABA, glycine, taurine and β-alanine which generally hyperpolarise neurones. Even though they are responsible for transmission at the majority of central synapses it is only in recent years that knowledge of their actions has really accumulated. Part of the reasons why amino acids did not receive much attention as neurotransmitters was that they are present in all cells as part of the amino acid pool for protein synthesis - identifying glutamate containing neurones was not thought to be much evidence for it acting as a transmitter. Research was biased towards the study of monoamines and acetylcholine since the earliest work was achieved through studying the peripheral transmission at the neuromuscular junction, neurocardiac junctions and ganglia. As research tackled the more difficult task of decyphering the CNS it was armed with the tools and knowledge gained from peripheral tissue. For example, the use of histochemical techniques such as glyoxalic acid fluorescence allowed the distribution of these transmitters to be determined in the brain. An array of pharmacological tools were also available.

2. GLUTAMATE.

Glutamate and aspartate occur in very high concentrations in brain tissue, they were first discovered to be excitatory when applied to neurones (Curtis & Watkins, 1960). Whether a neurone is using glutamate or aspartate can often be unclear and

most people consider glutamate to be the most likely transmitter at EAA synapses, therefore the term glutamate or glutamatergic does not rule out that it could be a different substance.

a. Glutamate receptors - different subtypes.

The actions of glutamate are mediated through more than one type of glutamate receptor. It was discovered that some of the actions of glutamate could be mimicked by analogues such as NMDA (N-methyl-D-aspartate), kainate and quisqualate. A different receptor subtype was thought to underlie the differences between these agonists, these putative receptors were named after the defining agonists. Confirmation that there was more than one receptor type came from the actions of selective antagonists, D-α-aminoadipate (DAA; Biscoe et al., 1977) and Mg^{2+} (Ault et al., 1980), which inhibited actions of NMDA only. The development of D-2-amino-5-phosphonopentanoate (D-AP5; Davies et al., 1981), a much more potent NMDA antagonist, allowed the physiological investigations of NMDA receptor function. The NMDA receptor has revealed itself to be complex and crucially important in terms of brain function (see Watkins & Collingridge, 1989). Lack of a clear distinction between quisqualate and kainate actions led to the all encompassing term 'non-NMDA receptors'.

b. NMDA receptors.

i. <u>Mg^{2+} block and voltage dependence.</u>

As mentioned above the NMDA receptor could be blocked by the divalent cation Mg^{2+}. In fact, at resting membrane potential the NMDA receptor is blocked by Mg^{2+} and very little current flows through even though it is activated. If Mg^{2+} is not present in the extracellular medium the NMDA receptor is then free to respond

to an agonist. Another intriguing thing is that the NMDA receptor is voltage dependent. A plot of the current flow at various membrane potentials reveals a strange 'J'-shaped curve. Only at positive membrane potentials is the response ohmic. The I-V curve in Mg^{2+}-free conditions was found to be almost ohmic and it was realised that the Mg^{2+} ions are the cause of the voltage dependence of the receptor. At resting membrane potentials the channel is blocked with Mg^{2+} ions and as the membrane potential decreases the Mg^{2+} is freed from the channel and allows current to flow.

ii. Glycine is a coagonist at the NMDA receptor.

This aspect of the receptor will be discussed in more detail in section III(D) below. Briefly, it was shown that glycine was necessary for activation of the NMDA receptor.

iii. NMDA receptor channel conducts Ca^{2+}.

It was shown that NMDA induced a rise of $[Ca^{2+}]_i$ in cultured spinal neurones using the Ca^{2+}-indicator dye arsenazo III (MacDermott *et al.*, 1986). The contribution of voltage-sensitive Ca^{2+}-channels was ruled out and the results explained by the fact that the NMDA channel itself allows influx of Ca^{2+}. The major charge carrier through the NMDA receptor is Na^+, Ca^{2+} is thought to contribute less than 10% to the current (Schneggenburger, Zhou, Konnerth & Neher, 1993). Although the number of Ca^{2+} ions entering is fairly small the importance comes from the role of Ca^{2+} as a second messenger not a charge carrier. The amplitude of the rise in $[Ca^{2+}]_i$ is governed by the internal volume of the cytoplasm compartment relative to the number of NMDA channels opened. The temporal characteristics are mainly governed by free diffusion of Ca^{2+} away from

the site of entry and the ability of the cell to clear the Ca^{2+} using pumps or Ca^{2+} buffering. Spines located on the dendrites of many central neurones are thought to provide a biochemical compartment that allows the $[Ca^{2+}]_i$ to be limited to the postsynaptic site of an active synapse (Koch & Zador, 1993). Microfluorometric measurements of $[Ca^{2+}]_i$ using Fura-2 in CA1 pyramidal cells has shown that $[Ca^{2+}]_i$ can rise to a few μM in the dendrites during a short train of synaptic impulses and that the majority of this is AP5-sensitive (Regehr & Tank, 1992).

iv. Molecular cloning of the NMDA receptors.

Towards the end of 1991 a report describing successful cloning of the elusive NMDA receptor was published (Moriyoshi *et al.*, 1991). It showed sequence similarity to the non-NMDA receptors previously cloned (see below). It had four likely transmembrane domains and when expressed in *Xenopus* oocytes coded for a functional receptor that had many of the characteristics of naturally expressed NMDA receptors. *In situ* hybridisation indicated that the receptor could potentially be expressed in almost all brain areas. There are splice variants that possess different pharmacological properties: for zinc (Hollman *et al.*, 1993); polyamines and PKC (Durand *et al.*, 1992) and ethanol (Koltchine *et al.*, 1993), in addition electrophysiological difference have been picked up (Nakanishi, Axel & Shneider, 1992). Further NMDA subunits were discovered, called NMDAR2 to discriminate them from the previous NMDAR1 subunit (Meguro *et al.*, 1992; Monyer *et al.*, 1992 and Kutsawada *et al.*, 1992). There are four subunits NMDAR2A, NMDAR2B, NMDAR2C and NMDAR2D. When any one of them is expressed with NMDAR1 there is a huge increase in the whole-cell current relative to homomeric NMDAR1 receptors. The NMDAR2 subunits confer slightly different

characteristics to the NMDA channels (Stern, Behe, Schoepfer and Colquhoun, 1993). The NMDAR2 subunits are found in different areas of the brain strikingly the NMDAR2C is only expressed in cerebellar granule cells. The NMDAR1 sequence only has about 22% homology with the non-NMDA clones and only about 18% with the NMDAR2 sequences. Even with this low homology there are some common characteristics. They all have the four transmembrane domains and remarkable similarity in the active portion of the probable channel lining TM2 section. The NMDAR1 and the NMDAR2 have an asparagine conserved (at the same position as the the glutamine/arginine site of non-NMDA channels, see below) which is crucial for the Mg^{2+} block and the Ca^{2+}-permeability. Transposing glutamine for the asparagine in NMDAR1 leads to a decrease in Ca^{2+}-permeability but does not alter the Mg^{2+}-permeability, whereas if the same thing is done to the NMDAR2 subunit the Mg^{2+}-permeabilty increases and leaves the Ca^{2+}-permeability unaffected (Burnashev *et al.*, 1992; Sakurada, Masu & Nakanishi, 1993). The prediction of different types of NMDA receptors predated the molecular biology. Differences in the ability of antagonists and antagonists to displace NMDA-binding and the effects of glycine (Monaghon, Olverman, Nguyen, Watkins & Cotman, 1988), blockade by MK-801 (Ebert, Wong & Krogsgaard-Larsen, 1991) and sensitivity to the endogenously occuring NMDA agonist quinolinate (Monaghan & Beaton, 1991; Monaghan & Beaton, 1992) all pointed towards different NMDA receptors. The most outstanding difference was shown by the NMDA receptor of the cerebellar granule cell layer. It is not yet clear which NMDA receptor subunits are responsible for the differences seen in native receptors.

v. <u>The NMDA receptor plays a crucial role in synaptic plasticity.</u>

The phenomenon of long term potentiation (LTP) in the hippocampus was discovered twenty years ago as characterised by Bliss & Lømo in 1973. A brief tetanus can lead to an almost permanent increase in the synaptic strength of subsequent single impulses. It is thought to represent a 'cellular memory' for past activities. The properties of the phenomenon are: 1) *in vivo* it can last for days if not weeks; 2) it is generated by a stimulus which is physiologically relevant; 3) it is specific to the synapses that are involved; 4) there is a threshold for its activation, thereby stopping it occuring 'accidently' due to synaptic noise; and 5) it shows associativity, that is, the system is able to encode the pairing of two independent events. If, as is believed, LTP underlies some forms of learning, the associativity is a very crucial part. Associativity is a phenomenon common to many forms of learning in animals and man, it is more complex than habituation and sensitisation and potentially could underlie the most complex learning. The work of Pavlov in the 1920's showed that an animal could learn to associate an irrelevant stimulus with an event simply by pairing the two within a certain time window and for a certain number of times. The psychologist has long searched for the neuronal substrates for such events. It was Donald Hebb in his book '*The Organization of Behaviour*' in 1949 who is most remembered for stating that 'when an axon of cell A is near enough to excite a cell B and repeatedly or persistantly takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased' - Hebb's rule. The NMDA receptor can by itself detect associativity, conduction is dependent on two things occuring simultaneously, glutamate (and glycine) to bind and depolarisation of the cell to remove the Mg^{2+}-block. The precise mechanism of

LTP is not known (Bliss & Collingridge, 1993), what is known is that it is dependent on a rise in postsynaptic $[Ca^{2+}]_i$, as shown by the blockade of LTP with injection of Ca^{2+} chelators. It is the NMDA receptors job to allow influx of Ca^{2+} during the high frequency firing that is capable of removing the Mg^{2+}-block. The pivotal role of NMDA receptors has been shown by the ability of AP5 to block LTP but have little effect on the basal transmission, mediated through AMPA receptors. The precise function of postsynaptic Ca^{2+} is not known. It is a matter of some controversy whether the potentiation of synaptic transmission is due to postsynaptic events (increase in AMPA mediated epsp's) or presynaptic (increase in transmitter release), or both. If presynaptic mechanisms are invoked there must be some retrograde messenger which tells the presynaptic terminal that Ca^{2+} has increased in the postsynaptic element. There are two strong candidates both of which seem to be involved to some degree, arachidonic acid and nitric oxide (Schuman & Madison, 1993; Williams, Errington, Li, Lynch & Bliss, 1993). The picture is still not certain, but at least some knowledge of the mechanisms specific to LTP has allowed attempts to tie LTP with learning in the whole animal. AP5 was the first drug to be tested to try and block a form of spatial learning that is highly dependent on the hippocampus. An infusion of AP5 into the ventricles of freely moving rats was shown to block the ability of these animals to learn the location of a submerged platform in an opaque swimming pool (Morris, Anderson, Lynch & Baudry, 1986; Morris, 1989). These experiments are not without interpretational difficulties (see debate by: Keith & Rudy, 1990; Gallagher, 1990; Morris, 1990; Staubli, 1990 and Rudy & Keith, 1990). Perhaps if the details of molecular mechanisms specific to LTP are found this will allow the kind of work

initiated by Morris to continue to try and bridge the gap between cellular phonomena and behaviour, this is essential if neuroscience is to mature into real theories of brain function.

vi. NMDA receptors in development.

Neurones in many brain areas show a supersensitivity to NMDA during development. What the NMDA receptors are doing is not really known. It may be that they are involved in the sorting and differentiation that occurs during migration and synapse formation (McDonald & Johnston, 1990; Rabacchi, Bailly, Delhaye-Bouchard & Mariani, 1992). The development of maps and stripes in the brain has been shown to be blocked by NMDA antagonists. The crucial signal is probably the Ca^{2+} influx that accompanies NMDA channel opening. The Ca^{2+} probably causes changes in the phosphorylation state or regulation of gene transcription. The fact that NMDA receptors are crucial in development and in synaptic plasticity in the adult is not a surprise as both are really different aspects of the same thing.

c. Non-NMDA receptors.

Molecular biology has confirmed the pharmacological findings for the NMDA receptor. The situation for other glutamate receptors was not so clear. The actions of the prototypical agonists kainate and quisqualate did not correlate precisely and there were suspicians that this represented a heterogenious group of receptors - termed the non-NMDA receptors. The discovery of antagonists selective for non-NMDA receptors, GAMS (γ-glutamylaminomethylsulphonate) and Glu-Tau (γ-glutamyltaurine), and later by the much improved CNQX (6-cyano-7-nitroquinoxaline-2,3-dione; Honoré *et al.*, 1988) and NBQX (2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline; Sheardown *et al.*, 1990) did not help separate

non-NMDA receptors into groups. Small variations in the effects of γ-DGG (γ-D-glutamylglycine; Davies & Watkins, 1981), GAMS (Zhou, Hammerland & Parks, 1993) and GDEE (glutamate diethyl ester; see Turner & Meldrum, 1991) which seemed to prefer responses to kainate or quisqualate, respectively, and the discovery of a 'cleaner' and more potent replacement for quisqualate - AMPA (α-amino-3-hydroxy-5-methyl-5-isoxazolepropionate; Krogsgaard-Larsen *et al.*, 1980) did not convincingly show consistent heterogeneity. The debate had to wait for the cloning of these receptors for resolution of the problem.

i. Cloning of the non-NMDA receptors.

The very first glutamate receptor was cloned by functional expression in 1989 (Hollman, O'Shea-Greenfield, Rogers & Heinemann, 1989). Using that sequence many others have been found by homology screening (see Seeberg, 1993, for references). The information has confirmed that there are indeed AMPA and kainate preferring classes and that not only do they vary in terms of pharmacology but they also vary in terms of function. The non-NMDA receptors are a much more diverse group of receptors than had previously been imagined.

ii. AMPA receptors - molecular biology.

There are at least four subunits belonging to the AMPA-preferring class, $GluR_{1-4}$ (or $GluR_{A,B,C\ \&\ D}$) (Boulter *et al.*, 1990; Nakanishi N., Shneider & Axel, 1990). These subunits can form functional receptors on their own or mix to form heteromeric receptors with different properties. The genes express receptors about 900 amino acids in length and are highly homologous. These receptor subunits were then located in the brain using *in situ* hybridisation (Sato, Kiyama & Tohyama, 1993) and anti-peptide antibodies (Hampson, Huang, Oberdorfer, Goh,

Auyeung & Wenthold, 1992; Petralia & Wenthold, 1992). The main conclusion about the location of these receptor subunits is that they are expressed in most brain areas in agreement with AMPA binding autoradiography.

iii. Alternative splicing creates more variation in receptors.

Even greater variation and diversity for AMPA receptors was uncovered when it was realised that the mRNA could undergo alternative splicing which formed modified but still fully functional receptors termed flip and flop (Sommer *et al.*, 1990). These were seen to vary in expression during development, implicating a role for AMPA receptor in developmental regulation as well as NMDA receptors (Monyer, Seeburg & Wisden, 1991).

iv. Ca^{2+}-permeability.

Interestingly the $GluR_2$ subunit has some peculiar features. When receptors are formed from the other subunits (1,3 & 4) they are highly permeable to Ca^{2+} (Hollman, Hartley & Heinemann, 1991). The ability of $GluR_2$ to make the channel Ca^{2+}-impermeable is due to a single amino acid residue on the TM2 region of the receptor (Burnashev, Monyer, Seeberg & Sakmann, 1992; Hume, Dingledine & Heinemann, 1991). In $GluR_2$ it is an arginine whilst in the others it is a glutamine residue. If the arginine is replaced with a glutamine the channel then becomes permeable to Ca^{2+}. We would predict that cells that do not express $GluR_2$ but express other subunits would possess Ca^{2+}-permeable AMPA channels. The only cell type that clearly fits this category, so far, is the Bergmann glial cells (Petralia & Wenthold, 1992) and indeed that has been found to be the case (Burnashev *et al.*, 1992; Müller *et al.*, 1992).

v. Kainate receptors.

The $GluR_{5,6\ \&\ 7}$ subunits and two other subunits KA1 and KA2 are capable of forming high affinity kainate receptors and are spread throughout the brain in a heterogeneous manner (Bettler *et al.*, 1990; Egebjerg, Bettler, Hermans-Borgmeyer & Heinemann, 1991; Werner *et al.*, 1991 and Sakimura, Morita, Kushiya & Mishina, 1992). $GluR_5$ and $GluR_6$ can form functional homomeric receptors whilst $GluR_7$ cannot. KA1 and KA2 are also incapable of forming functional homomeric receptors. When KA1 or KA2 form heteromeric structures with $GluR_6$ or $GluR_7$ the characteristics of the channel are much changed. Desensitisation and Ca^{2+}-permeability are dependent on the subunit composition but another level of complexity is added by RNA editing (Seeburg, 1993).

vi. Comments on AMPA/kainate receptors.

The molecular biology has opened up hidden depths to the operation of the non-NMDA receptor which once lagged behind its cousin the NMDA receptor in terms of the interest neuroscientists gave it. The question is how is it best to approach such a complex system? Expression of subunit varieties in cells and then trying to create a selective pharmacology would be one way of then finding out what the nervous system needs all these different varieties of receptor.

d. A new class of glutamate receptors - metabotropic.

i. History.

In the mid-1980's it was discovered that glutamate and quisqualate could stimulate phosphoinositide turnover (Sladeczek *et al.*, 1985; Sladeczek, Recasens & Bockaert, 1988). Quisqualate acts on AMPA receptors (and perhaps at NMDA receptors, but this may be due to glutamate contamination) but since AMPA was much less potent than quisqualate at hydrolysing PI it was assumed that there must

be a new form of glutamate receptor, called the Qp receptor, initially. The discovery of a selective Qp or metabotropic glutamate receptor (mGluR) agonist provided a tool for researching this new area - *t*-ACPD (t-1-amino-1,3-cyclopentanedicarboxylic acid; Palmer, Monaghan & Cotman, 1989). It was then quickly discovered that mGluR's are coupled to the $InsP_3$/DAG/PKC pathway, release internal stores of Ca^{2+} and can excite cells (see Miller, 1991). Moving away from biochemistry to physiology it was found that *t*-ACPD could block presynaptically in the hippocampus (Baskys & Malenka, 1991) and excited postsynaptically by blocking a K^+-conductance (Charpak, Gähwiler, Do and Knöpfel, 1990). Then molecular biology stepped in...

ii. Molecular cloning.

The first metabotropic glutamate receptor cloned was in 1991 (Masu *et al.*, 1991; Houamed *et al.*, 1991) there are now at least six with alternatively splices variants of $mGluR_1$ and $mGluR_5$ (Tanabe *et al.*, 1992; Abe *et al.*, 1992; Pin *et al.*, 1992; Minakami, Katsuki & Sugiyama, 1993). They are all closely related but show very little homology to the G-protein linked superfamily. The family of six divides up into three pairs by virtue of the sequence homology and transduction mechanism. $mGluR_1$ and $mGluR_5$ are both thought to be linked to $InsP_3$/DAG, $mGluR_2$ and $mGluR_3$ are however thought to inhibit cAMP formation, $mGluR_4$ and $mGluR_6$ are slightly different. The $mGluR_4$ is thought to represent the APB presynaptic receptor found in some parts of the brain and is also linked to inhibition of cAMP. $mGluR_6$ is the APB receptor found in the retinal bipolar cells (Schoepp & Conn, 1993).

e. Uptake and release of glutamate.

Regulation of the release and the rapid uptake of glutamate from the synaptic cleft

are essential for it to fulfil its role as an often rapid transmitter in the CNS. Three high-affinity glutamate transporters have recently been cloned (see Kanai, Smith & Hediger, 1993). They are unrelated to the transporters for GABA or other transmitters. They are all Na^+-dependent but have different tissue distributions one is neurone specific the others glial. They use the Na^+ gradient to drive transport of glutamate up a concentration gradient from about 1 μM outside the cell to 10 mM inside the cell (Nicholls & Attwell, 1990). Release of glutamate is through the Ca^{2+}-dependent fusion of vesicles into the synaptic cleft. The peak concentration in the cleft has been calculated to be around 1 mM and this lasts for a very short time, about 1 msec (Clements, Lester, Tong, Jahr & Westbrook, 1992). The mechanism of uptake must be very efficient to keep the ambient levels down.

3. GABA.

a. GABA is the major inhibitory transmitter in the CNS.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system, not only in mammals but also in most other animals. Most, if not all, neurones either use GABA or can respond to GABA. GABA is formed from glutamate by the marker enzyme L-glutamic acid decarboxylase (GAD) since this pathway plays no role in the metabolism of the cell it is a good marker for a GABAergic neurone. GABA is one of the most proven candidates as a neurotransmitter: it is released in a Ca^{2+}-dependent manner and there is a high-affinity uptake system for it.

b. GABA acts at two classes of receptor.

GABA was originally found to be inhibitory through a mechanism of increased conductance to Cl^-, not necessarily associated with any hyperpolarisation (Eccles,

Schmidt & Willis, 1963). This action of GABA is now known to act through a subtype of GABA receptors called $GABA_A$. These have been cloned and consist of the typical five subunit heteromeric ion channel of the ionotropic receptor superfamily. GABA also has actions at another receptor class the $GABA_B$ type. This receptor is a metabotropic G-protein linked receptor, although it has not been cloned it could well belong to the superfamily of this type of receptor. The $GABA_B$ can increase K^+-conductance, decrease Ca^{2+}-conductance or modulate cAMP levels and thereby modify the excitability of the cell or regulate the release of transmitters from the terminal (Bowery, 1993).

c. Multiple $GABA_A$ subunits create diversity of action.

There are currently several α-subunits, 3 β-subunits, 3 γ-subuints and a δ-subunit (Olsen & Tobin, 1990). The distributions of the subunits is well documented (Wisden, Laurie, Monyer & Seeburg, 1992; Laurie, Seeburg & Wisden, 1992 and Laurie, Wisden & Seeburg, 1992) but it does not tell us which subunits get together in a particular cell. The expression also changes with development and other factors (Laurie, Wisden & Seeburg, 1992). The different compositions differ in terms of modulatory influences: diazepam, pentobarbital and picrotoxin binding (Sigel *et al.*, 1990) and there is particular interest in a composition that is expressed in cerebellar granule cells and binds the alcohol antagonist imidazobenzodiazepine (Lüddens *et al.*, 1990). The huge diversity will hopefully allow selective interactions with particular receptor subunit combinations.

d. $GABA_B$ receptors.

These receptors are expressed both on postsynaptic membranes and, where they were originally found, on presynaptic terminals. They too can offer diversity by the

way they link up with the complex intracellular messengers.

SECTION II - METHODS

A. BRAIN SLICES

The factors that determine the survival of cells within a slice of brain tissue are mainly concerned with ensuring that the cells are provided with a suitable ionic environment and an adequate supply of oxygen to meet their metabolic needs (Jiang, Agulian and Haddad, 1991). During the preparation of brain slices the main source of danger for the cells is from anoxia. All living animal cells require oxygen to survive. Neurones, especially from the central nervous system, are particularly susceptible to anoxic damage. Once disconnected from the blood supply it is important to minimise the dissection time. It is of equal importance to reduce the physical trauma the tissue has to endure (Alger *et al.*, 1984). Cells damaged by cutting release excitatory amino acids, amongst other substances, which can cause excitotoxic deaths in neighbouring cells. The two factors, anoxia and physical trauma, also act in collusion since one of the first systems to fail when anoxia sets in is the uptake systems for the excitatory amino acids. Most of the damage done is only transient and reverses to what seems, at the structural level, to be normal under correct recovery conditions.

1. AGE.

The age of the animal used for preparation of the brain slices seems to be one important factor. In the cerebellum, it has been clearly demonstrated that for sagittal slices the use of a crude tissue chopper, such as the McIlwain, is highly unsuitable for adult tissue (Garthwaite *et al.*, 1979). The Campden vibroslice has been found to be a much more successful tool and although not essential for neonatal tissue, this was used throughout. Slices from neonatal rats tend to have

much better preserved cells (Garthwaite *et al.*, 1980). This is probably due to a more robust metabolism or the fact that most of the axons are non-myelinated and easier to cut.

2. THICKNESS.

The well-being of the cells in parasagittal slices taken from the vermis of the rat cerebellum have been shown to be optimised at around 400 μm thick. If the slices are thicker the pO_2 drops below that required for metabolic activity and the cells, especially in the centre, die an anoxic death. If the slices are cut thinner to avoid this, the proportion of cells unavoidably killed by the cutting procedure to live cells is increased.

3. TEMPERATURE

The slices were cut at a reduced temperature, generally 10-12°C. This is thought to help the cells to survive the unavoidable period of ischemia/anoxia by lowering their metabolic rate, thus reducing the demand for oxygen and glucose. It is not clear why the slices do not prefer to be cut at even lower temperatures. It may be that the shock of immersing tissue at 37°C into ice-cold solution cannot be tolerated by the tissue.

B. DEPOLARISATIONS.

1. THE GREASE-GAP METHOD

The technique of grease-gap recording has been applied to many neuronal preparations, including cerebral cortex (Harrison & Simmonds, 1985), hippocampus (Blake, Brown & Collingridge, 1988), the superior cervical ganglion (Brown & Dunn, 1983), spinal cord (Evans, 1989) and cerebellum (Garthwaite, Garthwaite & Hajós, 1986). The method relies on a high resistance seal between two ends of a bundle of neurones. Changes in membrane potential, due to application of exogenous receptor agonists or synaptic stimulation, are recorded across the neurones. The bundle of neurones can be thought of as a leaky conductor. When one end is depolarised positive current is conducted electrotonically up the axons leaking out into the extracellular space, which is mainly due to the high resting permeability to potassium ions. This is is picked up as a positive potential in the side chamber relative to the earthed central chamber. The electrical resistance of the seal produced by the grease determines the amplitude of the response, since the potential registered is the product of the current and the resistance between the chambers. The positioning of the tissue is also a critical determinant of the response size, because the passively propagated current diminishes rapidly with distance, the site of current injection (i.e. the ion channels) needs to be as close as possible to the side chamber to be picked up by the recording arrangement. The potential, Vx, at a distance, x, from the site of current injection is given by the exponential equation:

$$Vx = Voe^{-x/lambda}$$ where lambda = space constant and Vo = the potential at site of current injection.

For any given slice lambda should be constant since it depends solely on the longitudinal resistance through the cytoplasm and the resistance of the cell membrane. This assumes that the extracellular longitudinal resistance is negligible. The grease seal in fact restricts the extracellular space to a degree but this is advantageous because it increases lambda thus allowing us to record a larger signal. The potential recorded should therefore be directly proportional to the change in membrane potential occurring in the cell body for any individual slice. This assumes that the membrane resistance in the rest of the cell stays fairly constant.

The cerebellar cortex avails itself to this technique since it has a very regular structure and the homogeneous populations of neurones. The simple structure permits the cutting of two different slices of tissue in which we can study the effects of the direct application of receptor agonists on a single type of cerebellar neurone. Cutting in the sagittal plane preserves the flat dendritic tree of the Purkinje cell and the fibres running in the white matter. Cutting the pial surface at right angles to this preserves the granule cell bodies and their elongated axons, the parallel fibres.

2. TECHNIQUE OF CUTTING SAGITTAL SLICES

The rats were stunned and decapitated. The cerebellum was exposed by making two lateral cuts 15-20 mm long and lifting this portion of the skull. Cold and freshly bubbled Krebs solution was dripped onto the cerebellar surface. The peduncles and any pial matter was cut and the whole cerebellum was removed and placed on filter paper saturated with cold Krebs. The hemispheres were dissected off using a razor blade. The cut face of the vermis was then glued onto the stage of

a Campden Instruments vibroslice with cyanoacrylate glue. The stage was then placed in the bath, immersed in cold Krebs and mid-vermal slices cut at 400 μm intervals. Care was taken to cut the slices at as near as possible right angles to the folia, thus ensuring that the maximum number of intact Purkinje cells were contained within the slice. A single lobule, usually lobule VIa, was trimmed with a micro-knife (Beaver) such that the grey matter at the most distal end of the lobule was left intact with the connecting white matter attached.

3. TECHNIQUE OF CUTTING PIAL SLICES

The dissection is the same as above except that the whole cerebellum including the hemispheres is glued onto the stage with the vermal folia at right angles to the blade. The blade is lined up with the apex of the vermis, the cut is made 400 μm below, resulting in a slice that is 400 μm in the middle but due to the natural curvature of the vermis tapers at both ends. This slice should therefore contain granule cell bodies in the central portion with their axons extending out to the ends of the tapers.

4. THE RECORDING BATH

The tissue was installed in the right hand side of the chamber as shown in Figure II(8). The end of the tissue in the side chamber was supported while the end in the central chamber was not. Although this lead to small artefacts when the flow was switched, it allowed the drug solutions free access to all sides of the slice enabling fast equilibriation of drugs. The partition wall had a grease-lined notch that was cut to match the shape of the tissue.

5. THE Ag/AgCl ELECTRODES

Silver wire about 200 mm long and 0.25 mm diameter (Goodfellow, Cambridge)

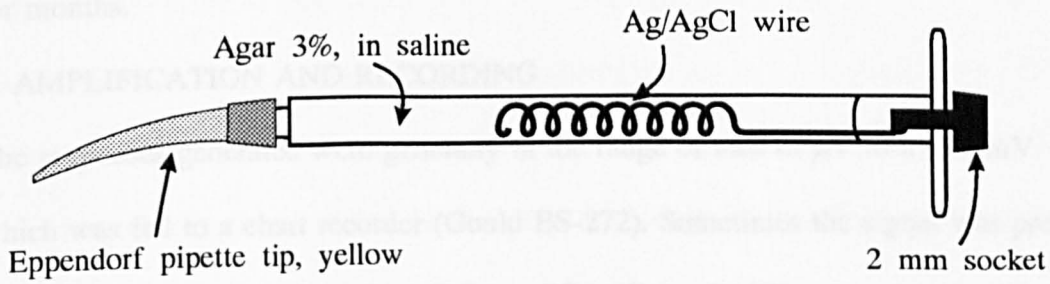


Figure II(1): The home made Ag/AgCl electrodes. For long term d.c. recordings the stability of the electrodes was essential. In this respect the large contact area between the chlorided-silver wire and the agar (dissolved in physiological saline) seemed advantageous.

was soldered to a 2 mm socket and electroplated with chloride from a NaCl solution (0.9 % at 25 °C) using two 1.5 V cells for 2-3 minutes, the correct thickness was estimated to occur when the coating was a black/red colour. A yellow Eppendorf pipette tip was glued to the end of a 1 ml syringe body and filled with a solution of hot Agar (3% in 0.9% NaCl). The coated wire was coiled and inserted into the Agar and the socket glued in place (Figure II(1)). These electrodes provided the stability required to record small DC shifts, and could last for months.

6. AMPLIFICATION AND RECORDING

The responses generated were generally in the range of tens of μV to a few mV which was fed to a chart recorder (Gould BS-272). Sometimes the signal was pre-amplified 100 or 1000 times by a DC amplifier (Grass P16D).

C. SYNAPTICS

The usefulness of the grease-gap method is greatly enhanced by the ability to record synaptic potentials as well as the responses to exogenous agonists (Garthwaite, 1986; Blake, Brown & Collingridge, 1988). The three-dimensional structure of the cerebellar cortex can be preserved by cutting unique biplanar slices. Basically, these comprise of a sagittal slice and a pial slice combined. They are cut on a specially constructed device on a standard Campden vibroslice.

1. CUTTING BIPLANAR SLICES

The cerebellum was excised and the hemispheres cut off as for sagittal slices. The cut edge of the vermis was glued to the small rotatable platform so that lobule VIa was lined up with the centre of the flat edge. The whole rig was then placed in a modified vibroslice bath and immersed in cold, freshly bubbled Krebs. The Perspex cutting guide was wound towards the tissue and allowed to gently touch it. A cut was made through the vermis about 100 μm above the top of the guide [Fig II(2)]. A second cut was made 350-450 μm below this, but stopped short of the guide. The blade was withdrawn and a fine tungsten wire (25 μm), held under tension in a specially constructed frame, was lined up with the cut by eye. The vibrating wire was then then used to cut down from the end of the cut made by the blade, following the natural curve of the vermis as dictated by the curved guide [Fig II(3)]. When the wire reached the platform the vibroslice was turned off and the guide withdrawn a few mm to allow the wire to be gently removed. The biplanar slice was gently separated from the rest of the tissue and the platform with the aid of a pair of fine iris scissors and a scalpel. The slice was then transferred to the recovery bath.

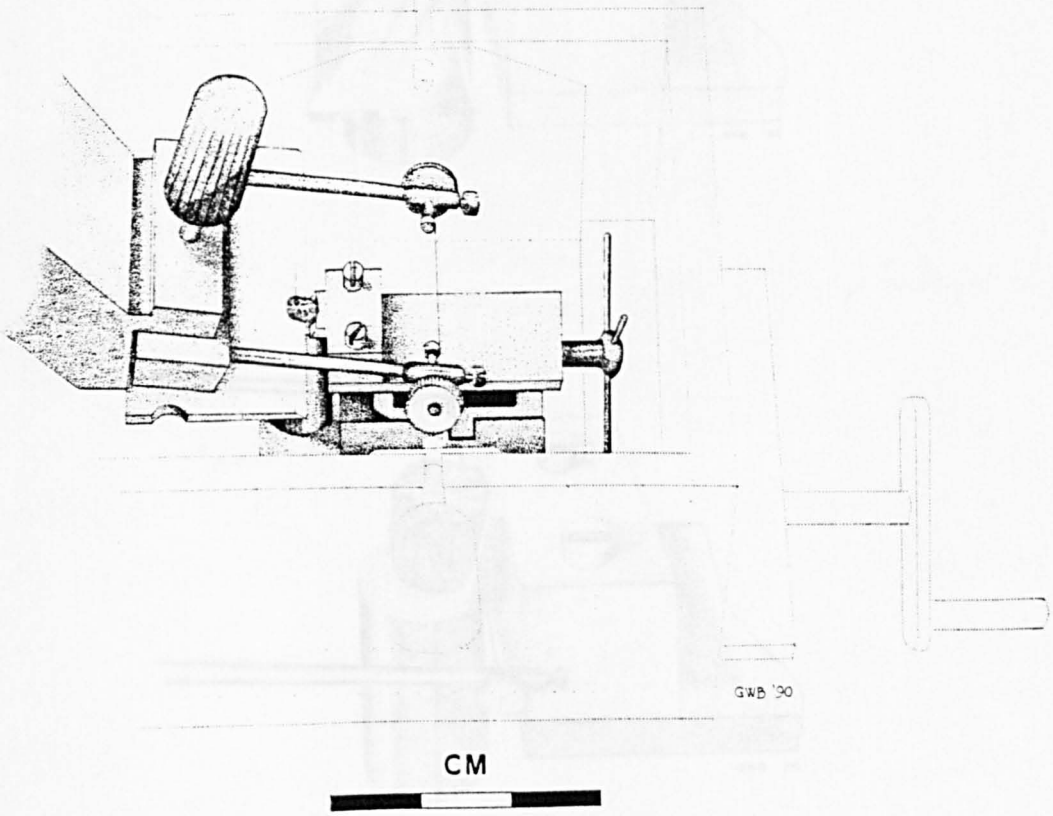


Figure II(2): Adaptation of the Campden vibroslice and the special holder necessary for cutting biplanar slices. A blade and the tungsten cutting wire are held in the arm of the vibroslice. The tissue is mounted on the small rotatable platform against the perspex guide. Drawings in this figure and the following six are courtesy of G. W. Batchelor.

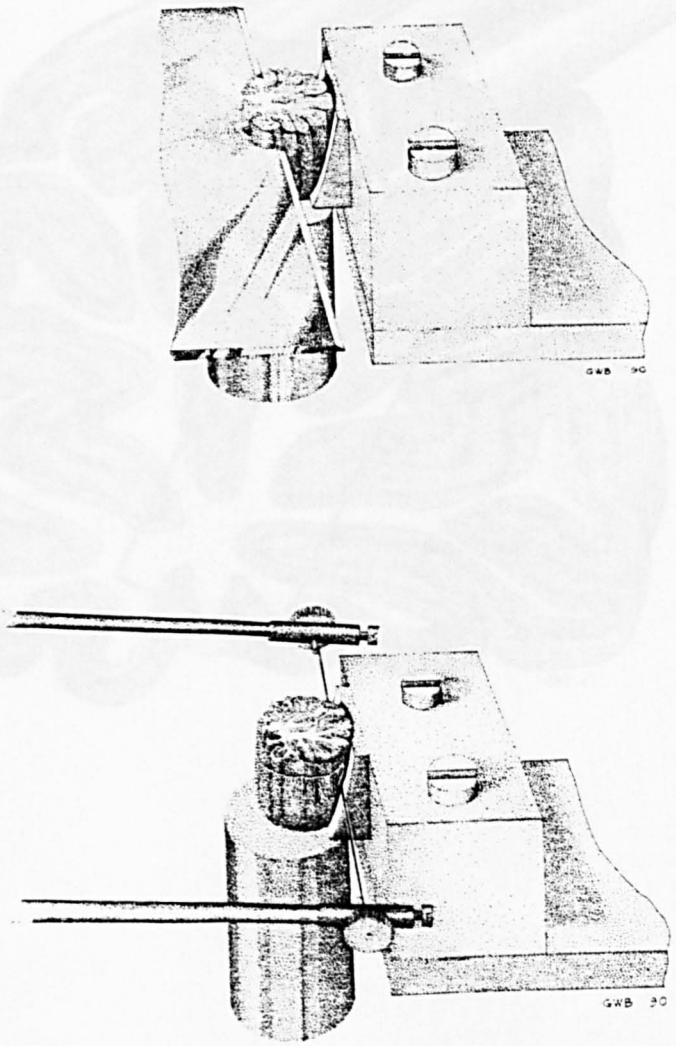


Figure II(3): Cutting a biplanar cerebellar slice. As shown in figure II(2) the first cut is made just above the perspex guide. A second cut is made 400 μm below but stops short of the guide. The blade is withdrawn and the wire inserted along the cut to the end where it makes a curving downward cut governed by the shape of the guide. The wire is then withdrawn and the slice gently cut from the base.

