The Effects of Persistent Peripheral Inflammation on the Ultrastructural Localization of Spinal Cord Dorsal Horn Group I Metabotropic Glutamate Receptors

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October 2005

A thesis submitted to McGill University in partial fulfillment of the requirements for the degree of Master of Science

Your file Votre référence
ISBN: 978-0-494-24771-6
Our file Notre référence
ISBN: 978-0-494-24771-6

Abstract

Persistent peripheral inflammation is thought to induce functional plasticity of spinal dorsal horn neurons, and may produce changes in glutamate receptor expression. Alterations in expression and cellular localization of group I metabotropic glutamate receptors (mGluR1α and mGluR5) is important in various neuronal systems, and these receptors are also known to modulate nociceptive neurotransmission in the spinal dorsal horn. The aim of the present study is to determine whether persistent inflammation produces alterations in ultrastructural localization of mGluR1α and mGluR5 in the dorsal horn of the spinal cord. Persistent inflammation was induced in rats by an intraplantar hindpaw injection of complete Freund's adjuvant (CFA). Three days after the CFA injection, rats were perfused with fixatives, and spinal cords were removed and the lumbar segments L3-L5 were sectioned using a vibratome. Using pre-embedding immunocytochemical protocols for electron microscopy, we quantified immunogold-labeled mGluR1α and mGluR5, in lamina IV-V and I-II, respectively, in the spinal dorsal horn of both CFA-treated rats and untreated control rats. Compared to untreated rats, CFA-treated rats had a significant increase in membrane-associated mGluR5 immunogold-labeled particles in lamina I-II neurons. No change in the ratio of membrane vs. intracellular mGluR1α receptors was found in CFA-treated rats, however membrane-bound mGluR1α moved closer to the synapse in CFA-treated as compared with untreated rats. These findings suggest that persistent peripheral inflammation elicits increased availability of spinal dorsal horn group I mGluR's for synaptically-released glutamate binding. Thus, trafficking of Group I mGluR's may underlie the development of plastic

changes in spinal dorsal horn neurons and associated persistent inflammation-induced chronic pain.

Résumé

L'inflammation périphérique persistante induirait une plasticité fonctionnelle des neurones de la corne dorsale médullaire et pourrait produire des changements dans l'expression des récepteurs de glutamate. Les altérations dans l'expression et la localisation cellulaire des récepteurs métabotropiques de glutamate de groupe I (mGluR1α et mGluR5) sont importants pour plusieurs systèmes neuronaux. Ces récepteurs sont connus pour moduler la neurotransmission nociceptive au niveau de la corne dorsale de la moelle épinière. Dans cette recherche, l'objectif est de déterminer l'effet de l'inflammation périphérique persistante sur la localisation ultrastructurale des récepteurs mGluR1α et de mGluR5 de la corne dorsale médullaire. Une inflammation persistante a été induite chez des rats par une injection intraplantaire de Complete Freund's Adjuvant (CFA) dans la patte arrière gauche. Trois jours post-injection, les rats étaient perfusés avec des fixatifs, puis la moelle épinière était prélevée pour sectionner, au vibratome, les segments L3-L5. C'est au microscope électronique, avec le protocole d'immunocytochimie de pré-inclusion, qu'ont été quantifié les récepteurs immunologiquement marqués de mGluR1α et mGluR5, pour la lamina IV-V et I-II, respectivement. La procédure a été exécutée dans la corne dorsale de la moelle des rats injectés au CFA ainsi que dans celle des rats contrôles (non-injectés). Au niveau des neurones de la lamina I-II, les rats injectés avaient une augmentation significative des particules immunologiquement marquées pour les récepteurs membranaires de mGluR5, et ce, par rapport aux rats contrôles. Pour les récepteurs mGluR1α, le ratio d'expression membranaire vs. intracellulaire n'avait pas changé chez les rats traités au CFA,

toutefois les marqueurs des récepteurs membranaires de mGluR1α se retrouvaient plus près de la synapse chez les rats traités comparativement aux contrôles. Ces observations suggèrent que l'inflammation périphérique persistante suscite une augmentation de la disponibilité des récepteurs mGluR1 de la corne dorsale médullaire possiblement pour une éventuelle liaison glutamatergique au niveau synaptique. Donc, le déplacement des récepteurs mGluR de la groupe I pourrait signifier un processus de plasticité au niveau des neurones de la corne dorsale et être associé à l'inflammation persistante liée à la douleur chronique.

Acknowledgements

Dr. Terence Coderre

Supervisor, conceptualization of experiment and experimental design, editing of thesis. Unbelievably patient, supportive, and with great advice.

Dr. Alfredo Ribeiro da Silva

Kind use of his laboratory and materials, as well as valuable advice and support.

Manon St. Louis

Frequent technical help, advice, and support in performing experiments. Close supervision of my laboratory work, invariably saving me valuable time and effort.

Dr. Isabella Ruocco

Kindly teaching me the basics of immunocytochemistry, as well as how to do an experiment properly, cleanly, sparingly, and safely.

My Laboratory Colleagues

Support, advice, valuable lessons about laboratory etiquette.

My Family

Support in all I have done and want to do. Thanks to my brother for first providing the intellectual spark and then fanning the flames of my career.

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1 Background

1.1 Brief history

Glutamate's involvement in sensory processing in the spinal cord has been apparent since the late 1950's when Curtis and Watkins (1959, 1960a/b) showed that exogenous glutamate activated spinal cord neurons. Further evidence followed in 1967 when Graham demonstrated that glutamate was present in the central terminals of primary afferent fibres. Since then, a multitude of studies has implicated glutamate as the main excitatory transmitter in the mammalian central nervous system, and correspondingly, glutamate receptors to be critical to learning and memory in the brain (Riedel et al. 2003) and chronic nociceptive processing in the spinal cord (Dickinson and Sullivan, 1987; Ji & Woolf 2000; Ji et al. 2003).

1.2 Ionotropic Glutamate Receptors (iGluR's)

In 1979, Eccles and McGeer coined the term ionotropic glutamate receptors (iGluR's) to contrast the fast-activating, ligand-gated cation channels from glutamate receptors which stimulate the activity of various intracellular messengers, termed metabotropic glutamate receptors (mGluR's; See section 1.3). Three types of ionotropic glutamate receptor have been identified to date, and their nomenclature reflects the compounds that activate them most selectively. Hence, NMDA (N-methyl-D-aspartic acid), kainate, and AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors (the latter two sometimes described as AMPA/kainate or non-NMDA). Numerous subunits exist for each of the iGluR's, each conferring distinct properties to the homo- or

heteromeric assembly (see below) (Cull-Candy et al. 2001; Ruscheweyh & Sandkuhler, 2002; Bredt & Nicoll, 2003)

1.2.1 AMPA Receptors

Functional AMPA receptors are composed of various combinations of four subunits, GluR1-4, most commonly either homomers of GluR1 or GluR3, or symmetric dimers of GluR2 and GluR1 or GluR3 (Kandel, Schwartz & Jessell, 2000). AMPA receptors, permeable to Na^+, K^+ and even Ca^{2+} ions (Ozawa et al. 1998), are highly expressed in the postsynaptic region of excitatory synapses (Sheng, 2001). Mediating the fast, early component of the excitatory post-synaptic current (EPSC; Ozawa et al. 1998), AMPA receptors in spinal cord areas associated with nociceptive processing are activated by low and high frequency input, such as innocuous sensory and acute nociceptive stimulation (Garry et al. 2003). Moreover, noxious peripheral stimulation persistently activating spinal AMPA receptors provides sufficient neuronal depolarization to activate NMDA receptors (Ji & Woolf, 2001), a crucial player in plasticity (see section 1.2.3). As such, AMPA receptors are considered the main determinant of post-synaptic excitability (Bredt & Nicoll 2003). Due to their prominent contribution to fast synaptic transmission throughout the brain, it is not unreasonable to expect that AMPA receptor antagonism could elicit undesirable side effects (Riedel et al. 2003). Behaviorally, inhibition of AMPA receptor activation exhibits significant anti-nociceptive effects, at the price of side effects such as ataxia and sedation (Chizh 2003).

Consistent with their critical role in synaptic transmission, AMPA receptor density at the synapse is under tight control: In addition to lateral movement

within the plasma membrane (Borgdorff & Choquet, 2002), rapid, activity-dependent regulation of postsynaptic AMPA receptors occurs between cytoplasm and cell membrane (Carroll et al., 1999; Luscher et al. 2001), and is thought to underlie various forms of plasticity at excitatory synapses (Sheng & Lee, 2001; Malinow & Malenka 2002). For example, some central synapses express only NMDA receptors without the usual complement of AMPA receptors (Gomperts et al. 1998; Takumi et al. 1999). Characterized by NMDA currents but no AMPA currents, these "silent synapses" (Isaac et al. 1995; Liao et al. 1995) are unmasked by high frequency afferent stimulation, such that AMPA receptors are recruited in an activity-dependent manner to these previously AMPA receptor-lacking synapses (Shi et al. 1999). Furthermore, following persistent nociceptive input, activity-dependent changes in AMPA receptor expression can occur in the spinal cord (Harris et al. 1996; Zhou et al. 2001; Garry et al. 2003; Galan et al. 2004).

1.2.2 Kainate Receptors

Kainate receptors are composed of various combinations of five subunits: GluR5-7, and KA1 and KA2. Ion selectivity of kainate receptors emulates AMPA receptor permeability to K^+ and Na^+. However, depending on subunit composition, kainate receptors may also be permeable to Ca^{2+} or Cl^- (Ruscheweyh & Sandkuhler, 2002). Although not prominently expressed in spinal cord (Tolle et al. 1993; Petralia et al 1994), spinal cord kainate receptors can be expressed pre-synaptically, located on inhibitory interneurons (Kerchner et al. 2001) or co-localized with markers of nociceptive afferent fibres, suggesting pre-synaptic localization on afferent fibres, likely serving as autoreceptors to

modulate glutamate release (Bonnot et al. 1996; Yung, 1998). Post-synaptic kainate receptors in spinal cord regions involved in nociceptive neurotransmission (Furuyama et al. 1993; Li et al. 1999) are selectively activated by high-threshold stimulation likely mediated by nociceptive primary afferents (Li et al. 1999). Although few behavioral studies on the subject are currently available, inhibition of spinal kainate receptors seems to elicit increased withdrawal thresholds to mechanical and heat stimulation alone (Li et al. 1999) or following nerve injury (Sutton et al. 1999).

1.2.3 NMDA Receptors

Functional cell-surface NMDA receptors are heteromeric complexes composed of NR1 and NR2A-D subunits (McIlhinney et al. 1998; Ozawa et al. 1998). Although NR3 subunits also exist, their contribution to synaptic transmission is unclear (Cull-Candy et al. 2001). Receptor subunit composition determines channel properties such as ligand affinity, deactivation and desensitization, and antagonist sensitivity (McBain & Mayer, 1994; Bredt & Nicoll 2003). NMDA receptor activity is modulated through numerous binding sites, including sites for D-serine, polyamines, Mg^{2+}, zinc, glycine and glutamate. For channel activation to occur, functional cell-surface NMDA receptors require glycine. Although normally an inhibitory transmitter at Cl^- gating Gly1 receptors, glycine also binds to the NR1 subunit of the NMDA receptor complex (sometimes know as a Gly2 receptor), as an excitatory co-transmitter to glutamate, which binds to the NR2 subunit (Johnson & Acher, 1987; Laube et al. 1997). Moreover, NMDA receptor activation is also voltage-sensitive: at resting membrane potentials Mg^{2+} blocks the ion channel (Nowak et al.1984), significantly curbing its involvement in low

frequency synaptic transmission (Collingridge & Bliss, 1987). High frequency neuronal activity first activates AMPA receptors or receptors for neuropeptides or other transmitters, which provide the membrane depolarization required to relieve the Mg^{2+} block (Madison et al. 1991; Bredt & Nicoll 2003). This liberates the NMDA-receptor operated ion channel for current flow (Nowak et al., 1984) of $Ca^{2+,}$ Na^{+}, and K^{+} (Nicoll & Malenka, 1999). Consequently, NMDA receptors mediate the slower, late component of the EPSC (Ozawa et al. 1998). While NMDA receptor antagonism is effective in chronic inflammatory or neuropathic pain states (Fisher et al. 2000), their effects on either innocuous sensory or acute pain processing is limited (Chizh et al. 1997). Due mainly to their adverse side effect profile, such as sedation and ataxia, the use of NMDA receptor antagonists in a clinical setting is a thorny issue (Chizh, 2002).

Like AMPA receptors, NMDA receptors are widely distributed in the spinal cord (Ye & Westlund, 1996) and are mainly post-synaptic (Sheng, 2001). Despite their stable linkage to the cytoskeleton (Scannevin & Huganir, 2000; Carroll & Zukin, 2002), post-synaptic cell-surface NMDA receptors can undergo activity-dependent lateral diffusion (Tovar & Westbrook, 2002) as well as activity-dependent alterations in expression levels in brain (Snyder 2001; Montgomery & Madison, 2002) and spinal cord (Caudle et al. 2003: Tao et al. 2003).

1.3 Metabotropic Glutamate Receptors (mGluR's)

The mGluR's belong to the class C G protein-coupled receptor (GPCR) family. In addition to the metabotropic glutamate receptors, other receptors such as the γ-amino butyric acid (GABA) receptors, calcium-sensing receptors, receptors for sweet and umami tasting substances, and pheromone receptors

also belong to class C GPCR's (Mitri et al. 2004). Similar to other class C G protein-coupled receptors (GPCR's), metabotropic glutamate receptors share a seven transmembrane domain motif with long extracellular N terminal and intracellular carboxyl (C) terminal tails (Ozawa et al. 1998). Ligand binding occurs on the mGluR extracellular N-terminus, which is considerably longer than other GPCR's (Schoepp et al. 1999). G protein coupling takes place on the second intracellular loop, governing the transduction mechanism for the receptor subtype (Schoepp et al. 1999). Recent structural analysis indicates that the mGluR1 subunit forms homo-dimeric assemblies (Kunishima et al. 2000; Tsuchiya et al. 2002), a prerequisite for their function (Pin et al. 2004.). Indeed, activation of metabotropic glutamate receptor 1 dimers requires glutamate binding in both subunits (Kammermeier & Yun, 2005). The functional significance of mGluR dimerization, however, remains elusive (Goudet et al. 2005).

To date, eight metabotropic glutamate receptors have been identified and split into three groups based on pharmacology, signal transduction mechanisms, and sequence homology (Pin and Duvoisin, 1995; Knopfel et al. 1995). Overall, the mGluR's share approximately 40% amino acid sequence homology between groups, and about 60% within groups (Pin & Duvoisin, 1995; Knopfel et al. 1995; Anwyl 1999). Specifically, group I mGluR's are comprised of mGluR1 and mGluR5, while group II include both mGluR2 and mGluR3. The remaining, mGluR4, mGluR6, mGluR7 and mGluR8, make up group III (Anwyl, 1999).

1.3.1 Group I mGluR's

Group I mGluR's (mGluR1 & 5) share approximately 62% sequence homology with each other at the amino acid level (Pin & Duvoisin, 1995; Knopfel et al.

1995). Four splice variants have been described for mGluR1 (mGluR1α-δ: Houamed et al. 1991; Masu et al. 1991; Pin et al. 1992; Tanake et al. 1992; Laurie et al. 1996) and two for mGluR5 (mGluR5a/b: Abe et al. 1992; Minakami et al. 1993; Joly et al. 1995). The longer intracellular C-terminal tails of mGluR1α and mGluR5a/b determine G protein-coupled intracellular cascades as well as trafficking and anchoring within the cell (Alvarez et al. 2000).

