PLASTICITY OF LOCAL INHIBITION IN THE SUPERFICIAL DORSAL HORN AND ITS CONTRIBUTION TO NEUROPATHIC PAIN

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ABSTRACT

Neurons found in the superficial dorsal horn (SDH), particularly in the first lamina, are critical for mediating transmission of pain-related information to the brain, and thus influence pain perception. Excitability control of these neurons derives largely from a network of inhibitory interneurons that render dynamic inhibition via the GABA (γ-aminobutyric acid) and glycine neurotransmitter systems. However, the processes that contribute to the regulation of these systems under normal and pathophysiological conditions have not been well elucidated. To shed light on these issues, our studies employed a variety of techniques to examine mechanisms of plasticity of intrinsic inhibition in the SDH. We began this survey by examining the organization of GABA- and glycine-mediated inhibition in SDH neurons from developmentally immature rats; this study was undertaken to understand the early arrangements of local inhibition in the pain pathway, a critical study for a system that is phylogenetically primitive. We found that in slices taken from neonates, corelease of GABA and glycine from terminals coactivated postsynaptic $GABA_A$ and glycine receptors ($GABA_ARs$ and GlyRs) on lamina I and II neurons. In contrast, at all ages older than three weeks, quantal release of GABA and glycine never coactivated $GABA_A$ and glycine receptors at synapses. Because neurobiological processes will often revert to a developmental state when subjected to insult, we next assessed whether lamina I neuronal inhibitory synapses would assume an immature-like configuration after nerve injury. We found that $GABA_ARs$ and GlyRs did indeed comediate quantal postsynaptic events in adult lamina I neurons after peripheral nerve injury (PNI); moreover, we revealed a shift in neuronal anion gradient that translated to $GABA_AR$/GlyR-mediated postsynaptic currents that were depolarizing. This shift was caused by the downregulation of the anion cotransporter KCC2, and was shown to directly trigger sensory hypersensitivity in animal models. The shift in anion gradient was further demonstrated to result from the actions of microglia-derived BDNF. Specifically, BDNF was demonstrated to tonically depolarize the anion reversal potential in lamina I neurons through its stimulation of TrkB and, consequently, of cAMP-dependent kinase. The results presented here significantly increase the understanding of inhibitory control of SDH neurons, and identify several loci where therapeutic manipulation may correct conditions of disinhibition.

RÉSUMÉ

Les neurones des couches superficielles de la corne dorsale, et en particulier les neurones de la couche I, jouent un rôle majeur dans la transmission des informations nociceptives périphériques vers le cerveau et pourraient, par conséquent, également influencer la perception de la douleur. L'excitabilité de ces neurones est contrôlée par un réseau d'interneurones inhibiteurs utilisant le GABA (acide γ– aminobutyrique) et la glycine comme neurotransmetteurs. Cette inhibition est finement régulée tant dans les conditions normales que dans des conditions pathologiques, mais les voies de régulation demeurent encore mal connues. Afin de mieux comprendre les mécanismes de plasticité du système inhibiteur dans la corne dorsale, nous avons utilisé différentes approches techniques chez le rat.

Dans un premier temps, nous avons étudié l'évolution de l'inhibition GABAergique et glycinergique au cours du développement postnatal et ainsi mis en évidence les étapes de la mise en place de l'inhibition dans les voies spinales nociceptives, qui constituent un système phylogénetiquement conservé. En utilisant des tranches de moelle épinière de rats immatures, nous avons montré que, dans les couches I et II, le GABA et la glycine sont co-libérés à partir d'une même vésicule et vont co-activer les récepteurs $GABA_A$ et de la glycine, co-localisés au niveau post-synaptique. En revanche, après 3 semaines post-natales, une telle co-détection n'a jamais été observée.

Il a été montré dans différents systèmes neurobiologiques qu'un état comparable à un état immature peut être observé suite à une lésion. Dans un modèle de lésion d'un nerf, connu pour engendrer une désinhibition significative des neurones de la corne dorsale, nous avons cherché à savoir si le système inhibiteur allait être comparable à celui observé chez les animaux immatures. En effet, une co-libération du GABA et la glycine est à nouveau observée. De plus, une inversion du gradient des ions chlorures est observé, rendant les courants postsynaptiques $GABA_AR$/GlyR dépolarisant. Ce changement découle d'une régulation négative de l'expression du co-transporteur KCC2 qui expulse les ions chlorure à l'extérieur de la cellule. *In vivo*, une telle régulation cause une hypersensibilité sensorielle.

Cette inversion du gradient des ions chlorures résulte de l'action du BDNF, libéré par les microglies. En effet, *in vitro,* l'application de BDNF dépolarise le potentiel d'inversion des ions chlorures dans les neurones de la lamina I suite à l'activation des récepteurs Trk-B et des kinases AMPc-dépendantes.

L'ensemble de nos résultats permet de mieux comprendre les voies de régulations de l'inhibition dans la corne dorsale dans une situation pathologique et d'identifier plusieurs cibles thérapeutiques potentielles qui pourraient corriger le défaut d'inhibition observé dans des conditions pathologiques.

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CONTRIBUTION OF AUTHORS

In accordance with McGill University's "Guidelines for Thesis Preparation", this is a manuscript-based thesis. The results presented in chapters 2, 3, 4 and 5 are composed of text and figures that are duplicated from manuscripts that are under revision, published or to be submitted for publication. The contribution made by each author is stated in the following:

Chapter 2. Region-specific developmental specialization of GABA-glycine cosynapses in laminas I-II of the rat spinal dorsal horn. Keller, A.F., Coull, J.A.M., Chéry, N., Poisbeau, P. and Y. De Koninck. *Journal of Neuroscience*, 21(20): 7871-80.

For this work, Dr. Florence Keller and I are co-first authors. I performed all of the electrophysiological analyses of lamina I neurons, while Dr. Keller carried out the analyses of lamina II neurons. I further wrote the full manuscript and prepared all figures on which this chapter is based. Professors De Koninck and Poisbeau provided revisions to text and figures.

Chapter 3. *Trans*synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. Coull, J.A.M., Boudreau, D., Bachand, K., Prescott, S.A., Nault, F., Sik, A., De Koninck, P. and Y. De Koninck. *Nature*, 424(6951): 938-42.

I performed all of the electrophysiological analyses presented in this report; I further directed all other aspects of the study. Dominic Boudreau performed calcium imaging, Karine Bachand prepared and tested experimental animal models, Steven Prescott executed the computer simulations, Francine Nault carried out the immunoblots, and Dr. Atilla Sik performed the electron microscopy. I was also responsible for drafting the entire manuscript and preparing the figures on which this chapter is based; Professors Yves and Paul De Koninck provided supervision and editions to the text and figures.

Chapter 4. Tonic repression of spinal inhibition by microglia-derived BDNF as a substrate of neuropathic pain. Coull, J.A.M., Beggs, S., Boivin, D., Boudreau, D., Zhang, J., Salter, M.W. and Y. De Koninck. To be submitted for publication.

For this study, I performed all of the electrophysiological analyses and directed the calcium imaging, which was carried out by Dominic Boudreau, and the behavioural pharmacology, which was executed by Dominick Boivin. Preparation and manipulation of microglia cultures was performed by Dr. Simon Beggs, and immunohistochemical stainings were carried out by Dr. Ji Zhang. I wrote the manuscript and prepared all figures on which this chapter is based. Professors Salter and De Koninck provided supervision and revisions to the manuscript.

Chapter 5. Cyclic-AMP dependent kinase activation is necessary for the disruption of anion gradient in spinal lamina I neurons after nerve injury. Coull, J.A.M., Boivin, D. and Yves De Koninck. To be submitted for publication.

I carried out all electrophysiological analyses described in this work and prepared all figures and text composing the manuscript. Dominick Boivin prepared experimental animal models and performed behavioural pharmacology, while Professor De Koninick provided overall supervision and revisions to the manuscript.

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LIST OF ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
AMP	Adenosine monophosphate
AMPA	Aminomethylphosphonic acid
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
CaMK	Calcium/calmodulin-dependent kinase
cAMP	Cyclic AMP
CNS	Central nervous system
DRG	Dorsal root ganglion
EPSC/P/G	Excitatory postsynaptic current/potential/conductance
GABA	γ-aminobutyric acid
$GABA_AR$	$GABA_A$ receptor
GABA-LI	GABA-like immunoreactivity
GAT	GABA transporter
GluR	Glutamate receptor
GlyR	Glycine receptor
Gly-LI	Glycine-like immunoreactivity
GLYT	Glycine transporter
IPSC/P/G	Inhibitory postsynaptic current/potential/conductance
LI/II/III…	Lamina I/II/III…
mPSC	Miniature postsynaptic current
mRNA	Messenger RNA
NMDA	*N*-methyl-*D*-aspartate
PKA	Protein kinase A; cAMP-dependent kinase
PKC	Protein kinase C
PNI	Peripheral nerve injury
PNS	Peripheral nervous system
RNA	Ribonucleic acid
SDH	Superficial dorsal horn
STT	Spinothalamic tract

TrkA/B/C	Tyrosine receptor kinase A/B/C
TTX	Tetrodotoxin
VIAAT/VGAT	Vesicular GABA/glycine uptake transporter

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Of the five modalities of our sentience, pain represents the sensation that is least well understood. Part of this lack of understanding stems from a long-held ignorance regarding the causes of pain: until recently, pain was thought to exist solely as a secondary symptom of actual or potential tissue damage resulting from injury or disease (Wall and Melzack, 2000). Yet, although conventional wisdom certainly bears out the fact that (acute) pain may result from tissue damage, it has become clear that some forms of pain may not be directly coupled to a second pathology. Indeed, forms of pain that persist chronically (by definition, longer than 3-6 months) are now thought to represent a disease in and of themselves (Wall and Melzack, 2000).

More specifically, contemporary research has identified the origin of certain forms of chronic pain to be a disturbance, not to tissue, but within the nervous system (Woolf and Mannion, 1999) – hence earning the condition the term neuropathic pain. Neuropathic pain may result from neuropathy of the central nervous system, especially of the spinal cord, often in addition to neuropathy of the peripheral nervous system (PNS).

In most cases these neuropathies are closely associated with a sensitization event whereby neurons experience a profound enhancement of their excitability (Coderre et al., 1993;Woolf, 1983). In the PNS, sensitization is manifested as an increase in the excitability of the terminal membrane of nociceptors – sensory afferents that carry pain-related information from the periphery to the spinal dorsal horn – whereby the amount of depolarization required to trigger action potential discharge is reduced (Woolf and Salter, 2000).

Peripheral sensitization has been documented to result from several sensitizing agents, including inflammatory mediators, neurotrophins and kinases. Among the generators of peripheral sensitization is the neuron-specific sodium channel SNS (sensory neuron-specific), which upon phosphorylation expresses a decreased threshold of activation along with an increased magnitude of sodium conductance to depolarization (Fitzgerald et al., 1999).

The central consequence of peripheral sensitization is an increased output of nociceptors at the first sensory synapse often located in the superficial laminae of the dorsal horn (Chul et al., 2000). Postsynaptic neurons, which in some cases transmit pain directly to the brain, respond to this heightened input by themselves increasing their excitability leading to a central sensitization that either outlasts the initiating input or requires a low-level peripheral drive to maintain it (Melzack et al., 2001).

The principal results of such central sensitization are facilitated excitatory synaptic responses in these neurons, leading to a state where normally subthreshold input stemming from noxious or innocuous peripheral stimulation becomes suprathresold causing neurons to fire in response to inappropriate stimulation (Woolf and Salter, 2000).

The causes of central sensitization are controversial. For example, some groups have suggested that the response of pain transmission neurons to inappropriate (*ie.* innocuous) stimulation that occurs after a peripheral nerve injury (PNI) may result from a sprouting of low-threshold (Aβ) sensory afferents into regions of the superficial dorsal horn (SDH) that are critical for pain transmission (Woolf et al., 1992;Chou et al., 2002;Kohama et al., 2000). However, other groups have failed to observe this phenomenon, and surmise that technical artifacts may account for the original findings (Hughes et al., 2003;Shehab et al., 2003;Shehab et al., 2004). Additionally, anatomical alterations fail to account for the rapid onset of central sensitization following an insult to the peripheral nervous system (T. Dickenson, 2004, personal communication).

Others have suggested that increased excitability in central neurons may result from a potentiation of AMPA glutamate receptor-mediated excitatory synaptic responses, as high-frequency stimulation (100 Hz) of nociceptors leads to this situation and to a state resembling neuropathic pain (Liu and Sandkuhler, 1997;Randic et al., 1993). However, this theory has been questioned on the basis of the observation that C-fiber nociceptors have never been observed to fire at anywhere near these frequencies under physiological conditions (Ji et al., 2003).

Several studies have documented the importance of NMDA receptor activation to the development and maintenance of central sensitization. For example, inhibitors of NMDA channels can both prevent the onset, and reduce the severity of neuropathic pain in animals and humans (Fisher and Hagen, 1999;Rabben et al., 1999;Yashpal et al., 2001). The mechanisms that lead to this enhancement of NMDA function, however, are unclear.

Some studies have demonstrated that a phosphorylation of NMDA subunits (especially NR1 and NR2B) may enhance channel activity (Brenner et al., 2004;Guo et al., 2002). The activation of requisite phosphorylation mechanisms in pain transmission neurons has been attributed to such systems as metabatropic glutamate receptors. However, both knock-down and pharmacological studies have shown that modulation of group I-III mGluRs has only modest influence over the development of nociceptive hypersensitivity (Fisher et al., 2002;Noda et al., 2003). Substantial evidence also supports the notion that TrkB activation by BDNF may trigger NMDA receptor phosphorylation (Kerr et al., 1999), however it is unclear where the said BDNF may originate from, especially considering a recent study reporting that there is no increase in the release of BDNF from nociceptors following PNI (Lever et al., 2003).

On the other hand, modern pain theory indicates that a decrease in the function of local spinal interneurons that serve to inhibit pain transmission neurons (a disinhibition) is critical for the development of central sensitization (Melzack and Wall, 1965). Yet, the mechanisms that may underlie this disinhibition have remained elusive. A few studies have suggested that PNI may trigger *trans*synaptic degeneration of these inhibitory interneurons, leading to net spinal disinhibition (Sugimoto et al., 1990;Coggeshall et al., 2001;Moore et al., 2002). This theory is strengthened by a study that shows that pharmacological inhibition of glycine receptors, which is known to produce a nociceptive hypersensitivity similar to that which develops after PNI, enhances nerve transection-evoked neuronal degeneration (Sugimoto et al., 1985).

However, a recent, comprehensive study failed to find signs of a relationship between nociceptive hypersensitivity and cell death in the dorsal horn after PNI, further arguing

that it is neither necessary nor sufficient for the subsequent development of hypersensitivity (Polgar et al., 2003b).

Another mechanism that could underlie disinhibition in the dorsal horn is a reduction of $GABA_A$ and/or glycine receptor expression. In models of PNI, several analyses of protein and mRNA expression have indicated that there does indeed occur an associated reduction of both $GABA_A$ and glycine receptor expression (Fukuoka et al., 1998;Simpson and Huang, 1998;Ibuki et al., 1997). Yet a very recent study employing *in situ* hybridization techniques found increases in both $GABA_A$ and glycine receptor mRNA after nerve transection (Yang et al., 2004b). Similarly, it is unclear how levels and/or release of GABA and glycine in the SDH might change after PNI. Following PNI, some groups have shown a decrease in GABA and/or glycine expression (Castro-Lopes et al., 1993;Eaton et al., 1998), some have found no change (Somers and Clemente, 2002) while others have actually found an increase (Satoh and Omote, 1996).

The uncertainty surrounding the role of the above phenomena in disinhibition may raise the question of whether a more subtle synaptic reorganization may be important for the diminishment of $GABA_A$R- and/or GlyR-mediated inhibition. In other systems, studies have suggested that neuronal injury can lead to a shift in the very mode of $GABA_A$R- and GlyR-mediated influence; that is, in some cases trauma has been reported to result in an alteration of ion levels within neurons – leading to $GABA_A$R- and GlyR-associated excitation, a situation that closely resembles the configuration of developmentally immature synapses (Nabekura et al., 2002;Toyoda et al., 2003). This type of reversion may not be surprising, considering that the pain system is thought to be phylogenetically primitive (Furue et al., 2004).

To shed some light on the mechanisms of plasticity that occur at nominally inhibitory SDH synapses following PNI, therefore, one might first endeavour to understand these processes in the immature SDH. However, very little is known about inhibitory synaptic function in the SDH; indeed, the very functions of GABA and glycine here are poorly understood.

Taken together, it is clear that the organization, as well as the role of $GABA_AR$ and GlyR synapses in PNI-evoked neuronal hyperexcitability in the SDH, are poorly understood. Therefore, the objective of this thesis was to examine these mechanisms from a functional perspective. Specifically, I have endeavoured to examine the plasticity of local inhibitory synapses in the SDH in order to answer such central questions as: (a) how does the contribution of $GABA_ARs$ and/or GlyRs to postsynaptic excitability control in the SDH change with development? (b) Does a significant plastic change occur after PNI? (c) What are the mechanisms of such a plastic change, and do there exist alterations that are similar to developmental plasticity at these inhibitory synapses? (d) Is PNI-evoked disinhibition sufficient to account for the neuronal hyperexcitability that manifests central sensitization?

To establish the basis on which I have empirically examined these issues, I will broadly review the current thinking regarding the properties of, and contributors to neuronal networks in the SDH. Moreover, I will focus on what has been reported in respect to the organization and coordination, development and plasticity of nominally inhibitory, $GABA_A$ and glycine receptor synapses in the CNS, with particular emphasis on the SDH – and LI neurons – wherever possible.

2. ORGANIZATION OF THE ADULT SUPERFICIAL DORSAL HORN

Early characterization of much of the spinal cord, including the substantia gelatinosa, was performed by Rolando in 1824 and later quoted by Cajal in 1909. More recently, it was Rexed (1952) that divided the grey matter of the cat spinal cord into the familiar sequence of laminae. In his scheme, lamina I (LI) corresponds to the marginal zone, lamina II (LII) corresponds to the substantia gelatinosa and the other four laminae (LIII-LVI) compose the balance of the dorsal horn. The SDH (LI-III), particularly the first two laminae, is well established to be one of the most critical regions for the somatosensory processing of pain (Light, 1992;Todd, 2002). For example, in rats, where LI is just 20 μm wide (Molander et al., 1989), a significant majority of nociceptors have been demonstrated to terminate in the SDH (Light and Perl, 1979;Woolf and Fitzgerald, 1983;Lawson, 2002).

2.1 Primary afferent input to the superficial dorsal horn.

Primary afferent input to the SDH largely stems from nociceptors, widely characterized as small afferents with axons that are either lightly myelinated (Aδ) or not myelinated at all (C) (Light and Perl, 1979;Sugiura et al., 1986). These nociceptors enter the SDH from the surrounding white matter via the tract of Lissauer (Wall et al., 1999). In LI, it was originally thought that afferent terminals originated predominantly from Aδ fibers (Beal and Bicknell, 1981;Light et al., 1979); although other studies support this finding (Woolf and Fitzgerald, 1983), it is now generally accepted that LI neurons, like LII neurons, can also respond to electrical activation of C-fibers (Gobel et al., 1981;Christensen and Perl, 1970). LI neurons have further been shown to respond to stimulation of large-diameter myelinated Aβ fibers usually associated with the transmission of innocuous sensory input (Woolf and Fitzgerald, 1983;Bester et al., 2000). However, anatomical studies have failed to reveal an Aβ projection pattern to LI (Marshall et al., 1996;Todd, 2002;Brown and Fyffe, 1981). Hence, it is probable that Aβ stimulation of LI neurons occurs via polysynaptic pathways, or via synaptic contacts between Aβ fibers and LI-II neuronal dendrites in the deeper laminae (Woolf and Fitzgerald, 1983).

Significant amounts of the sensory afferents terminating in LI are peptidergic, always expressing calcitonin gene related peptide (CGRP) (Hunt et al., 1992;Kruger et al., 1989). Peptidergic afferents (C-fiber and Aδ) containing substance P (SP) have been physiologically characterized as nociceptors (Lawson et al., 1997;Cuello, 1987) and generally terminate in LI and LII, although an additional population does terminate in the deep dorsal horn (Todd, 2002). Non-peptidergic C-fibers have also been identified; these afferents are distinguished by their propensity to bind the lectin *Bandeiraea simplicifolia* isolectin B4 (Silverman and Kruger, 1990). Although a role for IB4-binding C-fibers in pain transmission has not been fully elucidated, a preliminary report suggests that some may act as polymodal nociceptors (Gerke and Plenderleith, 2001).

2.2 Neurons in lamina I.

Neurons in the first lamina of the dorsal horn have been examined in several species of mammal, including rats (Lima and Coimbra, 1986;Spike et al., 2003), cats (Han et al., 1998), monkeys (Yu et al., 1999) and humans (Schoenen, 1982). Although several differences exist among species, it appears that LI neurons serve a discrete number of functions. That is, in all species examined there appear to exist neurons in LI that respond solely to noxious stimulation (nociceptive specific and polymodal nociceptive neurons), exclusively to innocuous stimulation (innocuous thermoreceptive neurons) and/or to the two (wide dynamic range neurons) (Dostrovsky and Craig, 1996). All of these neurons may further confine their axons to propriospinal regions, or project their axons to the brain via a plethora of spinal tracts. In the following, I will expand on what is known about the function of LI neurons, and relate this to morphology, biophysical properties and projection patterns within the central nervous system.

Nociceptive-specific neurons

Nociceptive-specific (NS) neurons have been defined as neurons possessing a small receptive field and that respond only to noxious thermal, mechanical or chemical cutaneous stimulation (Light, 1992;Blomqvist and Craig, 2000). In the rat, the majority of NS neurons are found in LI (Woolf and Fitzgerald, 1983), where up to 61% of neurons are classified as NS (Seagrove et al., 2004). Similarly, the cell population of the feline LI is composed of roughly 40% NS neurons (Han et al., 1998); NS neurons have also been revealed to compose a large proportion of the neurons found in the first lamina of the primate dorsal horn (Dostrovsky and Craig, 1996).

Although not explicitly defined in the rat or monkey, NS neurons have been identified using combined biocytin intracellular labeling and single-unit recording as typically having a fusiform morphology in the cat dorsal horn (Han et al., 1998). This observation is in agreement with immunostaining studies for both the rat and the monkey, where a substantial number of fusiform cells were reported to express the substance P receptor, NK-1 (Yu et al., 1999), which has been documented to be implicated in the transmission

of nociceptive information (Henry, 1982;Mantyh et al., 1997;Nichols et al., 1999). NS neurons were further described in the cat as displaying high ongoing discharge rates, and a significantly slower conduction velocity as compared to the other neurons found in LI (Han et al., 1998).

Taken together with the latter observation that NS neurons possess a slower conduction velocity, a finding that fusiform neurons in rat LI project unmyelinated axons, whereas pyramidal and multipolar neurons project myelinated axons (Gobel, 1978;Lima and Coimbra, 1986), may indicate a link between the fusiform morphology and NS function in rats. This link is underscored by another recent finding in the rat where a majority of fusiform LI neurons were reported to show tonic spiking in response to a depolarizing intracellular pulse (Prescott and de Koninck, 2002), and may be in keeping with the observation of high ongoing discharge rates in NS neurons in the cat.

Polymodal nociceptive neurons

A second group of neurons found in the first lamina of the dorsal horn are referred to as polymodal nociceptive neurons. Responsive to noxious **h**eat, **p**inch and **c**old stimuli [and therefore often referred to as HPC neurons (Craig et al., 2001)], this type of cell has been documented to respond to C-fiber nociceptor stimulation, and to display graded responses to increasing intensities of noxious stimulation (Light, 1992;Dostrovsky and Craig, 1996), but no frequency adaptation in spiking (Han et al., 1998).

Single-unit extracellular recordings have suggested that HPC cells make up approximately 18% of neurons in rat LI (Seagrove et al., 2004), whereas in the cat LI, HPC cells could account for as little as 16% of the neuronal population (Han et al., 1998).

HPC cells have further been reported to exhibit a multipolar morphology in the cat (Han et al., 1998). While an explicit link has not been established in the monkey, evidence of a correlation between HPC function and the multipolar morphology may stem from the observation that roughly one-half of multipolar LI neurons in the monkey express the

NK-1 receptor (Yu et al., 1999). A large proportion of multipolar neurons in the rat LI have also been revealed to express the NK-1 receptor (Polgar et al., 1999b).

Wide dynamic range neurons

Although not reported in the cat marginal zone (Steedman et al., 1985;Craig and Kniffki, 1985), wide dynamic range (WDR) neurons have been documented in both rat and monkey LI (Dostrovsky and Craig, 1996;Woolf and King, 1987). WDR neurons, which have also been observed in deeper regions of the dorsal horn (Lin et al., 1997), have been classified as those LI neurons that can be activated by low-threshold mechanoreceptors (innocuous stimulation), as well as by Aδ and C-fiber nociceptors (noxious stimulation) (Woolf and Fitzgerald, 1983;Light, 1992). In the rat, a recent report suggested that WDR neurons could account for as many as 26% of neurons in LI (Seagrove et al., 2004).

Innocuous thermoreceptive neurons

Although not reported to exist in the rat SDH (Seagrove et al., 2004), in cat and monkey LI neurons, a class of neurons that respond only to innocuous cooling (COLD cells) have been identified (Craig and Kniffki, 1985;Craig and Dostrovsky, 2001;Dostrovsky and Craig, 1996). COLD cells have been reported to be inhibited by radiant heat, being activated by even modest decreases in the temperature of the skin surface; additionally, COLD cells are said to display erratic spontaneous discharge at room temperature and do not display frequency adaptation (Dostrovsky and Craig, 1996;Han et al., 1998).

The morphology of LI COLD cells responding to innocuous cooling has been determined to be pyramidal-like in the cat (Han et al., 1998), which fits well with the observation that very few LI pyramidal cells express the pain-related NK-1 receptor in the monkey (Yu et al., 1999).

2.3 Projection patterns of lamina I neurons.

The use of retrograde tracers has allowed for detailed studies of the projection patterns of many neurons in the dorsal horn. Although in the deeper laminae (LIII-VI) many populations of neurons have been demonstrated to project rostrally (Kobayashi, 1998;Gauriau and Bernard, 2002), neurons that project to the brain are most densely packed in LI (Todd, 2002). LI neurons were first shown to project to the thalamus in the spinothalamic tract (Trevino and Carstens, 1975), however, it is now thought that LI neurons may project contra- and bilaterally to a number of discrete loci, including the thalamus, periaqueductal grey matter (PAG), solitary tract nucleus, medullary reticular formation and lateral parabrachial area (Giesler, Jr. et al., 1979;Cechetto et al., 1985;Menetrey and Besson, 1982;Burstein et al., 1990;Lima and Coimbra, 1988;Todd, 2002).

In the rat, a recent study by Todd et al. (2000) using cholera toxin subunit b (CTb) retrograde labeling identified the caudal ventrolateral medulla (CVLM) and the lateral parabrachial area (LPb) to be the principal termination points for LI projection neurons. The proportion of LI neurons that were observed to project to the PAG and thalamus was significantly less [see also Lima and Coimbra (1988) and Marshall (1996)] with the majority of neurons that project to the PAG (Spike et al., 2003) and to the thalamus (Hylden et al., 1989) also projecting to either the CVLM or the LPb.

The CVLM has also been identified as a major termination point for axons of LI neurons in cats and monkeys (Craig, 1995). However, it appears that a greater proportion of LI neurons project in the feline spinothalamic tract, as cholera toxin subunit b retrograde labeling from either the ventrolateral medulla or the thalamus identified equal numbers of neurons in the marginal zone (Andrew et al., 2003).

The proportion of LI projection neurons implicated in nociceptive processing is very high. Todd et al. (2000) provided indirect evidence of this by reporting that as many as 80% of LI neurons projecting to regions of the brain important for nociceptive integration, such as the CVLM and LPb, express the NK1 receptor [note that few NK-1+

LI neurons were documented to project solely to the PAG (Spike et al., 2003)]. Additionally, Bester et al. (2000) used antidromic stimulation protocols coupled with extracellular recordings to reveal that for rat LI neurons that projected to the parabrachial nuclei, 75% responded only to noxious stimulation (NS), whereas the remaining 25% responded to both noxious and innocuous stimulation (WDR). The proportion of nociceptive-specific LI neurons projecting in the feline spinothalamic tract was found to be somewhat different: only 43% of neurons were nociceptive-specific or HPC cells, while 56% were identified as COLD cells (Craig and Dostrovsky, 2001).

Various studies have further examined the morphology of cells projecting from LI to the different areas of the brain. In the rat, all three common morphologies of LI neurons (pyramidal, fusiform and multipolar) have been reported to project axons to the CVLM, LPb, PAG and thalamus – in equal proportions (Spike et al., 2003). However, these observation are in sharp contract with those of Lima and Coimbra (1989) and Lima et al. (1991), who, using retrograde CTb staining, reported that rat LI fusiform neurons projected mainly to the CVLM and LPb, whereas LI pyramidal neurons projected to the PAG. There is no apparent explanation for this difference of observation in the rat.

Gamboa-Esteves et al. (2001) investigated the immunochemical profiles of LI neurons projecting to the nucleus tractus solitarii (NTS) in the rat using CTb retrograde labeling. This group found that 38% of projecting LI neurons possessed a flattened morphology, while 37% were pyramidal and 25% were fusiform (Esteves et al., 1993). Interestingly, this group reported that only ~5% of retrogradely labeled neurons were NK-1r immunoreactive (Gamboa-Esteves et al., 2001), raising questions regarding the role neurons projecting to the NTS from LI in pain processing.

Fusiform, multipolar and pyramidal LI neurons were observed to project in both the feline spinothalamic and spinomedullary tracts; in both cases, fusiform neurons represented the greatest proportion of projecting cells (Andrew et al., 2003;Craig and Dostrovsky, 2001).

In the monkey, all three cellular morphologies in LI have been reported to send axons to the thalamus. Of these neurons, 25% of pyramidal neurons, and 75% of multipolar and fusiform neurons were NK-1r immunoreactive (Yu et al., 1999). However, for STT neurons that were found to be NK-1r-IR, 48% were fusiform, 33% were multipolar and 10% were found to be of pyramidal morphology (Yu et al., 1999). These findings were proposed to be consistent with the idea that most primate fusiform and multipolar LI neurons are nociceptive, whereas the majority of pyramidal LI neurons are non-nociceptive. It should be noted, however, that this study also provides evidence that not all nociceptive LI neurons are NK-1r-IR.

2.4 Interlaminae communications between LI neurons and other neurons in the dorsal horn.

For quite some time it has been known that neurons in LI can send out, and receive neurites from deeper laminae (Lima and Coimbra, 1986). For example, Polgar et al. (1999a) demonstrated that neuropeptide Y positive axons likely extending from GABAergic interneurons in LI-II (Todd and Spike, 1993;Rowan et al., 1993) could form synapses with NK1+ neurons found in the deep dorsal horn. Interestingly, it also appears that dendrites from these same deep NK1+ neurons can project to LI, where they receive numerous synaptic contacts from substance P-containing primary afferents (Naim et al., 1997;de Koninck et al., 1992;Ma et al., 1996). LI neurons have also been shown to inhibit deep dorsal horn neurons using paired extracellular recordings (Biella et al., 1997). A new study suggests that LI neurons may participate in polysynaptic pathways that positively modulate deep (LV) dorsal horn neurons (Nakatsuka et al., 2002). Specifically, this group showed that capsaicin-sensitive afferent input could first activate interneurons in the superficial laminae, after which excitatory activity was transmitted onto lamina V neurons.

Alternatively, many studies indicate that neurons residing in the deeper layers of the dorsal horn can modulate the activity of LI neurons via projection of axon collaterals. Paired electrophysiological recordings and anatomical observations have illustrated that stalked cells, whose cell bodies are located in LII, have axons that arborize predominantly

in LI and can positively modulate LI neurons (Bennett et al., 1980;Gobel, 1978;Bennett et al., 1982). Stalked cells projecting to LI have further been documented to receive direct input from high-threshold sensory afferents, and have recently been reported to be hyperpolarized by the μ-opioid receptor agonist DAMGO (Eckert, III et al., 2003). Taken together, all of these findings underscore the idea that the processing of nociceptive information in the dorsal horn is a dynamic process, governed by the actions of a network of neurons, as opposed to the actions of a single group of neurons found in one region.

2.5 GABA and glycine.

In the spinal cord, GABA and glycine were first shown to depress the excitability of spinal neurons forty years ago (Curtis et al., 1968a;Curtis et al., 1968b). More recently, electrophysiological studies have demonstrated that both GABA and glycine can inhibit second order neurons (including spinothalamic tract neurons) in the SDH that respond to exogenous substance P (Murase et al., 1989) and to high-threshold (pinch) stimulation of sensory afferents (Zieglgansberger and Sutor, 1983;Willcockson et al., 1984). These results strongly suggest that GABA and glycine can mediate excitability control of pain pathways in the SDH, and therefore can ultimately influence if and how pain is perceived. Moreover, it appears that in several cases GABA and glycine execute this function in concert (Chery and de Koninck, 1999); however, to fully appreciate the contributions of GABA and glycine to spinal inhibition, it would be most useful to briefly review the basic properties of each transmitter system.

GABA and the $GABA_A$ receptor

GABA, or γ-aminobutyric acid, is an amino acid neurotransmitter that is derived from glutamate via the enzymatic actions of glutamic acid decarboxylase (GAD) (Guthmann et al., 1998). In the spinal cord, glutamate can be converted to GABA by two isoforms of GAD: GAD67 and GAD65. Although protein levels are similar for the two GAD isoforms in the normal SDH (Feldblum et al., 1995), it has been suggested that they may subserve distinctive roles. For example, GAD67 has been reported to be found primarily in the cytosol, localized to neuronal cell bodies and dendrites; GAD65, on the other hand,

is preferentially targeted to membranes and nerve endings where it has been suggested to preferentially synthesize GABA for vesicular release (Soghomonian and Martin, 1998).

GABA activates the ionotropic receptor $GABA_A$. $GABA_A$ receptors ($GABA_ARs$) are hetero-oligomeric channels that are composed of five subunits, each of which containing four *trans*membrane hydrophobic sequences (Barnard et al., 1998;Macdonald and Olsen, 1994). In the mammalian nervous system seven $GABA_AR$ subunits have been classified on the basis of sequence homology. These subunits may be further divided into disparate subtypes: in total six subtypes have been described so far: α (α1-6), four β (β1-4), four γ (γ1-4), one δ, one ε, one π and at least three ρ subunits (Mehta and Ticku, 1999;Barnard et al., 1998;Smith, 2001).

In the SDH *in situ* hybridization and immunohistochemical studies suggest that more than one $GABA_AR$ isoform may exist (Persohn et al., 1991), perhaps indicating functional diversity (see below). For example, the terminals of some sensory neurons found in LII have been reported to express the α6 $GABA_AR$ subunit (Gutierrez et al., 1996), but not β2/3 (Alvarez et al., 1996). In contrast, immunohistochemical studies have identified robust postsynaptic expression of α2, α3, β2, β3 and γ2 subunits in LI-II (Gutierrez et al., 1996;Persohn et al., 1991;Alvarez et al., 1996;Bohlhalter et al., 1996). Curiously, detection of the α1 $GABA_AR$ subunit was almost exclusively restricted to the deep dorsal horn and intermediate zone (Bohlhalter et al., 1996).

Activation of all $GABA_AR$ isoforms (and the associated anion flux – see below) is thought to require at least two GABA molecules to stimulate the extracellular binding sites (Macdonald and Olsen, 1994). Competitive antagonism may be accomplished using the selective $GABA_AR$ antagonist bicuculline, in addition to certain arylaminopyridazines (Sieghart, 1995). Following activation, GABA efficiently diffuses away from the synaptic cleft (Thompson and Gahwiler, 1992) and is either uptaken by presynaptic terminals in the SDH via the **GABA t**ransporter GAT-1, which is driven by the *trans*membrane Na^+ gradient (Borden, 1996), or by neuroglia, which have been suggested

to express similar isoforms of high-affinity GABA-uptake transporters (Schon and Kelly, 1975).

In presynaptic terminals, cytosolic GABA loading into synaptic vesicles is mediated by the proton ATPase transporter VIAAT [**v**esicular **i**nhibitory **a**mino **a**cid **t**ransporter – also referred to as the **v**esicular **g**lycine/GABA **t**ransporter - VGAT (Dumoulin et al., 2000;Eiden, 2000;Gammelsaeter et al., 2004)].

Glycine and the glycine receptor

Glycine is the simplest amino acid by structure, yet has a plethora of metabolic functions in the body (Legendre, 2001). As a neurotransmitter system, glycine has been found mostly in the spinal cord and brainstem (Breitinger and Becker, 2002); particularly dense staining for glycine is found in all laminae of the dorsal horn (van den Pol and Gorcs, 1988).

In the CNS, glycine can trigger a *trans*membrane anion flux via activation of the glycine receptor (GlyR)(Kirsch and Betz, 1998;Legendre, 2001). Similar to the $GABA_AR$, the GlyR is an ionotropic receptor comprised of five α and β subunits, usually with an α:β stoichiometry of 3:2 in the adult dorsal horn (Langosch et al., 1988). Four splice variants of the α subunit have been cloned (Betz et al., 1994). In the adult dorsal horn, it is usually the α1 subunit that forms functional GlyRs together with the ubiquitous β subunit (Takahashi et al., 1992;Betz et al., 1994).

The ligand binding site on the GlyR is has been demonstrated to be located on the α subunit (Legendre, 2001). Competitive antagonism at this site is rendered by the plant alkaloid strychnine and its derivatives (Curtis et al., 1968a;Rajendra et al., 1997).

Following activation of the GlyR, glycine may diffuse away from the synaptic cleft and be taken up by presynaptic terminals. Such uptake is predominantly mediated in the dorsal horn by the GLYT2 (**gly**cine **t**ransporter 2), a transporter that, similar to the GABA

transporters, is driven by the *trans*membrane sodium gradient (Puskar et al., 2001;Kanner, 1994).

In the presynaptic terminal, glycine loading into synaptic vesicles has been demonstrated using *in situ* hybridization to be mediated by the VIAAT, the same vesicular transporter responsible for the vesicular uptake of GABA (Dumoulin et al., 1999;Gasnier, 2000). This intriguing observation confirmed previous studies that have suggested that there exists vesicular colocalization of GABA and glycine (Burger et al., 1991;Chaudhry et al., 1998), and represented one of primary lines of evidence that argued in favour of a corelease of GABA and glycine from the same terminals in the SDH.

Relationship between GABA and glycine in the SDH

In addition to the affinity of the vesicular transporter VGAT for both GABA and glycine, it has been demonstrated using immunohistochemical techniques that the glycine transporter GLYT2 is often colocalized with GAD in axons located in LI (Puskar et al., 2001). The notion that GABA and glycine can be localized in the terminals of intrinsic neurons in the SDH is now well accepted (Todd and Spike, 1993). Both of these transmitters have been shown to exist in the terminals of descending medullospinal neurons in LI-II (Antal et al., 1996), however a transection of these fibers in the cat spinal cord altered neither the concentration of GABA nor glycine significantly (Rizzoli, 1968), underscoring the importance of local neurons as the principal origin of the transmitters.

Many immunohistochemical analyses have demonstrated the colocalization of GABA and glycine in neurons of the SDH (Todd et al., 1994;Laing et al., 1994;Wang et al., 2000b;Sutherland et al., 2002) including in LI (Kerr et al., 1998;Todd and Sullivan, 1990). More precisely, (Todd and Sullivan, 1990) demonstrated that although 28% of LI neurons were GABA-IR, 9% were both GABA- and glycine-IR. Glycine-IR was shown to exist at increased concentrations in LII and LIII.

Both myelinated and unmyelinated primary afferents have been observed to innervate neurons in the SDH immunoreactive for both GABA and glycine (Yoshimura and Nishi,

1995b). Indeed, a high proportion of neurons postsynaptic to substance P-IR terminals were observed to contain both GABA and glycine (Wang et al., 2000a).

Todd (1996) has suggested that two types of synaptic glomeruli may be described in the SDH on the basis of their expression of GABA and glycine. Profiles within type I glomeruli were defined as being GABA-IR only, whereas those within type II glomeruli are GABA- and glycine-IR, suggesting that different types of inhibitory interneuron in the dorsal horn are associated with the two types of glomerulus.

The presence of mixed GABA- and glycine-IR terminals presynaptic to primary afferents, including substance P-IR terminals, has been confirmed with other studies (Wang et al., 2000b;Sutherland et al., 2002).