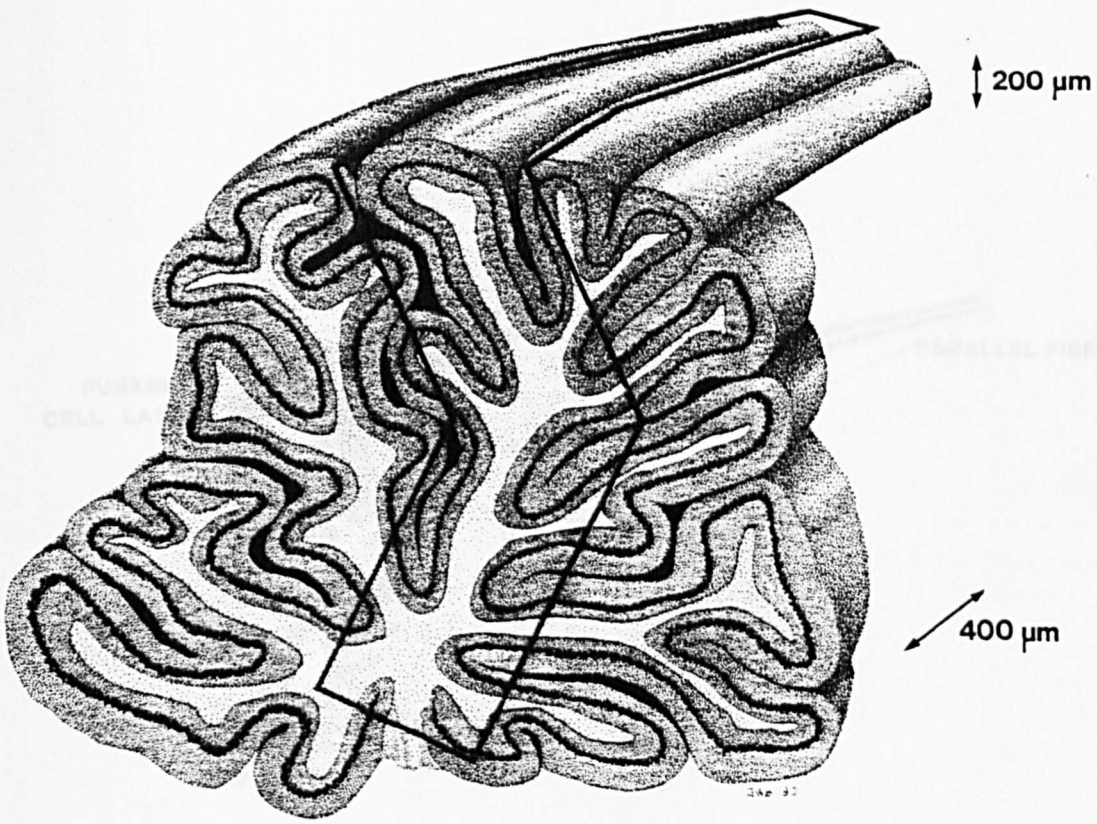


Figure II(4): A biplanar cerebellar slice. The slice is formed from a standard mid-vermal sagittal slice and a pial slice in one. There is a recess in the perspex guide which governs how thick the parallel fibre section is (approx. 200 µM.)

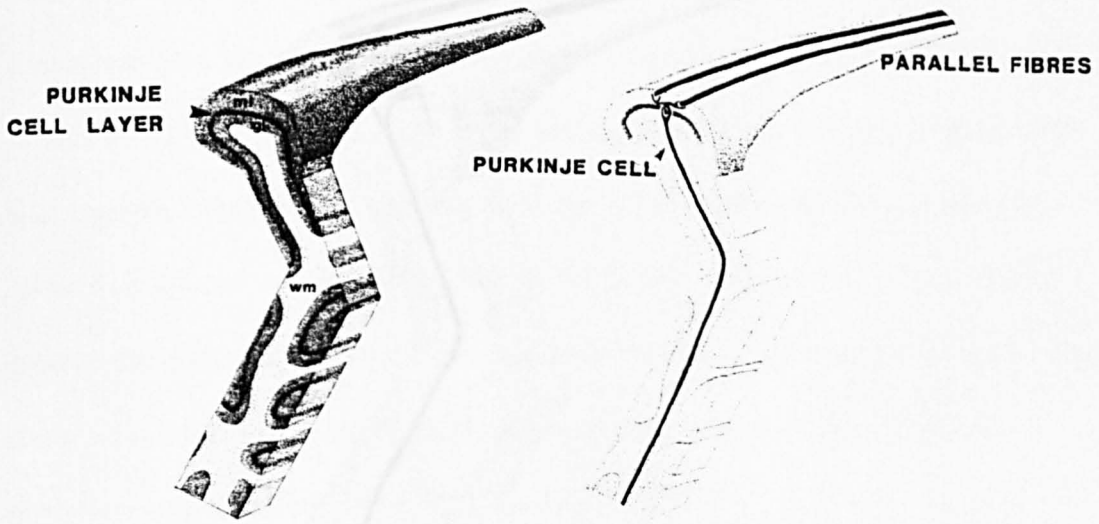


Figure II(5): Only a single lobule is used. The slice is trimmed along the line shown in figure II(4). The resulting slice contains the whole parallel fibre pathway intact. A bundle of parallel fibres contained in the surface section of the slice synapse onto the dendritic tree of the Purkinje cells. The Purkinje cells send their axon down the white matter.

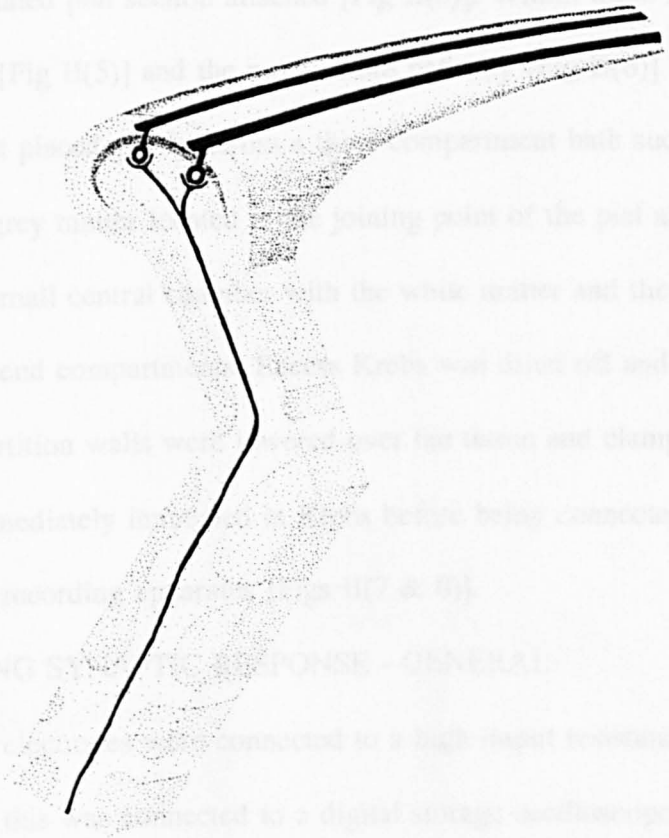


Figure II(6): The same slice preserves the mossy fibre pathway as well. The mossy fibres ascend the white matter and synapse onto granule cells whose axons travel along the surface section of the slice.

2. TRIMMING AND INSTALLING THE BIPLANAR SLICE

The whole biplanar slice was allowed to recover for at least one hour in oxygenated Krebs solution at room temperature, supported on a mesh with the pial section projecting upwards [Fig II(4)]. Lobule VIa was dissected out, as above, with the associated pial section attached [Fig II(5)]. Within these slices the parallel fibre pathway [Fig II(5)] and the mossy fibre pathway [Fig II(6)] are intact. The tissue was then placed gently across a three-compartment bath such that the cell bodies in the grey matter located at the joining point of the pial and sagittal slice was over the small central chamber with the white matter and the pial section located in the end compartments. Excess Krebs was dried off and grease coated gaps in the partition walls were lowered over the tissue and clamped in place. The tissue was immediately immersed in Krebs before being connected up to the perfusion and recording apparatus [Figs II(7 & 8)].

3. RECORDING SYNAPTIC RESPONSE - GENERAL

The Ag/AgCl electrodes were connected to a high imput resistance DC amplifier (Grass P16D) this was connected to a digital storage oscilloscope (Tektronix 2230) and to a FM tape recorder (Racal Store 4). The oscilloscope was connected via a IEEE 488 interface to an IBM compatible computer (Opus 286) running the signal acquisition program DADisp 1.1. Traces were exported from DADisp to a spreadsheet (Lotus-123) and generally into CorelDraw for publication quality figures. Each trace contained either 1 kbyte or 4 kbytes. A bipolar stimulating electrode was positioned with a micromanipulator onto the pial section to stimulate parallel fibres whilst the recording electrodes were placed across the other two chambers to record the parallel fibre response [Fig II(7)]. Alternatively the white

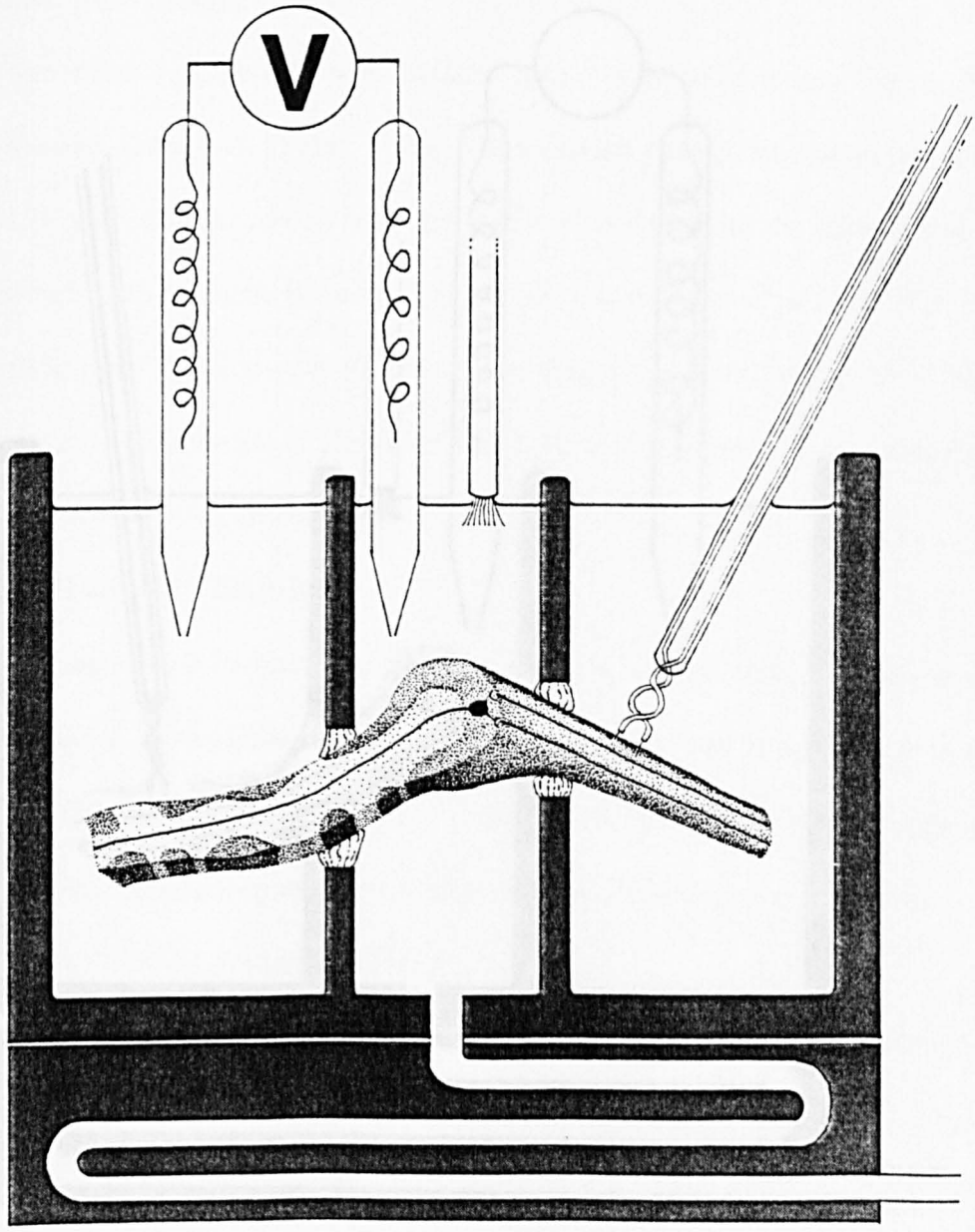


Figure II(7): The trimmed lobule is installed in the three chambered bath. The intersection between the surface and the sagittal part of the slice is positioned in the middle chamber where it is continuously perfused. Here the parallel fibres are stimulated and the response of the Purkinje cells relative to their axons recorded on an oscilloscope, V.

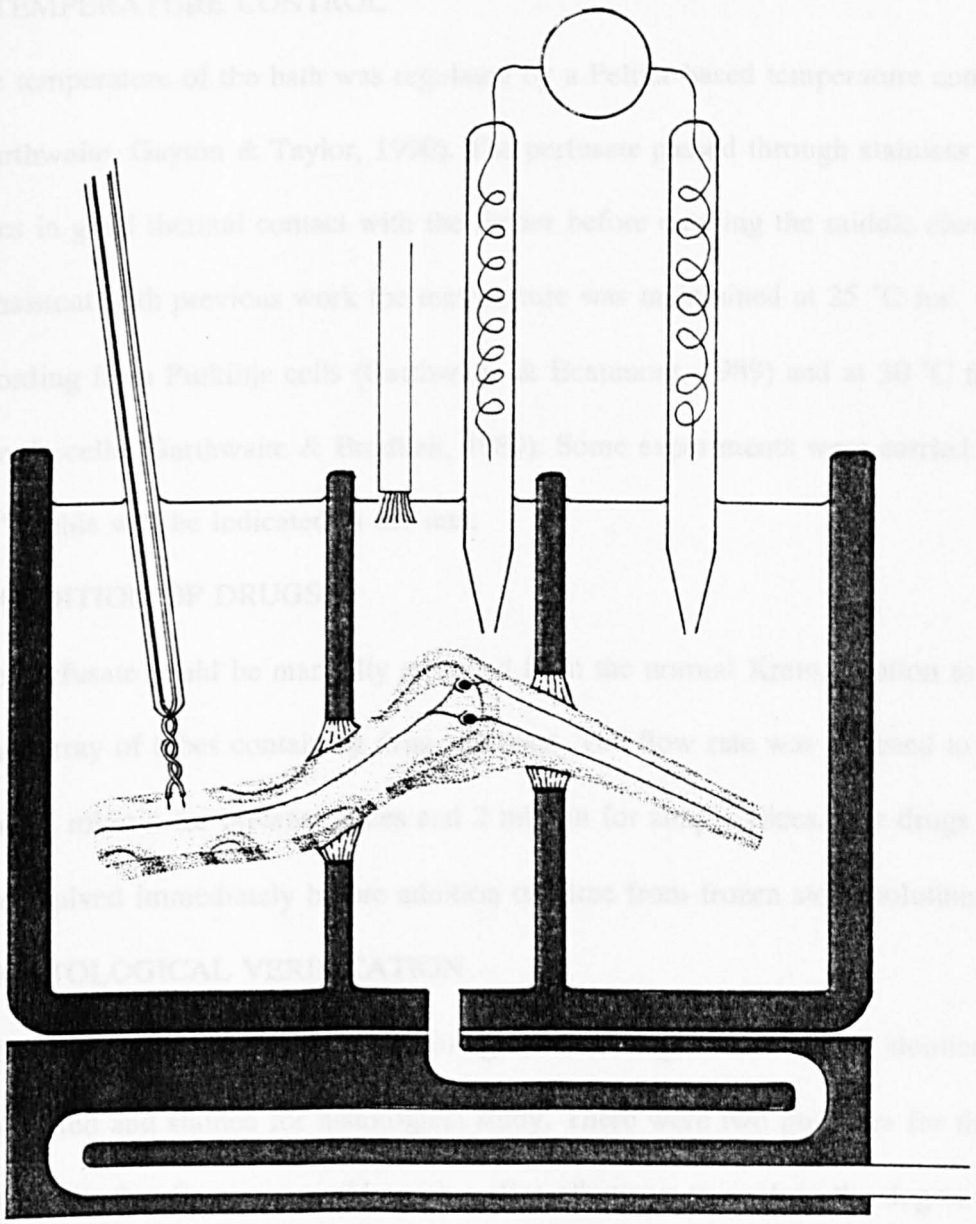


Figure II(8): The same slice can also be used for studying the mossy fibre response. The granule cells are in the central perfused chamber. The white matter including the mossy fibres are stimulated and the response of the granule cells is recorded relative to their axons going through into the side chamber.

matter could be stimulated to activate mossy fibres and the response of the granule cells, the mossy fibre response [Fig II(8)].

4. TEMPERATURE CONTROL

The temperature of the bath was regulated by a Peltier-based temperature controller (Garthwaite, Gayton & Taylor, 1990). The perfusate passed through stainless steel tubes in good thermal contact with the Peltier before entering the middle chamber. Consistent with previous work the temperature was maintained at 25 °C for recording from Purkinje cells (Garthwaite & Beaumont, 1989) and at 30 °C for granule cells (Garthwaite & Brodbelt, 1989). Some experiments were carried out at 37 °C, this will be indicated in the text.

5. ADDITION OF DRUGS

The perfusate could be manually switched from the normal Krebs solution to any of an array of tubes containing drug solutions. The flow rate was adjusted to give about 1 ml/min for biplanar slices and 2 ml/min for simple slices. The drugs were all dissolved immediately before addition or came from frozen stock solutions.

6. HISTOLOGICAL VERIFICATION

Some slices not used for electophysiological recording, but otherwise identical, were fixed and stained for histological study. There were two purposes for this. Firstly, the fast fixation possible with a slice allows us to analyse the degree of unavoidable cellular damage brought about by the cutting procedure and to examine the viability of the remaining cells. The second reason, was to verify that the cells contained within the slice were as expected and that the pre-blocking and the dimensions were set correctly. The slices were recovered as normal and then fixed in a mixture of 4 % paraformaldehyde and 2.5 % gluteraldehyde in 0.1 M

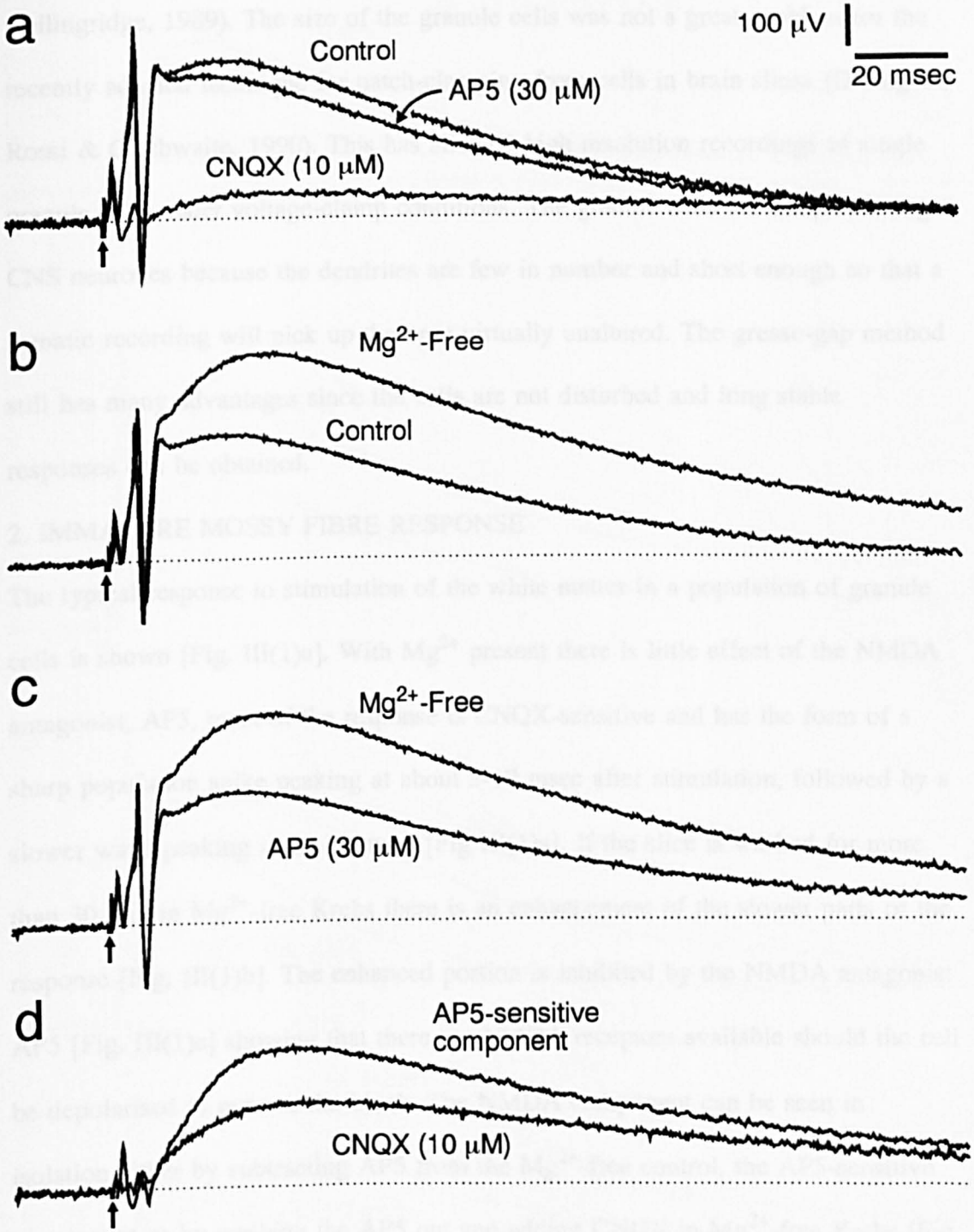
phosphate buffer (pH 7.4). They were then embedded in epoxy resin and semithin sections (μm) were cut and stained with toludine blue. The cells looked very healthy except for a few dead cells around the edges cut with the blade. The parallel fibre section was remarkably well preserved.

SECTION III : RESULTS (MOSSY FIBRE PATHWAY)

A. THE MOSSY FIBRE RESPONSE

1. INTRODUCTION

Mossy fibres course through the white matter and synapse with granule cells in the specialised regions called glomeruli, where one mossy fibre rosette contacts numerous granule cell dendrites and the terminals of Golgi inhibitory cells encapsulated by a layer of glial cells (Eccles, Ito & Szentágothai, 1967). The electrophysiology of the response of granule cells to mossy fibre activation aka. *the mossy fibre response*, has been difficult to study because of the tiny size of the granule cell bodies, 6 μm in diameter in rat. It has proved too difficult to use intracellular sharp electrodes, and until recently, could only be studied using extracellular electrodes (Eccles Llinás & Sasaki, 1966). The response recorded in the granule cell layer was composed of a biphasic positive-negative, P_1N_1, wave, followed by a large long-lasting negative wave N_2 which probably represents the fiber volley of the mossy fibres followed by the granule cell epsp. The transmitter substance was long thought to be acetylcholine because the mossy fibres were positive for choline acetyltransferase, a marker for cholinergic pathways (Ito, 1984). Adaptation of a grease-gap method used for recording from isolated ganglia allowed recordings from special biplanar cerebellar slices. This for the first time allowed the response of a population of granule cells to stimuation of mossy fibres to be recorded. The pharmacological manipulations indicated that the major transmitter was an excitatory amino acid, probably glutamate, which acted at a mixture of NMDA and non-NMDA receptors (Garthwaite & Brodbelt, 1989) not unlike those recorded from hippocampal CA1 pyramidal cells (Davies &

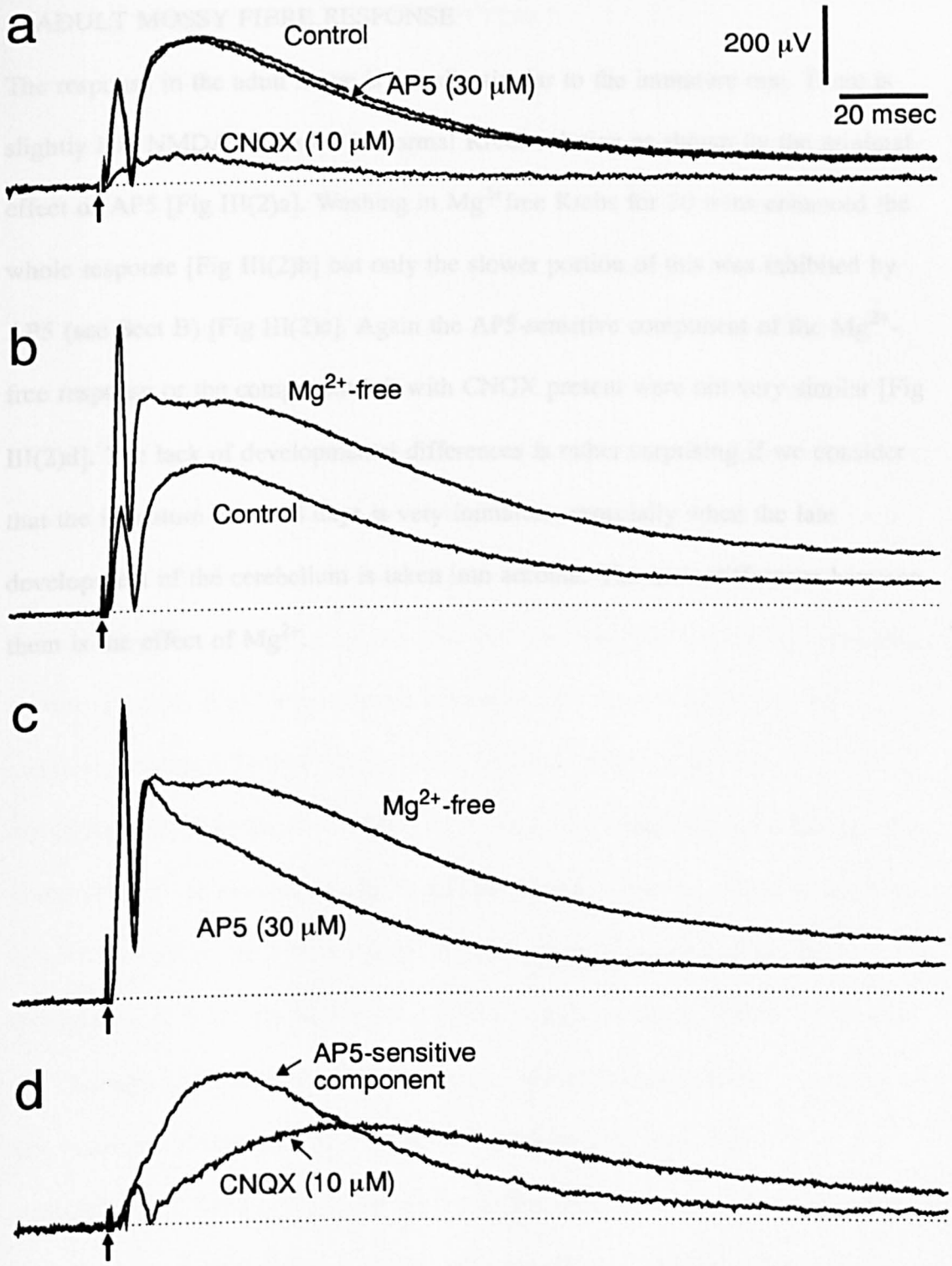


FigureIII(1): Mossy fibre response in slices from immature rat. a. In normal Krebs there is a two component response, one peaking after several msec and another at 40 msec. Both are almost unaffected by AP5 yet blocked by CNQX so are presumed to be AMPA receptor mediated. b. Washing with Mg^{2+}-free krebs increases a slow component which is AP5-sensitive (c). In d the response in Mg^{2+}-free plus CNQX is compared with the AP5-sensitive wave calculated by waveform sutraction of the two traces in (c). The arrow indicates the point of stimulation.

Collingridge, 1989). The size of the granule cells was not a great problem for the recently adapted technique for patch-clamping from cells in brain slices (D'Angelo, Rossi & Garthwaite, 1990). This has allowed high resolution recordings of single granule cells under voltage-clamp conditions. The granule cells are unique amongst CNS neurones because the dendrites are few in number and short enough so that a somatic recording will pick up the epsc virtually unaltered. The grease-gap method still has many advantages since the cells are not disturbed and long stable responses can be obtained.

2. IMMATURE MOSSY FIBRE RESPONSE

The typical response to stimulation of the white matter in a population of granule cells is shown [Fig. III(1)a]. With Mg^{2+} present there is little effect of the NMDA antagonist, AP5, most of the response is CNQX-sensitive and has the form of a sharp population spike peaking at about 5-10 msec after stimulation, followed by a slower wave peaking at 20-30 msec [Fig III(1)a]. If the slice is washed for more than 30 min in Mg^{2+}-free Krebs there is an enhancement of the slower parts of the response [Fig. III(1)b]. The enhanced portion is inhibited by the NMDA antagonist AP5 [Fig. III(1)c] showing that there are NMDA receptors available should the cell be depolarised to remove the block. The NMDA component can be seen in isolation either by subtracting AP5 from the Mg^{2+}-free control, the AP5-sensitive component or by washing the AP5 out and adding CNQX in Mg^{2+}-free Krebs [Fig III(1)d]. The fact that they are not identical could be due to a number of factors, including: the voltage dependency of the NMDA receptor due to residual Mg^{2+} and other effects of removing Mg^{2+} - destabilisation of membrane proteins and increased flux through Ca^{2+}-channels.



FigureIII(2): Mossy fibre response in adult tissue. The response is similar to that in the immature rat. In (a) AP5 has a negligable effect whereas CNQX blocks most of the response. In (b) perfusing with Mg^{2+}-free krebs enhances both the spike and the slow wave cf. immature and this is not reversed by AP5 (c, see text). The AP5-sensitive component from (c) was calculated and is superimposed on the Mg^{2+}-free plus CNQX.

3. ADULT MOSSY FIBRE RESPONSE

The response in the adult tissue is broadly similar to the immature one. There is slightly less NMDA activation in normal Krebs solution as shown by the minimal effect of AP5 [Fig III(2)a]. Washing in Mg^{2+}free Krebs for 30 mins enhanced the whole response [Fig III(2)b] but only the slower portion of this was inhibited by AP5 (see Sect B) [Fig III(2)c]. Again the AP5-sensitive component of the Mg^{2+}-free response or the component left with CNQX present were not very similar [Fig III(2)d]. The lack of developmental differences is rather surprising if we consider that the immature rat at 14 days is very immature, especially when the late development of the cerebellum is taken into account. The main difference between them is the effect of Mg^{2+}.

B. A Mg^{2+}-SENSITIVE AMPA RECEPTOR ?

In figure III(3) the effect of Mg^{2+}-free conditions on the mossy fibre responses obtained from an immature and an adult rat in the presence of AP5 (to rule out the NMDA effects) is shown. Comparing the fast portions of the response the adult is enhanced by 2 - 3 times whereas there is only a small change in the immature. The Mg^{2+} effect was also manifest in the effect of exogenous AMPA mediated depolarisations in adult granule cells (not shown), but this was less reliable an effect. Another strange effect of Mg^{2+} that caught my attention was that the actions of adenosine were depressed when the Mg^{2+} was removed from the extracellular medium (Bartrup & Stone, 1988). Thus, theoretically, adenosine could be tonically depressing the mossy fibre response (and that this was developmentally regulated) so that when the Mg^{2+} was removed adenosine no longer inhibited and the response increased. To test this we added DPCPX (8-cyclopentyl-1,3-dipropylxanthine; Bruns *et al.*, 1987) an A1 selective antagonist but it had no effect in the presence or absence of Mg^{2+} (not shown). More general effects of Mg^{2+} are its ability to affect the surface charge of the membrane to bring about the stabilisation of it and its proteinous contents, and the ability to inhibit the flow of Ca^{2+} through Ca^{2+} channels. It seems hard to reconcile these effects, especially the developmental differences with the results obtained. One possibility not yet excluded is that there could be an Mg^{2+}-sensitive AMPA receptor. Granule cells are known to express multiple AMPA receptors (Wyllie, Traynelis & Cull-Candy, 1991) and a strange AMPA receptor in the retina is blocked by Mg^{2+} (Gilbertson, Scobey & Wilson, 1991). The question probably deserves attention at the single-cell or even channel level.

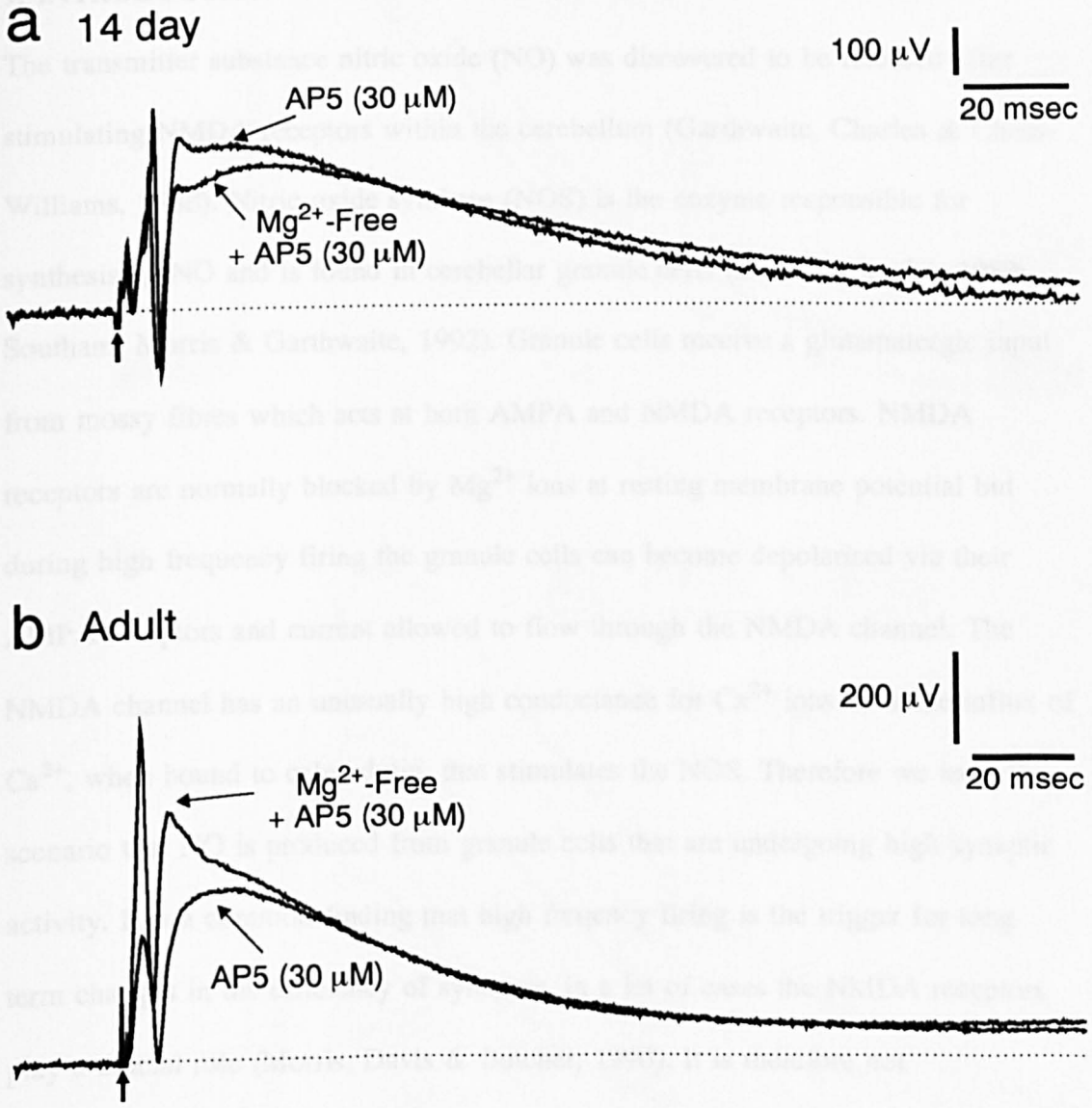


Figure III(3): Developmental difference in sensitivity of AMPA receptor mediated components to Mg^{2+}. All experiments were carried out in AP5 to block out the well recognised effects of Mg^{2+} on NMDA receptor mediated components. In the immature mossy fibre response (a) Mg^{2+} has a small effect on the response. In the adult (b), under comparable conditions, there is a large potentiation of the CNQX-sensitive, see FigIII(2), component. Same slices as shown in FigIII(1) & (2).