Group I mGluR's couple primarily to Gq-like G-proteins (Anwyl 1999). Group I mGluR activation stimulates the effector molecule phospholipase C (PLC), which cleaves phosphatidylinositol1,4,5-biphosphate (PIP_2) into diaglyceral (DAG) and inositol 1,4,5-triphosphate (IP_3), leading to protein kinase C (PKC) activation, phosphoinositide (PI) hydrolysis and intracellular Ca^{2+} mobilization; events known to contribute to nociception (Igwe & Ning, 1994; Mao et al. 1995, Coutinho & Knöpfel, 2002), as well as the potentiation of NMDA receptor activation and exocytosis (Cerne and Randic, 1992; Lan et al. 2001a). In Chinese hamster ovary (CHO) cells, transfected mGluR1 also stimulates cAMP formation as well as arachidonic acid release (Aramori & Nakanishi, 1992), whereas mGluR5 does not couple with the stimulatory cAMP pathway (Abe et al., 1992). However, evidence from some expression systems exists for group I mGluR coupling to other types of G-proteins implicated in other intracellular effector molecules such as adenylate cyclase, tyrosine kinase, and MAP kinase (Valenti et al. 2002), also implicated in nociceptive neurotransmission (Cerne et al. 1992; Pezet et al. 2001; Karim et al. 2001).

In comparison to the iGluR's, group I mGluR-mediated excitatory post-synaptic currents (EPSC's) and potentials (EPSP's) are both slower to appear and slower

in time course (Schoepp et al. 1994; Doherty et al. 1997; Anwyl, 1999). Group I mGluR's are expressed throughout the peripheral and central nervous system (Hay et al. 1999; Bhave et al. 2001; Alvarez et al. 2001). Within the central nervous system, group I mGluR activation has been implicated in neuronal plasticity (Anwyl, 1999; Coutinho & Knopfel, 2002). Of the three mGluR groups, group I mGluR's are most strongly associated with increased neuronal excitability (Pin & Duvoisin 1995; Conn & Pin 1997; Anwyl 1999) and learning and memory processes in the brain (Riedel et al. 1996; Riedel et al. 2003), as well as persistent nociceptive neurotransmission in the spinal cord dorsal horn (Neugebauer et al. 1999; Varney & Gereau, 2002).

1.3.2 Group II & III mGluR's

Group II and III mGluR's are negatively linked to adenylate cyclase through Gi/Go-type G proteins, thus elicit reduced intracellular Ca^{2+} and cyclic AMP (cAMP). Localized both pre- and post-synaptically, group II mGluR's are generally found at the periphery of the synapse, indicating that high synaptic concentrations of glutamate, due to repeated or high frequency afferent stimulation, are required for activation (Shigemoto et al. 1997). Group III mGluR's, however, are mainly pre-synaptic, and thus act as autoreceptors, regulating the release of glutamate or other neurotransmitters (Cartmell, 2000). Stimulation of group II and III mGluR's generally results in decreased neuronal excitability and synaptic transmission (Conn & Pin, 1997; Schoepp et al., 1999). Although spinal cord localization of group II and III mGluR's is limited (Berthele et al. 1999; Jia et al. 1999), recent evidence supports an anti-nociceptive effect of group III mGluR activation in spinal cord neurons (Chen et al. 2005).

2 Group I mGluR Involvement in Spinal Nociceptive Processing

2.1 Group I mGluR Localization in Spinal Cord

In the spinal cord dorsal horn, the first modulatory site of nociceptive processing before the brain (Willis & Coggeshall, 1991), group I mGluR's can be found pre-synaptically, at the termination sites of primary afferent fibres (Jia et al. 1999; Tang & Sim, 1999; Tao et al. 2000; Park et al. 2004). However, in the spinal cord both mGluR1 and mGluR5 are mainly post-synaptic, often on dendrites and soma of dorsal horn neurons (Vidnyanszky et al 1994; Jia et al. 1999; Tang & Sim, 1999; Wang & Tseng, 2004). Relative to the neuronal plasma membrane, group I mGluR's can be found either intracellularly, or in an annular formation (Lujan et al. 1996; Lujan et al. 1997) at peri-synaptic or extra-synaptic locations (Vidnyanszky et al 1994; Alvarez et al. 2000). Interestingly, immunogold studies in the rat substantia nigra demonstrate that mGluR1α immunoreactivity is mainly associated with the plasma membrane, while most mGluR5 immunoreactivity is intracellular (Hubert et al. 2001).

In accordance with spinal cord regions known to receive nociceptive input from Aδ- and C- primary afferent fibres, the highest mGluR5 immunoreactivity (IR) is found in inner lamina II (lamina II_i), however moderate-to-abundant mGluR5-IR is found in lamina I and outer lamina II (lamina II_o) as well (Valerio et al. 1997; Alvarez et al. 2000). One study has demonstrated mGluR1α-IR in the superficial laminae of the spinal cord (Yung, 1998), however the antibody used has since been shown to bind to both mGluR1 and mGluR5 (Alvarez et al. 2000). Thus, other studies using antibodies directed to different amino acid sequences on the intracellular C-terminal tail show mGluR1α-IR only in deeper lamina (i.e, lamina

III-V) (Jia et al. 1999; Alvarez et al. 2000). Tao et al. (2000) have shown that approximately 20% of Aδ- and C-fibre central terminals appose mGluR5-labeled dendrites, while 15% of mGluR5-labeled dendrites receive synaptic contacts from Aδ- or C-fibres in normal rats.

2.2 Role of Group I mGluR's in Nociceptive Processing in Spinal Cord Dorsal Horn

Metabotropic glutamate receptors play a modulatory role in neurotransmission. Electrophysiological evidence in brain indicates that pre-synaptic group I mGluR's may act as autoreceptors, having either facilitatory or inhibitory effects on glutamate release (Anwyl, 1999; Cartmell & Schoepp 2000). However, in light of the relative paucity of pre-synaptic group I mGluR's in the spinal cord, more emphasis is placed on the excitatory, pro-nociceptive effects of post-synaptic group I mGluR activation.

2.2.1 Effects of Spinal Administration of Group I mGluR Agonists (Electrophysiology and Nociceptive Behaviours)

Spinal administration of both DHPG ((RS)-3,5-dihydroxyphenyl-glycine) and CHPG ((RS)-2-chloro-5-hydroxyphenylglycine), agonists to mGluR1/mGluR5 and mGluR5 respectively, potentiate Aδ- and C-fibre-mediated polysynaptic EPSP's (excitatory post-synaptic potentials). Spinal administration of the mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP), as well as the group I mGluR antagonist/mGluR2 agonist (S)-4-carboxyphenylglycine ((S)-4-CPG), attenuates this facilitation (Zhong et al. 2000). In naïve rodents, intrathecal (i.t.) administration of the broad-spectrum mGluR agonist (1S,3R)-

aminocyclopentane-1,3-dicarboxylic acid ((1S, 3R)-ACPD), as well as DHPG, excite dorsal horn neurons (Neugebauer et al. 1994; Young et al. 1997) and induce spontaneous nociceptive behaviors (SNB's) such as hind paw licking, shaking or biting (Fisher & Coderre, 1996a). I.t. treatment with either the group I-II mGluR antagonist (S)-α methyl-4-carboxyphenylglycine ((S)-α-MCPG), or the mGluR1-specific antagonist 7-hydroximinocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt), attenuates SNB's after DHPG (Fisher & Coderre, 1996a; Karim et al. 2001). Young et al. (1997) demonstrated that i.t. treatment with an mGluR1-specific antagonist cyclothiazide (CTZ), reduces dorsal horn neuronal responses to DHPG. Accordingly, Fundytus et al. (1998) found that i.t. administration of antibodies directed against the intracellular C-terminal tail of either mGluR1 or mGluR5 attenuates SNB's following i.t. DHPG, further demonstrating the major role group I mGluR's play in spinal nociceptive processing. Although it could be argued that these antibodies should not reach their targets *in vivo*, it has been shown that antibodies directed to intracellular regions can penetrate living cells by an unknown mechanism (Ruiz-Arguelles et al. 2003). On the other hand, no behavioral effects follow either i.t. administration of specific group II or III mGluR agonists (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate ((2R,4R)-APDC) and L-2-amino-4-phosphonobutyrate (L-AP4), respectively), or the selective mGluR5 agonist *trans*-azetidine dicarboxylic acid (*trans*-ADA); Fisher & Coderre, 1996a), indicating that DHPG-induced SNB's are due mainly to activity at mGluR1. Interestingly, i.t. DHPG activates 40 - 53% of neurons examined, while t-ADA activates 19 - 23% of dorsal horn neurons examined (Young et al. 1997). Moreover, Lorrain et al. (2002) showed that while

i.t. DHPG elicits SNB's as well as increased glutamate release in the spinal cord in a dose-dependent manner, pretreatment with the mGluR5-specific antagonist MPEP attenuates SNB's and decreases glutamate release following DHPG. Thus, combined mGluR1/mGluR5 activation elicits both dorsal horn neuronal excitation and pain behaviors, which are attenuated by either mGluR1 or mGluR5 antagonists. However, while activation of spinal mGluR5 alone elicits significant neuronal excitation, no behavioral correlate appears.

2.2.1.1 Innocuous Sensory Processing & Acute Noxious Stimulation

Agonists of group I mGluR's appear to exert biphasic neuronal responses to innocuous stimulation in the spinal cord, such that a low concentration of the group I mGluR-specific agonist DHPG potentiates neuronal response to innocuous mechanical stimulation, while higher doses inhibit these responses. In the same study, Neugebauer et al. (1999) showed that the mGluR5-specific agonist CHPG also inhibits neuronal responses to innocuous stimulation, suggesting the inhibitory effect may be mGluR5-mediated.

Although dorsal horn neuronal responses to innocuous stimulation can be modulated by group I mGluR activation, antagonists of group I mGluR's do not appear to have an effect. Antisense oligonucleotides directed against mGluR1 (Young et al. 1998) and the mGluR1-specific antagonists (RS)-1-aminoindan-1,5-dicarboxylic acid (AIDA), CPCCOEt and CTZ do not affect the level of neuronal activation following innocuous stimulation (Young et al. 1997; Neugebauer et al. 1999). Overall, innocuous sensory processing does not seem to require group I mGluR activation, since antagonists have no effect. However, while no substantial changes to normal sensation follow mGluR1 activation, mGluR5

activation apparently hinders normal sensory processing through an unknown mechanism.

Similar to dorsal horn neuronal responses to innocuous stimulation, low doses of spinally administered DHPG potentiates dorsal horn neuronal responses to noxious mechanical stimulation, while higher doses inhibit these responses. Interestingly, the mGluR5-specific agonist CHPG also inhibited noxious mechanical stimulation (Neugebauer et al. 1999). I.t. administration of DHPG also produces heat hyperalgesia and mechanical allodynia (Fisher & Coderre, 1998). I.t. pretreatment with the mGluR5-specific antagonist MPEP blocks cold allodynia induced by i.t. DHPG (Hama, 2003).

In normal rodents, i.t. administration of the group I mGluR antagonist (*S*)-4-carboxy-3-hydroxyphenylglycine ((S)-CHPG) increases thermal latencies and mechanical thresholds, while the weak non-selective mGluR antagonist L-AP3 had no effect (Young et al. 1997). Furthermore, when injected intrathecally, neither antibodies against mGluR1 nor mGluR5 significantly altered the response latency to noxious thermal heat to the rat hind paw (Fundytus et al. 1998).

2.2.1.2 Inflammation

When injected in the hind paw of the rat, numerous substances produce a local inflammatory response, spontaneous nociceptive behaviors and thermal and mechanical hypersensitivity lasting for minutes, hours or even days. The effects of inflammatory agents such as formalin, mustard oil, capsaicin, carrageenan and complete Freund's adjuvant (CFA) have been extensively studied in the rat spinal cord dorsal horn, and it is generally understood that while they do not all act through identical neuronal pathways or elicit the same

behavioral responses, their behavioral and electrophysiological effects are all modulated by group I mGluR activation or inhibition.

2.2.1.2.1 Mustard Oil

Mustard oil (allyl isothiocyanate) causes neurogenic inflammation by selective activation of C-fibres, leading to enlarged receptive fields, enhanced dorsal horn neuronal activity and hypersensitivity to thermal and mechanical stimuli (Woolf & Wall, 1986; Woolf et al. 1994; Munro et al. 1994). Spinal application of the broad-spectrum mGluR antagonist R,S-CHPG as well as the group I mGluR antagonist L-AP3 reduce mustard oil-enhanced neuronal responses (Young et al. 1994; Young et al. 1995a). Furthermore, specific antagonism or antisense ablation of spinal mGluR1 reduces dorsal horn neuronal responses to mustard oil (Young et al. 1997; Young et al. 1998).

2.2.1.2.2 Capsaicin

A VR1 (vanilloid receptor 1) agonist, capsaicin is the principal "hot" component in hot peppers (Lesage, 2004). Expressed on C- and Ad-fibres (Ma, 2001), VR1 activation by capsaicin results in the release of glutamate and substance P into the spinal cord (Gamse et al. 1979; Sluka & Willis 1998), sensitization of spinal cord neurons as well as mechanical and thermal hypersensitivity (Willis, 2001). Intraplantar capsaicin potentiates dorsal horn neuronal responses to noxious mechanical stimulation, an effect that is reversed by the mGluR1-specific antagonists AIDA and CPCCOEt (Neugebauer et al. 1999). Both the mGluR1 antagonist AIDA and the mGluR5 antagonist MPEP reduce mechanical hypersensitivity after capsaicin, whereas neither reduce heat hypersensitivity

(Soliman et al. 2005). Together, these studies indicate group I mGluR involvement in capsaicin-mediated mechanical, but not heat hypersensitivity.

2.2.1.2.3 Formalin

Devised by Dubuisson and Dennis (1977), the formalin test involves intraplantar injection of formaldehyde diluted in saline, dubbed formalin. Intraplantar formalin generates spontaneous nociceptive behaviors in a bi-phasic pattern (Fisher & Coderre, 1996b). The first phase lasts approximately 15 minutes, while the second phase, preceded by a brief quiescent period, lasts for about 60 minutes (Coderre et al. 1990; Coderre & Melzack 1992; Fisher & Coderre 1996b). I.t. administration of the mGluR1 and mGluR5 antagonists CPCCOEt and MPEP, respectively, attenuates pain behavior associated with the early phase of the formalin test (Karim et al. 2001). Antisense knockdown of mGluR1 reduced the second but not the first phase pain behaviors (Noda et al. 2003). However, Fisher & Coderre (1996b) found that i.t. (S)-4CPG or (S)-CHPG, group I mGluR antagonists, result in a slight attenuation of nociceptive behaviors in the late phase of the formalin test. In addition, the group I mGluR agonist DHPG potentiates nociceptive behaviors during the late phase but not the early phase, an effect that can be reduced by prior i.t. administration of the group I antagonists (S)-α-MCPG and (S)-4C3HPG (Fisher & Coderre 1996b).

In contrast, spinal administration of antibodies directed against mGluR1 and mGluR5 had no effect on responses to intraplantar formalin (Fundytus et al. 1998). Furthermore, i.t. treatment with group I mGluR antagonist/mGluR2 agonist (S)-4CPG failed to deter nociceptive behaviours in the formalin test (Yashpal et al. 2001).

2.2.1.2.4 Carrageenan

Within three hours of intraplantar (hind paw) or intra-articular (knee joint) injection of the seaweed extract carrageenan, there are symptoms including localized inflammation, spontaneous nociceptive behaviors, increased mechanical and thermal hypersensitivity, as well as increased spinal cord dorsal horn glutamate release (Sluka & Westlund, 1992; Hargreaves et al. 1998; Dolan et al. 2003). Carrageenan-induced hypersensitivity is believed to be a result of sensitization of primary afferent nociceptors and spinal cord neurons (Schaible et al. 1985; Schaible et al. 1987). Spinal application of 2-amino-2-(3-*cis* and *trans*-carboxycyclobutyl)-3-(9*H*-thioxanthen-9-yl)propionic acid (LY393053), a group I mGluR antagonist, as well as the mGluR1-specific antagonists (+)-2-methyl-4-carboxyphenylglycine (LY367385) and AIDA reduce the thermal hyperalgesia induced by knee joint injection of carrageenan/kaolin (Zhang et al. 2002). I.t. administration of the group I mGluR antagonist AIDA either before or after intraplantar carrageenan also blocks development of mechanical hyperalgesia (Dolan & Nolan, 2002). Moreover, spinal application of the non-selective mGluR antagonist (S)-4-carboxy-3-hydroxyphenylglycine ((S)-CHPG) increases thermal latencies and mechanical thresholds in both naïve and carrageenan-inflamed rats. However, the group I mGluR-specific antagonist L-(+)-2-amino-3-phosphonopropionic acid (L-AP3) increases latencies and thresholds in only the carrageenan-treated rodents, suggesting that dorsal horn group I mGluR's play a more pronounced role in inflammatory pain (Young et al. 1995b; Young et al. 1997). These findings are in agreement with Neugebauer et al. (1994), who found

that the inhibitory effect of L-AP3 on dorsal horn neuronal activity is present only after peripheral inflammation.