It also appears that mixed GABA- and glycine-IR fibers can innervate projection neurons in the SDH. For example, in LI, both spinomedullary and spinoparabrachial tract neurons have been described as lying postsynaptic to GABA- and glycine-IR terminals (Maxwell et al., 1995;Wang et al., 2001).

$GABA_A$- and glycine receptors in the SDH

SDH neurons have been reported by many to respond to both GABA and glycine (Murase et al., 1989;Zieglgansberger and Sutor, 1983;Willcockson et al., 1984). More recently, it has been shown using electrophysiological analysis that miniature inhibitory postsynaptic currents (mIPSCs) could be mediated by either $GABA_A$Rs or GlyRs in neurons of the spinothalamic tract (Hori and Endo, 1992). Similarly, Narikawa et al. (2000) observed IPSCs evoked in *in vivo* rat SDH neurons by cutaneous mechanical stimulation that could be abolished by either bicuculline or strychnine. This same group also demonstrated *in vitro* that many IPSCs that result from stimulation of Aδ primary afferent fibers possess both a $GABA_A$R- and a GlyR-mediated component (Yoshimura and Nishi, 1995a).

Evidence of mixed synapses in the SDH first emerged through immunohistochemical studies, where $GABA_A$R α1-IR was shown to be present at postsynaptic densities

together with GlyR-IR in the dorsal horn (Bohlhalter et al., 1994). Todd et al. (1996) later demonstrated that the $GABA_AR$ subunit β3 colocalized with the GlyR subunit α1 at synapses in the dorsal horn. These studies, taken together with the affinity of the VGAT for both GABA and glycine, as well as the plethora of studies indicating that GABA and glycine are colocalized, suggested that $GABA_ARs$ and GlyRs may comediate postsynaptic inhibition at synapses in the dorsal horn.

Evidence of such comediation in the adult rat SDH was first presented by Chery and De Koninck (1999). Although they did not show codetection under control conditions, this group was able to show that the sensitization of $GABA_ARs$ using a benzodiazepine led to mIPSCs that possessed both a $GABA_AR$- and a GlyR-mediated component. Due to the very slow rise time of the isolated $GABA_AR$-mediated mIPSC, Chery and De Koninck (1999) theorized that the $GABA_AR$ component arose from a pool of extrajunctional $GABA_ARs$ that are not activated upon quantal release, whereas the faster GlyR-component arose from junctional GlyRs. The segregation of $GABA_AR$ and GlyR synapses in the adult murine SDH has also been reported by Graham et al. (2003), who showed that approximately 30% of neurons possess both $GABA_AR$- and GlyR-containing synapses.

2.6 Efficacy of postsynaptic excitability control of neurons by $GABA_A$ and glycine receptors.

The efficacy of a neurotransmitter, such as GABA or glycine, to modulate the excitability of a postsynaptic neuron is governed by two simple determinants: the amount of transmitter released from the presynaptic terminal, and the propensity of the postsynaptic receptors to cause a *trans*membrane flux of charge-carrying ions (Johnston and Wu, 1999).

Modulators of presynaptic GABA and glycine release in the SDH

For any given presynaptic terminal, the amount of transmitter release is defined by the probability of vesicular release (p_r), the number of presynaptic release sites or vesicles

(n), and the size of quanta (synaptic vesicle size or filling; q). For action-potential evoked release, where p_r is increased, the total probability (P_{tot}) that some number of vesicles will be released (k; known as quantal content) can be described by a binomial distribution:

$$P_{tot}(k) = \frac{n!}{k!(n-k)!} \cdot p_r^k (1-p)^{n-k}.$$

In the absence of an action-potential invasion of the presynaptic terminal, p_r may still be modified, usually via K^+-mediated depolarization or Ca^{2+}-dependent intracellular mechanisms. However, it should be noted that, as $p_r \rightarrow 0$, a Poisson distribution may better describe transmitter release:

$$P_{tot}(k) = \frac{m^k}{k!} \cdot \exp(-m), \text{ where } m = p_r \cdot n.$$

It appears that many agents that modulate the p_r of GABA and glycine vesicles act by regulating voltage-sensitive calcium entry via protein kinase C (PKC) and/or cyclic AMP-dependent kinase (PKA) (Shuntoh et al., 1989). Direct activation of PKC using phorbol esters, for example, has been shown to elicit an increased P_{tot} of GABA and glycine from local interneurons (Palecek et al., 1999;Gerber et al., 1989).

Activation of these kinases can be accomplished via neurotransmitter activation of presynaptic receptors. Among the agents that can modify GABA and glycine release in this way is GABA itself. In addition to binding $GABA_A$Rs in the SDH, GABA has been shown to activate the metabotropic receptor $GABA_B$, which is found at high levels on the presynaptic terminals of GABAergic/glycinergic interneurons (Price et al., 1984). Activation of the $GABA_B$ receptor by GABA or the high-affinity agonist baclofen, has been shown to reduce both the basal (including miniature) and stimulus-evoked release of GABA and glycine in the SDH (Chery and de Koninck, 2000;Yang et al., 2001;Teoh et al., 1996;Iyadomi et al., 2000). The mechanism underlying this reduction of p_r seems to involve both PKC and PKA-mediated alterations of voltage sensitive calcium entry in the

presynaptic terminal (Xi et al., 1997;Kamatchi and Ticku, 1990); but see Kubota et al. (2003).

On the other hand, interactions with the purinergic transmitter system have been demonstrated to increase GABA and glycine release in the SDH. ATP stimulation of P2X receptors located on both glycinergic and GABAergic terminals in the SDH has been reported to cause an increase in the probability of GABA and glycine release – manifested as a significant decrease in the interevent interval of miniature IPSCs (Hugel and Schlichter, 2000;Jang et al., 2001;Rhee et al., 2000). Although poorly understood, there is evidence that the effect of P2X receptors on GABA/glycine release is mediated by PKC and/or PKA (Wang et al., 2002a).

Other transmitter systems implicated in controlling the release of GABA and glycine include the serotonergic and opioidergic systems. Serotonin activation of presynaptic 5-HT_3 receptors in the SDH, for example, has been documented to cause a potentiation of GABA p_r (Kawamata et al., 2003), however the mechanisms that underlie this effect remain unclear.

The identified colocalization of GABA and [Met]enkephalin in SDH terminals (Todd et al., 1992) and obvious role for opioids in nociceptive processing prompted analyses of the function of presynaptic μ-opioid receptors located on GABAergic/glycinergic terminals. In various studies, stimulation of these μ-opioid receptors with synthetic opioids (*ie.* DAMGO) was shown to significantly decrease the p_r for GABA and glycine, both depressing miniature IPSC frequency and increasing the percentage of failures of evoked-GABAAR- and GlyR-mediated IPSCs – both measures indicating a presynaptic site of action for DAMGO (Kerchner and Zhuo, 2002;Teoh et al., 1995;Grudt and Henderson, 1998).

Modulators of postsynaptic $GABA_A$ and glycine receptor function

Activation of both $GABA_A$ and glycine receptors triggers an anion conductance through their associated ionophore, which forms a postsynaptic current (PSC). Namely, both the

$GABA_A$ and the glycine ionophores are selectively permeable to chloride and bicarbonate anions, with permeability ratios (P_{HCO3^-} / P_{Cl^-}) of 0.2 for the $GABA_A$ ionophore, and 0.1 for the glycine ionophore (Bormann et al., 1987). The net *trans*membrane anion flux that occurs during a PSC can be approximated by the synaptic charge (Q_s) transfer:

$$Q_s = \int_0^\infty PSC\,dt\,.$$

Or, more specifically:

$Q_s = \int_0^\infty I(t)\,dt$, where I is the amplitude of the PSC, t is time.

Upon integration, we may express synaptic charge as:

$Q_s = I\tau\left|\left(e^{\frac{-t_0}{\tau}} - 1\right)\right|$, where t_0 is the initial time, $\tau = (\tau_{decay} - \tau_{rise})$, and $\left|\left(e^{\frac{-t_0}{\tau}} - 1\right)\right|$ approaches 1.

Therefore, Q_s may be expressed simply as $I(\tau_{decay}\text{-}\tau_{rise})$ (Aradi et al., 2002).

Synaptic charge transfer is a critical parameter for neurons, as it has been well correlated with the propensity of a neuron to reach threshold from combined synaptic inputs (Aradi et al., 2002;Staley and Smith, 2001).

Many agents can modulate synaptic charge, either by prolonging time constant τ, or by increasing the peak amplitude of the PSC I. PSC amplitude I is established as a function of several synaptic properties including the probability of receptor channel opening, postsynaptic conductance (PSG) – which is governed by single channel conductance and the number of channels activated – and the mean driving force (DF_{ion}) applied to the permeant ions. DF_{ion} is calculated as the difference between an ion's *trans*membrane reversal potential (E_{ion}) and the membrane potential (E_{mp}) of the neuron ($DF_{ion} = E_{ion} - E_{mp}$).

Modulators of channel opening probability

The probability of $GABA_A$ or glycine ionophore opening can be modulated by altering either the agonist-dependent probability (frequency), or duration of channel opening (Twyman et al., 1989). Common agents that have been well-shown to allosterically increase the duration of $GABA_A$ channel opening are barbiturates (Twyman et al., 1989). This prolonging effect on channel opening appears to be mediated via barbiturate binding within the $GABA_A$ ionophore in a use-dependent manner (Macdonald and Olsen, 1994). Although barbiturates are typically thought of as allosteric modulators of $GABA_A$Rs, there is some evidence the barbiturates such as pentobarbital (Lu and Xu, 2002) and thiopental (Sudo et al., 2001) may have a prolonging effect on glycine channel open time in the dorsal horn. Some neurosteroids also appear to increase $GABA_A$ ionophore open time (Baulieu, 1998).

On the other hand, $GABA_A$ ionophore opening frequency can be potently regulated by the benzodiazepine class of drugs. Selectively binding a site distinct from that of agonist binding (Sieghart, 1995), the propensity of benzodiazepines to increase the opening frequency of $GABA_A$Rs may be associated with the observation that benzodiazepine binding can increase the affinity of the $GABA_A$ receptor for GABA, either by increasing the association rate, or decreasing the disassociation rate for GABA at the first GABA binding site (Macdonald and Olsen, 1994).

In the dorsal horn, several benzodiazepines, including midazolam (Kohno et al., 2000) and clonazepam (Rigo et al., 2002), and flunitrazepam (Chery and de Koninck, 1999;Park et al., 1999) have been documented to exert this allosteric modulation of postsynaptic $GABA_A$Rs. Interestingly, increasing p_o in this way does not generally lead to an increase in the amplitude of miniature PSCs (Hamon et al., 2003;Schlichter et al., 2000), suggesting that, in the SDH, release of a single vesicle of GABA and glycine saturates the apposing postsynaptic receptor density (Williams et al., 1998;Poncer et al., 1996;Nusser et al., 1997). Rather, benzodiazepine-mediated increases in $GABA_A$R opening probability tend to be manifested as a prolongation of the decay or deactivation phase of the mediated events (Johnston, 1996).

Although very few drugs have been documented to increase the frequency of glycine ionophore opening, PKA has shown some propensity to increase the opening frequency of GlyRs in the ventral horn (Song and Huang, 1990).

Modulators of postsynaptic conductance

Studies examining the modulation of $GABA_AR$- and GlyR-mediated postsynaptic conductance (PSG) have often focused on the role of PKC, likely because both the $GABA_AR$ and the GlyR possess well-known consensus sites for PKC phosphorylation (Ruiz-Gomez et al., 1991;Krishek et al., 1994). However, in the CNS, PKC phosphorylation of $GABA_ARs$ and GlyRs yields many conflicting results that may or may not be explained by receptor subunit composition (Macdonald and Olsen, 1994) or region (Poisbeau et al., 1999). In any event, little research on the effect of direct PKC activation on the PSG triggered by $GABA_ARs$ or GlyRs has been completed in the SDH.

Similar to the presynaptic terminal, transmitter-dependent activation of PKC has been demonstrated to modulate $GABA_AR$-mediated PSG. For example, the activation of A1 receptors with adenosine has been recently documented to trigger a PKC-dependent decrease in GABA-evoked inhibitory PSG (Wu et al., 2003;Jo and Schlichter, 1999). This finding may give reason to the observed corelease of GABA and the adenosine precursor, ATP, from some presynaptic terminals in the SDH (Hugel and Schlichter, 2003).

On the other hand, $5HT_2$ receptor activation has also been reported to lead to postsynaptic stimulation of PKC in the SDH; however in this case the outcome is the potentiation of both $GABA_AR$ and GlyR PSG (Li et al., 2000;Li et al., 2002). PKC (and downstream PKA) activation is also often linked to μ-opioid receptors (MORs) (Rubovitch et al., 2003;Kramer and Simon, 1999); however in the dorsal horn MOR activation has been observed to cause both an increase (Wang and Randic, 1994) and a decrease (Li et al., 2003) in $GABA_AR$-mediated PSG. Interestingly, this duplicity of actions may be explained by the observation that μ-opioid receptor-mediated actions are often lamina-specific in the dorsal horn (Magnuson and Dickenson, 1991).

Other kinases involved in modulating the postsynaptic conductance of these amino-acid receptors include calmodulin-dependent kinase II (CamKII) and PKA. For instance, intracellular perfusion of SDH neurons with the α-subunit of CamKII has been demonstrated in independent studies to increase both $GABA_AR$- (Wang et al., 1995) and GlyR- (Wang and Randic, 1996) PSG. Activation of PKA via PGE_2 stimulation of postsynaptic EP_2 receptors has also been shown to potentiate GlyR-evoked PSG in a population of SDH neurons (Ahmadi et al., 2002).

Many studies provide evidence that changes in PSG result directly from an increase or decrease in the number of $GABA_ARs$ or GlyRs in postsynaptic densities. For example, insulin and/or insulin growth factor-I stimulation of protein tyrosine kinase (PTK) has been shown to recruit $GABA_ARs$ to synapses in heterologous systems, resulting in increased PSG in as little as five minutes (Wan et al., 1997b;Luccardini et al., 2001). In this, and other studies (Castel et al., 2000) tyrosine phosphorylation as been reported to occur on the $GABA_AR$ β2 subunit [although tyrosine kinase consensus sequences have been identified on both the γ2 and the β1 subunits (Valenzuela et al., 1995)]. Stimulation of PTK using *src* peptides has also been demonstrated to modulate $GABA_ARs$ in a similar way (Boxall, 2000;Wan et al., 1997a), while an inhibition of $GABA_AR$-mediated PSG has been observed in the presence of PTK inhibitors, such as genistein (Valenzuela et al., 1995;Wan et al., 1997a).

Although poorly understood, GlyR clustering may also be possible via the actions of PTK, as a tyrosine phosphorylation consensus site has been identified on the GlyR β-subunit, which is known to be important for the association of GlyRs to the anchoring protein gephyrin (Rajendra et al., 1997).

Receptor tyrosine kinase (Trk) activation has further been demonstrated to influence the subcellular localization of $GABA_ARs$. TrkB activation by BDNF appears to both increase and decrease $GABA_AR$ surface expression, depending on the maturity and region of the effect (Mizoguchi et al., 2003a). For example, many studies have shown that BDNF-

mediated TrkB activation downregulates $GABA_AR$ subunits α1 (Penschuck et al., 1999), α2, β2/3 and γ2 (Brunig et al., 2001) and reduces mIPSC peak conductance (Tanaka et al., 1997) in mature hippocampal neurons. On the other hand, in primary cultures from immature cortex (Mizoguchi et al., 2003b) and slices of hippocampus (Yamada et al., 2002), BDNF application was reported to cause a rapid, and significantly increased expression of $GABA_ARs$ (β2/3 subunit) at the plasma membrane.

Modulators of anion driving force

Anion driving force (DF_{anion}) is computed simply as the difference between the *trans*membrane anion reversal potential (E_{anion}) and the membrane potential (E_{MP}) of a given neuron. Although modifications of either the E_{anion} or E_{MP} of a neuron may result in an altered DF_{anion}, for the sake of brevity and relevance, I will only focus on those agents or conditions that bring about changes in DF_{anion} via modulation of E_{anion} in this thesis. Hence, agents or conditions that alter *trans*membrane permeability or potassium gradient will not be discussed.

E_{anion} is a composite parameter, proportionally representing the chloride reversal potential (E_{Cl^-}) and that of bicarbonate (E_{HCO3^-}) – the two principal ionic species that travel through $GABA_A$ and glycine ionophores *in vivo* (Bormann et al., 1987). E_{anion} may be calculated as follows.

$$E_{anion} = \frac{RT}{nF} \ln\left(\frac{[Cl^-]_e + 0.2[HCO_3^-]_e}{[Cl^-]_i + 0.2[HCO_3^-]_i}\right)$$

Where R is the universal gas constant, T is the temperature (Kelvin), n is the valence of the anion and F is Faraday's constant. The coefficient 0.2 denotes that the permeability of the ionophore for bicarbonate is $^1/_5$ that for chloride (Bormann et al., 1987).

Evidently, changes to any of the non-constant parameters may evoke a hyper- or depolarization of E_{anion}; in eukaryotic cells alterations in E_{ion} have been attributed to

ambient temperature changes (Sesti et al., 1996), modifications in ionic permeability (Krylov et al., 2000;Usachev et al., 1995) and, of course, alterations of extracellular or intracellular ionic concentration (Kulik et al., 2000). In the following brief review, I will expand upon the various agents and conditions that have been documented to evoke changes in E_{anion} specifically in the central nervous system.

Perturbations in E_{HCO3}- have generally been shown to result from two conditions: an alteration of extraneuronal pH, or a modulation of the function of an enzyme known as carbonic anhydrase (Staley et al., 1995;Chesler and Kaila, 1992). Although a Na^+-dependent Cl^-/HCO_3- exchanger exists in the plasma membrane of some neurons (Virkki et al., 2003), it appears as if the majority of the replenishment of intraneuronal HCO_3- stems from a *trans*membrane passive diffusion of CO_2 (Deitmer, 2002), a process that is driven by the decrease in intraneuronal HCO_3- associated with synaptic events. Following the release efflux of HCO_3-, CO_2 is produced extracellularly through a dehydration reaction as follows (Staley et al., 1995).

$$HCO_3^- \xrightarrow{+H^+} H_2CO_3 \xrightarrow{-H_2O} CO_2$$

As shown, HCO_3- must undergo protonation before the dehydration of carbonic acid may occur. Extracellular pH, therefore, is the rate-limiting step in the production of CO_2; increases in extracellular pH will reduce the rate of reaction, and therefore reduce the amount of CO_2 available to be uptaken via diffusion (Pasternack et al., 1996).

Several groups have demonstrated empirically that there exists an inverse (although non-linear) relationship between extracellular pH and E_{HCO3}- in neurons of the central nervous system (Kaila, 1994;Kaila et al., 1990;Pasternack et al., 1996;Bonnet and Bingmann, 1995), as an increase in pH (decrease in $[H^+]$) decreases the rate of CO_2 produced for reuptake.

Following uptake, CO_2 rapidly undergoes a hydrolytic transformation back to HCO_3- in the cytosol, a process that is the reciprocal of that shown above.

$$CO_2 \xrightarrow{+H_2O} H_2CO_3 \xrightarrow{-H^+} HCO_3^-$$

The deprotonation of carbonic acid is catalyzed by the enzyme carbonic anhydrase (Kaila et al., 1990). Studies have shown that if carbonic anhydrase is blocked, usually using the diuretic acetazolamide, intraneuronal HCO_3- concentration will decrease and therefore E_{HCO3}- will be driven down (Bonnet and Bingmann, 1995;Staley et al., 1995;Amos et al., 1996).

The regulation of chloride homeostasis and E_{Cl}- in a neuron is more complex. Chloride homeostasis is maintained by a system of passive and active integral (co)transporters. Under normal conditions, *trans*membrane chloride import in central neurons is mediated largely by a Na^+-dependent potassium-chloride cotransporter known as NKCC1 (Payne et al., 2003;Sung et al., 2000). Loop diuretics, such as furosemide and bumetanide, have been shown to block NKCC1, thus hyperpolarizing E_{Cl}- in the affected neuron (Babila et al., 1989;Rohrbough and Spitzer, 1996;Xie et al., 2003).

Although much focus has been attributed to the mechanisms involving phosphorylation that regulate NKCC1 activity and expression in the kidney, little is known about the role of phosphorylation for the NKCC1 isoform located in the central nervous system. Using a phospho-specific antibody, however, three threonine residues have been recently identified in the N-terminus of the protein that appear to influence the function of NKCC1 in the brain (Flemmer et al., 2002;Darman and Forbush, 2002). Certain stress-related (serine-threonine) kinases, such as Ste20-related-proline-alanine-rich kinase (SPAK) and oxidative stress response 1 (OSR1) have been shown to act at these loci to increase NKCC1 function and therefore depolarize E_{Cl}-; contrariwise, inhibitors of phophorylation, such as calyculin A, inhibit NKCC1 function in the brain, decreasing $[Cl^-]_i$ and rendering E_{Cl}- more negative (Piechotta et al., 2003;Piechotta et al., 2002).

Although varying from neuron to neuron (Gulacsi et al., 2003), non-synaptic chloride efflux from neurons is accomplished largely by a neuron-specific potassium-chloride

cotransporter (KCC2) and a chloride-bicarbonate exchanger (CHE) (Deitmer, 2002;Xie et al., 2003). The CHE is activated by ATP, and therefore is presumptively an active transport mechanism (Soleimani and Burnham, 2001). However, a study has shown that, although binding of ATP is necessary for the activation of the exchanger, ATP hydrolysis is not (Boron et al., 1988), suggesting that ATP may act more in the role of a "ligand".

The CHE has been reported to exchange extracellular Na^+ and HCO_3^- for intracellular Cl^- (Soleimani and Burnham, 2001). Hence, agents that are well known to block this exchanger, such as disulphonic acid derivatives like SITS and DIDS, as well as ethacrynic acid, effectively trigger an intracellular accumulation of chloride (Heisler and Jeandel, 1989;Brett et al., 2002;Bonnet and Bingmann, 1995). Certain protein kinases have also been suggested to play a role in regulating the function of the CHE (Wang et al., 2002b), however the precise mechanisms are unclear.

As with the NKCC1, the KCC2 cotransporter, which transports chloride and potassium from intracellular to extracellular compartments (Payne, 1997), is partially inhibited by certain loop diuretics, including furosemide (Kelsch et al., 2001) and DIOA (Shen et al., 2001). Blockade of KCC2 ultimately causes an intraneuronal accumulation of chloride and hence a depolarization of E_{Cl^-}.

Also similar to NKCC1, regulation of KCC2 function appears to be governed by the phosphorylation of certain consensus sequences, such as a tyrosine kinase consensus sequence found on the carboxy terminus of the protein (Strange et al., 2000). Functionally, tyrosine kinase phosphorylation has been shown in neurons to increase the function of KCC2, especially during development (Kelsch et al., 2001) [but see Stein et al. (2004)] thus catalyzing the hyperpolarizing shift in E_{Cl^-} common to many neurons of the developing CNS (see Development section).

The activation of certain receptor tyrosine kinases has further been shown to influence the activation state and/or expression level of the KCC2 cotransporter in neurons. For example, in the developing brain, TrkB activation by BDNF has been reported to trigger a

hyperpolarization of E_{Cl}- via an increased KCC2 expression (Aguado et al., 2003). Interestingly, this same protocol, when carried out in mature rat brain, has yielded resuls showing that TrkB activation leads to a depolarization of E_{Cl}- (Wardle and Poo, 2003), likely resulting from a rapid downregulation of KCC2 membrane expression (Rivera et al., 2002). Although a resolution between these two disparate results is not forthcoming, it is interesting to note the in mature rats, an injury to a neuron (*ie.* axotomy) has also been documented to evoke a decrease in KCC2 expression and the associated depolarization of E_{Cl}- (Toyoda et al., 2003;Nabekura et al., 2002). This change has not been observed in DRG neurons following a PNI; nor would we expect a depolarization of E_{Cl}- as KCC2 mRNA is not found in neurons of the DRG (Kanaka et al., 2001). However, whether PNI – which is known to increase the ambient levels of BDNF in the dorsal horn (Miletic and Miletic, 2002) – may affect KCC2 expression levels and E_{Cl}- *trans*synaptically in second order spinal neurons is not known.

3. DEVELOPMENT OF INHIBITORY SYNAPSES

3.1 Pre- and perinatal expression of $GABA_A$ and glycine receptors.

Although synaptogenesis, and notably the formation of inhibitory synapses, occurs predominantly postnatally in the CNS, isoforms of both the $GABA_AR$ and the GlyR can be detected in the embryonic CNS (Moss and Smart, 2001).

For example, as early as E15, GlyR expression has been detected in the spinal ventral horn using immunocytochemical methods (Colin et al., 1998). In the embryonic state, the α2 subunit has been reported to compose the predominant isoforms of the GlyR in the spinal cord and brainstem (Triller et al., 1990), as well as in the cortex and hippocampus (Kuhse et al., 1991), and have been shown to be homomeric. The ion channels formed by homomeric α subunits have been demonstrated to be functional (Betz et al., 1999;Moss and Smart, 2001) and may participate in synaptic transmission, considering the absence of β subunit expression at early stages of neuronal development (Becker et al., 1988). It does appear, however, that homomeric GlyRs are largely confined to extrasynaptic domains (Kneussel and Betz, 2000;Takahashi et al., 1992).

The heterogeneity of the GlyR was first revealed biochemically by the finding that the α2 neonatal subunit (expressed in the absence of the larger 58 kDa β subunit) was of greater molecular weight (49 kDa) than the adult α subunits (48 kDa; (Becker et al., 1988)). Moreover, the α2 homomeric GlyRs were found to possess a decreased affinity for strychnine binding and different immunological properties compared to the adult polypeptide (Becker et al., 1988;Legendre, 2001). Two cDNA splice variants of the rat α2 subunit were later described: α2A and α2B (Kuhse et al., 1991). The two variants have similar distributions in the CNS in the perinatal state, and both experience decreased expression with development (Legendre, 2001). No differences in the functional properties of GlyRs corresponding to the isoforms have been detected as of yet.

In the spinal cord, switching between the neonatal α2 homomer GlyR and the adult GlyR isoform, which contains α1 and β subunits in the more familiar stoichiometry of 3α:2β (Moss and Smart, 2001;Legendre, 2001), occurs at approximately three weeks after birth (Becker et al., 1988;Akagi and Miledi, 1988). In addition to conferring strychnine-sensitivity upon the GlyRs, this switch has also been demonstrated to significantly shorten mean channel life time causing the kinetics of GlyR-mediated inhibitory postsynaptic currents to be accelerated (Takahashi et al., 1992).

Although the early development of the glycinergic system has been poorly studied in the SDH, it has been demonstrated that in lamina II and III binding sites for [^{3}H]strychnine are weakly expressed as early as birth (Bruning et al., 1990). An increase of this specific binding density with age was described, peaking at P21 and reaching a steady state (adult levels) shortly thereafter.

GABA and isoforms of the $GABA_AR$ have been detected as early as E13-14 in the rodent CNS (Poulter et al., 1992;Moss and Smart, 2001;Schaffner et al., 1993;Fritschy et al., 1994). It is apparent that, although bicuculline-sensitive, functional $GABA_ARs$ are detected at E14 in many regions of the CNS including the dorsal horn (Mandler et al., 1990), there occurs a widespread $GABA_AR$ isoform switch at perinatal stages, which has

been suggested to greatly alter the efficacy of GABA's action (Levitan et al., 1988;Puia et al., 1991). Specifically, between E17 and P5, there occurs a dramatic decrease in the expression of the α2 subunit, which is paralleled by an increase in the expression of the α1 subunit throughout the CNS; β2/3 immunostaining does not vary during ontogeny, and colocalization of β2/3 with α2 was demonstrated to transition to colocalization with α1 subunits (Fritschy et al., 1994). In the spinal dorsal horn, the increase in α1 subunit expression occurs after P5 with wide expression continuing into adulthood; yet, reduced α2 subunit expression is also observed in adult rats, suggesting that the two subunits may constitute isoforms of $GABA_AR$s with different functions/properties (Poulter et al., 1992).

Generally, the shift in α2- to α1-containing $GABA_AR$ isoforms can be tracked functionally in the CNS by assessing the sensitivity of the $GABA_AR$ to different benzodiazepines. A shift in the preponderance of "benzodiazepine type I" versus "type II" binding sites has been observed thoughout the brain with ontogeny (Lippa et al., 1981;Chisholm et al., 1983). Because type I benzodiazepines include those drugs (such as lorazepam, zolpidem and zalaplon) that modulate $GABA_AR$ isoforms containing the α1 subunit, whereas type II benzodiazepines (including flumazenil) modulate α2-containing $GABA_AR$s (Sanna et al., 2003;Pawelzik et al., 2003), such a shift from benzodiazepine type II to type I binding sites has been postulated to reflect the shift from α2 to α1 expression in the CNS.

Binding studies using radioactive ligands have further mapped the patterns of $GABA_AR$ expression in the dorsal horn. Interestingly, in the rat dorsal horn the number of binding sites for [^{3}H]muscimol (B_{max}) was reported to decrease 2-fold between P1 and P60 (Saito et al., 1983). Further analysis using immunohistochemical techniques yielded a similar result, yet found that $GABA_AR$ expression increased from birth until P14, and thereafter fell to the lower level expression that is maintained into adulthood. These studies are important because they suggest that there occurs a major reorganization of the GABAergic transmitter system in the dorsal horn with development. Other studies (Mandler et al., 1990;Schaffner et al., 1993) have further provided evidence that such postnatal alterations in receptor expression in the dorsal horn may be associated with

synaptogenesis; indeed, Schaffner et al. (1993) reported that after P10-12 the reduction in $GABA_AR$ expression correlates with the development of punctate $GABA_AR$ labeling on the soma and proximal dendrites of neurons, as well as with the colocalization of $GABA_ARs$ with synaptophysin.

3.2 Synaptogenesis of inhibitory synapses.

The configuration of adult GABAergic and glycinergic synapses in the spinal cord has been proposed to occur between two and three weeks after birth in the rodent (Wheal et al., 1998;Thomas, 1986;Grantyn et al., 1995). Gephyrin, a 93 kDa protein (Prior et al., 1992), has been demonstrated to act as a critical determinant of the formation of both $GABA_AR$ and GlyR synapses (Moss and Smart, 2001). Gephyrin immunoreactivity can be detected at embryonic stages in the CNS – even before significant GlyR-LI – and has been reported to begin to colocalize with GlyRs around the second postnatal week (Colin et al., 1998). Ubiquitous expression of gephyrin continues into adulthood in the CNS; interestingly, at many loci of gephyrin expression there is no evidence of GlyR expression, suggesting that gephyrin may serve additional purposes in the CNS (Betz et al., 1999;Kirsch and Betz, 1993).

Evidence of a direct association between GlyRs and gephyrin first stemmed from the observation that the two copurify in spinal cord extracts (Schmitt et al., 1987). Although multiple isoforms of the gephyrin protein have been proposed to exist, it appears that it is only the P1 isoform that links to GlyRs – specifically to the β subunit [no isoforms link to the GlyR α subunit, or to the $GABA_AR$ β subunit (Meyer et al., 1995;Kins et al., 1999)]. In addition to its affinity for the β subunit of GlyRs, gephyrin displays high affinity for many cytoskeletal protein including microtubules and actin according to Kirsch et al. (1995), underscoring its importance as an anchoring protein for GlyRs.

Gephyrin has further been demonstrated to elicit the clustering of GlyRs on postsynaptic membranes. For example, antisense knockdown of gephyrin in cultured spinal neurons was reported to result in a loss of GlyR clusters (Kirsch et al., 1993;Legendre, 2001)

[such a finding may be system-dependent, however, as in other systems homomeric GlyRs have been documented to cluster independently of gephyrin (Meier et al., 2000)]. Interestingly, recent evidence suggests that the clustering of gephyrin and GlyRs may be mutually-dependent. In spinal cord neurons, Kirsch and Betz (1998) demonstrated that the calcium entry via voltage-sensitive calcium mechanisms triggered by GlyR-mediated depolarization (discussed below) was necessary for the aggregation of gephyrin at the postsynaptic membrane. This aggregation of gephyrin then contributed to the formation of GlyR synapses. This form of activity-dependent synaptogenesis is further supported by another study that used a coculture of spinal motoneurons and glycine interneurons (Levi et al., 1999). The authors found that accumulation of GlyRs and gephyrin clusters was only observed on motoneurons apposed to the cocultured interneurons.

Although gephyrin imunoreactivity had long been used as a marker of GlyR location, it has recently come to light that gephyrin may colocalize with $GABA_A$Rs in many areas of the CNS, including the spinal cord (Triller et al., 1987;Cabot et al., 1995)[Any suggestion that gephyrin is common to all synapses may be ruled out by the fact that gephyrin immunoreactivity is not found at glutamatergic synapses (Craig et al., 1996)]. Here gephyrin also appears to play a role in the recruitment of $GABA_A$Rs to synapses: a recent study reported that gephyrin knockout mice displayed significantly less $GABA_A$R clustering (Kneussel et al., 1999). The link between gephyrin and $GABA_A$Rs has been demonstrated to be the $\gamma 2$ subunit, as $\gamma 2$ knockout mice also fail to accumulate gephyrin or $GABA_A$Rs (Essrich et al., 1998). However, it does not appear that there exists a direct association between the two proteins as $GABA_A$Rs and gephyrin fail to copurify (Moss and Smart, 2001). This observation, among other things, led to the search for, and ultimate discovery of another protein that seems to bridge gephyrin and $GABA_A$Rs.

Known as the $GABA_A$R-associated protein (or GABARAP), this 17 kDa protein was discovered to both copurify with $GABA_A$Rs and to interact with microtubules (Wang et al., 1999a;Wang and Olsen, 2000). Linking with the $GABA_A$R $\gamma 2$ subunit (Wang et al., 1999a), GABARAP was further documented to promote $GABA_A$R clustering (Chen et al., 2000;Phillips and Froehner, 2002). GABARAP was also shown to interact with

gephyrin in spinal neurons (Kneussel et al., 2000), yet did not colocalize to a significant extent with gephyrin at synapses, suggesting that GABARAP may be important for the synaptic targeting, but not the anchoring of $GABA_A$Rs. Further support for this hypothesis stems from the observation that GABARAP interacts extensively with NSF, a protein critical for intracellular trafficking in neurons (Kittler et al., 2001).

The identification of the GABARAP has led to the suggestion that the embryonic gephyrin expression that precedes GlyR expression in the spinal cord may be associated with the early placement of $GABA_A$Rs (Colin et al., 1998). Such an observation could account for the results of a seminal study in the dorsal horn that showed that the B_{max} of [^{3}H]flunitrazepam (binding $GABA_A$Rs) at birth is four times greater than the B_{max} of [^{3}H]strychnine (binding GlyRs), whereas, by P10 GlyRs predominated, with the B_{max} of [^{3}H]strychnine being 2-fold greater than that of [^{3}H]flunitrazepam (Bruning et al., 1990). A transient predominance of $GABA_A$Rs at early developmental stages has been likewise reported in other systems in the brain (Laurie et al., 1992;Poulter et al., 1992;Kotak et al., 1998).

The mechanisms that underlie developmental shifts in $GABA_A$R- and GlyR-mediated postsynaptic excitability control are controversial. A recent study in the auditory system, for example, reported that the oft-studied developmental transition from $GABA_A$R- to GlyR-mediated postsynaptic inhibition at lateral superior olive (LSO) synapses could be explained by a concurrent decrease in intraterminal GABA-like immunoreactivity and increase in intraterminal glycine-LI (Nabekura et al., 2004). However, this study is at odds with other studies that documented a close parallel between the functional shift in the contribution of GABA and glycine at LSO synapses, and an increase in gephyrin-LI and decrease in $GABA_A$R β2/3-LI (Kotak et al., 1998;Korada and Schwartz, 1999) – the latter study further providing evidence that intraterminal staining of GABA and glycine does not change with ontogeny.

Yet other studies have documented synaptic reorganization as the mediator of such synaptic shifts. For example, Koulen (1999) showed that, while functional $GABA_A$Rs

were present on retinal neurons long before GlyRs, they were distributed as random clusters on the postsynaptic membrane. As synaptic GlyR expression increased (at ~P14), there was no observed decrease in the expression of $GABA_ARs$; instead, the $GABA_AR$ staining pattern became much more diffuse, suggesting that $GABA_ARs$ had adopted an extrasynaptic localization. In the spinal ventral horn and brain stem, two studies have illustrated that in immature motoneurons (<P14), GABA and glycine coreleased from interneurons can activate both postsynaptic $GABA_ARs$ and GlyRs simultaneously, and at the same synapse (Jonas et al., 1998;O'Brien and Berger, 1999). Whether this coactivation dissipates with development is unclear; reciprocally, it is unknown whether postsynaptic $GABA_AR$/GlyR codetection can occur at early developmental stages in the dorsal horn. In the adult, although the corelease of GABA and glycine from interneurons in the SDH has been shown, $GABA_AR$-synapses appear to be segregated from GlyR-synapses in neurons (Chery and de Koninck, 1999).

Whether or not protein expression at postsynaptic junctions underlies the transition from $GABA_AR$- to GlyR-mediated postsynaptic control, it is highly likely that a subunit switch in one or both of the receptors underlies any reconfiguration. As previously mentioned, in the dorsal horn, the α1-containing $GABA_AR$ has been documented to largely, though not completely, replace the α2-containing isoform in rats aged P5 and older (Poulter et al., 1992). Although not evaluated in the dorsal horn, a switch from α2 to α1-containing GlyRs likewise occurs in the ventral horn at approximately the same age (Takahashi et al., 1992).

The implication of these switches for subcellular localization in the spinal cord is unclear. However, reports have been forthcoming in other regions suggesting distinct targeting of different receptor isoforms to the plasma membrane. For example, studies in many regions of the brain (Sassoe-Pognetto et al., 2000), and in particular in cerebellar granule cells (Nusser et al., 1998;Brickley et al., 1999) indicate that $GABA_ARs$ containing the γ subunit are preferentially clustered at synapses. In one study, -/- γ2 mice were shown to be unable to cluster $GABA_ARs$ at synapses in both the hippocampus and the cortex (Crestani et al., 1999). Contrariwise, reports have suggested that the α3 subunit in the

cortex (Hutcheon et al., 2000) and the δ subunit in the cerebellum (Nusser et al., 1998) may be important for the extrasynaptic localization of $GABA_AR$s.

3.3 Alterations in $GABA_A$ and glycine receptor function that parallel synaptogenesis.

Although receiving little examination in the spinal cord, a change in the functional properties of $GABA_AR$s and GlyRs accompanies the formation of GABAergic and glycinergic synapses in many regions of the CNS. For GlyRs, receptor clustering has been reported to affect sensitivity to both glycine and strychnine. In one study using recombinant GlyR α1 subunits expressed in *Xenopus* oocytes, a reduced EC_{50} for glycine and increased IC_{50} for strychnine correlated positively with the level of GlyR expression (Taleb and Betz, 1994).

The clustering of $GABA_AR$s has also been demonstrated to alter the EC_{50} for GABA (Chen et al., 2000). However, in this case the EC_{50} for GABA, in addition to the deactivation rate of $GABA_AR$s, increased with $GABA_AR$ clustering. Why this trend in EC_{50} differs from that for GlyRs is unclear. However, an acceleration of deactivation kinetics with synaptogenesis has been documented elsewhere for GlyRs (Ye, 2000), as well as for $GABA_AR$s (Dunning et al., 1999;Tia et al., 1996;Gao and Ziskind-Conhaim, 1995).

Perhaps the largest developmental change to $GABA_AR$ and GlyR function that has been documented throughout the CNS involves the transition from mediating depolarizing to hyperpolarizing anionic postsynaptic events (Bixby and Spitzer, 1982;Ben Ari et al., 1989;Swann et al., 1989;Staley and Smith, 2001). It now appears that the basis of these anionic depolarizing events is a high intracellular chloride level (less negative reversal potential for chloride), whereby GABA or glycine stimulation of their respective receptors causes an outward flux of chloride (Staley and Smith, 2001;Kaila, 1994). One of the consequences of this outward chloride flux is the triggering of calcium entry in neurons, via both NMDA receptors (Ganguly et al., 2001) and voltage-sensitive calcium channels (Owens et al., 1996). The resultant calcium transients have been documented to

mediate all manner of effects important for the growth and development of the neuron, including neurite arborization (Behar et al., 1996a;Spoerri, 1988), neuronal differentiation (Owens and Kriegstein, 2002) and the maturation of glutamatergic transmitter systems (Leinekugel et al., 1999), possibly through the activation of intracellular messengers such as cAMP-dependent kinase (Obrietan et al., 2002).

