CONTRIBUTION OF METABOTROPIC GLUTAMATE RECEPTORS TO SPINAL NOCICEPTIVE TRANSMISSION IN THE RAT SPINAL CORD

by

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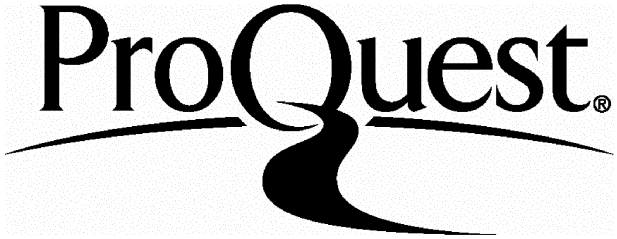

ABSTRACT

In this thesis I have examined the contribution of metabotropic glutamate receptors (mGluRs) to spinal nociception in control conditions and following inflammatory hyperalgesia in the rat. *In situ* hybridisation studies showed mGluR mRNA expression in all areas of the adult spinal cord with distribution being specific for each subtype. There was a general decrease in subtype expression during development, with the most marked changes in mGluR3 and mGluR5 mRNAs. Following the induction of inflammatory hyperalgesia in juvenile (postnatal day 11-13) rats, no changes were seen in the levels of expression of mGluR1, 2, 4, 5 and 7 mRNAs. However, a significant bilateral increase in mGluR3 mRNA expression in the superficial dorsal laminae was seen 1 day after the onset of inflammation. This change in expression mirrored the time course for mechanical hyperalgesia and allodynia in behavioural studies.

In an *in vitro* preparation of the hemisected spinal cord, the specific mGluR agonist, (1S,3R)-ACPD, produced a concentration-dependent, reversible ventral root depolarisation, which was enhanced in UV-treated animals. The specific, non-selective mGluR antagonist MCPG attenuated (1S,3R)-ACPD responses in naïve and UV-treated animals. In naïve preparations, the NMDA receptor antagonist, D-AP5, was ineffective against (1S,3R)-ACPD responses while in UV-treated animals it decreased the maximum response. MCCG (group II antagonist) enhanced (1S,3R)-ACPD responses in naïve animals and attenuated the same response in UV-treated animals. The more group II selective agonists, (1S,3S)-ACPD and L-CCG-I evoked responses in both naïve and hyperalgesic animals. MCCG attenuated responses to (1S,3S)-ACPD in both preparations. These data suggest an mGluR component in the spinal cord and provide evidence for a tonic NMDA component during hyperalgesia. Spinal reflex activity was also studied in the hemisected spinal cord. Both single shock and train-evoked responses revealed an mGluR component, although in established hyperalgesia, no further mGluR component was detected. These findings suggest that mGluRs may contribute to the development of enhanced spinal activity, however their importance could be shadowed by NMDA receptor activity in established hyperalgesic states.

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1. CHAPTER 1

INTRODUCTION

1.1 Introduction

Glutamic acid is an amino acid that has long been known to play an important neurotransmitter role in the central nervous system (CNS). Glutamate was first shown to play a role as a neurotransmitter in the early 1960s when some acidic amino acids were discovered to induce behavioural convulsions when topically applied to the cortex, and to excite a wide variety of central neurones (Curtis and Watkins 1960; 1962). It was soon recognised that glutamate was a major neurotransmitter in a vast majority of excitatory synapses in the brain and it is now clear that most central neuronal circuits involve glutamate at some level (Monaghan *et al* 1989).

Early studies suggested that most of glutamate's actions as a neurotransmitter were mediated via activation of ligand gated cation channels (Biscoe *et al* 1977; Hall *et al* 1977; Johnston *et al* 1974). However, several subsequent studies showed that certain actions of glutamate could not be explained by direct opening of glutamate-gated channels (Fagg *et al* 1982; Hori *et al* 1981; Monaghan *et al* 1983). It was not until the mid-1980s that the first convincing evidence for the existence of G-protein-coupled (metabotropic) glutamate receptors began to appear (Nicoletti *et al* 1986a; Sladeczek *et al* 1985; Sugiyama *et al* 1987). Since then it has emerged that a large family of metabotropic glutamate receptors (mGluRs) exists, with members being heterogeneously distributed throughout the CNS and coupled to a wide variety of second messenger systems.

The discovery of mGluRs has altered the traditional view of glutamatergic neurotransmission as activation of these receptors provides a mechanism for glutamate to modulate or fine-tune activity at the same synapse at which it can elicit a fast synaptic response. Because of the ubiquitous distribution of glutamatergic synapses, mGluRs have the potential to participate in many CNS functions. Indeed, mGluRs have been implicated in a variety of processes, including motor control, learning and memory, developmental plasticity, vision, sensory processing, nociception, epileptogenesis and responses to neuronal injury. The wide diversity and heterogeneity gives an opportunity to develop pharmacological agents which selectively interact with mGluRs involved in a number of CNS processes. Obtaining a detailed understanding of the specific roles of these receptors will help with the development of understanding and treating neurological disorders.

Glutamate has been well established in mediating nociceptive transmission in the spinal cord, and the involvement of NMDA and AMPA receptors is well defined.

However, to date very little is known about the role of mGluRs in nociception. This thesis is therefore concerned with elucidating the potential role and mechanism involving mGluRs in the rat spinal cord during nociceptive processing.

1.2 Glutamate as a Neurotransmitter

L-glutamate, as well as being involved in metabolic processes, fulfils all the criteria for classification as a neurotransmitter in the CNS; figure 1.1 summarises the synthetic pathway, storage, release and uptake. Glutamate is synthesised in the nerve terminals either from glucose via the citric acid cycle, or from glutamine that is synthesised in glial cells, transported to the nerve terminals and converted to glutamate by glutaminase. Over-production of glutamate is prevented by the activity of glutaminase, uptake and accumulation of glutamine and end-product inhibition. Within the nerve terminal, glutamate is stored in small clear synaptic vesicles and released by calcium-dependent exocytosis when the nerve becomes depolarised. The action of glutamate is terminated by a high affinity uptake process via a glutamate transporter on the presynaptic nerve terminal or neighbouring glial cells. Glutamate is co-transported with two Na^{2+} and one H^+ in exchange for one K^+, giving a net positive uptake. Glutamate that is taken up into glia is converted back to glutamine by the enzyme glutamine synthetase, and transported by a low-affinity process to neighbouring nerve terminals where it is recycled and used as a precursor for glutamate synthesis.

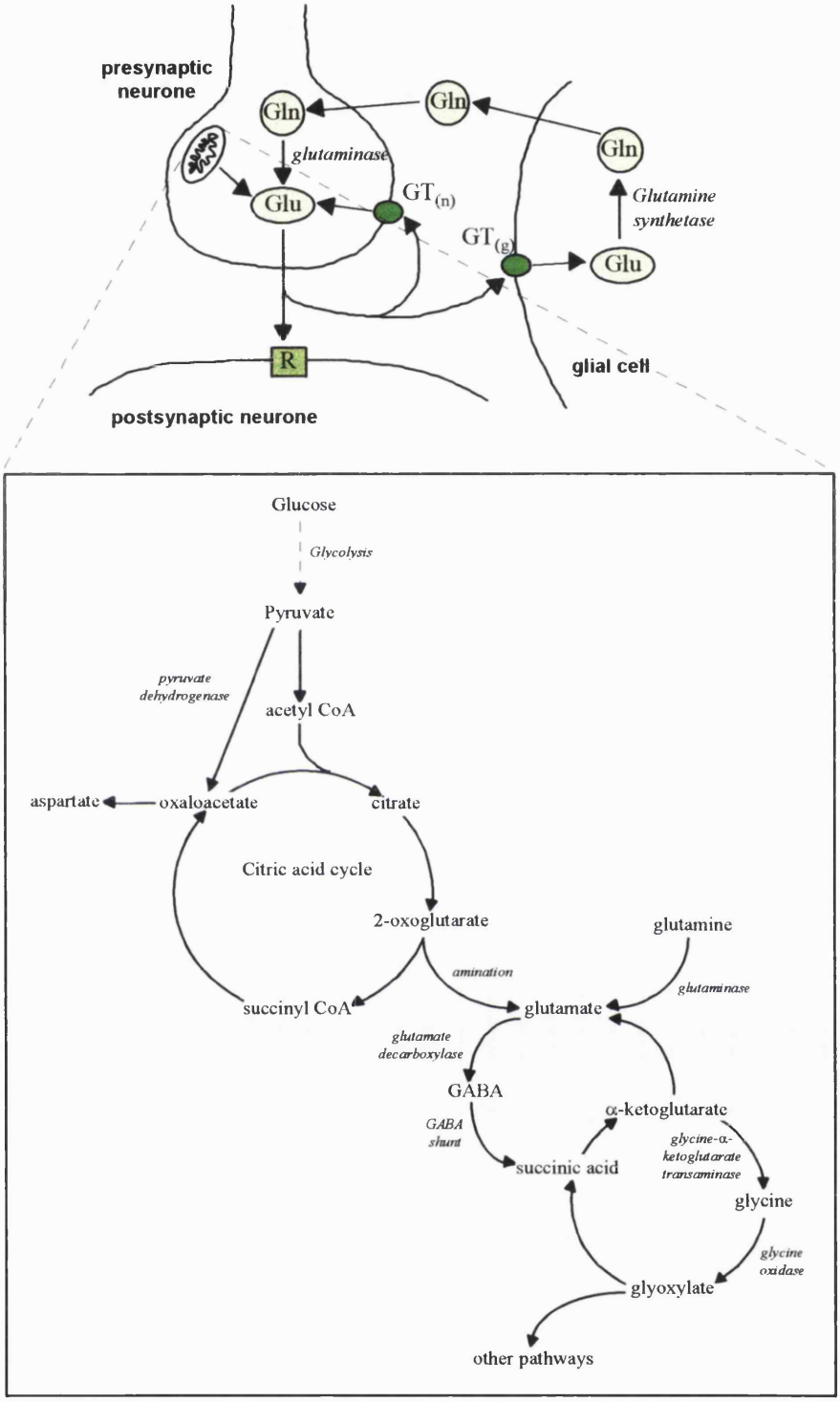


Figure 1.1 *Diagram illustrating the synthesis, release, uptake and storage of glutamate in the central nervous system.* Glu, *L-glutamate;* Gln, *glutamine;* $GT_{(g)}$, *glial glutamate transporter;* $GT_{(n)}$, *neuronal glutamate transporter;* R, *receptor.*

1.3 Glutamate Receptors

Glutamate activates receptors which can be divided into two distinct groups according to their signal transduction mechanisms. (1) Ionotropic glutamate receptors are directly coupled to the opening of cation channels (MacDermott *et al* 1986; Murphy *et al* 1987). These receptors have been further classified according to their ligand specificity for *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-1-isoxazole-4-propionic acid (AMPA) and kainic acid (See figure 1.2). (2) Metabotropic glutamate receptors (mGluRs) are coupled to a variety of second messenger systems via G-proteins (Mayer and Miller 1990; Monaghan *et al* 1989; Nahorski and Potter 1989; Schoepp and Conn 1993).

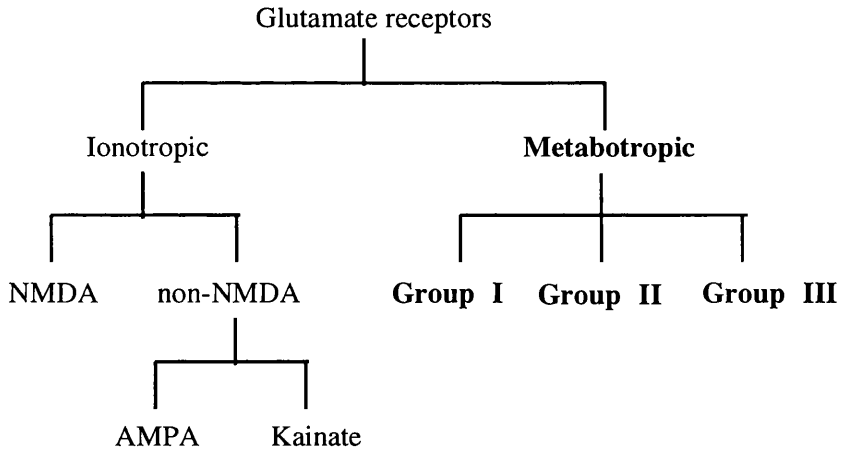


Figure 1.2 *The general classification of the glutamate receptors.*

1.3.1 The NMDA receptor

The NMDA receptor is a heteromeric ion channel complex that is highly permeable to Ca^{2+} as well as Na^{+} and K^{+} ions, with the resultant increase in intracellular [Ca^{2+}] thought to be responsible for evoking both neuronal plasticity and neurotoxicity (Hollmann and Heinemann 1994; Monaghan *et al* 1989). The receptor can be distinguished from other glutamate receptors by its pharmacological profile and unique properties, including modulation by glycine, a voltage-dependent channel block by Mg^{2+}, polyamine activation, and Zn^{2+} inhibition (Monaghan *et al* 1989; Nakanishi 1992; Watkins *et al* 1990).

The receptor channel complex is a heterooligomeric assembly of related subunits, the NMDAR1 subunit (Moriyoshi *et al* 1991) and at least one of the four subunits NMDAR2A-NMDAR2D (Ishii *et al* 1993; Kutsuwada *et al* 1992; Monyer *et al* 1992). The NMDAR1 gene is expressed in a regionally specific manner in the rat brain and the mRNA has the possibility to produce eight splice variants by alternative splicing (Moriyoshi *et al* 1991; Nakanishi *et al* 1992). Co-expression of NMDAR1 subunits with NMDAR2A-D in heterologous systems generates heteromeric ion channels with distinct electrophysiological and pharmacological properties, including altered deactivation kinetics, sensitivities to agonists and antagonists, glycine potentiation and strength of the Mg^{2+} block (Ishii *et al* 1993; Kutsuwada *et al* 1992; Meguro *et al* 1992; Monyer *et al* 1992; Nakanishi 1992).

Activation of NMDA receptors in the spinal cord and higher brain nuclei is important for both general "housekeeping" and pathological functions. The Mg^{2+} block and prolonged opening time of the NMDA-gated channel are essential for the generation of bursting patterns in the spinal cord (Grillner and Matsushima 1991), which are involved in the execution of regular movements (Grillner and Matsushima 1991). Under pathological conditions, regulation of the voltage-dependent Mg^{2+} ion block is, in part, responsible for the central sensitisation of pain processing (Chen and Huang 1991; Davies and Lodge 1987; Woolf and Thompson 1991). NMDA receptors are also critical in the phenomenon of "windup" - the increasing response of central pain-reporting neurones to repeated stimulation of peripheral nociceptive fibres (Davies and Lodge 1987; Mendell and Wall 1965; Schouenberg and Dickenson 1988; Woolf and Thompson 1991). NMDA receptors are also thought to be involved in mechanisms that underlie the induction of LTP in the hippocampus (Bliss and Collingridge 1993; Monaghan *et al* 1989). Weak stimulation only slightly activates NMDA receptors because of the channel block by Mg^{2+}. Strong stimulation (tetanic) reduces the Mg^{2+} block via depolarisation following activation of AMPA-kainate receptors, allowing Ca^{2+} permeability. This Ca^{2+} entry triggers a sequence of events that results in the enhancement of synaptic efficacy (Bliss and Collingridge 1993).

1.3.2 AMPA and kainate receptors

AMPA and kainate receptors were originally collectively referred to as non-NMDA receptors until radioligand binding studies revealed specific high affinity binding sites for NMDA, as well as AMPA, and low and high affinity kainate binding sites (Monaghan *et al* 1989; Young and Fagg 1990).

AMPA receptors consist of heteromeric assemblies of GluR-A to -D (GluR-1 to -4) subunits, the composition of which varies with cell type and developmental stage (Keinänen *et al* 1990; Monyer *et al* 1991; Petralia and Wenthold 1992). Each of the subunits exists as a Flip and Flop variant, producing different responses to applied glutamate and AMPA (Sommer *et al* 1990). AMPA receptors often co-localise with NMDA receptors in the same synapse in the CNS and mediate fast excitatory transmission (Bekkers and Stevens 1989; Bettler and Mulle 1995).

Kainate receptors are made up of a homomeric or heteromeric combination from two classes of subunits: GluR-5, -6 and -7 and KA-1 and -2, with the GluR5/-6/-7 subunits constituting the low affinity kainate binding sites and the KA-1/-2 subunits the high affinity kainate binding sites seen in neuronal membranes (for review see Bettler and Mulle 1995). Kainate receptors rapidly desensitise in the presence of kainate and have an unknown physiological function (Bettler and Mulle 1995). It has been suggested that they may play a modulatory role at the synapse, acting as presynaptic autoreceptors regulating transmitter release (Headley and Grillner 1990; Lerma *et al* 1997; Represa *et al* 1987).

1.4 Metabotropic Glutamate Receptors (mGluRs)

The existence of mammalian mGluR was predicted by Sladeczek *et al.* (1985) who suggested that glutamate and quisqualate stimulated phospholipase C in cultured striatal neurones through a novel type of excitatory amino acid receptor. A similar effect was also seen in hippocampal slices (Nicoletti *et al* 1986a,b), cultured cerebellar granule cells (Nicoletti *et al* 1986c), and cultured astrocytes (Pearce *et al* 1986). Since then, our knowledge of the biochemical and physiological roles of these receptors has increased dramatically with the development of selective ligands and the cloning and expression of the mGluR family. A wide range of studies have demonstrated a ubiquitous distribution throughout the brain (see section 1.4.3) and mGluR activation is now known to affect many aspects of neuronal function. These include changes in the activities of

phospholipase C, phospholipase D, adenylyl cyclase, calcium and potassium channels, as well as excitatory postsynaptic potentials (EPSPs), long -term potentiation (LTP) and long-term depression (LTD; Pin and Duvoisin 1995). The functional roles of the mGluRs will be discussed in more detail in section 1.4.5.

1.4.1 Molecular characteristics of the mGluRs

The first mGluR cDNA was independently cloned by Houamed *et al* and Masu *et al* in 1991. *Xenopus* oocytes were injected with RNA prepared from rat cerebellar cDNA clones, and tested for oscillatory Cl^- currents in response to glutamate, quisqualate and *trans*-ACPD application. Successful screening identified what we now know as the quisqualate-sensitive mGluR1a. Since then seven further genes encoding mGluRs have been identified, as well as several which generate different mRNA through alternative splicing. Based on sequence homology, synthetic ligand selectivity and coupling mechanisms, mGluRs can be subdivided into three groups as shown in figure 1.3.

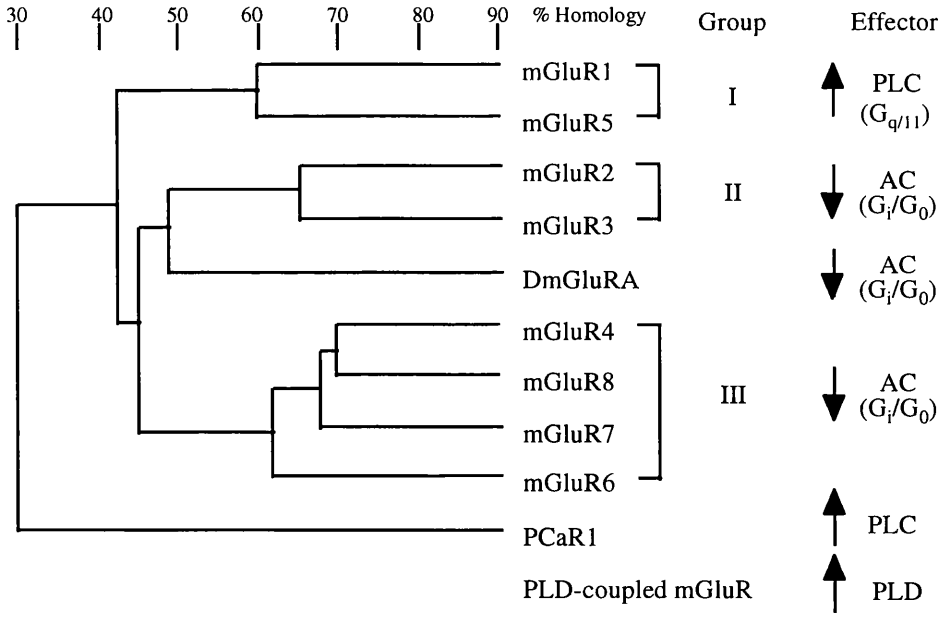


Figure 1.3 *mGlu receptor homology, classification and coupling.* AC, *adenylyl cyclase;* PLC, *phospholipase C;* PLD, *phospholipase D. Modified from Pin and Duvoisin 1995, Toms and Roberts 1997.*

Group I comprises mGluR1 and mGluR5; group II: mGluR2 and mGluR3; and group III: mGluR4, mGluR6, mGluR7 and mGluR8. Splice variants have been found for mGluR1 (a,b,c,d,e; Pin *et al* 1992; Tanabe *et al* 1992), mGluR4 (a,b; Saugstad *et al* 1994),

mGluR5 (a,b; Joly *et al* 1995; Minakami *et al* 1995) and mGluR7 (a,b; Corti *et al* 1996; Okamoto *et al* 1994; see also Pin and Duvoisin 1995). In addition, a receptor from Drosophila, DmGluRA, has recently been cloned which shows high sequence homology to the mammalian group II mGluRs (Parmentier *et al* 1996). This receptor also shows very similar pharmacology and transduction mechanisms to group II mGluRs, suggesting that the function of these receptors has been highly conserved during evolution.

All the mGluR subtypes belong to the G protein-coupled superfamily of receptors, possessing the typical seven transmembrane regions and an intracellular G protein binding domain. They share about 75% homology in their amino acid sequence within, and about 40% homology between groups (for review see Nakanishi 1992). However, the mGluR family shows little sequence homology with other G protein-coupled receptors, with the possible exceptions of a CNS Ca^{2+}-sensing receptor (PCaR) and the recently cloned $GABA_B$ receptor (Brown *et al* 1993; Kaupmann *et al* 1997). These both show 20-30% homology with the mGluR family, comparable to the level of homology seen between the distant subunits of the ionotropic glutamate gene family. The mGluRs possess an unusually large extracellular domain, preceded by a signal peptide (figure 1.4). In contrast to other G protein coupled receptors, this extracellular domain appears to be involved in glutamate binding and determines agonist selectivity (Takahashi *et al* 1993; Tones *et al* 1995).

The third intracellular loop has been shown to be important in functional coupling for other G protein coupled receptors. This region is highly conserved between all the mGluR subtypes, despite their diverse coupling to second messenger systems. However, studies using chimeric mGluRs have shown that the less conserved second intracellular loop and the region immediately following the seventh transmembrane region are important for the G protein coupling (Gomeza *et al* 1996a; Pin *et al* 1994). The group I mGluRs have a very long carboxy-terminal region with multiple serine and threonine residues and several phosphorylation sites suggesting that this domain may be important in the regulation of the receptor (Prézeau *et al* 1996). Recent work by Romano *et al* (1996) has suggested that mGluR5 may exist as a homodimer. They propose that mGluR5 may be linked by a disulphide bridge at the N terminal of the extracellular domain. This may facilitate phosphorylation of each monomer to initiate intracellular signal-transduction events. Other authors have also noted high molecular weight forms of the

mGluRs (Abe *et al* 1992; Baude *et al* 1993; Hampson *et al* 1994) suggesting possible dimerisation of other subtypes.

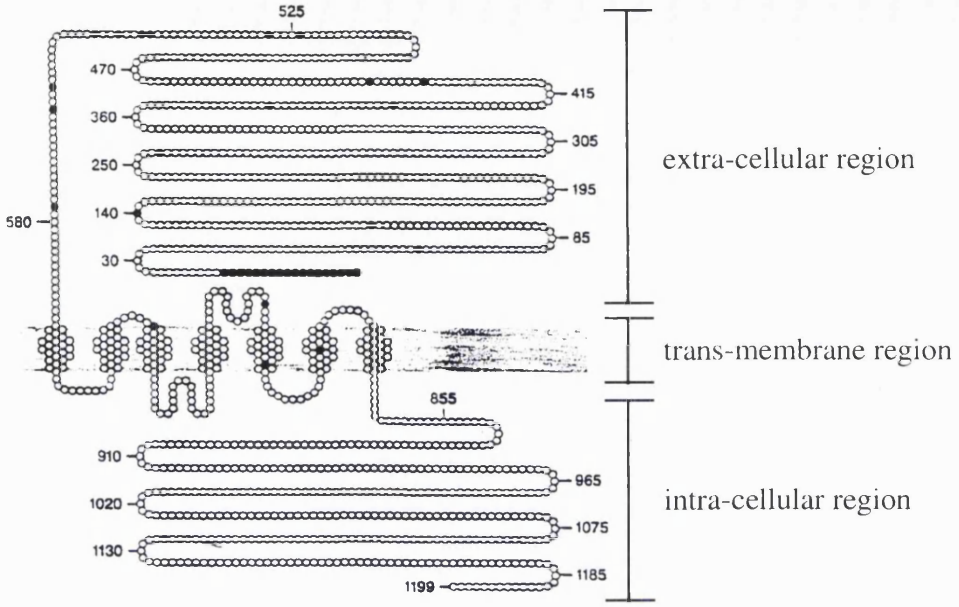


Figure 1.4 *Schematic representation of mGluR1a. The first twenty amino acid residues correspond to the signal peptide. Conserved cysteine residues are marked as filled circles. The putative glutamate binding domain is thought to be between amino acid residues 140 and 195 while the extracellular domain forms two globular lobes, hinged at the glutamate binding region (adapted from Suzdak et al 1994).*

1.4.2 Transduction mechanisms of mGluRs

The grouping of the mGluRs in terms of sequence is matched by their apparent transduction mechanisms. The mGluRs are coupled to multiple second messenger systems including activation of phosphoinositide hydrolysis, inhibition or activation of adenylyl cyclase, stimulation of phosphodiesterase, and activation of phospholipase A_2 and D (see figure 1.3; for review see Pin and Duvoisin 1995). In this way mGluR activation has the potential to affect many cellular processes.

The group I receptors (mGluR1, mGluR5) are found pre- and postsynaptically (Glaum and Miller 1994; Pin and Duvoisin 1995) and are coupled via $G_{q/11}$ to phosphoinositide hydrolysis and production of the second messengers inositol trisphosphate (IP_3) and diacyl glycerol (DAG). These then release Ca^{2+} from intracellular

stores and activate protein kinase C (PKC), respectively, resulting in the activation or phosphorylation of a variety of intracellular or membrane proteins such as protein kinases, phosphatases and ion channels (figure 1.5).

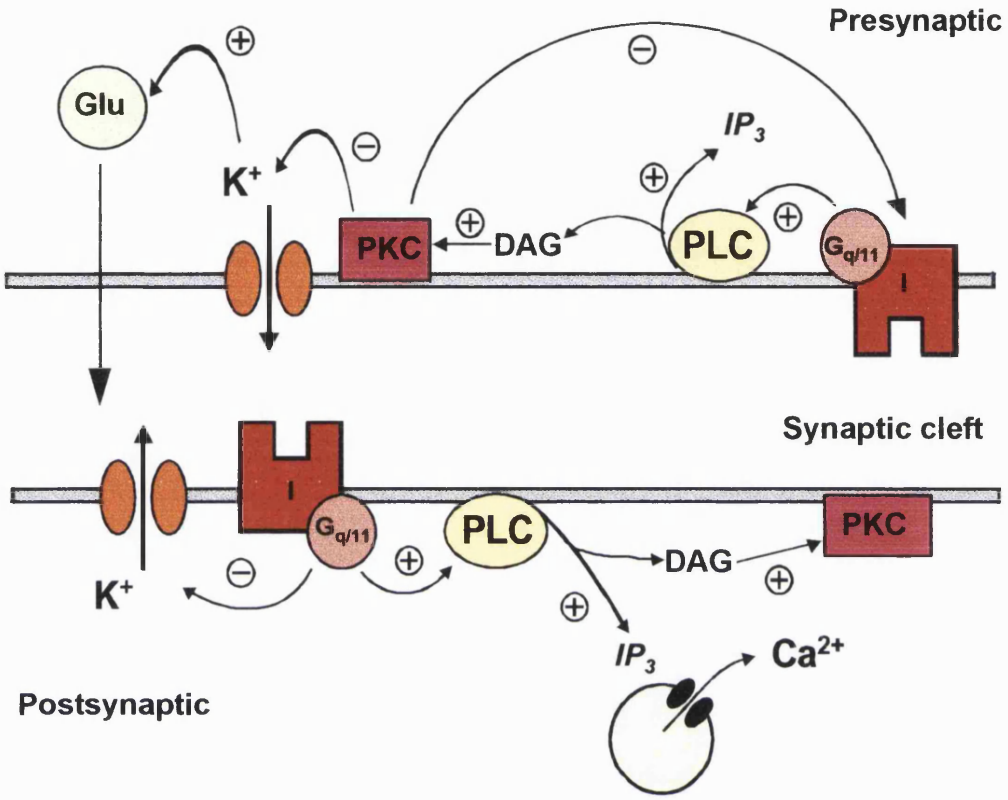


Figure 1.5 *Diagrammatic illustration of the intracellular effects of group I mGluR activation.* I, *group I mGluR;* DAG, *diacyl glycerol;* Glu, *glutamate;* IP_3, *inositol trisphosphate;* PKC, *protein kinase C;* PLC, *phospholipase C.*

Presynaptic group I mGluRs enhance glutamate release via a PKC-mediated inhibition of K^+ channels (Herrero *et al* 1992; Ikeda *et al* 1995). Excess glutamate release is prevented by a rapid PKC-mediated desensitisation of the presynaptic group I receptors. Postsynaptic group I mGluRs mediate a slow depolarisation and increase cell firing via direct G protein-mediated inhibition of IK_{AHP} and IK_M K^+ currents (Charpak *et al* 1990; Gerber *et al* 1992). These receptors also modulate ionotropic (NMDA, AMPA) glutamate receptor-mediated currents via mechanisms involving PKC, including reduction of the voltage-dependent Mg^{2+} block of the NMDA receptor (Aniksztejn *et al* 1992; Bleakman *et al* 1992; Chen and Huang 1992). These actions of group I mGluRs would all tend to increase neuronal excitability. This is supported by the finding that mGluR1a is located on the periphery of subsynaptic cerebellar and hippocampal membranes, in contrast to

ionotropic glutamate receptors which are found in the core of the synapse (Baude *et al* 1993), suggesting that mGluR1 may be activated by excess glutamate released during synaptic hyperactivity.

The group II (mGluR2, mGluR3) and group III (mGluR4, mGluR7, mGluR8) mGluRs are mainly found presynaptically and are negatively coupled to adenylyl cyclase (Prézeau *et al* 1994; Tanabe *et al* 1992, 1993; figure 1.6). At present, the physiological implications for mGluR-mediated reduction in cAMP accumulation are not known, although cAMP has important regulatory function in the CNS. Presynaptic group II and III mGluRs also inhibit transmitter release via direct G protein-mediated inhibition of L- and N-type Ca^{2+} channels (Chavis *et al* 1994; Swartz and Bean 1992; Swartz *et al* 1993; Trombley and Westbrook 1992). In contrast, group II agonists have also been shown to potentiate an increase in adenylyl cyclase produced by other receptors (Winder and Conn 1992). A possible mechanism for this may be via the $\beta\gamma$ subunits of G_i. Although they inhibit type I adenylyl cyclase, these subunits also potentiate type II adenylyl cyclase activation via the α subunit of G_s (Tang and Gilman 1991).

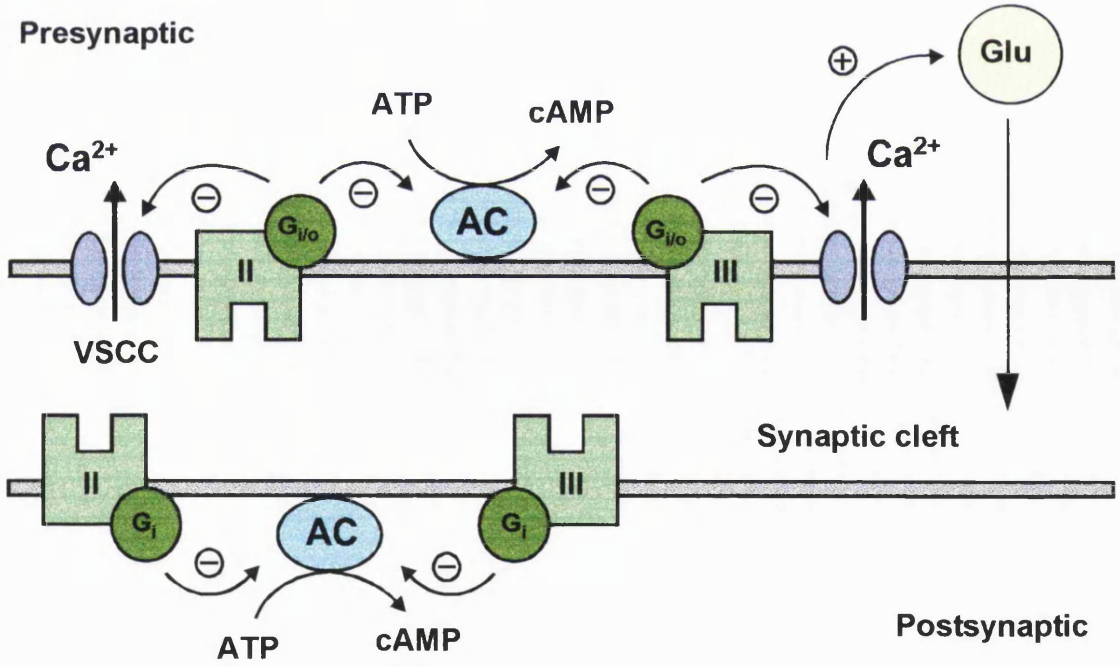


Figure 1.6 *Diagrammatic illustration of the intracellular effects of group II and III mGluR activation.* II, *group II mGlu receptor;* III, *group III mGlu receptor;* AC, *adenylyl cyclase;* cAMP, *cyclic adenosine monophosphate;* ATP, *adenosine trisphosphate;* Glu, *glutamate;* VSCC, *voltage sensitive calcium channel.*

mGluR6 also belongs to group III in the classification of mGluRs and is involved in glutamate transmission in the visual system. This subtype is specifically localised postsynaptically in retinal bipolar cell layer on ON-bipolar cells, causing glutamate-induced hyperpolarisation (Nakajima *et al* 1993; Nomura *et al* 1994). mGluR6 is linked to activation of a cGMP phosphodiesterase leading to a decrease in cGMP concentration and closure of cGMP-gated channels (Nawy and Jahr 1990; Nomura *et al* 1994; Shiells and Falk 1990). Light falling on the retina causes a reduction in glutamate release and depolarisation of the ON bipolar cell via stimulation of a cGMP-gated ion channel. mGluR6 therefore converts hyperpolarisation into depolarisation and amplifies the visual signal through second messenger pathways. This is the only example of a G-protein coupled receptor which is responsible for fast synaptic transmission.

Recent studies in rat hippocampal slices have revealed a novel mGluR, ~~based on sequence homology~~, which is coupled to activation of phospholipase D (PLD; Boss *et al* 1994; Holler *et al* 1993). PLD hydrolyses phosphatidylcholine to choline and DAG, which then activates various isoforms of PKC beginning a cascade of intracellular events. The PLD-coupled mGluR has high affinity for another endogenous excitatory amino acid, L-cysteine-sulfinic acid (L-CSA), but little affinity for glutamate and no cross sensitivity for ionotropic glutamate ligands (Boss *et al* 1994). If L-CSA, rather than glutamate is found to be the endogenous ligand for this receptor, this suggests that specific receptor families may exist for the different endogenous excitatory amino acids, creating more diversity than previously thought.

It is apparent that mGluRs have the potential to modulate fast synaptic transmission, either as presynaptic autoreceptors or as part of postsynaptic assemblies. Their many transduction mechanisms, producing inhibition or potentiation of glutamatergic transmission, provide opportunities to develop selective pharmacological tools to study their functions in more detail.

1.4.3 Distribution of mGluRs in the brain

In situ hybridisation and immunolocalisation studies have shown a ubiquitous but subtype specific distribution of the mGluRs throughout the CNS. Most studies have

concentrated on the brain, which will be discussed briefly here. The spinal cord distribution will be discussed in chapter 4.

In general mGluR1, 3 and 5 expression is diffuse throughout the adult mammalian brain (Abe *et al* 1992; Ohishi *et al* 1993a; Romano *et al* 1995; Shigemoto *et al* 1992; Tanabe *et al* 1993; Testa *et al* 1994a) whereas mGluR2 and 4 show marked regional distribution (Ohishi 1993b; Tanabe *et al* 1993; Testa *et al* 1994a). mGluR7 has a wider distribution than mGluR2 and 4 and expression tends to be intense or moderate in regions where there is no mGluR4 expression (Ohishi *et al* 1995a). mGluR8 shows low, presynaptic expression, localised to the olfactory bulb and retina (Duvoisin *et al* 1995; Kinoshita *et al* 1996a). mGluR3 shows strong labelling in glial cells as well as neurones (Jeffery *et al* 1996; Petralia *et al* 1996).

1.4.3.1 Hippocampus

mGluRs in the hippocampus are generally thought to play a role in long-term potentiation (LTP) and neuronal injury. mGluR5 is the most prominently expressed receptor subtype, and the highest expression of mGluR5 throughout the brain is seen in the hippocampus (Abe *et al* 1992; Romano *et al* 1995). mGluR5 is expressed throughout most regions of the hippocampus and entorhinal cortex, suggesting that it is the major mGluR subtype mediating the excitatory actions of glutamate in the CA1 region of the hippocampus (Fotuhi *et al* 1994; Shigemoto *et al* 1993; see figure 1.7). Therefore the main function of mGluR5 within the brain can be suggested to contribute to increases in intracellular calcium accumulation, a crucial process underlying LTP.

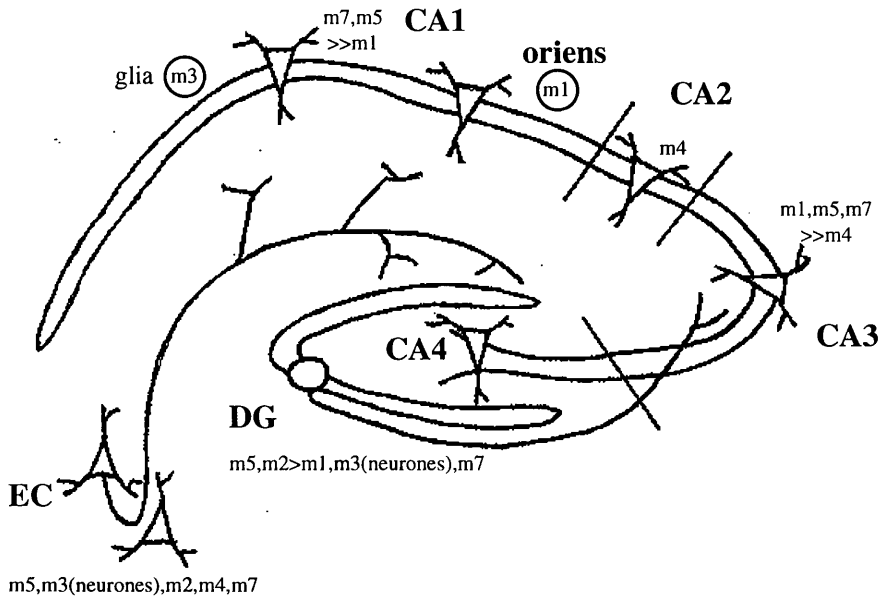


Figure 1.7 *Schematic diagram of the cellular localisation of mGluR gene expression in the hippocampus. mRNA for each of the mGluR1-5,7 receptors (designated m1-5,7) is indicated in the region of the cell bodies in which they are found. Levels of the receptor subtypes indicated are based on comparison of relative in situ hybridisation signal levels, not quantitative measurement of mRNA levels.* CA1, CA2, CA3 *and* CA4 *delineate the standard subdivisions of the Ammon's horn;* DG, *dentate gyrus;* EC, *entorhinal cortex;* oriens, *stratum oriens of CA1. (Modified from Testa et al 1994a.).*

The remaining mGluRs are all represented to varying degrees in the hippocampus, each having fairly discrete locations as shown in figure 1.7. The location and distribution of mGluR subtypes in the hippocampus suggests that the excitatory actions of glutamate occur in all regions of the hippocampus, but the inhibitory actions mediated by mGluRs are restricted to the dentate gyrus or presynaptic terminals (Fotuhi *et al* 1994).

