ANALYSIS OF EXCITATORY AMINO ACID RECEPTORS IN THE RAT SPINAL CORD IN VIVO AND IN VITRO

by

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Abstract

Several endogenous amino acids including L-glutamate and L-aspartate have potent excitatory effects in the central nervous system. They are thought to act as synaptic transmitters in many neural pathways including those in the spinal cord. Three distinct receptors have been described through which these excitatory amino acids exert their effects. These are referred to as quisqualate, kainate and N-methyl-D-aspartate (NMDA) receptors, after the exogenous excitants most specific for each. In addition, sub-types of the NMDA receptor have been proposed to account for differences observed in the actions of the endogenous excitant quinolinate (2,3-pyridine dicarboxylate) in various regions of the nervous system. The characterization of excitant amino acid receptors has been accomplished primarily using two or more potent antagonists which include D-(-)-2-amino-5-phosphonovalerate (APV), a specific NMDA antagonist, and kynurenate, a compound related to quinolinate which potently attenuates the actions of NMDA- and kainate-like excitants.

Structure-activity studies of amino acid receptors were undertaken using standard extracellular recording and iontophoretic techniques in the dorsal horn of the spinal cord in vivo, and compared with the neocortex of the rat. In addition, a spinal cord slice preparation was developed wherein dorso-ventral longitudinal slices were prepared from the lumbar enlargement of weanling rats (50 - 125 g). The slices were maintained in an "interface" tissue bath of novel design. Extracellular recording of several hours duration and up to 8 hours after slice

preparation were routinely possible.

Conformationally restricted analogues of glutamate, aspartate and quinolinate were examined for agonist and antagonist actions in the rat spinal cord in vivo and in vitro. Compounds found to be excitants were compared directly with quisqualate, kainate, and NMDA for sensitivity to blockade by APV and kynurenate applied both iontophoretically and in the bathing medium; antagonist dose-response curves were constructed for the actions of APV and kynurenate against quisqualate, kainate, quinolinate and NMDA. The conformationally restricted compounds found to be antagonists were examined to determine their potency and specificity against excitations elicited by quisqualate, kainate, quinolinate and NMDA.

Although quinolinate is known to be NMDA-like in the hippocampus and cortex, when compared to quisqualate, kainate and NMDA in the spinal cord in vitro, it proved to be unique. A fourth receptor (the "QUIN" receptor) is proposed to account for its actions in the spinal cord.

Three of the isomers of 1-amino-1,3-cyclopentane dicarboxylate (ACPD), conformationally restricted analogues of glutamate, were potently blocked by APV and KYNA and were therefore classified as NMDA-like. The fourth, D-trans-ACPD, was indistinguishable from quinolinate in terms of both potency and sensitivity to antagonists. The (-) isomer of trans-1-amino-1,2-cyclopentane dicarboxylate proved to be an antagonist with greater potency against excitations

elicited by quisqualate and kainate than those of NMDA. These findings are, in many ways, different from what has been observed in the hippocampal slice.

Several pyridine derivatives were examined; 2,5- and 2,6-pyridine dicarboxylate were weak excitants behaving like quisqualate in the presence of APV and kynurenate. No other pyridines were excitatory; however 2,4-pyridine dicarboxylate was observed to be a weak, non-specific antagonist similar in action to acridinate (an antagonist closely related to kynurenate). None of the pyridine derivatives, save quinolinate, are excitatory in the hippocampus.

Structural analysis of the active compounds tested, in consideration of previous studies, shows that three points of attachment (two carboxyl and one amino group) are necessary for activation of NMDA, quisqualate and quinolinate receptors in the spinal cord. The location of the distal or γ-carboxyl group relative to the α ionic groups appears to be the primary factor determining the activity of a conformationally restricted compound. The absolute distance between the γ-carboxyl and α-carbon appears to play a secondary rôle in determining the action of a compound.

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I. Introduction

A. History of excitatory amino acid research

Historically, interest in amino acids in the nervous system was initiated by Hayashi (1954) who applied glutamate and aspartate topically to the cortex of dogs and observed overt convulsive behaviour. In a review of his work (1959) Hayashi discussed several possible rôles for glutamate in the central nervous system in the light of results reported by Waelsch (1955) who found that glutamate was accumulated in the brain, and appeared to be taken up selectively across the blood brain barrier. Although Hayashi stated that convulsions produced by topically applied glutamate are physiological rather than pharmacological (since the threshold concentration was similar to the actual "wet weight" glutamate content of the brain, ie. it was applied at a physiological concentration), he argued mainly against the proposition that glutamate is a major excitatory transmitter because of the apparent involvement of glutamate in the energy supply of the brain. It was then thought that glutamate was an energy source for the central nervous system (secondary to glucose). However, on the basis that topically applied glutamate produced convulsions with a delay of 19 minutes, Hayashi did postulate that glutamate was linked metabolically to a compound which was the elusive excitatory transmitter.

The discovery in 1959 by Curtis, Phillis and Watkins that γ-aminobutyric acid (GABA; a neutral amino acid) could alter the electrical activity of neurones led to

the idea that the other amino acids, in particular aspartic, glutamic (Fig. 1, A & B) and cysteic, may in fact have rôles other than metabolic ones in the central nervous system. Systematic investigations into the excitation of central neurones by amino acids began in 1960 with the endogenous and readily available naturally occuring substances (Curtis et al., 1960a). That exogenous and synthetic analogues of glutamate and aspartate could possess even greater abilities than glutamate itself to excite neurones was discovered early in the '60's, and by 1963 several basic structural requirements for excitation were recognized. The ability to excite "lay in the possession by a compound of one amino and two acidic groups, the latter being separated from each other by a distance equal to that occupied by a chain of two to three carbon atoms, whilst the amino group should optimally be situated in the alpha position with respect to one of the acidic groups" (Curtis and Watkins, 1963). In addition, Curtis and Watkins (1963) reported that the D-isomers of most of the compounds tested were of a similar potency to their L counterparts, but that the addition of a methyl group to the nitrogen of D-aspartate resulted in the extremely potent excitant N-methyl-D-aspartate (NMDA). With hind-sight, the basic structural requirements elucidated in the early 1960's are compatible with current models. Curtis and his colleagues were convinced, however, that the action of glutamate and other excitatory amino acids was completely non-specific and "that the site of action is a fundamental structural component of the membranes of all central neurones" (Curtis and Watkins, 1963).

The criteria for identification of transmitters at that time were based entirely

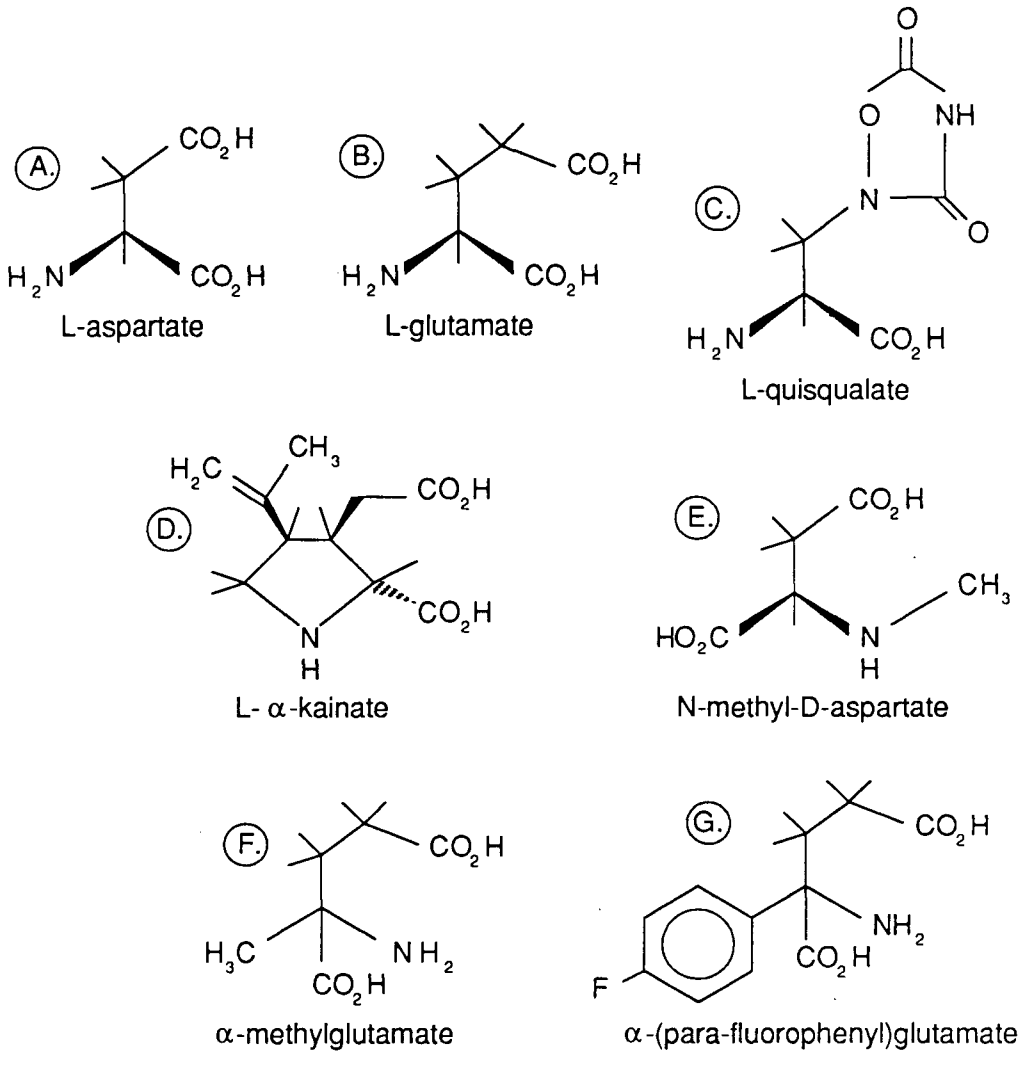


Figure 1. Diagrammatic representation of the structures of A. L-aspartate, B. L-glutamate, C. L-quisqualate, D. L-α-kainate, E. N-methyl-D-aspartate, F. α-methylglutamate and G. α-para-fluorophenylglutamate

on what was known of acetylcholine; thus an excitatory transmitter should have specific actions only on those neurones which were exposed to it physiologically. That glutamate was able to excite virtually every neurone tested, even the cholinoceptive Renshaw cells of the spinal cord, posed a problem and seemed to indicate that glutamate was unable to fulfill this criterion. Furthermore, Curtis, Phillis and Watkins (1960a) showed that the time-courses of amino acid excitations, in particular the "intervals preceding the cessation of cell reponses after the termination of the iontophoretic currents", (Curtis et al., 1960a) were identical for the D & L forms of glutamate, aspartate and cysteate. The time courses were not altered in the presence of a large variety of enzyme inhibitors. This argued against a transmitter rôle for amino acids since there were apparently no catabolic enzymes, analogous to acetylcholinesterase, present to terminate the activity of the excitants. Furthermore, it was thought unlikely that any enzymes present could accommodate the variety of amino acid structures known to be active. Curtis (1962) also presented evidence showing that depolarization of motoneurones by glutamate had equilibrium potentials different from those of synaptically evoked depolarization. This was thought to be definitive evidence against any claim that glutamate was the excitatory transmitter for the monosynaptic reflex, since it failed to mimic the endogenous synaptic response.

The opinion that glutamate does indeed act as an excitatory neurotransmitter in the brain and spinal cord was not widely held until the 1970's, and even then the debate continued. A key discovery which aided the change in opinion regarding

glutamate occurred in the late 1960's, but was largely overlooked at the time, was the finding that glutamate was compartmentalized in neurones, and existed as two pools linked only indirectly (Berl et al., 1961). A second important observation was made by Bennet et al. in 1973, who showed that many of the amino acids, including glutamate, aspartate and cysteate, were rapidly removed from the extracellular fluid of the brain and spinal cord by neurones and glia. It is now believed that this high affinity uptake system acts to terminate the actions of the amino acid transmitters.

More recent experiments involving the use of excitatory analogues of glutamate have shown them to mimic accurately the postsynaptic membrane events during synaptic transmission in the spinal cord and brain. In addition, it is now possible to block synaptic transmission in the CNS with potent amino acid antagonists at doses which do not affect depolarizations elicited by other agonists but which do attenuate the actions of an exogenously applied specific amino acid excitant. Finally, the release of glutamate from presynaptic terminals has been convincingly demonstrated (see for example Potashner, 1978). It is now believed that the major criteria for acting as a transmitter are met by glutamate: it is present in, and released from presynaptic neurones under conditions of physiological stimulation (see for review Fonnum, 1984); it acts post-synaptically in a manner similar to the endogenously released substance, including displaying a comparable sensitivity to antagonists; and the response is rapidly terminated by a system for its removal from the area of the

synapse. Some of this evidence will be discussed in more detail later in this section.

The characterization of the receptor(s) for the amino acids did not proceed rapidly, and not until the late 1960's did any major developments occur. McLennan et al. (1968) and Duggan and Johnston (1970) then showed that regional differences existed in the relative potencies of the various glutamate analogues available, which started a debate regarding the possible existence of multiple receptors for the amino acids. In 1972, Haldeman and McLennan (1972) found support for the multiple receptor theory by showing L-glutamate diethylester (GDEE) to have different effects on the excitations elicited by various amino acids. Specifically, GDEE was found to block the excitatory action of L-glutamate (L-GLU) more effectively than that of DL-homocysteate in the thalamus and spinal cord. Then in 1978, McLennan and Hall reported that a long chain glutamate analogue, D-α-aminoadipate (DαAA), had an antagonistic effect opposite to that of GDEE in that NMDA and DL-homocysteate were blocked more effectively than L-GLU and kainate (KAIN). This and subsequent studies in the thalamus and spinal cord of rats showed that when the agonists were ranked according to susceptibility to blockade by GDEE and DαAA, they appeared to fall into two or possibly three groups thus providing substantial support for the multiple receptor theory (McLennan and Hall, 1978). Several other long chain analogues, including D-α-aminopimelic and D-α-aminosuberic acid (Davies and Watkins, 1979), were also tested in the late 1970's and were found to be potent antagonists of amino acids

with actions similar to DαAA. This prompted the obvious suggestion that the extension of the carbon chain separating the ω-carboxyl from the α-ionic groups beyond the length of glutamate, and a D-configuration, were the structural characteristics necessary for antagonism of amino acids in the CNS. An exception to this trend, 1-hydroxy-3-amino-2-pyrrolidone (HA-966), a cyclic analogue (5-membered ring) devoid of carboxyl groups was first tested by Davis and Watkins in 1972, and found to be a moderately potent antagonist of amino acids. A subsequent study by Evans et al., 1978 showed that HA-966 acted in a similar fashion to DαAA (being more potent against NMDA-like activity than against that of other agonist) despite its lack of carboxyl groups and its cyclic structure.

The theory that more than one "receptor" mediates the actions of the excitatory amino acids gained support, and efforts were increased towards finding specific antagonists for the proposed receptors. Prior to 1981, several antagonists were discovered and reported, but none had a greater impact on amino acid research than did 2-amino-5-phosphonovalerate, discovered by Davies et al., (1981b) and separated into its L (inactive) and potent D-isomers by Stone et al. (1981) and by Davies and Watkins (1982). This compound was first examined as a racemate in the spinal cord in vivo, and was found to block specifically excitations elicited by the iontophoretic application of NMDA while having little effect on firing caused by other excitant amino acids or acetylcholine (Davies et al., 1981). Also discovered in 1981 was the conformationally restricted antagonist cis-2,3-piperidine dicarboxylate (PDA; Fig. 2, F.) which was first examined by Davies et al. (1981a).

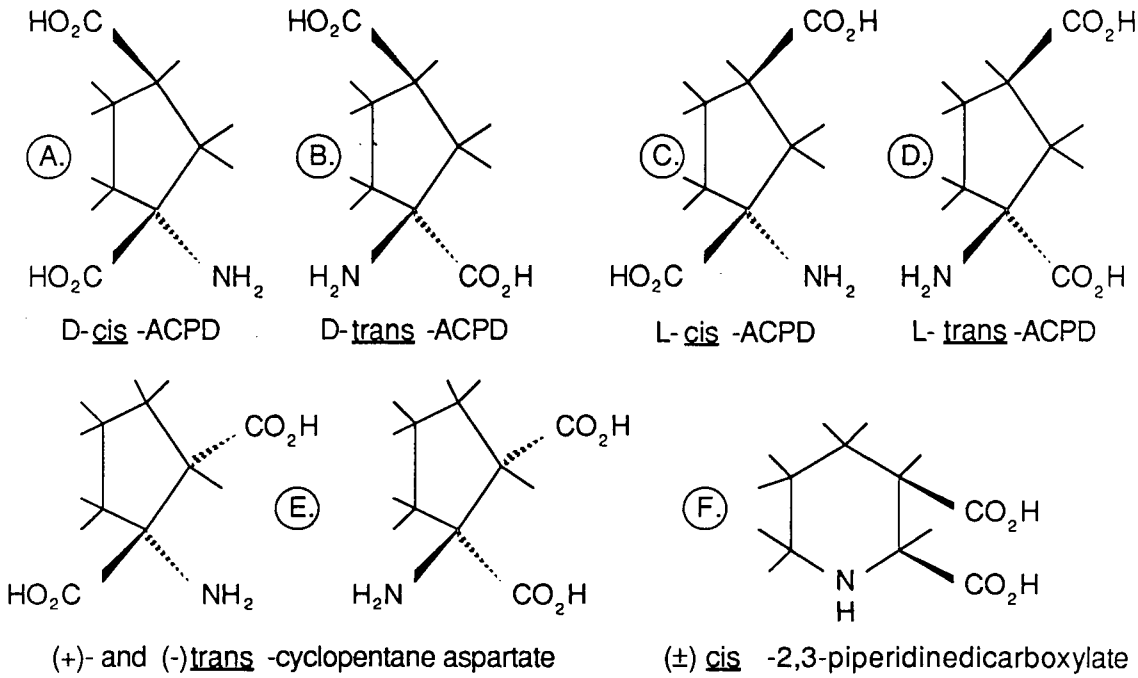


Figure 2. Diagrammatic representation of the structures of A.- D.; D- and L-, cis- and trans-1-amino-1,3-cyclopentane-dicarboxylate A. D-cis-ACPD; B. L-trans-ACPD; C. D-trans-ACPD; D. L-cis-ACPD; E. (+) and (-)trans-CPA and F. cis-2,3-piperidine dicarboxylate.

They reported it to be largely non-specific reducing NMDA-elicited firing of frog and cat spinal neurones only slightly more than those of quisqualate (QUIS) and KAIN. In addition they showed that when ejected onto motoneurones in the neonatal rat spinal cord in vitro, PDA caused depolarizations and firing that was APV sensitive and therefore thought to be mediated by NMDA receptors. These studies followed the discovery by Evans et al. (1977) that NMDA-induced excitation of frog and neonatal rat spinal neurones could also be selectively reduced by magnesium ion (Mg^{2+}), a finding which was subsequently demonstrated in the cat spinal cord in vivo by Davies and Watkins (1977). By 1981 considerable evidence existed suggesting that at least two receptors were responsible for the actions of the amino acids in the spinal cord. In addition, a third receptor mediating KAIN excitations in the spinal cord was proposed, since these were relatively insensitive to Mg^{2+}, D-(-)-2-amino-5-phosphono-valerate (APV) and GDEE, but were blocked along with NMDA by the dipeptide γ-D-glutamylglycine (γDGG; Davies and Watkins, 1981). By inference then, using APV to block NMDA-like compounds, γDGG to block NMDA- and KAIN-like compounds, and GDEE with a greater effect against L-GLU and other compounds including QUIS, three receptors commonly referred to as NMDA, KAIN and QUIS receptors, are now believed to be responsible for the bulk of the excitatory actions exhibited by amino acids and amino acid-like compounds in the spinal cord and presumably elsewhere in the central nervous system (Fig. 1, C, D & E). Certain precautions are necessary, however, when interpreting results from different areas of the nervous system because of the

increasing evidence, which will be dealt with extensively in this thesis, for differences in the structural requirements for activation of the amino acid receptors in various regions. For example, McLennan and Liu (1981), reported that the excitation of spinal cord neurones by a racemic mixture of trans-1-amino-1,3-cyclopentane-dicarboxylate (±trans-ACPD) was relatively insensitive to any of the antagonists γDGG, APV and GDEE. Based on these results a fourth receptor for the amino acids in the spinal cord was proposed.

An additional line of research into the excitatory amino acids in the CNS was initiated by Anis et al. (1983), who examined the effects of a pair of dissociative anaesthetics, phencyclidine and ketamine, on excitatory amino acid responses of dorsal and ventral horn neurones. These compounds had been shown previously to block L-GLU in the hippocampus, amygdala and thalamus (Sinclair and Tien, 1979) and polysynaptic responses in the spinal cord (Tang and Schroeder, 1973). Anis and his colleagues (1983) showed that both of these compounds selectively reduced NMDA-elicited firing of spinal cord neurones at doses which had little effect on activity induced by QUIS or KAIN. Ketamine in particular has been used extensively in research since the majority of the antagonists which are analogues of glutamate will not cross the blood brain barrier whereas ketamine will.

B. Other endogenous excitatory amino acids and antagonists

The recent addition of the tryptophan metabolites quinolinate (QUIN) and kynurenate (KYNA); Perkins and Stone, 1982; Fig. 3, A and F) to the list of neurally

active amino acid analogues has spawned a volume of research into their possible rôles as an endogenous amino acid agonist and antagonist respectively. KYNA is a potent, conformationally restricted antagonist of compounds which act similarly to NMDA or KAIN, while QUIS-like compounds are less affected. For QUIN, no uptake or enzymatic degradative system has been described, nor has release of QUIN from nerve terminals been demonstrated; nevertheless a potential rôle as a modulator cannot be ruled out. Perkins and Stone (1982) suggested that neurally active members of the kynurenine metabolic pathway may potentially "play a part in the overall control of CNS excitability..."; in particular, that the balance between extracellular levels of QUIN and KYNA may conceivably provide a long-term regulation of neuronal excitability. Acridinate (ACRA, Fig. 3, E.; Curry et al., 1986) a synthetic analogue closely related to KYNA is also an antagonist of amino acid excitations. It is, however, completely non-specific reducing the responses to QUIS, KAIN and NMDA comparably.

As early as 1960 (Curtis and Watkins, 1960b) the endogenous sulphur containing cysteic, cysteic sulphinic and homocysteic acids were known to be potent amino acid excitants. Interest in these compounds as possible endogenous ligands for NMDA receptors has been recently rekindled due to the results obtained by Do et al. (1986) who showed cysteine sulfinic and homocysteic acids to be released in a calcium dependent manner from a variety of brain regions. Convincing evidence for their participation as CNS excitatory transmitters has not yet been accumulated, but neither have results been reported to refute this suggestion.

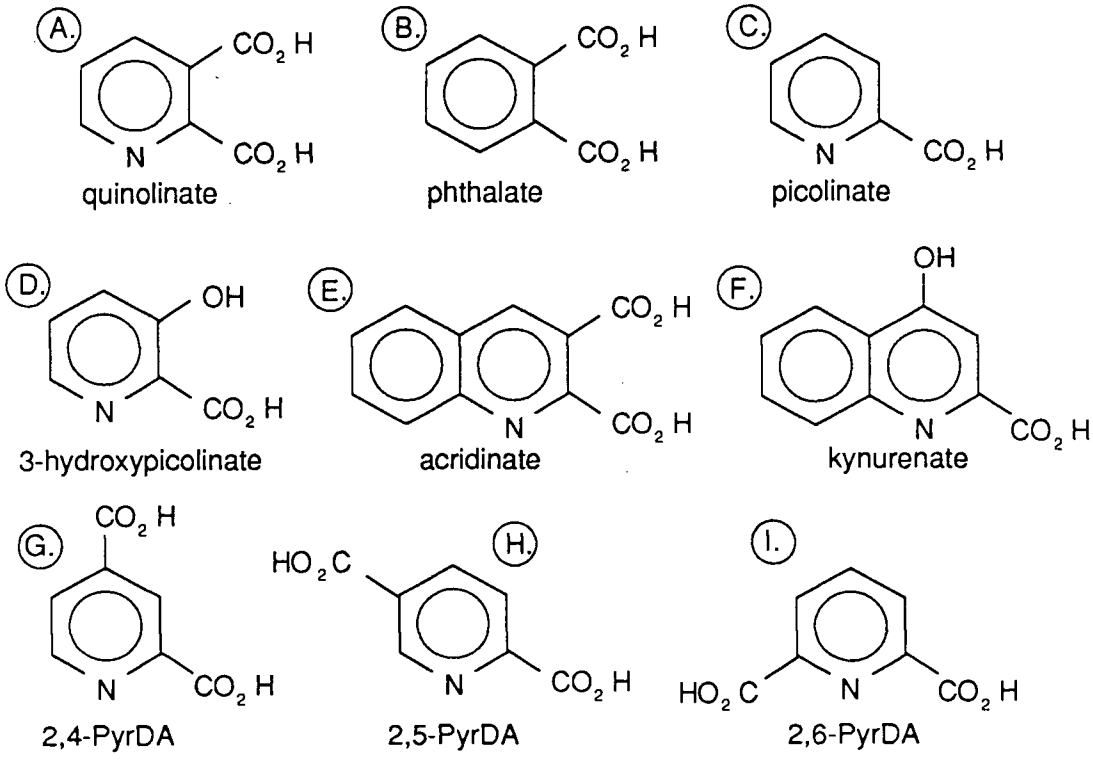


Figure 3. Diagrammatic representation of the structures of
A. 2,3-pyridine dicarboxylate (quinolinate),
B. 1,2-benzene dicarboxylate (phthalate),
C. 2-pyridine carboxylate (picolinate),
D. 3-hydroxy-2-pyridine carboxylate (3-hydroxy picolinate),
E. 2,3-quinoline dicarboxylate (acridinate),
F. 4-hydroxy-2-quinoline carboxylate (kynurenate) and
G. 2,4-pyridine dicarboxylate (2,4-PyrDA),
H. 2,5-pyridine dicarboxylate (2,5-PyrDA) and
I. 2,6-pyridine dicarboxylate (2,6-PyrDA).

C. Intracellular studies in the hippocampus and cortex

The development of the hippocampal and cortical slice preparations has permitted a more detailed characterization of the three "archetypal" (QUIS, KAIN and NMDA) amino acid receptors by intracellular recording from neurones in these regions of the CNS. In pyramidal cells of the hippocampal CA1 region, the amino acid agonists can be characterized by the pattern of depolarization and firing which they elicit in addition to differential antagonism by APV and KYNA. When exposed to low iontophoretic doses of NMDA-like compounds, these neurones display a slowly rising depolarization followed by a bursting pattern of firing consisting of "tetrodotoxin (TTX)-sensitive spikes (usually three to four) superimposed on an underlying depolarizing shift in membrane potential" (Flatman et al., 1983; Peet et al., 1986), usually followed by a pronounced hyperpolarization. With higher iontophoretic doses causing depolarizations greater than 15 mV, a transition occurs from the bursting pattern of firing to high frequency spikes superimposed on the depolarization. Continued application of high levels of NMDA-like compounds often causes extreme depolarization and spike inactivation (Peet et al., 1986). This entire sequence of response is blocked by APV and KYNA. When exposed to KAIN-like compounds, the cells display TTX-sensitive spikes upon a slowly rising depolarization which increases throughout the application of the agonist and often leads to inactivation of the spike-generating mechanism. These effects are potently blocked by KYNA but not by APV, and several conformationally restricted compounds which evoke this type of firing are known (Curry et al., 1987). Finally,

when exposed to QUIS-like compounds, including L-GLU, the neurones display TTX-sensitive spikes and a depolarization which reaches a plateau quickly and does not increase further even when excess agonist is applied.

D. Excitatory amino acid receptor channels

In vitro studies have also provided insight into the mechanisms of activation by the three classes of amino acid excitants. Using whole-cell patch-clamp recording and voltage-clamp techniques, Mayer et al. (1987) showed that when cultured spinal cord neurones loaded with the Ca^{2+}-sensitive dye, arsenazo III, were clamped near resting levels of polarization, with μM concentrations of Mg^{2+}, NMDA caused a large influx of Ca^{2+} while QUIS and KAIN elicited much smaller increases in intracellular Ca^{2+}. The NMDA-induced Ca^{2+} influx became voltage-sensitive with physiological (mM) levels of Mg^{2+}, peaking at -30 mV. By clamping TTX-treated neurones at various levels (-70 to +70 mV) in the absence of agonists, they found the voltage-sensitive Ca^{2+} influx to peak at +10 mV. These findings, and the fact that increased extracellular Mg^{2+} reduced NMDA-like excitations without affecting those elicited by agonists of the other two classes, suggested that the channel opened by NMDA-like agonists permitted the flow of sodium, potassium, calcium and magnesium ions, while the channel(s) opened by QUIS- and KAIN-like compounds were more selective in that divalent cations were excluded. The studies mentioned previously, support suggestions that calcium ion influx may be involved with the well known toxic effects of this class of excitant, and that neuronal

death resulting from ischaemia may in part result from amino acid-induced toxicity. In addition, the involvement of excitatory amino acid-induced calcium ion influx in long-term potentiation, first proposed by Baudry and Lynch, (1980) underscores the importance of studies involving NMDA and NMDA antagonists (see for review Collingridge and Bliss, 1987).

