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INHIBITORY CONTROL OF NEURONS IN THE MARGINAL ZONE (LAMINA I) OF THE RAT SPINAL CORD

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ABSTRACT

Lamina I neurons constitute a major output pathway from the dorsal horn of the spinal cord, relaying sensory information to the brain. Investigating how the excitability of these neurons is controlled is important for the understanding of nociceptive processing. The extensive colocalization of the major inhibitory neurotransmitters GABA (γ-aminobutyric acid) and glycine in the marginal zone of the rat spinal cord, raises the issue of their respective role in this spinal area. Therefore, I developed a spinal cord slice preparation that allowed us to clearly delineate lamina I neurons for *in vitro* studies. I used *whole cell* patch clamp recording in identified lamina I neurons to study spontaneously occurring inhibitory postsynaptic currents (IPSCs), and stimulus evoked IPSCs. In particular, spontaneous miniature (action potential independent) IPSCs (mIPSCs) were studied as they are thought to reflect transmitter release from single vesicles. An important initial finding was that lamina I neurons were almost exclusively bombarded by glycine receptor (GlyR)-mediated mIPSCs. However, all cells appeared to express both $GABA_A$ receptors ($GABA_ARs$) and GlyRs. Moreover, recording of mIPSCs in the presence of a benzodiazepine revealed $GABA_AR$-mediated mIPSCs with kinetic properties consistent with the interpretation that $GABA_ARs$ are likely located at extrasynaptic sites in lamina I neurons. Thus, GABA and glycine appeared to be co-released from the same synaptic vesicles. Together with IPSCs evoked from low- and high-intensity electrical stimulations, the results indicated that glycine is responsible for most tonic inhibitory control in lamina I, while $GABA_AR$-mediated inhibition may be more relevant for the control of evoked input. Paired-pulse stimulations indicated that

tonically released GABA appeared to first serve to activate presynaptic $GABA_B$ autoreceptors. To test the plasticity of GABAergic and glycinergic systems in lamina I, I have investigated their possible alteration in a rat with experimental neuropathy (injury of the sciatic nerve). The results indicated an imbalance between excitation and inhibition, and a possible reduction of activity in inhibitory interneurons leading to a disinhibition (and eventually a hyperexcitability) of lamina I neurons. Importantly, while glycine receptor-mediated mIPSCs were significantly reduced in amplitude, an additional $GABA_A$ component to the mIPSCs was revealed, possibly serving as a partial compensatory mechanism for the loss of glycinergic input. These results provide a better understanding of inhibitory control in lamina I neurons, and will ultimately allow the manipulation of selective aspects of inhibition, using specific drugs for the prevention of altered inhibition in the spinal cord, and sensory disorders in general in the CNS.

RÉSUMÉ

Les neurones de la lamina I de la corne dorsale de la moelle épinière constituent une voie principale de relai d'information nociceptive vers le cerveau. Il est donc important d'étudier les mécanismes de contrôle de leur excitabilité, pour comprendre la physiologie de la nociception. Les rapports sur la co-localisation des neurotransmetteurs inhibiteurs principaux, que sont le GABA (γ-aminobutyric acid) et la glycine, dans la *zone marginale* de la moelle épinière de rat, nous portent à questionner leur rôle respectif dans cette région spinale. Par conséquent, j'ai développé une préparation de tranche de moelle épinière permettant de bien définir les limites de la lamina I pour des études *in vitro* des neurones de cette couche. J'ai utilisé la méthode de patch clamp en configuration *whole cell* pour étudier, chez des cellules identifiées de la lamina I, les courants postsynaptiques inhibiteurs (CPSIs) spontanés, et les CPSIs évoqués. J'ai étudié en particulier les IPSCs miniatures, (qui sont indépendants des potentiels d'action), comme ils réflètent la libération de transmetteurs par des vésicules synaptiques unitaires. Un résultat initial important est que, dans la lamina I, les CPSIs miniatures sont prequ'exclusivement dus à l'activation des récepteurs de la glycine (GlyR). Cependant, toutes les cellules semblaient exprimer les récepteurs $GABA_A$ ($GABA_ARs$) ainsi que les GlyRs. De plus, l'enregistrement de CPSIs miniatures en présence de benzodiazépine a révélé des CPSIs miniatures dus à l'activation des $GABA_ARs$, dont les propriétés cinétiques indiquent que les $GABA_ARs$ se trouveraient à des sites extrasynaptiques chez les neurones de la lamina I. Ainsi, le GABA et la glycine seraient co-libérés à partir des mêmes vesicules synaptiques. Avec l'enregistrement des CPSIs évoqués, ces résultats

suggèrent que la glycine serait principalement responsable de l'inhibition tonique dans la lamina I, alors que l'inhibition de type $GABA_AR$ serait plus importante pour le contrôle des évènements évoqués. Les résultats de stimulations couplées ont indiqué que le GABA libéré lors d'activité tonique servirait d' abord pour l'activation des autorécepteurs $GABA_B$. Pour étudier la plasticité des systèmes GABAergiques et glycinergiques dans la lamina I, j'ai utilisé des rats ayant une neuropathie expérimentale (suite à une constriction du nerf sciatique). Mes résultats ont suggéré un déséquilibre entre excitation et inhibition dans la lamina I, et une réduction importante de l'activité des interneurones inhibiteurs, ayant possiblement entraîné une disinhibition (et éventuellement une hyperexcitabilité) des neurones de la couche I. Alors que les CPSIs miniatures dus à l'activation des GlyRs étaient réduits de façon importante, une composante additionnelle $GABA_A$ au CPSIs miniatures a été révélée, et pourrait probablement servir de compensation partielle pour la perte de contrôle glycinergique. Ces résultats permettent de mieux comprendre les mécanismes de contrôle inhibiteur des neurones de la lamina I, et pourraient servir à cibler certains aspects de l'inhibition, afin de développer des traitements spécifiques pour la prévention, non seulement d'altérations des systèmes d'inhibition dans la moelle épinière, mais aussi d'autres désordres sensoriels du système nerveux central.

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CONTRIBUTION OF AUTHORS

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

Chapter 2. Visualization of lamina I of the dorsal horn in live adult rat spinal cord slices. Chéry, N., Yu X.H. and De Koninck, Y., *Journal of Neuroscience Methods* (under revision).

I performed most of the work described in this chapter, under the supervision of Dr. De Koninck. I wrote the manuscript on which this chapter is based, and revisions to the text and figures were provided by Dr. De Koninck. The immunocytochemical experiments with NK1 (figure 2) were performed by Dr. Xiao Hong Yu.

Chapter 3. Junctional versus extrajunctional glycine and $GABA_A$ receptor-mediated IPSCs in identified lamina I neurons of the adult rat spinal cord. Chéry, N. and De Koninck, Y., *Journal of Neuroscience*, 19 (17) 7342-7355.

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Chapter 4. $GABA_B$ receptors are the first target of released GABA at lamina I inhibitory synapses in the rat spinal cord. Chéry, N. and De Koninck, Y. (to be submitted).

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Chapter 5. A shift in the balance of excitatory and inhibitory drive to lamina I neurons in neuropathic rats. Chéry, N. and De Koninck, Y. (to be submitted).

I performed most of the work described in this chapter, and wrote the manuscript on which this chapter is based under the supervision of Dr De Koninck. Ms Annie Constantin performed the sciatic nerve injury described and the behavioral tests.

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LIST OF ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
AMP	Adenosine MonoPhosphate
ATP	Adenosine Triphosphate
cAMP	Cyclic AMP
CNS	Central Nervous System
DRG	Dorsal Root Ganglion
G protein	Guanine-Nucleotide-Binding protein
GABA	γ-Aminobutyric acid
GABA-LI	GABA-Like Immunoreactivity
GAT	GABA Transporter
Glycine-LI	Glycine-Like Immunoreactivity
GlyR	Glycine Receptor
GLYT	Glycine Transporter
IPSC	Inhibitory PostSynaptic current
mIPSCs	Miniature IPSCs
mRNA	Messenger RNA
NMDA	N-methyl-D-aspartate
PKA	Protein Kinase A
PKC	Protein Kinase C
pS	PicoSiemens
RNA	RiboNucleic Acid
sIPSCs	Spontaneous IPSCs

STT	SpinoThalamic Tract
TTX	Tetrodotoxin
VIAAT	Vesicular Inhibitory Amino Acid Transporter

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Pain is a complex sensation that remains difficult to describe. The International Association for the Study of Pain (IASP) defines it as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Melzack, Wall, 1989). There appears to be two distinct aspects of pain. One aspect, acute pain, is thought to occur following a stimulus sufficiently intense to cause tissue injury (Woolf, 1991; Fields, Basbaum, 1994). For example, a pinch or firm pressure, an excess of cold/heat, or chemical irritants to the skin, generate acute pain. The unpleasant, (usually transient) sensation results from the activation of high threshold primary afferent fibers called nociceptors (Willis, Coggeshall, 1991). These nociceptors convey the nociceptive (pain-related) information from their sensory ending in the tissue, to their central synaptic terminals in the dorsal horn of the spinal cord, where the integration of nociceptive information occurs, before being relayed to higher brain centers (Willis, Coggeshall, 1991; Devor, 1994). It is generally believed that the role of acute pain is to warn the body of potential threats to its integrity; accordingly, acute pain can be present during an injury but should disappear after healing (Woolf, 1991).

In contrast, chronic pain may persist for months or even years after healing (Bennett, 1994). It is thought to result from lesions not only (or necessarily) to tissue, but also to the nervous system (Bennett, 1994). The mechanisms involved may include chemical and phenotypic alterations in primary sensory fibers (Neumann et al., 1996; Coggeshall et al., 1997), enhanced excitability or reduced inhibition (disinhibition) of spinal dorsal horn neurons, or other sensory regions in the CNS (Simone et al., 1989; Woolf et al., 1994; Devor, 1994; Sivilotti, Woolf, 1994; Woolf, 1995).

The superficial layers of the dorsal horn, particularly lamina I (the marginal zone) and lamina II (also called *substantia gelatinosa*), represent the first site for the integration and relay of nociceptive sensory input from primary afferent fibers (Willis, Coggeshall, 1991; Light, 1992). In contrast to neurons in lamina II which are mostly propriospinal (Willis, Coggeshall, 1991), many lamina I neurons have long axons reaching specific brain structures that have been shown to play an important role in the processing of pain-related information (Willis, Coggeshall, 1991; Light, 1992; Craig et al., 1994). For instance, lamina I neurons contribute to approximately half of the cells that project to the thalamus via the spinothalamic tract (STT), which is considered as a critical pathway for the perception of pain and temperature in mammals (Craig, Jr. et al., 1989; Craig et al., 1994; Willis et al., 1995; Zhang, Craig, 1997); thus, lamina I represents a major source of output from the dorsal horn. Moreover, several studies have shown that lamina I neurons can receive input directly from nociceptive, thermoreceptive and mechanical afferent fibers (Christensen, Perl, 1970; Light, Perl, 1979b; Light et al., 1979; Woolf, Fitzgerald, 1983; Han et al., 1998).

Neurons in lamina I are subjected to both excitatory and inhibitory influences (Willis, Coggeshall, 1991), the balance of which determines how a noxious input will be relayed to the brain, and thus how it will eventually be perceived by an individual. Many of the investigations on the physiology of dorsal horn neurons involved in nociception focused on the excitatory input to those cells, probably because these cells display an increased excitability following nerve/tissue damage [which can lead to chronic pain states (Coderre et al., 1993; Bennett, 1994)]. However, the results of other studies suggest that inhibitory input to dorsal horn neurons plays a crucial role in the processing of nociceptive information. For instance, intrathecal application of sub-convulsive doses of

bicuculline or strychnine, which antagonize inhibitory transmission mediated by γ-aminobutyric acid (GABA) and glycine, respectively, was shown to produce nociceptive responses to normally innocuous tactile stimuli, a characteristic of some chronic neuropathic pain syndromes (Yaksh, 1989; Yamamoto, Yaksh, 1993; Sherman, Loomis, 1996; Sorkin et al., 1998). Thus, the observed increase in excitability in certain chronic pain conditions (for example, following a peripheral nerve injury (Bennett, Xie, 1988)) could be the result of a decrease in inhibitory control of lamina I neurons, in such a way that the balance of input would favor excitation, or a previously suppressed excitatory pathway could be unmasked, leading to exaggerated responses to noxious stimuli, or even the occurrence of responses to innocuous stimuli in neurons normally only responsive to noxious stimuli (Simone et al., 1989; Woolf et al., 1994; Sivilotti, Woolf, 1994). Consequently, the inhibition mediated by GABA and glycine, the two main inhibitory transmitters in the central nervous system (CNS) (Sivilotti, Nistri, 1991; Betz et al., 1994), is likely to play a major role in the processing of nociceptive sensory information in lamina I. Yet, the inhibitory control mediated by these neurotransmitters on neurons involved in nociceptive processing remains incompletely understood. It is known that GABA and glycine can depress the activity of nociceptive neurons (Curtis et al., 1969; Curtis et al., 1971), and inhibitory currents have been recorded in the dorsal horn in response to activation of sensory fibers (Yoshimura, Nishi, 1993; De Koninck, Henry, 1994; Baba et al., 1999). Moreover, immunocytochemical data is available on the co-existence of GABA and glycine in many lamina I neurons, and the greatest concentration of spinal GABA appears to occur in laminae I-III (Todd, Sullivan, 1990; Todd et al., 1996b). Furthermore, in animal models of chronic pain, hypersensitivity to innocuous

tactile stimuli is exacerbated by bicuculline and strychnine (Yamamoto, Yaksh, 1993), and findings of a reduction in GABA-immunoreactive neurons in laminae I-III, in animals with peripheral nerve injury, adds further support to the determinant inhibitory role of GABA and glycine in nociceptive transmission in the superficial dorsal horn (Ibuki et al., 1997).

Despite a number of *in vivo* investigations of inhibitory mechanisms in lamina I (Christensen, Perl, 1970; Light, Perl, 1979b; Light et al., 1979; Woolf, Fitzgerald, 1983; Han et al., 1998), a direct study of inhibitory control of nociceptive input by GABA and glycine in this important spinal area is not available. Studies of inhibition in the dorsal horn have focused on inhibitory postsynaptic potentials (IPSPs) and currents (IPSCs) secondary to the activation of sensory nerves, thus the recorded inhibitory events are probably of polysynaptic (indirect) nature (Yoshimura, Nishi, 1993). Direct information is particularly lacking on several aspects of inhibitory transmission in lamina I, such as the pharmacological properties, modulation and distribution of inhibitory events among the different types of neurons. Direct studies of inhibition in lamina I have been hampered by the difficulty in accessing the small neurons in this area and recording their activity without damaging them, as well as the difficulty to resolve elementary components of synaptic transmission in the CNS. With the development of the patch clamp technique and CNS slices, it is now possible to record synaptic currents with a high level of resolution (Sakmann et al., 1989; Neher, Sakmann, 1992). In combination with pharmacological tools, patch clamp recording allows to study the properties of inhibitory receptor/channels during their normal physiological function at individual synapses in live, adult CNS tissue.

Therefore, the main objective in this thesis was to characterize the inhibitory currents mediated by GABA and glycine in lamina I, using the patch clamp technique. In particular, miniature (action potential independent) IPSCs (mIPSCs), which reflect transmitter release due to terminal activity alone, were studied. These mIPSCs are thought to represent responses to the release of single vesicles of transmitter (Edwards et al., 1990; Mody et al., 1994), and are considered as powerful tools to study elementary components of inhibitory transmission.

In the following sections, I will review the properties of lamina I neurons, focussing mainly on their physiological characteristics, and I will also provide background information on the mechanisms of GABA- and glycine-mediated inhibitory control in the CNS, with a particular emphasis on the spinal cord.

2. THE MARGINAL ZONE OF THE SPINAL CORD

Lamina I was described by Rexed as "a thin veil of gray substance, forming the dorsal-most part of the spinal gray matter" (Rexed, 1952). It is identical to the marginal zone characterized by Waldeyer over a century ago (Light, 1992). In rats, this region has a thickness of approximately 20 μm only (Molander et al., 1989), and many of its neurons receive input from different types of primary afferent fibers as demonstrated by histochemical and electrophysiological studies (Light, Perl, 1979a; Light et al., 1979; Woolf, Fitzgerald, 1983). Such a variety of afferent input probably underlies the different functions of neurons in this layer, and also indicates a complex integration of sensory input in lamina I.

2.1. Classification of lamina I neurons

2.1.2 Cellular morphology.

Several lamina I cell types have been described in different species, based on reconstruction of cell bodies and proximal dendrites of neurons stained with the Golgi impregnation technique (Gobel, 1978b; Beal et al., 1981; Lima, Coimbra, 1986), as well as with retrograde and direct intracellular labeling of the neurons using different markers, (Light et al., 1979; Bennett et al., 1981; Molony et al., 1981; Woolf, Fitzgerald, 1983). Three major cell types were reported for various species: fusiform neurons appear to have a longitudinal soma and two dendrites arising from each end of the soma; multipolar cells were reported to have a dense dendritic tree (composed of four or more dendrites arising from the cell body), and pyramidal neurons would have a triangular shape, with three dendrites arising from each pole of the triangle. The main components of this classification appear to be consistent between species such as the rat (Lima, Coimbra, 1986), the cat (Gobel, 1978b; Light et al., 1979), the monkey (Beal et al., 1981) and the human (Schoenen, 1982). Further subdivision of cell types has been proposed based on the occurrence or not of ventrally directed dendrites (Lima, Coimbra, 1986).

2.1.3 Functional classes

Primary afferent input. The sensory information that lamina I neurons relay to the brain comes directly or indirectly from primary afferent fibers. A large number of thinly myelinated fibers enter lamina I from a pathway known as the tract of Lissauer and

from the surrounding white matter, and form a structure called the marginal plexus (Willis, Coggeshall, 1991). About 50% of these fibers are primary afferents, and the remaining appear to arise from propriospinal and descending neurons (Willis, Coggeshall, 1991). It has been suggested that the primary afferent terminals in lamina I originate predominantly from the Aδ type (thinly myelinated) nociceptive fibers (Beal, Bicknell, 1981). This was supported by electrophysiological recordings of responses of lamina I neurons to activation of sensory fibers, and by reconstructions of labeled axons that were found to have an impulse conduction speed consistent with that of Aδ fibers (Light, Perl, 1979b; Light et al., 1979; Woolf, Fitzgerald, 1983). Many lamina I neurons were found to be activated by stimulation of thinly myelinated Aδ fibers; however, they could also respond to electrical activation of nociceptive unmyelinated C fibers (Christensen, Perl, 1970; Light et al., 1979; Gobel et al., 1981; Woolf, Fitzgerald, 1983; Fitzgerald, Wall, 1980). This suggests that lamina I neurons are able to respond to a wide range of nociceptive modalities, such as noxious mechanical, and/or noxious thermal stimulation. Intracellular and extracellular recordings provided evidence of an additional input from large Aβ fibers, which are usually thought to be associated with innocuous tactile and proprioceptive sensory information (Light et al., 1979; Woolf, Fitzgerald, 1983), although anatomical studies report no finding of large primary afferent fibers entering this layer (Brown, 1981b). It was therefore proposed that the source of Aβ input was likely via polysynaptic pathways (Woolf, Fitzgerald, 1983). Alternatively, these lamina I neurons responsive to innocuous stimulation could themselves have dendrites reaching deeper laminae where Aβ fibers terminate, and thus receive direct or indirect input from those

fibers at that level, although there is no electrophysiological study supporting this hypothesis.

Non-nociceptive C fibers are found in peripheral cutaneous nerves. Some are likely innocuous thermal receptors responding to slight increases or decreases in skin temperature (Hensel, Iggo, 1971; Hensel et al., 1974; Bessou et al., 1971; Light, 1992; Dostrovsky, Craig, 1996; Han et al., 1998). There is also a substantial population of low threshold C mechanoreceptors in cutaneous nerves (Light, 1992)

Nociceptive-specific neurons. A large proportion of neurons in the dorsal horn that are activated only by noxious stimulation are found in the marginal zone (Christensen, Perl, 1970; Woolf, Fitzgerald, 1983). Some cells respond exclusively to noxious mechanical, noxious thermal, or both and they have a small receptive field (Light, 1992). They constitute a significant proportion of the lamina I cell population in the cat and the monkey (Light, 1992; Dostrovsky, Craig, 1996). In the rat, a few marginal layer cells appear to be activated only by Aδ-fiber stimulation, while the majority responds to both Aδ and C-fiber nociceptive input (Woolf, Fitzgerald, 1983).

Multireceptive/wide-dynamic-range neurons. Another class of lamina I neurons was shown to respond to various types of inputs. They could be activated by low-threshold mechanoreceptors, thinly myelinated and/or C-fiber nociceptors (Woolf, Fitzgerald, 1983; Woolf, Fitzgerald, 1983; Light, 1992). They are often described as wide-dynamic-range neurons. Most authors agree that very few neurons in lamina I have

wide-dynamic-range properties (i.e responsive to both innocuous and noxious stimuli) (Steedman et al., 1985; Craig, Kniffki, 1985; Light, 1992; Woolf, Fitzgerald, 1983).

Neurons activated by innocuous thermal stimuli. It was demonstrated that some lamina I neurons in the monkey and in the cat were activated specifically by innocuous cooling (Light, 1992; Dostrovsky, Craig, 1996; Han et al., 1998). The C fiber type activated by innocuous thermal stimuli appear to have a faster conduction velocity than the C nociceptors, and their percentage varies between species (Light, 1992).

Polymodal nociceptive neurons. Another type of lamina I neurons may respond to noxious heat, pinch and cold stimuli (Dostrovsky, Craig, 1996; Han et al., 1998), following the activation of C polymodal nociceptors. Like other types of C fibers, C polymodal nociceptors activation threshold is greater than that of myelinated nociceptors. They display graded responses to increasing intensities of stimuli (Light, 1992).

Thus, lamina I neurons appear to be arranged in subsets that have distinct functional and anatomical characteristics. Nevertheless, the physiological properties of the neurons may not be absolute, as they do not depend only on their monosynaptic contacts with afferents, but also on polysynaptic interactions with different pathways. Moreover, activity in the various pathways can be modulated by past experience as well as ongoing activity from different brain regions (Wall, 1989; Perl, 1998). For example, nociceptive-specific lamina I neurons can start to respond to innocuous stimuli under certain circumstances (Woolf, Fitzgerald, 1983; Woolf et al., 1994).

2.2 Supraspinal projection pattern

Many lamina I neurons, which receive input from primary afferents, relay the sensory information through long axons projecting to rostral sites (Willis, Coggeshall, 1991). This was demonstrated by retrograde and anterograde labeling techniques, using various tracers. Some lamina I neurons project to the thalamus, forming the spinothalamic tract, a major pathway for information from neurons signaling pain and temperature (Lima, Coimbra, 1988; Willis, Coggeshall, 1991; Dostrovsky, Craig, 1996; Sherman et al., 1997a; Zhang, Craig, 1997). Some of the neurons reaching the thalamus may send collaterals to midbrain and medullary nuclei involved in nociception, and neurons projecting to the midbrain and medulla also send collaterals to neighboring regions (Swett et al., 1985; McMahon, Wall, 1985; Lima, Coimbra, 1989; Hylden et al., 1989). These specific supraspinal sites were also demonstrated by activating nerve terminals in specific brain regions while recording the antidromic responses to such activation in the dorsal horn in rats, cats and monkeys (Menetrey et al., 1980; Light, 1988; Hylden et al., 1985; Hylden et al., 1986; Dostrovsky, Craig, 1996).

2.3 Interaction with other laminae in the dorsal horn

A certain proportion of lamina I neurons have axon collaterals extending to deeper laminae (Lima, Coimbra, 1986), suggesting that they may directly interact with deeper dorsal horn neurons. Indeed, paired extracellular recordings from rat deep dorsal horn cells following the stimulation of neurons in the superficial layers suggested that lamina I neurons could modulate the activity of neurons in other laminae (Biella et al., 1997). Conversely, some neurons in lamina II and lamina III were shown to have axonal and dendritic processes reaching lamina I, and it was suggested that they might relay

excitatory input to the marginal zone (Gobel, 1978a; Bennett et al., 1980; Naim et al., 1998). For example, anatomical observations and electrophysiological recordings from pairs of neurons in lamina I and II in monkeys indicated that stalked cells in lamina II would be excitatory interneurons receiving input on their dendrites from primary afferents (with endings in layers II and III) and relaying it to lamina I projection neurons (Gobel, 1978a; Price et al., 1979). This may represent a substrate for the Aβ fiber input to lamina I (since Aβ fibers are thought to terminate mainly in deeper dorsal horn layers) (Woolf, Fitzgerald, 1983) (see section **2.1.3**).

Lamina I neurons may also receive inhibitory input from deeper dorsal horn neurons. This issue is covered in the following sections.

3. CONTROL OF NEURONAL ACTIVITY IN LAMINA I

In addition to the cells with long projecting axons, some neurons in lamina I have axons that appear to arborize locally (Willis, Coggeshall, 1991). Intrinsic neurons, which are found mainly in lamina II, (Willis, Coggeshall, 1991) are thought to play an important role in the modulation of incoming somatosensory information before it is relayed to higher centers, and thus they have been implicated in several theories of pain and analgesia (Melzack, Wall, 1965; Basbaum, Fields, 1978). For example, Melzack and Wall (1965) proposed that activity in large afferent fibers could inhibit synaptic transmission in neurons activated by small afferent fibers that carry pain signals, and suggested that inhibitory neurons in lamina II act as a gating mechanism to control afferent input before it affects nociceptive transmission cells (which are found in a large proportion in lamina I).

I will focus on the control of lamina I neuronal activity mediated GABA and glycine, the major inhibitory amino acids in the CNS (Sivilotti, Nistri, 1991), as many studies strongly suggest that they play a dominant role in the modulation of nociception in the dorsal horn (Curtis et al., 1969; Curtis et al., 1971; Yaksh, 1989; Yoshimura, Nishi, 1993; De Koninck, Henry, 1994; Sherman, Loomis, 1996; Sorkin et al., 1998).

3.1 GABA and glycine

GABA and glycine usually mediate fast inhibitory synaptic events (Sivilotti, Nistri, 1991) and they are found throughout the spinal cord (Todd, Spike, 1993). GABA and glycine appear to be localized in many superficial dorsal horn neurons (van den Pol, Gorcs, 1988; Todd, McKenzie, 1989; Todd, Sullivan, 1990; Proudlock et al., 1993; Mitchell et al., 1993; Bohlhalter et al., 1994; Todd et al., 1995a; Todd et al., 1996b; Bohlhalter et al., 1996). Since it was shown that many spinal neurons can be inhibited by GABA and glycine (Curtis et al., 1968), and that these inhibitory effects can be blocked by bicuculline and strychnine (the antagonists at $GABA_A$ and glycine receptors, respectively), neurons that contain these transmitters were assumed to have an inhibitory function. Recent studies have focussed on GABA- and glycine-mediated inhibitory postsynaptic events (IPSPs and IPSCs) in lamina II. The inhibitory events studied involved di- or multisynaptic inhibition (polysynaptic) as they were elicited following the stimulation of afferent fibers (Yoshimura, Nishi, 1995). It was found that some of the events were mediated by glycine only, others by GABA only, and some were mixed GABA- and glycine-mediated events (Yoshimura, Nishi, 1993; Yoshimura, Nishi, 1995). Thus, these data indicated that GABA and glycine can mediate distinct synaptic events in the superficial dorsal horn, even though they are frequently co-localized (Todd et al.,

1996b). Yet, their frequent co-localization and mode of action indicate a synergistic inhibitory role for GABA and glycine. The fact that they could be contained in the same synaptic vesicles and be co-released does not necessarily preclude a distinct role for these inhibitory transmitters. Because even if co-released, GABA and glycine may act on their respective receptors in a distinct manner, and their effect also depends on the presence and/or synaptic localization of their respective receptors. To address these issues, a study of miniature IPSCs should prove most useful, as they reflect the activation of postsynaptic receptors by single vesicles of transmitter (Edwards et al., 1990; Mody et al., 1994).

The following is a background review of the properties of GABA and glycine in the CNS, with particular emphasis on the spinal cord.

3.2 GABA

GABA is an amino acid derived from glutamate, it is formed by the enzymatic action of glutamic acid decarboxylase (GAD) (Ottersen et al., 1995). GABA-containing structures were first identified with the use of antibodies to GAD (McLaughlin et al., 1975). More recently, antibodies were raised against conjugates of GABA and were used as markers for GABAergic profiles in the CNS (Hodgson et al., 1985).

Following its release, GABA interacts with the receptor subtypes: $GABA_A$, a ligand-gated Cl^- channel (Sivilotti, Nistri, 1991), and $GABA_B$, a G protein-coupled receptor associated with K^+ and /or Ca^{2+} channels (Bowery, 1993). GABA may also bind to a third subtype, the $GABA_C$ receptor, a Cl^- ion channel like the $GABA_A$ type, but unlike the latter, $GABA_C$ receptors are insensitive to bicuculline (Johnston, 1996).

The inhibitory role of GABA was established from experiments showing its depressant action on the electrical activity in the brain [for reviews, see (Sivilotti, Nistri, 1991; Kaila, 1994)]. Early experiments on mammalian spinal cord neurons demonstrated an important depressant effect of iontophoretically applied GABA (Curtis et al., 1959). Some histochemical studies have indicated that several types of primary afferent terminals within lamina I are postsynaptic to GABAergic profiles. For example, it was shown that Aδ nociceptive fibers in cat and monkey are subjected to GABAergic presynaptic inhibition (Alvarez et al., 1992). However, the main afferents receiving GABA input appear to be the Aβ myelinated fibers (Wall, Devor, 1981; Woolf, Wall, 1982; Laird, Bennett, 1992). This GABAergic control of neuronal firing at axo-axonic synapses on primary afferents is referred to as primary afferent depolarization (Jankowska et al., 1981; Davidoff, Hackman, 1985). It was described as a phenomenon whereby afferent volleys entering the dorsal grey matter initiate an intense synaptic inhibitory process, the resulting effect of which is to reduce the release of excitatory transmitters from afferent terminals (Davidoff, Hackman, 1985).

Despite the importance of primary afferent depolarization for the control of sensory input, most of the GABAergic terminals in the spinal cord are presynaptic to intrinsic neurons (Todd, Spike, 1993). Inhibitory control of dorsal horn neurons, particularly lamina I, has not been studied at the cellular level. Because lamina I represents a major output pathway from the dorsal horn, relaying nociceptive sensory information to the brain [(Willis, Coggeshall, 1991) and see section **1.**], a study of inhibitory control of neurons in this layer plays an important role in determining how noxious information is processed.

3.2.1 Source of GABA in lamina I.

In the mammalian spinal cord, the highest concentration of GABA is found in the superficial laminae I-III (Todd, Spike, 1993). GABA is present in 28% of lamina I neurons, 9% of which also contain glycine (Todd, Sullivan, 1990). GABAergic terminals within lamina I appear to originate mainly from local neurons or other spinal neurons (from deeper layers or other segments) (Todd, Spike, 1993). Evidence has been presented for GABAergic axonal projections from the medulla to the dorsal horn (Jones et al., 1991; Antal et al., 1996) and some of these medullo-spinal fibers immunoreactive for GABA may also contain glycine. However, it was shown that complete transection of cat thoracic spinal cord did not cause a significant decrease in GABA concentration in the lumbar segment (Rizzoli, 1968). Thus, it can be inferred that most of the GABA present in the spinal cord would be from local/segmental origin.

3.2.2 Regulation of GABA release and uptake.

Membrane transporters. The clearance of GABA from the synaptic cleft is achieved by diffusion of this transmitter (Thompson, 1994) and by specific, high-affinity membrane transport systems, located on the presynaptic terminals and neighboring glial cells (Borden, 1996). GABA transporters belong to the family of Na^+- and Cl^--coupled transporters. The coupling of the neurotransmitter transport to the movement of sodium ions occurs against an electrochemical gradient. At least three different types of GABA transporters have been cloned from the human, rat and mouse (Borden, 1996). *In situ* hybridization studies revealed that one of these types, the GAT-1 (GABA Transporter 1) subtype is the most abundant and widespread in the CNS (Borden, 1996). In the spinal

cord, GAT-1 is expressed at the highest level in the dorsal horn, particularly in laminae I, II, IV (Borden, 1996).

Nipecotic acid (a GABA analog) and its derivatives inhibit GABA transport, and allowed to investigate how membrane transporters regulate the time course of GABA in the extracellular space. Most of these GABA uptake inhibitors display a greater affinity for GAT-1 (Borden, 1996), and their effect is to prolong the presence of GABA in the synaptic cleft (Krogsgaard-Larsen et al., 1987; Isaacson et al., 1993; Thompson, 1994). In some studies it was shown that tiagabine, a widely used GABA uptake blocker, produced a significant prolongation of the time course of $GABA_A$ receptor-mediated IPSCs evoked by electrical stimulation of rat hippocampal neurons; however, the uptake inhibitor did not affect the characteristics of $GABA_A$ receptor-mediated spontaneous and miniature IPSCs (mIPSCs), which are thought to arise from basal (low intensity/frequency) synaptic activity (Thompson, Gahwiler, 1992; Isaacson et al., 1993). Therefore, it was suggested that diffusion alone could account for the fast clearance of GABA from the synaptic cleft during miniature events (Thompson, Gähwiler, 1992; Isaacson et al., 1993).