1.4.3.2 Cerebellum

Long-term depression (LTD), a process that underlies some forms of motor learning, can be induced in the cerebellum and involves postsynaptic inhibition of target Purkinje cells mediated by the release of GABA (Ito *et al* 1982). Cells in the cerebellum express several mGluRs, with the exception of mGluR7 (Ohishi *et al* 1995a,b), but nearly all cells express a single predominant subtype (figure 1.8). Overall, mGluR1 is the predominant subtype, with specific postsynaptic mGluR1a expression in the Purkinje cell layer and some labelling in the granule layer (Görcs *et al* 1993; Hampson *et al* 1994).

Immunostaining reveals a postsynaptic location of mGluR1 on dendrites and dendritic spines (Martin *et al* 1992), in keeping with a possible role in LTD. mGluR2 is exclusively and highly expressed in Golgi cells of the granule layer and shows both pre- and postsynaptic location (Neki *et al* 1996; Ohishi *et al* 1994). mGluR3 shows little labelling in the cerebellum, with the exception of a marked signal in stellate and basket cells in the molecular and Purkinje cell layers. Granule cells express the highest level of mGluR4 seen in the adult brain, with the splice variant, mGluR4a, located presynaptically on parallel fibres originating from granule cells (Kinoshita *et al* 1996a). In contrast, mGluR5 is more or less absent, showing only a low level of staining in Golgi cells (Romano *et al* 1995; Shigemoto *et al* 1993).

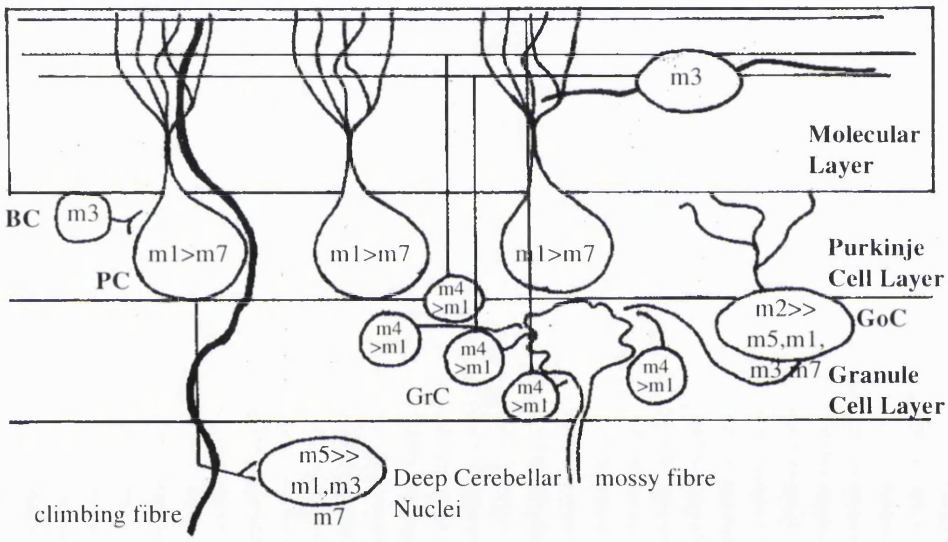


Figure 1.8 *Schematic diagram of the cellular localisation of mGluR gene expression in the cerebellum. The relative gene expression in each cell is indicated for mGluR1-5,7 (designated m1-5,7). The data represented are from comparison of relative in situ hybridisation signal levels, not quantitative measurement of mRNA levels.* BC, *basket cell;* GoC, *Golgi cell;* GrC, *granule cell;* PC, *Purkinje cell;* SC, *stellate cell. (Modified from Testa et al 1994a.)*

1.4.3.3 Basal ganglia

The basal ganglia are a group of deep forebrain nuclei whose major input is from the cortex, and regulates motor behaviour. mGluR5 shows the most prominant labelling

in the striatum and may be seen in the majority of neurones (figure 1.9). Those neurones not expressing mGluR5 are labelled by an mGluR2 probe and appear to be large cholinergic neurones (Testa *et al* 1994b). mGluR4 shows moderate but specific expression in the majority of medium-sized striatal neurones, whilst mGluR3 is quite prominently expressed in glia as well as neurones throughout the striatum. mGluRs in the striatum are thought to enhance glutamate neurotoxicity. This requires an increase in intracellular [Ca^{2+}], therefore the Group I mGluRs, especially with the abundance of mGluR5, are the most like candidate. LTD also occurs in the striatum and the widespread neuronal expression of mGluR1 suggests a potential role for this subtype in postsynaptic striatal LTD.

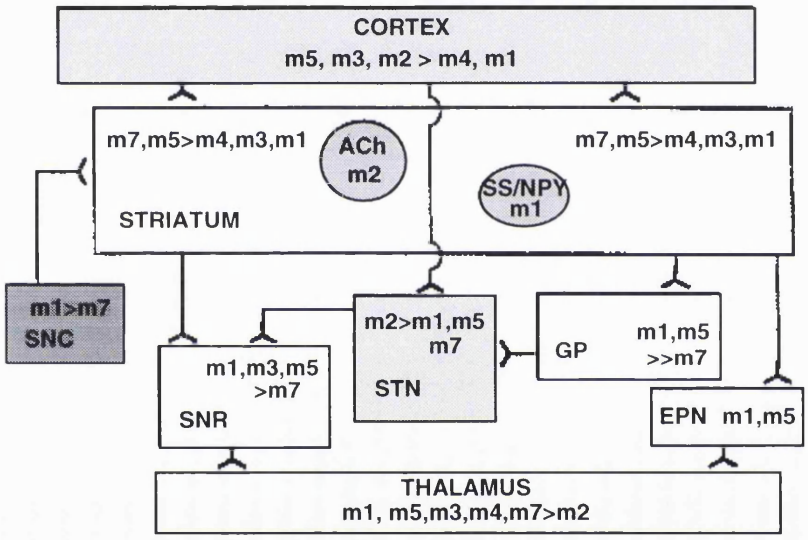


Figure 1.9 *Schematic diagram of the cellular localisation of mGluR gene expression in the basal ganglia. The relative subtype expression in each area is indicated for mGluR1-7 (designated m1-7). Levels of receptor subtypes are based on comparison of relative in situ hybridisation signal levels. Glutamatergic regions are pale grey; GABAergic nuclei are clear; the dopaminergic nucleus is dark grey. Striatal interneurones are moderately grey.* ACh, *acetylcholine interneurones; EPN, interpeduncular nucleus;* GP, *globus pallidus;* SNC, *substantia nigra pars compacta;* SNR, *substantia nigra pars reticulata;* SS/NPY, *somatostatin/neuropeptide Y interneurones;* STN, *subthalamic nucleus. (Modified from Testa et al 1994a.)*

In the other nuclei of the basal ganglia, mGluR1 is found presynaptically in the substantia nigra pars compacta in dopaminergic cells (Shigemoto *et al* 1992; Testa *et al* 1994b). Both mGluR1 and 5 are found together in the other nuclei (Testa *et al* 1994b), mGluR2 is expressed solely in subthalamic neurones, and mGluR3 is expressed in glia throughout the basal ganglia (Ohishi *et al* 1993a,b).

1.4.4 Pharmacology of the mGluRs

1.4.4.1 Agonists

L-Glutamate is the endogenous ligand for the mGluRs and all the ionotropic glutamate receptors. Early attempts at producing selective mGluR ligands were based around group substitutions of the L-glutamate molecule and synthetic compounds, such as quisqualate, were found to have fairly potent actions on mGluRs as well as having agonist effects mainly on AMPA receptors (Watkins *et al* 1990). Other compounds, e.g. ibotenic acid, were modelled on AMPA selective agonists and showed activity at metabotropic receptors as well as having potent NMDA agonist actions (Krogsgaard-Larsen *et al* 1980). Ibotenate and quisqualate were therefore the first mGluR agonists but they showed little or no selectivity for the mGluR subtypes as well as low potency. Moreover, their activity at NMDA and AMPA receptors limited their use as tools for studying the mGluRs.

In recent years there has been a large drive in the search for pharmacological agents for the study of mGluRs. Initially this was directed towards the development of compounds which would show selectivity for mGluRs over ionotropic receptors, but more recently compounds showing apparent selectivity between the mGluR groups have been described. The current aim is now to develop compounds that show selectivity within the mGluR groups. The first compounds to be developed showing selectivity for mGluRs were 1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) and L(+)-2-amino-4-phosphonobutyric acid (L-AP4).

The isomers *trans*-ACPD and *cis*-ACPD are conformationally restricted analogues of glutamate which show activity at mGluRs but have little effect on NMDA, AMPA and kainate receptors (Mistry and Challiss 1996; Palmer *et al* 1989). *trans*-ACPD consists of a racemic mixture of 1R,3S- and 1S,3R-stereoisomers. The racemate and active isomer 1S,3R-ACPD have been shown to stimulate phosphoinositide hydrolysis and increases in intracellular calcium in hippocampal slices (Irving *et al* 1990; Palmer *et al* 1989),

indicating actions at group I receptors. However, 1S,3R-ACPD also decreases cAMP in hippocampal slices (Schoepp *et al* 1992) and in CHO cells expressing mGluR2 (Tanabe *et al* 1992), although it has little effect on cells expressing mGluR4 (Tanabe *et al* 1993). *cis*-ACPD is similarly a mixture of 1R,3R- and 1S,3S-stereoisomers, the latter of which is the active isomer (Mistry and Challiss 1996). 1S,3S-ACPD again appears to show activity at group I and group II mGluRs, mediating increases in PI hydrolysis and inhibition of cAMP accumulation in guinea-pig cortical slices (Cartmell *et al* 1993). It also depresses ventral root depolarisations in the rat spinal cord via a presumed presynaptic action that is not affected by group III mGluR antagonists (Jane *et al* 1994; Pook *et al* 1992; Sunter *et al* 1991). This presynaptic inhibition has been proposed to be mediated via an interaction with a group of metabotropic receptors negatively coupled to adenylyl cyclase, presumably group II.

L-AP4 is also a synthetic analogue of glutamate. Early studies showed that it depressed glutamate transmission in the hippocampus and spinal cord (Evans *et al* 1982; Monaghan *et al* 1989), acting at presynaptic autoreceptors to regulate glutamate release. Its effects however were insensitive to ionotropic antagonists, and with recent cloning and molecular expression of the mGluRs, L-AP4 has been shown to act on group III mGluRs, with little activity at other receptors (Saugstad *et al* 1994; Tanabe *et al* 1993).

Following the introduction of ACPD and L-AP4, there has been the development of a range of agonists which show some selectivity for one or more of the mGluR groups. The activity of these compounds is summarised in Table 1.1.

(S)-3,5-dihydroxyphenylglycine ((S)-DHPG) is a selective agonist at the group I mGluRs based on experiments using *Xenopus* oocytes expressing rat mGluR1a (Ito *et al* 1992) and neonatal rat hippocampal slices (Baker *et al* 1995). DHPG stimulates phosphoinositide hydrolysis in adult and neonatal hippocampal slices with little effect on cAMP accumulation (Schoepp *et al* 1994), and acts as a partial agonist on cloned mGluR1a and 5a (Brabet *et al* 1995). DHPG is therefore regarded as a selective agonist and the tool of choice for the study of group I mGluRs.

Of the group II selective agonists L-CCG-I ((2S,1'R,2'R)-2-(carboxycyclopropyl)-glycine) and DCG-IV ((2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine) are the most characterised compounds. Expression of cloned mGluRs in CHO cells and functional studies have shown that L-CCG-I has a strong interaction with Group II mGluRs with

some activity at Group I (Cartmell *et al* 1994; Hayashi *et al* 1992). L-CCG-I causes a depolarisation in the neonatal rat hemisected spinal cord with a higher potency than glutamate and minimal effect on NMDA receptor activity (Shinozaki *et al* 1989). DCG-IV, an analogue of L-CCG-I, also has NMDA receptor activity (Ohfune *et al* 1993; Wilsch *et al* 1994). However, DCG-IV also depressed a monosynaptic excitation in neonatal rat motoneurones at concentrations lower than L-CCG-I or (1S,3R)-ACPD, suggesting a reduction in transmitter release from primary afferent terminals (Ishida *et al* 1993b). DCG-IV is the most potent group II agonist but it must be used with care due to its actions on ionotropic receptors. A novel Group II agonist, LY354740 (1S,2S,5R,6S-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate monohydrate), was recently described by Schoepp *et al* (1997) and appears to be highly selective for mGluR2 and mGluR3. LY354740 is highly potent in recombinant cells and rat hippocampal studies, making this a potentially very useful tool for investigating the function of this group of mGluRs.

Group	*mGluR subtype*	*Rank order of potency of agonists*
I	mGluR1 mGluR5	Quis > DHPG > glutamate > 1S,3R-ACPD = ibotenate > L-CCG-I > 3-HPG > t-ADA
II	mGluR2 mGluR3	DCG-IV = L-CCG-I > APDC> glutamate > 1S,3S-ACPD = 1S,3R-ACPD > 4C3HPG > ibotenate
III	mGluR4 mGluR6 mGluR7	L-AP4 > L-SOP > glutamate > L-CCG-I > 1S,3S-ACPD >> 1S,3R-ACPD
	mGluR8	glutamate > L-AP4

Table 1.1 *Rank order of potency of several agonists determined on cell lines expressing specific mGluR subtypes (mGluR1/5: Abe* et al *1992, Aramori and Nakanishi 1992, Brabet* et al *1995, Daggett* et al *1995, Flor* et al *1996, Hayashi* et al *1992, Houamed* et al *1991, Joly* et al *1995, Manahan-Vaughan* et al *1996, Masu* et al *1991; mGluR2/3: Flor* et al *1995, Gomeza* et al *1996b, Hayashi* et al *1992, Ishida* et al *1993a, Pin* et al *1994, Schoepp* et al *1995, Tanabe* et al *1993, Thomsen* et al *1994; mGluR4/6/7: Gereau and Conn 1995a, Gomeza* et al *1996b, Hayashi* et al *1994, Johansen* et al *1995, Nakajima* et al *1993, Okamoto* et al *1994, Saugstad* et al *1994, Tanabe* et al *1993; mGluR8: Duvoisin* et al *1995).*

Group III receptors, as previously mentioned, are selectively activated by L-AP4 and its analogue, L-serine-O-phosphate (L-SOP). L-SOP is an effective agonist at cloned mGluR4, 6 and 7 receptors with similar potency to L-AP4 (Eriksen and Thomsen 1995; Nakajima *et al* 1993; Okamoto *et al* 1994; Tanabe *et al* 1993) and shows activity at retinal L-AP4-sensitive receptors (Thoreson and Ulphani 1995).

1.4.4.2 Antagonists

Selective mGluR antagonists are shown in Table 1.2. The first mGluR antagonist to be described was L-2-amino-3-phosphonopropanoate (L-AP3; Schoepp and Johnson 1989a,b) which was reported to inhibit ibotenate-stimulated phosphoinositide hydrolysis in rat hippocampal slices with no effect on L-AP4-sensitive or ionotropic receptors indicating activity at group I receptors. Subsequent studies have shown that L-AP3 is a non-competitive inhibitor of mGluR-mediated responses (Mistry *et al* 1996; Schoepp *et al* 1990) but its usefulness is limited by its low potency. Its mechanism of action is not clearly understood as it appears to act as a partial agonist on phosphoinositide turnover (Mistry *et al* 1996) as well as having diverse effects on cAMP formation (see Schoepp 1994). Until recently, L-AP3 was nevertheless the primary antagonist for the mGluRs. However, in 1993 Birse *et al* published a series of phenylglycine derivatives which are competitive antagonists at mGluRs with no effect on ionotropic glutamate receptors (see Table 1.2). These compounds have proved to be invaluable in delineating the functional roles of mGluRs in the CNS.

(+)-α-methyl-4-carboxyphenylglycine (MCPG), but not (-)-MCPG, was one of the first derivatives found to have specific competitive antagonist action on mGluRs, albeit with low potency. Thus, it has been shown to antagonise the actions of L-glutamate on CHO cells expressing mGluR1, mGluR5 or mGluR2 but not mGluR4 (Hayashi *et al* 1994; Kingston *et al* 1995). In isolated tissues, (+)-MCPG selectively reduces ACPD-induced depolarisation in the neonatal rat spinal cord (Jones *et al* 1993; Sunter *et al* 1991) and ACPD-induced excitation in rat thalamic neurones (Eaton *et al* 1993a; Jane *et al* 1993). Overall, MCPG does not discriminate between the groups of mGluRs, antagonising ACPD-induced phosphoinositide hydrolysis as well as reversing L-CCG-I- and L-AP4-induced inhibition of forskolin-stimulated cAMP formation in rat cortical slices (Bedingfield *et al* 1995).

(S)-4-carboxy-3-hydroxyphenylglycine ((S)-4C3HPG) and more recently, (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA) were developed as selective group I mGluR antagonists. Again, (S)-4C3HPG inhibits ACPD-induced depolarisation in neonatal rat motoneurones and excitation of rat thalamic neurones with no effect at ionotropic receptors (Birse *et al* 1993). However, it also shows weak agonist activity on phosphoinositide hydrolysis in rat cerebral cortex slices (Birse *et al* 1993) as well as at some group II receptors (Hayashi *et al* 1994; Pellicciari *et al* 1995). Interestingly, (S)-4C3HPG has less activity in assays using cells expressing mGluR5a, indicating that it can discriminate between mGluR subtypes within the same group (Brabet *et al* 1995; Kingston *et al* 1995). The last of the group I antagonists, AIDA (UPF 523) shows higher potency at these receptors while having no effect on group II or III mGluRs or ionotropic glutamate receptors (Pellicciari *et al* 1995).

Group II-selective antagonists include α-methyl-L-CCG-I (MCCG) and (2S)-α-ethylglutamic acid (EGLU). Both compounds antagonise presynaptic inhibition of the rat spinal cord induced by 1S,3S-ACPD or L-CCG-I, with minimal effect on L-AP4-induced depression (Jane *et al* 1994, 1996; Thomas *et al* 1996b). Neither compound shows appreciable activity at postsynaptic NMDA and AMPA receptors (Jane *et al* 1996). In addition, MCCG has no effect on quisqualate-induced intracellular [Ca^{2+}] increase in CHO cells expressing human mGluR1b, or forskolin-stimulated cAMP formation in cells expressing mGluR4a. However, it inhibits (1S,3R)-ACPD-induced cAMP formation in cells expressing human mGluR2 (Knöpfel *et al* 1995). Interestingly, MCCG acts as a weak agonist on mGluRs negatively coupled to cAMP formation in the rat cortex suggesting a difference between group II and III mGluRs which mediate this effect (Kemp *et al* 1996). Accumulating evidence suggests that MCCG is a relatively selective antagonist for presynaptic mGluRs which are most likely to be group II.

MAP4 is an α-methyl derivative of L-AP4 and is, accordingly, a potent and selective antagonist at L-AP4 sensitive (Group III) mGluRs. MAP4 reverses L-AP4-induced depression in primary afferents in the neonatal rat spinal cord and reduces the L-AP4-induced inhibition on GABA transmission in rat thalamic neurones, and has little antagonistic effects against L-CCG-I at postsynaptic mGluRs or ionotropic glutamate receptors (Jane *et al* 1994; Salt and Eaton 1995). MAP4 has also been shown to have some agonist activity on forskolin-induced cAMP formation in rat cortical slices (Kemp

et al 1996). However, expression cloning experiments generally suggest that MAP4 acts as a potent antagonist at the presynaptic L-AP4-sensitive mGluRs negatively coupled to cAMP formation. An exception is mGluR6 on ON-bipolar cells where MAP4 has been reported to have agonist activity (Sekiyama *et al* 1996). The disparity between agonist and antagonist actions of this compound may be due to differences in receptor density and distribution resulting from the different tissues used.

Finally, (R,S)-α-methylserine-o-phosphate (MSOP) is the α-methyl derivative of Group III mGluR agonist, L-SOP. Like MAP4, MSOP shows similar potency and selectivity for the L-AP4-sensitive mGluRs compared to (1S,3S)-ACPD-sensitive receptors, with no activity at postsynaptic mGluRs or ionotropic receptors (Thomas *et al* 1995, 1996a). More pharmacological characterisation of this compound is needed.

The discovery of these novel mGluR ligands has advanced rapidly over the last few years but there are still no compounds available which are able to distinguish between individual subtypes within the three groups. The compounds discussed above are only a selection of many that have been tried and tested, and most are specifically relevant to this thesis. Others are shown in Table 1.2. The ever-enlarging catalogue of available tools will help to fully elucidate the role of the mGluRs in many systems within the CNS in the future.

Group	*mGluR subtype*	*Rank order of potency of antagonists*
I	mGluR1	
	mGluR5	AIDA > 4C3HPG > 4CPG > MCPG > 3C4HPG >> L-AP3
II	mGluR2	
	mGluR3	MCCG = MPPG > MSPG > MCPG = MTPG = MAP4
III	mGluR4	MPPG > MAP4 = MSOP >> MSPG
	mGluR6	
	mGluR7	not determined
	mGluR8	

Table 1.2 *Rank order of potency of several antagonists determined on cell lines expressing specific mGluR subtypes (mGluR1/5: Aramori* et al *1992, Brabet* et al *1995, Ferraguti* et al *1994, Hayashi* et al *1994, Houamed* et al *1991, Joly* et al *1995, Kingston* et al *1995, Moroni* et al *1997, Pellicciari* et al *1995, Sekiyama* et al *1996; mGluR2/3: Cavanni* et al *1994, Gomeza* et al *1996b, Sekiyama* et al *1996; mGluR4: Cavanni* et al *1994, Gomeza* et al *1996b, Johansen and Robinson 1995).*

1.4.5 Physiological roles of the mGluRs

Taken together, available evidence suggests that Group I mGluRs enhance glutamate release and increase neuronal excitation and excitability, whereas activation of Group II and III mGluRs will reduce glutamate release and synaptic excitation (for review see Nicoletti *et al* 1996). There are, as ever, exceptions to this. For example, Group I agonists inhibit synaptic transmission in the CA1 region of the hippocampus through a presynaptic mechanism (Gereau and Conn 1995b; Manzoni and Bockaert 1995), whereas activation of Group II receptors on nerve terminals co-expressing $GABA_A$ in the nucleus of the tractus solitarius will lead to neuronal disinhibition (Glaum and Miller 1993). The actions are described in greater detail below.

1.4.5.1 Excitatory effects

mGluRs enhance synaptic transmission through modulatory effects on voltage- and ligand-gated ion channels. Studies have paid special attention to K^+ conductances and ionotropic glutamate and GABA receptors (for review see Pin and Duvoisin 1995). The

mGluRs themselves have been shown to mediate postsynaptic excitatory transmission in the CNS either via direct mGluR agonist application or after high frequency electrical stimulation in the presence of ionotropic glutamate receptor antagonists.

The slow modulatory action of mGluRs was first observed in hippocampal CA1 neurones as enhanced excitability evoked by quisqualate or trans-ACPD (Charpak *et al* 1990; Stratton *et al* 1989). This effect is due to inhibition of two K^+ currents, I_M and I_{AHP}, which control the resting membrane potential of the neurone. Inhibition of these currents leads to increased neuronal excitability and cell firing, and block of accommodation. These effects can be blocked by MCPG (Gerber *et al* 1993) suggesting an mGluR involvement, although the group involved is not yet known. Interestingly, Glaum and Miller (1992) have shown that ACPD-induced depolarisation may not necessarily be mediated by inhibition of K^+ channels but by an inward current mediated by a Na^+/Ca^{2+} exchanger, resulting in an increase in intracellular $[Ca^{2+}]$. However, Charpak *et al* (1990) have shown that the inhibition of the K^+ currents is sometimes independent of Ca^{2+}.

mGluRs also regulate fast synaptic transmission through their effects on ionotropic glutamate and GABA receptors. ACPD potentiates NMDA- and AMPA-mediated currents in spinal cord neurones (Bleakman *et al* 1992; Cerne and Randic 1992), and NMDA-mediated responses only in CA1 pyramidal neurones (Aniksztejn *et al* 1992). Activation of PKC, via Group I mGluRs, reduces the Mg^{2+} block in the NMDA receptor channel, potentiating NMDA-induced currents (Chen and Huang 1992). This enhancement can be blocked by selective PKC inhibitors adding further support for a Group I involvement (Aniksztejn *et al* 1992). Glaum and Miller (1993) have also shown that ACPD facilitates synaptic transmission by inhibiting a postsynaptic $GABA_A$ response and potentiating an AMPA response in the nucleus tractus solitarius.

Stimulation of a presynaptic PLC-coupled mGluR also generates DAG which activates PKC only in the presence of arachidonic acid (Herrero *et al* 1992). This may result in direct or indirect phosphorylation and activation of presynaptic Ca^{2+} channels responsible for prolonged depolarisation. Enhanced Ca^{2+} entry will therefore increase the quantal release of glutamate. There is also evidence that presynaptic release may be enhanced via Group I mGluR activation of voltage-sensitive L-type Ca^{2+} channels (Chavis *et al* 1995).

Group I mGluR agonists cause neuronal depolarisation and excitation in transfected cells (Hayashi *et al* 1994; Thomsen *et al* 1994), in neonatal rat motoneurones and rat thalamic and hippocampal neurones (Ito *et al* 1992; Schoepp and Conn 1993; Watkins and Collingridge 1994; see also Roberts 1995 for review). These effects are inhibited by selective antagonists and are not seen when group II or III compounds are used, suggesting that activation of the group I mGluRs underlies depolarisation and excitation.

1.4.5.2 Inhibitory effects

Despite the classification of glutamate as an excitatory transmitter, the activation of certain mGluRs is now known to cause synaptic inhibition. The first inhibitory effects were described in the retina where the group III agonist, L-AP4 caused hyperpolarisation of ON bipolar cells via closure of a non-selective cGMP-activated cation channel. Immunolocalisation has confirmed that this effect was due to activation of postsynaptic mGluR6, which is exclusively expressed in the retina (Nakajima *et al* 1993).

Modulation of voltage-dependent Ca^{2+} channels regulates transmitter release, and activation of certain mGluRs inhibits these channels (Lester and Jahr 1990; Vranesic *et al* 1993). Thus, L-AP4 has been shown to inhibit presynaptic Ca^{2+} currents resulting in reduced transmitter release in hippocampal slices and cultured olfactory bulb neurones (Cotman *et al* 1986; Trombley and Westbrook 1992), and mGluRs have similarly been shown to inhibit N-type Ca^{2+} channels in the hippocampus (Sahara and Westbrook 1993; Swartz and Bean 1992; Swartz *et al* 1993; Wheeler *et al* 1994). This effect is mediated selectively by quisqualate pointing to the involvement of group I mGluRs (Swartz and Bean 1992). The inhibition of the N-type Ca^{2+} channels is fast, mediated via a G-protein and reversed by the activation of PKC, thus regulating glutamate accommodation (Swartz and Bean 1992; Swartz *et al* 1993).

L-type Ca^{2+} channels are also inhibited by mGluRs in cultured neurones (Sahara and Westbrook 1993; Sayer *et al* 1992). Inhibition by group I mGluRs is slow, mediated through G-proteins and does not involve protein kinases (Sahara and Westbrook 1993) and pharmacological experiments suggest that mGluR5 is responsible (Chavis *et al* 1995). Group II and III mGluRs inhibit L-type Ca^{2+} channels via activation of a PTX-sensitive G-protein, probably G_o (Chavis *et al* 1994; Trombley and Westbrook 1992). mGluRs may

also indirectly decrease Ca^{2+} currents and neurotransmitter release by activation of presynaptic K^+ channels (Sladeczek *et al* 1993).

Both ACPD and L-AP4 reduce excitatory postsynaptic potentials in various brain regions (Schoepp and Conn 1993). Inhibition by ACPD occurs via a presynaptic autoreceptor; explaining why L-AP4 was originally thought to be acting as an antagonist on a glutamate receptor. It has since been shown that L-AP4 acts directly via a G-protein coupled receptor. The pharmacological properties and synaptic distribution of the group II and III mGluRs support the idea that these subtypes play an important role in the modulation of glutamate release and the release of other transmitters.

1.4.5.3 Synaptic plasticity

The modulation of synaptic transmission by mGluRs and their discrete distribution throughout the CNS suggest a broad role for these receptors in learning and memory, neuronal death and nociceptive processing.

LTP is the most extensively studied form of synaptic plasticity and is thought to underlie memory formation and learning. NMDA receptor activation and Ca^{2+} influx are crucial for the induction of LTP, which can be facilitated by activating mGluRs with (1S,3R)-ACPD in hippocampal slices (Bortolotto and Collingridge 1993). The non-selective mGluR antagonist, MCPG, can prevent this facilitation (Bashir *et al* 1993) but only if the pathway under investigation has not previously experienced LTP (Bortolotto *et al* 1994). This suggests that mGluRs may act as a 'molecular switch', potentiating the NMDA current (O'Connor *et al* 1994). Once the pathway has been conditioned, the mGluRs are no longer required for further induction. On the other hand LTP could still be induced in mGluR1 knockout mice in the CA1 region of the hippocampus, but show reduced potentiation in CA3, suggesting that it is mGluR5 rather than mGluR1 which is involved in CA1 LTP. This has been further supported by immunolocalisation of the receptors in normal animals (Fotuhi *et al* 1993; Romano *et al* 1995).

LTD is the counterpart for LTP whereby low frequency electrical stimulation leads to a persistent depression of synaptic transmission. LTD of parallel fibre Purkinje cell synapses is well defined in the cerebellum and is thought to involve co-activation of AMPA and mGlu receptors, eliciting an increase in intracellular $[Ca^{2+}]$ (Linden *et al* 1991). mGluR1 is particularly prominent in this region and use of specific antibodies

(Shigemoto *et al* 1994) and knockout mice (Aiba *et al* 1994a) implicate mGluR1 in LTD. LTD is more prominent after induction of LTP and therefore can also be called depotentiation (Bortolotto *et al* 1994). Depotentiation can be prevented by MCPG (Bashir *et al* 1993; Bashir and Collingridge 1994; Bortolotto *et al* 1995), which can also reverse deconditioning of a previously conditioned pathway (Bortolotto *et al* 1994), further implicating the involvement of mGluRs in this phenomenon.

Glutamate is the main excitotoxic agent in the brain and excessive action results in cell death. Because of their association with high Ca^{2+} permeability channels, NMDA receptors play a large role in these toxic effects (Choi 1985, 1987). However, there is also evidence for the involvement of mGluRs. Thus, (1S,3R)-ACPD applied at high doses directly to brain tissue can itself induce neurodegeneration (Sacaan and Schoepp 1993), but may also enhance glutamate-, NMDA- or kainate-induced neurodegeneration (Koh *et al* 1991). Group I agonists such as quisqualate and DHPG similarly enhance NMDA toxicity in cultured neurones (Bruno *et al* 1995b; Buisson and Choi 1995). Conversely, group I and group II agonists have also been shown to be neuroprotective in cerebellar, hippocampal and striatal and neurones (Bruno *et al* 1994, 1995a; Opitz and Reymann 1995; Orlando *et al* 1996; Pizzi *et al* 1993) suggesting that a complex mechanism is involved. Selective agonists have also been shown to be neuroprotective, even when administered after an excitotoxic insult. The neuroprotective activation of group II mGluRs can be prevented by inhibitors of protein synthesis and appears to be mediated by astrocytic mGluR3 (Bruno *et al* 1996a,b). L-AP4 and L-SOP have also recently been shown to have neuroprotective effects in cultured neurones and brain slices (Bruno *et al* 1995a; Maiese *et al* 1995) and are reversed by selective group III antagonists (Bruno *et al* 1996a).

1.4.6 Summary

Over the last 15 years the original idea of glutamate as an excitatory neurotransmitter, exerting its actions via ligand gated ion channels, has been tested and a new family of G-protein coupled metabotropic receptors has emerged. The mGluRs represent a highly unique, diverse family of receptors that show little homology with other G-protein coupled receptors. They are widely distributed in the CNS and have the potential to modulate a variety of intracellular transduction events as well as fast

excitatory neurotransmission in the CNS, either as presynaptic autoreceptors or as part of postsynaptic transmission. Modulation can be either potentiation or inhibition of glutamate-mediated transmission with consequences as diverse as neuronal cell death, neuronal development and memory and learning. The continuing development of selective and potent ligands for the mGluR groups, and eventually individual subtypes, will facilitate the pharmacological characterisation and definition of the functional roles of these receptors within the CNS.

1.5 The Spinal Cord

The spinal cord extends from the medulla oblongata to the upper lumbar vertebrae, and its function is to process and relay information to and from the periphery and brain. Sensory information from the periphery is relayed, via the highly specialised transduction properties of primary sensory neurones, to the dorsal horn of the spinal cord. The dorsal horn consists of primary afferent terminals, intrinsic neurones and inputs and outputs from the rest of the CNS. Information is transmitted back to the periphery via efferent fibres in ventral roots. Ascending pathways in the spinal cord transmit information received from sensory receptors in the periphery to the brain, and descending pathways transmit motor and modulatory information from the brain to the spinal cord.

1.5.1 Primary afferents

One of the main functions of the nervous system is to provide information about the state of the periphery, including the occurrence or threat of injury. The apparatus responsible for this must be sensitive to many different modalities (thermal, mechanical, chemical) and provide information regarding the location and intensity of the stimulus. Primary afferent fibres are highly specialised sensory fibres which relay this information to the spinal cord via the dorsal roots. The cell bodies of these neurones are in dorsal root ganglia (DRG), located between the vertebrae. These give rise to pseudo-unipolar processes which branch into the spinal cord via dorsal roots, and into the periphery via a peripheral nerve. Some primary afferents (mostly unmyelinated) enter the spinal cord via the ventral root, terminating in the superficial dorsal horn (Coggeshall *et al* 1977; Light and Metz 1978).

Afferent input to the dorsal horn can be classified according to peripheral origin, which can be cutaneous, muscular or visceral. Somatosensory afferents can be further classified by their cell size, lack or type of myelination and their modalities although there is a certain amount of overlap in these properties, especially in modality, making rigid characterisation difficult. As a result there is some disagreement over classification and this broad review will follow the guidelines of Willis and Coggeshall (1991).

Aα fibres relay proprioceptive information from muscle spindles and Golgi organs in tendons, terminating in laminae IV-VII. Aβ fibres are large myelinated, low threshold mechanosensitive fibres that conduct at a velocity of 30-100m/s, relaying non-noxious

mechanical information from the skin and muscle to the deep dorsal horn (lamina III-VI) as well as the interneurone and motoneurone pools of laminae VII and IX (Brown and Fyffe 1978, 1979). Aδ fibres are thinly myelinated, mechano-heat sensitive fibres and conduct at a slower rate than the Aβ fibres - 4-30m/s. In general, these are high threshold fibres which relay noxious information to laminae I and V as well as non-noxious information to laminae III and IV, and the inner layer of lamina II of the dorsal horn (Light and Perl 1979). Finally, C fibres are unmyelinated slowly conducting fibres (conduction velocity <2.5m/s) with a high input threshold, primarily conveying nociceptive information after activation by thermal, mechanical and/or chemical stimuli in the noxious range. C fibres, and a proportion of Aδ-fibres, are activated by many stimulus modalities and are hence called polymodal nociceptors. They terminate almost exclusively in laminae I and II, with some input into lamina V. Visceral afferents are also unmyelinated and terminate more widely throughout the dorsal horn. They represent only a small proportion of the total afferent input into the spinal cord.

A- and C-fibres play significantly different roles in sensory function, with the C-fibres showing especially interesting properties. Many dorsal horn neurones appear to be driven by C-fibre input as seen by a general increase in excitability ("windup"; Mendell and Wall 1965), and many biologically active peptides are located within C-fibre terminals (see section 1.5.3). Cutaneous C fibres (and a subpopulation of Aδ nociceptors), responsive to noxious heat, mechanical and thermal stimuli, are also selectively activated by capsaicin, the pungent ingredient in a variety of *Capsicum* red peppers. This chemical provides a tool to study the role of these fibres in physiological and pathophysiological processes (Holzer 1991).

1.5.2 Spinal cord cytoarchitecture

The spinal cord is made up of grey matter surrounded by white matter. The white matter is so called through its appearance due to the presence of myelinated axons whilst the grey matter largely consists of cell bodies and their processes. The grey matter has an H-or butterfly-shaped outline when seen in coronal section, formed from the dorsal and ventral horns linked by an intermediate zone made up of interneurones and bundles of fibres. Some interneurones project supraspinally but the majority are involved in inter- and polysegmental loops within the spinal cord. The cord structure varies according to the

cranio-caudal level, reflecting the input and output requirements at each segment. For example the ventral horns of the lumbar and cervical enlargements are especially prominent due to the large number of motoneurones supplying the musculature of the lower and upper limbs.

1.5.2.1 White matter

This section gives a brief overview of the organisation of the white matter with no aim of completeness (for review see Tracey 1995). The white matter surrounds the grey matter and supports ascending and descending columns and tracts within the spinal cord. Neuronal cell bodies are not generally present although there are blood vessels and neuroglia, including oligodendrocytes, astrocytes and microglia. Dorsal columns are sensory in nature and carry information on tactile and spatial sensation via myelinated axons of ipsilateral dorsal root ganglia (DRG) cells from peripheral mechanoreceptors. Lateral columns lie in the region between the dorsal root entry zone and the ventral root exit zone and are made up of a number of discrete fibre systems, both ascending and descending whereas ventral columns originate in the brain stem and include the vestibulospinal system. Lissauer's tract carries mainly small unmyelinated primary afferent fibres into the spinal cord, terminating in the marginal zone at the edge of the dorsal column, and substantia gelatinosa (laminae I and II; LaMotte 1977; Molander 1984). The spinocerebellar tract runs along the peripheral edge of the grey matter and transmits information from muscle spindles and Golgi tendon organs to the cerebellum. The dorsal part of the tract arises from large cells of the dorsal nucleus of Clarke and the cells that make up the ventral part originate from lamina V-VII in the grey matter and project uniformly large fibres conveying information about posture or movement of limbs (Oscarsson 1965). The corticospinal tract originates in the cortex and descends to the spinal cord, terminating at the base of the dorsal horn in laminae IV, V and VI. This tract is involved in the control of movement and exerts its effects through interneurones and motoneurones (Liang *et al* 1991). The spinothalamic tract is the main pathway for the relay of pain and temperature to the brain stem and thalamus via small and medium-sized fibres. These fibres originate from neurones in laminae V-VIII in the grey matter and send axons across the midline of the spinal cord to form the contralateral spinothalamic tract.

1.5.2.2 Grey matter

The grey matter contains neuronal cell bodies and dendritic processes, along with axons, blood vessels and neuroglia. The high proportion of cell bodies gives this area a grey colour in fresh tissue. The cytoarchitecture of the grey matter was originally described in detail in the cat by Rexed (1952) and has been confirmed in the rat by many others, including Wall *et al* (1967) who provided physiological evidence to confirm Rexed's laminae in the rat dorsal horn. Rexed used Nissl staining to map cell bodies and divided the grey matter into ten distinct laminar divisions which run along the whole length of the spinal cord. The laminae each have discrete cell types and functions and the borders between them are indistinct and thought of as zones of transition rather than rigid borderlines (figure 1.10).