2.2.1.2.5 Zymosan

Zymosan is comprised of particles of the yeast *Saccharomyces cerevisiae* cell wall, containing mainly polysaccharides (DiCarlo et al. 1957). Zymosan induces the release of inflammatory products including TNF-α, IL-8, hydrogen peroxide, and arachidonic acid (Daum & Rohrbach, 1992; Noble et al. 1993; Okazaki et al. 1996). Zymosan also induces protein phosphorylation and inositol phosphate formation (Bondeson, 1996). Peripheral (hind paw) administration of zymosan produces a robust local inflammatory response (Guhring et al. 2001), as well as thermal and mechanical hyperalgesia (Meller & Gebhart, 1997). In addition to enhanced dorsal horn glutamate release, peripheral zymosan injection increases metabolic activity and possibly disturbs membrane integrity of spinal cord dorsal horn neurons (Vetter et al. 1996).

Intrathecal injection of the group I mGluR antagonist AP3 reduces mechanical withdrawal thresholds of zymosan-treated rats. However, spinal AP3 has no effect on zymosan-induced thermal hyperalgesia (Turnbach & Randich, 2001). In contrast, ionotophoretic application of AP3 decreases spinal wide dynamic range (WDR) neuronal responses to thermal stimulation of the hind paw in zymosan-treated but not saline-treated rats. AP3, however, does not affect neuronal activity in nociceptive-specific (NS) neurons after thermal stimulation in either the zymosan- or saline-treated rats (Spraggins et al. 2001).

2.2.1.2.6 Complete Freund's Adjuvant (CFA)

Complete Freund's adjuvant (CFA) is a suspension of heat-killed mycobacterium tuberculosum or butyricum in mineral oil. When injected in the the rodent hind paw, the classical "inflammatory triad" of swelling (tumor), heat (calor) and redness (rubor) appear, as well as expanded receptive fields of dorsal horn neurons (Hylden et al. 1989; Su & Urban, 2005) and mechanical (Stein et al. 1988) and thermal hypersensitivity (Hylden et al. 1989). Considered by many to be a model of persistent inflammation due to the activation and infiltration of immune cells, hypersensitivity to mechanical and thermal stimulation develops within 24 hours of injection, often lasting for at least several days (Hylden et al. 1989).

Intrathecal DHPG, administered 7 days after CFA-treatment, enhances both mechanical hypersensitivity and SNB's (Adwanikar et al. 2004). Antisense oligonucleotide knockdown of dorsal horn mGluR1 in rats, begun either before or after CFA-treatment, resulted in significant attenuation of both heat hyperalgesia and mechanical allodynia for up to 8 days post-CFA (Fundytus et al. 2002). However, Walker et al. (2001) found that intrathecal MPEP did not reduce mechanical hyperalgesia 24 hours following intraplantar injection of a small volume (2.5 uL) of CFA.

2.2.1.3 Neuropathy

In addition to traumatic accidents involving tissue and nerve damage, illnesses such as diabetes as well as certain chemotherapeutic drugs can give rise to neuropathic pain, which is often refractory to treatment. Animal models of

neuropathic pain often involve injury of peripheral nerves. Nerve injury-induced aberrant activity in both the peripheral and central nervous system can elicit hyperalgesia (exaggerated response to noxious stimulus) and allodynia (pain produced by normally innocuous stimulus), as well as spontaneous pain (Ji & Strichartz, 2004).

2.2.1.3.1 Peripheral Neuropathy

The chronic constriction injury (CCI) is widely used model of neuropathic pain involving a loose ligation of the rat sciatic nerve with either chromic catgut (Bennett & Xie 1988) or a polyethylene cuff (Mosconi & Kruger, 1996). Requiring approximately two weeks for maximal behavioral hypersensitivity to develop, the chronic constriction injury functionally axotomizes most large myelinated A-fibres, while sparing the majority of small unmyelinated C-fibres (Mosconi & Kruger, 1996). CCI-induced thermal and mechanical hypersensitivity are associated with ectopic firing of damaged fibres (Kajander & Bennett, 1992). Furthermore, nerve injury produces long term potentiation (LTP; see section 3.1) in spinal cord dorsal horn neurons (Sandkuhler & Liu, 1998)

Spinal administration of MPEP reduces spontaneous and noxious stimulation-evoked dorsal horn neuronal activity in CCI rats, while higher doses reduce evoked responses but not spontaneous neuronal activity in sham rats (Sotgiu et al. 2003), indicating a role for mGluR5 in neuropathic pain. Correspondingly, pretreatment with AIDA and MPEP, antagonists at mGluR1 and mGluR5 respectively, reduces the development of mechanical hypersensitivity following CCI. AIDA is also effective in reducing the development of cold hypersensitivity (Fisher et al. 2002). Antisense knockdown of spinal mGluR1 in CCI rats produces

decreased cold and heat hyperalgesia, as well as mechanical allodynia (Fundytus et al. 2001). However, where Fundytus et al. (1998) demonstrated that i.t. administration of antibodies directed against the intracellular C-terminal tail of mGluR1 and mGluR5 attenuates cold allodynia after CCI, spinal administration of MPEP does not (Fisher et al. 2002; Hama 2003; Urban et al. 2003). Prolonged post-operative treatment with the group I mGluR antagonist (S)-4-CPG attenuates mechanical allodynia and cold hyperalgesia (Fisher et al. 1998; Yashpal et al. 2001). In addition, early (S)-4CPG treatment (twice-daily injections on days 0-3 post-CCI) attenuates the development of mechanical allodynia and cold hyperalgesia. However, late treatment (twice-daily injections on days 8-11 post-CCI) has no effect on mechanical or cold hypersensitivity (Fisher et al. 1998). In another model of peripheral neuropathy, rats with spinal nerve ligation (SNL) show enhanced mechanical and heat hyperalgesia. Intrathecal 6-methyl-2-(phenylazo)-3-pyridinol (SIB-1757), an mGluR5 antagonist, partially reverses mechanical allodynia and fully reverses heat hyperalgesia associated with SNL (Dogrul et al. 2000).

2.2.1.3.2 Spinal Cord Injury

A significant reduction in amplitude of compound action potentials follows compression injury of the isolated spinal cord section. While bath application of the group I/II agonist trans-ACPD and group I agonist DHPG aggravate the reduction in amplitude, blockade of group I mGluR receptors with MCPG, 4CPG, or the mGluR1 antagonist AIDA returns amplitude to near baseline levels (Agrawal et al. 1998).

Impact-induced spinal cord injury (SCI) in rats leads to an increase in the concentration of extracellular glutamate, giving rise to glutamate receptor-mediated excitotoxicity. Intrathecal treatment with the mGluR1-specific antagonist AIDA significantly decreases extracellular glutamate concentration, improves locomoter scores, and significantly attenuates the development of mechanical but not thermal allodynia (Mills et al. 2000; Mills et al. 2001b; Mills et al. 2002b). Furthermore, i.t. MPEP also reduces glutamate concentration and attenuates the development of thermal hyperalgesia, with no effect on locomoter scores and mechanical allodynia (Mills et al. 2001b; Mills et al. 2002b). Combined spinal application the mGluR1 antagonist LY 367385 and the mGluR5 antagonist MPEP results in a decrease of glutamate concentration greater than either agent alone (Mills et al. 2001b). LY 367385-treated rats show improved locomotor scores and decreased mechanical allodynia. Unexpectedly, LY 367385 potentiates the development of thermal hyperalgesia (Mills et al. 2002b).

3 Group I mGluR Involvement in Activity-Dependent Receptor Trafficking

3.1 Role of Group I mGluR's in Spinal Cord Plasticity

In *Organization of Behavior* (1949), Donald Olding Hebb stated that *"when an axon of cell A is near enough to excite B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased."* In other words, the efficacy of synaptic transmission changes following certain types of afferent input. Bliss and Lomo's (1973) seminal paper provided the basis for "Hebb's synapse" by demonstrating that high frequency stimulation can

potentiate synaptic efficacy in a relatively stable and persistent fashion, a process dubbed long term potentiation (LTP). Since then, under the rubric of synaptic plasticity, many forms of activity-dependent changes in synaptic efficacy throughout the central nervous system have been described.

Depending on the conditioning stimulus, various forms of plasticity occur in the spinal cord dorsal horn (Sheng & Kim, 2002). Following peripheral capsaicin application or repeated high-frequency C-fibre-strength electrical stimulation of the sciatic nerve, wind up of C-fibre-mediated synaptic potentials results in dorsal horn neuronal responses progressively reaching a transient plateau response much higher than the original (Mendell & Wall, 1965; Dickenson, 1990; Silivotti et al. 1993; Thompson et al. 1990; Ji et al. 2003). Wind up is a short-lived, activity-dependent facilitation of excitability in spinal cord neurons, resulting in a cumulative depolarization, repetitive spiking (wind-up) and a prolonged (seconds to minutes) after-discharge of the cell post-synaptic to the high frequency afferent input. Spinal long term potentiation (LTP), however, is a long-lasting (minutes to hours) activity-dependent facilitation of EPSP's following brief, high frequency trains of electrical stimulation or intense nociceptor activity due to nerve injury or inflammation (Randic et al. 1993; Randic 1996; Sandkuhler et al. 1997; Sandkuhler & Liu, 1998; Sandkuhler, 2000). Induction of spinal LTP requires NMDA receptor activation as well as increased intracellular Ca^{2+} levels (Randic et al. 1993; Sandkuhler et al. 1997; Ji et al. 2003). As high frequency, C-fibre strength stimulation persists, neurons become sensitized, a condition commonly known as central sensitization. Enhanced spontaneous and evoked activity, expanded receptive fields and increased efficacy of nociceptive

neurotransmission of spinal dorsal horn neurons characterize this facilitated state of processing after persistent noxious input (Ji et al. 2003). The spinal mechanisms responsible for central sensitization and behavioral hypersensitivity associated with persistent nociceptive input involve activity-dependent alterations in synaptic efficacy (Dubner & Ruda, 1992; Ji et al. 2003). In particular, receptor trafficking and altered receptor/ion channel activity seems to be cardinal (Woolf & Salter, 2000).

Wind-up is enhanced by the broad spectrum mGluR agonist (1S,3R)-ACPD (Budai & Larson 1998), and is inhibited by the group I/II antagonist (S)-α-MCPG as well as the mGluR5-selective antagonist MPEP (Boxall et al. 1996; Bordi et al. 2000). In the same vein, antagonists specific to group I mGluR's, intrathecally delivered prior to electrical stimulation, impair the development of spinal LTP (Azkue et al. 2003). In the absence of repetitive pre-synaptic stimulation, direct activation of group I mGluR's by the group I / II agonist (1S,3R)-ACPD, the group I agonist DHPG and the mGluR5-specific agonist CHPG induce LTP in spinal cord slice neurons. These effects are partially reversed by the group I mGluR antagonist S4-CPG and the mGluR5 antagonist MPEP, and totally reversed by the NMDA receptor antagonist D(-)-2-amino-5-phosphonopentanoic acid (AP5; Zhong et al. 2000).

3.2 Role of Receptor Trafficking in Synaptic Plasticity

Current thought holds both pre- and post-synaptic mechanisms responsible for plasticity in the central nervous system. However, due to phenomena such as activity-dependent recruitment of AMPA receptors to previously "silent synapses", modifications to the post-synaptic neuron are increasingly emphasized (Malenka

& Nicoll, 1999; Sheng & Kim, 2002). Accordingly, the once-prevalent paradigm that post-synaptic glutamate receptors are locked in place has shifted to the idea that alterations in the number of functional, membrane-bound receptors participate in activity-dependent plasticity of synaptic transmission (Scannevin and Huganir, 2000; Carroll et al., 2001; Ji et al. 2003). Since the concentration gradient of pre-synaptically released neurotransmitter decreases with distance from the release site, the quantity and proximity of post-synaptic receptors to the synapse affects the likelihood of receptor activation (Bergles et al. 1999; Trimmer, 1999). Thus, post-synaptic neuronal activity is modulated by variation of the quantity of plasma membrane-associated receptors and/or the distance of a plasma membrane-associated receptor to the synapse (Bergles et al. 1999). Collectively termed receptor trafficking, the processes of endocytosis (internalization) and exocytosis, as well as lateral diffusion along the plasma membrane, are taken to be the general mechanisms responsible for variations in number and/or type of receptors at synapses (Carroll et al. 2001; Triller & Choquet, 2003).

3.2.1 Endocytosis and Exocytosis

Proper signaling requires the maintenance of a delicate balance of endocytosis and exocytosis (Triller & Choquet, 2003). Endocytosis, also known as internalization, is the process whereby receptors are removed from the cell-surface. Generally, endocytosis of cell surface mGluR's occurs through an agonist-dependent, clathrin-mediated pathway (Mundell et al. 2001; Albasanz et al. 2002; Pula et al. 2004). Ligand-bound receptors become desensitized (Bohm et al. 1997) and diffuse laterally to an extra- or peri-synaptic internalization zone

or "hot-spot" (Blanpied et al. 2002). The receptor-ligand complexes are then invaginated in clathrin-coated pits, followed by the formation of coated vesicles that are internalized and pinched off with the participation of the GTPase dynamin (Sweitzer et al. 1998; Mundell et al. 2001). Once internalized, clathrin-coated vesicles fuse with endosomes where ligand and receptor are separated. Receptors are then degraded into lysosomes or recycled to the cellular surface (Ferguson et al. 1998; Ceresa et al. 2000). Agonist-independent group I mGluR internalization also occurs (Dale et al. 2001; Pula et al. 2004), however the underlying mechanism is not clear.

Exocytosis is not the inverse process of endocytosis. However, current understanding of glutamate receptor exocytosis is limited, involving mainly AMPA receptors. Intracellular AMPA receptors are generally assembled and retained in the endoplasmic reticulum, transited through the Golgi and *trans*-Golgi into transport vesicles, and sent to axonal or dendritic zones for exocytosis. The exact mechanism underlying the trafficking of intracellular AMPA receptors to the membrane remains unclear, although a targeting role for scaffolding proteins containing PDZ domains has been proposed (see section 3.5). Recent studies indicate that AMPA receptors are initially delivered to the extra-synaptic region where lateral diffusion brings them to the synapse (Song & Huganir, 2002; Bredt & Nicoll, 2003).

3.2.2 Lateral Diffusion

Despite its important role in endo- and exocytosis of AMPA receptors, for instance, lateral diffusion is an activity-dependent form of receptor trafficking unto itself. Axelrod et al. (1976), originally described lateral diffusion, or the movement

of receptors along the plasma membrane, to occur on acetylcholine receptors at the neuromuscular junction, a process later postulated to underlie synapse formation (Young & Poo, 1983). Almost two decades later, Rao et al. (1998) suggested that lateral diffusion might alter the concentration of AMPA and NMDA receptors at central synapses, a phenomenon now known to occur in an activity-dependent manner (Tovar & Westbrook, 2002; Tardin et al. 2003; Groc et al. 2004). While mGluR5 is laterally mobile in an agonist-dependent manner in cultured hippocampal neurons (Serge et al. 2002), in the spinal cord, only evidence for the lateral mobility of glycine receptors exists (Srinivasan et al. 1990; Dahan et al. 2003).