The ever-increasing use of cultured neurones and techniques such as patch-clamp recording has added greatly to our knowledge of the mechanisms underlying the excitation of neurones by amino acids. Recent studies by Jahr and Stevens (1987), and by Cull-Candy and Usowicz (1987a, b) have described the actions of low concentrations of excitatory amino acids on small patches of membrane from cultured hippocampal and cerebellar neurones respectively. Using outside-out patches, Jahr and Stevens have found four distinct single-channel currents evoked by application of glutamate or its analogues; channel openings resulting in each of these currents appear to be dependent on which analogue is being applied. If these currents were the result of activation of four distinct receptor complexes, one would expect to find patches lacking one or more of the conductance states, and in patches where two or more of these complexes were present, one should expect to see currents resulting from the simultaneous opening of two or more of the channels. Instead, these researchers have observed direct transitions between each of the four conductance states in a single patch on several occasions, suggesting that all the various activities of the identified amino acid receptors may be accounted for by one complex molecular entity with three or

more separate binding sites. The largest conductance state, capable of permitting the passage of divalent cations, has a much higher rate of opening when exposed to NMDA-like agonists, and spends proportionately more time in the open state when bathed in magnesium-free medium. These characteristics are not observed for the lower conductance states seen more often with QUIS- and KAIN-like agonists.

Also using outside-out patches, but from cultured cerebellar neurones, Cull-Candy and Usowicz (1987a, b) have demonstrated a clear difference between the range of conductance states seen in these cells compared with hippocampal neurones. They have identified five different conductance states, and have observed transitions between all five, suggesting again that a complex consisting of one or two channels and two or more separate binding sites mediated the actions of the amino acids in these cells. Observed in both the hippocampal and cerebellar neurones, however, is the largest conductance state which results from NMDA activation, and which shows increased open times with magnesium-free medium.

E. Synaptic transmission in the spinal cord

The potential rôles of glutamate and aspartate as transmitters in the spinal cord were also being examined as early as 1972. Haldeman and McLennan (1972) demonstrated that iontophoretically applied GDEE simultaneously blocked dorsal root stimulated monosynaptic excitations and L-GLU-induced firing of spinal

interneurones, while firing induced by L-aspartate (L-ASP) was largely unaffected. Biscoe et al. (1977), Hall et al. (1977) and Davies and Watkins (1978), reported that D-α-aminoadipate (DαAA) ejected at only slightly higher iontophoretic currents than those required to block NMDA-excitations (38 nA vs 26 nA), blocked the dorsal root stimulation of Renshaw cells in the spinal cord, while the cholinergic excitation induced by ventral root stimulation was unaffected. The results outlined above (DαAA- & APV-sensitive and GDEE-sensitive receptors) were clarified in 1983 by Peet et al. who showed that both the polysynaptic excitation of dorsal horn neurones and the dorsal root excitation of Renshaw cells of cat spinal cord were blocked by a range of NMDA antagonists including DL-APV. Neither were the dorsal root-elicited monosynaptic excitations of dorsal horn neurones sensitive to antagonism by DL-APV nor were they blocked by PDA, a compound which attenuated KAIN-induced firing almost to the same extent as that of NMDA (Davies et al., 1981a). Studies by Davies and Watkins (1983, 1985) in the rat spinal cord in vivo also suggested that the low threshold primary afferent monosynaptic input (Ia) onto motoneurones was mediated by receptors insensitive to NMDA antagonists, and this conclusion is supported by the more recent intracellular studies by Jahr and Yoshioka (1986) using hemisected neonatal rat spinal cord. Furthermore, in the cat, iontophoretic doses of APV much higher than those needed to abolish completely the activity of iontophoretically applied NMDA did not significantly reduce the Ia excitatory postsynaptic potential (EPSP) recorded intracellularly from motoneurones in vivo (Flatman et al., 1986). In the cat, therefore, it appears that

non-NMDA receptors may also be involved in the monosynaptic input onto motoneurones. In summary, monosynaptic inputs onto dorsal horn cells and motoneurones appear to be mediated by nonNMDA-receptors while polysynaptic dorsal root inputs onto both dorsal horn cells and Renshaw cells probably involve NMDA receptors.

Recent intracellular recordings by King et al. (1987) of dorsal horn cells in transverse slices of neonatal rat spinal cord have shown that orthodromic stimulation of dorsal roots elicits (in 64% of neurones tested) excitatory responses consisting of short- and long-lasting components. The short component was an APV-insensitive fast-rising EPSP with typically one or two TTX-sensitive spikes superimposed, while an APV-sensitive longer latency "after depolarization" follows lasting up to 200 msec. It appears, therefore, that amino acid receptors play important rôles in the excitatory transmission of sensory information in the dorsal horn and also in the mediation of motor reflexes in the ventral horn.

F. Synaptic rôles in the hippocampus and cortex

The hippocampus is a highly organized part of the higher CNS, the pathways of which have been well studied. It has two main inputs, the perforant pathways projecting to the dentate granule cells from the ipsilateral entorhinal cortex, and the Schaffer-collateral commissural pathways (SCC) projecting from the CA3 to the CA1 pyramidal neurones of the ipsilateral and contralateral hippocampi. An additional internal pathway, the mossy fibres, project from the dentate granule cells

to the CA3 pyramidal neurones. A study by Collingridge, Kehl and McLennan (1983a) described amino acid excitation of hippocampal neurones and synaptic transmission in the major pathways of the hippocampal slice, in vitro, using a range of excitant amino acid antagonists. They found that APV specifically and potently blocked NMDA elicited firing of hippocampal neurones, and that γDGG was capable, at slightly higher iontophoretic doses than required to block NMDA and KAIN-induced firing, of also consistently blocking QUIS and L-GLU elicited activity of any CA1 hippocampal neurone. Having thus established the criteria to distinguish between NMDA and non-NMDA receptor mediated firing, they proceeded to examine the major synaptic pathways of the hippocampus and their susceptibility to these two antagonists. None of the excitatory inputs onto hippocampal pyramidal neurones could be blocked by DL-APV at "doses" which block NMDA, but γDGG was effective at reducing these inputs at doses which reduced QUIS and KAIN excitations. A subsequent study (Collingridge et al., 1983b) concentrated mainly on the SCC input onto CA1 pyramidal neurones. They found that the long-term potentiation (LTP) of synaptic efficacy resulting from high-frequency stimulation (100 Hz for 1 sec) of this pathway was blocked by APV, despite the fact that APV had little or no effect on the extracellularly recorded field EPSP. It appears then, that although NMDA receptors are not involved directly in low frequency transmission in the hippocampus, they are important during the induction of LTP. Wigström and Gustafsson (1984) discovered that the application of GABA antagonists promoted LTP induction. Specifically, while recording

extracellularly from the dendritic region (stratum radiatum) of the CA1 area, and stimulating the SCC at various frequencies and durations, they found a current source in the dendritic region which was enhanced by picrotoxin (a GABA antagonist) and depressed by APV. These findings along with those of Collingridge et al. (1983a, b) led Wigström and Gustafsson (1984) to suggest that tetanic stimulation of the excitatory pathways sufficiently depolarized the cells so as to remove the voltage-sensitive Mg^{2+} blockade of NMDA channels leading to large influxes of Ca^{2+}. The Ca^{2+} is thought to be responsible, at least in part, for the induction of LTP.

Intracellular recordings from pyramidal cells of the somatosensory cortex of the rat in vitro, have been used by Thomson (1987) and demonstrate that excitatory inputs onto these cells are mediated in part by NMDA receptors. In coronal slices of cortex maintained under standard in vitro conditions, the response due to the iontophoretic application of NMDA and the synaptic response resulting from stimulation of the corpus callosum were similarly altered by changes in the Mg^{2+} concentration and to the application of NMDA antagonists including APV and ketamine. In contrast, Oka et al (1987) reported that thalamic excitation of extracellularly recorded cortical neurones in the cat in vivo was not attenuated by the iontophoretic application of APV at doses which blocked NMDA elicited activity. Instead, high doses of less selective antagonists such as KYNA or PDA were required suggesting that this pathway was mediated by non-NMDA receptors. It appears then, that one can tentatively assign callosal excitation of cortical

neurones to be mediated, at least in part, by NMDA receptors (Thomson, 1986) while the thalamocortical inputs may be mediated by non-NMDA receptors. Recent experiments by Avoli and Olivier (1987) using excised epileptic human neocortical slices maintained in vitro, suggest that NMDA receptors play an important rôle during seizure activity, since synaptically generated interictal spiking could be blocked by APV at doses (50 to 100 μM) which block NMDA elicited activity, but did not reduce the intracellularly recorded synaptic EPSP resulting from electrical stimulation of the underlying white matter. In summary, NMDA receptors appear to be involved in the induction of LTP in the hippocampus, and in certain of the excitatory inputs onto cortical neurones. Non-NMDA receptors appear to be responsible for much of the low frequency transmission in the hippocampus and the thalamic inputs onto cortical neurones.

G. Actions of glutamate and aspartate

As the list of potent amino acid agonists has grown the use of glutamate and aspartate to study the receptors which supposedly are responsible for their activities as transmitters has declined. Results obtained when undertaking examinations of glutamate and aspartate are difficult to interpret due to several factors. First of all, both are rapidly taken up by neurones and glia (Logan and Snyder, 1971; Bennet et al., 1973; Balcar et al., 1977; Fonnum, 1984), while many analogues are not, and the efficiency of these uptake systems may not necessarily be similar from region to region in the CNS. Secondly, binding experiments have

shown both glutamate and aspartate (the L-isomers) to be displaced by different groups of compounds acting specifically at more than one of the three archetypal amino acid receptors. Electrophysiological evidence also suggests that the actions of glutamate and aspartate are mediated via combinations of the different receptor types; glutamate and aspartate are generally thought to be mixed agonists under most circumstances, although many exceptions to this generalization can be found (Davies et al., 1980).

The rôles played by L-GLU and L-ASP as transmitters, some of which have already been mentioned, are thought to occur via both NMDA and non-NMDA (presumably QUIS) receptors. There is little evidence for KAIN-receptor mediated synaptic events in the mammalian CNS, although Grillner et al. (1987) have suggested that in the lamprey cord certain of the pathways involved in locomotion are mediated by KAIN receptors.

It is apparent that changes in extracellular Mg^{2+}, kynurenate (KYNA) or a KYNA-like compound may influence the post-synaptic expression of a presynaptically released "mixed agonist" like glutamate (Perkins and Stone, 1982, 1984). Changes in the extracellular fluid content of excitotoxic amino acids have been considered as possible contributors to a variety of pathologies including epilepsy, Huntington's disease and ischaemic cytolysis (Coyle et al., 1981).

H. Quinolinate and other NMDA-like agonists

Since the discovery of the tryptophan metabolite quinolinate (QUIN) in the CNS (Wolfensberger et al., 1983), it has become increasingly evident that it should be considered a possible excitatory amino acid transmitter. QUIN has been found, along with anabolic and catabolic enzymes, in many CNS regions in a wide range of concentrations (Wolfensberger et al., 1983; Moroni et al., 1984; Foster et al., 1985). Stone and Perkins (1981) showed that in the sensorimotor cortex of the rat in vivo, QUIN had a potency about one quarter that of NMDA and one tenth that of QUIS. This early study into the action of QUIN also disclosed that in the cortex at least, it was indistinguishable from NMDA in its sensitivity to APV.

Subsequently, studies by Perkins and Stone (1983) and McLennan (1984) compared the potency of QUIN in the cortex and spinal cord in vivo. In these studies, QUIN was found to be at least five-fold less potent in the spinal cord than in the cortex, and few if any cells in the cord could be excited sufficiently to examine its pharmacological responsiveness. Therefore, there appears to be an anomaly: although NMDA is equipotent in cortex and spinal cord relative to QUIS, (Perkins and Stone, 1983; McLennan, 1984) and QUIN appears to act at the NMDA receptor, QUIN is extremely weak in the spinal cord.

A more recent study examined the activity of QUIN in the piriform cortex in vitro, and compared its action with L-ASP and NMDA (ffrench-Mullen et al., 1986). APV applied in the bathing medium at a concentration of 10^{-6} M blocked NMDA-elicited spikes in preference to those caused by QUIN and L-ASP. These

authors commented that the receptors responsible for the actions of QUIN and NMDA in the piriform cortex have some common characteristics but are pharmacologically distinguishable.

I. Cyclopentane and pyridine derivatives

Identification of compounds reacting with NMDA receptors in the hippocampus has been accomplished primarily using the specific antagonist APV and observing the characteristic bursts produced, as described earlier. An extensive structure-activity analysis for these receptors in hippocampal CA1 pyramidal cells has recently been reported (Peet et al., 1987). Using a series of conformationally restricted and substituted analogues, it was shown that only the 2,3 positioning of the carboxyl groups on a pyridine ring, as in QUIN, permits activation of these hippocampal receptors. Furthermore the nitrogen atom present on NMDA (secondary amine) or on QUIN (aromatic) was not essential for evocation of burst firing since phthalate (1,2-benzene dicarboxylate; Fig. 3, B) and itaconate (methylene succinate) were both weak agonists of NMDA receptors. No compounds have been reported to elicit NMDA-bursting which are not blocked by APV.

The cyclopentane glutamate analogues were first examined as racemic mixtures (DL-cis- and DL-trans-1-amino-1,3-cyclopentanedicarboxylate; ±cis- and ±trans-ACPD) in the thalamus in vivo by McLennan and Wheal (1978) and Hall et al. (1979) who found them to be very potent agonists, cis-ACPD being more

sensitive to DαAA (the most potent NMDA antagonist at that time) and relatively insensitive to GDEE while trans-ACPD not being greatly affected by either antagonist.

J. Structure of the dorsal horn

The dorsal horn cells of the spinal cord are arranged in several layers (laminae I through VII; Rexed, 1952; see Fig. 4). They receive afferent information from the dorsal roots and relay it to other regions of the spinal cord, to the medulla and to the ventroposterolateral (VPL) nucleus of the thalamus. Processing of the afferent information within the dorsal horn is thought to occur through local circuits between cells of the substantia gelatinosa or SG (laminae II and III), which receive a proportion of the primary afferent fibre input, and the lower laminae (IV - VII). Although the histology and major pathways of the spinal cord are well known, and extensive somatotopic maps have been made for certain areas of the spinal cord, the local circuits and electrophysiological characteristics of the cells involved are largely unknown (Wall, 1967; Wilson et al., 1986).

Information enters the spinal cord via the primary afferent fibres which have their cell bodies in the dorsal root ganglia. The peripheral processes originate from sensory receptors of all types, and centrally the fibres terminate in the dorsal horn of the cord. Within the cord, each central process divides into ascending and descending branches in either the zone of Lissauer (small myelinated and unmyelinated fibres) or the dorsal columns (large myelinated fibres). These

branches give off collaterals when they enter the cord and at various levels rostral and caudal to their point of entry. The largest and most medial of these fibres may ascend in the dorsal columns as far as the medulla. Collaterals from these larger myelinated fibres pass through laminae I and II or bypass these laminae medially, and appear to terminate in the lower part of the SG on the dendrites of neurones with somata in lamina IV, or directly onto inhibitory interneurones of the lower laminae (Jankowska and Roberts, 1972). The collaterals from the small myelinated and unmyelinated fibres for the most part terminate directly onto cells within lamina I and the SG (Cervero and Iggo, 1980).

In general, the cells of the dorsal horn are arranged in a dorsoventral fashion. Their dendrites extend dorsally into the adjacent laminae, and their axons either travel ventrally to lower laminae, or pass into the white matter to ascend or descend to other levels of the cord, or directly to higher centres. The exceptions to this are the cells of lamina I (the marginal zone), which are spindle shaped and lie parallel to the dorsal surface of the cord, with processes extending mediolaterally for several tens of microns (Cervero and Iggo, 1980).

K. In vitro slice preparations

Since the initial studies by Li and McIlwain in 1957, the in vitro slice preparation has been modified to suit a variety of applications in many areas of the nervous system. However, because of the relative softness of the tissue even at low temperatures, the adult mammalian spinal cord has proved difficult to prepare

for in vitro studies. These difficulties are especially evident when transverse slices are attempted although Zieglgänsberger and Sutor (1983) provided some evidence for the feasibility of this preparation. Other laboratories are currently studying the spinal cord prepared from neonatal rats in vitro. Hemisected cord (Jahr and Yoshioka, 1985; Fulton and Walton, 1986), or horizontal (Murase et al., 1982) and transverse slices (Ma and Dun, 1985; Miletic and Randic, 1982; Takahashi, 1978) of the lumbar region have been used for both intra- and extracellular electrophysiological studies with the majority of the work to date concentrating on the substantia gelatinosa, an area easily recognizable as a translucent band in slices less than 600 μM thick. The use of neonatal tissue for the study of the excitatory amino acid receptors in the spinal cord may not be entirely appropriate however, since a number of recent studies have shown that several populations of spinal receptors such as those for 5-HT (Lau et al., 1985), GABA (Saito et al., 1982) and glycine (Benavides et al., 1981) do not reach adult levels until the 14th, 15th and 30th postnatal day respectively. We have therefore performed studies on spinal cord slices prepared from weanling rats (24 to 30 days old) rather than from neonatal animals.

Several reviews available on slice preparations stress that the slice of nervous tissue should contain, intact, as many of the known synaptic pathways as possible (see for example Richards, 1981). Although many of the synaptic circuits in the dorsal horn are local, the ideal slice would have both the dorsoventrally oriented cells of the SG and lamina IV and V, and the rostrocaudally oriented

primary afferent branches in the zone of Lissauer and dorsal columns, intact. This has led to the use of a dorsoventral longitudinal slice (sagittal) which extends rostrocaudally for 8 or more mm; it should therefore contain at least three complete spinal segments of the lumbar enlargement of the rat cord. The advantages of this preparation lie in the potential for application of antagonists at a known concentration in the perfusion medium and in the opportunity for future intracellular investigation of spinal neurones. This preparation should also permit the study of synaptic activity both intra- and intersegmentally within the spinal cord since several complete segments are contained within one slice.

II. Aims of the Present Studies

Excitatory amino acid receptors are involved in synaptic transmission in many regions of the mammalian CNS. Several of the pathways mediated by these receptors have already been discussed, their functional significance ranging from the possible involvement of NMDA receptors in learning and memory (Collingridge, 1987) to the transmission of sensory and reflex information in the dorsal horn of the spinal cord (Headley et al., 1987). The primary aim of these studies was to contribute to the knowledge of how the amino acid receptors in the spinal cord differ, in terms of their structural requirements for activation, from those elsewhere in the CNS, and to provide a foundation for future study aimed at designing specific

antagonists for QUIS, KAIN and QUIN spinal cord receptors. In order to determine the structural requirements for activation of amino acid receptors in the spinal cord, in vivo and in vitro experiments were used to examine the activities of several groups of glutamate, aspartate and cyclic amino acid analogues.

The rationale for synthesizing and testing the first group of compounds was based on the premise that substituents of a bulky aliphatic or aromatic nature may produce analogues of high potency either as agonists or antagonists. Such compounds may bind to the receptor and, because of the substitutions, be unable to activate, or conversely, may activate but may have difficulty disengaging from the receptor. Several α-substituted analogues of glutamate were investigated including the methyl, phenyl, and para-substituted phenyl variations (examples Fig. 1, F and G: page 3).

Since the excitatory amino acids are believed to interact with their receptors in the spinal cord by a three-point attachment (the two acidic groups and the amino group), the relative position in space of these groups becomes exceedingly important to an understanding of the structural requirements of the receptors. A valuable compound to study would therefore be a potent agonist or antagonist which possessed a rigid structure with little possibility for conformational change. The cyclopentane-derived analogues of glutamate and aspartate are ideal for this type of investigation, and several different compounds of this type were examined (Fig. 2, A - E: page 8). As with the α-substituted compounds, they were tested initially using the in vivo preparation. Because all of the isomers available had interesting

Full Names and Abbreviations of All Compounds Tested

abbreviation	full name of compound	concentration (mM) -in vivo	-in vitro	source
Agonists:				
QUIS	quisqualate	5*	5*	Sigma
KAIN	kainate	50*	5*	Sigma
NMDA	N-methyl-D-aspartate	50*	20*	Sigma
QUIN	2,3-pyridine dicarboxylate (quinolinate)	200	20	Aldrich
(DL)t-ACPD	(DL)trans-1-amino-1,3-cyclopentane dicarboxylate	200	n.t.	K. C.
D-cis-ACPD	D-cis-1-amino-1,3-cyclopentane dicarboxylate	n.t.	200	K. C.
L-cis-ACPD	L-cis-1-amino-1,3-cyclopentane dicarboxylate	n.t.	200	K. C.
L-trans-ACPD	L-trans-1-amino-1,3-cyclopentane dicarboxylate	n.t.	200	K. C.
D-trans-ACPD	D-trans-1-amino-1,3-cyclopentane dicarboxylate	n.t.	200	K. C.
L-GLU	L-glutamate	500	100†	Sigma
D-GLU	D-glutamate	500	200	Sigma
L-ASP	L-aspartate	500	n.t.	Sigma
2,5-PyrDA	2,5-pyridine dicarboxylate	n.t.	200	Aldrich
2,6-PyrDA	2,6-pyridine dicarboxylate (dipicolinate)	n.t.	200	Aldrich
3-HPA	3-hydroxypyridine-2-carboxylate	n.t.	200	Aldrich
ITCA	methylene succinate (itaconate)	n.t.	200	Aldrich
PHTA	1,2-benzene dicarboxylate (phthalate)	n.t.	200	Aldrich
Antagonists:				
KYNA	4-hydroxyquinoline-2-carboxylate (kynurenate)	100†	20*	Aldrich
APV	D-(-)-2-amino-5-phosphonovalerate	10*	2.5*	T. N.
ACRA	2,3-quinoline dicarboxylate (acridinate)	200	200	K. C.
2,4-PyrDA	2,4-pyridine dicarboxylate	n.t.	200	Aldrich
PDA	(±)cis-2,3-piperidine dicarboxylate	100†	n.t.	K. C.
GDEE	glutamate diethylester	200	n.t.	Sigma
αPG	α-phenylglutamate	200	n.t.	K. C.
αMG	α-methylglutamate	200	n.t.	K. C.
αpFPG	α-(para-fluorophenyl)glutamate	200	n.t.	K. C.
αpCPG	α-(para-chlorophenyl)glutamate	200	n.t.	K. C.
(±)trans-CPA	(±)trans-1-amino-1,2-cyclopentane dicarboxylate	200	n.t.	K. C.
(-)trans-CPA	(-)trans-1-amino-1,2-cyclopentane dicarboxylate	200	n.t.	K. C.
(+)trans-CPA	(+)trans-1-amino-1,2-cyclopentane dicarboxylate	200	n.t.	K. C.

n.t. -not tested
K. C. -synthesized by Dr. K. Curry
T. N. -supplied commercially by Tocris Neuramin

* - in 150 mM NaCl
† - in 75 mM NaCl

Table 1. Complete list of all compounds tested; the abbreviations used in the text, their full name, concentrations used for iontophoresis in vivo and in vitro, and the source of the compound. All compounds dissolved in distilled water unless stated otherwise.

activities in vivo, the four isomers of cyclopentane glutamate (D- and L-, cis- and trans-1-amino-cyclopentane-1,3-dicarboxylate; D- and L-, cis- and trans-ACPD) were examined extensively in vitro. The two isomers of trans-cyclopentane aspartate, [(+) and (-)trans-1-amino-1,2-cyclopentane-dicarboxylate; (+) and (-)trans-CPA] were not synthesized in sufficient quantities to examine them in vitro. The separated optical isomers of cis-1-amino-1,2 cyclopentane-dicarboxylate have proved difficult to synthesize and were not available to study.

The third series of analogues examined were based on the aforementioned endogenous excitant 2, 3-pyridine dicarboxylic acid (quinolinic acid; QUIN, Fig. 3, A: page 12). A lengthy study of the agonist activity of QUIN in the spinal cord was done in order to characterize its action with respect to the well-known agonists and antagonists. Additional experiments looked at several other pyridine derivatives including the 2,4-, 2,5-, 2,6- (Fig. 3, G-I: page 12) and 3,4-dicarboxylate variations. The substituted pyridines 3- (Fig. 3, D: page 12) and 4-hydroxy-2-pyridine dicarboxylate and 1,2-benzenedicarboxylate (Fig. 3, B) were also examined.

A complete list of all the compounds used in these studies is shown in Table 1; the abbreviations used and suppliers of the compounds are listed for reference.

III. Methods

A. Spinal cord and cortex in vivo

In vivo studies were carried out using male Wistar rats (200-350 g) anaesthetized with urethane (1.5 g-kg^{-1} i.p.). A mid-dorsal incision of the skin from the scapulae to the sacrum initiated the surgery. Parallel incisions of the muscle 2 to 3 mm on either side of the vertebral dorsal spinous processes were then made from the fat pad of the back (T_9 or T_{10}) to the sacrum. The medial strip of muscle, along with the spinous processes, was removed with scissors and toothed forceps. Small blunt scissors were then used to clear adhering muscle from the lateral edges of the vertebrae, and the dorsal surfaces were scraped clean using a periosteal elevator. Small Friedman rongeurs were then employed to make a window in the rostral dorsolateral corners of the T_{10} vertebra permitting the insertion of small bone cutters to sever the vertebral laminae. The dorsal portion of the vertebra was removed exposing the underlying dura and spinal cord. This process was repeated, save the use of the rongeurs, for the remaining vertebrae. Exposed tissues, muscle and dura were periodically moistened with warmed, oxygenated artificial cerebrospinal fluid (ACSF) throughout the procedure. Under a dissecting microscope, the dura was removed and/or reflected using fine forceps and iridectomy scissors. Large vertebral clamps were then placed on the most rostral and caudal exposed vertebrae to immobilize the column and cord. The incised skin flaps were raised and tied to an oval frame in order to form a pool which was subsequently filled with warm paraffin

oil to prevent desiccation of the exposed tissues. A rectal probe and heating bed were used to maintain a body temperature of 35° C.

Surgery to expose the cortex began with a rostrocaudal skin incision of 2.5 cm in length, 0.5 cm to the left of midline, exposing the cranium overlying the left sensorimotor cortex. Connective tissue and muscle were removed, and the cranium was roughly wiped with a dry sponge and bone wax. A window approximately 1.0 x 1.0 cm was made in the cranium using a dental drill. Care was taken not to touch the underlying dura and cortex as slight disturbances often resulted in severe swelling and bleeding. As with the spinal cord, the dura was reflected, the skin flaps tied to a circular frame (about 4 cm in diameter) and warm paraffin oil was added to cover the exposed tissues.

B. Spinal cord slice preparation

Male Wistar rats (50 - 100 g) were anaesthetized with urethane (1.5 g-kg^{-1} i.p.). The spinal cord was exposed from T_{10} to S_1 by dorsal laminectomy. Using a small pair of curved scissors, parallel incisions (2 to 2.5 mm) were made in the lateral portions of the most rostral exposed vertebra. The portion of vertebra between these two incisions was then carefully removed with small curved haemostats. The lateral aspects of this vertebra were removed with a small pair of Friedman rongeurs. The dorsal lamina of each subsequent vertebra was then removed using small bone cutters and curved haemostats as described for the in vivo preparation. Bleeding was carefully controlled with slivers of sponge or gelfoam, and all bleeding was

staunched before proceeding further into the preparation of the slices. The dura was reflected and the dorsal and ventral roots cut as close to their spinal origins as possible. Although care was taken to avoid pressure upon the dorsal surface of the cord during surgery, the viability of the preparation was not compromised by the occasional light touch. The cord was then cooled by the gentle application of oxygenated ACSF at 3°C (3 - 4 ml). When the anterior and posterior ends of the exposed cord were sectioned, the entire length could be transferred to a dish of ACSF at 3°C. After 60 - 90 s., during which time any adhering dura, blood clot or roots were carefully dissected away, the length of cord was placed on the chilled cutting stage of a McIlwain tissue chopper. The stage was modified by the addition of a raised wall ca. 3 x 5 cm and 0.75 cm deep which permitted the tissue slices, once cut, to be submerged in ACSF prior to their removal to the recording chamber (see below).

Using the blade of the chopper (a standard platinum-chrome double-edged razor blade), the ends of the cord were cut away leaving a 4 - 8 mm segment which included the bulk of the lumbar enlargement. This piece of cord was then realigned longitudinally, ventral side up. Eight or nine 400 μm slices were cut in quick succession, leaving each slice in place to provide support for subsequent slices. After cutting, the well was filled with ACSF (3°C), and the slices were gently separated with a fine paint brush. Each slice was transferred by wide mouth pipette onto the taut nylon mesh support of a low-volume, continuously perfused tissue chamber. The slices were maintained at the interface between a warm moist

atmosphere of 95% O_2 - 5% CO_2 and ACSF at 33°C. Up to 4 slices per cord, originating 600 - 1400 µm on either side of midline, demonstrated a sharply-defined dorsal root region and substantia gelatinosa, as well as a consistently high degree of neural activity. The most medial and lateral slices contained very little grey matter and were discarded (Fig. 4). An incubation period of at least 90 minutes was necessary prior to the start of any experiment since extracellular recording from slices showed greatly reduced neural activity until this time. In general, a gradual increase in activity was observed for 3 h after preparation of the slices. No differences in viability or neuronal responses were observed between slices prepared from rats weighing less than 100 g; however decreased activity was observed in slices prepared from rats 125 g or larger.