C. SNP BLOCKS NMDA COMPONENT

1. INTRODUCTION

The transmitter substance nitric oxide (NO) was discovered to be released after stimulating NMDA receptors within the cerebellum (Garthwaite, Charles & Chess-Williams, 1988). Nitric oxide synthase (NOS) is the enzyme responsible for synthesising NO and is found in cerebellar granule cells (Bredt & Snyder, 1989; Southam, Morris & Garthwaite, 1992). Granule cells receive a glutamatergic input from mossy fibres which acts at both AMPA and NMDA receptors. NMDA receptors are normally blocked by Mg^{2+} ions at resting membrane potential but during high frequency firing the granule cells can become depolarised via their AMPA receptors and current allowed to flow through the NMDA channel. The NMDA channel has an unusually high conductance for Ca^{2+} ions. It is the influx of Ca^{2+}, when bound to calmodulin, that stimulates the NOS. Therefore we have the scenario that NO is produced from granule cells that are undergoing high synaptic activity. It is a common finding that high freqency firing is the trigger for long term changes in the efficiency of synapses, in a lot of cases the NMDA receptors play a crucial role (Morris, Davis & Butcher, 1990). It is therefore not unreasonable to predict that NO might be involved in long term regulation of the mossy fibre to granule cell synapse, as has been found in other areas of the brain (Schuman & Madison, 1993). During the initial 20 - 30 minutes of recording the mossy fibre response at a slow stimulation rate (single shocks at 0.05 Hz or less) there is a tendency for the overall size of the response to increase. During a number of experiments carried out to test whether NO played a role in this phenomenon, it was noticed that the NO donor sodium nitroprusside (SNP;

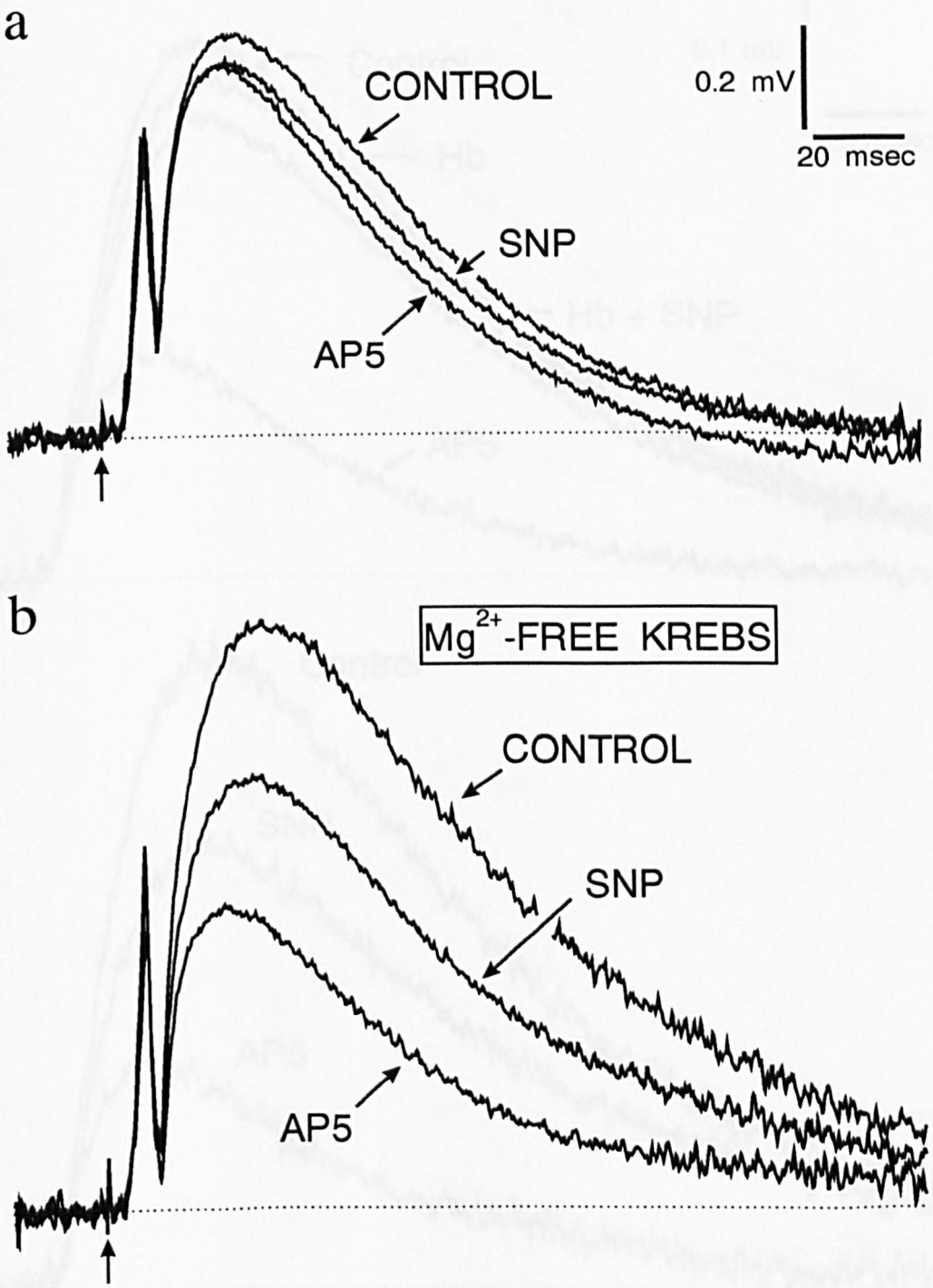


Figure III(4): The mossy fibre response is partially blocked by SNP. **a.** In normal Krebs solution, perfusion of SNP (300 μM) for 10 min reduces the response, particularly the late part of the slower wave, by a small amount. AP5 (30 μM; 8 min) has a slightly greater effect on the same part of the response. **b.** In Mg^{2+}-free conditions (35 min wash) there is a much larger AP5-sensitive component and SNP reduces this by about 50 %. Notice that the spike is not affected by AP5 or SNP. Scales apply to a and b.

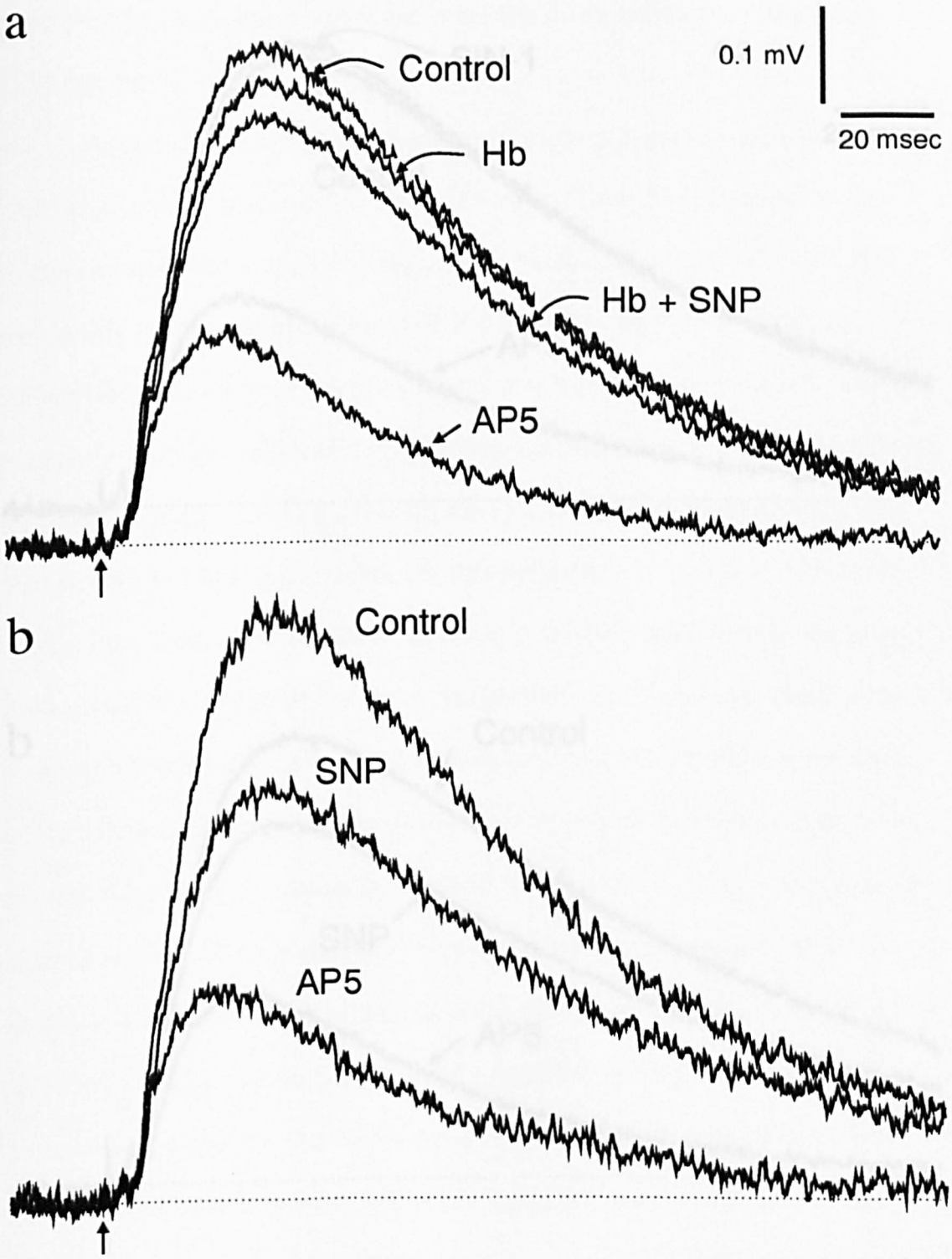


Figure III(5): Inhibition by SNP is blocked by haemoglobin. **a.** Haemoglobin (Hb; 30 μM) was applied for 5 mins with little effect. The addition of 300 μM SNP caused a further slight inhibition, less than 10% of AP5-sensitive component. **b.** In the same slice after washout of Hb for 25 min, perfusion of SNP (300 μM) for 7 min inhibited the AP5-sensitive component by about 45%. This experiment was carried out in Mg^{2+}-free conditions.

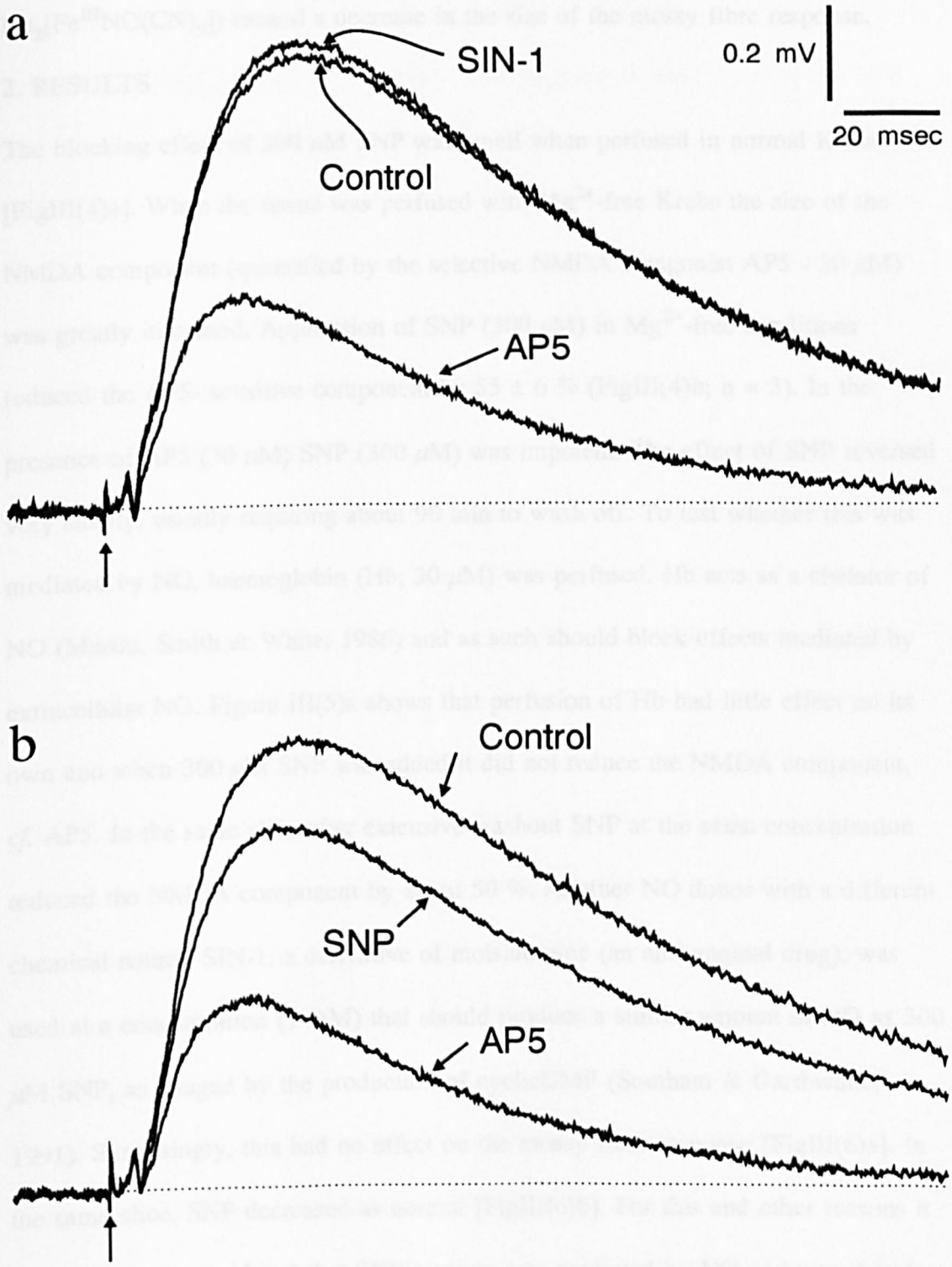


Figure III(6): The NO donor SIN-1 does not depress the synaptic response. **a.** SIN-1 (1 mM) had little or no effect on the mossy fibre response. **b.** In the same slice SNP (300 μM, 7min) inhibited the AP5-sensitive component by 35%. This experiment was carried out in Mg^{2+}-free conditions.

$Na_2[Fe^{III}NO(CN)_5]$) caused a decrease in the size of the mossy fibre response.

2. RESULTS

The blocking effect of 300 μM SNP was small when perfused in normal Krebs [FigIII(4)a]. When the tissue was perfused with Mg^{2+}-free Krebs the size of the NMDA component (quantified by the selective NMDA antagonist AP5 - 30 μM) was greatly increased. Application of SNP (300 μM) in Mg^{2+}-free conditions reduced the AP5- sensitive component by 55 ± 6 % (FigIII(4)b; n = 3). In the presence of AP5 (30 μM) SNP (300 μM) was impotent. The effect of SNP reversed very slowly, usually requiring about 90 min to wash off. To test whether this was mediated by NO, haemoglobin (Hb; 30 μM) was perfused. Hb acts as a chelator of NO (Martin, Smith & White, 1986) and as such should block effects mediated by extracellular NO. Figure III(5)a shows that perfusion of Hb had little effect on its own and when 300 μM SNP was added it did not reduce the NMDA component, *cf.* AP5. In the same slice after extensive washout SNP at the same concentration reduced the NMDA component by about 50 %. Another NO donor with a different chemical nature, SIN-1, a derivative of molsidomine (an anti-anginal drug), was used at a concentration (1 mM) that should produce a similar amount of NO as 300 μM SNP, as guaged by the production of cyclicGMP (Southam & Garthwaite, 1991). Surprisingly, this had no effect on the mossy fibre response [FigIII(6)a]. In the same slice, SNP decreased as normal [FigIII(6)b]. For this and other reasons it was no longer considered that SNP's action was mediated by NO and was therefore of doubtful physiological relevance. The chemical effects were studied further by S.J.East using exogenous NMDA and published as East, Batchelor and Garthwaite (1991).

3. DISCUSSION

SNP could reduce the size of the mossy fibre response in several ways. The fact that it had little effect when either Mg^{2+} or AP5 were present suggests that it was affecting the NMDA receptors selectively. The size of the AMPA/kainate receptor mediated component was undisturbed by the presence of SNP therefore ruling out some effect on synaptic transmission. The failure of SIN-1, S-nitrosoacetylpenicillamine (SNAP; 1mM; East et al., 1991) and S-nitrosoglutathione (SNOG; 1mM; unpublished observations) to block the NMDA component at concentrations that evoke large increases in cGMP can be explained if SNP is somehow acting in a NO-independent fashion. The probable explanation of SNP's effect is that it is acting at the redox site of the NMDA receptor. The reducing agent dithiothreitol enhances the effects of NMDA receptor activation, this can be reversed by the oxidising agent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; Aizenman, Lipton & Loring, 1989). SNP has long been known to act at thiol groups (Jocelyn, 1972). In agreement with this thinking, hexacyanoferrate(III), $K_3[Fe^{III}(CN)_6]$, which is structurally similar to SNP, except the nitroso group is replaced with another cyano group, also inhibits the depolarisation to exogenous NMDA (East et al., 1991). This still leaves the question of why Hb reversed the effects of SNP. This too could be explained by a redox effect. Hb contains two thiol groups (Jocelyn, 1972) which could interact with the SNP and render it ineffective. Further strength to this argument comes from the fact that methaemoglobin (metHb) was as effective as Hb in counteracting the action of SNP (East et al., 1991), whereas metHb should be much less effective than Hb at absorbing NO (Martin et al., 1986). There have been many subsequent reports of

the actions of NO donors acting at NMDA receptors. There is broad agreement that SNP blocks NMDA receptor action (Manzoni, Prezeau, Desagher, Sahuquet, Sladeczek, Bockaert and Fagni, 1992; Kiedrowski, Costa & Wroblewski, 1992). What is not clear is the mechanism. Wroblewski et al. (1992) found that SNP and $K_4[Fe^{II}(CN)_6]$ blocked NMDA receptor action whereas $K_3[Fe^{III}(CN)_6]$, $FeCl_2$ and $FeCl_3$ were inactive. The inhibition was reduced by haemoglobin and SNAP was ineffective. They came to a similar conclusion as ourselves and pointed towards some redox interaction. The only disagreement was that we found that $K_3[Fe^{III}(CN)_6]$ inhibited NMDA receptor action but did not try $K_4[Fe^{II}(CN)_6]$. Manzoni et al. (1992) found that SNP and $K_4[Fe^{II}(CN)_6]$ inhibited NMDA action and that since this was mimicked by $Fe^{II}SO_4$ they propose that the Fe^{2+} ion was somehow responsible. It is now becoming clear that other NO donors can also have actions at NMDA receptors and the evidence seems to point towards NO itself as the active moiety. NO released from SIN-1, 1-nitrosopyrrolidine and $NaNO_2$ was found to block NMDA receptor actions in striatal and cerebellar granule cell cultures (Manzoni, Prezeau, Marin, Deshager, Bockaert & Fagni, 1992). Degraded SIN-1 was inactive and haemoglobin partially reduced the effects of the NO donors. Interestingly, from a physiological point, is that haemoglobin itself potentiated NMDA responses. One possible explanation of this is that haemoglobin soaks up endogenous NO produced which normally provides a tonic inhibition of the NMDA receptor responses. The authors did not try the crucial test, i.e. adding NO-synthase inhibitors. It could be due to a similar effect as serum albumin (Eimerl & Schramm, 1991). There is evidence that inhibition by NO is due to modulation of a redox site (Lei, Pan, Aggarwal, Chen, Hartman, Sucher & Lipton,

1992). The authors opened up disulphide bonds in the extracellular side of the NMDA receptors and alkylated free thiol groups with N- ethylmaleimide and found that this blocked the previously inhibitory effect of the NO donors glyceryltrinitrite and S-nitrosocysteine, as well as the powerful oxidant DTNB. They assume that S-nitrosocysteine is only acting through NO, since the breakdown product cystine had no effect. In conclusion, although there are disagreements, it seems that NO donors under the right cicumstances can reduce NMDA receptor actions. SNP seems to have actions independent of NO. Differences between preparations, my experiments were carried out in slices whereas all the other reports use cultured neurones, and different buffers may explain the disagreements. The question of whether or not this is functionally significant is still open at the present time.

D. GLYCINE REGULATION OF NMDA RECEPTORS IN MOSSY FIBRE PATHWAY

1. INTRODUCTION

a. Glycine - an inhibitory transmitter.

Glycine is the simplest amino acid, possessing a single proton as a side chain, it is therefore achiral. It is one of the constituent amino acids which are strung together to form proteins, as well as the backbone of the porphyrin series of molecules. Its role as a neurotransmitter only became apparent in the 1960's. It is probably the major inhibitory transmitter within the spinal cord and brainstem, whereas GABA is predominant in higher regions of the brain. The classic antagonist of the inhibitory actions of glycine is the alkaloid strychnine. Stimulation of glycine receptors leads to an increase in the conductivity of Cl^- through the cell membrane, similar to the $GABA_A$ receptor.

b. Strychnine-insensitive glycine binding.

A comparison of strychnine and glycine binding sites revealed that there were huge differences in distribution throughout the brain (Bristow, Bowery & Woodruff, 1986). Strychnine binding sites were most dense in various brainstem nuclei, moderately dense in various hindbrain areas and apparently absent from the striatum, cerebral cortex, hippocampus and cerebellum. In contrast, glycine was found to densely label many additional areas, especially in the higher brain regions. Most densely labelled were parts of the hippocampal/amygdala complex and subiculum, moderate labelling was observed in the caudate putamen and the granule cell layer of the cerebellum. Low density glycine binding was also seen in the lateral cerebellar nuclei, dorsal cochlear nuclei and the trigeminal nucleus.

From this information it was clear that there was some form of strychnine-insensitive binding site with high affinity in higher brain regions. This study was carried out in Na^+-free conditions to stop binding to the glycine uptake sytems, therefore the binding site most likely represented a novel class of glycine receptors. A novel role for glycine - modulation of NMDA receptor function. A functional role for the strychnine-insensitive glycine receptors remained elusive. Johnson and Ascher (1987) were investigating the reason why the amplitude of NMDA responses in cultured cortical neurones was greater when a slow perfusion rate was used. They proposed that some factor was effusing from the neurones and was washed away at higher perfusion rates. The addition of 'conditioned medium', i.e. medium that had been in prolonged contact with the neurones, potentiated the response to NMDA when co-applied. The factor in the conditioned medium was heat stable and had a relative molecular mass of less than 700. A screen of the common amino acids showed that glycine was able to mimic the conditioned medium most closely.

c. Absolute requirement for glycine at NMDA receptors.

Initially, glycine was thought to be a modulator of the NMDA receptor but it soon became apparent that there was an absolute requirement for glycine. Expression of NMDA receptors in Xenopus oocytes allowed the levels of glycine contaminating the extracellular solution to be reduced to very low levels. Under these conditions NMDA had little effect unless exogenous glycine was co-applied (Kleckner & Dingledine, 1988). *In vivo*, slices or cultures added glycine was not required to obtain a response to NMDA because of the unavoidable levels of glycine contamination.

d. Pharmacology of the glycine site of the NMDA receptor.

Confirmation of the co-agonist role of glycine has come from antagonists of the glycine site of the NMDA receptor. The naturally occurring tryptophan metabolite kynurenate, a broad-spectrum glutamate antagonist (Ganong, Lanthorn & Cotman, 1983), was found to compete with glycine at the NMDA receptor (Kessler, Baudry, Terramini & Lynch, 1987). The halogenated derivative, 7-chlorokynurenate is much more potent and specific for the glycine site of the NMDA receptor (Kemp, Foster, Leeson, Priestly, Tridgett, Iverson & Woodruff, 1988) and has now been superseded by a whole range of derivatives (Foster, Kemp, Leeson, Grimwood, Donald, Marshall, Priestly, Smith & Carling, 1992). In addition to the kynurenic acid derivatives a number of other compounds have antagonistic activity at the NMDA-glycine site (see Lodge and Johnson, 1990; Thomson, 1990). Compounds with agonist activity at the NMDA-glycine site are 1-aminocyclopropane-1-carboxylic acid (ACC) > glycine > D-serine > D-alanine > L-serine, in order of potency. Of particular usefulness is the unnatural D-isomer of serine which is not a substrate for amino acid uptake mechanisms (see Thomson, 1990). The strychnine-insensitive glycine binding that was such a mystery in 1986 (Bristow et al., 1986), colocalises with NMDA binding and, in addition, is displaced by the antagonist 7-chlorokynurenate (McDonald, Penney, Johnston and Young, 1990), and therefore undoubtedly represents the glycine site of the NMDA receptor.

e. Is the glycine site of the NMDA receptor continuously saturated or not?

The problem concerning glycine as a functional co-agonist at the NMDA receptor is that there is evidence that the concentration of endogenous glycine is above that

needed to saturate the site. It is known that levels of glycine in the cerebrospinal fluid is about 10 μM (Ferraro & Hare, 1985; Skilling, Smullin, Beitz & Larson, 1988) which is greater than several hundred nanomolar needed to saturate the glycine site. In agreement with this view is the fact that addition of glycine site agonists often has no effect on NMDA receptor mediated responses. There is also contradictory evidence that levels are in fact subsaturating and addition of glycine agonists augment NMDA responses. How can the receptors not be saturated if the levels in the cerebrospinal fluid are so high? Presumably the concentration in the cerebrospinal fluid is not an accurate guide to local levels at NMDA receptors. The easiest way to conceive of this occuring is if glycine is actively removed from around the receptors. There is no definitive answer to the question of endogenous glycine levels. The electrophysiological data is not conclusive. Cultured or slice preparations could appear falsely sub-saturated due to diffusion of glycine into the bathing medium. On the otherhand application of exogenous agonists or stimulating large bundles of fibres in a given area could artificially promote the release of glycine which would make the glycine site seem falsely saturated. At this point it is assumed the glycine site is not continuously saturated under physiological conditions and that the glycine site of the NMDA receptor allows another level of complexity in the control of NMDA receptor mediated functions.

f. The cerebellum is the ideal place to look for glycine modulation of NMDA receptors.

It has already been shown that NMDA receptors are present on granule cell dendrites and are synaptically activated by the mossy fibres (Garthwaite & Brodbelt, 1989; D'Angelo, Rossi & Garthwaite, 1990; Silver, Traynelis &

Cull-Candy, 1992). In addition, some Golgi cell terminals contain large amounts of glycine (Ottersen, Storm- Mathisen & Somogyi, 1988) and also have a mechanism for high affinity uptake of [^{3}H]glycine (Wilkin, Csillag, Balázs, Kingsbury, Wilson & Johnson, 1981). Recently this finding has been strengthened by the demonstration of high levels of *in situ* hybridisation with a probe for the cloned high-affinity glycine transporter in the granule cell layer (Smith, Borden, Hartig, Branchek & Weinshank, 1992). Stimulation of cerebellar slices preloaded with labelled glycine using high potassium led to the calcium dependent release of glycine (Fletcher, Wilkin & Bowery, 1989). Molecular biology reveals inhibitory glycine receptor subunits in the cerebellum. Multiple subunits of the glycine receptor have been cloned and sequenced (see Betz, 1991). They share sequence homology with many other receptors and belong to the superfamily of ligand-gated ion-channel proteins (Betz, 1990). The quaternary structure is thought to be pentameric with a stoichiometry of $\alpha 3$-$\beta 2$. Three different α subunits have been sequenced for rats and humans ($\alpha 4$ has been cloned from mice), and only one β subunit. *In situ* hybridisation in the rat indicates that $\alpha 1$ is located in spinal cord, brainstem and the colliculi, whereas $\alpha 2$ is also located in parts of the forebrain. The mRNA for the $\alpha 3$ subunit is only sparsely distributed being discernible in olfactory bulb, hippocampus and particularly in the granule cell layer of the cerebellum. Messenger RNA for the β subunit was found in many brain regions, even in those with little or no discernable expression of an α subunit. Particularly strong hybridisation with the β-probe was seen in the Purkinje cells and also the granule cell layer of the cerebellum (Malosio, Marquèze-Pouey, Kuhse & Betz, 1991; Fujita, Sato, Sato, Imoue, Kozuka & Tohyama, 1991). The β subunit is

thought to possess a low affinity glycine binding site, leading to a suggestion that it forms part of the NMDA receptor complex bestowing it with glycine sensitivity (Malosio et al., 1991). Subsequent cloning of the NMDA receptor has shown that the NMDA receptor has intrinsic glycine sensitivity. The enigma of why the β-subunit is expressed in the cerebellum has to be left open. But, it has been postulated that it could form functional receptors with as yet undiscovered α-subunits, or could perhaps form up with subunits of the $GABA_A$ receptor (Malosio et al., 1991).

2. [^{3}H]GLYCINE RELEASE

a. Introduction

It has already been shown that slices preloaded with [^{3}H]glycine, when stimulated with high K^+, would release it in a calcium dependent manner (Fletcher et al., 1989). The aim of these experiments was to demonstrate that glycine could be released following electrical stimulation of the white matter. This should lead to stimulation of the Golgi cells via the parallel fibres. It is likely that all cell types are activated in this preparation but electrical stimulation of a tissue more closely resembles physiological conditions than high K^+ does. High K^+ depolarises glia as well as neurones and will affect different voltage-sensitive channels than those stimuated by action potentials.

b. Method.

The protocol for loading the slices with [^{3}H]glycine was similar to that used by Fletcher et al. (1989). The biplanar slices were cut and recovered for at least 60 min and then incubated in 5 ml of 100 nM [^{3}H]glycine in a flask in a shaking water bath at 37 °C. The slice was preincubated for 35 min but this was doubled to

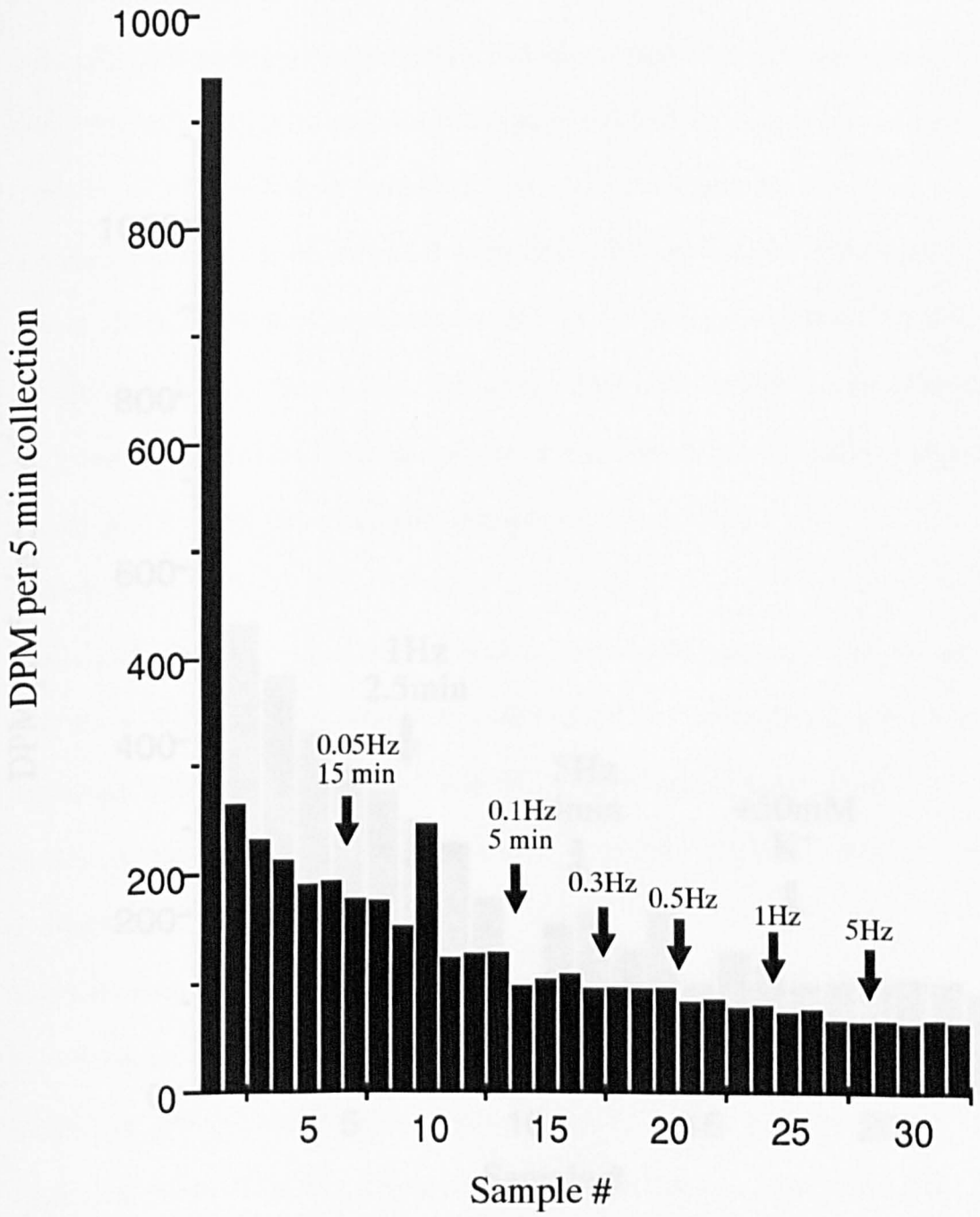


Figure III(7): Effect of electrical stimulation on the release of preloaded [3H]-glycine from a cerebellar slice. A variety of stimulations caused no noticeable increase in [3H]-glycine efflux.

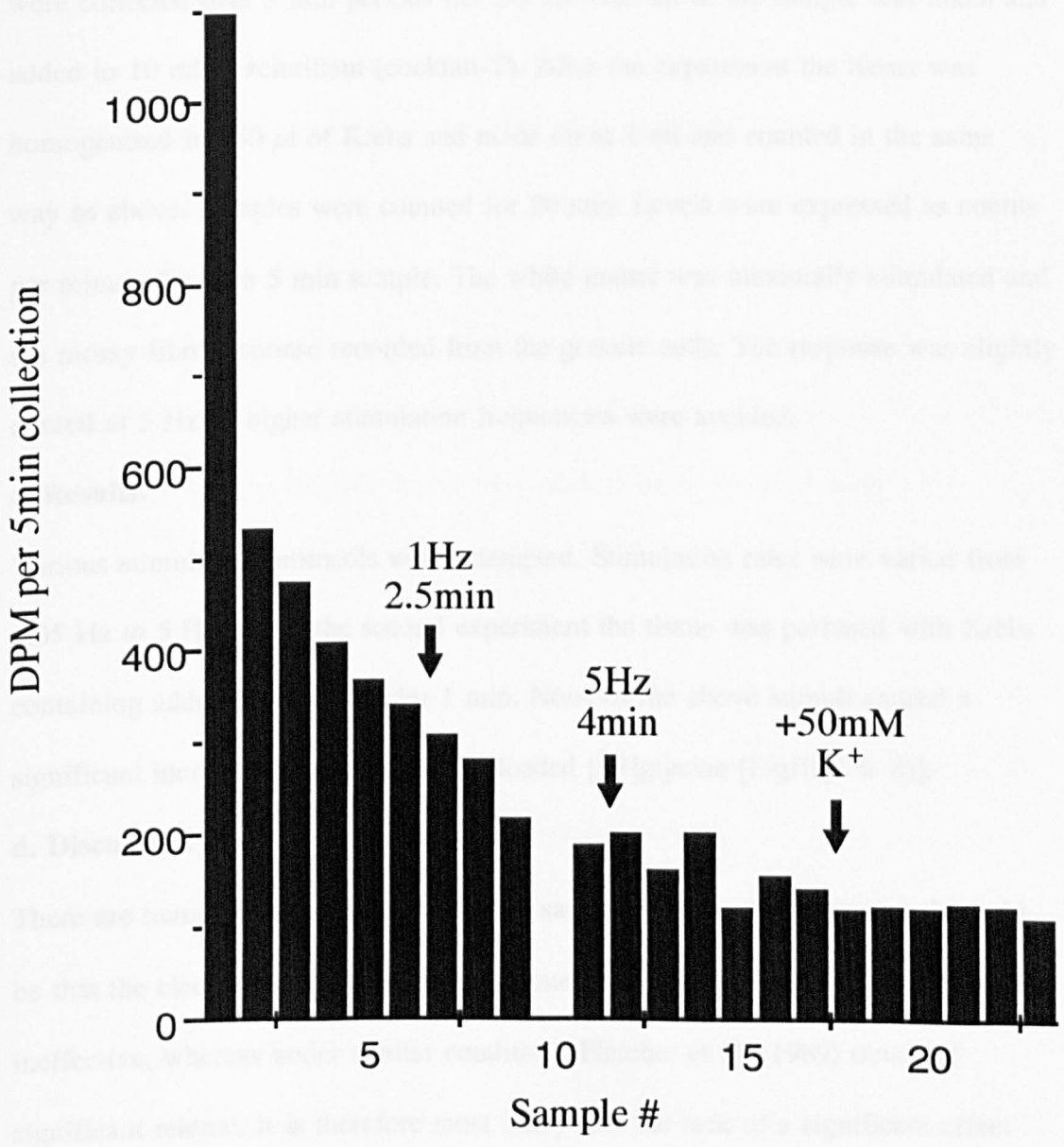


Figure III(8): Effect of electrical and high K^+ stimulation on the release of preloaded [^{3}H]-glycine from a cerebellar slice.