3.3 Role of Receptor Trafficking in Spinal Cord Nociceptive Processing

Evidence for alterations in the trafficking of receptors in spinal cord dorsal horn is to this date fairly limited. After CFA injection in the rat hind paw, Zhou et al. (2001) reported increased expression of AMPA receptors in the spinal cord. Cahill et al. (2003) showed that in response to intraplantar administration of CFA, hypersensitivity to thermal stimulation is observed, as well as increased levels of plasma membrane-associated δ-opioid receptors (Cahill et al. 2003). More recently, Galan et al. (2004) found that dorsal horn AMPA receptors are recruited to the plasma membrane following intracolonic capsaicin, contributing to increased pain sensitivity.

3.4 Group I mGluR Expression and Trafficking

Using an animal model of epilepsy, Akbar et al. (1996) show an initial up-regulation in hippocampal mGluR1 followed by a return to baseline levels within

one month. In contrast, although levels of mGluR5 initially decrease, persistent, region-specific up- or down-regulation of mGluR5 mRNA occurs. In another model of epilepsy in rats, mGluR5 protein expression increases markedly in hippocampal reactive astrocytes by one week after treatment, an effect lasting several months. In accordance, both Aronica et al. (2000) and Ulas et al. (2000) find persistent increases in astrocytic mGluR5 following stimulation-induced hippocampal seizures. Similarly, spinal cords of humans suffering from amyotrophic lateral sclerosis show a marked increase in mGluR1α and mGluR5 in reactive astrocytes compared to controls (Aronica et al. 2001). Exposure of astrocytes to specific growth factors increases both the level of astrocytic mGluR5 and the ability of group I mGluR agonists to stimulate phosphoinositide hydrolysis (Miller et al. 1995). Chronic amphetamine exposure in rats causes a transient increase in striatal mGluR1α, yet a sustained decrease is seen in striatal mGluR5 (Mao & Wang, 2001). An up-regulation of mGluR5 was observed in the cerebral cortex of Down's syndrome cases (Oka & Takashima, 1999). In a mouse model of glaucoma, expression of both mGluR1α and mGluR5 increase in the retina and ganglion cell layers of the eye (Dyka et al. 2004). Enteric neurons express both iGluR's (Burns et al. 1994; Liu et al. 1997; Kirchgessner et al. 1997) as well as mGluR's (Liu & Kirchgessner, 2000; Tong & Kirchgessner, 2003), both which are involved in sensation (Kirchgessner et al. 1992) and enteric motility (Wiley et al. 1991). In response to either agonist application or enteric reflex, plasma membrane-associated mGluR5 is internalized (Liu & Kirchgessner, 2000).

Interestingly, despite the fact that group I mGluR's play important roles in various processes in the body, it is surprising that so little has been done to examine whether these alterations in group I mGluR expression level reflect sub-cellular trafficking.

3.4.1 Changes in Group I Spinal Cord mGluR Expression Associated with Nociception or Spinal Cord Injury

Two weeks after sciatic nerve section, a peripheral neuropathic injury, Hudson et al. (2002) found increase in mGluR5 immunoreactivity in lamina II of the L4-L5 region of the spinal cord, particularly in regions of the superficial lamina (lamina I-II) receiving non-peptidergic afferent C-fibre input. Measuring mGluR1α and mGluR5 mRNA in sheep spinal cord, Dolan et al. (2003) found no change in dorsal horn mGluR1 and mGluR5 mRNA after carrageenan-induced acute inflammatory pain. However, after persistent inflammatory pain due to a bacterial infection of the foot, a dramatic increase in dorsal horn lamina I-II mGluR5 mRNA is reported. No change in dorsal horn mGluR1 mRNA is detected. Dolan et al. (2004) showed an up-regulation of mGluR5 mRNA and protein one day after abdominal surgery in sheep. No changes were seen in mGluR1. In a rat model of diabetic neuropathy, elevated levels of both mGluR1 and mGluR5 mRNA are detected throughout the spinal cord dorsal horn (Tomiyama et al. 2005). In contrast, no changes in dorsal horn mGluR1 or mGluR5 expression are seen seven days after intraplantar CFA, despite the finding that intrathecal DHPG strongly potentiates mechanical allodynia in these mice (Adwanikar et al. 2004).

Following crush-induced spinal cord injury (SCI), Mills et al. (2001) observed increased levels of mGluR1 in spinal cord segments rostral and caudal to the

injury site between seven and 60 days post-SCI. Levels of mGluR5 immunoreactivity in the superficial lamina of the spinal cord were significantly elevated on day 1 post-SCI but this increase was not present on day 60. Similarly, Mills and Hulsebosch (2002) demonstrated an increase in mGluR1 immunoreactivity in the peri-lesion area on spino-thalamic tract cells after spinal cord crush injury. Gwak & Hulsebosch (2005), also using SCI, found increased expression of both mGluR1 and mGluR5 on spinal cord neurons and astrocytes. Unilateral transection of the spinal cord lateral funiculus produces changes in group I mGluR expression: Levels of mGluR1 underwent an initial decrease, followed by a brief increase. However, mGluR1 expression again decreased, remaining low for up to two years. Levels of mGluR5, on the other hand, underwent a dramatic up-regulation between two and four weeks post-injury, and decreased to control levels, where it remained for up to two years (Wang & Tseng, 2004). In general, peripheral nerve or tissue insult seems to affect mainly spinal mGluR5 expression levels, while direct spinal cord injury results primarily in alterations of mGluR1 expression in the spinal cord neurons.

3.5 Scaffold Proteins Involved in Group I mGluR Trafficking

While it is clear that the level of group I mGluR expression is altered in various tissues in response to various stimuli, including spinal cord group I mGluR's in response to persistent nociception, very little is understood about the cellular mechanism(s) responsible.

Multi-molecular protein assembly through protein-protein interaction is vital for diverse cellular functions in neuronal cells (Scannevin & Huganir, 2000; Sheng & Sala, 2001). Scaffolding proteins with numerous protein-protein interaction

domains are central to this process. In neurons, the PDZ ((PSD-95)1/discs-large/ZO-1) domain is an important protein-binding domain (Kornau et al. 1995; Songyang et al. 1997; Bredt & Nicoll, 2003). Scaffolding proteins containing the PDZ domain are crucial to intracellular trafficking of their partner proteins (Kim, 1997; Sheng & Sala, 2001). With respect to group I mGluR's, two recently identified intracellular scaffolding proteins, Homer and Tamalin, hold promise.

3.5.1 Homer

Discovered by Brakeman et al. (1997), the intracellular scaffolding protein Homer has recently been implicated in dynamic effects on group I mGluR expression and function (Ciruela et al. 1999; Roche et al. 1999; Ango et al. 2000). Three genes encode Homer proteins, Homer1-3. Homer proteins comprise an N-terminal domain homologous to the EVH1 domain of the Ena/VASP family of proteins and a C-terminal domain containing a leucine zipper motif responsible for their dimerization (Kato et al. 1997; Xiao et al. 1998). Homer 1b/c and 3 are found in the cytoplasm at the lateral margin of the post-synaptic density (Xiao et al. 1998). Homer1 proteins interact with the C-terminus of the mGluR1α, mGluR5a, mGuR5b, IP3, and ryanodine receptors (Brakeman et al. 1997; Tu et al. 1998; Kato et al. 1998; Xiao et al. 1998). Homer proteins also interact with the post-synaptic density protein Shank, a PDZ domain-containing protein binding to the GKAP/PSD-95/NMDA receptor complex (Naisbitt et al. 1999; Tu et al. 1999; Valenti et al. 2002), through which group I mGluR's and NMDA receptors cluster in the post-synaptic density and, in hippocampal neurons, NMDA receptor activity is potentiated (Fitzjohn et al. 1996). While Homer1b and Homer1c are constitutively expressed (Fagni et al. 2004),

Homer1a, the only isoform lacking the long C-terminal motif, is the product of an immediate early gene (IEG) induced by intense neuronal activity such as convulsive seizures or LTP (Brakeman et al. 1997; Kato et al. 1997).

Homer1a is considered a dominant negative binding protein (Fagni et al. 2004). Despite Homer1a's short C-terminal tail, it competes with Homer1b/c for the same binding site on group I mGluR's (Tu et al. 1998) and antagonizes mGluR1α binding to Homer1c (Tadokoro et al. 1999). Furthermore, Homer 1a over-expression in transgenic mice interferes with Homer 1b binding to mGluR5 (Xiao et al. 1998). However, as Homer1a cannot form dimers, it disrupts the intracellular linkages provided by the long Homer proteins, yielding significant effects on group I mGluR localization and signaling properties. In line with this, Homer1a seems to facilitate group I mGluR coupling to their G protein (Kammermeier et al. 2000) as well as inducing basal constitutive activity of mGluR1α and mGluR5a/b without the presence of a ligand (Prezeau et al. 1996; Litschig et al. 1999; Ango et al. 2001). In addition, Homer 1a controls the agonist-mediated Ca^{2+} response of mGluR1α by increasing the latency of the mGluR1α-mediated intracellular Ca^{2+} response (Tu et al. 1998; Ango et al. 2002), as well as amplifying the amplitude of the mGluR1α-mediated intracellular Ca^{2+} response (Ango et al. 2002). Therefore, it is reasonable to suggest that in the absence of agonist, constitutively expressed Homer1b/c keep group I mGluR's silent, and activity-dependent induction of Homer1a, replacing the long forms of Homer, prompts spontaneous activation of the receptors, as well as enhancing intracellular Ca^{2+} levels (Fagni et al. 2004).

In terms of receptor trafficking, Homer1a and Homer1c increase the level of plasma membrane-associated mGluR1α in cultured HEK 293 cells (Ciruela et al. 1999a; Ciruela et al. 2000). In cultured cerebellar granule cells transfected with mGluR5 alone or with Homer1a, mGluR5 is exclusively localized on the somatic cell surface (Roche et al. 1999; Ango et al. 2002). However, when transfected with mGluR5 and either Homer1b or Homer1c, mGluR5 is retained in the cytoplasm at dendritic synaptic sites (Ango et al. 2000; Ango et al. 2002), in the endoplasmic reticulum (ER; Roche et al. 1999). Interestingly, intense neuronal activity due to NMDA and kainate application produces a transient expression of endogenous Homer1a and persistent membrane localization of mGluR5 at the same synaptic sites, even when co-expressed with Homer1b or Homer1c (Ango et al. 2000; Ango et al. 2002). In cultured hippocampal neurons, agonist application enhances the lateral mobility of mGluR5, an effect diminished by Homer1b (Serge et al. 2002). Together, these results suggest a mechanism whereby constitutively expressed Homer1b and Homer1c retain mGluR5 within the cytoplasm of the post-synaptic neuron until neuronal activity, sufficient to induce Homer1a synthesis, promotes trafficking of intracellular mGluR5 to the membrane (Fagni et al. 2004), corroborating the finding that Homer proteins are involved in enhancing synaptic transmission (Hennou et al. 2001).

3.5.2 Tamalin

In both rat and monkey substantia nigra neurons, most mGluR1α is found on the neuronal membrane, while mGluR5 is generally intracellular (Hubert et al. 2001). Furthermore, in comparison to mGluR5, Homer1 involvement with mGluR1α localization and function is meager, suggesting an additional contender

for mGluR1α trafficking. Enriched in the post-synaptic density, Tamalin is a scaffold protein with numerous protein-interacting domains, such as a PDZ domain, a leucine-zipper region, a proline-rich region, and a C-terminal PDZ binding motif (Nevrivy et al. 2000; Kitano et al. 2002; Kitano et al. 2003a). The PDZ domain of tamalin binds with the intracellular C-terminal of group 1 mGluR's, whereas the leucine-zipper region binds to cytohesins (Nevrivy et al. 2000; Kitano et al. 2002). Accordingly, mGluR1α/Tamalin/cytohesin complexes are found in both cultured cells and in rat brain (Kitano et al. 2002; Kitano et al. 2003b). Cytohesins, which belong to the guanylate nucleotide exchange factor (GEF) family, activate ADP-ribosylation factors (ARFs), small G proteins that control the vesicular intracellular transport (Moepps & Fagni, 2003). Through its interaction with cytohesin, Tamalin enhances cell-surface expression of the mGluR1α, as well as facilitating the dendritic and axonal distribution of endogenous mGluR5 in cultured hippocampal neurons (Kitano et al. 2002). Thus, it is not unreasonable to suppose that Tamalin is involved in intracellular trafficking of mGlu1a and mGlu5 receptors.

3.7 Aim

Receptor trafficking has recently become a focal point in the study of plastic changes to spinal cord dorsal horn neurons following persistent nociceptive input. Studies examining the roles of AMPA and NMDA receptors in nociception and spinal plasticity, often termed central sensitization, are plentiful. However, group I mGluR's have received comparatively little attention. Alterations in spinal group I mGluR expression occurs following persistent noxious input. Changes in protein or mRNA levels of group I mGluR's are reported. However, these techniques

cannot specify whether changes occur on the cell membrane or within the cytoplasm. In contrast, visualization of the ultrastructural localization of group I mGluR's on spinal cord dorsal horn neurons allows the direct measurement of receptor density on the cell membrane versus in the cytoplasm, not to mention other informative parameters such as distance of plasma membrane-associated receptors to the synapse. In this thesis, through the use of immunocytochemical protocols for confocal and electron microscopy, the ultrastructural localization of mGluR1α and mGluR5 will be assessed in neurons from specific regions of the spinal cord dorsal horn of both CFA-treated and untreated rats. This will enable us to determine whether group I mGluR trafficking correlates with the development of behavioral hyperalgesia in an animal model of persistent inflammation.

4 Methods

4.1 Animals and Model of Peripheral Inflammation

Male Long Evans rats (Charles River) between 250 – 300 grams were used in all experiments. All surgical and behavioral testing interventions were in accordance with the guidelines of *The Care and Use of Experimental Animals* of the Canadian Council on Animal Care as well as with the McGill University Animal Care Committee standards. Persistent peripheral inflammation was induced by intraplantar injection of 100 microliters of complete Freund's adjuvant (CFA; 1 mg/ml) into the left hind paw while under brief halothane anesthesia. Control animals received no intraplantar injection.

4.2 Experiment 1: Behavioral Effects of CFA-Induced Peripheral Inflammation

4.2.1 Mechanical Threshold and Heat Withdrawal Latencies following CFA-Induced Peripheral Inflammation

Both withdrawal latency to a heat stimulus and mechanical withdrawal threshold were measured before intraplantar CFA (baseline), as well as 1, 2, 3, 4, 5, 7, and 10 days after CFA (n=10). A group of untreated rats was also tested at the same time points (n=11). To evaluate behavioral responses to heat, thermal withdrawal latencies were measured using the plantar apparatus. Each rat was placed in a 10x40x30 cm Plexiglas® box over a transparent platform through which a noxious radiant heat source was aimed at the plantar surface of the rat's left hind paw, and the time was measured until the rat exhibited a brisk flexion (withdrawal) reflex. For mechanical thresholds, calibrated von Frey filaments between 0.25 and 15 g were applied according to a modified up-down method (Chaplan et al. 1994). Briefly, beginning with the smallest, filaments were applied in ascending order to the rat's hind paw until a positive response was obtained, at which point lower filaments were applied in descending order until a negative response was obtained, and so on until the experimental response pattern corresponded with one of the response patterns described in Chaplan et al. (1994). A positive response was considered a brisk withdrawal from at least two out of three stimulations, less than two was considered a negative response. For each stimulation, the filament held in contact with the rats paw for approximately 5 seconds, with approximately 10-15 seconds between stimulations.

4.2.2 Analysis

Two-way repeated-measures analysis of variance (ANOVA) was used to compare thermal withdrawal latencies followed by Tukey's post hoc tests. The non-parametric Mann-Whitney U-test was used to compare mechanical withdrawal thresholds. For all analyses, $p<0.05$ was considered statistically significant.

4.3 Experiment 2: Ultrastructural Localization of Group I mGluR's in Spinal Cord Dorsal Horn Neurons of Naïve and CFA-Treated Rats

4.3.1 Heat Withdrawal Latencies

Compared to thermal withdrawal latencies, mechanical withdrawal thresholds of CFA-treated rats were not robust in Experiment 1, as reported in the results section (section 5.1.2). Thus, only thermal withdrawal thresholds were measured for Experiment 2. Measurements were as described in section 4.2.1, however performed only on day 3 after CFA. Maximum heat hypersensitivity occurred three days after CFA injection, thus spinal cords were harvested at this time point.