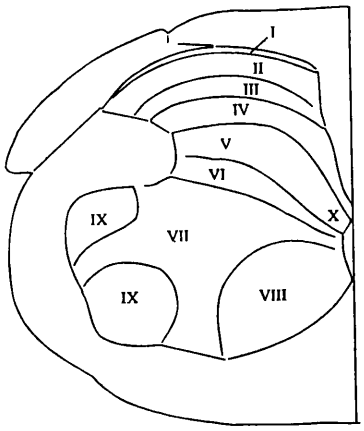


Figure 1.10 *Schematic diagram of the laminar organisation of the lumbar spinal cord as described by Rexed (1952).*

Lamina I was originally termed the marginal layer or zone, and it can be seen as a very thin layer around the top of the dorsal horn. The main feature of this layer is given by horizontally arranged axons, and four characteristic cell types: fusiform, multipolar, pyramidal and flattened (Lima and Coimbra 1986). Electrophysiological studies have shown that a large proportion of lamina I neurones appear to be wide dynamic range, being activated by low and high intensity stimulation (McMahon and Wall 1983; Woolf and Fitzgerald 1983). Others respond to either low or high threshold stimulation. Fine primary afferent fibres enter lamina I via Lissauer's tract (LaMotte 1977; Light and Perl 1977) and are collaterals mainly from Aδ fibres with some input from C-fibres (Gobel and Binck 1977; Gobel *et al* 1981; Light and Perl 1979; Nagy and Hunt 1983; see figure

1.11). Axons of lamina I neurones project mainly to the thalamus (Lima and Coimbra 1988), which is the major area of nociceptive processing and perception in the brain.

Lamina II runs parallel and ventral to lamina I and is commonly known as the *substantia gelatinosa* (Cervero and Iggo 1980). The name was coined by Rolando (1824) due to the high concentration of small neurones and distinct lack of myelinated axons which give a gelatinous appearance in transverse sections. This lamina can be divided into outer, dorsal (II_o) and inner, ventral (II_i) laminae. Terminology and separation of cell types in lamina II is somewhat confusing but the cells seem to fall into two basic categories - stalked and islet cells. Stalked cells, originally classified as limiting cells (Ramon y Cajal 1909) are located in II_o. They are interlaminar cells with dendrites projecting mainly into lamina I as well as deeper laminae. Recordings from these cells suggest they are mainly excitatory and either nociceptive specific or wide dynamic range cells (Bennett *et al* 1980). Islet cells, previously known as central cells (Ramon y Cajal 1909), have small cell bodies and tend to be located in clusters in the lamina. Dendrites from the cell bodies arbourise mainly in lamina III in a rostrocaudal plane (see figure 1.11). The axons terminate largely within lamina II and have been suggested to be inhibitory, playing an important role in pre- and postsynaptic control (Gobel 1978). Islet cells located in II_o are reported to be nociceptive specific whilst those in II_i are mechanoreceptive (Bennet *et al* 1980). A third cell type, arboreal cells, are a subytpe of islet cell. They are predominantly interneurones found in II_o and have extensive dendritic trees which reach to laminae I and II_i. A pronounced anatomical feature of the substantia gelatinosa is the presence of glomeruli, found predominantly in II_i. The glomeruli consist of a central sensory axon surrounded by dendrites from spinal cord interneurones, and axonal boutons from presynaptic neurones intrinsic to the dorsal horn. The central boutons are capsaicin sensitive implicating them as unmyelinated nociceptive fibres (Ribeiro da Silva and Coimbra 1984), and the glomeruli are thought to play an important role in the modulation of sensory synaptic transmission. The primary afferent input to lamina II is predominantly from nociceptive C fibres with some input also from Aδ fibres and non-cutaneous, unmyelinated (visceral) afferents, hence lamina II is the major area of termination for nociceptive primary afferents.

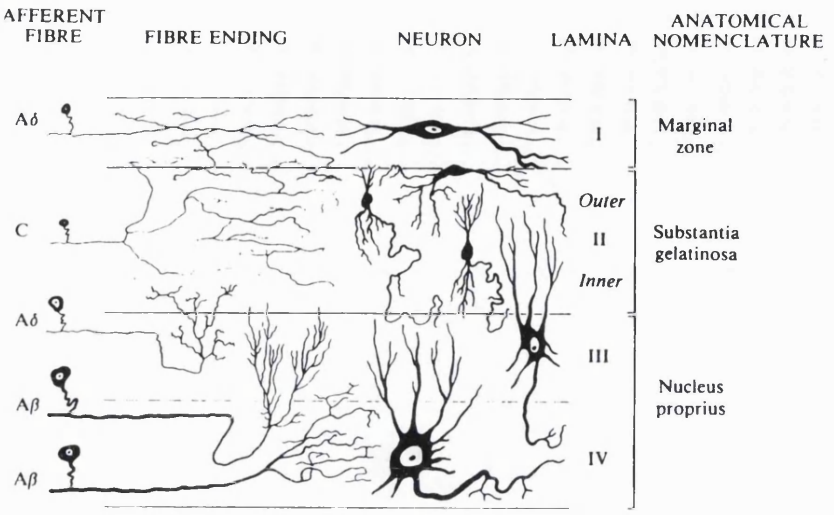


Figure 1.11 *Schematic diagram of the neuronal organisation of, and input to the superficial dorsal horn. The following cell types are illustrated (from top to bottom): a marginal cell, a substantia gelatinosa stalked cell, two substantia gelatinosa Islet cells, and two neurones of the nucleus propius, the most superficial of which has dendrites extending to lamina II (adapted from Cervero and Iggo 1980).*

Lamina III forms a wide band below lamina II and was originally thought of as being an extension of lamina II. It has slightly larger and more widely spaced cells and contains many myelinated fibres compared to lamina II. Two cell types have been identified in lamina III: postsynaptic dorsal column cells and spinocervical tract cells. The cells show similar morphology to lamina II cells but have a greater dendritic field, extending both dorsally and ventrally, and are orientated rostrocaudally (figure 1.11). Some axons cross the midline and terminate in the opposite dorsal horn, whilst others enter the white matter and terminate in another segment in laminae II or III. Lamina III has a major primary afferent input, mainly from coarse (Aβ) afferents, and again possesses many glomeruli.

Laminae IV-VI make up the bulk of the deep dorsal horn. *Lamina IV* is a wide layer with a heterogeneous population of small and large cells. Large cells in this layer have dendrites which pass laterally, dorsally and medially. Dendrites which enter the substantia gelatinosa are contacted by axons from islet cells and may represent the output from these cells. Small cells have dendrites that travel in all directions from the soma. The cell population is a mixture of spinocervical and postsynaptic dorsal column cells as well

as local interneurones. Axons from these cells pass into the white matter, either in the same segment or ascending before they cross the midline, while others cross the midline and join the opposite spinothalamic tract. The majority of primary afferent axons which enter lamina IV belong to large myelinated (Aβ) fibres and no fine unmyelinated fibres are found in this lamina (La Motte 1977; Light and Perl 1977).

Lamina V is a thick band across the neck of the dorsal horn and has an even more heterogeneous cell population than lamina IV. The cells here tend to be multireceptive i.e. wide dynamic range, responding to noxious and innocuous stimulation (Ritz and Greenspan 1985). Neurones have extensively branching dendritic trees and the outside edge of this lamina has a distinct mesh-like appearance due to bundles of myelinated axons (McClung and Castro 1976; Schrøder 1977). Axons from lamina V project mainly to the contralateral thalamus, as well as to dorsal column nuclei and local destinations within the cord, either into deeper laminae or the white matter. Primary afferent input consists of fine visceral and muscle afferents as well as large myelinated fibres (Molander and Grant 1987; Woolf 1987).

Lamina VI marks the bottom of the dorsal horn. The cells found here are smaller and more regularly arranged compared to those in lamina V. The dendritic arrangement is similar to lamina V, although the dendrites never enter lamina I or II and are therefore not directly exposed to fine primary afferent input. Axons generally project locally and, to a lesser extent, to the thalamus. Many primary afferent fibres terminating on ventral horn cells send collaterals to lamina VI. The cells in this lamina primarily respond to noxious stimuli while others appear to be wide dynamic range neurones (Cervero *et al* 1988).

Laminae VII to X make up the ventral horn. *Lamina VII* is the intermediate zone of the grey matter. It is the site of cell pools communicating between different parts of the CNS as well as a source of general visceral efferent innervation for most of the body, receiving projections from muscle and large cutaneous afferents. These cells are large with prominent Nissl granules and axons projecting into the spinocerebellar tract. Other cells either project within the spinal cord or to the thalamus (Burstein *et al* 1990; Chaouch *et al* 1983). *Lamina VIII* is located ventrally in the ventral horn and consists entirely of interneurones. The cells project mainly within the spinal cord and to the thalamus (Burstein *et al* 1990). The most important function of these cells is to act as receptive sites for supraspinal fibres whose excitation is transferred to motoneurones. *Lamina IX* consists

of discrete groups (nuclei) of somatic motoneurones, segregated according to function. The neurones are large (25-10μm) and multipolar. The larger cells are known as α motoneurones and innervate striatal muscle fibres, whereas the smaller cells are known as γ motoneurones and innervate muscle spindles. Dendrites from motoneurones vary in their patterns and some are widely distributed, reaching as far dorsally as lamina III (Cook and Woolf 1985). Axons leave the motoneurone nuclei individually and leave the spinal cord as small rootlets and join to form the ventral root. *Lamina X* surrounds the central canal and contains cells that project to the thalamus (Burstein *et al* 1990). Many of these neurones respond to noxious stimuli (Nahin *et al* 1983).

1.5.3 Neurotransmitters in the spinal cord

There are a large number of neurotransmitters in the spinal cord found either in intrinsic cells of the spinal cord or transported from cell bodies in the DRG to primary afferent terminals. Neurotransmitters either play a direct role in signalling or are involved in modulation of sensory processing. Information processing does not, however, depend solely on the specificity of neurotransmitter release as many terminals have been shown to store and release more than one substance.

1.5.3.1 Excitatory neurotransmitters

1.5.3.1.1 Glutamate

Excitatory amino acids (EAAs), most notably glutamate, are the major neurotransmitters in the CNS with the primary afferents and the dorsal horn being no exception. The excitatory effects of glutamate on the spinal cord were first described in the cat by Curtis (Curtis *et al* 1959; Curtis and Watkins 1960), who showed that iontophoretically applied glutamate caused repetitive discharges in the dorsal horn. Glutamate and its metabolic precursors and enzymes are found in many DRG cells (Willis and Coggeshall 1991) as well as primary afferent central terminals (De Biasi and Rustioni 1988, Hökfelt 1991). Stimulation of primary afferents causes the release of endogenous glutamate (Jeftinija *et al* 1991; Roberts 1974). Lesions of the dorsal columns reduce glutamate uptake (Roberts and Hill 1978) suggesting a role for glutamate as a transmitter in the large myelinated primary afferents which project to laminae III and IV. Dorsal roots

have also been shown to contain more glutamate than ventral roots implying a primary role in primary afferents (Duggan and Johnston 1970; Graham *et al* 1967).

Glutamate is released after acute (Kangrga and Randic 1991) and prolonged (Skilling *et al* 1988) peripheral noxious stimulation and has an excitatory effect on ~~DRG cells and~~ the spinal cord, causing depolarisation via NMDA, AMPA and metabotropic receptors. Autoradiography shows the highest density of [^{3}H]-glutamate binding sites in the substantia gelatinosa, especially in the cervical and lumbar regions, with low level binding in the other laminae (Greenamyre *et al* 1984).

AMPA receptor subunits show discrete localisation in the spinal cord. The mRNAs for the GluR-A and -B subunits are concentrated in laminae I and II of the dorsal horn, with the GluR-A gene being preferentially expressed in laminae I and II_o and the GluR-B gene being more evenly expressed throughout the superficial laminae. GluR-B, -C and -D mRNAs are present in the ventral horn, with GluR-C and -D being the most prominently expressed (Tölle *et al* 1993; 1995b). Of the NMDA subunits, only NMDAR1, NMDAR2C and NMDA2D mRNAs are detectable in the lumbar spinal cord of the rat (Tölle *et al* 1993). NMDAR1 mRNA is abundantly expressed throughout the spinal cord, with splice variants showing distinct expression patterns (Tölle *et al* 1995a). The NMDAR2 subunit mRNA is expressed at a much lower level compared to NMDAR1, with the -C splice variant mRNA being expressed in scattered cells in laminae I and II, while the -D splice variant mRNA is expressed at a low level throughout the grey matter. Acute inputs do not activate NMDA receptors due to the voltage-dependent Mg^{2+} block (Mayer *et al* 1984), which may be removed by altered membrane potential after activation of non-NMDA and peptidergic receptors (MacDonald and Nowak 1990). The NMDA receptor complex also requires the presence of glycine before glutamate binding occurs, therefore binding of glutamate alone is insufficient to activate the channel. The NMDA receptor-channel complex therefore probably plays only a minor role in "normal" synaptic transmission. Repetitive stimulation recruits NMDA receptors (Davies and Lodge 1987) and this is thought to underlie the hyperexcitability associated with hyperalgesia. NMDA antagonists indeed abolish the polysynaptic component of EPSPs (Jeftinija 1989; King *et al* 1988).

The distribution of the mGluR subtypes has, to date, not been studied in much detail in the spinal cord. All the subtypes are present, with the exception of mGluR6,

which is purely retinal, and mGluR2 which is not detectable (Nakajima *et al* 1993; Ohishi *et al* 1993b). The other subtypes are predominantly expressed in the dorsal horn, with the exception of mGluR4 which shows discrete mRNA localisation in motoneurones (Ohishi *et al* 1995a). Both mGluR5- and mGluR7-like immunoreactivity are discretely localised in the substantia gelatinosa (Ohishi *et al* 1995a,b; Vidnyanszky *et al* 1994) with mGluR7-like immunoreactivity being co-localised with substance P-like immunoreactivity, suggesting localisation on nociceptive inputs (Li *et al* 1997). The mGluRs are both pre- and postsynaptically located in the spinal cord and modulate glutamate release either by presynaptic inhibition, or via postsynaptic depolarisation or enhancement of ionotropic responses. They have been implicated in nociceptive transmission in the dorsal horn of the spinal cord (Neugebauer *et al* 1994; Young *et al* 1994) and their are discussed later (see section 1.6).

Low frequency electrical stimulation of primary afferents activates postsynaptic AMPA receptors, which are responsible for most excitatory transmission, including acute and tonic noxious inputs (Dickenson 1994; Thompson *et al* 1992). AMPA receptors have also been shown to mediate an NMDA-insensitive monosynaptic "fast" component of the dorsal root evoked ventral root potential (DR-VRP; Evans *et al* 1987; Thompson *et al* 1992) via excitation of motoneurones (Davies *et al* 1982).

1.5.3.1.2 Tachykinins

Considerable evidence indicates that substance P (SP) is a neurotransmitter in nociceptive afferent fibres (Nicholl *et al* 1980). It is synthesised in a distinct population of small DRG cells forming approximately 20% of the population (Hökfelt *et al* 1975, 1976) and transported to peripheral and central terminals by axonal flow (Takahashi and Otsuka 1975), as well as being found in intrinsic cells of the dorsal horn (Tessler *et al* 1980, 1981). It is released in the spinal cord in response to electrical afferent stimulation of Aδ and unmyelinated fibres (Akagi *et al* 1980; Duggan *et al* 1987; Nagy *et al* 1993; Yaksh *et al* 1980), inflammation (Duggan *et al* 1988; Schaible *et al* 1990), or high extracellular $[K^+]$ (Otsuka *et al* 1976), and its effects are blocked by specific antagonists (Courteix *et al* 1993; Fleetwood-Walker *et al* 1993; Lepre *et al* 1994). Release of SP is calcium dependent (Jessell 1978; Schenker *et al* 1976). SP acts predominantly via NK1 receptors which are located postsynaptically in laminae I and II of the dorsal horn (Yashpal *et al*

1990), although a presynaptic action has also been described (Hu *et al* 1997). The distribution of SP is not as widespread as glutamate although it has been shown to be co-localised with glutamate in the superficial dorsal horn (de Biasi and Rustioni 1988) and may participate in spinal hyperexcitability via indirect modulation of NMDA receptor function (Urban *et al* 1994a,b). Iontophoretic application of SP to dorsal horn neurones and spinal motoneurones has excitatory effects, causing a depolarisation by inactivating K^+ currents (Hösli *et al* 1981; Nowak and MacDonald 1982), or by increasing Na^+ or Ca^{2+} currents (Konishi *et al* 1974; Murase and Randic 1984; Murase *et al* 1989). High frequency primary afferent stimulation also evokes a slow depolarisation in SP-sensitive neurones in laminae II-V (Urban and Randic 1984), which can be blocked by NK1 antagonists, as can the slow polysynaptic component of the ventral root potential (Thompson *et al* 1993a), suggesting a major role for SP in nociception.

Other tachykinins are neurokinin A (NK-A) and B (NK-B) which act primarily via NK2 and NK3 receptors respectively (Yashpal *et al* 1990). NK-A is co-localised with substance P (Dälsgaard *et al* 1985) and released in the dorsal horn where there are high basal levels in the absence of stimulation (Duggan *et al* 1990; Hope *et al* 1990; Thompson *et al* 1993a). It is also released in response to noxious stimulation, and specific antagonists have been shown to attenuate noxious inputs (Fleetwood-Walker *et al* 1990, 1993). NK-B is not expressed in sensory neurones.

1.5.3.1.3 CGRP

CGRP is another peptide which is abundant in the spinal cord where it has a modulatory role. It is found in primary afferent terminals throughout the dorsal horn and can be seen in both myelinated and unmyelinated fibres in laminae I and II_o and Lissauer's tract. It is also found in many cells in the ventral, but not the dorsal horn (Willis and Coggeshall 1991). Small diameter DRG cells contain CGRP, along with SP-like immunoreactivity, and dorsal rhizotomy depletes CGRP in the spinal cord, indicating primary afferent origin (Gibson *et al* 1984; Traub *et al* 1989; Wiesenfeld-Hallin *et al* 1984). CGRP potentiates the release of glutamate and substance P in the dorsal horn *in vitro* (Kangrga and Randic 1990; Le Greves *et al* 1985; Oku *et al* 1987), possibly via its actions on increasing calcium levels in the nerve terminals thus enhancing transmitter release (Oku *et al* 1988). CGRP and substance P have also been shown to act

synergistically to modulate the nociceptive flexor reflex, presumably through actions on glutamate (Woolf and Wiesenfeld-Hallin 1986; Schneider and Perl 1985). CGRP therefore has a modulatory role in the spinal cord.

1.5.3.1.4 Other peptides

Like substance P, CCK and VIP are found in the superficial laminae of the dorsal horn, in small diameter fibres. The peptides are found in primary afferent terminals as well as intrinsic neurones and descending axons, with the exception of VIP which does not appear to be present in cells in the dorsal horn. Cells which do label for VIP are thought to belong to visceral afferents. Capsaicin sensitivity of these afferents suggests small soma size with unmyelinated fibres (Jancso *et al* 1977). Both VIP and CCK co-exist with substance P, and their Ca^{2+}-dependent release causes excitation and depolarisation as well as enhancing responses to noxious stimuli (Cridland and Henry 1988; Jeftinija *et al* 1982; Willetts *et al* 1985). However, CCK alone has little effect on postsynaptic membrane potential but potentiates substance P responses. CCK has therefore been suggested to have a modulatory effect rather than a direct effect as a neurotransmitter (Willetts *et al* 1985).

1.5.3.2 Inhibitory neurotransmitters

1.5.3.2.1 GABA

GABA and glycine are inhibitory amino acids: GABA is found in the intrinsic cells of laminae I-III in the spinal cord, and glycine is primarily located in lamina III. GABA is occasionally seen in the deeper laminae and it is present in the ventral horn in terminals surrounding unlabelled motoneurones (Magoul *et al* 1987). Both GABA and glycine are found together in presynaptic axonal varicosities or small dendrites (Magoul *et al* 1987) and myelinated primary afferents (Todd 1990). Ultrastructural localisation shows GABA in glomeruli around both myelinated and unmyelinated axons, GABA being located in axons and dendrites from local inhibitory neurones. Some glomeruli show only GABA immunoreactivity and others show GABA and glycine immunoreactivity, suggesting that there are different populations of interneurones mediating presynaptic inhibition (Ribeiro da Silva and Coimbra 1980; Todd 1996). There is no evidence for GABA-containing cells in the DRG (Price *et al* 1987). GABA mediates its effects via

$GABA_A$ and $GABA_B$ receptors. $GABA_A$ receptors are heteromeric ion channels while $GABA_B$ receptors are metabotropic, being negatively coupled to adenylyl cyclase activity (Knight and Bowery 1996; Wojcik and Neff 1984). The $GABA_B$ receptor has recently been cloned and shows no sequence similarity between and other GABA receptors nor other G protein-coupled receptors, with the exception of the mGluRs (Kaupman *et al* 1997). $GABA_A$ and $GABA_B$ receptors have been shown to co-exist on Aδ and C primary afferent fibres (Désarmenien *et al* 1984), although the $GABA_A$ receptors show a general distribution over the dorsal horn, whereas $GABA_B$ receptors appear to be discretely localised in laminae I and II (Price *et al* 1984). Dorsal rhizotomy or capsaicin pretreatment reduces both GABA receptor subtypes, although $GABA_A$ is reduced to a lesser extent (Price *et al* 1984; 1987; Singer and Placheta 1980). This suggests presynaptic localisation on nociceptive primary afferent terminals for both receptor subtypes, and a greater role for $GABA_B$ receptors in axo-axonic transmission in the spinal cord compared to $GABA_A$ (Price *et al* 1984). GABA causes hyperpolarisation via $GABA_A$ receptors and presynaptic closure of voltage-dependent Ca^{2+} channels, or facilitating the opening of K^+ channels via $GABA_B$ receptors (Bowery 1993; Davies 1981). Both GABA and glycine cause pre- and postsynaptic inhibition in the spinal cord via axo-axonal and axo-somatic or axo-dendritic synapses (Magoul *et al* 1987). Antagonists of these receptors therefore induce altered patterns of neuronal firing (Curtis 1969; Curtis *et al* 1968; Duggan *et al* 1981; Sivilotti and Woolf 1994).

1.5.3.2.2 Opioids

Morphine and opioid receptors have long been implicated in anti-nociception. There are three families of endogenous opioid peptides; the endorphins (END), enkephalins (ENK) and dynorphins (DYN) which act via mu, delta and kappa receptors respectively, although none of the peptides are specific for any one receptor (Kosterlitz 1985). Opiate receptors are located mainly in laminae I and II of the dorsal horn (Besse *et al* 1990; LaMotte *et al* 1976) and they appear to be located presynaptically around C-fibre terminal zones. Cells containing DYN and ENK show a similar distribution to the opiate receptors (Cho and Basbaum 1989; Glazer and Basbaum 1981; Hökfelt *et al* 1977a,b; Hunt *et al* 1981; Ruda *et al* 1988). Cells containing ENK are of many morphological types indicating a wide range of functions (Bennett *et al* 1982). Fibres labelled for

endogenous opiates terminate in laminae I and II predominantly on dendrites as well as on fine primary afferents suggesting both pre- and postsynaptic action (Cho and Basbaum 1989; Glazer and Basbaum 1982; Hökfelt 1977a,b). Opioids primarily act to inhibit synaptic transmission, although low concentrations of agonists have been shown to have a direct receptor-mediated excitatory effect on sensory neurones (Crain and Shen 1990). They may also produce excitatory effects mediated via disinhibition, reflecting inhibition of GABAergic neurones (Duggan and North 1984; Magnusson and Dickenson 1991).

The analgesic action of opioids is spinal as well as supraspinal, acting via inhibition of transmitter release (Jessell and Iversen 1977; Yaksh *et al* 1980). Activation of presynaptic kappa receptors causes a decrease in neurotransmitter release via closure of Ca^{2+} channels, whilst mu and delta activation has an inhibitory effect by opening K^{+} channels (Duggan and North 1984; McFadzean 1988; North 1989). Opioids are primarily implicated in nociception, especially those acting via mu receptors which produce antinociception (Dickenson 1991; Dickenson and Sullivan 1986, 1987; Dickenson *et al* 1987; Fleetwood-Walker *et al* 1988; Iadarola *et al* 1988a,b) and have little if any effect on non-noxious inputs (Dickenson and Sullivan 1986; Duggan and North 1984).

1.5.3.2.3 Galanin

Another peptide that has inhibitory effects on spinal transmission is galanin. Galanin has been found in DRG cells and cells and terminals in small neurones in laminae I and II of the dorsal horn (Melander *et al* 1986; Xu *et al* 1996). After axotomy it is strongly upregulated in DRG cells (Hökfelt *et al* 1987; Villar *et al* 1989) and has been suggested to suppress pain by acting as an endogenous inhibitor of substance P and CGRP related mechanisms (Wall and Woolf 1984; Xu *et al* 1990).

1.5.3.2.4 5-HT

5-HT is widely distributed in the CNS in ascending and descending pathways which originate in the Raphe nuclei (see Törk 1990). This system plays an important but complex role in the central and spinal modulation of peripheral noxious inputs, although little is known of its role in nociception or chronic pain states. A large number of 5-HT receptors have been identified, although it is unclear as to which are found in the spinal cord. Initially 5-HT1, 5-HT2, 5-HT3 and 5-HT4 receptors were described on the basis of

pharmacological criteria. With the advent of molecular biology, a number of other receptors have been cloned such that seven different receptors are now accepted with subtypes within some families e.g. 5-HT_{1a-f}. However, selective tools for each subtype are not yet available, making delineation of physical and pathophysiological roles difficult (for review see Hoyer and Martin 1996). Although 5-HT has well characterised sensitising actions on peripheral endings of afferent fibres (see section 1.5.4.3.2.), a role in the spinal cord is less clear. However, 5-HT_{1b} agonists have been shown to have antinociceptive effects centrally as well as in the dorsal horn (El-Yassir *et al* 1988; El-Yassir and Fleetwood-Walker 1990) suggesting a role for these receptors in nociceptive processing.

1.5.3.2.5 Other transmitters

Other neuromodulators in the spinal cord include adenosine and nitric oxide (NO). Enzymes responsible for adenosine synthesis have been found in DRG cells and substantia gelatinosa (Nagy and Daddona 1985). Adenosine is stored and released from small diameter fibres and interneurones in the dorsal horn (Braas *et al* 1986) and acts via A_1 and A_2 receptors. These are mainly expressed on intrinsic cells of the dorsal horn (Choca *et al* 1988; Geiger *et al* 1984; Li and Perl 1994), and ventral horn, respectively (Choca *et al* 1988). The location of adenosine and its receptors suggests a role in the processing of sensory information to maintain a balanced state between excitatory and inhibitory inputs. Adenosine has also been implicated in nociception such that selective agonists for the A_2 receptor are antinociceptive in *in vivo* animal models (Reeve and Dickenson 1995).

Nitric oxide (NO) has been shown to be synthesised in response to increased activity in intrinsic cells of the dorsal horn (Haley *et al* 1992). NO synthase (NOS) is found in DRG cells as well as the dorsal horn (Anderson 1992; Morris *et al* 1992) and NO is produced by a calcium dependent process. One target for released NO is primary afferent terminals where NO activates soluble guanylyate cyclase, increasing cGMP and release of glutamate (Garthwaite *et al* 1988). Inhibitors of NOS have been shown to be analgesic in animal models (Haley *et al* 1992; Moore *et al* 1991, 1993), as well as reducing formalin-evoked c-*fos* expression in the spinal cord (Chapman *et al* 1995) thereby implicating NO in nociception. NO does not appear to contribute to acutely evoked spinal reflex activity (Thompson *et al* 1995a) but is likely to be involved in

chronic processing via NMDA receptor activation which results in an influx of Ca^{2+} and subsequent production of NO (Meller *et al* 1992).

1.6 Nociceptive Transmission

1.6.1 Pain

Nociception is the perception of signals in the CNS evoked by activation of specialised sensory receptors (nociceptors) that provide information about potential or actual tissue damage (Sherrington 1906). Not all noxious stimuli are experienced as pain. Pain is the perception of an aversive or unpleasant sensation that originates from a specific region of the body and is associated with potential or actual tissue damage. Pain that results, under normal conditions, from intense or potentially damaging noxious stimuli activating nociceptors, is called physiological (or nociceptive) pain. It is transient and highly localised if no tissue damage occurs, and its role is to warn the individual of potential danger and initiate a defensive flexor withdrawal reflex. This pain has distinct mechanical, thermal and chemical thresholds and its amplitude is related to its intensity. Physiological pain is driven by functionally specialised nociceptor afferent fibres which encode aspects of noxious stimuli such as modality, intensity, duration and location. Once the threshold of these afferents is reached, action potentials in Aδ and C fibres to the spinal cord are generated, eliciting a flexor withdrawal reflex, changes in heart rate and blood pressure via the sympathetic system, and segmental responses, causing changes in local blood flow, piloerection and sweating. These responses are accompanied by pain sensation and pain-like behaviour such as escape response, vocalisation (Chapman *et al* 1985). Physiological pain is therefore regarded as nociceptor-mediated pain.

Pathological pain occurs when pain is caused by low intensity or otherwise innocuous stimuli following tissue or nerve damage. It is characterised by a disruption of normal sensory mechanisms such that pain occurs without a clear stimulus; in response to innocuous stimuli and in an exaggerated and prolonged manner to noxious stimuli (Dubner and Bennett 1983). These represent an increase in the sensitivity of the somatosensory system with a decrease in pain thresholds (allodynia), an amplification of nociceptive responsiveness (hyperalgesia) and prolonged post-stimulus sensations (hyperpathia). An increased sensitivity of the system promotes repair by minimising

further injury by avoiding all contact rather than just preventing contact with noxious stimuli.

1.6.2 Nociceptors

Cutaneous nociceptors are free nerve endings which can be grouped into two main categories according to the primary afferents they are connected to (Aδ and C fibres; Bessou and Perl 1969; Perl 1968). They can be distinguished by the conduction velocity of their connecting primary afferent fibre and the nature of the stimuli they respond to. As well as receptors in the skin which are available to be activated at all times, there is also a group of nociceptors, known as "silent" nociceptors, which do not respond to thermal or mechanical stimuli under normal conditions (Lynn and Carpenter 1982). It is believed that they must first become sensitised, e.g. during inflammation, before they can be activated. Their activity appears to be associated with hyperalgesia (McMahon and Koltzenburg 1990; Reeh *et al* 1987).

Mechanical nociceptors, connected to Aδ fibres, respond primarily to mechanical stimuli that damage the skin, and are therefore termed high threshold-mechanoreceptors (Burgess and Perl 1967; Lynn and Shakhanbeh 1988). High-threshold mechanoreceptors respond to strong mechanical stimuli without being activated by chemical or thermal stimulation under normal conditions (Lynn and Carpenter 1982). Some fine myelinated cutaneous units can be excited by noxious mechanical and thermal (cold and warm) stimuli and are therefore termed Aδ mechanoheat/mechanocold nociceptors (Adrieansen *et al* 1983). Their threshold for thermal activation, however, tends to be higher than that for thermoreceptive C fibres (LaMotte 1984). A significant proportion of Aδ mechanoheat nociceptors also respond to chemical stimuli (Adrieansen *et al* 1983; Lang *et al* 1990) and may be classified as polymodal nociceptors, a term commonly used to characterise a subclass of nociceptive C fibres.

C fibres have a mechanical threshold 10 to 100 times greater than the intensity which is generally considered as light touch and respond to potentially tissue damaging or noxious stimuli (Georgopoulos 1976). Some of these high threshold mechanoreceptors do not respond to heat or chemical stimulation (Georgopoulos 1976; Lang *et al* 1990). but a significant number respond to skin surface temperatures above 40°C (Bessou and Perl 1969). This receptor type is therefore called the C fibre mechano-thermal nociceptor. A

small population of this receptor type may also transmit cold painful sensation (LaMotte and Thalhammer 1982). The majority of C fibre mechano-thermal nociceptors also respond to chemical agents such as bradykinin, 5-hydroxytryptamine (Lang *et al* 1990) or capsaicin (Szolcsányi 1987). This class of nerve endings, which responds to mechnical, thermal and chemical stimulation is termed polymodal nociceptor (Bessou and Perl 1969).

1.6.2.1 Hyperalgesia

High intensity noxious stimuli that cause tissue damage, produce a hypersensitivity whereby a painful stimulus not only generates pain but alters the system such that responses are exaggerated, amplified and prolonged. This hypersensitivity is termed hyperalgesia and is a major symptom of inflammation and tissue injury. Hyperalgesia is characterised by a decrease in pain threshold and an increase in the pain response to noxious stimuli. There are two distinct types, primary and secondary, which can be distinguished by their difference in location relative to the injury site (Lewis 1935). Primary hyperalgesia refers to changes that occur within the site of injury due to peripheral alterations, and is especially sensitive to thermal stimuli. Secondary hyperalgesia refers to changes occurring in the undamaged area surrounding the injury site as a result of changes in central mechanisms, and tends to be mechanically sensitive (Woolf 1995).

Hypersensitivity also causes allodynia, which is where there is a sufficient increase in sensitivity such that a stimulus which would not normally generate pain (innocuous) produces pain. This is thought to be due to peripheral sensitisation of large A fibres as well as changes in central circuitry. Processes underlying hyperalgesia are complicated, involving changes in sensitivity at peripheral and central levels, as well as long term changes in afferent properties.

1.6.2.2 Peripheral sensitisation

Inflammation recruits a range of inflammatory mediators which act on nociceptors, altering their sensitivity resulting in primary hyperalgesia. The transduction sensitivity of the nociceptors is modified such that, as well as noxious stimuli, normal innocuous stimuli also activate the nociceptors. This is still a nociceptor-driven pain

mechanism but the stimulus required to activate it has changed. This phenomenon is termed peripheral sensitisation.

Tissue damage triggers a cascade of events involving a range of chemical agents which sensitise or excite nociceptive nerve endings. These mediators can be released from non-neuronal cells, the afferent fibres themselves and from products triggered by activation of defence mechanisms. They include bradykinin, prostanoids, histamine, 5-HT, neuropeptides, ATP and protons (figure 1.12). They form the so called "inflammatory soup" which provides a complex chemical environment for the maintenance of inflammation (Dray 1994). Components of the soup are regarded as either algogenic (protons, bradykinin, prostaglandins), or able to produce algogenic substances (SP, NK-A, CGRP).

Prostaglandins and leukotrienes are released from cells following lesion or damage to the cell membranes (Martin *et al* 1987). Prostaglandins sensitise nociceptor nerve endings via activation of membrane bound receptors and modulation of tetrodotoxin-resistant Na^+ channels (Birrell *et al* 1992; England *et al* 1996; Gold *et al* 1996; Rueff and Dray 1993; Schepelmann *et al* 1992; Taiwo *et al* 1989). Leukotrienes, in particular LTB4, induce sensitisation by releasing hydroxy acids from polymorphonuclear leucocytes (Levine *et al* 1986; Martin *et al* 1988). In addition, leukotrienes, as well as histamine released from mast cells, cause vasodilation and plasma extravasation thus facilitating cell migration (e.g. platelets) from the blood into the inflamed tissue (Simone *et al* 1991). Tissue damage may also release ATP as it is a ubiquitous component of cells. ATP may act via receptors on macrophages to release various cytokines and prostanoids, as well as activating sensory neurones and increasing their permeability to cations (Dray 1995; Rang *et al* 1991).

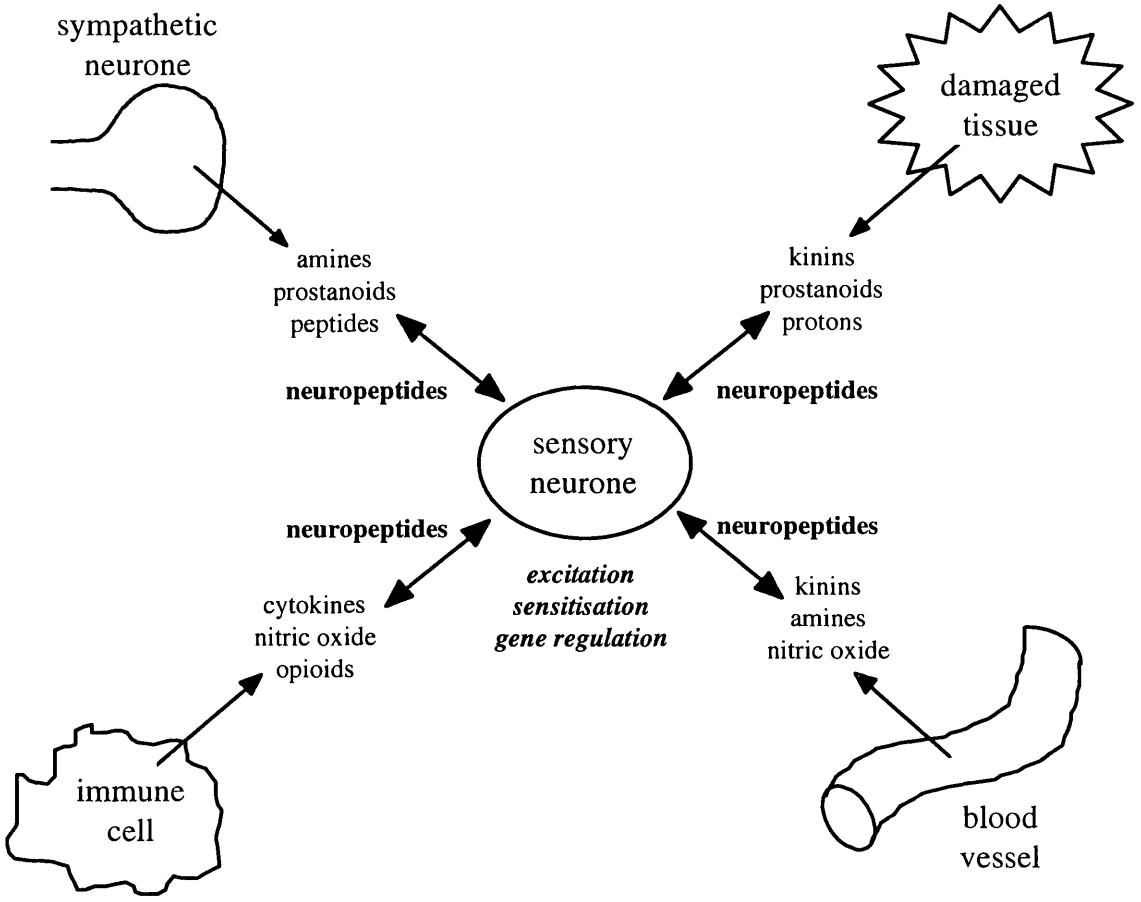


Figure 1.12 *Schematic representation of peripheral sensitisation. (Adapted from Dray* et al *1994.)*

Bradykinin is synthesised and released from the microvasculature as part of the blood clotting cascade (see Dray 1995). It has a variety of effects, but in particular, has been shown both to excite and sensitise sensory neurones (Correa and Calixto 1993; Dray *et al* 1992a; Rueff and Dray 1993). It acts via B_1 and B_2 receptors, although to date, sensory neurone effects appear to be mediated via B_2 receptors. Sensitisation by bradykinin may be indirect, via cAMP-mediated prostaglandin release (Cui and Nicol 1995; Lang *et al* 1990; Schepelmann *et al* 1992). Mast cell degranulation also causes the release of platelet activating factor (PAF) which releases 5-HT from platelets. Activation of distinct 5-HT receptors mediates sensitisation and excitation of the nociceptive nerve ending by increasing Na^+ permeability (Dray 1995; Mense 1981; Rang *et al* 1991; Rueff and Dray 1992). Under inflammatory conditions, inflammatory mediators produce vascular changes which include oedema, vasodilation and local acidosis. Protons are found to increase in inflammation and may activate nociceptors directly as well as sensitising them to mechanical stimulation (Steen *et al* 1992, 1995) They cause a brief

depolarisation by increasing Na^+ and K^+ ion conductances (Krishtal and Pidoplichko 1980), as well as causing a sustained depolarisation in capsaicin-sensitive neurones (Bevan and Yeats 1991).