70 min for the second experiment. The slice was washed by shaking it in two aliquots of fresh Krebs for 5 min, it was then installed in the recording chamber as usual and allowed to perfuse for 30 min at a rate of about 0.5 ml/min, samples were collected over 5 min periods i.e. 2-3 ml. One ml of the sample was taken and added to 10 ml of scintillant (cocktail-T). After the experiment the tissue was homogenised in 250 μl of Krebs and made up to 1 ml and counted in the same way as above. Samples were counted for 20 min. Levels were expressed as counts per minute for each 5 min sample. The white matter was maximally stimulated and the mossy fibre response recorded from the granule cells. The response was slightly altered at 5 Hz so higher stimulation frequencies were avoided.

c. Results.

Various stimulation protocols were attempted. Stimulation rates were varied from 0.05 Hz to 5 Hz and in the second experiment the tissue was perfused with Krebs containing added 50 mM KCl for 1 min. None of the above stimuli caused a significant increase in the efflux of preloaded [^{3}H]glycine [FigIII(7 & 8)].

d. Discussion.

There are numerous explanations why we saw no release of [^{3}H]glycine. It could be that the electrical stimulation was not intense enough but high K^+ was also ineffective, whereas under similar conditions Fletcher et al. (1989) obtained significant release. It is therefore most likely that the lack of a significant effect was due to the fact that we were only dealing with a very small piece of tissue compared with the whole slices that Fletcher et al. (1989) used. Since it was not simple to change the amount of tissue used in my experiments it was decided to try and load up the slices with a greater amount of labelled glycine, hence the

preincubation time was doubled. The sensitivity of the detection could be enhanced by pooling the samples from large numbers of experiments and by designing the bath so that more tissue could be used, or that a slower low rate could be used. Since there was not even a suggestion of a rise in labelled glycine release it was decided to curtail this approach to the problem.

3. ENDOGENOUS GLYCINE RELEASE - HPLC DETECTION.

a. Introduction.

There are problems with the technique of loading slices with labelled transmitter. One assumption is that bathing the tissue in the presence of the labelled substance, glycine in this instance, will lead to the tissue taking up the glycine and distributing it evenly amongst the possible metabolic pools. If, as suggested, glycine is released from synaptic terminals it is likely there is a specialised pool, which may not have initial access to the labelled glycine that has been taken up. Another possible problem is that the radiolabelled substance measured may not be glycine. It is very likely a proportion of the glycine is metabolised and it may be one of these metabolites we are measuring the release of.

b. Method.

A biplanar slice was cut and installed in a modified recording chamber. The central chamber was much smaller to reduce the dilution factor of substances released from the tissue, yet, keep the flow rate high enough for adequate oxygenation of the tissue. The chamber volume was about 100 μl and the flow rate was usually 300 μl/min, the chamber volume was therefore replaced every 20 sec. The Krebs-Henseleit solution was made fresh shortly before the experiments using the purest quality salts (supra-pur or puriss from Merck or Fluka) dissolved in HPLC

grade water (Milli-Q, Millipore, Bedford, MA). The very fine measurment of amino- acids requires great care to avoid contamination. Rubber gloves were worn at all times and the glassware was thoroughly washed in 10% ethanoic acid and rinsed extensively with 'Milli-Q' water. The slice was installed and a few pulses were required to position the stimulating electrode, the slice was then allowed to settle for 20-30 min before samples of the perfusate were collected. Fractions were collected on ice every minute into preweighed glass vials. Usually four fractions were taken as a measure of basal efflux before stimulation, during stimulation and for several minutes after stimulation. The vials were then weighed again and 5 pmol of norvaline in 5 μl of water was added as an internal standard. The samples were sometimes concentrated by drying the samples in a spinning-vucuum dessicator and redissolving in a smaller volume. The HPLC analysis consisted of a chromatograph (Hewlett-Packard 1090), equipped with a autoinjector, a cooled autosampler (4 °C) and a programmable fluorescence detector (Hewlett-Packard 1046A). Analytical hyperchrome columns were packed with Spherisorb ODS II 5 μm particles (Phase Sep, CT, USA). The samples (20 μl) were automatically derivatised with o-phthaladehyde-mercaptoethanol in borate buffer (20 μl) on-line, and injected onto the column. The isoindole derivatives are rather unstable so the automated procedure reduces variation between samples. A linear elution gradient of was used at 0.5 ml/min flow rate (70 min per sample). The fluorescent isoindole derivatives of the amino-acids were excited at 230 nm and the emissions at 455 nm were monitored. The detection has limits of about 10-50 fmol. The results were normalised with respect to isoleucine, the levels of isoleucine release have previously been found to be relatively unaffected by stimulation and therefore

control for any variation due to factors such as flow rate.

c. Results

The results were all negative we could not see a rise in any of the amino-acids that we might expect to see i.e. glutamate, GABA and aspartate.

d. Discussion

The negative results were probably due to the fact that the piece of tissue we were using was too small and we could not detect the levels released. Alternatively the uptake mechanisms may be so good that we are not seeing any release into the extrasynaptic space, or perhaps it is the glomeruli that are forming a little 'glycine microenvironment'.

SECTION IV - RESULTS

(PARALLEL FIBRE PATHWAY)

A. THE PARALLEL FIBRE RESPONSE

1. STRUCTURE

The ontology of the granule cells is a fascinating example of neuronal development (Burgoyne & Cambray-Deakin, 1988). The immature granule cells find themselves out on the fringe of the cerebellar cortex around birth in the external granule cell layer. In the rat there are about 10^8 granule cells produced (Harvey & Napper, 1991). During the first 2-3 weeks of postnatal life these cells make a remarkable migration to their mature domain below the Purkinje cells, where they form synapses with the encroaching mossy fibres. In approximate terms, this means that about 500 granule cells complete this journey every second! As the cells descend through the rapidly developing Purkinje dendrites they leave a neurite behind which forms the axon of these cells. The axon bifurcates and spreads itself in a direction orthogonal to the planar Purkinje dendrites, all the axons lie in the same direction, hence their name 'parallel fibres'. The parallel fibres make *en passent* synapses with the Purkinje dendrites and those belonging to the three types of inhibitory interneurones found in the cerebellar cortex: basket; stellate and Golgi cells.

2. FUNCTION

The 'parallel fibre response' refers to the response recorded from Purkinje cells on stimulating the associated parallel fibres. It includes the disynaptic, feedforward inhibition from the basket and stellate cells. There is a lot of evidence that the

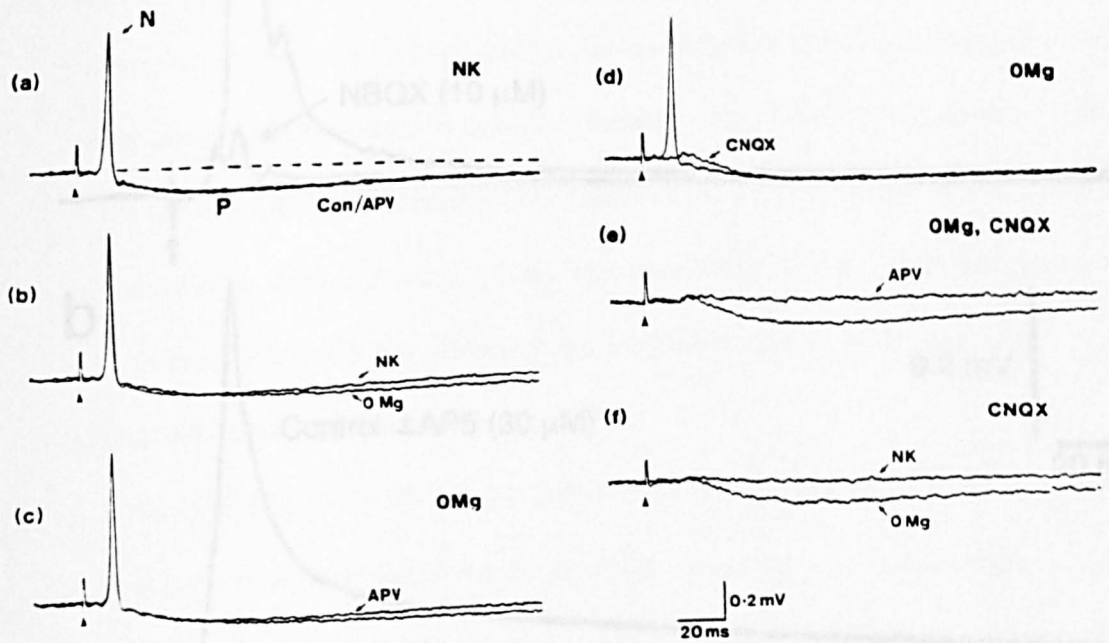


Figure IV(1): Pharmacology of the immature parallel fibre response. a. Perfusion of AP5 (30 μM) had little effect on the response. b. Washing in Mg^{2+}-free Krebs prolonged the inhibitory wave which was reversed by AP5 (c). d. Addition of CNQX blocked the population spike completely but the inhibitory wave was left intact. The inhibitory wave could now be blocked by AP5 (e) or Mg^{2+} (f). NK = normal Krebs. This figure was extracted from Garthwaite & Beaumont, 1989.

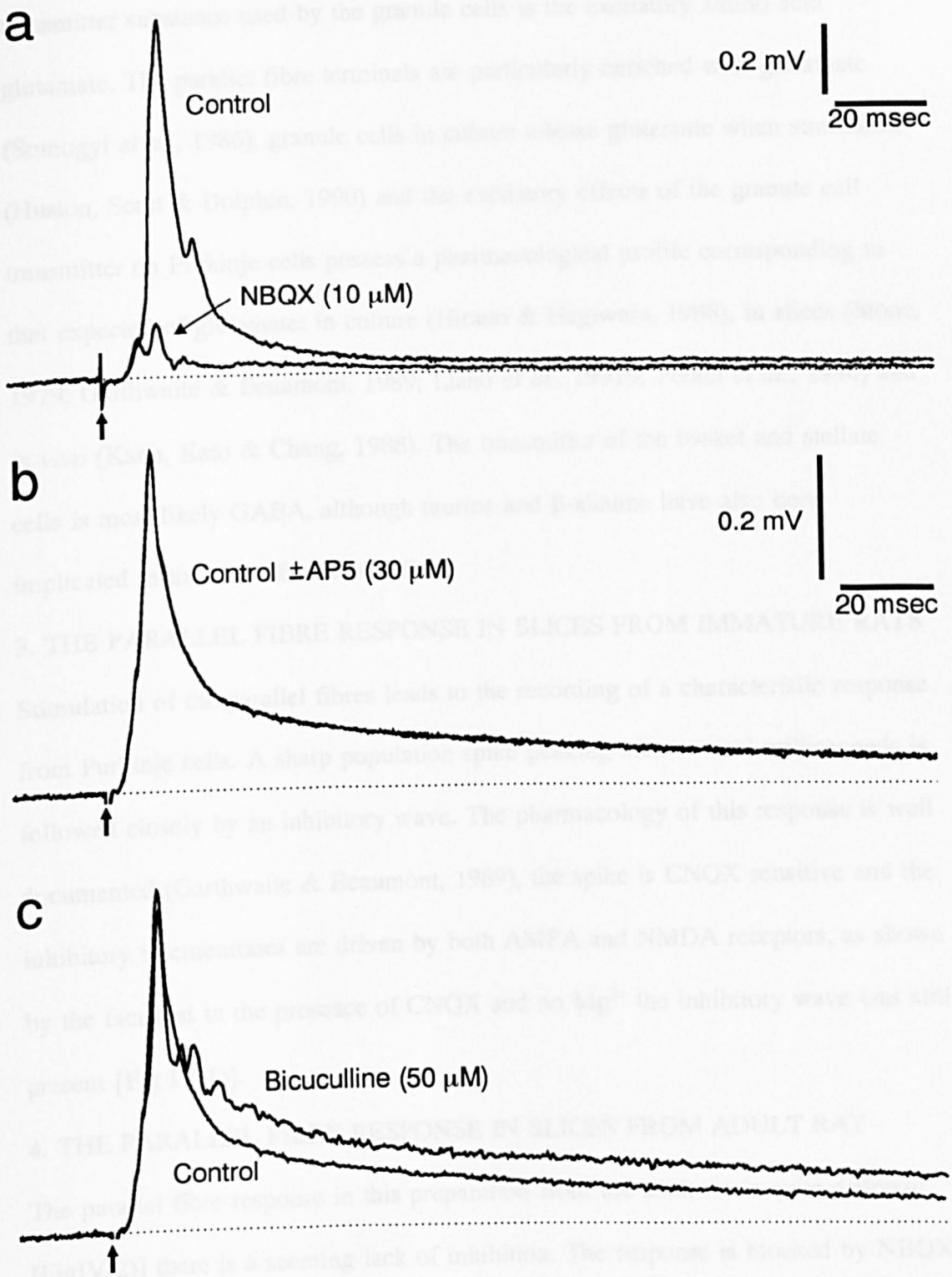


Figure IV(2): Typical example of the parallel fibre response from adult tissue. In (a) the response is almost completely blocked by the AMPA receptor antagonist, NBQX, whereas the NMDA receptor antagonist, AP5, has no noticeable effect (b), even in Mg^{2+} free conditions (not shown). Addition of the $GABA_A$ receptor antagonist bicuculline MeCl has little effect on the peak response but prolongs the later phases. Traces in (a) are from a different slice to those in (b) & (c). Scale in (b) also applies to (c).

transmitter substance used by the granule cells is the excitatory amino acid glutamate. The parallel fibre terminals are particularly enriched with glutamate (Somogyi *et al.*, 1986), granule cells in culture release glutamate when stimulated (Huston, Scott & Dolphin, 1990) and the excitatory effects of the granule cell transmitter on Purkinje cells possess a pharmacological profile corresponding to that expected of glutamate: in culture (Hirano & Hagiwara, 1988), in slices (Stone, 1979; Garthwaite & Beaumont, 1989; Llano *et al.*, 1991b; Perkel *et al.*, 1990) and *in vivo* (Kano, Kato & Chang, 1988). The transmitter of the basket and stellate cells is most likely GABA, although taurine and β-alanine have also been implicated in the case of stellate cells.

3. THE PARALLEL FIBRE RESPONSE IN SLICES FROM IMMATURE RATS

Stimulation of the parallel fibres leads to the recording of a characteristic response from Purkinje cells. A sharp population spike peaking after several milliseconds is followed closely by an inhibitory wave. The pharmacology of this response is well documented (Garthwaite & Beaumont, 1989), the spike is CNQX sensitive and the inhibitory interneurones are driven by both AMPA and NMDA receptors, as shown by the fact that in the presence of CNQX and no Mg^{2+} the inhibitory wave was still present [Fig IV(1)].

4. THE PARALLEL FIBRE RESPONSE IN SLICES FROM ADULT RAT

The parallel fibre response in this preparation from the adult rat is quite different [FigIV(2)] there is a seeming lack of inhibition. The response is blocked by NBQX and AP5 has no effect - consistent with the fact that only AMPA receptors are stimulated on Purkinje cells. The addition of the $GABA_A$ antagonist bicuculline reveals that there is a small inhibitory influence over quite a long time course.

Compare figure IV(2)c with figure IV(3).

B. $GABA_B$

1. INTRODUCTION

a. $GABA_B$ receptors in the cerebellum

In addition to its action at the $GABA_A$ receptor, GABA is also known to act at a second type, $GABA_B$ receptors. Suspicions that there were more than one type of GABA receptor arose from the finding that GABA acting presynaptically to reduce the evoked release of transmitter from a nerve preparation was insensitive to bicuculline. Almost all other actions of GABA were successfully antagonised by bicuculline. In addition, a GABA mimetic, baclofen (para-chlorophenol-GABA), was found to act stereospecifically at these 'bicuculline-insensitive GABA receptors'. The most convincing piece of evidence that there was indeed two separate populations of receptors came from the study of the distribution of binding sites (Hill & Bowery, 1981). Interestingly, the most prominent differential binding was that seen in the cerebellum. The level of $GABA_B$ binding in the molecular layer was one of the highest in any brain area (Wilkin, Hudson, Hill & Bowery, 1981; Bowery, Hudson & Price, 1987; Chu, Albin, Young & Penney, 1990; Martinelli, Holstein, Pasik & Cohen, 1992). In the intervening ten years there have been various attempts to discover exactly where these $GABA_B$ receptors are located. Mice with various mutations which selectively affect the development of different cell populations within the cerebellum suggest that $GABA_B$ binding is located presynaptically on granule cell terminals and also postsynaptically on Purkinje cell dendrites (Bowery, Price, Turnbull & Wilkin, 1983). Activation of $GABA_B$ receptors in the cerebellum and other brain areas results in a decrease in cAMP levels. Using mutant mice, Wojcik and Neff (1984) showed that cerebellar

slices from granule cell deficient Weaver mouse had a significantly reduced inhibition of cAMP when baclofen was added, and deduced that the $GABA_B$ receptors were located on parallel fibre terminals. Lesioning the climbing fibres reduced the level of $GABA_B$ binding in the cerebellum (Kato & Fukada, 1985) indicating that the receptors might also be located on the climbing fibre terminals. The problem with all these reports is that they use rather indirect methods. It is not known how removing one set of cells affects the other cells, especially in the mutant mice, where the whole development of the cerebellum is in abnormal conditions. Some evidence, of a much less intrusive nature, has come from the development of a remarkable monoclonal antibody that recognises the baclofen molecule when it is bound to the $GABA_B$ receptor in fixed tissue (Martinelli *et al.*, 1992). This has allowed study of the distribution of $GABA_B$ at the level of the electron microscope for the first time. The majority of binding in the molecular layer of the cerebellum was dendritic in nature, although some was axonal in nature.

b. Function of $GABA_B$ receptors in the CNS

The functions of $GABA_B$ receptors in the central nervous system have been most fully studied using electrophysiological techniques in the hippocampus, where they are both post- and presynaptically located. The existance of postsynaptic receptors on pyramidal cells was first indicated by the hyperpolarisation to an exogenously applied $GABA_B$ agonist (Newberry & Nicoll, 1984b) mediated by an increase in K^+-conductance (Gähwiler & Brown, 1985). The synaptically evoked slow hyperpolarisation that follows the faster $GABA_A$ component recorded from pyramidal cells was proposed to be due to activation of postsynaptic $GABA_B$

receptors (Newberry & Nicoll, 1984a), this was confirmed (Dutar & Nicoll, 1988a) with the development of a selective $GABA_B$ antagonist, a phosphono derivative of baclofen, named phaclofen (Kerr, Ong, Prager, Gynther and Curtis, 1987). This receptor couples to the K^+-channel by a Pertussis toxin-sensitive g-protein (Andrade, Malenka & Nicoll, 1986; Thalmann, 1987).

Activation of presynaptic $GABA_B$ receptors in the hippocampus suppress synaptic transmission (Lanthorn & Cotman, 1981). The apparent lack of effect of phaclofen to antagonise the presynaptic actions of baclofen at concentrations which blocked postsynaptic effects was taken as an indication of the existance of more than one $GABA_B$ receptor (Dutar & Nicoll, 1988b; Harrison, 1990). This probably stems from its lack of potency, since further studies showed that high (1 mM) doses of phaclofen had some antagonist effect (Davies, Davies & Collingridge, 1990; Harrison, Lovinger, Lambert, Teyler, Prager, Ong & Kerr, 1990). Newer and more potent $GABA_B$ antagonists, 2-hydroxysaclofen (Kerr, Ong, Johnston, Abbenante & Prager, 1988) and CGP 35348 (Olpe, Karlsson, Pozza, Brugger, Steinman, van Reizen, Fagg, Hall, Froestl & Bittiger, 1990) unequivocally reduces baclofen's presynaptic effect (Harrison *et al.*, 1990; Davies, Davies & Collingridge, 1990; Davies, Starkey, Pozza & Collingridge, 1991; Thompson & Gähwiler, 1992). Although all three antagonists share the same order of potency at both the post- and presynaptic $GABA_B$ receptors they are always more potent at the postsynaptic sites. Whether this is due to two different receptor subtypes or just different affinity states of the same receptor molecule is not known. A definite answer awaits the molecular cloning of the receptor(s).

There is still debate as to whether or not the presynaptic actions are

mediated via a Pertussis toxin sensitive G-protein due, in part, to the difficulty of interpreting a negative result (see Thompson & Gähwiler, 1992). It is still an open question which of two possible mechanisms presynaptic $GABA_B$ receptors engage to inhibit transmitter release. Either, the activated G-protein decreases the influx of Ca^{2+} that enters the terminal through voltage-activated channels (Dunlap & Fischbach, 1981), or alternatively an increase in K^+ conduction reduces the depolarising effect of action potentials as they enter the synaptic terminal (Thompson & Gähwiler, 1992). It is also possible that different cells use different mechanisms or that one cell can use more than one mechanism.

I set out to find some electrophysiological evidence for the location and function of this type of receptor, therefore explaining the very high density of binding in the molecular layer.

2. POSTSYNAPTIC $GABA_B$ RECEPTORS ON PURKINJE CELLS

The parallel fibre response from immature rats contains a large monophasic hyperpolarising wave which is blocked by the $GABA_A$ antagonist bicuculline [Fig IV(3 & 4)]. Perfusion of the selective $GABA_B$ antagonists phaclofen and 2-hydroxysaclofen did not have any noticeable effect. Suprisingly, when the $GABA_A$ component was nearly completely blocked with 30-50 μM bicuculline a shallow and slow wave was revealed (n=17; fig IV(5)a). The wave peaked at around 350-400 msec and sometimes lasted as long as 1 sec. This wave was found to be blocked in a concentration dependent manner by the $GABA_B$ antagonists [Fig IV(5)b & c and Fig IV(6)]. In the examples illustrated there is still a hyperpolarising component that persists in the presence of high doses of $GABA_A$ and $GABA_B$ antagonists.

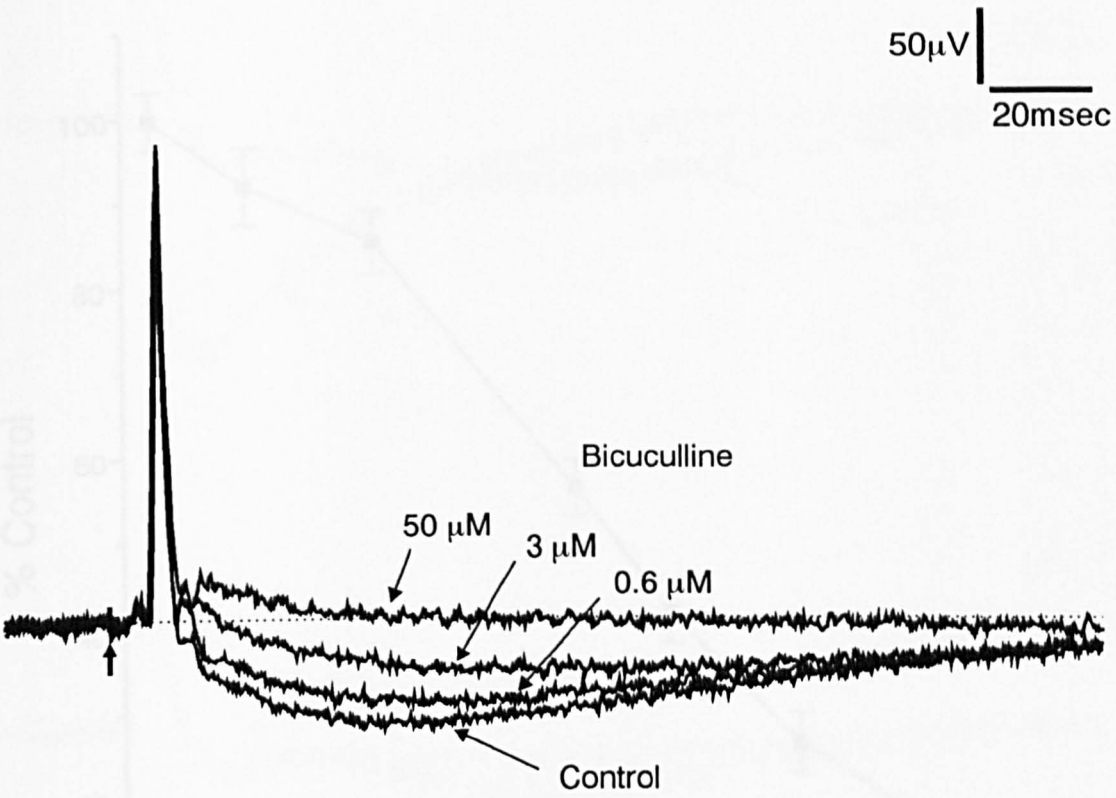


Figure IV(3): The inhibitory wave of the immature parallel fibre response is inhibited in a concentration dependent manner by bicuculline MeCl. Bicuculline was applied for periods of 5- 10 min until a steady state was reached.

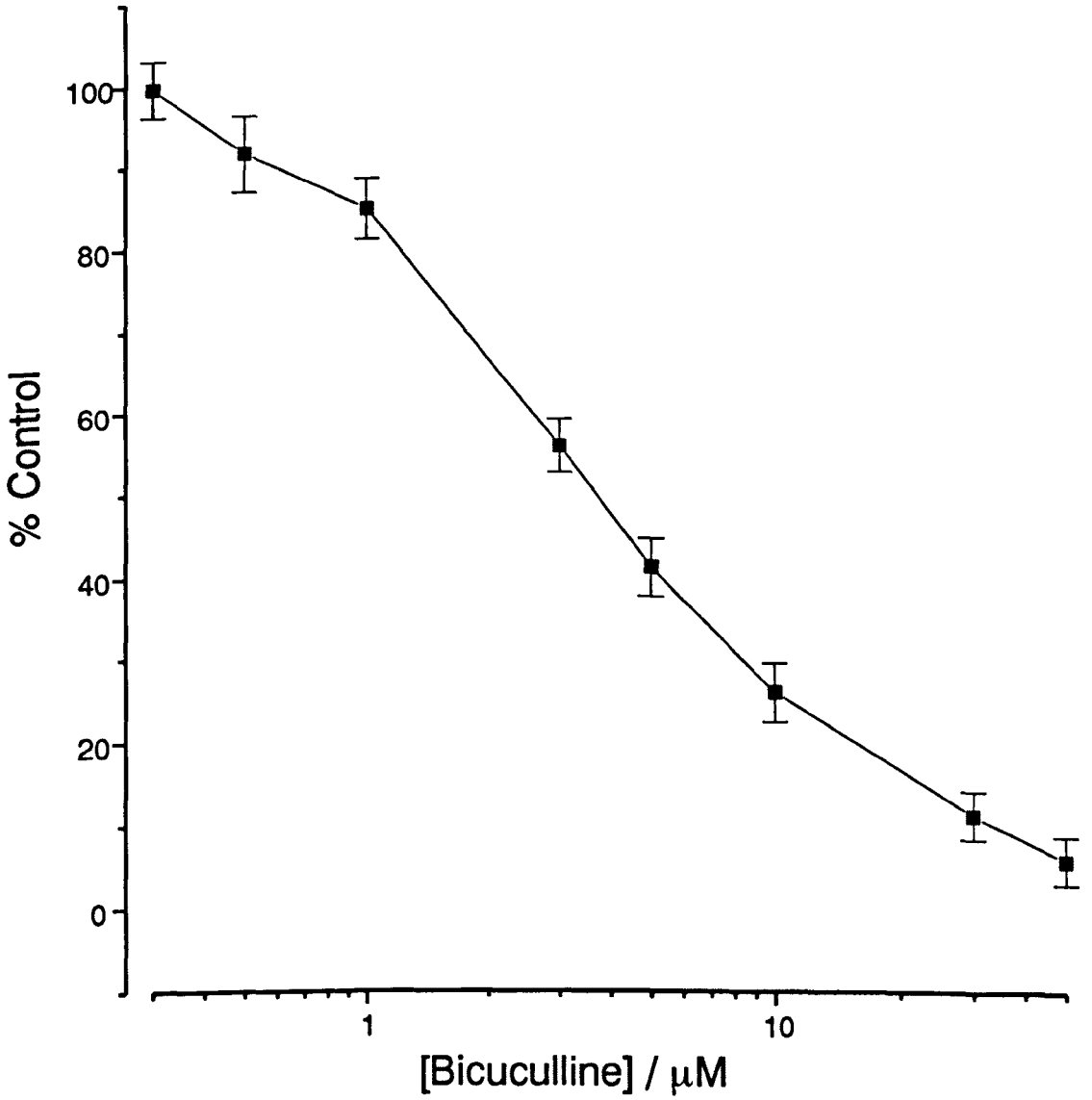


Figure IV(4): Inhibition curve for the fast hyperpolarising wave. The peak of the hyperpolarising wave was measure relative to baseline from three to five slices. The IC_{50} was approximately 3.5 μM. Bicuculline MeCl was applied for 5-10 min to reach a stable response.

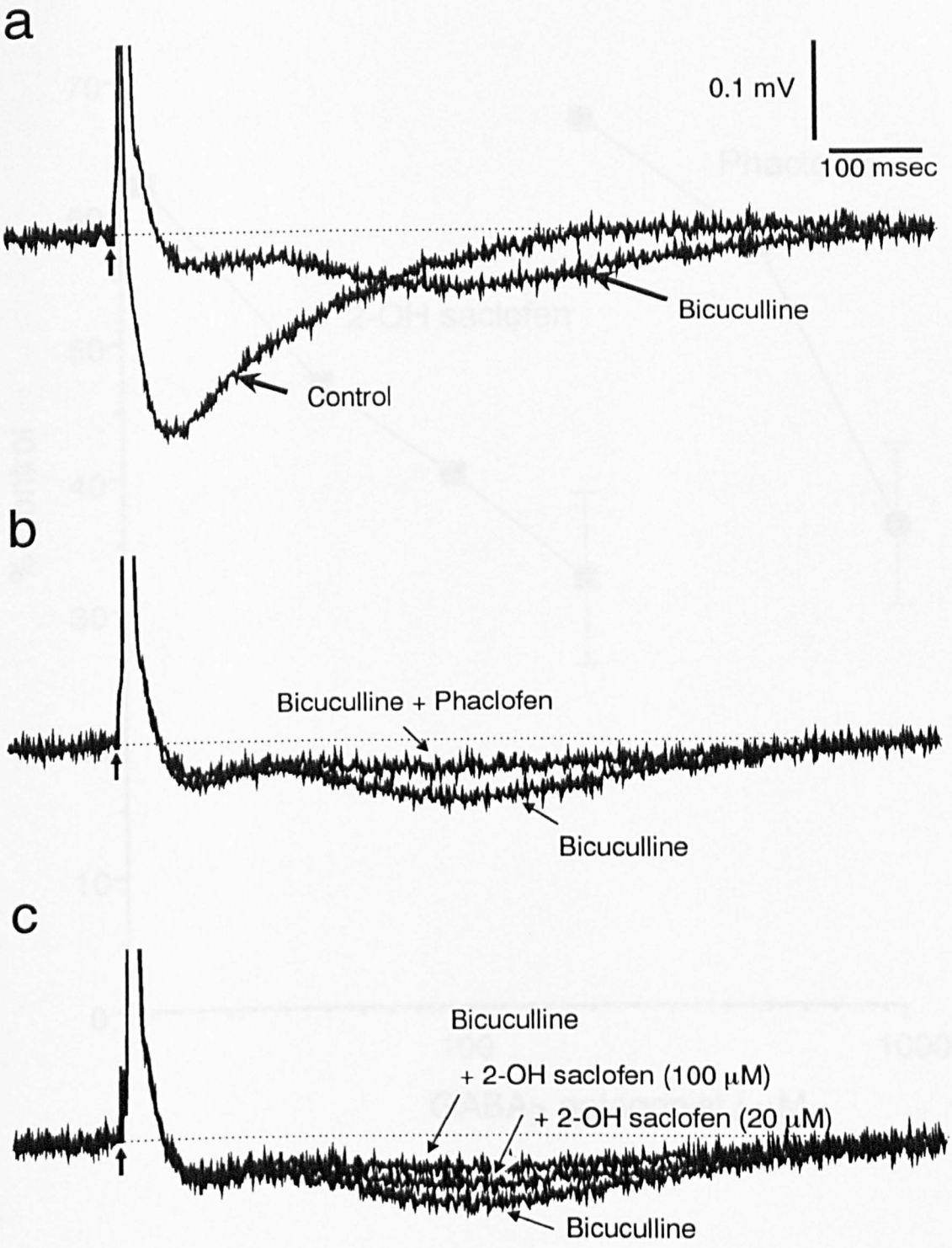


Figure IV(5): A slow bicuculline-insensitive wave is inhibited by two $GABA_B$ antagonists. Addition of bicuculline (50 μM) inhibited the fast inhibition and revealed a slow hyperpolarising wave. The wave was sensitive to phaclofen (1 mM; b) and 2-hydroxysaclofen (c). The population spikes are truncated.

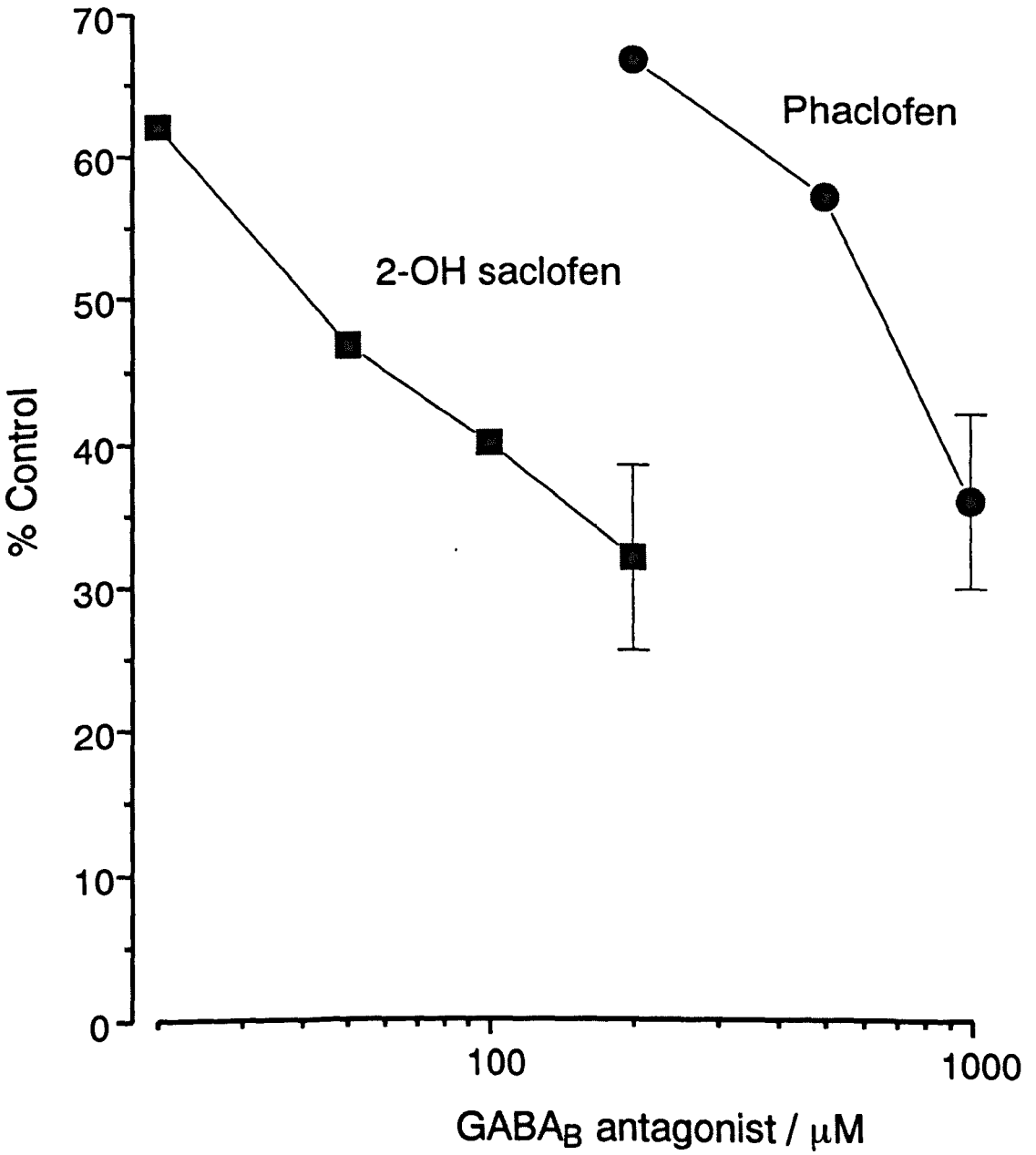


Figure IV(6): Inhibition of the slow wave by GABA$_B$ antagonists. Saclofen was about 10 times more potent at blocking the slow hyperpolarisation than phaclofen. The point measured was the peak of the wave relative to baseline.