Vesicular transporters. Cytosolic GABA is taken up into synaptic vesicles for further release by exocytosis (Maycox et al., 1990). Synaptic vesicles contain specific transporters for amino acid transmitters. They display a lower affinity for the neurotransmitters than the membrane transporters, and unlike the latter, require energy provided by a proton ATPase (Maycox et al., 1990). The vesicular GABA transporter was cloned from the nematode *Caenorhabditis elegans unc-47* gene (McIntire et al., 1997). Biochemical experiments have suggested that the mammalian vesicular transporters of GABA and glycine are functionally similar (Burger et al., 1991; Christensen et al., 1991; Christensen, Fonnum, 1991), although other studies suggested that GABA and glycine

could have distinct vesicular transporters (Kish et al., 1989; McIntire et al., 1997). There is immunocytochemical data showing that a rodent homologue of the *unc-47* mRNA, named Vesicular Inhibitory Amino Acid Transporter (VIAAT), is localized at GABAergic as well as glycinergic nuclei in the rat brain (Sagne et al., 1997). And recently, Dumoulin and collaborators reported that VIAAT is localized in GABAergic and glycinergic synaptic boutons in the rat spinal cord (Dumoulin et al., 1999). That GABA and glycine are taken up by the same vesicular transporter was also supported by electron microscopy studies from rat brain (Chaudhry et al., 1998), and by electrophysiological studies (Jonas et al., 1998). Thus, it can be inferred from these findings that GABA and glycine can be stored in the same synaptic vesicles, and therefore can be co-released and act together at the same synaptic junction via their respective receptors.

3.2.3 Receptor targets of GABA in the marginal zone.

The major GABA receptor targets studied are the $GABA_A$ and $GABA_B$ subtypes (Macdonald, Olsen, 1994).

3.3 $GABA_A$ receptors

3.3.1 Structural characteristics and spinal localization.

The $GABA_A$ receptor has been studied extensively (Macdonald, Olsen, 1994), and several aspects of its function were elucidated before its cloning about a decade ago. The $GABA_A$ receptor was shown to be a member of the ligand-gated ion channel family,

which also includes nicotinic acetylcholine, glycine and serotonin type 3 ($5\text{-}HT_3$) receptors (Schofield et al., 1987). It is a hetero-oligomeric chloride channel that is encoded by several subunit genes (Macdonald, Olsen, 1994; Barnard et al., 1998). On the basis of sequence homology, the different mammalian subunits have been divided into distinct subtypes. These comprise six α, four β, three γ, one δ, one ε, one π and three ρ (Barnard et al., 1998). The subunit heterogeneity generates multiple receptor subtypes, which also differ in functional properties (Macdonald, Olsen, 1994), although certain subunit combinations appear to be naturally favored (McKernan, Whiting, 1996).

A functional $GABA_A$ receptor consists of five subunits, each composed of four membrane-spanning hydrophobic sequences (Macdonald, Olsen, 1994). At least two GABA molecules must bind to fully activate the receptor-channel (Macdonald, Olsen, 1994). *In situ* hybridization studies of the $GABA_A$ subunit expression in the spinal cord have reported strong signals for $\alpha 2$, $\alpha 3$, $\beta 3$ and $\gamma 2$, a weak expression of $\alpha 1$, $\alpha 5$, $\beta 1$, $\beta 2$, $\gamma 1$, and $\gamma 3$, whereas the $\alpha 6$ and δ subunit mRNAs were not detected (Persohn et al., 1991; Wisden et al., 1991; Ma et al., 1993). More recently, immunohistochemical studies have shown an intense labeling of $\alpha 2$, $\alpha 3$, $\beta 2$, $\beta 3$ and $\gamma 2$ in laminae I-II (Bohlhalter et al., 1996). The selective distribution of these subunits in the superficial layers of the spinal cord suggests a differential modulation of GABAergic transmission in this area. Whether the different subunits found in lamina I belong to cells in this layer or to axonal endings of afferent fibers remains to be determined. This could be achieved by immunocytochemical detection of the different subunits on identified lamina I neurons.

3.3.2 Properties of $GABA_A$ receptor/channels.

Different approaches have been used to examine the biophysical properties of $GABA_A$ receptors in the CNS. The conductance values for the channel were obtained from single channel studies and noise analysis of whole cell current in different vertebrate species (Hamill et al., 1983; Huck, Lux, 1987; Allen, Albuquerque, 1987; Bormann et al., 1987; Edwards et al., 1989; Macdonald et al., 1989; Edwards et al., 1990; Mistry, Hablitz, 1990; Maconochie et al., 1994; De Koninck, Mody, 1994; Brickley et al., 1999). From these studies a wide range of conductance states have been observed for the $GABA_A$ channel, ranging from 3 to 71 pS. In general, the conductance of the channel increases with temperature (De Koninck, Mody, 1994), and appears to depend on the subunit composition of the receptor/channel (Macdonald, Olsen, 1994). The conductance of $GABA_A$ channels may vary also with voltage, but this voltage dependence of conductance depends on the different cell types studied. In some cells, the conductance of the channel does not appear to depend on the voltage (Allen, Albuquerque, 1987; Otis, Mody, 1992a), in other neurons $GABA_A$ channels display inward rectification (Gray, Johnston, 1985), while in some other cells they show outward rectification (Fatima-Shad, Barry, 1992).

The kinetic properties of $GABA_A$ receptors also appear to be influenced by the subunit composition of the channels and the cell type (Macdonald et al., 1989; Macdonald, Olsen, 1994; Puia et al., 1994; Soltesz, Mody, 1994; Sieghart, 1995; Poisbeau et al., 1999), potentially conferring distinct kinetics to $GABA_A$ receptor-mediated IPSCs in different cell types.

3.3.3 Modulation of $GABA_A$ receptor function.

Binding studies, electrophysiological and behavioral experiments indicated that the inhibitory action of GABA at $GABA_A$ receptors is subject to modulation by various allosteric ligands, including benzodiazepines, barbiturates (Study, Barker, 1981; Twyman et al., 1989a) as well as steroids (Macdonald, Olsen, 1994). $GABA_A$ receptor activity can also be altered by interaction with inverse agonists, for example β-carbolines (Sieghart, 1995). On the other hand, $GABA_A$ receptor function can be affected by antagonists like bicuculline, a competitive blocker of the channel (Davidoff, Hackman, 1985) and picrotoxin, which acts in a non-competitive manner (Barker et al., 1983).
Another modulatory mechanism of receptor function in the CNS is phosphorylation, and $GABA_A$ receptors are also subject to this type of modulation (Macdonald, Olsen, 1994; Sieghart, 1995).

Benzodiazepines. These compounds produce an anticonvulsant, anti-anxiety or sedative effect when administered clinically (Macdonald, Olsen, 1994). Electrophysiological experiments have shown that benzodiazepines like diazepam or flunitrazepam enhance the actions of GABA at $GABA_A$ receptor channels; their mechanism of action appears to be via an increase in the frequency of chloride channel opening without altering channel open time or conductance (Study, Barker, 1981) and, specifically, an increased occurrence of bursting activity, with no effect on burst duration (Twyman et al., 1989a). Benzodiazepines bind at a site distinct from the GABA recognition site on the $GABA_A$ receptor (Sieghart, 1995), but they are unable to activate $GABA_A$ channels in the absence of GABA (Study, Barker, 1981). It has been suggested

that they could also favor an increased association rate or a decreased dissociation rate of GABA at the first GABA binding site (Macdonald, Olsen, 1994). In summary, benzodiazepines are thought to enhance the binding of GABA to $GABA_A$ receptors sites by increasing the affinity of the receptor for its ligand. It must be kept in mind that the action of benzodiazepines on $GABA_A$ receptors varies with the subunit composition of the channel, and appears to depend mostly on the presence of the α and γ subunit subtypes (Sieghart, 1995).

$GABA_A$ receptor-mediated currents can be reduced by inverse agonists such as β-carbolines, which bind at the benzodiazepine site of the receptor. Single-channel recordings from excised membrane patches demonstrated that β-carbolines appear to decrease the opening and burst frequencies of the channel, without affecting its conductance or mean opening and burst duration (Macdonald, Olsen, 1994). These results were interpreted to indicate that β-carbolines reduce the affinity of GABA binding at the first GABA binding site.

Barbiturates. Barbiturates such as pentobarbital, phenobarbital or secobarbital have a sedative hypnotic effect (Macdonald, Olsen, 1994). The results from electrophysiological studies suggest that barbiturates enhance the actions of GABA by increasing the average channel open time of the $GABA_A$ receptor, without modifying receptor conductance or opening frequency (Study, Barker, 1981). Unlike benzodiazepines, barbiturates appear to able to mimic the action of GABA at high concentrations (>50 μM) by opening the $GABA_A$ channel in the absence of GABA (Macdonald, Olsen, 1994).

Steroids. Steroids such as androsterone or pregnanolone might enhance $GABA_A$ function in a manner similar to that of barbiturates (Macdonald, Olsen, 1994). They appear to have a distinct site of action on the $GABA_A$ receptor complex; their mechanism of action may be to prolong the average open time and burst duration of the channels. In addition, they appear to induce an increase in open channel frequency (Macdonald, Olsen, 1994).

Other agonists. Muscimol and isoguvacine are able to activate chloride conductance and to allosterically modulate the binding of ligands to other binding sites on the $GABA_A$ receptor in a manner similar to that of GABA. Other agents such as THIP (4,5,6,7-tetrahydro-isoxazolo-[4,5-c]pyridine-3-ol), 4-PIOL (5-(4-piperidyl)isoxazol-3-ol), or piperidine 4-sulphonic acid, display a weaker effect on $GABA_A$ receptor action (Sieghart, 1995).

Bicuculline. This compound is a plant toxin with convulsant properties (Macdonald, Olsen, 1994). It displays a selective competitive antagonist action on $GABA_A$ receptors (Curtis et al., 1971). Bicuculline can reduce $GABA_A$ currents by decreasing the open frequency and mean open duration of the channel (Macdonald et al., 1989).

Picrotoxin. This toxin is an equimolar mixture of the inactive picrotin and the active compound picrotoxinin (Sivilotti, Nistri, 1991). It can exert a non-competitive inhibition of $GABA_A$ channels via a site within the channel. The blockade of $GABA_A$

receptors by picrotoxin requires an open receptor/channel. Single channel recordings revealed that picrotoxin might reduce the average GABA-evoked open channel duration and burst duration (Twyman et al., 1989b).

Other antagonists. Several other compounds competitively inhibit $GABA_A$ receptor function. They include the arylaminopyridazines SR 95103 and SR 95531, which may have a greater potency than bicuculline. Some muscimol derivatives with higher potency than bicuculline have also been synthesized (Sieghart, 1995).

Other agents. $GABA_A$ receptor responses can be inhibited by a number of cations such as Zn^{2+}, and to a lesser extent some metal cations: Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+} (Sieghart, 1995). Patch clamp recordings suggested that the zinc binding site on $GABA_A$ receptors could be localized extracellularly and distinct from the benzodiazepine, barbiturate, picrotoxin and steroid recognition sites (Smart, 1992). On the other hand, $GABA_A$ currents may be stimulated by La^{3+} and other lanthanides, which may act at a distinct site on the receptor channel (Sieghart, 1995).

Ethanol seems to potentiate GABAergic transmission in a manner similar to that of barbiturates and benzodiazepines (Sieghart, 1995). Its mechanism of action on the $GABA_A$ receptor remains controversial.

The antibiotic penicillin can inhibit $GABA_A$ currents and its mechanism of action appears to be an open-channel blockade, as indicated by single-channel recordings from mouse spinal neurons in culture (Twyman et al., 1992).

3.3.4 Modulation by phosphorylation/dephosphorylation.

The function of $GABA_A$ channels may be modified by agents that activate or inhibit protein phosphorylation. Different effects of phosphorylation on $GABA_A$ receptor channels have been reported. Physiological studies have suggested that the activation of different kinases either potentiates, inhibits or does not affect $GABA_A$ receptor function (Sigel, Baur, 1988; Moss et al., 1992; Kano, Konnerth, 1992; Porter et al., 1990). This discrepancy may be due to the various types of neuronal preparations used, or to the variable $GABA_A$ subunit combinations found in different neuronal systems. For instance, the intracellular domain between the third and fourth membrane-spanning region of $GABA_A$ receptor subunits contain potential consensus sequences for phosphorylation by different protein kinases, but the number and the position of the phosphorylation sites vary in the different subunits, and in the different subtypes of the same subunit (Macdonald, Olsen, 1994; McKernan, Whiting, 1996). It was shown from various neuronal preparations that stimulation of endogenous or exogenous cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA), Ca^{2+}-phospholipid-dependent protein kinase C (PKC), or protein tyrosine kinases, and other kinases and phosphatases modulate $GABA_A$ receptor function (Chen et al., 1990; Sigel, Baur, 1988; Stelzer et al., 1988; Macdonald, Olsen, 1994; Wan et al., 1997; Jones, Westbrook, 1997; Poisbeau et al., 1999).

Phosphorylation by PKA and PKC usually occurs on serine/threonine residues and the best substrates appear to be the β subunits (Macdonald, Olsen, 1994). It was demonstrated that while $GABA_A$ currents mediated by receptors containing the β1 or β3 subunits are reduced or increased by PKA activation, respectively, when the channel is

composed of β2 subtypes, its function is not affected by PKA (McDonald et al., 1998), and mutagenesis of specific residues on the β subunits β1 and β3 eliminates the PKA-induced modulation of $GABA_A$ currents (Moss et al., 1992; McDonald et al., 1998), indicating that the subunit composition of the receptor affects its response to phosphorylation.

The effect of PKC activation on $GABA_A$ receptors has also been suggested to be dependant on the subunit composition of the receptor. For instance, site-directed mutagenesis studies indicated that PKC (stimulated by phorbol esters) phosphorylation of recombinant $GABA_A$ receptor β2 subunit subtype expressed in oocytes resulted in an inhibition of the receptor function (Macdonald, Olsen, 1994), whereas in rat hippocampal preparations the activation of PKC was either without effect or enhanced the amplitude of $GABA_A$-mediated mIPSCs, depending on the hippocampal area studied (Poisbeau et al., 1999).

In addition to PKA and PKC, tyrosine phosphorylation, through endogenous and/or exogenous protein tyrosine kinase activation, may enhance the activity of neuronal and recombinant $GABA_A$ receptors, because it was demonstrated that the whole-cell GABA response was increased by tyrosine phosphorylation (Wan et al., 1997; Wan et al., 1997). Conversely, inhibitors of protein tyrosine kinase function (like genistein) eliminate the effect of protein tyrosine kinase activation on $GABA_A$ currents (Wan et al., 1997; Wan et al., 1997).

In addition to *in vivo* and *in vitro* regulation of $GABA_A$ receptor function, phosphorylation appears to be a mechanism that prevents rundown of $GABA_A$ receptor currents during whole-cell patch clamp recordings. Rundown is a phenomenon

characterized by a time-dependent decline in ion channel activity (distinct from desensitization which appears to be use-dependent) that has been observed during patch clamp recordings for several ion channels (Akaike, 1995; Rosenmund, Westbrook, 1993). Indeed, it was shown that low intracellular concentration of adenosine triphosphate (ATP) and high intracellular calcium are involved in rundown of several ion channels (Rosenmund, Westbrook, 1993). It was suggested that ATP may limit rundown by acting as a substrate for protein phosphorylation (Stelzer et al., 1988). The mechanism of the Ca^{2+}-mediated rundown, although unclear, was proposed to be through a direct inhibition of the receptor/channel complex or through activation of a Ca^{2+}/calmodulin-dependent phosphatase (Behrends et al., 1988; Jones, Westbrook, 1997). However, rundown is not observed in many CNS preparations [see for example, (De Koninck, Mody, 1994; Lewis, Faber, 1996)].

Because of the various phosphorylation/dephosphorylation systems involved in the modulation of $GABA_A$ receptors, drugs could be used that interfere selectively with these regulatory mechanisms to produce specific effects on GABAergic transmission in the CNS. For instance, the activity of PKA and PKC appear to be enhanced in pathological conditions leading to hyperexcitability of dorsal horn neurons (Coderre, 1992; Mao et al., 1992; Bennett, 1994; Lin et al., 1996). In such cases, selective phosphorylation of $GABA_A$ receptors could potentiate $GABA_A$ currents and allow a better control of the excitability of dorsal horn cells.

3.3.5 Desensitization of $GABA_A$ receptors.

Characteristics of receptor desensitization are continuously debated. It appears that prolonged neuronal exposure to GABA or repetitive neuronal stimulation may lead to a reduction in GABA-induced currents. This phenomenon is termed desensitization (Segal, Barker, 1984; Verdoorn et al., 1990; Sieghart, 1995). As for some other modulatory processes, the rate of and recovery from desensitization depends on the subunit composition of the $GABA_A$ receptor (Jones, Westbrook, 1995; Tia et al., 1996).

Chronic exposure of neurons to GABA could result in a decrease in the number of agonist binding sites (Sieghart, 1995). With chronic exposure to GABA, the rate of desensitization and the affinity of the receptors for the agonist appear to be unchanged; thus desensitization might rather represent a down-regulation of the $GABA_A$ receptors (Sieghart, 1995).

Hence, the diversity of $GABA_A$ receptors confers specific properties to the various subtypes, and provides an opportunity for selective modulation of these receptors in specific cell types.

3.4 $GABA_B$ receptors

3.4.1 Structural characteristics and spinal localization.

Although the $GABA_B$ receptor was the last of the major neurotransmitter receptors to be cloned (Kaupmann et al., 1997), its presence was discovered approximately twenty years ago (Bowery et al., 1980) and it was postulated to be a member of the guanine-nucleotide-binding (G)-protein-coupled receptor superfamily

(Bowery, 1993). Indeed, the cloned receptor, termed GRB1 was found to inhibit adenylyl cyclase activity in a pertussis-toxin-sensitive manner (indicating that the receptor interacts with G-proteins) and displayed structural similarities with metabotropic receptors (Kaupmann et al., 1997). However, it was recently shown that the cloned GRB1 was able to activate potassium currents (a key feature of $GABA_B$ receptor function, see below) only through heterodimeric interaction with another protein, GRB2 (Kuner et al., 1999).

In the spinal cord, $GABA_B$ receptors are found predominantly in the superficial laminae of the dorsal horn (Price et al., 1987), and it was reported that surgical or chemical denervation of primary afferents cause a 50% reduction in the number of $GABA_B$ binding sites (Price et al., 1984), indicating the presynaptic occurrence of these receptors on afferent fibers. The remaining density of $GABA_B$ receptors would be found either on axon endings of neurons intrinsic to the dorsal horn and/or on their soma and dendrites. $GABA_B$ receptors on GABAergic terminals are termed autoreceptors, by opposition to the $GABA_B$ heteroreceptors located on glutamate- or other neurotransmitter-containing terminals (Bowery, 1993). It has been proposed from agonist and antagonist binding studies on dorsal horn slices and spinal cord synaptosomes that $GABA_B$ autoreceptors may be distinct from the heteroreceptors (Teoh et al., 1996; Bonanno et al., 1998).

3.4.2 Functional properties.

The preferential arrangement of $GABA_B$ receptors on axon terminals suggested that their main function is to inhibit, neurotransmitter release (Bowery, 1993). Some of the first observations of $GABA_B$ receptor function were from dorsal root ganglion (DRG)

cells. Stimulation of $GABA_B$ receptors in DRG neurons showed a reduction in membrane Ca^{2+} conductance; (Desarmenien et al., 1984). The effect of $GABA_B$ activation on voltage-gated Ca^{2+} channels supports the evidence that baclofen (an analog of GABA which can activate $GABA_B$ receptors) and GABA can reduce the evoked release of neurotransmitters from synaptic terminals in spinal cord slices or synaptosomes (Bowery et al., 1980; Hill, Bowery, 1981; Teoh et al., 1996; Bonanno et al., 1998) and appears to be mediated through a direct coupling with pertussis toxin-sensitive G-proteins (Bowery, 1993).

Another important effect of $GABA_B$ receptor regulation of neurotransmitter release is to cause an increase in K^+ channel conductance in the presynaptic terminal (Dutar, Nicoll, 1988b; Kangrga et al., 1991; Bowery, 1993). Activation of $GABA_B$ receptors may cause a sufficient number of K^+ channels in the presynaptic ending to open, which might "shunt" an incoming action potential in such a way that it is less effective in depolarizing the terminal. Thus, fewer Ca^{2+} channels would activate, causing less calcium influx in the terminal, thus an inhibition of neurotransmitter release (Gage, 1992). $GABA_B$ receptor-mediated regulation of transmitter release has been studied by recording the depression of a test postsynaptic response following a conditioning response at different intervals between the stimuli. The phenomenon observed is referred to as paired pulse depression (Davies et al., 1990). It is generally accepted that the mechanism of paired pulse depression is presynaptic inhibition resulting in a reduction of transmitter release. This phenomenon appears to be mediated by presynaptic $GABA_B$ receptors since paired pulse depression can be blocked by specific $GABA_B$ receptor antagonists (Davies et al., 1990; Otis et al., 1993).

3.4.3 Pharmacology.

Agonists. Baclofen (β-p-chlorophenyl-GABA) is a GABA mimetic which activates $GABA_B$ receptors, but not $GABA_A$, with an EC_{50} in the micromolar range. Phosphonic analogs of baclofen have also been synthesized and shown to act as agonists at $GABA_B$ receptor sites; some of them display 10-100 times greater affinity for $GABA_B$ receptors than baclofen, but their potency varies with the different functional assays, indicating that there might be some heterogeneity within the $GABA_B$ receptor population (Bowery, 1993).

Antagonists. Selective $GABA_B$ receptor antagonists include 3-aminopropane sulphonic acid (3-APS) with its modified versions saclofen and the 2-hydroxy derivative (Kerr et al., 1988). A phosphonic derivative of baclofen, phaclofen was the first, albeit weak (IC_{50} in the 100 μM range), selective $GABA_B$ receptor antagonist (Kerr et al., 1988). Other antagonists with a better brain-penetration properties have been developed by Ciba-Geigy; they include CGP 35348 (3-amino-propyl(diethoxymethyl) phosphinic acid) and other more potent compounds, the most commonly used in spinal cord studies being CGP 52432 ([3-[[3,4-dichlorophenyl)methyl] amino]propyl] (diethoxymethyl) phosphinic acid), and CGP 56999A ([3[[1-(R)-(3-carboxyphenyl)ethyl] amino] 2-(S)-hydroxy-propyl]-cyclohexyl-methyl-phosphinic acid (Teoh et al., 1996; Bonanno et al., 1998). With the use of these compounds, it was shown that GABAergic and glutamatergic spinal nerve terminals possess $GABA_B$ autoreceptors and heteroreceptors, respectively, which display distinct pharmacological profiles (Teoh et al., 1996; Bonanno

et al., 1998), opening the avenue to the development of selective ligands able to modulate specific $GABA_B$ receptors.

3.4.4 Regulation of $GABA_B$ receptors.

$GABA_B$ receptor number in the rat spinal dorsal horn can be altered by chronic treatment with ligands (Malcangio et al., 1993). It was demonstrated that $GABA_B$ receptor binding in the spinal cord can be increased using selective antagonists, or down-regulated when an agonist is used (Malcangio et al., 1993; Malcangio et al., 1995). Down-regulation of these receptors could account for the development of tolerance to baclofen-induced anti-nociception in rodents (Bowery, 1993), whereas the up-regulation by treatment with antagonists might explain the GCP 35348-induced hyperalgesia, for instance (Malcangio et al., 1991).

The modulation of $GABA_B$ receptors present on primary afferents and excitatory transmitter-releasing terminals in the dorsal horn could have an effect opposite to the regulation of $GABA_B$ receptors on GABAergic terminals. For instance, the activation of $GABA_B$ autoreceptors may depress inhibition by reducing the release of GABA and in turn facilitate excitatory transmission. This could have important physiological implications, particularly during hyperexcitability in the dorsal horn, a characteristic of some neuropathic pain conditions. On the other hand, a selective modulation of $GABA_B$ heteroreceptors may potentially reduce the release of excitatory transmitters, and could play an important role in the mechanisms of analgesia (Henry, 1982).

3.5 Glycine

Aprison and collaborators were the first to provide evidence that glycine is released from cat spinal ventral horn interneurons to produce a postsynaptic inhibition of motoneurons (Aprison, Werman, 1965; Werman et al., 1967). Following this work, glycine (acting at strychnine-sensitive glycine receptors) was established as an inhibitory transmitter not only in the spinal cord but also in other brainstem structures (Ottersen et al., 1995). Although this small neutral amino acid fulfils metabolic functions throughout the body, it appears to be present at very high concentration in restricted regions of the CNS, namely the spinal cord (van den Pol, Gorcs, 1988). Autoradiographic and immunostaining studies confirmed that glycine was localized in spinal cord synaptic regions (Matus, Dennison, 1971; van den Pol, Gorcs, 1988; Todd, Sullivan, 1990). The distribution of glycine within the rat dorsal horn has been investigated in several studies at both the light and electron microscopy level. Glycine-like immunoreactivity (glycine-LI) was found to be present in lower concentrations in the superficial laminae I-II than in lamina III and deeper dorsal horn layers. In fact, in laminae I, II, III, the proportion of cells that contain glycine is 9%, 14%, and 30%, respectively.

3.5.1 Relationship to GABA in the spinal cord.

Nearly all of the cells immunoreactive for glycine in laminae I-III also showed immunoreactivity to GABA (Todd, Sullivan, 1990). Moreover, the two transmitters were shown to co-exist at many synapses in the spinal cord (Todd et al., 1996b). The extensive colocalization of these two inhibitory neurotransmitters in the superficial dorsal horn suggested that they have synergistic effects on neuronal function in this area of the spinal

cord. Indeed, several electrophysiological studies indicated that many spinal neurons are inhibited by both GABA and glycine (Curtis et al., 1968; Game, Lodge, 1975; Rudomin et al., 1990; Yoshimura, Nishi, 1995; Grudt, Henderson, 1998). Furthermore, evidence from immunocytochemical and electrophysiological studies indicate that glycine and GABA are contained in the same synaptic vesicles in several regions of the CNS (Burger et al., 1991; Jonas et al., 1998; Chaudhry et al., 1998; Dumoulin et al., 1999). This raises the issue of the respective roles of GABA and glycine in the superficial dorsal horn. The co-localization and co-release of GABA and glycine suggest that these transmitters might act together at the same synaptic junction. However, their inhibitory action depends on the presence and localization of their respective receptors in the postsynaptic membrane, and distinct pattern of activation of their receptors may confer distinct roles to GABA and glycine.

3.5.2 Source of glycine in lamina I.

As for GABA, lamina I profiles that contain glycine appear to originate mainly from local spinal cord neurons. Glycine was reported to be present in approximately 10% of rostral ventromedial medulla fibers projecting to the dorsal horn (Antal et al., 1996). However, since transection of the cat spinal cord at the thoracic level failed to cause a significant decrease in glycine concentration at the lumbar level (Rizzoli, 1968), it is likely that most of the glycinergic source in lamina I is from intrinsic spinal cord neurons. In fact, it was also shown that in laminae I, II, III, the density of glycine-immunoreactive cell bodies matches that of immunoreactive terminals (van den Pol, Gorcs, 1988; Todd, Sullivan, 1990).

Glycine exerts its inhibitory effect by binding to the glycine receptor (GlyR), a member of the ligand-gated ion channel superfamily which, like the $GABA_A$ receptor, is permeable to Cl^- ions (Rajendra et al., 1997).

3.5.3 Uptake of glycine.

Glycine is thought to be cleared from the synaptic cleft by diffusion following its release (Titmus et al., 1996), and by the specific membrane transporters GLYT1 (for glycine transporter 1), which is distributed throughout the CNS, and GLYT2, which is present selectively in the brainstem and spinal cord and was shown to correlate clearly with glycine density in synaptic terminals (Zafra et al., 1997; Spike et al., 1997). The function of GLYT2 has not been evaluated directly, as specific inhibitors of this glycine transporter have yet to be developed. Glycine transporters appear to belong to the extracellular $[Na^+]$-dependent family of transporters (Kanner, 1989).

3.6 Glycine receptors

3.6.1 Structural properties and spinal localization.

The strychnine-sensitive glycine receptor (GlyR) was the first neurotransmitter receptor to be purified from the rat spinal cord (Pfeiffer, Betz, 1981; Pfeiffer et al., 1982). GlyR is a pentamer composed of α and β subunits, with an α:β stoichiometry of 3:2 (Langosch et al., 1988). Four variants of the α subunit have been cloned (Betz et al., 1994). In contrast to the α_2 and β subunits that are expressed at embryonic stages, the α_1

and α_3 variants appear only postnatally. Alternate splicing of exons in the GlyR pre-mRNA generates other variants of the α1 and α2 isoforms (Betz et al., 1994).

There is evidence from cultures of rat and mouse spinal neurons that immature recombinant GlyRs are homomers composed of five α2 subunits, the functional properties of which are consistent with that of native embryonic GlyRs (Takahashi et al., 1992), while β subunits do not readily form homomers, but efficiently combine with α1, α2 and α3 subunits to produce heteromers (Rajendra et al., 1997).

The distribution of GlyRs in the CNS was determined from autoradiographic binding studies using the tritiated GlyR antagonist strychnine or tritiated glycine (Matus, Dennison, 1971; Young, Snyder, 1973), and from immunocytochemical studies using monoclonal antibodies generated against GlyR subunits (Triller et al., 1985; van den Pol, Gorcs, 1988). The GlyR distribution pattern was found to be consistent with that of endogenous glycine in the CNS (Rajendra et al., 1997). While the α_1 subunit is mainly restricted to the spinal cord and brainstem, α_2 and α_3 have a wider but lower-level of distribution, and the β subunit is found throughout the CNS (Betz et al., 1994). A peripheral membrane protein, named gephyrin, anchors the receptor to the cytoskeleton by binding to microtubules, and is thought to be essential for the synaptic localization of GlyRs (Betz et al., 1994; Todd et al., 1995a; Feng et al., 1998; Kirsch, Betz, 1998). However, some reports indicate that gephyrin is not specific to, or essential for GlyR clusters at synapses, and may also play a role in the anchoring of $GABA_A$ receptors in the CNS (Sassoe-Pognetto et al., 1995; Craig et al., 1996; Lévi et al., 1999). The glycine receptors appear to be organized in discrete clusters, and to be concentrated in postsynaptic membranes in close apposition to presynaptic terminals (Triller et al., 1985;

van den Pol, Gorcs, 1988; Betz et al., 1994; Kirsch, Betz, 1998). It was recently reported that GlyR cluster size on postsynaptic membranes correlates with the extent of presynaptic innervation (Dumoulin et al., 1999). The α and β subunits from human, mouse and rat share considerable sequence homology to each other and to subunits of the $GABA_A$ receptor (Rajendra et al., 1997). Photoaffinity labeling experiments with tritiated strychnine provided evidence that the ligand binding site on the GlyR is located on the α subunit (Betz et al., 1994).

3.6.2 Functional properties.

Glycine receptors mediate fast (millisecond range) inhibitory synaptic events (Akaike, Kaneda, 1989; Jonas et al., 1998). Studies of IPSCs in spinal neurons and cultures of embryonic mouse neurons revealed that GlyRs are permeable to anions such as Br^-, NO_3^-, I^- and SCN^-, in addition to Cl^- ions (Bormann et al., 1987). The GlyR displayed various conductance states, some of which are similar to those of $GABA_A$ receptors (Hamill et al., 1983; Bormann et al., 1987). The conductance states of the receptor was obtained from single-channel analysis and recordings of mIPSCs from spinal native and recombinant GlyRs in rodents (Twyman, Macdonald, 1991; Takahashi, Momiyama, 1991; Takahashi et al., 1992). The conductance appears to vary from 12 to 112 pS, and this variation in conductance level is probably due to heterogeneity in the GlyR subunit composition (Rajendra et al., 1997).

The subunit composition of the receptor also influences its kinetics. For instance, the mean open time of mature spinal GlyRs (containing α1) was shown to be faster than the decay of fetal GlyR-mediated chloride currents (containing α2) (Takahashi et al.,

1992). This also indicates the importance of working with CNS tissue from adult animals when studying the biophysical properties of the mature GlyRs. Electrophysiological investigations have also reported that GlyR-mediated mIPSCs, as well as spontaneous and evoked IPSCs, have faster decay kinetics than $GABA_A$ receptor-mediated inhibitory events in lamina II of the adult rat dorsal horn or in immature motoneurons (Yoshimura, Nishi, 1995; Grudt, Henderson, 1998; Jonas et al., 1998). In contrast, GlyR currents display slower decay kinetics than $GABA_AR$ mediated IPSCs in the retina (Protti et al., 1997). This difference, albeit slight compared to the similarities shared by the two inhibitory receptors, indicates that GABA and glycine may play distinct roles in the control of the excitability of spinal cord neurons. The slower decay of the $GABA_A$ receptor-mediated currents suggests that $GABA_ARs$ could mediate a more sustained inhibition than glycine receptors. This could be useful to control prolonged or repetitive discharges.

3.6.3 Pharmacological characteristics

Agonists. In addition to glycine, several simple amino acids may inhibit spinal neuron firing by activating the GlyR in a strychnine-sensitive manner (Curtis et al., 1968). Binding studies revealed that, in decreasing order of potency, these agonists were β-alanine, taurine, DL-β-aminoisobutyric acid, L-α-alanine, L-serine, and L-proline (Young, Snyder, 1973). Although glycine is the most potent compound able to activate GlyRs, the EC_{50} values range from 20 to >400 μM (Lewis, Faber, 1993; Kaneda et al., 1995; Jonas et al., 1998; Gu, Huang, 1998) . This reflects variations in subunit composition of the receptor. In a study of the human GlyR channel activation properties

using the *Xenopus* oocyte expression system, Schmieden and collaborators found that chloride currents activated by β-alanine and taurine in α2-containing GlyRs had a significantly smaller amplitude than those from GlyRs containing the α1 subunit; however there was no difference in the kinetics of the inhibitory currents (Schmieden et al., 1992). Since taurine concentrations are high in the newborn CNS (Huxtable, 1992), these results were interpreted as indicating an adaptation to alterations in the endogenous levels of taurine and β-alanine during development (Schmieden et al., 1992).

Unlike the $GABA_A$ receptor, a narrow range of chemical agents are able to allosterically modulate GlyR function. They include zinc, which potentiates glycine currents by enhancing the apparent affinity of the GlyR at concentrations between 20 nM and 10 mM, but at concentrations >20 mM zinc decreases glycine affinity (Laube et al., 1995; Rajendra et al., 1997). Other allosteric modulators include ethanol (at high concentrations), the inhalation anaesthetic isoflurane, and hydrocortisol (Rajendra et al., 1997).