Interestingly, it also appears that $GABA_AR$- and GlyR-mediated depolarization may mediate the ontogenic formation of inhibitory synapses in two ways. Firstly, it has been reported that the transition to hyperpolarizing $GABA_AR$-mediated events, which occurs when the level of intracellular chloride drops to adult levels by the end of the first postnatal week in the brain (Ben Ari et al., 1989;Cherubini et al., 1991), is mediated by the increased expression of a potassium-chloride exporter called KCC2 (Rivera et al., 1999). Recently, the increased expression of this transporter has been demonstrated to be driven by depolarizing $GABA_AR$ activity in the hippocampus (Ganguly et al., 2001), which is linked to the expression of BDNF (Aguado et al., 2003;Obrietan et al., 2002). Secondly, the calcium transients that result from depolarizing anionic fluxes have been linked to the regulation of $GABA_AR$ subunit mRNA expression in the cerebellum (Poulter and Brown, 1999). Similarly, the calcium influx triggered by GlyR activation in perinatal ventral horn has been shown to effect gephyrin aggregation, and thus the recruitment of GlyRs to the synapse (Kirsch and Betz, 1998).

In cultures of spinal dorsal horn neurons, a depolarizing effect mediated by both glycine and GABA has also been observed in a proportion of cells (Reichling et al., 1994). However, these *in vitro* experiments failed to detect a developmental shift in GABA- and glycine-mediated polarization (Wang et al., 1994), leaving the question of whether such shifts occur *in situ* in the SDH unanswered.

4. PLASTICITY OF GABA- AND GLYCINE-MEDIATED SIGNALLING FOLLOWING PERIPHERAL INSULT

Ongoing nociceptive hypersensitivity in many species of animals (Lin et al., 1996;Wall and Melzack, 2000), including humans (Melzack et al., 2001), is proposed to stem in part from an increase in the excitability of neurons located in the spinal dorsal horn. This increase in excitability, characterized by such modifications as heightened spontaneous discharge, increased excitatory inputs and an alteration of input thresholds (Ferrington et al., 1987) for SDH neurons, has been termed central sensitization (Woolf, 1983;Ji et al., 2003).

The development and maintenance of central sensitization, and the associated nociceptive hypersensitivity, almost certainly involves an attenuation of GABA- and glycine-mediated inhibition. Strong evidence for this hypothesis stems from reports that local spinal delivery of either strychnine, a competitive GlyR antagonist, or bicuculline, a $GABA_AR$ antagonist, to rodents produce many of the physiological correlates of central sensitization; these include increases in Aδ and C-fiber evoked activity (Kontinen et al., 2001;Baba et al., 2003), background- and after-discharges (Sorkin et al., 1998), and in the paw withdrawal response to repeated low-intensity touch stimuli (Sivilotti and Woolf, 1994), as well as the expression of extracellular stress-related kinases [ERK; Baba et al. (2003)], an enzyme commonly observed to be upregulated in response to PNI (Ji et al., 1999;Pezet et al., 2002). Furthermore, strychnine administration to rats has been demonstrated to increase the response of thalamic nociceptive-specific neurons to tactile stimuli, including brush stimulation (Sherman et al., 1997).

In addition, many studies have illustrated the importance of GABA and glycine neurotransmission for regulation of pain sensitivity in rodents. For example, administration of either strychnine (Sorkin and Puig, 1996;Sherman and Loomis, 1994) or bicuculline (Reeve et al., 1998;Zhang et al., 2001) by intrathecal catheter can produce a dramatic tactile allodynia in rats. Moreover, it has been sugessted that the local blockade of the two receptor populations, concurrently, elicits a synergistic effect, with the resulting allodynia being significantly more pronounced than that mediated via blockade

of either GlyRs or $GABA_ARs$ alone (Loomis et al., 2001). Indeed, the nature of the nociceptive hypersensitivity resulting from such pharmacological manipulations resembles PNI-elicited hypersensitivity so closely that many researchers now consider strychnine- or bicuculline-induced allodynia as a model of neuropathic pain (Hall et al., 1999;Khandwala et al., 1998), exhibiting many of the cardinal signs of the disease such as a resistance to opiate receptor agonist pharmacotherapy (Sorkin and Puig, 1996).

Despite all of this, the mechanisms underlying the attenuation of GABA- and glycine-mediated inhibition that occur after inflammation or PNI remain unclear and controversial. Although much of the published data is simply contradictory (Polgar et al., 2003b;Sugimoto et al., 1990;Moore et al., 2002), one of the reasons for this lack of clarity may be that the alterations at SDH inhibitory synapses that underlie nociceptive hypersensitivity are dependent on the type of peripheral insult (Castro-Lopes et al., 1995).

In the following sections I will review the role and plasticity of GABAergic and glycinergic synapses in the SDH that occur after a variety of peripheral insults.

4.1 Inflammation.

Experimental models of peripheral inflammation commonly use formalin, carrageenan or complete Freund's adjuvant (CFA), typically injected into the plantar surface of the paw or an articulation, to precipitate an inflammatory response (Wall and Melzack, 2000). In animals, this inflammatory response usually produces nociceptive hypersensitivity that is short-lived in comparison with that resulting from PNI.

Perhaps the most commonly used model of peripheral inflammation, the formalin test generally produces a bi-phasic hypersensitivity response: an early phase is usually interpreted as resulting from C-fiber activation due to the peripheral stimulus, while a later, and more prolonged phase, appears to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord (Tjolsen et al., 1992;Coderre and Melzack, 1992). Although data is lacking regarding the role of GlyRs in the mediation of formalin-evoked hypersensitivity,

several studies have demonstrated that $GABA_A$ receptors are critical for (especially the second phase of) the formalin response. For example, bicuculline delivered via intrathecal catheter either before, or together with formalin causes a significant augmentation of nociceptive hypersensitivity in the second phase of the response (John et al., 1998;Kaneko and Hammond, 1997). Similarly, intrathecal bicuculline applied concurrently with intraplantar formalin was observed to increase single unit discharge properties in the SDH during phase II (Green and Dickenson, 1997). Hence, it seems likely that a lack of $GABA_AR$ activation contributes to this central component of formalin-induced inflammatory pain [but see Tatsuo et al. (1999)]. This hypothesis agrees well with one of the few available studies evaluating modifications to GABAergic signaling in the SDH after formalin: Hu et al. (2003) reported that intraplantar formalin triggers a long-lasting increase in GABA uptake via the GAT-1 transporter, and furthermore, that GAT1 selective inhibitors effect a pronounced antinociceptive effect during the second phase of formalin-induced hypersensitivity in rodents.

Somewhat contrary to the effect rendered in the formalin model, local spinal delivery of bicuculline has been shown to decrease the short-term nociceptive hypersensitivity resulting from articular (knee-joint) injection of carrageenan (Sluka et al., 1993;Sluka et al., 1994) – a second model of inflammatory pain that well represents many of the signs of arthritis (Morris, 2003). Additionally, spinal bicuculline has been shown to inhibit the development of hyperexcitability (wind-up) in the SDH neurons of arthritic rodents (Weng et al., 1998). Paradoxically, this antinociceptive effect of bicuculline may result from a suppression of GABA and glycine upregulation in the SDH: while it has been demonstrated that both GABA- and glycine-like immunoreactivities (LI) increase in the SDH subsequent to an articular injection of carrageenan (Castro-Lopes et al., 1994a;Sorkin et al., 1992), it appears that the action of bicuculline to attenuate nociceptive hypersensitivity is closely related temporally to its propensity to inhibit increases in GABA- and glycine-LI in the SDH (Sluka et al., 1994). The idea that, in this model of arthritis, an excess of GlyR or $GABA_AR$ activation contributes to the development of nociceptive hypersensitivity is further supported by the observation that

the benzodiazepine midazolam increases the expression of the hyperexcitability-related proto-oncogene, *c-fos* (Castro et al., 1999).

In a third, and more protracted model of inflammatory pain, the nociceptive hypersensitivity displayed by rodents 2-3 weeks after intraarticular injection of complete Freund's adjuvant (CFA) (Billiau and Matthys, 2001) also appears to arise from modifications of GABA and glycine neurotransmission in the SDH. However, the implications of $GABA_AR$ and GlyR activation in the development of nociceptive hypersensitivity are unclear. Reports suggest that CFA injection leads to a striking increase in GABA-LI expression and release in the SDH that well parallels the course of hypersensitivity (Castro-Lopes et al., 1992;Castro-Lopes et al., 1995). Moreover, this increased expression may result from a CFA-evoked increase in glutamic acid decarboxylase (GAD) mRNA in neurons of the SHD (Castro-Lopes et al., 1994b), and may be driven by augmented NMDA receptor activation in these neurons (Gu and Huang, 2002). However, notwithstanding these increases in GABA, local spinal delivery of a positive allosteric modulator of $GABA_ARs$, desipramine, was observed to attenuate CFA-evoked hypersensitivity, whereas blockade of $GABA_AR$ and GlyR ionophores using intrathecally-delivered picrotoxin facilitated the hypersensitivity (Asahi and Yonehara, 2001). Although difficult to reconcile for GABA, this finding may agree with a recent discovery that the frequency of GlyR-mediated miniature IPSCs recorded from SDH neurons decreases after CFA injection (Muller et al., 2003), suggesting that a decrease in the release of glycine from SDH presynaptic terminals may contribute to CFA-elicited hypersensitivity.

4.2 Nerve Injury.

There exist several experimental models of PNI, all of which are designed to induce a peripheral neuropathy and the accompanying neuropathic pain. A majority of the more common models employ an injury to certain spinal nerves in the rodent. Spinal nerve injury models may be broadly divided into three classes: (a) tight ligation of the L5 and L6 spinal nerves ["Chung Model", Kim and Chung (1992)], (b) loosely constrictive ligations ["Bennett Model", (1988)] or a loose polyethylene cuff ["Kruger Model",

(1996)] around the common sciatic nerve, and (c) full transection of the sciatic nerve (Sugimoto et al., 1985).

Tight constrictive ligation

In the Chung Model, spinal nerve ligation produces a robust and sustained central sensitization (Kontinen et al., 2001) that is accompanied by a long-lasting tactile and thermal hypersensitivity within a few days (Kim and Chung, 1992). Subsequent to the development of nociceptive hypersensitivity, pharmacological studies have shown that intrathecal delivery of $GABA_A$ receptor agonists, such as isoguvacine (Malan et al., 2002), and muscimol (Hwang and Yaksh, 1997;Rashid and Ueda, 2002) significantly attenuate both allodynia and hyperalgesia in rats. The effect of locally-delivered bicuculline is unclear (Hwang and Yaksh, 1997). Intrathecal glycine has also been observed to reduce both thermal and tactile hypersensitivity (Simpson, Jr. et al., 1996;Simpson, Jr. et al., 1997), however, interestingly, in these same studies strychnine administration was shown to augment tactile allodynia only, displaying no effect on the development or maintenance of thermal hypersensitivity.

Physiological explanations for these *in vivo* results may stem from the analysis of primary afferent-evoked responses in the SDH. Many studies suggest that the $GABA_AR$ contribution to primary afferent depolarization, especially for C-fibers, is modified following L5-6 spinal nerve ligation (Sokal and Chapman, 2003). For example, local spinal delivery of bicuculline via intrathecal catheter was shown to have no effect on the magnitude of C-fiber-evoked synaptic responses in naïve animals [although triggering an augmentation of A-fiber-evoked responses (Baba et al., 2003)], yet produced a substantial increase in C-fiber-evoked activity in nerve injured animals [strychnine did not elicit these responses in either condition (Kontinen et al., 2001)]. Similarly, the subcutaneous administration of the benzodiazepine midazolam reduced C-fiber-evoked synaptic activity only in nerve injured animals, never in naïve animals (Kontinen and Dickenson, 2000).

While falling short of explaining the increased ability of $GABA_AR$ modulators to affect C-fiber-evoked activity, a reduction of $GABA_AR$-mediated primary afferent

depolarization associated with a loss of $GABA_AR$ numbers on terminals in the SDH may help to explain these results. Towards this end, Fukuoka et al. (1998) demonstrated using *in situ* hybridization that there occurs a significant reduction of $GABA_AR$ $\gamma 2$ subunit mRNA on terminals in the SDH after tight ligation of the L5-6 spinal nerves. On the other hand, the efficacy of glycine to attenuate nociceptive hypersensitivity in this model may be explained by a bilateral loss of glycine receptors in the SDH after PNI (Simpson, Jr. and Huang, 1998).

Loose constrictive ligation

Observations relating to the development of hypersensitivity following loose ligation of the sciatic nerve are also strongly suggestive of a pivotal role for GABA and glycine signaling, however the nature of the plasticity of these systems is controversial.

As in the Chung model, $GABA_AR$ agonists are generally shown to decrease nociceptive hypersensitivity following loose ligation injuries, while $GABA_AR$ antagonists increase hypersensitivity. For example, Zarrindast et al. (2001) demonstrated that intrathecal muscimol effectively diminishes nociceptive hypersensitivity, while other groups (Yamamoto and Yaksh, 1993;Satoh and Omote, 1996) observed both bicuculline and strychnine to exert an amplifying effect both on the development and sustainment of nociceptive hypersensitivity subsequent to PNI.

Further evidence that loose constrictive ligation results in disinhibition may be derived from a study that demonstrated that after PNI, the threshold intensity for eliciting A-fiber-mediated EPSCs was substantially reduced (Kohno et al., 2003). The mechanisms that subserve this disinhibition, however, remain unclear. Some studies indicate that a decrease in $GABA_AR$ and/or GlyR function must underlie PNI-evoked hypersensitivity, as decreases in GABA- and glycine-like immunoreactivities in the SDH (Satoh and Omote, 1996) [also see Somers and Clement (2003)], and the expression of GAT-1 (GABA uptake transporter) in terminals of the SDH (Miletic et al., 2003) have not been observed after PNI. Moreover, Moore et al. (2002) report that the magnitude and duration of afferent-evoked $GABA_AR$-mediated IPSCs in LII neurons is significantly

reduced in animals with perirpheral nerve injury – further underscoring the hypothesis that a decrease in receptor function accounts for disinhibition. However, on the contrary, several other studies employing immunohistochemical detection have reported a (often dramatically) decreased number of GABAergic profiles (Ibuki et al., 1997;Eaton et al., 1998), in addition to GAD-65 expression (Moore et al., 2002). This apparent quandary is further complicated by two recent studies that suggest that subsequent to loose sciatic constriction, and in parallel to nociceptive hypersensitivity, there occur no changes at all in either the expression of GABA and glycine, or the existence of GABAergic and glycinergic neurons in the SDH (Somers and Clemente, 2002;Polgar et al., 2003b).

Transection

In the most severe model of peripheral neuropathy, a peripheral nerve (usually the sciatic nerve) is completely transected and ligated in a rodent, leading to a level of nociceptive hypersensitivity that is significantly higher than that produced by other models of PNI, as well as other dramatic forms of pain-related behaviour such as autotomy (Abdulla and Smith, 2001). Once again, it is unclear how $GABA_A$- or glycine receptor signaling contribute to the manifestation of nociceptive hypersensitivity. As with the other models of PNI, intrathecal strychnine has been shown to increase the occurrence of pain-related behaviours following sciatic transection (Seltzer et al., 1991), suggesting that the cause of nociceptive hypersensitivity may be linked to a loss of GlyR activation in the SDH. However, one of the few studies of GlyR expression after nerve transection, which employed cDNA microarrays and *in situ* hybridization, reports that an *increase* in GlyR α2A subunit mRNA in the SDH accompanies post-injury nociceptive hypersensitivity (Yang et al., 2004b).

The situation relating to GABAergic transmission in the SDH after sciatic transection is likewise confusing. The same cDNA microarray study cited above reports an increase in the expression of $GABA_AR$ α5 subunit mRNA (Yang et al., 2004b), however most other studies support the conclusion that there is decreased, or at best unchanged, GABA signaling in the SDH after transection. For example, Lever et al. (2003) show that subsequent to nerve transection, there is decreased GABA released in the SDH as a

consequence of A-fiber or A- and C-fiber stimulation, despite reports that GABA mediates decreased primary afferent depolarization post-injury (Kingery et al., 1988). These reports are both in agreement with immunohistochemical studies that show a substantial transection-elicited reduction of GABA-like immunoreactivity in the SDH, as well as decreased GABA concentration in homogenates of the SDH (Castro-Lopes et al., 1993). However, in one of the few electrophysiolgical analyses of $GABA_AR$-mediated synaptic events in the SDH after sciatic nerve transection, Moore et al. (2002) report that the magnitude and duration of primary afferent-driven IPSCs recorded from LII neurons are unchanged following sciatic nerve transection (note that this is in contrast to what this group observed after chronic constriction injury of the same nerve).

Hence, the contribution of GABA and glycine signaling to PNI-evoked nociceptive hypersensitivity remains unsure in all of the experimental models discussed. One of the reasons why this may be is that there have been few studies that address the actual function of $GABA_ARs$ and GlyRs in the post-injury state. That is, there are very few analyses of the propensity of these amino-acid receptors to induce neuronal inhibition, or more precisely, a hyperpolarization of membrane potential in SDH neurons after PNI. Moreover, the implications of changes in $GABA_AR$ or GlyR expression in SDH neurons have not been made clear. Reports of increased receptor expression are functionally meaningless if they do not enunciate whether this expression is postsynaptic to a GABA or glycine release site, for example. In the same vein, analyses of plasticity of $GABA_AR$ or GlyR function generally fail to address critical questions, such as whether injury-evoked changes in receptor contribution to postsynaptic inhibition stem from a modification of *de novo* synthesis, or simply from a synaptic reorganization, whether it be a shuttling of new receptors to or from the synaptic junction, or a modulation of the intrinsic properties of receptors to regulate postsynaptic excitability. Therefore, in this thesis, I will examine many of these issues pertaining to the strength and influence of $GABA_AR$ and GlyR-mediated signaling after PNI.

5. OBJECTIVES OF THE THESIS

Despite the impressive progress made in the understanding of nociceptive processing in recent years, the contribution of inhibitory systems in the SDH to the regulation of pain sensitivity remains poorly understood. More precisely, the role of local $GABA_AR$- and GlyR-mediated postsynaptic inhibition in the maintenance of normal neuronal (*e.g.* LI) excitability, as well as in the generation of the neuronal hyperexcitability that underlies states of neuropathic pain, has not been well elucidated. Hence, **the principal objective of this thesis was to broadly examine the physiological function of $GABA_AR$- and GlyR-mediated inhibition in both immature and mature states, and also after peripheral insults that have been postulated to result in an attenuation of SDH neuronal inhibition.**

In the study presented in the chapter 2, our aim was to **investigate the organization of inhibitory synapses in the rat SDH during development**. Notwithstanding observations that GABA and glycine are coreleased from terminals in the SDH, in the adult rat, only GlyRs mediate quantal postsynaptic inhibition in LI neurons, whereas both $GABA_ARs$ and GlyRs mediate quantal inhibition in LII neurons – yet never together at the same synapse (Chery and de Koninck, 1999). Other studies of spinal neurons taken from immature rats also report that GABA and glycine are coreleased, however these studies further reported that GABA and glycine could be codetected by their respective ionotropic receptors at the same synapses in LI-II (Jonas et al., 1998;O'Brien and Berger, 1999). Therefore, we used electrophysiological techniques to examine whether coreleased GABA and glycine could be codetected at immature SDH synapses, and if they were, what was the process by which $GABA_ARs$ cease to contribute to postsynaptic inhibition with development. We further assessed the functional implications of GlyR/$GABA_AR$ comediation of postsynaptic inhibition.

In the studies presented in the third, fourth and fifth chapters **we endeavoured to evaluate the relationships between nerve injury, local inhibition in the SDH and behavioural hypersensitivity (neuropathic pain)**. Specifically, in the investigation presented in the third chapter, we aimed to reveal how LI inhibitory synapses change after

PNI, and whether these changes could contribute significantly to the development and maintenance of nociceptive hypersensitivity in animal models. The initial impetus for this study stemmed from the understanding that after peripheral insult, synapses often assume an immature, or more primitive, configuration (Chong et al., 1992); therefore, one investigation assessed whether PNI evoked the recruitment of $GABA_ARs$ to postsynaptic excitability control. We further used several different empirical techniques (including calcium imaging, electrophysiology, immunoblotting and behavioural pharmacology) to survey the plasticity mechanisms that could contribute to an attenuation of inhibition, without relying on an alteration of presynaptic inhibitory neuronal function or existence – phenomena that are highly controversial (Sugimoto et al., 1985;Polgar et al., 2003b).

As we identified a recruitment of $GABA_ARs$ to postsynaptic excitability control and a collapse of the anion gradient in LI neurons as two principal plasticity mechanisms contributing to PNI-evoked neuronal hyperexcitability, we next **focused our efforts on the identification of upstream agents that mediate the decrease in KCC2 function – and hence collapse of anion gradient in LI neurons – in response to PNI**. The results of these analyses are presented in chapter 4. Brain-derived neurotrophic factor (BDNF) had recently been reported to trigger a rapid downregulation of KCC2 in hippocampal neurons (Rivera et al., 2002), and was widely documented to play a major neuromodulatory role in the development of neuronal hyperexcitability after nerve injury (Thompson et al., 1999). **Our goal, therefore, was to examine whether BDNF could trigger the disruption of anion gradient in LI neurons that occurs subsequently to PNI**. We discovered that endogenous BDNF released in the SDH did indeed trigger this shift; however, it was unclear what the source of this BDNF was, as a recent study reported that BDNF release from neuronal sources did not increase after PNI (Lever et al., 2003). To resolve this quandary, **we endeavoured to assess the contribution of other cells to the increased concentration of BDNF in the SDH after PNI**.

In the last chapter, I discuss our findings from **a study designed to assess mediators in the pathway linking TrkB activation with alterations with anion gradient**. This study was important, as a clear picture of the intracellular mediators involved in processing

disruptions of anion gradient in LI neurons after PNI would be necessary for the future development of therapeutics designed to correct anionic imbalances after PNI.

CHAPTER 2

REGION-SPECIFIC DEVELOPMENTAL SPECIALIZATION OF GABA/GLYCINE COSYNAPSES IN LAMINAE I-II OF THE RAT SPINAL DORSAL HORN

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ABSTRACT

The spinal dorsal horn is the first level in the central nervous system where nociceptive input from sensory afferents is integrated and transmitted. Although inhibitory control in this region has a crucial impact on pain transmission, the respective contribution of GABA and glycine to this inhibition remains elusive. We have previously documented corelease of GABA and glycine at the same inhibitory synapse in spinal laminae I-II of adult rats (>P30). However, despite this corelease, individual miniature inhibitory post-synaptic currents (mIPSCs) were mediated by either glycine receptors (GlyR) or $GABA_A$ receptors ($GABA_AR$), yet never by the two together. In contrast, recent studies of ventral horn immature inhibitory synapses (≤P21) reported individual mIPSCs mediated by both $GABA_ARs$ and GlyRs. This raises the question of whether mixed mIPSCs are present in immature lamina I-II neurons yet are lost through a maturation-dependent synaptic specialization. To test this, we recorded mIPSCs using patch-clamp techniques in lamina I-II neurons in spinal slices taken at different stages of development. We found that, in neurons younger than P23, both GlyR-only and $GABA_AR$-only mIPSCs could be recorded, in addition to mixed $GABA_AR$/GlyR mIPSCs. With maturation however, both lamina I-II neurons gradually discontinued to exhibit mixed mIPSCs, although with differing patterns of specialization. Yet, at all development stages, benzodiazepine administration could unmask mixed mIPSCs. Taken together, these findings indicate that, while GABA and glycine are continually coreleased throughout development, junctional codetection ceases by adulthood. This indicates an age-dependent postsynaptic tuning of inhibitory synapses that occurs in a region-specific manner.

INTRODUCTION

Laminae I and II of the spinal dorsal horn are key areas in the central nervous system where pain-related, or nociceptive information carried by sensory afferents is integrated and relayed to higher brain structures. Thus, the control of excitability of lamina I-II neurons has a major impact on pain perception. Glycine and γ-aminobutyric acid (GABA) are the main transmitters responsible for inhibitory control in this region and therefore play a crucial role in nociception. For example, blocking $GABA_A$ or glycine receptors ($GABA_A$Rs or GlyRs, respectively) in the superficial dorsal horn can cause conditions of hyperexcitability characteristic of neuropathic pain syndromes (Yaksh, 1989;Sherman and Loomis, 1996;Sorkin and Puig, 1996). Yet, the exact respective contributions of GABA and glycine to inhibitory control in this area remain largely unknown.

Immunocytochemical studies have provided substantial evidence in favour of a colocalization of GABA and glycine as well as their respective receptors in the dorsal horn (Todd et al., 1996). For example, the vesicular inhibitory amino acid transporter (VIAAT) which is known to carry both glycine and GABA [for review see Gasnier (2000)] has been shown in almost every inhibitory bouton in laminae I-III (Dumoulin et al., 1999;Chaudhry et al., 1998). Not surprisingly, virtually all glycinergic neurons in this region are also immunoreactive for GABA (Todd and Sullivan, 1990;Mitchell et al., 1993). Recent reports have demonstrated the corelease of GABA and glycine from individual vesicles at some synapses (Jonas et al., 1998;O'Brien and Berger, 1999;Chery and de Koninck, 1999). More precisely, the studies by Jonas et al. (1998) and O'Brien and Berger (1999) have shown that GABA and glycine can be coreleased from interneurons in the immature ventral horn to activate both post-synaptic $GABA_A$Rs and GlyRs simultaneously. In contrast, while we have also recently demonstrated the corelease of GABA and glycine in adult laminae I-II (Chery and de Koninck, 1999), our results indicated that quantal postsynaptic currents were either GlyR- or $GABA_A$R-mediated – never both in these adult cells. Taken together, these findings could suggest a specialization of inhibitory synapses with maturation.

We have investigated this possibility in the present study using whole-cell patch-clamp techniques on transverse and parasagittal slices of spinal cord to record and analyze action-potential independent miniature inhibitory post-synaptic currents (mIPSCs), which are thought to reflect the release of single vesicles of transmitter. We found that in immature rats [postnatal day 8 (P8) to postnatal-day 23 (P23)], most neurons in laminae I-II not only exhibited both $GABA_AR$- and GlyR-mediated mIPSCs, but also mixed $GABA_AR$/GlyR mIPSCs. These mixed mIPSCs decreased in proportion with age, disappearing by P23 – at which time neurons could be differentiated as displaying either GlyR-only or $GABA_AR$-only mIPSCs, but with lamina-specific patterns of specialization. Additionally, both $GABA_AR$- and GlyR-mediated mIPSCs displayed a quickening of decay kinetics with age, especially in the case of $GABA_ARs$. This allowed for a region-specific tuning of inhibitory charge carried during quantal events via an age-dependent specialization.

MATERIALS & METHODS

In this study we used slices from immature (P8-P23) or adult male (>P23) Wistar or Sprague Dawley rats. These slices were either cut in the parasagittal or transverse plane following removal of spinal cord by laminectomy or hydraulic extrusion. All dissections were carried out in the presence of ice-cold ($\leq 4^{\circ}C$) saccharose-artificial cerebrospinal fluid (S-ACSF) containing (in mM): 248 saccharose, 11 glucose, 2 $NaHCO_3$, 2 KCl, 1.25 KH_2PO_4, 2 $CaCl_2$, 2 $MgSO_4$ (pH 7.35 ± 0.05). S-ACSF was bubbled continuously with 95% O_2 – 5% CO_2.

Laminectomy. Rats were deeply anaesthetized with a cocktail of ketamine (75mg/kg i.p.) and xylazine (5mg/kg, i.p.). In order to limit hemorrhage and excitotoxicity during the dissection procedure, we systematically performed bilateral injections of xylocaine/adrenaline (2%) in the spine muscles of the thoraco-lumbar spinal cord segments. Vertebral laminectomy was then carried out, followed by dorsal and ventral root transection and in situ meninges removal.

Hydraulic Extrusion. We have previously described the protocol for hydraulic extrusion of rat spinal cords (Chery et al., 2000). Briefly, rats were anesthetized with sodium pentobarbital (30 mg/kg); adult rats were then intracardially perfused with ice-cold S-ACSF, while immature rats were immersed in ice-cold water for 4-5 minutes. Following either perfusion or immersion, both groups of rats were rapidly decapitated. The sacral vertebral column was transected and a syringe was used to inject S-ACSF into the foramen thus ejecting the spinal cord.

Slice Preparation. Cervical and lumbar segments (0.5-2 cm long) were isolated and glued with cyanoacrylate cement, either lateral side down (allowing for parasagittal slicing) or vertically, supported by an agarose block (allowing for transverse slicing), to the platform of a Vibratome chamber filled with oxygenated ice-cold sucrose ACSF. Slices, 300-400 μm in thickness, were cut, incubated in S-ACSF at room temperature (23-28°C) for 30 min, and transferred to normal ACSF for at least one hour prior to electrophysiological recordings. Finally, slices were transferred to a recording chamber under a Zeiss Axioscope equipped with infrared differential interference contrast (IR-DIC) and water immersion-objectives for visualization of neurons in thick live tissue. The slices were continuously perfused with oxygenated normal ACSF, where 125 mM NaCl was substituted for saccharose. To increase the frequency of occurrence of mIPSCs, 100 μM ruthenium red (Sigma) was applied to the bath chamber for some slices (n = 25). Ruthenium red is a polyvalent cation that blocks voltage-dependent calcium channels and enhances mIPSC frequency via a Ca^{2+}-independent mechanism (Trudeau et al., 1996;Sciancalepore et al., 1998;Hoffman and Lupica, 2000).

Electrophysiological recordings, data acquisition and analysis. All recordings were made at room temperature. For voltage-clamp experiments, patch pipettes were obtained by pulling borosilicate glass capillaries with inner filament using a horizontal laser puller (P-2000, Sutter instrument Co., USA) or a two-stage vertical puller (Narishige PP-83). The pipettes were filled with a solution containing (in mM): 130 CsCl , 2 MgCl2, 10 HEPES (pH=7.3, adjusted with CsOH). Approximately 60% of recordings were made with 0.4 mM GTP and 2 mM ATP (Sigma) added to the intracellular solution. As no

differences in mIPSC characteristics were observed among the two conditions all data were pooled. Whole-cell patch-clamp recordings were obtained using an Axopatch 200B amplifier (Axon Instruments, Foster city, CA) with >80% series resistance compensation. Recordings were low-pass filtered (5-10 kHz), digitized and stored on videotape. Off-line, recordings were filtered at 2KHz and digitized at 4-10 kHz on an Intel-Pentium®-based computer. Data were acquired and analyzed using the Strathclyde electrophysiology software CDR (courtesy of Dr. J. Dempster, U. of Strathclyde, Glasgow, UK) and analysis software designed by YDK.

Detection of individual mIPSCs was performed using a software trigger previously described in detail (Poisbeau et al., 1999;Chery and de Koninck, 1999). Greater than 95% of events that satisfied the trigger criteria were detected, even during compound mIPSCs. For each experiment, all detected events were examined individually and any noise which spuriously met trigger specification was rejected.

Statistical analysis and curve fitting. Peak amplitudes, rise times, and decay time constants were calculated for each of several hundreds of mIPSCs per cell, using an automated algorithm (de Koninck and Mody, 1996). Averages of several hundred mIPSCs were also superimposed for comparison. Decay time constants were fitted using a least square method based on a simplex algorithm as previously described (de Koninck and Mody, 1994). The goodness of fit was evaluated on the basis of fitting subsets of points drawn from the whole set of data points as well as from evaluation of the reduced χ^2: $\chi_\nu^2 = \chi^2/\nu$, where the factor $\nu = N - n$ is the number of degrees of freedom left after fitting N data points to the n parameters. The necessity to introduce additional exponential components to the fits was judged first on the basis of visual inspection of the fitted curves superimposed onto the data. When the merit of additional components was not obvious, an F test was used to assess how the additional component improved the value of the reduced χ^2: $F_\chi = \Delta\chi^2/\chi_\nu^2$ ($df_1 = 1$ and $df_2 = \nu$). The critical value for the merit of additional components was set at a low level ($p < 0.01$) to favor parsimony of the fitted function. When focusing on comparisons of the late component of mIPSCs, fits were started at a fixed interval after the peak of the event to allow for nonequivocal

monoexponential fits that provide an easier and fairer reference when dealing with nonaveraged, individual traces (de Koninck and Mody, 1994;Chery and de Koninck, 1999). This also avoided contamination of the values of decay time constants with variable weighting factors. Student *t* tests were used to analyze the differences between parameters of the $GABA_A$R- and GlyR-mediated IPSCs. Cumulative probability distributions were compared using the Kolmogorov-Smirnov test. χ^2 tests for contingency tables were performed to determine the correlation between the laminar location of the cells and the presence of $GABA_A$R- and GlyR-mediated sIPSCs. The critical value for statistical significance was set at $p < 0.05$. All the data are expressed as mean ± SEM, unless otherwise indicated.

Drug application. Slices were continually perfused with oxygenated ACSF containing tetrodotoxin (TTX, 0.5μM, Sigma) and either kynurenic acid (KA, 2mM, Fluka) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM, Tocris Cookson) and D2-amino-5-phosphonovaleric acid (D-AP5, 40 μM, Tocris Cookson). For selective blockade of glycine receptors, strychnine hydrochloride (200nM – 1 μM, Research Biochemicals) was used. For selective blockade of $GABA_A$ receptors, bicuculline methiodide (10 μM, Research Biochemicals) or SR-95531 (Gabazine; 3 μM; Research Biochemicals) were used. All drugs were prepared as 1000X concentrated frozen stock solution aliquots. Diazepam (Sigma) was diluted in 96% ethanol while all other drugs were prepared in distilled water.

RESULTS

Whole-cell voltage-clamp recordings of miniature inhibitory postsynaptic currents (mIPSCs) from 68 adult (>P30) and 78 immature (P8-P23) lamina I and II neurons are included in this report. Neurons were selected if they could be recorded from for a sufficient duration (>15 min) with stable access resistance throughout (7-20 MΩ). Spinal slices were obtained from either lumbar or cervical spinal enlargements. Consistent with our previous reports, no differences were observed in recordings between the two regions, in terms of mIPSC frequency or kinetics (Chery and de Koninck, 1999).

Lamina II neurons were accessed using either a parasagittal or a transverse spinal slice preparation. Optimal identification of lamina I neurons required the use of a parasagittal slice. As previously described (Chery et al., 2000), we defined lamina I neurons as those found within 30 μm of the outlying dorsal white matter in the parasagittal configuration. No significant differences were observed in recordings obtained using the different slice preparations, and thus data were pooled.

In some cases, we bath-applied 100 μM ruthenium red, a polyvalent cation that blocks voltage-dependent calcium channels, in order to increase mIPSC frequency ($n = 25$). We observed no significant differences in recordings from cells in the absence or presence of ruthenium red, in terms of decay time constant ($GABA_A$R-only: 23.5 ± 11.2 ms *vs.* 27.5 ± 1.8 ms; GlyR-only: 9.7 ± 1.0 ms *vs.* 10.0 ± 1.3 ms) or 10-90% rise time ($GABA_A$R-only: 1.1 ± 0.3 ms *vs.* 1.0 ± 0.1 ms; GlyR-only: 1.1 ± 0.2 ms *vs.* 0.9 ± 0.2 ms; tested in 16 cells all taken at P14).

GlyR- and $GABA_A$R-mediated mIPSCs

Prior to P23, all neurons in lamina I (100%, $n = 12$) and the vast majority of neurons in lamina II (72%, $n = 47$) exhibited quantal events mediated by GlyRs and by $GABA_A$Rs, as well as by both types of receptors concurrently (see below). The remaining lamina II neurons displayed events mediated either solely by glycine receptors (termed hereafter GlyR-only mIPSCs; $n = 3$ or 4% of the cells) or by $GABA_A$ receptors (termed hereafter $GABA_A$R-only mIPSCs; $n = 16$ or 24% of cells). In neurons displaying both GlyR- and $GABA_A$R-mediated events, the simultaneous presence of bicuculline (10 μM) and strychnine (500 nM) was required to completely and reversibly abolish miniature events (Fig. 1A). Kinetic analysis revealed that the decay phase of the bicuculline-sensitive $GABA_A$R-only mIPSCs was significantly slower than that of strychnine-sensitive GlyR-only mIPSCs (Fig. 1B). This is further highlighted in Figure 1C, which shows the cumulative probability distribution of mIPSC decay time constants in two neurons. In control conditions, a clear bimodal distribution of decay time constants indicated two kinetically-distinct populations of mIPSCs within this cell. Each of the two peaks

represented by this bimodal distribution could be selectively abolished by the administration of bicuculline or strychnine, respectively. Generally, pharmacologically isolated $GABA_AR$- and GlyR-only mIPSCs possessed decay phases that could be appropriately fitted by a monoexponential function (Fig. 1B).

Mixed $GABA_AR$/GlyR-mediated mIPSCs

In the absence of inhibitory amino acid receptor antagonists, a population of individual mIPSCs with a prominent dual component could be observed in neurons in both laminae I and II. As shown at the bottom of Figure 1B, these mIPSCs represented as many as 27% of the total number of mIPSCs displayed by an immature neuron. This proportion of dual component mIPSCs was further found to be not significantly different when superficial neurons were recorded using Cs_2SO_4 containing electrodes ($E_{Cl} \sim -60$ mV) at a holding potential of 0 mV ($n = 9$, data not shown). The relative contribution of the fast decay component to these mIPSCs (τ_1) was consistently 66 ± 10% compared to that of the slow decay component (τ_2), which was 33 ± 6% ($n = 10$; Fig. 1E).

Importantly, biexponential mIPSCs were absent in the presence of either bicuculline or strychnine, and when fitted with double exponential decay functions yielded components that had kinetics comparable to that of their pharmacologically-isolated counterparts ($GABA_AR$-only and GlyR-only mIPSCs). In addition, the frequency of pharmacologically-isolated monoexponential mIPSCs was found to be similar to the sum of the frequencies of the monoexponential mIPSCs and biexponential mIPSCs when isolated kinetically in neurons younger than P23 (Fig. 1D). That is, the frequency of GlyR-only mIPSCs in the presence of bicuculline (0.43 ± 0.1 Hz) was not significantly different from the sum of the frequencies of kinetically-isolated fast monoexponentially decaying mIPSCs and dual component mIPSCs (0.42 ± 0.09 Hz, $n = 8$, $p > 0.05$). Similarly, after strychnine application, the frequency of pharmacologically-isolated $GABA_AR$-only mIPSCs (0.33 ± 0.09 Hz) was equivalent to the sum of the mean frequencies of kinetically-isolated slow monoexponentially decaying mIPSCs and dual component mIPSCs (0.36 ± 0.1, $n = 17$, $p > 0.05$). These results are consistent with the

interpretation that, in these cells, mIPSCs with prominent biexponential decays possess both a $GABA_AR$ and a GlyR component, and can be reduced to a monoexponential mIPSC of either variety simply by blocking the other component. Consistent with this observation is also the fact that dual component mIPSCs had peak amplitudes that were on average twice that of the GlyR-only mIPSC or the $GABA_AR$-only mIPSC (confirmed in all of 51 neurons tested; ages P8 – P22; Fig. 2). Taken together, all of these results suggest the synchronous coactivation of synaptic $GABA_ARs$ and GlyRs during a subgroup of quantal inhibitory events in lamina I-II neurons. These events will henceforth be referred to as mixed $GABA_AR$/GlyR mIPSCs.

Age-dependent disappearance of mixed $GABA_AR$/GlyR mIPSCs

We observed a disappearance of mixed $GABA_AR$/GlyR mIPSCs with development. In lamina I (Fig. 3A), the proportion of these events peaked at P14 (27%), and then decreased in a linear fashion, becoming virtually undetectable by approximately P23. Interestingly, and similar to the mixed mIPSCs, $GABA_AR$-only mIPSCs were also gradually lost in lamina I with maturation and were virtually absent beyond P23. Thus, consistent with our previous results (Chery and de Koninck, 1999), adult lamina I neurons only expressed GlyR-only mIPSCs ($n = 39$).

A similar trend in the disappearance of mixed $GABA_AR$/GlyR mIPSCs was also observed in lamina II neurons (Fig. 3B). Again, mixed events were excluded by approximately P23. In contrast to lamina I, however, the synaptic events recorded in adult lamina II neurons were evenly divided into $GABA_AR$-only mIPSCs and GlyR-only mIPSCs (52% *vs.* 48%, respectively, $n = 33$).