4.3.2 Immunocytochemistry

Immunocytochemical methods were used to label neurons immunoreactive for mGluR1α and mGluR5 for both confocal and electron microscopy. Both the mGluR1α (AB1595) and mGluR5 (AB5675) antibodies were purchased from Chemicon (Temecula, CA). The specificities of the mGlur1α and mGluR5 antibodies for their respective receptors were confirmed by western blot in transfected cells by Chemicon. Confocal microscopy was used qualitatively to

determine the laminar distribution of mGluR1α and mGluR5 on spinal cord neurons, and electron microscopy was used to determine the ultrastructural location and quantity of neurons with mGluR1α and mGluR5-IR. Different methods were used to prepare tissue for confocal and electron microscopy.

4.3.2.1 Confocal Microscopy

Confocal microscopic immunocytochemistry was used to confirm the laminar distribution of mGluR1α and mGluR5. Rats were anesthetized deeply with sodium pentobarbital (100 mg/kg) and then perfused intracardically with 500 ml of 4% paraformaldehyde and 15% saturated picric acid (v/v) in 0.1 M phosphate buffer (PB). The spinal cord were removed following laminectomy, post-fixed in the same fixative, and then cryoprotected in a 30% sucrose solution in PB overnight. Transverse sections (50 μm) of lumbar cord were cut on a freezing microtome. Unspecific staining was blocked with 5% normal goat serum in 0.1 M PB saline with 0.2% Triton X-100 for 1 hr prior to incubation with primary antibodies: rabbit anti-mGluR1α (1:150) or anti-mGluR5 (1:400) antibodies, at 4°C overnight. Sections were thoroughly washed and incubated in a Rhodamine Red-X-conjugated goat anti-rabbit secondary antibody for 1 hr at room temperature. Then the tissue was washed and mounted on gelatin-coated slides, cover-slipped with an anti-fading medium, and viewed by with a Zeiss LSM 510 confocal microscope with integrated software.

4.3.2.2 Electron Microscopy

Briefly, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and then perfused intracardially with 4% paraformaldehyde and 0.05%

glutaraldehyde. Spinal cords were removed following laminectomy and post-fixed for 2 hours in 4% paraformaldehyde (PFA) followed by cryoprotection for 24 hours in 30% sucrose. 50-micrometer transverse sections were cut using a vibratome and placed in a plastic 24-well plate at 8-10 sections per well, containing 0.1 M PB.

Pre-embedding immunocytochemical protocols for electron microscopy were used for all receptor quantification studies. Immediately following vibratome sectioning, spinal cord sections were immersed in sodium borohydride for 30 minutes, followed by four 15 minute rinses in phosphate buffered saline (PBS). Sections were then placed in 0.5% bovine serum albumin (BSA) followed by a 30 minute incubation in the primary antibody anti-mGluR1 (Chemicon; 1:150) or anti-mGluR5 (Chemicon; 1:400) at room temperature. Sections were then incubated overnight in primary antibody at 4°C. Sections were then rinsed twice for 15 minutes in PBS followed by 10 minutes in washing buffer. Sections were incubated overnight at 4°C in goat-anti-rabbit gold-conjugated (1 nanometer) IgG (Amersham Bioscience). Sections were placed in washing buffer for 5 minutes, followed by three 5-minute rinses in PBS. At this point, sections were placed in 2% glutaraldehyde for 10 minutes, followed by three 5-minute rinses in PBS. Sections were then transferred to glass vials containing citrate buffer for 10 minutes. Next, silver intensification of the sections was performed using the IntenSE-M kit (Amersham Bioscience), for 15 minutes. Sections were washed twice for 5 minutes in citrate buffer followed by a single wash in 0.1 M PB. Sections were then incubated at 4°C in 1% osmium tetroxide with vial caps tightly screwed on, followed by dehydration in ascending concentrations of ethanol,

ending with 10 minutes propylene oxide. Sections were immersed for 2 hours in 1:1 Epon/propylene oxide, then 2 hours 2:1 Epon/propylene oxide, and finally 2 hours pure Epon. At this point, sections were removed from the vials and flat-embedded using Epon on acetate sheets and left at 60°C for 24 hours. Flat-embedded sections were re-embedded in modified eppendorf vials and filled with Epon. Re-embedded sections were left at 60°C for 48 hours. Ultrathin sections were cut. Counterstaining using uranyl acetate and lead citrate was then performed.

4.3.3 Quantification

Electron microscopy was performed using a Philips 410 LS electron microscope and MegaView II software. Spinal cord sections were trimmed such that only the target lamina were available for viewing on the electron microscope, that is to say lamina I-II (mGluR5) and lamina V (mGluR1α). All electron micrographs were taken at 10,200 times magnification according to a pre-determined method. Beginning at the upper left hand corner of the section, an image was captured every two rotations (720°) of the vertical axis control. At the edge of the section, the horizontal axis control was rotated twice (720°) downwards and the process repeated in a descending zig-zag pattern. Minor adjustments to the field were sometimes required to exclude blood vessels or damage to the section. As lamina I-II sections were larger than lamina V sections, 40 or 50 images were acquired per section respectively, for mGluR5 or mGluR1. Correspondingly, a total of 240 or 300 images per experimental condition (i.e., 6 rats per group) were used for quantification.

Immunogold particles were classified according to the type of neuronal process on which they were located, such as soma, dendrite, or axon. Immunogold grains located on cell soma or in myelinated fibers (axons) were not included in quantification. Six parameters were measured: (1) The number of immunogold-labeled neuronal processes, (2) the number of cell-surface immunogold grains, (3) the number of intracellular grains, (4) the shortest distance of cell-surface grains to a synapse, (5) the shortest distance of intracellular grains to the membrane, and in the same axis, (6) the total distance across the process. From these six parameters, seven variables were derived: (1) the number of cell-surface grains, (2) the number of intracellular grains, (3) the total number of grains, (4) the cell-surface: intracellular proportion, (5) the total number of labeled processes, (6) the mean distance to synapse of cell-surface grains, (7) and, in order to account for the variation in size of neuronal processes, the percent distance to the cell-surface of intracellular grains. This was calculated by dividing the distance to the cell-surface by the total distance across the neuronal process along the same axis, multiplied by 100. The results were separated into three bins, near (0.1-17.9%), intermediate (18-32.9%) and far (33-50%).

4.3.4 Analysis

Frequencies were assessed by visual inspection of electron microscope images, while distances, measured using calibrated Image Pro V software, were in nanometers (nm). All quantification was done blind to experimental condition. Neuronal processes containing one or more immunogold particles were considered labeled. A particle in contact with the plasma membrane was

considered a cell surface receptor. An intracellular particle at any distance from the cell membrane was considered intracellular. For the measurement of gold-particle distance from the synapse, all micrographs containing labeled neuronal processes containing at least one synapse were included. If more than one synapse was present on the process, distance of the particle to the nearest synapse was measured.

Chi Square (χ^2) tests were used to compare differences in number of labeled processes, plasma membrane-associated particles, intracellular particles, and total particles between untreated and CFA-treated rats. The effects of chronic inflammation on receptor localization (comparisons of the counts for particles near, intermediate and far from the closest membrane) were examined using contingency tables (2 X 3 χ^2 tests). Independent t-tests were used to evaluate whether the mean distance of a cell-surface grain from the nearest synapse was altered by experimental condition. ANOVA was used to compare thermal withdrawal latencies between untreated and CFA-treated rats. For all statistical analyses, $p<0.05$ was considered statistically significant.

5 Results

5.1 Experiment 1: Behavioral Effects of CFA-Induced Peripheral Inflammation

5.1.1 Heat Withdrawal Latencies

Average baseline withdrawal latencies were approximately 10 seconds. As shown in Fig. 1A, treatment with CFA resulted in lowered latencies for the first few days after treatment. Two-way repeated measures analysis of variance

(ANOVA) showed significant main effects for both treatment ($F(1,147) = 31.69$, $p < 0.01$) and time ($F(7,147) = 2.67$, $p < 0.05$), as well as a significant interaction ($F(7,147) = 8.12$, $p < 0.01$). Post-hoc Tukey tests revealed that baseline withdrawal latencies were not significantly different between CFA-treated and untreated rats ($mean_{untreated} = 9.4 \pm 0.5$ sec. vs. $mean_{CFA\text{-}treated} = 9.3 \pm 0.5$ sec., $p > 0.05$). Withdrawal latencies of CFA-treated rats were significantly lower than untreated rats on days 1 ($mean_{untreated} = 10.3 \pm 1.1$ sec. vs. $mean_{CFA\text{-}treated} = 5.7 \pm 0.6$ sec., $p < 0.01$), 2 ($mean_{untreated} = 8.3 \pm 0.4$ sec. vs. $mean_{CFA\text{-}treated} = 4.6 \pm 0.6$ sec., $p < 0.01$), and 3 ($mean_{untreated} = 10.4 \pm 0.8$ sec. vs. $mean_{CFA\text{-}treated} = 4.5 \pm 0.2$ sec., $p < 0.01$) post-treatment.

5.1.2 Mechanical Withdrawal Thresholds

Baseline mechanical withdrawal thresholds were approximately 15 g, and as shown in Fig. 1B were lowered early after CFA treatment. Mann-Whitney U-tests reveal that baseline withdrawal thresholds between CFA-treated (mean = 14.7 g, n = 10) and untreated (mean = 14.6 g, n = 11) rats were not significantly different ($U = 55.0$, $p > 0.05$). However, withdrawal thresholds of CFA-treated rats were significantly lower than untreated rats on day 1 ($mean_{untreated}$ = 13.6 g vs. $mean_{CFA\text{-}treated}$ = 6.6 g, $U = 0.5$, $p < 0.01$), day 2 ($mean_{untreated}$ = 15.0 g vs. $mean_{CFA\text{-}treated}$ = 8.3 g, $U = 0.0$, $p < 0.01$) and day 3 ($mean_{untreated}$ = 14.7 g vs. $mean_{CFA\text{-}treated}$ = 9.4 g, $U = 6.5$, $p < 0.01$).

5.2 Experiment 2: Ultrastructural Localization of Group I mGluR's in Spinal Cord Dorsal Horn Neurons of Naïve and CFA-Treated Rats

5.2.1 mGluR1 Experiment

5.2.1.1 Heat Withdrawal Latencies

Baseline latencies for both untreated and CFA-treated rats were again approximately 10 seconds. At day three post-CFA, while the latencies of untreated rats remained comparable to their baseline, CFA-treated rats showed a significant decrease in latency ($F(3,4) = 22.17$, $p < 0.01$) (Fig. 2A), to 3.0 ± 0.4 seconds. As both untreated and CFA-treated rats underwent identical behavioral testing procedures, any differences of mGluR1α localization are entirely attributable to CFA-treatment.

5.2.1.2 Laminar Distribution of mGluR1α-IR

Confocal microscopy immunocytochemistry confirmed the localization of mGluR1α in the spinal cord dorsal horn as defined by Alvarez et al. (2000). As shown in Fig. 3A, mGluR1α is virtually absent in the superficial dorsal horn, but expressed heavily throughout the remainder of the spinal cord dorsal horn, most prominently in lamina V.

5.2.1.3 Quantification of mGluR1α Immunogold Labeled Particles

At the electron microscopic level of analysis, mGluR1α was found predominantly in dendrites, although immunogold particles were occasionally found presynaptically, sometimes in myelinated afferent fibers (Fig. 4A). Also, as depicited in Fig 4A, immunogold labeled particles were often found in association with the plasma membrane, even in untreated control rats.

No significant differences were found in either the number of labeled neuronal processes ($\chi^2(1) = 0.56$, $p > 0.05$), or the total number of mGluR1-labeled particles ($\chi^2(1) = 2.70$, $p > 0.05$), between untreated and CFA-treated rats (Table 1). Accordingly, no significant differences in levels of either plasma membrane-

associated (χ^2 (1) = 0.65, $p > 0.05$) or intracellular (χ^2 (1) = 2.27, $p > 0.05$) mGluR1α were detected in lamina V spinal dorsal horn neurons between experimental conditions (Table 1). In addition, the proportion of plasma membrane-associated to intracellular mGluR1α did not differ significantly between untreated (1.14:1) and CFA-treated rats (1.09:1; χ^2 (1) = 0.29, $p > 0.05$), indicating a lack of specific mGluR1α sequestration to the plasma membrane following chronic inflammation (Table 2, Fig. 6A/B). Correspondingly, the proportion of intracellular mGluR1α located near, intermediate or far from the plasma membrane did not change following CFA treatment (χ^2 (1) = 1.66, $p > 0.05$) (Table 2). Interestingly, the distance of membrane-associated mGluR1α from the nearest synapse differed significantly between untreated (mean = 758.47 ± 84.69 nm) and CFA-treated (mean = 237.51 ± 9.95 nm) rats (t (357) = 6.83, $p > 0.05$), with a significantly shorter distance after CFA-treatment.

5.2.2 mGluR5 Experiment

5.2.2.1 Heat Hyperalgesia

Baseline testing showed both treatment groups to have similar thermal withdrawal latencies of approximately 10 seconds. Three days after intraplantar injection of CFA, thermal withdrawal latencies were 3.4 ± 0.5 seconds, a significant decrease ($F(3,8) = 37.22$, $p < 0.01$) (Fig. 2B). Again, as both untreated and CFA-treated rats underwent identical behavioral testing procedures, any differences of mGluR5 localization are entirely attributable to CFA-treatment.

5.2.2.2 Laminar Distribution of mGluR5-IR

Confocal microscopy immunocytochemistry also confirmed the localization of mGluR5 in the spinal cord dorsal horn as defined by Alvarez et al. (2000). As shown in Fig. 3B, mGluR5 is expressed most prominently in the superficial lamina (I-II), with considerably less immunostaining observed in the deeper lamina.

5.2.2.3 Quantification of mGluR5 Immunogold Labeled Particles

At the electron microscopic level of analysis, mGluR5 was also found predominantly in dendrites, although immunogold particles were again occasionally found presynaptically, sometimes in myelinated afferent fibers (Fig. 4B). However, as depicited in Fig 4B, immunogold labeled particles were not always found in association with the plasma membrane. Immunogold particles were also observed on occasion in processes that were associated with glomerular axonal endings. Fig. 5 shows an example of a type IIa glomerulus in lamina II (typically a termination of a myelinated primary afferent) apposed to various mGluR5 immunolabeled dendrites with obvious axo-dendritic synapses, and occasionally dendro-dendritic synapses, as well as in axons exhibiting axo-axonic synapses. The mGluR5 gold particles are often intracellular, or may be on the plasma membrane, in either perisynaptic or extrasynaptic locations.

Both the total number of mGluR5 labeled neuronal processes and the total number of mGluR5-labeled particles were significantly increased in CFA-treated rats compared to untreated rats (χ^2 (1) = 49.38, $p < 0.01$ and (χ^2 (1) = 28.44, $p < 0.01$, respectively) (Table 3, Fig. 6C/D). Compared to untreated rats, CFA-treated rats had significantly increased levels of mGluR5 on the plasma membrane (χ^2

(1) = 77.44, $p < 0.01$), but not in the cytoplasm (χ^2 (1) = 0.00, $p > 0.05$) (Table 3) of neurons in lamina I-II of the spinal cord dorsal horn. Correspondingly, there was a significant increase in the ratio of plasma membrane-associated particles to number of intracellular particles in CFA-treated rats (0.71:1) compared to untreated rats (0.47:1; χ^2 (1) = 49.60, $p < 0.01$), indicating specific targeting of mGluR5 to the plasma membrane following chronic peripheral inflammation (Table 4, Fig. 6D). On the other hand, the proportions of intracellular mGluR5 located near, intermediate or far from the plasma membrane did not change significantly following CFA-treatment (χ^2 (2) = 2.29, $p > 0.05$) (Table 4). In addition, the distance of plasma membrane-associated mGluR5 from the nearest synapse did not differ significantly between untreated (mean = 248.94 ± 19.87 nm) and CFA-treated (mean = 221.10 ± 15.69 nm) rats (t (165) = 0.99, $p > 0.05$.