Antagonists. The neurotransmitter nature of glycine was established mainly with the use of strychnine, a plant alkaloid, which displays selective and potent competitive antagonism of glycine, β-alanine and taurine actions on the GlyR (Curtis et al., 1968; Young, Snyder, 1973). Strychnine sensitivities of recombinant GlyRs containing the α1 subunit were shown to be similar to that of α2-containing GlyRs. However, a rat-specific variant of the α2 subunit which displays a ~40-fold reduction in glycine affinity, has a ~560-fold decrease in strychnine sensitivity (Kuhse et al., 1990). Other strychnine-related compounds have been developed that have similar or reduced potencies compared to that

of strychnine; they include pseudostrychnine, 2-nitrostrychnine, and brucine, to name a few (Rajendra et al., 1997).

A series of benzodiazepines such as flunitrazepam, bromazepam and nitrazepam were shown to be able to displace tritiated strychnine in binding studies. However, the IC_{50} value of up to 1 mM, suggesting that these benzodiazepines might have antagonistic actions on GlyRs at very high concentrations (Rajendra et al., 1997).

GlyR function can also be altered by a few opiate alkaloids related to morphine that also affect central opioid receptors, but display significantly less effect on $GABA_A$ receptor function. These compounds were shown to inhibit tritiated strychnine binding in spinal cord synaptosomes with different potencies; they include thebaine, methadone, codeine and morphine (Curtis, Duggan, 1969; Goldinger et al., 1981).

A well-known antagonist of $GABA_A$ receptors that also modulates GlyR function is the plant alkaloid picrotoxin (Curtis et al., 1969). In contrast to its noncompetitive actions on $GABA_A$ receptors, picrotoxin appears to be a competitive antagonist of GlyRs, according to its action on recombinant homomeric α1-containing GlyRs. Picrotoxin antagonism is more pronounced in homomeric (α1) receptors than on heteromeric (α1/β) GlyRs, in both human and rat (Rajendra et al., 1997).

Other substances with antagonistic activity include steroids such as progesterone, and antidepressants such as imipramine. Cyanotriphenylborate (CTB) has also been shown to act as a GlyR blocker (Rajendra et al., 1997).

3.6.4 Regulation of GlyR function

Phosphorylation. Glycine receptors contain phosphorylation sites on their α and β subunits. Like in other ligand-gated ion channels, these sites are located on the intracellular loop between the third and fourth transmembrane domains of GlyRs (Rajendra et al., 1997). The effects of phosphorylation by PKA, PKC and protein tyrosine kinases appear to depend on the regional and developmental distribution of GlyR subunits. For example, PKA has been reported to enhance glycine currents in *Xenopus* oocytes transfected with GlyR mRNA (Vaello et al., 1994) and to increase channel open probability in trigeminal neurons of the rat spinal cord (Song, Huang, 1990), while reducing the open probability of GlyRs in acutely isolated rat ventromedial hypothalamic neurons (Agopyan et al., 1993). On the other hand, PKC has been shown to increase glycine responses in rat substantia nigra (Nabekura et al., 1996), and in rat sacral dorsal commissural neurons (Xu et al., 1996), whereas inhibitory currents mediated by glycine receptors expressed in *Xenopus* oocytes were depressed by PKC (Vaello et al., 1994). Moreover, it was recently reported that PKC action on GlyR function in acutely dissociated trigeminal neurons is conditional upon the modulation of GlyR currents by PKA and vice versa (Gu, Huang, 1998).

A putative tyrosine phosphorylation site is also present on the β subunit of GlyRs; however, its function remains to be investigated. It might be involved in the clustering of the receptors, since the β subunit is associated to and co-purifies with the anchoring protein gephyrin (Rajendra et al., 1997).

Tissue damage causes spinal neurons to develop hypersensitivity to noxious and non-noxious stimuli (Bennett, 1994). Concomitant with this phenomenon, the levels and

activity of PKA and PKC may be enhanced (Coderre, 1992; Mao et al., 1992; Lin et al., 1996). In this situation, phosphorylation of GlyRs may cause a reduction in GlyR activity, thus a disinhibition of neurons normally under glycinergic control, and this may account for the increase in excitability observed following nerve lesion, for example (Yamamoto, Yaksh, 1993). Alternatively, a selective modulation of phosphorylation mechanisms that enhance GlyR activity might favor a more effective inhibitory control in chronic pain conditions.

Desensitization. Data from various neuronal preparations suggest that desensitization of GlyRs occur over a biphasic time course (Akaike, Kaneda, 1989; Lewis, Faber, 1993). In general, the decay time constant of GlyR-mediated currents shortens with increased concentration of the transmitter, and the receptor function may recover from desensitization within 60 seconds (Akaike, Kaneda, 1989).

Desensitization of GlyRs may depend on subunit composition of the receptor. For instance, Takahashi and collaborators reported that GlyRs containing the $\alpha 1$ subunits had their channel open time significantly shorter and desensitized faster than receptors containing the $\alpha 2$ subunits (Takahashi et al., 1992).

4. PERTUBATIONS IN GABA- AND GLYCINE-MEDIATED INHIBITION

From behavioral studies it was reported that after intrathecal administration of sub-convulsive doses of the $GABA_A$ receptor antagonist bicuculline or the GlyR antagonist strychnine in rodents, a light tactile stimulus, which would normally produce only an orientation response, triggers vigorous scratching and biting of the stimulated site, vocalization and attempts to escape, indicating the occurrence of a nociceptive event

(Yaksh, 1989; Sherman, Loomis, 1996). *In vivo* electrophysiological recordings also indicated that a reduction in local segmental inhibitory mechanisms (induced by bicuculline or strychnine) could produce a substantial increase in the paw withdrawal response to repeated low-intensity touch stimuli and noxious stimuli (Sivilotti, Woolf, 1994). The hypersensitivity (or strong nociceptive responses) to previously non-noxious stimuli, termed allodynia (Melzack, Wall, 1989), appears to occur in response to activation of myelinated Aβ fibers (Woolf, Fitzgerald, 1983). These fibers are thought to carry innocuous sensory information (Willis, Coggeshall, 1991), and the evidence that neonatal treatment of primary afferents with capsaicin (which destroys Aδ/C fibers) did not affect strychnine-induced allodynia (Sherman, Loomis, 1996) supports the involvement of Aβ fibers in this hypersensitivity condition. This suggest that GABAergic and glycinergic neurons in the dorsal horn also play an important role in the control of non-noxious information in the spinal cord. Alteration in lamina I neuronal function following loss or impairment of inhibitory input has not been addressed specifically.

4.1 When do alterations in the spinal GABAergic and glycinergic mechanisms occur?

This is an important question since disinhibition may be one of the causes leading to abnormal processing of sensory information (Sivilotti, Woolf, 1994). Beside the action of pharmacological agents, a major physiological cause of perturbation in GABAergic and glycinergic inhibition appears to be nerve injury (Bennett, 1994). Injury to a peripheral nerve may lead to chronic neuropathic pain, one of the most difficult pain syndromes to treat (Bennett, 1994). Nerve injury appears to generate a state of

hyperexcitability in the dorsal horn, which involves activity at glutamatergic (excitatory) synapses, particularly the NMDA (N-methyl-D-aspartate) types (Woolf, Thompson, 1991). It is known that intense activity at NMDA synapses can induce excitotoxicity and neuronal death (Choi, Rothman, 1990). Similar excitotoxic events might occur in the superficial dorsal horn following peripheral nerve injury and intrathecal treatment with strychnine (Sugimoto et al., 1990). Loss of inhibitory interneurons or disinhibition could alter the normal responses of lamina I neurons to excitatory input, in such a way that innocuous sensory input to lamina I becomes unmasked, and this innocuous information relayed by lamina I neurons may become perceived as painful. The finding that nociceptive-specific cells in the medial thalamus were able to respond to innocuous tactile input after intrathecal administration of strychnine also supports this hypothesis (Sherman et al., 1997a).

4.2 How are GABA and glycine characteristics altered in chronic pain?

The difficulty of treating neuropathic pain syndromes prompted the development of animal models to gain a better understanding of this pathological condition (Bennett, Xie, 1988; Seltzer et al., 1990; Kim, Chung, 1992; Mosconi, Kruger, 1996). These models of neuropathic pain allowed to investigate changes in GABA and glycine immunoreactivity (Ibuki et al., 1997; Ibuki, Tanaka, 1998; Simpson, Huang, 1998) as well as alterations in other systems (*e.g.* NMDA receptors, opioids and other peptides) (Goff et al., 1998). In the sciatic nerve constriction model (Bennett, Xie, 1988) and in the spinal nerve injury model (Kim, Chung, 1992), a loss of immunoreactivity in the superficial dorsal horn (laminae I-III) was reported for both GABA and glycine. The decrease in staining was ipsilateral (and to a lesser but significant extent contralateral) to

the site of injury (Ibuki et al., 1997; Ibuki, Tanaka, 1998). A reduction in receptor number in the superficial dorsal horn was also reported for GlyRs in the sciatic nerve constriction model (Simpson, Huang, 1998), as well as a possible down-regulation of $GABA_A$ receptors on primary afferents, in the spinal nerve ligation model, from evidence of reduction in their $\gamma 2$ (and possibly $\alpha 2$) subunit mRNA staining revealed by *in situ* hybridization in dorsal root ganglion cell bodies (Fukuoka et al., 1998). These results add further support to the prediction that disinhibition may underlie exaggerated sensory processing, notably allodynia. Indeed, the maximal loss of GABA and glycine staining coincides with the time of peak of allodynic response and the maximal loss of large myelinated ($A\beta$) afferent fibers (Mosconi, Kruger, 1996).

However, reports of increases in $GABA_A$ receptor binding in the spinal cord in animal models of inflammation and nerve transection should also be taken into consideration (Castro-Lopes et al., 1995), although the opposite changes were previously shown (Bihsitkul et al., 1990). But nerve transection injury represents an experimental condition in which the signs displayed by the animals do not always correlate with those observed in clinical practice from patients with deafferentation (Woolf, Wall, 1982). No enhancement in glycinergic mechanisms following nerve injury has been investigated.

5. OBJECTIVES OF THE THESIS.

Despite the considerable progress in the understanding of nociceptive processing in the spinal dorsal horn over the past years, the cellular determinants of inhibitory control in this area of the spinal cord remain incompletely understood. Investigation of inhibitory mechanisms mediated by GABA and glycine in lamina I in particular is lacking. Hence, the aim of this thesis was to study the properties of inhibitory synaptic events mediated by GABA and glycine at the cellular level, in the marginal zone (lamina I).

The issue of the extensive colocalization of GABA and glycine as well as their respective Cl^--gated receptor channels at dorsal horn synapses raises several questions: Are GABA and glycine co-released following the invasion of an action potential in lamina I terminals? Are GABA and glycine contained in the same synaptic vesicles? Do these inhibitory transmitters act together on their respective receptors at the same synaptic junction? What are the respective inhibitory roles of GABA and glycine in lamina I?

To address these questions, I first had to develop a spinal cord slice preparation (in which synaptic connections are maintained intact), to record GABA- and glycine-mediated inhibitory postsynaptic events in identified lamina I neurons. Indeed, the study of physiological activity in the superficial dorsal horn has been hampered to a large extent by the difficulty to identify this layer in slices and to record from the small cells in this spinal area without damaging them. The description of the approach I developed to obtain a spinal slice preparation for electrophysiological recordings in identified lamina I neurons is presented in Chapter 2.

With the spinal cord slice preparation, I was able to carry out experiments to answer the questions addressed above by using the whole cell patch clamp recording technique which provides a high resolution recording of synaptic events (Neher, Sakmann, 1992). Thus, I studied miniature IPSCs (*i.e.* spontaneously occurring even after blocking action potential propagation to the terminals). These miniature IPSCs are thought to represent synaptic currents arising from the release of single vesicles of transmitter (Edwards et al., 1990; Mody et al., 1994). I also studied TTX-sensitive spontaneous IPSCs, which result from activity at both the terminals and the soma of interneurons. An initial finding indicated that spontaneous IPSCs in lamina I were almost exclusively mediated by glycine receptors (GlyRs). However, all cells appeared to express both GlyRs and $GABA_A$Rs, and studies of monosynaptically evoked IPSCs indicated that $GABA_A$Rs are activated following intense electrical stimuli. Moreover, experiments designed to increase $GABA_A$R sensitivity (with a benzodiazepine) indicated that GABA and glycine are likely contained in the same synaptic vesicles and are co-released.

These results appeared to be in contrast with the findings that $GABA_A$Rs activation was not detected following low-intensity stimuli. Thus, I studied whether GABA released under such conditions could serve to activate other receptor targets, such as $GABA_B$ receptors. A detailed account of my findings on these topics is presented in Chapters 3 and 4.

Finally, I investigated how GABAergic and glycinergic inhibitory mechanisms are altered in an experimental model of chronic pain, to test the plasticity of these inhibitory mechanisms in the marginal zone. This work is described in Chapter 5.

CHAPTER 2

Visualization of lamina I of the dorsal horn in live adult rat spinal cord slices.

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Abstract

The superficial dorsal horn of the spinal cord, particularly lamina I, plays a key role in the integration and relay of pain related sensory input. To study the physiology of lamina I neurons in slices, a clear delineation of this layer can be greatly advantageous. Yet, it has remained difficult to distinguish this layer in live tissue in conventional transverse spinal slices because of its very narrow thickness at the edge of the dorsal horn. We describe here the criteria we used to delineate lamina I in live tissue using gradient contrast videomicroscopy in 400μm-thick parasagittal spinal cord slices from adult rats (30-60 day-old). Because of the longitudinal orientation of the neurons in this layer, the resulting distinctive reticulated appearance of lamina I made it possible to readily distinguish it from lamina II. The usefulness of this distinguishing parameter is demonstrated by our ability to contrast synaptic properties of neurons in lamina I from those in lamina II. Complete morphological identification of lamina I neurons however also requires visualization of the cell in the horizontal plane. To maintain compatibility with the parasagittal slice, we used 3D reconstructions from confocal images of the recorded neurons. Rotation of the neuron in space allowed for its morphological characterization in all three planes (horizontal, parasagittal, and transverse). This approach therefore presents optimal conditions for systematic electrophysiological recording from visually identified lamina I neurons.

1. Introduction

Lamina I, the thin marginal layer of neurons at the edge of the spinal cord dorsal horn (Rexed, 1952), is a crucial component of the central representation of pain and temperature sensibility and is one of the main output pathways from the spinal cord to higher structures (brainstem and thalamus) (Perl, 1984; Willis, 1985; Light, 1992; Craig, 1996).

Intracellular recordings from neurons in the marginal zone *in vivo* have remained limited [*e.g.*, (Light et al., 1979; Woolf, Fitzgerald, 1983; De Koninck et al., 1992; Han et al., 1998)] mainly because they have relatively small soma and because of the very narrow size (in the dorso-ventral axis) of this superficial layer of the dorsal horn. Spinal slice preparations are particularly useful for obtaining stable recordings, for placement of multiple electrodes in the vicinity of a single neuron and to control the extracellular milieu for detailed studies of synaptic physiology. Yet, in contrast to lamina II, virtually no systematic recording from lamina I neurons has been achieved in slice preparations. This is again because of the difficulty to delineate this layer of neurons in conventional transverse live slice preparations and without proper conditions for its visualization.

An optimal slice for studying the physiology of lamina I neurons should respect the natural orientation of cells in this layer. Most of the neurons in lamina I have their cell body and dendrites oriented in the rostrocaudal axis, extending up to 800 μm in this orientation (Lima, Coimbra, 1986; Light et al., 1979; Woolf, Fitzgerald, 1983; De Koninck et al., 1992; Zhang et al., 1996; Zhang, Craig, 1997; Han et al., 1998; Yu et al., 1999). Thus, a longitudinal plane of slicing is more appropriate than the conventional 400-500 μm-thick transverse slices, in which the dendritic arborization of lamina I

neurons can easily be truncated. In horizontal sections, however, lamina I cannot be visually delineated from other layers, precluding targeted recording from these cells. Therefore, we show here how the use of a parasagittal spinal cord slice (Magnuson et al., 1987; Bentley, Gent, 1994) can be exploited for a direct visualization of lamina I in live tissue using infrared gradient contrast videomicroscopy (Dodt, Zieglgänsberger, 1994; Dodt et al., 1998).

Proper morphological identification of lamina I neurons nevertheless also requires a view in the horizontal plane. A classification of lamina I neurons into distinct morphological types has emerged based on cell body shape and the characteristics of proximal dendritic branches, most of which are extending horizontally (Gobel, 1978b; Lima, Coimbra, 1986). The main categories of this classification appear to be maintained across species (Zhang et al., 1996; Zhang, Craig, 1997; Yu et al., 1999). Importantly, growing evidence indicates a correspondence between these morphological types and the functional classes of neurons in lamina I (Craig, Kniffki, 1985; Han et al., 1998; Craig et al., 1999). Similarly, each morphological type may be associated with distinct transmitter and receptor phenotypes (Yu et al., 1999; Nagy et al., 1994; Lima et al., 1993; Standaert et al., 1986). This stresses the importance of being able to properly identify lamina I neurons recorded in slices on the basis of their morphology.

To maintain the advantages of the parasagittal slice as well as to be able to obtain a proper morphological identification of the cells, we therefore used intracellular labeling with a fluorescent dye followed by 3D reconstructions from serial confocal images. The neurons could then be rotated in space to obtain an image in all three major planes (horizontal, parasagittal and transverse) . The horizontal view was used for the classification of the neurons on the basis of cell body shape and main proximal dendrites,

while vertical views served to determine whether the neurons had ventrally oriented dendrites (Lima, Coimbra, 1986).

Finally, to confirm that we can properly delineate laminae I in live tissue, we show that using our criteria to distinguish lamina I from lamina II in live slices, a differential distribution of $GABA_A$ and glycine receptor mediated miniature inhibitory postsynaptic currents (mIPSCs) can be found. This approach therefore presents optimal conditions for systematic recording from visually identified lamina I neurons. Preliminary accounts of this work have been reported (De Koninck, Chéry, 1998).

2. Materials and methods

2.1. Slices

Parasagittal slices were prepared as previously described (Bentley, Gent, 1994) with the following modifications. Fully mature male Sprague Dawley rats were used (30-60 day-old, 120-250g). They were anesthetized with Na^+-pentobarbital (30 mg/kg). In some cases, a laminectomy was performed at this stage to expose the lumbar segments. The spinal cords were excised either by hydraulic extrusion (after decapitation) or after a laminectomy. Prior to excision, the animals were briefly perfused intracardially with ice-cold oxygenated (95%O_2, 5% CO_2) sucrose-substituted ACSF (S-ACSF) containing (in mM): 252 sucrose, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 5 kynurenic acid, (pH 7.35; 340-350 mOsm). The spinal cord was then immersed in ice-cold S-ACSF. Relevant segments (lumbar or cervical; 2 cm-long) were isolated and sliced in a Vibratome into 400 μm-thick parasagittal sections. The slices were then

incubated in S-ACSF at room temperature for 30 minutes, and subsequently transferred to a storage chamber filled with oxygenated normal ACSF (126 mM NaCl instead of sucrose and no kynurenic acid; 300-310 mOsm).

2.2. Infrared imaging

After a minimum incubation of one hour, the slices were transferred to a recording chamber under a Zeiss Axioscope equipped with the newly developed infrared "gradient contrast" system (Dodt, Zieglgansberger, 1994; Dodt et al., 1998). This gradient contrast allows good visualization depth of up to 100 μm into the slice and provides higher contrast and resolution for visualization of neurons in fresh tissue than conventional differential interference contrast (DIC). It is also more compatible for combination with epifluorescence because no contrast element has to be placed behind the objectives (Dodt et al., 1998). A 40X water immersion-objective optimized for near infrared wavelength (Achroplan; N.A. 0.8, W.D. 3mm) was used for improved visualization in thick adult live tissue. Dark-field illumination was used to delineate the white matter overlaying lamina I (Snyder, 1982; Nagy, Hunt, 1983; Light, 1992; Todd et al., 1998).

2.3. Recording and data acquisition

For whole-cell patch voltage-clamp recordings, patch pipettes were pulled from borosilicate glass capillaries. The pipettes were coated with Sylgard (Dow Corning) to reduce their capacitance, and filled with an intracellular solution composed of (in mM): 100 CsCl, 10 HEPES, 2 $MgCl_2$, 2 ATP, 0.4 GTP, 11 BAPTA (all from Sigma) and 1 $CaCl_2$ (pH 7.2; osmolarity 260-280 mOsm). Recordings were obtained by lowering the

patch electrode onto the surface of visually identified neurons in lamina I or II. Neurons with a healthy appearance presented a smooth surface, and the cell body and parts of the dendrites could be clearly seen. These neurons also had fusiform or oval cell bodies (usually 10-20 μm in length). An Axopatch 200B amplifier (Axon Instruments) with > 80% series resistance compensation was used for the recording. The access resistance was monitored throughout each experiment. Only recordings with access resistance between 7-20 MΩ (average 14 ± 1 MΩ, mean ± SEM) were considered acceptable for analysis of IPSCs. Data acquisition and analysis of miniature IPSCs was performed as previously described (De Koninck, Mody, 1994) with locally designed software (Y. De Koninck).

2.4. Drugs

For recording of miniature $GABA_A$R- and GlyR-mediated inhibitory postsynaptic currents (mIPSCs), the following drugs were added to the ACSF: the glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM; Tocris Cookson), and D-2-amino-5-phosphonovaleric acid (D-AP5, 40 μM; Tocris Cookson) as well as the sodium channel blocker tetrodotoxin (1 μM; TTX; Alomone labs). For the identification of mIPSCs, strychnine (100nM) was bath applied for the selective antagonism of glycine receptors and bicuculline (10μM) or SR-95531 (3μM) for $GABA_A$ receptors.

2.5. Retrograde labeling and immunocytochemistry

To confirm proper delineation of lamina I with infrared gradient contrast imaging, we used retrograde labeling of projection neurons and immunocytochemical labeling of

the neurokinin-1 (NK-1) receptor. For retrograde labeling, under stereotaxic positioning, a Hamilton syringe was used to inject cholera toxin subunit b (CTb; 1 μL in parabrachial; 5 μL in thalamus) (Lima, Coimbra, 1989; Lima, Coimbra, 1988) or latex microspheres (1-2 μL) (Huang, 1989; Huang, 1987; Tseng et al., 1991; Tseng, Prince, 1993) in the thalamus or the parabrachial nucleus. The locations of the injection sites were confirmed by postmortem examination. The animals were allowed to survive 7-14 days following the injections. To reveal the CTb or NK-1 receptor immunoreactivity, the rats were anesthetized and perfused transcardially with a solution of 4% paraformaldehyde in PB. The brain and spinal cord were removed and postfixed in 4% paraformaldehyde in PB for 4 hours and then transferred to 30% sucrose in PB and stored at 4° C for later immunocytochemical processing. Serial 100 μm-thick parasagittal sections were cut and incubated in 10% normal donkey serum for 1 hr, and then for 48-72 hours with either a rabbit anti-NK-1 receptor or a goat anti-CTb antibody polyclonal antibody at 4° C (Yu et al., 1999; Marshall et al., 1996). After several rinses in PBS-T, for detection of NK-1 receptor immunoreactivity, the sections were incubated with a biotinylated donkey anti-rabbit IgG and revealed with streptavidin conjugated to Alexa 488 (Molecular Probes). For detection of CTb immunoreactivity, a donkey anti-goat IgG conjugated to Rhodamine Red-X (Jackson ImmunoResearch Labs) was used. All antibodies were diluted in PBS-T containing 5% normal donkey serum. Finally, the sections were washed, mounted on gelatin-subbed slides, and coverslipped with an anti-fading mounting medium (Dako).

2.6. Intracellular labeling and 3D reconstructions

All neurons were labeled during the recordings by including Lucifer Yellow (Dipotassium salt; 0.1-0.5%; Sigma) in the patch pipette. Simple diffusion of the dye from the pipette into the cell during the course of the recording was sufficient to obtain complete labeling. Immediately after the end of the recording, the slice was placed between wet filter paper to prevent wrinkling and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer at 4 °C. Subsequently, the slices were cryoprotected by infiltration with 30% sucrose in 0.1M phosphate buffer, overnight at 4 °C, and processed later for confocal light microscopy. The slices were examined under a Zeiss LSM 410 inverted Laser scanning microscope equipped with argon/krypton and helium/neon lasers. From 40 to 60 serial optical sections (1 μm apart) of the Lucifer yellow labeled cell were obtained. Using an M4 image analysis system (MCID software; Imaging Research Inc.), 3D reconstructions were performed from the sets of confocal image. Rotations in space were performed to view the cell in all three major planes: parasagittal, horizontal and transverse. With this approach, it was possible to identify the full morphological features of each neuron.

3. Results

3.1. Visualization of lamina I with infrared gradient contrast

Figure 1 illustrates the appearance of the slices at low and high power. The translucent band corresponding to lamina II or *substantia galatinosa* can be clearly seen at low power (Fig. 1, *top*). At higher magnification, using infrared gradient contrast

videomiscroscopy (Dodt et al., 1998), the distinctive reticulated appearance of lamina I neurons in the parasagittal plane allowed us to easily identify and delineate this layer from the deeper layers (Fig. 1 *bottom*).

To confirm that the striated band of the neurons that is clearly seen with infrared gradient contrast videomiscroscopy corresponds to lamina I, we combined this visualization with dark field illumination as well as complementary markers of lamina I, namely NK-1 receptor immunolabeling (Fig. 2) and retrograde labeling of spinothalamic tract (STT) and spinoparabrachial tract lamina I neurons (Fig. 3). In the superficial layers of the rat dorsal horn, using the antibody developed by Shigemoto against the NK-1 receptors, the labeling was found densest in lamina I (Nakaya et al., 1994). Dark-field illumination (Figs. 2 & 3), which highlights the white matter, allowed us to delineate the dorsal edge of lamina I (Light, 1992; Todd et al., 1998). Figure 2 also illustrates the correspondence between the dense NK-1 immunofluorescent labeling of lamina I (Fig. 2, *bottom*) and the reticulated area viewed with the infrared gradient contrast (Fig. 2, *top*). The complete dendritic labeling obtained with this NK-1 receptor labeling also emphasizes the longitudinal orientation of cells in this region. In the superficial layers, STT neurons virtually all originate from lamina I (Lima, Coimbra, 1988), thus providing another means of identifying this layer. Again, there was a good correspondence between the area of labeled STT neurons and the narrow reticulated band seen with gradient contrast below the area of greatest brightness with dark field illumination (Fig. 3).

In addition to providing better delineation of lamina I, the parasagittal slices also have the advantage that they can span an entire segment or more, maximizing the number of lamina I cells in a single slice. This is illustrated in figure 4. Note the narrow string of retrogradely labeled lamina I neurons along the entire length of the slice, which contrast

with small number of labeled neurons that would be contained in a single transverse slice (*e.g.*, between dotted lines in Fig. 4). This labeling also emphasizes the primary longitudinal orientation of the dendritic tree of the neurons, optimally preserved in this plane.

The dorso-ventral thickness of lamina I in these live slices typically ranged between 15-50 μm, depending on the sections and exact orientation of the plane of slicing, because the lateral sections have a thicker lamina I due to the curving of the superficial layers at the lateral border of the dorsal horn and because slices are not always cut with a perfect parasagittal angle (*i.e.*, a slightly oblique angle will result in a thicker lamina I).

3.2. Three dimensional reconstructions of lamina I neurons

Lamina I neurons can be morphologically identified into 4 major types in the rat on the basis of cell body shape and characteristics of the proximal dendritic tree. The 4 main types are: fusiform, flattened, multipolar and pyramidal (Lima, Coimbra, 1986). Because the dendritic tree of lamina I mainly extends in rostro-caudal and medio-lateral axis, proper identification of the neurons also requires a view in the horizontal plane (Gobel, 1978b; Lima, Coimbra, 1986; Zhang et al., 1996; Zhang, Craig, 1997; Yu et al., 1999). Therefore, during the recordings, the neurons were filled with Lucifer Yellow to obtain serial confocal images of the entire cell. Figure 5 illustrates examples of 3D reconstructions from the different morphological types of intracellularly labeled lamina I neurons. Using this approach, it was possible to rotate the neuron in space and obtain a view of the same cell in the horizontal, parasagittal and transverse planes. We were able

to identify the neurons on the basis of the criteria established by Lima & Coimbra (1986). Fusiform and multipolar neurons were divided into two subtypes, on the basis of whether they had ventrally directed dendrites extending into lamina II. The parasagittal view could therefore be used to identify these dendrites. For example, the multipolar neuron in figure 5 (*bottom*) clearly had dendrites oriented ventrally, making it a type IIB neuron, in sharp contrast with flattened neurons (Fig. 5, *middle*).

3.3 Differential synaptic mechanisms recorded in visually identified laminae I and II

With the infrared gradient contrast, individual lamina I neurons could be clearly distinguished in the parasagittal plane. Viewed in this plane, most neurons had a mainly bipolar shape, with dendrites extending rostro-caudally, but a few had dendrites extending dorso-ventrally (Fig. 1). The neurons usually had small oval cell bodies: 10-20 μm-long in the rostro-caudal plane and 5-10 μm-wide in the dorso-ventral axis.

A sample whole cell recording from a lamina I neuron is illustrated in figure 6. The traces illustrate the high signal-to-noise ratio that can be obtained with such an approach. Synaptic events could be clearly separated from background noise as illustrated by the amplitude histogram in figure 6.

To test whether our method of delineating lamina I in live parasagittal slices was accurate, we contrasted recordings between laminae I and II neurons using our identification criteria. The occurrence of both $GABA_AR$- and GlyR-mediated synaptic events was previously observed using blind patch recordings in the *substantia gelatinosa* (lamina II) (Yoshimura, Nishi, 1993; Yoshimura, Nishi, 1995). However, no correlation was reported between the presence of $GABA_AR$- and GlyR-mediated synaptic events and

specific populations of cells in this area. To test for the possibility that $GABA_AR$- and GlyR-mediated inhibition in lamina I may differ from that in lamina II, we performed a systematic comparison of miniature IPSCs (in the presence 10 μM CNQX, 40 μM APV and 1 μM TTX) recorded in these two layers. To identify the chemical nature of these miniature events we used the selective $GABA_AR$ antagonists bicuculline (10 μM) or SR-95531 (3 μM) and the GlyR antagonist strychnine (100 nM). We compared the distribution of $GABA_AR$- and GlyR-mediated mIPSCs, and found that almost all lamina I neurons (30/31) displayed exclusively GlyR-mediated mIPSCs. In contrast, approximately half (9/20) of the neurons recorded from lamina II had GlyR-mediated mIPSCs and the other half (11/20) $GABA_AR$-mediated mIPSCs. These findings indicated a differential distribution of these two types of synaptic events in laminae I-II neurons, suggesting distinct inhibitory roles for GABA and glycine in the superficial dorsal horn.

4. Discussion

4.1. Visualization of lamina I in live slices

We have shown how a parasagittal spinal cord slice preparation can be exploited to clearly delineate lamina I neurons by infrared gradient contrast videomicroscopy for patch clamp recordings in adult live tissue. The characteristic reticulated appearance of lamina I in this plane of section results from the longitudinal orientation of its neurons and the parallel arrangement of the thin (Aδ) myelinated fibers that enter this layer (Willis, Coggeshall, 1991). Taking advantage of this feature, lamina I could easily be distinguished from deeper laminae. That these landmarks in live slices are reliable was

confirmed with histological methods such as retrograde labeling from the thalamus and the parabrachial nucleus and immunolabeling for the NK-1 receptor. Both of these methods had been previously shown to label specifically lamina I neurons in the superficial layers (Lima, Coimbra, 1989; Lima, Coimbra, 1988; Nakaya et al., 1994). Furthermore, our finding of a differential distribution of GlyR- and $GABA_AR$-mediated mIPSCs in neurons we identified as belonging to lamina I *vs.* lamina II indicates that our approach to separate the two layers is successful.

Because of the longitudinal arrangement of lamina I, sectioning the spinal cord in the parasagittal plane may also be preferable for optimal physiology of this layer. Indeed, with 400 μm-thick transverse slices, the dendritic tree of lamina I neurons is likely to be often truncated, as most of the neurons are oriented rostrocaudally with their dendrites extending up to 800 μm in the rostrocaudal direction, while rarely spanning more than 160 μm in the transverse plane (Lima, Coimbra, 1986; Light et al., 1979; Woolf, Fitzgerald, 1983). This is a significant advantage because minimizing dendritic damage is important for the quality of the physiology of neurons (*e.g.*, maintain the integrity of synaptic events) and the electrophysiological recordings (Edwards, Konnerth, 1992; Soltesz, Mody, 1995).

Horizontal slices or explants have been used with success to record from dorsal horn neurons (Schneider, 1992). They also have the advantage of preserving intact longitudinally oriented cells. Direct visual delineation of lamina I is however not possible with this preparation. For a systematic study of the properties of lamina I neurons, the possibility of targeting specifically this layer at the time of the recording can greatly increase the yield. In fact, it is interesting to note that, in many respects, the organization

of afferent inputs and inter-laminar connections follows the parasagittal plane in the spinal cord, similar to a stack of parallel bands along the latero-medial axis (Brown, 1981b; Willis, Coggeshall, 1991). Thus, the parasagittal plane also facilitates studies of inputs from deeper layers to lamina I. Finally, because lamina I neurons are organized as string of cells along the rostro-caudal axis, the parasagittal plane of section also provides a greater sample of retrogradely labeled lamina I cells per slice, which may be useful to maximize the yield for recording from retrogradely labeled neurons in slices (Huang, 1989; Huang, 1987).

Previous spinal cord slices studies of the superficial dorsal horn have mainly focused on lamina II (*substantia gelatinosa*) or did not attempt to distinguish differences between laminae I and II. Yet, lamina I is a crucial component for the relay of nociceptive information to higher structures (Perl, 1984; Willis, 1985; Light, 1992; Craig, 1996). Indeed, in contrast to lamina II neurons which are mostly propriospinal, many neurons in lamina I project to supraspinal sites, thus constituting a major output from the dorsal horn. A large proportion of the neurons of the spinothalamic tract originate from lamina I (Lima, Coimbra, 1986; Zhang et al., 1996; Zhang, Craig, 1997; Yu et al., 1999). Thus, systematic studies of lamina I neurons in slices will prove very valuable for the understanding of nociceptive processing. With the technique we describe here, the selective study of lamina I neurons becomes more feasible and this will improve the yield for systematic recordings from lamina I, as illustrated by our results on $GABA_AR$- vs. GlyR-mediated mIPSCs.