Under normal conditions, neuropeptides (neurokinins, CGRP) are released from sensory nerve endings and exert efferent and trophic effects on target tissues (Dray 1994; Holzer 1988). During inflammation however, the neuropeptide content of sensory nerves is increased by the actions of neurotrophins (e.g. nerve growth factor (NGF; Donnerer *et al* 1992) such that the effects of substance P and neurokinin A (NKA) are more pronounced causing vasodilation, plasma extravasation and mast cell degranulation, allowing the release of other inflammatory mediators (Lewin *et al* 1994). CGRP does not produce peripheral plasma extravasation itself but acts synergistically with SP as a vasodilator (Brain and Williams 1985; Gamse and Saria 1985). Neurokinins may also act on sensory neurones directly, depolarising them by reducing K^+ permeability (Dray and Pinnock 1982).

A variety of cytokines, such as IL-1β, IL-6, IL-8, TNFα, are released from immune and inflammatory cells and are important pro-inflammatory agents which stimulate the synthesis and release of other proinflammatory agents, such as NO, prostaglandins and substance P (Cunha *et al* 1992; Dray 1995; Jonakait 1993). Cytokines cause behavioural hyperalgesia, although they have not been shown to have direct effects on afferent fibres (Cunha *et al* 1991; Safieh-Garabedian *et al* 1995; Woolf *et al* 1997). They also cause the release nerve growth factor (NGF; Donnerer *et al* 1992), which itself has been shown to be important in inflammation. NGF contributes to the generation of hyperalgesia and anti-NGF has been shown to prevent hyperalgesia induced by complete Freund's adjuvant (Lewin *et al* 1994; Woolf *et al* 1994). It also enhances the synthesis of other neuropeptides including neurokinins and CGRP (Lindsay *et al* 1989; Winter *et al* 1988; see Dray 1995) and regulates other proteins such as the capsaicin receptor, Na^+ channels and proton-activated ion channels (Bevan and Geppetti 1994; Rang *et al* 1994).

While sensory nerves respond to a variety of physical and chemical stimuli under normal conditions, their activity and metabolism is drastically altered by various mediators generated by tissue injury and inflammation. The inflammatory mediators activate or sensitise primary afferents by changing membrane ion channels which are directly coupled via receptors or regulated by receptor-coupled second messenger

systems. The latter have the potential to alter gene transcription inducing long-term changes in the biochemistry of the sensory neurones as well as altering the properties of the nociceptors and their ability to transmit pain signals.

1.6.2.3 Central sensitisation

Central sensitisation is a C fibre-triggered alteration in the excitability of spinal neurones (Woolf 1983). As far as we know, it is only these afferent fibres which are able to induce prolonged changes in the excitability of dorsal horn neurones. Central sensitisation has a distinct induction phase, initiated by C fibre input, and a maintainance phase which is independent of input (Ma and Woolf 1995). It is thought to be induced by temporal summation of EAA- and neuropeptide-mediated slow synaptic potentials, a reduction of tonic inhibition, and an increase in intracellular [Ca^{2+}] or activation of PLC or PKC.

C fibre activation elicits the release of glutamate and substance P which generate a slow synaptic potential (Thompson *et al* 1990; Urban and Randic 1984). The long duration of these synaptic potentials enables temporal summation to occur. Low frequency stimulation causes a steady build-up of membrane potential resulting in a cumulative depolarisation ("windup"; Sivilotti *et al* 1993; Thompson *et al* 1990). Summation causes a sustained increase in membrane excitability which persists for several minutes, during which time any input results in an augmented response (Lozier and Kendig 1995; Thompson *et al* 1993b). This occurs via a mechanism involving heterosynaptic facilitation (Thompson *et al* 1993b) which seems to be due to the initiation of second messenger changes in dorsal horn neurones. Changes include calcium influxes through voltage dependent ion channels and NMDA receptor activation (Mayer and Miller 1990). Activation of metabotropic glutamate and neurokinin receptors also indirectly increases Ca^{2+} flux and activates intracellular enzymes via PLC-mediated production of diacylglycerol and inositol phosphate (Miller 1991) potentiating the release and effects of glutamate (Gerber *et al* 1991; Kangrga and Randic 1990; Rusin *et al* 1993).

Raised intracellular [Ca^{2+}] has many effects including activation of protein kinases, phospholipases and nitric oxide synthase (Garthwaite *et al* 1988). Activation of these enzymes can result in changes that would alter membrane excitability for prolonged periods (Coderre 1992; Gerber *et al* 1989). For example, PKC can phosphorylate a

number of proteins, including the NMDA receptor (Chen and Huang 1992), which changes the Mg^{2+} binding kinetics of the receptor and promotes an enhanced glutamate response.

Maintainance of central sensitisation appears to be due to the alteration in NMDA receptor sensitivity to glutamate. Once the receptor complex has been phosphorylated and the voltage dependence of the Mg^{2+} blockade removed, glutamate sensitivity is increased as receptors no longer rely on depolarisation for activation.

Under normal conditions, activation of large myelinated A fibres does not cause pain. However under pathological conditions central sensitisation of dorsal horn neurones provides an altered central circuitry for the peripheral input. Activation of nociceptors either directly by noxious stimuli or following sensitisation via peptides such as nerve growth factor (NGF; Thompson *et al* 1995b), produces an activity dependent alteration in the dorsal horn neurones so they respond in an abnormal or exaggerated way to Aβ afferent input. Activation of these afferents can then evoke flexor withdrawal responses, autonomic responses and pain behaviour and is thought to underlie the phenomenon of allodynia.

1.6.2.3.1 Changes in phenotype

It is apparent that during hyperalgesia there may be more long term changes in the phenotypes of primary afferents and architecture of the spinal cord. Following hyperalgesia induced by axotomy, changes in peptide levels have been demonstrated in DRG and dorsal horn neurones (for review see Hökfelt *et al* 1994). Substance P, calcitonin gene-related peptide and somatostatin levels decrease, whereas vasointestinal polypeptide, neuropeptide Y and galanin levels are increased. Therefore axotomy causes a decrease in the excitatory mediators, SP and CGRP which are replaced by VIP, while galanin antagonises the excitatory effects of VIP. In general the changes may serve to decrease the magnitude of the hyperalgesia.

Enhanced production and increased release of substance P and neurokinin A occurs from sensitised and often spontaneously active fibres (Andreev *et al* 1994; Schaible and Schmidt 1988) during inflammatory hyperalgesia (Donnerer *et al* 1992; Gibbs and Kendig 1992; Hope *et al* 1990; Schaible *et al* 1990). Furthermore, evidence suggests that there may be an increase in binding sites for NK1 receptors in the dorsal

horn (McCarson and Krause 1994; Stucky *et al* 1993). During acute noxious stimulation activation of both NK2 (Laird *et al* 1993; Munro *et al* 1993; Nagy *et al* 1993; Thompson *et al* 1993a, 1994) and NK1 receptors (De Koninck and Henry 1991; Yamamoto and Yaksh 1991) have been described. Antagonist studies have also shown that activation of NK2 receptors occurs after brief noxious input to the spinal cord (Thompson *et al* 1993a), as well as NK1 receptor activation after prolonged hyperalgesia (Thompson *et al* 1994).

Neumann *et al* (1996) have suggested that inflammation may cause a phenotypic change in Aβ afferent fibres so that they, like C-fibres, gain the ability to express substance P and enhance synaptic transmission, thereby exaggerating the central response to innocuous stimuli. There is also evidence that, in peripheral neuropathy, Aβ fibres sprout into lamina II of the dorsal horn, where there has previously been little input from this fibre type (Lekan *et al* 1996; Woolf *et al* 1992, 1995). Sprouting has also been shown to occur from intact myelinated primary afferents following section of neighbouring peripheral nerves (Doubell *et al* 1997) or topical capsaicin treatment (Mannion *et al* 1996).

Altered gene expression, as demonstrated for immediate early genes (Abbadie and Besson 1992, 1993; Hunt *et al* 1987; Jennings and Fitzgerald 1996; for review see Fitzgerald 1990), preprotachykinin mRNA (Leslie *et al* 1995; Lindsay and Harmar 1989; Noguchi and Ruda 1992) and dynorphin (Iadarola *et al* 1988a; Noguchi *et al* 1991) may produce even longer lasting effects, thus transforming brief electrical inputs into long lasting changes in sensory neurones in the CNS.

1.7 mGluRs in Spinal Nociception

The mGluRs have been strongly implicated in synaptic plasticity and, although there is relatively little experimental evidence for an involvement of the mGluRs in spinal nociceptive processing, there is accumulating data to support the theory. mGluR activation directly excites spinal neurones and also potentiates NMDA- and AMPA-induced currents (Bleakman *et al* 1992; Cerne and Randic 1992; Lodge *et al* 1996b; Meller *et al* 1993). This cooperativity may be physiologically relevant as co-activation will enhance glutamate transmission and has been shown to elicit mechanical hyperalgesia (Meller *et al* 1993) and enhance nociceptive responses to formalin (Coderre *et al* 1993). The mGluRs have also been shown to enhance ionotropic glutamate receptor-

mediated responses in spinal neurones through an enhancement of cellular excitability rather than a specific interaction with the ionotropic receptors (Jones and Headley 1995). Selective mGluR antagonists inhibit sustained activation of dorsal horn neurones *in vivo* elicited by mustard oil (Young *et al* 1994), and limit the expression of inflammation-evoked hyperexcitability (Neugebauer *et al* 1994). mGluRs have also been shown to contribute to behavioural and formalin-induced nociception, probably acting via an interaction with NMDA receptors (Fisher and Coderre 1996a,b). The mGluRs have been suggested to be involved in intracellular windup in cervical dorsal horn cells, mediating a plateau potential via L-type Ca^{2+} channels (Morriset and Nagy 1996). However, the N and P type, but not L-type voltage sensitive Ca^{2+} channels have been implicated in formalin-induced behavioural hyperalgesia (Malmberg and Yaksh 1994). Inhibition of phospholipase C and treatment with protein kinase C inhibitors decreases nociceptive responses in the formalin test, whereas activation of protein kinase C enhances the nociceptive response (Coderre 1992). Coderre *et al* (1993) have also suggested that protein phosphorylation may have a role in the enhancement of NMDA-activated currents in central sensitisation by noxious stimuli. This may very well occur via activation of the mGluRs and enhanced glutamate release. The activation of mGluRs at specific synapses has also revealed a role for these receptors in nociception (Eaton *et al* 1993; Young *et al* 1994).

Given the outstanding evidence, the aim of this thesis has been to further explore the role of the mGluRs in sensory synaptic transmission in the spinal cord of the rat. I have used basic pharmacological tools as well as *in situ* hybridisation to investigate the presence, involvement and any changes that may occur under both normal and pathological conditions using a model of peripheral inflammation.

2. CHAPTER 2

BEHAVIOURAL CHARACTERISATION OF UV-INDUCED INFLAMMATORY HYPERALGESIA

2.1 Introduction

Mechanistic studies of nociception and the development and evaluation of analgesic drugs have, in the past, mainly been made on animal models using acute, brief noxious stimuli, e.g. tail-flick, tail-pinch or hot-plate tests (Ramabadran and Bansinath 1986). However, it has been recognised for a number of years that there are major differences between acute and chronic pain states (Wall 1984). Indeed, prolonged activation of nociceptive fibres can induce plastic changes in peripheral and central components of sensory pathways, and these changes may be short lived or long lasting (Dubner and Ruda 1992; Wall and Woolf 1984). Such events could be of major significance in chronic pain states, including inflammatory hyperalgesia, neuropathic pain and sympathetically mediated pain. If such pain states remain untreated, other physiological and psychological compensatory mechanisms may be induced as well as potentially irreversible morphological changes to the nervous system, (LaMotte *et al* 1991; Liebeskind 1991; Macrae *et al* 1992; Sugimoto *et al* 1990; Woolf 1983).

The extent of plastic changes occurring in chronic pain conditions has highlighted the shortcomings of model systems in which brief activation of nociceptive pathways has been used. This has prompted the development of animal models which provide more appropriate information relevant to chronic pain conditions (Dubner 1991). The methods currently being developed involve the administration of irritant chemicals (capsaicin, formalin, mustard oil) to induce prolonged stimulation of nociceptive afferents (Dubuisson and Dennis 1977; LaMotte *et al* 1991), immune stimulants (yeast, carrageenan, complete Freund's adjuvant) to induce prolonged inflammatory hyperalgesia (Stein *et al* 1988), and peripheral and central nerve lesions to induce neuropathic pain (Bennett and Xie 1988; Hao *et al* 1991; Kim and Chung 1992).

Pain hypersensitivity is associated with tissue damage and inflammation, which recruits a range of inflammatory mediators which in turn act on sensory neurones both directly and indirectly. This may result in altered primary afferent sensitivity as well as changing their phenotype, causing upregulation of transmitters and receptors within peripheral nerves and the spinal cord. Of the various animal models that exist for the study of inflammatory pain, the most commonly used include the formalin test, carageenan-induced inflammation, complete Freund's adjuvant and ultra-violet irradiation.

The formalin test is a model of acute tissue injury-induced pain, lasting upto 1 hour in rats, whereby dilute formalin is injected subcutaneously into one paw of the animal (Abbott *et al* 1995; Dubuisson and Dennis 1977; Tjølsen *et al* 1992). This induces a biphasic pain behaviour such as paw elevation, licking or biting. The initial phase (3-5 minutes) is thought to be due to direct chemical stimulation of C fibre nociceptors (Dubuisson and Dennis 1977; Heapy *et al* 1987; Hunskaar *et al* 1985) and is insensitive to non-steroidal anti-inflammatory drugs (NSAIDS; Hunskaar and Hole 1987; Rosland *et al* 1990). The second phase starts approximately 15-20 minutes after formalin injection, lasting for 20-40 minutes, and can be reduced by NSAIDS (Hunskaar and Hole 1987; Rosland *et al* 1990; Tjølsen *et al* 1992). Experimental evidence suggests that substance P, bradykinin, histamine, 5-HT and prostaglandins are involved in this phase (Shibata *et al* 1989).

Carageenan is a mucopolysaccharide, extracted from Irish sea moss, *Chondrus*. When injected into the plantar side of a paw, it produces an oedema which peaks after 3 to 5 hours and lasts upto 4 days (Vinegar *et al* 1987; Winter *et al* 1962). It also produces a unilateral mechanical and thermal hyperalgesia which lasts for up to seven hours after injection of carrageenan (Hargreaves *et al* 1988), and a decrease in vocalisation threshold to mechnical stimulation which persists for 96 hours after carrageenan administration (Kayser and Guilbaud 1987). This model therefore produces an acute inflammatory response, accompanied by hyperalgesia, which is more prolonged than that seen induced by formalin. The levels of opioid peptides and transcription markers have been shown to be elevated after carrageenan-induced inflammation (Iadarola *et al* 1988a) suggesting peripheral and central sensitivities.

Complete Freund's adjuvant induces an immunopathological disease that is similar to arthritis. Adjuvant arthritis is produced by inoculation, into the base of the tail or into the plantar surface of a hindpaw, with *Mycobacterium butyricum* suspended in oil, producing an initial inflammatory response in the area of the inoculation, as well as long lasting (up to several months) swelling of joints, particularly in the hind paws (for review see Colpaert 1987). The inflammation results in hyperalgesia that lasts for at least 20 days and can be attenuated by opiates and NSAIDs (Capetola *et al* 1980; Winter and Nuss 1966).

A model of relatively mild pain and hyperalgesia was developed using UVA irradiation of the paw of adult rats (Perkins *et al* 1993a). While this model involves local, light inflammation of the glaborous and hairy skin of the hindpaw, animals always show a long lasting thermal and mechanical hyperalgesia with medium severity. Previous studies have shown that UV irradiation induces several phases of hyperalgesia including an initial phase of inflammatory hyperalgesia, which can be reduced by NSAIDs, and a later, NSAID-resistant phase where hyperalgesia persists without inflammation (Perkins *et al* 1993b). This suggests that other components, possibly with central origins, are responsible for the maintainance of the hyperalgesia. Thompson *et al* (1994) adapted the model to young animals and characterised the thermal and mechanical hyperexcitability as well as using this model to study the changes in spinal cord sensory transmission during hyperalgesia *in vitro*.

Model	**Time course**
Formalin	Early phase lasting 0-5 minutes
	Late phase occurs 15-20 minutes after formalin injection lasting for 20-40 minutes
Carrageenan	Hyperalgesia lasting 7-96 hours
	Oedema lasting upto 4 days
Ultra-violet irradiation	Hyperalgesia lasting upto 9 days (adults)
Complete Freund's adjuvant	Hyperalgesia lasting >20 days
	Severe pain for 2-3 weeks
	Chronic pain for upto 4 weeks
	Joint deterioration for upto 8 weeks

Table 2.1 *Comparison of animal models of peripheral inflammation.*

UV-irradiation of the hindpaw does not cause the widespread systemic effects created by adjuvant-induced arthritis, but causes a relatively naturally-induced, persistent hyperalgesia with a relatively rapid onset. Therefore I have used this model of

inflammatory pain in juvenile rats, and this chapter characterises the development of mechanical hyperalgesia evoked over time as a behavioural adjunct to the data presented in the following chapters.

2.2 Methods

2.2.1 Induction of hyperalgesia

Mechanical hyperalgesia was induced using the method previously described in detail for adult and juvenile rats (Perkins *et al* 1993a; Thompson *et al* 1994). Both surfaces of the left hindpaw of 9 day old rats (Sprague-Dawley, Charles River) were exposed to a cold UVA light source (365nm, 69nW/cm^2) for 90 secs. This exposure was repeated after 18 hours.

Animals that were irradiated were taken from the same litter and kept with their mother and litter mates throughout the period of testing. All experiments were carried out in accordance with Home Office Regulations on use of experimental animals.

2.2.2 Behavioural experiments

UV-irradiated animals (n=10) were tested two hours after the final dose of UV irradiation and 24 hours subsequently for four days. Contralateral hindpaws were tested in parallel to ipsilateral hindpaws in UV-treated animals and age-matched naïve rats (n=17). Mechanical hypersensitivity was characterised using von Frey hairs (allodynia; von Frey 1906) and the Randall-Sellito paw pressure test (mechanical hyperalgesia; Randall and Sellito 1957). Von Frey hairs were used, in ascending order, on the upper surface of the ipsi- and contralateral hind paws of unrestrained animals at one minute intervals. Threshold mechanosensitivity was taken when a clear withdrawal reflex occurred. This procedure was repeated four times for each animal at every time point and the values averaged. For the paw pressure test, the hindpaw was placed on a Perspex platform and pressure (up to 100g) applied to the upper surface of the paw using a flat probe. The pressure applied was gradually increased until a withdrawal reflex was seen.

Care was taken to standardise the behavioural testing as far as possible. All behavioural tests were carried out in the same room under subdued light conditions and the time that the pups were kept away from their mother was reduced to the minimum.

Results are given as mean +/- S.E.M. of the theoretical log weight (converted from Newtons) of the von Frey hairs, and the weight tolerated as measured by the Randall-Sellito apparatus. A one-way ANOVA followed by a posthoc analysis of means (Tukey's test) was used for statistical analysis.

2.3 Results

UV-irradiation of both surfaces of the left hindpaw produced a cutaneous inflammation which was characterised by redness and swelling of the irradiated area. No blistering or skin erosion developed and general behaviour and weight gain were not different to untreated control animals. On the other hand, when mechanical sensitivity was tested, animals in the UV-irradiated group showed a significantly decreased withdrawal threshold to von Frey hair stimulation and a decrease in load tolerated in the Randall Sellito paw pressure test when compared to untreated control animals (figure 2.1).

Values for the withdrawal threshold to von Frey hair stimulation in control animals steadily increased during the 4 days of observation (postnatal day 11-15) from 2.77±0.27g to 7.49±0.109g (n=17), with no significant difference between the two sides. Von Frey thresholds in the UV-treated animals decreased significantly on both sides with a maximum drop in threshold on the first day after irradiation (ipsilateral: 0.20±0.02g; contralateral: 0.84±0.09g; $p<0.001$; n=10). Within 3 days of UV-irradiation, threshold levels recovered to normal in the contralateral paw while full recovery on the ipsilateral side did not occur within the time course studied (figure 2.1/A). The Randall Sellito test provided a similar profile of results, with maximum hyperalgesia present 1 day after UV-irradiation on both sides (see figure 2.1/B) and gradual recovery 3 days post-UV treatment.

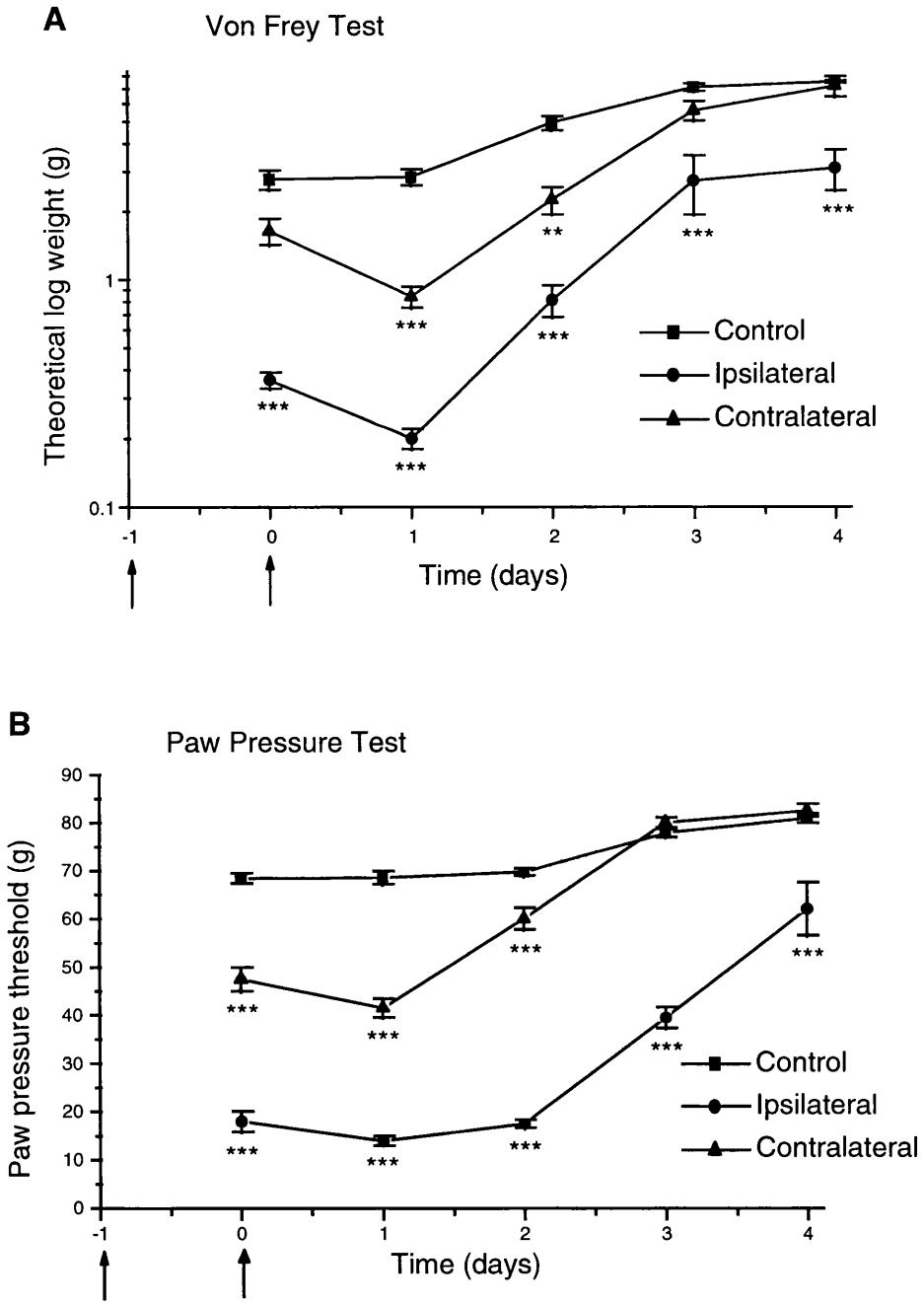


Figure 2.1 *UV-irradiation induced allodynia and hyperalgesia in the rat hindpaw. After a double exposure of UVA irradiation (18 hours apart; for details see methods) of both dorsal and ventral surfaces of the left hindpaw of 9 day-old rats, allodynia, measured by von Frey hair stimulation (**A**) and mechanical hyperalgesia, measured by the Randall-Sellito test (**B**) develops within one day. Arrows represent the time of irradiation. ***p<0.001; **p<0.01; n=10 and 17 for UV-irradiated and control animals, respectively.*

2.4 Discussion

These results show that an acute, unilateral insult of UVA light induces mechanical hyperalgesia in the ipsilateral hindlimb and, in addition, milder but parallel hyperalgesia in the contralateral limb of the juvenile rat. Hyperalgesia and allodynia in the inflamed hindpaw had almost returned to control levels four days after UV-irradiation, and the contralateral paw had completely recovered within this time. UV irradiation has been used previously to induce acute sensitisation of afferent fibres (Szolcsányi 1987) and to produce acute inflammation of the hindpaw in adult animals (Gillardon *et al* 1991). Recently this model has also been shown to display features of both acute and prolonged hyperalgesia (Perkins *et al* 1993b) as well as mechanical and thermal hyperalgesia (Perkins *et al* 1993b; Thompson *et al* 1994). In the present study mechnical hypersensitivity was measured in the ipsilateral, treated hindpaw as well as in the contralateral untreated paw. This has also been seen by others using this model with juvenile animals (Thompson *et al* 1994) but not with adults, although adult animals show a contralateral thermal hyperalgesia (Perkins *et al* 1993b). The pronounced contralateral effect seen in juvenile animals may be due to the high level of synaptic plasticity at this age (Fitzgerald *et al* 1987a), resulting in a larger ascending/descending component or due to spreading sensitisation across the spinal cord into the contralateral side. The tissue damage caused by the UV-irradiation may also be greater in the juvenile animals as tissues are generally more delicate and the paws are not yet protected by hairs. The time course of the hypersensitivity in the contralateral paw mirrored that of the ipsilateral paw, and both returned faster to control levels than observed in the adult animals (Perkins *et al* 1993b). The significantly shorter duration of hyperalgesia in this model in comparison to adults may be explained by higher plasticity and metabolism in the young animals. Contralateral hyperalgesia and reductions in contralateral flexor reflex thresholds have also been demonstrated in adult animals following a localised thermal injury to the hindpaw (Woolf 1984). Cutaneous and deep tissue injury also produce an increase in ipsilateral and contralateral flexion withdrawal reflex in response to noxious pinch of the hindpaw (Woolf 1983; Woolf and McMahon 1985). The characteristics of the contralateral reflex were not examined further in the present experiments *in vitro* and require further analysis.

The scores for both tests increase steadily in the control animals due to their fast growth rate at this age. The animals gain about 2g per day as well as growing substantial fur. Furthermore, the animals become accustomed to handling and responding to the test apparatus.

Extrapolation of these results to adult conditions may be complicated because of the relative immaturity of the animals. However, I have used pups towards the end of the second postnatal week, the time at which the neurochemical and anatomical appearance of C fibre terminals in the dorsal horn of the spinal cord approaches that of the adult (Fitzgerald and Gibson 1984), the physiological function of the C fibres is virtually established (Fitzgerald 1985) and the appearance of the cutaneous afferent responses closely resembles that of the adult (Fitzgerald 1985). At this age, however, the flexion reflex threshold is still lower than in the adult (Fitzgerald *et al* 1987b) but is beyond the time (PN day 0-7) whereby innocuous as well as noxious peripheral stimuli may evoke a flexion reflex (Fitzgerald *et al* 1987b). By using age matched untreated pups as controls, however, I have demonstrated that the hindlimb reflex shows a reliable and prolonged sensitisation in response to injury.

Mechanical hyperalgesia and allodynia were investigated using a limb-withdrawal reflex, where the end point is taken when the animal withdraws the foot from the weight. This simple reflex allows the animal to control the magnitude of the stimulus as well as the level of pain. Once the animals were accustomed to the procedure, the end point was very clear and was often accompanied by vocalisation. In the event of no reflex, the end point was set at the maximum load at which the foot could still be removed. Any sensitisation to the repeated tests was monitored and the tests repeated after a period of time, allowing for recovery. The Randall-Sellito paw pressure test is generally recognised to be a reliable measure of mechanical hyperalgesia but the question arises as to whether the von Frey test is a measure of mechanical hyperalgesia or allodynia. Mechanical hyperalgesia manifests itself as a lowering of the threshold of the load tolerated, which is indeed seen with the von Frey test as less pressure is needed to elicit a response. Equally, a weight which normally causes no response elicits a reflex after the induction of hyperalgesia and may be termed allodynia. The parameter investigated here is more likely to be mechanical hyperalgesia rather than allodynia but different experimental design, such as using only one weight of hair, may have been a better method to investigate

allodynia. Thermal hyperalgesia was not investigated as initial results were erratic and difficult to obtain due to the age of the animals.

As a model, UV irradiation has many benefits when compared to the existing models of inflammation. It is non-invasive, therefore any inflammatory response is due to local tissue damage rather than being driven by non-physiological chemicals and/or toxins. It is reproducible in that it does not have the complication of vehicle effects, or volumes and concentrations of solutions. It is an ethical model evoking a mild pain that the animals seem easily able to cope with, and the insult is brief yet long enough to evoke central as well as peripheral changes. It is also a clinically relevant model with the increasing incidence of skin cancers and burns due to UV irradiation, and the need to control the pain that accompanies these conditions. Experimentally, this is a valid model as UV inflammation has been shown to elicit an increased excitability in peripheral nerves and dorsal horn neurones (Urban *et al* 1993). UV-induced inflammation has also been shown to evoke an enhanced contribution of NMDA receptors and a novel contribution of NK1 receptors to the electrically evoked spinal reflex (Thompson *et al* 1994).

One consideration that must not be forgotten is that the study presented in this chapter was carried out using juvenile animals. The nociceptive system of these animals has almost reached adult levels but there are bound to be differences when compared to adults, such as the speed of development of hyperalgesia. What is important, though is that these young animals can generate an inflammatory response, which appears to have a more pronounced central component, as manifested by a contralateral hyperalgesia. This may suggest that the treatment of chronic inflammation in children and babies is very important as the consequences are likely to be more pronounced compared to adults. This has, of course, been expounded by the many years of work on paediatric pain carried out by Maria Fitzgerald (for review see Fitzgerald 1994).

2.5 Conclusion

Unilateral UVA irradiation to the left hindpaw of the juvenile rat produces a pronounced bilateral mechanical hyperalgesia and allodynia. The contralateral hindpaw had recovered four days after the insult and the ipsilateral paw had nearly returned to control levels within this time. UV irradiation is therefore a useful model for studying nociceptive behaviour in peripheral inflammation.

3. CHAPTER 3

mGluR mRNA EXPRESSION IN THE LUMBAR SPINAL CORD USING *in situ* HYBRIDISATION

3.1 Introduction

There is substantial information about the distribution of individual metabotropic glutamate receptor (mGluR) mRNA subtypes and their splice variant expression in the central nervous system. However their distribution and function in the spinal cord during development and under pathological conditions, is, in comparison, poorly understood.

Molecular biology studies confirmed the expression of mRNAs for mGluR1,2,3,4,5 and 7 in brain (Abe *et al* 1992; Ohishi *et al* 1993a,b, 1995a; Saugstad *et al* 1994; Shigemoto *et al* 1992; Tanabe *et al* 1993), while mGluR6 expression was found to be specific for the retina (Akazawa *et al* 1994; Nakajima *et al* 1993). Receptor proteins have been described in the central nervous system for mGluR1,2,3,4a,5,7 and 8 subtypes (Bradley *et al* 1996; Görcs *et al* 1993; Hampson *et al* 1994; Kinoshita *et al* 1996a,b; Li *et al* 1996; Neki *et al* 1996; Ohishi *et al* 1995b; Petralia *et al* 1996; Romano *et al* 1995). These appear to be localised on neurones both pre- and postsynaptically (Baude *et al* 1993; Kinoshita *et al* 1996a,b; Li *et al* 1996; Neki *et al* 1996; Ohishi *et al* 1994, 1995b; Petralia *et al* 1996; Romano *et al* 1995) and also appear in glial cells (Catania *et al* 1994; Fotuhi *et al* 1994; Petralia *et al* 1996; Porter and McCarthy 1995). The presence of mGluR1,3 and 5 mRNA in the adult rat spinal cord has been demonstrated using *in situ* hybridisation techniques (Anneser *et al* 1995; Ohishi *et al* 1993a; Shigemoto *et al* 1992). mGluR2 has also been studied (Ohishi *et al* 1993b), however no distinct signal has been shown in the spinal cord.

In general, the mGluR family strongly contributes to synaptic modulation in the central nervous system (see chapter 1). Thus it is not surprising that changes in mGluR receptor function have been suggested to underly changes during development and neuropathological conditions (Bruno *et al* 1994; Buisson and Choi 1995; Nicoletti *et al* 1996; Tizzano *et al* 1995).

Although most of our knowledge about mGluR distribution in the brain has been derived from studies in the adult animal, there is evidence that the pattern of distribution is age dependent. Catania *et al* (1994) have described changes in the distribution of the mGluRs in the developing brain and changes in mGluR expression have been associated with functional consequences during development e.g. synaptogenesis. However, there is no similar study to date, concerning changes of mGluR expression during development.

It is well known that both motor and sensory functions in the spinal cord undergo an intensive maturation process after birth in the rat (Dekkers *et al* 1994; Fitzgerald 1991, 1995; Fitzgerald and Gibson 1984; Fitzgerald and Swett 1983; Fitzgerald *et al* 1987a; Lisovoski *et al* 1994; Seebach and Mendell 1996). Changes in different transmitter systems have also been described during spinal development, e.g. NMDA (Hori and Kanda 1994), AMPA (Jakowec *et al* 1995) and glycine (Watanabe and Akagi 1995), and it has been suggested that each receptor-ligand system is selectively regulated during maturation of the spinal cord (Kar and Quirion 1995).

Pharmacological and physiological studies of the spinal cord reflex suggest that mGluRs could both attenuate (Ishida *et al* 1995; Jane *et al* 1995; Pook *et al* 1993) or enhance (Aniksztejn *et al* 1992; Bleakman *et al* 1992; Bond and Lodge 1995; Jones and Headley 1995; Young *et al* 1994) the motor output of the spinal cord. Indeed both AMPA and/or NMDA receptor mediated currents are facilitated in spinal dorsal horn neurones by mGluR ligands (Bleakman *et al* 1992; Cerne and Randic 1992; Lodge *et al* 1996a; Meller *et al* 1993). Intracellular studies have revealed that membrane properties of wide dynamic range interneurones (Morisset and Nagy 1996) and ventral horn neurones (Liu and King 1995) are also directly affected by mGluR activation.

Recent findings suggest that group II and/or III mGluRs may be distributed on primary afferent terminals and could inhibit transmitter release, hence modulate the primary afferent transmission in the spinal cord (Jane *et al* 1995; Kemp *et al* 1994).

In vivo observations revealed that mGluR activation contributes to the development of spinal hyperexcitability (Meller *et al* 1993; Neugebauer *et al* 1994; Young *et al* 1994; see chapter 4 for details). These observations present the first evidence for mGluR activation during hyperalgesia associated with peripheral inflammation. However, the lack of subtype-selective antagonists with high affinity did not allow the precise determination of mGluR subtypes involved in these changes.

This issue has been addressed in the study presented in this chapter. I have used oligonucleotide probes to characterise changes in the mRNA expression of a wide array of mGluR subtypes during development of the rat spinal cord and during the course of ultra-violet (UV)-induced peripheral inflammation in young rats. The work presented in this

chapter was carried out by me in collaboration with Prof. W. Zieglgänsberger, Dr. T.R. Tölle and Dr. A. Berthele at the Max Planck Institute of Psychiatry in Munich, Germany.

3.2 Methods

3.2.1 Construction of probes

Radioactive *in situ* hybridisation was carried out using synthetic oligodeoxynucleotide probes. Each probe was a 45-mer and was 3'-end labelled with [α-^{35}S]-dATP (1200 Ci/mmol; NEN Du Pont) and terminal deoxyribonucleotide transferase (Boehringer Mannheim) using a 30:1 molar ratio of dATP to oligonucleotide. The probes were designed to be complementary to sequences encoding 15 amino acids in the region approximately 100 amino acids prior to the first transmembrane region. These amino acid sequences are as follows:

rmGluR1: QYTEANRYDYVHVGT

rmGluR3: VFNLQQTGGKYSYLK

rmGluR4: YQYQLRNGSAEYKVI

rmGluR5: WDNGELKMDDDEVWS

rmGluR6: YQAVGQWAEALRLDM

rmGluR7: YRLIGQWTDELQLNI

The rmGluR2 probe was identical to that described by Fotuhi *et al* (1994).

3.2.2 In situ hybridisation

In situ hybridisation was carried out according to the methods of Tölle *et al* . (1993) and Wisden and Morris (1994). For the developmental experiments, lumbar portions (L3-5) of spinal cords were removed from terminally anaesthetised (Enflurane or Halothane), non-perfusion-fixed Sprague Dawley rats at postnatal (PN) day 1, 7, 12 and 21 (n=3 per group). For the comparison between naïve and hyperalgesic rats, lumbar portions (L3-5) of spinal cords were removed from terminally anaesthetised (Enflurane), non-perfusion-fixed control and UV-treated Sprague-Dawley rat pups at postnatal day 11, 12 and 13 (n=6 per group). Blocks of spinal cord were rapidly frozen on dry ice. Sections (14 μm) were cut on a cryostat at -20°C, and thaw-mounted onto poly-L-lysine coated glass slides. The sections were fixed in 4% paraformaldehyde and stored in 96% ethanol.

The labelled probe was diluted in hybridisation buffer (50% formamide, 4x sodium-saline citrate (SSC) and 10% dextran sulfate) to a concentration of 1pg/ml. Sections were hybridised at 42°C for 17 hours, and washed in 1x SSC at 55°C for 30 minutes. The slides were then either exposed to Kodak Biomax film (for 4 weeks) or dipped in Ilford K5 nuclear emulsion (for 8 weeks). Developed sections were then counterstained with cresyl violet. Non-specific labelling was assessed by incubating sections with the corresponding unlabelled probe in 100-fold excess. The resulting images were indistinguishable from background.

3.2.3 Data analysis

For quantitative measurements (Tölle *et al* 1995a) three sections from each animal were analysed. Microdensitometry by counting silver grains in emulsion-dipped sections was evaluated using a computer-assisted image analyser (Optimas-Bioscan) fitted with a Zeiss Axioplan microscope and a Sony CCD camera. For the developmental study, three areas from each section were chosen for analysis, representing the laminar distribution of mGluR expression: area 1 corresponding to laminae I, II and III, area 2 to laminae II, III and IV and area 3 to laminae IV and V. Optical densities from autoradiographs were measured and each area was averaged and values compared over the time course. Statistical significance was measured using a one-way ANOVA followed by posthoc analysis of means (Tukey's test) and compared to PN day 1. For the comparative study between naïve and hyperalgesic animals, five areas from each section (on both sides of the spinal cord) were chosen for quantitative analysis (figure 3.1): area 1 corresponding to laminae I, II and III, area 2 to laminae II, III and IV, area 3 to laminae IV and V, area 4 to interneurones of lamina VII and area 5 to a population of motoneurones (lamina IX).