During the development of this procedure, a variety of different approaches to the problem of getting viable slices from the spinal cord of young adult rats were utilized. The initial attempts focussed on transverse slices of the lumbar or cervical enlargements or of the thoracic cord. Some success was experienced with all three of these approaches. Problems were encountered, however, with the longevity of the slice, and with the consistency of the preparation (ie. some slices would have few or no live cells, with no plausible explanation). When examined using a high-power (500 X) dissecting microscope, transverse slices of cord in the tissue bath appeared

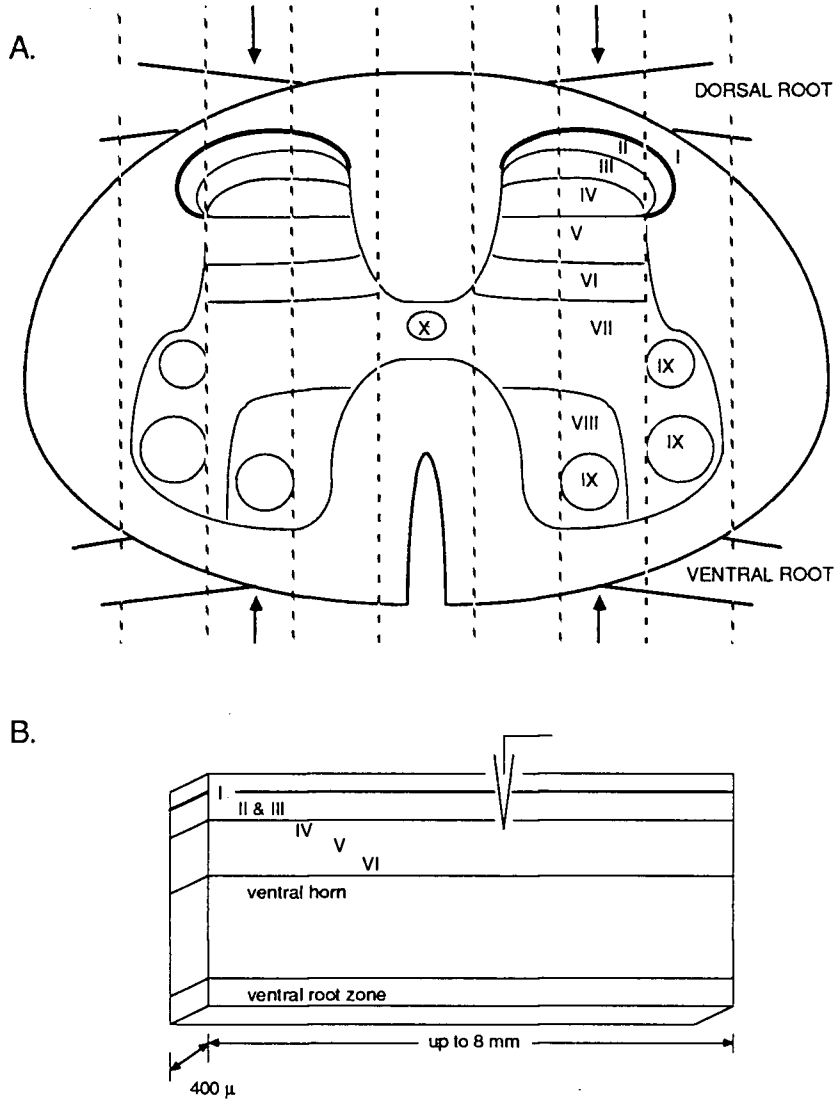


Figure 4. A. Diagrammatic representation of the lumbar enlargement of the rat in cross section, and the approximate lines of passage of the chopping blade through the cord. B. Diagrammatic representation of one slice as it would appear during an experiment with the visible structures indicated.

to leak the cytoplasm of damaged cells or cut axons into the ACSF. In a slice 400 μm thick, it seems probable that the cut ends of rostro-caudally oriented axons do not seal but rather empty themselves of cytoplasm, which must flow through and over the rest of the slice. This suggestion is supported by the observation that the viability of the dorsal horn cells depended on their relative orientation in the bath with respect to the flow of ACSF. On several occasions, simultaneously cut slices were placed in the bath, some with the dorsal half upstream (ventral horns oriented towards the suction) and others with the dorsal half downstream (dorsal horns oriented towards the suction). It was observed that the slices with their dorsal horns placed downstream, such that the ACSF first flowed over the ventral horns, were routinely of poorer quality relative to the others. Longitudinal slices, on the other hand, would have many of the rostro-caudal axons intact for distances up to the entire length of the slice (6 - 8 mm). Microscopic examination of these slices once in the bath showed that some leaking of cytoplasm still occurred, but to a lesser degree and for a much shorter time.

Several successful experiments were done with slices cut, either longitudinally or transversely, from the cervical enlargement. The most successful approach to this preparation was to decapitate and surgically isolate the cord by laminectomy, keeping the cord chilled from the moment of exposure. The viability of the slices was inconsistent, however, for several possible reasons. The cervical enlargement is larger than, and contains relatively more white matter than does the lumbar enlargement. It was therefore more difficult to cut, and if the procedure did not run

entirely smoothly such that the time taken between decapitation and the slices arriving in the bath was much over 6 - 8 minutes, the results were unsatisfactory (these strict requirements were by comparison quite relaxed when working with the lumbar enlargement). Viability was achieved more consistently with cervical slices from animals of 25 to 50 g. However, for the reasons given earlier the use of such young animals was unacceptable, and experiments with the cervical region were not performed.

Slices of the thoracic cord of rats above 50 g. in weight were also successfully prepared. The thoracic cord, 2.2 - 2.6 mm in diameter by comparison with the lumbar enlargement (3.3 - 3.9 mm), was troublesome to work with. It was very difficult to get more than a single longitudinal slice with the correct orientation from each piece of cord. Transverse slices of thoracic cord were also prepared with some success, although they still suffered from the problems outlined above. In addition, the small size of the dorsal horn and lack of cell numbers made it less than optimal for the studies undertaken here.

C. Maintaining spinal cord slices

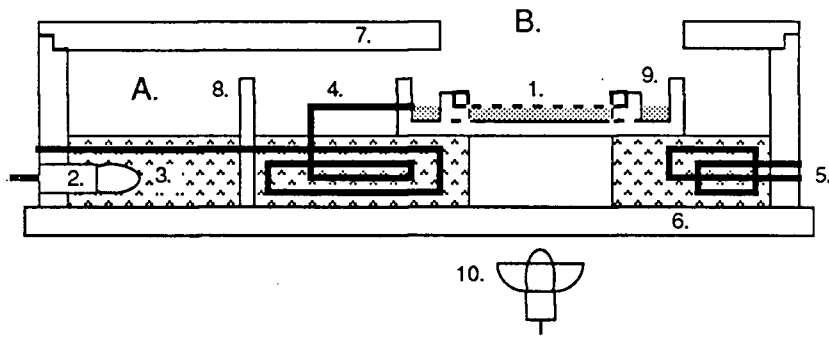
During the development of the slice technique, it became apparent that the environmental requirements (ie. ACSF flow and O_2 content, temperature and humidity) were different and more rigid for the spinal cord than for the hippocampus. The tissue bath used for hippocampal slices in the laboratory was of a simple, single chamber design where the slice chamber and the chamber

holding the heated distilled water used to humidfy the O_2/CO_2 were continuous (Fig. 5). When placed in the single chamber bath, slices of the spinal cord survived for 3 to 4 hours at most. In an effort to improve the viability of the slices, a slice chamber of completely new design was employed.

D. Design of tissue chamber

The viability of spinal cord slices once introduced into the in vitro chamber depends on an adequate flow of warmed and oxygenated ACSF below, and moisture-laden 95% O_2 - 5% CO_2 above. The amount of moisture carried by the gases (humidity) is dependent on the temperature of the fluid through which they are bubbled and is a critical component of slice maintenance which cannot be controlled in single chamber baths of conventional design. If the distilled water used to humidify the gases is separated from the tissue chamber it can then be maintained at a temperature different from that of the slices. Thus a two chamber bath was designed which permits saturation of the gases at any temperature prior to introduction into the slice chamber. The amount of condensation in the tissue chamber is then very accurately controlled by varying the temperature of the water used to humidify the gases.

The construction of the bath itself is also unique. A heated base and outer sleeve of aluminum encases a tissue chamber constructed of lexan (Fig. 6). A pair of transistors, embedded in the aluminum base, are used as a source of heat, the current to which is externally controlled by a microthermistor also embedded in the

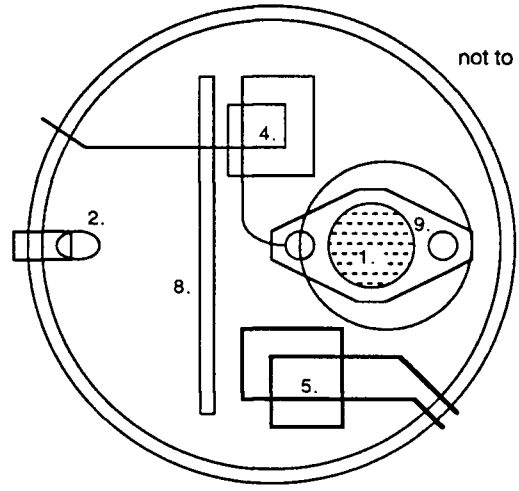


-ACSF -plexiglass -distilled water

-ACSF tubing (left) -heating coil (right)

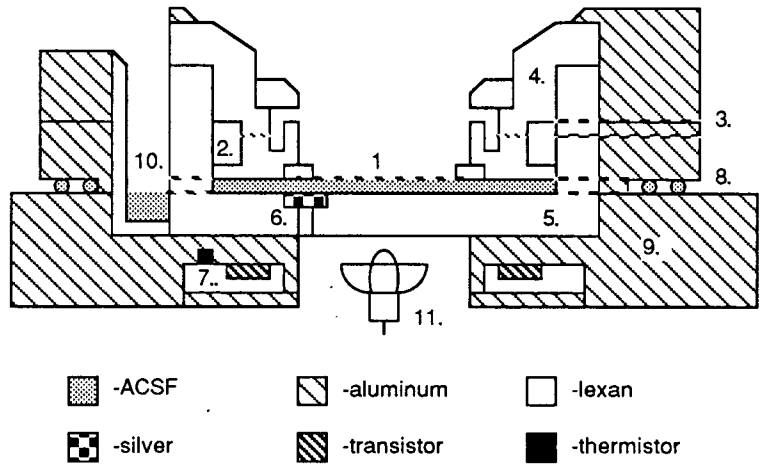
TOP VIEW

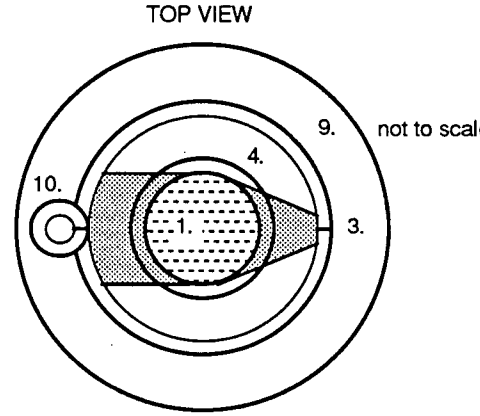
not to scale



1. -lexan ring and taut nylon mesh to support slices
2. -airstone inlet for 95% O2 - 5% CO2
3. -distilled water or ACSF
4. -ACSF tubing
5. -nichrome wire heating coils (in teflon tubing)
6. -plexiglass bottom and sides
7. -plexiglass lid
8. -bubble stop
9. -suction chamber
10. -light source

Figure 5. Diagram (not to scale) of a single chamber tissue bath of conventional design. The chamber holding the water through which the gases are bubbled (A) and the tissue chamber itself (B) are contiguous.





1. -lexan ring and taut nylon mesh to support slices
2. -oxygen chamber
3. -inlet port for warm/moist 95% O2 - 5% CO2
4. -inner lexan sleeve
5. -outer lexan sleeve
6. -silver ground
7. -temperature probe and transistors
8. -tubing and inlet port for ACSF
9. -aluminum baseplate and housing
10. -suction chamber
11. -light source

Figure 6. Diagram (not to scale) of a two-chambered tissue bath of novel design. The water through which the gases are bubbled is held in a separate container which is maintained at a temperature which provides the optimal humidity in the slice chamber.

base. The tissue chamber maintains an ACSF depth beneath the slices of less than 1.2 mm and has a total volume of less than 1 ml. The working area is circular with a diameter of 18 mm permitting up to three electrodes to be introduced into the chamber at one time. The total volume of the tissue chamber from reservoir to suction, including the tubing is less than 3 ml. This permitted the application of compounds in the perfusate with rapid turnover of the fluid in the chamber. Tryptamine, which has a specific ultraviolet absorbance at 640 nm, was added to the perfusate in order to observe the rate of change of concentration of compounds applied. Samples of 6 µl. were taken every 30 seconds from the fluid surface at the centre of the bath, diluted to 1 ml., and the absorbance measured. Figure 7 shows that the concentration reached 66 % of the theoretical maximum within 3.5 minutes. When applied in 10 ml. volumes at the standard rate of 1.5 ml.-min.$^{-1}$, the "washout curve" shows that a maximum concentration of ca. 92 % was obtained, while a level of 66 % was maintained for approximately 6 minutes (Fig. 7). During some experiments consistent firing rates for two or three agonist cycles (see below) were not obtained with a 6 minute application (particularily when 4 or more agonists were being tested at one time), and antagonists were therefore applied in volumes of 20 ml. or more. In these situations it was assumed that the maximum theoretical concentration of antagonist (100 %) was obtained and accounted for the observed reduction in the size of the responses.

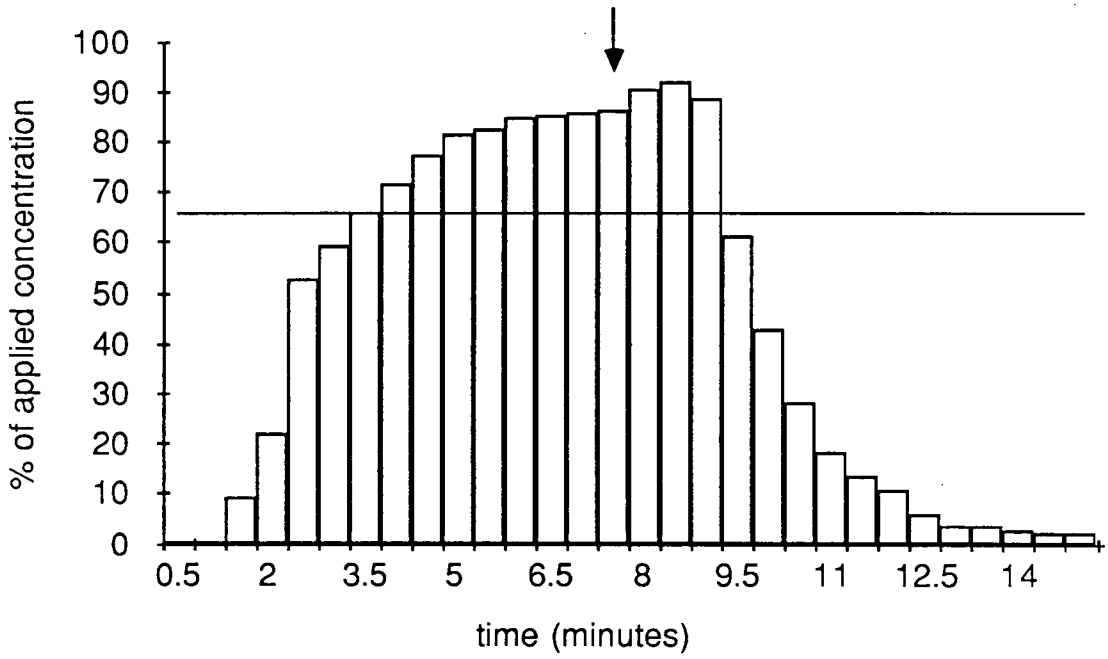


Figure 7. Bar graph of the absorbance (% of control) at 640 nm of tryptamine solution sampled (6 µl. every 30 sec.) from the middle of the perfusate surface. Stock solution of tryptamine was diluted in 10 ml. of ACSF which was applied at the standard rate of 1.5 ml.- min.$^{-1}$. Arrow indicates when the 10 ml. volume of antagonist solution ends and is replaced by normal ACSF at the fluid pump.

E. Microiontophoresis

Seven barrelled glass microelectrodes prepared from omega-dot tubing were purchased as blanks from Vancouver Scientific Glass Blowing. The blanks were pulled on a vertical electrode puller, and broken back to a final diameter of 5 - 8 μm under visual control with a Leitz micromanipulator. Compounds were applied iontophoretically with currents ranging from 1 to 100 nA from the outer 6 barrels of the 7-barrelled microelectrodes. Microiontophoresis is based on the principle that ions will move in solution when a charge is applied to that solution, resulting in current flow. If the ion to be ejected is negatively charged at the pH used, and is the "only" ion in the solution, it can be assumed that any current resulting from an applied charge is predominantly carried by the desired ion.

The outer barrels containing the amino acids to be ejected had, in these experiments, resistances between 50 and 200 MΩ. To prevent the barrels from having much higher resistances when attempting to apply very small amounts of a potent agonist or antagonist, the ion to be ejected comprised only a percentage of the total ionic strength of the solution. Thus for example, QUIS was commonly used at 5 mM in 150 mM NaCl. The total ionic concentration of the solution is therefore 155 mM, with the ion of interest comprising only about 3.2 % of the total (Table 1: page 30). Therefore, when ejecting QUIS with a current of 100 nA, the current carried by quisqualate molecules is approximately 3 nA. Another complication arises when determining how much amino acid is leaving the iontophoretic barrel. Proportionately more of the current will be carried by the

chloride ions than by the amino acid due to the lower mobility of the latter. Variability in the iontophoretic efficiency of individual electrode barrels and other factors make it very difficult to use the technique of iontophoresis for quantitative estimates of the relative potencies of agonists and antagonists. Over a large number of experiments, however, using many electrodes and testing numerous cells, it is possible to estimate the relative potency of an agonist based on the average firing rate achieved per nA of current applied, taking into account the concentration of agonist and NaCl in the solutions used. Antagonist potency can be more accurately assessed by application in the superfusate, as will be explained later. Although current balancing was not used, cells which displayed rapid changes in firing with the onset or offset of current were not included. A complete list of all compounds tested, and the concentrations used for iontophoresis both in vivo and in vitro, is shown in Table 1 on page 30. The amino acid solutions for iontophoresis were adjusted to pH 7.6 - 8.0 using 0.1 M NaOH, allowing the compounds to be ejected as anions.

F. Recording electrical activity

The extracellular electrical activity of dorsal horn and cortical neurones was recorded from the 4 M NaCl-containing central barrels of the electrode assemblies, and had resistances of less than 4 MΩ. The electrical activity of the neurones was passed through a 100 gain preamplifier to an oscilloscope and was also displayed on a second oscilloscope after passing through a variable filter (Fig. 8). Action

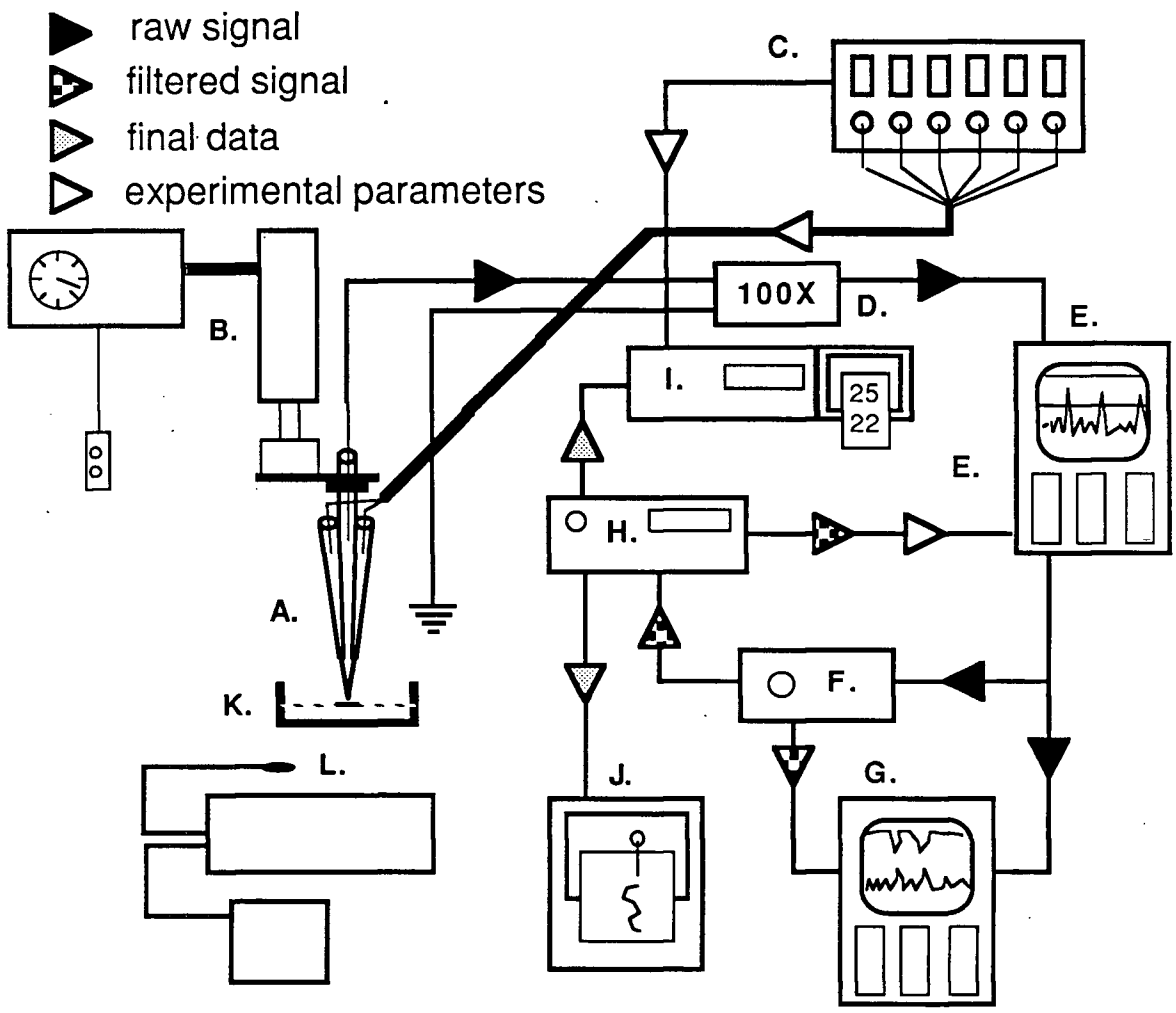


Figure 8. A) Seven-barrel glass microelectrode. B) Piezoelectric microdrive with depth indicator and remote control. C) Iontophoretic current generator with automated timer. D) 100 gain preamplifier grounded to the perfusate of the tissue chamber. E) Channel A of oscilloscope #1. F) Variable filter. G) Oscilloscope #2. H) Winston Instruments rate meter / window discriminator. E) Channel B of oscilloscope #1. I) Hewlett Packard thermal printer. J) Spikes - $sec.^{-1}$ plotted on a chart recorder. Recordings were made of the electrical activity of neurones maintained in vitro at 33° C in a two-chambered tissue bath K), or in vivo with the animals body temperature maintained at 35° C by L) a rectal thermistor and heating bed.

potentials of 1 mV in height were routinely recorded both in vivo and in vitro; spikes from single neurones were isolated with a window discriminator and rate meter records were made. The numbers used to express the activity of the cells were counts of the action potentials generated during each agonist ejection period only. No estimates were attempted of the various times of onset or offset of cell activity. Care was taken however, to adjust the position of the electrode relative to the cell and magnitude of the ejection currents used, to achieve rate meter peak heights and durations, as well as action potential numbers, that were similar for each agonist tested. This was not difficult to accomplish for most cells and for most agonists. Agonists were usually ejected for 10 or 15 seconds followed by a 10 or 15 second recovery period. A typical cycle would test two, three or sometimes four agonists against one or two antagonists. The agonists were applied in timed automated sequences, while the ejection currents for the antagonists were turned on and off manually. In general, three complete agonist cycles showing a minimum of variation were completed as controls prior to the addition of an antagonist. The antagonist would then be applied until the responses to one or more of the agonists was reduced by more than 50%, or until two or more cycles showing little variation were complete.

The antagonists were also applied in the superfusate in 10 ml. volumes at 1.5 ml-min^{-1}, which provided a constant concentration of the antagonist for about 6 minutes. On occasion, recordings were made during which 4 or 5 agonists were cycled and topically applied antagonists only were used: this necessitated using larger volumes of antagonist (as mentioned previously) to accommodate the prolonged agonist cycle times.

IV. Results

A. Presentation of the data

Figure 9, A. shows a ratemeter recording of the activity of a single dorsal horn neurone in vivo elicited by ejection of QUIS at 35 nA, L-GLU at 60 nA and NMDA at 40 nA. Each of these agonists were automatically applied in turn (indicated by horizontal bars beneath each peak) with 20 seconds of recovery allowed between each ejection period. The instantaneous firing rate of the cell can be estimated by comparing the ratemeter peak with the vertical scale bar on the left. The antagonist, PDA in this case, was turned on manually and applied for 4 complete agonist cycles; the horizontal bar above indicates the final cycle (three ratemeter peaks) recorded during the ejection of the antagonist. Only the spikes elicited during the ejection of the agonists were counted and used to represent the activity of the cells. The ratemeter record, however, shows all cell activity before, during and after the application of each agonist.

The tabular data is presented in three forms. First of all, it is depicted as the mean % reduction of control firing, pooled for all cells (Table 2 middle column). For example, in Figure 9, A. firing elicited by QUIS (393 spikes), L-GLU (383 spikes) and NMDA (293 spikes) is lower during the ejection of PDA; ie. a response reduction of 3% (377 spikes-QUIS), 10% (358 spikes-L-GLU) and 76% (75 spikes-NMDA) occurred. The % reduction by PDA of firing elicited by each of the agonists is averaged for all the cells examined and is presented as the mean %

reduction plus or minus the standard deviation with the numbers of cells tested given in parentheses (Table 2, middle column).

Secondly, the % reduction by an antagonist of firing elicited by an agonist is compared directly with the reduction of NMDA-elicited firing for each cell tested. This paired presentation in effect normalizes the data to a standard agonist, NMDA. For example in Figure 9, A. the 3% reduction of QUIS-induced activity by PDA is compared as a ratio to the 65% reduction of NMDA-elicited firing to give 0.05. The difference between the reduction of QUIS- and NMDA-elicited firing by PDA for this cell is greater than the average shown in Table 2, but clearly demonstrates the separation between these two agonists which, on some but not all occasions, can be obtained with PDA. The specificity of PDA is moderate for NMDA-elicited firing and the degree of separation between that of QUIS and NMDA with PDA depends on achieving modest and stable responses to the agonists coupled with relatively low iontophoretic currents of PDA for extended periods. The first number in the right hand column of Table 2 for PDA indicates an average X/NMDA ratio for QUIS of 0.58. This indicates that for a number of cells the reduction by PDA of the firing induced by QUIS will be on average 58 % of the reduction of NMDA-elicited activity. These ratios are averaged over the total number of cells on which an agonist was compared directly with NMDA, and are presented as the X/NMDA ratio plus or minus the standard deviation with the numbers of cells tested given in parentheses (Table 2, right hand column).

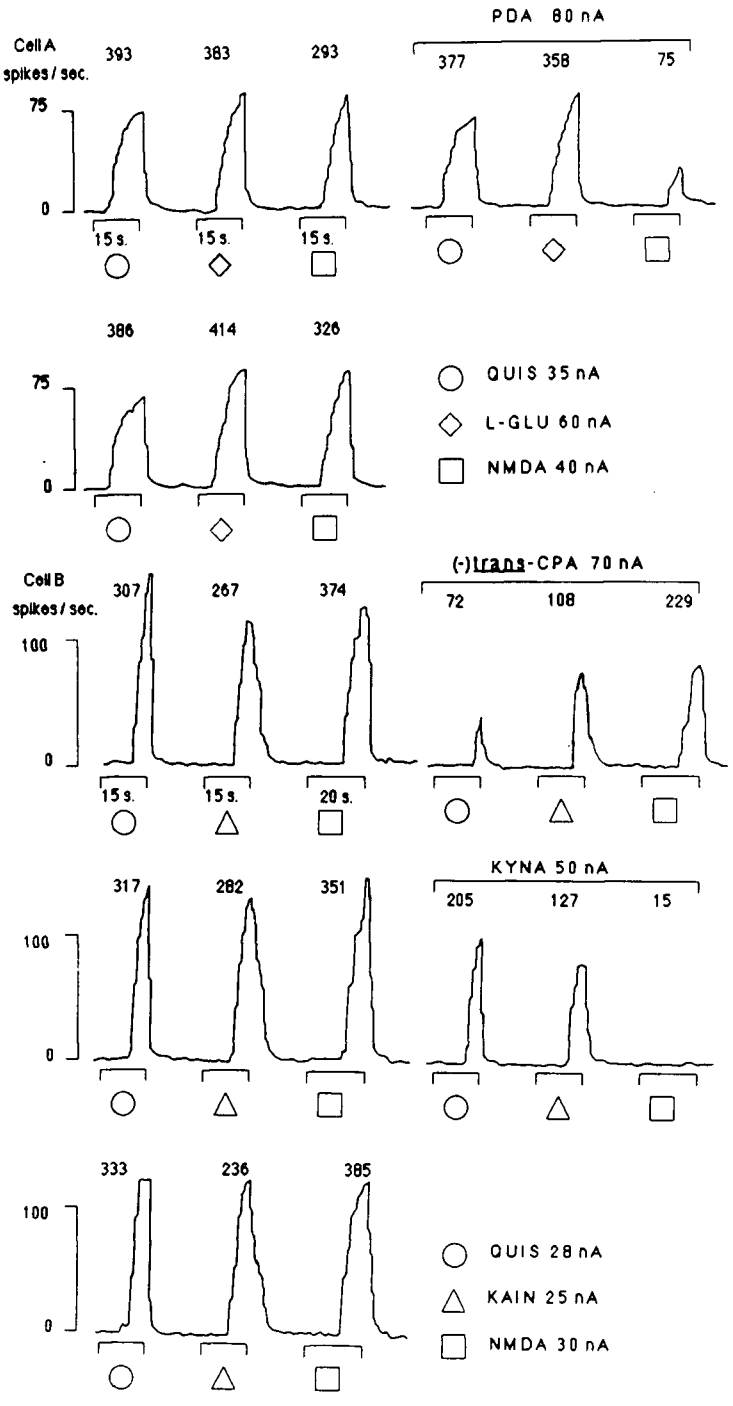


Figure 9. Segments of two continuous ratemeter records of the responses of single dorsal horn neurones in vivo to: cell A; QUIS (35 nA), L-GLU (60 nA) and NMDA (40 nA) and the antagonism of those responses by PDA applied iontophoretically at 80 nA and cell B; QUIS (28 nA), KAIN (25 nA) and NMDA (30 nA) and the antagonism of those responses by (-)trans-CPA and KYNA applied iontophoretically at 70 and 50 nA respectively. Horizontal bars indicate the ejection period of compounds; the agonists being applied in an automated-timed sequence and the antagonist currents being turned on and off manually. The total numbers of spikes elicited during each agonist ejection period are shown above each record.