Additionally, the decrease in the proportion of mixed mIPSCs in lamina I and II neurons was not correlated with a significant change in the relative contribution of the fast decaying component to the peak amplitude of mixed events (P8-15: $A_{GlyR}/A_{Total} = 58.5 \pm 9.6$, $n = 36$; P20-22: $A_{GlyR}/A_{Total} = 69.7 \pm 9.6$, $n = 5$, $p > 0.05$).

Diazepam revealed that corelease still occurred during GlyR-only mIPSCs at immature synapses

Our finding of mixed $GABA_AR$/GlyR mIPSCs that are excluded with maturation in both laminae I and II in favour of GlyR- or $GABA_AR$-only mIPSCs, raises the question of whether this specialization reflects a reorganization of presynaptic release or of postsynaptic receptor expression. To address this question we used the benzodiazepine diazepam (DZP, 1 μM) to increase the affinity of $GABA_ARs$ in lamina I and II neurons to test for the possibility that GABA may be coreleased with glycine but remain subliminal to post-synaptic $GABA_ARs$. DZP was perfused on immature neurons ($n = 5$) with synaptic events being initially categorized into GlyR-only, $GABA_AR$-only and mixed $GABA_AR$/GlyR mIPSCs on the basis of their decay kinetics (as described above). At the end of each recording, the validity of these categories was confirmed using strychnine and bicuculline administration sequentially and following full recovery from each application. Figure 4 illustrates the effect of diazepam administration to each of these types of mIPSCs. As shown in Figure 4A, GlyR-only mIPSC kinetics were not affected by perfusion of DZP. In the 5 cells tested, the decay time constant remained stable (Control: 9.8 ± 1.4 ms; DZP: 10 ± 1.7 ms) in addition to the 10-90% rise time (Control: 0.5 ± 0.3 ms; DZP: 0.7 ± 0.3 ms; data not shown) and the peak amplitude (Control: 69.6 ± 37.6 pA; DZP: 55.4 ± 23.2 pA). However, the inter-event interval of fast-decaying mIPSCs was significantly prolonged, corresponding to a reduction in frequency from 0.24 ± 0.16 Hz to 0.09 ± 0.06 Hz, for control and DZP respectively ($p < 0.05$; $n = 5$).

DZP also induced an increase in the decay time constant of $GABA_AR$-only mIPSCs which grew from 30.2 ± 7.0 ms (control) to 58.7 ± 14.2 ms (n = 5; Fig. 4B). None of the other kinetic parameters were significantly affected. Upon assessing the effects of DZP on mixed $GABA_AR$/GlyR mIPSCs (Fig. 4C) we found that the fast decay component remained stable (11.2 ± 3.3 ms *vs.* 10.1 ± 3.1 ms), while the slow component doubled from 31.1 ± 4.8 ms to 67.4 ± 16.9 ms ($n = 5$). The relative contribution of the fast-decaying component (GlyR-mediated) to the peak amplitude of mixed $GABA_A$/GlyR mIPSCs was also observed not to change with DZP application (Control: $A_{GlyR} / A_{Total} =$

58.5 ± 9.6%, $n = 36$; DZP: A_{GlyR}/A_{Total} = 57.3 ± 5.6%, $n = 7$, $p > 0.05$). Together, these results reinforce our conclusion that dual-component mIPSCs correspond to mixed $GABA_AR$/GlyR-mediated mIPSCs with the slow component being attributed to $GABA_AR$ activation. More importantly, the frequency of mixed mIPSCs was significantly higher after DZP perfusion with values of 0.20 ± 0.1 Hz and 0.31 ± 0.13 Hz in control and DZP, respectively ($p < 0.01$; n = 5). The total frequency of events was not significantly different between the control and DZP conditions (0.21 ± 0.06 Hz *vs.* 0.19 ± 0.06 Hz, $p > 0.05$). Consistent with this, the reduction in GlyR-only mIPSC frequency was similar to the increase in mixed $GABA_AR$/GlyR mIPSC (for greater power of the test, comparison was made for each cell; the mean difference was 0.036 ± 0.056 Hz, not significantly different from zero; $p > 0.05$). We can thus conclude that the increase in mixed $GABA_AR$/GlyR mIPSC frequency directly results from the DZP-induced conversion of GlyR-only mIPSCs into mixed $GABA_AR$/GlyR mIPSCs.

Comparable potentiation of $GABA_AR$-mediated mIPSCs in immature *vs.* adult neurons by diazepam

To test for changes in $GABA_AR$ subunit composition with maturation (Laurie et al., 1992), we applied DZP at the same concentration on isolated $GABA_AR$-only events from both immature and adult superficial dorsal horn neurons. Perfusion of diazepam (1 μM) induced a comparable ~2-fold increase in the decay time constant of pharmacologically isolated $GABA_AR$-mediated mIPSCs in both immature (control: 30.2 ± 7.0 ms *vs.* diazepam: 58.7 ± 14.2 ms; $n = 5$) and adult neurons (control: 14.4 ± 0.6 ms *vs.* diazepam: 27.3 ± 0.9 ms; $n = 6$). Figure 5 illustrates such a potentiation for isolated $GABA_AR$-mediated mIPSCs in lamina II neurons. This finding indicates that the sensitivity of immature and adult $GABA_ARs$ to diazepam was not significantly different.

Age-dependence of mIPSC kinetics

Although no change was observed for the benzodiazepine sensitivity of $GABA_ARs$ with maturation, we found significant alterations in the decay time course of both

pharmacologically-isolated $GABA_AR$-only and GlyR-only mIPSCs with maturation. Specifically, $GABA_AR$-only mIPSCs showed a linear three-fold quickening of decay time between P8 and P23 (from 35.0 ± 3.0 ms to 10.0 ± 0.6 ms; Fig. 6B). In contrast GlyR-only mIPSCs exhibited a smaller decrease in decay time with maturation (from 9.8 ± 1.0 ms to 5.8 ± 0.3 ms; Fig. 6B). Interestingly, this decrease in the decay time constant for GlyR-only mIPSCs appeared to coincide more closely with the period in which the proportion of mixed mIPSCs declined, from P15 to P23. There was also a decrease in the 10-90% rise time of both $GABA_AR$-only and GlyR-only mIPSCs (Fig. 6A). The fact that the ratio of $GABA_AR$-only/GlyR-only rise time remained constant at ~1.3 (see also Fig. 8), however, suggests that the shortening of both rise times may be due to a similar mechanism, such as a change in the degree of electrotonic filtering with maturation. Alternatively, the ratio of $GABA_AR$-only to GlyR-only decay time constant was not constant, falling from ~4.5 at P8 to ~2 at P23 (Fig. 6C), indicating that changes in electrotonic filtering are not sufficient to explain this differential change in decay kinetics.

In addition to changes in kinetics, mIPSCs in adult animals were significantly larger than in immature animals. In lamina I, GlyR-mediated mIPSCs were 24.4 ± 4.8pA (n = 10) in immature animals vs. 74.4 ± 10.2pA (n = 12) in adults. A similar increase in mIPSCs amplitude was observed in lamina II for both isolated GlyR- (47.1 ± 3.5 pA; n = 39 *vs.* 76.8 ± 5.8 pA; n = 32) and $GABA_AR$-mediated mIPSCs (44.7 ± 3.3 pA; n = 39 vs. 56.1 ± 6.3 pA; n = 22).

Change in net inhibitory charge carried by mIPSCs

As a direct consequence of the reduced duration of $GABA_AR$-only mIPSCs, in addition to the loss of mixed mIPSCs in the adult superficial dorsal horn, the net inhibitory charge carried by individual mIPSCs recorded from lamina I neurons was dramatically decreased. Figure 7 illustrates that in P8 lamina I neurons, which principally develop GlyR-dominant synaptic junctions, individual mIPSCs carried a mean net inhibitory charge of 668.7 ± 72.1 fC (n = 7). This net charge is roughly 50% greater than that

carried by mIPSCs in P20 lamina I neurons (445.0 ± 26.9 fC; $n = 3$; $p < 0.05$), despite comparable mIPSC amplitudes (P8: 23.5 ± 1.8 pA; $n = 7$ *vs.* P20: 25.6 ± 1.2 pA; $n = 3$). Even in adult lamina I neurons, which display mIPSCs with significantly greater amplitude (86.5 ± 2.5 pA; $n = 3$), the net inhibitory charge carried by mIPSCs (509.7 ± 69.2 fC; $n = 3$) remains less than that of P8 lamina I neurons.

Analysis of the development of net quantal inhibition in lamina II neurons was difficult because age-dependent specialization results in a mixed population in this area (*i.e.*, adult cells display varying proportions of GlyR-only *vs.* $GABA_AR$-only mIPSCs). It is interesting to note however, that for cells with a greater proportion of $GABA_ARs$ mIPSCs, the reduction in net quantal inhibition with maturation is tempered because of the more prolonged decay kinetics of these synaptic currents in adult animals (Fig. 6). Thus, these cells appear to develop a smaller deficit of inhibition with maturation than neurons where quantal inhibition is predominantly mediated by GlyRs. These observations emphasize the impact of the change in mIPSC kinetics and composition on the net inhibition of the cell during the maturation process, and further demonstrate the distinction in development among lamina I and II neurons.

Comparison of mIPSC rise times

In our previous study of adult lamina I neurons, we found that the $GABA_AR$-component unveiled with the use of a benzodiazepine had a much slower (>10X) rise time than its GlyR counterpart. Because the two components were part of the same mIPSC, and thus originating from corelease from the same presynaptic terminal, we had hypothesized that this may indicate that $GABA_ARs$ may be located perisynaptically at lamina I inhibitory synapses. We thus decided to contrast this result with values of rise time at different stages of development.

Interestingly, we found that individual 10-90% rise times from the GlyR-only and $GABA_AR$-only mIPSCs were similar in immature neurons, displaying a constant mean ratio of approximately 1.3 ($GABA_AR$ rise/GlyR rise) in both laminae ($n = 11$; Fig. 8). In the adult, despite the disappearance of mixed mIPSCs, the rise time ratio between

$GABA_A$-only and GlyR-only mIPSCs also remained stable in lamina II. This result was in sharp contrast with that from adult lamina I neurons which normally only display GlyR-mediated mIPSCs. In the latter case, benzodiazepine-revealed $GABA_AR$-component of mIPSCs had a much slower rise time (>10X) than that of its GlyR counterpart or that of $GABA_AR$ events recorded from lamina II neurons [Fig. 8; also see Chery and De Koninick (1999)].

DISCUSSION

In this study we show that, in contrast to what is found in the adult, immature lamina I-II inhibitory synapses exhibit individual miniature IPSCs possessing both a GlyR- and a $GABA_AR$-mediated component in normal conditions. More importantly, while our results confirm previous evidence of copackaging and thus corelease of GABA and glycine from the same terminals in the spinal cord (Jonas et al., 1998;O'Brien and Berger, 1999;Chery and de Koninck, 1999) they also show that despite continued corelease of these transmitters throughout development, codetection ceases at lamina I-II adult synapses. This indicates an age-dependent postsynaptic tuning of inhibitory synapses in this area. Furthermore, we demonstrate that this pattern of development differs among lamina I, a predominantly output layer, and the more propriospinal lamina II, suggesting that synaptic tuning may be dictated by functional requirements.

Maintenance of corelease, loss of codetection

The observation of dual-component mIPSCs throughout maturation, and the fact that benzodiazepines can continually unmask mixed quantal events [this study and Chery and De Koninck (1999)], argues for the continuous copackaging and corelease of GABA and glycine at all ages. These mixed mIPSCs also indicate the coactivation of kinetically distinct junctional receptor/channel complexes and, because corelease appears to persist, the switch we observe is likely reflecting a postsynaptic change in transmitter detection at synaptic junctions. Although we cannot completely rule out a presynaptic reorganization to explain these developmentally-mediated changes, several lines of evidence argue against this possibility. First, immunocytochemical evidence indicates that GABA/glycine colocalization persists uninterupted in adulthood (Todd et al., 1996).

Second, a decrease in the relative vesicular concentration of GABA is unlikely given that the affinity of VIAAT for GABA is three times greater than that for glycine (Bedet et al., 2000). While these arguments do not allow direct estimation of vesicular concentration, the fact that the amplitude of $GABA_AR$-only mIPSCs and of the $GABA_AR$ component of mixed mIPSCs was not increased by diazepam (even though they were prolonged) suggests that the amount of GABA released during these quantal events was saturating (de Koninck and Mody, 1994), arguing against low vesicular content.

The observation that all three combinations, mixed $GABA_AR$/GlyR mIPSCs, GlyR-only mIPSCs and $GABA_AR$-only mIPSCs, are present in the same cell and the fact that benzodiazepine can convert many GlyR-only into mixed $GABA_AR$/GlyR mIPSCs suggests the existence of a heterogeneous junctional distribution of $GABA_ARs$ and GlyRs in spinal laminae I and II. A differential distribution of receptor subtypes or subunits at distinct junctions within the same cell has previously been reported for other receptors (Dodt et al., 1998;Toth and McBain, 1998). Furthermore, developmental shift from GABAergic to glycinergic transmission has been observed in some systems (Kotak et al., 1998). The alteration described in the latter study also appeared to occur at the postsynaptic level, but at earlier stages (P3-P12) than what we observed in spinal laminae I-II (P14-P23).

The pattern of distribution of mIPSC type among lamina I-II neurons was also different. In lamina I, for example, where virtually all cells will display only GlyR-mediated mIPSCs in the adult, as little as 29% GlyR-only events were present at P8. The reason for this is unknown, but it is interesting to note that a similar pattern of development in the rat ventral horn has been reported (Colin et al., 1998). In that study, it was shown that, at birth, the points of gephyrin-immunoreactivity (IR) outnumber GlyR-IR significantly. GlyR-IR only matched gephyrin-IR by P10, with proliferation of these GlyR-gephyrin microdomains occurring gradually with development. In light of reports linking the $GABA_AR$ to gephyrin (Sassoe-Pognetto et al., 2000;Bohlhalter et al., 1994;Todd et al., 1996) this same group suggested that early unmatched gephyrin sites may be associated with the expression of a perinatal $GABA_AR$. Indeed it does now seem

that gephyrin is required for the postsynaptic localization of $GABA_A$Rs (for review see Kneussel et al. (1999), perhaps via cytoskeletal interactions (Wang et al., 1999b). Transient expression of $GABA_A$Rs at early postnatal stages has also been reported in rat retina (Koulen, 1999) and other regions of the brain (Laurie et al., 1992;Poulter et al., 1992). These findings could explain why neurons that appear to only express junctional GlyRs in the adult stage display a significant proportion of mIPSCs with a $GABA_A$R-component at developmental stages.

Mechanisms of synaptic switch

The loss of codetection of coreleased GABA and glycine likely involves a change in affinity of the receptors or of their expression/subsynaptic distribution. It is reasonable to hypothesize that these changes are linked to altered subunit expression and/or intracellular regulatory mechanisms. Many reports, for example, suggest that differences in subunit composition affect subcellular localization (Nusser et al., 1998;Crestani et al., 1999;Sassoe-Pognetto et al., 2000;Brickley et al., 1999;Hutcheon et al., 2000). Similarly, phosphorylation mechanisms have been reported to interfere with translocation of $GABA_A$Rs to postsynaptic domains (Wan et al., 1997b).

A switch in receptor subunit expression has been proposed to underlie changes in kinetics of GlyRs during development (Takahashi et al., 1992). Although we also observe a shortening GlyR-mediated mIPSCs, the change reported by Takahashi et al. (1992) appears to be largely completed by the beginning of the second postnatal week, which does not fit with the time frame reported here (between P15 and P23). Similarly, we observed a shortening of the decay kinetics of $GABA_A$R mIPSCs, consistent with that previously reported in other areas (Dunning et al., 1999;Tia et al., 1996;Hollrigel and Soltesz, 1997;Gao and Ziskind-Conhaim, 1995). The possibility that this change may be related to altered subunit expression during maturation in the spinal cord (Poulter et al., 1992), must be considered given the recent data associating shortening of $GABA_A$R-mIPSCs decay with an increase in $\alpha 1$ subunit expression in cerebellar neurons (Vicini et al., 2001). This subunit however appears to be absent in adult laminae I-II (Persohn et al., 1991;Bohlhalter et al., 1996) suggesting that it may not underlie kinetic changes observed

in the spinal cord. Our results also indicate that any switch should not involve an altered subunit composition affecting diazepam sensitivity.

Functional significance

The significance of the transient occurrence of mixed $GABA_AR$/GlyR quantal events during the maturation process may lie within the development of synapses themselves. Recently, it has been shown that the postsynaptic clustering of glycine receptors is activity-dependent (Kirsch and Betz, 1998). More specifically, the release of glycine from presynaptic terminals and subsequent depolarization elicited by perinatal GlyRs [<P7; (Wang et al., 1994)] is required for the aggregation of gephyrin which clusters GlyRs. The presence of $GABA_ARs$, which are also depolarizing prior to ~P7 (Obrietan and van den Pol, 1995;Boehm et al., 1997;Serafini et al., 1995) may augment this activity dependent formation of GlyR synapses.

Furthermore, transient $GABA_AR$ expression at developing glycinergic synaptic junctions might also play a more ancillary role. GABAergic signalling has been shown to be important in early arborization (Behar et al., 1996b;Spoerri, 1988) and is known to display a synergistic relationship with developing glutamatergic transmitter systems (Leinekugel et al., 1999).

Alternatively, coexpression of $GABA_ARs$ and GlyRs at the same junctions may allow for optimal activity-dependent formation of $GABA_AR$ synapses, as it is now clear that gephyrin plays some role in the clustering of $GABA_ARs$ [for review see Kneussel and Betz (2000)]. Poulter et al. (1997) have reported that synapse formation itself may regulate the selective trafficking of $GABA_AR$ subunit mRNA during synaptogenesis. This finding is of particular importance in the interpretation of our results. That is, perhaps in lamina I $GABA_ARs$ are first required to be expressed at synaptic junctions in immature dorsal horn in order to initiate activity-dependent migration to extrajunctional sites in the adult. The possibility that this migration is mediated by a subunit switch is supported by a second study from the same group that suggests that perinatal $GABA_A$

receptor activity may regulate the developmental switchover of $GABA_A$ receptor subunit mRNA expression (Poulter and Brown, 1999).

The possibility that the transient occurrence of junctional $GABA_ARs$ and GlyRs coactivation represents a protective mechanism must also be considered. It has been reported, for example, that supraspinal descending inhibitory pathways do not become fully functional until the third postnatal week (Falcon et al., 1996;Van Praag et al., 1993). A greater net inhibitory charge carried by quantal mIPSCs could therefore allow the intrinsic circuitry to accomplish sufficient inhibition of neuronal excitability in the absence of extrinsic inhibitory input. Alternatively, a greater net charge could also serve as a protective mechanism during the development of glutamatergic transmission, as it has been reported that $GABA_A$ synapses precede glutamatergic synapses (Tyzio et al., 1999).

Previous evidence suggests that $GABA_A$ synapses can be made silent in a rapidly reversible manner (Poisbeau et al., 1997). Knowing that the functional properties of synaptic $GABA_ARs$ are regulated by intracellular mechanisms (Poisbeau et al., 1999;Wan et al., 1997a), it is possible that the loss of a GABAergic contribution to quantal inhibitory events with maturation may represent a type of contingency organization. For instance, in normal adult lamina I $GABA_ARs$ do not appear to contribute to basal inhibition, yet they may be brought into play in conditions such as the hyperexcitability characteristic of chronic pain (Woolf, 1983).

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FIGURES

Figure 1. Three distinct types of mIPSCs could be recorded from immature (P8-P23) neurons in the superficial dorsal horn. *A*, Raw traces of spontaneously occurring mIPSCs in a P14 lamina I neuron in control conditions, in the presence of either strychnine (500 nM) or bicuculline (10 μM) as well as in the presence of both antagonists. While strychnine or bicuculline alone only blocked a subpopulation of mIPSCs, no events were left in the concurrent presence of both antagonists. Note how the events remaining in the presence of strychnine have a distinctly slower decay time course than those remaining in the presence of bicuculline. Holding membrane potential (V_H) was –60 mV. *B*, The mIPSCs could be separated into three distinct categories on the basis of their decay kinetics: events with a rapid monoexponential decay phase, which persisted in the presence of bicuculline, thus apparently being mediated by GlyRs (termed GlyR-only); events with a slow monoexponential decay phase, which persisted in strychnine, thus apparently being mediated by $GABA_A$Rs (termed $GABA_A$R-only); and events with clear double decay kinetics which were absent in the presence of either strychnine or bicuculline, and therefore involving activation of both $GABA_A$Rs and GlyRs (termed mixed $GABA_A$R/GlyR mIPSCs). Traces on the *left* are superimposed individual events (*n* = 3, each). Traces to the *right* are averages of 100 consecutive events. *C*, Cumulative probability plots of decay time constants of mIPSCs recorded from two P8 neurons. The plots were constructed by forcing monoexponential fits to all individual mIPSCs recorded under control conditions (**dashed line**) and then in the presence of either 500 nM strychnine (**solid black line)** in one neuron (*top*) or 10 μM bicuculline (**solid grey line)** in another neuron (*bottom*). A clear bimodal distribution of decay times is observable in control conditions, while strychnine and bicuculline selectively abolish the early or late mode, respectively. *D*, The sum of the frequencies of kinetically-isolated monoexponential mIPSCs and biexponential mIPSCs was similar to the frequency of that same monoexponential mIPSC when isolated pharmacologically. For illustration, individual examples are provided: in an immature lamina II neuron (P14), in control conditions (C), kinetically-isolated slow monoexponentially decaying mIPSCs (**solid black bar**) occurred at a frequency of 0.15 Hz while that for mixed mIPSCs (**solid white**

bar) was 0.18 Hz. The sum of these frequencies (0.34Hz) was comparable to that of $GABA_AR$-only mIPSCs recorded in the presence of strychnine (S; 0.30 Hz). A similar example is provided using bicuculline to isolate GlyR events (C vs. B). See text in RESULTS for further details, for population data and statistical analysis. *E*, In mixed $GABA_AR$/GlyR mIPSCs, the mean relative contribution (Rel. Contn.) of the fast (τ_1) and the slow decay component (τ_2) was 65 ± 10% and 35 ± 6%, respectively (n = 10).

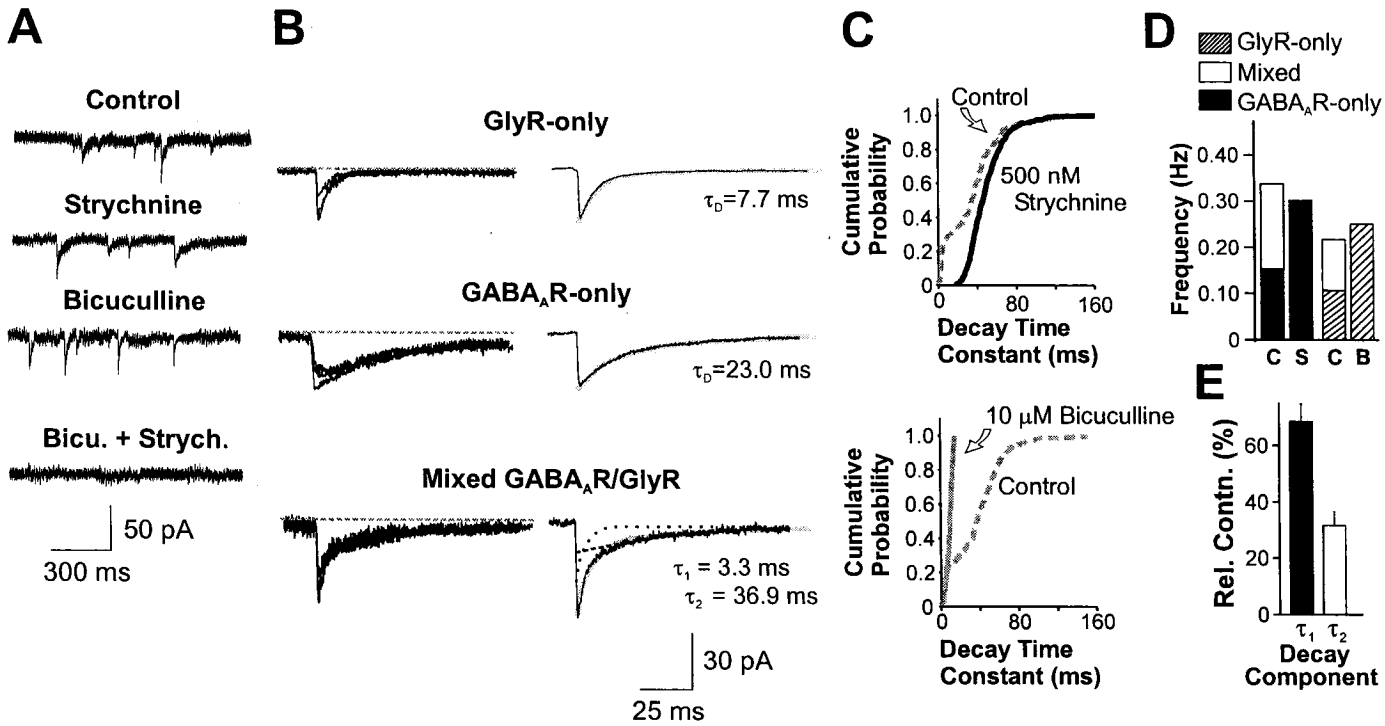

A
Control
Strychnine
Bicuculline
Bicu. + Strych.
50 pA
300 ms
B
GlyR-only
τ_D=7.7 ms
GABA$_A$R-only
τ_D=23.0 ms
Mixed GABA$_A$R/GlyR
τ_1 = 3.3 ms
τ_2 = 36.9 ms
30 pA
25 ms
C
Control
500 nM Strychnine
Cumulative Probability
Decay Time Constant (ms)
10 μM Bicuculline
Control
Cumulative Probability
Decay Time Constant (ms)
D
GlyR-only
Mixed
GABA$_A$R-only
Frequency (Hz)
C S C B
E
Rel. Contn. (%)
τ_1 τ_2
Decay Component

Figure 2. Mixed $GABA_AR$/GlyR mIPSCs have significantly greater peak amplitudes than either $GABA_AR$-only or GlyR-only mIPSCs. *A*, Cumulative probability plot highlighting the difference in peak amplitude distribution between mixed $GABA_AR$/GlyR mIPSCs (**solid black line**) and GlyR-only (**dashed line**) and $GABA_AR$-only mIPSCs (**solid grey line**) recorded from the same lamina II neuron (age P17; each neuron is used as its own control to avoid confounding effects of cell-cell variation in mean mIPSC amplitude; the same analysis was replicated in $n = 10$ neurons and yielded similar results). *B*, From the same neuron described in A, the mean peak amplitude of mixed $GABA_AR$/GlyR mIPSCs was 47 ± 4 pA, representing an amplitude ~113% greater than that of $GABA_AR$-only mIPSCs (22 ± 2 pA; $p < 0.05$) and ~74% greater than the mean peak amplitude of GlyR-only mIPSCs (27 ± 2 pA; $p < 0.05$). The insets above each bar are representative traces of mIPSCs from each category.

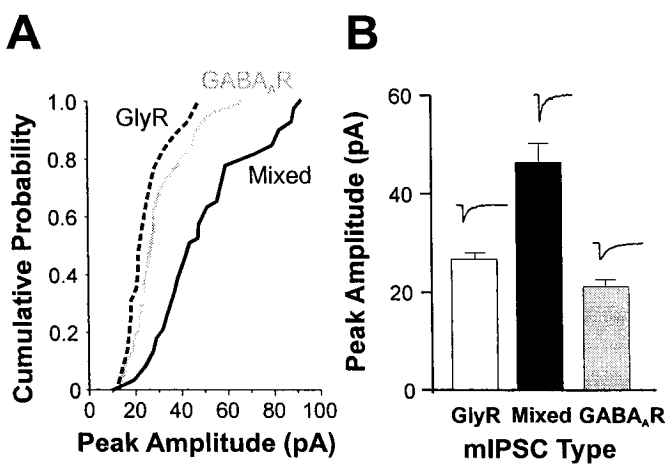

A
B
GABA$_A$R
GlyR
Mixed
Cumulative Probability
1.0
0.8
0.6
0.4
0.2
0
0 20 40 60 80 100
Peak Amplitude (pA)
Peak Amplitude (pA)
60
40
20
0
GlyR Mixed GABA$_A$R
mIPSC Type

Figure 3. The proportion of mixed $GABA_AR$/GlyR mIPSCs decreased with maturation in both lamina I and II neurons. A, In lamina I neurons, mixed $GABA_AR$/GlyR mIPSCs (**solid triangles**) virtually disappeared along with $GABA_AR$-only mIPSCs (**open circles**) by approximately P23, after which, only GlyR-only mIPSCs (**solid squares**) could be detected. B, Lamina II neurons also discontinued to display mixed $GABA_AR$/GlyR mIPSCs by ~P23. However, in contrast to lamina I neurons, mIPSCs recorded in adult lamina II were evenly divided into $GABA_AR$-only and GlyR-only. All data points, $3 \leq n \leq 6$.

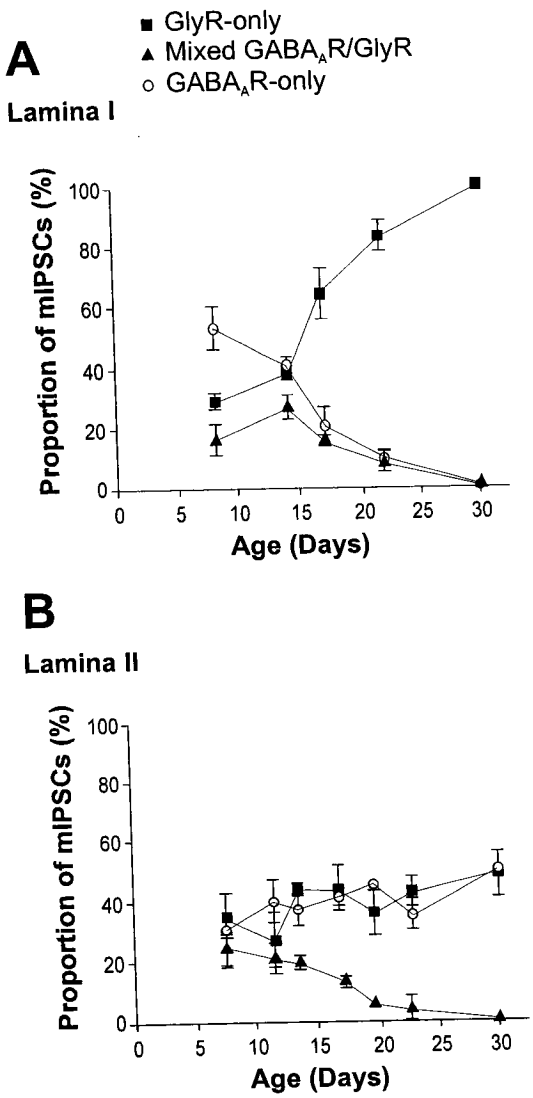

A
GlyR-only
Mixed GABA$_A$R/GlyR
GABA$_A$R-only
Lamina I
Proportion of mIPSCs (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
Age (Days)
B
Lamina II
Proportion of mIPSCs (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
Age (Days)

Figure 4. Benzodiazepine revealed that corelease still occurred during GlyR-only mIPSCs. Example of the modulatory effect of benzodiazepine diazepam (DZP, 1μM; measurements made under steady state conditions) on mIPSCs in a P13 lamina II neuron. *A*, Neither the cumulative distribution of peak amplitudes (*right*), nor the decay time constant of the group of mIPSCs characterized by a rapid monoexponential decay phase (GlyR-only) was changed significantly with the administration of DZP (*center, inset*). Inter-event interval (*left*) was, however prolonged with DZP yielding a significant decrease in its reciprocal parameter, instantaneous frequency (control, 1.4 Hz *vs.* DZP, 0.37 Hz; $p < 0.05$; *center*). *B* and *C*, In contrast, the groups of mIPSCs with a slower monoexponential decay phase ($GABA_AR$-only) and with a dual exponential decay phase (mixed $GABA_AR$/GlyR mIPSCs) were prolonged in the presence of DZP (*respective center insets*). Mean decay of $GABA_AR$-only mIPSCs was prolonged from 30.2ms to 58.7ms (*B, inset*). Mixed $GABA_AR$/GlyR mIPSCs only exhibited an increase in τ_2, from 31.1ms to 67.4ms, while τ_1 remained stable (*C, inset*). Note that the mean peak amplitude of $GABA_AR$-only (*B, right*) and mixed $GABA_AR$/GlyR (*C, right*) was not significantly affected by the perfusion of DZP. There was, however, a significant four-fold increase in the instantaneous frequency of mixed $GABA_AR$/GlyR mIPSCs which grew from 0.36 Hz in control conditions to 1.11 Hz with the administration of DZP ($p < 0.01$; *C, center*). No similar change in instantaneous frequency was noted for $GABA_AR$-only mIPSCs (*B, center*). The adequacy of the categorization of GlyR-only, $GABA_AR$-only and mixed $GABA_AR$/GlyR mIPSCs on the basis of their decay kinetics was confirmed with the sequential use of strychnine and bicuculline at the end of the recording (not shown). Parameters under the influence of diazepam are indicated by **grey lines** or **hatched bars**, where appropriate. **Cum. Prob.**: cumulative probability, **Inst. Freq.**: instantaneous frequency, **Amp.**: peak amplitude. * $p < 0.05$.

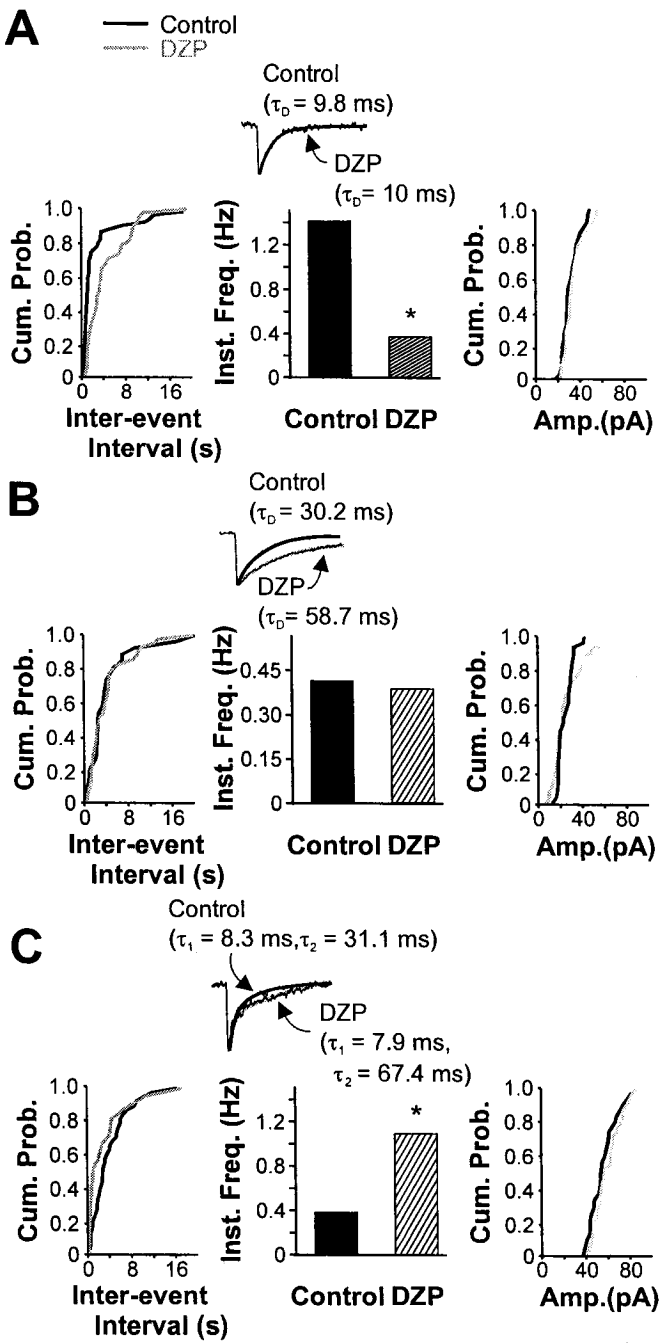

A
Control
DZP
Control
(τ_D = 9.8 ms)
DZP
(τ_D= 10 ms)
Cum. Prob.
Inter-event Interval (s)
Inst. Freq. (Hz)
Control DZP
Cum. Prob.
Amp.(pA)
B
Control
(τ_D = 30.2 ms)
DZP
(τ_D= 58.7 ms)
Cum. Prob.
Inter-event Interval (s)
Inst. Freq. (Hz)
Control DZP
Cum. Prob.
Amp.(pA)
C
Control
(τ_1 = 8.3 ms,τ_2 = 31.1 ms)
DZP
(τ_1 = 7.9 ms,
τ_2 = 67.4 ms)
Cum. Prob.
Inter-event Interval (s)
Inst. Freq. (Hz)
Control DZP
Cum. Prob.
Amp.(pA)

Figure 5. The diazepam-induced prolongation of the decay phase of $GABA_AR$-only mIPSCs was similar among neurons from immature (P8-P23) and adult superficial dorsal horn. *A*, This cumulative probability plot illustrates the ~100% potentiation of isolated $GABA_AR$-only mIPSCs recorded from a P13 lamina II neuron after application of DZP (control, 36.6ms, **solid grey line**, *vs.* DZP, 73.3ms, **solid black line**). *B*, A similar potentiation (117%) was observed in adult neurons with an increase in the decay time constant from 13.5 ms (**dashed grey line**) to 29.3 ms (**dashed black line**). Both cumulative probability plots show a similar rightward shift in decay distribution suggesting that the whole population of $GABA_AR$ mIPSCs were affected by DZP. The *insets* in *A* and *B* show averaged representative traces illustrating the potentiation by DZP. Each trace was appropriately fitted by a monoexponential function. **Cum. Prob.**: cumulative probability, **Decay Const.**: decay time constant.

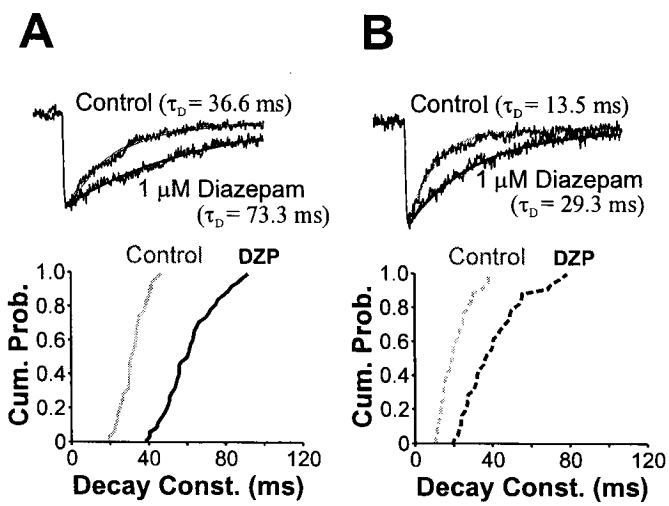

A
Control (τ_D= 36.6 ms)
1 μM Diazepam
(τ_D= 73.3 ms)
Control
DZP
Cum. Prob.
1.0
0.8
0.6
0.4
0.2
0
0
40
80
120
Decay Const. (ms)
B
Control (τ_D= 13.5 ms)
1 μM Diazepam
(τ_D= 29.3 ms)
Control
DZP
Cum. Prob.
1.0
0.8
0.6
0.4
0.2
0
0
40
80
120
Decay Const. (ms)

Figure 6. The kinetics of both $GABA_A$R-only and GlyR-only mIPSCs changed with maturation. *A*, Both $GABA_A$R-only and GlyR-only mIPSCs displayed a decrease in 10-90% rise time over the course of maturation. This decrease in rise time was however similar among $GABA_A$R-only and GlyR-only mIPSCs, and thus the ratio of rise times between these two type of mIPSCs remained constant throughout development (all data points, $2 \leq n \leq 4$). *B*, $GABA_A$R-only mIPSCs displayed a four-fold reduction in decay time constant (from 35.0 ± 3.0 ms to 10.0 ± 0.6 ms) between P8 and P23 (**closed square**). After showing a slight increase, GlyR-only mIPSC decay time decreases two-fold (from 9.8 ± 1.0 ms to 5.8 ± 0.3 ms) between P14 and P23 (**open square**); all data points, $2 \leq n \leq 4$. *C*, The ratio of the $GABA_A$R-only to GlyR-only decay time constant thus decreased from 4.5 at P8 to ~2 at P23 and onward. Note that P23 (indicated by a dotted line) represents the time point by which mixed $GABA_A$R/GlyR mIPSCs virtually disappear in all neurons from laminae I and II.