The transition zone between lamina I and the outer portion of lamina II (lamina IIo) remains often difficult to distinguish in the rat. In fact, lamina IIo in the rat differs in several respects from this same layer in cat and monkey (Ribeiro-da-Silva,

1995). Thus, because the possibility remains that some of the layer we identified as lamina I included a portion of lamina IIo, we decided to restrict our lamina I recordings to the first 20 μm from the dorsal edge between the gray and the white matter. This represents a safe minimal thickness (Ribeiro-da-Silva, 1995), as lamina I should be at least 20 μm-thick, because some portions of lamina I may be thicker (*i.e.*, lateral slices can have thicker lamina I because of the curving of this layer and some evidence suggest a thickening of the dorsal horn next to Lissauer's tract (Todd et al., 1998; Molander et al., 1984; Snyder, 1982; Nagy, Hunt, 1983).

4.2. Morphological identification of lamina I neurons

Early studies failed to find a correlation between the functional properties of lamina I neurons and their morphological characteristics (Woolf, Fitzgerald, 1983; Light et al., 1979). This may have been due in part to the difficulty to distinguish the different morphological types of lamina I neurons in the parasagittal plane (the plane of choice for *camera lucida* reconstructions in these previous studies). Since then, the classification scheme of lamina I neurons that has emerged clearly calls for a view of the cells also in the horizontal plane (Lima, Coimbra, 1986; Zhang et al., 1996; Zhang, Craig, 1997; Yu et al., 1999). Three dimensional reconstructions from confocal images thus offers an ideal tool for the complete morphological identification of lamina I neurons. The excellent correspondence between the morphological characteristics of our intracellularly labeled cells and that described by Lima & Coimbra (1986) further confirms the validity of our approach for targeting lamina I in live spinal cord slices.

In conclusion, visualization of lamina I with infrared gradient contrast videomicroscopy from parasagittal spinal cord slices presents an efficient method to target specifically neurons in this layer. Combined with complete 3D reconstruction of intracellularly labeled neurons, this approach will prove very useful for systematic investigations from visually identified lamina I neurons in live tissue.

Acknowledgements

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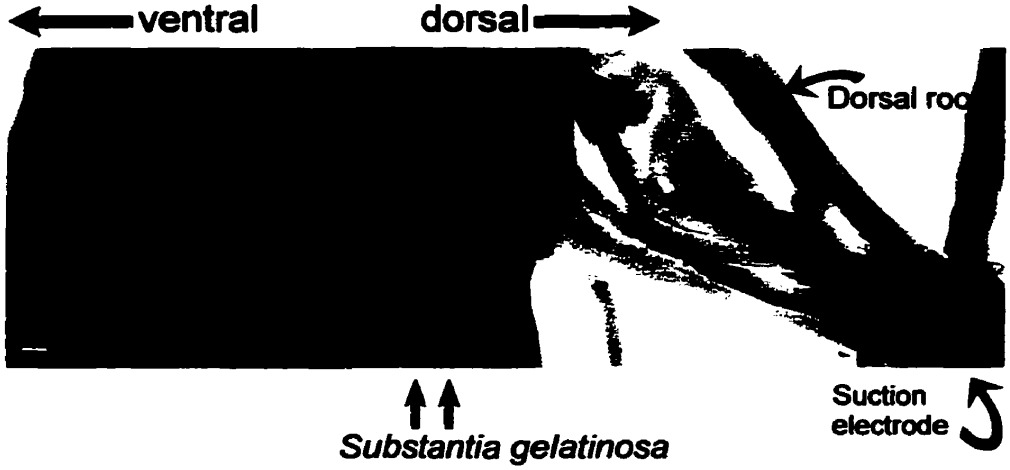
Figure legends

Figure 1. Photomicrographs illustrating a parasagittal spinal cord slice. Dorsal rootlets were attached (*curved arrow*). The clear band corresponds to the *substantia gelatinosa* (lamina II, *arrows*), just beneath the dorsal column and marginal zone (lamina I). The lower micrograph illustrates a higher power image using infrared gradient contrast (Dodt et al., 1998). Note the striated appearance of lamina I (*empty arrows*) which allows us to delineate it from lamina II and from the dorsal white matter. Most of the cell bodies are small, with a longitudinal morphology (*arrowheads*). Scale bars: top = 100μm; bottom = 50μm.

Fig. 1


ventral
dorsal
Dorsal roo
Suction
electrode
Substantia gelatinosa

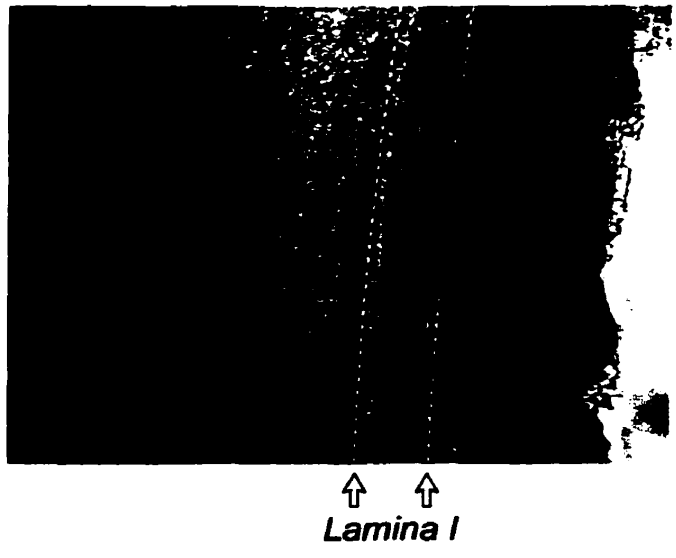

Lamina I

Figure 2. The layer of neurons with reticulated appearance below the while matter corresponds to that of intense NK-1 receptor labeling. The three micrographs are from the same field. *Top*. Infrared gradient contrast (GC) image emphasizing the longitudinal orientation of lamina I neurons allowing its clear delineation from lamina II in the parasagittal plane. (Scale bar = 20 μm). *Middle*, Dark field (DF) image highlighting the myelin-rich white matter delineating the dorsal edge of lamina I. *Bottom*, NK-1 receptor labeling. With this antibody, the intense labeling is restricted to lamina I (Nakaya et al., 1994). The complete dendritic labeling obtained with this receptor staining emphasizes the longitudinal orientation of cells in this region.

Fig. 2

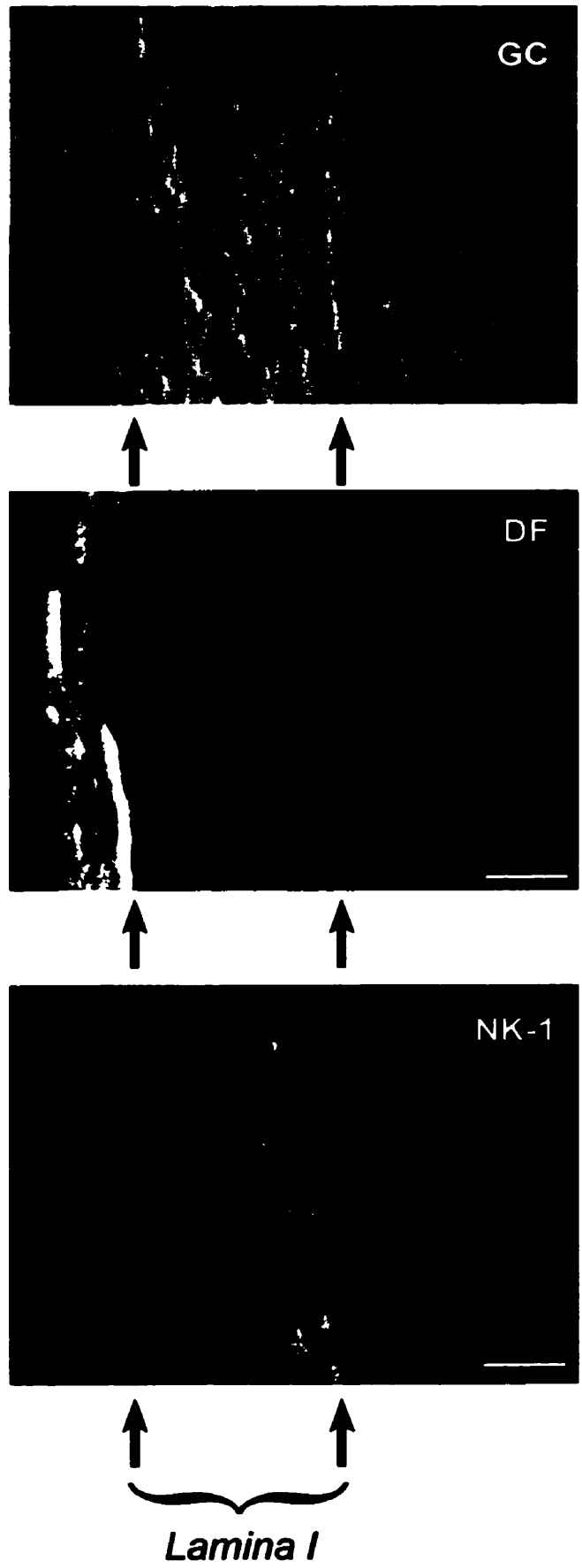


Figure 3. The layer of neurons with reticulated appearance below the while matter corresponds to that of where retrogradely labeled spinothalamic tract (STT) lamina I neurons are found. The three micrographs are from the same field. *Top*. Infrared gradient contrast (GC) image emphasizing the longitudinal orientation of lamina I neurons allowing its clear delineation from lamina II in the parasagittal plane. (Scale bar = 30 μm). *Middle*, Dark field (DF) image highlighting the myelin-rich white matter delineating the dorsal edge of lamina I. *Bottom*, retrogradely labeled lamina I STT neurons. In the superficial dorsal horn, STT neurons virtually all originate from lamina I (Lima, Coimbra, 1988).

Fig. 3

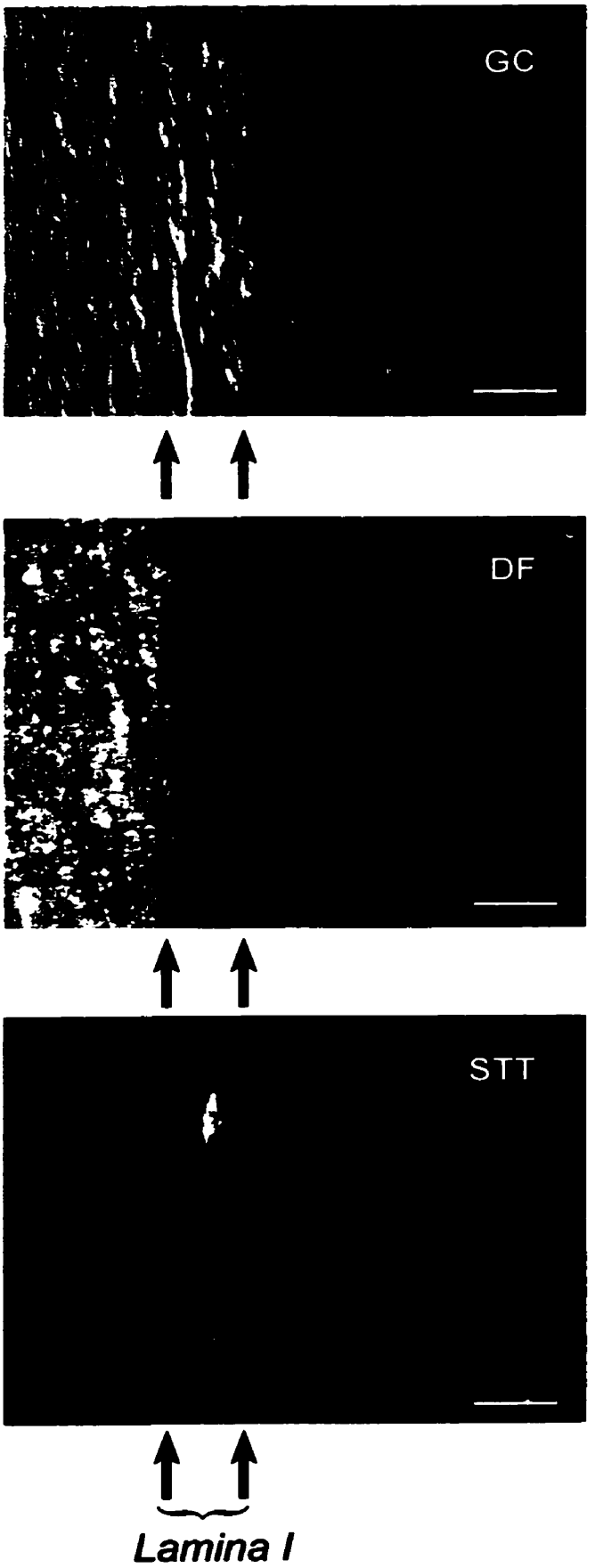
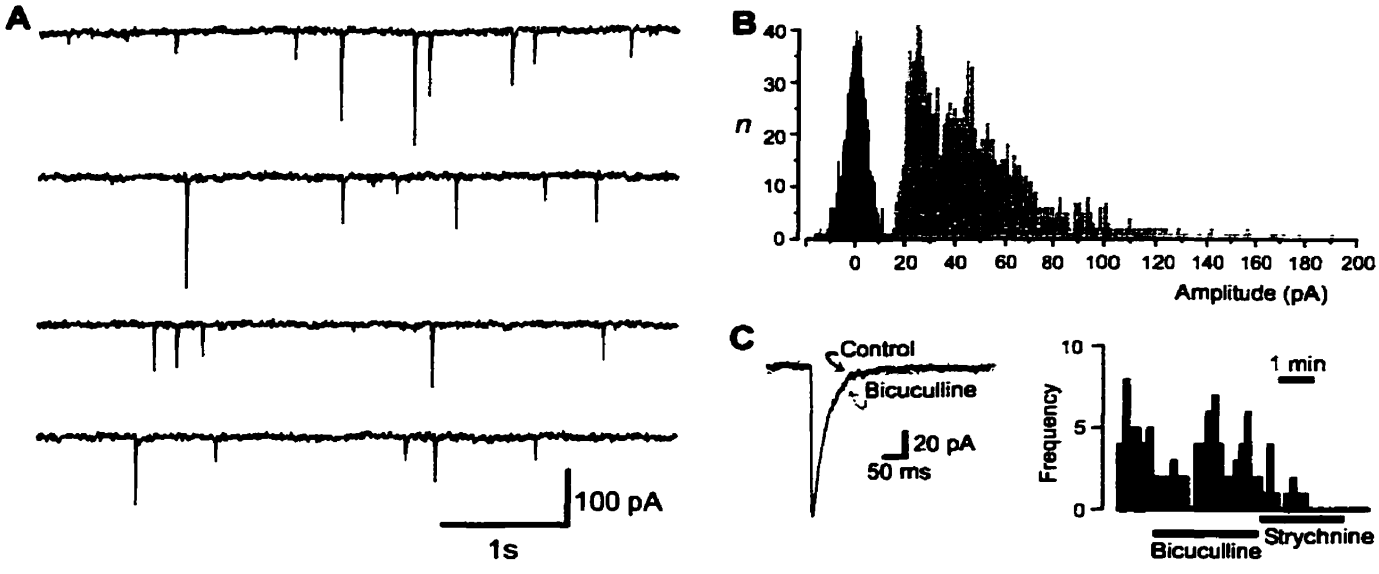


Figure 4. Photomicrographs of retrogradely labeled spinothalamic and spinoparabrachial tract neurons in parasagittal sections of the spinal cord. Note the string of longitudinally oriented labeled neurons in lamina I that can be detected in single sections. In contrast, only a small subset of the lamina I neurons would be found in a 400 μm-thick transverse section (represented by the dashed horizontal lines). The insets at the bottom are high magnification of the areas outlined by the dotted squares in the micrographs above. Dark field (DF) illumination highlights the myelin-rich white matter delineating the dorsal edge of lamina I.

Figure 5. Three dimensional reconstructions of Lucifer Yellow labeled lamina I neurons for their proper morphological identification following the scheme described by Lima and Coimbra (1986). Each neuron was rotated in space for a view in the parasagittal, horizontal and transverse planes, as illustrated by the diagrams at the top (C = caudal, R = rostral, D = Dorsal, V = ventral, M = medial, L= lateral). Note how these cell types are best identified in the horizontal plane. *Top*. A representative fusiform cell with longitudinally oriented and ventral dendrites is clearly observed in the parasagittal and horizontal planes. In the transverse plane, the morphology of this neuron is not readily defined. *Middle*, Example of a flattened neuron. In the parasagittal plane the cell appears fusiform, but rotation to the horizontal plane reveals the multipolar organization of its dendrites, classifying it as a flattened neuron. Note the poor visualization of the cells is in the transverse plane. *Bottom*, Typical multipolar neuron. This neuron has a multipolar morphology in both the parasagittal and horizontal planes and has numerous spines, corresponding to the multipolar type (type IIB of Lima and Coimbra (1986)). Again in the transverse plane, the morphology of this neuron cannot be easily defined. (Scale bars = 10μm)

Figure 6. Whole-cell *patch clamp* recording from a lamina I neuron. (A) Continuous traces of glycine receptor-mediated mIPSCs. The intracellular solution contained 140 mM CsCl, thus the IPSCs are inward when recorded at a holding potential of –65 mV. (B) Amplitude histogram illustrating the high signal-to-noise ratio of the whole cell patch clamp recording in these slices. The mIPSCs (open bars; bin width = 1 pA) can be clearly separated from the background noise (filled bars; bin width = 0.25 pA). (C) In lamina I neurons, the mIPSCs virtually always involved activation of glycine receptors only. Addition of 10 μM bicuculline failed to affect the amplitude and kinetics of these mIPSCs (superimposed average mIPSCs on the *left*), nor did it affect the frequency of these mIPSCs (time histogram on the *right*). Addition of 100nM strychnine, on the other hand, abolished all the mIPSCs, confirming their mediation by activation of glycine receptors.

Fig. 6


A
100 pA
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Amplitude (pA)
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20 pA
50 ms
10
5
0
Frequency
1 min
Bicuculline
Strychnine

PREFACE TO CHAPTER 3

With a spinal cord slice preparation in hands, allowing the direct visualization and delineation of lamina I neurons, I was able to perform electrophysiological recordings to address specific questions regarding inhibitory control in this layer. To determine whether the previously reported coexistence of GABA and glycine (Todd, Sullivan, 1990) means that both inhibitory transmitters are contained in the same synaptic vesicles and co-released, I recorded mIPSCs, as they are thought to represent the activation of receptors by single vesicles of transmitter (Edwards et al., 1990). The results of this study are presented in the next chapter.

CHAPTER 3

Junctional *vs.* Extrajunctional Glycine and $GABA_A$ Receptor-Mediated IPSCs in Identified Lamina I Neurons of the Adult Rat Spinal Cord.

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ABSTRACT

Co-localization of GABA and glycine in synaptic terminals of the superficial dorsal horn raises the question of their relative contribution to inhibition of different classes of neurons in this area. To address this issue, spontaneously occurring miniature (action potential-independent) inhibitory post-synaptic currents (mIPSCs) mediated via $GABA_A$ receptors ($GABA_A$Rs) and glycine receptors (GlyRs) were recorded from identified laminae I-II neurons in adult rat spinal cord slices. $GABA_A$R-mediated mIPSCs had similar amplitude and rise time, but significantly slower decay kinetics than GlyR-mediated mIPSCs. Lamina I cells appeared to receive almost exclusively GlyR-mediated mIPSCs, even upon application of hypertonic solutions. In contrast, in lamina II, approximately half of the cells received either $GABA_A$R- or GlyR-mediated mIPSCs. Yet, all neurons responded to exogenous applications of both GABA and glycine, in a bicuculline- and strychnine-dependent manner, indicating that they all expressed both $GABA_A$Rs and GlyRs. Given that virtually all glycinergic interneurons also contain GABA, the possibility was examined that $GABA_A$Rs may be located extrasynaptically in lamina I neurons. Large-evoked, but not minimally evoked monosynaptic IPSCs, had both $GABA_A$R- and GlyR-mediated components. The $GABA_A$R component had comparable latency, but significantly slower rise and decay kinetics than the GlyR component. Administration of the benzodiazepine flunitrazepam unmasked a $GABA_A$R component to most mIPSCs in lamina I neurons, suggesting that both transmitters were released from the same vesicle. The isolated $GABA_A$R component of these mIPSCs were characterized by particularly slow rising kinetics (*e.g.*, 10 times slower than that of lamina I GlyR mIPSCs or of $GABA_A$R mIPSCs in deeper layers). Finally, while GABA uptake blockers failed to prolong mIPSCs in lamina I, they markedly prolonged evoked IPSCs.

It is concluded that, while GABA and glycine are likely released from the same vesicle of transmitter in lamina I, $GABA_A$Rs appear to be located extrasynaptically.

Thus, glycine mediates most of the tonic inhibition at these synapses. This differential distribution of $GABA_A$Rs and GlyRs confers distinct functional properties to inhibition mediated by these two transmitters in lamina I.

INTRODUCTION

The superficial laminae I and II of the dorsal horn play a pivotal role in the integration and relay of pain related information (Perl, 1984; Willis, 1985; Light, 1992; Craig, 1996) and thus elucidating the nature of inhibitory control in this area is crucial for our understanding of nociceptive processing. Both GABA and glycine function as inhibitory neurotransmitters in the mammalian spinal cord (for review, see Todd and Spike (1993)) and blocking either of these control mechanisms produces a hypersensitivity characteristic of neuropathic pain syndromes (Sivilotti, Woolf, 1994; Yaksh, 1989; Sherman, Loomis, 1996; Sorkin, Puig, 1996). Previous studies report the co-existence of GABA and glycine as well as their respective receptors at many synapses in the superficial dorsal horn of the rat spinal cord (van den Pol, Gorcs, 1988; Bohlhalter et al., 1994; Todd et al., 1995a; Todd et al., 1996b; Bohlhalter et al., 1996) and it appears that nearly all glycine-immunoreactive cells in this area are also GABA-immunoreactive (while only half of GABAergic cells contain glycine) (Todd, Sullivan, 1990; Mitchell et al., 1993). $GABA_A$ receptors ($GABA_ARs$) and glycine receptors (GlyRs) were also found co-localized on the post-synaptic membranes of dorsal horn neurons (Todd et al., 1996b; Bohlhalter et al., 1996; Alvarez et al., 1996). Together with recent evidence of the possible co-storage of GABA and glycine within the same vesicles at some synapses (Burger et al., 1991; Christensen, Fonnum, 1991; Chaudhry et al., 1998; Jonas et al., 1998), these findings raise the question of whether GABA and glycine are contained within the same synaptic vesicles, co-released, and act together at the same synaptic junction in the superficial dorsal horn and therefore whether they play distinct roles in inhibition of laminae I and II neurons.

Previous studies of $GABA_AR$- and GlyR-mediated inhibitory events focussed on lamina II of the spinal dorsal horn (Yoshimura, Nishi, 1995) or trigeminal medulla (Grudt, Williams, 1994). They did not directly address the question of whether GABA and glycine were co-released from the same vesicles and whether inhibition mediated by

these two transmitters differed among identified classes of neurons, in part because they did not identify lamina I neurons. Thus, data is lacking on this important layer that represents one of the main spinal nociceptive output pathways (Willis, 1989; Light, 1992; Craig, 1994).

Recordings from lamina I in slices have remained limited by the difficulty to maintain and delineate this thin layer in conventional preparations. To overcome this, we used a parasagittal slice of adult rat spinal cords that respects the natural rostro-caudal orientation of marginal layer neurons (Light et al., 1979; Woolf, Fitzgerald, 1983; Lima, Coimbra, 1986; De Koninck et al., 1992; Ma et al., 1996), thus allowing visual identification of lamina I (Chéry, De Koninck, 1997; De Koninck, Chéry, 1998) while still providing visual access to deeper layers. Using this approach, we performed whole cell patch clamp recordings in identified spinal laminae I-II neurons to study stimulus evoked, and spontaneously occurring inhibitory postsynaptic currents (IPSCs). In particular, spontaneous miniature (action potential independent) IPSCs were studied as they are thought to reflect transmitter release from single vesicles (Edwards et al., 1990).

An important initial finding was that GABA and glycine appeared to mediate separate mIPSCs that were differentially distributed among distinct classes of neurons. In particular, lamina I neurons were almost exclusively bombarded by GlyR-mediated mIPSCs even though all cells appeared to express both $GABA_A$Rs and GlyRs, suggesting that perhaps $GABA_A$Rs were not located at synaptic junctions in this layer. To test this hypothesis more directly, we manipulated release, receptor sensitivity, and uptake system using electrical stimulation, benzodiazepines, and GABA uptake inhibitors. The results are consistent with the interpretation that $GABA_A$Rs are likely located at extrasynaptic sites in lamina I. Thus, while glycine may be responsible for most tonic inhibitory control in this layer, $GABA_A$R-mediated inhibition may be only effective when sufficient summation of extrasynaptic GABA *spillover* occurs as seen with synchronous input

(Isaacson et al., 1993). Some of these results have been reported in preliminary form (Chéry, De Koninck, 1997; Chéry, De Koninck, 1998).

MATERIALS AND METHODS

Slice preparation. Adult male Sprague Dawley rats (30-60 day-old) were anesthetized with Na^+-pentobarbital (30 mg/kg), and perfused intracardially for 15-20 s with ice-cold oxygenated (95%O_2, 5% CO_2) sucrose-substituted ACSF (S-ACSF) containing (in mM): 252 sucrose, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 5 kynurenic acid, (pH 7.35; 340-350 mOsm). The rats were then rapidly decapitated and the spinal cord immediately removed by hydraulic extrusion and immersed in ice-cold S-ACSF for 1-2 minutes. In some cases, a laminectomy was performed prior to the perfusion for subsequent surgical extraction of the cord rather than by hydraulic extrusion. Lumbar and cervical segments (2 cm-long) were isolated, glued, lateral side down, on a brass platform with cyanoacrylate cement, in a chamber filled with oxygenated ice-cold S-ACSF, and 400 μm-thick parasagittal sections were cut. The slices were then incubated in S-ACSF at room temperature (23-28°C) for 30 minutes, and subsequently transferred to a storage chamber filled with oxygenated normal ACSF (126 mM NaCl instead of sucrose, 300-310 mOsm) at room temperature. After a minimum incubation of one hour, the 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 perfused at ~2 ml/min with oxygenated ACSF containing the glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM; Tocris Cookson), and D-2-amino-5-phosphonovaleric acid (D-AP5, 40 μM; Tocris Cookson).

Labeling and reconstruction of neurons. All neurons were labeled during the recordings by including Lucifer Yellow (dipotassium salt, 0.5-1%, Sigma) in the recording pipette. Simple diffusion of the dye from the pipette into the cell during the

course of the recording was sufficient to obtain complete labeling. Immediately after the end of the recording, the slice was placed between wet filter paper to prevent wrinkling and fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer. Subsequently, the slices were cryoprotected by infiltration with 30% sucrose in 0.1M phosphate buffer, overnight at 4 °C, and processed later for confocal light microscopy. The slices were examined under a Zeiss LSM 410 inverted Laser scanning microscope equipped with argon/krypton and helium/neon lasers. From 40 to 60 serial optical sections (1 μm apart) of the Lucifer yellow-labeled cell were obtained. With this approach, it was possible to reconstruct the entire dendritic tree and perform 3D rotations in different planes for full morphological identification of each neuron.

Drug application. Bicuculline methiodide (10 μM; RBI), SR-95531 (3 μM; RBI) and strychnine hydrochloride (100 nM - 1μM; RBI) were added to the ACSF from frozen, aliquoted stock solutions. For recording of miniature (action potential independent) IPSCs (mIPSCs), 1 μM tetrodotoxin (TTX; RBI) was added to the bathing solution. GABA and glycine were prepared similarly and dissolved in ACSF containing 10 mM N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid (HEPES) in replacement for the bicarbonate buffer, to reach a concentration of 1 mM. These amino acids were applied locally onto lamina I-II dorsal horn neurons by pressure ejection through glass micropipettes. Two pipettes, connected to a two-channel Picospritzer, were positioned close to the cells and contained GABA and glycine, respectively, except in control experiments where one of the pipettes contained only vehicle solution. In some cases, GABA and glycine were bath-applied. In experiments aimed at forcing additional vesicle release, pressure-application of hypertonic ACSF onto the recorded neurons was used (sucrose was added to HEPES-buffered ACSF to obtain an osmolality of 590-610 mOsm). In some experiments, to potentiate possible subliminal $GABA_AR$-mediated events, the benzodiazepine flunitrazepam (Sigma) was bath applied (1 μM). The GABA uptake blockers tiagabine (25 μM; Abbott Laboratories) and 1-(2-

(((Diphenylmethylene)imino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridine-caboxylic acid hydrochloride (NO-711; 5 μM; RBI) were also used to study accumulation of synaptically released GABA.

Whole cell recording and data acquisition. For whole-cell voltage-clamp recordings, patch pipettes were pulled from borosilicate glass capillaries (with an inner filament, WPI) using a two-stage vertical puller (Narishige PP-83). The pipettes were filled with an intracellular solution composed of (in mM): 140 CsCl, 10 HEPES, 2 $MgCl_2$, and 0.5% Lucifer Yellow (Sigma). The pH was adjusted to 7.2 with CsOH, and the osmolarity ranged from 260-280 mOsm (pipette resistance 3 MΩ). In more than 80% of the recordings, we added 2 mM ATP, 0.4 mM GTP, 11 mM BAPTA (all from Sigma) and 1 mM $CaCl_2$ to the intracellular solution. Recordings were obtained by lowering the patch electrode onto the surface of visually identified neurons in lamina I or II. Neurons with a healthy appearance presented a smooth surface, and the cell body and parts of the dendrites could be clearly seen. These neurons also had fusiform or oval cell bodies (usually 10-20 μm in length). Neurons with round, swollen cell bodies were avoided as recording from them revealed low resting membrane potential and poor membrane integrity. While monitoring current responses to 5 mV pulses, a brief suction was applied to form >GΩ seals. An Axopatch 200B amplifier (Axon Instruments) with > 80% series resistance compensation was used for the recording. The access resistance was monitored throughout each experiment. Only recordings with access resistance between 7-20 MΩ (average 14 ± 1 MΩ, mean ± SEM) were considered acceptable for analysis of IPSCs and only recording with stable access throughout the entire administration of antagonists were used for classification of $GABA_AR$- and GlyR-mediated IPSCs. Traces were low-pass filtered at 10 kHz and stored on a videotape, using a digital data recorder (VR-10B, Instrutech Corp.). Off-line, the recordings were low-pass filtered at 2-3 kHz and sampled at 10-20 kHz, on an Intel Pentium-based computer using the Strathclyde

Electrophysiology software (CDR, by J. Dempster, Dept. of Physiology & Pharmacology, Univ. of Strathclyde, Glasgow, U.K.).

Eliciting monosynaptic IPSCs and detection of spontaneous IPSCs. Monosynaptic $GABA_AR$- and GlyR-mediated IPSCs were evoked by electrical stimulation via either bipolar tungsten electrodes for large intensity stimuli, or a patch micropipette for focal stimulations. Square-wave constant pulses (200-300 μs duration) were applied at a frequency of ≤ 0.3 Hz. The electrode was placed within 20-50 μm of the neuron cell body (for proximal stimuli) or within 20-50 μm of a distal branch of a dendrite at 150-300 μm away from the soma (for distal stimulation) along the bipolar axis of the dendritic tree of lamina I neurons. Individual spontaneously occurring IPSCs (sIPSCs and mIPSCs) were detected off-line using a software trigger as previously described (De Koninck, Mody, 1994). For each experiment, the detected events were examined; any noise, which spuriously met the trigger specifications, was rejected. For analysis of the decay phase of sIPSCs or mIPSCs, the events were selected on the basis of the following criteria: *1)* traces containing multiple events were discarded. *2)* only events, which had stable baselines before the rise and after the end of the decay, were kept for analysis. Rise times were determined between 10 and 90% of the peak amplitude of the IPSCs. For averaging of IPSCs, the events were software aligned by their initial rising phase. All software for analysis was designed by Y. De Koninck.

Statistical analysis and curve fitting. Peak amplitudes, rise times and decay time constants were calculated for each of several hundreds of sIPSCs or mIPSCs per cell, using an automated algorithm (De Koninck, Mody, 1996; De Koninck, Mody, 1994). Averages of several hundred mIPSC 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, 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 chi squared $\chi_\nu^2 = \chi^2/\nu$, 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 chi-square: $F_\chi = \Delta\chi^2 / \chi_\nu^2$ ($df_1 = 1$ 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 focussing on comparisons of the late component of mIPSCs, fits were started at a fixed interval following the peak of the event to allow for non-equivocal monoexponential fits that provide an easier and fairer reference when dealing with non-averaged, individual traces (De Koninck, Mody, 1994; Williams et al., 1998). This also avoided contamination of the values of decay time constants with variable weighting factors. Membrane time constants were estimated in voltage clamp mode from analysis of current transients following 5mV, 200 ms-long voltage pulses (Rall, 1969; Jackson, 1992; Spruston, Johnston, 1992).

Student t tests, were used to analyze the differences between parameter of the $GABA_A$R- and GlyR-mediated IPSCs. Analysis of variance (ANOVA) was used to determine the differences in rise times of proximally *vs.* distally evoked IPSCs, and *post hoc* tests were obtained with Bonferroni or Tukey corrections. To evaluate the relationship between different parameters, we used the Pearson correlation matrix, and the significance of the r value was determined with an ANOVA followed by a t test using a Bonferroni correction. Chi squared 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.