6 Discussion and Conclusions

Functional group I mGluR's are expressed in the spinal cord dorsal horn where they play an important modulatory role in processing nociceptive input. In naïve rodents, group I mGluR activation evokes post-synaptic depolarization and excitation of dorsal horn neurons, however only mGluR1 activation yields nociceptive behaviors. Agonists of mGluR1/5 enhance inflammation-induced thermal and mechanical hypersensitivity in addition to potentiation of dorsal horn neuronal activity; effects inhibited by group I mGluR antagonists. Innocuous and acute noxious stimulation are generally impervious to group I mGluR inhibition. Blockade of spinal group I mGluR's preferentially attenuates processing of persistent neuropathic or inflammation-induced noxious input. In spinal cord slice,

bath application of DHPG and CHPG, mGluR1 and mGluR5 agonists, respectively, induce LTP (Zhong et al. 2000). Both mGluR1/5 and mGluR5-specific antagonists attenuate the induction of LTP. Together, these findings provide a clear role for spinal cord dorsal horn group I mGluR's mediating noxious input associated with persistent pain states. The nature of this involvement in persistent nociceptive input, at the expense of innocuous or acute nociceptive input, however, is not as apparent.

In this study, we determined the effect of peripheral inflammation on trafficking of group I mGluR's in the dorsal horn of the rat spinal cord. First, we determined the time course of behavioral hypersensitivity associated with CFA-treatment. Using confocal microscopy, we next verified the laminar distribution of the both group I mGluR subtypes in the spinal cord dorsal horn. Finally, using electron microscopy, we examined the subcellular distribution of immunolabeled mGluR1α and mGluR5 in spinal lamina V and lamina I-II, respectively, of CFA-treated and untreated rats.

6.1 Experiment 1: Behavioral Effects of CFA-Induced Peripheral Inflammation

For up to three days following intraplantar CFA injection, rats show significant mechanical allodynia and heat hyperalgesia when compared to untreated rats. The mechanical withdrawal threshold of CFA-treated rats was approximately 15 g at baseline testing, diminishing to approximately 6 g one day after treatment. Response threshold then steadily increased to 8 g day three, after CFA-treatment. Response thresholds were no longer significantly different from

untreated rats by four days after CFA-treatment. Normally innocuous mechanical stimulation induced a brisk withdrawal of the inflamed paw, often followed by increased guarding and licking of the paw. CFA-treatment had similar effects on thermal withdrawal latencies. Specifically, while baseline latencies were comparable for both groups, CFA-treatment reduced latencies from about 10 seconds at baseline to 5.6 seconds, 4.6 seconds and 4.5 seconds, on days one, two and three, respectively.

To correspond with our interest in the effects of persistent nociception, it was important to take into consideration both the duration of hypersensitivity as well as the time point of maximal hypersensitivity. Since both thermal and mechanical hypersensitivity were no longer detectable four days after CFA-treatment, and hypersensitivity to thermal stimulation was most intense on day three, we chose to harvest spinal cords for immunocytochemical processing on day three after treatment. Furthermore, to eliminate undue stress on the rats, only thermal latencies were tested to confirm the presence of hyperalgesia in rats to be used for tissue harvesting.

6.2 Laminar Distribution of Spinal Group I mGluR's

As shown in Fig. 3B, we found a high concentration of mGluR5 in the superficial dorsal horn (lamina I-II), as has been previously demonstrated (Alvarez et al. 2000; Berthele et al. 1999; Tang & Sim, 1999; Yung, 1998). In contrast, mGluR1α was absent in lamina I-II, but expressed throughout the rest of the dorsal horn, most densely in lamina V (Fig. 3A), again, as demonstrated previously (Alvarez et al. 2000; Berthele et al. 1999). Although, previous studies

have suggested mGluR1α is present in the superficial dorsal horn (Yung, 1998; Tang & Sim, 1999), it was suggested that this expression was mistakenly observed due to the use of non-selective antibodies that cross-reacted with mGluR5 (Alvarez et al., 2000). Our observations demonstrate a selective expression of mGluR5 in the superficial dorsal horn, consistent with that reported by Alvarez et al. (2000) and Berthele et al. (1999). Given that the specificity of the commercially-available antibodies used in the present study has been confirmed in transfected cells by Chemicon, and that the localization of these receptors, determined here, is entirely consistent with the literature, further validation of antibody specificity was deemed unnecessary. Therefore, based on the high density of mGluR5 in the superficial dorsal horn and of mGluR1α in lamina V, we targeted these areas, respectively, for our electron microscopic immunocytochemistry studies of mGluR1α and mGluR5.

6.3 Experiment 2: Ultrastructural Localization of Group I mGluR's in Spinal Cord Dorsal Horn Neurons of Naïve and CFA-Treated Rats

Ultrastructural studies support previous studies indicating that both mGluR1α and mGluR5 are predominantly expressed in dendrites, as has previously been shown for mGluR1α (Tang & Sim, 1999; Alvarez et al., 2000) and mGluR5 (Jia et al., 1999; Valerio et al., 1997). We did, however, occasionally find both mGluR1α and mGluR5 immunogold particles presynaptically in myelinated primary afferent fiber axons, again as shown previously (Valerio et al., 1997; Alvarez et al., 1999; Jia et al., 1999; Tang & Sim, 1999). We also found a few cases of immunogold labeled mGluR5 particles in processes associated with primary afferent

glomerular fiber endings (see Fig. 5). In the case shown, mGluR5 immunogold particles were observed intracellularly and on the plasma membrane both perisynaptically and extrasynaptically. Since type IIa glomeruli are typically found on axonal endings of myelinated primary afferents, this provides support that mGluR5 is located in dendrites targeted by A fiber primary afferents (although we do not know whether it is an Aβ or Aδ fiber). Additional double-labeling immunochemistry for confocal microscopy would illuminate to which afferent fiber type mGluR5 is apposed. Previously, mGluR5 has been found in dendrites targeted by primary afferent Aδ and C-fiber terminals (Vidnyansky et al., 1994; Jia et al., 1999; Tao et al. 2000). Interestingly, in this particular glomerulus it appears that mGluR5 is contained not only in dendrites making axo-dendritic contact with the axonal ending, but also in associated dendrites that make dendro-dendritic contacts, and an associated axon that made an axo-axonic contact with another axon, as well as apparently with the glomerular ending. In these cases, the mGluR5 particles appear perisynaptic in both axo-axonal synapses.

6.3.1 Group I mGluR Trafficking

The main goal of this study was to determine if there were changes in the overall numbers of mGluR1α- and mGluR5-immunolabeled particles between untreated and CFA-treated rats, and if so, to determine the ultrastructural localization of these alterations. It is clear from table 1 that while there was no change in any measure of mGluR1α expression levels in CFA-treated rats, mGluR1α moved significantly closer to the synapse. Specifically, the average distance of cell surface mGluR1α to the nearest synapse was dramatically

reduced from 750 nm to 250 nm, a 3.2-fold decrease. In contrast to the lack of change in either cell-surface or intracellular mGluR1α expression following CFA-treatment, there was a global up-regulation of mGluR5 due entirely to an increase of plasma membrane-associated mGluR5 in lamina I-II (Table 3). Specifically, for mGluR5 there was a 34% increase in the total number of immunolabeled neuronal processes, a 17% increase in overall mGluR5 expression, a 53% increase in plasma membrane-associated particles, and no change in the number of intracellular particles. Our present observations, showing no overall change in mGluR1α expression levels in addition to a significant mGluR5 up-regulation following CFA-treatment, correspond well with previous studies. Hudson et al. (2002) found increased mGluR5 immunoreactivity after sciatic nerve section. Similarly, mRNA levels for mGluR5, but not mGluR1, are elevated in the spinal dorsal horn in sheep suffering from persistent peripheral inflammation or abdominal surgery (Dolan et al. 2003; Dolan et al. 2004). More importantly, our findings extend current understanding of group I mGluR involvement in persistent pain states. Ango et al. (2000) showed than intense neuronal activity results in mGluR5 trafficking to the membrane of cultured cerebellar granule cells. We demonstrated that, in response to peripheral CFA-treatment, a dramatic recruitment of cell-surface mGluR5 occurs, as well as lateral movement of cell-surface mGluR1α toward the synapse.

Our data suggests that CFA treatment leads to enhanced synaptic efficiency of glutamate transmission at mGluR1α as well as mGluR5. As the concentration of neurotransmitter diminishes with distance from the synaptic release site, the

likelihood of extra-synaptic receptor activation is relatively slim (Bergles et al. 1999; Trimmer, 1999). The probability of activation, however, can be enhanced either by increasing the number of extra-synaptic receptors as we showed after CFA treatment for mGluR5, or by decreasing the receptors' distance from the synapse as we showed after CFA treatment for mGluR1α. Our findings strongly support both possibilities.

Our laboratory has previously shown that activation of spinal cord group I mGluR's is associated with spontaneous nociceptive behaviors as well as exaggerated responses to mechanical and thermal stimulation (Fisher & Coderre, 1996a; Fisher & Coderre, 1998). In experiment 1, we observed that the development of mechanical and heat hypersensitivity followed different patterns, indicating divergent underlying mechanisms. Specifically, our findings demonstrate that thermal hypersensitivity steadily increases to a maximum by day three following CFA-treatment, while mechanical hypersensitivity is maximal on day one, followed by a progressive decrease. Unmyelinated nociceptive afferent C-fibres terminating in the superficial lamina have been suggested to mediate primarily thermal hyperalgesia, while myelinated A-fibres terminating in the deeper lamina mediate mainly mechanical hyperalgesia (Torebjork et al. 1992; Lesage, 2004). Taken together, our behavioral and immunocytochemical results suggest that thermal hypersensitivity observed after peripheral application of CFA could reflect the progressive enhancement of cell-surface mGluR5 on spinal lamina I-II neurons. Furthermore, the pattern of mechanical hypersensitivity observed in the present study points toward an initial activation of

peripheral mechanoreceptors by the CFA injection and plasma extravasation, for example. The steady decrease in mechanical hypersensitivity could indicate a desensitization of peripheral nociceptors (Simone et al. 1991). To study the role of spinal group I mGluR's, examination of the ultrastructural localization of mGluR1α and mGluR5 at numerous time points during the development of CFA-induced thermal and mechanical hypersensitivity would be enlightening.

In our study, CFA treatment led to both trafficking to the cell membrane of mGluR5 and lateral diffusion of mGluR1α. Although both Homer and Tamalin proteins are implicated in mGluR1α trafficking, there is an absence of information concerning the lateral mobility of mGluR1α. Interestingly, agonist application enhances the lateral mobility of mGluR5 in cultured hippocampal neurons, an effect hindered by transfection of Homer1b (Serge et al. 2002). As Homer1a is induced by intense neuronal activity (Brakeman et al. 1997; Kato et al. 1997), it is conceivable that CFA treatment could also induce Homer1a, possibly mediating the lateral diffusion of mGluR1α described here. Furthermore, there is evidence that constitutively expressed Homer1b/c retains mGluR5 in the cytoplasm, while intense neuronal depolarization induces the expression of the dominant negative isoform Homer1a, triggering the targeting of mGluR5 to the axons and dendrites of transfected cultured cerebellar granule cells (Ango et al. 2000; Fagni et al. 2004). Considering that Homer1a is associated with synaptic plasticity in the brain (Hennou et al. 2001) and Homer1b/c with persistent pain states in the spinal cord dorsal horn (Miletic et al. 2005), it is reasonable to expect that persistent, inflammation-induced afferent input would promote the trafficking of

mGluR5 to the plasma membrane in lamina V spinal cord dorsal horn neurons. The involvement of Homer1a in spinal nociceptive processing may go further than "simply" mediating group I mGluR trafficking. Homer1a is known to facilitate of group I mGluR coupling to their G protein (Kammermeier et al. 2000), enhance the amplitude of the mGluR1α Ca^{2+} response (Ango et al. 2002), not to mention induce agonist-independent constitutive activation of mGluR1α and mGluR5 (Prezeau et al. 1996; Litschig et al. 1999; Ango et al. 2001). Conceivably, noxious stimulus-induced Homer1a could (1) trigger mGluR1α lateral diffusion toward the synapse, (2) target mGluR5 to the neuronal membrane, (3) enhance the activity of functional, cell-surface group I mGluR subunits resulting in behavioral hypersensitivity to thermal and mechanical stimulation.

Overall, the replacement of longer Homer1 isoforms by the immediate early gene Homer1a provides an enticing theoretical foundation on which to base our present findings. We have strongly emphasized Homer1a's involvement in the trafficking of group I mGluR's described in this study. Logically, the next step would be to directly examine the role Homer1a plays in CFA-induced behavioral hypersensitivity and group I mGluR trafficking. Techniques such as antisense oligonucleotide or small interfering RNA (siRNA) knockdown of spinal Homer1a would be effective means of evaluating the role Homer1a plays in CFA-induced group I mGluR trafficking. Specifically, we expect that removal of spinal Homer1a would reduce CFA-induced hypersensitivity to thermal stimulation as well as the group I mGluR trafficking described here. Another interesting approach would be to selectively overexpress Homer1a in naïve rats and examine both behavioral responses to heat and mechanical stimulation as well as the ultrastructural

localization of group I mGlur's. We expect that this procedure would result both in behavioral hypersensitivity to stimulation and group I mGluR trafficking.

Despite the significant sequence homology and common Gq protein-coupling intracellular effector mechanism shared by mGluR1α and mGluR5, numerous studies indicate a certain heterogeneity of function in spinal nociceptive processing between the two group I mGluR subtypes. Our findings provide an attractive theoretical framework with which we can explain these issues. Both DHPG, a group I mGluR agonist, and trans-ADA, an mGluR5-specific agonist, activate dorsal horn neurons (Young et al. 1997). Yet DHPG, but not trans-ADA, produces spontaneous nociceptive behaviors (Fisher & Coderre, 1996a). Together these studies suggest that either DHPG acts mainly at mGluR1, or that activation of mGluR5 in naïve rats is not sufficient to yield nociceptive behaviors. The increase in cell-surface mGluR5 following CFA-treatment described in the present study supports both hypotheses: DHPG likely acts at both mGluR1 and mGluR5. However, in naïve rats the behavioral effect is due to preferential activation of mGluR1 as result of a relative paucity of mGluR5 on the cell membrane. Consequently, DHPG would be expected to strongly potentiate CFA-induced behavioral hypersensitivity, which is precisely the case (Adwanikar et al. 2004).

Group I mGluR's do not seem to be involved in processing either innocuous or acute noxious stimulation. Although glutamate concentration in the spinal cord dorsal horn is briefly elevated in response to acute noxious stimulation (Dmitrieva et al. 2004), the synaptic concentration of glutamate is tightly regulated by rapid reuptake via glutamate transporters (Palmada & Centelles, 1998). Therefore, it

seems improbable that group I mGluR's, generally located extra-synaptically, could be activated by synaptically released glutamate following innocuous or acute noxious stimulation. However, increased cell-surface mGluR5 in addition to mGluR1α located much closer to the synapse, as we observe here after CFA-treatment, would enhance the likelihood of group I mGluR activation in response to stimulation. Thus, the present study supports a relatively minor role for spinal group I mGluR's in processing innocuous or acute noxious input in naive rats, and extends current understanding by demonstrating the more prominent role mGluR1α and mGluR5 play in persistent pain states.