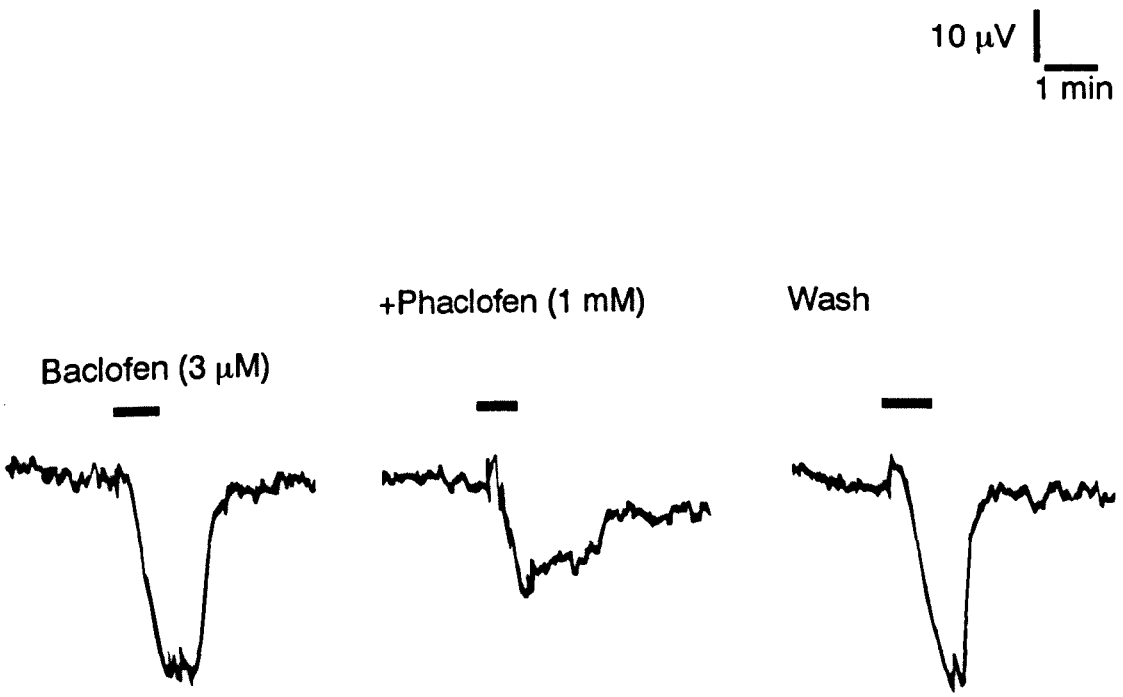


Figure IV(7): Purkinje cells hyperpolarise to baclofen and this is inhibited by a $GABA_B$ antagonist. Baclofen was applied for around 1 min and caused a small but distinct hyperpolarisation. Reapplication in the presence of the $GABA_B$ antagonist phaclofen inhibited the response in a reversible manner (wash).

The production of the slow hyperpolarising wave suggests that there are indeed $GABA_B$ receptors on Purkinje cells. To test this further we directly applied the $GABA_B$ agonist baclofen to Purkinje cells in sagittal cerebellar slices in the presence of tetrodotoxin. Perfusion of a range of concentrations of baclofen (1-30 μM) caused a hyperpolarisation of the Purkinje cells and this was partially blocked by phaclofen (1 mM) in a reversible manner [Fig IV(7)].

In slices from adult cerebellum the form of the parallel fibre response is quite different. The most obvious difference is the apparent lack of a hyperpolarisation, but, perfusion of bicuculline shows that $GABA_A$ receptors do play a role [FigIV(8)], this normally being obscured by the prolonged depolarising phase. Addition of a high dose of 2-hydroxysaclofen (500 μM) had no noticeable effect ([Fig IV(8)], n=4). In agreement with this, the direct effects of exogenous baclofen were much less discernible in the adult. There is definitely a small effect but unlike the immature slices it was hardly above the baseline fluctuations.

3. PRESYNAPTIC $GABA_B$ RECEPTORS ON PARALLEL FIBRE TERMINALS

In addition to the direct hyperpolarising effects of baclofen on the postsynaptic neurones, perfusion during synaptic stimulation blocked the parallel fibre response. The baclofen blocked both the depolarising and the hyperpolarising phases indistinguishably. The IC_{50} of this effect was similar in the young and adult, although the apparent maximal effect was less in the adult [Fig IV(9)]. The effect of baclofen was reversed by the subsequent addition of the antagonist 2-hydroxysaclofen (500 μM; Fig IV(10)). Curiously, in the adult the effect of baclofen was not completely reversible on washout, whereas it was in the immature tissue.

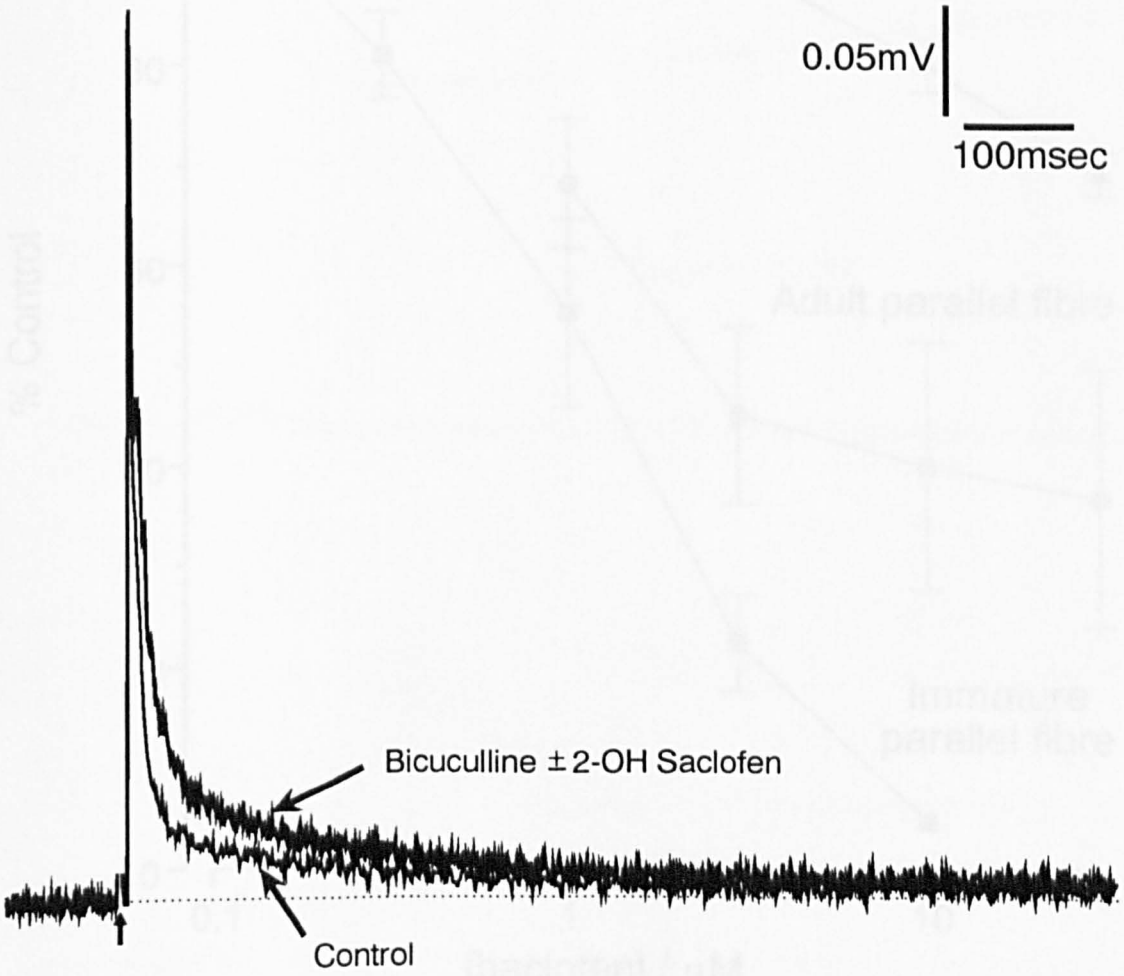


Figure IV(8): The parallel fibre response in adult rats did not reveal a $GABA_B$ wave. Addition of bicuculline MeCl (50 μM) did not reveal a slow wave, unlike the immature response, and the addition of 2-hydroxysaclofen (500 μM) had no apparent effect.

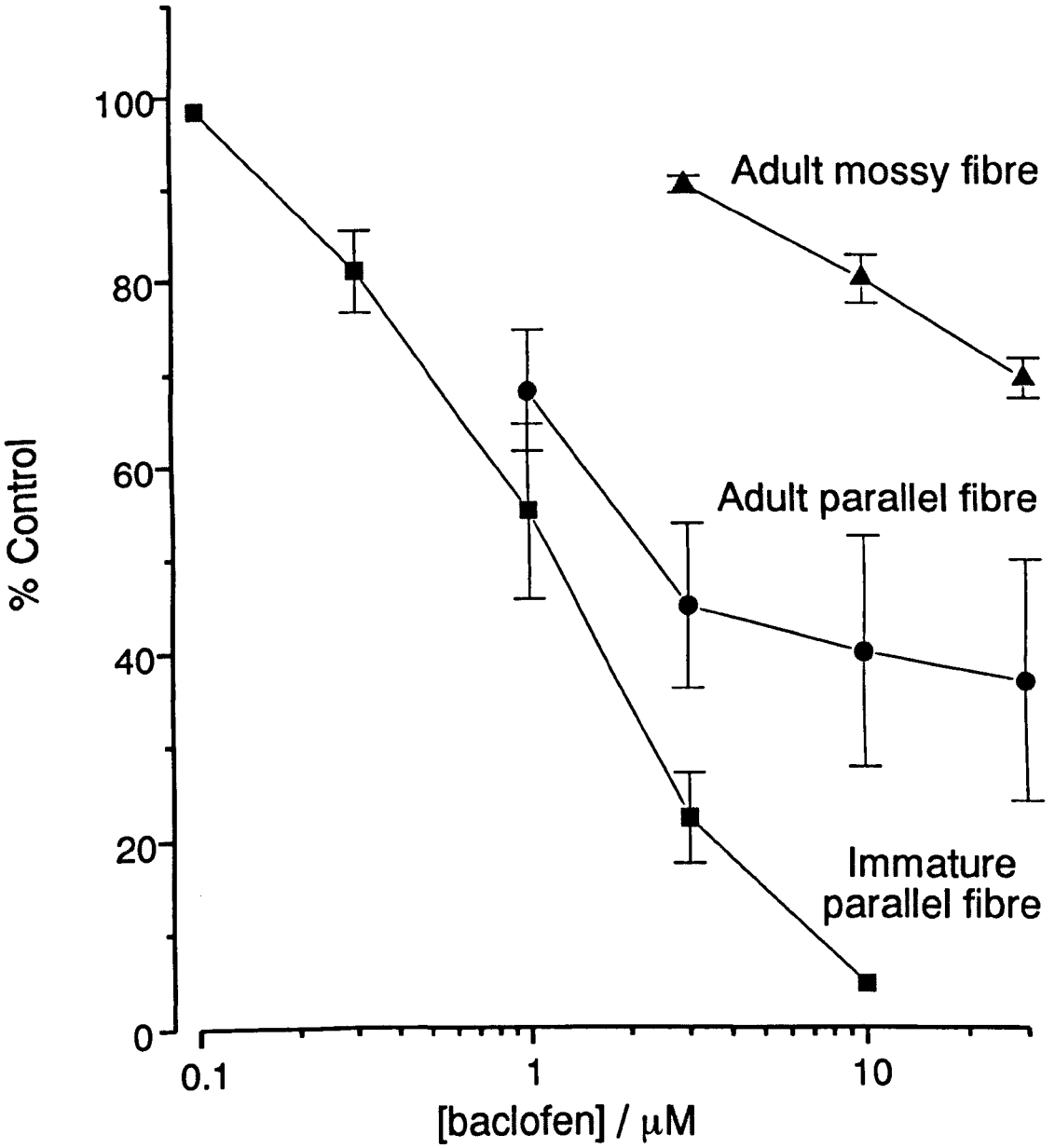


Figure IV(9): Baclofen affects the mossy fibre and the parallel fibre responses differently. Baclofen was quite a potent inhibitor of the parallel fibre response with IC50 of about 1 μM. Even at the highest doses of baclofen (30 μM) the mossy fibre response was only depressed by 30 %. n > 3.

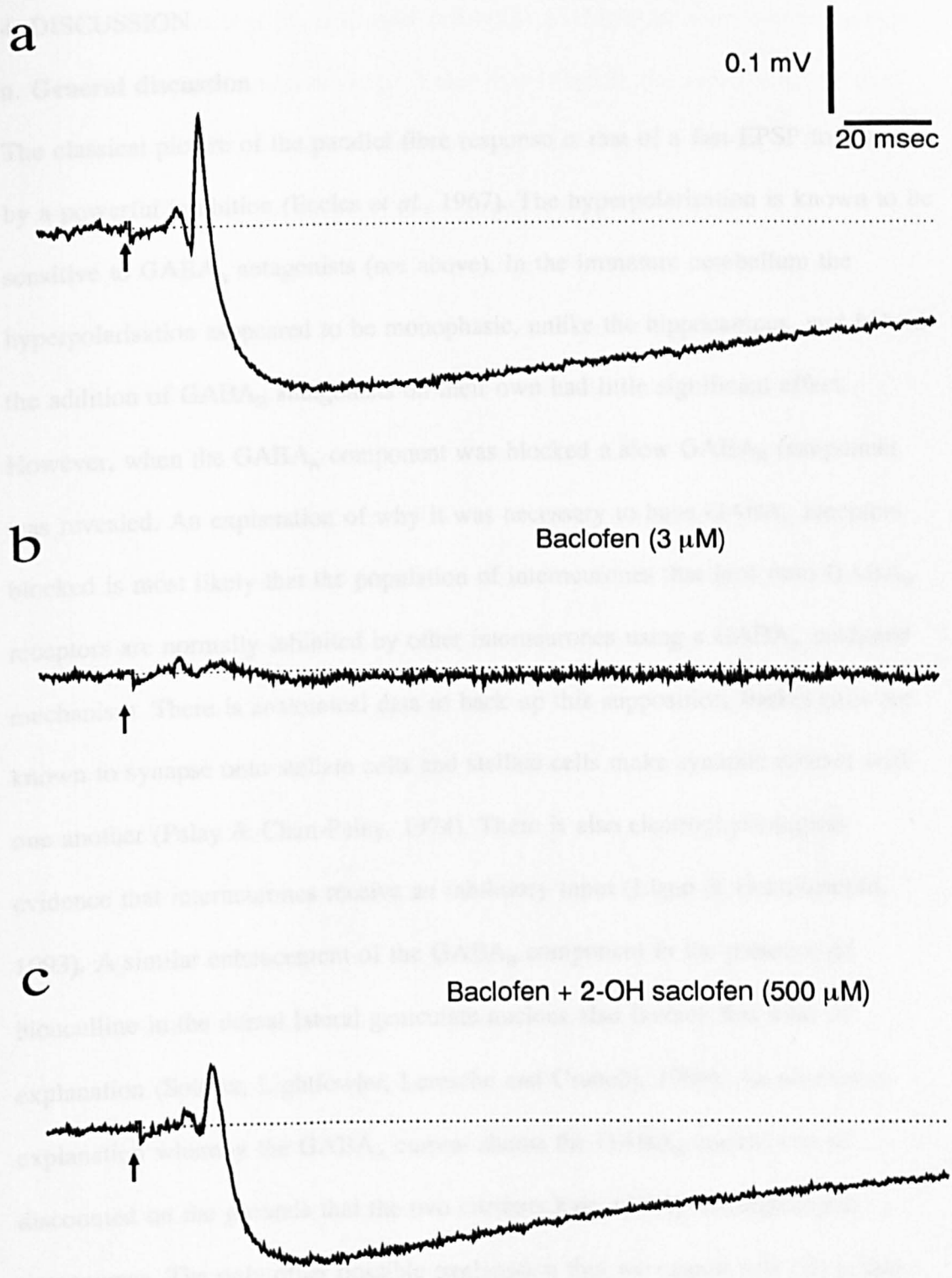


Figure IV(10): Baclofen inhibits the parallel fibre response and this can be reversed by a $GABA_B$ receptor antagonist. (a) Control response from an immature rat. Baclofen potently inhibits all parts of the parallel fibre response (b) and this can be reversed by adding 2-OH saclofen (500 μM) in the continued presence of baclofen.

4. DISCUSSION

a. General discussion

The classical picture of the parallel fibre response is that of a fast EPSP followed by a powerful inhibition (Eccles *et al.*, 1967). The hyperpolarisation is known to be sensitive to $GABA_A$ antagonists (see above). In the immature cerebellum the hyperpolarisation asppeared to be monophasic, unlike the hippocampus, and indeed the addition of $GABA_B$ antagonists on their own had little significant effect. However, when the $GABA_A$ component was blocked a slow $GABA_B$ component was revealed. An explanation of why it was necessary to have $GABA_A$ receptors blocked is most likely that the population of interneurones that feed onto $GABA_B$ receptors are normally inhibited by other interneurones using a $GABA_A$ mediated mechanism. There is anatomical data to back up this supposition, basket cells are known to synapse onto stellate cells and stellate cells make synaptic contact with one another (Palay & Chan-Palay, 1974). There is also electrophysiological evidence that interneurones receive an inhibitory input (Llano & Gerschenfeld, 1993). A similar enhancement of the $GABA_B$ component in the presence of bicuculline in the dorsal lateral geniculate nucleus also favours this kind of explanation (Soltesz, Lightfowler, Leresche and Crunelli, 1989). An alternative explanation whereby the $GABA_A$ current shunts the $GABA_B$ current can be discounted on the grounds that the two currents have clearly distinguishable timecourses. The only other possible explanation that we cannot rule out is that somehow the Cl^- ions that enter the cell through the $GABA_A$ channel disrupt the coupling of the $GABA_B$ receptor with its effector mechanism.

The discovery of this $GABA_B$ mediated potential in is interesting for a

number of reasons. It is the first clear indication of a functional second messenger linked receptor in Purkinje cells and it also helps explain the mystery of the very high levels of $GABA_B$ binding in the molecular layer. The likelihood is that the current evoked by activating $GABA_B$ receptors is due to an increase in K^+-conductance (Ogata, 1990). Qian and Sejnowski (1990) have argued that a $GABA_A$-mediated Cl^--conductance is likely to be impotent out in the fine dendrites, due to the limited intracellular space, whereas a K^+-conductance linked to $GABA_B$ receptors would be much more effective. The Purkinje cells receive inhibitory synapses from the stellate cells, whereas the basket cells synapse at the level of the soma and initial segment. One interesting possibility is that perhaps the stellate cells predominantly synapse onto $GABA_B$ receptors and basket cells onto $GABA_A$ receptors. It has already been suggested that two different cell populations differentially synapse onto either $GABA_A$ or $GABA_B$ receptors elsewhere in the brain (Sugita *et al.*, 1992; Otis & Mody, 1992). In terms of the information processing within the cerebellar cortex the discovery of a second level of processing in the inhibitory input to Purkinje cells allows much more complex processing than would otherwise have been expected.

In the adult, there was apparently no slow hyperpolarising wave, even in the presence of bicuculline. This could mean that the receptors are only present in the immature rats but this seems unlikely since the studies of Martinelli *et al.* (1992) and Bowery *et al.* (1983) were performed in adult rat. Perhaps in the adult, activation of the receptor does not lead to the opening of an ion channel or that perhaps the channel is located in the distal dendrites where it escapes detection. This last suggestion would conform with the hypothesis that the $GABA_B$ receptors

were exclusively dendritic. The fact that we see no synaptic activation of postsynaptic $GABA_B$ receptors does not mean that they are not there and indeed cannot rule out that they are activated.

The addition of baclofen blocked transmission at the parallel fibre synapse in the adult as well as in the immature slices. This was presumably due to the activation of presynaptic $GABA_B$ receptors which are known to suppress synaptic transmission in many parts of the nervous system. Baclofen has been shown to inhibit the release of various neurotransmitters from the CNS, including GABA itself (see Waldmeier & Baumann, 1990). With reference to the cerebellum, baclofen is known to block the release of glutamate from cultured granule cells (Huston, Scott & Dolphin, 1990). The IC_{50} was about 5 μM for the active isomer, R-baclofen, as compared with the IC_{50} of about 1 μM for the R,S-baclofen used in my experiments. The maximal inhibition of glutamate (using 100 μM R-baclofen) was only about 25 % compared with almost complete block using 30 μm R,S-baclofen in my experiments. In addition, although the inhibition of glutamate release was partially reversed by phaclofen the authors saw little effect of 2-hydroxysaclofen (500 μM) again contrary to my findings. This clearly shows a pharmacological difference between my preparation and theirs. Probably, the source of the difference is that high K^+-evoked depolarisation and action potentials arriving at the synaptic terminals engage distinct mechanisms that are affected differently by baclofen. This leaves unanswered the mechanism of baclofen's 2-hydroxysaclofen-resistant action.

Using whole-cell patch clamping of cultured granule cells Wojcik *et al.* (1990) found that R,S-baclofen blocked the current through what looked like an L-

type calcium channel by up to 60 % with concentrations as low as 200 nM. Neither these nor the release studies are really studying what happens during synaptic transmission. They are still observing effects of $GABA_B$ receptor activation but the conditions under which they become physiologically appropriate is not known.

b. Possible role of $GABA_B$ receptors in the cerebellum

i. Postsynaptic

Before a function for the postsynaptic $GABA_B$ receptors located on Purkinje cells in the cerebellum can be contemplated, it has to be explained why their effect is only evident under certain circumstances. Obviously, *in vivo* there is no bicuculline present so will they $GABA_B$ receptors ever get stimulated? A possible explanation is that the pattern of stimulation of the parallel fibres in my experiments is somewhat artificial. *In vivo*, the parallel fibres will probably be firing asynchronously. If we assume that under the correct conditions $GABA_B$ receptors do indeed become activated, then what is their role in the function of the cerebellum? *Temporal processing.* The $GABA_B$ mediated hyperpolarisation, by virtue of its longevity, would allow the effect of inhibitory interneurone activity to be registered for a longer period in the processing of synaptic inputs by a Purkinje cell. *Spatial processing.* Stellate cells and basket cell dendrites occupy different parts of the molecular layer. The stellate cells contact parallel fibres over a much larger area and do so primarily in the superficial molecular layer. They two populations of interneurones therefore receive a different set of signals, and if, as suggested above, $GABA_B$ receptors are selectively activated by stellate cells, this means that the set of parallel fibres that activate $GABA_B$ receptors is different from that which activates $GABA_A$ receptors for a given Purkinje cell. This is obviously

an oversimplification, there may be interactions between these sets of interneurones, but what cannot be denied is that Purkinje cells acquire a much more complicated inhibitory input than has been previously thought.

ii. Presynaptic.

A function for presynaptic receptors relies on there being an endogenous source of transmitter. Presynaptic receptors which can be stimulated by the transmitter released from the terminal on which they are located are termed autoreceptors. $GABA_B$ receptors are thought to act as autoreceptors in many areas of the nervous system (Davies, Davies & Collingridge, 1990; Mott, Lewis, Ferrari, Wilson & Swartzwelder, 1990). During a single synaptic event GABA is released and is quickly taken up by highly efficient systems, GABA only remains in the vicinity of the receptors for a matter of milliseconds. During this time, the presynaptic as well as the postsynaptic receptors are stimulated. Should another action potential invade the terminal within a few hundred milliseconds it will provoke a second but smaller release of transmitter. This leads to the phenomenon of paired-pulse depression of inhibitory transmission, if this is indeed mediated through $GABA_B$ receptors it would be expected that blocking the actions of the autoreceptors with a $GABA_B$ antagonist would eliminate this depression; as has been observed in the hippocampus (Davies, Davies & Collingridge, 1990; Nathan & Lambert, 1991; Brucato, Morrisett, Wilson & Swartzwelder, 1992). Partially switching off inhibition is one of the ways of controlling frequency-dependent events such as long-term potentiation (Davies *et al.*, 1991; Mott & Lewis, 1991). There is a lot of interest in the role $GABA_B$ receptors in epilepsy, especially after it was shown that $GABA_B$ antagonists could prevent absence seizures in mice (Hosford *et al.*, 1992).

This is thought to be a rather good model for a similar phenomenon in man (Marescaux, Vergnes & Bernasconi, 1992). This provides hope for the future of developing effective anticonvulsants.

C. METABOTROPIC GLUTAMATE RECEPTORS IN THE CEREBELLUM

Glutamate acts at two different kinds of receptors; ionotropic and metabotropic glutamate receptors. Ionotropic glutamate receptors namely, AMPA, kainate and NMDA have an integral ion channel formed by the receptor proteins which is opened by the conformational change that occurs on binding an agonist. These receptors are generally thought to be resposible for the fast synaptic transmissions, in the range of a few milliseconds to severel tens of milliseconds. A second and more recently discovered family are the metabotropic glutamate receptors (mGluR's). There are now at least six members of this family, $mGluR_{1-6}$ (Masu *et al.*, 1991; Houamed *et al.*, 1991; Tanabe *et al.*, 1992; Abe, Sugihara, Nawa, Shigemoto, Mizuno & Nakanishi, 1992) with the $mGluR_1$ having two alternatively spliced versions alpha and beta (Tanabe *et al.*, 1992). These receptor molecules do not have an integral ion channel but instead modulate cell function by interacting with the intracellular or second messengers. They too can lead to the opening of independent ion channels but, in addition, they can lead to many other responses including closure of channels, liberation of Ca^{2+} from internal stores, activation of kinases even regulation of gene expression. These receptors act much more slowly from several tens of milliseconds to permanent changes. The first action thought to be mediated by metabotropic glutamate receptors was the stimulation of IP_3 production

D. ACPD-INDUCED CALCIUM ELEVATIONS IN PURKINJE CELLS

1. INTRODUCTION

Of the six known metabotropic glutamate receptors (mGluR's) cerebellar Purkinje cells most clearly express mRNA for the first metabotropic glutamate receptor cloned (Masu *et al.*, 1991; Houamed *et al.* 1991), now called $mGluR_{1\alpha}$ to distinguish it from the alternatively spliced $mGluR_{1beta}$ (Tanabe *et al.*, 1992). This receptor couples to the $InsP_3/Ca^{2+}$ transduction pathway when expressed in *Xenopus* oocytes (Masu *et al.*, 1991). Expression in Chinese hamster ovary cells also stimulates the $InsP_3/Ca^{2+}$ pathway, but interestingly was found to stimulate cAMP and arachidonic acid production as well. Pertussis toxin pretreatment reduced $InsP_3$ by about 50%, increased cAMP by 2-3 times and abolished the arachidonic acid production. This suggests that $mGluR_1$ can modulate different second-messenger pathways probably by interacting with different G proteins. Pharmacologically, $mGluR_1$ receptors were activated in rank order by quisqualate > L-glutamate > t-ACPD and were little effected by the putative antagonists AP3 and AP4 (Aramori & Nakanishi, 1992).

In addition to the discovery of mRNA encoding an mGluR in Purkinje cells there are three pieces of evidence that connect Purkinje cells to an mGluR, most likely $InsP_3$-linked. Firstly, it was known that [^{3}H]-glutamate bound to a site which was insensitive to AMPA or NMDA but was displaced by quisqualate, ibotenate and t-ACPD, and that this site was particularly dense in the molecular layer. It was proposed that this binding represented binding to an mGluR. Furthermore, in Purkinje cell deficient mutant mice the binding was reduced, indicating that this site may be expressed on Purkinje cells (Cha, Makowiec, Penney & Young, 1990).

Secondly, the glutamate stimulated production of phosphoinositides in cerebellar slices is much reduced in Purkinje cell deficient mice (Olson, Greenamyre, Penney & Young, 1987); Blackstone, Supattapone & Snyder, 1989). Thirdly, evidence that there is a $InsP_3/Ca^{2+}$ linked mGluR in Purkinje cells comes from the autoradiographic localisation of a insoluble product of the $InsP_3$/DAG bifurcating pathway, namely [^{3}H]cytidine diphosphate diacylglycerol. Slices of cerebellum were prelabelled with [^{3}H]cytidine and stimulated with t-ACPD. The product was particularly enriched in the molecular and Purkinje cell layers of the (immature) cerebellum (Hwang, Bredt and Snyder, 1990).

Recording the membrane potential of cerebellar Purkinje cells while stimulating with the selective mGluR agonist, t-ACPD, resulted in a transient depolarisation (Ito & Karachot, 1990; Crepel, Daniel, Hemart & Jaillard, 1991) or an inward current in voltage-clamped Purkinje cells in slice-culture (Knöpfel & Staub, 1991; Staub, Vranesic and Knöpfel, 1992). In acute slices, the active isomer, 1S,3R-ACPD, produced a depolarisation which was relatively larger in the adult compared to the immature (East & Garthwaite, 1992). This last finding seemed contrary to the biochemical data which showed a decrease in phosphoinositide turnover in response to *t*-ACPD during development (Palmer, Nangel-Taylor, Krause, Roxas & Cotman, 1990) but was in agreement with the molecular biology which showed an approximate doubling in mRNA for $mGluR_1$ in the cerebellum over the same period (Condorelli, Dell'Albani, Amico, Casabona, Genazzani, Sortino & Nicoletti, 1992).

The ionic mechanism underlying the depolarisation to *t*-ACPD is not known, in the most extensive study Staub *et al.* (1992) suggest either a Ca^{2+}-

activated non-specific cation channel or activation of an electrogenic sodium/calcium exchanger. If *t*-ACPD does indeed activate the $InsP_3$/DAG pathway we would expect to see a rise in intracellular Ca^{2+} ion concentration, $[Ca^{2+}]_i$. It is possible to do this by using the Ca^{2+} indicator Fura-2. Purkinje cells in culture mobilise intracellular Ca^{2+} in response to quisqualate > glu > ibotenate = *t*-ACPD. In response to quisqualate, $[Ca^{2+}]_i$ increased most in the soma (Yuzaki & Mikoshiba, 1992). This was different from the report from whole-cell voltage-clamped Purkinje cells in acute slices of cerebellum, where the increase in $[Ca^{2+}]_i$ to quisqualate or glutamate was greatest in the dendrites (Llano, Dreesen, Kano & Konnerth, 1991a).

Therefore the situation was not clear. Most papers reported an action of *t*-ACPD whereas Llano *et al.* (1991a) did not.

2. METHODS AND MATERIALS

These experiments were carried out at the Brain Research Institute of the University of Zürich in one of the groups under Professor B.H. Gähwiler. The group is lead by Dr. med. Thomas Knöpfel and these experiments were shared amongst myself, Dr. Knöpfel and his graduate student Ivo Vranesic. The data was analysed with the help of another graduate student Christophe Staub.

a. Slices

Sprague-Dawley rats aged 13-23 days were used. Thin saggital slices of the centre of the vermis were cut on a vibroslice. The chances of obtaining a good stable recording seemed optimised from slices around 150 μm thick. The slices were allowed to recover in continuously bubbled Krebs solution of the same composition as before. The slices were then transferred to the temperature controlled recording

chamber of volume 0.5 cm^3 where they were perfused with warmed (32 oC) Tyrode's solution containing (mM): Na^+ 148.9; K^+ 2.7; Ca^{2+} 3.8; Mg^{2+} 1.0; Cl^- 149.2; $H_2PO_4^-$ 0.4; HCO_3^- 11.6 and D-glucose 5.6, at a rate of 1.5 cm^3min^{-1}.

b. Electrophysiological recordings

Intracellular recordings were made with sharp electrodes with a resistance of 70-90 MOhms when the tip was filled with 1.5 mM fura-2 (Molecular probes) dissolved in 0.5 M K_2MeSO_4 and backfilled with 2 M K_2MeSO_4. The electrode holder was mounted on the movement of a hydrolic stepper (Kopf Instruments). An Axoclamp-2 amplifier (Axon Instruments) was used in current-clamp and single-electrode voltage clamp modes.

c. Simultaneous microfluorometric recordings of $[Ca^{2+}]_i$

The Purkinje cells were penetrated during negative current steps, immediately a cell was penetrated hyperpolarising current of up to 1 nA were applied. This aided the injection of the fura-2 as well as counteracting the leakage. It took about 15 min for the dendritic tree to fill with dye. The dye was excited with a mercury arc lamp (Osram 100 W) at two wavelengths, 366 and 405 nm. The fluorescence was collected through a Neofluar x40 objective (Zeiss) which was projected onto a CCD camera for high spatial resolution. It was possible to capture CCD images at 30 Hz with an approximate resolution of 1 μm. For higher temporal resolution the fluorescence was passed to a 10 X 10 array of photodiodes, of which only 3 X 3 was used in these experiments, giving a temporal resolution of 250 μs (4 kHz) with a spatial resolution of 35 μm. For technical details and the calibration of the ratio see Vranesic and Knöpfel (1991).

3. RESULTS

Recordings were obtained from nine Purkinje cells that were stable enough to allow full experiments to be carried out. Cells with input resistance greater than 40 MOhms were accepted for analysis. The tip of the electrode was lined up with the Purkinje cell layer and stepped through at a shallow angle of 35 - 40°, the Cells were identified as being Purkinje cells because of the unique firing pattern - in particular the anomalous rectifier and the pattern of fast Na^+ spikes followed by slower Ca^{2+} spikes (Llinas & Sugimori, 1980) their identity was confirmed once the fluorescent dye diffused into the spectacular dendritic tree.

Simultaneous voltage clamp and microflurometric quantification of $[Ca^{2+}]_i$ was carried out in the presence of tetrodotoxin (0.5 μM), the membrane was held at around -70 mV, the resting level of $[Ca^{2+}]_i$ was around 40 nM. Bath application of 100 μM t-ACPD for 60 sec caused a transient inward current which reached 497 ± 145 pA (n = 18) accompanied by a rise in intrasomatic $[Ca^{2+}]$ up to about 500 nM [Fig IV(11)]. In contrast, application of 1 μM AMPA for 30 sec produced a similar inward current (625 ± 132 pA; n = 3) but without any detectable increase in $[Ca^{2+}]_i$. CCD images were also obtained before, during and after application of 100 μM t-ACPD, the calculated $[Ca^{2+}]_i$ is displayed as a pseudocolour image [Fig IV(12)] with high spatial resolution. Again the rise in $[Ca^{2+}]_i$ seems to be mainly restricted to the soma, with a small rise in the distal dendrites.