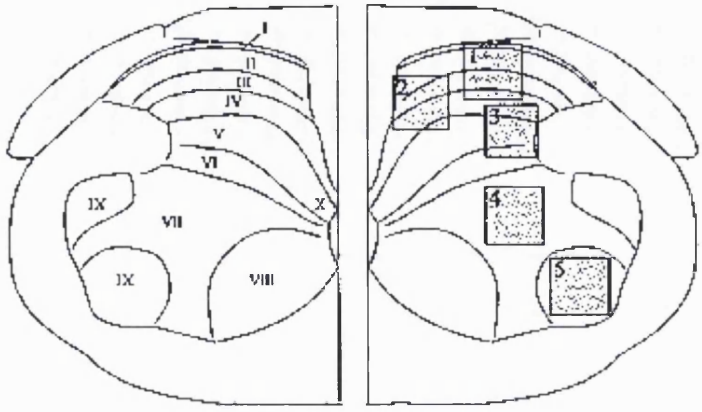


Figure 3.1 *Diagram illustrating the Rexed laminar organisation of the rat lumbar spinal cord (left panel) and an illustration of the areas quantified in the comparative study between naïve and hyperalgesic animals (right panel). For further details see text.*

Readings of silver grain density from each area were averaged and values compared between control and UV-treated animals over a three day time course. All data are presented as mean ± S.E.M of the silver grain density above background. A comparison between the ipsi- and contralateral sides of each spinal cord section was made by a paired Student's t-test. A one-way ANOVA followed by posthoc analysis of means (Tukey's test) was used to compare data from control and UV-treated animals over the three day time course.

3.2.4 Induction of hyperalgesia

Hyperalgesia was induced using the method previously described in chapter 2. Briefly, both surfaces of the left hindpaw of 9 day old rats were exposed to a cold UVA light source for 90 secs. This exposure was repeated after 18 hours, the animals were sacrificed one, two or three days later (n=6 at each time point) and their spinal cords used for *in situ* hybridisation. In parallel, age matched groups of rats (n=6) at each time point were used as controls.

All animals were housed with their mother in bedded cages. All experiments were carried out in accordance with Home Office Regulations.

3.3 Results

3.3.1 Distribution of mGluR subtype mRNA in the adult spinal cord

Of the known mGluR subtypes, all show mRNA expression in the lumbar spinal cord except mGluR6, which was purely retinal (Nakajima *et al* 1993). mGluR2 shows very low mRNA expression which is barely detectable with the methods used here (Ohishi *et al* 1993b; figure 3.2). In this section, the mGluR distribution will be outlined according to regions of the spinal cord.

3.3.1.1 Dorsal horn

The dorsal horn shows most of the mGluR mRNA expression (refer to figures 3.2 and 3.3). mGluR1 mRNA is evenly distributed at a relatively low level over laminae I-IV. Cells covered with silver grains were mainly observed in the deeper laminae of the dorsal horn (III-VII), and in the intermediate grey matter. mGluR3 mRNA appears to be almost exclusively localised in the dorsal horn (laminae II-V) with some labelling being apparent in the white matter also. The signal for mGluR4 mRNA was of a lower level in the superficial laminae (laminae I-II) of the dorsal horn, but a strong, diffuse signal was observed in the rest of the spinal grey matter, particularly in dorsal horn interneurones. mGluR5 shows the strongest signal of the mGluR subtypes in the dorsal horn with the highest density in lamina I-IV forming a broad band over these laminae. However, in terms of medio-lateral expression, mGluR5 is unevenly expressed, with a higher density of expression at the medial edge of the grey matter. mGluR7 mRNA was also strongly expressed in the dorsal horn. The highest density was found in lamina II and laminae III and IV also contained high levels of mGluR7 mRNA.

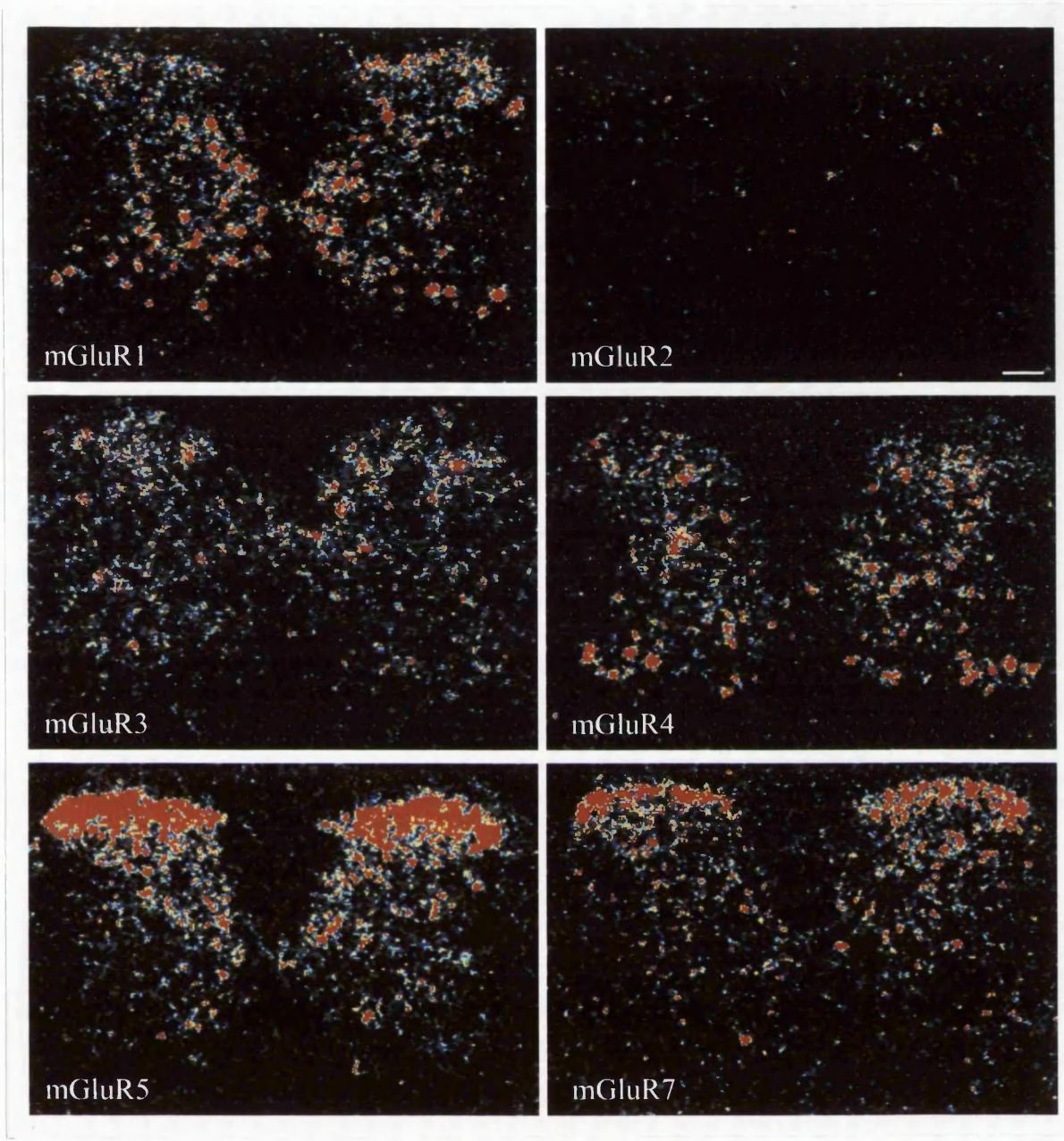
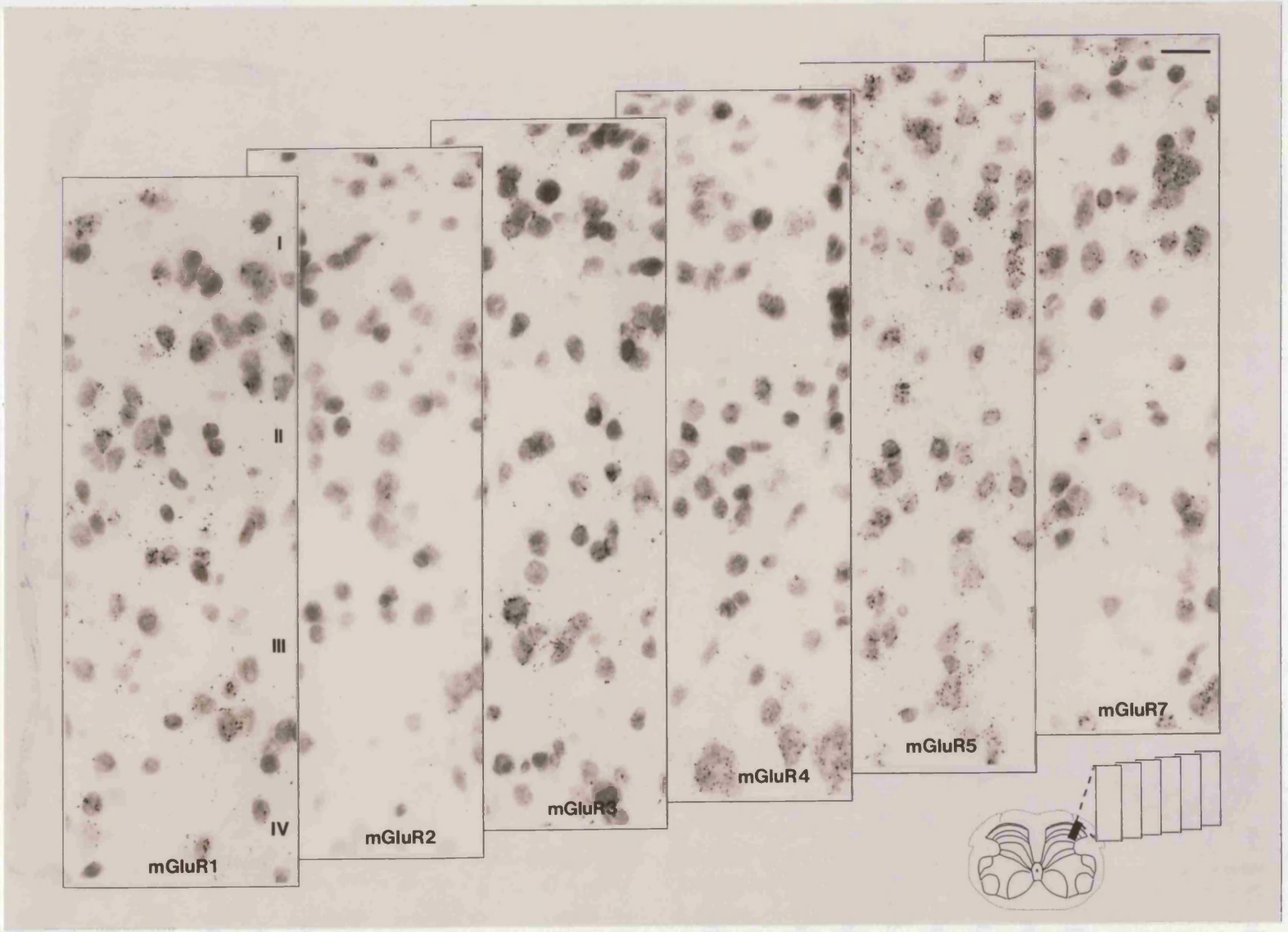


Figure 3.2 (This page) *Expression of mGluR subtype mRNAs in the lumbar spinal cord (L4-5) of adult rats. Micrographs show the whole spinal cord after in situ hybridisation with ^{35}S-labelled oligonucleotides exposed to x-ray film. Scale bar=200μm.*

Figure 3.3 (Next page) *Cellular expression of mGluR1-5,7 mRNAs in the dorsal horn of the adult lumbar (L4-5) spinal cord taken from whole spinal cords exposed to photographic emulsion. Roman numerals indicate the approximate position of the laminae of the dorsal horn. Scale bar=30μm.*


I
II
III
IV
mGluR1
mGluR2
mGluR3
mGluR4
mGluR5
mGluR7

3.3.1.2 Intermediate grey matter

mGluR1 and 5 mRNAs show the highest levels of expression, especially around the central canal, while mGluR1,4 and 7 do not show particularly high expression in this area. mGluR1 is expressed in the intermediate grey matter in large cells.

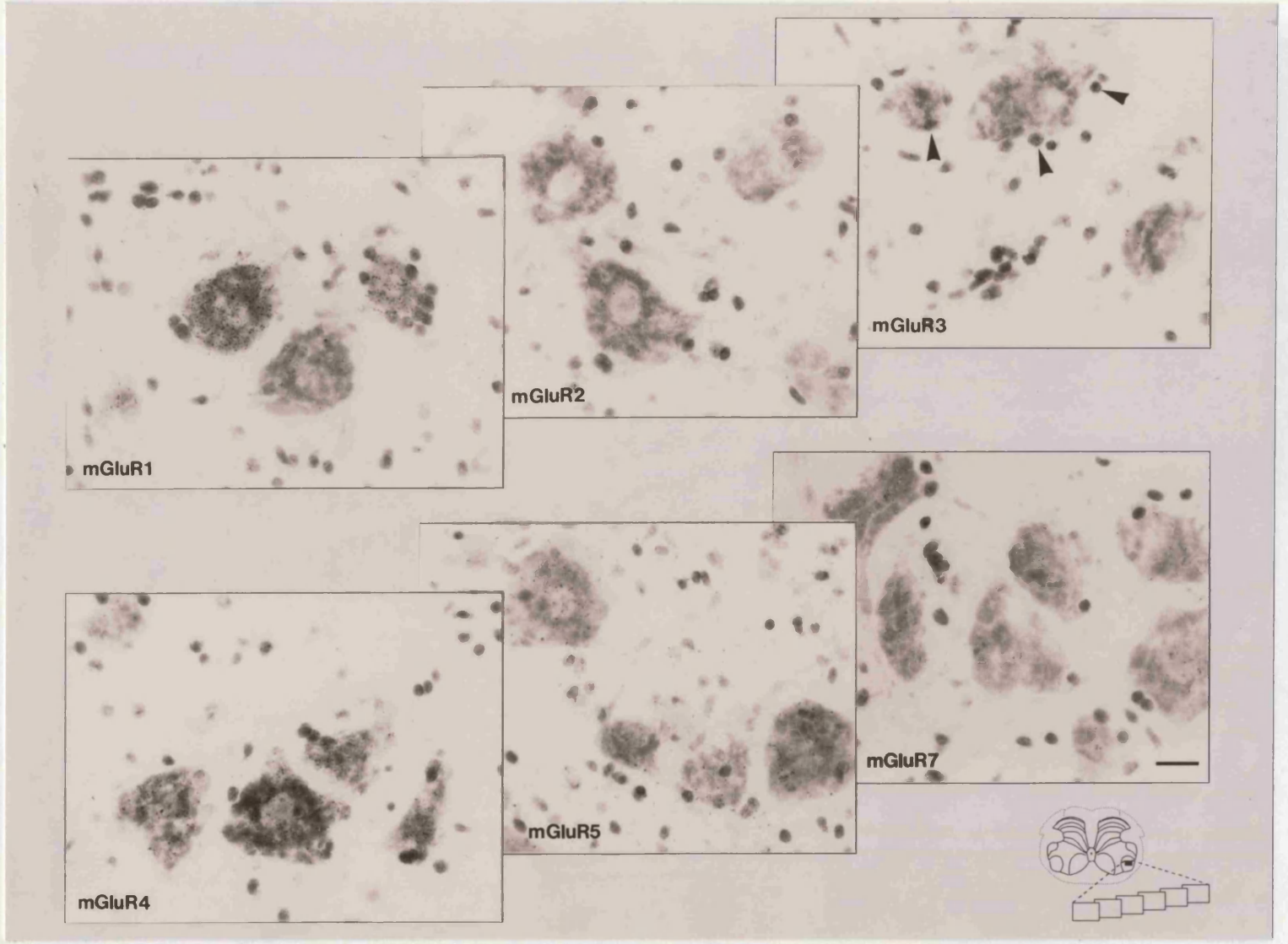
3.3.1.3 Ventral horn

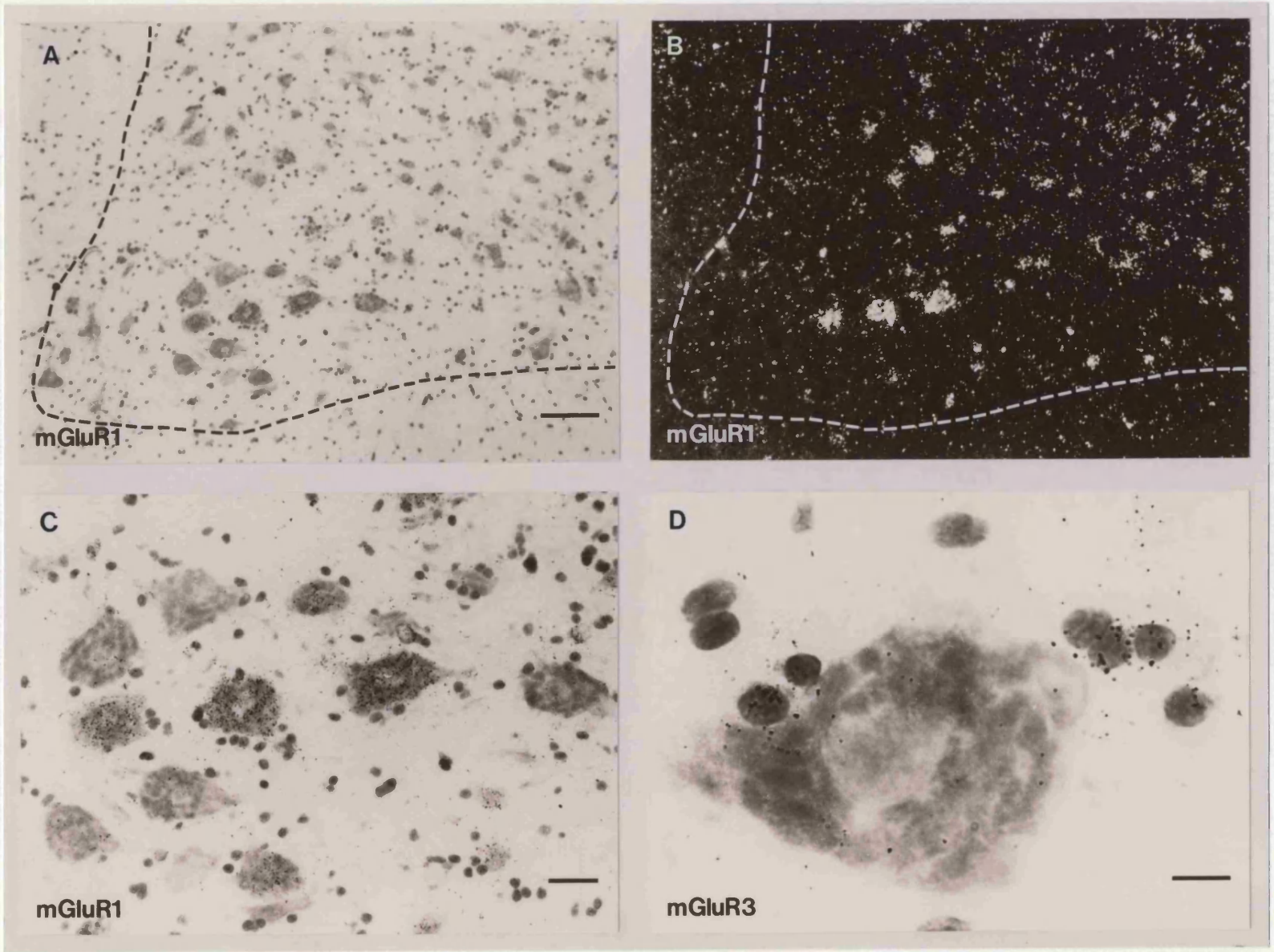
mGluR1 and mGluR4 show strong mRNA expression in motoneurones, while mGluR2 and 7 mRNAs appear to be absent and mGluR5 mRNA is expressed at a low level. The ventral horn showed scattered expression of mGluR3 mRNA which appears to be present in small cells surrounding motoneurones with no expression in the motoneurones themselves (figure 3.4). This may indicate glial expression. mGluR1 mRNA expression is patchy in motoneurones. The motoneurone pool seems to be divided into two types of cells: those expressing mGluR1 mRNA and those not (figure 3.4 and 3.5).

The splice variants of mGluR1, 4, 5 and 7 are known to be expressed at different levels in the adult spinal cord, but this will not be covered in this thesis.

Figure 3.4 (Next page) *Cellular expression of mGluR1-5,7 mRNAs in motoneurones of the adult lumbar (L4-5) spinal cord taken from whole spinal cords exposed to photographic emulsion. Arrows indicate mGluR3 expression in small cells surrounding motoneurones. Scale bar=20μm.*

Figure 3.5 (Page 87) *Expression of the mGluR1 subtype gene is either highly expressed or missing in neighbouring motoneurones (boxes **A-C**). **D)** The mGluR3 subtype gene is expressed in small cells in the direct vicinity of large motor neurones. A/B, scale bar=100μm; C, scale bar=30μm; D, scale bar=10μm.*


mGluR1
mGluR2
mGluR3
mGluR4
mGluR5
mGluR7


A
mGluR1
B
mGluR1
C
mGluR1
D
mGluR3

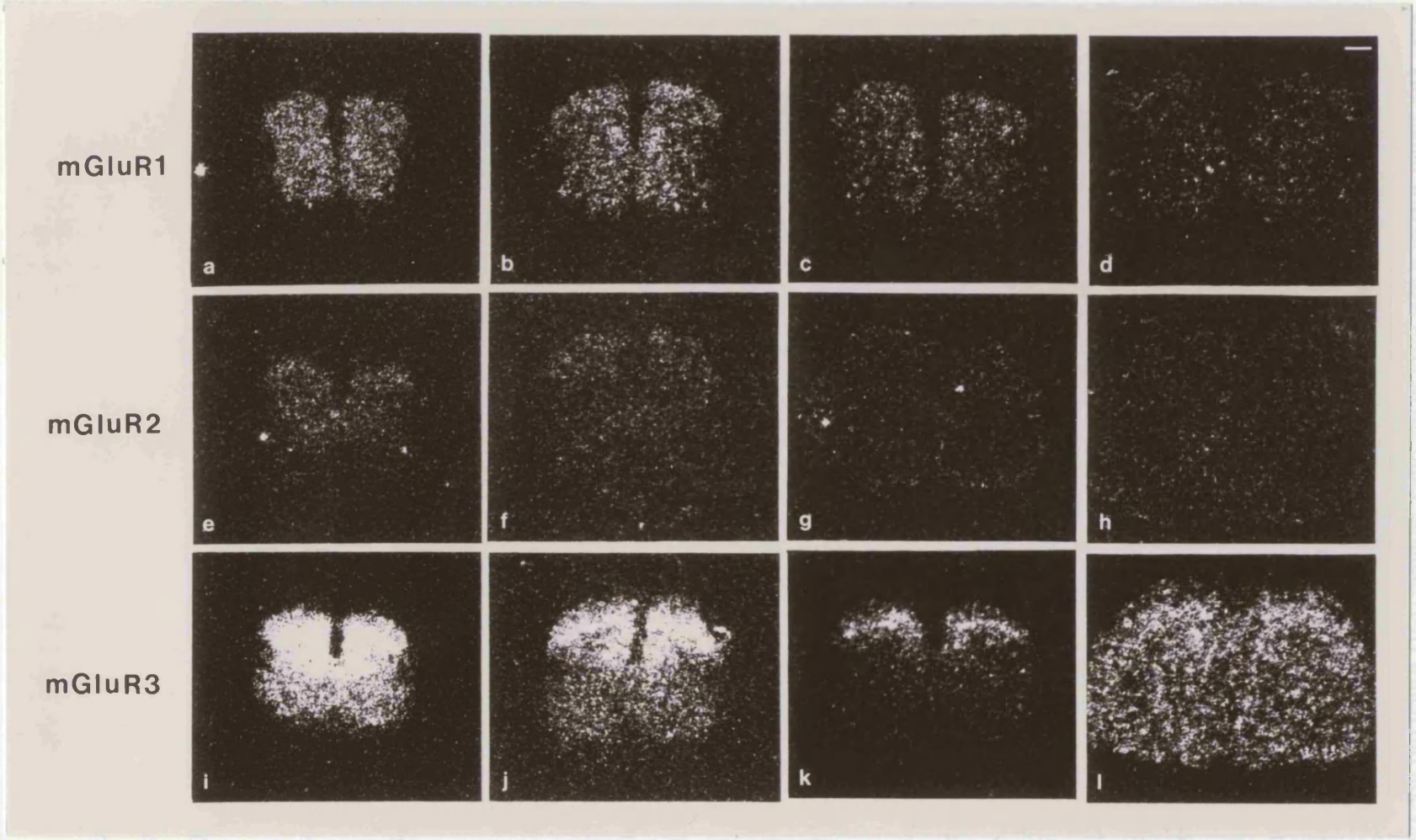
3.3.2 Distribution of mGluR subtype mRNA during development

All the mGluR mRNA subtypes showed significant changes in distribution in rat lumbar (L4-5) spinal cords during development. mGluR3 and 5 showed the most significant decrease in signal with an emergence of mGluR3 in the white matter after postnatal (PN) day 21 (refer to figures 3.6, 3.7 and 3.8).

In general, mGluR1 mRNA showed a diffuse signal over the dorsal horn with a slightly higher signal in the motoneurone pool (figure 3.6a-e). Expression in laminae I-III showed little change during early development, although a highly significant reduction in mRNA expression occurred between PN day 12 and 21. The deeper laminae of the dorsal horn showed a highly significant decrease in signal after PN day 1, remaining at a similar level until after PN day 12 when the signal decreased further to adult levels. The signal in the motoneurones showed minimal change with development (figure 3.8A), although expression in the ventral horn generally decreased, the number of labelled motoneurones had increased by PN day 21.

mGluR2 mRNA was seen at a barely detectable level in the dorsal horn of spinal cords taken from PN day 1 animals and this minimal expression had almost disappeared by PN day 7 (figures 3.6f-i and 3.8B). This finding is in agreement with published studies (Ohishi *et al* 1993). As it was not possible to reliably identify the motoneurone region on the autoradiographs due to such a low signal, this area was not investigated for mGluR2.

The change in mGluR3 mRNA expression was dramatic during the course of development. In general, mGluR3 mRNA expression was very high at PN day 1. At this stage of development the density of mRNA expression was highest in laminae II-IV, but high levels were also seen throughout the rest of the dorsal horn. These levels had significantly and gradually decreased by PN day 21. The deep laminae (lamina IV-V) showed the most dramatic decrease by PN day 7 when the level of expression appeared to stabilise (see figure 3.8C). The ventral horn showed lower expression of mGluR3 mRNA in comparison to the deep dorsal horn. A significant decrease in the level of expression was measured between PN day 1 and 7. There was a further decrease by PN day 12 and interestingly a significant increase was seen by PN day 21. At PN day 21 the mGluR3 signal became notably dispersed and could also be seen in the grey as well as the white matter. The latter may be a glial component of the signal (figures 3.6j-m and 3.8C).


mGluR1
mGluR2
mGluR3
a
b
c
d
e
f
g
h
i
j
k
l

The expression of mGluR4 mRNA in the dorsal horn showed little change with age. The signal was of a low level in the outer laminae (I-II) which did not vary with age. A slightly stronger signal was seen in the deeper laminae (laminae IV-V), which decreased in optical density during the third week of development (figure 3.8D). A distinct signal in the motoneurone pool developed with age and a significant mGluR4 mRNA labelling could be seen in the adult spinal cord (figures 3.7a-e and 3.8D).

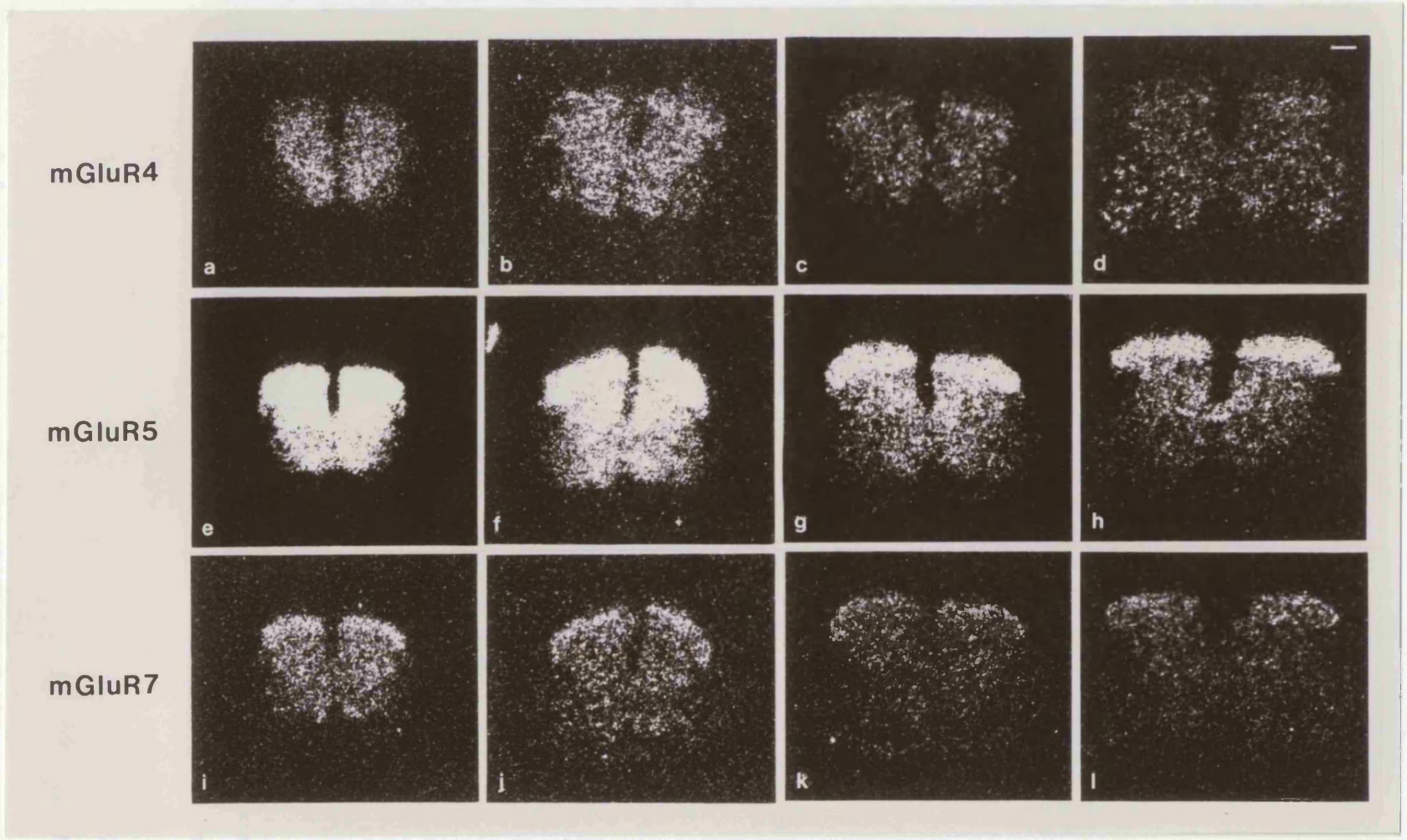
At PN day 1 mGluR5 mRNA showed a very strong signal in the dorsal horn. The outer laminae showed a relatively small but significant decrease in signal which remained high during the whole course of development. The most marked decrease in signal was detected in the deeper laminae, leaving a band of mGluR5 mRNA labelling in the outer laminae of the adult spinal cord. mGluR5 mRNA expression produced a low signal in the ventral horn, and the motoneurone pool (lamina IX) showed little change during development with the exception of a small significant decrease in signal between PN day 12 and 21 (figures 3.7f-i and 3.8E).

As shown previously, mGluR6 mRNA was undetectable in the spinal cord at all ages studied (data not shown). This was in agreement with published data (Nakajima *et al* 1993).

mGluR7 mRNA was relatively strongly expressed in the dorsal horn with the highest density found in the outer laminae (laminae I-II) and a low signal seen throughout the rest of the spinal cord, including the motoneurones. There was a tendency for a decrease in the dorsal horn signal with maturity while the motoneurones showed no alteration in expression throughout (figures 3.7j-m and 3.8F).

Figure 3.6 (Previous page) *Expression of mGluR subtype mRNAs in the lumbar (L4-5) spinal cord from postnatal day 0 to 21.* ***a-d)*** *mGluR1, PN day 0, 7, 12 and 21 respectively;* ***e-h)*** *mGluR2; i-l) mGluR3 mRNA-labelled coronal sections, respectively. Scale bar=260μm.*

Figure 3.7 (Next page) *Expression of mGluR subtype mRNAs in the lumbar (L4-5) spinal cord from postnatal day 0 to 21.* ***a-d)*** *mGluR4, PN day 0, 7, 12 and 21 respectively;* ***e-h)*** *mGluR5;* ***i-l)*** *mGluR7 mRNA-labelled coronal sections, respectively. Scale bar=260μm.*


mGluR4
mGluR5
mGluR7
a
b
c
d
e
f
g
h
i
j
k
l

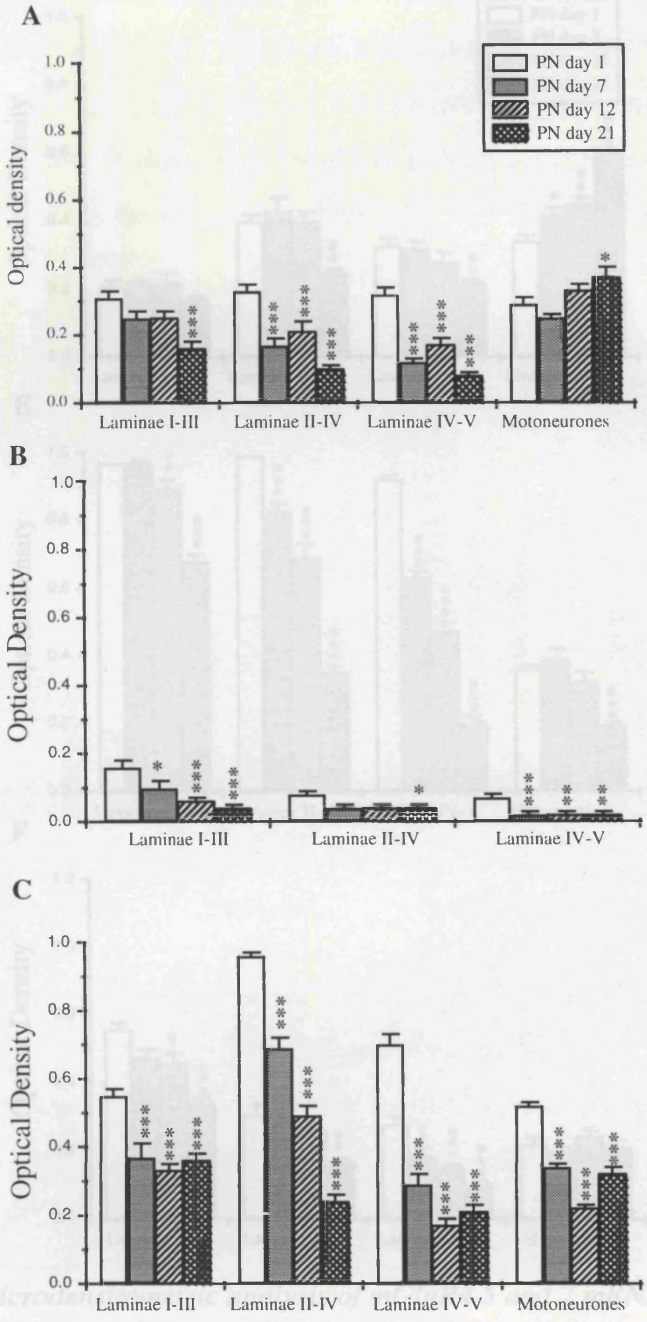


Figure 3.8 *Microdensitometric analysis of mGluR1,2 and 3 mRNA expression in the lumbar spinal cord of the rat during development. Each bar represents mean optical density ± SEM (n=3) measurements from different laminae or the spinal cord. Graphs are plotted on the same scale to illustrate the relative quantities of each mGlu receptor subtype.* **A)** *mGluR1;* **B)** *mGluR2;* **C)** *mGluR3. * p<0.05, *** p<0.001, ANOVA. Statistical significance is compared to PN day 1.*

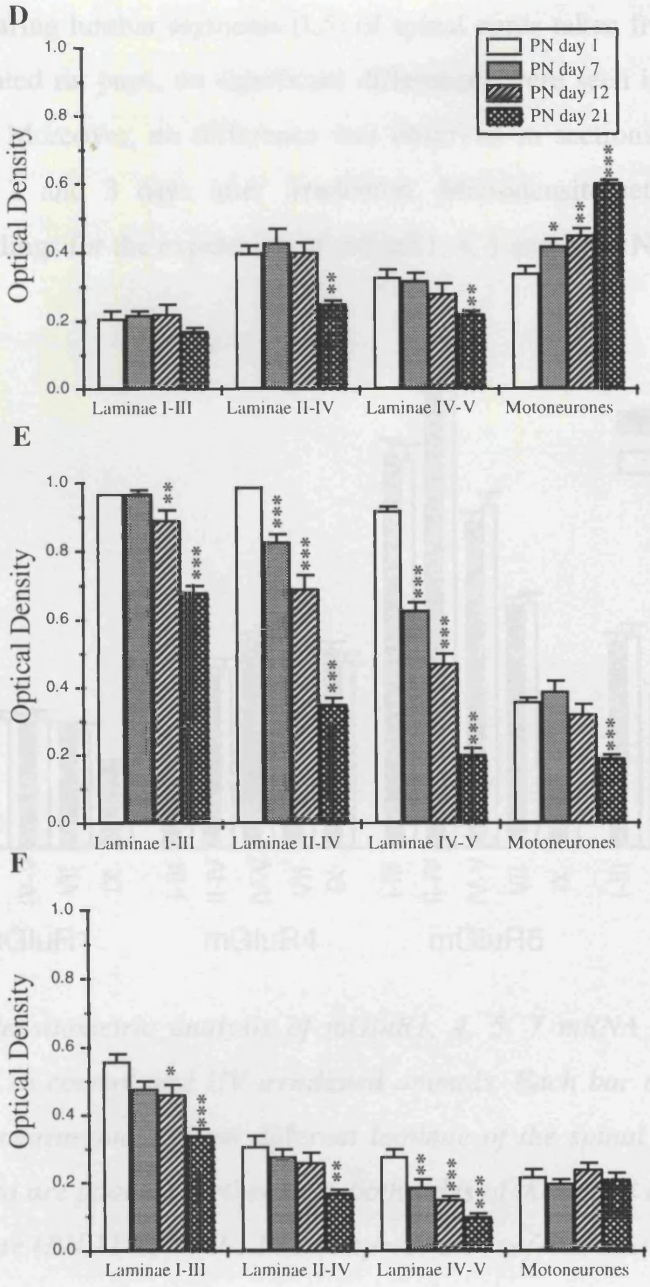


Figure 3.8 cont. *Microdensitometric analysis of mGluR4,5 and 7 mRNA expression in the lumbar spinal cord of the rat during development. Each bar represents mean optical density ± SEM (n=3) measurements from different laminae or the spinal cord. Graphs are plotted on the same scale to illustrate the relative quantities of each mGlu receptor subtype.* **D)** *mGluR4;* **E)** *mGluR5;* **F)** *mGluR7. * p<0.05, ** p<0.01, *** p<0.001, ANOVA. Statistical significance is compared to PN day 1.*

3.3.3 Changes in mGluR mRNA expression during inflammatory hyperalgesia

When comparing lumbar segments (L5) of spinal cords taken from PN day 11-13 control and UV-treated rat pups, no significant differences were seen in mGluR1, 2, 4-7 mRNA expression. Moreover, no difference was observed in sections taken from UV-treated animals 1, 2 and 3 days after irradiation. Microdensitometric analysis fully confirmed these findings for the expression of mGluR1, 4, 5 and 7 mRNA (figure 3.9).