The crucial role of NMDA receptors in central nervous system plasticity is well documented. Activation of group I mGluR's potentiates NMDA receptor-mediated EPSC's in numerous brain regions (Aniksztejn et al. 1992; Challiss et al. 1994; Pisani et al., 1997). Furthermore, knockout mice for both mGluR1 (Aiba et al. 1994a/b; Conquet et al. 1994; Bordi, 1996) and mGluR5 (Lu et al. 1997) demonstrate reductions of NMDA-dependent hippocampal LTP. A similar association in the spinal cord dorsal horn could be expected. Correspondingly, group I mGluR activation enhances spinal cord NMDA receptor activity (Bleakman et al., 1992; Holohean et al. 1999) and expression (Lan et al. 2001a) through various pathways. For example, PKC, triggered by group I mGluR activation, stimulates enhanced NMDA receptor activation (Chen & Huang, 1992; Kelso et al., 1992; Kitamura et al., 1993; Harvey & Collingridge, 1993; Raymond et al., 1994) and expression (Lan et al. 2001b). In consequence, antisense knockdown of mGluR1 attenuates increased sensitivity to i.t. NMDA exhibited by neuropathic rats, as well as reducing the level of spinal cord dorsal horn PKC

(Fundytus et al. 2001). Furthermore, group I mGluR activity enhances the intracellular Ca^{2+} concentration through IP_3 receptor activation (Abe et al. 1992; Nakanishi et al. 1998). Tyrosine phosphorylation of the NMDA receptor, induced by elevated levels of intracellular Ca^{2+} (Guo et al. 2004), is associated with noxious input and the development of behavioral hypersensitivity (Guo et al. 2002). CFA-induced NMDA receptor tyrosine phosphorylation is attenuated by both group I mGluR and IP_3 receptor inhibition (Guo et al. 2004), further implicating group I mGluR's as upstream modulators of NMDA receptor activity. Consequently, as a result of the enhanced probability of group I mGluR activation after CFA, various intracellular mechanisms likely increase NMDA receptor expression and activity, with important effects on noxious stimulation-induced plasticity of spinal cord dorsal horn neurons.

The association between activity-dependent changes to synaptic efficacy and altered behavioral responses is not new. It has been over three decades since plastic changes in the brain were first elicited in an activity-dependent manner (Bliss & Lomo, 1973), an epiphany for modern neuroscience. Since then, long-term changes in synaptic efficacy have been shown to occur in the spinal cord in response to electrical stimulation as well as intense nociceptive input due to neuropathy or inflammation (Sandkuhler & Liu, 1998; Sandkuhler, 2000). The sub-cellular mechanisms underlying plasticity are receiving focused attention. Two main mechanisms of plasticity in the spinal cord have recently been proposed: changes in ion channel / receptor activity and receptor trafficking (Woolf & Salter, 2000). The activity of functional ion channels and receptors can be altered through numerous mechanisms. For example, phosphorylation of the

AMPA or NMDA receptor results in a striking potentiation of activity (Sandkuhler, 2000; Guo et al. 2002). Targeted trafficking of receptors to the cell surface enhances neuronal activity by simply making more receptors available to synaptically released neurotransmitter.

In the present study, we have shown that peripheral CFA-treatment induces severe hypersensitivity to mechanical and thermal stimulation. In addition, paralleling the time point of maximal thermal hypersensitivity, the number of cell-surface mGluR5 increased by more than 50% while cell-surface mGluR1α moved more than threefold closer to the synapse. This is the first evidence that chronic nociception leads to elevated expression of mGluR5 receptors at the cell surface of spinal dorsal horn neurons. This study is also among the first to demonstrate that trafficking of glutamate receptors contributes to synaptic plasticity in spinal cord dorsal horn associated with chronic nociception. Thus, the present results suggest that the increased expression of cell surface mGluR5 as well as the increased proximity of mGluR1α to the synapse in the spinal dorsal horn of CFA-treated rats may be an important factor in the generation and maintenance of synaptic plasticity that leads to persistent nociception and ultimately the chronic debilitating pain that is often associated with peripheral inflammation.

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8 Appendix

8.1 Tables

Table 1: Counts of mGluR1α immunolabeled particles.

mGluR1α	Number of labeled processes	Total number of particles	Number of plasma membrane-associated particles	Number of intracellular particles
Untreated	683	948	506	442
CFA-treated	712	1022	533	489

Table 2: Proportion of cell surface versus intracellular mGluR1α immunolabeled particles, and distance of particles to membrane by category.

mGluR1α	Proportion cell surface/intracellular particles	Distance to nearest membrane		
		Near	Intermediate	Far
Untreated	1.14:1	138 (43.7%)	110 (34.8%)	68 (21.5%)
CFA-treated	1.09:1	156 (48.1%)	109 (33.6%)	59 (18.2%)

Table 3: Counts of mGluR5 immunolabeled particles.

mGluR5	Number of labeled processes	Total number of particles	Number of plasma membrane-associated particles	Number of intracellular particles
Untreated	1000	2222	707	1515
CFA-treated	1341	2593	1080	1513

Table 4: Proportion of cell surface versus intracellular mGluR5 immunolabeled particles, and distance of particles to membrane by category.

mGluR5	Proportion cell surface/intracellular particles	Distance to nearest membrane		
		Near	Intermediate	Far
Untreated	0.47:1	429 (36.7%)	484 (41.3%)	258 (22.0%)
CFA-treated	0.71:1	497 (38.9%)	477 (37.4%)	303 (23.7%)

8.2 Figures

Fig. 1: Paw withdrawal latencies (A) and mechanical withdrawal thresholds (B) for CFA-treated (filled bars) and untreated (open bars) rats in experiment 1. Untreated rats (n = 11) display neither thermal (A) nor mechanical (B) hypersensitivity at any time point. However, CFA-treated rats (n = 10) display significant hypersensitivity to thermal (A) and mechanical stimulation (B) on days 1, 2 and 3 after treatment.

Fig. 1A

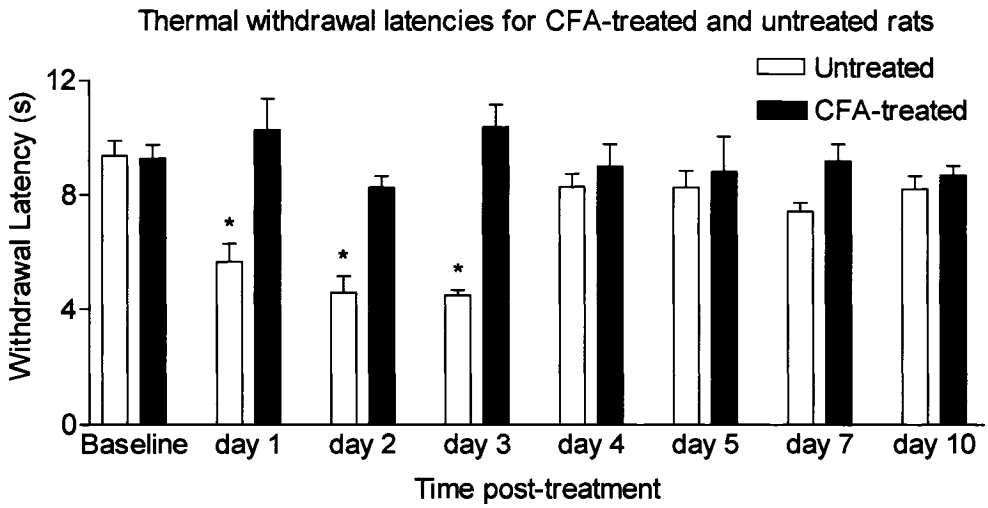

Thermal withdrawal latencies for CFA-treated and untreated rats
Untreated
CFA-treated
Withdrawal Latency (s)
12
8
4
0
*
*
*
Baseline
day 1
day 2
day 3
day 4
day 5
day 7
day 10
Time post-treatment

Fig. 1B

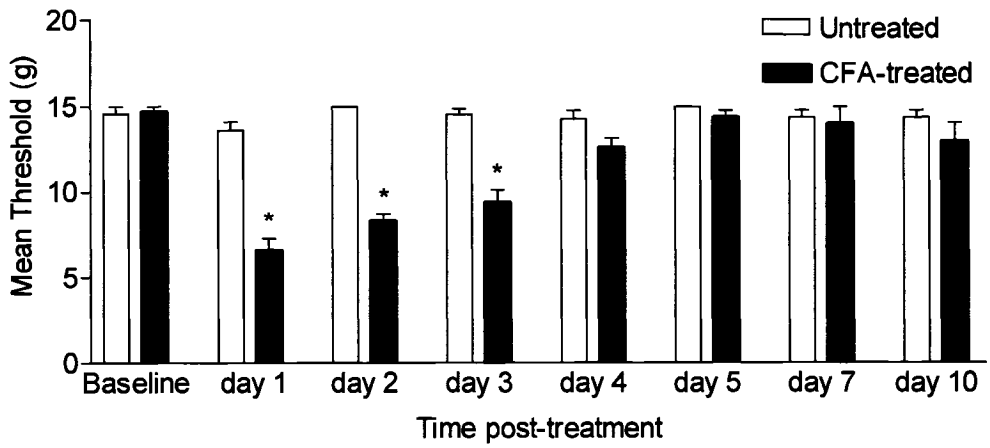

Mechanical withdrawal thresholds for CFA-treated and untreated rats
Untreated
CFA-treated
Mean Threshold (g)
20
15
10
5
0
*
*
*
Baseline
day 1
day 2
day 3
day 4
day 5
day 7
day 10
Time post-treatment

Fig.2: Paw withdrawal latencies in the plantar test in untreated (open bars) rats or in CFA-treated (filled bars) rats before (baseline) and after (post-test) hind paw injection of CFA for rats included in the mGluR1α (A) or mGluR5 (B) immunocytochemistry groups.

Fig. 2A

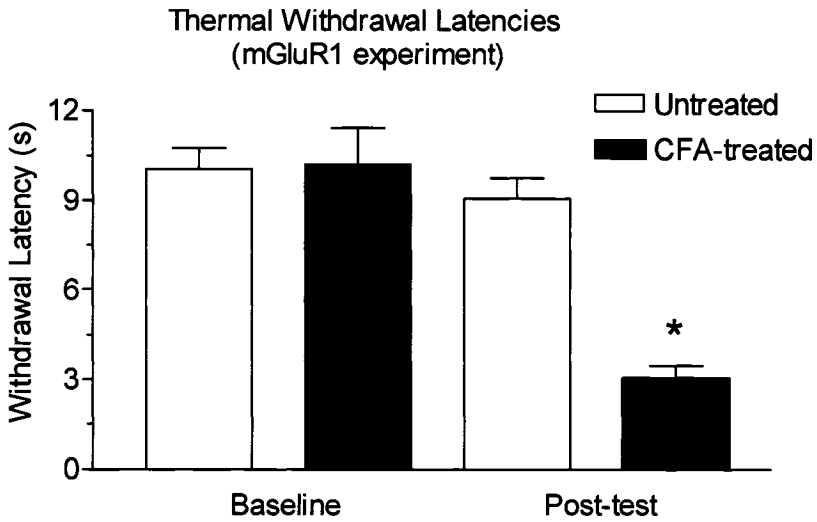

Thermal Withdrawal Latencies
(mGluR1 experiment)
Untreated
CFA-treated
Withdrawal Latency (s)
12
9
6
3
0
*
Baseline
Post-test

Fig. 2B

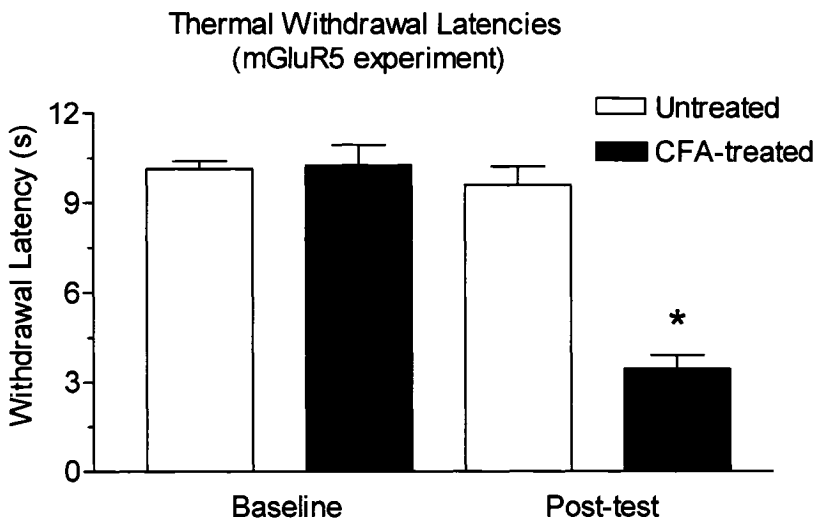

Thermal Withdrawal Latencies
(mGluR5 experiment)
Untreated
CFA-treated
Withdrawal Latency (s)
12
9
6
3
0
*
Baseline
Post-test

Fig. 3: Distribution of mGluR1α and mGluR5 in lumbar spinal cord dorsal horn of untreated control rats for sections immunolabeled with (A) anti-mGluR1α or (B) anti-mGluR5 and Rhodamine Red-X-conjugated secondary antibody.

Fig. 3

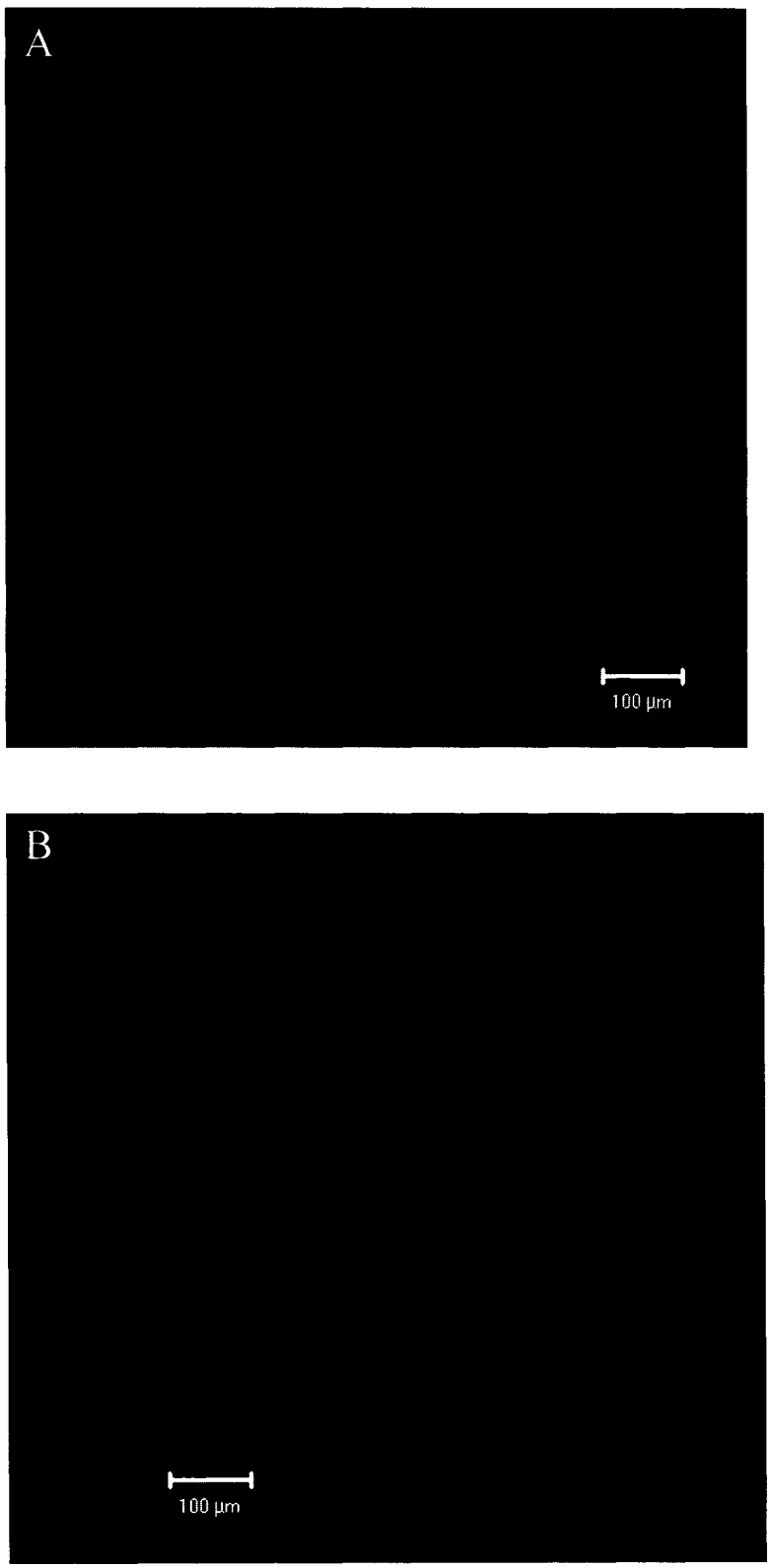


Fig. 4: Electron micrograph of mGluR1α (A) and mGluR5 (B) immunolabeled dendrites (white arrows) and axons (black arrows) in lamina V and lamina I-II of the spinal cord dorsal horn of naive rats, respectively.