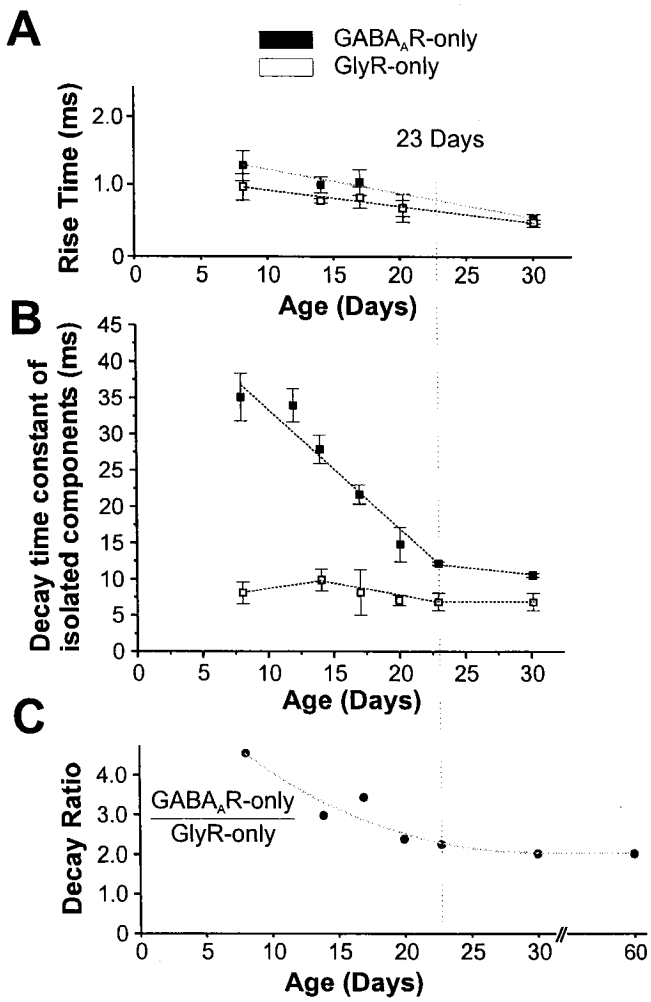

A
GABA$_A$R-only
GlyR-only
23 Days
Rise Time (ms)
2.0
1.0
0
0 5 10 15 20 25 30
Age (Days)
B
Decay time constant of isolated components (ms)
45 40 35 30 25 20 15 10 5 0
0 5 10 15 20 25 30
Age (Days)
C
Decay Ratio
GABA$_A$R-only / GlyR-only
4.0 3.0 2.0 1.0 0
0 5 10 15 20 25 30 60
Age (Days)

Figure 7. The loss of mixed $GABA_AR$/GlyR mIPSCs, in addition to the decrease in mIPSC duration with development resulted in a reduction in the net inhibitory charge carried by individual mIPSCs. *A*, *left,* Lamina I neurons of age P8 **(solid black)** displayed mIPSCs that carried significantly greater net inhibitory charge (668.7 ± 72.1 fC; $n = 7$) than either P20 lamina I neurons (**hatched**; 445.0 ± 26.9 fC; $n = 3$; $p < 0.05$) or adult lamina I neurons (**solid white**; 509.7 ± 69.2 fC; $n = 3$; $p < 0.05$). A representative cumulative probability distribution of mIPSC net inhibitory charge is shown on the *right* for three neurons, each taken from one of the age groups. *B*, *left*, The greater net charge of mIPSCs displayed by P8 neurons when compared to P20 and adult neurons occurred despite similar peak amplitudes of mIPSCs among P8 and P20 lamina I neurons (P8: 23.5 ± 1.8 pA; $n = 7$ *vs.* P20: 25.6 ± 1.2 pA; $n = 3$; $p > 0.05$) and a significantly greater peak amplitude of mIPSCs in adult lamina I (86.5 ± 2.5 pA; $n = 3$; $p < 0.01$). *Right*, A representative cumulative probability of mIPSC peak amplitudes is shown for three neurons, each taken from one of the age groups. * $p < 0.05$.

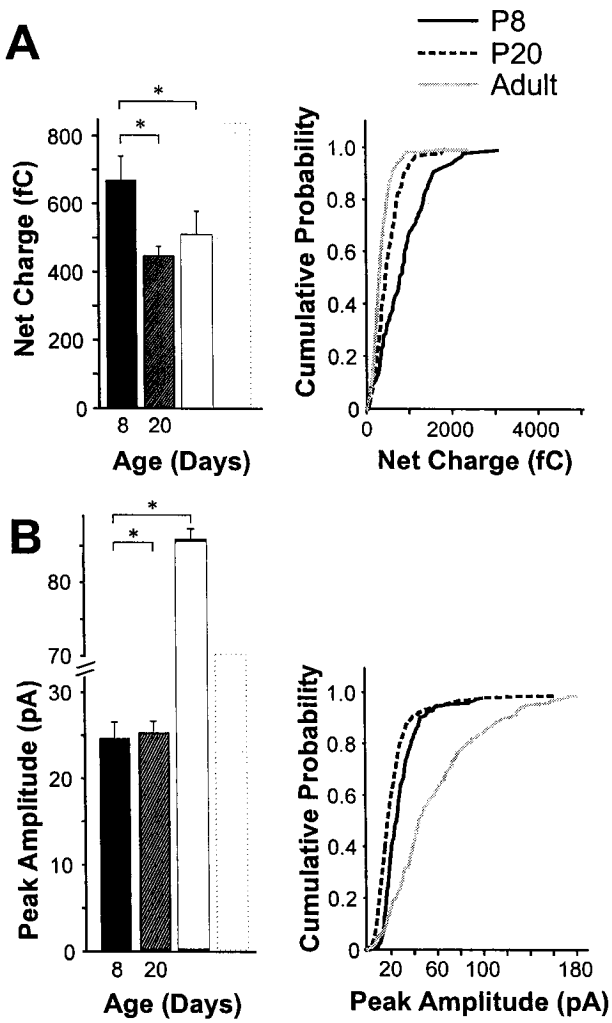
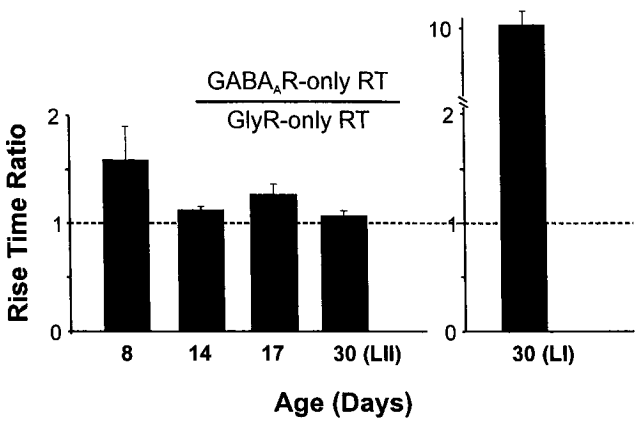

A
P8
P20
Adult
Net Charge (fC)
800
600
400
200
0
8 20
Age (Days)
Cumulative Probability
1.0
0.8
0.6
0.4
0.2
0
0 2000 4000
Net Charge (fC)
B
Peak Amplitude (pA)
80
70
30
20
10
0
8 20
Age (Days)
Cumulative Probability
1.0
0.8
0.6
0.4
0.2
0
20 60 100 180
Peak Amplitude (pA)

Figure 8. Difference in the ratio of $GABA_A$R-only to GlyR-only mIPSC rise time in immature and adult lamina I-II neurons. In immature neurons (P8-P23) the ratio of $GABA_A$R-only mIPSCs 10-90% rise time to that of GlyR-only mIPSCs was relatively stable, with a mean ratio of 1.3. This ratio was also not significantly different in adult lamina II neurons. In contrast, $GABA_A$R-only mIPSCs revealed by 1 μM flunitazepam in adult lamina I exhibited a rise time (4.1 ± 0.9 ms) that was >10X that of GlyR-only mIPSCs (0.4 ± 0.04 ms).


GABA$_A$R-only RT
GlyR-only RT
Rise Time Ratio
2
1
0
10
2
1
0
8
14
17
30 (LII)
30 (LI)
Age (Days)

PREFACE TO CHAPTER 3

For the most part, studies of spinal disinhibition after PNI have focused on contributions (or lack thereof) stemming from alterations in presynaptic inhibitory interneuron function (Moore et al., 2002;Polgar et al., 2003b). These studies are controversial; so too are the few studies that examine postsynaptic SDH neuronal function after PNI – virtually all of which focused on the expression of postsynaptic $GABA_A$Rs and GlyRs (Simpson and Huang, 1998;Yang et al., 2004b).

Having elucidated a novel plastic mechanism involving the reorganization of SDH inhibitory synapses with maturation, and in keeping with reports that PNI may cause synapses to adopt an immature configuration (Chong et al., 1992), I assessed in the following study whether there occurred a simple reorganization of GlyRs and $GABA_A$Rs at inhibitory synapses after PNI. Furthermore, below I reveal another novel mechanism that profoundly attenuates inhibition after PNI: a collapse of LI neuronal anion gradient.

CHAPTER 3

*TRANS*SYNAPTIC SHIFT IN ANION GRADIENT IN SPINAL LAMINA I NEURONS AS A MECHANISM OF NEUROPATHIC PAIN

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***Nature*, 424: 938-942, 2003.**

ABSTRACT & INTRODUCTION

Modern pain control theory (Melzack and Wall, 1965) predicts that a loss of inhibition (disinhibition) in the dorsal horn of the spinal cord should be a critical substrate for chronic pain syndromes (Woolf and Salter, 2000). However, evidence for the mechanisms underlying such disinhibition have remained controversial (Kontinen et al., 2001;Moore et al., 2002;Somers and Clemente, 2002;Polgar et al., 2003a). Here we show a novel mechanism of disinhibition following peripheral nerve injury. It involves a transsynaptic reduction in the expression of the potassium-chloride exporter KCC2 and the consequent disruption of anion homeostasis in neurons of lamina I of the superficial dorsal horn (SDH), one of the main spinal nociceptive output pathways (Light, 1992). The resulting shift in the transmembrane anion gradient caused normally inhibitory anionic synaptic currents to be excitatory, substantially driving up the net excitability of lamina I neurons. Local blockade or knock-down of the spinal KCC2 exporter in intact rats markedly reduced nociceptive threshold, confirming that the reported disruption of anion homeostasis in lamina I neurons was sufficient to cause neuropathic pain.

RESULTS

Peripheral neuropathy was induced in rats by chronically constricting the sciatic nerve (Fig.1a). Recent data indicate that injury to neurons can cause hyperexcitability via a modification of the transmembrane anion gradient (van den Pol et al., 1996), but it is unknown whether this can occur transsynaptically (downstream from the injured neuron). To test whether the hyperexcitability (sensitization) of SDH neurons that follows peripheral nerve injury (Pitcher and Henry, 2000) (PNI) is associated with a modification of the anion gradient (∇_{anion}), we measured anion reversal potential (E_{anion}) using gramicidin-perforated patch clamp recording. This technique avoids disrupting the intracellular anion concentration (Ebihara et al., 1995). Responses to exogenous GABA application showed that the anion reversal potential (E_{anion}) of lamina I (LI) neurons taken from PNI rats was –49.0 ± 2.3 mV ($n = 9$) compared to -72.6 ± 3.5 mV ($n = 5$; $p < 0.005$) in LI neurons from naïve rats (Fig.1b-d). Resting membrane potential was not significantly different between PNI (-62 ± 4 mV, $n = 7$) and naïve rat LI neurons (-61 ± 2 mV, $n = 16$; $p > 0.1$). Spontaneous and evoked postsynaptic currents (PSCs), recorded from PNI rat LI neurons in the presence of fast glutamate receptor (GluR) blockers were also inward (depolarizing from rest), their mean reversal potential increasing by 16.1 mV relative to that in lamina I neurons from naïve rats ($n = 6$, PNI; $n = 4$, naïve).

We next tested whether other properties of synaptic transmission were altered in the SDH after PNI. Inhibitory miniature PSCs (mPSCs) in LI neurons from naïve rats are mediated by glycine receptors (GlyRs) alone despite GABA and glycine corelease from local inhibitory interneurons (Keller et al., 2001) (Fig.2a). While GluR-mediated mPSCs were unaffected by PNI (Fig.2b), in all cells tested from PNI rats, a population of outward mPSCs at 0 mV persisted in the presence of the GlyR antagonist strychnine (up to 1 μM; $n = 4$). These remaining mPSCs were mediated by $GABA_A$Rs, as they were blocked by bicuculline (10 μM) and displayed prolonged decay kinetics compared to the GlyR-mediated component ($\tau_{D(GABA_AR)}$ = 34.0 ± 2.9 ms, $n = 5$, *vs.* $\tau_{D(GlyR)}$ = 11.3 ± 1.3 ms, $n = 6$; $p < 0.01$; Fig.2C).

Kinetic analysis further revealed that the decay phase of 36.9 ± 2.3% of mPSCs followed a dual exponential function (τ_{D1} = 7.5 ± 2.0 ms and τ_{D2} = 51.3 ± 7.9 ms; n = 6; Fig.2c). These events possessed both a $GABA_AR$ and a GlyR-mediated component, as either strychnine or bicuculline could lead to the abolition of their respective components (n = 4). Therefore, in parallel with the collapsed ∇_{anion}, PNI caused reorganization at LI synapses thereby unmasking a $GABA_AR$ component. This synaptic organization is similar to that observed in immature LI-II neurons (Keller et al., 2001). The net effect of this synaptic switch is that it yielded a population of quantal synaptic events with significantly longer decay kinetics.

To examine the function of the PNI-induced $GABA_AR$-mediated contribution to mPSCs, we analysed both the peak conductance and the frequency of mPSCs. This was performed using CsCl-filled pipettes to clamp the E_{anion} at 0 mV in both LI neurons taken from PNI and naïve rats to prevent biased detection of mPSCs resulting from changes in driving force. Peak conductance of GlyR-only mPSCs recorded in LI neurons taken from PNI rats was significantly smaller (~2-fold) than that recorded from naïve rat LI neurons (Fig.2d). The addition of $GABA_AR$-mediated events in the PNI condition, however, partially compensated the decrease in GlyR-only conductance. The peak conductance of GluR-mediated quantal events was not significantly different between LI neurons taken from naïve and PNI rats (Fig.2d).

Factoring together the changes in peak conductance, kinetics, and driving force, the net charge carried by GlyR-only mPSCs at resting membrane potential in LI neurons taken from PNI rats was nearly 3-fold smaller than that in naive rats (Fig.2e). With the contribution of $GABA_ARs$, however, the net charge carried by mPSCs in PNI rats rose back to a level similar to that mediated by GlyRs in naïve rats.

The frequency of GlyR-only mPSCs recorded in LI neurons from PNI rats was observed to be significantly less (0.13 ± 0.04 Hz, $n = 5$) than that for GlyR-only mPSCs in naive rat LI neurons (0.18 ± 0.04 Hz, $n = 6$; $p < 0.05$; Fig.2f). As with peak conductance, however, the addition of the $GABA_AR$-mediated mPSCs compensated the PNI-induced decrease in frequency (0.22 ± 0.10 Hz, $n = 4$, for all $GABA_AR$ and/or GlyR-mediated events combined; $p > 0.5$). In contrast, there was no significant change in the frequency of GluR-mediated events in LI neurons isolated from PNI rats (1.51 ± 0.90 Hz, $n = 9$) compared to LI neurons from naïve rats (0.82 ± 0.40 Hz, $n = 5$; $p > 0.3$; Fig.2f). Thus, the result of these synaptic analyses revealed a shift from GlyR- towards $GABA_AR$-dominated synaptic transmission after PNI.

If depolarizing $GABA_AR$/GlyR-mediated postsynaptic currents exert a net excitatory influence in PNI LI neurons, they should directly evoke action potentials, and consequently lead to Ca^{2+} influx(Nabekura et al., 2002). To test this hypothesis, we first employed Ca^{2+}-imaging using Fura-2-AM loaded LI neurons in slice to obtain a large data set. Administration of exogenous GABA to neuronal somata caused a significant increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in 19% of LI neurons ($n = 53$; Fig.3a,c) lying ipsilateral to the site of PNI. This represents a seven-fold increase compared to LI neurons found in naïve and/or contralateral dorsal horn (where only 1 of 37 neurons showed an increase in $[Ca^{2+}]_i$; Fig.3b,c). These responses were blocked by bicuculline (10 μM; $n = 5$) and by the voltage sensitive sodium channel blocker tetrodotoxin (TTX; 1 μM; $n = 31$). We then further confirmed electrophysiologically that applied GABA and synaptically elicited anionic postsynaptic potentials can directly evoke action potentials (Fig.3d,e). These results indicate that postsynaptic anion fluxes can cause net excitation in lamina I neurons in PNI rats.

What cellular mechanism underlies this shift in ∇_{anion}? Recent studies at early developmental stages in the brain have shown a link between the expression of the potassium-chloride exporter KCC2 and neuronal $[Cl^-]_i$ (Hubner et al., 2001;Ganguly et al., 2001). We therefore compared KCC2 protein levels by immunoblotting on horizontal slices of SDH. The KCC2 expression level in the lumbar SDH ipsilateral to the PNI was significantly reduced (>2-fold) relative to the side contralateral to the injury (Fig.3f). In naive rats, there was no significant difference between the two sides ($n = 3$; not shown).

If a decrease in the expression of the KCC2 exporter leads to an increase in neuronal $[Cl^-]_i$ and, in turn, $GABA_AR$/GlyR-mediated depolarization, a pharmacological blockade of the KCC2 exporter in LI neurons from naïve rats should have the same effect. To test for this possibility, we bath applied the selective KCC2 blocker DIOA (30 μM) to naïve spinal slices. As in the PNI condition, GABA application in the presence of DIOA caused an increase in $[Ca^{2+}]_i$ in 30% of naïve LI neurons tested (Fig.3b,c).

Although we have shown that, after PNI, $GABA_ARs$ and GlyRs mediated postsynaptic depolarization, the predictive value of miniature and evoked PSCs for overall neuronal excitability is limited. This stems from the distinct input-output properties of neurons in intact animals resulting from the multi-fold greater frequency and magnitude of spontaneous PSCs bombarding cells (Bernander et al., 1991). Therefore, to assess whether the empirically determined changes in $GABA_AR$/GlyR-mediated postsynaptic control were sufficient to account for the central sensitization that is known to follow PNI (Pitcher and Henry, 2000), we simulated *in vivo* conditions using a biophysically realistic neuron model (Supplemental Information Fig.1). The simulation confirmed that, after PNI, the extent of LI neuronal sensitization varied as a function of their E_{anion}, ranging from slight disinhibition to a net hyperexcitation.

To test whether this predicted hyperexcitability (sensitization) would result in a decrease in the stimulus threshold to evoke a nociceptive withdrawal reflex, we administered DIOA (15-30 μg) directly to the lumbar enlargement of the spinal cord in intact rats via an intrathecal catheter. DIOA caused a rapid, and reversible decrease in nociceptive threshold to both mechanical and thermal stimuli (Fig.4a-b). This effect of DIOA did not occur via an alteration of anion conductance (Supplemental Information Fig.2). A similar decrease in nociceptive threshold was elicited via selective knock-down of the exporter using spinal administration of an antisense oligodeoxynucleotide against KCC2 mRNA (Fig.4c), further confirming the functional impact of KCC2 dowregulation.

DISCUSSION

The results show that the neuropathic pain that follows PNI can be explained by a downregulation of the KCC2 exporter and the resultant shift in the ∇_{anion} in spinal LI neurons. They also demonstrate that such a modification of ∇_{anion} in adult animals can occur in a neuron transsynaptic to an injury site. Our findings thus provide a new avenue to understand disinhibition in nociceptive pathways. The inversion in polarity of the $GABA_AR$/GlyR-mediated postsynaptic action forces a reinterpretration of data related to changes in the efficacy of GABAergic transmission and provides a potential mechanistic basis for many of the reported consequences of central sensitization (Cook et al., 1987). It is interesting to note, in this context, that LI nociceptive specific neurons have been shown to respond to innocuous input when sensitized (Blomqvist and Craig, 2000).

A critical feature of the spinal cord is that it employs two very distinct GABAergic inhibitory mechanisms: GABAergic control of the central terminals of sensory fibres already involves a depolarizing mechanism (Rudomin and Schmidt, 1999), in contrast to dorsal horn cells where GABAergic inhibition involves hyperpolarization. Thus, the

change in KCC2 expression reported here affects the polarity of GABA action in only one of the two inhibitory mechanisms controlling sensory input. This is confirmed by the fact that primary afferents lack expression of KCC2 (Fig.4d,e; also see Supplemental Information Fig.3). This may explain the seemingly contradictory antiallodynic effect of intrathecally-applied $GABA_AR$ agonists (Hwang and Yaksh, 1997;Malan et al., 2002).

GABA/glycine-mediated depolarization may also serve as a gating mechanism to enable excitation via voltage sensitive Ca^{2+} channels (VSCCs) and NMDA receptor/channels (Staley et al., 1995). Ca^{2+} influx via these channels is thought to be critical for the sensitization of spinal neurons (Coderre and Melzack, 1992). Indeed, blocking these Ca^{2+} channels in humans by drugs such as gabapentin and ketamine has proven highly efficacious in the treatment of neuropathic pain (Serpell, 2002;Rabben et al., 1999;Martin and Eisenach, 2001). However, use of Ca^{2+} channel blockers, particularly ketamine and other NMDA antagonists, is associated with many undesirable side effects (Martin and Eisenach, 2001;Farber et al., 2002). Our identification of the pivotal role played by appropriate anion homeostasis in SDH neurons in maintaining adequate nociceptive gate control opens new avenues for the development of more selective therapeutic strategies in the management of chronic neuropathic pain syndromes.

METHODS

Nerve Injury. Briefly, peripheral nerve injury was induced by surgically implanting a polyethylene cuff (~2 mm long, 0.7 mm inner diameter) around the sciatic nerve of adult, male, Spague-Dawley rats as previously described (Mosconi and Kruger, 1996). A group of rats also received sham surgery. Only animals that showed a gradual decrease in mechanical threshold (over 14-17 days) down to 2.0 g or less were used for further experiments.

Behavioural Testing. Thermal and mechanical threshold for nociceptive withdrawal reflexes were tested as previously described (Coderre and Van, I, 1994).

Slice preparation. Parasagittal slices (300-350 μm) of spinal cord were prepared from adult (>50 days old) male rats as previously described (Keller et al., 2001). Slices were continually superfused (2-3 $ml \cdot min^{-1}$) with artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 26 $NaHCO_3$, 10 glucose, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 0.001 TTX (bubbled with 95% O_2 – 5% CO_2, pH~7.4); when measuring $GABA_A$/GlyR-mediated currents, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 40 μM D2-amino-5-phosphonovaleric acid (APV) were added to block fast glutamatergic transmission.

Recordings. All recordings were preformed at room temperature. For perforated patch recordings, the pipette tip was filled with a solution containing (in mM): 130 cesium gluconate (CsGlu), 5 CsCl, 2 $MgCl_2$, 11 BAPTA, 1 $CaCl_2$, 4 ATP, 0.4 GTP, 10 HEPES (pH~7.4). The pipette was back-filled with this same solution supplemented with 25 μg/ml gramicidin D (gramicidin stock was at 10 mg/ml in DMSO). Recordings in this mode were selected when access resistance was stable between 25-45 MΩ. For whole-cell voltage-clamp recordings, pipettes were filled with the above solution without gramicidin D. For whole-cell current-clamp recordings, pipettes were filled with the same intracellular solution as for voltage-clamp, except potassium methyl sulfate ($KMeSO_4$) was used to replace CsGlu. To clamp E_{anion} at 0 mV, CsGlu was replaced with 110 mM CsCl in the intracellular solution. All whole-cell recordings at E_{anion} = 0 mV were made at V_m = -60 mV in the presence of GluR-blockers. GABA (1 mM) was applied locally for 30-250 ms by pressure ejection through a patch micropipette. Data acquisition and analysis of PSCs was performed as previously described (Keller et al., 2001). All measurements are given as means ± SEM, except where indicated. Statistical significance was tested using Student's *t*-tests for comparison of mean values, chi-square

tests for contingency tables, and mixed design ANOVAs (post-hoc – Tukey's HSD) for repeated measures.

Calcium Imaging. Slices were prepared as detailed above. After 15 min incubation in ACSF, slices were loaded with 10 μM Fura-2-AM, in HEPES-buffered saline (+10% DMSO) for 1 hour. Slices were washed for ~15 min with ACSF before being mounted in the recording chamber, where they continued to be superfused by ACSF (2-3 $ml \cdot min^{-1}$). $[Ca^{2+}]_i$ was fluorometrically measured using a 40X water immersion objective on a Zeiss Axioscope equipped with epifluorescence optics. Images were acquired using a TILL Photonics monochromator coupled to a CCD camera and regions of interest (for ratioing) were drawn on clearly distinct neuronal cell bodies.

Immunoblotting. Horizontal slices (150 μm) of the SDH were made from the lumbar enlargement of both PNI and naïve adult rats. Tissue extracts were prepared by homogenizing the slices with a Teflon pestle in a buffer containing 0.32 M sucrose, 0.5 mM Tris-HCl, pH 7.5, 2 mM ethylenediaminetetracetic acid (EDTA), 2.5 mM β-mercaptoethanol, and a cocktail of protease inhibitors (Complete, Roche Diagnostics). Supernatants from 3,000 *g* (20 min) and 10,000 *g* (30 min) centrifugations were collected. Equal amounts of proteins (20 μg/lane) diluted in sample buffer were preheated at 37 °C for 30 min, resolved by SDS-PAGE, and electroblotted onto nitrocellulose membranes. Membranes were blocked 30 min in 5% nonfat dry milk in TBST buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.05% Tween-20) and incubated overnight at 4° C with a rabbit anti-KCC2 antibody (1:1000, Upstate Biotechnology). After several washes in TBST, membranes were incubated for 30 min at room temperature with peroxidase-labeled goat anti-rabbit antibody (1:2000). Chemiluminescent bands were detected using Super Signal Femto (Pierce Biotechnology). Digital images were captured with the VersaDoc imaging system and analysed with Quantity One software (BioRad).

Oligodeoxynucleotides. KCC2 antisense and scrambled oligodeoxynucleotides, phosphorothioated at all positions, were designed as previously described (Rivera et al., 1999): antisense, 5'-TCTCCTTGGGATTGCCGTCA-3' (+59 relative to the initial ATG); scrambled, 5'-TCTTCTTGAGACTGCAGTCA-3'.

Intrathecal Injections. At least three days prior to drug administration, rats were anaesthetized with sodium pentobarbital (65 mg kg^{-1}) and a lumbar spinal catheter was inserted into the intrathecal space, as previously described (Coderre and Melzack, 1992). Upon recovery from surgery, lower body paralysis was induced via i.t. lidocaine (2%, 30 µl) injection to confirm proper catheter localization. Only animals exhibiting appropriate, transient paralysis to lidocaine, as well a lack of motor deficits were used for behavioural testing. Following drug/vehicle administration, animals were sacrificed and their vertebral column dissected to visually confirm correct placement of the catheter. Drugs included DIOA (10-30 µg, in 0.9% NaCl, 10 % DMSO) and oligodeoxynucleotides (single doses of 2 µg at 0h, 12h & 24h; 0.9% NaCl). Behavioural testing was performed as above (under blind conditions); normal (~15 g) mechanical threshold for withdrawal responses was confirmed in naïve rats prior to receiving drug or vehicle. At the doses used, none of the compounds produced motor disturbances or sedation as assessed by grasping, righting and placing reflexes and behavioral observations (Coderre and Van, I, 1994).

ACKNOWLEDGEMENTS

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measurements, Dr. L. Pellegrini for valuable advice on quantification of immunoblotting and O. Szalay for expert technical assistance. We also thank Drs. F.J. Alvarez, M. Deschênes, P. Drapeau and M.W. Salter for critical comments on the manuscript.

FIGURES

Figure 1. PNI induced a collapse of the ∇_{anion} in LI neurons in the ipsilateral SDH. **a**, PNI (n = 23), but not sham surgery (n = 11), caused a significant reduction in the 50% nociceptive withdrawal threshold to mechanical stimulation of the hindpaw in rats ($p <$ 0.01). **b,** E_{anion} recorded from LI neurons of naïve (△) and PNI (○) rats. Solid symbol = mean E_{anion} ± SEM. **c**, All classes of LI neurons (*i.e.* with phasic (P), single-spike (SS) and tonic (T) firing properties (Prescott and de Koninck, 2002)) showed a shift in E_{anion} in response to PNI. Scale(y,x)=50mV,150ms. **d,** Mean peak current measured in LI neurons from naïve (▲) and PNI (●) rats in response to applied GABA at various V_m. Horizontal standard error bars represent inter-neuron differences in recording pipette offset. **Inset** – Representative raw traces. Scale(y,x)=0.6 nA,1.0 s.

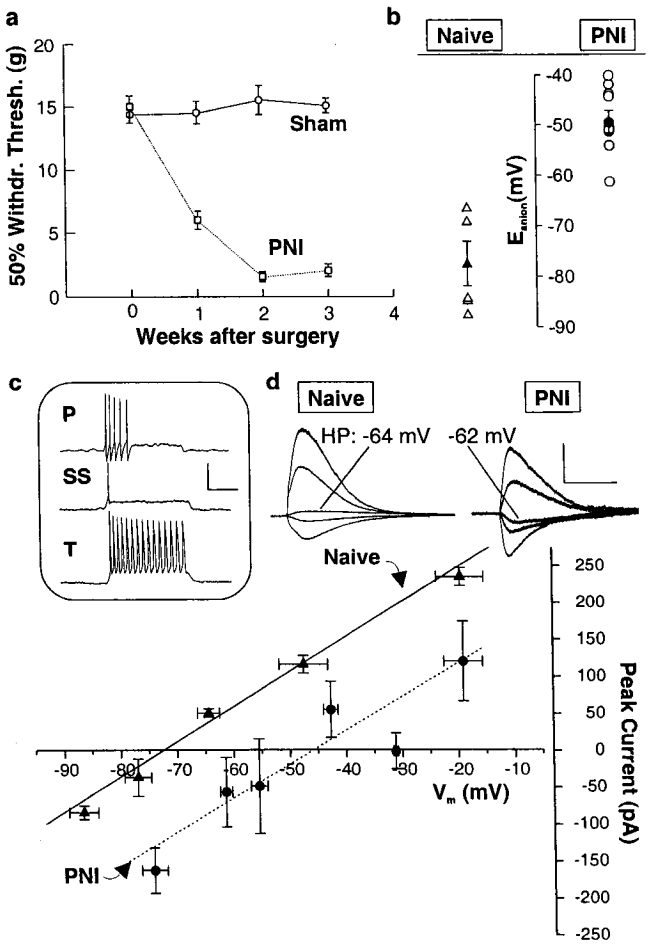

a
50% Withdr. Thresh. (g)
Sham
PNI
Weeks after surgery
b
Naive
PNI
E_{anion} (mV)
c
P
SS
T
d
Naive
PNI
HP: -64 mV
-62 mV
Naive
PNI
V_m (mV)
Peak Current (pA)

Figure 2. Switch from GlyR-only to mixed $GABA_AR$- and GlyR-mediated mPSCs following PNI in LI neurons. **a,b,** Pharmacological identification of outward (**left**) and inward (**right**) miniature synaptic events recorded at 0 mV and–60mV, respectively, from naïve (**a**) and PNI (**b**) rat LI neurons. Note the appearance of bicuculline-sensitive mPSCs in PNI rats. HP – Holding Potential. Scale(y,x)=20 pA,300 ms. **c, left** – Superimposed individual mPSCs recorded from PNI rat LI neurons. GlyR-only and $GABA_AR$-only and mixed $GABA_AR$/GlyR-mediated were clearly identifiable by their sensitivity to strychnine and/or bicuculline as previously described (Keller et al., 2001) (see also text). **Right** – Averages of > 100 GlyR- and $GABA_AR$-mediated mPSCs recorded from a PNI rat LI neuron. Scale(y,x)=15 pA,20 ms. **d,** Mean peak conductance of mPSCs recorded from naïve (N; $n = 10$ for GlyR; $n = 5$ for GluR) and PNI (P; $n = 9$ for GlyR; $n = 8$ for GluR) LI neurons. P(B) indicates GlyR-mediated mPSCs recorded in PNI rat LI neurons ($n = 12$) at 0 mV in the presence of bicuculline. **e,** Net charge carried by GlyR-mediated mPSCs in naïve rats ($n = 6$), by bicuculline-isolated GlyR-mediated mPSCs in PNI rats [PNI(Bic); $n = 4$], and by mixed $GABA_AR$/GlyR-mediated mPSCs in PNI rat LI neurons (PNI; $n = 12$). **f,** Cumulative probability plot of isolated GlyR-only mPSC inter-event interval (I.E.I.) in naïve LI neurons compared to PNI rat LI neurons [PNI(Bic)]. **Inset** – No effect of PNI on the frequency of GluR-mediated mPSCs.

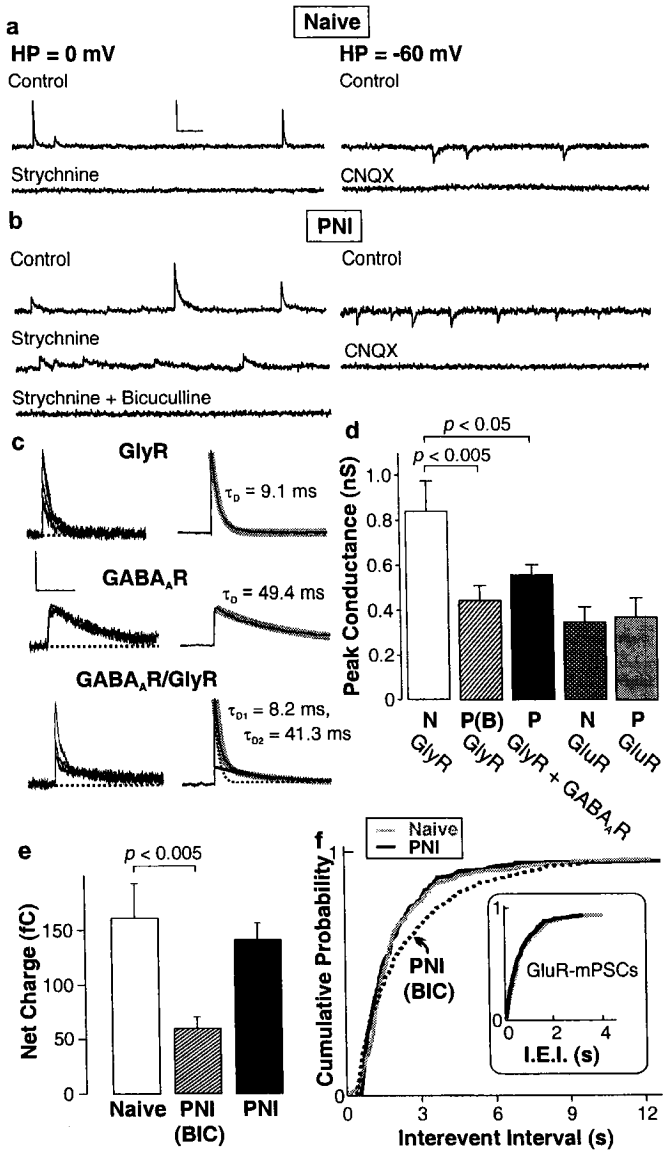

a
Naive
HP = 0 mV
Control
Strychnine
HP = -60 mV
Control
CNQX
b
PNI
Control
Strychnine
Strychnine + Bicuculline
Control
CNQX
c
GlyR
τ_D = 9.1 ms
GABA$_A$R
τ_D = 49.4 ms
GABA$_A$R/GlyR
τ_{D1} = 8.2 ms,
τ_{D2} = 41.3 ms
d
p < 0.05
p < 0.005
Peak Conductance (nS)
1.0
0.8
0.6
0.4
0.2
0
N GlyR
P(B) GlyR
P GlyR + GABA$_A$R
N GluR
P GluR
e
p < 0.005
Net Charge (fC)
150
100
50
0
Naive
PNI (BIC)
PNI
f
Naive
PNI
Cumulative Probability
1
0
PNI (BIC)
GluR-mPSCs
0
2
4
I.E.I. (s)
0
3
6
9
12
Interevent Interval (s)

Figure 3. PNI-induced downregulation of KCC2 in SDH lamina I neurons ipsilateral to PNI led to GlyR/$GABA_AR$-mediated excitation. a, Brief GABA application caused a TTX- and bicuculline-sensitive rise in $[Ca^{2+}]_i$ in a LI neuron from a Fura-2-AM loaded slice from a PNI rat. b, KCl, but not GABA application caused an increase in $[Ca^{2+}]_i$ in a naïve rat LI neuron. In the presence of the KCC2-specific antagonist DIOA, however, GABA application did elicit a rise in $[Ca^{2+}]_i$ in a naïve rat LI neuron. Scale(y,x)=0.02,10 s. c, Compared to control naïve rats, the proportion of LI neurons displaying a GABA-evoked elevation in $[Ca^{2+}]_I$ was significantly increased after PNI ($\chi^2_{corrected} = 3.91$) or in the presence of DIOA ($\chi^2_{corrected} = 4.43$),. d, Brief GABA application could repeatedly elicit action potentials in a LI neuron from a PNI rat. Upper scale(y,x)=5 mV,200 ms. Lower scale(y,x)=30 mV,4 s. Inset, response to a depolarizing pulse illustrating that this was a single-spike neuron (Prescott and de Koninck, 2002). Scale(y,x)=20 mV,300 ms. e, Focal electrical stimuli (in the presence of CNQX/APV) could elicit bicuculline-sensitive monosynaptic excitatory postsynaptic potentials in a PNI rat LI neuron. Scale(y,x)=5 mV,250 ms. Inset, response to a depolarizing pulse showing that this was a phasic neuron (Prescott and de Koninck, 2002). Scale(y,x)=20 mV,300 ms. f, Left, Immunoblotting revealed that KCC2 levels were decreased in the lumbar SDH lying ipsilateral (Ipsi), but not contralateral (Con), to the site of the PNI. Right, Quantification of KCC2 protein levels (normalized to actin) measured from immunoblots ($n = 4$) as in left (Ipsi normalized to Con).