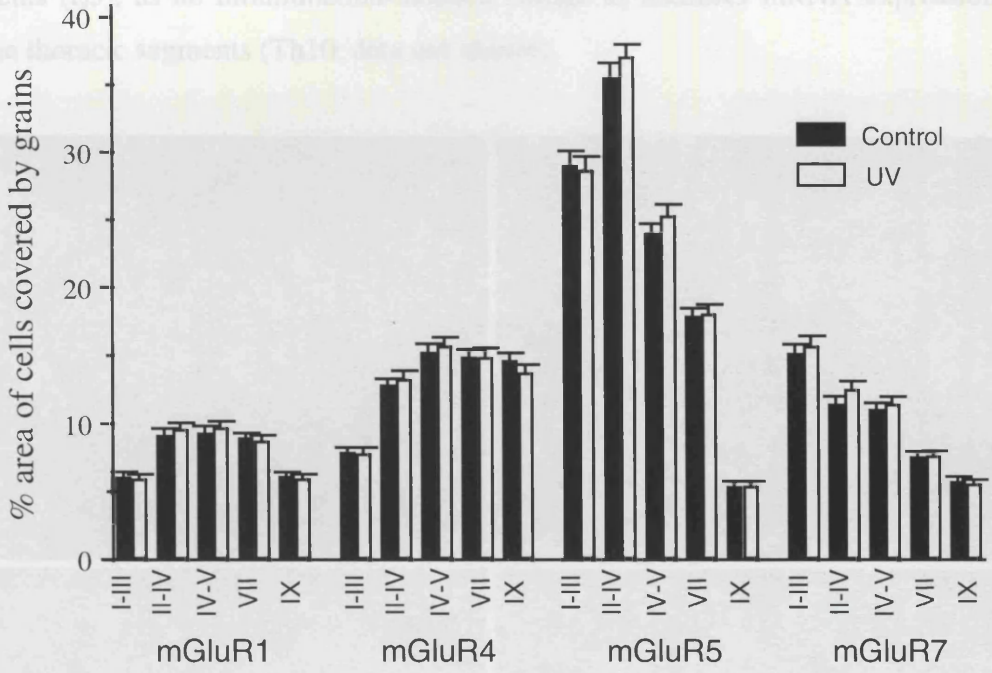


Figure 3.9 *Microdensitometric analysis of mGluR1, 4, 5, 7 mRNA expression in the lumbar spinal cord in control and UV irradiated animals. Each bar represents mean ± S.E.M. (n=17-18) measurements from different laminae of the spinal cord (see. Figure 4.1 for details). Data are pooled together from both sides of the spinal cords and over the three day time course (PN 11-13). Filled bars represent data from control, and open bars from UV-irradiated animals. There is no change in expression in any mGluR mRNA group. Changes in mGluR3 expression are discussed separately in Figures 3.10, 3.11.*

On the other hand, one day after the onset of inflammation, a significant increase was detected in the expression of mGluR3 mRNA in the superficial dorsal laminae and in the deep dorsal horn, on both sides of the spinal cord (figures 3.10 and 3.11). Ipsi- and contralateral changes in mGluR3 mRNA expression remained identical for the duration of the experiments, therefore quantified data from the two sides were pooled into one group.

On the first day after UV-irradiation, the level of mGluR3 mRNA was significantly increased in all regions of the spinal grey matter except lamina IX. The increase was transient and most pronounced in the superficial dorsal horn (laminae II-IV) and by the third day after UV-irradiation, there was no significant difference in mGluR3 mRNA expression compared to control in any area of the spinal cord (Figs. 3.10, 3.11).

Change in mGluR3 mRNA expression was restricted to the relevant lumbar segments (L5), as no inflammation-induced change in mGluR3 mRNA expression was seen in thoracic segments (Th10; data not shown).

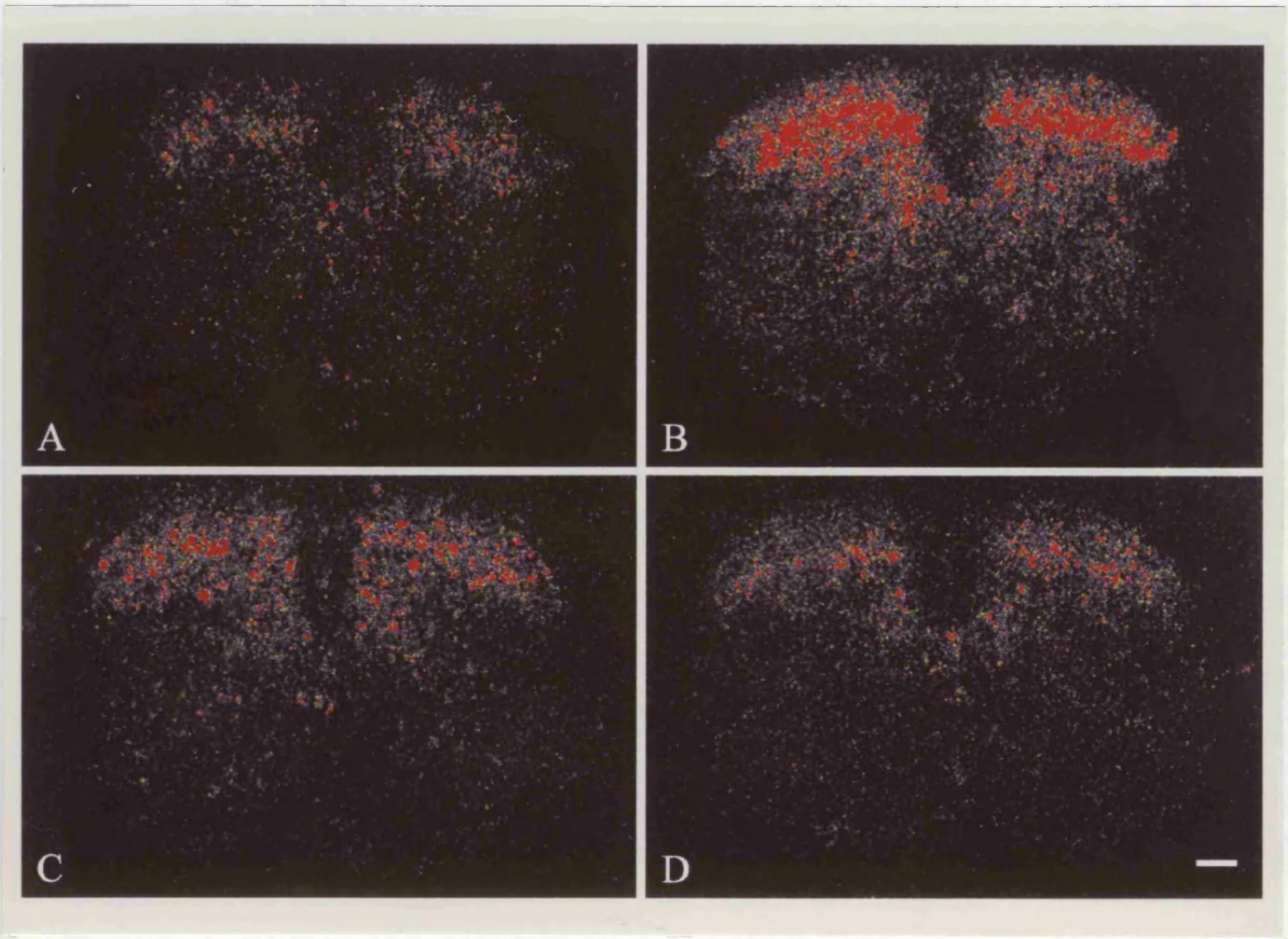


Figure 3.10 *Image analysis of changes in mGluR3 mRNA expression during the course of UV-induced hyperalgesia. In comparison to control (A), there is increase in mGluR3 mRNA expression 1 (B) and 2 (C) days after UV-irradiation with recovery at day 3 (D) post-UV. The pseudo-colours cover all grey values representing significant (>background) expression (red:maximum-yellow-green-blue:minimum). Scale bar= 200μm.*

The significant time-dependent change in mGluR3 mRNA expression followed a similar time course to the development of hyperalgesia after UV-irradiation in the whole animal. The most significant change in the expression of mGluR3 message coincided with the peak of mechanical hyperalgesia/allodynia on the first day after UV-irradiation. Ipsilateral hyperalgesia was maintained for a longer duration than changes in mRNA expression in the spinal cord (see chapter 2, figure 2.1).

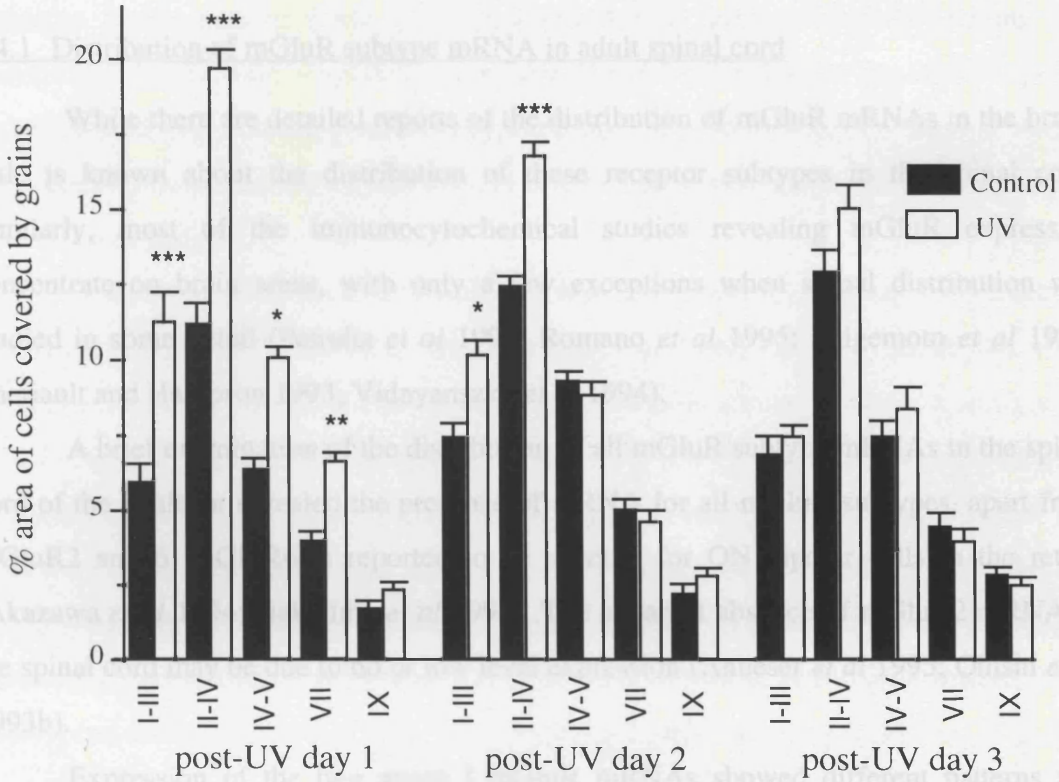


Figure 3.11 *Microdensitometric analysis of mGluR3 mRNA expression in the lumbar spinal cord in control and UV-irradiated animals. Each bar represents mean±S.E.M. (n=4-6) measurements from different laminae of the spinal cord (see. Figure 3.1 for details). Data are pooled together from both sides of the spinal cords. Filled bars represent data from control, and open bars from UV-irradiated animals. ***p<0.001; **p<0.01; *p<0.05, ANOVA.*

3.4 Discussion

This chapter shows a broad spectrum analysis of mGluR subtype mRNA expression in the lumbar spinal cord of rats during development, and describes changes in spinal distribution of mGluR mRNA in young rats (postnatal day 11-13) after UV-irradiation of the hindpaw.

3.4.1 Distribution of mGluR subtype mRNA in adult spinal cord

While there are detailed reports of the distribution of mGluR mRNAs in the brain, little is known about the distribution of these receptor subtypes in the spinal cord. Similarly, most of the immunocytochemical studies revealing mGluR expression concentrate on brain areas, with only a few exceptions when spinal distribution was studied in some detail (Petralia *et al* 1996; Romano *et al* 1995; Shigemoto *et al* 1992; Theriault and Hampson 1993; Vidnyanszky *et al* 1994).

A brief examination of the distribution of all mGluR subtype mRNAs in the spinal cord of the adult rat revealed the presence of mRNA for all mGluR subtypes, apart from mGluR2 and 6. mGluR6 is reported to be specific for ON bipolar cells in the retina (Akazawa *et al* 1994; Nakajima *et al* 1993). The apparent absence of mGluR2 mRNA in the spinal cord may be due to no or low level expression (Anneser *et al* 1995; Ohishi *et al* 1993b).

Expression of the two group I mGluR mRNAs showed different patterns and levels of distribution. While the signal for mGluR1 mRNA was relatively low and broad in the spinal grey matter, the expression of mGluR5 mRNA was very strong and comparatively localised to the dorsal horn.

mGluR3 mRNA (a Group II receptor subtype) showed expression throughout the dorsal horn but not in motoneurones. This is in good agreement with a study published by Ohishi *et al* (1993a). Interestingly, high magnification shows a discrete mGluR3 mRNA signal in small cells surrounding the motoneurones. These small cells may be glial but this has yet to be confirmed with specific co-localisation and glial markers.

Expression of mGluR4 and mGluR7 receptor mRNAs (Group III) showed different distributions. mGluR4 mRNA was expressed in a disperse manner over the grey matter. In contrast, mGluR7 mRNA was localised with a high concentration within the superficial dorsal horn, and to a somewhat lesser extent in the deep dorsal horn and the

lateral motoneuronal nucleus in the ventral horn. mGluR7 was found in previous studies on primary afferent fibres in the spinal dorsal horn (Ohishi *et al* 1995b) and also described by immunocytochemical methods in a broad population of dorsal root ganglia (DRG) cells which contain glutamate/aspartate (Li *et al* 1996). Based on these data, mGluR7 receptors have been suggested to act as presynaptic autoreceptors. Other mGluRs (mGluR4 and 8) have been localised on presynaptic terminals in the brain (Kinoshita *et al* 1996a,b). In the present study it was not possible to differentiate between pre- and postsynaptic localisation of mGluR expression. However, the distribution of silver grains was measured over cellular profiles suggesting postsynaptic localisation. Further studies are needed to determine the expression and distribution of presynaptic mGluR expression in dorsal root ganglia.

In addition to neurones, mGluR3 is also expressed in glial cells in the brain (Catania *et al* 1994; Fotuhi *et al* 1994; Jeffery *et al* 1996; Ohishi *et al* 1993a, 1994; Petralia *et al* 1996; Testa *et al* 1994b), however there is no similar evidence for this in the spinal cord. The method of staining used in this study (cresyl violet) allows identification of nerve cells but no distinction between glia and other cell types such as fibroblasts and cells from small capillaries. Film autoradiography did not show mGluR mRNA labelling in the white matter of the spinal cord of young animals. At present it is not possible to speculate on the existence and proportion of glial expression of any mGluR mRNA in the adult spinal cord.

3.4.2 Distribution of mGluR subtype mRNA during development of the spinal cord

Differences in receptor expression during development have been described in details for several types of receptors, eg. $GABA_A$ (Ma *et al* 1993; Poulter *et al* 1993), and NMDA (Hori and Kanda 1996; Laurie and Seeburg 1994; Portera-Cailliau *et al* 1996), however there is no comprehensive study, to date, on the age-dependence of mGluR expression in the spinal cord.

mGluR1 shows no change during development of the brain (Catania et al 1994), however there is a decrease in mRNA expression in the dorsal horn of the spinal cord with age. This decrease appears to be almost biphasic, with an initial highly significant drop in expression during the first week in the deeper laminae. Expression drops further throughout the dorsal horn during the third week of development. In contrast, the other

group I receptor, mGluR5, shows very high mRNA expression in the dorsal horn which decreases dramatically during development. The most pronounced decrease is in the deeper laminae of the dorsal horn. There is also a decrease in mRNA expression in motoneurones. Recent studies have suggested that mGluR5 may play an important role in early postnatal life (Nicoletti *et al* 1996) since stimulation of phosphoinositide hydrolysis by excitatory amino acids is greatest after birth and declines progressively during postnatal development (Nicoletti *et al* 1986). This early peak in phosphoinositide hydrolysis has since been shown to be due to mGluR5a (Bruno *et al* 1995c). The expression of mGluR5a-receptor mRNA in the brain is higher in early postnatal life than in adult life, when mGluR5b mRNA becomes predominant (Minakami *et al* 1995). It has been suggested that the mGluR5 subtype may play a role in neuronal survival as studies *in vivo* have shown that inhibition of group I mGluRs in infant rodents produced widespread degenerative effects in the retina (Price *et al* 1995). If mGluR5 plays a similar role in the developing spinal cord, the decrease in the mRNA expression of this subtype may be due to the switching of the splice variant from mGluR5a to mGluR5b, with mGluR5b showing a more localised distribution in the spinal cord compared to mGluR5a.

mGluR3 mRNA expression appears to decrease with age in the brain (Catania *et al* 1994) and this can also be seen in the dorsal horn of the spinal cord. The outer (laminae I-III) and deep (laminae IV-V) laminae show an initial decrease in mRNA expression after PN day 1, and this level of expression is sustained throughout development. Laminae II-IV show a very high level of mRNA expression at birth which then significantly decreases as the dorsal horn matures. The different rate of reduction in the mRNA signal in laminae II-IV compared to their bordering laminae, may suggest differing roles for mGluR3 in the different laminae. mGluR3 and mGluR5 in this area may participate in the same process as the two subtypes show parallel decreases in mRNA expression. mGluR3 is known to be present in glia in the brain, and the emergence of mGluR3 mRNA uniformly throughout both the white and grey matter at PN day 21 may suggest a glial component at this time point. Interestingly, Catania *et al* (1994) also describe the appearance of mGluR3 mRNA in small cells surrounding Purkinje cells in the cerebellum at PN day 21, but not before, suggesting a similar time course for a glial component in the brain. For a conclusive answer to this, the co-localisation of mGluR3 with a glial marker, such as GFAP, must be investigated.

mGluR4 shows little change in mRNA expression in the dorsal horn, but a marked increase in signal in the motoneurones. Catania *et al* (1994) suggested that this mGlu receptor subtype may be involved in the maintainance of synaptic function. mGluR7 mRNA is expressed at a relatively low level throughout the spinal cord and shows a small decrease in laminae I-IV (PN day 21). A significant decrease in mRNA expression was detected in the deep dorsal horn. There was no change in the level of expression in the motoneurone pool. mGluR7-like immunoreactivity has also been shown in primary afferent neurones (Ohishi *et al* 1995b) and mGluR7 mRNA expression in a subpopulation of larger DRG cells (Kinzie *et al* 1995). The localisation of the mRNA in the spinal cord suggests a role for this subtype in nociceptive processing, and the mRNA expression in the DRG implies that mGluR7 may be expressed in small diameter A fibres, possibly Aδ fibres. The role of mGluR7 at the postsynaptic site as suggested here is not clear at present.

The findings of this study are based on autoradiographical images which does not allow detailed cellular analysis. It is therefore not possible to determine which cell types express certain mGluR mRNA subtypes and indeed to localise specific changes during development. Another drawback of this method of visualisation is that it is not possible to take into account any changes in cell density with age. By definition, the spinal cords from PN day 1 animals are much smaller, but a brief histological overview shows that these spinal cords have approximately the same number of cells as the adult cords. As the X-ray signal is, by virtue, quite scattered, a dense cell population may give rise to a stronger radiographic signal compared to one produced by more dispersed cells. As it is not possible to evaluate the difference in cell density under the circumstances, I have assumed that the cell number per area is the same in neonatal and adult animals. With the results presented here it is also only possible to speculate on the potential functional role of changes in mGluR mRNA expression during development. The small changes in mRNA expression may or may not be seen with translation to receptor proteins or even manifest themselves physiologically. Autoradiographical studies investigating receptor levels may provide a clearer picture.

3.4.3 Changes in mRNA expression during inflammation

Several studies have reported early gene expression in the spinal cord after nociceptive stimulation (Tölle *et al* 1994a,b). Other studies have clearly indicated the induction of protein synthesis during painful peripheral pathological conditions (Hunt *et al* 1987). Indeed, an upregulation of certain transmitter receptors in the spinal dorsal horn, during peripheral inflammation has been shown (Dubner and Ruda 1992; McCarson and Krause 1994; Schäfer *et al* 1993; Zieglgänsberger and Tölle 1993). The prominence of mGluR3,5 and 7 mRNA in the superficial dorsal horn of the spinal cord suggests that these mGluRs may be involved in nociceptive processing. However, there has been no report on corresponding changes in mGluR expression during inflammatory hyperalgesia.

One day after irradiation, comparison of spinal cord sections taken from control and UV-irradiated animals showed an upregulation of mGluR3 mRNA expression in all areas of the spinal cord, except in motoneurones. No increase in mGluR3 mRNA expression was seen in lower thoracic segments taken from animals after UV-irradiation of the hindpaw, suggesting highly specific segmental localisation. None of the other mGluR subtype mRNAs showed significant alteration in distribution during the course of hyperalgesia and allodynia.

The most pronounced changes in mGluR3 mRNA expression were measured in the area where most C- and A-delta afferent fibres terminate in the superficial laminae (Cervero and Iggo 1980; Sugiura *et al* 1986). Upregulation of mGluR3 mRNA occurred in both sides of the spinal cord. These molecular observations coincide with behavioural data which showed mechanical hyperalgesia in both ipsi- and contralateral hindpaws in animals of the same age. Significant mechanical hyperalgesia and allodynia was measured up to three days after the second UV treatment. The time course of mGluR3 mRNA upregulation shows a rapid onset and similar duration to nociceptive behavioural changes. The rapid time course of changes is not surprising as the duration of hyperalgesia induced by UV-irradiation is about 50-70% shorter in 10-14 day old than in adult animals (Perkins *et al* 1993a; Thompson *et al* 1995b), therefore age dependent variation should be taken into consideration. In addition, in *in vivo* experiments, using anaesthetised adult rats, changes in mGluR-dependent nociceptive reflex responses took place within 6 hours after induction of inflammation in the knee joint (Neugebauer *et al* 1994).

Based on the present data it is not possible to state whether upregulation is related to neuronal and/or glial changes in mGluR mRNA expression. Recent evidence suggests that mGluR5 receptor expression is induced by growth factors in astrocytes (Miller *et al* 1995). Nevertheless, the lack of labelling with the mGluR3 probe in the white matter throughout the three days of hyperalgesia suggests that glial expression is not important in this phenomenon. Further experiments are needed to decide on this issue.

To date there are no suggestions for the role of changes in mGluR3 mRNA expression during hyperalgesia in the spinal cord. The area of upregulation suggests strong involvement in nociceptive transmission, but it is not known whether mGluR3 may serve as part of a compensatory inhibitory mechanism or contribute to the general hyperexcitability of wide dynamic range cells. Also, in the absence of immunohistochemical evidence regarding mGluR3 expression during inflammation, it is only possible to speculate that the changes in the expression of mGluR3 mRNA materialise in protein synthesis, thus production of functional receptors occurs. Bruno *et al* (1996) have shown that mGluR3 in glia is neuroprotective against NMDA excitotoxicity, and this neuroprotection can be prevented by blocking protein synthesis. *In vivo* data (Neugebauer *et al* 1994) suggest that novel activation of mGluRs happens during the development of hyperalgesia, and Tölle *et al* (1996) have shown a decrease in mGluR1 and mGluR4 mRNA in motoneurones 1 week following neurectomy. Activation of mGluRs also underlie chemically induced hyperexcitability of dorsal horn neurones which results in hyperalgesia (Young *et al* 1994). The reason for this increased involvement of mGluRs could be manyfold: upregulation (eg. NK1; McCarson and Krause 1994); changes in receptor phosphorylation (Urban *et al* 1994b; Zieglgänsberger and Tölle 1993); changes in mGluR-ionotropic EAA receptor interaction (Ambrosini *et al* 1995; Bruno *et al* 1995c; Colwell *et al* 1996; Kojic and Randic 1993; Meller *et al* 1993). There is no direct evidence for the upregulation of mGluR mRNA under pathological conditions, with the exception of mGluR4 upregulation in vulnerable regions of the rat brain following global ischemia (Iversen *et al* 1994).

None of the *in vivo* spinal cord studies (Neugebauer *et al* 1994; Young *et al* 1994) used selective receptor ligands, therefore nothing is known about the mGluR subtypes which contribute to the development of spinal hyperactivity. The relative lack of results concerning the effects of mGluR activation lies in the limited number of selective

pharmacological tools. *In vivo* experiments are further hindered by the low potency of selective ligands. Most information about the spinal pharmacology of mGluRs is derived from *in vitro* experiments. In spinal neurones mGluR activation contributes to activity dependent spinal hyperexcitability during windup (see chapter 3) and LTP (Kojic and Randic 1993). The exact mechanism and the involvement of specific mGluRs in these phenomena are not known at present. Single cell intracellular studies indicate that mGluR activation, in general, could enhance the activity of ionotropic excitatory amino acid receptors (Aniksztejn *et al* 1992; Bleakman *et al* 1992; Jones and Headley 1995) and modulate L-type Ca^{2+} currents (Morisset and Nagy 1996). None of these effects were firmly associated with the activation of any particular mGluR subtype.

mGluR3 belongs to Group II mGluRs which are negatively coupled to adenylyl cyclase (Prezeau *et al* 1992). Inhibition of cyclic AMP may down regulate transcription and signal transduction (Schoepp and True 1992), which in turn reduces the receptor's own transduction. This may explain the rapid decrease in upregulation that is seen with time. Group II receptors can also potentiate an increase in cAMP formation, independently of kinase activity, possibly via synergistic interactions by G-protein $\beta\gamma$ subunits with G_S-coupled receptors (Gereau and Conn 1994; Winder and Conn 1995) through Group I mGluRs and/or adenosine receptors (Schoepp *et al* 1996). Lodge *et al* (1996b) have shown synergy between Group I and Group II agonists in the neonatal rat spinal cord with respect to potentiation of ionotropic responses but no pharmacological data exist on Group II mGluR contribution during hyperalgesic conditions. Therefore, at this stage of the experiments it is highly speculative to suggest a precise physiological function of the upregulation of mGluR3 mRNA during inflammation.

3.5 Conclusion

In summary, experiments have shown that mGluR1,3,4,5,7 mRNA is expressed in the spinal cord of the adult rat. In general, there is a decrease in mGluR mRNA expression during early postnatal development with the exception of mGluR4 mRNA expression in motoneurones, which increases. Highly significant changes occur to the expression of mGluR3 and 5 during development, suggesting a role for these receptors in the maturation of the spinal cord and reflecting different developmental stages in spinal functions. Peripheral injury produced by UV-irradiation of the hindpaw induced

upregulation of mGluR3 mRNA specifically in the lumbar segments (L4-5) on both sides of the spinal cord. The time course of this upregulation was similar to that of mechanical hypersensitivity described after UV-irradiation. The change in mGluR3 expression may contribute to the development of spinal hyperexcitability during peripheral inflammatory conditions.

4. CHAPTER 4

PHARMACOLOGICAL CHARACTERISATION OF mGluRs IN THE *in vitro* HEMISECTED SPINAL CORD

4.1 Introduction

Although the presence of metabotropic glutamate receptors (mGluR) in the spinal cord have been shown by molecular biological and physiological methods (see Chapter 1), comparatively little is known about their contribution to spinal somatosensory transmission. The diverse distribution and second messenger coupling of these receptors in the CNS may induce excitation (McBain *et al* 1994) or inhibition (Gereau and Conn 1994) of synaptic events, depending on the presence of the subtype and location of the mGluR, indicating the potential for a diverse function for these receptors within the CNS.

A major role for the mGluR has been suggested in plastic changes within the CNS. Both long term potentiation (LTP; Bashir *et al* 1993) and long term depression (LTD; Aiba *et al* 1994b; Pin and Duvoisin 1995) may depend on the activation of mGluRs, and the effects of their activation have been extensively studied in the hippocampus, cortex and cerebellum (Pin and Bockaert 1995; Schoepp *et al* 1991). However, experiments which have studied the role of mGluRs in spinal excitability changes have been conflicting. Firstly, it has been shown that the monosynaptic activation of spinal motoneurones was depressed by the activation of mGluRs (Ishida *et al* 1993b). This was thought to be via a presynaptic site on the primary afferents as mGluR agonists did not have any direct effect on depolarising responses elicited by ionotropic glutamate agonists in spinal motoneurones. On the other hand, the selective mGluR agonist, trans-ACPD, potentiated AMPA and NMDA-evoked responses in dorsal horn cells (Bleakman *et al* 1992; Cerne and Randic 1992; Meller *et al* 1993; Palacek *et al* 1994). Although all of these findings implicate a modulatory role for mGluRs in sensory processing in the spinal cord, other experiments using the non-selective mGluR antagonist, L-AP3, failed to support the hypothesis (Birse *et al* 1993; Thompson *et al* 1992). More light was shed on this discrepancy by Neugebauer *et al* (1994), who showed the functional presence of mGluRs in the spinal dorsal horn by iontophorising trans-ACPD, and revealed that the mGluR contribution to reflex transmission was dependent on the development of peripheral inflammation. Similarly, the contribution of mGluR to spinal nociceptive processing was also seen following mustard oil induced skin inflammation (Young *et al* 1994). It was apparent from these experiments that mGluR activation may play an important role in the maintenance of spinal hyperexcitability resulting in hyperalgesia and pain, with a less important role in "normal" spinal sensory processing.

NMDA and NK receptor activation has been implicated in the hyperexcitability of spinal circuitry following peripheral inflammation (see chapter 1; Coderre and Melzack 1992; Thompson *et al* 1994; Woolf and Thompson 1991). As a result of this and the mounting evidence for a contribution of the G protein-coupled mGluRs in sensory processing following peripheral hyperalgesia, I have investigated the role of these receptors in the spinal nociceptive reflex. I have used an *in vitro* preparation of the juvenile rat hemisected spinal cord to investigate the selectivity of some commercially available mGluR ligands. I have then used these ligands to investigate whether mGluRs are recruited during sensory nociceptive transmission and whether or not there are any changes in mGluR function in conditions of spinal hyperexcitability following peripheral UV-induced inflammation.

4.2 Methods

Hemisected spinal cords were prepared as previously described (Thompson *et al* 1992) from 10-14 day old rat pups (weight 24-26g). Following Enflurane anaesthesia animals were killed by decapitation and spinal cords removed and placed into aerated (95% O_2 and 5% CO_2) and cooled ACSF (in mM: 138 NaCl; 3.35 KCl; 21.0 $NaHCO_3$; 0.58 NaH_2PO_4; 10.0 glucose; 1.16 $MgCl_2$; 1.26 $CaCl_2$; pH 7.4). Spinal cords were hemisected and transferred to a recording chamber (figure 3.1) and superfused at approximately 5ml/min with aerated ACSF solution at room temperature (18-22^oC). Spinal reflex activity was evoked by either dorsal root stimulation or drug application to the spinal cord and measured as a ventral root potential (VRP). VRPs were recorded with close-fitting suction electrodes from the L_5 ventral root following electrical stimulation of the ipsilateral L_5 dorsal root. The stimulus intensity used in the present experiments (50V, 200μs) was equivalent to that used in a previous study (Thompson *et al.*, 1994). This stimulation intensity evokes a compound action potential in the dorsal root which corresponds to recruitment of afferent fibres conducting within the C afferent fibre range as well as Aβ and Aδ afferents. Single electrical pulses or short duration (20 secs) trains at 1, 5 and 10Hz were used throughout these experiments. Ventral root responses were amplified (Neurolog), digitised using a Labmaster DMA interface and recorded onto a PC using the SCAN software (J. Dempster, Strathclyde University).

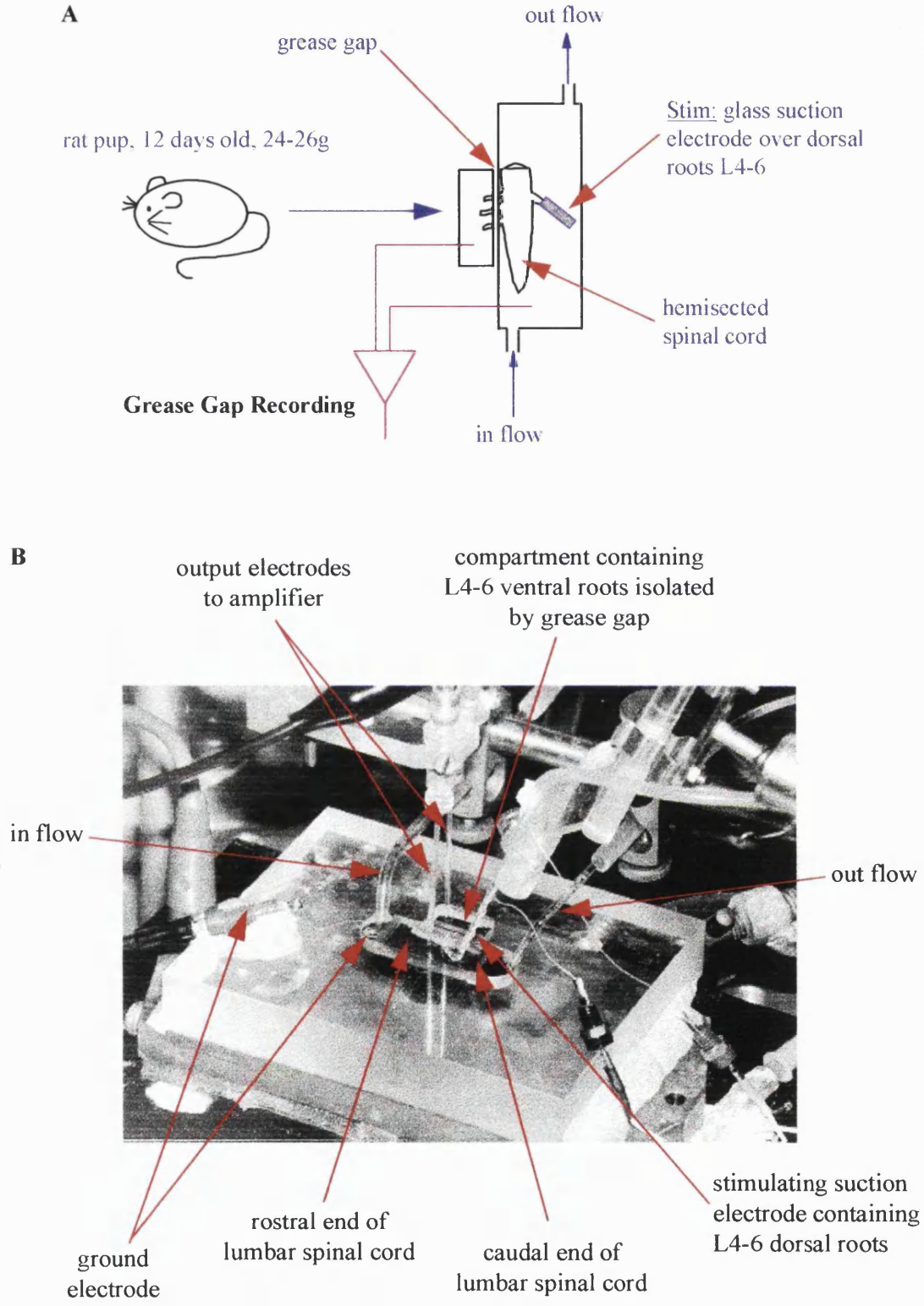


Figure 4.1 A) *Diagram showing the set-up for the hemisected spinal cord.*

B) *Photograph showing details of the hemisected spinal cord set-up.*

4.2.1 Drug induced responses

The effect of selective excitatory amino acid agonists and antagonists was investigated by superfusing ligands over the spinal cord. Dose response curves for agonists were constructed by superfusing increasing concentrations until a maximum effect was reached. Responses were calculated as a percentage of a preceding windup response (50V, 200μs, 5Hz), which was taken as the maximum response of the tissue. The inhibitory effects of antagonists were investigated using increasing concentrations against a single, near maximal concentration of an agonist. The responses were measured as the amplitude of the response to the agonist in the presence of the antagonist compared to an averaged initial and final dose of the agonist in the absence of the antagonist. Agonists were superfused into the spinal cord compartment for 30 seconds. Antagonists were superfused for 15 minutes prior to the addition of agonists and were continuously present throughout superfusion of the agonist. Complete recovery was obtained between doses. Responses were recorded as a ventral root response from the L_5 ventral root using the grease gap method, and the maximum amplitude of the response was measured (figure 4.2).

Figure 4.2 *Typical trace from a 30 second application of the selective mGluR agonist, 1S,3R-ACPD to the spinal cord. The peak amplitude of the response is measured as indicated by the arrows.*

In a separate set of experiments, instead of electrical stimulation, a selective excitant, capsaicin, was used to characterise spinal cord activity induced by C- and polymodal Aδ-fibre activation. Capsaicin selectively excites polymodal nociceptive Aδ- and C-fibres (see Bevan and Szolcsányi 1990) and therefore provides an excellent tool to study the spinal nociceptive input. Capsaicin was superfused for 30 seconds in the presence of Concanavalin A. Concanavalin-A (1.0 μM) was superfused to the spinal cord 15 minutes prior to, during and after capsaicin application to prevent desensitisation as previously described (Dray *et al* 1992b; Nagy *et al* 1993). A wash-out period of 60 minutes was maintained between all drug applications.

All data are expressed as mean ± S.E.M. Statistical comparisons were performed using a paired Student's *t*-test.

4.2.2 Single shock electrical stimulation

The afferent-evoked ventral root potential (DR-VRP) shows progressive recruitment of several distinct waves which correspond to the recruitment of the different groups of primary afferent fibres. High intensity stimulation evokes a compound action potential (figure 4.3) which has been described and characterised by Thompson *et al* (1992). The response has an early phase which is a short duration response, corresponding to the recruitment of large myelinated fibres, and a late phase which is a long duration response. The late phase can be divided into slow and prolonged phases corresponding to the recruitment of Aδ and C fibres respectively. The early phase can be attenuated by the AMPA antagonist, 6-nitro-cyanoquinoxaline-2,3-dione (CNQX), and the slow peak of the late response can be significantly reduced by the NMDA antagonist, D-AP5, with both compounds being selective for their respective responses (Thompson *et al* 1992). The prolonged response can be reduced by tachykinin antagonists, suggesting that this response may be mediated by neurokinin receptors (NK1 and NK2; Thompson *et al* 1993a). As tachykinin antagonists do not completely abolish the prolonged response, other types of G-protein coupled receptors may also be involved. The waveform of the VRP closely resembles the time course and frequency of postsynaptic activity of segmentally activated motoneurones thus providing a convenient and accurate measure of spinal excitability associated with the nociceptive flexor reflex. Following single shock electrical stimulation, the integrated area between 0 and 8 seconds was measured, encompassing the majority of the prolonged response as described elsewhere in detail (Thompson *et al* 1994). A fibre evoked responses were not investigated in this thesis.

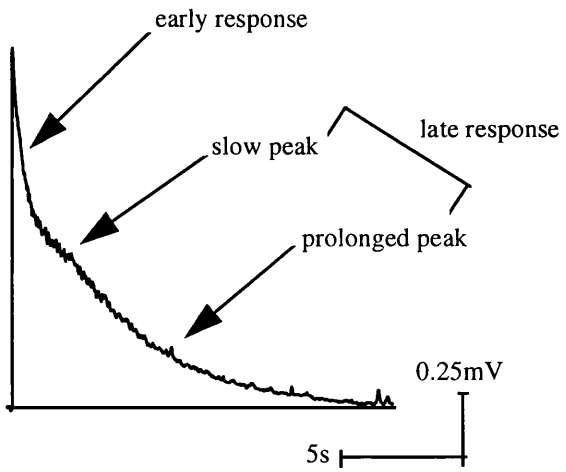


Figure 4.3 *Typical afferent-evoked ventral root potential recorded following single shock stimulation of the L5 dorsal root at 50V, 200μs. Arrowheads indicate approximate positions of the slow and prolonged peaks.*

4.2.3 Cumulative electrical stimulation ("windup")

Low frequency (1-10Hz, 20 secs. duration) stimulation of high threshold dorsal root afferents was also investigated. Repetitive stimulation at 1-10Hz evoked a temporal summation of synaptic activity generating a progressively depolarising VRP whose amplitude was graded with the frequency of stimulation (figure 4.4). This depolarisation, known as "windup", is thought to mimic the mechanisms responsible for postinjury hypersensitivity and is not seen with low intensity stimulation under normal conditions (Mendell 1966; Thompson *et al* 1994). In the experiments presented here, the peak amplitude of the cumulative ventral root responses was measured (see figure 4.4).