Finally, the data is presented in a tabular form in which the agonists are ranked in order of susceptibility to a given antagonist for each cell tested using a plus or minus 15 % distinction value. The first number of each pair in Table 3 represents those cells for which the firing elicited by the agonist at the head of the column was reduced by at least 15 % more than was that of the agonist at the front of the row. The number contained in parentheses indicates the number of cells for which the two agonists could not be distinguished. This method of presentation allows each agonist to be compared directly to every other agonist for sensitivity to an antagonist. Thus, the trends that can be seen at a glance are very helpful. For example, in Table 3 for PDA, the second pair of numbers from the top on the far right [11(3)] indicate that for 11 cells on which both NMDA and QUIS were tested with PDA, the NMDA-elicited firing was reduced by at least 15 % more than was that of QUIS, while for three additional cells no distinction between NMDA and QUIS could be made. The comparison between NMDA and QUIS with PDA is made complete by examining the pair of numbers at the bottom of the second row from the left [0(3)], which indicates that firing elicited by QUIS was never more sensitive to antagonism by PDA than was that induced by NMDA, while the same three cells are indicated for which no distinction between QUIS and NMDA could be made. The conclusion, therefore, is, that of a total of 14 cells where the blockade of QUIS and NMDA was directly compared using the antagonist PDA, on 11 of these NMDA-induced activity was more potently attenuated than was that of QUIS, while on three cells no distinction could be made.

Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones In vivo by Cis-2,3-piperidinedicarboxylate, Kynurenate and Acridinate

Agonist	% reduction of control firing	X / NMDA ratio
PDA		
QUIS	39.4 ± 17.7 (17)	0.58 ± 0.27 (14)
KAIN	49.6 ± 22.8 (8)	0.59 ± 0.23 (4)
NMDA	72.7 ± 16.7 (15)	1.00
KYNA		
QUIS	68.9 ± 24.9 (28)	0.70 ± 0.22 (24)
KAIN	75.9 ± 25.3 (18)	0.91 ± 0.13 (11)
NMDA	83.1 ± 21.6 (37)	1.00
ACRA		
QUIS	54.6 ± 19.8 (29)	0.82 ± 0.32 (23)
NMDA	69.7 ± 19.3 (26)	1.00
KAIN	73.7 ± 20.3 (17)	1.13 ± 0.32 (9)

Table 2. Reduction of amino acid-induced firing of dorsal horn neurones in vivo by iontophoretically applied PDA, KYNA and ACRA expressed as the % reduction of control firing ± S.D. Also, for each cell, the reduction of QUIS or KAIN induced firing by each of the antagonists is compared directly with the reduction of NMDA-induced activity and is expressed as the ratio: % reduction of X over the % reduction of NMDA ± S.D. (X / NMDA ratio). The numbers of cells tested are given in brackets. L-GLU was not tested on a sufficient number of cells to be included in this table, but does appear in Table 3.

Ranked Order of Paired Data for the Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones In vivo by Cis-2,3-piperidinedicarboxylate, Kynurenate and Acridinate

PDA

	L-GLU	QUIS	KAIN	NMDA
L-GLU		3 (0)	3 (0)	2 (0)
QUIS	0 (0)		0 (2)	11 (3)
KAIN	0 (0)	1 (2)		3 (1)
NMDA	0 (0)	0 (3)	0 (1)	

KYNA

	L-GLU	QUIS	KAIN	NMDA
L-GLU		1 (3)	2 (2)	3 (3)
QUIS	2 (3)		3 (8)	10 (14)
KAIN	1 (2)	0 (8)		3 (8)
NMDA	1 (3)	0 (14)	0 (8)	

ACRA

	QUIS	L- GLU	NMDA	KAIN
QUIS		-	12 (9)	8 (4)
L-GLU	-		-	3 (1)
NMDA	2 (9)	-		3 (4)
KAIN	0 (4)	0 (1)	2 (4)	

Table 3. Antagonism of the amino acid-induced excitation of spinal neurones by PDA, KYNA and ACRA in vivo . The figures in each column show the number of occasions on which excitation by the compound listed at the head of the column was selectively blocked when compared directly with the excitant listed at the left. Numbers in parentheses show the trials in which the two compounds could not be distinguished (±15 %). The excitants are listed from left to right, and from above downward in order of increasing susceptibility to blockade. A bar indicates that the two compounds were never directly compared.

The majority of the results and discussion to follow will deal with the paired X/NMDA ratios: decimal ratios will be given for agonists other than NMDA without referring constantly to the fact that they have been compared directly with NMDA, and that the reduction of agonist-induced activity has been normalized to NMDA = 1.00. Additional reference will be made to tables of ranked-paired data in order to support conclusions, to indicate trends, or to make the groupings of the agonists more apparent.

B. Archetypal agonists and antagonism by PDA, KYNA and ACRA in the spinal cord in vivo

In the first series of experiments, excitation of dorsal horn neurones by the archetypal agonists, QUIS, KAIN and NMDA, and the reduction of these responses by two previously studied and one novel antagonist were examined in vivo. Figure 9 shows two ratemeter records of single dorsal horn neurones in vivo, demonstrating the actions of PDA, (-)trans-1-amino-1,2-cyclopentane dicarboxylate [(-)trans-CPA] and KYNA. Cell A shows the reduction of QUIS- (3 %), L-GLU- (10 %) and NMDA- (65 %) elicited firing resulting from the application of PDA at 80 nA. The reduction of firing induced by QUIS (37 %) , KAIN (51 %) and NMDA (96 %) by KYNA applied at 50 nA is seen for cell B. Also shown for cell B is the reduction of agonist-induced firing by iontophoretically applied (-)trans-CPA which will be discussed in more detail later in this section.

PDA, when ejected at 40 to 80 nA from a 100 mM solution, was found to

reduce firing elicited by QUIS, L-GLU and KAIN, but more potently attenuated activity due to NMDA. Table 2 shows that the ratios of the reduction of QUIS- and KAIN-elicited activities by PDA were 0.58 and 0.59 (ie. the reduction of QUIS- and KAIN-elicited firing by any given iontophoretic dose of PDA would be on average 60% that of NMDA-induced acitivity). This finding concurs with results reported by Davies et al. (1981) for the cat spinal cord, although the separation between NMDA and QUIS or KAIN appeared to be less in the cat.

KYNA was ejected at 25 to 70 nA from a 100 mM solution and was found to reduce KAIN and NMDA excitations comparably, while QUIS activity was less affected. Direct comparison with NMDA showed activity induced by KAIN and QUIS to be reduced 0.91 and 0.70 respectively (Table 2). These data also are in general agreement with previous reports by Stone and Perkins (1984), Ganong et al. (1985) and Peet et al. (1986) in the hippocampus, Perkins and Stone (1984) in the cortex, Elmslie and Yoshikami (1985) in the frog spinal cord, and Ganong et al. (1983) in the rat spinal cord.

The third antagonist tested in this series of experiments was acridinate (ACRA), a previously untested compound (synthesized by Dr. K. Curry). It was found to be less potent than KYNA and PDA, (ejected at 37 to 80 nA from a 200 mM solution) with little ability to distinguish between the three archetypal agonists. Table 2 shows that when compared directly with the reduction of NMDA-elicited firing, QUIS and KAIN activities were altered to 0.82 and 1.13 respectively.

The ranked-paired data in Table 3 show that for 11 of 14 cells tested with PDA, NMDA-induced firing was reduced by at least 15 % more than was QUIS-induced firing, while for 2 of 3 cells, QUIS- and KAIN-activity was reduced by a similar amount. The agonists are shown from right to left and from the top down in order of increasing susceptibility to antagonism. The trends apparent in Table 3 do not appear to contradict the conclusions gained from Table 2. Thus, with PDA, the agonists appear to fall into two categories: (QUIS and KAIN) < NMDA. Two categories of agonists are also apparent when using KYNA, however, KAIN is more like NMDA than QUIS in its susceptibility to this antagonist, hence: QUIS < (KAIN and NMDA).

C. Archetypal agonists and antagonism by PDA, KYNA and ACRA in the cortex in vivo

In these experiments the excitation of cortical neurones in vivo by QUIN, in addition to the archetypal agonists QUIS, KAIN and NMDA, and the antagonism of these responses by iontophoretically applied KYNA and ACRA were examined.

Figure 10 shows two ratemeter records from single cortical neurones in vivo which demonstrate for cell A the degree of separation between QUIS- and NMDA-elicited activity which can be achieved using KYNA, and for cell B, that responses to QUIN and NMDA are often comparably reduced by KYNA in the cortex.

As in the spinal cord in vivo, ACRA (ejected at 50 to 80 nA from a 200 mM solution; Table 1: page 30) was found to be a relatively weak, nonspecific

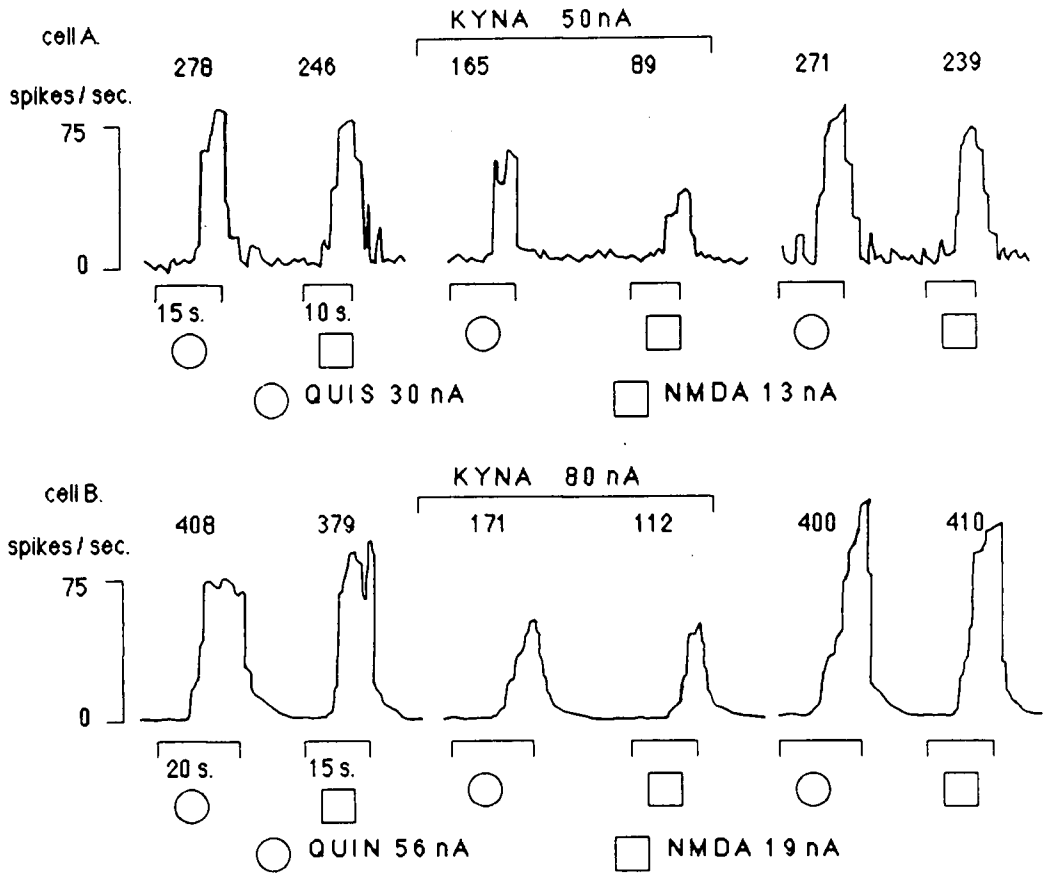


Figure 10. Segments of two continuous ratemeter records of the responses of single cortical neurones in vivo to: cell A; QUIS (30 nA) and NMDA (13 nA) and the antagonism of those responses by KYNA applied iontophoretically at 50 nA and cell B; QUIN (56 nA) and NMDA (19 nA) and the antagonism of those responses by KYNA applied iontophoretically at 80 nA. Conventions as in Figure 9 (page 50).

antagonist for which QUIN, KAIN and QUIS activity had reduction ratios of 0.85, 0.98 and 1.01 respectively (Table 4). Table 5 demonstrates using ranked-paired data that for the majority of cells no distinction between amino acid agonists could be made using ACRA (± 15 %).

When ejected at 50 to 70 nA from a 100 mM solution (Table 1: page 30), KYNA reduced excitations elicited by all four agonists tested, with QUIN, KAIN and NMDA being affected almost equally for most cells (0.96 and 1.13 for QUIN and KAIN; Table 4). QUIS-elicited activity was on average slightly less susceptible to antagonism by KYNA in the cortex in vivo (0.77, Table 4). Table 5 shows that the agonists could be distinguished for a somewhat larger proportion of cells with KYNA than with ACRA; QUIN, KAIN and NMDA appearing to fall into one group, with QUIS the sole member of a second group. The degree of separation between NMDA/QUIN/KAIN and QUIS is not sufficient, however, to show a statistical difference between these two groups of compounds.

The data presented so far for the spinal cord and cortex in vivo have been published in a slightly different form (Curry et al., 1986) and are in general agreement with results reported by many other authors (Davies et al., 1981; McLennan and Liu, 1981; Perkins and Stone, 1982; Ganong et al., 1983; McLennan, 1984; Elmslie and Yoshikami, 1985).

Reduction of Amino Acid-Induced Activity of Cortical Neurones In vivo by Kynurenate and Acridinate

Agonist	% reduction of control firing	X / NMDA ratio
KYNA		
QUIS	42.1 ± 16.6 (24)	0.77 ± 0.36 (18)
QUIN	45.5 ± 13.8 (12)	0.96 ± 0.49 (10)
NMDA	51.5 ± 17.1 (18)	1.00
KAIN	56.5 ± 23.0 (12)	1.13 ± 0.39 (12)
ACRA		
QUIN	40.3 ± 18.6 (8)	0.85 ± 0.25 (4)
KAIN	43.3 ± 15.8 (12)	0.98 ± 0.44 (11)
NMDA	48.9 ± 13.7 (13)	1.00
QUIS	41.7 ± 12.4 (20)	1.01 ± 0.32 (13)

Table 4. Reduction of amino acid-induced firing of cortical neurones in vivo by KYNA and ACRA expressed as the % reduction of control firing ± S.D. and the X / NMDA ratio as described for Table 2 (page 52). The numbers of cells tested are given in brackets.

Ranked Order of Paired Data for the Reduction of Amino Acid-Induced Activity of Cortical Neurones In vivo by Kynurenate and Acridinate

KYNA

	QUIS	QUIN	NMDA	KAIN
QUIS		2 (10)	6 (11)	7 (3)
QUIN	1 (10)		5 (4)	-
NMDA	1 (11)	1 (4)		4 (6)
KAIN	1 (3)	-	2 (6)	

ACRA

	QUIN	QUIS	NMDA	KAIN
QUIN		3 (5)	1 (3)	1 (0)
QUIS	1 (5)		2 (10)	3 (7)
NMDA	0 (3)	1 (10)		4 (4)
KAIN	0 (0)	3 (7)	3 (4)	

Table 5. Antagonism of the amino acid-induced excitation of cortical neurones by KYNA and ACRA in vivo. Conventions are as described for Table 3 (page 53). The excitants are listed from left to right, and from above downward in order of increasing susceptibility to blockade. A bar indicates that the two compounds were never directly compared.

D. α-Substituted analogues in the spinal cord in vivo

In this series of experiments substituted analogues of L-GLU were tested for agonist and antagonist properties in the spinal cord in vivo. α-Methyl, -phenyl and para-substituted-phenyl varieties were tested, but only the para-chloro (αpCPG) and para-fluoro (αpFPG; Fig. 1, F and G: page 3) substituted analogues had noticeable actions: these two were found to have weakly antagonistic effects. Table 6 shows the effects of αpCPG and αpFPG on dorsal horn neurone activity elicited by the iontophoretic application of NMDA, KAIN, QUIS, L-GLU and AMPA in vivo. The analogue αpFPG reduced L-GLU-elicited firing more than that induced by NMDA. Table 7 demonstrates that for all 7 cells on which both L-GLU and NMDA were tested, the activity of L-GLU was reduced more than that of NMDA (± 15 %). All of the α-substituted analogues tested were ejected from 200 mM solutions (Table 1: page 30) and αpFPG required iontophoretic currents averaging over 70 nA to cause any noticeable reduction of amino acid-induced firing.

E. (+) and (-) Trans-cyclopentane aspartate in the spinal cord in vivo

The optical isomers of a previously untested analogue of aspartate were examined in these experiments for agonist or antagonist activities in the spinal cord in vivo. This compound, trans-1-amino-1,2-cyclopentane dicarboxylate (trans-cyclopentane aspartate; trans-CPA), was synthesized and resolved into its optical isomers by Dr. K. Curry. On no occasion did either isomer of trans-CPA cause excitation by itself or an enhancement of firing elicited by another compound; on

Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones In vivo by α-Substituted Analolgues of Glutamate

Agonist	αpCPG	αpFPG
NMDA	-7.1 ± 19.8 (7)	+19.1 ±27.8 (11)
KAIN	-15.0 ± 13.2 (3)	-2.7 ± 4.6 (3)
QUIS	-6.3 ± 11.5 (7)	-7.2 ± 8.1 (6)
AMPA	n. t.	-9.4 ± 17.9 (8)
L-GLU	-6.5 ± 7.5 (4)	-23.7 ± 11.3 (12)

Table 6. Effects of α-(para-chlorophenyl)glutamate and α-(para-fluorophenyl)-glutamate on amino acid induced firing of dorsal horn neurones in vivo shown as the % change in control firing rate ± S.D. observed with the iontophoretic application of the α-substituted analogue, with the numbers of cells tested given in brackets.

Ranked Order of Paired Data for the Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones In vivo by α-(para-fluorophenyl)glutamate

	NMDA	KAIN	QUIS	AMPA	L-GLU
NMDA		1 (1)	2 (0)	2 (0)	7 (0)
KAIN	0 (1)		-	-	2 (0)
QUIS	0 (0)	-		2 (3)	3 (2)
AMPA	0 (0)	-	0 (3)		2 (1)
L-GLU	0 (0)	0 (0)	0 (2)	1 (1)	

Table 7. Antagonism of the amino acid-induced excitation of cortical neurones by KYNA and ACRA in vivo. Conventions are as described for Table 3 (page 53). The excitants are listed from left to right, and from above downward in order of increasing susceptibility to blockade. A bar indicates that the two compounds were never directly compared.

the contrary both were found to reduce amino acid-elicited firing in the dorsal horn.

Figure 11 is a ratemeter recording of a single dorsal horn neurone in vivo showing the reduction of amino acid-induced activity by iontophoretically applied (-)trans-CPA. When applied at 50 nA from a 200 mM solution (Table 1: page 30), (-)trans-CPA completely blocked the firing of this neurone elicited by QUIS and L-GLU, caused a 90 % reduction of that induced by KAIN while reducing NMDA's activity by only 26 %. This pattern of antagonism can also be seen in Figure 9, B. (page 50) where (-)trans-CPA ejected at 70 nA caused 77 and 61 % reductions of QUIS- and KAIN-activities respectively while reducing NMDA-elicited firing by only 37 %. When tested on that same neurone in Figure 9, B., KYNA caused 37 and 51 % reductions of QUIS- and KAIN-activities and almost completely blocked (96 %) the firing elicited by NMDA.

Table 8 compares the antagonist activity exhibited by (+) and (-)trans-CPA on amino acid-induced firing of dorsal horn neurones. When ejected at 25 to 90 nA from a 200 mM solution (Table 1: page 30) both isomers were capable of reducing firing elicited by all of the agonists tested. QUIS, L-GLU and KAIN elicited activities were substantially more susceptible to antagonism by (-)trans-CPA than was NMDA-induced firing. Paired students t-tests show that QUIS, KAIN and L-GLU are not significantly different from one another in sensitivity to antagonism by (-)trans-CPA, but are significantly different from NMDA in that respect ($p < 0.05$). Table 9 supports these conclusions by demonstrating that for 15 of 16 neurones tested, the activity of QUIS was reduced more than that of NMDA by at least 15 %.

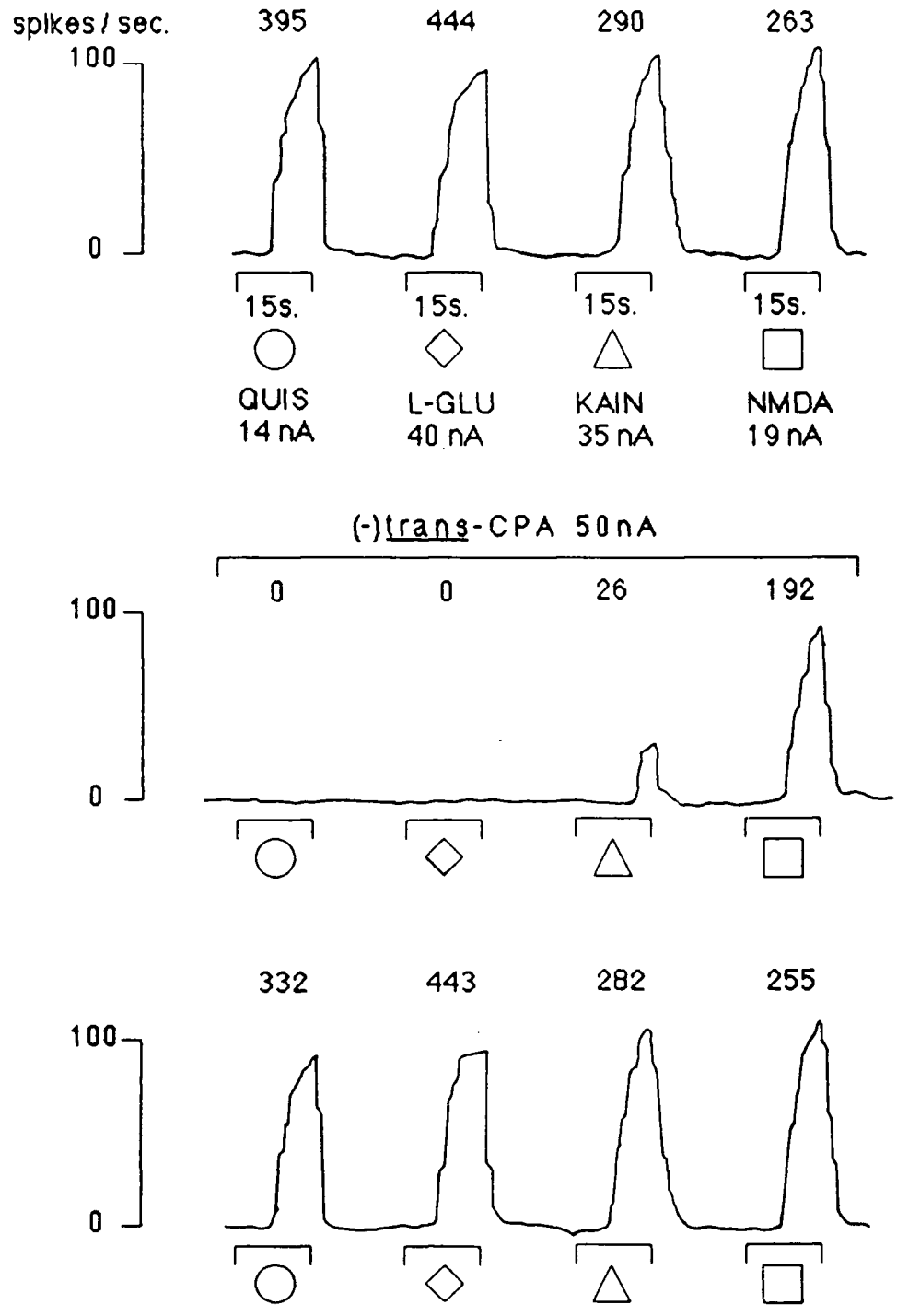


Figure 11. Segments of a continuous ratemeter record of the responses of a single dorsal horn neurone in vivo to QUIS (30 nA), L-GLU (40 nA), KAIN (35 nA) and NMDA (19 nA) and the antagonism of those responses by (-)trans-CPA applied iontophoretically at 50 nA.
Conventions as in Figure 9 (page 50).

In addition, Table 9 shows that despite the statistical similarity, in 9 of 13 cells tested QUIS-induced firing was more sensitive to blockade by (-)trans-CPA than was that of KAIN (± 15 %).

F. Archetypal agonists and antagonism by

APV, PDA, KYNA and ACRA in the spinal cord in vitro

All the remaining experiments to be discussed were done using the spinal cord slice preparation. The first goal was to examine many of the compounds which had already been tested in vivo in order to determine whether or not their actions were similar in vitro. Table 10 shows the results for the first 30 cells from which records were obtained in the spinal cord in vitro and compares them with the in vivo results already described. KYNA and PDA were found to have similar profiles of antagonism in vitro as in vivo, in that the order of susceptibility of agonists to these antagonists was identical in the two preparations. The difference in the effect of KYNA and PDA on excitations elicited by the most sensitive group of compounds and those of the least sensitive compounds is apparently greater in vitro than in vivo. For example, in Table 10, NMDA and KAIN excitations are the most sensitive of any tested to blockade by KYNA, while those of QUIS are the least. When examined in vivo the reduction ratios are 1.00 for NMDA, 0.91 for KAIN and 0.70 for QUIS. The separation between QUIS and KAIN is, therefore, 0.19. When studied in vitro, however, this separation grows to 0.30 (0.96 for KAIN and 0.66 for QUIS; Table 10). Similarly for PDA, both QUIS- and KAIN-elicited

Reduction of Amino Acid-Induced Firing of Dorsal Horn Neurones In vivo by the Optical Isomers of Trans-1-amino-1,2-cyclopentane dicarboxylate

	(-)trans-CPA	(+)trans-CPA
agonist	% reduction of control firing	% reduction of control firing
QUIS	73.4 ± 21.5 (24)	21.9 ± 24.2 (18)
L-GLU	68.6 ± 19.8 (13)	30.8 ± 34.2 (7)
KAIN	62.6 ± 21.9 (16)	39.4 ± 29.1 (11)
NMDA	29.8 ± 16.2 (20)	9.0 ± 24.4 (13)

Table 8. Average iontophoretic current used: 63.5 and 72.3 nA respectively for (-) and (+)trans-CPA. Data given as the percent reduction of control firing resulting from iontophoretic application of the antagonists ± S.D. with the numbers of cells tested given in brackets.

Ranked Order of Paired Data for the Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones In vivo by (-)Trans-1-amino-1,2-cyclopentane dicarboxylate

	NMDA	KAIN	L-GLU	QUIS
NMDA		4 (4)	6 (1)	15 (0)
KAIN	0 (4)		2 (7)	9 (2)
L-GLU	0 (1)	2 (7)		4 (5)
QUIS	1 (0)	2 (2)	1 (5)	

Table 9. Antagonism of the amino acid-induced excitation of spinal neurons by (-)trans-CPA in vivo. Conventions are as described for Table 3 (page 53). The excitants are listed from left to right, and from above downward in order of increasing susceptibility to blockade.

firing was less sensitive to blockade in vitro than in vivo relative to NMDA. ACRA, on the other hand, was as unspecific an antagonist in vitro as it was in vivo. Because PDA proved to have a profile of antagonism comparable to that reported for APV, but with a lower potency and specificity for reducing NMDA-elicited firing compared to APV (ie. the separation between NMDA and QUIS and KAIN was not as great), APV was used for the remaining experiments in addition to, and often to the exclusion of, PDA.