Fig. 4

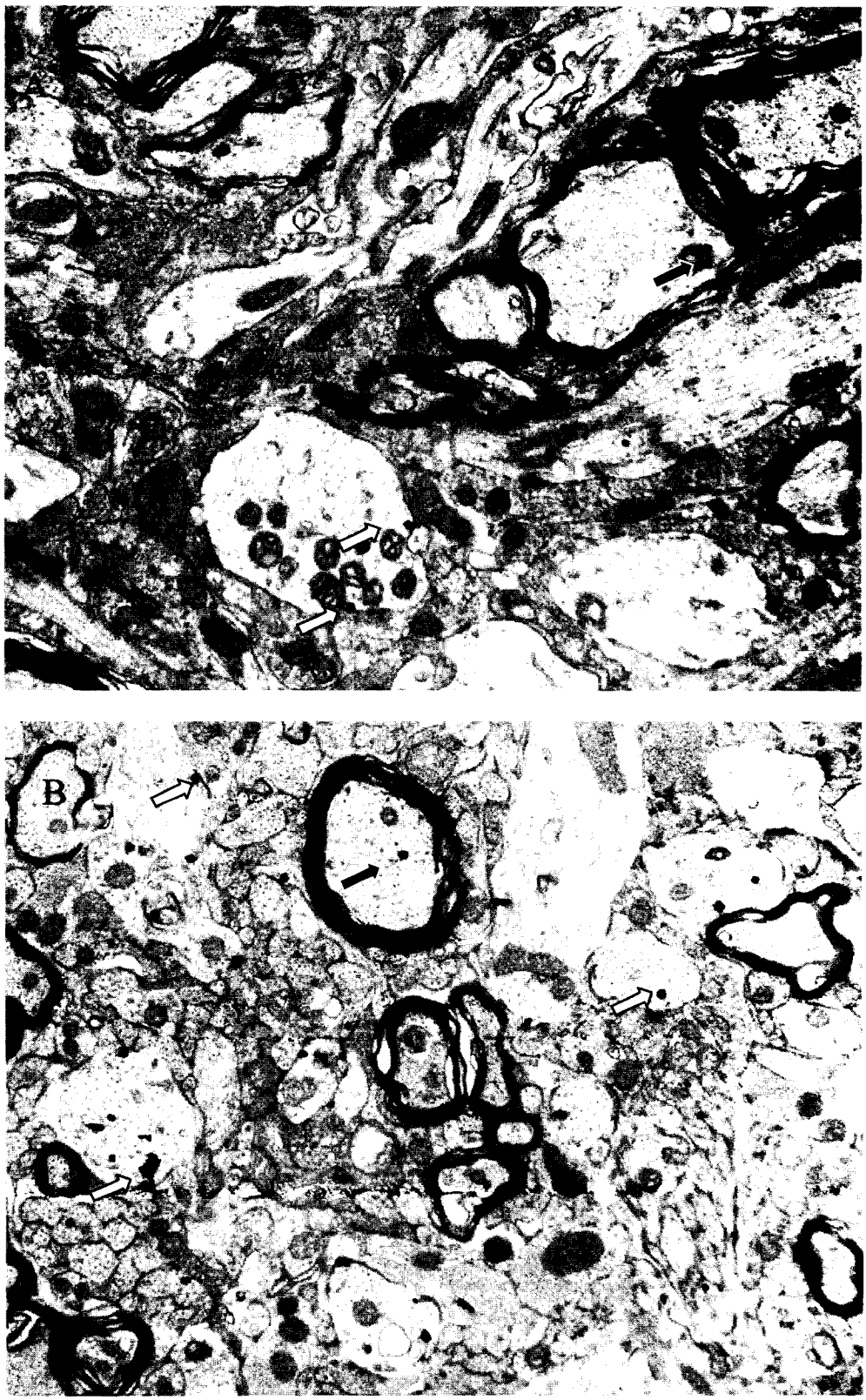


Fig. 5: Electon micrograph showing a type IIa glomerulus (IIa) from the superficial dorsal horn. Micrograph illustrates mGluR5 immunogold labeled particles in dendrites with axo-dendritic synapses (black arrows), dendrites with a dendro-dendritic synapse (white arrow head), and axons with axo-axonic synapses (white arrow).

Fig. 5

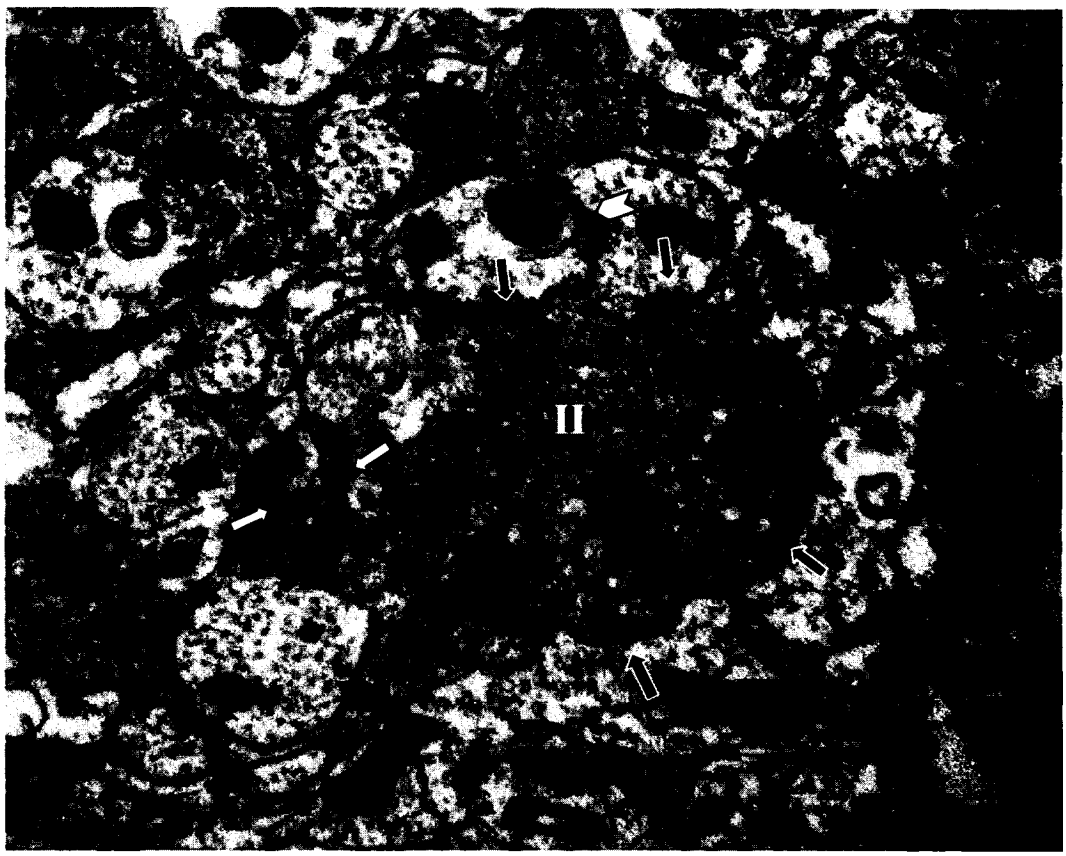


Figure 6: Representative electron micrographs of mGluR1α - and mGluR5-immunolabeled dendrites in dorsal horn of the spinal cord in untreated and CFA-treated rats. In the untreated rat, many mGluR1α (A) but few mGluR5 (C) immunogold particles are evident on the plasma membrane. In the CFA-treated rat, there is no change in the relative number of cell surface versus intracellular mGluR1α (B). In contrast, there is an upregulation of mGluR5 in the CFA-treated rat (D), and many more immunogold particles are associated with the plasma membrane compared with the untreated rat (scale bar A, B, C, D: 1 μm).

Fig. 6A/B

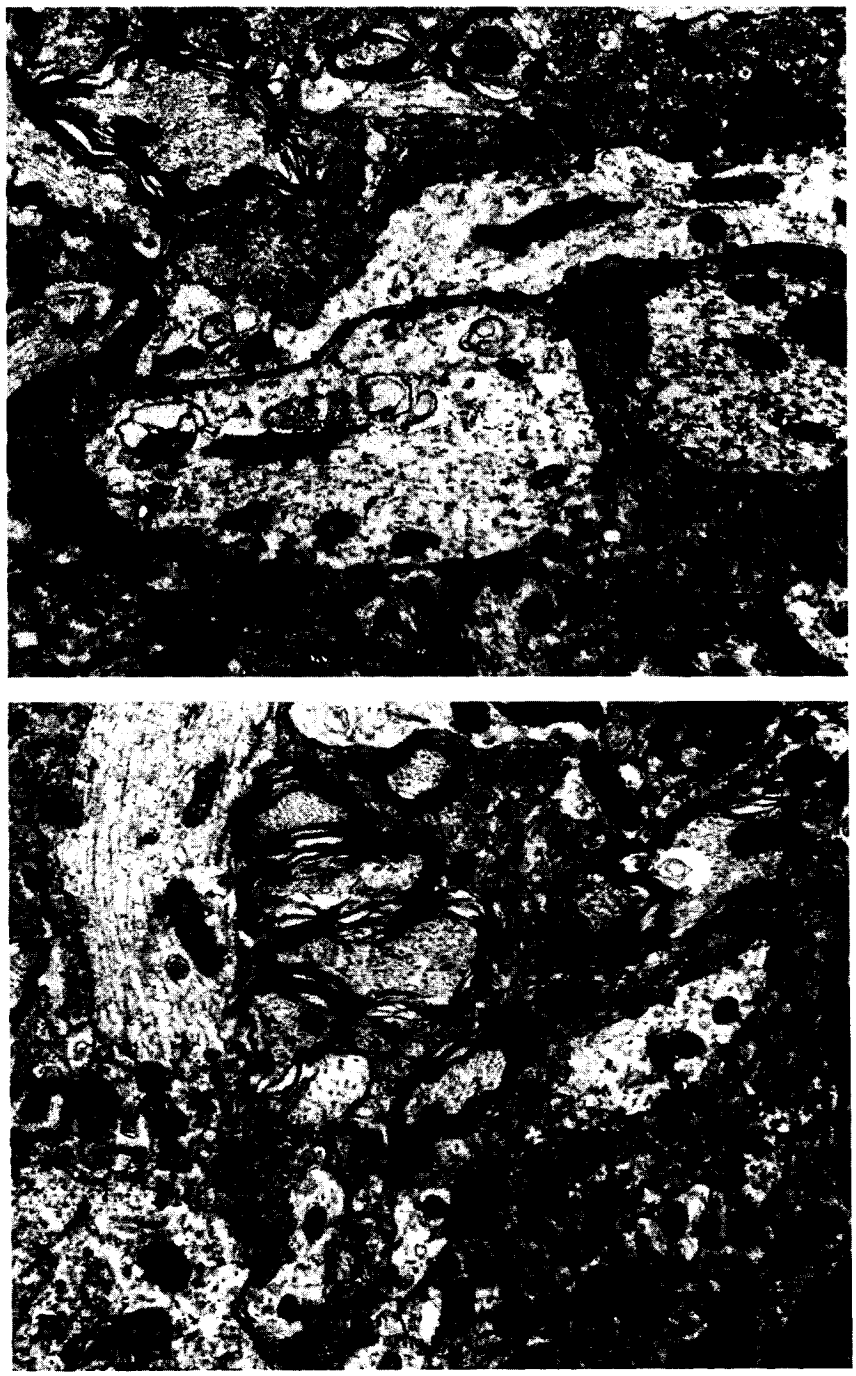
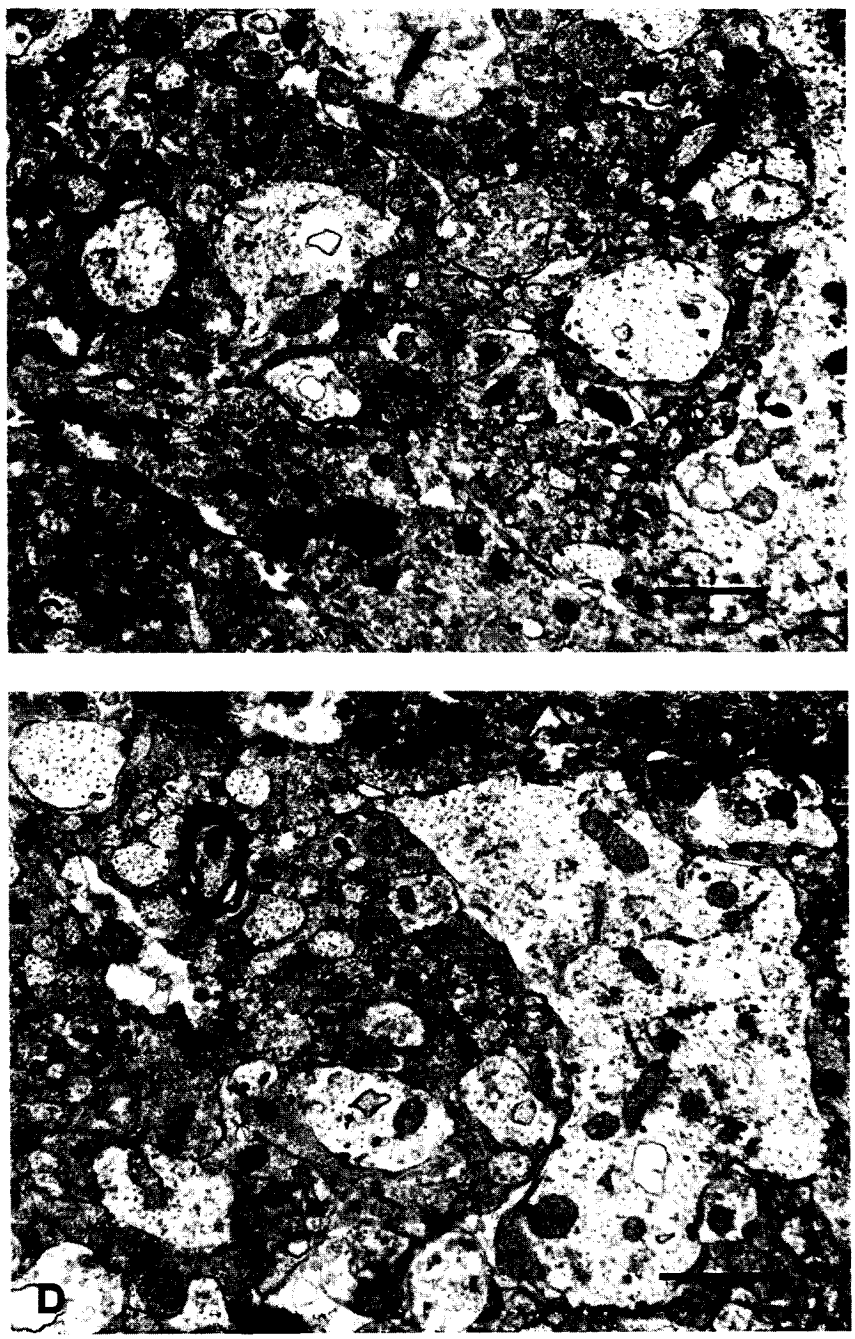


Fig. 6C/D



8.3 Ethics Certificate for Research Involving Animal Subjects