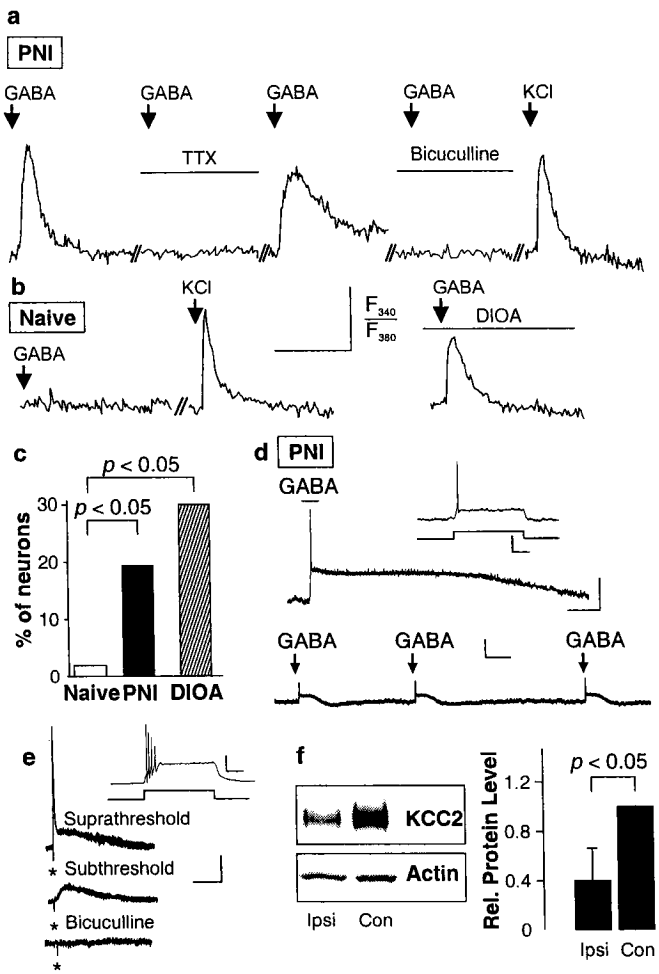
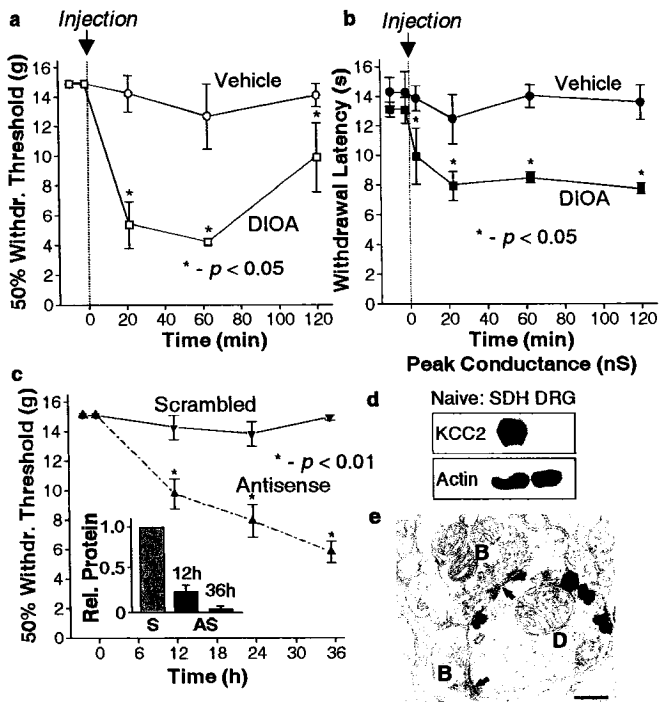

a
PNI
GABA
GABA
GABA
GABA
KCl
TTX
Bicuculline
b
Naive
KCl
GABA
GABA
DIOA
F340/F380
c
p < 0.05
p < 0.05
% of neurons
30
20
10
0
Naive
PNI
DIOA
d
PNI
GABA
GABA
GABA
GABA
e
Suprathreshold
Subthreshold
Bicuculline
f
KCC2
Actin
Ipsi
Con
Rel. Protein Level
1.2
0.8
0.4
0
p < 0.05
Ipsi
Con

Figure 4. Selective blockade or knock-down of the postsynaptic KCC2 exporter in the SDH significantly reduced nociceptive threshold. a, Tactile nociceptive withdrawal threshold as a function of time after intrathecal injections of DIOA (n = 5) or vehicle (n = 3). b, Thermal nociceptive withdrawal latency as a function of time after intrathecal injections of DIOA (n = 3) or vehicle (n = 3). Upon withdrawal, the rats often licked their paw indicating nociception. c, A significant decrease in the tactile nociceptive withdrawal threshold in naïve rats was observed following local lumbar spinal (intrathecal) administration of a KCC2 antisense (n = 8), but not scrambled (n = 7) oligodeoxynucleotide (0h, 12h & 24h). Inset, Decrease in spinal KCC2 protein levels (measured by immunoblots) following antisense (AS, 12h or 36h) or scrambled (S, 36h) oligodeoxynucleotide treatment. d, Lack of KCC2 immunoreactivity in dorsal root ganglia (DRG) in a naïve rat, compared to SDH. e, Electron micrograph illustrating the selective expression of KCC2 in SDH dendrites (D), but not synaptic boutons (B) (for quantitative details see Supplemental Information Fig.3). Arrows point to synapses. Scale=0.2μm.

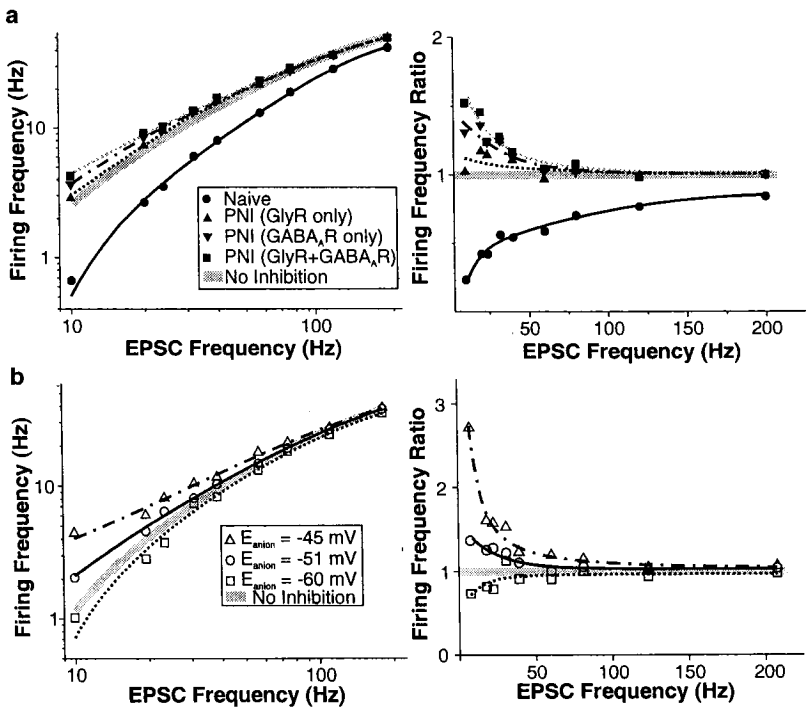

a
Injection
Vehicle
DIOA
* - p < 0.05
50% Withdr. Threshold (g)
Time (min)
b
Injection
Vehicle
DIOA
* - p < 0.05
Withdrawal Latency (s)
Time (min)
Peak Conductance (nS)
c
Scrambled
* - p < 0.01
Antisense
50% Withdr. Threshold (g)
Rel. Protein
12h
36h
S
AS
Time (h)
d
Naive: SDH DRG
KCC2
Actin
e
B
D
B

SUPPLEMENTARY FIGURES

Supp. Figure 1. Computer simulations of *in vivo* synaptic conditions confirmed that sensitization of lamina I neurons occurred as a function of the shift in the E_{anion}. **A, Left,** Computer simulations using a model neuron (see Methods below) demonstrated how PNI-induced changes to GlyR- and $GABA_AR$-mediated PSCs [PNI(GlyR+$GABA_AR$)] affect the output firing frequency of LI neurons as a function of GluR-mediated PSC frequency. Also shown is the result expected in LI neurons after PNI if only considering the effect of GlyR-mediated [PNI(GlyR-only)] or $GABA_AR$-mediated [PNI($GABA_AR$-only)] synaptic events. **Right** – Same data as shown in the left panel, but expressed in terms of firing frequency ratio, which was calculated as the quotient of a specific data set divided by the No Inhibition data set (*i.e.*, a firing frequency ratio of one is equivalent to no inhibition). While the normally hyperpolarizing GlyR-mediated PSCs (mean E_{anion} = -72.8 mV in naïve rats) had a net inhibitory effect on the output firing frequency (f_{out}), depolarizing GlyR-mediated PSCs (mean E_{anion} = -49.0 mV in PNI rats), enhanced f_{out} beyond that predicted to result with no inhibition, demonstrating a net excitatory effect. This excitatory effect was more prevalent when the $GABA_AR$ component was incorporated due to the increased charge carried by $GABA_AR$-mediated PSCs. **B, Left** – Effect of different values of E_{anion} (over the range observed in our study) on the firing frequency of a LI neuron after PNI. **Right** – Same data as left panel expressed in terms of firing frequency ratio (as above).

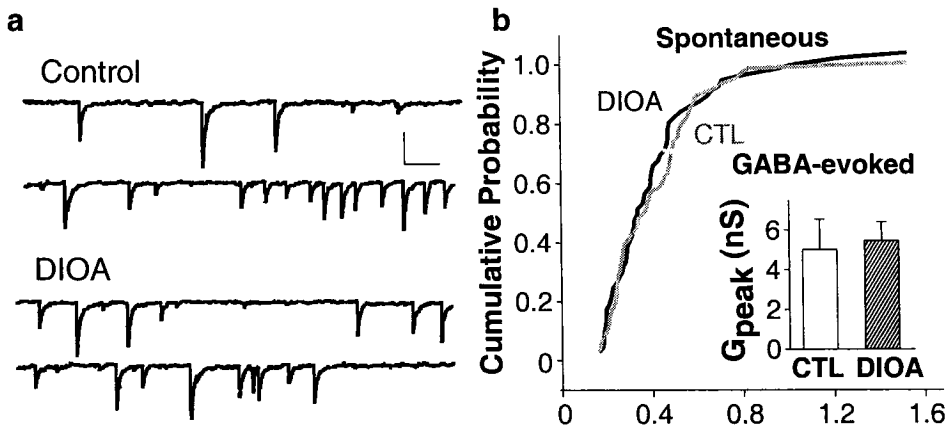
Methods

All simulations were performed with NEURON 4.3.1 using a compartment model of a generic spinal lamina I fusiform neuron with morphology and passive membrane properties based on (Prescott and de Koninck, 2002). Dendrites bifurcated up to fourth order and an axon similar to that described in (Mainen et al., 1995) were attached to the soma. Fast Na^+ and delayed rectifier K^+ currents based on (Traub and Miles, 1991) were inserted at 0.1 and 0.01 S/cm^2, respectively, in the soma and axon initial segment and nodes; voltage threshold for spiking was -49 mV. Two sets of inhibitory synapses were distributed randomly in the perisomatic region and four sets of excitatory synapses were more distal; each set was driven by an independent Poisson process at rates extrapolated from (Narikawa et al., 2000;Furue et al., 1999).


a
Firing Frequency (Hz)
10
1
10
100
EPSC Frequency (Hz)
Naive
PNI (GlyR only)
PNI (GABA$_A$R only)
PNI (GlyR+GABA$_A$R)
No Inhibition
Firing Frequency Ratio
2
1
50
100
150
200
EPSC Frequency (Hz)
b
Firing Frequency (Hz)
10
1
10
100
EPSC Frequency (Hz)
Δ E_{anion} = -45 mV
o E_{anion} = -51 mV
□ E_{anion} = -60 mV
No Inhibition
Firing Frequency Ratio
3
2
1
0
50
100
150
200
EPSC Frequency (Hz)

Supp. Figure 2. DIOA did not alter anion conductance. To ensure that the facilitation of the nociceptive reflex by intrathecal administration of DIOA was due to blockade of KCC2 and did not result from inhibition of $GABA_A$ or glycine receptor/channels, we tested the effect of DIOA on spontaneous and evoked $GABA_AR$/GlyR-mediated PSCs. To exclude any alterations in amplitude that would result from a change in E_{anion}, we performed whole cell recordings with CsCl filled pipettes to obtain symmetrical Cl^- concentrations (i.e. clamp E_{anion} at 0mV; see also Methods).

A, Spontaneous mPSCs recorded with a CsCl pipette in a LI neuron in the presence and absence of DIOA. Scale(y,x)=20pA,300ms. **B,** Cumulative probability plot ($n = 4$ neurons x 50 mPSCs) demonstrating that DIOA neither affected the peak conductance of synaptic events ($p > 0.5$), nor GABA-evoked responses ($n = 5$; $p > 0.5$, **Inset**) and therefore does not act on GlyRs nor $GABA_ARs$. G_{peak} = peak conductance.


a
Control
DIOA
b
Spontaneous
DIOA
CTL
GABA-evoked
Cumulative Probability
1.0
0.8
0.6
0.4
0.2
0
0
0.4
0.8
1.2
1.6
Gpeak (nS)
6
4
2
0
CTL
DIOA

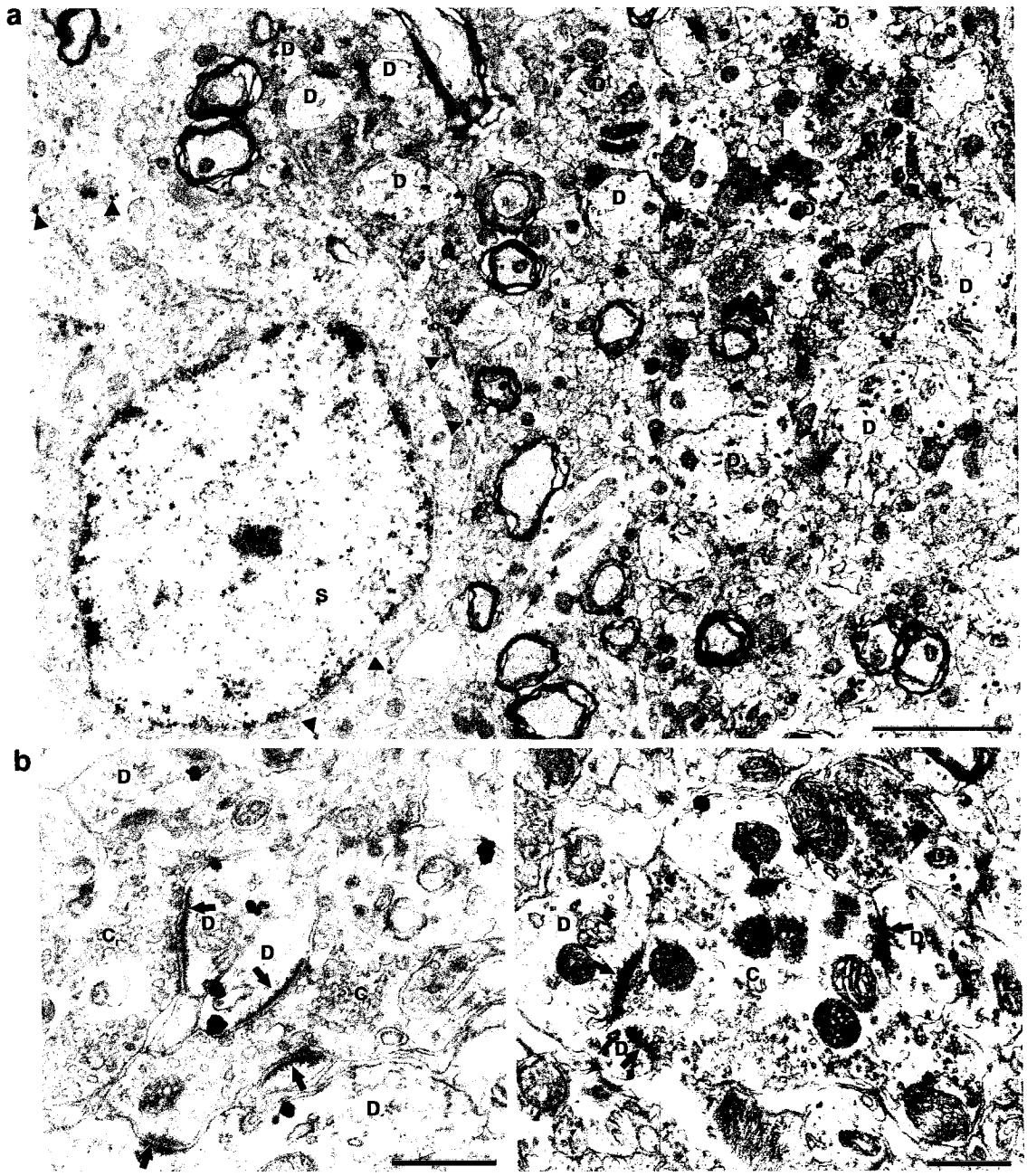
Supp. Figure 3. KCC2 exporter expression is restricted to dorsal horn neurons, not sensory fibres. Although the KCC2 levels are below detection by immunoblotting from DRG (Fig. 4f), we verified whether KCC2 could be preferentially shuttled away from cell bodies to central terminals of primary afferents.

A, Electron micrograph illustrating the presence of KCC2 on dendrites (D) in lamina I of the dorsal horn. Membrane-delimited immunogold staining on the soma (S) of a lamina I neurons is also shown (arrowheads). In contrast, no KCC2 immunostaining was observed in any of the randomly selected synaptic profiles examined (n = 171). **B**, KCC2 immunoreactiticity was also absent from central boutons (n = 42 randomly selected central boutons) of synaptic glomeruli in laminae I and II (type I: C_I; left; type II: C_{II}; right; arrows indicate excitatory synapses, D: dendrite) that unequivocally correspond to central terminals of primary afferents [A- and C-fibres (Ribeiro-da-Silva and Coimbra, 1982;Ribeiro-da-Silva, 1995)]. Scale bars: a: 2 μm; b; 0.5 μm (left), 0.2 μm (right).

Methods

Tissue was prepared for ultrastructural analysis as previously described (Sik et al., 2000). Briefly, rats were perfused through the aortic arch with 0.9% NaCl followed by a fixative solution containing 4% paraformaldehyde (Sigma-Aldrich, Germany). After perfusion, spinal cords were removed, coronal blocks were dissected, then 60μm thin sections were cut cryoprotected and freeze-thawed over liquid nitrogen and rinsed several times in phosphate buffer before incubation in the primary antiserum. After incubation in blocking solution containing 2% bovine serum albumin (BSA), sections were incubated in rabbit anti-KCC2 (1:500, Upstate Biotechnology, USA) for 48 hours at 4 °C. After extensive washing, sections were incubated with 1 nm gold-conjugated anti-rabbit secondary antibody (1:250, Aurion) for 12 hours at 4 °C followed by silver intensification (SE-EM, Aurion). Sections were treated with 0.5% OsO_4 (20 min), dehydrated in graded ethanol, then in propylene oxide and embedded in Durcupan ACM (Fluka). After ultra-sectioning (Ultracut UCT, Leica, Germany), specimens were examined using an electron microscope (Philips Tecnai 12, equipped with MegaView CCD camera). Non-consecutive (spacing > 3 μm) ultrathin sections were analyzed in the electron microscope.

Boutons with synaptic profiles were randomly selected and analyzed in laminae I & II and white matter for the expression of the KCC2 protein (Gulyas et al., 2001).


a
D
S
b
C

PREFACE TO CHAPTER 4

In the following chapter, I present the findings of my investigation into the upstream mechanisms responsible for the disruption of anion homeostasis in LI neurons after PNI.

I initially focus on brain-derived neurotrophic (BDNF) factor for three reasons:

(a) The apparent return of inhibitory receptor configuration at LI synapses to that found at developmentally immature synapses suggests a possible role for a growth factor; TrkB (BDNF receptor) is expressed postsynaptically by LI neurons, whereas TrkA and TrkC are not (Zhou et al., 1993);
(b) A recent study in the hippocampus reported that BDNF activation of TrkB could rapidly trigger a downregulation of KCC2 (Rivera et al., 2002);
(c) BDNF has been reported by several studies to evoke hyperexcitability in SDH neurons (Thompson et al., 1999), and BDNF sequestration before and after PNI has been reported to attenuate some types of nociceptive hypersensitivity (Groth and Aanonsen, 2002;Kerr et al., 1999).

Therefore, I endeavoured to examine the role of TrkB activation by BDNF in LI neurons in the alteration of transmembrane anion gradient for the first part of my analysis. For the second part, I performed experiments aimed at discovering the source of BDNF.

My initial hypothesis was that BDNF was released from primary afferents after PNI. However, a very recent study suggested that after PNI there was no increase in BDNF release from primary afferents (Lever et al., 2003); moreover, it became clear that stimulus-evoked BDNF release from afferents could not be sustained to the same degree as the disruption of anion gradient, which was shown early to require tonic TrkB (Lever et al., 2001).

Therefore, I looked at microglia as a source of BDNF. Microglia have recently been shown to be critical in the induction of nociceptive hypersensitivity (Tsuda et al., 2003),

and, in the hyperactive phenotype, have been documented to upregulate BDNF materially.

CHAPTER 4

TONIC REPRESSION OF SPINAL INHIBITION BY MICROGLIA-DERIVED BDNF AS A SUBSTRATE OF NEUROPATHIC PAIN

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To be submitted for publication.

ABSTRACT & INTRODUCTION

Following peripheral nerve injury, microglia in the spinal dorsal horn (SDH) have been implicated in the induction of allodynia (Inoue et al., 2004;Tsuda et al., 2003). However, the mechanisms by which microglia mediate such nociceptive hypersensitivity remain unclear. Given our recent demonstration that an alteration of anion homeostasis in pain-related SDH neurons leads to dramatic hyperexcitability, we investigated whether a similar mechanism may explain microglia-evoked hypersensitivity. We report that in spinal slices taken from rats in which the prior spinal delivery of ATP-stimulated microglia – but not resting microglia – caused significant allodynia, the *trans*membrane anion gradient of lamina I (LI) neurons was determined to be significantly decreased. This decrease of anion gradient occurred to the extent that GABA-mediated synaptic responses were depolarizing – similar to the effect of GABA on LI neurons from rats that received a nerve injury. Administration of anti-TrkB antibodies to both nerve injured- and stimulated microglia-injected rats attenuated both the concomitant allodynia, as well as the underlying alteration of anion gradient in LI neurons suggesting that the suppression of spinal inhibition required the tonic activation of TrkB. Additionally, knock-down of BDNF in ATP-stimulated microglia using interfering RNA reduced allodynia in rats that subsequently received intrathecal injection of these microglia. Taken together, these results demonstrate that microglia activated by nerve injury contribute to the ensuing nociceptive hypersensitivity by triggering a collapse of anion gradient in LI neurons via the release of BDNF.

RESULTS

Experimental peripheral nerve injury (PNI) elicits a significant reduction of 50% withdrawal threshold to mechanical stimulation (WD_{50}) in rats (Fig. 1A). In many models of PNI, including our adopted model of loose sciatic ligation (Mosconi and Kruger, 1996), nociceptive hypersensitivity may be consequential to a strong activation of microglia in the superficial dorsal horn (Fig. 1C). To investigate the mechanisms by which activated spinal microglia trigger allodynia in rats, we administered either ATP (50 μM)-stimulated or resting microglia (30 μl: 1000 cells/μl) locally via intrathecal catheter to naïve rats ($WD_{50} \geq 15$ g). As shown in Fig. 1D, the WD_{50} of rats that had been administered ATP-stimulated – but not resting – microglia was confirmed to progressively drop, reaching a minimum of 4.6 ± 1.6 g after 5 hours, a value significantly lower than the initial WD_{50} of 14.7 ± 0.3 g ($n = 8$; $p < 0.05$).

To assess whether the development of allodynia could result from a shift in the anion gradient of pain-related lamina I (LI) neurons, we sacrificed the microglia-injected rats and used perforated-patch and whole-cell clamp recording techniques to measure the E_{anion} of LI neurons in slice. For LI neurons taken from rats injected with resting microglia, the E_{anion} was measured to be -68.3 ± 1.8 mV ($n = 6$), not significantly different from that recorded from LI neurons from non-injected naïve rats (-73.2 ± 4.0 mV, $n = 9$, $p > 0.5$, Fig. 1E,F). However, LI neurons from rats that had developed allodynia in response to activated microglia administration displayed a significantly altered E_{anion} of -61.6 ± 1.1 mV ($n = 16$) – high enough that brief applications of GABA to neurons at resting potential often evoked depolarization, in contrast to neurons under control conditions, where GABA was always hyperpolarizing from rest (Fig. 1E).

In a recent work that documented the potential of activated microglia to trigger allodynia in rats, it was shown that the stimulation of $P2X_4$ receptors expressed by microglia was necessary to prime these cells to evoke and sustain nociceptive hypersensitivity (Tsuda et al., 2003). To test whether the requirement of $P2X_4$ receptor activation for nociceptive hypersensitivity arising from PNI may derive from an indirect modulatory action on the

E_{anion} of LI neurons, we applied the $P2X_4$ antagonist TNP-ATP (1 μM) to spinal slices taken from rats that had developed allodynia ($WD_{50} \leq 2$ g) in response to PNI. In the presence of TNP-ATP, the E_{anion} of LI neurons was determined to be -59.3 ± 1.8 mV (n = 6), significantly more negative than that measured from control LI neurons taken from PNI rats (-49.3 ± 4.5 mV, $n = 6$, $p < 0.05$, see Supplemental Material). Application of the non-selective microglia inhibitor minocycline (50 μM), on the other hand, failed to alter the E_{anion} in LI neurons taken from PNI rats (-47.2 ± 0.6 mV, $n = 4$, $p > 0.5$).

We next addressed the question of what factors mediate the propensity of activated microglia to modify E_{anion} in LI neurons. Of the many substances secreted by activated microglia, few have been demonstrated to be important for the induction of hyperexcitability in dorsal horn neurons after PNI. However, a neurotrophin that is persistently released from activated spinal microglia (Dougherty et al., 2000), brain-derived neurotrophic factor (BDNF), has been well-documented to play a critical, yet unclear role in this process (Thompson et al., 1999). We therefore hypothesized that BDNF may catalyze the influence of microglia on the E_{anion} of LI neurons. In a first test of this hypothesis, we assessed if serial administration of exogenous human recombinant BDNF to rats via intrathecal catheter would replicate the allodynia observed to result from injection of activated microglia.

As shown in Figure 2A, rats that were given repeated intrathecal injections of BDNF (10 μg/day) quickly developed a robust allodynia – the WD_{50} after 24 hrs being significantly less than that measured in rats prior to injection [BDNF: pre-injection, 15.0 ± 0 g, 24 hrs, 6.8 ± 0.9 g ($p < 0.05$), 48 hrs, 6.0 ± 1.3 g ($p < 0.05$); $n = 4$]. Interestingly, the minimum WD_{50} of rats that had received BDNF (after 4 days) was not significantly different than that measured in rats that had received ATP-stimulated microglia (after 5 hours; $p > 0.5$).

To test if BDNF-induced allodynia could result from a depolarization of the E_{anion} of LI neurons, we incubated spinal slices taken from naïve rats in BDNF (50 ng/ml), and subsequently measured the steady-state (> 90 minutes BDNF) E_{anion} of LI neurons by analyzing responses to brief puffs (5-15 ms) of GABA (1mM). We found that the E_{anion}

of LI neurons incubated in BDNF was significantly less negative than that measured neurons under control conditions (BDNF, –52.9 ± 4.7 mV, $n = 9$, *vs.* control, -73.2 ± 4.0 mV, $n = 9$; $p < 0.005$). Interestingly, this shift commenced after as little as 60 minutes incubation in BDNF, and at steady-state was often substantial enough to invert the polarity of anion driving force from negative values (hyperpolarizing from rest) to positive values (depolarizing from rest).

Another consequence of these depolarizing GABA-mediated postsynaptic currents was the activation of voltage-sensitive calcium mechanisms in LI neurons (Fig. 2F). Specifically, calcium imaging of Fura-2-AM-loaded LI neurons in slices taken from naïve rats and exposed to BDNF (50 ng/ml) demonstrated that 26% of neurons ($n = 5$ of 16 neurons responding to KCl-mediated depolarization) responded to exogenous GABA application with a bicuculline-sensitive (10 μM; $n = 18$) influx of Ca^{2+}. This finding is contrasted to control conditions, where GABA triggered Ca^{2+} influx in but 1 of 37 neurons ($\chi^2_{corrected} = 6.45$, $p < 0.05$).

Our finding that exogenous BDNF triggered both an alteration of E_{anion} in LI neurons and nociceptive hypersensitivity in intact rats does not directly indicate that BDNF is necessary or sufficient to mediate nociceptive hypersensitivity in rats that have received PNI. Nor does this finding address the question of whether tonic activation of TrkB receptors by BDNF is necessary for the maintenance of these two phenomena in PNI rats. To more appropriately address these points, we first performed serial spinal delivery of a specific antibody that blocks TrkB receptors (Jiang et al., 2003), anti-TrkB, to PNI rats via intrathecal catheter. After as little as 2 hours (injections of 12 μg anti-TrkB every 2 hours), the WD_{50} of the PNI rats rose significantly from an initial value of 2.3 ± 0.2 g to 8.7 ± 1.4 g ($n = 17$, $p < 0.05$; Fig. 3A). PNI rats that had received serial injections of saline showed no such change in WD_{50} after 2 hours (2 hrs, saline, 4.3 ± 0.8 g *vs.* pre-injection, 2.1 ± 0.4 g; $n = 4$, $p < 0.05$).

In spinal slices from PNI rats, the administration of anti-TrkB antibodies (1 μg/ml) was further shown to hyperpolarize the E_{anion} of LI neurons to –59.2 ± 3.2 mV ($n = 7$), a

significant 10 mV hyperpolarizing shift in E_{anion} compared to that measured under control conditions from LI neurons taken from PNI rats (-49.3 ± 4.5 mV, $n = 6, p = 0.0494$; Fig. 3B,C). A more dramatic shift was observed with the use of the less selective receptor tyrosine kinase inhibitor, K252a (200 nM): LI neuronal E_{anion} in the presence of K-252a was measured to be -79.6 ± 5.9 mV ($n = 7, p = 0.0001$; data not shown). Both anti-TrkB-IgG (Fig. 3B) and K-252a perfusion led to GABA-evoked anion currents that were hyperpolarizing from rest, whereas GABA-evoked anion currents were depolarizing from rest in control LI neurons taken from PNI rats.

In a final set of experiments we examined whether inhibition of BDNF/TrkB signaling was sufficient to prevent nociceptive hypersensitivity in rats, as well as the underlying alteration of LI neuronal E_{anion}, triggered by ATP-stimulated microglia. To test this hypothesis, we first injected rats with both ATP-stimulated microglia and anti-TrkB antibodies by intrathecal catheter. This cocktail did not evoke any change in nociceptive threshold to mechanical stimulation after 5 hours, compared to the pre-injection WD_{50} (ATP + anti-TrkB, 15.0 ± 0 g *vs.* pre-injection, 15.0 ± 0 g; $n = 8, p > 0.5$; Fig. 4A).

To confirm that this effect resulted from a blockade of TrkB activation by BDNF directly released from microglia, we knocked-down BDNF in microglia via lipofection of anti-BDNF interfering RNA. Following BDNF deletion and incubation with ATP, microglia were injected intrathecally into intact rats. This preparation failed to evoke a significant drop in WD_{50} after five hours (ATP + anti-BDNF siRNA, 14.6 ± 0.4 g *vs.* pre-injection, 15.0 ± 0 g; $n = 7, p > 0.5$), suggesting that BDNF release from microglia is necessary to trigger nociceptive hypersensitivity. To confirm that it was not the siRNA itself that prevented the development of allodynia in response to ATP-stimulated microglia, we incubated microglia with a scrambled version of the siRNA. Upon stimulation with ATP, this preparation was injected into rats, and after five hours elicited a significant allodynia (scrambled siRNA: pre-injection, 15.0 ± 0 g *vs.* 5 hours, 6.9 ± 2.4 g; $n = 4, p < 0.05$; Fig. 4A).

Does the failure of ATP-stimulated microglia to effect nociceptive hypersensitivity when in the presence of anti-TrkB antibodies or anti-BDNF siRNA result from an inability to modulate E_{anion} in LI neurons? We answered this question by assessing the E_{anion} in LI neurons taken from both rats that had received injections of the ATP-stimulated microglia / anti-TrkB cocktail and ATP-stimulated microglia where BDNF had been knocked-down. As shown in Figures 4B and C, the E_{anion} measured from LI neurons taken from the former group of rats was not significantly different from that measured from LI neurons taken from control naïve rats (ATP + microglia + anti-TrkB, -70.0 ± 1.5 mV, n = 8 *vs.* naïve, -73.2 ± 4.0 mV, $n = 9$; $p > 0.5$). Similarly, the E_{anion} measured from LI neurons taken from rats that had received ATP-stimulated microglia exposed to anti-BDNF siRNA, -66.7 ± 1.1 mV ($n = 7$), was not significantly different from that recorded from control naïve LI neurons ($p > 0.1$; Fig. 4B,C).

To be certain that both the anti-BDNF siRNA and the anti-TrkB antibodies attenuated the propensity of microglia to modulate LI neuronal E_{anion} via an interruption of BDNF signaling, and not via an inhibition of the ability of ATP to stimulate microglia, we performed control calcium imaging experiments. In the first set of experiments, we administered puffs of ATP (5 ms) to primary cultures of microglia loaded with the fura-2-AM indicator, in the presence or not of anti-TrkB. As shown in Fig. 4D, there was no significant difference between the proportion of microglia that displayed an increase in $[Ca^{2+}]_i$ to ATP application in the presence of anti-TrkB (17 of 21), and the proportion that showed this increase under control conditions (19 of 27; $\chi^2 = 0.7$, $p > 0.5$). Similarly, no fewer microglia showed increased $[Ca^{2+}]_i$ to ATP in the presence of anti-BDNF siRNA (12 of 14) compared to control conditions (18 of 18; $\chi^2_{corr} = 0.85$, $p > 0.5$). Additionally, neither anti-TrkB nor anti-BDNF siRNA affected the amplitude or area of ATP-evoked calcium fluxes in microglia (inset, Fig. 4D).

DISCUSSION

These results demonstrate that BDNF tonically released from microglia, whether activated by exogenous ATP or PNI, triggers a shift in the E_{anion} of LI neurons. The shift of E_{anion} in these pain-related neurons translates to an inversion of the influence of local GABAergic/glycinergic interneurons, from hyperpolarizing to depolarizing – therefore making the LI neurons hyperexcitable and triggering nociceptive hypersensitivity. Although a link between microglia and nociceptive hypersensitivity has been previously established (Inoue et al., 2004;Tsuda et al., 2003), this report provides the mechanistic link between activated microglia and a mechanism that, in and of itself, is known to generate hyperexcitability in spinal neurons (Coull et al., 2003). Moreover, we identify the diffusible messenger by which microglia influence the anion gradient of LI neurons to be BDNF.

BDNF has been previously documented to be critical for the central neuroplasticity that follows PNI (Thompson et al., 1999), BDNF sequestration causing an attenuation of the development (Yajima et al., 2002), and even the maintenance (Kerr et al., 1999), of nociceptive hypersensitivity. As confirmed by our experiments, it further appears that tonic activation of TrkB receptors by BDNF is required for the sustenance of central sensitization. This contention is well supported by previous work that demonstrated that the regional increase in the expression of BDNF in the SDH after PNI well correlates with the expression of nociceptive hypersensitivity (Miletic and Miletic, 2002). The continuous release of BDNF by activated spinal microglia (Dougherty et al., 2000), in contrast to stimulus-evoked BDNF release by sensory afferents (Lever et al., 2001), would seem to fulfill this requirement for tonic TrkB activation in LI neurons. Moreover, the potentiation of voltage-sensitive calcium mechanisms that is known to be critical for central sensitization may be largely attributed to TrkB activation by microglia-derived BDNF. This statement is confirmed by the observation that both BDNF and ATP-stimulated microglia were shown here to evoke a shift in LI neuronal E_{anion} that was sufficient to enable GABA-mediated calcium influx, and by the previous reports that both activated microglia and exogenous BDNF may effect a similar potentiation of NMDA responses (Moriguchi et al., 2003;Kerr et al., 1999;Groth and Aanonsen, 2002).

In the context of PNI-evoked neuroplasticity, BDNF released from microglia may be viewed as *too much of a good thing*. It is clear that BDNF of neuronal origin is required for the normal tuning of inhibitory synapses in the brain (Wardle and Poo, 2003) and spinal cord (Skup et al., 2002); indeed, patterns of stimulation known to trigger long-term postsynaptic plasticity have been demonstrated to elicit the release of BDNF from primary afferents in the SDH (Lever et al., 2001). However, it appears that only brief activation of TrkB receptors is necessary for normal plasticity, as the application of BDNF sequestering antibodies has been documented to attenuate only the induction of long-term plasticity, having no effect on maintenance (Chen et al., 1999). In contrast, the pathophysiological repression of inhibition may require the repetitive activation of TrkB receptors: TrkB inhibition by application of a neutralizing antibody (anti-TrkB) was shown here to rapidly attenuate nociceptive hypersensitivity, as well as decreases in LI neuronal anion gradient – stemming from nerve injury. This finding highlights the appeal of targeting microglia-derived BDNF as a means to effect therapeutic intervention of neuropathic pain, rather than manipulating all BDNF action, because it represents a strategy to eliminate processes catalyzing the disease, while leaving processes (*i.e.* neuronal pools of BDNF) critical for normal neuronal function intact.

METHODS

Behavioural Studies. Except where indicated, all drugs and reagents were obtained from Sigma (St. Louis, MO). Peripheral nerve injury was induced by surgically implanting a polyethylene cuff (~ 2 mm in length, inner diameter 0.7 mm) around the sciatic nerve of adult male Sprague–Dawley rats, as previously described (Mosconi and Kruger, 1996). A group of rats also received sham surgery. The 50 % withdrawal threshold to mechanical stimulation was assessed as previously described (Chaplan et al., 1994a). Subsequent to nerve injury, only animals that showed a gradual decrease in mechanical threshold (over 14–17 days) down to 2 g or less were used for further experiments.

Slice preparation. Parasagittal slices (300–350 μm) of spinal cord were prepared from adult (> 50 days old) male rats as previously described. Slices were continually

superfused (2–3 ml min^{-1}) with artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 26 $NaHCO_3$, 10 glucose, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4 (bubbled with 95% O_2/5% CO_2, pH ~ 7.4).

Recordings. For perforated-patch recordings, the pipette tip was filled with a solution containing (in mM): 130 caesium gluconate, 5 CsCl, 2 $MgCl_2$, 11 BAPTA, 1 $CaCl_2$, 4 ATP, 0.4 GTP, 10 HEPES (~pH 7.4). The pipette was back-filled with this same solution supplemented with 25 μg ml^{-1} gramicidin D [gramicidin stock was at 10 mg ml^{-1} in dimethylsulphoxide (DMSO)]. Recordings in this mode were selected when access resistance was stable between 25–45 MΩ. For whole-cell voltage-clamp recordings, pipettes were filled with the above solution without gramicidin D. For whole-cell current-clamp recordings, pipettes were filled with the same intracellular solution as for voltage clamp, except that potassium methyl sulphate ($KMeSO_4$) was used instead of caesium gluconate. GABA was applied locally for 10–100 ms by pressure ejection through a micro-pipette. Data acquisition and analysis of PSCs were performed as previously described (Keller et al., 2001); membrane potential measurements were corrected pursuant to (Tyzio et al., 2003). Neither input resistance nor resting membrane potential of LI neurons was affected by any of the drugs or protocols used in this study. All measurements are given as means ± SEM, except where indicated. Statistical significance was tested using Student's *t*-tests for comparison of mean values, chi-squared tests for contingency tables, and mixed-design analyses of variance (post-hoc Tukey's HSD test) for repeated measures.

Immunohistochemistry. Immunohistochemistry was performed on perfused, free-floating sections. OX-42 (Cedarlane, 1:1000) which labels CR3/CD11b was used as a specific marker for microglia. After overnight incubation at 4°C with the primary antibody, sections were rinsed and incubated with biotinylated anti-mouse IgG (1:1000) for 1h at room temperature. Sections were then rinsed again and immersed for 1h in an avidin-biotin-peroxydase complex (Vector Laboratories). Finally positive labelling was visualized with 0.05% 3,3'-diaminobenzidine (DAB) containing 0.003% hydrogen peroxide.

Microglial cultures. Rat primary cultured microglia were prepared under standard conditions as described previously (Nakajima et al., 1992). In brief, mixed glial culture was prepared from neonatal Wistar rats and maintained for 10–16 days in DMEM medium with 10% fetal bovine serum. Microglia were separated from the primary culture by gentle shaking of the flask and replated on plastic dishes. The cells were removed from the dish surface using a cell scraper and collected in 100ul of PBS; subsequently, the cell density of microglia was measured using a cell counter and the volume of PBS adjusted to give a final density of 1000 cells/10μl. This method produces microglia cultures of >95% purity.

Intrathecal injections. At least three days before drug administration, rats were anaesthetized with sodium pentobarbital (65 mg kg^{-1}), and a lumbar spinal catheter (PE-10 polyethylene tube) was inserted into the intrathecal space, as previously described (Yaksh et al., 1980). On recovery from surgery, lower-body paralysis was induced through intrathecal lidocaine (2%, 30 μl) injection to confirm proper catheter localization. Only animals exhibiting appropriate, transient paralysis to lidocaine, as well as a lack of motor deficits, were used for behavioural testing. Following drug/vehicle administration, animals were killed and their vertebral column dissected to visually confirm correct placement of the catheter. Drugs included BDNF (10 μg/day) and anti-TrkB (12 μg each 2 hrs), both of which were prepared in saline + 10% (v/v) DMSO. At the doses used, none of the compounds produced motor disturbances or sedation, as assessed by grasping, righting and placing reflexes and behavioural observations (Coderre and Melzack, 1992). For experiments in which microglia were lipofected with small interfering RNA (siRNA), anti-BDNF and scrambled siRNA were obtained from Dharmacon Inc. The BDNF siRNA consisted of four pooled 21-nucleotide duplexes. The sequences of the four duplexes were as follows (Baker-Herman et al., 2004):

1) TCGAAGAGCTGCTGGATGA
2) TATGTACACTGACCATTAA
3) GAGCGTGTGTGACAGTATT

4) GAACTACCCAATCGTATGT

Microglia cultures were transfected with BDNF or scrambled siRNA with Lipofectamine 2000 following the manufacturer's instructions. Briefly, siRNA and lipofectamine were diluted in serum-free medium, mixed and added to the microglial cultures. Transfection was allowed to occur for 5 hours and the microglia collected as above for subsequent intrathecal injection.