During these first experiments using the spinal cord preparation in vitro it was discovered that 41 to 60 % lower agonist ejection currents were required to achieve similar firing rates when compared to the situation in vivo. For QUIS, NMDA and QUIN the mean iontophoretic currents required to elicit a control firing rate of 15 to 25 Hz. were significantly lower in vitro than in vivo ($p < .001$, t-test; Table 11). For subsequent experiments involving KAIN and NMDA a lower concentration of agonist in the iontophoretic pipette was used in vitro compared to in vivo (Table 1: page 30). This increase in potency was also found for antagonists, so that on average significantly lower iontophoretic currents were used in vitro to achieve reductions of QUIS- or NMDA-induced firing comparable to those in vivo (66 % lower for PDA, 63 % lower for KYNA; $p < .001$, t-test; Table 11). These findings are shown in Table 11, where the mean iontophoretic currents used to eject QUIS, QUIN, NMDA, KYNA and PDA are shown for many in vivo and in vitro experiments. The increased neuronal sensitivity in vitro meant that QUIN, applied at 20 to 80 nA from a 200 mM solution (Table 1: page 30), was of sufficient potency

Reduction of Amino Acid-Induced Firing of Dorsal Horn Neurones In vivo and In vitro by Kynurenate, Acridinate, Cis-2,3-piperidine dicarboxylate and D-(-)-2-amino-5-phosphonovalerate

compound	QUIS	QUIN	KAIN
KYNA			
in vivo	0.70 ± 0.22 (17)	---	0.91 ± 0.13 (10
in vitro	0.66 ± 0.29 (16)	0.65 ± 0.17 (8)	0.96 ± 0.13 (11)
ACRA			
in vivo	0.82 ± 0.32 (21)	---	1.13 ± 0.32 (9)
in vitro	1.13 ± 0.39 (11)	1.02 ± 0.49 (7)	1.10 ± 0.37 (7)
PDA			
in vivo	0.58 ± 0.27 (14)	---	0.59 ± 0.23 (4)
in vitro	0.49 ± 0.34 (7)	---	0.51 ± 0.43 (6)
APV			
in vivo	0.37 ± 0.23 (46)[1]	---	0.22 ± 0.28 (33)[1]
in vitro	0.23 ± 0.21 (14)	0.45 ± 0.34 (7)	0.18 ± 0.18 (7)

Table 10. For each cell the effect of iontophoretically applied KYNA, ACRA, PDA and APV has been expressed as the X / NMDA ratio as described for Table 2 (page 52). The numbers of cells tested are given in brackets. QUIN was not able to excite cells sufficiently to be tested in vivo. The effect of PDA on QUIN-induced firing was not examined.
1 -data from experiments by H. McLennan.

to be examined in this preparation; although of all the cells which were excited by QUIS and NMDA, only 60 - 70 % would respond adequately to QUIN. This is in contrast to its failure to excite more than a very small proportion (< 10 %) of spinal neurones in vivo (observations of the author and cf. Stone and Perkins, 1981; McLennan, 1984).

These initial experiments showed that the neurones being examined in the spinal cord slice respond to the amino acid agonists and antagonists in a manner qualitatively similar to what had been observed in vivo. They also demonstrated that QUIN's action in the spinal cord in vitro is unlike that of NMDA, in that its blockade by KYNA was significantly less than that of NMDA (Table 10). This is in contrast to what has already been described herein for the actions of NMDA and QUIN in the cortex where they were comparable in their sensitivity to blockade by KYNA (Tables 4 and 5: pages 59 and 60). QUIN has been reported by others to be qualitatively indistinguishable from NMDA in the cortex (Perkins and Stone, 1982; McLennan, 1984), and hippocampus (Ganong and Cotman, 1986; Peet et al., 1986).

Comparison of Agonist and Antagonist Ejection Currents Used in the Spinal Cord In vivo & In vitro

compound	In Vivo (nA)	In Vitro (nA)
QUIS (5mM)	22.4 ± 9.9 (109)	12.5 ± 8.7 (51)
mean firing rate	20.1 Hz	19.1 Hz
NMDA (50mM)	30.8 ± 14.6 (117)	18.2 ± 10.4 (94)
mean firing rate	19.4 Hz	18.9 Hz
QUIN (200mM)	94.0 ± 23.0 (5)	38.1 ± 16.9 (41)
mean firing rate	18.4 Hz	19.9 Hz
KYNA (20mM)	51.0 ± 10.6 (26)	34.2 ± 16.3 (36)
mean firing rate reduction	68.1 %	74.2 %
PDA (100mM)	54.0 ± 11.3 (14)	19.8 ± 8.4 (18)
mean firing rate reduction	68.7 %	67.5 %

In each case the firing rate achieved with the degree of reduction induced is not significantly different in vivo vs in vitro. The mean currents needed to elicit the effects however were always less in vitro ($p < 0.001$, t-test).

Table 11. QUIS, NMDA and QUIN: The average iontophoretic current (± S.D.) required to evoke a control firing rate of between 15 and 25 Hz. KYNA and PDA: The average iontophoretic current required to achieve a 50 to 80% reduction of QUIS- or NMDA-induced firing. The numbers of cells tested are given in brackets.

G. L- and D-glutamate in the spinal cord in vitro

Both the L- and D-isomers of glutamate were examined on a number of cells using the spinal cord preparation in vitro. These results are shown as the pooled % reduction and paired X/NMDA ratios in Table 12 and as ranked-order paired data in Table 13. Paired t-tests determined that the blockade of D-GLU-induced activity was significantly greater than that of QUIS but significantly less than that of NMDA for both APV and KYNA (paired t-tests; Table 12). In contrast, no significant difference could be shown between the reductions of QUIS- and L-GLU-elicited firing by either APV or by KYNA. This grouping, with L-GLU and QUIS being the least, D-GLU intermediate and NMDA being the most sensitive to antagonism by APV and KYNA can also be seen in Table 13. Most notably, D-GLU-elicited firing was more sensitive (± 15 %) to antagonism by both APV and KYNA than were those of QUIS and L-GLU on all cells examined (top two pairs of numbers in the third column of Table 13 for APV and KYNA). That D-GLU is not purely NMDA-like was also seen, however, since for 8 of 9 and 3 of 5 cells examined, it was less sensitive to antagonism by APV and KYNA respectively than was NMDA (Table 13). When the iontophoretic currents required to evoke a similar firing rate from a given neurone were compared for these agonists, QUIS was found to be 43 times and D-GLU 0.43 times as potent as L-GLU (Table 12). These conclusions are in agreement with earlier reports on the activity of D-GLU (Hicks et al., 1978; Hall et al., 1979).

Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones In vitro by D-(-)-2-amino-5-phosphonovalerate and Kynurenate

compound	potency*	average % reduction	X / NMDA (paired)
APV			
D-GLU	0.43	56.6 ± 18.1 (10)	[a] 0.64 ± 0.15 (9)
L-GLU	1.00	23.4 ± 14.3 (10)	[b] 0.26 ± 0.17 (10)
QUIS	43.0	23.1 ± 17.7 (15)	[b] 0.29 ± 0.21 (12)
KYNA			
D-GLU		46.2 ± 15.7 (5)	[a] 0.70 ± 0.24 (5)
L-GLU		30.3 ± 16.9 (7)	[b] 0.48 ± 0.26 (6)
QUIS		32.5 ± 15.7 (15)	[b] 0.51 ± 0.24 (10)

a -significantly different from QUIS and NMDA. paired t-tests, $p < 0.05$.
b -significantly different from NMDA. paired t-tests, $p < 0.005$.

Table 12. Reduction of amino acid-induced firing of dorsal horn neurones in vitro by KYNA and APV expressed as the % reduction of control firing ± S.D. and as the X / NMDA ratio as described for Table 2 (page 52). The numbers of cells are given in brackets.
*Potency: agonist potency compared to L-GLU = 1.00. For example, D-GLU requires about 2.3 X the ejection current needed by L-GLU to achieve a similar effect on a given neurone, making allowance for the total ionic strength of the solutions.

Ranked Order of Paired Data for the Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones In vitro by D-(-)-2-amino-5-phosphonovalerate and Kynurenate

APV

	QUIS	L-GLU	D-GLU	NMDA
QUIS		0 (2)	2 (0)	12 (0)
L-GLU	0 (2)		8 (0)	10 (0)
D-GLU	0 (0)	0 (0)		8 (1)
NMDA	0 (0)	0 (0)	0 (1)	

KYNA

	QUIS	L-GLU	D-GLU	NMDA
QUIS		0 (2)	2 (0)	9 (1)
L-GLU	1 (2)		4 (0)	5 (1)
D-GLU	0 (0)	0 (0)		3 (2)
NMDA	0 (1)	0 (1)	0 (2)	

Table 13. Antagonism of the amino acid-induced excitation of dorsal horn neurones in vitro by APV and KYNA. Conventions are as described for Table 3 (page 53). The excitants are listed from left to right, and from above downward in order of increasing susceptibility to blockade.

H. Quinolinate in the spinal cord in vitro:

antagonists applied iontophoretically and topically

Figure 12 shows typical segments of a continuous ratemeter record which compares the reduction of amino acid-induced firing in a single lamina IV neurone in vitro by APV and ACRA applied iontophoretically or via the superfusate, and by KYNA applied in the superfusate. For this cell APV applied iontophoretically at 9 nA reduced NMDA-evoked firing (94 %), had a minimal effect on the response elicited by QUIS (26 %), and had an intermediate effect on QUIN-induced firing (51 %). Similarly, APV applied in the superfusate at 4×10^{-6}M abolished the response to NMDA (100 %) while reducing the QUIS-elicited firing 45 % and that of QUIN 60 % (Fig. 12). KYNA was less specific than APV, thus, when topically applied at 5×10^{-5}M, firing elicited by NMDA was reduced 85 % while that of QUIS and QUIN was reduced 57 and 61 % respectively. The nonspecific antagonist ACRA applied at 4×10^{-4}M caused approximately a 50% reduction of the activities elicited by all three agonists.

In Table 14, the reduction of amino acid-induced firing of dorsal horn neurones in vitro by iontophoretically applied antagonists is shown, as it was in part in Table 10. For each cell examined, the reduction of QUIS-, QUIN- and KAIN-induced firing, expressed as a percent of control, is compared with the reduction of NMDA-elicited activity. Responses to QUIS and KAIN are largely unaffected by APV (0.23 and 0.18 respectively) at ejection currents which reduced NMDA-evoked responses on average by 93% (n = 18). The antagonism of QUIN-activity by APV

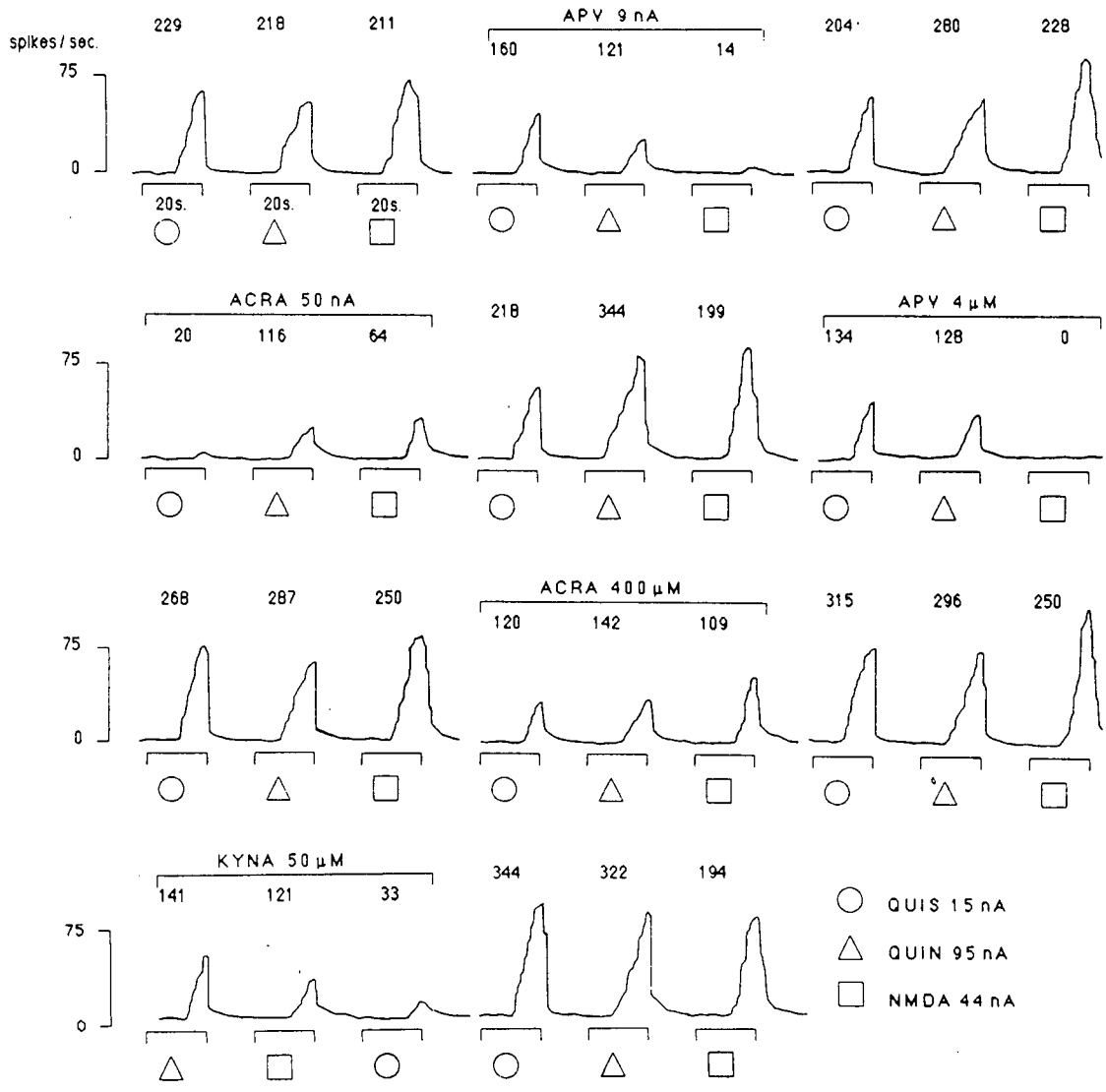


Figure 12. Segments of a continuous ratemeter record of the responses of a single dorsal horn neurone <u>in vitro</u> to QUIS (15 nA), QUIN (95 nA) and NMDA (44 nA) and the antagonism of those responses by: APV applied iontophoretically at 9 nA and in the superfusate at 4 µM, ACRA applied iontophoretically at 50 nA and in the superfusate at 400 µM and KYNA applied in the superfusate at 50 µM. Conventions as in Figure 9 (page 50). Stock solutions of the antagonists were diluted in 10 ml. of gased ACSF and applied at the standard superfusion rate of 1.5 ml.- min.$^{-1}$.

Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones

In vitro by D-(-)-2-amino-5-phosphonovalerate, Kynurenate and Acridinate

Agonist	% reduction of control firing	X / NMDA ratio
APV		
KAIN	18.5 ± 16.3 (10)	(1)0.18 ± 0.18 (7)
QUIS	20.1 ± 19.1 (19)	(1)0.23 ± 0.21 (14)
QUIN	47.1 ± 31.0 (11)	(2)0.45 ± 0.34 (7)
NMDA	93.0 ± 8.6 (16)	(3)1.00
KYNA		
QUIN	53.0 ± 26.8 (14)	(2)0.65 ± 0.17 (8)
QUIS	57.7 ± 36.2 (27)	(2)0.66 ± 0.29 (16)
KAIN	81.7 ± 25.9 (15)	(3)0.96 ± 0.13 (11)
NMDA	87.3 ± 22.0 (30)	(3)1.00
ACRA		
QUIN	64.1 ± 22.4 (9)	1.02 ± 0.49 (7)
NMDA	71.5 ± 24.0 (15)	1.00
KAIN	79.5 ± 26.8 (6)	1.10 ± 0.37 (7)
QUIS	69.5 ± 32.0 (12)	1.13 ± 0.39 (11)

(1) significantly different from QUIN and NMDA
(2) significantly different from KAIN and NMDA
(3) significantly different from QUIN and QUIS

paired t-tests ($p<0.05$)

Table 14. Reduction of amino acid-induced firing of dorsal horn neurones in vitro by iontophoretically applied APV, KYNA and ACRA expressed as the % reduction of control firing ± S.D. and as the X / NMDA ratio as described for Table 2 (page 52). The numbers of cells tested are given in brackets.

was found to be 0.45 averaged for 7 cells, which was significantly greater than that of QUIS- and KAIN-induced firing but significantly less than that of NMDA ($p < .001$, t-test).

Using APV as an antagonist, the agonists can be separated into three significantly different groups with QUIS and KAIN the least susceptible, QUIN intermediately affected and NMDA the most sensitive to blockade. In contrast, iontophoretically applied KYNA caused similar reductions of QUIS- and QUIN-evoked responses (0.66 and 0.65 respectively), while KAIN-elicited firing could not be distinguished from NMDA (0.96 compared with NMDA = 1.00). Thus, only two significantly separable categories are apparent when using KYNA: QUIS and QUIN are the least affected while KAIN and NMDA are much more susceptible. Reductions of agonist-induced firing by ACRA were similar for all four of the agonists studied and no statistical differences could be shown for any of the excitatory compounds tested.

Figure 13 shows the dose-response curves for KYNA and APV applied in the bathing medium. The concentration range used for KYNA is precisely 10-fold larger than that of APV which gives a good indication of the relative potencies of these two antagonists against NMDA activity. The blockade of QUIN, QUIS, and KAIN-induced firing is incomplete at 20 μM APV however, and the slopes of the curves at this concentration suggest that doses of APV several fold higher would have to be used to block completely the actions of these agonists. In contrast, the highest dose of KYNA shown in Figure 13 of 200 μM was sufficient to abolish the

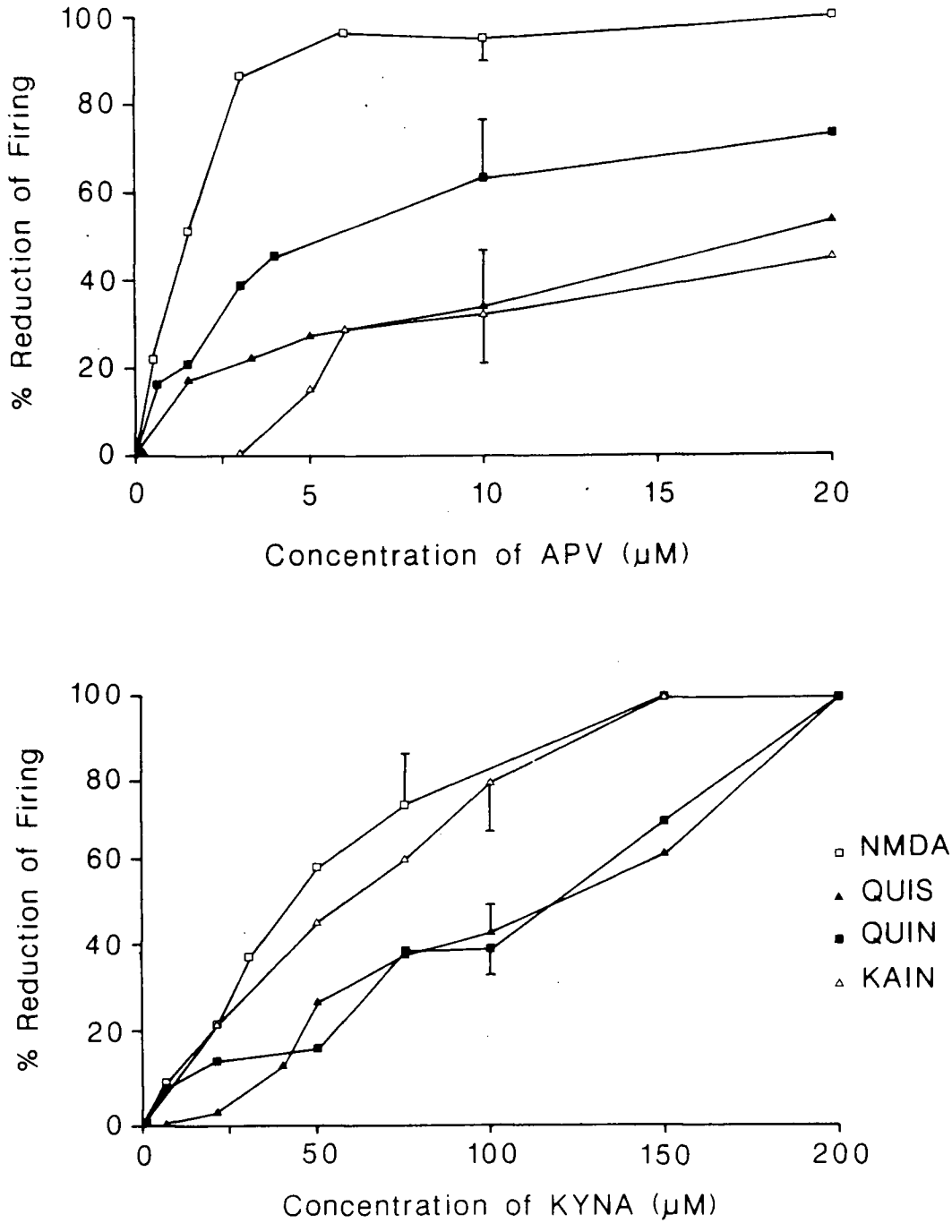


Figure 13. Dose-response curves for the actions of kynurenate (KYNA; bottom) and D-(-)-2-amino-5-phosphonovalerate (APV; top) against excitations elicited by the iontophoretic application of QUIS, QUIN, KAIN and NMDA. Points on the middle part of each curve are means of a minimum of 3 cells. The standard deviation is indicated for one point on each curve.

activity of all the agonists on virtually every neurone examined. Furthermore, the larger separation between the APV dose-response curves for the various agonists demonstrates the greater separation of effects which can be achieved with APV compared to KYNA. Table 15 contains the IC50's determined directly from the dose-response curves which represents the average concentration of antagonist required to reduce agonist-induced firing by 50%. The Figure 13 potency orders determined from the graph are identical to those calculated from the iontophoretic data (NMDA = KAIN) > (QUIS = QUIN) for KYNA and NMDA > QUIN > (QUIS = KAIN) for APV.

Action of Perfusate Applied Antagonists: IC50's

Antagonist	Agonist	Cells Tested	IC50 (μM)	Range
KYNA	QUIS	19	120.0	(6 - 200 μM)
	QUIN	15	120.0	
	KAIN	14	50.0	
	NMDA	19	40.0	
APV	QUIS	17	18.0	(0.5 - 20 μM)
	QUIN	18	7.0	
	KAIN	10	20.0	
	NMDA	18	2.0	

Table 15. IC50's were determined directly from the dose-response curves for the two antagonists. Cell numbers are totals for all concentrations used. Note that the majority of cells were tested at more than a single antagonist concentration.

These results can be seen as ranked order of paired data in Table 16. This Table shows that for 6 of 7 and 8 of 8 cells respectively, the action of NMDA was more sensitive to APV and KYNA than was that produced by QUIN. In addition, for 6 of 11 cells tested with APV, QUIN-activity was reduced more than was QUIS-induced firing while the converse never occurred.

On several occasions the normal bathing medium was replaced with one in which additional sodium ions were substituted for the normal concentration of magnesium ions (Mg^{2+}). The reduction of NMDA-elicited firing by physiological levels of Mg^{2+} (Davies and Watkins, 1977) was thereby removed, which resulted in a two-fold increase in the size of NMDA-induced responses. Table 17 shows that for six of the eight cells studied with Mg^{2+}-free medium, QUIN-responses were compared directly with those of NMDA and found to increase an average of 104 %. Firing elicited by QUIS was also examined for 7 of the 8 cells tested with NMDA and were observed to increase an average of 42 % giving an X/NMDA ratio of 0.52. Paired t-tests disclosed that QUIN and NMDA responses increased comparable amounts in Mg^{2+}-free medium ($p > 0.375$), while those of QUIS were increased a significantly smaller amount ($p < 0.05$). Despite the ability of APV and KYNA to distinguish between the activities of QUIN and NMDA in the dorsal horn in vitro, removal of Mg^{2+} from the medium does not do so.

Ranked Order of Paired Data for the Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones In vitro by D-(-)-2-amino-5-phosphonovalerate, Kynurenate and Acridinate

APV

	QUIS	KAIN	QUIN	NMDA
QUIS		2 (8)	6 (5)	14 (0)
KAIN	1 (8)		1 (2)	7 (0)
QUIN	0 (5)	0 (2)		6 (1)
NMDA	0 (0)	0 (0)	0 (1)	

KYNA

	QUIS	QUIN	KAIN	NMDA
QUIS		1 (5)	5 (1)	13 (0)
QUIN	2 (5)		4 (1)	8 (0)
KAIN	0 (1)	0 (1)		2 (4)
NMDA	0 (0)	0 (0)	1 (4)	

ACRA

	QUIS	QUIN	KAIN	NMDA
QUIS		2 (4)	-	3 (3)
QUIN	2 (4)		0 (2)	1 (6)
KAIN	-	2 (2)		1 (3)
NMDA	2 (3)	1 (6)	2 (3)	

Table 16. Antagonism of the amino acid-induced excitation of spinal neurones, in vitro, by APV, KYNA and ACRA. Conventions are as described for Table 3 (page 53). The excitants are listed from left to right, and from above downward in order of increasing susceptibility to blockade. A bar indicates that the two compounds were never directly compared.

The Effect of Magnesium-Free Bathing Medium on Dorsal Horn Neurone Firing Elicited by Quisqualate, Quinolinate and NMDA In vitro

	QUIS	QUIN	NMDA
A. % increase	42 ± 37 (8)	104 ± 58 (6)	102 ± 63 (8)
B. X / NMDA	0.52 ± 0.22 (7)	1.04 ± 0.20 (6)	1.00

Table 17. Data presented in line A as the percent increase in agonist-elicited firing in response to magnesium-free bathing medium (± S.D.) and in line B as the X / NMDA ratio as described for Table 2 (page 52). The numbers of cells tested are given in brackets. Paired t-tests show QUIS to be significantly different from both QUIN and NMDA ($p < 0.005$) while QUIN and NMDA are not significantly different ($p > 0.375$).

I. Conformationally restricted analogues:

1-amino-1,3-cyclopentane dicarboxylate and pyridine dicarboxylate

The final series of experiments examined the actions of the optical and geometric isomers of 1-amino-1,3-cyclopentane dicarboxylate (ACPD) and several derivatives of pyridine closely related to QUIN. The isomers of ACPD were first examined in the in vivo preparation and were found to be excitants. They were then studied in vitro using primarily iontophoretically applied APV and KYNA as antagonists, but several experiments using topically administered antagonists were done to confirm the iontophoretic results. On no occasion were discrepancies detected.

J. Compounds acting at NMDA receptors

Figure 14 shows a series of ratemeter records of the firing of single dorsal horn neurones. For cells A and B, firing elicited by NMDA is compared directly with that of two ACPD isomers for susceptibility to iontophoretically applied APV and KYNA and topical APV. For cell A, the activities elicited by D-cis- and L-cis-ACPD and NMDA were reduced similarly by APV applied at 3 nA (ca. 85 %). Topical APV also resulted in reductions of similar magnitudes for these three agonists (ca. 55 %). In contrast, firing induced by D-trans-ACPD remained at more than 60 % of control (less than a 40 % reduction) during the iontophoretic application of KYNA at 10 nA, a dose which, on this particular cell, completely blocked the action of D-cis-ACPD and reduced the firing resulting from NMDA by ca. 75 %.

The results obtained with all of the isomers of ACPD are included in Table 18 for APV and Table 20 for KYNA, and a comparison of their potencies with those of many other agonists is also found in Table 18. When ejection currents required to evoke a similar level of firing in a number of neurones were compared, D-cis-, L-trans-, and L-cis-ACPD were 1.8, 0.58 and 0.32 times as potent as L-GLU respectively, while NMDA was found to have a potency ratio of 5 (Table 18). When compared to NMDA, therefore, D-cis-, L-trans-, and L-cis-ACPD were found to be 3, 9 and 16 times less potent. The pattern of potency observed for these compounds appeared to parallel their sensitivity to APV; L-trans- and particularly L-cis-ACPD often retained a small degree of residual activity in the presence of doses of APV or KYNA which completely blocked NMDA and D-cis-ACPD.