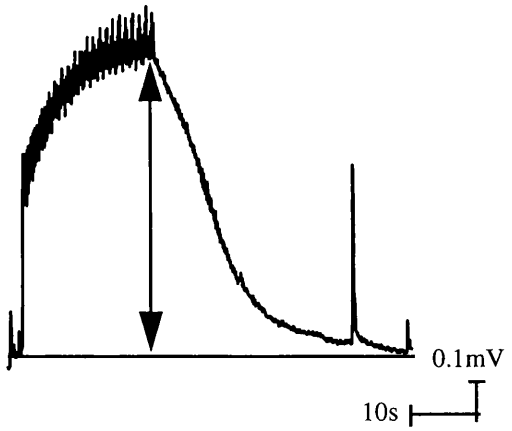


Figure 4.4 *Typical cumulative ventral root potential following low frequency (5 Hz) C-fibre stimulation of the dorsal root for 20 seconds. The arrows indicate the peak amplitude of the cumulative VRP which is measured.*

4.2.4 Experiments carried out on hyperalgesic animals

Similar experiments were carried out using spinal cords taken from naïve animals as well as those that had been irradiated by UVA light, thus rendering them hyperalgesic (see chapter 2). For experiments with hyperalgesic animals I used the same *in vitro* protocol as for naïve animals as explained above. The left hindpaw was irradiated at postnatal day 10 and again after 18 hours (see protocol for chapter 2). The animals were decapitated under deep anaesthesia and dissected 24 hours after the second dose of UV irradiation.

4.2.5 Compounds used

The NMDA receptor agonist, *N*-methyl-D-aspartic acid (NMDA), the mGluR agonists (1S,3R)-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD), (1S,3S)-ACPD and (2S,1'S,2'S)-2-(carboxycylopropyl)glycine (L-CCG-I), the selective NMDA receptor antagonist D(-)-amino-5-phosphonopentanoic acid (D-AP5), the stereospecific selective mGluR antagonists, (S)-α-methyl-4-carboxyphenylglycine (MCPG; Jane *et al* 1993), L(+)-2-amino-3-phosphonopropionic acid (L-AP3) and (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine (MCCG), and capsaicin were all obtained from commercial sources (Sigma, Tocris) and freshly prepared prior to each experiment. Drugs were

dissolved in ACSF from stock and superfused into the recording chamber at the same flow rate as control ACSF.

4.3 Results

4.3.1 Pharmacological characterisation of mGluR ligands in control and inflammatory conditions

In this section the activity of mGluRs in control and inflammatory conditions will be addressed. Neugebauer *et al* (1994) reported that the contribution of the mGluRs to sensory processing was only revealed during the development of peripheral inflammation. They showed that, although mGluR agonists excited spinal cord neurones, responses evoked by peripheral mechanical stimulation were attenuated only during the development of inflammation in the area of articular innervation. My experiments were therefore designed to re-examine the contribution of mGluRs to sensory transmission in the isolated spinal cord under control conditions and to investigate changes in an inflammatory hyperalgesia model of the rat.

I have investigated whether the agonists and antagonists of mGluRs acted in a concentration dependent manner in the isolated spinal cord, and established EC_{50} and IC_{50} values respectively. These values were then used as guidelines for functional experiments (see section 4.3.2 and 4.3.3). Due to the low potency of the available mGluR ligands and consequent high concentrations used, selectivity of the compounds was questioned. Therefore, initial experiments were carried out to characterise the selectivity and specificity of the compounds.

The selective mGluR agonist, (1S,3R)-ACPD, superfused to the naïve spinal cord for 30 seconds produced reproducible and concentration-dependent ventral root depolarisations with a maximum amplitude reached at 500μM (EC_{50}=58 ± 7μM; n= 4) (figure 4.5).

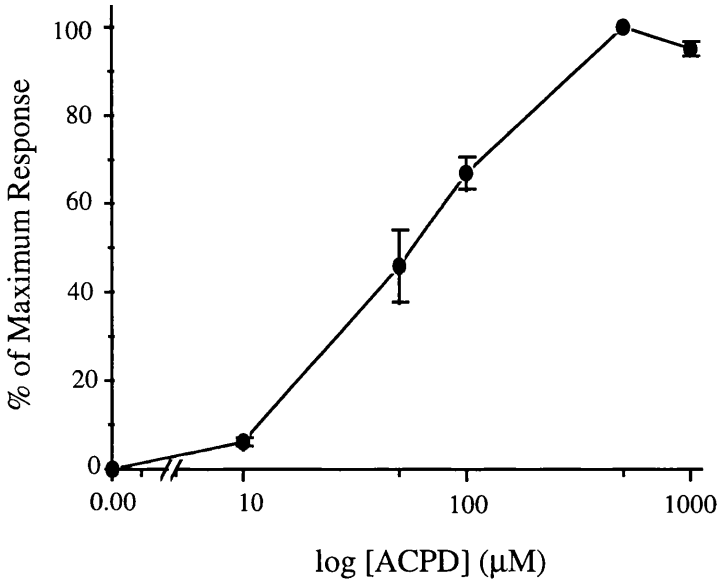


Figure 4.5 *Concentration-dependent depolarisation of the spinal ventral root to bath application of (1S,3R)-ACPD (10μM - 1.0mM). Values are expressed as percentage of the maximum response to (1S,3R)-ACPD in the same preparation.* EC_{50}*=58.1 ± 7.2μM (n=4).*

The potency of selective ionotropic and metabotropic glutamate receptor antagonists was subsequently determined against concentrations of agonists which evoked submaximal ventral root responses (NMDA: 100μM (Thompson *et al* 1994); (1S,3R)-ACPD: 100μM). NMDA-induced ventral root responses were blocked in a concentration-dependent manner by D-AP5 in the concentration range of 1-100μM (IC_{50}=15.8 ± 3.57μM, n=3-5), consistent with earlier observations (Thompson *et al* 1990; figure 4.6B). The selective mGluR antagonist, MCPG (100μM - 5mM), had no effect on the NMDA-induced ventral root responses (Fig. 4.6A/B). (1S,3R)-ACPD-evoked ventral root responses were blocked by MCPG (IC_{50}=243 ± 61μM; n=4) in a concentration-dependent manner (figure 4.7A/B). Correspondingly, D-AP5 (10μM - 1.0mM) had no effect on (1S,3R)-ACPD evoked ventral root responses (figure 4.7B). Neither antagonist affected the baseline potential of the ventral root (n=5). From these experiments 40μM D-AP5 and

100μM - 5mM MCPG were confirmed as being suitably specific and effective concentrations to study the contribution of the NMDA and mGlu receptors to segmental reflex mechanisms.

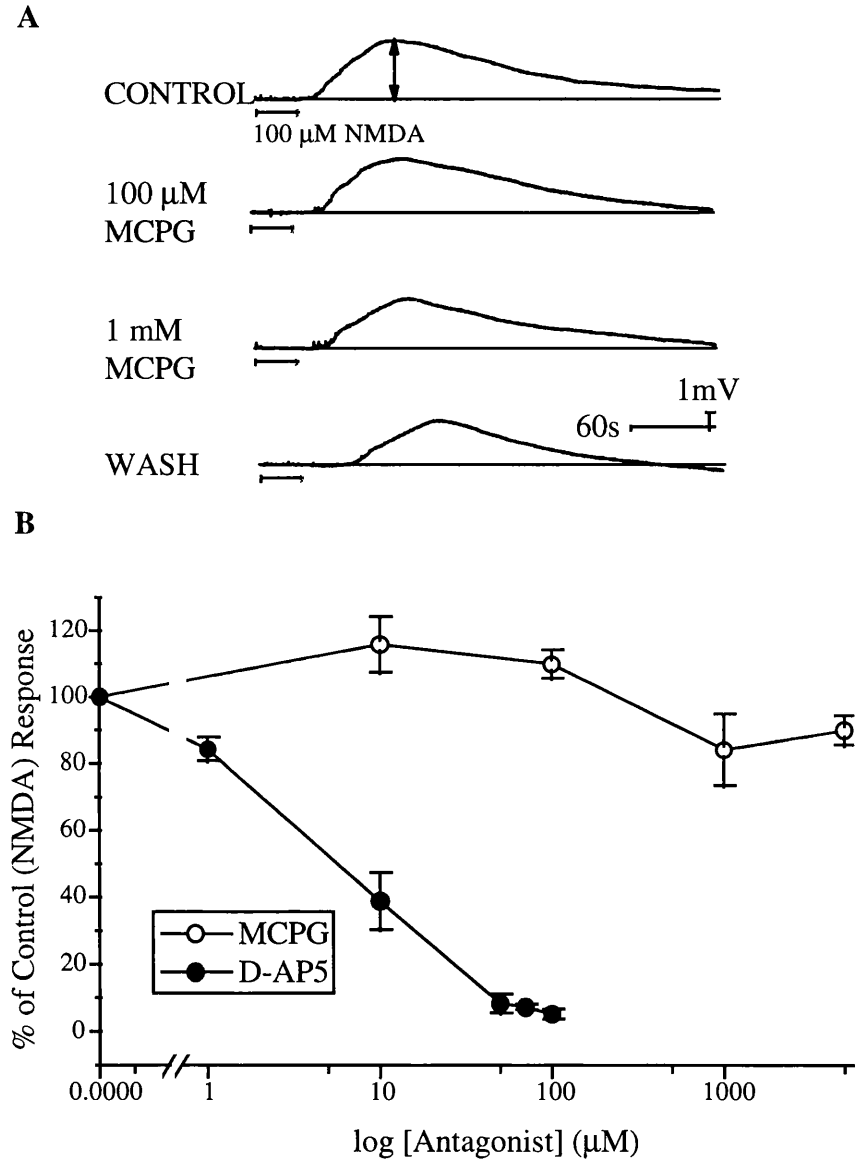


Figure 4.6 *Selective, concentration-dependent effects of D-AP5 and MCPG on the peak amplitude of the ventral root depolarisations evoked by NMDA.*

A). *Representative traces demonstrating the lack of effect of MCPG to block NMDA-induced ventral root depolarisation in the hemisected rat spinal cord. (100μM NMDA was superfused for 30 sec as indicated by the bars below the traces).*

B). *Mean data (n=3-5) show inhibition of the amplitude of the NMDA-evoked ventral root depolarisation by the selective NMDA receptor antagonist, D-AP5 (1 - 100μM) but not by the selective mGluR antagonist, MCPG (1.0μM - 5.0mM).*

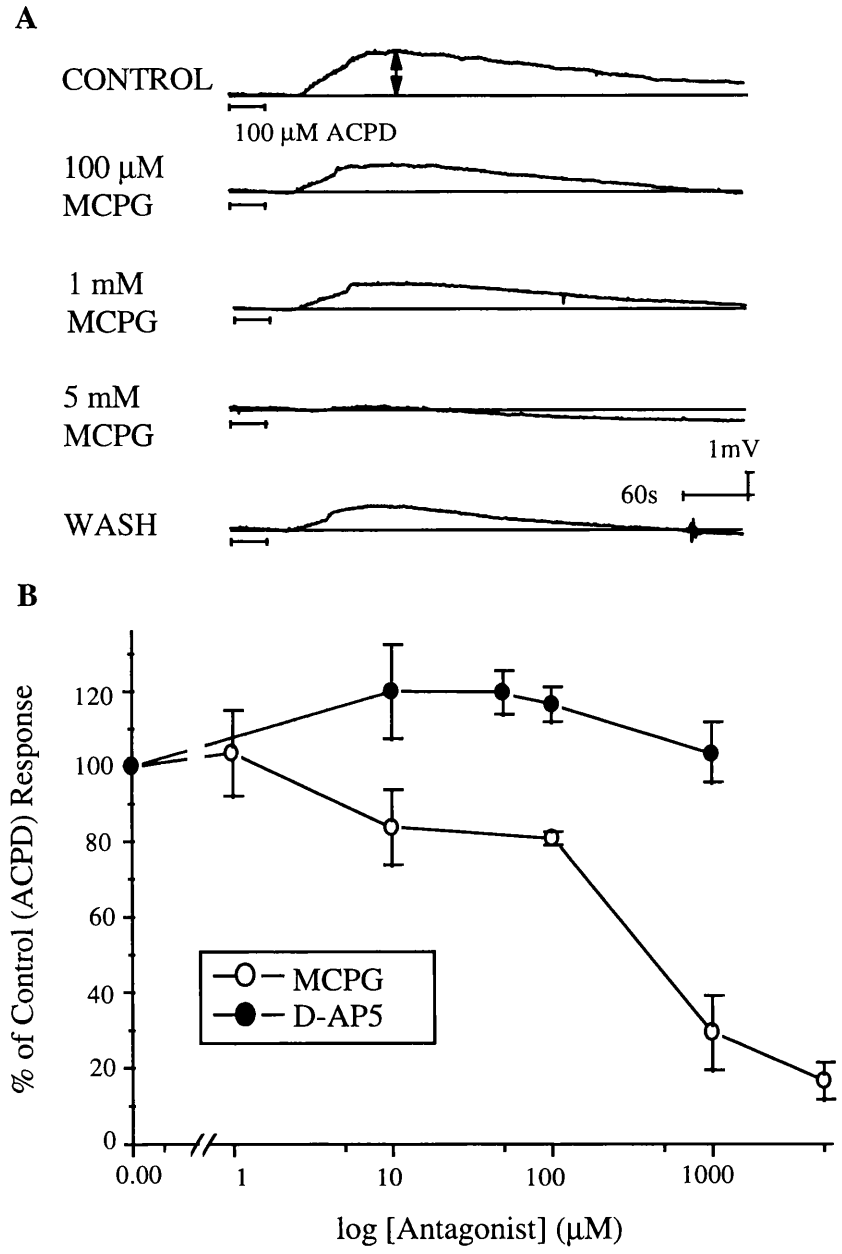


Figure 4.7 *Selective, concentration-dependent effects of MCPG on the peak amplitude of the ventral root depolarisations evoked by (1S,3R)-ACPD.*

A). *Characteristic traces demonstrating the dose-dependent inhibitory effect of MCPG on (1S,3R)-ACPD-evoked ventral root depolarisation. (100μM (1S,3R)-ACPD was superfused for 30 sec as indicated below the traces).*

B). *Mean data (n=3-5) show inhibition of the amplitude of (1S,3R)-ACPD-evoked ventral root depolarisation by MCPG (1.0μM - 5.0mM) but not by the selective NMDA receptor antagonist, D-AP5 (10μM - 1.0mM).*

The next series of experiments compared the effects of the selective, non-specific mGluR agonist, (1S,3R)-ACPD, in the spinal cord of naïve rats and animals with inflammatory hyperalgesia. Superfusion of (1S,3R)-ACPD to the spinal cord produced a concentration-dependent, reversible ventral root depolarisation in both naïve and hyperalgesic animals with no significant difference in EC_{50} values (55.5 ± 6.36μM and 51.0 ± 5.76μM respectively, n=4; figure 4.8; table 4.1). However, the maximum amplitude of (1S,3R)-ACPD-induced responses in spinal cords dissected from hyperalgesic animals was significantly enhanced by 23% ($p<0.05$) compared to control animals.

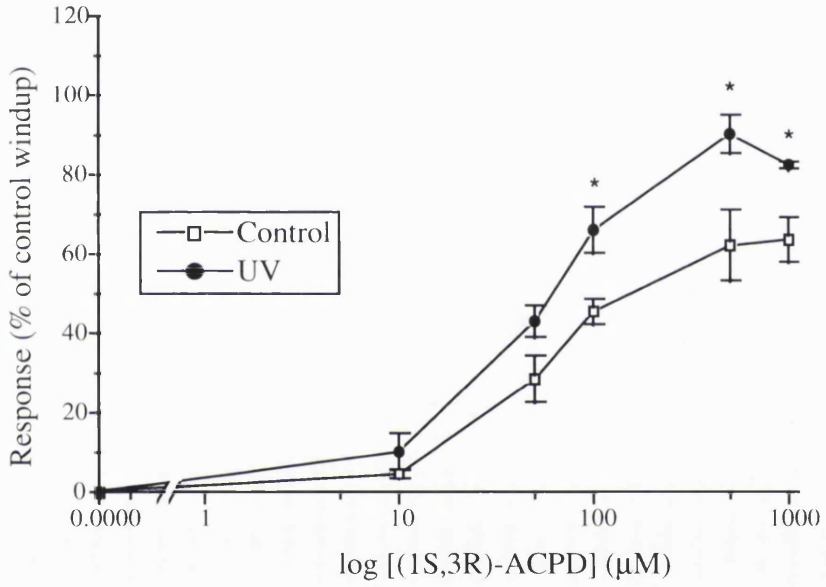


Figure 4.8 *Concentration-dependent depolarisation of the spinal ventral root to bath application of the non-selective mGluR agonist, (1S,3R)-ACPD (1.0μM - 1.0mM), in control and UV-treated rats. Values are expressed as mean percentage ± SEM of the maximum amplitude of the windup response (5 Hz) in the same preparation in the absence of any ligands (n=3-4). *=p<0.01, ANOVA.*

The increase in maximal response to (1S,3R)-ACPD in UV-treated animals was abolished by the selective NMDA receptor antagonist, D-AP5 (40μM), bringing the concentration response curve to the level seen in control animals. On the other hand, D-AP5 did not affect the concentration response curve produced by (1S,3R)-ACPD in control animals, or the EC_{50} values in both control and UV-treated animals (EC_{50}=43.5 ±

9.74μM and 48.2 ± 5.75μM respectively, in the presence of 40μM D-AP5; figure 4.9; table 4.1).

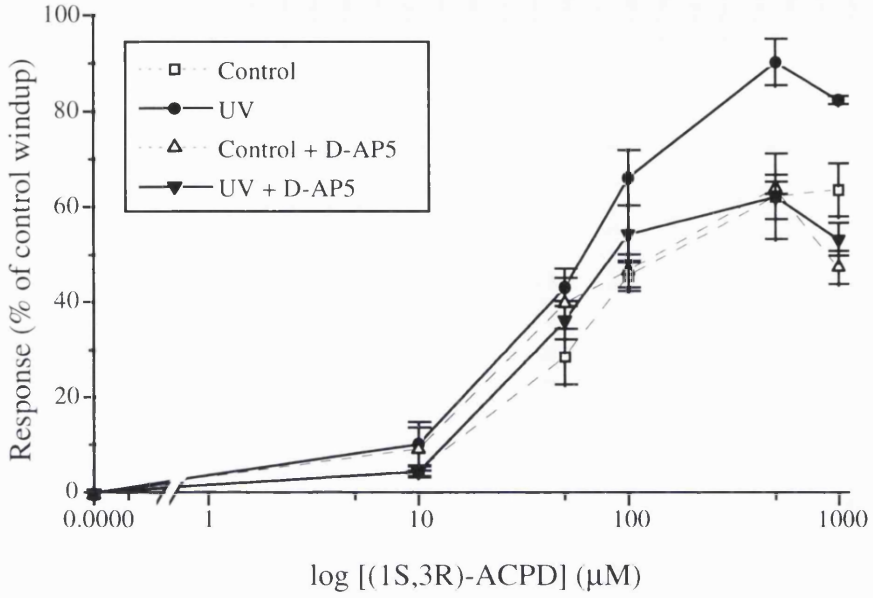


Figure 4.9 *Concentration response curve to (1S,3R)-ACPD (10μM - 1.0mM) in control and UV-treated rats in the presence of the selective NMDA receptor antagonist, D-AP5 (40μM). Values are expressed as a mean percentage ± SEM of the maximum amplitude of the windup response (5 Hz) in the same preparation in the absence of any ligands (n=4-6).*

When (1S,3R)-ACPD (100μM) was superfused to the spinal cord in the presence of increasing concentrations of the selective, non-specific mGluR antagonist, MCPG (1.0μM-5.0mM), a similar concentration-dependent reduction was revealed in naïve and UV-treated animals. The IC_{50} values were calculated as 289 ± 63.4μM and 288 ± 66.7μM respectively (figure 4.10). There was no significant difference between naïve and UV-treated animals.

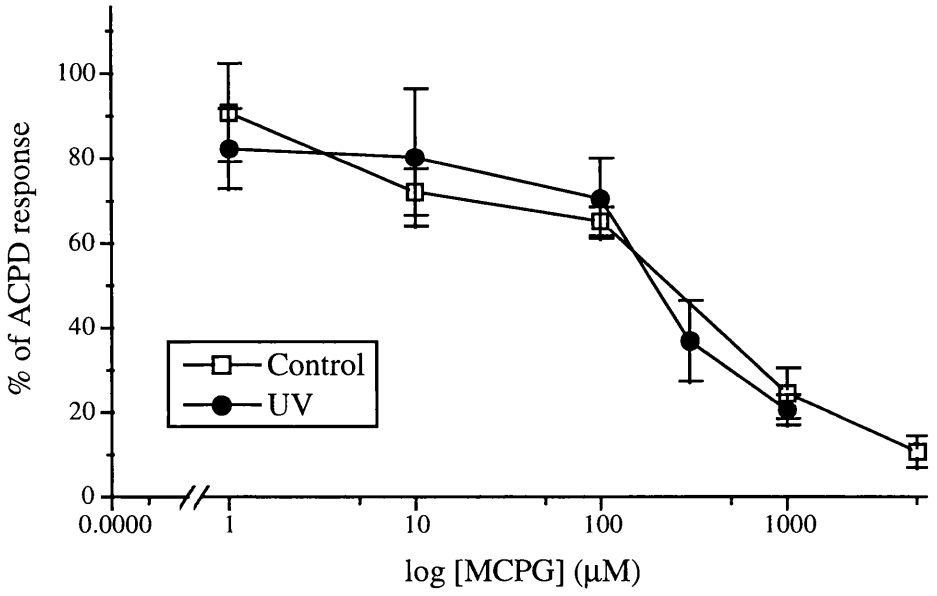


Figure 4.10 *Dose-dependent inhibitory effect of MCPG (1.0μM - 5.0mM) on the peak amplitude of the ventral root depolarisations evoked by 100μM (1S,3R)-ACPD in control and UV-treated animals. Values are expressed as mean percentage ± SEM of the maximum response to (1S,3R)-ACPD in the same preparation (n=3-5).*

Experiments with ligands differentiating only between NMDA and mGlu receptors have therefore provided evidence for the presence of mGluRs in the spinal cord, and revealed a tonic NMDA component in spinal cords taken from hyperalgesic animals. However, no further details were revealed about the contribution of mGluR subtypes in spinal transmission. The next set of experiments were therefore designed to investigate any detectable changes in the contribution of specific mGluR groups to the ventral root response. As the *in situ* hybridisation experiments had shown an increase in mGluR3 mRNA (chapter 3), group II receptors, (1S,3S)-ACPD and L-CCG-I were chosen as more selective tools for the study of group II mGluRs. (1S,3S)-ACPD produced a similar concentration-dependent depolarisation in both naïve and UV-treated animals with EC_{50} values of 184 ± 37.8μM and 204 ± 5.0μM (n=4) respectively (table 4.4; figure 4.11).

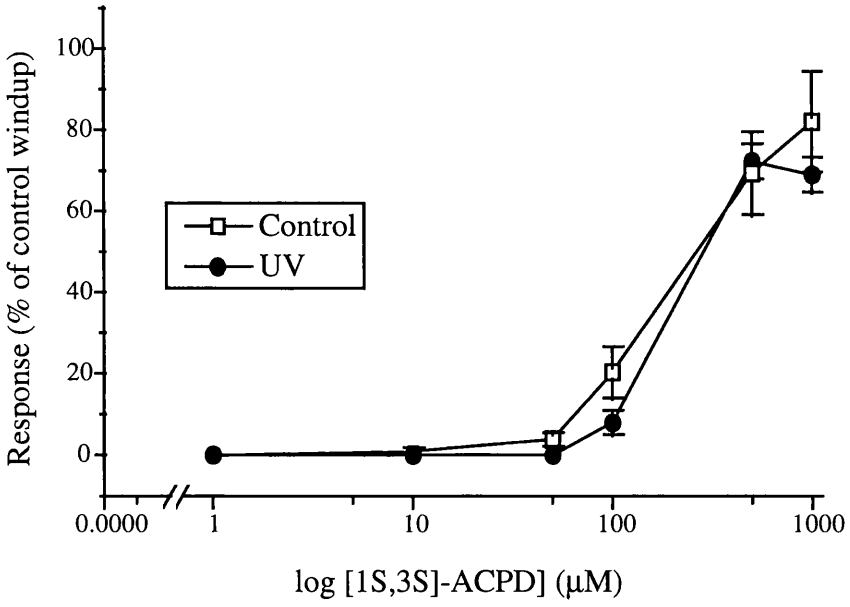


Figure 4.11 *Concentration response curve to the selective group II agonist (1S,3S)-ACPD (1μM - 1mM) in control and UV-treated rats. Values are expressed as a mean percentage ± SEM of the maximum amplitude of the windup response (5 Hz) in the same preparation in the absence of any ligands (n=4).*

L-CCG-I, a selective agonist for presynaptic mGluRs, also produced a dose dependent depolarisation of the spinal ventral roots in control animals, but with a higher degree of potency than (1S,3S)-ACPD (EC_{50}=50.2 ± 10.0μM). L-CCG-I produced an almost identical EC_{50} concentration in UV-treated rats (EC_{50}=44.3 ± 5.04μM; figure 4.12).

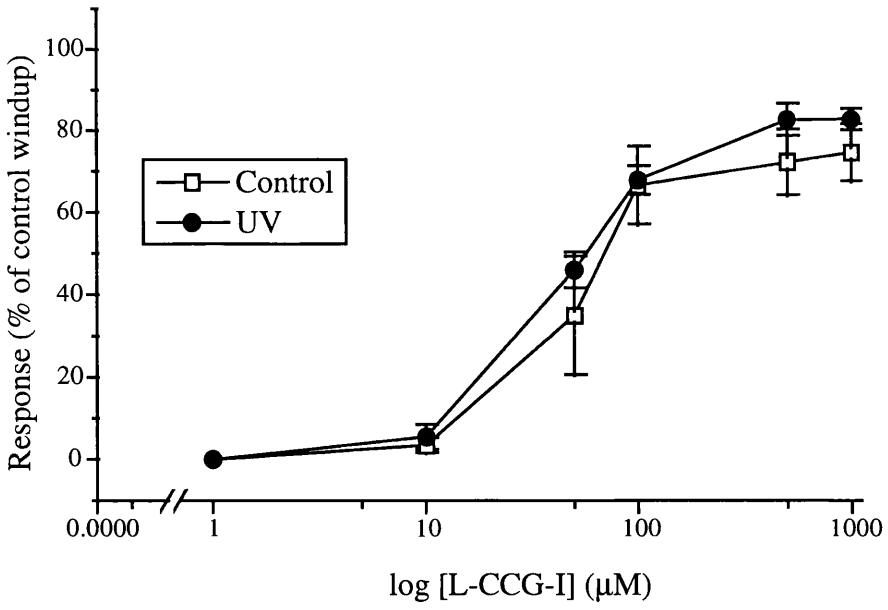


Figure 4.12 *Concentration response curve to the selective group II agonist L-CCG-I (1μM - 1mM) in control and UV-treated rats. Values are expressed as a mean percentage ± SEM of the maximum amplitude of the windup response (5 Hz) in the same preparation in the absence of any ligands (n=4-5).*

Agonist	Control EC_{50} (μM)	UV EC_{50} (μM)
(1S,3R)-ACPD	55.5±6.36	51.0±5.76
(1S,3S)-ACPD	184±37.8	204±5.0
L-CCG-I	50.2±10.0	44.25±5.04

Table 4.1 *Table of comparison of EC_{50} values for the non-selective mGluR agonist, (1S,3R)-ACPD, and the group II selective agonists, (1S,3S)-ACPD and L-CCG-I in spinal cords taken from control and UV-treated animals.*

The specific group II antagonist, MCCG, revealed an interesting discrepancy between naïve and hyperalgesic animals. In naïve animals, MCCG slightly enhanced the response to the selective, non-specifc agonist, (1S,3R)-ACPD, however the effect was not significant. On the other hand, in hyperalgesic animals the same concentration of (1S,3R)-ACPD (100μM) was significantly and concentration-dependently reduced by MCCG, producing an IC_{50} value of 522 ± 40.5μM (n=4-5; figure 4.13).

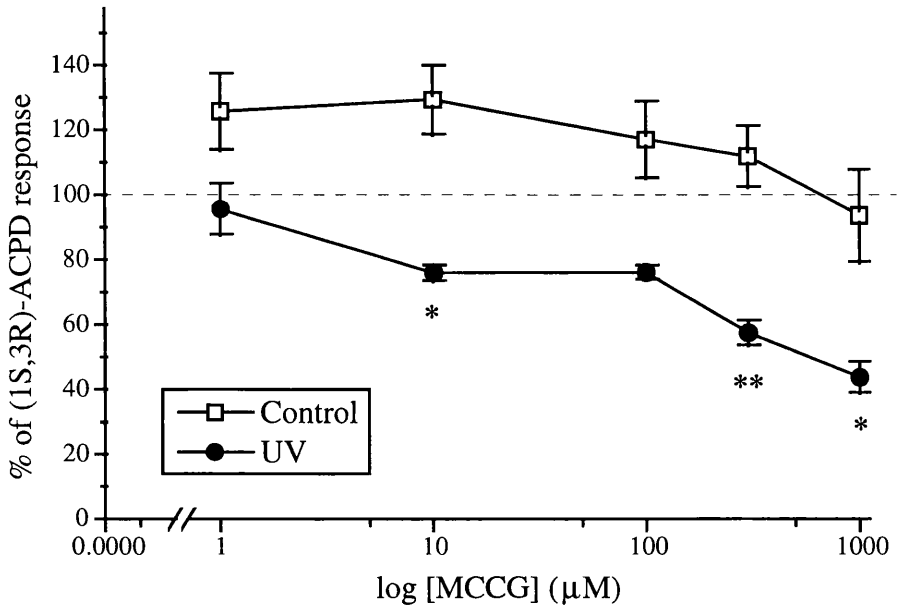


Figure 4.13 *Effects of the group II selective antagonist, MCCG (10μM - 1.0mM) on the peak amplitude of the ventral root depolarisation evoked by 100mM (1S,3R)-ACPD in control and UV-treated animals. Values are expressed as mean percentage ± SEM of the maximum response to (1S,3R)-ACPD in the absence of MCCG in the same preparation. *=p<0.05, **=p<0.01, ANOVA. Horizontal line indicates the (1S,3R)-ACPD response in the absence of MCCG as 100%.*

When (1S,3R)-ACPD was replaced by (1S,3S)-ACPD (300μM), the more group II mGluR specific agonist, MCCG (1μM - 1mM) dose-dependently reduced ventral root depolarisations, with calculated IC_{50} values of 522 ± 40.5μM and >1mM in control and UV-treated animals, respectively. Although the IC_{50} concentration for MCCG was higher in UV treated animals, there was no significant difference in effect or efficacy when compared between naïve and UV-treated animals (figure 4.14).

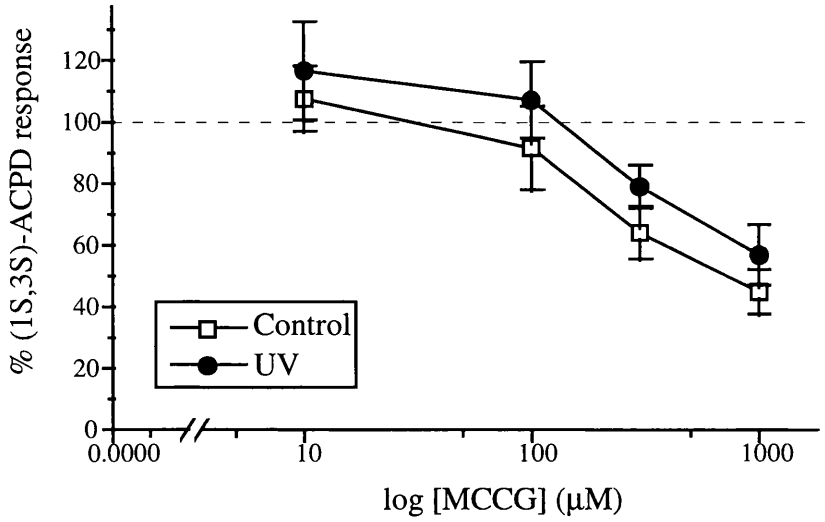


Figure 4.14 *Dose dependent inhibitory effect of the group II selective antagonist, MCCG (10μM - 1.0mM) on the peak amplitude of the ventral root depolarisation evoked by 300μM (1S,3S)-ACPD in control and UV-treated animals. Values are expressed as mean percentage ± SEM of the maximum response to (1S,3S)-ACPD in the absence of MCCG in the same preparation. Horizontal line indicates 100% level.*

4.3.2 The Contribution of mGluRs to the ventral root potential

In spinal cords dissected from naïve animals, the ventral root potential evoked by single shock electrical stimulation of the L_5 dorsal root was reduced following perfusion with the selective NMDA receptor antagonist, D-AP5 (40μM) (figure 4.15A and Table 4.2). This was consistent with previous findings (Thompson *et al* 1990). The reduction of the amplitude of the VRP occurred within 8 seconds after electrical stimulation, leaving the late, prolonged phase of the response intact.

Perfusion of the selective, non-specific mGluR antagonist, MCPG, produced a significant concentration-dependent inhibition of the C-fibre evoked ventral root response (IC_{50}=2.9 ± 0.2mM; n=3-5; table 4.2; figure 4.15B). The maximum reduction was 43 ± 0.4% of control at 5.0mM (n=3; $p<0.001$). However, unlike D-AP5, MCPG appeared to reduce the prolonged, as well as the slow component of the VRP (For comparison, see figure 4.15).

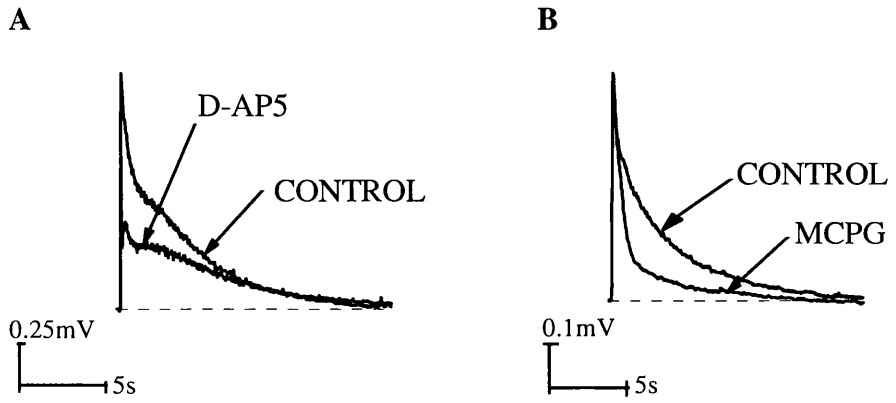


Figure 4.15 *Representative traces compare the inhibitory effects of D-AP5 and MCPG on high intensity, single shock electrical stimulation (50V, 200μs) of the dorsal root. Fifteen minutes perfusion of 40μM D-AP5 (**A**) or 5mM MCPG (**B**) reduces the amplitude (and consequently the area under the curve between 0 - 8 sec; see Table 3.1) of the late (polysynaptic) phase of the single shock-evoked VRP in the isolated hemisected spinal cord of the rat.*

In separate experiments (n=9) 100μM L-AP3, a low potency, selective, non-specific mGluR antagonist, did not affect ventral root depolarisations induced by the selective, non-specific mGluR agonist, (1S,3R)-ACPD, and did not alter ventral root potentials evoked by electrical stimulation (Table 4.2).

Low frequency (1.0 - 10 Hz), short duration (20s), repetitive electrical stimulation of the L_5 dorsal root at C-fibre intensities evoked a temporal summation of the VRP that resulted in a cumulative VRP (figure 4.16). This response closely represents the temporal summation of EPSP's and windup recorded intracellularly from spinal cord neurones *in vivo* and *in vitro* (Thompson *et al* 1990; Woolf and King 1987).

The maximum amplitude of the cumulative VRP (windup), following 1, 5 and 10Hz stimulation, was reduced to 58.9 ± 2.7%, 66.2 ± 4.4% and 68.0 ± 2.2% (n=10 and $p<0.001$ for each value) of control value following superfusion with D-AP5 (40μM), respectively. MCPG also produced a significant, concentration-dependent reduction of the amplitude of the cumulative VRP throughout the same frequency range (see Table 4.2) with a maximum reduction to 79.7 ± 4.5% (at 1Hz; n=4; $p<0.05$) of control value at 1.0mM, and to 69.5 ± 0.4% of control value at 5mM concentration (at 10Hz; n=3; $p<0.001$), but at 100μM, MCPG produced a consistent reduction at 1 and 5Hz stimulus frequencies which were not significantly different from control. At 10Hz stimulus

frequency the value was 82.6 ± 2.5% of the control (n=5; $p<0.05$). There was no significant correlation between the degree of inhibition by both NMDA and mGlu receptor antagonists of the cumulative VRP and the frequency of the electrical stimulation in the range between 1.0 - 10.0Hz.

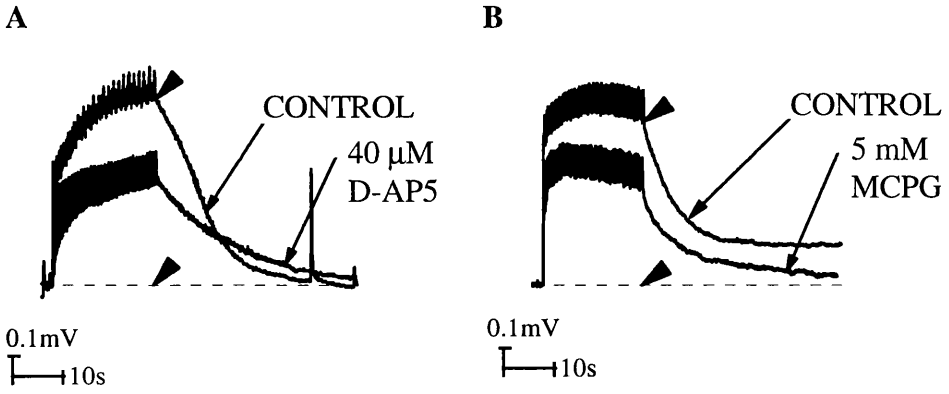


Figure 4.16 *Representative traces compare the inhibitory effects of D-AP5 and MCPG on high intensity, cumulative depolarisation ("windup") of the ventral root evoked by 20 seconds of 5Hz repetitive electrical stimulation of the L_5 dorsal root. Traces represent different experiments. Fifteen minutes perfusion of 40µM D-AP5 (**A**) or 5mM MCPG (**B**) reduces the amplitude of the cumulative ventral root potential. Arrowheads indicate the time when amplitude was measured. For further details and cumulative data see text and Table 4.2.*

Antagonist	Single shock	Windup		
		1 Hz	5 Hz	10 Hz
D-AP5 40μM (n=10)	68.7±5.13***	58.9±2.65***	66.2±4.40***	68.0±2.02***
MCPG 100μM (n=5)	83.2±4.18**	90.8±10.7	88.5±7.55	82.6±3.52*
MCPG 1mM (n=4)	59.0±8.47**	79.7±4.49*	89.8±2.57*	87.9±2.47*
MCPG 5mM (n=3)	42.7±0.46***	78.1±8.09*	74.2±5.67**	69.5±0.38***
L-AP3 100μM (n=9)	107.7±5.12	100.4±4.57	99.6±4.14	102.2±4.89

Table 4.2 *Comparison of the effects of D-AP5, L-AP3 and MCPG against ventral root responses evoked by high intensity single shock and repetitive electrical stimulation of the L_5 dorsal root. Values are given as percentage of the control response. Effects on the single shock-evoked VR responses were measured as reduction of the area under the curve between 0 - 8 sec after stimulation. Change in the cumulative response (windup) was measured as decrease of the amplitude at the end of the 20 sec. stimulus train. See methods for further details of response measurement.* $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$, *unpaired* t *test.*

After the induction of hyperalgesia by UVA irradiation, the characteristics of the ventral root response evoked by primary afferent stimulation changed. Ventral root responses evoked by high intensity single shock stimulation became significantly prolonged with the area under the response (0-8 secs.) increasing from 1.14 ± 0.17mV.sec in naïve animals to 2.95 ± 0.39mV.sec in UV-treated animals (n=19; p<0.001, ANOVA; figure 4.17).