It could be argued that for some reason the $[Ca^{2+}]_i$ was simply not being detected in the dendrites, but in one cell, while still in current clamp, we showed that there was a clear dendritic signal during the Ca^{2+} spikes evoked by a prolonged depolarising pulse [Fig IV(11)]. The depolarising pulse evoked fast Na^+ spikes which cease and are replaced by Ca^{2+} spikes which are recognised by the large

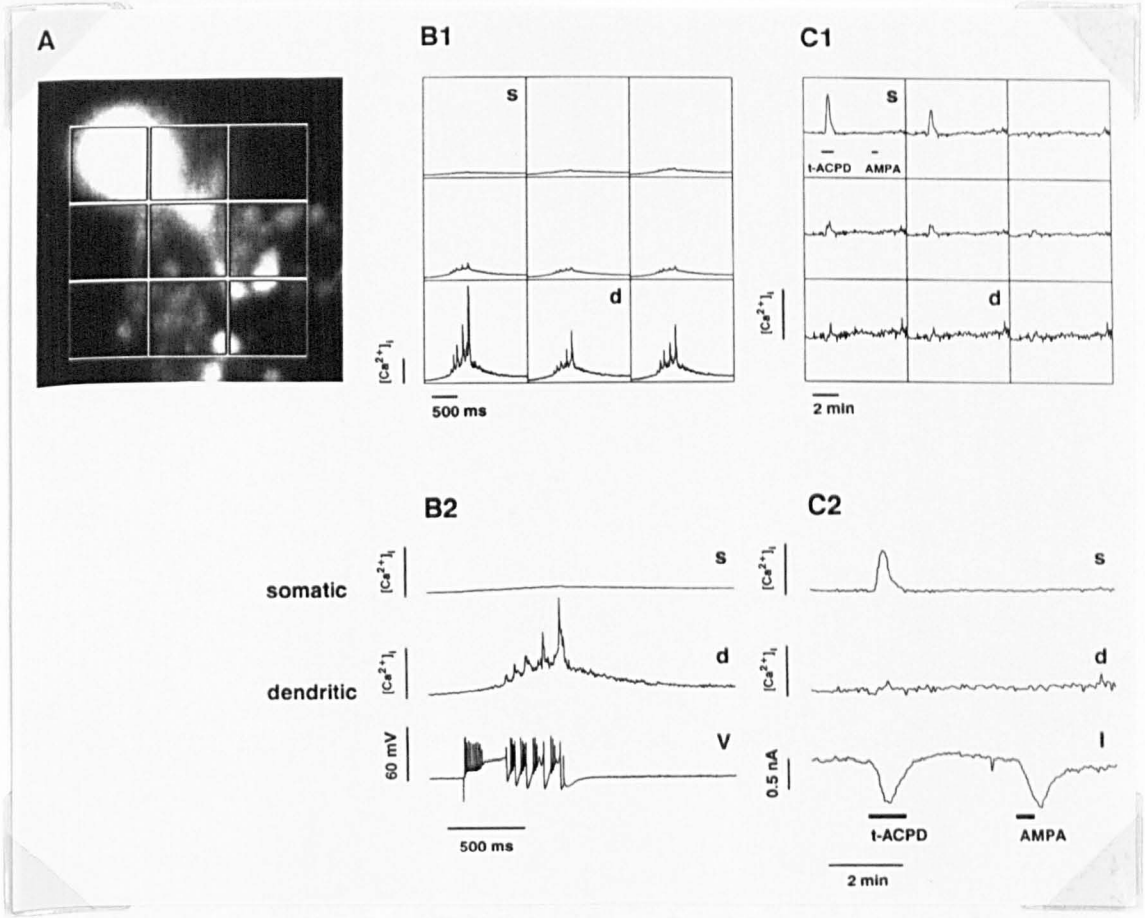


Figure IV(11): Simultaneous electrophysiological and microfluorometric analysis of immature Purkinje cell during applications of *t*-ACPD or AMPA and during calcium spikes.

A. A single Purkinje cell is filled with Fura-2 - raw fluorescence image. The grid refers to the photodiode array.

B. In current-clamp a current step caused a complex pattern of Na^+ spikes and Ca^{2+} spikes (B2, v). The $[Ca^{2+}]_i$ during this time was captured by the photodiodes with high temporal resolution (B1). A photodiode over the dendrites (d) showed clear Ca^{2+} signals correlated with the spikes whilst the one over the soma (s) only showed a slow rise.

C. In voltage-clamp application of AMPA (1 μM, 1min) or *t*-ACPD (100 μM, 1 min) caused a clear inward current (C2, I) whereas the $[Ca^{2+}]_i$ rise only occured during the *t*-ACPD application and this was centered over the soma (C1 & C2, s). Scale bar for $[Ca^{2+}]i$ is 250 nM.

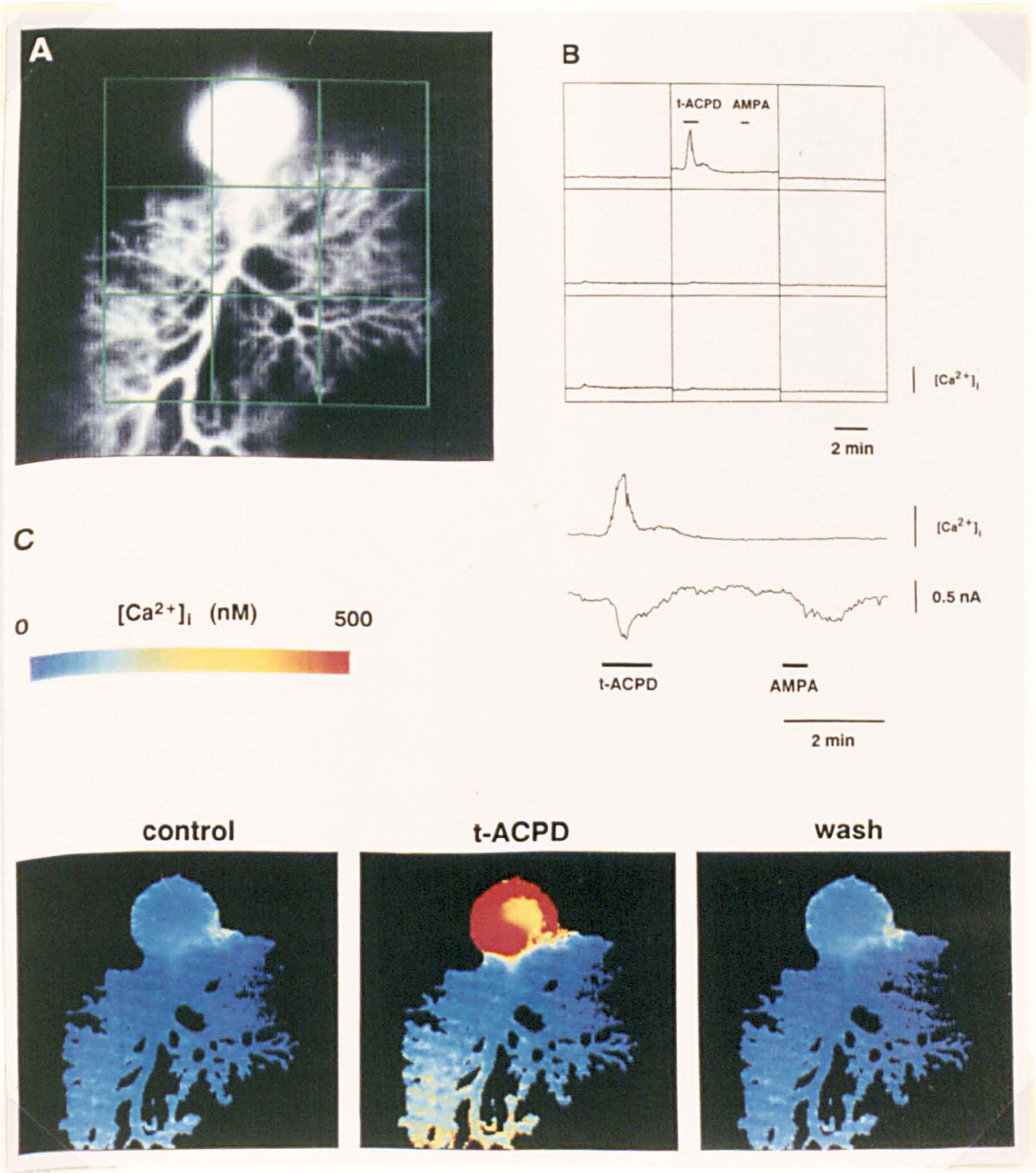


Figure IV(12): High resolution spatial images of somatic $[Ca^{2+}]_i$ rise induced in immature Purkinje cells by *t*-ACPD.
A. Raw fluorescence image of Fura-2 injected Purkinje cell.
B. Application of AMPA (1 μM, 1 min) and t-ACPD (100 μM, 1min) as before caused inward currents and an increase in the somatic $[Ca^{2+}]_i$. Scale bar for $[Ca^{2+}]_i$ is 250 nM.
C. High spatial resolution ratio images were taken before (control), at the peak of the *t*-ACPD induced Ca^{2+} rise and after (wash). The images are pseudocoloured, the relationship between $[Ca^{2+}]_i$ and colour is indicated in the scale bar.

Ca^{2+}-activated hyperpolarisation. There was a high degree of synchronicity between the Ca^{2+} spikes and the large increase in $[Ca^{2+}]_i$ [Fig IV(11) B2]. This cell was then voltage-clamped and immersed in tetrodotoxin and responded to t-ACPD and AMPA in a similar fashion to the others.

4. DISCUSSION

The main finding of this work was that t-ACPD as well as inducing an inward current concomitantly raised $[Ca^{2+}]_i$ as predicted, but unexpectedly this was mainly found in the soma.

The source of the rise in $[Ca^{2+}]_i$ could be extracellular or from intracellular stores. The clearest way to distinguish between them is to carry out the experiments in Ca^{2+}-free solution. We attempted this on a few occasions but were unable to keep stable recordings, the cells being lost within a number of minutes. The solution contained raised Mg^{2+}, and has been used successfully both by myself (see below) and a number of authors, although in more mature Purkinje cells prolonged extracellular Ca^{2+} deprivation can kill the cells, it is more likely that the cells were lost because the seal between the electrode and the plasma membrane deteriorated. It is possible that the voltage-clamp was not complete out at the distal dendrites and that ACPD could depolarise the membrane and activate voltage-sensitive calcium channels but this seems unlikely to explain our results, firstly, AMPA which caused a similar inward current did not increase $[Ca^{2+}]_i$ in the dendrites or anywhere else and secondly, it would not explain the somatic location of the increase. In the nearest comparable study (Llano *et al.*, 1991) use whole-cell patch recordings and only looked at the dendrites. They got no response to ACPD but instead used quisqualate and glutamate, which provoked an increase in

dendritic $[Ca^{2+}]_i$. They obtained the same results in Ca^{2+}-free conditions so presume that the Ca^{2+} came from internal stores. The reason for the differences is not known. There are two main differences, firstly the age of the rats was generally younger in our case (13 - 23 days old compared to 17 - 33 days). But Crepel *et al.* (1991) got membrane responses with ACPD in his experiments which were similar in age to Llano *et al.* (22 - 35 days) and East and Garthwaite (1992) got clear responses at both 14 days and adult (28 - 42 days). The second difference is that intracellular constituents are much more likely to diffuse into the larger patch electrodes than the sharp electrodes used by ourselves and Crepel *et al.*. We did not use quisqualate because it generally caused a much smaller inward current (in the presence of CNQX) than ACPD, although it was not studied with the fura-2 technique. So it could well be that quisqualate acts at a different mGluR or that in older Purkinje cells the mGluR's move out to the dendrites.

E. SLOW SYNAPTIC RESPONSE IN PURKINJE CELLS - POSSIBLE ACTIVATION OF METABOTROPIC GLUTAMATE RECEPTORS

1. INTRODUCTION

As discussed above, application of the exogenous metabotropic agonist, t-ACPD, induces a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and a concomitant inward current in rat cerebellar Purkinje cells (Vranesic, Batchelor, Gähwiler, Garthwaite, Staub, Knöpfel, 1991). If metabotropic glutamate receptors on Purkinje cells are physiologically relevant they would either receive their transmitter from climbing fibres or parallel fibres. Both of these inputs use an excitatory amino acid, which is most probably glutamate. Fast synaptic actions at these two synapses are blocked by CNQX which blocks AMPA/kainate type of glutamate receptors.

When climbing fibres are stimulated in slice culture preparations, both the increase in $[Ca^{2+}]_i$ and the membrane potential changes in Purkinje cells are completely blocked by CNQX (Knöpfel, Audinat & Gähwiler, 1990; Knöpfel, Vranesic, Staub & Gähwiler, 1991), suggesting, by default, that the parallel fibres are the most likely activators of the metabotropic glutamate receptors on Purkinje cells, as has already been suggested on the basis of indirect evidence (Blackstone, Supattapone & Snyder, 1989). The aim of the present study was to test this premise.

2. RESULTS

a. Potentials resistant to ionotropic glutamate and GABA receptor blockade

Single stimuli (0.05 Hz) applied to parallel fibres in cerebellar slices from adult rats evoked a sharp population spike from Purkinje cells. Perfusion for 10-15 min with 10 μM NBQX (2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline), a

selective antagonist for the AMPA/kainate subtypes of ionotropic glutamate receptor (Sheardown, Nielsen, Hansen, Jacobsen & Honoré, 1990) inhibited most of the response [FigIV(13)]; an increase the concentration to 20 μM gave no further reduction indicating maximal effect. It was impracticable to routinely obtain full recovery from 10 μM NBQX, as this took 1-2 hours or more.

The small potentials remaining in the presence of 10 μM NBQX were, with single shocks, unaffected by the addition of the NMDA antagonist, AP5 (D-2-amino-5-phosphopentanoate; 30 μM), or the $GABA_A$ antagonist, bicuculline (50 μM). The fastest of the residual potentials, designated R1, coincided with the initial part of the population spike; it peaked 9.3 ± 0.8 msec (mean ± SEM, n = 6) after the stimulus artefact. There was also a slower, lower amplitude wave, R2, peaking at 45 ± 5 msec (n = 5; b). Increasing the stimulus frequency (20-100 Hz) revealed a third, still slower component (R3) peaking at 447 ± 36 msec (n = 6; [FigIV(13)c]. Even in averaged traces, it was evident that the R3 wave was associated with increased background noise. The addition of 0.4 μM tetrodotoxin to all the compartments of the chamber completely abolished R1, R2 and R3. A further wave, R4, is described below.

Various stimulation protocols were tested. With twin stimuli, a stimulation frequency of 30-50 Hz was required before R3 became just visible. At 50 Hz, R2 and R3 responded differently to increasing numbers of stimuli: the amplitude of R2 seemed to increase linearly, whereas R3 increased steeply to become maximal after 5-6 pulses. The same number of shocks delivered at 100 Hz evoked no additional increase in the size of R3 and so we chose to apply 6 pulses at 50 Hz in subsequent experiments.

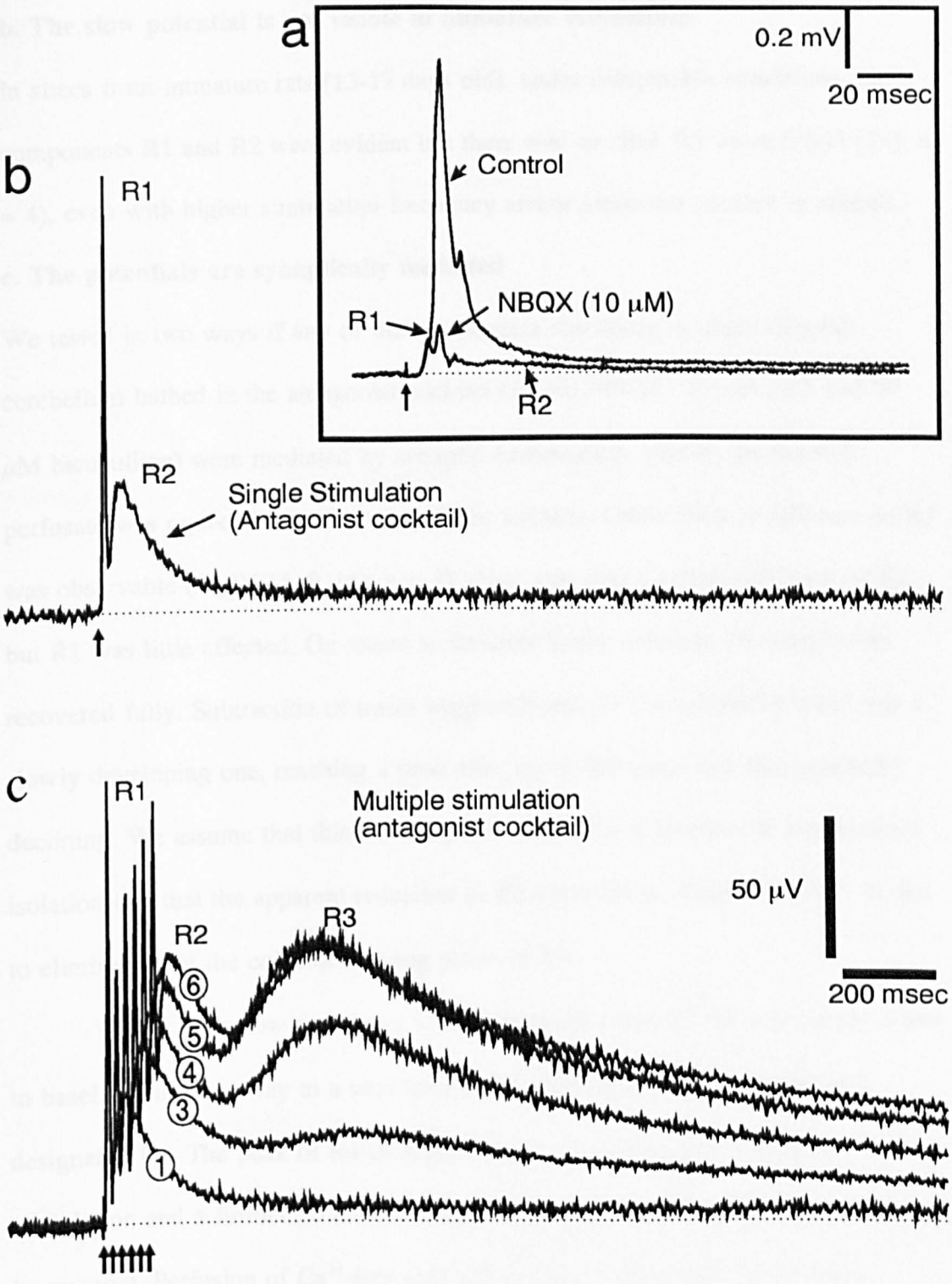


Figure IV(13): Novel potentials, resistant to ionotropic glutamate and GABA receptor antagonists, due to parallel fibre stimulation. In (a) The control response is almost completely inhibited by NBQX. Addition of AP5 and bicuculline, as well as NBQX - 'antagonist cocktail', left two components, designated R1 and R2 (b & c are at different amplification and timebase from a). Multiple stimulation at 50 Hz revealed a third component R3, this was stimulus dependent getting larger with 1, 3, 4, 5, & 6 pulses.

b. The slow potential is not visible in immature cerebellum

In slices from immature rats (13-17 days old), under comparable conditions, the components R1 and R2 were evident but there was no clear R3 wave (FigIV(14); n = 4), even with higher stimulation frequency and/or increased number of stimuli.

c. The potentials are synaptically mediated

We tested in two ways if any of the components remaining in slices of adult cerebellum bathed in the antagonist cocktail (10 μM NBQX, 30 μM AP5 and 50 μM bicuculline) were mediated by synaptic transmission. Firstly, the normal perfusate was replaced with Ca^{2+}-free Krebs solution. Under these conditions no R3 was observable (FigIV(15 & 16); n = 3); there was also a partial reduction of R2 but R1 was little affected. On return to standard Krebs solution, all components recovered fully. Subtraction of traces suggested that the Ca^{2+}-sensitive wave was a slowly developing one, reaching a peak after about 400 msec and then gradually declining. We assume that this wave represents the R3 component in approximate isolation and that the apparent reduction in R2 observed on removal of Ca^{2+} is due to elimination of the coincident rising phase of R3.

With a very slow time base, it became evident that R3 did not simply return to baseline but gave way to a very long-lasting hyperpolarisation [FigIV(16)], designated R4. The peak of R4 took place some 30 to 40 seconds after the initial stimulation and a further 30 to 40 seconds were needed for the baseline potential to be restored. Perfusion of Ca^{2+}-free solution reversibly abolished the R4 wave. Subtraction of the Ca^{2+}-free trace from the control indicated that the decay of the R3 wave may have more than one phase; in particular a small hump appeared to precede the transition to R4.

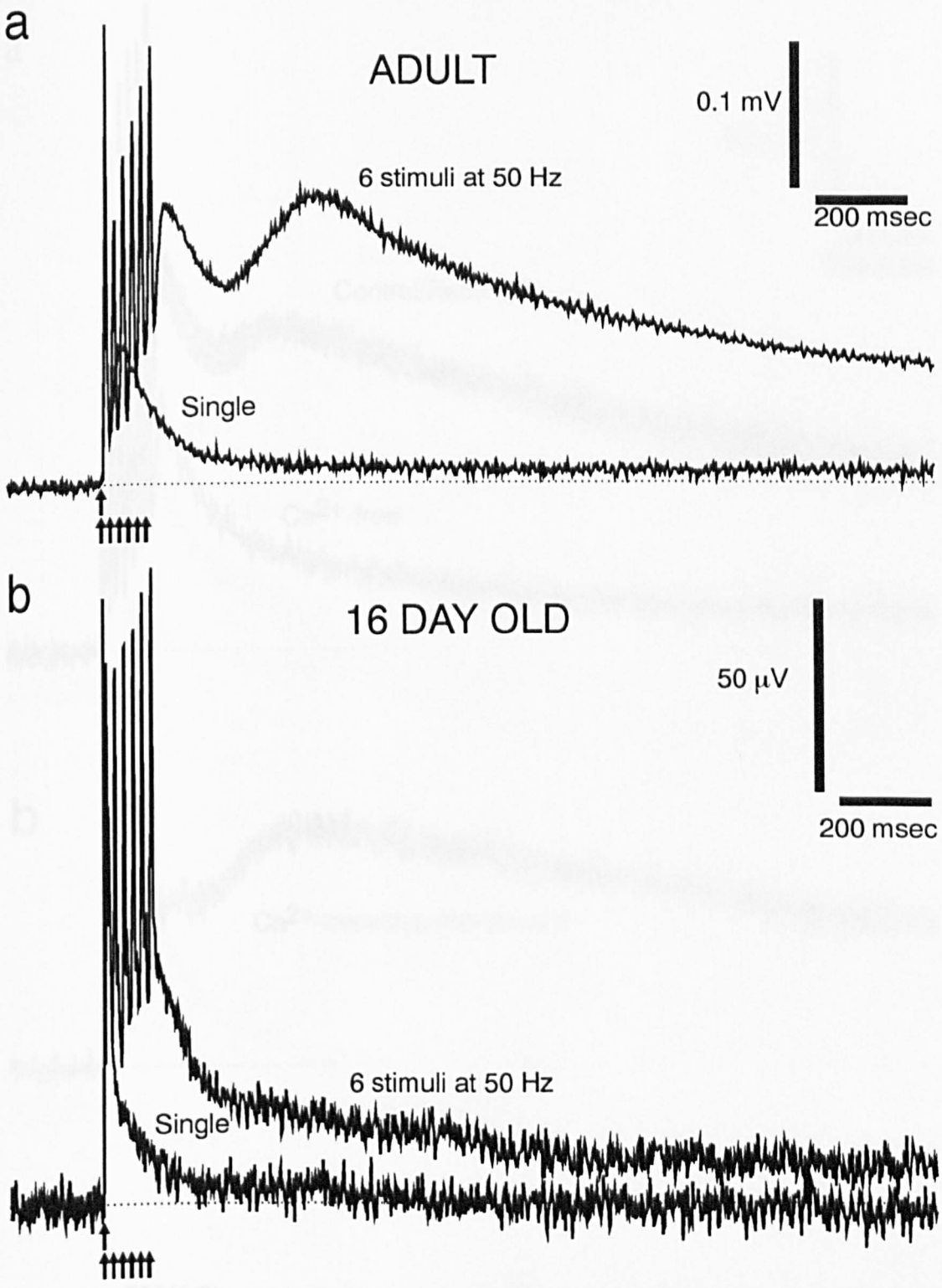


Figure IV(14): There is no R3 wave apparent in immature animals. The response to one or six stimuli is shown for both adult (a) and immature (b). The traces in (a) are from Fig.IV(13).

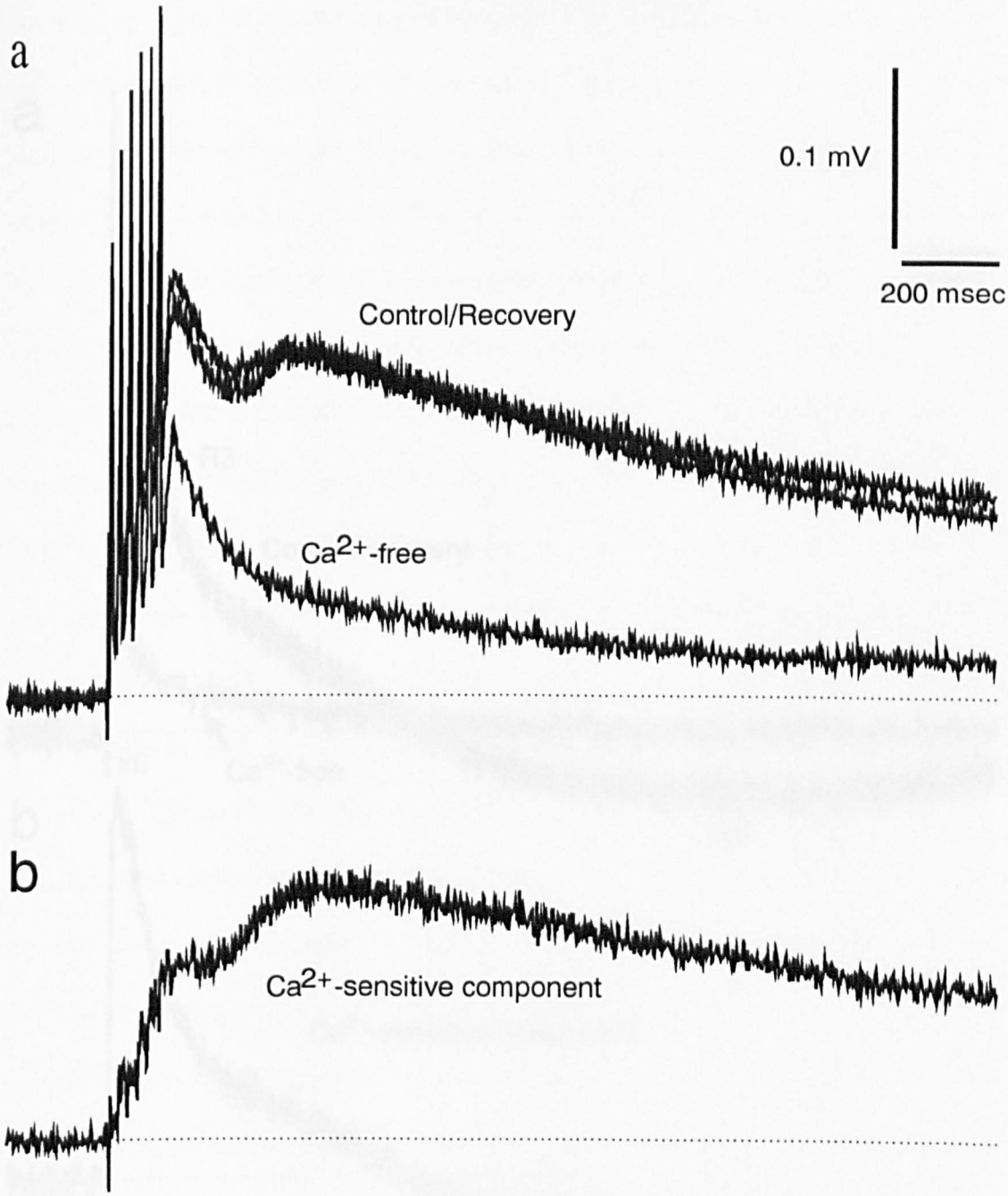


Figure IV(15): R3 is Ca^{2+}-dependent. The parallel fibres were stimulated at 6 pulses at 50 Hz in the presence of the antagonist cocktail. Perfusion with Ca^{2+}-free Krebs (no added Ca^{2+}, extra $MgCl_2$ 3mM and EGTA 200 μM) for 6 minutes selectively abolished R3 (a). Waveform subtraction of Ca^{2+}free from control gives the Ca^{2+}-dependent part (b).

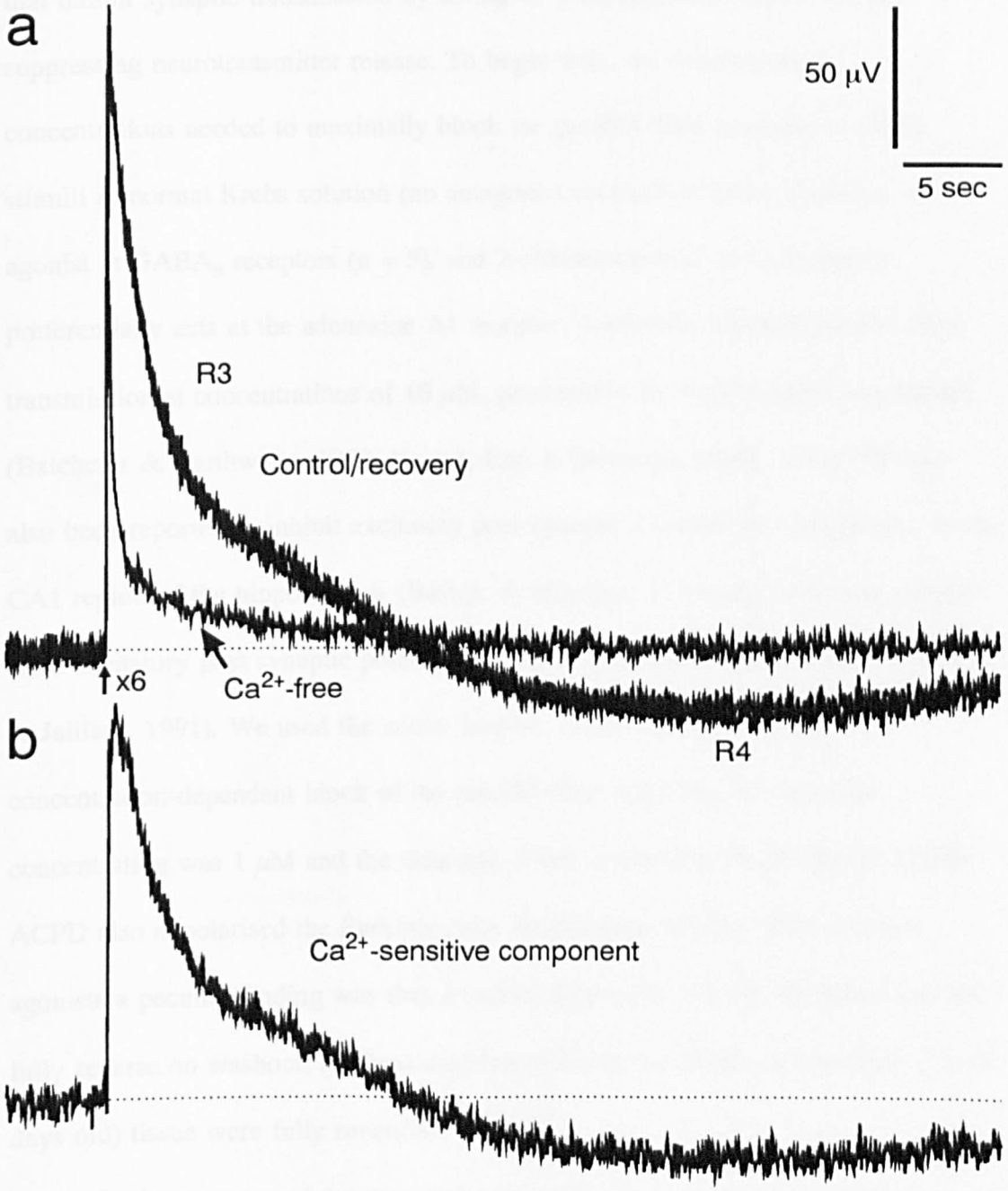


Figure IV(16): At a slow timebase a further wave (R4) is apparent. The wave peaked at around 30- 40 sec after stimulation (six stimuli at 50 Hz in the presence of the antagonist cocktail). Waveform subtraction reveals the Ca^{2+}-dependent portion (b).

As well as using the classical test of removing Ca^{2+}, we also applied some agonists that inhibit synaptic transmission by acting on presynaptic receptors, thereby suppressing neurotransmitter release. To begin with, we determined the concentrations needed to maximally block the parallel fibre response to single stimuli in normal Krebs solution (no antagonist cocktail present). Baclofen, an agonist at $GABA_B$ receptors (n = 5), and 2-chloroadenosine (n = 3), which preferentially acts at the adenosine A1 receptor, maximally blocked parallel fibre transmission at concentrations of 10 μM, presumably by a presynaptic mechanism (Batchelor & Garthwaite, 1992; Kocsis, Eng & Bhisitkul, 1984). (±)-ACPD has also been reported to inhibit excitatory post synaptic currents presynaptically, in the CA1 region of the hippocampus (Baskys & Malenka, 1991) and to reduce parallel fibre excitatory post synaptic potentials in the cerebellum (Crepel, Daniel, Hemert & Jaillard, 1991). We used the active isomer, 1S,3R-ACPD, and found a concentration-dependent block of the parallel fibre response; the threshold concentration was 1 μM and the maximal effect occurred at 50 μM (n=2). 1S,3R-ACPD also depolarised the Purkinje cells directly (see below). With all three agonists a peculiar finding was that, in slices from adult rat, the inhibition did not fully reverse on washout, (> 2hrs) whereas their similar effects in immature (13-15 days old) tissue were fully reversible (15 min).

In the presence of the antagonist cocktail, all of the above agents had comparable actions [FigIV(17)]. They had little or no influence on R1. The first application of a maximal dose of one of the agents reduced the size of R2 and abolished R3. (The R4 wave was not studied in these experiments.) As in unblocked slices, extensive washing could not fully reverse the effect. However,

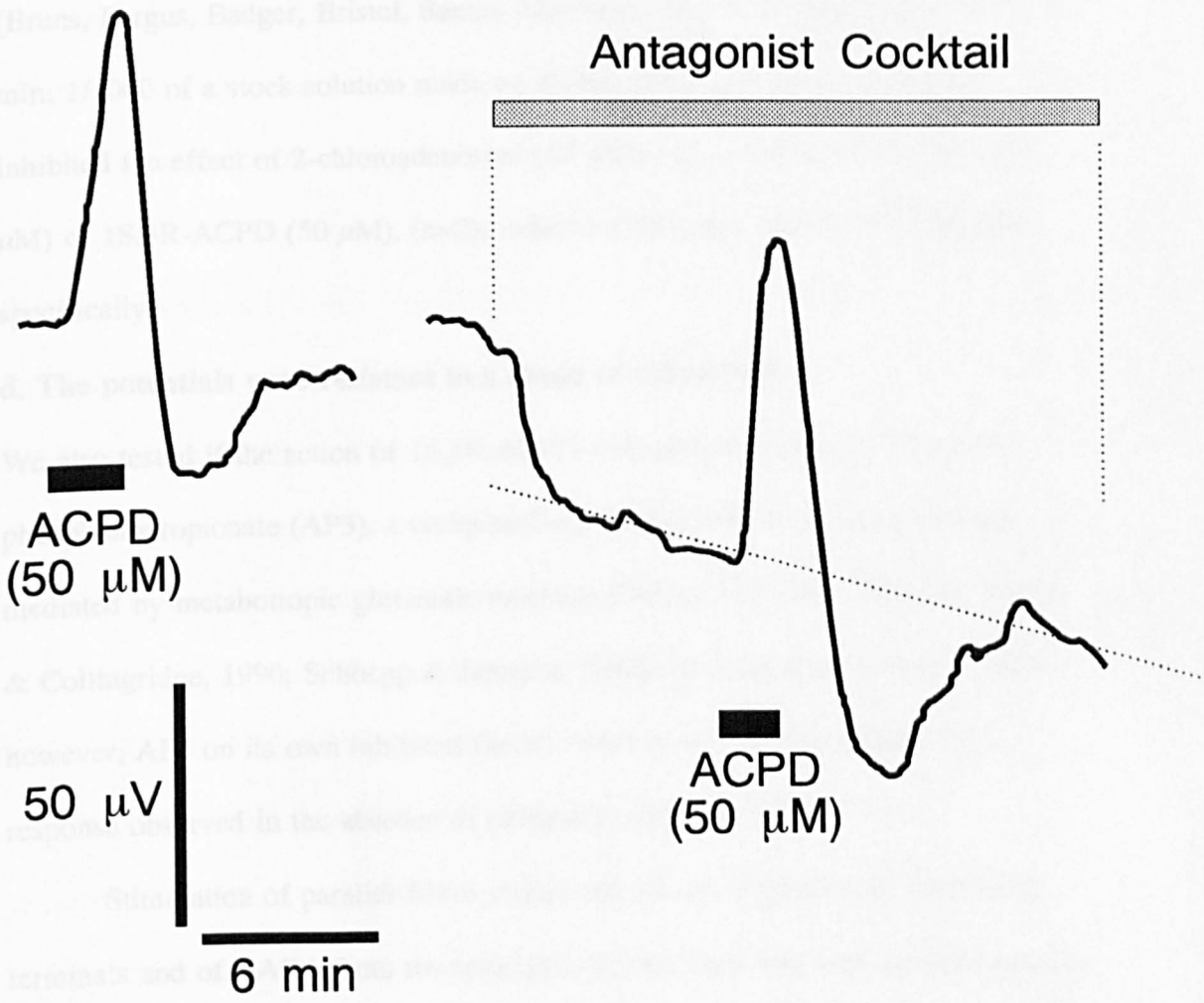


Figure IV(18): ACPD mimics the form of the R3-R4 wave and is resistant to the antagonist cocktail. ACPD was applied for the periods indicated. The control application was identical to the one in the presence of the antagonist cocktail (NBQX, AP5 & bicuculline). Notice that the application ten minutes before the ACPD caused a small hyperpolarisation.

this state of partial recovery was maintained following further applications. The highly selective adenosine A1 antagonist, 8-cyclopentyl-1,3-dipropylxanthine (Bruns, Fergus, Badger, Bristol, Santay, Hartman, Hays & Huang, 1987; 5 μM, 10 min; 1/1000 of a stock solution made up in dimethylsulphoxide), completely inhibited the effect of 2-chloroadenosine (10 μM), but not those of baclofen (10 μM) or 1S,3R-ACPD (50 μM), (n=2), indicating that they were not acting non-specifically.

d. The potentials were resistant to a range of antagonists

We also tested if the action of 1S,3R-ACPD was antagonised by L-2-amino-3-phosphonopropionate (AP3), a compound reported to inhibit certain responses mediated by metabotropic glutamate receptors (Irving, Schofield, Watkins, Sunter & Collingridge, 1990; Schoepp & Johnson, 1989). At a concentration of 1 mM, however, AP3 on its own inhibited the R3 wave as well as the parallel fibre response observed in the absence of antagonist cocktail (n=2).