RESULTS

Recordings from identified laminae I and II neurons

With our slice preparation, laminae I and II were easily identified. Lamina I neurons have a distinct rostro-caudal orientation (Light et al., 1979; Woolf, Fitzgerald, 1983; Lima, Coimbra, 1986; De Koninck et al., 1992; Ma et al., 1996; Zhang, Craig, 1997; Zhang et al., 1996), and appeared densely packed (see Fig. 1). Most of the cells in lamina I had a bipolar morphology in the parasagittal plane, with rostro-caudal dendritic arborization (Fig. 1), but a few neurons with dendrites extending dorsally and/or ventrally could also be clearly identified. The cell bodies in lamina I were usually small (5-20 μm in length, in the longitudinal axis). Lamina II neurons, in contrast had larger cell bodies (20-50 μm in length). Consistent with previous reports, the thickness of lamina I ranged from 15-50 μm (Ribeiro-da-Silva, 1995; Todd et al., 1998), being closer to 20 μm in the medial slices, while the more lateral sections had thicker lamina I because of the curving of the superficial layers at the lateral border of the dorsal horn (Fig. 1). The recorded lamina I neurons were at an average distance of 10 ± 2 μm from the dorsal border between the gray and white matter, indicating that these neurons clearly belonged to the marginal layer (Fig. 1). In contrast, the recorded lamina II neurons were located at an average distance of 67 ± 9 μm from the dorsal border of the gray matter to ensure a clear distinction between the sample of cells belonging to these two laminae.

Differential distribution of $GABA_A$R- and GlyR-mediated mIPSCs in laminae I-II neurons

Spontaneous mIPSCs were observed in 322 of 387 recorded laminae I-II neurons of the dorsal horn, in the presence of 10 μM CNQX, 40 μM APV and 1 μM TTX, with a recording time varying from 2-90 minutes. To determine whether these miniature events were mediated by activation of $GABA_A$Rs or GlyRs, we used the selective $GABA_A$R antagonists bicuculline (10 μM) or SR-95531 (3 μM) and the glycine receptor antagonist

strychnine (100 nM; Fig. 2). For a comparison of the distribution of $GABA_AR$- and GlyR-mediated mIPSCs, only data from 51 neurons (31 in lamina I and 20 in lamina II) were retained, because 1) the recordings were of sufficient duration, 2) the access resistance was stable throughout the testing of antagonists, and 3) it was possible to test the effects of both bicuculline (or SR-95531) and strychnine. Almost all lamina I neurons (30/31) displayed mIPSCs which could be entirely, selectively and reversibly abolished by 100 nM strychnine, indicating that they were mediated by glycine (Table 1). In contrast, 11 of the 20 neurons in lamina II received exclusively SR-95531- or bicuculline-sensitive mIPSCs, suggesting that they were mediated via activation of $GABA_ARs$ (Fig. 2B); the remaining neurons in lamina II displayed exclusively strychnine-sensitive mIPSCs, indicating their mediation by glycine (Fig. 2A and Table 1). None of the cells tested in laminae I and II displayed both GlyR- and $GABA_AR$-mediated mIPSCs together. In cells with GlyR-mediated mIPSCs (that were selectively blocked by 100 nM strychnine; Fig. 2A) administration of 10 μM bicuculline or 3 μM SR-95531 did not decrease the frequency of events (Fig. 2A) nor affect their amplitude, rise time and decay kinetics ($n = 34$; $p > 0.2$; paired comparisons; Fig. 2C). To test for the possibility that, in these cases, bicuculline might have decreased the frequency of the events by an undetectable amount (i.e. a very small proportion of the events might have been GABAergic), in some experiments we added 100 nM strychnine first, and in all cases it completely blocked all of the mIPSCs ($n = 5$). Conversely, in cells with GABAergic mIPSCs, the synaptic events were selectively abolished by 10 μM bicuculline (Fig. 2B) while up to 1 μM strychnine failed to affect these synaptic events or decrease their frequency ($n = 12$; $p > 0.2$; Fig. 2B). These findings indicate that mIPSCs have distinct distributions in laminae I-II neurons.

Additional experiments were performed in the absence of TTX to record spontaneous IPSCs (sIPSCs; $n = 9$). As for mIPSCs, virtually all of these sIPSCs were

also completely blocked by strychnine in lamina I. Thus, results with sIPSCs were pooled with those from mIPSCs (Table 1).

Using our recording technique, no apparent run down of either type of mIPSCs was observed even with recordings lasting over 2 hours as we previously reported in other tissues in absence of ATP or additional calcium buffers in the recording pipettes (De Koninck, Mody, 1996; De Koninck, Mody, 1994; Otis et al., 1994). Nevertheless, because of the possibility that some $GABA_AR$-mediated currents may be more sensitive to the lack of ATP and calcium buffering [(Chen et al., 1990); however, see (De Koninck, Mody, 1996)], after the initial recordings, we always added 2 mM ATP, 0.4 mM GTP, 11 mM BAPTA and 1 mM $CaCl_2$ to the intracellular solution for all subsequent recordings. We found no difference in the recordings obtained with the two different intracellular solutions; thus all the data were pooled.

Distinct kinetic properties of $GABA_AR$- and GlyR-mediated mIPSCs

Miniature IPSCs mediated by GlyRs in lamina I had amplitude and kinetic properties that were not different from those in lamina II and therefore were pooled for comparison with properties of $GABA_AR$-mediated mIPSCs recorded in lamina II.

A number of parameters were similar for both GlyR- and $GABA_AR$-mediated mIPSCs. Their frequency was variable with an average of 1.0 ± 0.2 Hz (range 0.1-5.0 Hz, n = 19) for GlyR mIPSCs, and an average frequency of 0.6 ± 0.2 Hz (range 0.1-1.8 Hz, n = 17) for $GABA_AR$ mIPSCs. The difference in frequency was not significant between these two populations of mIPSCs ($p > 0.05$, see also Table 2). In all cases, the mIPSCs amplitude distribution was skewed. The mean amplitude of GlyR-mediated events was 87.5 ± 6.1 pA (mean ± SD, n = 30). The $GABA_AR$-mediated mIPSCs had a mean amplitude of 72.9 ± 10.3 pA (n = 11), not significantly different from that of GlyR-mediated mIPSCs (Fig. 3 and Table 2). Similarly, the average 10-90% rise time for

$GABA_AR$-mediated mIPSCs was comparable to that of GlyR-mediated events (455 ± 64 μs *vs.* 403 ± 36 μs, respectively; $p > 0.5$).

The decay time course of $GABA_AR$-mediated mIPSCs was however significantly slower than that of GlyR-mediated mIPSCs (Fig. 3 and Table 2). For quantitative comparison, the decay phase of individual mIPSCs were fit by a monoexponential function (De Koninck, Mody, 1996; De Koninck, Mody, 1994; Williams et al., 1998). Figures 3*Ab* and 3*Bb* illustrate the difference in kinetics of the two populations of mIPSCs. The mean decay time constant (τ_D) of GlyR-mediated mIPSCs was 5.8 ± 0.3 ms *vs.* 10.5 ± 0.6 ms for $GABA_AR$-mediated mIPSCs ($p < 0.001$). The average τ_D at +40 mV were 19.4 ± 1.0 ms for the GlyR-mediated mIPSCs against 25.9 ± 2.4 ms for $GABA_AR$-mediated mIPSCs, respectively ($p < 0.05$). Figure 3 illustrates representative distributions of the rise time, decay time constant and amplitude of GlyR- and $GABA_AR$-mediated mIPSCs, respectively. The average access resistances during the recordings of $GABA_AR$- and GlyR-mediated inhibitory events were equivalent (14.0 ± 1.8 MΩ *vs.* 13.9 ± 1.1 MΩ, respectively). No correlation was found between the access resistance and the rise times for each type of mIPSC ($r = 0.236$, $p > 0.2$). In addition, the input resistance of the neurons displaying $GABA_AR$-mediated mIPSCs was not different from that of cells showing GlyR-mediated mIPSCs (544 ± 240 MΩ vs. 422 ± 69 MΩ, respectively, $p > 0.5$). Finally, we found no correlation between the rise time and decay values of miniature $GABA_AR$- and GlyR-mediated events ($r = 0.335$, $p > 0.4$), indicating that the differences in decay time constants could not be accounted for by differences in electrotonic distance from the soma for these two types of mIPSCs (i.e. $GABA_AR$-mediated mIPSCs are unlikely to occur at synapses further away from the soma than GlyR-mediated mIPSCs).

Co-occurrence of both $GABA_AR$- and GlyR-mediated mIPSCs in deeper neurons

Because miniature synaptic currents are thought to reflect release from single vesicles of transmitters, they can serve as a useful tool to test whether GABA and glycine are contained within the same vesicle. For example, if individual mIPSCs included both a $GABA_AR$ and GlyR-mediated component it could indicate co-packaging of GABA and glycine. In the neurons tested in laminae I and II , the mIPSCs were completely blocked by either strychnine or bicuculline/SR-95531. This cannot rule out the possibility of co-packaging as it may be a consequence of selective expression of one of the two respective receptors in these cells. Because there is clearer evidence that the same cell in lamina III expressed $GABA_AR$ and GlyRs (many dendrites in lamina I-II do not belong to laminae I-II cells) (Todd et al., 1996b), we recorded mIPSCs in deeper (lamina III) dorsal horn neurons ($n = 5$; Fig. 4). In these cells, two populations of mIPSCs were identified on the basis of their decay kinetics (distribution of decay time constants best fitted by two Gaussians; *e.g.*, Fig. 4B). Upon administration of strychnine or bicuculline, only one of the two Gaussian populations of decay time constant remained; for example, in Fig. 4, the faster mIPSCs were blocked by application of strychnine. No mIPSC with the combined fast and slow kinetics were found indicating that separate populations of $GABA_AR$- and GlyR-mediated mIPSCs were found within the same cell. This indicated that, for these cells, either GABA and glycine were likely released from separate vesicles of transmitter or that $GABA_ARs$ and GlyRs were clustered at separate synaptic junctions.

Responses to exogenous applications of GABA and glycine

To determine whether the fact that GABA and glycine mediated mIPSCs were never observed jointly within the same cell was due to a differential expression of their respective postsynaptic receptors among laminae I-II neurons, GABA and glycine were locally applied by pressure ejection to 27 cells. Interestingly, all neurons tested responded to both GABA and glycine (1mM) regardless of the type of mIPSCs they

displayed. Figure 5A illustrates an example of responses induced by application of GABA and glycine in a lamina I cell showing exclusively glycinergic mIPSCs. GABA- and glycine-induced currents could be blocked by bicuculline/SR-95531 or strychnine, respectively (Fig. 5B-D), indicating that the responses to these agonists involved receptors similar to those mediating the mIPSCs.

Hypertonic solution-induced release of inhibitory neurotransmitters

To test whether the lack of detection of either $GABA_AR$- or GlyR-mediated mIPSCs reflected a low frequency of release of one of the two transmitter, in some experiments we applied hypertonic ACSF (590-610 mOsm, adjusted with sucrose) onto laminae I-II neurons (n = 14). The hypertonic stress appears to provoke exocytosis of the releasable pool of vesicles and thus substitutes for the calcium-induced release following action potential invasion (Rosenmund, Stevens, 1996). Yet, as the frequency of the synaptic events was increased but remained non-synchronized, it was still possible to continue recording spontaneous action potential-independent IPSCs and thus compare the results with those from the control conditions. A 25-fold increase in frequency of mIPSCs was observed on average (range 17-33 fold increase). In all cases, the mIPSCs were blocked either exclusively by bicuculline or by strychnine (Fig. 6).

Large-evoked IPSCs in lamina I

The observation that lamina I neurons displayed only GlyR-mediated mIPSCs, yet appeared to express both GlyRs and $GABA_ARs$, together with the finding that virtually all glycine-immunoreactive interneurons in the superficial dorsal horn also express GABA (Todd, Sullivan, 1990), suggest that $GABA_ARs$ may not be located at synaptic junctions in lamina I neurons. By recruiting more synaptic input synchronously, accumulation of transmitter may lead to sufficient spillover from synapses to activate distant receptors (Isaacson et al., 1993). To test this possibility, we recorded monosynaptic evoked IPSCs

in lamina I by placing a bipolar tungsten electrode within 20-50 μm of the recorded neurons. Large intensity stimulation (200-500 μA; 200-300 μs duration) was used, at a frequency of ≤ 0.3 Hz. The evoked IPSCs (eIPSCs) were only partially attenuated by 1-2 μM strychnine; the remaining component was blocked by 20 μM bicuculline (n = 5, Fig. 7A). Thus, in lamina I, there was a $GABA_A$R-mediated component of large eIPSCs. The rise time of the $GABA_A$R-mediated component of these eIPSCs was significantly slower than that of the GlyR-mediated component (6.2 ± 0.5 ms *vs.* 2.8 ± 0.6 ms, respectively; $p < 0.005$, Fig. 7A). The decay time course of the GlyR-mediated component (17.1 ± 3.2 ms), was also significantly faster than that of the $GABA_A$R component (80.4 ± 30.3 ms; $p < 0.05$).

Minimal *vs.* large evoked IPSCs in lamina I

To investigate whether the differences in rise times of the evoked GlyR- and $GABA_A$R-mediated IPSCs could reflect a difference in the location of their respective receptors, we used minimal stimuli (producing all-or-none IPSCs), using patch micropipettes placed either close to the soma (within 20-50 μm) or close to a dendrite at a distance from the cell body (150-300 μm). As most of the neurons in lamina I have a bipolar morphology in the parasagittal plane (Lima, Coimbra, 1986; Light et al., 1979; Woolf, Fitzgerald, 1983; Ma et al., 1996), the positioning of the stimulating electrode could be achieved to reach specifically one portion of the dendrite of the recorded neuron (see the diagram in Fig. 8). In all cases tested, minimally evoked IPSCs (meIPSCs) obtained from stimulations within 20 μm from the cell body were completely blocked by 1 μM strychnine (n = 12, Figs. 7B & 8). Following complete block by strychnine, upon a 5-10 fold increase the stimulus intensity, a slower IPSC could be evoked that was blocked by 10-20 μM bicuculline (Figs. 7B & 8). The rise time of the $GABA_A$R-mediated IPSCs was 2.2 ± 1.2 ms, significantly slower that that of GlyR-mediated meIPSCs (0.9 ± 0.5 ms; see inset in Fig. 7B).

The $GABA_AR$ evoked IPSCs may have had slower rise times because they systematically originated at a more distant site from the soma than the GlyR evoked IPSCs. For this to be the case, the $GABA_AR$ evoked IPSCs resulting from stimulation proximal to the soma would have to have a significantly longer latency than 1) GlyR evoked IPSCs and than 2) events in response to distant stimulation (i.e. proximal stimuli producing $GABA_AR$ IPSCs would have to activate fibers that travel a longer distance to reach distal portions of the postsynaptic cell). We thus compared the latencies of the different components with each type of stimuli.

The latency of both GlyR and $GABA_AR$-mediated components was comparable (1.2 ± 0.2 ms versus 1.7 ± 0.4 ms, $p > 0.1$). Similarly, the latency of GlyR-mediated meIPSCs elicited by stimulation close to the soma *vs.* that in the vicinity of a distal portion of a dendrite were not significantly different (1.2 ± 0.2 *vs.* 1.7 ± 0.6 ms, respectively). Moreover, stimulation at a >100 μm distance from the soma but also away from the vicinity of a dendrite always failed to produce IPSCs. Evoked $GABA_AR$-mediated IPSCs (using larger stimulus intensities) at these same proximal and distal sites of stimulation had also comparable latencies (1.7 ± 0.4 versus 2.1 ± 0.9 ms, respectively). Finally, a comparison of the rise time of GlyR-mediated meIPSCs originating at a site proximal or distal to the soma revealed little prolongation (0.7 ± 0.6 ms *vs.* 0.9 ± 0.5 ms, respectively; Fig. 8A), consistent with the short membrane time constant of these cells (15.2 ± 6.8 ms; range 2.5-45.3 ms). Proximal and distal $GABA_AR$-mediated IPSCs also had comparable rise times (2.2 ± 1.2 ms *vs.* 2.3 ± 1.1 ms, respectively; Fig. 8B). The decay time constant of the GlyR and $GABA_AR$ components evoked by these focal stimulations were 10.8 ± 1.7 ms *vs.* 42.7 ± 6.0 ms, respectively.

Thus, it appeared that both $GABA_AR$- and GlyR-mediated IPSCs could originate at similar electrotonic distance from the soma. Eliciting $GABA_AR$-mediated IPSCs, however, always required stronger stimulus intensities that produced synaptic events with significantly slower rising and decaying kinetics than their GlyR-mediated counterpart.

Taken together, these results indicated that the slower rising phase of evoked $GABA_AR$-mediated IPSCs appeared not to be due to a more distant site of origin but rather a slower, likely diffuse or distant activation of $GABA_AR$ upon sufficient accumulation of released GABA.

Effect of flunitrazepam on miniature IPSCs in lamina I

The results from mIPSCs and stimulus-evoked IPSCs suggested that either different threshold for glycine and GABA release or sub-threshold activation of $GABA_ARs$ by single vesicles of GABA. To distinguish between these two possibilities, we aimed at raising the sensitivity of $GABA_ARs$ with the benzodiazepine flunitrazepam, to potentiate possible subliminal responses to GABA released by single vesicles of transmitter. In the presence of 1 μM flunitrazepam, the rise time of mIPSCs was significantly prolonged to 2.4 ± 0.3 ms and the decay time constant increased to 27 ± 4.2 ms (vs. 0.6 ± 0.1 & 6.0 ± 0.3 in control; $n = 7$; Fig. 9). Close examination of the distribution of rise times and decay time constants (Fig. 9B-D) indicates that the large majority of mIPSCs had their kinetics prolonged by flunitrazepam. Addition of 100 nM strychnine to the bath solution containing flunitrazepam revealed mIPSCs with very slow rise time (4.1 ± 0.9 ms); 10 times more prolonged than that of GlyR mIPSCs in lamina I neurons and of $GABA_AR$ mIPSCs in neurons of deeper laminae. Similarly, the decay kinetics (52.8 ± 8.9 ms) were approximately 10 times slower than that of the GlyR mIPSCs in lamina I neurons and 5 times slower that of $GABA_AR$ mIPSCs in lamina II. Addition of 10 μM bicuculline completely abolished all of these events indicating that they were $GABA_AR$-mediated. The frequency of mIPSCs in flunitrazepam was slightly increased (20-30%) over that in control conditions, most likely because, at some junctions, no synaptic glycine receptor were present. Importantly however, the frequency of the very slow $GABA_AR$ mIPSCs that persisted in the presence of strychnine and flunitrazepam was always over 75% that of the number of events in control conditions

and more than twice the increase in frequency observed in the presence of flunitrazepam alone (Fig. 9B & C). There was also less than 15% overlap in areas between the distributions of decay time constants in control *vs.* flunitrazepam (Fig. 9D). This therefore indicated that the majority of individual mIPSCs had a dual GlyR and $GABA_AR$ component. Given the fact that mIPSCs are likely reflecting responses to single vesicles of transmitter, these results indicate co-storage of GABA and glycine in the same synaptic vesicles.

Effects of GABA uptake inhibitors on IPSCs in lamina I.

Because GABA uptake inhibitors do not affect the amplitude or the time course of mIPSCs, it is thought that clearance of GABA from the synaptic cleft is not a limiting factor determining the decay time course of mIPSCs (Thompson, Gahwiler, 1992; Isaacson et al., 1993). On the other hand, the uptake blockers prolong large evoked $GABA_AR$-mediated IPSCs. If the GABA released by single vesicles at lamina I synapse is insufficient to activate distant $GABA_ARs$, blocking uptake should not significantly affect mIPSCs. On the other hand, with "spillover" of GABA from several neighboring synapses activated synchronously, blocking uptake may favor temporal summation of extracellular GABA originating from adjacent sources (Isaacson et al., 1993). We first tested the effects of tiagabine (25-50 μM) and NO-711 (10-30 μM) of normal mIPSCs in lamina I ($n = 7$). Neither uptake blocker had a detectable effect on these mIPSCs (Fig. 10). Following tiagabine or NO-711 administration, eIPSCs had significantly reduced peak amplitude and their decay time courses prolonged ($n = 5$; mean increase in decay of the late component of 44 ± 3 ms; $p < 0.01$; Fig. 10).

Uptake may be important in limiting the extent of extrasynaptic receptor activation (Isaacson et al., 1993; Asztely et al., 1997). Yet, failure to detect an effect of the uptake inhibitor on miniature events may simply reflect that the amount of "spillover" during a miniature event is subliminal for activation of these receptors. At glutamate

synapses for example, spillover of transmitter upon release of a single vesicle appears to be relevant only for NMDA receptors because they have a much higher affinity for the transmitter than AMPA receptors. It may be expected therefore that raising the affinity of extrasynaptic $GABA_A$ receptors with a benzodiazepine could reveal a significant effect of the uptake blocker on mIPSCs. To test this, we recorded pure $GABA_AR$ mIPSCs in lamina I revealed in the presence of 1 μM flunitrazepam and 100 nM strychnine to test for the effect of the uptake inhibitors. Tiagabine and NO-711 administration decreased the frequency of mIPSCs, and significantly prolonged their decay time course (from 62.3 ± 8.7 to 106.7 ± 10.2; $p < 0.05$; $n = 5$; Fig. 10). In all cases, subsequent addition of 10 μM bicuculline or 3 μM SR-95531 abolished all of these mIPSCs.

DISCUSSION

Our results indicate that GABA and glycine mediate separate miniature IPSCs with distinct kinetics in superficial dorsal horn neurons of the rat spinal cord and these cells are usually bombarded exclusively by one type of mIPSC only. Even in cells with both types of mIPSCs present, these synaptic events represent separate populations. The differences in kinetics of the GlyR- *vs.* $GABA_AR$-mediated mIPSCs are consistent with previous findings in the dorsal horn; (Baba et al., 1994; Yoshimura, Nishi, 1993; Yoshimura, Nishi, 1995; Takahashi, Momiyama, 1991; Takahashi et al., 1992) and in medullary neurons (Grudt, Henderson, 1998; Lewis, Faber, 1996), in contrasts with retinal ganglion cells where $GABA_AR$-mediated IPSCs have a faster decay than GlyR-mediated ones (Protti et al., 1997).

Immunocytochemical studies by Todd and Sullivan (1990) demonstrated that the proportion of glycine-immunoreactive neurons in laminae I, II, and III were 9%, 14%, and 30%, respectively. The proportions of GABA-immunoreactive neurons in laminae I, II, and III were higher: 28%, 31%, and 46%, respectively. They also reported that virtually all of the glycine-immunoreactive cells in this area were also GABA-

immunoreactive, but many GABA-immunoreactive cells do not show immunoreactivity to glycine. Thus, while recording from laminae I-II, one would expect that the neurons displaying GlyR-mediated mIPSCs would also show $GABA_A$R-mediated mIPSCs. On the contrary, we found namely that lamina I neurons were bombarded exclusively by GlyR-mediated mIPSCs in normal conditions. Thus, glycine appears to be solely responsible for tonic inhibition of second order neurons in this layer.

Co-release of glycine and GABA from the same vesicle

Unmasking of a very slow rising and decaying $GABA_A$ component of mIPSCs in lamina I with flunitrazepam, provided an ideal tool to address the issue of co-storage of GABA and glycine in the same vesicles. Importantly, the whole distribution of rise times and decay time constants was shifted in the presence of the benzodiazepine with little overlap with the control distribution, indicating that the majority of the events were affected. Accordingly, the increase in frequency of events observed in the presence of the benzodiazepine was much lower than the number of $GABA_A$R-mediated mIPSCs remaining in the presence strychnine. Thus, it can be concluded that the majority of mIPSCs had a dual GlyR and $GABA_A$R-mediated component. Because mIPSCs appear to reflect postsynaptic responses to the release of single vesicles of transmitter, the data strongly indicated the co-storage of glycine and GABA in the same vesicles. This is consistent with similar recent evidence at the immature motoneuron synapse (Jonas et al., 1998) as well as with the observation that both transmitters can be transported by the same vesicular transporter (Burger et al., 1991; Christensen, Fonnum, 1991) present at both glycinergic and GABAergic synapses (Chaudhry et al., 1998). While the evidence presented by Jonas et al. (1998) relied on template fits of the decay phase of mIPSCs, our evidence has the added advantage that the rising kinetics of the $GABA_A$ component was also dramatically slower than that of the GlyR component and that the events were sufficiently altered that is was possible to quantify the degree of overlap between the

distributions of events for each of kinetic parameter, providing an even more compelling argument for the co-storage of GABA and glycine.

Extrajunctional $GABA_AR$ activation in lamina I

Several pieces of evidence converge to indicate that, in contrast to GlyRs, $GABA_ARs$ are likely located extrasynaptically in lamina I: 1) the absence of $GABA_AR$-mediated spontaneous and miniature IPSCs while the cells received GlyR-mediated mIPSC and all responded to exogenous application of GABA in a bicuculline dependent manner; 2) activation of several inhibitory synapses synchronously (large evoked IPSCs) revealed a $GABA_A$ component; 3) the evoked $GABA_AR$ component had a similar latency, yet slower rising kinetics than the GlyR component; 4) benzodiazepines can unmask a $GABA_A$ component to individual mIPSCs and this component has extremely slow rising kinetics (10X slower than GlyR mIPSCs in lamina I or $GABA_AR$ mIPSCs in deeper laminae); 5) specific blockers of GABA uptake did not affect normal mIPSCs in lamina I but significantly prolonged the decay kinetics of evoked IPSCs that involve synchronous activation of neighboring synapses as well as of $GABA_AR$ mIPSCs unmasked by flunitrazepam (in the presence of strychnine).

The slow kinetics of $GABA_AR$-mediated IPSCs in lamina I could suggest that they specifically originate at distant sites from the soma (therefore subject to greater space clamp attenuation (Spruston et al., 1994). However, the short time constant of lamina I neurons and the lack of difference in the kinetics of proximally vs. distally evoked IPSCs argues against this possibility. More importantly, the dual-component mIPSCs in flunitrazepam clearly show that the slow $GABA_A$ component originated from the same release sites as the fast rising GlyR components.

Extrasynaptic distribution of $GABA_A$ receptor subunits have often been described in the spinal cord and other brain regions (Bohlhalter et al., 1994; Nusser et al., 1998; Soltesz et al., 1990; Somogyi et al., 1989). In the dorsal horn, the β_2/β_3 subunit

immunoreactivity, which appear to be one of the most widely expressed subunit in the spinal cord, appeared often extrasynaptically (Alvarez et al., 1996). It is however difficult to draw definitive conclusions from studies on the subcellular distribution of $GABA_A$Rs with pre-embedding approaches because of the limited access of some antigenic sites (Nusser et al., 1995). Nevertheless, even with pre-embedding approaches, antibodies directed against an intracellular loop of the receptor may have better access to postsynaptic densities (active zones) [see (Todd et al., 1996b)]. Interestingly, while Todd et al (1996b) found punctate staining for the β_3 subunit in deeper laminae, the labeling often extended beyond active sites in laminae I-II. In fact, it was often difficult to localize precisely the β_3 staining in these laminae (Todd, personal communication). For this reason, they refrained from further quantifying β_3 *vs.* gephyrin immunoreactivity in lamina I. Thus, while immunocytochemical evidence may be consistent with the possibility of a prominent extrasynaptic distribution of $GABA_A$Rs in lamina I, no direct data is available.

Evidence with benzodiazepines rule out the possibility of a specifically higher threshold for activation of interneurons releasing GABA. The very slow rise time of $GABA_A$R-mediated mIPSCs revealed by flunitrazepam indicate a very low concentration of agonist reaching the receptors, since the binding kinetics of the receptor are concentration dependent (Maconochie et al., 1994). This suggests that either very little GABA is contained in glycine containing synaptic vesicles or that the $GABA_A$Rs are located at a distance from the site of release. No evidence available to indicate that a very low level of GABA in synaptic vesicles may occur, in fact all available evidence suggest the opposite (Mody et al., 1994). Furthermore, this possibility would appear highly unlikely in the present case, because the vesicular transporter has a greater affinity for GABA than glycine (Burger et al., 1991; Christensen, Fonnum, 1991) and because substantial levels of immunoreactivity for GABA is detected in these cells (Todd, Sullivan, 1990) and their terminals (Todd et al., 1996b). Finally, while low concentration

of GABA may lead to slower rise times, it is not expected to generate longer decay time courses (Maconochie et al., 1994). Thus, the most plausible explanation for our results is that $GABA_A$Rs are located at a distance from the vesicle release site, suggesting a prominent extrasynaptic distribution of functional $GABA_A$Rs at these synapses.

Functional significance of the difference in $GABA_A$R and GlyR -mediated inhibition

Symptoms very similar to that observed in neuropathic pain models involving peripheral nerve constriction can also be obtained with intrathecal administration of subconvulsant doses of strychnine or bicuculline (Sivilotti, Woolf, 1994; Yaksh, 1989). Importantly, strychnine-induced hypersensitivity is selective to non-noxious input (allodynia) and is morphine insensitive (Sherman, Loomis, 1996; Sherman, Loomis, 1995; Sherman, Loomis, 1994; Sorkin, Puig, 1996). Moreover, the sensitization produced does not only affect spinal segmental nociceptive reflexes, but also ascending nociceptive pathways (Sherman et al., 1997b; Sherman et al., 1997a). Thus $GABA_A$ and glycine receptor-mediated inhibition appear to play an important role in the regulation of excitability in specific nociceptive sensory pathways in the dorsal horn and blockade of this control appears to unveil subliminal innocuous input to neurons in these pathways. The present finding of the differential distribution of $GABA_A$ and glycine receptor mediated mIPSCs among cell types, and especially that only glycine is responsible for the tonic inhibition of lamina I neurons, suggests separate role for these two inhibitory systems. The selective tonic inhibition exerted by glycine on lamina I neurons for example may be contrasted to evoked inhibition mediated by GABA acting on $GABA_A$ receptors. Blockade of either type of control may differentially affect nociceptive integration. For example, the difference in kinetics of the GABAergic versus glycinergic IPSCs and the type of activity that recruit them may match the nature of excitatory input they control. Interestingly, metabotropic receptor antagonists attenuate bicuculline-induced allodynia but are not effective against strychnine-induced allodynia (Onaka et al.,

1996). Because metabotropic glutamate receptors appear to be mainly located at extrasynaptic sites (Baude et al., 1993) and may thus be activated mainly upon release of a sufficient concentration of glutamate that can spillover the synapses (Rusakov, Kullmann, 1998), this property may match that guiding activation of $GABA_AR$s in lamina I.

ACKNOWLEDGEMENTS

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Table 1. Differential distribution of $GABA_A$- and glycine receptor-mediated spontaneous IPSCs (pooled sIPSCs with mIPSCs) in laminae I and II neurons of the dorsal horn. ($\chi^2 = 19.80$; degree of freedom = 1; $p < 0.001$)

	Glycinergic	GABAergic	**Total**
Lamina I	39	1	40
Lamina II	9	11	20
Total	48	12	60

Table 2. Summary of the properties of $GABA_A$- and glycine receptor-mediated mIPSCs recorded in laminae I-II neurons of the dorsal horn (expressed as mean ± S.E.M.)

	Peak amplitude (pA)	**Frequency (Hz)**	**Rise time (μs)**	**Decay time constant (ms)**	**Input resistance (MΩ)**	**Access resistance (MΩ)**
Glycinergic	87.5 ± 6.1	1.0 ± 0.2	403 ± 36	5.8 ± 0.3	422 ± 69	13.9 ± 1.1
GABAergic	72.9 ± 10.3	0.6 ± 0.2	455 ± 64	10.5 ± 0.6*	544 ± 240	14.0 ± 1.8

(* $p < 0.001$).

FIGURE LEGENDS

Figure 1. Visualization of the dorsal horn and delineation of laminae I and II in parasagittal 400 μm-thick slices of the spinal cord. The low-power micrograph at the top (*left*) illustrates a portion of the slice with dorsal rootlets attached. Note the clear band (*arrows*) corresponding to the *substantia gelatinosa* (lamina II). On the right side is a schematic drawing of the parasagittal plane of slicing, illustrating the lateral, intermediate and medial slices which can be obtained. The micrograph at the bottom (*left*) is a high power image of the superficial layers viewed with infrared differential interference contrast (IR-DIC) under a Zeiss Axioscope. *Arrows* point to distinctive lamina I neurons. Note the distinctive striated appearance of lamina I which allows us to delineate it from lamina II. The micrograph on the right side shows a higher power image of lamina I on which is superimposed a confocal reconstruction of a lamina I neuron that was filled with Lucifer Yellow during the recording. Note the typical rostro-caudal orientation of the dendritic tree of these neurons with their dendrites mainly confined to lamina I.