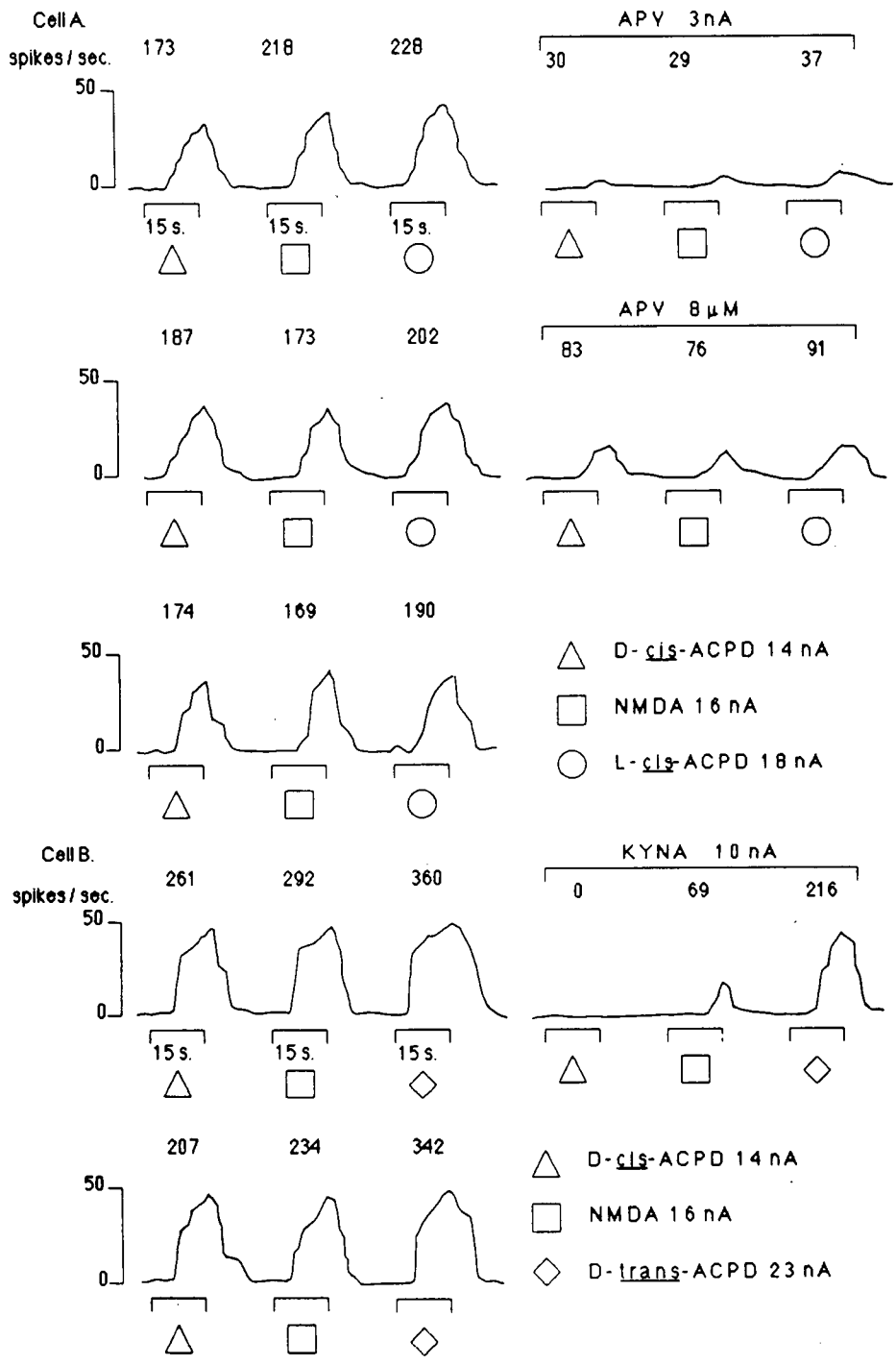


Figure 14. Segments of two continuous ratemeter records of the responses of single dorsal horn neurones in vitro to: Cell A. D-cis-ACPD (14 nA), NMDA (16 nA) and L-cis-ACPD (18 nA) and the antagonism of those responses by: APV applied iontophoretically at 3 nA and in the superfusate at 8 µM. Cell B. D-cis-ACPD (14 nA), NMDA (16 nA) and D-trans-ACPD (23 nA) and the antagonism of those responses by KYNA applied iontophoretically at 10 nA. Conventions as in Figure 9 (page 50).

Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones In vitro

by D-(-)-2-amino-5-phosphonovalerate

compound	potency*	average % reduction	X / NMDA (paired)
NMDA	5.0	97.1 ± 5.5 (20)	1.00
D-cis-ACPD	1.8	96.8 ± 6.7 (9)	(a) 1.00 ± 0.03 (9)
L-trans-ACPD	0.58	72.5 ± 13.6 (10)	(a) 0.92 ± 0.16 (10)
L-cis-ACPD	0.32	75.9 ± 19.4 (10)	(a) 0.87 ± 0.18 (10)
D-trans-ACPD	0.25	45.7 ± 26.1 (7)	(b) 0.51 ± 0.26 (7)
QUIN	0.24	47.3 ± 25.0 (14)	(b) 0.45 ± 0.34 (7)
L-GLU	1.00	23.4 ± 14.3 (10)	(b, d) 0.26 ± 0.17 (10)
QUIS	43	20.3 ± 16.0 (20)	(c) 0.23 ± 0.21 (14)
2,6-PyrDA	0.27	17.6 ± 22.6 (10)	(c) 0.22 ± 0.25 (10)
2,5-PyrDA	0.19	18.1 ± 13.9 (9)	(c) 0.19 ± 0.15 (8)

a -not significantly different from NMDA. paired t-tests $p > 0.375$.
b -significantly different from NMDA. paired t-tests $p < 0.05$.
c -significantly different from NMDA and QUIN. paired t-tests $p < 0.05$.
d -significantly different from QUIN unpaired t-test $p < 0.05$

Table 18. Reduction of amino acid-induced firing of dorsal horn neurones in vitro by APV expressed as the % reduction of control firing ± S.D. and as the X / NMDA ratio as described for Table 2 (page 52). The numbers of cells tested are given in brackets.

* potency: compared to L-GLU = 1.00. For example, both D-trans-ACPD and QUIN require 4 X the ejection current needed by L-GLU to achieve a similar effect on a given neurone, making allowance for the total ionic strength of the solutions. Not shown is KAIN = 20. When compared directly with NMDA, KAIN is approximately 4 X more potent, thus making it 20 X the potency of L-GLU.

The difference between the susceptibility of L-trans-, L-cis-ACPD and NMDA to blockade by APV is not statistically significant using paired t-tests ($p > 0.375$). These data appear as ranked order of paired results in Table 19, which clearly illustrates that D-cis-, L-cis- and L-trans-ACPD are NMDA-like. In the top right quadrant of the table for APV, which shows the results when these compounds were tested against QUIS, QUIN or D-trans-ACPD, the NMDA-like compounds were more strongly affected by APV in 34 of 36 cells (± 15 %). The bottom right quadrant of this table, where these compounds were compared against one another, shows that a distinction was made with APV between two members of this group only 7 times out of 61 trials, and all 7 of those experiments were when D-cis-ACPD or NMDA were compared directly with L-cis- or L-trans-ACPD, compounds found to retain some residual activity in the presence of antagonist.

Table 20 shows the data obtained while using iontophoretically applied KYNA. Activities elicited by D-cis-, L-cis- and L-trans-ACPD were consistently reduced to a comparable extent by KYNA, and no statistical differences could be shown between any of these compounds and NMDA. These data appear as ranked order of paired results in Table 19. It can be seen in this table that although KYNA was unable to distinguish between members of this group in 42 of 44 trials (bottom right quadrant), a distinction was made in 29 out of 30 experiments during which these compounds were tested directly with KYNA against QUIS, QUIN or D-trans-ACPD.

Ranked Order of Paired Data for the Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones In vitro by D-(-)-2-amino-5-phosphonovalerate and Kynurenate

APV

	QUIS	QUIN	DT	LC	LT	DC	NMDA
QUIS		-	1 (0)	3 (0)	6 (0)	3 (0)	11 (0)
QUIN	-		0 (2)	-	-	-	3 (1)
DT	0 (0)	1 (2)		-	-	2 (0)	6 (1)
LC	0 (0)	-	-		1 (1)	1 (2)	3 (7)
LT	0 (0)	-	-	0 (1)		-	2 (8)
DC	0 (0)	-	0 (0)	0 (2)	-		0 (9)
NMDA	0 (0)	0 (1)	0 (1)	0 (7)	0 (8)	0 (9)	

KYNA

	QUIS	QUIN	DT	LC	LT	DC	NMDA
QUIS		-	0 (0)	2 (0)	4 (0)	3 (0)	9 (0)
QUIN	-		0 (2)	-	-	-	2 (1)
DT	1 (0)	0 (2)		-	1 (0)	2 (0)	6 (0)
LC	0 (0)	-	-		0 (4)	-	0 (4)
LT	0 (0)	-	0 (0)	0 (4)		-	2 (7)
DC	0 (0)	-	0 (0)	-	-		0 (6)
NMDA	0 (0)	0 (1)	0 (0)	0 (4)	0 (7)	0 (6)	

Table 19. Antagonism of the amino acid-induced excitation of dorsal horn neurones in vitro by APV and KYNA. Conventions are as described for Table 3 (page 53). The excitants are listed from left to right, and from above downward in order of increasing susceptibility to blockade. A bar indicates that the two compounds were never directly compared. DT, LC, LT and DC represent D-trans-, L-cis-, L-trans- and D-cis-ACPD respectively.

K. Compounds acting at QUIS receptors

Of all the conformationally restricted compounds tested only 2,5- and 2,6-PyrDA elicited activity which was pharmacologically similar to that of L-GLU and QUIS. When these compounds were compared with QUIS-, KAIN- and NMDA-induced firing for susceptibility to blockade by APV and KYNA, activity evoked by 2,5- and 2,6-PyrDA was resistant to blockade by APV (Table 18, Figs. 15 and 16) and only moderately sensitive to KYNA (Table 20, Figs. 15 and 16).

Figures 15 and 16 are ratemeter records of single dorsal horn neurones recorded in vitro. In Figure 16, cell A., activity induced by QUIS was 18 % lower and that by 2,6-PyrDA 16 % lower than control firing during the iontophoretic application of APV at 9 nA while NMDA-elicited activity is reduced by 50 %. A qualitatively similar result is found in Figure 16, cell B., where QUIS and 2,6-PyrDA activities were reduced by approximately 50 % while those of NMDA were almost completely blocked when KYNA was applied at 7 nA. That cell activity elicited by QUIS and 2,5-PyrDA is less susceptible to antagonism by APV and KYNA than that of NMDA is shown in Figure 15. When tested with APV applied at 2 nA (cell A) QUIS- and 2,5-PyrDA-activities were reduced by 40 and 5 % respectively while the action of NMDA was lowered by 94 %. For cell B., KYNA applied at 5 nA resulted in a 22 % reduction of QUIS- and a 6 % reduction of 2,5-PyrDA-elicited firing while that induced by NMDA decreased by 68 %.

The pyridine analogues 2,5- and 2,6-PyrDA were the least susceptible to blockade by APV of all the conformationally restricted analogues tested and in this

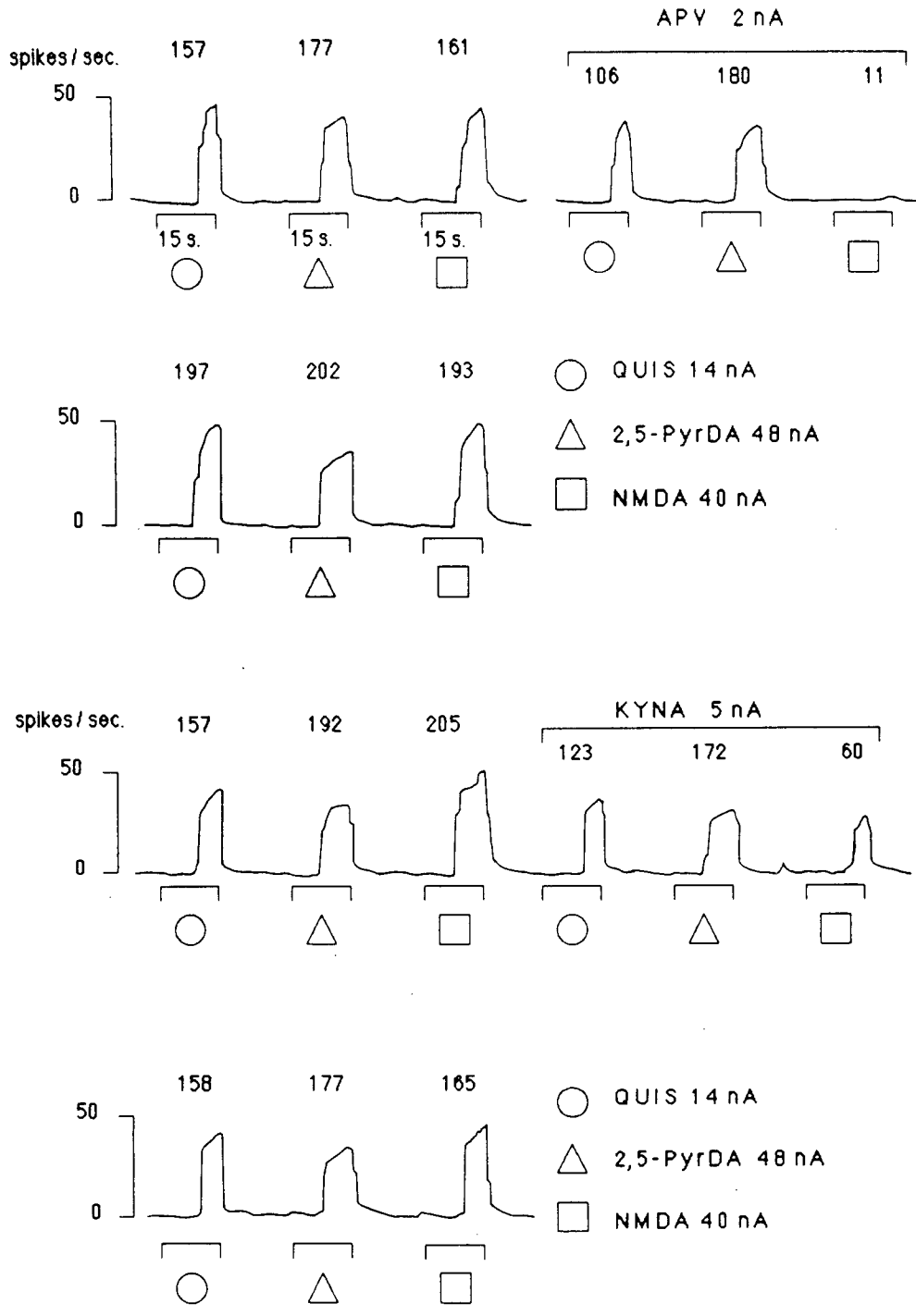


Figure 15. Segments of two continuous ratemeter records of the responses of single dorsal horn neurones in vitro to: Cell A. QUIS (4 nA), 2,6-PyrDA(24 nA) and NMDA (12 nA) and the antagonism of those responses by: APV applied iontophoretically at 9 nA, and cell B. QUIS (5 nA), 2,6-PyrDA (13 nA) and NMDA (12 nA) and the antagonism of those responses by KYNA applied iontophoretically at 7 nA. Conventions as in Figure 9 (page 50).

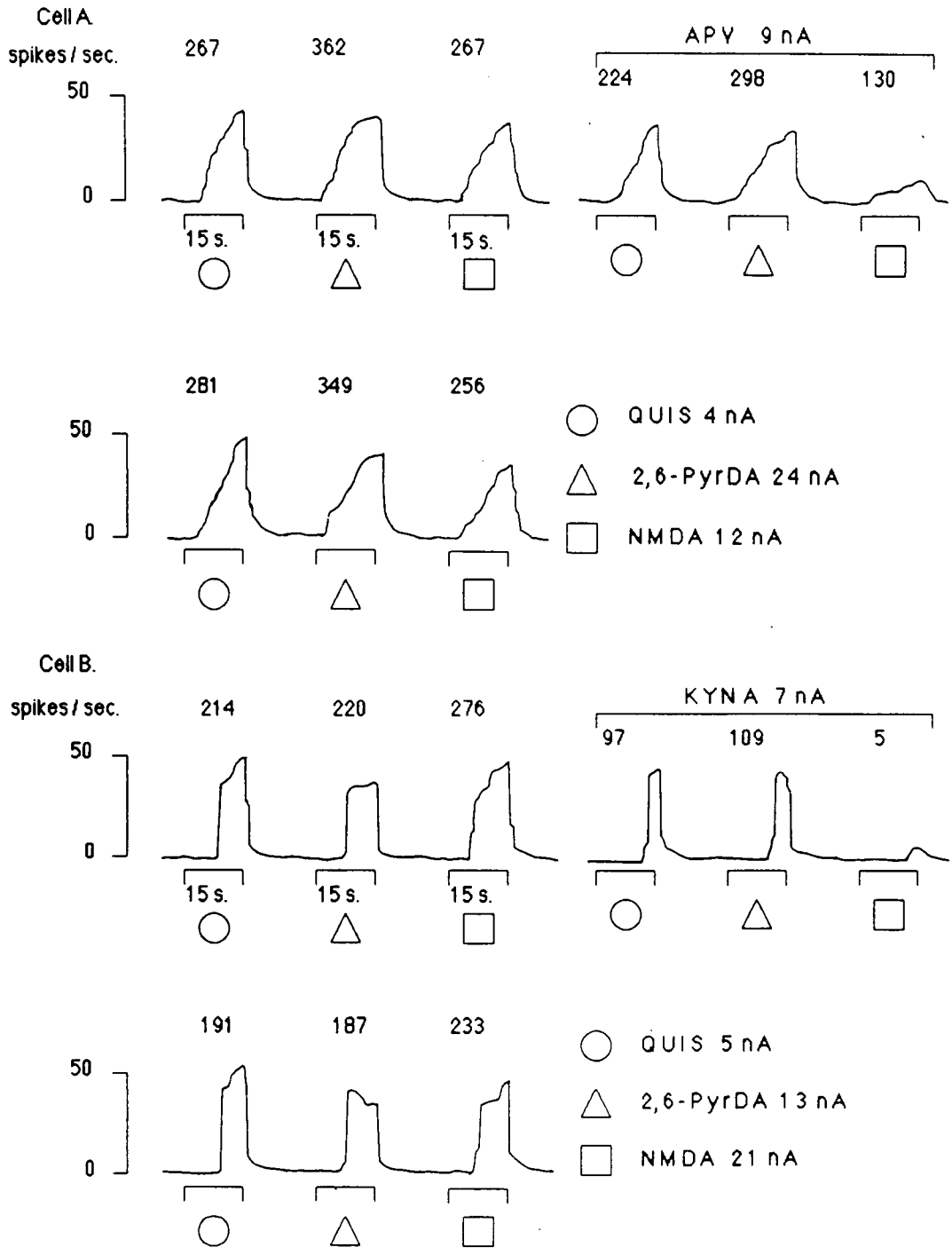


Figure 16. Segments of two continuous ratemeter records of the responses of single dorsal horn neurones in vitro to: Cell A. QUIS (14 nA), 2,5-PyrDA (48 nA) and NMDA (40 nA) and the antagonism of those responses by APV applied iontophoretically at 2 nA, and cell B. QUIS (4 nA), 2,5-PyrDA (48 nA) and NMDA (40 nA) and the antagonism of those responses by KYNA applied iontophoretically at 5 nA. Conventions as in Figure 9 (page 50).

respect are indistinguishable from L-GLU and QUIS, although less potent (potency ratios 0.27 and 0.19 compared to L-GLU respectively, Table 18). Paired t-tests could show no difference between either of these analogues and QUIS either on the basis of antagonism by APV or by KYNA. These compounds, like QUIS, are significantly less sensitive to antagonism by APV or by KYNA than is NMDA (paired t-tests; Table 18).

Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones

In vitro by Kynurenate

compound	average % reduction	X / NMDA (paired)
NMDA	91.6 ± 6.2 (18)	1.00
D-cis-ACPD	92.5 ± 8.9 (6)	[a]1.01 ± 0.05 (6)
L-trans-ACPD	72.2 ± 18.4 (9)	[a]0.94 ± 0.16 (9)
L-cis-ACPD	63.0 ± 13.6 (6)	[a]0.92 ± 0.10 (4)
QUIS	40.7 ± 16.9 (18)	[b]0.66 ± 0.29 (16)
QUIN	45.8 ± 23.1 (10)	[b]0.65 ± 0.17 (8)
2,5-PyrDA	47.0 ± 38.3 (7)	[b]0.55 ± 0.44 (7)
D-trans-ACPD	39.0 ± 25.6 (7)	[b]0.50 ± 0.52 (6)
L-GLU	30.3 ± 16.9 (9)	[b]0.48 ± 0.26 (6)
2,6-PyrDA	27.3 ± 23.9 (9)	[b]0.40 ± 0.36 (9)

a -not significantly different from NMDA. paired t-tests $p > 0.375$.
b -significantly different from NMDA. paired t-tests $p < 0.05$.

Table 20. Reduction of amino acid-induced firing of dorsal horn neurones in vitro by KYNA expressed as the % reduction of control firing ± S.D. and as the X / NMDA ratio as described for Table 2 (page 52). The numbers of cells tested are given in brackets.

These data are shown as the ranked order of paired results in Table 21. When members of this group of compounds (QUIS, 2,5- and 2,6-PyrDA) were compared with one another against APV and KYNA (top left quadrant of Table 21 for APV and KYNA), no distinction could be shown for 7 of 12 and 6 of 10 trials respectively. In contrast, when compared directly with NMDA against APV and KYNA, NMDA-activity was more potently reduced by these antagonists than were members of this group in 35 of 35 and 24 of 28 trials respectively (top right quadrant of Table 21 for APV and KYNA).

L. Quinolinate - like activity

Table 18 (page 85) shows that cell firing elicited by D-trans-ACPD was intermediately affected by APV and was similar to QUIN-induced firing in its sensitivity to blockade by this antagonist (it was more susceptible than QUIS but less so than NMDA). Furthermore, as previously described, Figure 14, cell B. (page 84), demonstrates that D-trans-ACPD can retain considerable activity in the presence of an iontophoretic dose of KYNA which strongly attenuates NMDA and the NMDA-like isomers of ACPD. D-trans-ACPD was indistinguishable from QUIN both on the basis of sensitivity to blockade by APV and KYNA, and iontophoretic potency; both of these compounds require approximately 4 times the current of L-GLU to achieve similar firing rates from a given neurone (Table 18: page 85). In Table 19 (page 87) the ranked order of paired results shows that where D-trans-ACPD was compared directly with QUIN no distinction could be made by

Ranked Order of Paired Data for the Reduction of Amino Acid-Induced Activity of Dorsal Horn Neurones In vitro by D-(-)-2-amino-5-phosphonovalerate and Kynurenate

APV

	QUIS	2,5-PyrDA	2,6-PyrDA	NMDA
QUIS		0 (3)	3 (4)	16 (0)
2,5-PyrDA	1 (3)		-	8 (0)
2,6-PyrDA	1 (4)	-		11 (0)
NMDA	0 (0)	0 (0)	0 (0)	

KYNA

	QUIS	2,5-PyrDA	2,6-PyrDA	NMDA
QUIS		2 (2)	1 (4)	11 (2)
2,5-PyrDA	1 (2)		0 (0)	5 (1)
2,6-PyrDA	0 (4)	1 (0)		8 (1)
NMDA	0 (2)	0 (1)	0 (1)	

Table 21. Antagonism of the amino acid-induced excitation of dorsal horn neurones in vitro by APV and KYNA. Conventions are as described for Table 3 (page 53). The excitants are listed from left to right, and from above downward in order of increasing susceptibility to blockade. A bar indicates that the two compounds were never directly compared.

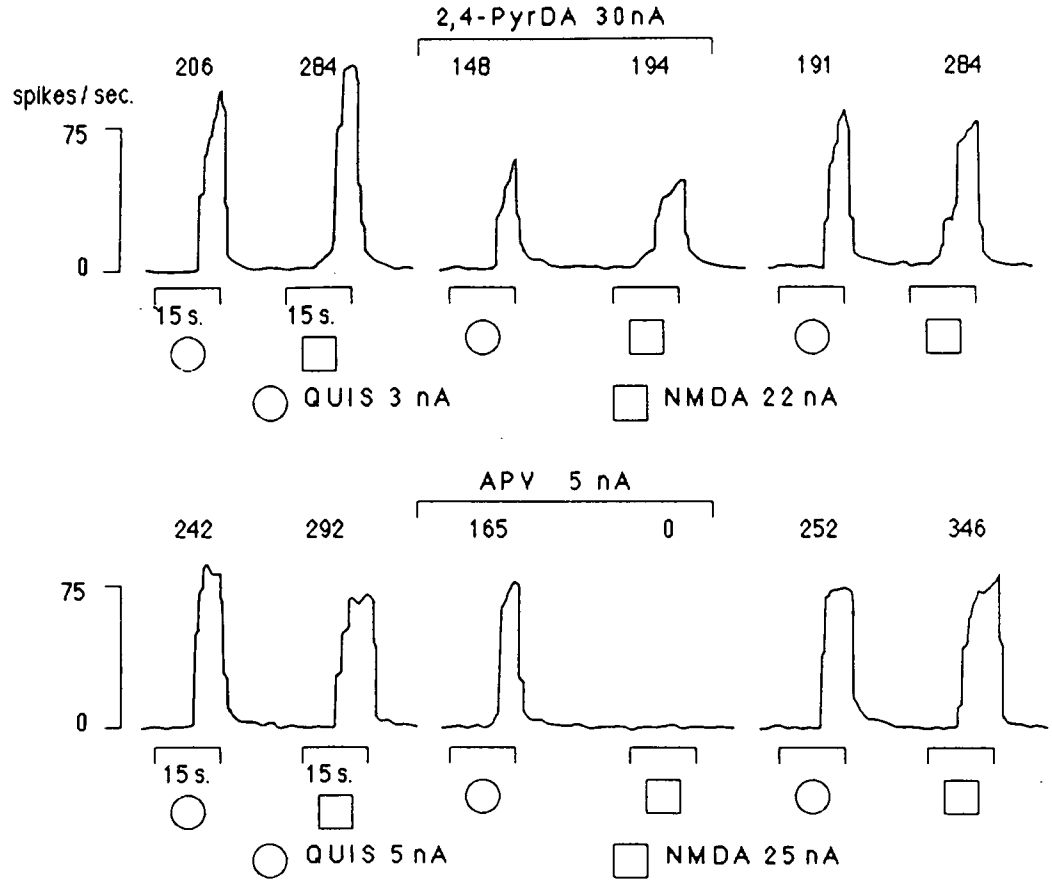


Figure 17. Segments of a continuous ratemeter record of the responses of a single dorsal horn neurone <u>in vitro</u> to QUIS (3 & 5 nA) and NMDA (22 & 25 nA) and the antagonism of those responses by: 2,4-PyrDA applied iontophoretically at 30 nA and APV applied iontophoretically at 30 nA. Conventions as in Figure 9 (page 50).

APV (2 of 3 cells) or KYNA (2 of 2 cells); and it was clearly distinguishable from NMDA and the other isomers of ACPD (Table 19: page 87).

Picolinate, 3-hydroxypicolinate, 2,4- and 3,4-PyrDA, phthalate and itaconate were also tested for activity in the spinal cord in vitro. These compounds were not able to excite dorsal horn neurones when ejected from 200 mM solutions with currents up to 100 nA. The pyridine analogue 2,4-PyrDA was, however, found to reduce neuronal firing elicited by QUIS, QUIN, KAIN and NMDA. Figure 17 shows that 2,4-PyrDA applied at 30 nA from a 200 mM solution caused 25 and 32 % reductions of the firing elicited by QUIS and NMDA respectively. A later trial with the same cell showed that APV applied at 5 nA completely blocked the firing induced by NMDA, while that by QUIS was 33 % lower than control. 2,4-PyrDA was found to be very weak, however, requiring at least 200 times the relative iontophoretic dose of KYNA to cause similar reductions of amino acid induced firing.

V. Discussion

A. Interpretation of data and results

All of the results to be discussed were obtained by extracellular recording of the electrical responses of dorsal horn and cortical neurones to iontophoretically applied amino acid excitants. The techniques used for iontophoresis and extracellular recording have some inherent features which should be noted prior to consideration of the results.

For each experiment, the cell to be recorded was located by lowering the 7-barrelled electrode assembly through the tissue while ejecting an agonist, typically QUIS or L-GLU. The agonist was often ejected continuously for several minutes such that many cells in the area were affected. The oscilloscope signal showed only those cells nearest the electrode, with the closest of those producing the largest signal. Because this process discriminated against smaller neurones, the total population sampled was biased in favour of the larger cells in the area under study. This phenomenon was aggravated by the use of a window discriminator / ratemeter which permitted the experimenter to easily isolate large spikes, but made the selection of smaller signals more difficult. If the cells in the dorsal horn and cortex consist of two or more populations which differ in average size and in their responses to the amino acid excitants, then this biased selection of cells could represent a real problem. There is very little evidence, however, that populations of dorsal horn (ventral to the substantia gelatinosa) or cortical neurones vary qualitatively in their responses to excitatory amino acids. Schneider

and Perl (1985) have reported that some superficial dorsal horn neurones do not respond to L-GLU, but no other supporting reports have been made. Furthermore, the author never observed neuronal responses to amino acid agonists or antagonists which would favour the supposition that the sampled cells represented heterogeneous populations which differed in their responses to the compounds examined (ie. the relative potency of agonists and antagonists was quite similar from neurone to neurone). Neurones which were particularly sensitive to excitation by one agonist invariably had higher than average sensitivities to all the agonists. The small variations in relative potency that were observed are likely due to the individual differences in current passing characteristics of each iontophoretic barrel and to the small differences in barrel-tip-to-cell distance for each of the six outer barrels of an electrode.

Extracellular recording from neurones in the dorsal horn and cortex also involve other uncertainties regarding the positioning of the recording electrode, and thus the location of the compound source (iontophoretic barrel) relative to the soma, dendrites and axon hillock of the cell under study. It is the opinion of the author that changes in the electrode to cell distance, and in the location of the electrode relative to the regions of the neurone mentioned above, changed the apparent potencies of the compounds being ejected (a cell located a short distance from the ejecting barrel required a smaller iontophoretic dose of compound) but did not alter the qualitative observations made from that cell. The variations in the responses between cells consisted entirely of small differences in the relative iontophoretic doses of agonists and antagonists needed to achieve the desired

effects. Cell to cell variations should not, and did not, result in observable qualitative differences in the excitation of the cells, or in the blockade of induced firing with antagonists. The experiments using antagonists applied in the superfusate were subject to small variations resulting from differences in the electrode to cell distance and the orientation of the electrode to the cell body: ie. a large cell some distance from the electrode will be excited by having a comparatively large area of membrane exposed to a low concentration of agonist which is easily blocked by antagonist in the perfusate. By contrast, a cell located a shorter distance from the electrode may be excited by having a comparatively small area of membrane exposed to a high concentration of agonist which therefore requires somewhat higher doses of antagonist relative to the earlier scenario. When care was taken to achieve stable, sub-maximal responses to the excitants, however, the blockade of the amino acid-evoked activity was also stable and consistent.