Stimulation of parallel fibres evokes the release of glutamate from their terminals and of GABA from the terminals of inhibitory interneurones (basket and stellate cells). Our antagonist cocktail blocks all the known ionotropic receptors for these transmitters but any activation of metabotropic glutamate or GABA ($GABA_B$) receptors should persist. Perfusion of another putative metabotropic glutamate receptor antagonist, AP4 (L-2-amino-4-phosphonobutyrate, 50 μM, 10 min) (Nicoletti, Iadarola, Wrobleski & Costa, 1986; Shoepp & Johnson, 1989), had no detectable effect on R3 (n = 2). Activation of postsynaptic $GABA_B$ receptors in central synapses is classically associated with a hyperpolarisation and so would not be expected to underlie the depolarising wave, R3. As predicted, the $GABA_B$

antagonist, 2-hydroxysaclofen, at a concentration (0.5 mM) found previously to maximally inhibit the $GABA_B$ receptor-mediated synaptic potential (hyperpolarisation) in immature Purkinje cells (Batchelor & Garthwaite, 1992) did not inhibit the R3 wave, nor did it attenuate the hyperpolarising R4 wave (n = 3). There is some evidence for cholinergic and adrenergic innervation of the cerebellar cortex (Ito, 1984) which might inadvertently be activated. Nevertheless, perfusion of 50 μM phentolamine plus 15 μM propanalol (α and β adrenergic antagonists) or 1 mM hexamethonium plus 1 μM atropine (nicotinic and muscarinic cholinergic antagonists) had no detectable effect. R3 was also resistant to kynurenic acid (3 mM), D-α-aminoadipate (3 mM) and L-glutamic acid diethyl ester (3 mM), which are all non-selective ionotropic glutamate receptor blockers.

e. Exogenous 1S,3R-ACPD emulates the R3/R4 waves

Application of 1S,3R-ACPD (50 μM), as well as inhibiting synaptic transmission, also induced a depolarisation of Purkinje cells [FigIV(18)], in agreement with previous reports (Crepel, Daniel, Hemart & Jaillard, 1991: East & Garthwaite, 1992). The amplitude was about 60% of the depolarisation produced by 1 μM AMPA in the same experiments. On washout, a hyperpolarisation was generated. Perfusion of the antagonist cocktail led to a small hyperpolarisation of the baseline potential, suggesting inhibition of spontaneous ionotropic glutamate and/or GABA receptor activity. However, the cocktail failed to affect the response to 1S,3R-ACPD [FigIV(18)].

3. DISCUSSION

We have found that blockade of ionotropic glutamate and GABA receptors does not eliminate all the potentials generated in Purkinje cells as a result of parallel

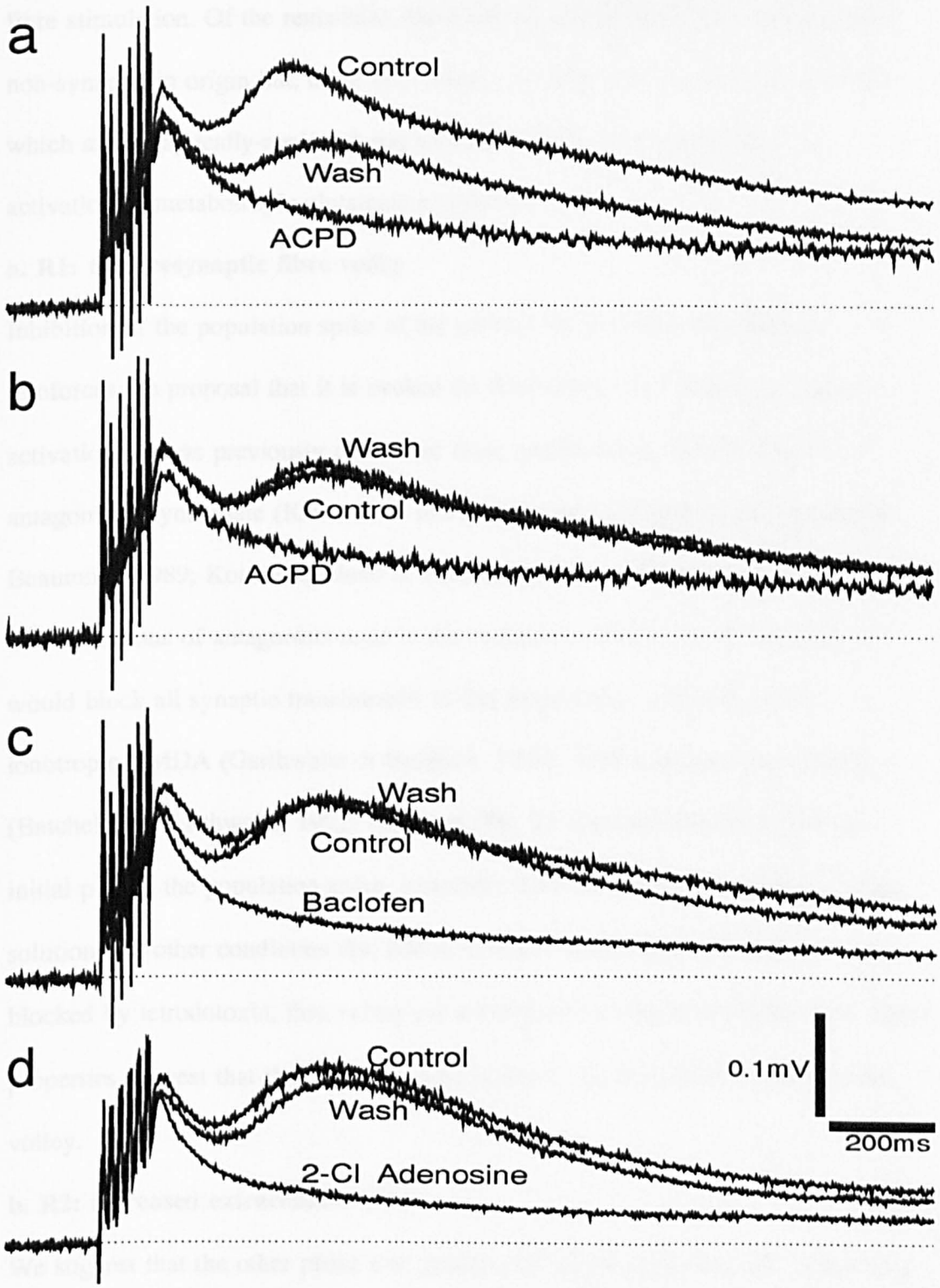


Figure IV(17): ACPD (50 μM), baclofen (10 μM) and 2-chloroadenosine (10 μM) inhibit the R3 wave. In the presence of the antagonist cocktail 6 stimuli were given at 50 Hz. The presumed presynaptic agojnists were all applied until a steady response was obtained, approximately 10 min. A strange but repeatable phenomenon was that the first one or two applications of the drugs was not fully reversible on washout, subsequent applications were fully reversible - compare the effects of ACPD in (a) with(b). Notice that all the drugs changed the response in a similar fashion except that during ACPD there was an increase in noise whereas with the others there was a decrease.

fibre stimulation. Of the remainder, there are two components which seem to be non-synaptic in origin but, more importantly, we have discovered slow potentials which are synaptically-mediated and which are likely to originate from the activation of metabotropic glutamate receptors.

a. R1: the presynaptic fibre volley

Inhibition of the population spike of the parallel fibre response by NBQX reinforces the proposal that it is evoked by AMPA/kainate glutamate receptor activation, as was previously concluded from studies using the less selective antagonists, kynurenate (Kano, Kato & Chang, 1988) and CNQX (Garthwaite & Beaumont, 1989; Konnerth, Llano & Armstrong, 1990; Perkel *et al.*, 1990). Concentrations of antagonists used in the antagonist cocktail were such that it would block all synaptic transmission in this preparation, mediated by the ionotropic NMDA (Garthwaite & Brodbelt, 1989), AMPA/kainate and $GABA_A$ (Batchelor & Garthwaite, 1992) receptors. The R1 remnant coincided with the initial part of the population spike, was short-lived and was resistant to Ca^{2+}-free solution and other conditions that inhibit synaptic transmission. However, it was blocked by tetrodotoxin, thus ruling out it being part of the stimulus artefact. These properties suggest that the R1 component is due to the presynaptic parallel fibre volley.

b. R2: increased extracellular [K^+]

We suggest that the other phase that persists in Ca^{2+}-free solution, R2, is due to a build up of extracellular K^+ ($[K^+]_o$). An increase in $[K^+]_o$, measured with K^+-sensitive electrodes, is known to occur in the molecular layer of the cerebellum following parallel fibre stimulation (Nicholson, ten Bruggencate & Senekowitsch,

1976; Nicholson, ten Bruggencate, Stöckle & Steinberg, 1978; Shibuki & Okada, 1990). After a single stimulus, $[K^+]_o$ measured in this way becomes elevated for about 2 sec, the peak increase being approximately 0.5 mM. With multiple stimuli there is initially a smooth summation of the $[K^+]_o$ signals (Kocsis, Malenka & Waxman, 1983). Such rises in $[K^+]_o$ would be expected to depolarise Purkinje cells. The amplitude and time course of the R2 phase are in agreement with it reflecting an increase in $[K^+]_o$. With single stimuli, the R2 component peaked at around 45 msec and was still not back to baseline after 1 sec. Repeated stimuli at 50 Hz caused summation of the individual responses, the decay of which was a smooth curve (but not a simple exponential) that lasted for several seconds. There is evidence that both presynaptic (parallel fibres and inhibitory interneurones) and postsynaptic (Purkinje cells) elements contribute substantially to the rise in $[K^+]_o$ (Nicholson *et al.*, 1978; Shibuki & Okada, 1990). In the absence of Ca^{2+}, or presence of the antagonist cocktail, the $[K^+]_o$ increase would be expected to originate mainly from the parallel fibres.

c. R3 and R4: novel synaptic potentials

The third component, R3, of the antagonist cocktail-insensitive response was not evident with a single stimulus, but sharply increased in size with additional ones, to attain a maximum amplitude after just five or six pulses at 50 Hz. This saturation behaviour is characteristic of receptor/enzyme-mediated phenomena. The sensitivity to Ca^{2+}-free solution and other conditions which suppress synaptic transmission constitute good evidence that this component is a receptor-mediated, synaptic response. The ensuing hyperpolarising wave, R4, was not studied so extensively but its Ca^{2+}-dependence strongly suggests that it, too, is synaptically generated and

could be due to the foregoing wave.

The antagonist cocktail is not expected to affect the release of glutamate from the parallel fibres, although it should prevent the synaptic activation of inhibitory interneurones and therefore restrict the release of GABA onto Purkinje cells. Further evidence that GABA is not involved came from the demonstration that a $GABA_B$ antagonist (together with the $GABA_A$ antagonist present in the cocktail) was without influence on R3 or R4. Pharmacological tests also argued against an involvement of acetylcholine and noradrenaline. Glutamate is strongly believed to be the transmitter released from parallel fibre terminals and is therefore the most likely effector molecule for the generation of these waves. The only known glutamate receptors that are resistant to the antagonist cocktail used are metabotropic glutamate receptors. Positive evidence also supports the involvement of these receptors: (i) the slow time courses of R3 and R4 are consistent with a mediation by second messengers rather than by direct channel opening; (ii) Purkinje cells are highly enriched in metabotropic glutamate receptors of the $mGluR_1$ subtype (Masu *et al.*, 1991); and (iii) direct activation of metabotropic glutamate receptors with 1S,3R-ACPD emulated the R3/R4 sequence, in terms of both the voltage changes and the resistance to the antagonist cocktail. The effects of 1S,3R-ACPD we observed on Purkinje cells (also see East & Garthwaite, 1992) are consistent with those found with intracellular recording techniques in the same neurones (Crepel *et al.*, 1991; Vranesic *et al.*, 1991). A slow excitatory synaptic wave has been observed previously in turtle Purkinje cells (Larson-Prior, McCrimmon & Slater, 1990) but it is different from the one we report here: for example, it was sensitive to CNQX, AP4 and kynurenate, all of which had no

effect on R3 in our preparation.

Our finding that the R3 component was not apparent in the immature cerebellum is in apparent conflict with the view that metabotropic glutamate receptors are specially active during brain development (Palmer, Nangel-Taylor, Krause, Roxas & Cotman, 1990; Schoepp, Bockaert & Sladeczek, 1990). However, contrary to this view, the mRNA for $mGluR_1$ in the cerebellum, has been found to increase markedly during development (Condorelli, Dell'Albani, Amico, Casabona, Genazzani, Sortino & Nicoletti, 1992). Moreover, the depolarising effect of 1S,3R-ACPD on Purkinje cells is, likewise, larger in adult than in the immature cerebellum (East & Garthwaite, 1992). In developing (14-21 day old) Purkinje cells, ACPD has been found to provoke a rise in $[Ca^{2+}]_i$ but this was most prominent in the soma (Vranesic *et al.*, 1991) rather than in dendrites. This could indicate that, during development, the receptors linked to Ca^{2+} mobilisation (presumably the $mGluR_1$ subtype) are located predominantly in the somatic membrane and are thus not accessed by the glutamate released from parallel fibres. It is also possible, however, that the electrophysiological response is independent of a $[Ca^{2+}]_i$ signal, as has already been suggested for the action of ACPD in the hippocampus (Charpak, Gähwiler, Do & Knöpfel, 1990) and/or that other metabotropic glutamate receptors (Tanabe *et al.*, 1992) contribute to, or are responsible for, the R3/R4 waves.

The two putative antagonists of metabotropic glutamate receptor responses, AP4 and AP3 (Nicoletti *et al.*, 1986; Schoepp & Johnson, 1989), were tested but AP4 was found to have no effect and AP3 depressed all components of the synaptic response, suggesting a presynaptic site of action. Although these

antagonists have been reported to attenuate the increased inositol polyphosphate turnover evoked by ACPD, electrophysiological responses to ACPD have generally been found to be insensitive to them. This applies to the depolarising action of 1S,3R-ACPD on Purkinje cells in cerebellar slices (East & Garthwaite, 1992).

Two other brain areas in which synaptic activation of metabotropic glutamate receptors have been suggested, so far, are the nucleus of the solitary tract (Glaum & Miller, 1992) and the hippocampus. In hippocampal slice cultures stimulation of the mossy fibre input to CA3 pyramidal neurones results in a slow excitation (due to blockade of K^+ channels) which is mimicked by exogenously-applied glutamate and (±)-ACPD (Charpak & Gähwiler, 1991). Additionally, these authors showed that exogenous (±)-ACPD could occlude the slow synaptic response. This was not an approach we could take since 1S,3R-ACPD inhibited synaptic transmission by acting (presumably) at presynaptic sites (Baskys & Malenka, 1991; Crepel, Daniel, Hemert & Jaillard, 1991).

d. Concluding comments

The main conclusion to be drawn from the present study is that parallel fibre-Purkinje cell synaptic transmission involves not only fast excitation mediated through AMPA/kainate receptors but also much slower potentials putatively generated through metabotropic glutamate receptors. The significance of these potentials is likely to be multifarious. Firstly, given that the presence, or otherwise, of the potentials, and their amplitude, depend on the stimulation frequency, the receptors probably equip Purkinje cells with an activity-dependent mechanism. Secondly, the duration of the potentials would be expected to impose alterations in the excitability of these neurones, and hence their responsiveness to subsequent

synaptic activity, for periods in the second-to-minute time scale. Finally, the implicit involvement of second messengers is likely to have repercussions in its own right, one of which may be the activation of enzymes required for synaptic plasticity. A familiar example of synaptic plasticity in the cerebellum is "long term depression" of parallel fibre-Purkinje cell synaptic transmission (Ito, 1984). This phenomenon is a putative cellular correlate of motor learning and it can be elicited either by conjoint climbing and parallel fibre stimulation or by co-application of depolarising stimuli and a metabotropic glutamate receptor agonist (Ito & Karachot, 1990; Linden, Dickinson, Smeyne & Connor, 1991).

SECTION V - CONCLUDING REMARKS

Discovery of the physiological mechanisms of the cerebellum has gone through several phases. Initially, there was the classic work of Sir John Eccles in the 1960's, the techniques used were *in vivo* extracellular and some intracellular recording. The work mainly told us which neurones were excitatory and which were inhibitory. With the increase in interest in amino acids following the work of Curtis and Watkins through the 1970's the pharmacology of the fast responses provided evidence of the transmitters at work. Here the tools were pharmacological. The modern era has been brought about by three techniques that have revolutionised modern neuroscience: the development of know-how concerning cutting and preserving brain slices; the patch clamp technique which has allowed higher resolution recordings of neuronal activity and even single channels and now in the last five years molecular biology. Throughout this time of cerebellar research theoreticians have used the available knowledge and have not been able to resist modelling the cerebellar network. Since the time of Eccles the knowledge of second messenger systems and receptors that couple to them has added another level of complexity to the functioning of the nervous system. No evidence of a synaptically activated second messenger linked receptor has previously been reported in the cerebellum. I have found two novel slow conductances, one excitatory and the other inhibitory. This obviously has implications for the the temporal processing of signals.

SECTION VI - REFERENCES

Abe T., Sugihara H., Nawa H., Shigemoto R., Mizuno N. and Nakanishi S. (1992) Molecular characterization of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca^{2+} signal transduction. J. Biol. Chem. 267: 13361-13368.

Adams P.R., Brown D.A. and Constanti A. (1982) M-currents and other potassium currents in bullfrog sympathetic neurones. J. Physiol. 330: 537-572.

Aizenman E., Lipton S.A. and Loring R.H. (1989) Selective modulation of NMDA responses by reduction and oxidation. Neuron 2: 1257-1263.

Alger B.E., Dhanjal S.S., Dingledine R., Garthwaite J., Henderson G., King G.L., Lipton P., North A., Schwartzkroin P.A., Sears T.A., Segal M., Whittingham T.S. and Williams J. (1984) Brain slice methods. In: R. Dingledine (ed.) Brain slices, Plenum Press, New York, pp 381-437.

Andrade R., Malenka R.C. and Nicoll R.A. (1986) A G protein couples serotonin and $GABA_B$ receptors to the same channels in hippocampus. Science 234: 1261-1265.

Aramori I. and Nakanishi S. (1992) Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. Neuron 8: 757-765.

Ault B., Evans R.H., Francis A.A., Oakes D.J. and Watkins J.C. (1980) Selective depression of excitatory amino acid induced depolarisations by magnesium ions in isolated spinal cord preparations. J. Physiol. 307: 413-428.

Bartrup J.T. and Stone T.W., (1988) Interactions of adenosine and magnesium on rat hippocampal slices. Brain Res. 463: 374-379.

Baskys A. and Malenka R.C. (1991) Agonists at metabotropic glutamate receptors presynaptically inhibit EPSCs in neonatal rat hippocampus. J. Physiol. 444: 687-701.

Batchelor A.M. and Garthwaite J. (1992) $GABA_B$ receptors in the parallel fibre pathway of rat cerebellum. Eur. J. Neurosci. 4:1059-1064.

Beavo J.A. (1990) Multiple phosphodiesterase isozymes: background, nomenclature and implications. In: J.A. Beavo and M.D. Houslay (Eds.) Cyclic Nucleotide Phosphodiesterases: Structure, regulation and Drug Action. Wiley, New York.

Bettler B., Boulter J., Hermans-Borgmeyer I., O'Shea-Greenfield A., Deneris E.S., Moll C., Borgmeyer U., Hollman M. and Heinemann S. (1990) Cloning of a novel glutamate receptor subunit, GluR5: expression in the nervous system during

development. Neuron 5: 583-595.

Betz H. (1990) Ligand-gated ion channels in the brain: the amino acid receptor superfamily. Neuron 5: 383-392.

Betz H. (1991) Glycine receptors: heterogeneous and widespread in the mammalian brain. Trends Neurosci. 14: 458-461.

Biscoe T.J., Evans R.H., Francis A.A., Martin M.R., Watkins J.C., Davies J. and Dray A. (1977) D-α-aminoadipate as a selective antagonist of amino-acid induced and synaptic excitation of mammalian spinal neurones. Nature 270: 743-745.

Blackstone C.D., Supattapone S. and Snyder S.H. (1989) Inositolphospholipid-linked glutamate receptors mediate cerebellar parallel-fibre-Purkinje-cell synaptic transmission. Proc. Natl. Acad. Sci. USA. 86: 4316-4320.

Blake J.F., Brown M.W. and Collingridge G.L. (1988) CNQX blocks acidic amino acid induced depolarisations and synaptic components mediated by non-NMDA receptors in rat hippocampal slices. Neurosci. Letts. 89: 182-186.

Bliss T.V.P. and Collingridge G.L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361: 31-39.

Bliss T.V.P. and Lømo T. (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetised rabbit following stimulation of the perforant path. J. Physiol. 232: 331-356.

Boulter J., Hollman M., O'Shea-Greenfield A., Hartley M., Deneris E., Maron C. and Heinemann S. (1990) Molecular cloning and functional expression of glutamate receptor subunit genes. Science 249: 1033-1037.

Bowery N.G. (1993) $GABA_B$ receptor pharmacology. Ann. Rev. Pharmacol. 33: 109-147.

Bowery N.G., Hudson A.L. and Price G.W. (1987) $GABA_A$ and $GABA_B$ receptor site distribution in the rat central nervous system. Neuroscience 20: 365-383.

Bowery N.G., Price G.W., Turnbull M.J. and Wilkin G.P. (1983) Evidence for the presence of $GABA_B$ receptors on cerebellar Purkinje dendrites. Br. J. Pharmacol. 79(Suppl.): 189P.

Bredt D.S., Hwang P.H., Glatt C., Lowenstein C., Reed R.R. and Snyder S.H. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reducctase. Nature 452: 714-718.

Bredt D.S. and Snyder S.H. (1990) Isolation of nitric oxide synthetase, a calmodulin requiring enzyme. Proc. Natl. Acad. Sci. 87: 682-685.

Bristow D.R., Bowery N.G. and Woodruff G.N. (1986) Light microscopic autoradiographic localisation of [^{3}H]glycine and [^{3}H]strychnine binding sites in rat brain. Eur.J.Pharmacol. 126: 303-307.

Brodal A. and Jahnsen J. (1946) The ponto-cerebellar projection in the rabbit and cat. J. Comp. Neurol. 84: 31-118.

Brown D.A. and Dunn P.M. (1983) Depolarization of rat isolated superior cervical ganglia mediated by β_2-adrenoceptors. Br. J. Pharmacol. 79: 429-439.

Brucato F.H., Morrisett R.A., Wilson W.A. and Swartzwelder H.S. (1992) The $GABA_B$ receptor antagonist, CGP-35348, inhibits paired-pulse disinhibition in the rat dentate gyrus in vivo. Brain Res. 588: 150-153.

Bruns R.F., Fergus J.H., Badger E.W., Bristol J.A., Santay J.D., Hartman J.D., Hays S.J. and Huang C.G. (1987) Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. Nauynn-Schmiedeberg's Arch. Pharmacol. 335: 59-63.

Buck L. and Axel R. (1991) A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65: 175-187.

Burgoyne R.D. and Cambray-Deakin M.A. (1988) The cellular neurobiology of neuronal development: the cerebellar granule cell. Brain Res. Rev. 13: 77-101.

Burnashev N., Khodorova A., Jonas P., Helm P.J., Wisden W., Monyer H., Seeburg P.H. and Sakmann B. (1992a) Calcium-permeable AMPA-kainate receptors in fusiform cerebllar glial cells. Science 256: 1566-1570.

Burnashev N., Monyer H., Seeburg P.H. and Sakmann B. (1992b) Divalent ion permability of AMPA receptor channels is dominated by the edited form of a single subunit. Neuron 8: 189-198.

Burnashev N., Schoepfer R., Monyer H., Ruppersberg J.P., Günther W., Seeburg P.H. and Sakmann B. (1992c) Control by asparagine residues of calcium permeability and magnesium blockade in the NMDA receptor. Science 257: 1415-1419.

Carafoli E. (1987) Intracellular calcium homeostasis. Ann. Rev. Biochem. 56: 395-433.

Cha J-H.J., Makowiec R.L., Penney J.B. and Young A.B. (1990) L-[^{3}H]Glutamate labels the metabotropic excitatory amino acid receptor in rodent brain. Neurosci. Letts. 113: 78-83.

Charpak S. and Gähwiler B.H. (1991) Glutamate mediates a slow synaptic response in hippocampal slice cultures. Proc. Roy. Soc. Lond. B. 243: 221-226.

Charpak S., Gähwiler B.H., Do K.Q. and Knöpfel T. (1990) Potassium conductances in hippocampal neurons blocked by excitatory amino-acid transmitters. Nature 347: 765-767.

Chu D.C.M., Albin R.L., Young A.B. and Penney J.B. (1990) Distribution and kinetics of $GABA_B$ binding sites in rat central nervous system: a quantitative autoradiographic study. Neuroscience 34: 341-357.

Clements J.D., Lester R.A.J., Tong G., Jahr C.E. and Westbrook G.L. (1992) The time course of glutamate in the synaptic cleft. Science 258: 1498-1501.

Cockcroft S.and Thomas G.M.H. (1992) Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. Biochem. J. 288: 1-14.

Cohen P. (1989) The structure and regulation of protein phosphatases. Ann. Rev. Biochem. 58: 453-508.

Cohen P. (1992) Signal integration at the level of protein kinases, protein phosphatases and their substrates. Trends Biochem. Sci. 17: 408-413.

Condorelli D.F., Dell'Albani P., Amico C., Casabona G., Genazzani A.A., Sortino M.A. and Nicoletti F. (1992) Developmental profile of metabotropic glutamate receptor mRNA in rat brain. Mol. Pharmacol. 41: 660-664.

Crepel F., Daniel H., Hemart N. and Jaillard D. (1991) Effects of ACPD and AP3 on parallel-fibre-mediated EPSPs of Purkinje cells in cerebellar slices in vitro. Expl. Brain Res. 86: 402-406.

Curtis D.R. and Watkins J.C. (1960) The excitation and depression of spinal neurones by structurally-related amino-acids. J. Neurochem. 6: 117-141.

D'Angelo E., Rossi P. and Garthwaite J. (1990) Dual-component NMDA receptor currents at a single central synapse. Nature 346: 467-470.

Davies C.H., Davies S.N. and Collingridge G.L. (1990) Paired-pulse depression of monosynaptic GABA-mediated inhibitory postsynaptic responses in rat hippocampus. J. Physiol. 424: 513-531.

Davies C.H., Starkey S.J., Pozza M.F. and Collingridge G.L. (1991) $GABA_B$ autoreceptors regulate the induction of LTP. Nature 349: 609-611.

Davies J.D., Francis A.A., Jones A.W. and Watkins J.C. (1981) 2-Amino-5-phosphonovalerate (2-APV), a potent and selective antagonist of amino-acid induced and synaptic excitation. Neurosci. Letts. 21: 77-81.

Davies J. and Watkins J.C. (1981) Differentiation of kainate and quisqualate receptors in the cat spinal cord by selective antagonism with γ-D-(and L-) glutamylglycine. Brain Res. 206: 172-177.

Davies S.N. and Collingridge G.L. (1989) Role of excitatory amino acid receptors in synaptic transmission in area CA1 of rat hippocampus. Proc. Roy. Soc. Lond. B236: 373-384.

Dawson D.A., Kusumoto K., Graham D.I.., McCulloch, J. and Macrae I.M. (1992) Inhibition of nitric oxide synthesis does not reduce infarct volume in a rat model of focal ischaemia. Neurosci. Letts. 142: 151-154.

Dawson V.L., Dawson T.M., Bartley D.A., Uhl G.R. and Snyder S.H. (1993) Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. J. Neurosci. 13: 2651-2661.

Dixon R.A.F., Kobilka B.K., Strader D.J., Benovic J.L., Dohlman H.G., Frielle T., Balanowski M.A., Bennett C.D., Rands E., Diehl R.E., Mumford R.A., Slater E.E., Sigal I.S., Caron M.G., Lefkowitz R.J. and Strader C.D. (1986) ** Nature 321: 75-79.

Doerner D. and Alger B.E. (1988) Cyclic GMP depresses hippocampal Ca^{2+} current through a mechanism independent of cGMP-dependent protein kinase. Neuron 1: 693-699.

Dohlman H.G., Caron M.G. and Lefkowitz R.J. (1987) A family of receptors coupled to guanine nucleotide regulatory proteins. Biochemistry 26: 2657-2664

Drøbak B.K. (1992) The plant phosphoinositide system. Biochem. J. 288: 697-712.

Dunlap K. and Fischbach G.D. (1981) Neurotransmitters decrease the calcium conductance activated by depolarisation of embryonic chick sensory neurones. J.Physiol. 317: 519-535.

Durand G.M, Gregor P., Zheng X., Bennett M.V., Uhl G.R. and Zukin R.S. (1992) Cloning of an apparent splice variant of the rat N-methyl-D-aspartate receptor NMDAR1 with altered sensitivity to polyamines and activators of protein kinase C. Proc. Natl. Acad. Sci. 89: 9359-9363.

Dutar P. and Nicoll R.A. (1988a) A physiological role for $GABA_B$ receptors in the CNS. Nature 332: 156-158.

Dutar P. and Nicoll R.A. (1988b) Pre- and postsynaptic $GABA_B$ receptors in the hippocampus have different pharmacological properties. Neuron 1: 585-591.

East S.J., Batchelor A.M. and Garthwaite J. (1991) Selective blockade of N-methyl-D-aspartate receptor function by the nitric oxide donor, nitroprusside. Eur. J. Pharmacol. 209: 119-121.

East S.J. and Garthwaite J. (1992) Actions of a metabotropic glutamate receptor agonist in immature and adult rat cerebellum. Eur. J. Pharmacol. 219: 395-400.

Ebert B., Wong E.H.F and Krogsgaard-Larsen P. (1991) identification of a novel NMDA receptor in rat cerebellum. Eur. J. Pharmacol. Mol. Pharmacol. 208: 49-52.

Eccles J.C., Ito M. and Szentagothai J. (1967) The cerebellum as a neuronal machine. Springer, Heidelberg.

Eccles J.C., Llinás R. and Sasaki K. (1966) The mossy fibre-granule cell relay in the cerebellum and its inhibition by Golgi cells. Exp. Brain Res. 1: 82-101.

Eccles J.C., Schmidt R.F. and Willis W.D. (1963) Pharmacological studies on presynaptic inhibition. J. Physiol. 168: 500-530.

Egebjerg J., Bettler B., Hermans-Borgmeyer I and Heinemann S. (1991) Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA. Nature 351: 745-748.

Eimerl S. and Schramm M. (1991) Serum albumin strongly potentiates glutamate neurotoxicity in cultured rat cerebellar granule cells. Neurosci. Letts. 130: 125-127.

Ellisman M.H., Deerinck T.J., Ouyang Y., Beck C.F., Tanksley S.J., Walton P.D., Airey J.A. and Sutko J.L. (1990) Identification and localisation of ryanodine binding proteins in the avian central nervous system. Neuron 5:135-146.

Evans R.H. (1989) *In vitro* preparations of spinal cord or brain which can be used for grease gap electrical recording of excitatory amino acid action. In: J.C. Watkins and G.L. Collingridge (Eds.) The NMDA Receptor. IRL Press, Oxford, pp65-75

Ferraro and Hare (1985)

Ferris C.D., Huganir R.L., Supattapone S. and Snyder S.H. (1989) Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstiuted lipid vesicles. Nature 342: 87-89.

Ferris C.D. and Snyder S.H. (1992) Inositol phosphate receptors and calcium disposition in the brain. J. Neurosci. 12: 1567-1574.

Fletcher A.E., Wilkin G.P. and Bowery N.G. (1989) A putative glycinergic cell in rat cerebellum which may modulate NMDA receptor function in granule cells. Br. J. Pharmacol. 96: 85P.

Foster A.C., Kemp J.A., Leeson P.D., Grimwood S., Donald A.E., Marshall G.R., Priestly T., Smith J.D. and Carling R.W. (1992) Kynurenic acid analogues with improved affinity and selectivity for the glycine site on the *N*-methyl-D-aspartate receptor from rat brain. Mol. Pharmacol. 41: 914-922.

Fujita M., Sato K., Sato M., Inoue T., Kozuka T. and Tohyama M. (1991) Regional distibution of the cells expressing glycine receptor beta subunit mRNA in the rat brain. Brain Res. 560: 23-37.

Furuichi T., Yoshikawa S., Miyawaki A., Wada K, Maeda N. and Mikoshiba K., (1989) Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P_{400}. Nature 342: 32-38.

Gähwiler B.H. and Brown D.A. (1985) $GABA_B$-receptor-activated K^+ current in voltage-clamped CA3 pyramidal cells in hippocampal cultures. Proc. Natl. Acad. Sci. USA. 82: 1558-1562.

Gallagher M. (1990) Robinson et al. (1989) deserves another look. Psychobiol. 118: 258-260.

Gally J.A., Montague P.R., Reeke G.N. and Edelman G.M. (1990) The NO hypothesis: possible effects of a short-lived, rapidly diffusible signal in the development and function of the nervous system. Proc. Natl. Acad. Sci. USA 87: 3547-3551.

Ganong A.H., Lanthorn T.H. and Cotman C.W. (1983) Kynurenic acid inhibits synaptic and acidic amino acid induced responses in the rat hippocampus and spinal cord. Brain Res. 273: 170-174.

Garthwaite J. and Beaumont P.S. (1989) Excitatory amino acid receptors in the parallel fibre pathway in rat cerebellar slices. Neurosci. Lett. 107: 151-156.

Garthwaite J. and Brodbelt A.R. (1989) Synaptic activation of *N*-methyl-D-aspartate and non-*N*-methyl-D-aspartate receptors in the mossy fibre pathway in adult and immature rat cerebellar slices. Neuroscience 29: 401-412.

Garthwaite J., Charles S.L. and Chess-Williams R. (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 336: 385-388.

Garthwaite J., Garthwaite G. and Hajós F. (1986) Amino acid neurotoxicity: relationship to neuronal depolarization in rat cerebellar slices. Neuroscience 18: 449-460.

Garthwaite J., Garthwaite G., Palmer R.M.J. and Moncada S. (1989) NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. Eur. J. Pharmacol. 172: 413-416.

Garthwaite J., Gayton R.J. and Taylor D.C.M. (1990) A Peltier-based system for controlling the temperature of a grease-gap slice chamber. J. Physiol. 424: 4P.

Garthwaite J., Woodhams P.L., Collins M.J. and Balázs R. (1979) On the preparation of brain slices: morphology and cyclic nucleotides. Brain Res. 173: 373-377.

Garthwaite J., Woodhams P.L., Collins M.J. and Balázs R. (1980) A morphological study of incubated slices of rat cerebellum in relation to postnatal age. Devel.

Neurosci. 3: 90-99.

Gilbertson T.A., Scobey R. and Wilson M. (1991) Permeation of calcium ions through non-NMDA glutamate channels in retinal bipolar cells. Science 251: 1613-1615.

Gilman A.G. (1987) G proteins: transducers of receptor-generated signals. Ann. Rev. Biochem. 56: 615-649.

Glaum S.R. and Miller R.J. (1992) Metabotropic glutamate receptors mediate excitatory transmission in the nucleus of the solitary tract. J. Neurosci. 12: 2251-2258.