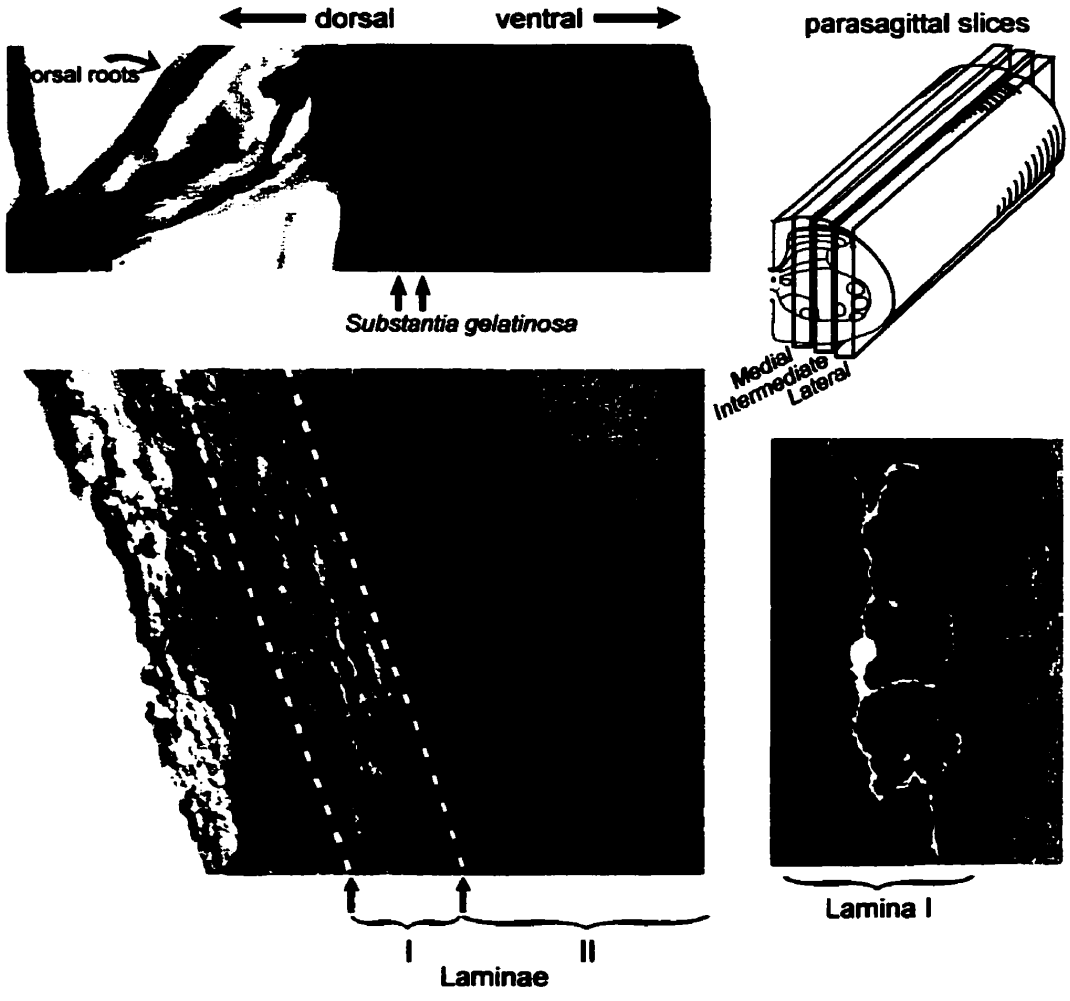

dorsal
ventral
orsal roots
Substantia gelatinosa
parasagittal slices
Medial
Intermediate
Lateral
Lamina I
I
II
Laminae

Figure 2. $GABA_AR$- and GlyR-mediated mIPSCs occur in distinct populations of neurons in the superficial laminae. The top micrographs in **A** and **B** illustrate confocal reconstructions of these lamina II neurons in the parasagittal plane (dorsal up, rostral to the right). Note the more stellar orientation of the dendritic tree of these cells in contrast to the lamina I neuron illustrated in Fig 1. **A,** GlyR-mediated mIPSCs are selectively blocked by strychnine. The raw traces are representative examples of GlyR-mediated mIPSCs taken at points indicated on the time histogram at the bottom (*bin width = 10 s*). The mean frequency was not significantly altered following bath application of 10 μM bicuculline (0.74 s^{-1} *vs.* 0.65 s^{-1}), but 100 nM strychnine completely blocked all the events. **B,** $GABA_AR$-mediated mIPSCs are selectively blocked by bicuculline. Representative traces are examples of $GABA_AR$-mediated mIPSCs taken at points indicated on the time histogram at the bottom (*bin width = 10 s*). **C,** the amplitude and kinetics parameters of the GlyR-mediated mIPSCs recorded in **A** were not altered following bath application of 10 μM bicuculline (mean amplitude -110 ± 13 pA *vs.* -130 ± 14 pA; $p > 0.1$; mean rise time 0.27 *vs.* 0.24 ms; $p > 0.5$; mean decay time 2.24 *vs.* 2.21 ms; $p > 0.5$). Similarly for $GABA_AR$-mediated mIPSCs, they were not affected by up to 1 μM strychnine (not shown; mean amplitude -79 ± 12 pA *vs.* -69 ± 15 pA; $p > 0.1$; mean rise time 0.71 *vs.* 0.84 ms; $p > 0.1$; mean decay time constant 11.19 *vs.* 12.33 ms; $p > 0.5$). Thus, GABA and glycine mediate separate mIPSCs in laminae I-II neurons. The holding membrane potential was –65 mV.

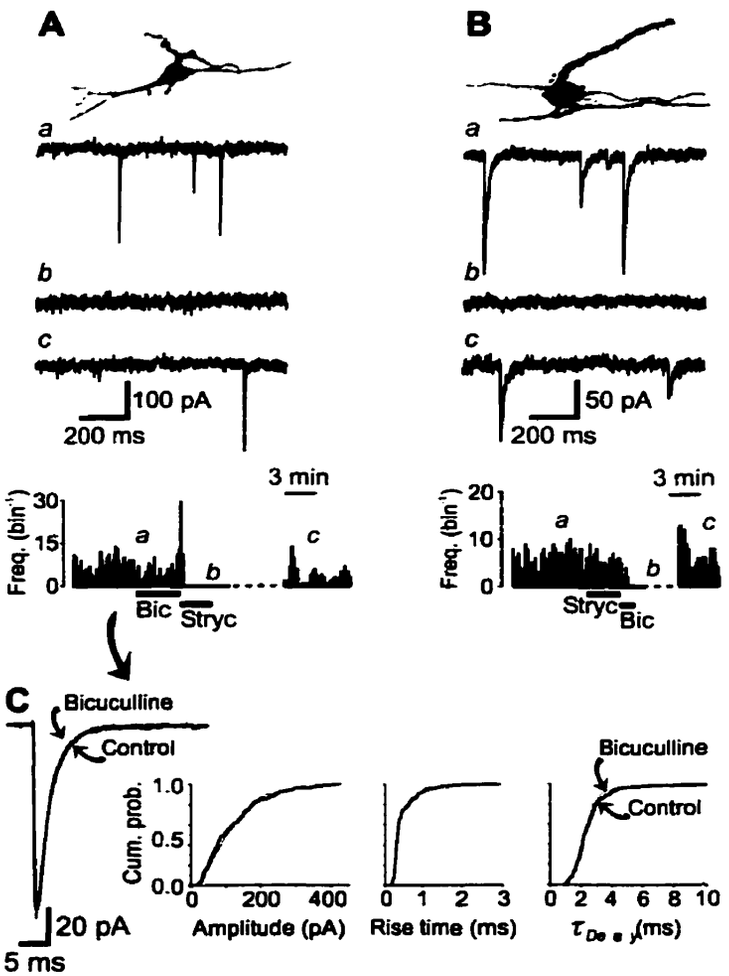

A
B
a
b
c
100 pA
200 ms
50 pA
200 ms
3 min
Freq. (bin⁻¹)
30
15
0
20
10
0
Bic
Stryc
Stryc
Bic
C
Bicuculline
Control
20 pA
5 ms
Cum. prob.
1.0
0.5
0.0
0
200
400
Amplitude (pA)
0 1 2 3
Rise time (ms)
0 2 4 6 8 10
τDecay (ms)

Figure 3. Distinct properties of $GABA_AR$- and GlyR-mediated mIPSCs in laminae I-II neurons. Comparison of the peak amplitudes and kinetic properties of glycinergic (**A**) and GABAergic (**B**) mIPSCs in two different cells. Amplitude distribution of GlyR- (**A***a*) and $GABA_AR$-mediated (**B***a*) mIPSCs (*empty bars, bin width 5 pA*) and corresponding noise distribution (*black bars, bin width 0.5 pA*); in both cells the mIPSCs amplitude distributions are skewed. The insets are superimposed representative traces of glycinergic mIPSCs (**A***a*) and GABAergic mIPSCs (**B***a*) recorded at -65 mV. The glycinergic mIPSCs were specifically blocked by 0.1 μM strychnine and not affected by 10 μM bicuculline (*not shown*), while 1 μM strychnine did not affect the $GABA_AR$-mediated mIPSCs, which were selectively blocked by 10 μM bicuculline (*not shown*). The mean amplitude of the GlyR-mediated mIPSCs in this cell was –111 pA, occurring at a frequency of 5.1 Hz , whereas the mean amplitude of $GABA_AR$-mediated mIPSCs in the other cell (**B**) was –70 pA with a frequency of 1.8 Hz. **A***b* and **B***b*, distribution of the decay time constant of GlyR- and $GABA_AR$-mediated mIPSCs, respectively. In both cells, the mIPSCs decays were normally distributed (bin *width 1 ms*). **A***c* and **B***c*, the mean 10-90% rise time of GlyR-mediated mIPSCs was 560 μs, against 630 μs for the $GABA_AR$-mediated mIPSCs (*bin width 100 μs*). The main difference between these two types of mIPSCs was their decay time course.

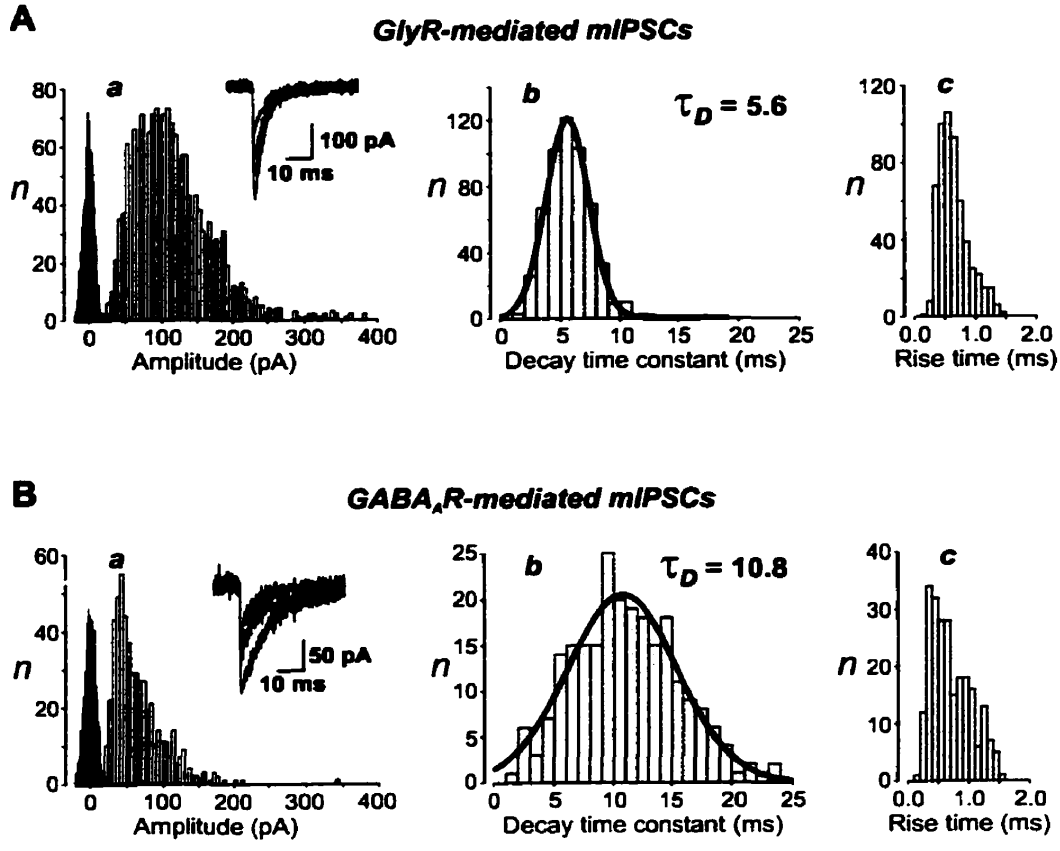

A
GlyR-mediated mIPSCs
a
100 pA
10 ms
n
Amplitude (pA)
b
τ_D = 5.6
Decay time constant (ms)
c
Rise time (ms)
B
GABA$_A$R-mediated mIPSCs
a
50 pA
10 ms
n
Amplitude (pA)
b
τ_D = 10.8
Decay time constant (ms)
c
Rise time (ms)

Figure 4. Separate $GABA_AR$- and GlyR-mediated mIPSCs within the same deep dorsal horn neuron. The trace in **A** is a continuous record showing the occurrence of spontaneous miniature IPSCs over 40 minutes of recording. Note the periodical testing of the access resistance to the cell (upward and downward dark areas) to ensure that changes in IPSC amplitude or kinetics is not due to increase in series resistance. Strychnine (100 nM) and bicuculline (10 μM) were added to the bathing solution as indicated by the horizontal bars above the trace. While strychnine did not appear to block the IPSCs on this time scale, bicuculline reversibly blocked the large, slow IPSCs (see expanded traces below). In **B,** closer inspection of the IPSC kinetics revealed a heterogeneous population of decays, which were best fitted by the sum of two Gaussians. The group of faster decays disappeared after administration of strychnine indicating the presence of two populations of mIPSCs in this neuron: GlyR-mediated mIPSCs and slower $GABA_AR$-mediated mIPSCs. The traces in **C** represent averages of 25 IPSCs automatically selected by computer for decay time constants of less (*left*) or more (*right*) than 20 ms thus illustrating the difference in kinetics of the two populations of mIPSCs. The rise times of each group of mIPSCs were not significantly different (0.8 ± 0.2 vs. 0.9 ± 0.5 ms, respectively). Thus, $GABA_AR$ and GlyR-mediated mIPSCs can be found within the same cell, but with distinct kinetics.

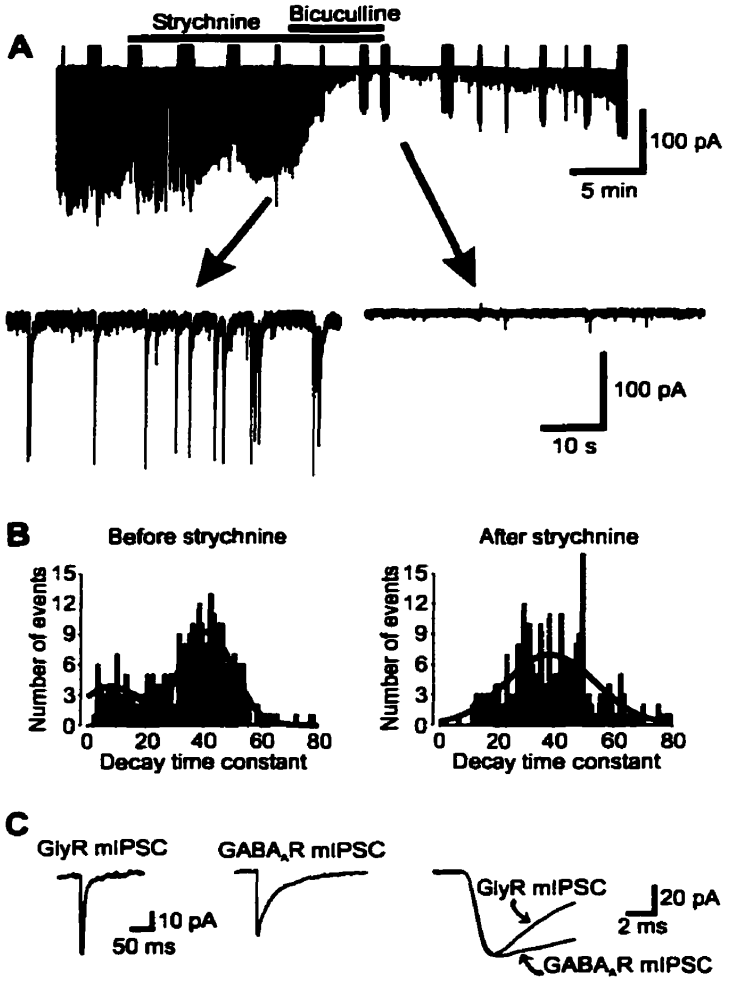

A
Strychnine
Bicuculline
100 pA
5 min
100 pA
10 s
B
Before strychnine
After strychnine
Number of events
15
12
9
6
3
0
0
20
40
60
80
Decay time constant
C
GlyR mIPSC
GABA$_A$R mIPSC
10 pA
50 ms
GlyR mIPSC
20 pA
2 ms
GABA$_A$R mIPSC

Figure 5. All laminae I-II neurons respond to exogenous application of both GABA and glycine. **A**, a lamina I neuron displaying only GlyR-mediated mIPSCs (**A***a*) because they were all selectively blocked by 100 nM strychnine (**A***b*). Recovery in 10 μM bicuculline (**A***c*),. Yet application of both GABA (1 mM) and glycine (1 mM) induced an inward current in this cell (**A***d*). Each of the agonists was applied by pressure ejection for 30 seconds, using a micropipette positioned near the recorded cell. The holding potential = -65 mV.

B, Both GABA-induced response and $GABA_AR$-mediated mIPSCs are blocked by bicuculline. *a*, The traces illustrates spontaneous mIPSCs recorded from a lamina II neuron. *b*, bath-application of 10 μM bicuculline blocked all the $GABA_AR$-mediated mIPSCs. *c*, puff-application of 1 mM GABA for 3 seconds induced an inward current in this cell. *d*, the response to 1 mM GABA was blocked by 10 μM bicuculline.

C, in this lamina II cell, the $GABA_AR$-mediated mIPSCs (*a*) are not affected by bath application of 1 μM strychnine (*b*), but selectively blocked by 3 μM SR-95531 (*c*); in the same cell, bath-applied 1 mM glycine induced an inward current, which is not affected by 3 μM SR-95531, but abolished by 1 μM strychnine (*d*).

D, GlyR-mediated mIPSCs (*a*) in this lamina I neuron are not affected by bath-applied 3 μM SR 95531 (*b*), but selectively blocked by 100 nM strychnine (*c*); in this lamina I neuron, bath-applied 1 mM GABA induced an inward current, which was abolished by 3 μM SR-95531, but not affected by 1 μM strychnine (*d*).

Chéry & De Koninck, Fig. 5

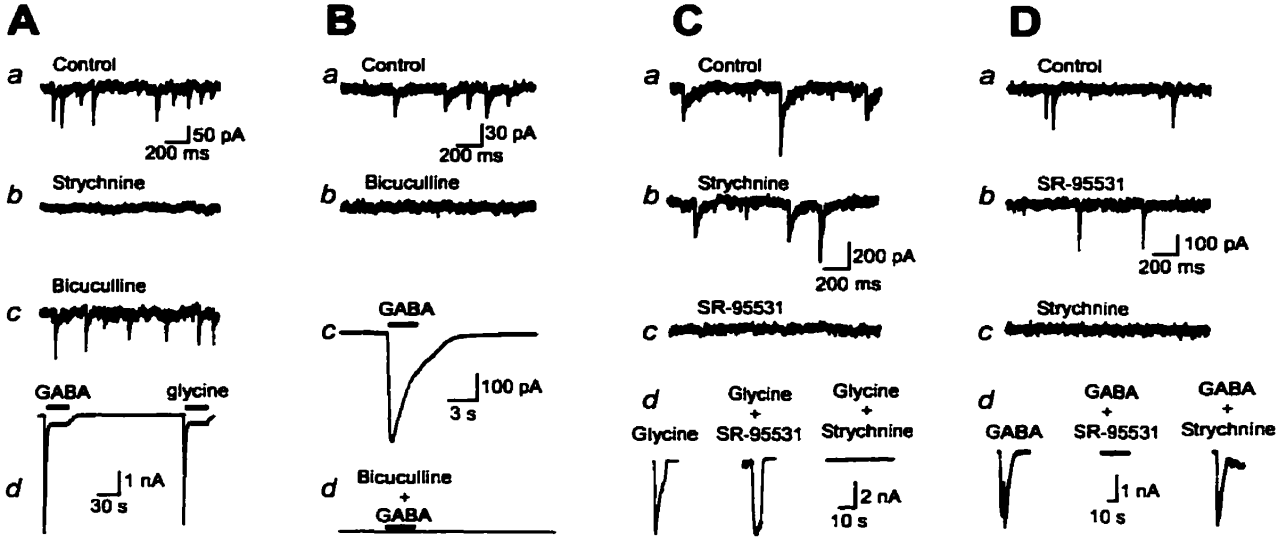


Figure 6. Hypertonic solution-induced increase in vesicular release failed to reveal a heterogeneous population of mIPSCs in laminae I-II. Pressure-application of hyperosmotic (610 mOsm) ACSF (middle traces) induced a 33-fold increase in the frequency of mIPSCs in this lamina I neuron. The mIPSCs were not affected by bath-application of 3 μM SR-95531, but selectively blocked by 100 nM strychnine. The superimposed averages in the inset show the lack of change in mIPSC kinetics in sucrose to further confirm that no new class of mIPSC was revealed by the hyperosmotic solution.

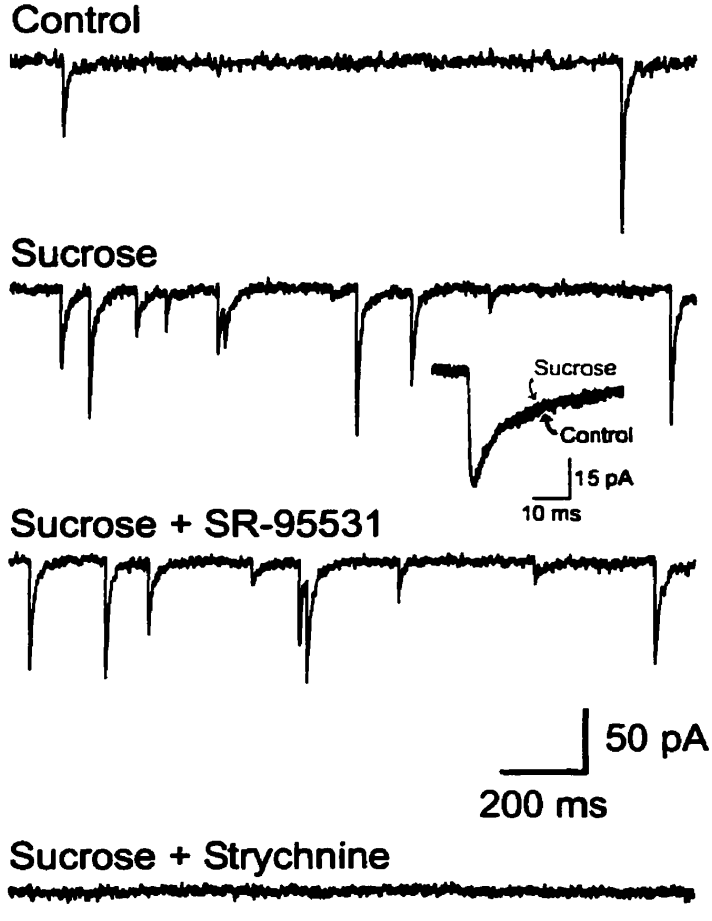

Control
Sucrose
Sucrose
Control
15 pA
10 ms
Sucrose + SR-95531
50 pA
200 ms
Sucrose + Strychnine

Figure 7. Large stimulus-evoked IPSCs in lamina I reveal a $GABA_AR$-mediated component. **A,** Average of 48 IPSCs elicited by placing a tungsten electrode within 300 μm from the cell body of the recorded neuron (*Combined*). The membrane potential was held at +40mV to avoid activation of voltage sensitive Na^+ currents. Following the application of 2 μM strychnine, a component remained (*GABA*) that could be blocked by 20 μM bicuculline. The GlyR-mediated component (*Glycine*) was obtained by subtraction of the *GABA* trace from the *Combined* trace. The inset shows the time course of block of evoked $GABA_AR$- and GlyR- -mediated IPSCs (quantified by the area under the curve, i.e. charge). **B,** (a) superimposed minimally-evoked IPSCs obtained by focal stimuli applied within 20 μm from the cell body using a patch micropipette. These meIPSCs were completely blocked by 1-2 μM strychnine (b, average of 25 traces). c, a 6 fold increase in stimulus intensity elicited $GABA_AR$-mediated IPSCs that could be blocked by addition of 10-20 μM bicuculline (d, average of 15 traces). The inset illustrates the $GABA_AR$- and GlyR-mediated components scaled to the same amplitude; note the slower rising and decay phases of the $GABA_AR$ component.

Chéry & De Koninck, Fig. 7

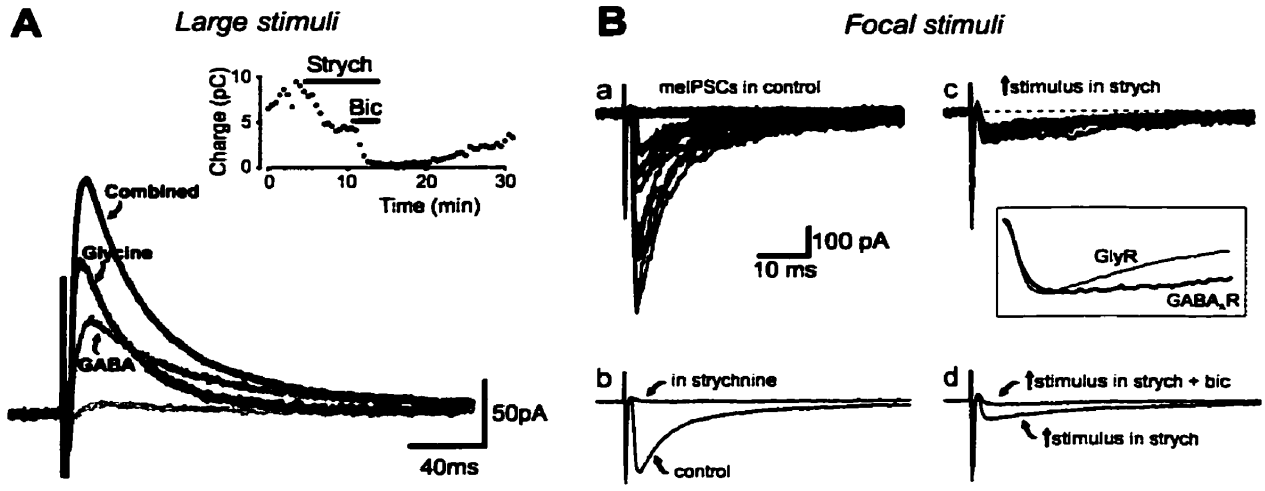


Figure 8. Comparable latencies, rise time and amplitude of proximal and distal $GABA_AR$- and GlyR-mediated meIPSCs. The diagram at the top illustrates how the micropipettes were positioned for focal stimulation along the bipolar axis of lamina I neurons. Focal stimuli were applied within 20 μm from the cell body (*Proximal*), and within 20-70 μm of a distal dendrite located at 150-300 μm distance from the soma (*Distal*). Top traces, superimposed GlyR-mediated meIPSCs elicited in the presence of 10 μM bicuculline. Bottom traces, in the presence of 1 μM strychnine, $GABA_AR$-mediated IPSCs with slower kinetics could be evoked. Note the slightly longer latencies for both the distal $GABA_AR$- and GlyR-mediated evoked IPSCs, but the very similar rise times and amplitudes. The only slight increase in latency of the distally-evoked IPSCs account for an approximately 2-fold conduction distance and thus indicated that these IPSCs still originated at a significant distance from the soma. In any case, the distally evoked $GABA_AR$-mediated events did not have a shorter latency, arguing against the possibility that $GABA_AR$ IPSCs are only occurring at distal points from the soma. Moreover the only slight slowing of the rising phase of the distal GlyR-mediated meIPSCs indicate little space clamp limitations in these cells and thus ruling out the possibility that $GABA_AR$-mediated mIPSCs may exist that could not be detected by somatic recording.

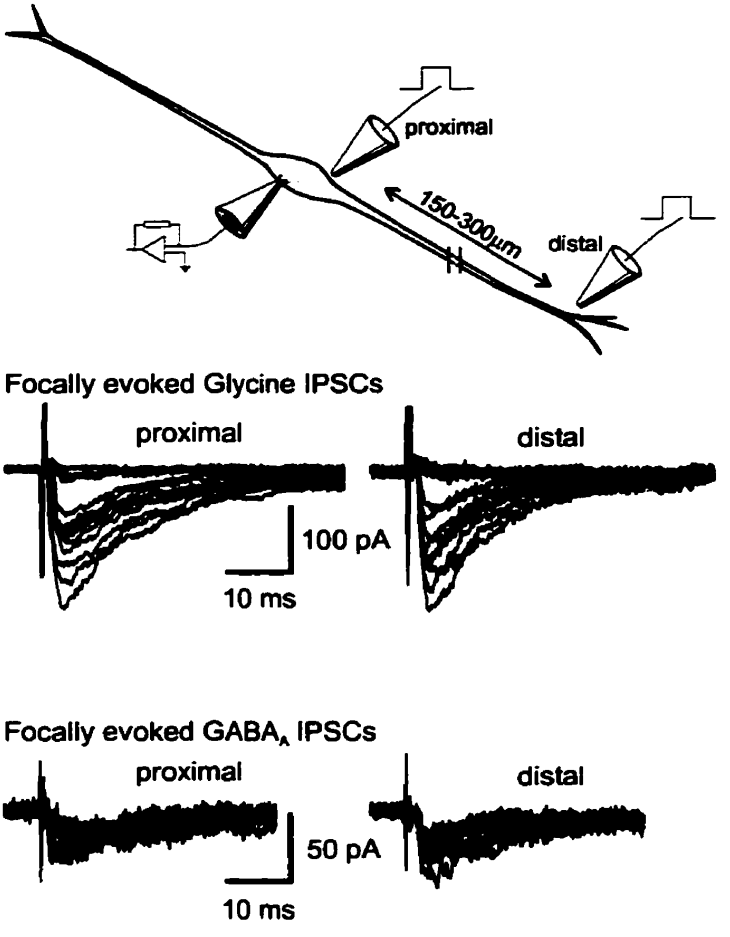

proximal
150-300µm
distal
Focally evoked Glycine IPSCs
proximal
distal
100 pA
10 ms
Focally evoked GABA$_A$ IPSCs
proximal
distal
50 pA
10 ms

Figure 9. The benzodiazepine flunitrazepam unmasks a slow $GABA_AR$-mediated component to mIPSC recorded in lamina I neurons. The traces in **A** are representative superimposed mIPSCs used to compile the histograms below each trace (**B** & **C**). The inset at the right top corner is a superimposition of the events scaled to the same amplitude to emphasize their difference in rising and decaying kinetics. The histograms in **C** & **D** illustrate the distributions of 10-90% rise times and decay time constants for all the events detected in each condition during a 7 minute window. Thus, the number of events in each histogram provides an estimate of the frequency of events in each condition. The curve in **D** illustrates the area overlap between the Gaussian curves fit to the histograms above. The overlap (gray area) was less than 11% of the area under the curve for the control histogram. The very small overlap between the distributions or rise times and decay time constants between control conditions and in the presence of flunitrazepam indicates that the majority of individual mIPSCs had their kinetics altered. Consistent with this observation, the number of events remaining in the presence of flunitrazepam and strychnine is greater than the difference between the number of events detected in flunitrazepam *vs.* control.

Addition of 10 μM bicuculline completely abolished all mIPSCs that remained in the presence of 100 nM strychnine. The graphs on the right are cumulative histograms illustrating the intermediate distribution of kinetics observed between control, flunitrazepam (Flu), and flunitrazepam plus 100 nM strychnine (Flu+strych) conditions.

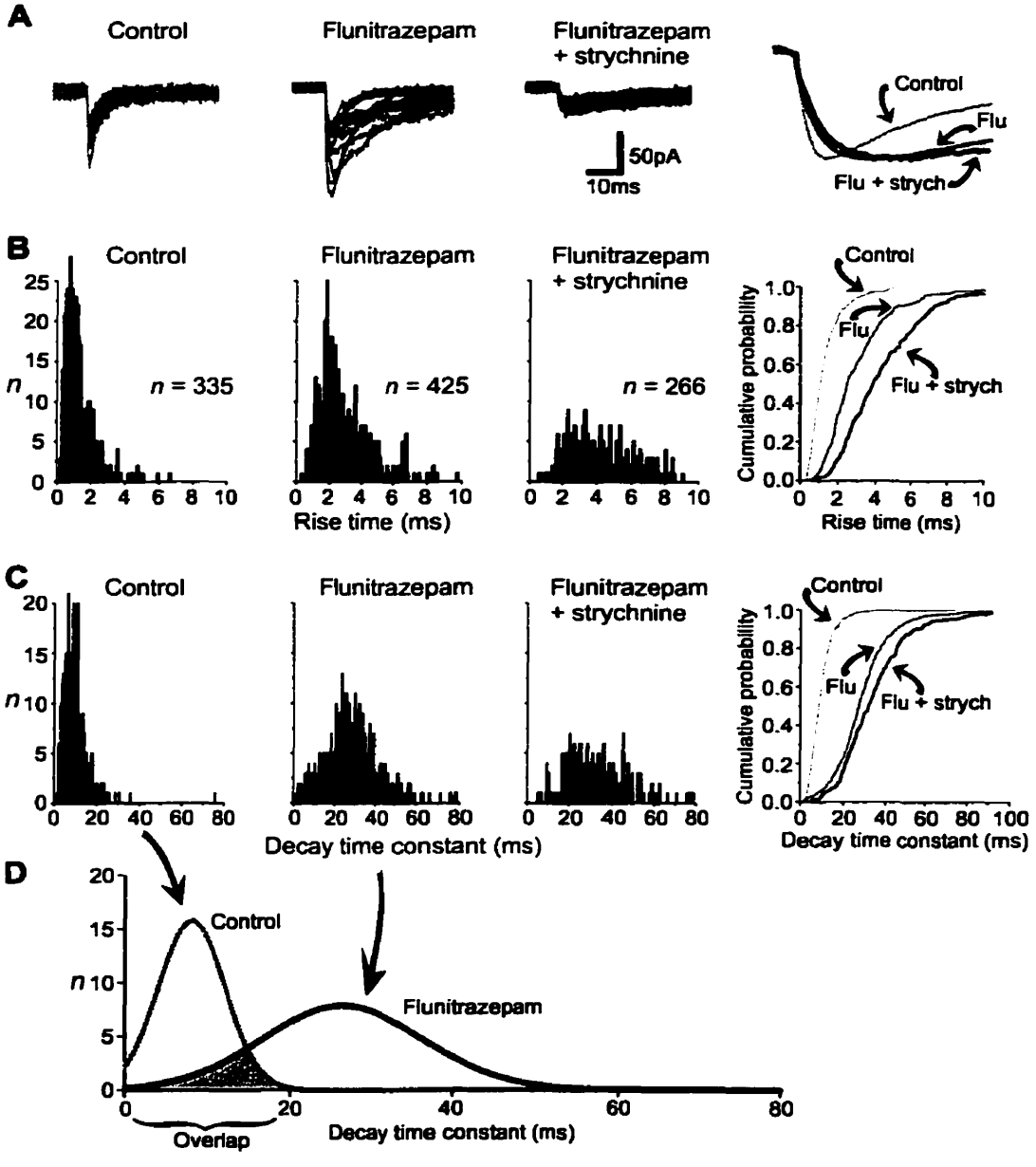

A
Control
Flunitrazepam
Flunitrazepam + strychnine
50pA
10ms
Control
Flu
Flu + strych
B
Control
Flunitrazepam
Flunitrazepam + strychnine
n = 335
n = 425
n = 266
n
Rise time (ms)
Cumulative probability
C
Decay time constant (ms)
D
Overlap

Figure 10. GABA uptake blockers do not significantly affect GlyR-mediated mIPSCs in lamina I but prolong the decay time course of evoked IPSCs and of $GABA_A$R-mediated mIPSCs revealed by flunitrazepam. The traces at the *top* are averages of 89 consecutive mIPSCs in control solution and 100 mIPSCs after 30 min of bathing in 30 μM NO-711. The traces in the middle are averages of 23 mIPSCs recorded in the presence of 1 μM flunitrazepam and 100nM strychnine before and after addition of 30 μM NO-711. The traces at the bottom are averages of 42 stimulus evoked IPSCs in control solution and 57 evoked IPSCs after 20 min of bathing in 10 μM NO-711. (10 mM QX-314 was added to the pipette solution to block voltage sensitive Na^+ channels).