In all cases, 30 μl microglia + supernatant were injected intrathecally in normal rats.

Calcium imaging. Primary cultures of microglia were prepared as above, transferred to standard cover slips and incubated with 10 μM Fura-2-AM in HEPES-buffered saline (+ 10% DMSO) for 1 h. Following fluorophore loading, calcium influx in individual microglia was evoked using brief (~5 ms) applications of ATP (12.5 – 50 μM) from a micro-pipette. $[Ca^{2+}]_i$ was fluorometrically measured using a X40 water-immersion objective on a Zeiss Axioscope equipped with epifluorescence optics. Images were acquired using a TILL Photonics monochromator coupled to a CCD camera, and regions of interest (for ratioing) were drawn on clearly distinct neuronal cell bodies.

FIGURES

Figure 1. ATP-stimulated microglia delivered to rats via intrathecal catheter evoked nociceptive hypersensitivity and a shift in the LI neuronal *trans*membrane anion similar to those triggered by PNI. *A*, Following PNI, but not sham surgery, the 50% withdrawal threshold to mechanical stimulation (WD_{50}) of adult rats dropped significantly over the course of 2-3 weeks. *B*, The mean anion reversal potential (E_{anion}) measured from LI neurons taken from PNI rats ($WD_{50} \leq 2$ g) was significantly less negative than that recorded from the LI neurons of naïve rats. V_M – membrane potential. *C*, Micrographs illustrating that OX-42 staining (indicative of activated microglia) is much more intense in the ipsilateral dorsal horn of PNI rats (right) compared to sham-operated rats (left). Scale bar is 0.2 mm; SDH ipsi. – superficial dorsal horn ipsilateral to PNI. *D*, In naïve rats, local spinal delivery of ATP-stimulated microglia, but not resting microglia, caused a significant decrease in the mean WD_{50}. *E*, Representative current-voltage plots from two lamina I neurons, one taken from a rat with $WD_{50} = 3.4$ g in response to the intrathecal injection of ATP-stimulated microglia, the other taken from a rat that showed no significant change in WD_{50} (12.6 g) after injection of resting microglia. Note that the E_{anion} (*x*-intercept) for the LI neuron from the allodynic rat is significantly less negative than that taken from the non-allodynic rat. V_M – membrane potential; MG – Microglia. *Inset*, Representative traces showing that, at resting membrane potential, the postsynaptic response to GABA was depolarizing in the LI neuron taken from rat with $WD_{50} = 3.4$ g, compared to the neuron from the rat with $WD_{50} = 12.6$ g, where GABA was hyperpolarizing. *F*, Bar graph comparing the mean E_{anion} recorded from the LI neurons of naïve, PNI, resting microglia- (MG – ATP) and ATP-stimulated microglia- (MG + ATP) injected rats.

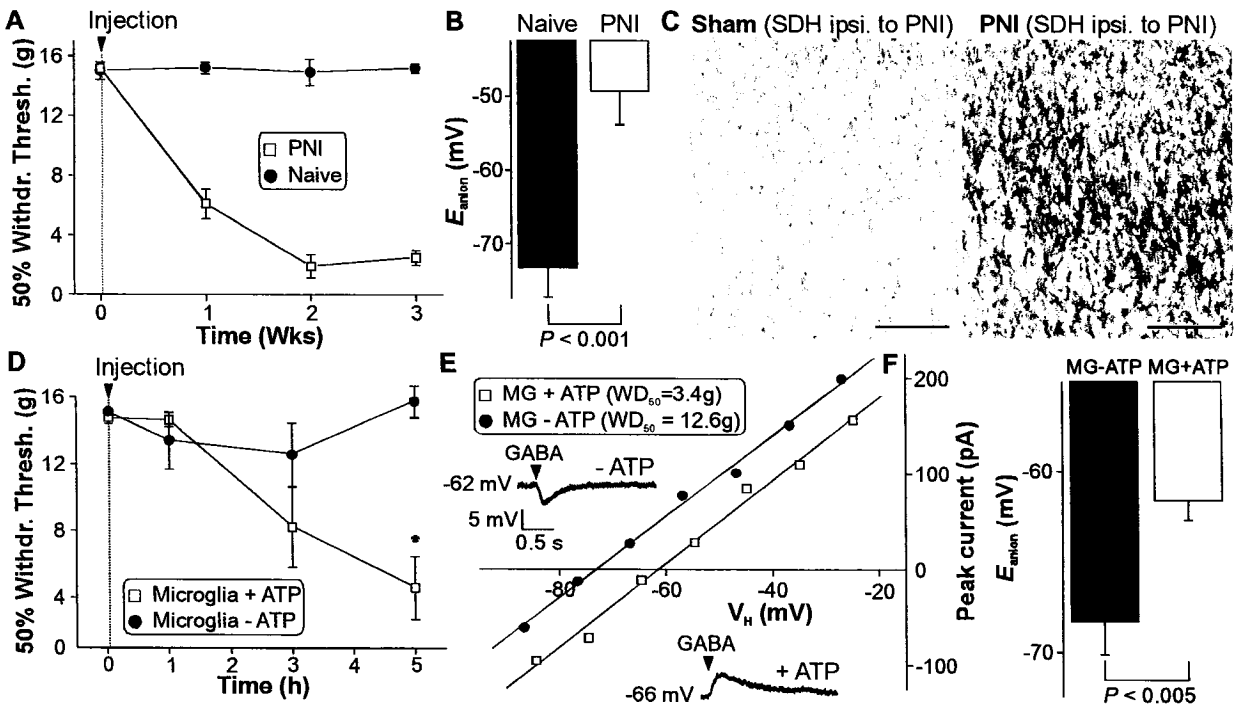

A
Injection
50% Withdr. Thresh. (g)
16
12
8
4
0
PNI
Naive
0
1
2
3
Time (Wks)
B
Naive
PNI
E_{anion} (mV)
-50
-60
-70
P < 0.001
C
Sham (SDH ipsi. to PNI)
PNI (SDH ipsi. to PNI)
D
Injection
50% Withdr. Thresh. (g)
16
12
8
4
0
Microglia + ATP
Microglia - ATP
0
1
2
3
4
5
Time (h)
E
MG + ATP (WD_{50}=3.4g)
MG - ATP (WD_{50} = 12.6g)
GABA
-62 mV
- ATP
5 mV
0.5 s
-80
-60
-40
-20
V_H (mV)
GABA
+ ATP
-66 mV
F
200
100
0
-100
Peak current (pA)
MG-ATP
MG+ATP
E_{anion} (mV)
-60
-70
P < 0.005

Figure 2. Serial spinal administration of BDNF to rats elicited nociceptive hypersensitivity via a shift in the *trans*membrane anion gradient of lamina I neurons. *A*, Serial intrathecal delivery of recombinant human BDNF (10 □g / day) to the lumbar dorsal horn of intact rats led to a significant decrease in the WD_{50} after 24 and 48 hours, compared to saline control where there was no significant decrease. *B*, Peak currents measured from lamina I neurons under conditions of ACSF (control) or BDNF perfusion and in response to exogenous GABA application at various values of membrane potential (V_M). *Inset*, representative traces that demonstrate that brief GABA application (5-30 ms) to lamina I neurons perfused with rhBDNF could elicit a depolarizing postsynaptic potential from resting membrane potential (V_{rest}; shown on the left). In control lamina I neurons GABA application always triggered hyperpolarizing postsynaptic potentials. *C*, Incubation of slices with BDNF (*cf.* ACSF) triggered a significant depolarization of mean E_{anion} in lamina I neurons taken from naïve rats. *D*, Representative plot demonstrating that bath-applied BDNF could trigger a shift in E_{anion} in lamina I neurons in as little as 60 minutes. *E*, The shift in E_{anion} triggered by BDNF application translated into a reversal in the polarity of anion driving force (DF = E_{anion} - resting membrane potential). *F*, Representative traces showing that brief GABA application to Fura-2-AM-loaded LI neurons in the presence of BDNF could cause a bicuculline-sensitive increase in intracellular calcium ($[Ca^{2+}]_i$). The viability of cells not responding to GABA was confirmed via KCl-mediated depolarization. *Bottom right*, The proportion of lamina I neurons showing GABA-mediated calcium influx was significantly greater after rhBDNF perfusion, compared to control neurons.

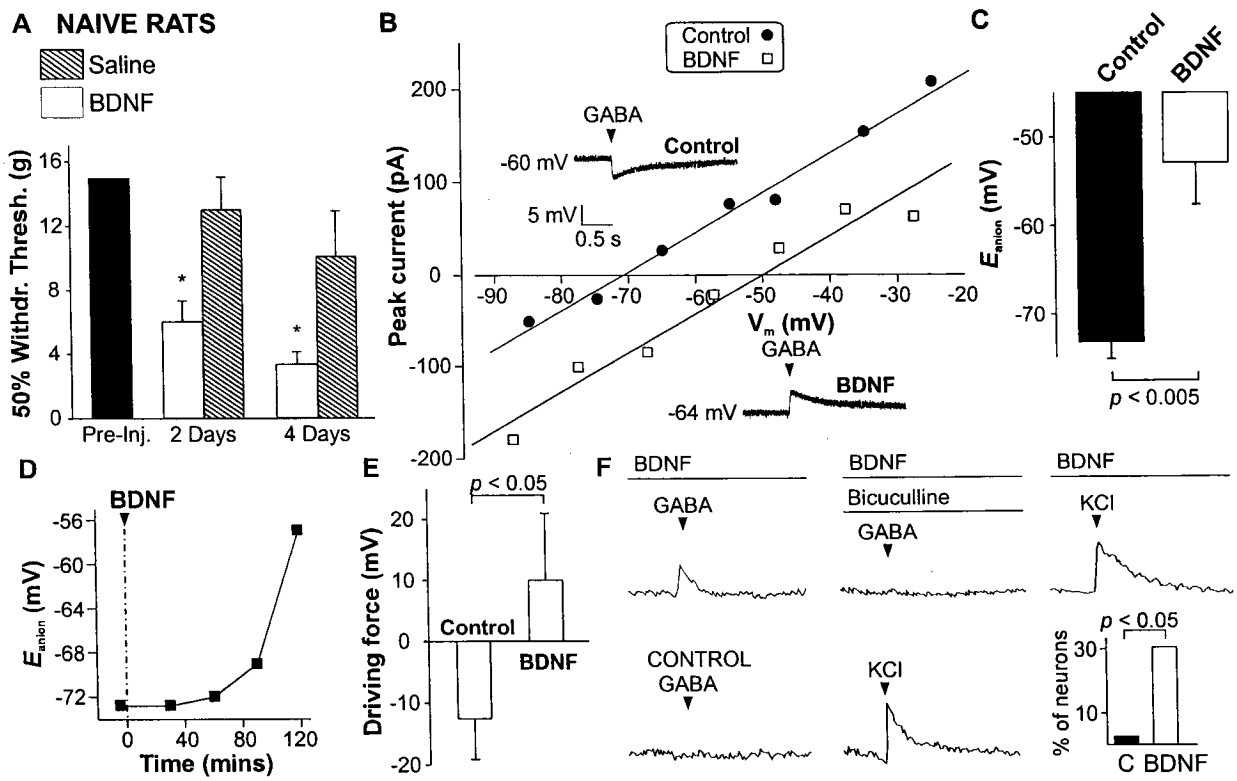

A NAIVE RATS
Saline
BDNF
50% Withdr. Thresh. (g)
Pre-Inj.
2 Days
4 Days
B
Control
BDNF
Peak current (pA)
GABA
-60 mV
Control
5 mV
0.5 s
V_m (mV)
GABA
BDNF
-64 mV
C
Control
BDNF
E_{anion} (mV)
p < 0.005
D
BDNF
E_{anion} (mV)
Time (mins)
E
p < 0.05
Driving force (mV)
Control
BDNF
F
BDNF
GABA
CONTROL
GABA
BDNF
Bicuculline
GABA
KCl
BDNF
KCl
p < 0.05
% of neurons
C BDNF

Figure 3. Functional inhibition of TrkB caused a significant reduction of allodynia in PNI rats and of the corresponding E_{anion} in lamina I neurons taken from PNI rats. *A*, Serial intrathecal delivery of anti-TrkB-IgG (0.8 ng / 2 hours), but not saline, to the lumbar dorsal horn of rats that displayed a robust allodynia in response to PNI caused a significant increase in the WD_{50} after as little as two hours. *B*, Peak currents measured from LI neurons under conditions of ACSF (control), or anti-TrkB-IgG perfusion and in response to exogenous GABA application at various values of membrane potential (V_M). *Inset*, Representative traces illustrating that GABA-evoked postsynaptic potentials were depolarizing from rest in LI neurons, whereas these potentials were hyperpolarizing from rest in LI neurons being perfused with anti-TrkB-IgG. *C*, Anti-TrkB-IgG perfusion of slices taken from rats that had received PNI elicited a significant hyperpolarization of E_{anion} in LI neurons.

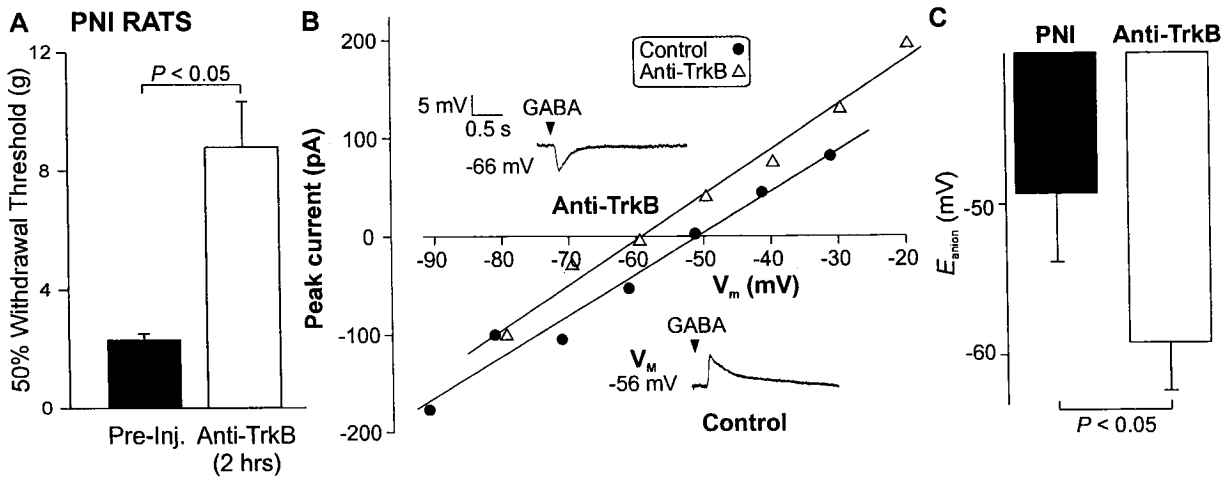
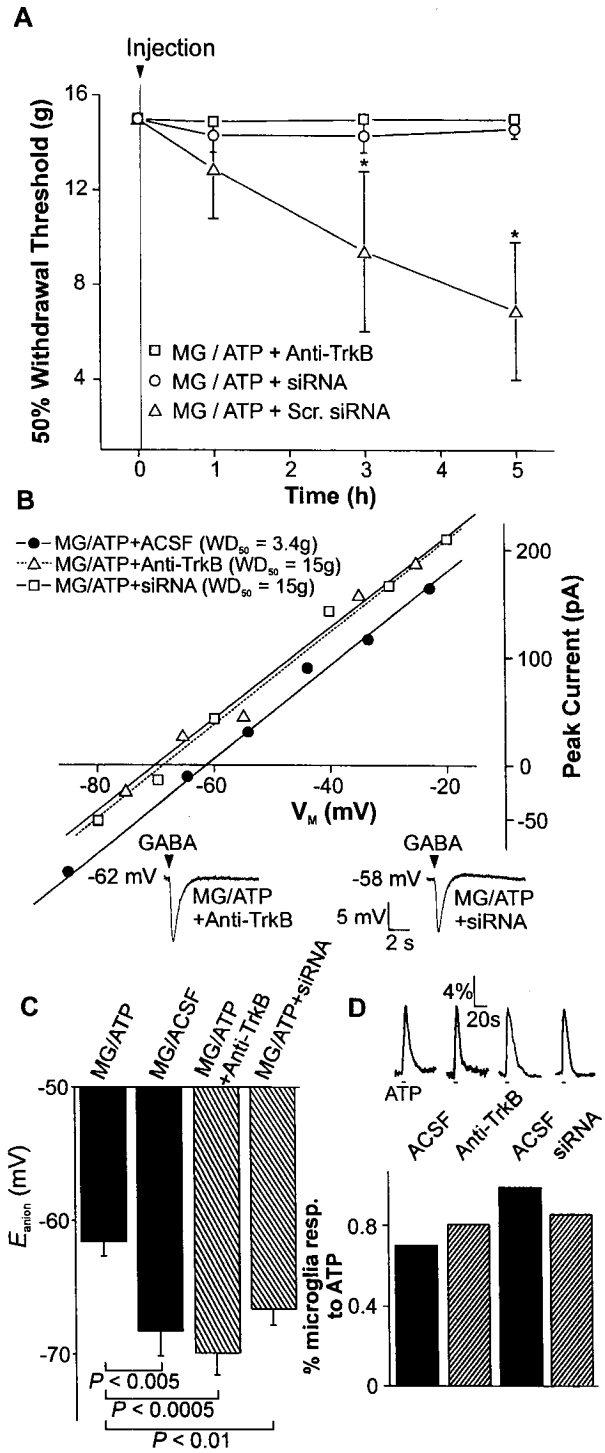

A
PNI RATS
50% Withdrawal Threshold (g)
12
8
4
0
P < 0.05
Pre-Inj.
Anti-TrkB
(2 hrs)
B
Peak current (pA)
200
100
0
-100
-200
Control
Anti-TrkB
5 mV
0.5 s
GABA
-66 mV
Anti-TrkB
-90
-80
-70
-60
-50
-40
-30
-20
V_m (mV)
GABA
V_M
-56 mV
Control
C
PNI
Anti-TrkB
E_anion (mV)
-50
-60
P < 0.05

Figure 4. Microglia-derived BDNF triggers nociceptive hypersensitivity in rats as well as the underlying shift in LI neuronal anion gradient. *A*, Local spinal delivery of neither ATP-stimulated microglia incubated with an anti-TrkB antibody nor lipofected with anti-BDNF interfering RNA (siRNA) caused a significant change in the WD_{50}. Lipofection of ATP-stimulated microglia with a scrambled version of the interfering RNA did cause the WD_{50} to drop significantly after five hours. MG – microglia; siRNA – anti-BDNF siRNA. *B*, Representative current-voltage plots from three lamina I neurons, one taken from a rat that displayed a significant decrease in WD_{50} (3.4 g) in response to the intrathecal injection of ATP-stimulated microglia, the other two taken from rats that showed no change in WD_{50} (15 g) after injection of ATP-stimulated microglia in addition to anti-TrkB and anti-BDNF siRNA. V_M – membrane potential. *Lower inset*, Representative traces showing that, from resting membrane potential, GABA evoked hyperpolarizing postsynaptic potentials in LI neurons taken from rats treated with either ATP-stimulated microglia and anti-TrkB antibodies, or ATP-stimulated microglia lipofected with anti-BDNF interfering RNA. *C*, The mean E_{anion} measured from LI neurons taken from rats that had received local spinal delivery of either ATP-stimulated microglia and anti-TrkB antibodies or ATP-stimulated microglia lipofected with anti-BDNF interfering RNA was significantly more negative than that measured from LI neurons from rats that were injected with ATP-stimulated microglia (MG/ATP), yet not significantly different than the E_{anion} measured from rats that had received resting mircroglia (MG/ACSF). *D*, Bar graph and representative traces illustrating that ATP (12.5 – 50 □M)-mediated postsynaptic calcium fluxes in fura-2-AM-loaded microglia were not affected by exposure of microglia to anti-TrkB antibodies or anti-BDNF siRNA.


A
Injection
50% Withdrawal Threshold (g)
16
12
8
4
0
1
2
3
4
5
Time (h)
MG / ATP + Anti-TrkB
MG / ATP + siRNA
MG / ATP + Scr. siRNA
B
MG/ATP+ACSF (WD50 = 3.4g)
MG/ATP+Anti-TrkB (WD50 = 15g)
MG/ATP+siRNA (WD50 = 15g)
200
100
0
-50
Peak Current (pA)
-80
-60
-40
-20
VM (mV)
GABA
-62 mV
MG/ATP
+Anti-TrkB
GABA
-58 mV
MG/ATP
+siRNA
5 mV
2 s
C
MG/ATP
MG/ACSF
MG/ATP
+Anti-TrkB
MG/ATP+siRNA
-50
-60
-70
Eanion (mV)
P < 0.005
P < 0.0005
P < 0.01
D
4%
20s
ATP
ACSF
Anti-TrkB
ACSF
siRNA
0.8
0.4
0
% microglia resp. to ATP

PREFACE TO CHAPTER 5

In chapter 4, I report our finding that BDNF stimulation of TrkB in LI neurons taken from adult rats can trigger a decrease in the *trans*membrane anion gradient. In the study composing chapter 5, I turn my attention to the intracellular pathways that mediate the relationship between TrkB and anion gradient in LI neurons.

We focussed on the calcium/calmodulin-dependent kinase (CaMK) – cAMP-dependent kinase pathway (PKA) linked to TrkB for two reasons:

a) This pathway has been associated with the induction of LTP in hippocampal neurons (Minichiello et al., 2002), a process that may require regulation of neuronal anion homeostasis (Woodin et al., 2003) and which resembles PNI-evoked sensitization of SDH neurons in many ways (Ji et al., 2003);

b) Both CaMK and PKA activation have been reported to be necessary for the induction of PNI-evoked central sensitization and nociceptive hypersensitivity in animal models (Fang et al., 2002;Ma et al., 2003).

CHAPTER 5

CYCLIC-AMP-DEPENDENT KINASE ACTIVATION IS NECESSARY FOR THE DISRUPTION OF ANION GRADIENT IN SPINAL LAMINA I NEURONS AFTER NERVE INJURY

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ABSTRACT

Previous studies have shown that a TrkB-mediated shift in the anion reversal potential (E_{anion}) of spinal lamina I (LI) neurons elicits a significant increase in the excitability of these pain-related neurons, leading to nociceptive hypersensitivity in rats. To determine whether this effect is mediated via activation of cAMP-dependent kinase (PKA), a messenger downstream of TrkB, we examined whether direct stimulation and inhibition of PKA in spinal slices affects the E_{anion} of LI neurons. Using perforated-patch and whole-cell recording techniques, we found that stimulation of PKA using sp-cAMPs perfusion led to a shift in naïve rat LI neuronal E_{anion} similar in magnitude to that observed following BDNF treatment. Alternatively, perfusion of spinal slices taken from nerve injured rats with H-89, a blocker of PKA, restored the E_{anion} to a level very close to the E_{anion} measured from LI neurons taken from naïve rats. Additionally, blockade of calcium/calmodulin-dependent kinase (CaMK) using KN-93 in LI neurons from nerve injured rats triggered a hyperpolarization of E_{anion}. The propensity of PKA inhibition to decrease nociceptive hypersensitivity was demonstrated in nerve injured rats, where local delivery of a small quantity of H-89 via intrathecal catheter increased the nociceptive withdrawal threshold significantly. These results suggest that PKA activation, possibly via CaMK, is necessary to maintain a less negative E_{anion} in LI neurons, as well as nociceptive hypersensitivity in nerve injured rats.

INTRODUCTION

Neurons found in the first lamina (LI) of the spinal dorsal horn (SDH) are critical for the integration and transmission of pain-related information and thus influence pain perception (Light, 1992;Ferrington et al., 1987). A key substrate contributing to pathological pain conditions involves a sensitization of LI neurons, manifested as an increase in the excitability of these neurons (Woolf et al., 1994). We have previously demonstrated that, subsequent to a peripheral nerve injury (PNI), a TrkB-regulated disruption of the *trans*membrane anion gradient of LI neurons contributes substantially to the generation of such hyperexcitability (Coull et al., 2003). The intracellular pathway linking TrkB activation with the regulation of the anion gradient in LI neurons, however, is poorly understood.

Among the intracellular pathways that transduce signals originating from TrkB activation is the calcium / calmodulin-dependent kinase (CaMK) – cAMP-dependent kinase (PKA) pathway (Minichiello et al., 2002). This pathway, which ultimately triggers CREB-mediated gene transcription in the dorsal horn (Miletic et al., 2004), has been reported to be activated following peripheral nerve injury (Miletic et al., 2002) and to contribute to the development of the ensuing central sensitization (Fang et al., 2002). However, the mechanisms by which the CaMK-PKA pathway trigger hyperexcitability in pain-related neurons after nerve injury have not been characterized.

In contrast, the mechanisms by which the CaMK-PKA pathway contribute to long-term potentiation (LTP) and epilepsy in the hippocampus have been reasonably well documented (Nguyen et al., 1994;Binder et al., 1999). For example, recently a group has suggested that the critical role for the CaMK-PKA pathway in epilepsy may revolve around its propensity to rapidly transduce TrkB-mediated control of KCC2 expression, and hence anion gradient, in CA1 and CA3 regions of the hippocampus (Rivera et al., 2004). This finding is intriguing when considered along with another recent report that suggests that CaMK and PKA participate in LTP of C-fiber responses in the SDH in a manner similar to that in the hippocampus (Yang et al., 2004a); taken together, these studies raise the possibility that the CaMK-PKA pathway may help to induce central

sensitization in the SDH via a disruption of the anion gradient of neurons. We have examined this possibility by pharmacologically-manipulating the CaMK-PKA pathway, and subsequently measuring the anion reversal potential of LI neurons in slices taken from both naïve rats and rats that had received a chronic constriction injury to the sciatic nerve.

METHODS

Behavioural studies. Peripheral nerve injury was induced by loosely ligating the sciatic nerve of adult male Sprague–Dawley rats, as previously described (Mosconi and Kruger, 1996). The 50 % withdrawal threshold to mechanical stimulation was assessed using Von Frey filaments and the "up-down" method, as previously described (Chaplan et al., 1994a). Subsequent to nerve injury, only animals that showed a gradual decrease in mechanical threshold (over 14–17 days) down to 2 g or less were used for further experiments. For intrathecal catheterization, a lumbar spinal catheter (PE-10 polyethylene tube) was inserted into the intrathecal space of anaesthetized adult rats, as previously described (Yaksh et al., 1980). Following H-89 (380 nmol in saline + 10% DMSO) or vehicle administration, animals were killed and their vertebral column dissected to visually confirm correct placement of the catheter. At the dose of H-89 used, none of the compounds produced motor disturbances or sedation, as assessed by grasping, righting and placing reflexes and behavioural observations (Coderre and Melzack, 1992).

Spinal cord removal. Briefly, adult Sprague-Dawley rats were anesthetized with sodium pentobarbital (30 mg/kg), perfused intracardially with ice-cold sucrose-substituted artificial cerebrospinal fluid (S-ACSF) and rapidly decapitated. The sacral vertebral column was transected, and a syringe was used to inject S-ACSF into the foramen, thus ejecting the spinal cord. S-ACSF consisted of (in mM): 248 sucrose, 11 glucose, 2 $NaHCO_3$, 2 KCl, 1.25 KH_2PO_4, 2 $CaCl_2$, 2 $MgSO_4$, pH 7.35 ± 0.05. S-ACSF was bubbled continuously with 95% O_2/5% CO_2.

Slicing. Parasagittal spinal slices (300-400 μm thick) were cut using a Leica Vibratome, incubated in S-ACSF at room temperature (23-28°C) for 30 min. Upon transfer to a

recording chamber under a Zeiss Axioscope, slices were continuously perfused with oxygenated normal ACSF in which 125 mM NaCl was substituted for sucrose.

Electrophysiological recordings. All recordings were made at room temperature. In all cases, patch pipettes were obtained by pulling borosilicate glass capillaries using a two-stage vertical puller (PP-83; Narishige, Tokyo, Japan). The pipettes were filled with a solution containing (in mM): 130 caesium gluconate, 5 CsCl, 2 $MgCl_2$, 11 BAPTA, 1 $CaCl_2$, 4 ATP, 0.4 GTP, 10 HEPES (~pH 7.4). For perforated-patch recording, pipettes were back-filled with this same solution supplemented with 25 $\mu g\ ml^{-1}$ gramicidin D [gramicidin stock was at 10 $mg\ ml^{-1}$ in dimethylsulphoxide (DMSO)]. Both voltage-clamp and current-clamp recordings were obtained using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) with >80% series resistance compensation.

γ-aminobutyric acid (GABA; 1 mM) was spritzed for brief durations (5-50 ms) onto the somas of neurons using a PicoSpritzer (General Valve, New York, NY). All drugs that were bath-applied, including BDNF (50 ng/ml), sp-cAMPs (50 □M), H-89 (15 □M) and KN-93 (5 μM) were purchased from EMD Biosciences (Calbiochem) and were prepared in deionized water as 1000 × concentrated frozen stock solution aliquots.

Data analysis & statistics. Following low-pass filtration and digitization, data were acquired and analyzed using the Strathclyde electrophysiology software CDR (courtesy of Dr. J. Dempster, Glasgow, UK) and analysis software designed by Y.D.K. All measurements are given as means ± SEM, except where indicated. Statistical significance was tested using Student's *t*-tests for comparison of mean values and mixed-design analyses of variance (post-hoc Tukey's HSD test) for repeated measures.

RESULTS

Data were obtained from 34 visually-identified lamina I (LI) neurons in slice using perforated-patch and whole-cell recording techniques. Of these neurons, 14 were taken from adult Sprague-Dawley rats that had received a chronic peripheral nerve injury (PNI) (Mosconi and Kruger, 1996) and displayed a 50% withdrawal threshold (WD_{50}) to

mechanical stimulation of ≤ 2 g. The other group of 20 LI neurons was taken from adult rats that had not received surgery and displayed a normal, and stable, WD_{50} of 15g (naïve). Previous analyses revealed that the WD_{50} of animals that received sham surgery was not significantly different from that of naïve animals (Coull et al., 2003). Additionally, we performed behavioural analyses of alert rats; as above, naïve rats were selected for these tests only if their initial $WD_{50} \geq 15$g, whereas PNI rats were selected provided their WD_{50} was ≤ 2g.

Direct cAMP-dependent kinase stimulation in LI neurons from naïve rats

As shown in Fig. 1A and B [and previously in (Coull et al., 2004)], acute incubation of spinal slices in BDNF resulted in a significant depolarization of the LI neuronal transmembrane anion reversal potential ($E_{anion} = E_{Cl^-} + E_{HCO3^-}$), as compared to LI neurons under control conditions (BDNF, -52.9 ± 4.7 mV ($n = 9$) *vs.* control, -73.2 ± 4 mV ($n = 9$), $p < 0.005$). To assess whether stimulation of the downstream mediator of TrkB, cyclic-dependent AMP-dependent kinase (PKA) (Minichiello et al., 2002), may be linked to this TrkB-mediated response, we perfused spinal slices taken from naïve rats with sp-cAMPs (50 μM), a membrane-permeable analogue of cAMP that has been reported to stimulate PKA activation (Mangiavacchi and Wolf, 2004). Following ~60 minutes incubation of slices taken from naïve rats with sp-cAMPs, the mean E_{anion} recorded from LI neurons was found to increase significantly to -55.5 ± 5.4 mV ($n = 4$, $p < 0.001$; Fig. 1C). This shift was large enough in magnitude to translate to an inversion of driving force: GABA-mediated post-synaptic potentials were depolarizing from resting potential in the presence of sp-cAMPs (DF = +7.2 ± 9.5 mV, $n = 4$), whereas, under control conditions, GABA-mediated events were always hyperpolarizing (DF = -12.5 ± 6.5 mV, $n = 9$; Fig. 1B).

Inhibition of PKA in LI neurons from PNI rats

The finding that direct PKA stimulation in LI neurons evokes a shift in E_{anion} does not necessarily suggest that PKA activation is sufficient or required for the depolarization of

E_{anion} in neurons taken from PNI (allodynic) rats. To more appropriately address this point, we perfused LI neurons taken from PNI rats with an inhibitor of PKA – H-89 (15 μM; Fig. 2A). Administration of H-89 caused a rapid hyper-polarization of E_{anion} in LI neurons, restoring the E_{anion} to a value very similar to that recorded from LI neurons taken from naïve rats (H-89, -73.2 ± 5.1 mV (n = 5) *vs.* PNI, -49.3 ± 4.5 mV (n = 6), $p < 0.005$; Fig. 2B,C). This value was also similar to the E_{anion} recorded from PNI rat LI neurons in the presence of K252a (200 nM), a direct blocker of TrkB autophosphorylation (K252a, -79.6 ± 5.9 mV, $n = 7$, $p > 0.5$; data not shown). As it is has been demonstrated that calcium / calmodulin-dependent kinase II and IV (CaMK) links TrkB activation with cAMP-dependent processes (Blanquet et al., 2003), we further tested whether the CaMK inhibitor KN-93 (5 μM) would affect the LI neuronal E_{anion} in slices taken from PNI rats. Similar to H-89 treatment, KN-93-mediated blockade of CaMK [KN-93 is reported to block both CaMK II and IV (P. De Koninck, Personal Communication, 2004)] resulted in a significant hyperpolarization of the E_{anion} in these LI neurons (-67.3 ± 7.2 mV; $n = 2$; Fig. 2B,C).

In another test of the role of PKA in the repression of local inhibition after PNI, the effect of H-89-mediated inhibition of PKA on pain sensitivity was assessed in PNI rats. Specifically, local spinal delivery of H-89 (up to 380 nmol) via intrathecal catheter to PNI rats ($n = 4$) resulted in a significant attenuation of tactile allodynia within 2 hours of injection, compared to PNI rats that received DMSO/saline vehicle, where no diminishment of allodynia (increase of WD_{50}) was observed (H-89 at 2 hours, WD_{50} = 5.5 ± 0.5 g, $p < 0.05$ – ANOVA; Fig. 2D).

DISCUSSION

The present results demonstrate that activation of the CaMK-PKA pathway is both sufficient and necessary to induce a shift in LI neuronal anion gradient, an alteration that we have reported to be linked to nociceptive hypersensitivity (Coull et al., 2003). That is, we show that direct activation of PKA using a membrane-permeable cAMP analogue is sufficient to rapidly evoke a depolarization of the E_{anion} in LI neurons, whereas inhibition of either CaMK or PKA can effect a significant reduction of the chronically heightened

E_{anion} in LI neurons taken from PNI rats. This latter effect may explain the documented ability of the PKA inhibitor, H-89, to reduce nociceptive hypersensitivity in neuropathic rats, and is consistent with a report from (Ma et al., 2003), who showed that spinal administration of cAMP response element binding protein (CREB) antisense oligodeoxynucleotides to nerve injured rats resulted in an attenuation of nociceptive hypersensitivity.

These results further support the idea that tonic activation of CREB via the TrkB-CaMK-PKA pathway is necessary to maintain a less negative E_{anion} in LI neurons, as acute pharmacological blockade of either CaMK or PKA could rapidly hyperpolarize E_{anion} in LI neurons from PNI rats (*i.e.* after establishment of allodynia). We have previously demonstrated that such tonic activation of TrkB-mediated mechanisms is necessary to maintain an abnormal anion gradient in LI neurons through our observation that a neutralizing anti-TrkB antibody could hyperpolarize E_{anion} in LI neurons taken from PNI rats (Coull et al., 2004). Moreover, we and others (Kerr et al., 1999) have demonstrated that local spinal blockade of either BDNF or TrkB could reverse nociceptive hypersensitivity in PNI rats.

The requirement for tonic activation of the TrKB-CaMK-PKA pathway to sustain the depolarization of the LI neuronal E_{anion} reinforces the notion that tyrosine phosphorylation is dynamic, being governed by the opposing actions of tyrosine kinases and tyrosine phosphatases (Burke, Jr. and Zhang, 1998;Hunter, 1995). In keeping with this theory, it has been shown that noxious peripheral stimulation (that produces short-lived nociceptive hypersensitivity) causes only brief phosphorylation of TrkB (Pezet et al., 2002), as well as other mediators found downstream of TrkB (Ji et al., 1999). On the other hand, both the expression of BDNF and the phosphorylation of CREB are sustained – along with the expression of nociceptive hypersensitivity – in rats that have received nerve injury (Miletic and Miletic, 2002;Miletic et al., 2002).

Our finding that direct PKA stimulation in LI neurons using sp-cAMPs triggers a shift in E_{anion} and thus increases in excitability further highlights the similarity among central

sensitization in the spinal cord and long-term potentiation (LTP) in the hippocampus, as classically, sp-cAMPs has been used to elicit transcription-dependent LTP (Nguyen et al., 1994). However, whereas tonic activation of TrkB-linked pathways is necessary for the maintenance of a less negative E_{anion} in LI neurons, BDNF has been reported to be necessary only for the induction of LTP in neurons of the hippocampus, having no prominent role in its long-term maintenance (Chen et al., 1999). Similarly, in the dorsal horn, reversal of LTP of C-fiber-evoked responses using inhibitors of CaMK or PKA was reported to be possible only shortly after LTP induction; indeed, application of a PKA inhibitor (Rp-CPT-cAMPs) had no effect on LTP when applied $\geq$ 15 minutes post-tetanic stimulation (Yang et al., 2004a). Taken in this context, the PNI-evoked depolarization of E_{anion} in LI neurons could be viewed as an exaggerated and/or prolonged LTP event, a possibility that is underscored by a recent report suggesting that modifications of E_{anion} may be important in some forms of long-term plasticity (Woodin et al., 2003).

In conclusion, the present observations strongly suggest that tonic activation of the TrkB-CaMK-PKA pathway is necessary for maintaining the hyperexcitability of LI neurons after nerve injury. While these findings provide key answers to many of the unknowns surrounding PNI-evoked central sensitization, they also shed light on many of the avenues whereby therapeutic intervention of neuropathic pain may occur.

FIGURES

Figure 1. Cyclic AMP stimulation triggers a rapid depolarization of anion reversal potential (E_{anion}) in LI neurons. ***A***, Representative current-voltage plot demonstrating that application of either BDNF or a membrane permeable analogue of cAMP, sp-cAMPs, to spinal slices taken from naïve rats elicited a significant depolarization of E_{anion} in LI neurons, in comparison with control conditions. Note that neither drug evoked a significant alteration of slope conductance. V_M – membrane potential. ***B***, Representative traces of postsynaptic potentials illustrating that, whereas GABA application evokes hyperpolarizing response in control LI neurons taken from naïve rats, in slices from naïve rats treated with either BDNF or sp-cAMPs, GABA evokes a depolarizing response from rest. ***C***, The mean E_{anion} recorded from LI neurons following acute sp-cAMPs perfusion was similar to that recorded from LI neurons after BDNF administration, and significantly less negative than the E_{anion} recorded from LI neurons under control conditions.