Hamori J., Mezey, E. and Szentagothai J. (1981) Electron microscopic identification of cerebellar nucleocortical mossy terminals in the rat. Exp. Brain Res. 44: 97-100.

Hampson D.R., Huang X.P., Oberdorfer M.D., Goh J.W., Auyeung A. and Wenthold R.J. (1992) Localisation of AMPA receptors in the hippocampus and cerebellum of the rat using an anti-receptor monoclonal antibody. Neurosci. 50: 11-22.

Harrison N.L. and Simmonds M.A. (1985) Quantitative studies on some antagonists of *N*-methyl-D-aspartate in slices of rat cerebral cortex. Br. J. Pharmacol. 84: 381-391.

Harrison N.L., Lovinger D.M., Lambert N.A., Teyler T.J., Prager R., Ong J. and Kerr D.I.B. (1990) The actions of 2-hydroxy-saclofen at presynaptic $GABA_B$ receptors in the rat hippocampus. Neurosci.Letts. 119: 272-276.

Harrison N.L. (1990) On the presynaptic action of baclofen at inhibitory synapses between cultured rat hippocampal neurones. J. Physiol. 422: 433-446.

Harvey R.J. and Napper R.M.A. (1991) Quantitative studies on the mammalian cerebellum. Prog. Neurobiol. 36: 437-463.

Henzi V. and MacDermott A.B. (1992) Characteristics and function of Ca^{2+}- and inositol 1,4,5-trisphosphate-releasable stores of Ca^{2+} in neurones. Neurosci. 46: 251-273.

Hepler J.R. and Gilman A.G. (1992) G proteins. Trends Biochem. Sci. 17: 383-393.

Herz J.M., Johnson D.A. and Taylor P. (1989) Distance between the agonist and noncompetitive inhibitor sites on the nicotinic acetylchloine receptor. J. Biol. Chem. 264: 12439-12448

Hill D.R. and Bowery N.G. (1981) ^{3}H-Baclofen and ^{3}H-GABA bind to bicuculline-insensitive $GABA_B$ sites in rat brain. Nature 290: 149-152.

Hille B. (1992) G protein-coupled nechanisms and nervous signaling. Neuron 9: 187-195.

Hirano T. and Hagiwara S. (1988) Synaptic transmission between rat cerebellar granule and Purkinje cells in dissociated cell culture: effects of excitatory-amino acid transmitter antagonists. Proc. Natl. Acad. Sci. USA 85: 934-938.

Hofman F., Dostmann W., Keilbach A., Landgraf W. and Ruth P. (1992) Structure and physiological role of cGMP-dependent protein kinase. Biochem. Biophys. Acta 1135: 51-60.

Hollman M., Hartley M. and Heinemann S. (1991) Calcium permeability of KA-AMPA-gated receptor channels: dependence on subunit composition. Science 252: 851-853.

Hollman M., Boulter J., Maron C., Beasley L., Sullivan J., Precht G. and Heinemann S. (1993) Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. Neuron 10: 943-954.

Hollman M., O'Shea-Greenfield A., Rogers S.W. and Heinemann S. (1989) Cloning by functional expresion of a member of the glutamate receptor family. Nature 342: 643-648.

Holmes G (1939) The cerebellum of man. Brain 62: 1-30.

Honoré T., Davies S.N., Dreger J., Fletcher E.J., Jacobsen P., Lodge D. and Neilsen F.E. (1988) Quinoxalinediones: potent competitive non-N-methyl-D-aspartate glutamate receptor antagonists. Science 241: 701-703.

Hosford D.A., Clark S., Cao Z., Wilson W.A., Lin. *et al.* (1992) The role of $GABA_B$ receptor activation in absence seizures of lethargic (lh/lh) mice. Science 257: 398-400.

Houamed K.M., Kuijper J.L., Gilbert T.L., Haldeman B.A., O'Hara P.J., Mulvihill E.R., Almers W. and Hagen F.S. (1991) Cloning, expression, and gene tructure of a G protein-coupled glutamate receptor from rat brain. Science 252: 1318-1321.

Hume R.I., Dingledine R and Heinemann S.F. (1991) Identification of a site in glutamate receptor subunits that controls calcium permeability. Science 253: 1028-1031.

Huston E., Scott R.H. and Dolphin A.C. (1990) A comparison of the effect of calcium channel ligands and $GABA_B$ agonists and antagonists on transmitter release and somatic calcium channel currents in cultured neurones. Neuroscience 38: 721-729.

Hwang P.M., Bredt D.S. and Snyder S.H. (1990) Autoradiographic imaging of phosphoinositide turnover in the brain. Science 249: 802-804.

Imoto K., Busch C., Sakmann B., Mishina M., Konno T., Nakai J., Bujo H., Mori Y., Fukuda K. and Numa S. (1988) Rings of negatively-charged amino-acids determine the acetylcholine receptor channel conductance. Nature 335: 645-648.

Irving A.J., Schofield J.G., Watkins J.C., Sunter D.C. and Collingridge G.L. (1990) 1S,3R-ACPD stimulates and L-AP3 blocks Ca^{2+} mobilization in rat cerebellar neurons. Eur. J. Pharmacol. 186: 363-365.

Ito M. (1984) The cerebellum and neural control. Raven Press, New York.

Ito M. and Karachot L. (1990) Messengers mediating long-term desensitization in cerebellar Purkinje cells. NeuroReport 1: 129-132.

Jiang C., Agulian S. and Haddad G.G. (1991) O_2 tension in adult and neonatal brain slices under several experimental conditions. Brain Res. 568: 159-164.

Jocelyn P.C. (1972) Biochemistry of the SH group. Academic Press, London.

Johnson J.W. and Ascher P. (1987) Glycine potentiates the NMDA response in cultured mouse brain neurones. Nature 325: 529-531.

Kanai Y., Smith C.P. and Hediger M.A. (1993) The elusive transporters with high affinity for glutamate. Trends Neurosci. 16: 365-370.

Kano M., Kato M. and Chang H.S. (1988) The glutamate receptor subtype mediating parallel fibre-Purkinje cell transmission in rabbit cerebellar cortex. Neurosci. Res. 5: 325-337.

Kato K. and Fukada H. (1985) Reduction of $GABA_B$ receptor binding induced by climbing fiber degeneration in the rat cerebellum. Life Sciences 37: 279-288.

Keith J.R. and Rudy J.W. (1990) Why NMDA-receptor-dependent long-term potentiation may not be a mechanism of learning and memory: reappraisal of the NMDA-receptor blockade strategy. Psychobiol. 18: 251-257.

Kelly R.B. (1993) Storage and release of neurotransmitters. Neuron 10 (supplement): 43-53.

Kemp J.A., Foster A.C., Leeson P.D., Priestly T., Tridgett R., Iverson L.L. and Woodruff G.N. (1988) 7-Chlorokynurenic acid is a selective antagonist at the glycine modulatory site of the *N*-methyl-D-aspartate receptor complex. Proc. Natl. Acad. Sci. USA 85: 6547-6550.

Kerr D.I.B., Ong J., Johnston G.A.R., Abbenante J. and Prager R.H. (1988) 2-Hydroxy-saclofen: an improved antagonist at central and peripheral $GABA_B$ receptors. Neurosci.Letts. 92: 92-96.

Kerr D.I.B., Ong J., Prager R.H., Gynther B.D. and Curtis D.R. (1987) Phaclofen:

a peripheral and central baclofen antagonist. Brain Res. 405: 150-154.

Kessler M., Baudry M., Terramini T and Lynch G. (1987) Complex interactions between a glycine binding site and NMDA receptor. Abs. Soc. Neurosci. 13: 209.18.

Kiedrowski L., Costa E. and Wroblewski J.T. (1992) Sodium nitroprusside inhibits *N*-methyl-D-aspartate-evoked calcium influx via a nitric oxide- and cGMP-independent mechanism. Mol. Pharmacol. 41: 779-784.

Kikkawa U., Kishimoto A. and Nishizuka Y. (1989) The protein kinase C family: heterogeneity and its implications. Ann. Rev. Biochem. 58: 31-44.

Kleckner N.W. and Dingledine R. (1988) Requirement for glycine in activation of NMDA receptors expressed in *Xenopus* oocytes. Science 241: 835-836.

Knöpfel T., Audinat E. and Gähwiler B.H. (1990) Climbing fibre responses in olivo-cerebellar slice cultures. i. Microelectrode recordings from Purkinje cells. Eur. J. Neurosci. 2: 726-732.

Knöpfel T. and Staub C. (1991) Activation of metabotropic excitatory amino acid receptors induce an inward current in cerebellar Purkinje cells. Pflügers Arch. 418(Suppl.): R12.

Knöpfel T., Vranesic I., Staub C. and Gähwiler B.H. (1991) Climbing fibre responses in olivo-cerebellar slice cultures. ii. Dynamics of cytosolic calcium in Purkinje cells. Eur. J. Neurosci. 3: 343-348.

Koch C. and Zador A. (1993) The function of dendritic spines: devices subserving biochemical rather than electrical compartmentalization. J. Neurosci. 13:413-422.

Kocsis J.D., Eng D.L. and Bhisitkul R.B. (1984) Adenosine selectively blocks parallel-fibre-mediated synaptic potentials in rat cerebellar cortex. Proc. Natl. Acad. Sci. USA. 81: 6531-6534.

Kocsis J.D., Malenka R.C. and Waxman S.G. (1983) Effects of extracellular potassium concentration on the excitability of the parallel fibres of the rat cerebellum. J. Physiol. 334: 225-244.

Koltchine V., Anantharam V., Wilson A., Baylay H. and Treistman S.N. (1993) Homomeric assemblies of NMDAR1 splice variants are sensitive to ethanol. 152: 13-16.

Konnerth A., Llano I. and Armstrong C.M. (1990) Synaptic currents in cerebellar Purkinje cells. Proc. Natl. Acad. Sci. USA. 87: 2662-2665.

Krogsgaard-Larsen P., Honoré T., Hansen J.J., Curtis D.R. and Lodge D. (1980) New class of glutamate agonist structurally related to ibotenic acid. Nature 284: 64-

66.

Kutsuwada T., Kashiwabuchi N., Mori H., Sakimura K., Kushiya E., Araki K., Megura H., Masaki H., Kumanishi T., Arakawa M. and Mishina M. (1992) Molecular diversity of the NMDA receptor channel. Nature 358: 36-41.

Lagnado L. and Baylor D. (1992) Signal flow in visual transduction. Neuron 8: 995-1002.

Lancaster B., Nicoll R.A. and Perkel D.J. (1991) Calcium activates two types of potassium channels in the rat hippocampal neurons in culture. J. Neurosci. 11; 23-30.

Larson-Prior L.J., McCrimmon D.R. and Slater N.T. (1990) Slow excitatory amino acid receptor-mediated synaptic transmission in turtle cerebellar Purkinje cells. J. Neurophysiol. 63: 637-650.

Laurie D.J., Seeburg P.H. and Wisden W. (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. J.Neurosci. 12: 1063-1076.

Laurie D.J., Wisden W. and Seeburg P.H. (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J. Neurosci. 12: 4151-4172

Lefkowitz R.J., Hausdorff W.P. and Caron M.G. (1990) Role of phosphorylation in desensitisation of the beta-adrenoceptor. Trends in Pharmacol. Sci. 11: 190-194.

Lei S.Z., Pan Z-H., Aggarwal S.K., Chen H-S.V., Hartman J., Sucher N.J. and Lipton S.A. (1992) Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex. Neuron 8: 1087-1099.

Linden D.J., Dickinson M.H., Smeyne M. and Connor J.A. (1991) A long-term depression of AMPA currents in cultured cerebellar Purkinje neurons. Neuron 7: 81-89.

Lindstrom J., Schoepfer R. and Whiting P. (1987) Molecular studies of the neuronal nicotinic acetylcholine receptor family. Mol. Neurobiol. 1: 281-239

Llano I., Dreesen J., Kano M. and Konnerth A. (1991a) Intradendritic release of calcium induced by glutamate in cerebellar Purkinje cells. Neuron 7: 577-583.

Llano I. and Gerschenfeld H.M. (1993) Inhibitory synaptic currents in stellate cells of rat cerebellar slices. J.Physiol. 468: 177-200.

Llano I., Marty A., Armstrong C.M. and Konnerth A. (1991b) Synaptic- and agonist-induced excitatory currents of Purkinje cells in rat cerebellar slices. J. Physiol. 434: 183-213.

Llinas R. and Hess R. (1976) Tetrodotoxin-resistant dendritic spikes in avian Purkinje cells. Proc. Natl. Acad. Sci. USA. 73: 2520-2523.

Llinas R. and Sugimori M. (1980) Electrophysiological properties of *in vitro* Purkinje cell somata in mammalian cerebellar slices. J. Physiol. 305: 171-195.

Llinas R., Sugimori M. and Silver R.B. (1992) Microdomains of high calcium concentration in a presynaptic terminal. Science 256: 677-679.

Lodge D. and Johnson K.M. (1990) Noncompetitive excitatory amino acid receptor antagonists. Trends Pharm. Sci. 11: 81-86.

Lüddens H., Pritchett D.B., Kohler M., Killisch T., Keinanen K., Monyer H., Sprengel R. and Seeburg P.H. (1990) Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. Nature 346: 648-651.

Lundberg A. (1971) Function of the ventral spinocerebellar tract - A new hypothesis. Exp. Brain Res. 12: 317-330.

MacDermott A.B., Mayer M.L., Westbrook G.L., Smith S.J. and Barker J.L. (1986) NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. Nature 321: 519-522.

Malosio M-L., Marquèze-Pouey B., Kuhse J. and Betz H. (1991) Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. EMBO J. 10: 2401-2409.

Manzoni O., Prezeau L., Desagher S., Sahuquet A., Sladeczek F., Bockaert J. and Fagni L. (1992) Sodium nitroprusside blocks NMDA receptors via formation of ferrocyanide ions. NeuroReport 3: 77-80.

Manzoni O., Prezeau L., Marin P., Deshager S., Bockaert J. and Fagni L. (1992) Nitric oxide-induced blockade of NMDA receptors. Neuron 8: 653-662.

Marescaux C., Vergnes M. and Bernasconi R. (1992) $GABA_B$ receptor antagonists: potential new anti-absence drugs. J. Neural Trans. (Suppl.) 35: 179-188.

Martin W., Smith J.A. and White D.G. (1986) The mechanisms by which haemoglobin inhibits the relaxation of rabbit aorta induced by nitrovasodilators, nitric oxide, or bovine retractor penis inhibitory factor. Br. J. Pharmacol. 89: 563-

Martinelli G.P., Holstein G.R., Pasik P. and Cohen B. (1992) Monoclonal antibodies for ultrastructural visualisation of L-baclofen-sensitive $GABA_B$ receptor sites. Neuroscience 46: 23-33.

Masu M., Tanabe Y., Tsuchida K., Shigemoto R. and Nakanishi S. (1991) Sequence and expression of a metabotropic glutamate receptor. Nature 349: 760-765.

Mayer B. (1993) Molecular characteristics and enzymology of nitric oxide synthase and soluble guanylyl cyclase in the CNS. Seminars Neurosci. 5: 197-205.

Mayer B., Klatt P., Böhme E and Schmidt K. (1992) Regulation of neuronal nitric oxide and cyclicGMP formation by Ca^{2+}. J. Neurochem. 59: 2024-2029.

McDonald J.W. and Johnston M.V. (1990) Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res. Rev. 15: 41-70

McDonald J.W., Penney J.B., Johnston M.V. and Young A.B. (1990) Characterization and regional distribution of strychnine-insensitive [^{3}H]glycine binding sites in rat brain by quantitative receptor autoradiography. Neuroscience 35: 653-668.

Meguro H., Mori H., Araki K., Kushiya E., Kutsawada T., Yamazaki M., Kumanishi T., Arakawa M., Sakimura K. and Mishina M. (1992) Functional characterisation of a heteromeric NMDA receptor channel expressed from cloned cDNAs. Nature 357: 70-74.

Mignery G.A., Sudhof T.C., Takei K. and De Camilli P. (1989) Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor. Nature 342: 192-195.

Mikoshiba K., Huchet M. and Changeux J.-P. (1979) Biochemical and immunological studies on the P_{400} protein, a protein characteristic of the Purkinje cell from mouse and rat cerebellum. Devl. Neurosci. 7: 179-187.

Miller R.J. (1991) Metabotropic excitatory amino acid receptors reveal their true colours. Trends Pharmacol. Sci. 12: 365-367.

Minakami R., Katsuki F. & Sugiyama H., (1993) A variant of metabotropic receptor subtype 5: an evolutionary conserved insertion with no termination codon. Biochem. Biophys. Res. Comm. 194: 622-627

Mishina M., Takai T., Imoto K., Noda M., Takahashi T., Numa S., Methfessel C. and Sakmann B. (1986) Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321: 406-411.

Monaghan D.T. and Beaton J.A. (1991) Quinolinate differentiates between forebrain and cerebellar NMDA receptors. Eur. J. Pharmacol 194: 123-125.

Monaghan D.T. and Beaton J.A. (1992) Pharmacologically-distinct NMDA receptor populations of the cerebellum, medial thalamic nuclei and forebrain. Mol. Neuropharmacol. 2: 71-75.

Monaghan D.T., Olverman H.J., Nguyen L., Watkins J.C. and Cotman C.W. (1988) Two classes of N-methyl-D-aspartate recognition sites: differential distribution and differential regulation by glycine. Proc. Natl. Acad. Sci. 85; 9836-9840.

Montminy M.R., Gonzalez G.A. and Yamamoto K.K. (1990) Regulation of cAMP-inducible genes by CREB. Trends Neurosci. 13: 184-188.

Monyer H., Sceburg P.H. and Wisden W. (1991) Glutamate-operated channels; dedvelopmentally early and mature forms arise by alternative splicing. Neuron 6: 799-810.

Monyer H., Sprengel R., Schoepfer R., Herb A., Higuchi M., Lomeli H., Burnashev N., Sakmann B. and Seeburg P.H. (1992) Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 256: 1217-1221.

Moriyoshi K., Masu M., Ishii T., Shigemoto R., Mizuno N. and Nakanishi S. (1991) Molecular cloning and characterisation of the rat NMDA receptor. Nature 354: 31-37.

Morris R.G.M., Anderson E., Lynch G.S. and Baudry M. (1986) Selective impairment of learning and blockade of lon-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. Nature 319: 774-776.

Morris R.G.M. (1989) Synaptic plasticity and learning: selective impairment of learning in rats and blockade of long-term potentiation *in vivo* by the N-methyl-D-aspartate receptor antagonist AP5. J. Neurosci. 9:3040-3057.

Morris R.G.M. (1990) It's heads they win, tails I lose! Psychobiol. 18: 261-266.

Morris R.G.M., Davis S. and Butcher S.P. (1990) Hippocampal synaptic plasticity and NMDA receptors: a role in information storage? Phil. Trans. Roy. Soc. Lond. B329: 187-204.

Mott D.D. and Lewis D.V. (1991) Facilitation of the induction of long-term potentiation by $GABA_B$ receptors. Science 252: 1718-1720.

Mott D.D., Lewis D.V., Ferrari C.M., Wilson W.A. and Swartzwelder H.S. (1990) Baclofen facilitates the development of long-term potentiation in the rat dentate gyrus. Neurosci. Letts. 113: 222-226

Müller T., Möller T., Berger T., Schnitzer J. and Kettenmann H. (1992) calcium entry through kainate receptors and resulting potassium-channel blockade in Bergmann glial cells. Science 256: 1563-1566.

Murphy S.N. and Miller R.J. (1988) A glutamate receptor regulates Ca^{2+} mobilization in hippocampal neurons. Proc. Natl. Acad. Sci. USA. 85: 8737-8741.

Nakade S., Maeda N. and Mikoshiba K. (1991) Involvement of the C-terminus of the inositol 1,4,5-trisphosphate receptor in Ca^{2+} release analysed using region-specific monoclonal antibodies. Biochem. J. 277: 125-131.

Nakanishi N., Shneider N.A. and Axel, R. (1990) A family of glutamate receptor

genes: evidence for the formation of heteromultimeric receptors with distinct channel properties. Neuron 5: 569-581.

Nathan T. and Lambert J.D. (1991) Depression of the fast IPSP underlies paired-pulse facilitation in area CA1 of the rat hippocampus. J. Neurophysiol. 66: 1704-1715.

Newberry N.R. and Nicoll R.A. (1984a) A bicuculline-resistant inhibitory postsynaptic potential in rat hippocampal pyramidal cells in vitro. J. Physiol. 348:239-254.

Newberry N.R. and Nicoll R.A. (1984b) Direct hyperpolarising effect of baclofen on hippocampal pyramidal cells. Nature 308: 450-452.

Nichols D. and Attwell D. (1990) The release and uptake of excitatory amino acids. Trends Pharmacol. Sci. 11: 462-468.

Nicholson C., ten Bruggencate G. and Senekowitsch R. (1976) Large potassium signals and slow potentials evoked during aminopyridine or barium superfusion in cat cerebellum. Brain Res. 113: 606-610.

Nicholson C., ten Bruggencate G., Stöckle H. and Steinberg R. (1978) Calcium and potassium changes in extracellular microenvironment of cat cerebellar cortex. J. Neurophysiol. 41: 1026-1039.

Nicoletti F., Iadarola M.J., Wrobleski J.T. & Costa E. (1986) Excitatory amino acid recognition sites coupled with inositol phospholipid metabolism: developmental changes and interaction with α_1-adrenoceptors. Proc. Natl. Acad. Sci USA. 83: 1931-1935.

Nicoll R.A., Madison D.V. and Lancaster B. (1987) Noradrenergic modulation of neuronal excitability in mammalian hippocampus. In: H.Y. Meltzer (Ed.) Psychopharmacology: the third generation of progress. Raven Press, New York, pp105-112.

Ogata N. (1991) Physiological and pharmacological charcterisation of $GABA_B$ receptor-mediated potassium conductance. In: Bowery N.G., Bittiger H. and Olpe H.-R. $GABA_B$ receptors in mammalian function John Wiley, Chichester, pp 274-291.

Olpe H.-R., Karlsson G., Pozza M.F., Brugger F., Steinman M., van Reizen H., Fagg G., Hall R.G., Froestl W. & Bittiger H., (1990) CGP 35 348: a centrally active blocker of $GABA_B$ receptors. Eur. J. Pharmacol. 187: 27-38.

Olsen R.W. and Tobin A.J. (1990) Molecular biology of $GABA_A$ receptors. FASEB J. 4: 1469-1480

Olson J.M., Greenamyre J.T., Penney J.B. and Young A.B. (1987)

Autoradiographic localization of cerebellar excitatory amino acid binding sites in the mouse. Neuroscience 22: 913-923.

Oscarsson O. (1973) Functional organisation of spinocerebellar paths. In: Iggo A. Handbook of sensory physiology. Vol. 2. Somatosensory system. Springer-Verlag, Berlin, 339-380.

Otis T.S. and Mody I. (1992) Differential activation of $GABA_A$ and $GABA_B$ receptors by spontaneously released transmitter. J. Neurophysiol. 67: 227-235.

Ottersen O.P., Storm-Mathisen J. and Somogyi P. (1988) Colocalisation of glycine-like and GABA-like immunoreactivities in Golgi cell terminals in the rat cerebellum: a postembedding light and electron microscopic study. Brain Res. 450: 342-353.

Palay S.L. and Chan-Palay V. (1974) Cerebellar cortex: cytology and organisation. Springer-Verlag, Berlin.

Palmer E., Nangel-Taylor K., Krause J.D., Roxas A. and Cotman C.W. (1990) Changes in excitatory amino acid modulation of phosphoinositide metabolism during development. Dev. Brain. Res. 51: 132-134.

Palmer E., Monaghan D.T. and Cotman C.W. (1989) Trans-ACPD, a selective agonist of the phosphoinositide-coupled excitatory amino acid receptor. Eur. J. Pharmacol. 166: 585-587.

Palmer R.M.J., Ferrige A.G. and Moncada S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327: 524-526.

Pazin M.J. and Williams L.T. (1992) Triggering signaling cascades by receptor tyrosine kinases.Trends Biochem. Sci. 17 374-378.

Perkel D.J., Hestrin S., Sah P. and Nicoll R.A. (1990) Excitatory synaptic currents in Purkinje cells. Proc. Roy. Soc. Lond. B241: 116-121.

Petralia R.S. and Wenthold R.J. (1992) Light and electron immunocytochemical localisation of AMPA-selective glutamate receptors in the rat brain. J. Comp. Neurol. 318: 329-354.

Peunova N. and Enikolopov G. (1993) Amplification of calcium-induced gene transcription by nitric oxide in neuronal cells. Nature 363: 450-453.

Pin J.P., Waeber C., Prezeau L., Bockaert J. and Heinemann S. (1992) Alternative splicing generates metabotropic glutamate receptors inducing different patterns of calcium release in *Xenopus* oocytes. Proc. Natl. Acad. Sci. USA 89: 10331-10335.

Qian N. and Sejnowski T.J. (1990) When is an inhibitory synapse effective? Proc.

Natl. Acad. Sci. USA 87: 8145-8149.

Rabacchi S., Bailly Y., Delhaye-Bouchard N. and Mariani J. (1992) Involvement of the N-methyl-D-aspartate (NMDA)receptor in synapse elimination during cerebellar development. Science 256: 1823-1825.

Regehr W.G. and Tank D.W. (1992) Calcium concentration dynamics produced by synaptic activation of CA1 hippocampal pyramidal cells. J. Neurosci. 12: 4202-4223.

Roberts A. and Bush B.M.H. (1981) Neurones without impulses: their significance for vertebrate and invertebrate nervous systems. Cambridge Univ. Press, Cambridge.

Role L.W. (1992) Diversity in primary structure and function of neuronal nicotinic acetylcholine receptor channels. Curr. Opin.Neurobiol. 2: 254-262.

Rudy J.W. and Keith J.R. (1990) Why NMDA-receptor-dependent long-term potentiation may not be a learning and memory mechanism, or is it Memorex? A reply to Morris, Gallagher, and Staubli. Psychobiol. 18: 269-272.

Sakimura K., Morito T., Kushiya E. and Mishina M. (1992) primary structure and expression of the γ2 subunit of the glutamate receptor channel selective for kainate. Neuron 8: 267-274.

Sakurada K., Masu M. and Nakanishi S. (1993) Alteration of Ca^{2+} permeability and sensitivity to Mg^{2+} and channel blockers by a single amino acid substitution in the N-methyl-D-aspartate receptor. J. Biol Chem. 268: 410-415.

Sato K., Kiyama H. and Tohyama M. (1993) The differential expression patterns of messenger RNAs encoding non-N-methyl-D-aspartate glutamate receptor subunits (GluR1-4) in the rat brain. Neurosci. 52: 515-539.

Schneggenburger R., Zhou Z., Konnerth A. and Neher E. (1993) Fractional contribution of calcium to the cation current through glutamate receptor channels. Neuron 11: 133-143.

Schoepp D., Bockaert J. and Sladeczek F. (1990) Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. Trends Pharmacol. Sci. 11: 508-515.

Schoepp D.D. and Conn P.J. (1993) Metabotropic glutamate receptors in brain function and pathology. Trends Pharmacol. Sci. 14: 13-20.

Schoepp D.D. and Johnson B.J. (1989) Inhibition of excitatory amino acid-stimulated phosphoinositide hydrolysis in the neonatal rat hippocampus by 2-amino-3-phosphonoproprionate. J. Neurochem. 53: 1865-1870.

Schuman E.M. and Madison D.V. (1993) Nitric oxide as an intercellular signalin long-term potentiation. Seminars Neurosci. 5: 207-215.

Seeburg P.H. (1993) The molecular biology of mammalian glutamate receptors. Trends Neurosci. 9: 359-365.

Sheardown M.J., Nielsen E.Ø., Hansen A.J., Jacobsen P. and Honoré T. (1990) 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline: a neuroprotectant for cerebral ischemia. Science 247: 571-574.

Shibuki K. and Okada D. (1990) Long-term changes in rat cerebellar slices reflected in extracellular K^+ activity. Neurosci. Lett. 113: 34-39.

Silver R.A., Traynelis S.F. and Cull-Candy S.G. (1992) Rapid-time-course miniture and evoked excitatory currents at cerebellar synapses *in situ*. Nature 355: 163-166.

Skilling S.R., Smullin D.H., Beitz A.J. and Larson A.A. (1988) Extracellular amino acid concentrations in the dorsal spinal cord of freely moving rats following veratridine and nociceptive stimulation. J. Neurochem. 51: 127-132.

Sladeczek F., Pin J-P., Récasens M., Bockaert J. and Weiss S. (1985) Glutamate stimulates inositol phosphate formation in striatal neurones. Nature 317: 717-719.

Sladeczek F., Récasens M. and Bockaert J. (1988) A new mechanism for glutamate receptor action: phosphoinositide hydrolysis. Trends Neurosci. 11: 545-549.

Smith K.E., Borden L.A., Hartig P.R., Branchek T. and Weinshank R.L. (1992) Cloning and expression of a glycine transporter reveal colocalisation with NMDA receptors. Neuron 8: 927-935.

Sobusiak T., Zimny R. and Matlosz Z. (1971) Primary glossopharyngeal and vagal afferent projection into the cerebellum in the dog. J. Hirnforsch. 13:117-134.

Soltesz I., Lightfowler S., Leresche N. and Crunelli V. (1989) On the properties and origin of the $GABA_B$ inhibitory postsynaptic potential recorded in morphologically identified projection cells of the cat dorsal lateral geniculate nucleus. Neuroscience 33: 23-33.

Sommer B., Keinanen K., Verdoon T.A., Wisden W., Burnashev N., Herb A., Köhler M., Takagi T., Sakmann B. and Seeburg P.H. (1990) Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. Science 249: 1580-1585.

Somogyi P., Halasy K., Somogyi J., Storm-Mathisen J. and Ottersen O.P. (1986) Quantification of immunogold labelling reveals enrichment of glutamate in mossy fibre and parallel fibre terminals in cat cerebellum. Neuroscience 19: 1045-1050.

Southam E. and Garthwaite J. (1991) Comparative effects of some nitric oxide

donors on cyclic GMP levels in rat cerebellar slices. Neurosci. Letts. 130: 107-111.

Southam E., Morris R. and Garthwaite J. (1992) Sources and targets of nitric oxide in rat cerebellum. Neurosci. Letts. 137: 241-244.

Starke K. (1981) Presynaptic receptors. Ann. Rev. Pharmacol. Toxicol. 21:7-30.

Staub C., Vranesic I. and Knöpfel T. (1992) Responses to metabotropic glutamate receptor activation of cerebellar Purkinje cells: induction of an inward current. Eur. J. Neurosci. in press

Staubli U. (1990) Behavioral reflections of the NMDA system. Psychobiol. 18: 267-268.

Stern P., Behe P., Schoepfer R. and Colquhoun D. (1993) Single-channel conductances of NMDA receptors expressed from cloned cDNAs: comprison with native receptors. Proc. Roy. Soc. Lond. B250: 271-277.

Stone T.W. (1979) Glutamate as the neurotransmitter of cerebellar granule cells in the rat: electrophysiological evidence. Br. J. Pharmacol. 66: 291-296.

Sugita S., Johnson S.W. and North R.A. (1992) Synaptic inputs to $GABA_A$ and $GABA_B$ receptors originate from discrete afferent neurones. Neurosci. Letts. 134: 207-211.

Sugiyama H., Ito I. and Hirono C. (1987) A new type of glutamate receptor linked to inositol phospholipid metabolism. Nature 325: 531-533.

Swope S.L., Moss S.J., Blackstone C.D. and Huganir R.L. (1992) Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity. FASEB J. 6: 2514-2523.

Takei K., Stukenbrok H., Metcalf A., Mignery G.A., Südhof T.C., Volpe P. and De Camilli P. (1992) Ca^{2+} stores in Purkinje neurons: endoplasmic reticulum subcompartments demonstrated by the heterogeneous distribution of the $InsP_3$ receptor, Ca^{2+}-ATPase, and calsequestrin. J. Neurosci. 12: 489-505.

Tanabe Y., Masu M., Ishii T., Shigemoto R. and Nakanishi S. (1992) A family of metabotropic glutamate receptors. Neuron 8: 169-179.

Thalmann R. (1987) Pertussis toxin blocks a late inhibitory postsynaptic potential in hippocampal CA3 neurons. Neurosci. Letts. 82: 41-46.

Thompson S.M., Copogna M. and Scanziani M. (1993) Presynaptic inhibition in the hippocampus. Trends Neurosci. 16: 222-227.

Thompson S.M. and Gähwiler B.H. (1992) Comparison of the actions of baclofen at pre- and postsynaptic receptors in the rat hippocampus *in vitro*. J. Physiol. 451:

329-345.

Thompson W.J. (1991) Cyclic nucleotide phosphodiesterases: pharmacology, biochemistry and function. Pharmac. Ther. 51: 13-33.

Thomson A.M. (1990) Glycine is a coagonist at the NMDA receptor/channel complex. Prog. Neurobiol. 35: 53-74.

Toyoshima C. and Unwin N. (1988) Ion channel of acetylcholine receptor reconstructed from images of postsynaptic membranes. Nature 336: 247-250.

Tsien R.W. and Tsien R.Y. (1990) Calcium channels, stores and oscillations. Ann. Rev. Cell Biol.6: 715-760.

Turner J.P. and Meldrum B.S. (1991) L-Glutamate diethyl ester and deaminated analogues as excitatory amino acid antagonists in rat cerebral cortex. Br. J. Pharmacol. 104: 445-451.

Vranesic I., Batchelor A.M., Gähwiler B.H., Garthwaite J., Staub C. and Knöpfel T. (1991) Trans-ACPD-induced Ca^{2+} signals in cerebellar Purkinje cells. NeuroReport 2: 759-762.

Vranesic I. and Knöpfel T. (1991) Calculation of calcium dynamics from single wavelength fura-2 fluorescence recordings. Pflügers Arch. 418: 184-189.

Wagner K.R., Mei L. and Huganir R.L. (1991) Protein tyrosine kinases and phosphatases in the nervous system. Current Opinion Neurobiol. 1: 65-73.

Watkins J.C. and Collingridge G.L. (1989) The NMDA receptor. IRL Press, Oxford.

Werner P., Voigt M., Keinänen K., Wisden W. and Seeburg P.H. (1991) Cloning of a putative high-affinity kainate receptor expressed predominantly in hippocampal CA3 cells. Nature 351: 742-744.

Wilkin G.P., Csillag A., Balázs R., Kingsbury A.E., Wilson J.E. and Johnson A.L. (1981) Localisation of high affinity [^{3}H]glycine transport sites in the cerebellar cortex. Brain Res. 216: 11-33.

Wilkin G.P., Hudson A.L., Hill D.R. and Bowery N.G. (1981) Autoradiographic localisation of $GABA_B$ receptors in rat cerebellum. Nature 290: 584-587.

Williams J.H., Errington M.L., Li Y.-G., Lynch M.A. and Bliss T.V.P. (1993) The search for the retrograde messengers in long-term potentiation. Seminars Neurosci. 5: 149-158.

Wisden W., Laurie D.J., Monyer H and Seeburg P.H. (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon,

mescencephalon. J. Neurosci. 12: 1040-1062.

Wojcik W.J. and Neff N.H. (1984) Gamma-aminobutyric acid B receptors are negatively coupled to adenylate cyclase in brain, and in the cerebellum these receptors may be associated with granule cells. Mol. Pharmacol. 25: 24-28.

Wojcik W.J., Travagli R.A., Costa E. and Bertolino M. (1990) Baclofen inhibits with high affinity an L-type-like voltage-dependent calcium channel in cerebellar granule cell cultures. Neuropharmacol. 29: 969-972.

Wroblewski J.T., Kiedrowski L., Raulli R. and Costa E. (1991) Nitric oxide: a messenger of cerebellar granule cells. In: V.I. Teichberg & L. Turski (Eds.) Excitatory amino acids and second messenger systems, Springer, Berlin, pp103-120.

Wyllie D.J.A., Traynelis S.F. and Cull-Candy S.G. (1991) Multiple non-NMDA glutamate receptors in rat cerebellar granule cells in explant culture. J. Physiol. 446: 178P

Xia Z., Refsdal C.D., Merchant K.M., Dorsa D.M. and Storm D.R. (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.

Yingcharoen K. and Rinvik E. (1982) Branched projections from the nucleus prepositus hypoglossi to the oculomotor nucleus and the cerebellum. A retrograde fluorescent double-labeling study in cat. Brain Res. 246: 133-136.

Yuzaki M, and Mikoshiba K. (1992) Pharmacological and immunocytochemical characterization of metabotropic glutamate receptors in cultured Purkinje cells. J.Neurosci. 12: 4253-4263.

Zhou N., Hammerland L.G. and Parks T.N. (1993) γ-D-Glutamylaminomethyl sulfonic acid (GAMS) distinguishes kainic acid- from AMPA-induced responses in *Xenopus* oocytes expressing chick brain glutamate receptors. Neuropharmacol. 32: 767-775.