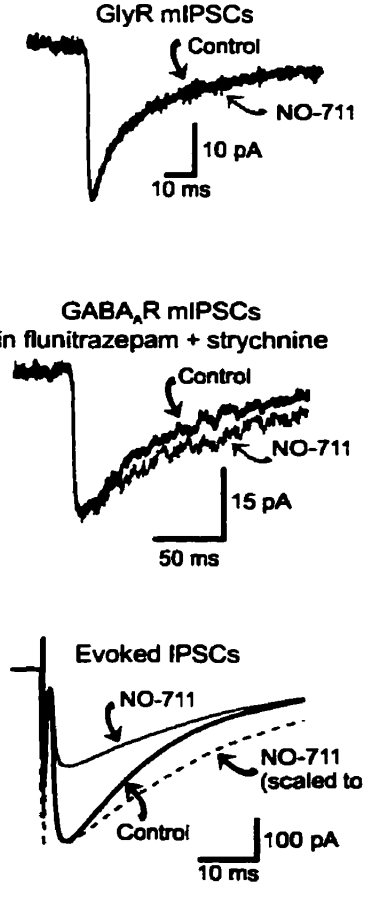
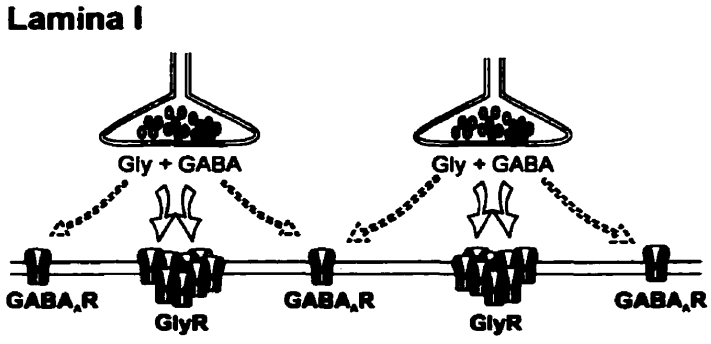

GlyR mIPSCs
Control
NO-711
10 pA
10 ms
GABA$_A$R mIPSCs
in flunitrazepam + strychnine
Control
NO-711
15 pA
50 ms
Evoked IPSCs
NO-711
NO-711
(scaled to peak)
Control
100 pA
10 ms

Figure 11. Proposed summary of glycine *vs.* $GABA_A$ receptor-mediated inhibition in lamina I. During normal basal activity, both glycine and GABA may be released, but only glycine receptors that are clustered at synapses will mediate the mIPSCs, while extrasynaptic $GABA_AR$s may require accumulation of GABA "spillover". Thus, in lamina I, glycine appear to mediate tonic inhibition from spontaneous vesicular release. In contrast, $GABA_AR$-mediated inhibition may be effective to control larger, synchronous (evoked) input.

Chéry & De Koninck, Fig. 11



PREFACE TO CHAPTER 4

The co-release of GABA with glycine following low-intensity input to lamina I neurons appears to be in contrast with the finding that $GABA_A$Rs activation was not detectable during small evoked activity. Could GABA released under such conditions serve to activate other receptor targets? This issue is addressed in the following chapter.

CHAPTER 4

$GABA_B$ receptors are the first target of released GABA at lamina I inhibitory synapses in the rat spinal cord.

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Abstract

We have previously provided functional evidence that glycine and GABA are contained in the same synaptic vesicles and co-released at the same synapses in lamina I of the rat spinal dorsal horn. Yet, while both glycine receptors (GlyRs) and $GABA_A$ receptors ($GABA_ARs$) are expressed on the postsynaptic target, under certain conditions, inhibitory events appeared to be mediated by GlyRs only. We therefore wanted to test whether $GABA_B$ receptors could be activated in conditions where GABA released was insufficient to activate $GABA_ARs$. Focal stimulation in the vicinity of visually identified lamina I neurons elicited monosynaptic IPSCs, in the presence of the glutamate receptor antagonists CNQX and APV. Pairs of stimuli were given at different interstimulus intervals (ISI), ranging from 25 ms to 1s to study the depression of the second of evoked IPSCs (paired pulse depression; PPD). Maximal PPD of IPSCs was 60 ± 14% (of the conditioning pulse amplitude), at ISI between 150-200 ms. PPD was observed with IPSCs evoked at stimulus intensities where they had no $GABA_AR$ component. PPD of small evoked IPSCs was not affected by the $GABA_AR$ antagonist bicuculline, but selectively abolished by 10-30 μM CGP52432, a specific $GABA_B$ receptor antagonist. These data indicate that, under conditions where GABA released is insufficient to affect postsynaptic $GABA_ARs$ in lamina I, significant activation of presynaptic $GABA_B$ autoreceptors can occur.

Introduction

We have recently shown that GABA and glycine are co-released from the same synaptic vesicles at inhibitory synapses on lamina I neurons of the spinal cord. However, we have also shown that miniature (action potential independent) or small evoked inhibitory postsynaptic currents (IPSCs) involve activation of glycine receptors (GlyRs) only, even though the postsynaptic neurons expressed both GlyRs and $GABA_A$ receptors ($GABA_ARs$). Activation of postsynaptic $GABA_A$ receptors could be detected only following stimuli of sufficient intensity to allow synchronous activation of a sufficient number of terminals, presumably because under such conditions GABA could spillover from synapses to reach extrasynaptic $GABA_ARs$ (Chéry, De Koninck, 1999). We therefore wanted to test whether presynaptic $GABA_B$ autoreceptors could be activated at stimulus intensities where GlyR-mediated IPSCs are elicited, but $GABA_AR$ activation is not detectable. This is because $GABA_B$ receptors ($GABA_BRs$) may display a greater affinity for GABA than do $GABA_ARs$ (Yoon, Rothman, 1991; Isaacson et al., 1993). In many regions of the CNS $GABA_BRs$ are often found localized on axonal endings (Bowery, 1993), indicating that they might play a role in the modulation of neurotransmitter release. A classical test of $GABA_BRs$ activation in the brain is the study of paired-pulse depression (PPD) of inhibitory synaptic events where one analyzes the response to a test stimulus following a conditioning stimulus at different interstimulus intervals (ISIs) (Davies et al., 1990; Otis et al., 1993). Thus, we studied PPD of IPSCs evoked by focal electrical stimuli applied in the vicinity of spinal lamina I neurons. Our findings indicate that, under those circumstances, the amount of GABA released from synaptic terminals first serves to activate $GABA_B$ autoreceptors. Preliminary accounts of this study have been reported in abstract form (De Koninck, Chéry, 1999).

Methods

Slicing procedure. Adult male Sprague-Dawley rats (weighing 150-250g) were anesthetized with Na^+-pentobarbital (30 mg/kg), and spinal cord slices were obtained as described previously (De Koninck, Chéry, 1998). Briefly, rats were perfused with and ice-cold sucrose-ACSF solution (in which 126 mM NaCl was replaced with 252 mM sucrose; see below for a description of normal ACSF), then rapidly decapitated. The spinal cord was removed by hydraulic extrusion, and the cervical and lumbar segments (2 cm-long) were isolated and glued, lateral side down, on a brass platform with cyanoacrylate cement, in a chamber filled with oxygenated ice-cold sucrose-ACSF. Parasagittal 400 μm-thick slices were cut, incubated in sucrose-ACSF at room temperature (23-28°C) for 30 minutes, and then transferred to normal ACSF for at least one hour prior to electrophysiological recordings. Next, the 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 perfused at ~2 ml/min with oxygenated ACSF containing (in mM): 126 NaCl, , 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 26 $NaHCO_3$, 1.25 NaH_2PO_4, (pH 7.35; 300-310 mOsm), and the glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 10 μM; Tocris Cookson) and D-2-amino-5-phosphonovaleric acid (D-AP5, 40 μM; Tocris Cookson).

Drug application. Bicuculline methiodide (10-20 μM; RBI), strychnine hydrochloride (100 nM - 1μM; RBI) and CGP52432 (([3-[[3,4-dichlorophenyl)methyl] amino]propyl] (diethoxymethyl) phosphinic acid; 10-30 μM, Ciba-Geigy) were used to block $GABA_A$Rs, GlyRs, and $GABA_B$Rs, respectively. The glutamate receptor antagonists CNQX and D-AP5 were used to isolate monosynaptic IPSCs. The action potential blocker tetrodotoxin (TTX, 1 μM; RBI) was used to record miniature IPSCs and

the benzodiazepine flunitrazepam (1 μM; Sigma) to potentiate the $GABA_A$ components of IPSCs.

Whole-cell recordings and data analysis. For whole-cell voltage-clamp recordings of IPSCs, patch pipettes were pulled from borosilicate glass capillaries (with an inner filament, WPI) using a two-stage vertical puller (Narishige PP-83). To record mIPSCs and evoked IPSCs, the pipettes were filled with an intracellular solution composed of (in mM): 110 CsCl, 10 HEPES, 2 $MgCl_2$, 2 mM ATP (Sigma), 0.4 mM GTP (Sigma), 11 mM BAPTA (Sigma), 1 mM $CaCl_2$ and 0.5% Lucifer Yellow (Sigma). The pH was adjusted to 7.2 with CsOH, and the osmolarity ranged from 260-280 mOsm (pipette resistance 3 MΩ). For the paired pulse experiments, 110 mM CsCl was replaced with 110 mM Cs-gluconate and 5 mM CsCl. Recordings were obtained by lowering the patch electrode onto the surface of visually identified neurons in lamina I. While monitoring current responses to 5 mV pulses, a brief suction was applied to form >GΩ seals. An Axopatch 200B amplifier (Axon Instruments) with > 80% series resistance compensation was used for the recording. The access resistance was monitored throughout each experiment. Only recordings with access resistance between 7-20 MΩ were considered acceptable for analysis of evoked IPSCs and only recording with stable access throughout the entire administration of antagonists were used for further analysis. Monosynaptic IPSCs were evoked by focal electrical stimulation using a patch micropipette. Square-wave constant paired-pulses (200-300 μs duration) were applied at a frequency of < 0.3 Hz, at different interstimulus intervals, ranging from 25 ms to 1s. The electrode was placed within 20-50 μm of cell body of lamina I neurons. For analysis of the data, traces were low-pass filtered at 10 kHz and stored on a videotape, using a digital data recorder (VR-10B, Instrutech Corp.). Off-line, the recordings were low-pass filtered at 2-3 kHz and sampled at 10-20 kHz, on an Intel Pentium-based computer and analyzed using

locally designed software (Y. De Koninck) (De Koninck, Mody, 1994; Chéry, De Koninck, 1999).

Statistical analysis. Student t tests were used to analyze the differences between the kinetic and amplitude parameters of the IPSCs. The critical value for statistical significance was set at $p < 0.05$. All the data are expressed as mean ± SEM, unless otherwise indicated.

Results

We have shown that lamina I neurons receive exclusively GlyR-mediated miniature IPSCs (mIPSCs) (Chéry, De Koninck, 1999) although GABA coexists with glycine in superficial dorsal horn neurons (Todd, Spike, 1993). Failure to detect a $GABA_A$R-mediated component to mIPSCs in lamina I neurons was due to a sub-threshold activation of $GABA_A$Rs (Chéry, De Koninck, 1999). We confirmed that both GlyRs and $GABA_A$Rs can be activated during mIPSCs by adding flunitrazepam to enhance the sensitivity of $GABA_A$Rs. Figure 1 illustrates that, while under normal conditions, all mIPSCs are antagonized by 100nM strychnine, in the presence of flunitrazepam, an additional slowly rising and slowly decaying $GABA_A$R-mediated component appeared in the large majority of events. Given that mIPSCs represent the activation of postsynaptic receptors by single vesicles of transmitter (Edwards et al., 1990), these results indicate co-release of GABA and glycine from the same synaptic vesicles and thus from the same terminals. This evidence is consistent with previous reports indicating that GABA and glycine are taken up by the same vesicular transporter (Burger et al., 1991; Chaudhry et al., 1998; Dumoulin et al., 1999) and with evidence at the motoneuron synapse that stimulation of single inhibitory interneurons produces mixed GlyRs and $GABA_A$R-mediated IPSCs (Jonas et al., 1998).

While under certain conditions, the GABA co-released with glycine may be subthreshold to activation of $GABA_A$Rs, it may still be sufficient to activate another receptor subtype, namely $GABA_B$ receptors. $GABA_B$ autoreceptors are found predominantly in laminae I-II of the dorsal horn (Bowery, 1993), and may have a greater affinity for the inhibitory transmitter than $GABA_A$Rs. To test this hypothesis, we sought to detect activation of $GABA_B$ receptors under conditions where $GABA_A$Rs are not activated, *i.e.* conditions in which inhibitory currents are mediated by GlyRs only. Figure 2 illustrates that IPSCs evoked by focal stimuli at low-intensity (<100 μA for 200 μs) in the vicinity of identified lamina I neurons were completely blocked by strychnine (Fig 2a; n=12). In the presence of strychnine, $GABA_A$R-mediated evoked IPSCs were only obtained upon increasing the stimulus intensity (Fig 2b).

Using such stimuli that resulted in pure GlyR-mediated IPSCs, we studied the paired-pulse depression (PPD) of IPSCs evoked in lamina I neurons. Paired-pulse depression is typically associated with activation of presynaptic $GABA_B$Rs (Davies et al., 1990). A conditioning current and a test current were applied focally at different interstimulus intervals (ISIs; see Fig 3). The ISIs ranged from 25 ms to 1 s. When the ISI was shorter than the decay of the conditioning IPSCs, an overlap in time of the conditioning and test currents was observed. Thus a digital subtraction was used to obtain accurate values for the peak of the test IPSCs, as previously described (Otis et al., 1993). Figure 3 illustrates PPD of evoked IPSCs in a lamina I neuron. The maximal depression of the test IPSCs (60±14% of the amplitude of the conditioning IPSC; $p<0.01$) was observed at 150-200 ms ISIs (n=6).

Up to 20 μM bicuculline failed to affect the amplitude of the conditioning pulse (Fig 4B; nor the PPD ratio), indicating that IPSCs evoked by minimal stimuli do not involve activation of postsynaptic $GABA_A$Rs. PPD of small evoked IPSCs was abolished following bath application of 10-30 μM CGP52432 (Fig 4C), a specific $GABA_B$ receptor antagonist. This suggests that $GABA_B$ autoreceptors appear to be the first target of GABA

released at inhibitory synapses in lamina I neurons. The small evoked IPSCs were completely abolished by strychnine (Fig 4B), confirming that they are selectively mediated by GlyRs.

Discussion

Our findings indicate that, while GABA and glycine appear to be released from the same synaptic terminals in lamina I, the amount of GABA released at inhibitory synapses may be subliminal to the activation of postsynaptic $GABA_A$ receptors, yet the released GABA may be sufficient to significantly activate presynaptic $GABA_B$ receptors.

The predominant localization of $GABA_B$ receptors in superficial laminae of the spinal cord (Malcangio et al., 1993), and their preferential occurrence on synaptic terminals in many CNS regions (Bowery, 1993) indicate that they may have an important role in the modulation of GABA release in the dorsal horn.

Given the evidence of both GABA and glycine are contained in the same synaptic vesicles [this study and (Burger et al., 1991; Chaudhry et al., 1998; Dumoulin et al., 1999; Jonas et al., 1998; Chéry, De Koninck, 1999)] and that small evoked IPSCs were mediated exclusively by glycine provided an ideal setting to test whether activation of $GABA_B$ receptors occurs in conditions where $GABA_AR$ activation is not detectable. In this study we were able to show $GABA_B$-mediated PPD of GlyR IPSCs. Thus, our results provide evidence that $GABA_B$ autoreceptors are present on glycinergic interneurons that also contain GABA (Todd, Spike, 1993), where they modulate the release of both inhibitory transmitters from interneurons terminals. This evidence is consistent with the recent demonstration of presynaptic inhibition of both GABA and glycine release at spinal interneuron-motoneuron synapses by the $GABA_BR$ agonist baclofen (Jonas et al., 1998).

The PPD ratio reported here is in general agreement with previous studies of PPD of GABA release in other regions of the CNS (Davies et al., 1990; Otis et al., 1993). Consistent with our present results, maximal reductions of the test pulse amplitude (up to 48% of the conditioning pulse) have been reported to occur at ISIs between 100-200 ms.

Manipulating GABA release at lamina I inhibitory synapses may be an important means to control excitability in this area and thus the relay of nociceptive input to the brain. Interestingly, according to the results of some binding studies with dorsal horn slices and spinal cord synaptosomes, $GABA_B$ receptors on GABAergic terminals would be distinct from the heteroreceptors present on glutamate-releasing terminals (Teoh et al., 1996; Bonanno et al., 1998). Since our recent study indicated that $GABA_A$Rs could be activated under conditions that promote spillover of GABA from synapses in lamina I (Chéry, De Koninck, 1999), selective antagonism of $GABA_B$ autoreceptors may prove useful to enhance inhibitory transmission mediated by both GABA and glycine in lamina I. On the other hand, increasing the activation of $GABA_B$ heteroreceptors present on glutamate-containing synaptic terminals may reduce glutamate release, thus further enhancing inhibitory control of lamina I neurons. This selective targeting of $GABA_B$ autoreceptors could have an important impact on the treatment of chronic pain states.

At most GABAergic synapses in the CNS, weak stimulation of interneurons preferentially activates $GABA_A$Rs likely located at synaptic sites, whereas activation of $GABA_B$ autoreceptors is rather detected following strong electrical stimulation which promotes GABA spillover from neighboring synapses (Dutar, Nicoll, 1988a; Otis, Mody, 1992b; Isaacson et al., 1993; Ouardouz, Lacaille, 1997; Nurse, Lacaille, 1997). The converse scenario appears to apply to lamina I inhibitory synapses, and could be attributed to the fact that in this case $GABA_A$Rs, like $GABA_B$Rs, are located at a distance from the release site (*i.e.*, a preferential extrasynaptic distribution of $GABA_A$Rs). Thus, GABAergic inhibition appears to be modulated in a selective manner in lamina I, whereby $GABA_B$ autoreceptors are the first target of GABA released in this spinal area.

Such regulation of GABA release may have important physiological implications, notably under conditions that favor hyperexcitability in the dorsal horn.

In summary, our present data indicate that under conditions where GABA release is insufficient to significantly affect postsynaptic $GABA_A$Rs on lamina I neurons, it may rather serve to activate $GABA_B$ autoreceptors to regulate the release of both glycine and GABA

Acknowledgements

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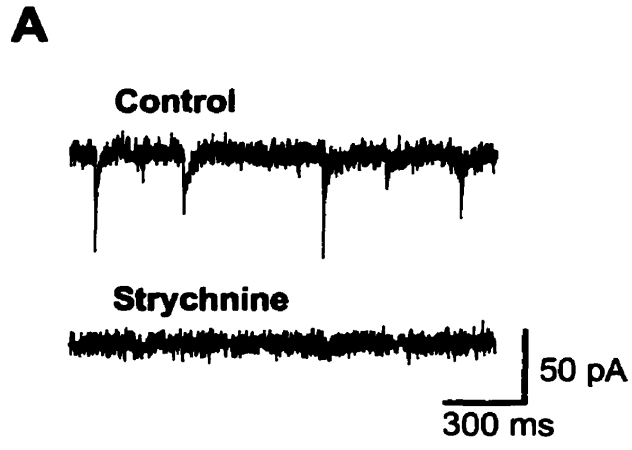
Figure legends

Figure 1. A, mIPSCs recorded in lamina I are completely blocked by strychnine. Thus they all mediated by glycine acting at GlyRs. B, superimposition of GlyR-mediated mIPSCs (*top trace*) illustrating their fast kinetics. In the presence of flunitrazepam (*middle trace*), the majority of mIPSCs display prolonged kinetics. Addition of strychnine to the bathing solution containing flunitrazepam (*bottom trace*) revealed mIPSCs with very slow kinetics; these mIPSCs were abolished by the $GABA_AR$ antagonist bicuculline (not shown). The prolonged kinetic of the mIPSCs in the presence of flunitrazepam and the antagonism of the mIPSCs remaining in the presence of flunitrazepam and strychnine by bicuculline suggest that they are mediated by both GABA and glycine. Thus the inhibitory transmitters appear to be contained in the same synaptic vesicles and co-released. Recordings were obtained with CsCl-filled pipettes at a holding potential of –60 mV.


A
Control
Strychnine
50 pA
300 ms

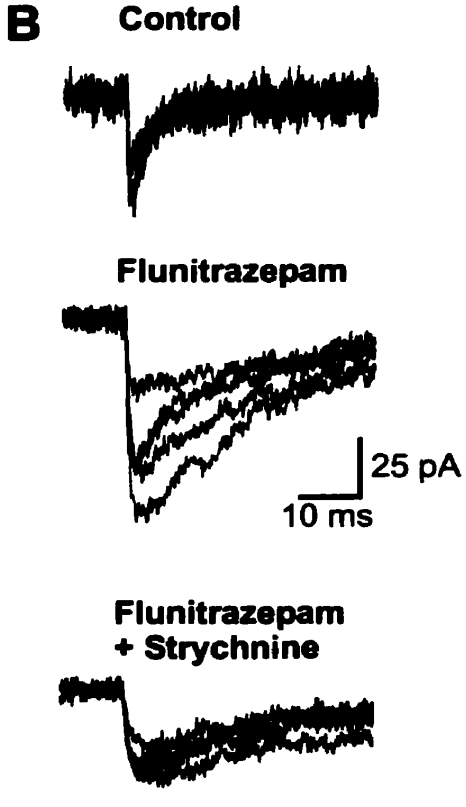

B
Control
Flunitrazepam
25 pA
10 ms
Flunitrazepam
+ Strychnine

Figure 2. Minimally evoked IPSCs (meIPSCs) are mediated exclusively by glycine. A, focal stimulation at an intensity of <100 μA in the vicinity of this lamina I neuron evoked monosynaptic IPSCs that were blocked by strychnine (average of 10 small evoked IPSCs traces). B, a four- to six-fold increase in the stimulus intensity in the presence of strychnine, elicited IPSCs with much slower kinetics (average of 10 traces). The latter IPSCs were antagonized by addition of bicuculline. Recordings were done in symmetrical Cl^- condition at a holding potential of –60 mV, the patch pipettes contained 10 mM QX-314.

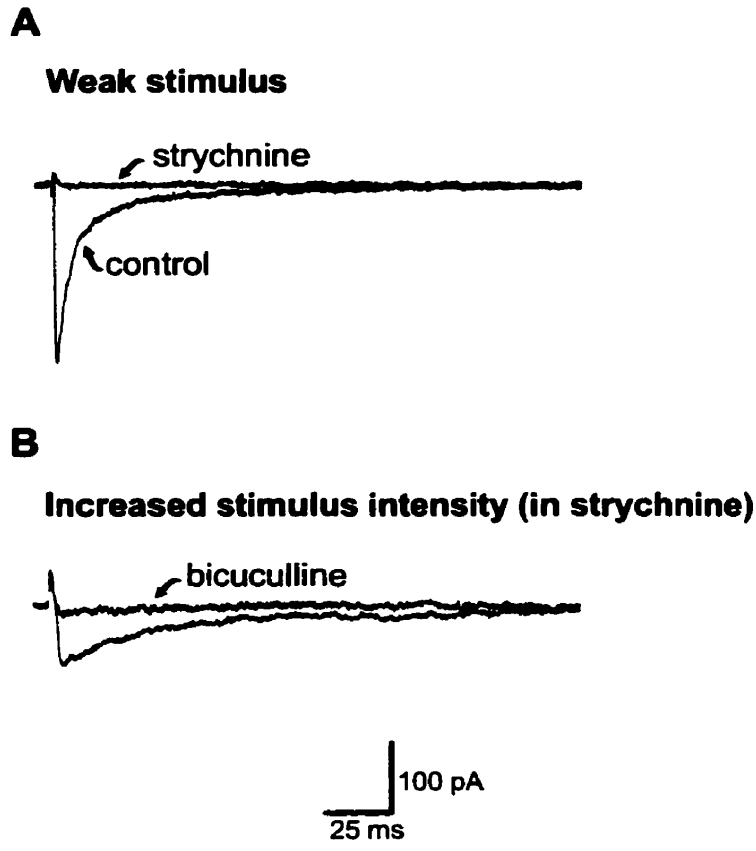

A
Weak stimulus
strychnine
control
B
Increased stimulus intensity (in strychnine)
bicuculline
100 pA
25 ms

Figure 3. Paired pulse depression (PPD) of meIPSCs in a lamina I neuron. A conditioning current (<100 μA) that elicited an all-or-none response was applied in the vicinity of an identified lamina I neuron, and an identical test stimulus was given subsequently to study the activation of presynaptic $GABA_B$ autoreceptors. To isolate the test response a subtraction procedure was used as described previously (Otis et al., 1993). The interstimulus interval (ISI) is indicated at the left of each trace. The maximal depression was observed at an ISI of 150 ms in this lamina I neuron. Each trace is the average of 10 to15 responses. The recording pipette contained Cs-gluconate. The holding membrane potential was 0 mV.

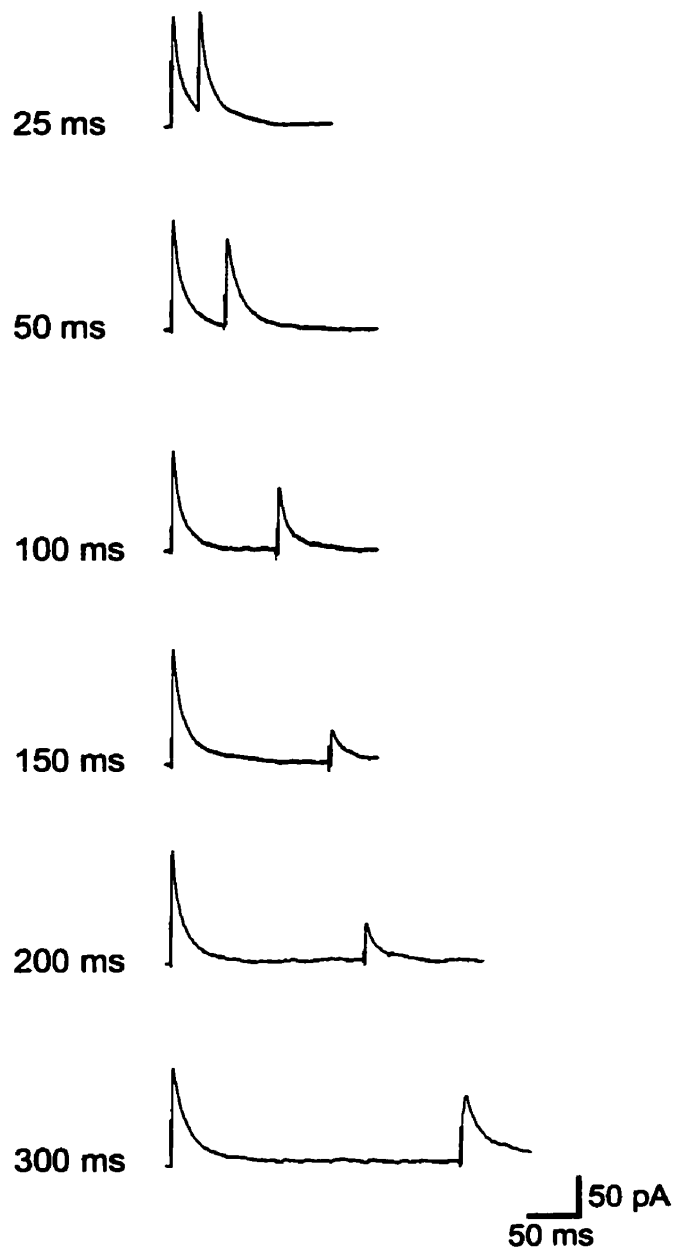
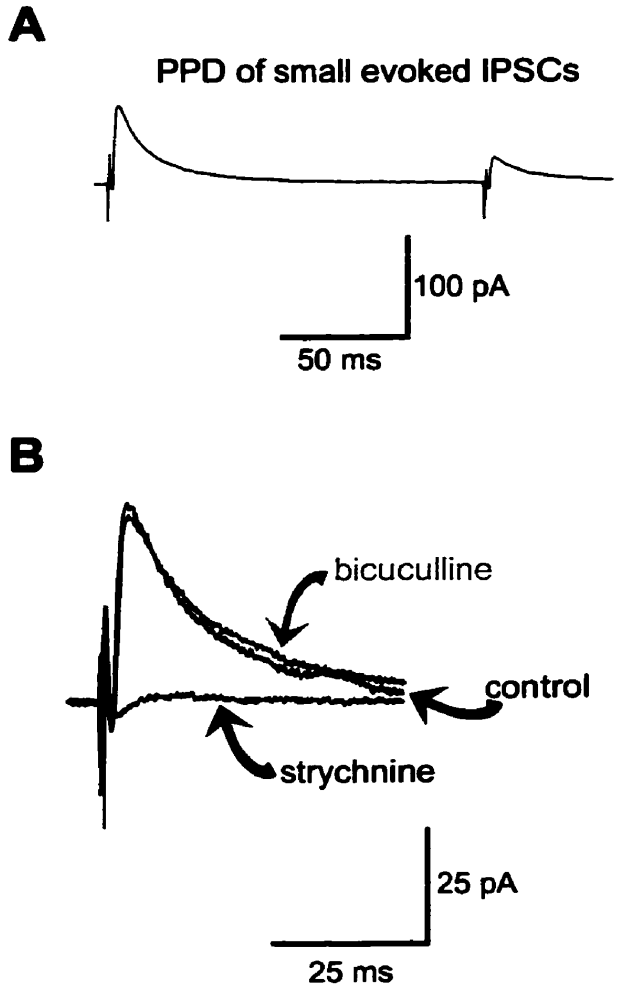

25 ms
50 ms
100 ms
150 ms
200 ms
300 ms
50 pA
50 ms

Figure 4. The paired pulse depression (PPD) of IPSCs evoked by low-intensity stimuli is due to the activation of presynaptic $GABA_B$ receptors. A, illustration of PPD of small evoked IPSCs in a lamina I neuron (average of 67 traces). B, the superimposed averages (traces in A) show that the amplitude and kinetics of the IPSC in response to the conditioning stimulus was not affected by bicuculline. On the other hand these IPSCs but they were completely antagonized by strychnine. C, the amplitude of the test IPSCs (P2; control) in control solution was significantly reduced compared to that of the conditioning IPSCs (P1; control). Bath application of CGP52432 reversed the depression of the test response (P2, *vs.* P1 in CGP52432), indicating that PPD is mediated by activation of presynaptic $GABA_B$ autoreceptors.


A
PPD of small evoked IPSCs
100 pA
50 ms
B
bicuculline
control
strychnine
25 pA
25 ms
C
100
0
Amplitude (pA)
*
P1
P2
Control
P1
P2
CGP52432

PREFACE TO CHAPTER 5

The studies presented so far addressed the inhibitory control of lamina I neurons by GABA and glycine in a normal situation. My next question was to study whether inhibition mediated by these transmitters is altered in pathological conditions, for instance, following a peripheral nerve injury.

CHAPTER 5

A shift in the balance of excitatory and inhibitory drive to lamina I neurons in neuropathic rats.

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To be submitted for publication

ABSTRACT

Following injury to a sensory nerve, hypersensitivity can develop in the form of painful sensation to normally innocuous stimuli (allodynia). Recording from neurons in spinal cord lamina I, a crucial area for integration and relay of pain-related signals, revealed that, in normal rats the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) was significantly reduced after blockade of action potential propagation. In contrast, the frequency of sIPSCs was not affected by this manipulation in rats with nerve injury, indicating a loss of activity in inhibitory interneurons presynaptic to lamina I cells. The frequency of spontaneous excitatory postsynaptic currents (sEPSCs) was significantly increased in nerve injured animals, while no change was detected in the frequency of action potential independent EPSCs. Furthermore, while sIPSCs are normally only mediated via glycine receptors in lamina I neurons, an additional $GABA_A$ receptor-mediated component appeared following nerve injury. The results indicate that disinhibition of spinal lamina I neurons occurs after nerve injury that is partially compensated by recruitment of GABAergic inhibition. This disinhibition may be a substrate for abnormal pain sensation in neuropathic syndromes.

INTRODUCTION

Peripheral tissue damage or nerve injury often leads to persistent pathological pain processes, such as hyperalgesia, allodynia and spontaneous pain. Although peripheral neural mechanisms such as nociceptor sensitization and spontaneous activity in sensory nerve fibers contribute to these pathological pain processes, aberrant processing of sensory input within the spinal dorsal horn is an important contributing factor (Woolf, Mannion, 1999; Bennett, 1994; Coderre et al., 1993).