The results showing the sensitivity of an agonist to blockade by an antagonist have been presented in three ways: the average % reduction of control firing pooled for all cells tested, the average % reduction of control firing compared directly with NMDA (X/NMDA ratio, paired), and the ranked agonists for each cell tested using a ± 15 % distinction value (every agonist is paired with every other on a small number of cells). The pooled data was subject to variations due to the differences in the electrode to cell distances and relative cell position and did not take those variations into account. The paired (X/NMDA) results were obtained primarily from experiments where sub-maximal blockade of agonist-elicited activity

was achieved. Because the reduction by an antagonist of the response of each agonist was compared directly to the reduction of NMDA for each cell, the experimental conditions were virtually identical for each agonist, and the variabilities due to electrode position and cell location were reduced. Similarly, for the ranked paired data, each number was generated by a direct comparison of two agonists under similar experimental conditions, thus removing the variations encountered while using extracellular recording and iontophoretic techniques.

Assessment of agonist or antagonist potency using iontophoretic currents as the measure is admittedly problematic. Table 11 (page 70) presents the average currents used during many experiments in vivo and in vitro to provide insight into and a possible explanation for the observation that QUIN is capable of eliciting activity in vitro despite its lack of potency in the spinal cord in vivo. Comparing potencies in this way could only be done, however, because the numbers of cells involved were high, and the electrodes, solutions, and iontophoretic and recording conditions were similar. All the data shown in Table 11 were obtained using the same experimental techniques, electrodes and compounds from the same suppliers, and, in many instances, agonist and antagonist dilutions derived from the same stock solutions. The differences in currents used, therefore, probably represented a real increase in potency in vitro compared to in vivo, without an observable alteration to the qualitative results obtained with the various compounds (Table 11). Furthermore, no increase in the numbers of spontaneously active neurones was observed in vitro compared to the situation in vivo. Reduced uptake of compounds, possibly due to destruction of glia during the preparation of

the slices, may have contributed to, but is unlikely to have accounted entirely for the enhanced potency since no uptake systems are known for several of the compounds. However, the increase in QUIN's potency (based on only a very few cells in vivo) appeared to be relatively greater than for the other agonists, and an observation made by the author that the potency of L-GLU was at least 3-fold higher (it was normally used at 500 mM in vivo but only 100 mM in 75 mM NaCl in vitro) would suggest that uptake efficiency for L-GLU and perhaps any degradative system available for QUIN was reduced in the slice compared to the situation in vivo.

Why are QUIS, NMDA, KYNA and PDA more potent in vitro? A possible suggestion is that the replacement of the normal extracellular fluid with artificial cerebrospinal fluid (ACSF) may, because it lacks amino acids and protein, enhance the diffusion of ions from the electrode to the cell, thereby increasing the effective concentration reached for a given electrode-to-cell distance and iontophoretic current applied. Other unknown factors may, admittedly, have something to do with this observation.

Determination of the relative potencies of agonists, as presented in Table 18 (page 85), presents less of a problem because much of the data needed were obtained from paired comparisons. Nonetheless, the results achieved must be regarded as rough estimates and should only be interpreted as such. More accurate estimates of the potencies of these and other agonists in the spinal cord will be possible in the future using intracellular recordings from cells where the rate of rise and extent of depolarization of a cell in response to a topically or

iontophoretically applied agonist can be observed.

B. Excitation of dorsal horn neurones by the archetypal agonists QUIS, KAIN and NMDA in vivo and in vitro

That the extracellularly recorded responses of dorsal horn neurones to the archetypal amino acid agonists QUIS, KAIN and NMDA are mediated by three distinct receptors can only be inferred using the circumstantial evidence of their differential sensitivity to blockade by antagonists. Unlike intracellular recordings from CA1 neurones in the hippocampus (Peet et al., 1986) and pyramidal neurones in the cortex (Flatman et al., 1983) where these agonists elicit qualitatively different types of depolarization and spike activation, no such definitive distinctions can be made while recording extracellularly. However, the responses of dorsal horn neurones to these three agonists did have certain characteristics that were fairly consistent from cell to cell. As in the hippocampus (Peet et al., 1986), QUIS-induced excitations had a fast onset (indicative of a fast-rising depolarization) and had the most stable firing pattern of the three excitants, ie. firing reached its maximum rate quickly and stabilized at that rate, often giving a ratemeter peak with a distinct plateau. Increasing the iontophoretic dose of QUIS sometimes led to overdepolarization (indicated by a reduced extracellular spike amplitude), but required a relatively greater increase in the dose than for KAIN or NMDA.

The responses to KAIN were unstable in comparison to those of QUIS; it was more difficult to find an iontophoretic dose which gave consistent sub-maximal

responses because small alterations in current could result in large changes in the integrated firing rate. In addition, too large a dose of KAIN easily resulted in overdepolarization and occasionally caused apparently irreversible damage to the cell. These observations are consistent with those seen in the hippocampus (Peet et al., 1986), in particular the difficulty in achieving stable responses. The firing rate seldom attained a plateau, but rather tended to increase for the entire duration of agonist application. Both onset and offset of the responses were slower than for the other agonists, and continued firing after termination of the KAIN ejecting current was particularly noticeable. For these reasons fewer cells were recorded with KAIN than for the other agonists, and when other analogues being examined were not KAIN-like in action it was not routinely tested (see for example Table 18: page 85).

Bursts of action potentials induced by NMDA commonly seen in other regions (Flatman et al., 1983; Peet et al., 1986) were never observed in the spinal cord. However, like KAIN, NMDA-elicited firing tended to increase in rate throughout the ejection period, although the offset was more rapid for NMDA. Toxic effects were seldom seen with high doses of NMDA, but overdepolarization occurred often with only moderate (15 - 20 %) increases in the iontophoretic dose, once a substantial but sub-maximal response had been obtained. In general, these observations are similar to those seen in the cortex and hippocampus (Flatman et al., 1983; Peet et al., 1986) despite the lack of NMDA-induced bursting in the spinal cord.

C. Antagonism of dorsal horn neurone responses to archetypal agonists by PDA, KYNA, ACRA and APV in vivo

To distinguish with confidence between the agonists acting at the different amino acid receptors in the spinal cord using extracellular recording requires the use of at least two of the available amino acid antagonists. Experiments in the hippocampus (Peet et al., 1986) and cortex (Flatman et al., 1983) have shown that all compounds which elicit NMDA-like bursting and thus presumably activate NMDA receptors, are very sensitive to blockade by APV, KYNA and PDA. In the spinal cord it is presumed that responses to a compound which are similar to those of NMDA in their sensitivity to blockade by these antagonists also act at NMDA receptors. In Table 2 (page 52), the antagonism of agonist-induced firing by PDA and KYNA is shown. PDA was first demonstrated to be an antagonist of amino acid excitations by Davies et al. (1981) in the cat spinal cord, where they found it to block NMDA excitations potently with lesser actions against the excitations produced by QUIS and KAIN. Their results were presented as pooled data showing NMDA responses to be reduced by 69 % with those of QUIS and KAIN by 54 and 55 % respectively. These data do not show a statistically significant difference between the agonists but nevertheless show trends similar to those presented in Table 2 (page 52). Other authors have reported that KAIN-induced excitations were similar in sensitivity to blockade by PDA as those of NMDA (McLennan and Liu, 1982). The order of agonist sensitivity to antagonism by PDA (the profile of antagonism) is similar to that of APV in the spinal cord, except the separation between agonists which can be achieved with PDA is less.

Cell firing evoked by both NMDA and KAIN was powerfully attenuated by KYNA, as shown in Table 2 (page 52); QUIS-elicited activity was also affected, but to a lesser degree. These findings corroborate those of Peet et al. (1986) in the hippocampus, but differ somewhat from several other reports. For example, Perkins and Stone (1982, 1984) found that KYNA had little ability to distinguish between NMDA and QUIS excitations of either cortical or spinal cord neurones, although in the hippocampus they found that NMDA and KAIN were both more sensitive to KYNA than was QUIS. Ganong et al. (1983) reported that KYNA was able to distinguish the activity of NMDA from that of QUIN and QUIS, and that the effects of KAIN were intermediately affected (more so than QUIS but less than NMDA) both in the hippocampus and immature rat spinal cord.

In conclusion, the use of PDA or APV to block NMDA-excitations and KYNA to reduce both NMDA- and KAIN-like activity provides the needed circumstantial evidence to infer that QUIS, KAIN and NMDA react with three distinct receptor types in the rat spinal cord in vivo.

D. Antagonism of cortical neurone responses to amino acid agonists by KYNA and ACRA in vivo

The inclusion of QUIN in the studies performed in the cortex in vivo was in response to the increasing interest in endogenous tryptophan-related compounds (including KYNA) and their potential rôles as modulators of CNS excitability (for review see Stone and Connick, 1985). Furthermore, it was not possible to examine the pharmacology of QUIN in the spinal cord in vivo because of its marked lack of

potency in this preparation (Table 11: page 70; Perkins and Stone, 1983; McLennan, 1984).

As reported by Perkins and Stone (1982, 1984) and McLennan (1984), QUIN- and NMDA-induced excitations were affected almost equally by KYNA in the cortex in vivo. In addition, the action of KYNA against KAIN activity was also very similar to its antagonism of NMDA-induced firing (Tables 4 and 5: pages 59 and 60). Only QUIS was slightly less affected by KYNA in this preparation. The results presented here, in conformity with those of Perkins and Stone (1982, 1984) and McLennan (1984), provide evidence for the supposition that QUIN exerts it excitatory action via NMDA receptors in the cortex as it does in the hippocampus (Peet et al., 1986). Because of the relatively poor separation between the excitations of the agonists provided by PDA and KYNA in the cortex, any further conclusions would be difficult to defend. ACRA (Curry et al., 1986) had a non-specific antagonistic affect against all four of the agonists tested in the cortex, and provided no additional information regarding the receptors utilized by the various amino acid excitants.

E. The aromatic antagonists ACRA and KYNA in the spinal cord and cortex

The antagonistic actions of ACRA and KYNA provide important structure-activity information regarding the requirements for antagonism of amino acid excitations. Removal of the benzene ring from ACRA (Fig. 3, E: page 12) yielded QUIN (Fig. 3, A), an excitant, even though the relative positions of the amino and carboxyl groups are unchanged. KYNA devoid of its second aromatic ring is

4-hydroxypicolinate (4-hydroxy-2-pyridine carboxylate) which was not tested, but 2,4-PyrDA which was examined was found to be a rather weak antagonist in vitro (Fig. 17: page 94). It may therefore be concluded that an aromatic agonist is converted into an antagonist either by the addition of a second aromatic ring and/or by a change in position of the second acidic substituent from C3 to C4. Presumably the antagonistic effects of ACRA, KYNA and 2,4-PyrDA are due to their ability to bind to but inability to activate the receptors because of steric factors (second ring) or the inappropriate placement of the distal carboxyl (C3 to C4) group.

F. α-Substituted analogues of glutamate

In 1963, Curtis and Watkins reported that N-substitution of aspartate and glutamate (methyl, ethyl, propyl, iminomethyl) did not significantly reduce the potency of these compounds, and in some cases dramatically enhanced it. It was considered possible that substitutions of this type made to the α-carbon rather than the nitrogen might enhance the binding of these compounds to receptors such that they would have potent actions either as agonists or antagonists. The analogues in this class that were synthesized and tested are listed in Table 1 (page 30). However, only the α-(parachlorophenyl)- and α-(parafluorophenyl)-substituted glutamates had any activity in the spinal cord in vivo ; they were weak antagonists with a preference for L-GLU-induced excitations (Tables 6 and 7: page 62). The iontophoretic potency of these compounds is low, but does increase when going from the para-chloro to the para-fluoro analogue, indicating that steric interactions

with the aromatic ring are not necessarily the cause of the antagonistic actions. The increased tendency of the fluoride moiety (compared to chloride) to pull electrons off the ring and ultimately from the α-carbon suggests that the electronic effects of the substituted halogens may be the characteristic resulting in antagonism.

G. (+) and (-) Trans-1-amino-1,2-cyclopentane dicarboxylate in vivo

Examination of (-)trans-CPA in the spinal cord shows that it is an antagonist with an action unlike any other compound previously reported save L-glutamate diethylester (GDEE). The iontophoretic potency of (-)trans-CPA is less than that of KYNA, but greater than that of ACRA, and it is significantly more potent against QUIS and KAIN activity than against that of NMDA (Table 8: page 66). The importance of this compound in the study of putative glutamatergic pathways in the spinal cord will take several years to determine, but its potential is great. Its action in the higher CNS, however, is entirely different from that in the spinal cord. Unpublished results obtained by intracellular recording from CA1 hippocampal neurones (M. J. Peet, personal communication) show (-)trans-CPA to be an agonist which elicits NMDA-like bursting and is blocked by APV. At no time did either isomer of trans-CPA show excitant behaviour in the spinal cord. It is hoped that future studies with this compound will provide valuable information regarding the regional differences in the receptors for the excitatory amino acids.

Figure 2, E and F (page 8) shows that there are two major differences in the structural characteristics of PDA and trans-CPA. First of all, PDA has a secondary

amino group forming part of the piperidine ring, while the amino group of CPA is primary. Secondly, the relatively flexible piperidine ring of PDA may assume many of the conformations of cyclohexane rings, thus the intercarboxyl distances can vary considerably. The five-membered ring of CPA, on the other hand, is confined to an envelope conformation with relatively fixed inter-group distances (Table 25: page 130). The transition from an NMDA antagonist (PDA) to an antagonist with preference for QUIS and KAIN-type excitations ((-)trans-CPA) might be a consequence of the exclusion of the amino nitrogen from the ring and the reduction of the flexibility of the molecule which limits the compound to intercarboxyl and distal carboxyl to amino distances only attainable by L-GLU in a folded conformation. If trans-CPA binds to the QUIS and/or KAIN receptors utilizing all three ionic groups, the spacing of the charged areas on the receptor is limited by the distance over which electrostatic interactions may be effective. The QUIS-like actions of 2,5- and 2,6-PyrDA (discussed in more detail later) suggest that the distal carboxyl group is preferentially situated in juxtaposition to the amino group; ie. it is closer to the amino group than to the α-carboxyl. Trans-CPA should therefore be capable of binding with all three charged groups to a receptor with this configuration. Nonetheless, trans-CPA binds with enough affinity to allow it to compete successfully with QUIS, L-GLU and KAIN for these receptors. This supports suggestions that a partially folded conformation is the preferred one for the QUIS (McLennan et al., 1982; McBain and Wheal,1984) and possibly for the KAIN receptor. That trans-CPA competes for QUIS and KAIN receptor sites but does not activate them suggests that a folded conformation alone is insufficient for

activation of these receptors. Once bound to the receptor site flexibility in the agonist molecule permitting increases in the intercarboxyl distance may be important, perhaps allowing or encouraging a conformational change in the receptor which is necessary for activation.

The need for specific and potent QUIS and KAIN antagonists has not been filled, even by GDEE, which has in the past been used extensively as a QUIS antagonist, but is being used less in recent years due to its somewhat unpredictable actions. More recent additions to the list of amino acid antagonists include several piperazine derivatives with potent NMDA-blocking capabilities (Davies et al., 1986) and an analogue related to the dipeptide γDGG, namely γ-D-glutamyl-aminomethyl-sulphonate (GAMS). GAMS has been used in the spinal cord in vitro (Davies and Watkins 1985) and in the thalamus (Salt, 1987) and was found in both regions to be a moderately potent antagonist with some selectivity for responses to KAIN. None of the recently discovered antagonists have conformationally restricted structures and thus little structure-activity analysis can be done. It is apparent, however, by the number of these compounds containing ω-phosphonate or sulphonate groups that this is a characteristic which confers potency to amino acid antagonists.

The additional need for conformationally restricted compounds active at these receptors is now being met by cyclic amino acids such as those described here. The preliminary examination of trans-CPA discussed earlier suggests that it may be a useful tool to distinguish between synaptic events in the spinal cord which utilize QUIS or KAIN receptors from those involving NMDA receptors.

Davies et al. (1982) reported on the actions of several piperidine derivatives, and concluded that the relative position of the carboxyl groups was the structural feature determining their effect. The 2,3 arrangement of carboxyl groups provided the most potent compounds in the series. Trans-2,3-piperidine dicarboxylate was reported to be a potent NMDA agonist antagonized by APV (Davies et al., 1982), while the cis isomer was, as also shown here (Tables 2 and 3: pages 52 and 53), an antagonist with a moderate preference for NMDA-excitations. Davies et al. (1982) also reported that PDA had a significant excitatory potency in the neonatal rat spinal cord in vitro, but not in the cords of frog or mature cat. Responses to this compound showed it to be more potent under these conditions than was L-GLU, and revealed that it was blocked by APV at iontophoretic currents which also blocked NMDA but had little effect on L-GLU. PDA was never found to be excitatory when applied alone or concomitantly with another agonist to dorsal horn neurones either in vivo or in vitro, and as described above it had a significant potency as an antagonist against all amino acid-induced firing. That no excitatory properties were ever observed with PDA provides circumstantial evidence to suggest that the spinal cord slices prepared from 24 - 30 day old rats were mature with respect to the amino acid receptor populations. Davies et al. (1982) further reported that (-)cis-PDA was responsible for both the agonistic and antagonistic actions of the racemic mixture. Also tested by Davies et al. (1982) was trans-2,4-piperidine dicarboxylate which proved to be a weak NMDA-like antagonist in both the rat and frog spinal cords. The flexibility of the piperidine ring, which can adopt many of the conformations of the cyclohexane ring, makes difficult

any structure-activity analysis beyond noting the importance of the 2,3- and 2,4-placement of carboxyl groups for agonistic and antagonistic interactions with NMDA receptors respectively.

H. Archetypal agonists and

blockade of their actions in the spinal cord in vitro

The results of the initial experiments performed in vitro showed that the profile of antagonism (order of agonist sensitivity to blockade) for PDA, KYNA and APV was identical to that found in vivo. Furthermore, Table 10 (page 68) shows that the separation or distinction made between QUIS/KAIN and NMDA by PDA and APV, and between QUIS and KAIN/NMDA by KYNA was greater in vitro than in vivo. This increase in selectivity allows the compounds to be divided into four statistically different groups (using paired Student's t-tests) on the basis of their susceptibility to antagonism (QUIS = L-GLU < KAIN < QUIN < NMDA; Table 14: page 76). These statistically significant differences show that QUIS, KAIN and NMDA all act in a qualitatively different manner in the spinal cord, supporting on pharmacological grounds the theory that their actions are mediated by at least three different receptors. Recent patch-clamp studies (Cull-Candy and Usowicz, 1987a, b) have indicated that two or more binding sites as part of a single receptor-channel complex are responsible for the variety of responses observed to the amino acid excitants.

I. Actions of L- and D-glutamate in the spinal cord in vitro

The investigations into the amino acid excitants in the early 1960's began by focussing on glutamate and aspartate, two compounds abundant in the CNS. The focus gradually shifted away from these endogenous excitants with the introduction of the more potent active compounds NMDA, and more recently, QUIS and KAIN, which are commonly used today in the nomenclature of the receptors responsible for the actions of the excitant amino acids. It is still believed by most researchers, however, that the majority of amino acid mediated excitatory synapses in the CNS use glutamate as the principal transmitter (see for review Puil, 1981; Fonnum, 1984). How this compound fits into contemporary ideas regarding the structural requirements for activation of the various receptor types should therefore be addressed. Tables 12 and 13 (pages 72 and 73) show the results of a series of experiments where L-GLU and D-GLU were compared directly with NMDA and QUIS for sensitivity to blockade by APV and KYNA. Paired t-tests disclosed that L-GLU activity was indistinguishable from that of QUIS with respect to antagonism by APV and KYNA, but that D-GLU was unlike either NMDA or QUIS since it was affected by both APV and KYNA (cf. Hicks et al., 1979). These results are most easily interpreted by suggesting that the structure of D-GLU allows it to activate either or both of the NMDA and KAIN types of receptor in addition to QUIS receptors. The D-configured glutamate molecule appears to have a higher affinity for NMDA receptors and weakly activates those responding to QUIS and KAIN. In the present experiments, L-GLU acted much more potently at QUIS receptors, although other studies have shown it to possess at least nominal affinity for the

NMDA or KAIN receptor varieties (for review see Fonnum, 1984). The flexibility of the glutamate molecule allows it to fit any receptor template based on the configuration of one or more of the known potent excitants.

It does not seem unbelievable to suppose that L-GLU may be responsible for mediating transmission at the bulk of "fast" excitatory synapses in the brain and spinal cord. The involvement of the different amino acid receptors in neuronal function would depend on their location, relative density, and balance of excitatory and inhibitory / antagonistic compounds in the extracellular fluid. The receptor type present at the highest concentration on the postsynaptic membrane would determine whether the postsynaptic response would be NMDA- or QUIS-like and therefore whether or not Ca^{2+} influx would accompany the Na^{+} influx during the EPSP. Many different rôles for the archetypal amino acid receptors in synaptic activity have been determined; a few, namely those in the hippocampus and spinal cord, were described in the introduction. Endogenous ligands other than L-GLU and L-ASP have been discovered, and in particular the tryptophan metabolites QUIN and KYNA are strong candidates for rôles as an endogenous agonist and antagonist respectively.

J. Does quinolinate act at a fourth receptor?

There has been considerable discussion of the possible reasons for QUIN's relative lack of potency in the spinal cord and cerebellum in vivo (cf. Perkins and Stone,1983). Perkins and Stone (1983) suggested that the observed differences in potency support a rôle for QUIN as a transmitter since only small regional

differences in potency could be shown for other excitatory amino acids, including L-GLU. These authors further suggested that these regional differences possibly indicate a heterogeneous population of NMDA receptors ($NMDA_1$ and $NMDA_2$, both APV sensitive) which by way of differential distribution in the CNS may reflect a neurotransmitter function for QUIN or a QUIN-like compound. Since there is no evidence for uptake of QUIN anywhere in the CNS (see the review by Stone and Connick, 1985) or for a rapid enzymatic degradation of QUIN (Foster et al., 1985), regional differences in such processes cannot readily be used to explain the differences in excitatory potency.

The results to be discussed here (Tables 14 and 16: pages 76 and 81) show that when using APV to block the NMDA-elicited activity in the spinal cord in vitro, the excitation evoked by QUIN was significantly less affected, but was more sensitive to blockade than those of QUIS and KAIN. Iontophoretically applied APV thereby permits the agonists to be divided into three distinct groups, QUIN being separated from the other agonists on the basis of its intermediate sensitivity to APV. The dose-response curves for APV (Fig. 13: page 78) support this conclusion by showing a large separation between the effects of this antagonist applied topically on firing induced by NMDA, QUIN, and QUIS/KAIN.

The IC50's determined from the dose-response curves for APV in vitro show that to reduce the activities of NMDA, QUIN and QUIS by 50%, concentrations of at least 2×10^{-6}, 7×10^{-6} and 1.8×10^{-5} M would be required respectively (Table 15: page 79). These data are similar to those presented by ffrench-Mullen et al. (1986) for the piriform cortex in vitro, where NMDA, QUIN and L-aspartate were compared

using the antagonist APV (reported IC50's of 3.5×10^{-6}, 3.5×10^{-5} and 3×10^{-4} M respectively). It is concluded, therefore, that on the basis of antagonism by APV, QUIN's action in the spinal cord in vitro is pharmacologically distinct from that of NMDA. This is in contrast to the actions of these compounds in the CA1 region of the hippocampus where QUIN and NMDA are identical in their sensitivity to APV (Peet et al., 1986, 1987)

The actions of KYNA in the spinal cord in vivo and in the hippocampus in vitro (Curry et al., 1986; Peet et al., 1987) are less specific than those of APV (i.e. it antagonizes both KAIN and NMDA responses) and are about 10-fold less potent. In the spinal cord in vitro, KYNA applied iontophoretically (Tables 14 and 16: pages 76 and 81) was equally effective against KAIN and NMDA responses, and had a smaller action on responses elicited by QUIS and QUIN, which were similarly reduced (Tables 14 and 16). When applied topically in a range of concentrations, KYNA reduced the activities of NMDA and KAIN in parallel with significantly greater potency than it did those of QUIN and QUIS which were also reduced in parallel. The pattern of antagonism exhibited by KYNA suggests that QUIN does not excite spinal cord cells by acting, even in part, at either or both of the NMDA or KAIN receptors, since it was indistinguishable from QUIS using KYNA. That portion of QUIN's activity which was antagonized by APV however, could not readily be accounted for by reaction with any of the known receptors since it was insensitive to KYNA, and thus it is suggested that a fourth amino acid receptor exists in the spinal cord, an inference which has also been made previously (Perkins and Stone, 1983).

A few experiments were performed using the spinal cord preparation in vitro with Mg^{2+}-free medium. As described earlier, Mg^{2+} specifically blocks the action of NMDA in a dose-dependent, voltage-sensitive manner (Evans et al., 1977; Davies and Watkins, 1977). Table 17 (page 82) shows that when Mg^{2+}-free medium was substituted for ACSF containing 2 mM Mg^{2+}, responses to both NMDA and QUIN were approximately doubled while those of QUIS were on average only 40 % larger. The inability of Mg^{2+}-free bathing medium to distinguish between NMDA and QUIN-induced firing in the spinal cord suggests that these two receptors may be related, perhaps utilizing the same channel complex as apparently is the case in the hippocampus, in spite of their obvious pharmacological differences.

K. The actions of the isomers of ACPD and several pyridine derivatives related to quinolinate

Two dicarboxylate derivatives of pyridine (2,5- and 2,6-PyrDA), and all four isomers of ACPD (D- and L-, cis- and trans-ACPD) were found to be excitants in the spinal cord in vitro. Their actions were compared with those of L-GLU, QUIS, QUIN, KAIN and NMDA for susceptibility to blockade by APV and KYNA. Using paired Student's t-tests, these agonists fall into three categories based on antagonism by APV (Table 18: page 85); D-cis-, L-cis- and L-trans-ACPD are statistically indistinguishable from NMDA, D-trans-ACPD and QUIN are similar to each other but statistically different from both NMDA and QUIS, while 2,5- and 2,6-PyrDA are similar to L-GLU and QUIS and statistically different from both NMDA and QUIN. Using KYNA as an antagonist, the agonists divide into two

distinct categories, D-cis-, L-cis- and L-trans-ACPD being statistically similar to NMDA, while the remaining five all differ from NMDA but not from each other (Table 20: page 91).

L. Analogues acting at spinal cord NMDA receptors.

The results of this study show that in the spinal cord, as in the hippocampus, D-cis-ACPD is a powerful excitant sensitive to both KYNA and APV. It is a structurally rigid D-amino acid with the two carboxyl groups in a cis configuration. Being the most potent conformationally restricted compound tested, it represents the best example of the preferred arrangement of charged groups for NMDA receptors in the spinal cord and hippocampus. However, the responses of dorsal horn neurones to L-cis- and L-trans-ACPD were also sensitive to APV, in contrast to the hippocampus where they, and D-trans-ACPD, are insensitive to APV but are blocked by KYNA suggesting that they act via kainate receptors (Curry et al., 1987). It has been shown here (Fig. 13: page 73) that KAIN-elicited firing of dorsal horn neurones was similar to QUIS-induced activity when APV was used and was blocked in parallel with NMDA by KYNA: none of the conformationally restricted compounds tested here showed this pattern of response.

In the hippocampus the KAIN-like L-cis-, L-trans- and D-trans-ACPD are 4 to 8 times less potent than KAIN itself (Curry et al., 1987). In the spinal cord in vitro, however, indirect potency comparisons (relative to NMDA) show them be 35, 62 and 80 times less potent than KAIN respectively (L-trans-ACPD, L-cis-ACPD and D-trans-ACPD; Table 18: page 85). The low potencies observed in the present

experiments compared to those found in the hippocampus suggest that these compounds may be unable to interact with KAIN receptors in the spinal cord, but when applied at higher concentrations are able to activate NMDA receptors. Thus, it appears that differences may also exist in the structural requirements of the KAIN receptor among various regions of the CNS, and currently, a lack of other structurally related, conformationally restricted active compounds precludes a closer examination of this receptor type. It remains to be determined whether or not the ability of L-trans- and L-cis-ACPD to activate the spinal cord NMDA receptor shows an effect which is not observed in the hippocampus merely because these compounds act at hippocampal KAIN receptors at lower concentrations.