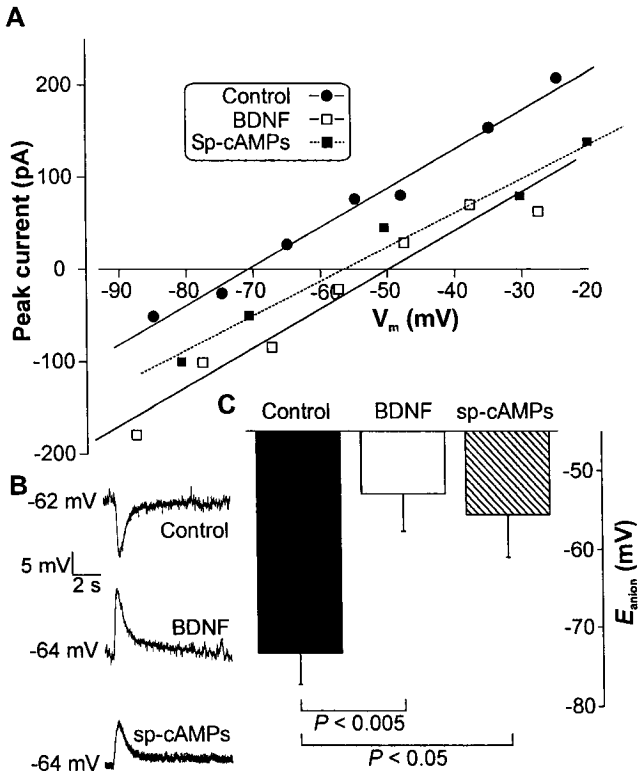
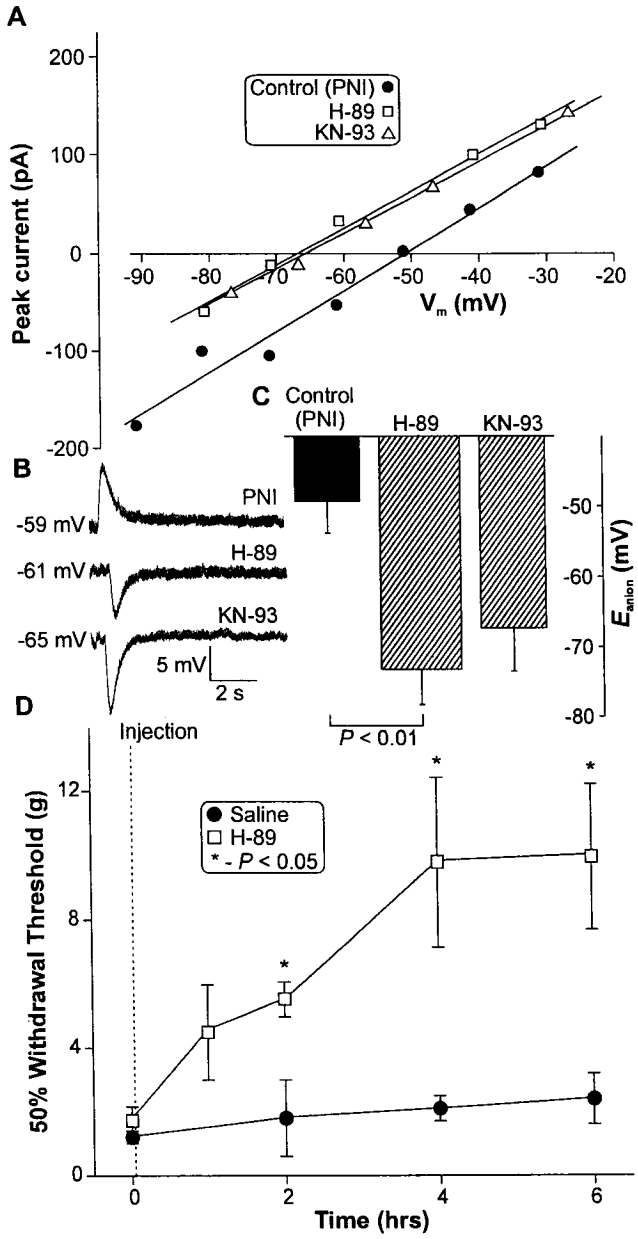

A
Control
BDNF
Sp-cAMPs
Peak current (pA)
200
100
0
-100
-200
-90
-80
-70
-60
-50
-40
-30
-20
V_m (mV)
B
-62 mV
Control
5 mV
2 s
BDNF
-64 mV
sp-cAMPs
-64 mV
C
Control
BDNF
sp-cAMPs
-50
-60
-70
-80
E_{anion} (mV)
P < 0.005
P < 0.05

Figure 2. Disruption of the CaMK-cAMP pathway evokes both a hyperpolarization of E_{anion} recorded from LI neurons taken from PNI rats, and a reduction in nociceptive hypersensitivity *in vivo* in PNI rats. ***A***, Representative I-V plot illustrating how the E_{anion} recorded from LI neurons in slices from PNI rats was rendered significantly more negative by acute administration of either H-89, an inhibitor of cAMP-dependent kinase (PKA), or KN-93, an inhibitor of calcium/calmodulin-dependent kinase (CaMK), in comparison with control conditions. ***B***, Representative current-clamp traces showing that GABA triggers a depolarizing response in LI neurons from control PNI rats, while triggering a hyperpolarizing response in these neurons incubated with a PKA inhibitor (H-89) or calcium / calmodulin-dependent kinase inhibitor (KN-93). ***C***, The mean E_{anion} recorded from LI neurons (from PNI rats) following acute perfusion of either H-89 or KN-93 was significantly more negative than that recorded from LI neurons perfused with ACSF alone. ***D***, Local spinal delivery of H-89 (380 nmol; 1 injection), but not saline, to PNI (allodynic) rats via intrathecal catheter elicited a significant increase in the nociceptive threshold to tactile stimulation.


A
Peak current (pA)
200
100
0
-100
-200
Control (PNI) ●
H-89 □
KN-93 △
-90
-80
-70
-60
-50
-40
-30
-20
V_m (mV)
B
PNI
-59 mV
H-89
-61 mV
KN-93
-65 mV
5 mV
2 s
C
Control (PNI)
H-89
KN-93
-50
-60
-70
-80
E_{anion} (mV)
P < 0.01
D
Injection
50% Withdrawal Threshold (g)
12
8
4
0
● Saline
□ H-89
* - P < 0.05
*
*
*
0
2
4
6
Time (hrs)

CHAPTER 6
DISCUSSION

6. DISCUSSION

6.1 Two mechanisms of plasticity at inhibitory synapses in the superficial dorsal horn

Plasticity of local inhibitory synapses in the superficial dorsal horn (SDH) is critical for regulating the integration and transmission of pain-related information at the first sensory synapse (Light, 1992). Although modern pain theory predicts that a decrease of inhibition at this locus should be a major contributor to states of cellular hyperexcitability (central sensitization) necessary for the induction of nociceptive hypersensitivity (Melzack and Wall, 1965), the processes that regulate inhibitory strength have been poorly characterized.

In this thesis, I present two principal mechanisms that contribute to such regulation – both in the context of neuropathic pain, as well as ontogeny. As will be discussed below, the first mechanism involves a disruption of anion homeostasis in lamina I (LI) neurons, which occurs following a peripheral nerve injury (PNI). The second mechanism comprises a reorganization of $GABA_A$ receptor-mediated postsynaptic responses at synapses in LI and LII.

6.2 Alterations of anion homeostasis in LI neurons

The disruption of LI neuronal anion homeostasis that occurs subsequent to PNI is manifested as a depolarization of the anion reversal potential (E_{anion}) – a change that results in depolarizing $GABA_A$- and glycine receptor-mediated responses (Chapter 3). The shift in E_{anion} was further documented to result from the tonic activation of cAMP-dependent kinase (Chapter 5) via TrkB stimulation by microglia-derived BDNF (Chapter 4).

Such lability of anion gradient in CNS neurons has been demonstrated in a number of studies using various experimental paradigms. For example, two independent groups recently showed that, in motor neurons, ectopic discharge stemming from axotomy could trigger a depolarization of E_{anion} to the extent that $GABA_A$ receptor activation led to

calcium influx (Toyoda et al., 2003;Nabekura et al., 2002). Other studies have illustrated the relationship between presynaptic input and postsynaptic anion gradient: in the hippocampus high frequency stimulation of presynaptic GABAergic interneurons was observed to elicit a rapid shift in the anion gradient of postsynaptic pyramidal neurons (Staley et al., 1995;Woodin et al., 2003). However, the question that has remained unanswered in the literature pertains to how exactly an injury to a presynaptic neuron may induce a *trans*synaptic modification of E_{anion} in a second neuron.

One of the principal quandaries pertaining to the nature of such *trans*synaptic processes is whether a post-injury increase in the presynaptic excitatory drive in the SDH is necessary. This has been documented to occur: several groups have shown that there is an increase in the excitatory output of sensory afferents in the SDH originating at the site of the injury (Tal and Eliav, 1996;Zimmermann, 2001) and arising from nociceptor sensitization (Mannion et al., 1999;Bridges et al., 2001) and/or a reduction of primary afferent depolarization (Kingery et al., 1988). Moreover, acute blockade of spinal nerves via topical application of bupivicaine prior to PNI caused a marked, though transient, reduction of the ensuing hypersensitivity (Abdi et al., 2000). Hence, we may reasonably surmise that such hyperactivity in the peripheral nervous system is necessary for the *trans*synaptic disruption of E_{anion}; yet what are the mediators that transmit this alteration?

As we have demonstrated that tonic BDNF stimulation of TrkB receptors in LI neurons subserves shifts in E_{anion}, it would be tempting to hypothesize that BDNF released from the central terminals of sensory afferents could represent the key neuromodulator. Indeed, several lines of evidence would indirectly support this contention: BDNF is released from sensory afferents in response to bursting artificial stimulation (Lever et al., 2001), migrates anterogradely from DRG to central terminals after nerve injury (Zhou and Rush, 1996) and is present in increased extracellular concentrations in the SDH after nerve injury (Miletic and Miletic, 2002). However, one of the few studies that has actually examined stimulus-evoked release of BDNF after PNI recently concluded that stimulation of dorsal roots at either C-fiber or A+C fiber strength did NOT elicit the release of BDNF (Lever et al., 2003). Moreover, the release of BDNF from neuronal

sources cannot account for the tonic activation of TrkB receptors necessary for the maintenance of a heightened E_{anion}, as the stimulation of sensory afferents (noxious natural, or artificial stimulation) necessary to evoke BDNF release also triggers a rapid depletion of intraterminal BDNF vesicles (Lever et al., 2001).

Instead, we showed that the BDNF necessary for the induction and early maintenance of a less negative E_{anion} in LI neurons derives from activated spinal microglia. Interestingly, a previous study demonstrated that the contribution of microglia to nociceptive hypersensitivity hinged on the activation of p38 MAP-kinase via ATP-stimulation of $P2X_4$ receptors (Inoue et al., 2004) – the activation of p38 MAP-kinase also reported to be a prerequisite of BDNF release from microglia (Imamura et al., 2003) [note that we used ATP to artificially stimulate microglia to adopt the hyperactive phenotype]. Taken together, these observations may indicate that it is ATP, released after PNI from sensory afferents (Li et al., 1998;Sawynok et al., 1993) that acts as *trans*synaptic messenger, connecting presynaptic hyperactivity with a postsynaptic disruption of anion homeostasis (Inoue et al., 2003). This possibility should be further explored, perhaps by pre-treating rats with a $P2X_4$ inhibitor, performing PNI and assessing whether the E_{anion} of LI neurons in slice is significantly altered.

As I have stressed throughout this thesis, it is the continuous release of BDNF from activated microglia that appears to be necessary to tonically stimulate TrkB receptors, and hence cause a decrease in the anion gradient of LI neurons. This finding supports previous observations that the expression of the hyperactive microglia phenotype (OX-42+) in the SDH correlates well with the onset of nociceptive hypersensitivity in rats after PNI (DeLeo and Yezierski, 2001). However, the contribution of microglia to the long-term maintenance of neuropathic pain is controversial. While some groups have reported that microglia activation parallels nociceptive hypersensitivity in rats as late as 90 days post-injury (Coyle, 1998), others suggest that hyperactive microglia are only expressed transiently subsequent to PNI, returning to a resting state by 28 days – a time when allodynia continues to persist (Tanga et al., 2004). This latter observation was underscored by observations from the same group that administration of an inhibitor of

microglia activation to rats could prevent the onset of – but not reverse full-blown – nociceptive hypersensitivity after PNI (Raghavendra et al., 2003). On the other hand, in one of the few studies of humans, activated microglia (detected by their expression of the peripheral benzodiazepine receptor) accompanied neuropathic pain in the ipsilateral spinal cord more than 20 years after nerve transection (Banati, 2002). In any event, it is generally agreed that microglia activation is usually accompanied by later-onset activation of astrocytes (Colburn et al., 1999), a glial cell that has also been reported to secrete significant quantities of BDNF upon activation (Dougherty et al., 2000). Whether or not astrocytes act without microglia to sustain long term nociceptive hypersensitivity, they do appear to contribute significantly, as glial inhibiting agents have been documented to reverse PNI-evoked allodynia (Sweitzer et al., 2001). The precise role of astrocytes in the maintenance of a less negative E_{anion} is unclear, however, and warrants further investigation.

6.3 Reorganization of $GABA_AR$-mediated excitability control at SDH synapses

Following PNI and during early development, we observed a contribution of $GABA_A$ receptors to miniature postsynaptic currents (mPSCs) in both LI and LII neurons. This contribution of $GABA_ARs$ resulted in a configuration whereby these neurons received input from both $GABA_ARs$ and GlyRs in response to the ongoing corelease of vesicular GABA and glycine. This organization is in contrast to that found in adult LI-II neurons, where only GlyRs mediate quantal events.

As demonstrated in several experiments, the decay time constant (τ_D) of $GABA_AR$-mediated PSCs in LI-II neurons is significantly longer than that of GlyR-mediated PSCs. Hence the addition of a $GABA_AR$-mediated component to synaptic events results in individual mPSCs with substantially longer decay kinetics, and therefore a greater ability to effect *trans*membrane charge transfer (Aradi et al., 2002). In our initial appraisal of the function of transient $GABA_AR$-mediated mPSCs in the developmentally immature rat, we proposed that the greater inhibitory charge transferred by mixed $GABA_AR$/GlyR events compensated for the relatively low frequency of GlyR-mediated mPSCs observed; this is

a view that has been echoed by others (Russier et al., 2002;Wu et al., 2002). This hypothesis is further underscored by studies in mice possessing a disruptive mutation in the gene that encodes the glycine receptor, where there occurs an augmentation of GABAergic transmission manifested as a dramatic increase in the proportion of neurons receiving $GABA_AR$-mediated mPSCs (Graham et al., 2003).

However, the situation is not as clear in the mature SDH after PNI, as the aforementioned shift in E_{anion} caused $GABA_ARs$ to mediate a depolarization of the postsynaptic membrane often generating action potential, instead of the typical hyperpolarization. That is, although the contribution of $GABA_ARs$ to synaptic events increases the net charge transfer, it increases the total anion flux out of the neuron – and hence the magnitude of depolarization. Using computer simulations we demonstrated that the addition of $GABA_AR$-mediated depolarization in LI neurons enhanced the output firing frequency as a function of input EPSC frequency in comparison with that elicited via GlyR-mediated depolarization alone. We further demonstrated in LI neurons taken from PNI rats (in the presence of glutamatergic blockers) that $GABA_AR$-mediated postsynaptic depolarization was necessary and sufficient to generate of action potentials, as focal stimuli evoked spiking in LI neurons – a phenomenon that was abolished by the $GABA_AR$ antagonist bicuculline.

The observation that $GABA_ARs$ mediated depolarization of LI neurons after PNI begs the question of whether they do the same in LI neurons taken from developmentally immature rats. To examine this query, we performed calcium-imaging techniques on Fura-2-AM-loaded LI neurons in slices taken from developmentally immature rats (see Methods, Chapter 3; $n \geq 3$ for each age shown). As shown in Figure 1 (unpublished observations), we found that from ~postnatal day 3 (P3) until ~P23, a gradually-decreasing proportion of LI neurons responded to GABA application with an increase in intracellular calcium concentration. As these responses were largely attenuated by either Ni^{2+} application, a metal cation known to non-selectively block voltage sensitive calcium mechanisms (Bao et al., 1998), or 0 $[Ca^{2+}]_e$ media (data not shown) we may conclude that

GABA mediated the entry of extracellular calcium via its depolarizing influence on the postsynaptic membrane.

These results confirm other reports of the propensity of GABA to trigger postsynaptic depolarization in SDH neurons taken from immature rats (Reichling et al., 1994;Wang et al., 1994;Baccei and Fitzgerald, 2004). It is further interesting to note that the complete loss of GABA-mediated depolarization occurs at a very similar time to that of $GABA_AR$-mediated synaptic events in LI neurons (~P23). This coincidence may suggest that the two phenomena are linked. A recent study of immature hippocampal neurons proposed just that: specifically, voltage-sensitive calcium entry mediated by depolarizing GABA was shown to be instrumental in the upregulation of KCC2, and therefore in the hyperpolarization of the E_{anion} (Ganguly et al., 2001;Staley and Smith, 2001).

The pathway linking GABA-mediated calcium entry and KCC2 gene expression, however, was not elucidated precisely. Towards filling this knowledge gap, a study of immature hypothalamic neurons revealed that GABA-mediated calcium influx triggered the activation of MAP kinase – CREB pathway (Obrietan et al., 2002), which is known to be an important regulator of KCC2 expression (Rivera et al., 2004). Moreover, the former study also demonstrated that CREB stimulation resulted in increased BDNF gene expression. This observation is intriguing because, although BDNF gene expression never occurs in second order neurons in the SDH (Michael et al., 1997), TrkB expression is stimulated by CREB phosphorylation (Deogracias et al., 2004). The upregulation of TrkB receptor expression in the SDH may then catalyze a BDNF-mediated increase in KCC2 expression, which has been documented in developing hippocampal neurons (Aguado et al., 2003). Support for the notion that TrkB upregulation is the key requirement for developmental BDNF regulation of KCC2 expression to occur may also be derived from: (a) immunohistochemical studies performed by Maisonpierre et al. (1990), who report that BDNF levels remain relatively constant in the spinal cord with ontogeny; and (b) the observation that spinal microglia do not display the hyperactive phenotype in neonates, and therefore do not likely secrete BDNF (S. Beggs, 2004, personal communication).

At the outset, BDNF-mediated upregulation of KCC2 appears paradoxical, considering that we, and others (Rivera et al., 2002;Rivera et al., 2004), showed that in neurons taken from naïve adult rats BDNF causes a rapid depolarization of E_{anion} and/or reduction of KCC2 protein expression. To preliminarily address the issue of this observational disparity, we took spinal slices from both developmentally immature (P7) and PNI adult rats (>P40), perfused them with an inhibitor of TrkB autophosphorylation [K-252a (200 nM)], and measured the steady-state E_{anion} from LI neurons. This approach allowed us to examine the role of endogenous BDNF, in slices (immature *vs.* PNI) where the state of the LI neuronal E_{anion} was similarly increased. As summarized in Figure 2 (unpublished observations), we found that in LI neurons taken from PNI adult rats, K-252a treatment caused a significant hyperpolarization of the mean E_{anion} (K-252a, -79.6 ± 5.9 mV (n = 7) *vs.* control, -49.3 ± 4.5 mV (n = 8); $p < 0.05$). On the contrary, when K-252a was administered to spinal slices taken from P7-8 rats, the E_{anion} of LI neurons increased from -59.8 ± 4.7 mV (n = 5) under control conditions, to -40 ± 4 mV (n = 2) with K-252a.

These results suggest that there occurs a switch in the TrkB-mediated regulation of E_{anion}: whereas BDNF mediates a hyperpolarizing shift in the E_{anion} of LI neurons taken from PNI adult rats, it triggers a depolarization in the E_{anion} of these neurons taken from immature rats. This finding is consistent with reports from the hippocampus, where in neurons taken from immature rats BDNF was reported to trigger increased KCC2 expression (Aguado et al., 2003), in contrast to hippocampal slices taken from adults, where evidence has been presented that BDNF causes a repression of the expression of KCC2 (Rivera et al., 2002). A third study from the hippocampus lends support to these findings by showing that the effect of BDNF on $GABA_AR$-mediated synaptic activity shifts from potentiating to suppressing with ontogeny (Mizoguchi et al., 2003a).

The mechanisms that underlie this switch in the function of TrkB have not been characterized. However, it is interesting to consider a recent study that suggests that TrkB-mediated regulation of KCC2 expression varies as a function of the intracellular pathways involved. More specifically, Rivera et al. (2004) showed that when the TrkB-

coupled Shc/FRS-2-associated pathway is stimulated in combination with a second downstream pathway (phospholipase C γ), KCC2 expression is decreased, whereas Shc/FRS-2 stimulation alone results in an increase in KCC2 expression (Rivera et al., 2004). Taken together, we may surmise that with maturation in the SDH there occurs a coupling of TrkB receptors with the PLCγ-associated pathway, thus mediating the switch in the regulation of KCC2 expression, and therefore E_{anion}, by TrkB. This matter warrants further investigation, possibly by evaluating whether blockade of PLCγ (using D609, for example) affects BDNF-mediated regulation of E_{anion} in immature neurons.

If TrkB activation by BDNF is indeed responsible for regulating E_{anion} in neurons taken from both adults and neonates, might the concomitant reorganization of $GABA_A$Rs, observed in both age groups, also be governed by TrkB? As a preliminary analysis of this question, we applied BDNF to slices taken from naïve adult rats and subsequently examined the components of miniature PSCs recorded from LI neurons. We found that, unlike mPSCs recorded under control conditions, which are solely mediated by glycine receptors (single exponential, $\tau_D = 6.5 \pm 0.4$ ms, $n = 3$), BDNF perfusion causes ~27% GlyR-mediated mPSCs to also express a more protracted, bicuculline-sensitive $GABA_A$R component (biexponential, $\tau_{D1} = 17.7 \pm 3.5$ ms, $\tau_{D2} = 138.5 \pm 18.8$ ms, $n = 5$; Fig. 3, unpublished observations).

These observations suggest that BDNF treatment does trigger a $GABA_A$R-contribution to mPSCs recorded from adult rat LI neurons; however, it is unclear whether BDNF acts by shuttling $GABA_A$Rs to synapses, activating pre-existing synaptic $GABA_A$Rs, sensitizing extra-synaptic $GABA_A$Rs, *de novo* synthesis of $GABA_A$Rs, modulating expression or release of GABA from presynaptic terminals, *etc.* Although any of the stated mechanisms may account for the increased contribution of $GABA_A$Rs to quantal synaptic events by LI neurons, recent studies may lend support to the increased expression hypothesis. For example, using radiolabeling assays, Mizoguchi et al. (2003b) showed that in rat cortical neurons in which GABA is hyperpolarizing, BDNF administration caused a rapid increase in the number of functional $GABA_A$Rs. A second study of hippocampal neurons, this time in primary culture (prepared from rat embryos 17 days

post-gestation), further underscores the duplicity of actions mediated by TrkB receptors in reporting that BDNF triggered a rapid downregulation of $GABA_AR$ surface expression (Brunig et al., 2001). Of course, this finding is also in keeping with our results showing that the contribution of $GABA_ARs$ to mPSCs in LI neurons disappears with maturation.

6.4 Summary

One of the principal objectives of this thesis was to characterize mechanisms that relate to plasticity at inhibitory synapses in the SDH in development and pathology. More specifically, we sought to describe consequential processes that underlie alterations in the influence of GABA and glycine on cellular function.

As shown schematically in Figure 4, we discovered two discrete synaptic mechanisms that fulfill this role. Firstly, we revealed that with ontogeny and subsequent to PNI, there occurs a reorganization of $GABA_A$-mediated transmission at LI neuronal synapses. That is, whereas only GlyRs were observed to mediate quantal synaptic events in LI neurons taken from control adults (Fig. 4 – ***1***), in LI neurons taken from developmentally immature (<P23) and PNI rats $GABA_ARs$ also mediated quantal postsynaptic currents (***2***). The functional result of this configuration is that miniature PSCs mediated by both $GABA_ARs$ and GlyRs carry substantially greater net charge than those mediated by GlyRs alone.

Secondly, we discovered that in both LI neurons taken from immature rats and from PNI rats, the anion gradient is significantly less than that measured from LI neurons taken from control, adult rats. Specifically, we found that in both of the former conditions the LI neuronal E_{anion} was increased (*i.e.* less negative) sufficiently to lead to $GABA_AR$- and GlyR-mediated depolarization (and in some cases net excitation; ***3***), in contrast to the typical GlyR-mediated hyperpolarization observed in LI neurons taken from adult rats (***4***). In the case of the PNI-mediated upset of anion homeostasis, it was found that the decreased expression of the KCC2 potassium-chloride exporter in LI neurons could account for the less negative E_{anion} measured. As demonstrated, a critical functional consequence of this increased E_{anion} in both neurons taken from immature and PNI rats,

was GABA-evoked calcium influx through voltage-sensitive calcium mechanisms. Such calcium influx has been well documented to be important for growth, differentiation, repair, as well as the maintenance of pathophysiological states, such as neuropathic pain (Zucker, 1999;Chaplan et al., 1994b).

For both of the described mechanisms of plasticity, we further found that BDNF-activation of TrkB receptors plays a key role. In the context of PNI, BDNF secreted by activated microglia (***5***) was demonstrated to trigger both a recruitment of a $GABA_A$ component to mPSCs and a depolarization of E_{anion} in LI neurons via the activation of TrkB receptors, which subsequently led to the stimulation of calcium/calmodulin-dependent kinase – PKA pathways. Similarly, BDNF activation of TrkB was also shown to play a role in the regulation of E_{anion} in LI neurons taken from neonate rats.

Overall, the plasticity mechanisms described in this thesis have been demonstrated to play a critical role in the regulation of the efficacy of inhibition at SDH synapses. As discussed below, the knowledge of neuronal excitability control created by these discoveries is expected to significantly advance the development of novel therapeutics to abolish pathophysiologies, such as neuropathic pain.

6.5 Potential therapeutic implications

The findings presented in this thesis identify several novel targets and strategies for the treatment of neuropathic pain. Specifically, we identify four novel loci where therapeutic manipulation may effectively reduce the pain associated with conditions of peripheral neuropathy. Firstly, we demonstrate that PNI leads to a decrease in the activity of the KCC2 exporter, and hence to a disruption of anion homeostasis. As *in vivo* pharmacological blockade of KCC2 exporters (using DIOA) triggered an allodynia in rats that was similar to that resulting from PNI, we predict that agents effecting the re-activation of KCC2 [perhaps through direct modulation of KCC2 phosphorylation (Payne, 1997)] will possess significant analgesic properties. Secondly, we have identified the TrkB-CaMK-PKA pathway to control the expression of KCC2 in SDH neurons; we have already demonstrated that manipulation of this pathway can rapidly attenuate

nociceptive hypersensitivity (*i.e.* using H-89 or anti-TrkB antibodies). Further research should yield more selective compounds that are effective at promoting KCC2 expression.

Thirdly, our identification of the recruitment of $GABA_ARs$ to postsynaptic excitability control in LI neurons subsequent to PNI suggests that, in countering anion-mediated depolarization in these pain-related neurons, it is not adequate to simply block glycine receptors. In my view, effective combat against states of sensitization in LI neurons will require blockers of $GABA_ARs$ and GlyRs; this complicates matters somewhat, as $GABA_ARs$ play a duplicity of roles in SDH synapses during PNI-evoked central sensitization: presynaptic inhibition via primary afferent depolarization (Rudomin and Schmidt, 1999), and postsynaptic excitation. Hence, general blockade of $GABA_ARs$ will serve to reduce both inhibition and excitation; this finding may explain why benzodiazepines are generally observed to render no clinical effect on neuropathic pain (King and Strain, 1990).

Lastly, we identify microglia-derived BDNF to be responsible for the induction and maintenance of PNI-evoked upsets in LI neuronal anion homeostasis, and the associated nociceptive hypersensitivity. This finding is critical, as it identifies an upstream mediator of nociceptive hypersensitivity where directed inhibition should not interfere with normal neuronal function. That is, because microglia are not expressed in the hyperactive phenotype under control conditions, BDNF release should not be associated with typical neuronal physiology. The BDNF that is necessary for normal synaptic tuning almost certainly derives from neuronal pools (Lever et al., 2001). Therefore, inhibiting the release of BDNF from microglia could represent a viable strategy to selectively modulate central hyperexcitability after PNI, while minimizing side-effects.

DISCUSSION FIGURES

Figure 1. In spinal slices taken from neonates, the proportion of LI neurons responding to exogenous GABA application with an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) decreased as a function of age. At 23 days and older, GABA never evoked increases in $[Ca^{2+}]_i$.

Discussion Figure 1

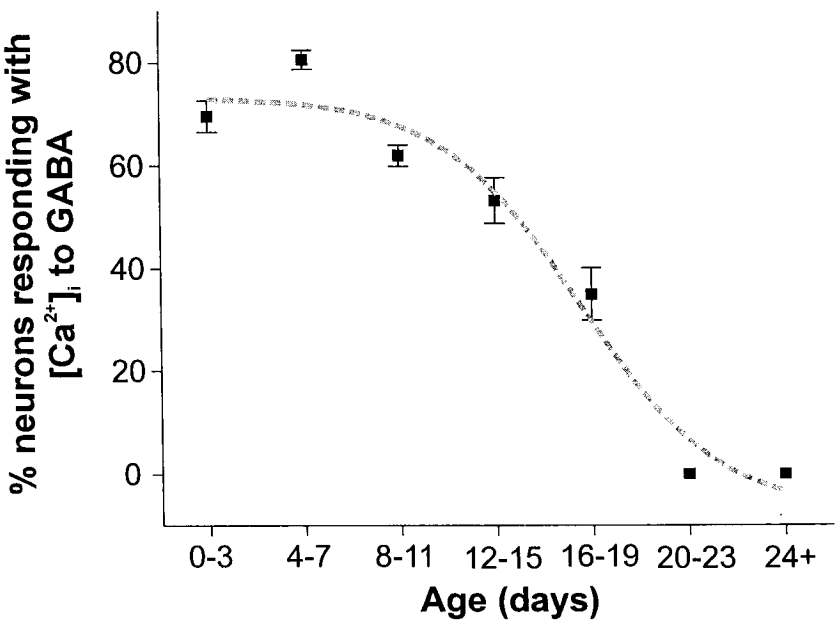


Figure 2. Bar graph illustrating the differential effect of K-252a, an inhibitor of TrkB autophosphorylation, on LI neuronal E_{anion} and anion driving force (DF) in slices taken from adult *versus* immature rats (P7). Whereas bath-applied K-252a was found to trigger a rapid increase in both E_{anion} and DF of LI neurons taken from a P7 rat, in LI neurons from adult rats, K-252a evoked a significant decrease of both of these parameters.

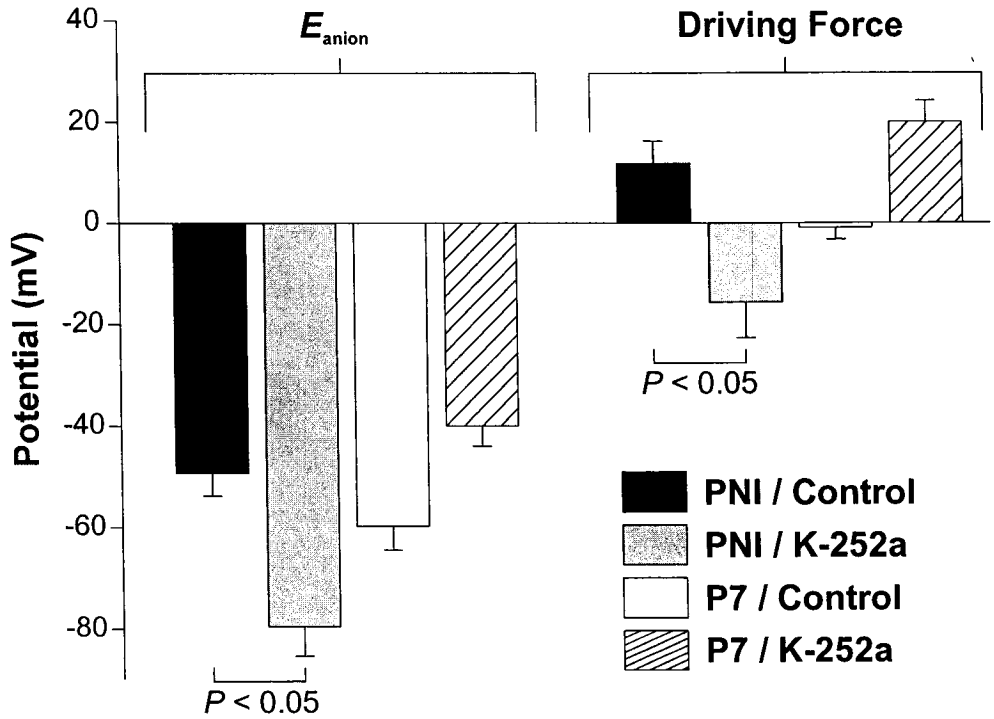

40
20
0
-20
-40
-60
-80
Potential (mV)
E_{anion}
Driving Force
P < 0.05
P < 0.05
PNI / Control
PNI / K-252a
P7 / Control
P7 / K-252a

Figure 3. Acute BDNF administration to spinal slices taken from an adult rat triggered the recruitment of a $GABA_AR$-mediated component to miniature PSCs recorded from LI neurons. ***A***, multiple raw traces of mPSCs (superimposed) recorded from LI neurons after exposure to BDNF for $\geq$ 60 minutes. ***B***, compound mPSC trace created by the averaging of several raw mPSCs recorded from LI neurons in the presence of BDNF. ***C***, in the presence of the GABAAR antagonist bicuculline, the more protracted decay component of the compound mPSC was abolished. ***D***, in the presence of the GlyR antagonist strychnine, the faster decay component of the compound mPSC was attenuated. ***E***, Two compound mPSCs (recorded in the presence of BDNF) superimposed; the grey mPSC is that recorded in the presence of bicuculline, while the black mPSC is recorded under control conditions of physiological saline (ACSF). Note the prolonged nature of the bicuculline-sensitive component ($GABA_AR$) of decay.

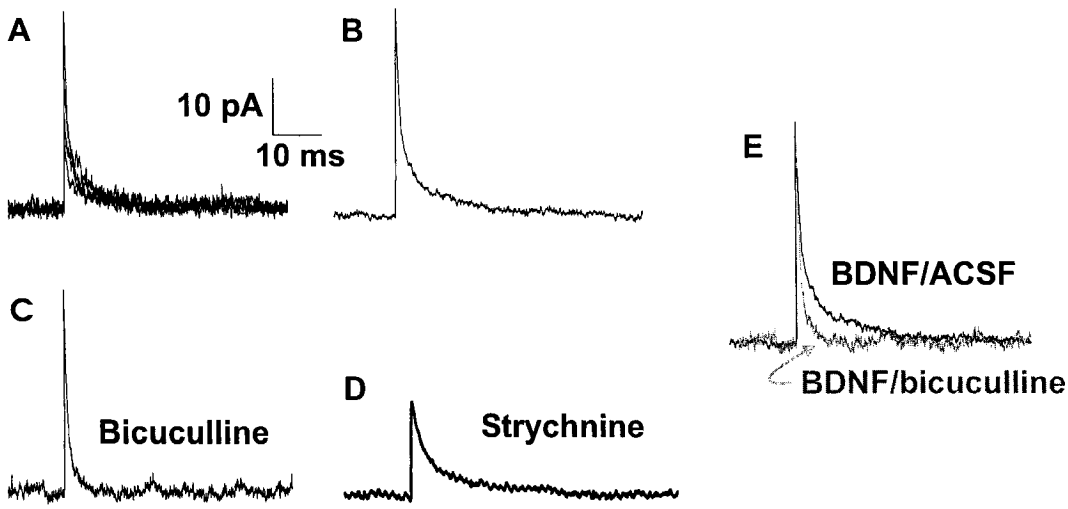
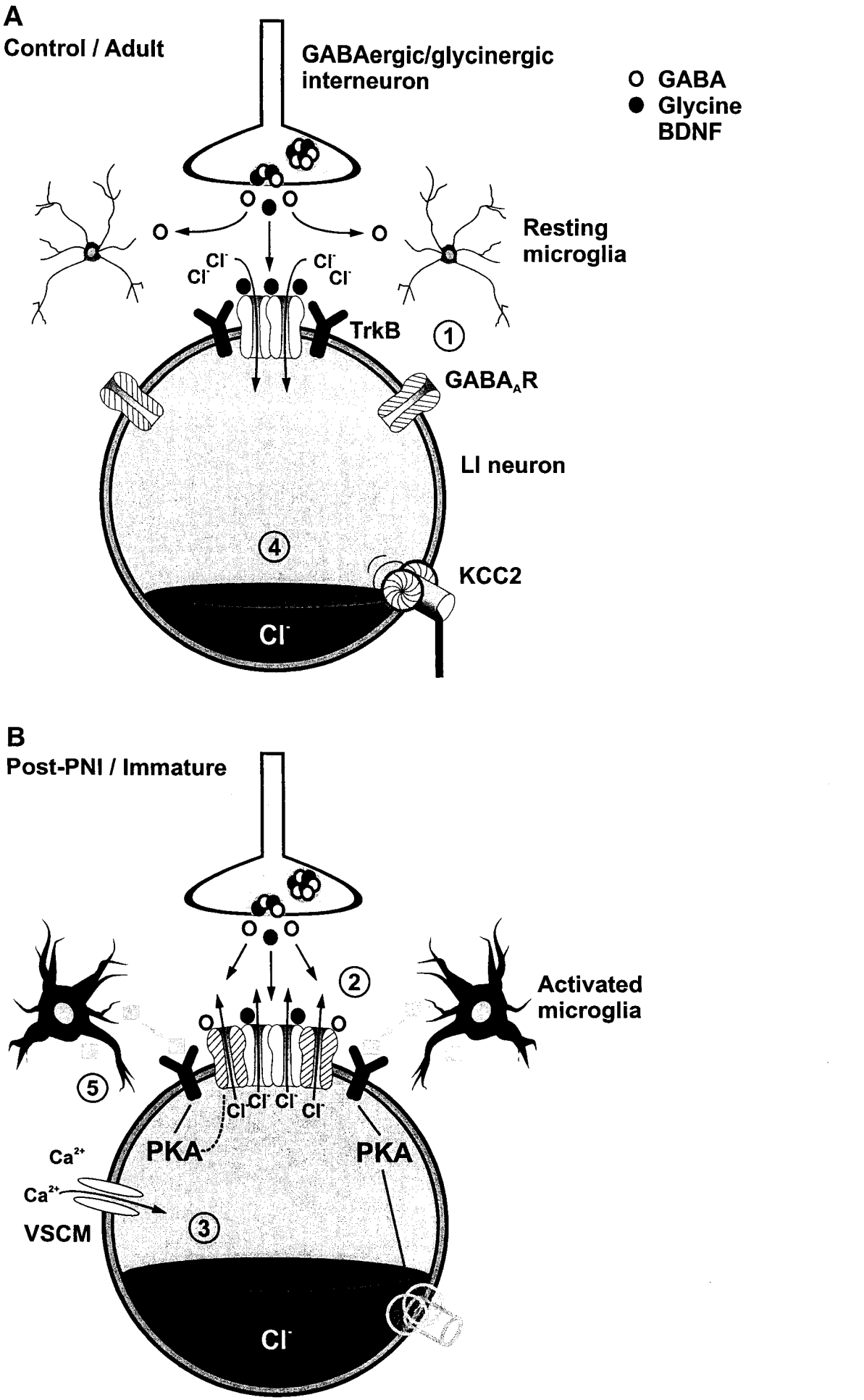

A
10 pA
10 ms
B
C
Bicuculline
D
Strychnine
E
BDNF/ACSF
BDNF/bicuculline

Figure 4. Schematic drawings illustrating the pathways responsible for mediating the effect of microglia-derived BDNF on LI neuronal *trans*membrane anion gradient subsequent to PNI. ***A***, under control conditions. ***B***, after PNI. For further details, please see DISCUSSION text.


A
Control / Adult
GABAergic/glycinergic interneuron
GABA
Glycine
BDNF
Resting microglia
Cl-
TrkB
1
GABA$_A$R
LI neuron
4
KCC2
Cl-
B
Post-PNI / Immature
2
Activated microglia
5
Cl-
PKA
PKA
Ca2+
Ca2+
VSCM
3
Cl-

ORIGINAL CONTRIBUTIONS

- I have provided evidence that the contribution of $GABA_A$ and glycine receptors ($GABA_ARs$ and GlyRs) to postsynaptic inhibition in lamina I and II neurons is different in developmentally immature rats compared to adult rats. Specifically, I have demonstrated that quantal corelease of GABA and glycine coactivates $GABA_ARs$ and GlyRs at lamina I neuronal synapses in slices taken from neonatal rats, whereas in slices from adult rats, coreleased GABA and glycine are never codetected at lamina I synapses.

- I have found that after peripheral nerve injury (PNI), adult rat lamina I neuronal synapses adopt a configuration similar to that detected in slices taken from immature rats, where coreleased GABA and glyine coactivate their respective receptors, $GABA_AR$ and GlyR.

- I have revealed a novel mechanism directly contributing to the generation of neuropathic pain. That is, I have shown that PNI causes a downregulation of the KCC2 potassium-chloride cotransporter, and hence effects a collapse of the lamina I neuronal transmembrane anion gradient. This shift in gradient was shown to result in depolarizing anionic currents mediated by $GABA_A$ and glycine receptors – thus contributing to substantial hyperexcitability in lamina I neurons.

- I provided evidence that subsequent to PNI, BDNF activation of TrkB in lamina I neurons was responsible for triggering the depolarization of anion gradient described above.

- Moreover, I demonstrated that the BDNF that triggered the shift in anion gradient after PNI was derived from microglia displaying a hyperactive phenotype.

- I elucidated the pathway linking TrkB activation with the decrease in anion gradient after PNI: such intracellular messengers as calcium/calmodulin-dependent kinase and cAMP-dependent kinase were identified to be critical for effecting the alteration of anion gradient in response to PNI.

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APPENDIX 1
CERTIFICATE OF COMPLIANCE TO STANDARD OF ANIMAL CARE

APPENDIX 2
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