The effect of low frequency (1-10 Hz) high intensity dorsal root stimulation for 20 seconds was also significantly enhanced in UV-treated preparations when compared to naïve. Stimulation at 1, 5 and 10Hz produced a cumulative VRP with amplitudes of 0.40 ± 0.06mV, 0.57 ± 0.06mV and 0.56 ± 0.08mV respectively in naïve preparations, in comparison to 0.99 ± 0.11mV, 1.41 ± 0.12mV and 1.35 ± 0.12mV respectively in UV-

treated animals (n=19; p<0.001, ANOVA; figure 4.17). The enhanced windup did not depend on the frequency of stimulation in either naïve or hyperalgesic animals.

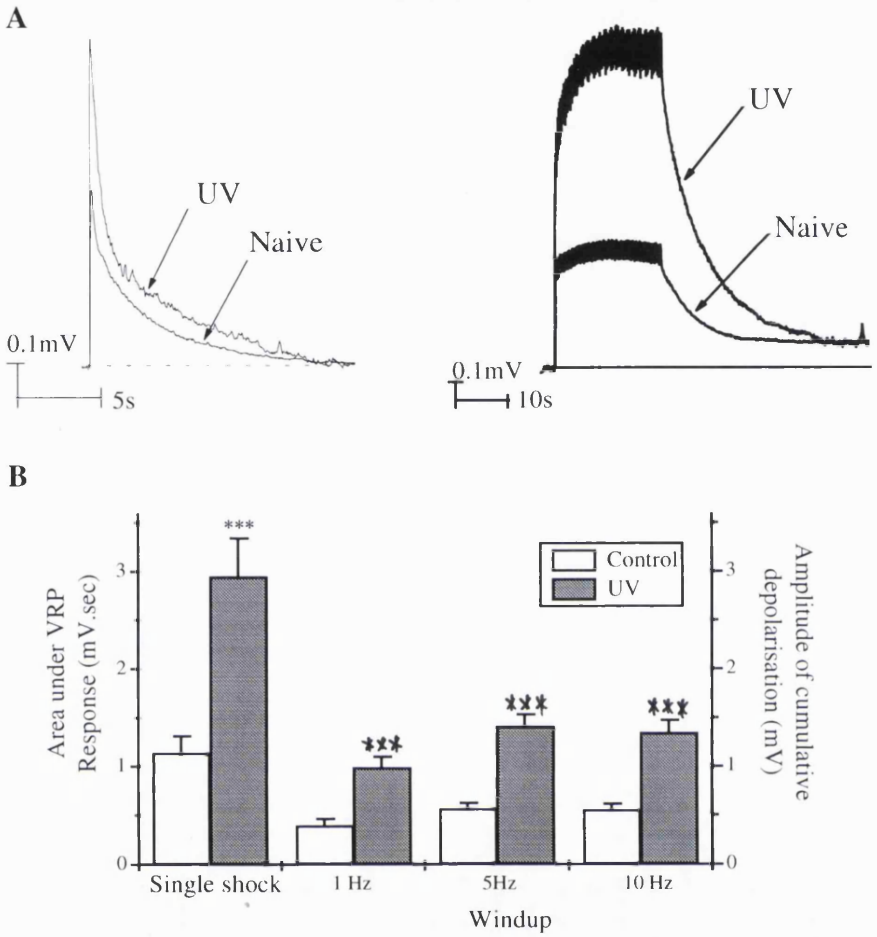


Figure 4.17 *Comparison of high-threshold afferent stimulation in naïve and UV-irradiated animals.*

A). *Single shock electrical stimulation (50V, 200ms) evokes a compound action potential which is significantly enhanced in UV-irradiated animals (left panel). Repetitive low-frequency (5 Hz) stimulation of high-threshold afferent fibres delivered for 20 seconds evoked a maintained summated VRP which is significantly larger in amplitude in UV-irradiated compared to naïve animals (right panel).*

B). *Graph comparing the area of the single shock and summated VRPs in naïve (open bars) and UV-irradiated (filled bars) animals. The area under the single shock-induced response is measured between 0 and 8 seconds and is expressed as mV.sec (left axis). The maximum amplitude of the summated VRP (windup) is measured and expressed as mV (right axis). Values are expressed as mean ± S.E.M. (n=19). *** p<0.001, ANOVA.*

Although the single shock C-fibre evoked VRP was significantly enhanced in spinal cords taken from hyperalgesic animals, neither the NMDA receptor antagonist, D-AP5, nor the low potency, selective, non-specific mGluR antagonist, L-AP3, produced any significantly different reduction of the responses when compared to naïve preparations (see Table 4.3).

The data using D-AP5 confirms an earlier study by Thompson *et al* (1994). The lack of effect with L-AP3 is not surprising due to its low potency at the mGluRs. On the other hand, MCPG, which is a non-selective antagonist across the groups of mGluRs, but which has a higher potency than L-AP3, showed a significantly different effect in UV-treated animals compared to naïve animals. While MCPG produced a concentration-dependent inhibition of the single shock evoked VRP in naïve animals, it was ineffective in the same concentration range in the UV-treated group.

Antagonist	**Naïve**	**UV day 1**
D-AP5	68.7 ± 5.13 (n=10)	57.9 ± 5.18 (n=5)
MCPG 100μM	83.2 ± 4.18 (n=5)	93.0 ± 2.75 (n=3)
MCPG 1mM	59.0 ± 8.47 (n=4)	**98.2 ± 5.47**** (n=3)
L-AP3	107.7 ± 5.15 (n=9)	101.8 ± 0.97 (n=4)

Table 4.3 *The effect of NMDA and mGlu receptor antagonists on the C-fibre evoked ventral root responses in naïve and UV-treated animals. Values are given as mean percentage ± SEM of the control response. Effects of the single shock-evoked VRPs were measured as reduction of the area under the curve (0-8 secs.) after stimulation. **=p<0.01, ANOVA.*

Surprisingly, none of the three antagonists (D-AP5, L-AP3, MCPG) showed significantly different effects against the summated VRP in UV-treated animals compared to naïve (Table 4.4). Thompson *et al* (1994) have shown an enhanced effect of D-AP5 against 1Hz stimulation in UV-treated animals which has not been reproduced in this set of experiments.

Antagonist		Windup 1 Hz	Windup 5 Hz	Windup 10 Hz
D-AP5 40μM	control (n=10)	58.9±2.65	66.2±4.40	68.0±2.02
	UV (n=5)	*64.8±3.54*	*71.5±6.2*	*68.1±8.60*
MCPG 100μM	control (n=5)	90.8±10.7	88.5±7.55	82.6±3.52
	UV (n=3)	*97.1±12.8*	*99.7±10.8*	*104.2±14.6*
MCPG 1mM	control (n=4)	79.7±4.49	89.8±2.57	87.9±2.47
	UV (n=3)	*88.3±13.7*	*86.3±14.1*	*84.0±12.8*
L-AP3 100μM	control (n=9)	100.4±4.57	99.6±4.14	102.2±4.89
	UV (n=4)	*95.2±4.74*	*109.0±4.52*	*112.0±6.64*

Table 4.4 *The effect of NMDA and mGlu receptor antagonists on the C-fibre evoked summated VRP in naïve and UV-treated animals. Values are expressed as mean percentage ± SEM of the control response. Change in the cumulative response (windup) was measured as the decrease of the amplitude at the end of the 20 second stimulus train. The number of experiments are indicated in brackets.*

4.3.3 Contribution of mGluRs to the chemically induced nociceptive response

Capsaicin (1.0μM; 30 seconds), in the presence of Concanavalin-A (1.0μM), evoked a reproducible and characteristic long duration ventral root potential (1.30 ± 0.26mV; n=13) in naïve preparations. Both D-AP5, and the selective mGluR antagonist, MCPG, reduced the capsaicin-induced depolarisations in a concentration-dependent manner (IC_{50}=143 ± 43μM and 809 ± 35μM, respectively; maximum depression of control response was 88.9 ± 7.5% and 55.5 ± 8.6% respectively (n=4); figure 4.18). The inhibitory effects of both antagonists were reversed following wash of the preparation with ACSF (30 minutes).

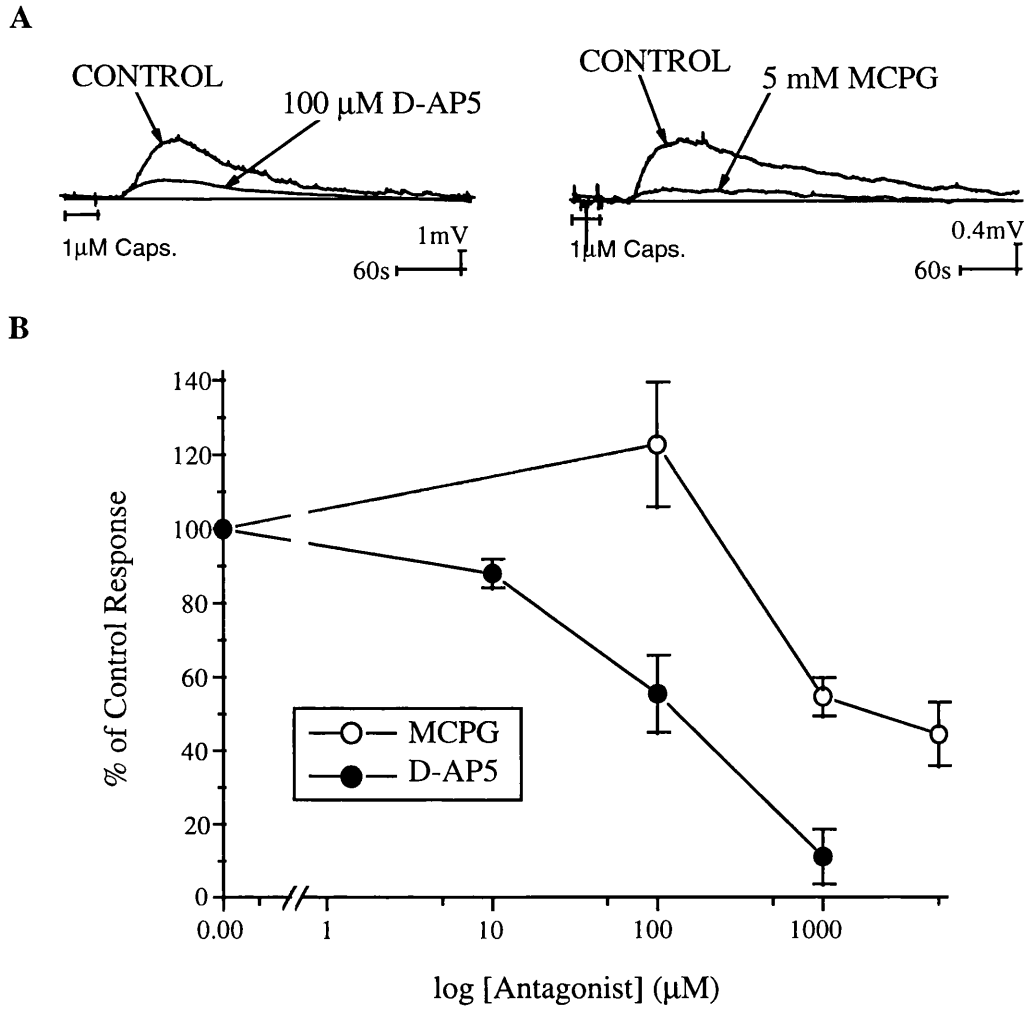


Figure 4.18 *Concentration-dependent inhibition of the capsaicin-evoked ventral root depolarisation by D-AP5 and MCPG. Traces (A) represent different experiments.*

A). *Representative traces demonstrate the effects of D-AP5 (100μM; left hand side panel) and MCPG (5mM; right hand side panel) on the amplitude of capsaicin-evoked ventral root depolarisation (1.0μM capsaicin was superfused for 30 sec in the presence of 1.0μM Concanavalin-A) as indicated by the bar under the curve. (Recovery of the response was obtained in all experiments).*

B). *Graph shows cumulative data on the concentration-dependent effects of the NMDA receptor antagonist, D-AP5 (10μM - 1.0mM) and the mGluR antagonist, MCPG (10μM - 5.0mM) on the amplitude of capsaicin-evoked (1.0μM) ventral root depolarisation (n=3-4).*

The effect of hyperalgesia on the capsaicin-evoked ventral root response was not investigated.

4.4 Discussion

In this chapter I have reviewed data concerning the contribution of EAA activation to the nociceptive spinal transmission and analysed my data obtained in the *in vitro* isolated spinal cord experiments. Finally I have made an attempt to draw conclusions on the nature of mGluR function in the rat spinal cord.

4.4.1 mGluR pharmacology in the spinal cord

4.4.1.1 Selectivity and Specificity of Ligands

Ventral root responses measured after dorsal root stimulation are complex events which involve the release of neuropeptides and excitatory amino acids, and activation of postsynaptic receptors (Thompson *et al* 1992). It has been previously determined that both ionotropic (non-NMDA and NMDA) and G-protein coupled metabotropic glutamate receptors contribute to the VRP (Birse *et al* 1993; Eaton *et al* 1993; King and Lopez-Garcia 1993; Thompson *et al* 1992). It was necessary to establish the basic pharmacology of the mGluR ligands, including concentration/response relationships, and determine EC_{50} and IC_{50} values for agonists and antagonists respectively, before the question of mGluR components could be addressed. (1S,3R)-ACPD was chosen as an agonist to mGluRs because it activates both phosphoinositide-coupled and cAMP-linked subtypes of metabotropic glutamate receptors without any known direct effect at ionotropic receptors (Jones and Headley 1995; Shigemoto *et al* 1992). (1S,3R)-ACPD evoked a concentration-dependent depolarisation of the ventral root, similar to that reported in the neonatal spinal cord (Birse *et al* 1993; Eaton *et al* 1993). D-AP5, the selective NMDA receptor antagonist (Davies *et al* 1981) did not affect (1S,3R)-ACPD-evoked ventral root depolarisation while these responses were reduced by MCPG, a selective mGluR antagonist (Birse *et al* 1993; Eaton *et al* 1993; Hayashi *et al* 1994; Watkins and Collingridge 1994). On the other hand, the non-selective, low potency mGluR antagonist, L-AP3 (100μM - 1.0mM), did not affect any drug- or electrically-induced ventral root responses, in good agreement with published data (Birse *et al* 1993; Thompson *et al* 1992). Primarily, these data confirmed previous observations that mGluR and NMDA receptors are present and are operational in the naïve rat spinal cord (Davies *et al* 1981; Thompson *et al* 1990), and also established

the selective concentration range for the antagonists for the study of mGluR contribution to the nociceptive segmental reflex.

MCPG significantly inhibited (1S,3R)-ACPD, C-fibre-evoked VRP, cumulative VRP and capsaicin-induced responses. However, there was a distinct variability in its potency against each of these responses. Whilst MCPG blocked the (1S,3R)-ACPD-evoked ventral root depolarisation with an IC_{50}=243 ± 61μM it was significantly less potent against capsaicin- and electrically-evoked ventral root responses. This finding may suggest that, although mGluR receptors are present in the spinal cord, their contribution is not dominant in various segmental reflexes in the naïve animal. This hypothesis is supported by Neugebauer *et al.*, (1994) who found mGluRs present but inactive in the spinal cord of anaesthetised, adult rats during stimulation of joint afferents, however activation of mGluRs was required for the generation of inflammation-induced spinal hyperexcitability. These data, together with the finding that mGluRs are situated anatomically adjacent to NMDA receptors at the edge of the synaptic cleft in the brain (Nusser *et al* 1994) suggest that mGluRs may be involved in the functional plasticity during inflammatory pathophysiological conditions.

4.4.1.2 Comparison of ligand effects between hyperalgesic and naïve animals

(1S,3R)-ACPD shows an increase in the maximum of the dose response curve in hyperalgesic animals but with no change in EC_{50} concentration. This implies that there is no increase in the number of binding sites, which would be reflected as a change in EC_{50} value or a shift in the dose response curve. The reasons for the increase in maximum are unclear but it would appear to involve the NMDA receptors at some level, as the increase in maximum response is abolished by the selective NMDA receptor antagonist, D-AP5. Since it may be assumed that (1S,3R)-ACPD does not act on NMDA receptors, it must be speculated that there is some interaction between the mGlu and NMDA receptors at an intracellular level to produce this enhancement. The most obvious possibility would be the phosphorylation of the NMDA receptor as a result of mGluR activation. In support of this hypothesis, intracellular recording from striatal neurones has shown an enhancement of NMDA responses by the group I mGluR agonist, DHPG, which could be reversed by the PKC inhibitor calphostin C (Pisani *et al* 1996). Lodge *et al* (1996) have also suggested that group I and group II mGluRs may act synergistically in the spinal cord to potentiate

ionotropic glutamate responses. This type of interaction has been proposed between peripheral G protein-coupled receptors and NMDA receptors (Urban *et al* 1994b). Central sensitisation produced by hyperalgesia may "prime" the NMDA receptors and enhanced coupling of mGluRs may further facilitate NMDA receptor activation. The most interesting aspect of this finding is that it supports evidence for a tonic enhancement of the NMDA component during hyperalgesic conditions.

The selective, non-specific mGluR antagonist, MCPG, shows no change in efficacy or potency against (1S,3R)-ACPD in hyperalgesic animals. As both ligands act on group I and group II mGluRs, any changes may be masked by the opposing effects of the two receptor subtypes. Using the more group II specific antagonist, MCCG, against (1S,3R)-ACPD does indeed reveal alterations between control and hyperalgesic states. In control animals MCCG enhances the response induced by 100μM (1S,3R)-ACPD. As the group II receptors are suggested to be predominantly presynaptic and negatively coupled to adenylyl cyclase, hence inhibiting transmitter release, inhibition of this effect will enhance neurotransmitter release, thus increasing the ventral root response. In hyperalgesic animals however, MCCG inhibits the response to 100μM (1S,3R)-ACPD. One possible explanation may be an upregulation of group II mGluRs on inhibitory spinal neurones after hyperalgesia resulting in disinhibition of neurotransmitter release. Once this has been revealed, ventral root responses would be decreased. This hypothesis is not supported by results from experiments where the two group II specific mGluR agonists, (1S,3S)-ACPD and L-CCG-I were used to evoke VRPs. Although they both produced a depolarisation, there was no change to the response after the induction of hyperalgesia, suggesting that the enhanced (1S,3R)-ACPD response maybe solely mediated by group I mGluRs. Another possibility may be an alteration in the coupling of mGluRs to intracellular messenger systems and/or changes in second messenger systems themselves. Furthermore, MCCG showed no change against 300μM (1S,3S)-ACPD between naïve and hyperalgesic animals. These data do not provide evidence for an increase in the number of receptor sites.

These findings do not support the *in situ* data shown in chapter 3, which provided evidence for enhanced mGluR3 mRNA expression following the induction of inflammatory hyperalgesia. Possible explanations are i) that mRNA expression is not manifested in protein synthesis and/or production of functional receptors. ii) There is no

Group II agonist pharmacology shows no difference between naïve and UV-treated animals, whereas (1S,3R)-ACPD revealed a clear NMDA component following inflammation. However, a true comparison of the mGluR components between naïve and UV-treated animals cannot be resolved in these experiments due to technical constraints. Experiments comparing windup between UV-treated and naïve animals were carried out using tightly sealed suction electrodes, and show a significant enhancement of the windup (figure 4.17). The agonist response curves were constructed using the grease gap method, using windup as a viability marker. The variability of the raw data produced by this technique is much greater, and may mask any likely changes in the contribution of mGluRs to spinal processing following inflammation. More experiments are therefore needed to fully resolve this issue.

"de novo" synthesis of mGluR3 following hyperalgesia in previously non-expressing cells but increased synthesis in originally positive cells. iii) Alternatively, the mGluR3 mRNA expression occurs in glial cells without major contribution to neuronal hyperexcitability. My data would also suggest a possible "excitatory" effect of mGluR3 during hyperalgesia, which is then blocked by MCCG. There is published evidence for the possible excitatory action of group II mGluR activation elsewhere in the CNS (Gereau and Conn 1994; Scheopp *et al* 1996; Winder and Conn 1992, 1995), however this assumption remains highly speculative. Further experiments are needed to decide on this question.

see facing page.

4.4.2 Electrical Stimulation

4.4.2.1 Naïve animals

Single shock electrical stimulation of the dorsal root under *in vitro* conditions is used to represent an acute noxious or innocuous stimulus depending on the stimulus strength. Previous work by Thompson *et al.* (1992) showed that D-AP5 reduced a well characterised NMDA component of the response. This finding has been confirmed by my results, which have also shown that MCPG attenuated the C-fibre evoked VRP. However, unlike D-AP5, MCPG appeared to block a later phase of the response. The late, prolonged component has been characterised as "peptidergic" (Thompson *et al* 1992) and was attenuated by neurokinin receptor antagonists (Thompson *et al* 1993b; 1994). It is likely that the late, prolonged phase of the VRP is dependent on slow synaptic events, which may involve second messenger systems probably through the activation of G-protein coupled receptors (Urban *et al* 1994a,b). My data suggest that mGluR activation contributes preferentially to this late, prolonged component of the C-fibre-evoked VRP (Thompson *et al* 1994). In this study the early (monosynaptic) phase of the VRP was not affected by MCPG suggesting that the generation of the fast, kainate and AMPA receptor-dependent monosynaptic component of the VRP did not involve the activation of mGluR. These results are in good agreement with the observations of Eaton *et al.* (1993) who showed that MCPG does not affect AMPA-evoked ventral root responses in the neonatal rat spinal cord. The same study also found that mGluR activation by superfusion of selective agonists inhibited the spinal monosynaptic reflex. The lack of effect of MCPG in the present study on the monosynaptic component of the VRP suggests that electrical

stimulation of the dorsal roots does not initiate this inhibitory phenomenon in the isolated spinal cord.

4.4.2.2 Hyperalgesic animals

After the induction of inflammatory hyperalgesia, the duration of the C fibre-evoked VRP was significantly enhanced when compared to naïve animals, confirming results published by Thompson *et al* (1994). Peripheral tissue injury, inflammation, or nerve injury produces many alterations in the properties of spinal cord neurones (see chapter 1). For example, the peripheral receptive fields of dorsal horn neurones increase in size following thermal or mechanical injury (Laird and Cervero 1989; McMahon and Wall 1984), or following the injection of chemical irritants into joints and skin (Hylden *et al* 1989; Schaible *et al* 1987; Simone *et al* 1989). These modifications in receptive field properties may also be mimicked by brief, high intensity electrical nerve stimulation (Cook *et al* 1987). The cutaneous receptive fields of flexor motoneurones also show similar dynamic changes following injury or high-threshold electrical nerve stimulation (Woolf 1983; Woolf and McMahon 1985; Woolf and Wall 1986). These changes are thought to be mediated by alterations in excitability within the spinal cord (for review see Treede *et al* 1992). Similar changes in receptive field properties were evoked by UV-induced inflammation of the hindpaw of the rat (Urban *et al* 1993). In the experiments shown here, enhanced afferent-evoked ventral root responses were recorded, suggesting that changes occur in central excitability.

After the induction of hyperalgesia, the NMDA receptor antagonist, D-AP5 significantly antagonised the polysynaptic component of the VRP, but to the same extent as in naïve animals. This suggest that the NMDA receptors activated by C-fibre evoked synaptic transmission show no disproportional change in their contribution to this reflex after the induction of hyperalgesia. Thompson *et al* (1994) showed that D-AP5 blocked a novel component of an A-fibre evoked response following hyperalgesia, suggesting that considerable NMDA receptor activation occurred when only A fibres were stimulated. It may be that the NMDA receptors are fully activated by A fibre recruitment so when the slower conducting C-fibres are stimulated, the NMDA receptors are already maximally activated. Also, it has to be taken into consideration that the absolute contribution of NMDA receptors has been increased. Taken together, it is possible to assume that, in

addition to the increased NMDA component, other receptor systems show enhanced activity (e.g. neurokinins, CGRP, other peptides).

The non-selective mGluR antagonist, L-AP3, had no effect on the C-fibre evoked VRP after the induction of hyperalgesia. This is surprising as Neugebauer *et al* (1994) found that L-AP3 reduced spinal hyperexcitability following acute inflammation of the knee joint. However, they found that L-AP3 was only effective when applied during the induction of hyperalgesia, suggesting a role for the mGluRs in the generation of inflammation-evoked spinal hyperexcitability. In my study, the contribution of the mGluRs is studied after the induction of peripheral hyperalgesia, at which time point the mGluRs may not directly contribute to spinal hyperexcitability. Other differences in the two studies occur with the ages of the animals used. I have used immature spinal cords whereas Neugebauer used adult animals. Neugebauer was also selectively stimulating joint afferents whereas the dorsal roots stimulated in the hemisected spinal cord preparation contain afferents from skin and muscle as well as joints, which make up only a small proportion of the total fibres. Iontophoresis, as used by Neugebauer et al (1994) also produces higher local concentrations compared to bath superfusion as I have used.

The more potent mGluR antagonist, MCPG, appeared not to be effective against any part of the C-fibre evoked VRP in UV-treated animals. As this is a non-selective antagonist, acting at all three groups of mGluRs, it may be that the opposing effects of the group I and group II/III receptors may mask each other, giving an overall outcome of no apparent mGluR activation. There is also an enhanced tachykinin component contributing to the prolonged phase of the VRP (Thompson *et al* 1994), which may also mask or alter any mGluR activation. One can also argue that mGluR activation has a major modulatory role in the development of spinal hyperexcitability and, in an established hyperexcitable phase, its importance is diminished (e.g. the NMDA component becomes established and dominating). Glutamate may also have a preferential action at ionotropic, including NMDA, receptors in conditions of spinal hyperexcitability.

Low frequency, repetitive stimulation of the dorsal root at high intensity evokes a progressive increase in the action potential discharge elicited in spinal neurones by each of the successive stimuli in a train (Sivilotti *et al* 1993). This complex phenomenon, termed "windup", may be used as an index of central sensitisation in the spinal cord (Meller *et al* 1993; Mendell 1966). The effect of D-AP5 on windup in naïve animals has

previously been characterised by Thompson *et al* (1992) and my results agree with these findings as D-AP5 reduced the amplitude of the cumulative response. MCPG shows a small, but significant dose-dependent reduction of the response which suggests mGluR involvement in the generation of "windup".

Following peripheral inflammation, my results show that the maximum amplitude of the summated VRP was significantly enhanced. This is in agreement with Thompson *et al* (1994) who also saw a summated VRP after low threshold stimulation of A fibre afferents. Spinal excitability changes evoked by A fibre stimulation may relate to allodynia, but prior conditioning of spinal circuitry by C fibres is a prerequisite (Gracely *et al* 1992; LaMotte *et al* 1992; Tørebjörk *et al* 1992). In my hands, neither NMDA nor mGlu receptor antagonists appear to have any altered effect on the windup response, suggesting that neither of these two glutamate receptor families show altered function after the induction of hyperalgesia. Thompson *et al* (1994) show a novel involvement of NK1 receptors following hyperaglesia, which may account for the enhanced amplitude of the cumulative VRP. However, they also show and enhanced NMDA component, which I could not repeat. The difference in my results may benefit from larger experiment numbers.

4.4.3 Capsaicin-induced Responses

Capsaicin selectively activates polymodal nociceptive C-fibres and induces a specific, sustained primary afferent input to dorsal horn neurones (Bevan and Szolcsányi 1990; Dray 1992; Simone *et al* 1987). D-AP5 and MCPG inhibited the capsaicin-evoked ventral root depolarisation indicating that both NMDA and mGlu receptor activation contributed to this response. This finding may also suggest that mGlu and NMDA receptors are expressed in the same set of interneurones.

The contribution of the mGluRs to the capsaicin response following peripheral inflammation has yet to be determined.

4.5 Conclusion

In naïve animals, in addition to D-AP5, MCPG is effective in reducing C-fibre induced ventral root responses, hence NMDA and mGlu receptor activation are involved in the generation of the segmental nociceptive reflex. After the induction of inflammatory

hyperalgesia, central changes occur, as seen by an enhanced nociceptive reflex, and alterations in the pharmacology of the mGluRs.

5. CHAPTER 5

GENERAL DISCUSSION

The study of the metabotropic glutamate receptors has, to date, been dominated by molecular biology, with functional and pharmacological studies following far behind. Currently, eight receptor subtypes have been cloned, some with splice variations, therefore there is a race to find a functional role for each subtype. In the absence of potent, selective ligands an option is the use of transgenic technology to produce knock-out animals, although this often has many drawbacks including lack of a clear phenotype, the time scale involved and the development of possible compensatory mechanisms.

Throughout my thesis I have tried to use a multidisciplinary approach to address the problem of the role of mGluRs in nociception. In the first instance I have examined the presence and role of the mGluRs in normal animals. *In situ* hybridisation with specific oligonucleotide probes revealed the presence of mGluR1,3-5,7 mRNAs in the spinal cord. Group I mGluRs (mGluR1,5) showed differing localisation with mGluR1 mRNA found at a low level throughout the spinal cord. On the other hand, mGluR5 shows a very high mRNA signal in the dorsal horn. Of the group II mGluRs (mGluR2,3), only mGluR3 mRNA is found in the spinal cord where it is expressed at a low level primarily in the dorsal horn. The group III mGluRs (mGluR4,6,7) show the most discrete mRNA expression of the family, although mGluR6 is not found in the spinal cord. mGluR4 mRNA is expressed at a low level in the dorsal horn, but shows a high signal in the motoneurones. mGluR7, however, shows a high mRNA signal in laminae I and II of the dorsal horn only. An immunohistochemical study has previously shown a similar localisation for mGluR7 suggesting a role for this subtype in nociception (Ohishi *et al* 1995b). The scattered localisation of some of the mGluR subtype mRNAs makes it difficult to speculate on their specific role. It must be remembered that this study shows the relative presence or absence of mRNA so we can only assume that the receptor proteins show the same localisation and level of expression. To date, immunohistochemical studies of mGluR in the spinal cord are sketchy and lacking in detail. As specific subtype antibodies become available it would be useful to tie the mRNA and protein studies together to give a clearer picture of the distribution of these receptors. It is also not clear whether neurones in the spinal cord co-express more than one mGluR.

As I have investigated the pharmacology of the mGluRs in juvenile (postnatal day 12-14) rats, I have also briefly looked at the developmental aspect of mGluR expression

in the spinal cord. This study confirmed that the level of mGluR mRNA expression in postnatal day 12 rats is similar to that seen in the adult. It also highlighted large changes in mGluR3 and 5 mRNA expression, which appear to be highly expressed at birth and down-regulated with age. This correlates well with a similar study carried out in the brain (Catania *et al* 1994). There is also an upregulation of mGluR4 mRNA in motoneurones with age, suggesting that this subtype may play a role in the maintenance of synaptic function in the adult spinal cord, rather than the formation of synapses. With the exception of mGluR3, changes in the distribution of mRNA expression are rather quantitative. The appearance of a probably glial signal over the spinal during at PN day 21 is remarkable, however its relevance remains unknown. This developmental study is limited, though, as autoradiography does not allow detailed cellular analysis, which would provide more information on the exact localisation and changes in mRNA expression.

Using an *in vitro* preparation of the hemisected rat spinal cord I was able to investigate the role of the mGluRs in sensory nociceptive processing. Previous studies have suggested that the mGluRs are present in the spinal cord but show little contribution to sensory processing unless there is a nociceptive input (Neugebauer *et al* 1994; Young *et al* 1994). However, my data show that the mGluRs do indeed contribute, as the non-selective mGluR antagonist, MCPG, reduced a specific component of the single shock electrical response. MCPG also reduces the amplitude of the windup response and depolarisation evoked by capsaicin, suggesting a mGluR contribution to nociceptive processing.

To study the role of mGluRs in pain I have used UVA irradiation of the hindpaw as a model of inflammation. Behavioural studies using juvenile rats (postnatal day 10-14) showed that irradiation of the hindpaw with UVA light produced an ipsilateral mechanical hyperalgesia and allodynia, as well as a less severe hyperalgesia and allodynia in the contralateral paw. The hyperalgesia induced by UV irradiation can be reduced in adult rats by morphine and NSAIDs (Perkins *et al* 1993b), verifying this as a suitable model of pain. My behavioural data confirm that this model induces hyperalgesia in juvenile animals, which correlates with other published data (Thompson *et al* 1994). Unfortunately, time constraints and the lack of potency of mGluR ligands *in vivo* meant that I was not able to examine the effect of mGluR antagonists on the development or reversal of hyperalgesia. However, *in vitro* pharmacology does suggest that these receptors may play a role.

Following UV-induced inflammation, *in situ* hybridisation revealed no changes in mGluR mRNA expression, with the exception of mGluR3. mGluR3 mRNA is fairly discretely localised in laminae III and IV of the dorsal horn in postnatal day 12 rats. This signal is significantly enhanced following inflammation and returns to control levels with a similar time course to that seen in the behavioural studies. The enhancement is also seen on both sides of the spinal cord, again, mirroring the behavioural data. This is an exciting result but must be treated with some caution as it is not yet known whether the upregulation of mRNA is translated into receptor protein. Also, there is a possibility that mGluR mRNA expression following inflammation may behave differently in the adult animal. This study also only gives us a view of the postsynaptic element and, as the changes following inflammation appear to be so discrete, it would be valuable to investigate any presynaptic changes in dorsal root ganglia.

The *in vitro* pharmacology of the mGluRs in the spinal cord following inflammation is less clear. The non-selective mGluR agonist, (1S,3R)-ACPD, shows an enhanced maximum following inflammation, but the more group II selective agonists, (1S,3S)-ACPD and L-CCG-I, show no change. The enhanced maximum seen with (1S,3R)-ACPD is entirely reversed by an NMDA antagonist, D-AP5. As NMDA receptors are thought to be phosphorylated following central sensitisation (Urban et al 1994b), and the group I mGluRs promote phosphorylation via their interaction with inositol phosphate and protein kinase C, it is possible to speculate that this may be a group I mGluR-mediated effect. Inflammation may cause changes in mGluR activity which do not only include an increase in receptor number, but alter interactions in second messenger pathways. This finding may be important as it sheds light on two aspects of inflammatory pain: i) the presence of a tonic NMDA component and ii) a relative unimportance of mGluR activity in established pain. The latter may reflect that the mGluR role is rather restricted to the developmental phase of hyperalgesia and pain.

Studies with mGluR antagonists produced some unexpected data which is difficult to interpret. The non-selective antagonist, MCPG, shows no change in efficacy or effect following inflammation. As this ligand is thought to act on all three groups of mGluRs, any changes may be masked by its non-selectivity. The more group II selective antagonist, MCCG, showed an interesting discrepancy between normal and hyperalgesic rats. In spinal cords taken from control animals, MCCG enhanced the depolarisation induced by

(1S,3R)-ACPD. As the group II receptors are thought to be negatively coupled to adenylate cyclase and mainly presynaptic, this result may be explained as presynaptic inhibition of inhibitory receptors. Following the induction of inflammation, MCCG attenuates the (1S,3R)-ACPD-induced depolarisation. As there is an upregulation of mGluR3 mRNA, a further enhancement of the (1S,3R)-ACPD response might be expected. However, if the upregulation occurs on inhibitory neurones, this result may be explained by disinhibition. It has been shown that inhibitory transmitter systems are upregulated in the spinal cord following some types of peripheral inflammation (Castro-Lopes *et al* 1992; Nahin *et al* 1989), which may manifest itself here. Inflammation may also alter the coupling of the mGluRs and the second messenger systems which they modulate.

This study has been limited by the specificity and potency of the ligands available. As new compounds are developed, the contribution of the three groups of mGluRs to nociceptive processing may become clearer. The next step would be to investigate mGluR ligands in behavioural models of hyperalgesia, especially as group I agonists have been suggested to be nociceptive, whereas group II and III agonists may be antinociceptive (Bruno *et al* 1994,1995; Buisson and Choi 1995; Fisher and Coderre 1996a,b; Maiese *et al* 1995; Opitz and Reymann 1995). Studies with more refined, selective mGluR ligands on the development of the enhanced spinal nociceptive reflex in vitro should follow and shed light on fine changes in the nociceptive system. As the molecular biology for these receptors is more advanced compared to the function and pharmacology in the spinal cord, the possible changes in the receptor proteins following inflammation, both pre- and postsynaptically, may highlight a possible role for these receptors. The synergy and between NMDA and mGlu receptors may be enhanced following inflammation, as well as alterations in the phosphorylated states of the two groups of receptors. If the NMDA receptors are strongly modulated by the mGluRs, development of selective mGluR compounds may be a route to NMDA antagonism without the side effects seen with selective NMDA antagonists.

This thesis has highlighted the complexity of the expression and function of the metabotropic glutamate receptors and implicated them in nociceptive processing in the rat spinal cord.

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PUBLICATIONS

Some of the work presented in this thesis has been published elsewhere.

PAPERS

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ABSTRACTS

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APPENDIX

List of Abbreviations

3-HPG	3-hydroxyphenylglycine
3C4HPG	3-carboxy-4-hydroxyphenylglycine
4C3HPG	4-carboxy-3-hydroxyphenylglycine
4CPG	4-carboxyphenylglycine
5-HT	5-hydroxytryptamine
ACPD	1-aminocyclopentane-1,3-dicarboxylic acid
ACSF	artificial cerebrospinal fluid
AIDA	1-aminoindan-1,5-dicarboxylic acid
AMPA	α-amino-3-hydroxyl-5-methyl-1-isoxazole-4-propionic acid
APDC	2R,4R-4-aminopyrrolidine-2,4-dicarboxylate
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene-related peptide
CHO	chinese hamster ovary
CNQX	6-nitro-cyanoquinoxaline-2,3-dione
D-AP5	D(-)-amino-5-phosphonopentanoic acid
DAG	diacyl glycerol
DCG-IV	(2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine
DHPG	(S)-3,5-dihydroxyphenylglycine
EAA	excitatory amino acid
EGLU	(2S)-α-ethylglutamic acid
EPSP	excitatory postsynaptic potential
GABA	γ-amino butyric acid
IL-1β	interleukin-1β
IP_3	inositol trisphosphate
L-AP3	L-2-amino-3-phosphonopropanoate
L-AP4	L(+)-2-amino-4-phosphonobutyric acid

L-CCG-I	(2S,1'R,2'R)-2-(carboxycyclopropyl)glycine
L-CSA	L-cysteine-sulfinic acid
L-SOP	L-serine-O-phosphate
L_5	lamina 5
LY354740	1S,2S,5R,6S-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate monohydrate
MAP4	α-methyl-2-amino-4-phosphonobutyric acid
MCCG	α-methyl-(2S,1'R,2'R)-2-(carboxycyclopropyl)glycine
MCPG	α-methyl-4-carboxyphenylglycine
MPPG	α-methyl-4-phosphonophenylglycine
mRNA	messenger ribonucleic acid
MSOP	α-methylserine-O-phosphate
MSPG	α-methyl-4-sulphonophenylglycine
MTPG	α-methyl-4-tetrazolylphenylglycine
NMDA	*N*-methyl-D-aspartate
PTX	pertussis toxin
Quis	quisqualate
t-ADA	trans-azetidine-2,4-dicarboxylic acid
TNFα	tumour necrosis factor α
VIP	vasointestinal polypeptide