M. Quinolinate-like compounds

As mentioned previously QUIN is pharmacologically indistinguishable from NMDA in the hippocampus (Peet et al., 1987) and cortex (McLennan, 1984). By contrast, in the spinal cord it was relatively much less potent, was not readily blocked by KYNA, and was not as sensitive to APV as was NMDA (Fig. 13, Tables 18 and 20: pages 78, 85 and 91). Phthalate (1,2-benzene dicarboxylate; Fig. 3, A: page 12), also NMDA-like in the hippocampus (Peet et al., 1987), is structurally similar to QUIN but without the aromatic nitrogen, was unable to elicit firing of dorsal horn neurones in vitro even when ejected at 70 to 100 nA from a 200mM solution. In addition, phthalate neither enhanced nor depressed the activity of other agonists when ejected concomitantly. It is concluded that the aromatic nitrogen of QUIN is a requirement for activation of amino acid receptors by

heterocyclic agonists in the spinal cord. Furthermore, picolinate (2-pyridine carboxylate) and 3-hydroxypicolinate (Fig. 3, D and C: page 12), which are inactive in the hippocampus (Peet et al., 1987), were also unable to elicit activity in the spinal cord in vitro; however 3-hydroxypicolinate weakly enhanced activity elicited by other agonists indicating that it possesses a very slight excitatory ability. Thus, it can be concluded that the carboxyl group on carbon 3 is important for the activity of heterocyclic agonists in the spinal cord, and substitution with an hydroxyl markedly reduces the ability of the compound to activate excitatory receptors.

The failure of QUIN to act at NMDA receptors in the spinal cord may be due to the fact that it has a shorter intercarboxyl distance than all the NMDA-mimetic compounds tested. D-trans-ACPD (Fig. 14: page 84) is the other substance which, possessing 1/4 the activity of L-GLU as an excitant in the cord, with respect to both potency and sensitivity to APV and KYNA appears also to act at the same receptor as does QUIN.

N. Quisqualate-like compounds

In the absence of a specific L-GLU / QUIS antagonist, activation of this receptor is inferred from the relative insensitivity of evoked excitations to both APV and KYNA. Both 2,6- and 2,5-PyrDA elicited firing in dorsal horn neurones in vitro which was reduced by APV and KYNA to a degree comparable to QUIS and L-GLU (Tables 18 and 20: pages 85 and 91). These compounds were found to possess about 1/5 the potency of L-GLU (Table 18); in contrast, QUIS was found to be 43 times more potent than L-GLU. The latter finding agrees with that reported by

several other authors (see e.g. Lodge et al., 1980). Despite the high iontophoretic doses of 2,6- and 2,5-PyrDA required, it is unlikely that any activity would be observed in response to these pyridine derivatives if the charged groups on the molecules were not appropriately positioned to interact with the receptor. 2,6-PyrDA was tested on cortical neurones by Perkins and Stone (1982) and was found to increase the spontaneous firing rate of a few cells but was extremely weak, and no report of the antagonism of the responses was given.

A folded conformation allows the charged groups of QUIS and L-GLU to approximate a template based on 2,5- and 2,6-PyrDA (discussed in more detail in the following section). It is concluded, therefore, that the active conformations of QUIS and L-GLU are those which approximate the conformations of the rigid 2,5- and 2,6-PyrDA. This suggests that the γ-carboxyl group (Cd) of L-GLU or the isoxazole ring of QUIS is preferentially situated in juxtaposition to the α-amino group rather than the α-carboxyl (Cp) to interact with the receptor molecule. The comparative lack of potency of 2,5- and 2,6-PyrDA may be due to the presence of the aromatic ring and hindrance between it and components of the receptor, or more likely that their arrangements of the charged groups are not optimal but nevertheless permit interaction with spinal cord QUIS receptors, assuming some conformational flexibility in the latter. The significant drop in potency observed when going from 2,6- to 2,5-PyrDA (t-test; $p < 0.005$) may thus reflect that the increased Cd - N distance in the latter compound places the distal carboxyl group in a less desireable position relative to the other charged groups than that seen in 2,6-PyrDA. The lack of flexibility of these pyridine analogues could also be

responsible for their low potency. If a conformational change in the receptor is necessary for activation, this may be in part prevented or hindered by the rigidity of the 2,5- and 2,6-PyrDA molecules, thus reducing their effectiveness as activators once bound to the receptor. This supposition is also supported by the finding that (-)trans-CPA apparently competes for QUIS receptor sites but is unable to cause excitation.

O. Regional differences in amino acid structure-activity relationships

In 1968, McLennan and his colleagues reported that neurones in certain regions of the thalamus displayed significantly different relative sensitivities to L-GLU and DL-homocysteic acid. This paper was the first to provide evidence for the existence of heterogenous populations of receptors for the amino acids, and led the way to further reports strengthening the case in favour of transmitter rôles for L-GLU and L-ASP. There has since been an accumulation of evidence to suggest that regional differences may exist not only in the distribution of the various receptors for the amino acids but also in their structure-function characteristics. Perkins and Stone (1983a) provided convincing data showing that neurones in two central nervous system regions in particular did not respond as expected to NMDA-mimetic agonists. Dorsal horn and cerebellar neurones, when recorded extracellularly in vivo, were virtually insensitive to QUIN despite its significant potency in the cortex and hippocampus. As already described, the increased potency of amino acid agonists in the spinal cord in vitro compared to in vivo enabled the pharmacology of QUIN to be examined in this tissue. The data already

discussed is in general similar to that presented by ffrench-Mullen et al. (1986) for the piriform cortex. An additional report by Herrling et al. (1983) places caudate neurones into the group typified by APV-sensitive bursts in response to both NMDA and QUIN.

It is tempting at this point, on the basis of the neuronal responses to NMDA and QUIN, to divide the regions of the CNS into two groups: those in which NMDA and QUIN are pharmacologically similar (cortex, hippocampus, striatum) and those in which QUIN is relatively much less potent and is distinguishable from NMDA using APV and/or KYNA (spinal cord, cerebellum, piriform cortex). Care must be taken, however, as pointed out by ffrench-Mullen et al. (1986), since although responses of piriform cortical neurones appeared to be very similar to those of spinal cord neurones with respect to many of the "standard" agonists and antagonists, when examining GDEE it was found to have no effect on any of the amino acid or synaptic responses in the piriform cortex despite its well known effects in the spinal cord and elsewhere. However, this observation may have been due to the methods used ie. exclusively applying the antagonists in the superfusate and only at concentrations from 10^{-7} to 10^{-3}, since in the experience of the author GDEE is not extremely potent when superfusate applied to spinal cord neurones in vitro. Nevertheless, GDEE's lack of activity in the piriform cortex provides a warning against categorizing the regions of the CNS into discrete groups on the basis of response to the amino acids.

VI. Structure-activity relationships, model analysis

A. NMDA-like compounds.

Figure 18 shows, diagramatically, a template based on D-cis-ACPD which represents an arrangement of charged groups with which NMDA-like compounds can react with in the spinal cord. Analysis of scale models of each of the analogues, using the bond length approximations shown in Table 22 , has shown that the distance between the carboxyl carbon atoms of NMDA can vary between 0.25 and 0.37 nm (Table 23). The 5-membered cyclopentane ring can adopt an envelope conformation where one carbon will extend out of the plane of the ring. For the cis-ACPD isomers, the flexibility of the ring allows the distance between the proximal carboxyl (Cp; corresponding to the α-carboxyl of glutamate) and the distal carboxyl (Cd; corresponding to the γ-carboxyl of glutamate) to vary between 0.33 and 0.45 nm (Table 23). In contrast, the distance between the carboxyl groups (Cd - Cp) of a model of L-trans-ACPD can vary only between 0.43 and 0.45 nm, while the Cd - N distance has a range of 0.33 to 0.45 nm (Table 23). These compounds (L-cis- and L-trans-ACPD) were ca. 3 and 9 times less potent than NMDA (Table 18: page 85) and any receptor template based on these compounds should be designed taking into account their relative potencies. The relative positions of the charged groups of NMDA, D-cis- and L-trans-ACPD correspond most closely if carbon 2 of D-cis-ACPD is out of the plane away from the amino group, allowing the separation of the carboxyl groups to approach its maximum of 0.45 nm (Table 23), while carbon 2 of L-trans-ACPD must also be out of the plane

of the ring but towards the amino group thus bringing the distal carboxyl to a position almost equidistant from the other charged groups. In this conformation, the distance separating the distal carboxyls from the amino groups of both ACPD isomers is about 0.44 nm. NMDA can adopt a conformation with an intercarboxyl distance of 0.35 nm (Table 23); in this conformation Cd is also virtually equidistant from both N and Cp and is approximately 0.09 nm closer to the proximal groups than in the D-cis-ACPD model.

Bond Lengths Used With the Orbit Molecular Building System

bond	interionic distance
- C - C -	0.15 nm
- C = C -	0.14 nm
- C - N -	0.14 nm
- C = N -	0.13 nm

Table 22. Bond length estimates used in the construction of chemical models for structure-activity analysis. Model system used is the Orbit molecular building system, Cochranes of Oxford.

L-cis-ACPD can fit precisely the template based on D-cis-ACPD (Fig. 18: page 133). It has a similar arrangement of charged groups, but has ring carbons extending into the plane of the presumed active site (L-cis carbons 4 and 5). The protrusion of the rings of this analogue into the binding region may sterically hinder its approach to the receptor site and be responsible for its lower potency compared to D-cis-ACPD. L-cis-ACPD was 16 times while D-cis-ACPD was 3 times less potent than NMDA (Table 18: page 85).

Interionic Distances Determined by Analysis of Molecular Scale Models

Compound	A. Cd - Cp (nm)	B. Cd - N (nm)	B. / A.
D-cis-ACPD *	0.45	0.44	0.98
range	0.33 - 0.45	0.43 - 0.45	0.98 - 1.30
L-cis-ACPD *	0.45	0.44	0.98
range	0.33 - 0.45	0.43 - 0.45	0.98 - 1.30
L-trans-ACPD *	0.44	0.45	1.02
range	0.43 - 0.45	0.33 - 0.45	0.77 - 1.02
NMDA *	0.35	0.33	0.94
range	0.25 - 0.37	0.25 - 0.37	0.71 - 1.40
QUIN	0.27	0.36	1.33
D-trans-ACPD *	0.44	0.45	1.02
range	0.43 - 0.46	0.33 - 0.46	0.77 - 1.02
2,6-PyrDA	0.47	0.23	0.49
2,5-PyrDA	0.55	0.36	0.65
QUIS *	0.38	0.22	0.58
range	0.18 - 0.50	0.18 - 0.50	0.48 - 2.06
L-GLU *	0.38	0.22	0.58
range	0.16 - 0.50	0.16 - 0.50	0.48 - 2.06

Table 23. Interionic distances estimated from molecular scale models constructed using the "Orbit Molecular Building System". Compounds in *italics* are those from which templates have been constructed for Figure 18 (page 133).

* bond lengths determined with molecule in conformation of best fit for templates in Figure 18. A C2-envelope conformation for ACPD analogues, an extended conformation for NMDA and a folded conformation for QUIS and L-GLU.

B. QUIN-like compounds.

Unlike the cases of the NMDA-like isomers of ACPD discussed above however, it is difficult to construct a simple template model which will accept both QUIN and D-trans-ACPD. The intercarboxyl distance Cd - Cp, and the Cd - N and Cp - N distances are fixed in QUIN at 0.27, 0.36 and 0.23 nm respectively, while the corresponding distances for D-trans-ACPD lie in the ranges 0.43 - 0.46 and 0.30 - 0.46 with the Cp - N length fixed at 0.24 nm (cf. Table 23), and there is no evident way in which the two molecules can be matched to a single template. Whether this betokens the existence of yet another receptor subtype, or whether the postulated QUIN receptor is sufficiently malleable when confronted with an unusual agonist that its configuration can change to accept a molecule with a quite different charge separation, is unclear. Neither alternative is particularly attractive.

C. QUIS-like compounds.

Analysis of scale models of L-GLU and QUIS demonstrates that the distances between the distal carboxyl and the amino groups (Cd - N) of the rigid pyridine analogues are 0.23 and 0.36 nm for 2,6- and 2,5-PyrDA respectively (Table 23). A template has been constructed based on these compounds (Fig. 18: page 85) and this has been compared to models of QUIS and L-GLU. Assuming that the isoxazole ring of QUIS plays the same role in binding as does the distal (γ) carboxyl group of L-GLU, the Cd - Cp and Cd - N distances of these compounds are shown in Table 23. A folded conformation allows the charged groups of QUIS and L-GLU to approximate a template based on 2,5- and 2,6-PyrDA, with a Cd - N

distance close to that of the latter but 0.14 nm shorter than the former compound.

These results place 2,5- and 2,6-PyrDA on the end of a short list of compounds known to interact with spinal cord QUIS receptors. Also included are L-GLU, L-cysteate, and a number of compounds structurally related to ibotenic acid, including (±)-α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA; Krogsgaard-Larsen et al., 1980; Krogsgaard-Larsen, et al., 1981). All of these compounds are structurally capable of achieving a conformation where the distal anionic group is in juxtaposition with the amino group, despite the conformational restrictions present in those related to AMPA.

The structural feature common to all the potent QUIS-like agonists, which is not observed in those compounds acting at the other amino acid receptors, is the ability of the compound to adopt a conformation where the distal carboxyl group (or isoxazole ring) is less than 0.24 nm distant from the amino group (ie. Cd - N ca. 0.24 nm). This feature is seen in L-GLU, QUIS and AMPA, three powerful excitants, and also in 2,6-PyrDA, all of which show similar profiles of sensitivity to antagonism (Krogsgaard-Larsen, 1980). Table 24 characterizes a series of analogues related to AMPA which have different potencies and sensitivities to antagonism in the cat spinal cord (Krogsgaard-Larsen et al., 1980; Lauridsen et al., 1985). AMPA- and homoibotenate-elicited activity is mediated via L-GLU / QUIS receptors as it is sensitive to blockade by GDEE while that of ibotenate is not. AMPA is approximately 3 times more potent than L-GLU while homoibotenate is slightly less; the activities of both are insensitive to blockade by the NMDA antagonist DαAA. The potency of ibotenate is twice that of L-GLU, but firing induced by this

compound is not blocked by GDEE but is susceptible to antagonism by DαAA. If the activity of these compounds is dependent on the intercarboxyl (Cd - Cp) distance, ibotenate should be more potent than homoibotenate (being closer to AMPA in this respect) as it is, but also should be GDEE-sensitive (QUIS-like), which it is not. A structure-activity parallel can be seen, however, for the potency and sensitivity to antagonism based on variation in the Cd - N distance, and/or in the ratio of Cd - N (B) / Cd - Cp (A) (Tables 23 and 24). AMPA has a minimum Cd - N of 0.21, a B/A ratio of 0.54, and is L-GLU-like in its sensitivity to GDEE. The minimum Cd - N distance seen in homoibotenate is 0.33 nm with a B/A ratio of 0.67, and it is described as being less potent than L-GLU but sensitive to GDEE. In contrast, ibotenate shows a minimum Cd - N distance of 0.40 nm, a B/A ratio of 0.91 and is not blocked by GDEE. Assuming that the isoxazole ring of homoibotenate has an area of electrostatic interaction larger than that of the carboxyl group of L-GLU (an assumption supported by the 43 fold increase in potency when going from L-GLU to QUIS), homoibotenate can conceivably interact with a receptor having the charged groups located ca. 0.24 nm apart. The antagonism of L-GLU and QUIS activity by (-)trans-CPA also supports these conjectures; it is limited to a Cd - N distance of 0.25 nm and has a B/A ratio of 0.69 (Table 25) and should be capable of interacting with the L-GLU / QUIS receptor template shown in Figure 18.

Interionic Distances of Other Conformationally Restricted Analogues Determined by Analysis of Molecular Scale Models

compound	A. Cd - Cp (nm)	B. Cd - N (nm)	potency*	GDEE*	DαAA*	B / A
cyclic analogues:						
AMPA[1]	0.38	0.21	++++++	yes	no	0.54
range	0.21 - 0.48	0.21 - 0.48				0.54 - 1.85
homoibotenate[1]	0.48	0.33	+(+)	yes	no	0.67
range	0.33 - 0.53	0.33 - 0.53				0.67 - 1.50
ibotenate[1]	0.44	0.40	++++	no	yes	0.91
range	0.40 - 0.44	0.40 - 0.44				0.91 - 1.10
AMAA	0.33	0.33	++	no	yes	1.00
range	0.28 - 0.39	0.28 - 0.39				0.70 - 1.40
bicyclic analogues:						
7-HPCA[2]	0.48	0.38	++++	yes	no	0.80
5-HPCA[2]	0.50	0.40	++++	yes	no	0.80
4-HPCA[2]	0.35	0.40	n.a.	inactive		1.14

Table 24. Interionic distances estimated from molecular scale models constructed using the "Orbit Molecular Building System".

* Potencies (relative to L-GLU = ++) and sensitivities to GDEE and DαAA in the cat spinal cord in vivo from Krogsgaard-Larsen et al., 1980, 1985.

1. bond lengths determined with molecule in conformation of best fit for the QUIS/L-GLU template in Figure 18 (page 133).
2. bond lengths determined with molecule in conformation described by Krogsgaard-Larsen et al., 1985.

Interionic Distances of Conformationally Restricted Antagonists
Determined by Analysis of Molecular Scale Models

Compound	A. Cd - Cp (nm)	B. Cd - N (nm)	QUIS*	NMDA*	B. / A.
(-)trans-CPA†	0.36	0.25	yes	no	0.69
range	0.33 - 0.36	0.24 - 0.26			0.69 - 0.76
PDA†	0.28	0.28	no	yes	1.00
range	0.25 - 0.28	0.28 - 0.37			1.00 - 1.36

Table 25. Interionic distances estimated from molecular scale models constructed using the "Orbit Molecular Building System".
† bond lengths determined with molecule in conformation of best fit for the QUIS / L-GLU template in Figure 18 (page 133).
* reduce excitations elicited by iontophoretic application of QUIS or NMDA

D. Other conformationally restricted compounds

Many other conformationally restricted compounds related to ibotenate have been synthesized and tested by Krogsgaard-Larsen and his colleagues (1980, 1981, 1985), the actions of which provide additional support for the structure-activity distinctions between QUIS and NMDA receptors discussed previously. (RS)-3-hydroxy-4,5,6,7-tetrahydroisoxazolo[5,4-c]- pyridine-7-carboxylic acid and 5-carboxylic acid (7-HPCA, 5-HPCA; Krogsgaard-Larsen et al., 1985), (RS)-3-hydroxy-4,5,6,7-tetrahydro- isoxazolo[5,4-c]pyridine-4-carboxylic acid (4-HPCA; Madsen et al., 1987) and α-amino-5-methyl-3-hydroxy-4-isoxazoleacetic acid (AMAA; Krogsgaard-Larsen et al., 1980; Honoré et al., 1981) are four conformationally very restricted compounds; 4-HPCA is inactive and AMAA is NMDA-like, while 5- and 7-HPCA are GDEE -sensitive but are unaffected by DαAA and are therefore QUIS-like. The charged groups of AMAA are held on an aspartate-like backbone which is confined by the isoxazole ring and the methyl

group on carbon 5 into an extended conformation (Krogsgaard-Larsen et al., 1980) with a B/A ratio near 1.0 (Table 24), hence its NMDA-like activity. The charged groups of 4-HPCA are separated by one additional carbon (thus with a glutamate backbone), however the bicyclic nature of this compound constrains the receptive groups into a configuration with a B/A ratio of ca. 1.2 (Table 24) apparently rendering it unable to activate either NMDA or QUIS receptors. Of this group of compounds only 5- and 7-HPCA are configured such that the intercarboxyl distance (Cd - Cp) is greater than that separating Cd from N, and an interaction with QUIS receptors is observed. These analogues have a B/A ratio of ca. 0.8 when in the conformations described by Krogsgaard-Larsen et al., (1985), which is greater than that possible in other analogues examined, but still allows a sufficient degree of folding for interaction with QUIS receptors. These compounds are unable to achieve a conformation where the B/A ratio is 1.0, thereby preventing their interaction with NMDA receptors. Neither 5- nor 7-HPCA has a potency comparable to QUIS or AMPA however, supporting further the suggestions made earlier in this discussion that a conformation allowing the distal anionic group to come into close juxtaposition with the amino group is preferred by QUIS receptors and that structural rigidity may not be entirely conducive to potency at this receptor type.

All of the agonists tested which are capable of achieving a B/A ratio less than 0.70 are found to be GDEE sensitive (Krogsgaard-Larsen et al., 1980) and/or are QUIS-like with respect to both KYNA and APV (Tables 23 and 24). Furthermore, those agonists confined to a conformation with a B/A ratio of 0.8 (5- and 7-HPCA)

have also been reported to be QUIS-like and presumably are unable to interact with NMDA receptors. In addition, the two conformationally restricted antagonists studied here, PDA and trans-CPA also fit this model, since PDA has a minimum B/A ratio of 1.00 and competes primarily with NMDA-like agonists while trans-CPA, which has a minimum B/A ratio of 0.69, effectively blocks QUIS-like agonists (Table 25).

VII. Contributions made to the study of the spinal cord and excitatory amino acids in the central nervous system

In vitro slice preparations have been used successfully to study the function of the central nervous system for more than 20 years. For several reasons, the number of successful attempts to examine the adult mammalian spinal cord as a slice, to date, is very small. First of all, the ease of preparation of slices of the cortex and hippocampus and their highly organized cytoarchitecture make them desirable subjects of study. Secondly, the spinal cord has been studied successfully in vivo for many years, and the structure itself and its afferent and efferent innervation are anatomically well defined and accessible. Finally, great difficulties are associated with the preparation of spinal cord slices because of the high proportion of white matter present, and the softness of the tissue even when chilled. The evolution of the techniques available to prevent damage during the cutting process led to the successful development of this preparation. It is hoped that this method of spinal cord slice preparation, or a modification thereof, will be successfully adopted by other laboratories wishing to study the function of the spinal cord.

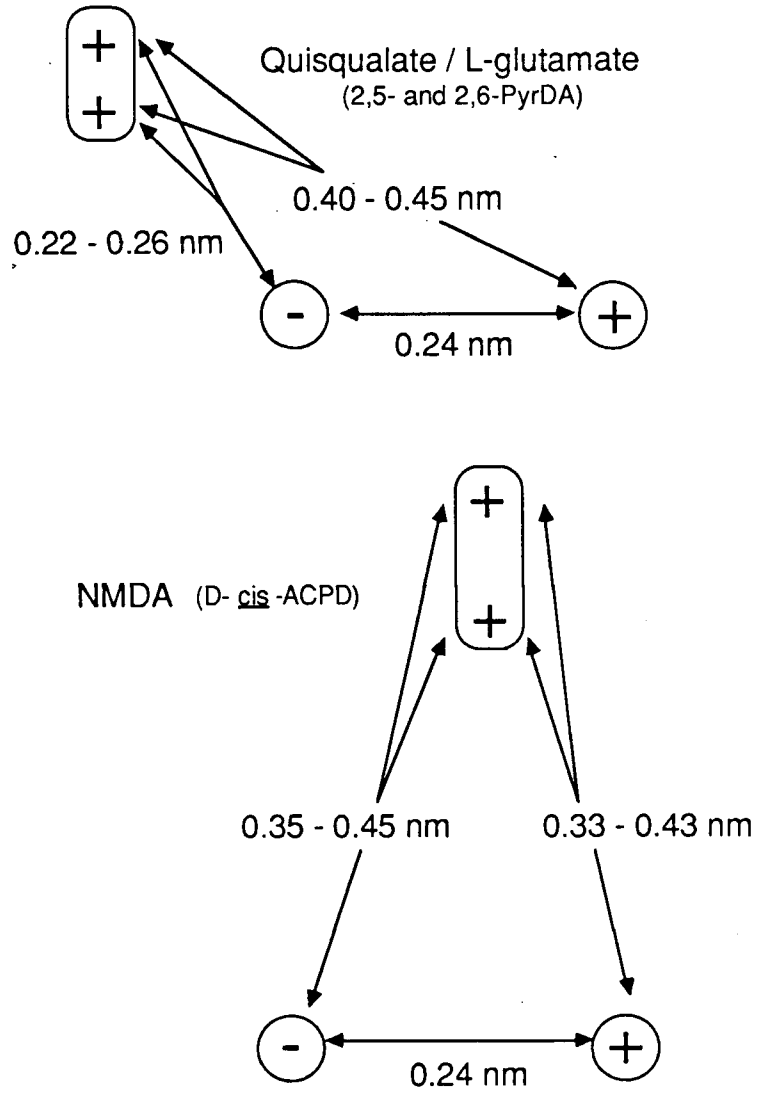


Figure 18. Diagrammatic representations of templates designed to accommodate the ligands acting at QUIS and NMDA spinal cord receptors. As drawn, the QUIS receptor template accommodates a range of Cd-N distances of 0.22 - 0.26 nm, and a B / A ratio of about 0.50 - 0.80. The NMDA receptor template has a B / A ratio near 1.00.

VIII. Structure-Activity Conclusions

It has been shown that the requirements for activation of NMDA receptors in the spinal cord involve an adjacent positioning of two carboxyl groups and the presence of an amino group, either primary or secondary, located in a D-configuration with respect to the carboxyl groups. Aromatic agonists (QUIN and pthalate) are not accepted by spinal cord NMDA receptors as they are in the hippocampus. Ring carbons extending into the region of the charged binding groups as in L-cis-ACPD appear to hinder the interaction of the agonist with the spinal cord receptor; and the addition of a second ring as in KYNA results in an antagonist for both NMDA and KAIN. All of the conformationally restricted analogues tested which were found to be APV sensitive are capable of achieving a conformation where the distal carboxyl group is approximately equidistant from the proximal charged groups giving a B/A ratio close to 1.0 (Table 23: page 125). Two isomers of ACPD which are KAIN-like in the hippocampus are NMDA-like in the spinal cord suggesting that differences also exist between spinal cord and hippocampal KAIN receptors.

Activation of the receptor(s) with which QUIN interacts (the "QUIN receptor") requires one cationic group and two rather widely spaced anionic groups (D-trans-ACPD), and substitution (3-hydroxypicolinate) or removal (picolinate) of the 3-carboxyl group greatly reduces or eliminates activity. Substitution of a sixth aromatic carbon for the aromatic nitrogen of QUIN (phthalate) also results in a loss of activity. Only QUIN and D-trans-ACPD are known to cause excitations in the

spinal cord which show this pattern of antagonism (QUIS-like in senstivity to KYNA, intermediately affected by APV). This pattern of antagonist sensitivity has not been observed for any agonist in the hippocampus, but has in part been described in the piriform cortex (ffrench-Mullen et al., 1986). With respect to the orientation of the three binding groups these two compounds are structurally dissimilar, and it is impossible to draw any defensible structure-activity conclusions beyond those mentioned above.

Spinal cord QUIS receptors also require one cationic and two anionic groups, but prefer the distal anionic group to be adjacent to the cationic group as it is in 2,5- and 2,6-PyrDA. This supports suggestions that QUIS and L-GLU interact with spinal cord QUIS receptors in a folded conformation (McLennan et al., 1982). Decreases in the potencies of QUIS-like agonists can be observed when the minimum Cd - N distance is greater than 0.24 nm, and/or when the B/A ratio described in Table 23 (page 125) is greater than 0.60 (AMPA to homoibotenate; 2,6-PyrDA to 2,5-PyrDA). Shifts from agonism via QUIS receptors to that via NMDA receptors is observed when going from L- to D-configured excitants (L-GLU to D-GLU) and/or when going from close analogues with B/A ratios increasing beyond 0.70 (homoibotenate to ibotenate; L-GLU to NMDA; Tables 23 and 24: pages 125 and 129).

The relatively large discrepencies in the Cd - Cp and Cd - N distances seen in compounds which apparently act at similar receptors in the spinal cord (L-trans-ACPD and D-cis-ACPD; 2,5- and 2,6-PyrDA) suggests that the tolerance of the receptor binding sites may be quite large, particularly for the distal or γ-carboxyl

moiety, allowing the relative positions of binding groups to vary by more than 0.10 nm in some situations. Although electrostatic interactions are thought to occur over relatively long distances, it is unlikely that three different arrangements of receptive groups carrying "single" charges similar to those associated with carboxyl and amino groups in solution at pH 7.4 could account for these observations. A greater degree of latitude could be imagined, however, if the charged groups on the receptors were larger, possibly composed of two or more similarly charged moieties, allowing the binding site to be considered a charged region or area rather than an individual group. Alternatively, the receptive groups may be composed of amino acids with little conformational restriction allowing them to adopt one of a number of different conformations in order to accommodate ligands of various sizes, or various conformations of a particular ligand. This explanation is made even more plausible when considering the large variety of acidic amino acids of varying chain length which are capable of interacting with these receptors. The similarity in the B/A ratios seen in those conformationally restricted compounds found here to act either at QUIS receptors (0.49 - 0.69) or NMDA receptors (0.77 - 1.02) supports the suggestion that the receptive group which interacts with the distal carboxyl is either mobile or large enough to accommodate compounds with different distances between this moiety and the α-carbon. Finally, there is evidence to suggest that more than one amino acid molecule is necessary to activate excitatory receptors, and thus some degree of cooperativity in the interaction of ligand(s) and receptor may exist (McLennan and Wheal, 1976).

In conclusion, the use of conformationally restricted analogues of glutamate, aspartate and quinolinate have provided new information regarding the structural requirements for activation of spinal cord amino acid receptors. The evidence in support of a fourth amino acid receptor, responsible for the activity of quinolinate in the spinal cord, has been strenghtened and details of the regional differences in the amino acid receptors have been accumulated. The fundamental differences in the NMDA receptors of the spinal cord and hippocampus and the possible involvement of the latter in learning and memory (via long-term potentiation) suggests that functional significance may, in the future, be attributed to the structural differences seen in these studies. Local patterns of distribution may also be an important correlate of function, as has been suggested for the cerebellum (Olsen et al., 1987).

IX. References

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