Several substrates have been proposed underlying the development of this hyperexcitability, including neurochemical changes (Coderre et al., 1993) as well as structural reorganization of sensory fibers in the superficial dorsal horn (Woolf et al., 1992; Nakatsuka et al., 1999). While a number of these mechanisms point to enhanced excitation, impaired inhibition is also a likely important factor (Woolf, Mannion, 1999; Woolf, Wall, 1982; Sugimoto et al., 1990). Blocking glycine or $GABA_A$ receptor-mediated inhibition at the spinal level has been shown to replicate in many respects the abnormal sensory responses characteristic of neuropathic pain (Yaksh, 1989; Sivilotti, Woolf, 1994; Sherman, Loomis, 1994; Sherman, Loomis, 1996; Sorkin et al., 1998), but the mechanism by which such potential disinhibition is expressed at the spinal level remains unknown. Information is lacking in particular as to the balance of excitatory and inhibitory input to nociceptive (pain-related) relay neurons in the dorsal horn.

An important area for the integration and relay of nociception in the central nervous system is the marginal layer or lamina I of the dorsal horn of the spinal cord. This area represents one of the main nociceptive spinal output pathways to the brain (Perl, 1984; Willis, 1985; Light, 1992; Craig, 1996). Thus, altered processing in these neurons will have an important impact on how sensory information will be relayed. To determine the substrate of alteration in the balance of input to lamina I neurons, we used a spinal cord slice preparation to perform whole cell patch clamp recordings of spontaneously occurring excitatory and inhibitory synaptic currents bombarding these cells in control

rats and in rats with a sciatic nerve injury (Mosconi, Kruger, 1996). This approach allowed us to identify whether an altered balance of input was the result of changes in the functional properties of the synapses on lamina I neurons or upstream (presynaptic) from these cells. The results revealed a loss of activity in inhibitory interneurons presynaptic to lamina I cells in rats with a nerve injury. This loss of activity was accompanied with a concurrent increase in the excitatory activity presynaptic to lamina I neurons, without any apparent change in the properties of excitatory synapses onto these cells. Furthermore, a switch occurred in the inhibitory synaptic receptors on these cells: from only glycine receptors (GlyRs), to a mix of GlyRs and $GABA_A$ receptors ($GABA_ARs$). Because $GABA_ARs$ have slower kinetics than GlyRs, this switch may represent a compensatory response from the lamina I neurons to counterbalance the increase in excitatory drive.

RESULTS

We examined spontaneously occurring excitatory and inhibitory postsynaptic currents (sEPSCs and sIPSCs, respectively) in visually identified lamina I neurons in parasagittal spinal cord slices (Chéry, De Koninck, 1999) obtained from control rats and from rats that had been subjected to an injury to the sciatic nerve (Mosconi, Kruger, 1996). Isolated EPSCs were recorded at a holding membrane potential of -60 mV (Fig 1a) and IPSCs were recorded at 0 mV (Fig 1b). In these cells, sEPSCs were reversibly antagonized by 10 μM CNQX (Fig 1a), whereas sIPSCs were entirely and reversibly blocked by 100 nM strychnine (Fig 1b), consistent with our previous report (Chéry, De Koninck, 1999).

Altered nociceptive reflexes

The time course of development of allodynia in animals with nerve injury was monitored over 3 weeks post-surgery and is illustrated in Fig 1c. The paw withdrawal

response to a mechanical stimulus was measured in control rats and in animals with sciatic nerve injury. For rats with nerve injury, there was a significant decrease in the 50% paw withdrawal threshold as early as the first week after the injury. This reduction in threshold reached a maximum at two weeks and was subsequently maintained; (means: 15.0±0.4g, 6.0±0.7g, 1.5±0.1g, 2.0±0.2g, at times 0, 1, 2, and 3 weeks post surgery, $p<0.0001$; $n=39$). The time course of development of hypersensitivity was consistent with that previously reported for this model (Mosconi, Kruger, 1996), and for other models of chronic constriction injury of the sciatic nerve (Bennett, Xie, 1988; Seltzer et al., 1990) or segmental spinal nerve ligation (Kim, Chung, 1992). In sham operated animals, the 50% paw withdrawal threshold remained unchanged (means: 14.4±0.6g, 14.5±0.6g, 15.6±0.6g, and 15.1±0.1g at times 0, 1, 2, and 3 weeks post-surgery respectively, $p>0.2$; $n=11$). To minimize variability within the group of rats with constricted nerve, only those that had a 50% paw withdrawal threshold at below 2g were used for slice experiments.

Since maximal allodynia occurred at 2 weeks post-surgery (Fig 1c), all recordings from nerve injured animals were performed at that time point. Spontaneously occurring postsynaptic currents were recorded in normal ACSF from 30 of 40 lamina I neurons in control (sham operated) rats, and from 16 of 22 lamina I neurons in animals that had been subjected to a nerve injury. Data from 11 neurons (from control rats) and 9 neurons (from nerve injured rats) were retained, because the recordings were of sufficient duration, and the access resistance was stable throughout the testing of antagonists.

Loss of interneuronal activity following nerve injury.

The calculated mean conductance of sIPSCs in control *vs.* nerve injured rats did not significantly differ (797±99 pS vs. 709±152 pS, respectively; $p>0.6$), nor did the mean mIPSCs conductance in both rat groups (753±82 pS vs. 584±43 pS, respectively; $p>0.3$; see Fig 2a,c).

The frequency of sIPSCs in control animals was also similar to that from nerve injured rats: 1.1±0.3 Hz (range 0.1-5.6 Hz) vs. 1.0±0.1 Hz (range 0.5-1.4 Hz), respectively ($p>0.7$). To test whether the activity in inhibitory interneurons was different between the two rat groups, we compared the frequency of sIPSCs with that in the presence of 1 μM tetrodotoxin (TTX; to block action potential propagation). Overall, in control animals, albeit lower, the frequency of miniature (action potential independent) IPSCs (0.8±0.2 Hz, Fig 2b,d) did not appear to significantly differ from that of average sIPSC frequency. The frequency of mIPSCs from nerve injured animals was slightly higher (1.0±0.2 Hz), albeit not significantly from that of mIPSCs in control animals (see Fig 2b,c). Because the frequency of sIPSCs and mIPSCs was variable from cell to cell, to apply a more powerful test, we used each cell as its own control and calculated the ratio between sIPSCs frequency after (F_{mIPSCs}) *vs.* before (F_{sIPSCs}) addition of TTX for each cell. Figure 2f illustrates that application of TTX caused a three-fold reduction in the frequency of sIPSCs in control rats (ratio 37±9% of control sIPSCs frequency). In contrast the frequency of IPSCs from nerve injured animals was not affected by TTX (ratio 108±27% of control sIPSCs frequency), suggesting that in these animals, all sIPSCs were in fact miniature (action potential independent) IPSCs, i.e., they all arose from activity at inhibitory interneuron terminals and did not involve activity at the level of the soma or dendrites of the interneurons (see diagram in Fig 2e). Thus, these results indicated a loss of activity in inhibitory interneurons presynaptic to lamina I neurons.

Three fold increase in sEPSCs frequency in lamina I neurons following nerve injury.

Spontaneously occurring EPSCs in lamina I neurons had a mean frequency of 0.9±0.2 Hz. With nerve injured animals, the frequency of sEPSCs was increased by a factor of three (2.7±0.6 Hz, $p<0.05$; Fig 3a,c), while the frequency of mEPSCs was not significantly different between the two groups (0.6±0.3 Hz *vs.* 0.7±0.3 Hz, for control *vs.* rats with nerve injury, respectively; Fig 3e). The mean conductance of sEPSCs from

lamina I neurons of rats with nerve injury was also significantly decreased compared to that of control animals (535±52 pS *vs.* 397±33 pS, respectively; $p<0.05$; Fig 3b,d). However, the average conductance of mEPSCs was similar in both rat groups (451±11 pS *vs.* 434±72 pS, for animals with nerve injury *vs.* control rats, respectively; $p>0.8$; Fig 3f).

Recruitment of functional synaptic $GABA_A$Rs in lamina I neurons following nerve injury.

We have previously reported that mIPSCs in lamina I are mediated via activation of GlyRs only (Chéry, De Koninck, 1999). A $GABA_A$R component to mIPSCs in these cells can however be unmasked in the presence of a benzodiazepine indicating that glycine and GABA are released from the same synaptic vesicle at these synapses (Chéry, De Koninck, 1999). To test for a change in functional synaptic GlyRs and $GABA_A$Rs in rats with a nerve injury, we therefore examined the pharmacology of mIPSCs in these animals. In control rats all sIPSCs were abolished by 100 nM strychnine (Fig 4a) (Chéry, De Koninck, 1999). In contrast, however, mIPSCs recorded from lamina I neurons of nerve injured rats were not completely blocked by up to 1μM strychnine; the remaining events were abolished by further application of 10 μM bicuculline or 3 μM SR-95531 (see Fig 4b), indicating a change in functional synaptic $GABA_A$Rs in animals with experimental neuropathy.

Properties of mIPSCs in lamina I neurons of rats with peripheral nerve injury.

The decay kinetics of mIPSCs from control rats was 8.5±1.0 ms, similar to the decay of the isolated GlyR component of mIPSCs in cells from neuropathic animals (7.0±1.4 ms; $p>0.4$; fig 4e,h). While the decay of the isolated $GABA_A$R component in nerve injured animals was significantly slower than that of GlyR-mediated mIPSCs (59.6±14.5 ms; $p<0.001$), both $GABA_A$R and GlyR components of mIPSCs recorded from animals with nerve injury had similar rise times (2.6±0.5 ms *vs.* 2.3±0.5 ms,

respectively, $p>0.7$; Fig 4g). The rise times of these two components of mIPSCs were also comparable to that of GlyR mIPSCs in control condition (1.6±0.2 ms; $p>0.2$). Interestingly, the rise time of the $GABA_AR$ component of mIPSCs in experimental condition was faster than that of the $GABA_AR$ component that was unveiled by a benzodiazepine in normal animals (Chéry, De Koninck, 1999) (2.6±0.5 ms *vs.* 4.1±0.9 ms, respectively, $p<0.05$; Fig 4g).

Sorting mIPSCs on the basis of template fits (Jonas et al., 1998) (templates built from isolated GlyRs and $GABA_AR$ components) we determined the relative distribution of mIPSCs, in animals with nerve injury, that had only a GlyR-mediated component, only a $GABA_AR$-mediated component or that were mixed and determined that each of all three combinations was significantly represented (Fig 4f).

Finally, further analysis indicated a decrease in the conductance of the isolated GlyR component of mIPSCs in lamina I neurons from rats with experimental neuropathy compared to GlyR mIPSCs in control conditions (Figs 4c and 4d 356±82 pS; $p<0.05$). The mean frequency of GlyR mIPSCs was also slightly albeit not significantly, decreased in nerve injured animals (0.5±0.1 Hz; $p>0.1$; see Fig 5b).

DISCUSSION

The results presented in this study indicate an altered balance of excitatory and inhibitory inputs to lamina I neurons in rats with peripheral nerve injury. This is a likely substrate for the hyperexcitability of these cells in this pathological condition. Enhanced excitatory drive to lamina I neurons was not counterbalanced by an increase in inhibition. On the contrary, we identified a loss of activity at the level of the soma and dendrites of inhibitory interneurons. The enhanced excitatory drive was due to increased activity in neurons presynaptic to lamina I cells because the number and/or properties of excitatory synapses on these latter cells did not appear to change (*i.e.*, lack of change in frequency or

amplitude of miniature EPSCs). Thus, the decrease in activity in interneurons likely contributed to the enhanced excitatory drive.

Previous reports have shown that intrathecal administration of subconvulsive doses of $GABA_A$ and/or glycine receptor (GlyR) antagonists induces hypersensitivity characteristic of neuropathic pain syndromes such as allodynia (Yaksh, 1989; Sivilotti, Woolf, 1994; Sherman, Loomis, 1996; Sherman, Loomis, 1994; Sorkin et al., 1998). While these observations indicate that these inhibitory mechanisms are important for the proper interpretation of innocuous input at the spinal level, they do not necessarily imply that such disinhibition does occur in the chronic pain condition. The loss of activity in inhibitory interneurons reported here indicates that this is a likely substrate.

The mechanism responsible for this loss of activity in inhibitory interneurons is unknown. It may result from a reduction in the number of excitatory fibers contacting them (either from primary afferents or neurons intrinsic to the spinal cord). Consistent with this is the findings of a ~10 fold loss of large myelinated sensory fibers in this model of neuropathy (Mosconi, Kruger, 1996). This may however be counterbalanced by the reported sprouting of Aβ fibers in lamina II (Woolf et al., 1992; Nakatsuka et al., 1999). Changes in membrane properties and receptor expression in inhibitory interneurons or modulatory input to them (*e.g.*, peptidergic input) may also affect their level of intrinsic activity. The net result of these changes is likely an uncoupling of interneurons from excitatory influences. A similar mechanism as been proposed in experimental models of temporal lobe epilepsy (Sloviter, 1987; Sloviter, 1991; Bekenstein, Lothman, 1993), where the hyperexcitability is thought to result mainly from the fact that GABAergic interneurons become dormant rather than being lost. Thus, the uncoupling of inhibitory interneurons from excitatory influences may be a common feature underlying pathological hyperexcitability in the CNS.

The decrease in amplitude of the GlyR-mediated mIPSCs suggests a loss of postsynaptic GlyRs at synaptic junctions. This may result in deaf postsynaptic junctions

for glycine. Given our evidence that glycine and GABA are contained in the same synaptic vesicles at these synapses (Chéry, De Koninck, 1999), the occurrence of ~25% of pure $GABA_AR$-mediated mIPSCs would be consistent with deaf junctions for glycine. On the other hand, pure $GABA_AR$-mediated mIPSCs may be the result of new input from GABA-only interneurons. Indeed, while nearly all glycinergic cells in this area appear to contain GABA, only half of GABAergic cells also contain glycine (Todd, Sullivan, 1990; Mitchell et al., 1993). However, a loss of GABA-immunoreactivity has been reported in a similar model of nerve injury (Ibuki et al., 1997; Ibuki, Tanaka, 1998). In any case, the occurrence of ~40% of mixed GlyR/$GABA_AR$ mIPSCs clearly indicates the recruitment of functional $GABA_ARs$ at junctions that normally do not contain detectable $GABA_ARs$. In fact, the results thus suggest a switch from GlyR to $GABA_AR$ synapses. The reasons for this shift are unknown. It may be speculated however that, because $GABA_AR$-mediated IPSCs have much more prolonged decay kinetics, they may be better suited to counterbalance repetitive activity. This may thus correspond to a compensatory response from the lamina I neurons to counterbalance the increased excitatory drive.

The rise in frequency of sEPSCs may originate from increased activity in primary afferents or feedforward excitatory interneurons. This increased activity may result from the loss of activity in inhibitory interneurons. It may also result from loss of GlyRs on dorsal horn neurons and/or $GABA_ARs$ on primary afferent terminals. This would be consistent with reports of down-regulation of $GABA_AR$ subunit mRNA (Fukuoka et al., 1998) and GlyR immunostaining (Simpson, Huang, 1998) in the superficial dorsal horn in animals with chronic constriction injury of the sciatic nerve. Finally, it may reflect increased intrinsic activity (independent of disinhibition) in excitatory interneurons or primary afferents.

In conclusion, the results of this study indicate that the balance of excitatory and inhibitory inputs to lamina I neurons is shifted towards excitation, probably subsequent to a reduced inhibition in this and deeper layer. The fact that this area of the spinal cord is

normally devoted mainly to nociceptive input (and that it gives rise to one of the main spinal nociceptive output pathway to the brain) suggests that its disinhibition may be an important substrate for aberrant nociceptive responses to innocuous input (allodynia) that results from peripheral nerve injury. Indeed, previous studies have shown that sensitization of nociceptive specific neurons can result in unmasking of non-nociceptive input to these cells (Simone et al., 1989; Woolf et al., 1994; Craig, Kniffki, 1985). Lamina I is mainly comprised of nociceptive specific neurons and constitutes the major part of the nociceptive specific spinothalamic tract [because many of the deep dorsal horn neurons that project to the thalamus have wide dynamic range properties; (Willis, Coggeshall, 1978)]. The fact that intrathecal administration strychnine at the lumbar level causes a transformation of nociceptive specific neurons within the thalamus to wide dynamic range cells (Sherman et al., 1997b; Sherman et al., 1997a) thus strongly suggest that the substrate for the allodynia is indeed due to unmasked innocuous input to lamina I neurons as a result of disinhibition to these cells. Furthermore, the net increase in on-going excitatory drive to lamina I neurons that we observed in the current study may underlie the spontaneous pain (*i.e.*, in absence of a stimulus) that occurs in these pathological conditions (Bennett, Xie, 1988).

METHODS

Sciatic nerve injury. Adult male Sprague Dawley rats weighing 130-150g at the time of surgery were used in this study. The nerve injury was performed according to the procedure described by (Mosconi, Kruger, 1996). Briefly, the rats were anesthetized with Na^+-pentobarbital (30 mg/kg). In one group of animals, the left sciatic nerve was exposed at the level of the thigh and freed from the surrounding connective tissue. Polyethylene cuffs (2 mm-long, inner diameter 0.030") were applied to the nerve, the wound was closed and the animals were allowed to recover for a day before testing of nociceptive

reflexes. A control group of sham operated animals was treated the same way except that no cuff was applied to the nerve.

Behavioral tests. The behavior of the animals was tested according to the method of (Chaplan et al., 1994). Briefly, during the day and at room temperature, the rats were placed in a cage with a wire mesh bottom, which allowed full access to the paws. The animals were allowed to accommodate to that environment for at least 10 minutes before the beginning of the test. A von Frey hair was approached perpendicular to the plantar surface of the paw until it slightly buckled against the paw, then it was held for ~6-8 s; von Frey hairs of gradual stiffness were presented until a sharp paw withdrawal was noted. The stimulus was presented in a consecutive manner, whether ascending or descending stiffness. When the rats did not display a paw withdrawal response to the hair selected initially, the paw was touched with a stronger hair. If paw withdrawal occurs, the next weaker hair was used. The pattern of positive and negative responses was tabulated using the convention: X = withdrawal, 0 = no withdrawal. Then, the 50% paw withdrawal threshold was determined using the formula: 50% g threshold = $(10^{[X_f + k\delta]})/10{,}000$, where X_f is the value (in log units) of the final von Frey hair used; k is the tabular value for the pattern of positive/negative responses, and δ is the mean difference (in log units) between stimuli (Chaplan et al., 1994). Animals were considered to have developed allodynia only if they displayed a 50% threshold value of 2g or less.

Slicing procedure. Animals were sacrificed between 10-15 days post-surgery. This is the time period at which minimal threshold to produce a nociceptive withdrawal response was observed for rats with constricted nerve. Each animal used for slicing procedures were first tested on the day of slicing, to confirm whether they displayed allodynia (for rats with cuffs) or not (for sham operated animals). The rats were re-anesthetized with Na^+-pentobarbital (30 mg/kg), and spinal cord slices were obtained as

described previously (De Koninck, Chéry, 1998). Briefly, rats were perfused with and ice-cold sucrose-ACSF solution (in which 126 mM NaCl was replaced with 252 mM sucrose; pH 7.35; 340-350 mOsm), then they were rapidly decapitated. The spinal cord was removed by hydraulic extrusion, and the cervical and lumbar segments (2 cm-long) were isolated and glued, lateral side down, on a brass platform with cyanoacrylate cement, in a chamber filled with oxygenated ice-cold sucrose-ACSF. Parasagittal 400 μm-thick slices were cut, incubated in sucrose-ACSF at room temperature (23-28°C) for 30 minutes, and then transferred to normal ACSF for at least one hour before electrophysiological recordings. Next, the slices were transferred to a recording chamber at room temperature (23-28°C) 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 perfused at ~2 ml/min with oxygenated ACSF. Recordings were obtained from lamina I neurons in lumbar sections ipsilateral to the injured nerve. Recordings from spinal slices of sham operated rats were not different from those from cervical sections of rats with nerve constriction, thus they were pooled.

Drug application. Bicuculline methiodide (10 μM; RBI), SR-95531 (3 μM; RBI) and strychnine hydrochloride (100 nM - 1μM; RBI) were added to the ACSF from frozen, aliquoted stock solutions. For recording of miniature (action potential independent) IPSCs (mIPSCs), 1 μM tetrodotoxin (TTX; RBI) was added to the bathing solution. The glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 10 μM; Tocris Cookson), and D-2-amino-5-phosphonovaleric acid (D-AP5, 40 μM; Tocris Cookson) were used from stock solutions kept at 4°C.

Whole cell recording and data analysis. The slice were transferred to a recording chamber and continuously perfused at room temperature with artificial

cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 5 kynurenic acid, (pH 7.35; 300-310 mOsm). For whole-cell voltage-clamp recordings, patch pipettes were pulled from borosilicate glass capillaries (with an inner filament, WPI) using a two-stage vertical puller (Narishige PP-83). The pipettes were filled with an intracellular solution composed of (in mM): 110 Cs-gluconate, 5 CsCl, 10 HEPES, 2 $MgCl_2$, 2 mM ATP, 0.4 mM GTP, 11 mM BAPTA, 1 mM $CaCl_2$ and 0.5% Lucifer Yellow (all from Sigma) (pH: 7.2; osmolarity: 260-280 mOsm; pipette resistance: 3 MΩ). Recordings were obtained by lowering the patch electrode onto the surface of visually identified neurons in lamina I. IPSCs were recorded at 0 mV and EPSCs at –60 mV. An Axopatch 200B amplifier (Axon Instruments) with > 80% series resistance compensation was used for the recording. The access resistance was monitored throughout each experiment. Only recordings with stable access resistance between 7-20 MΩ, and which remained constant throughout the entire administration of antagonists, were used for further analysis of IPSCs and EPSCs . Traces were low-pass filtered at 10 kHz and stored on a videotape, using a digital data recorder (VR-10B, Instrutech Corp.). Off-line, the recordings were low-pass filtered at 2-3 kHz and sampled at 10-20 kHz, on an Intel Pentium-based computer and analyzed using locally designed software (Y. De K.).

Statistical analysis. *Student t* tests were used to analyze the differences between the different parameters of IPSCs and EPSCs from neuropathic vs. sham operated rats. The critical value for statistical significance was set at $p < 0.05$. All the data are expressed as mean ± SEM, unless otherwise indicated.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Recordings of excitatory and inhibitory postsynaptic events in lamina I. **a**, Example of spontaneous EPSCs (sEPSCs) recorded from a lamina I neuron of a control rat. The patch pipettes were filled with Cs-gluconate, and the holding potential was set at –60 mV. The sEPSCs were blocked after application of 10 μM CNQX to the bathing solution. **b**, The traces illustrate sIPSCs from a lamina I neuron of a control rat. The sIPSCs were recorded at a holding potential of 0 mV, and they were completely blocked following bath application of 100 nM strychnine. **c**, Rats with experimental nerve injury displayed increasingly lower threshold to mechanical stimuli necessary to evoke a withdrawal responses. While the 50% paw withdrawal threshold value remained unchanged with time for control animals (on average 14.5±0.4 g), for rats with sciatic nerve injury the 50% threshold was significantly lower as early as 3 days following the surgical implant (mean 6.0±0.7 g, $p<0.0001$), and reached a peak by two weeks post-surgery (mean 1.5±0.1 g, $p<0.0001$). n=11 (sham) and 39 (nerve injury). All recordings were performed at two weeks, where the threshold for withdrawal response was lowest.

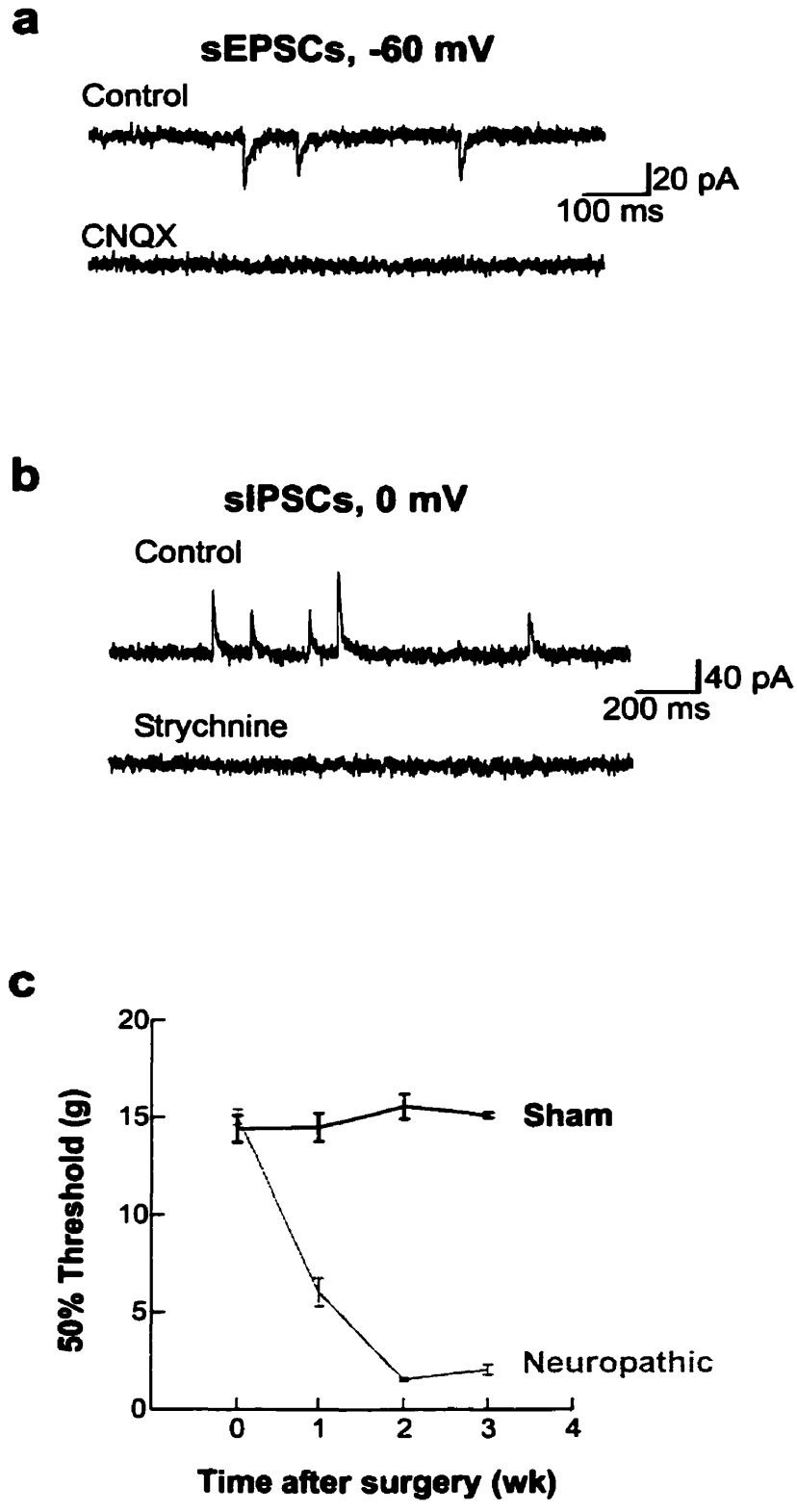

a
sEPSCs, -60 mV
Control
20 pA
100 ms
CNQX
b
sIPSCs, 0 mV
Control
40 pA
200 ms
Strychnine
c
50% Threshold (g)
20
15
10
5
0
Sham
Neuropathic
0
1
2
3
4
Time after surgery (wk)

Figure 2. Loss of interneuronal activity following nerve injury. sIPSCs (**a**) and mIPSCs (**c**) had a mean conductance in control animals similar to that in animals with nerve injury ($p>0.6$). **b**, The average frequency of sIPSCs and mIPSCs was also similar in both conditions (1.6±0.5 Hz vs. 1.0±0.1 Hz, respectively; $p>0.3$). **d**, The F_{mIPSCs} [average frequency after treatment with TTX] to F_{sIPSCs} [average frequency in control solution] ratio following the application of TTX was significantly decreased in lamina I neurons of control animals (mean 0.4±0.1). However, the frequency (ratio) of IPSCs in neurons from nerve injured rats was not affected by TTX (average F_{mIPSCs}/F_{sIPSCs} ratio 1.1±0.3). The effect of TTX was significantly different between the two experimental groups ($p<0.05$). This suggests that the mIPSCs recorded in nerve injured animals are due solely to activity at inhibitory interneurons terminals not activity in the soma or dendrite of the interneuron, as illustrated in **e**: The diagram illustrates the origin of the activity recorded under different conditions. In normal ACSF solution (*top*), the IPSCs recorded represent synaptic release due to activity at the level of the soma of inhibitory neurons as well as intrinsic to their terminals. The sIPSCs remaining after blockade of action potential propagation (*bottom*) reflected activity in the terminals only. Thus the ratio between the two (**f**) provide an estimate of somal activity.

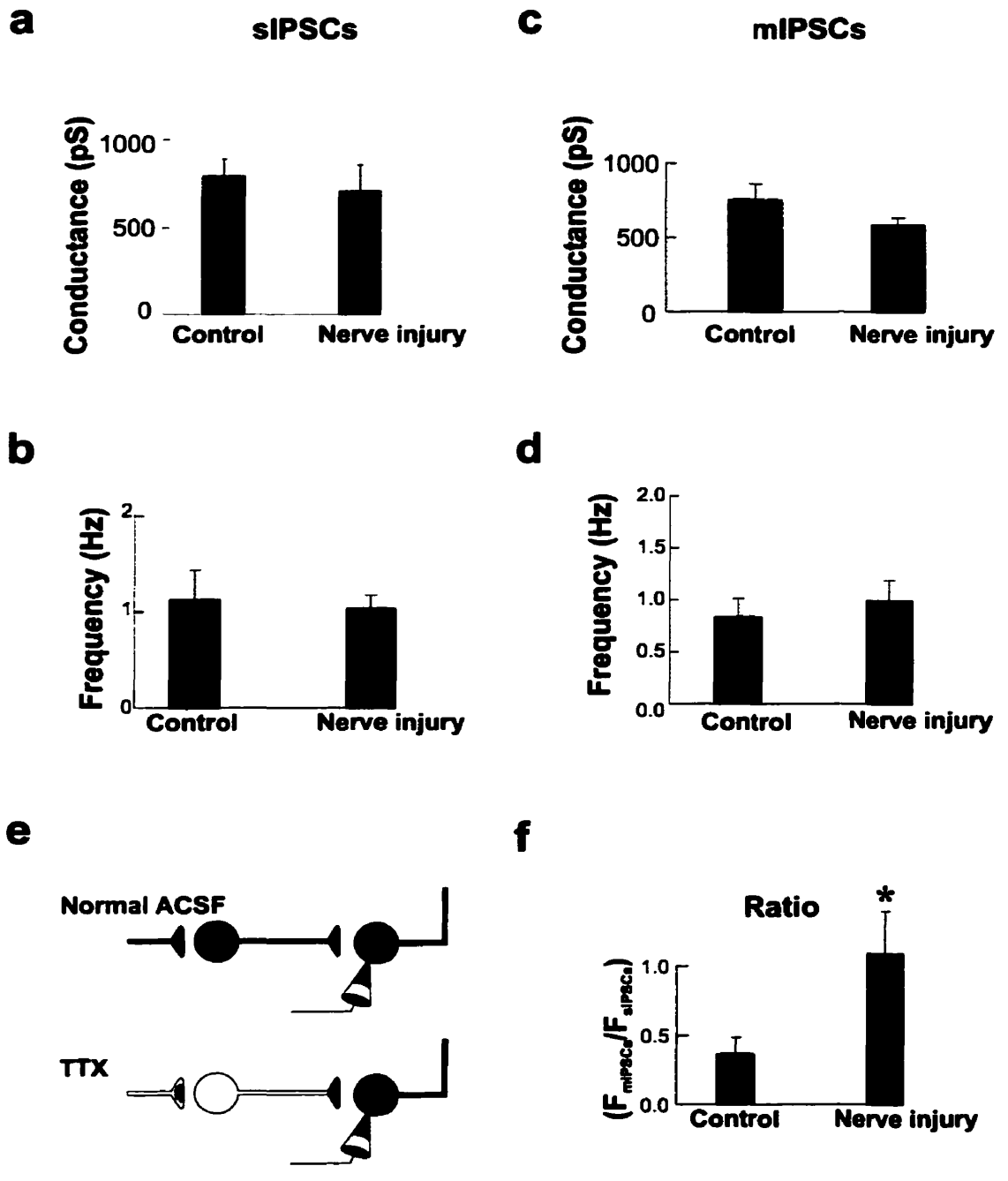

a
sIPSCs
Conductance (pS)
1000
500
0
Control
Nerve injury
c
mIPSCs
Conductance (pS)
1000
500
0
Control
Nerve injury
b
Frequency (Hz)
2
1
0
Control
Nerve injury
d
Frequency (Hz)
2.0
1.5
1.0
0.5
0.0
Control
Nerve injury
e
Normal ACSF
TTX
f
Ratio
*
1.0
0.5
0.0
Control
Nerve injury

Figure 3. Three fold increase in spontaneous but not miniature EPSCs following nerve injury. **a**, The average frequency of sEPSCs in neurons from animals with nerve injury was significantly higher than in control conditions (mean 2.7±0.6 Hz *vs.* 0.9±0.2 Hz, $p<0.05$). The inset diagram in **a** illustrates that recordings of sEPCS in normal ACSF solution represent on-going activity at the cell body and terminals of neurons. **c**, The histograms illustrate a comparison of the inter-event intervals in control and nerve injured animals; both distributions could be fitted by a monoexponential function. **b**, The mean conductance of sEPSCs from lamina I neurons of rats with nerve injury was significantly decreased ($p<0.05$). In **d**, the histograms illustrates the distribution of sEPSCs conductance in control (*top histogram*) and animals with nerve injury (*bottom histogram*). The inset is a cumulative distribution of the sEPSCs conductances in both rat groups. On the other hand, the average frequency of mEPSCs (**e**) and the mean conductance of mEPSCs (**f**) were similar between the two groups.

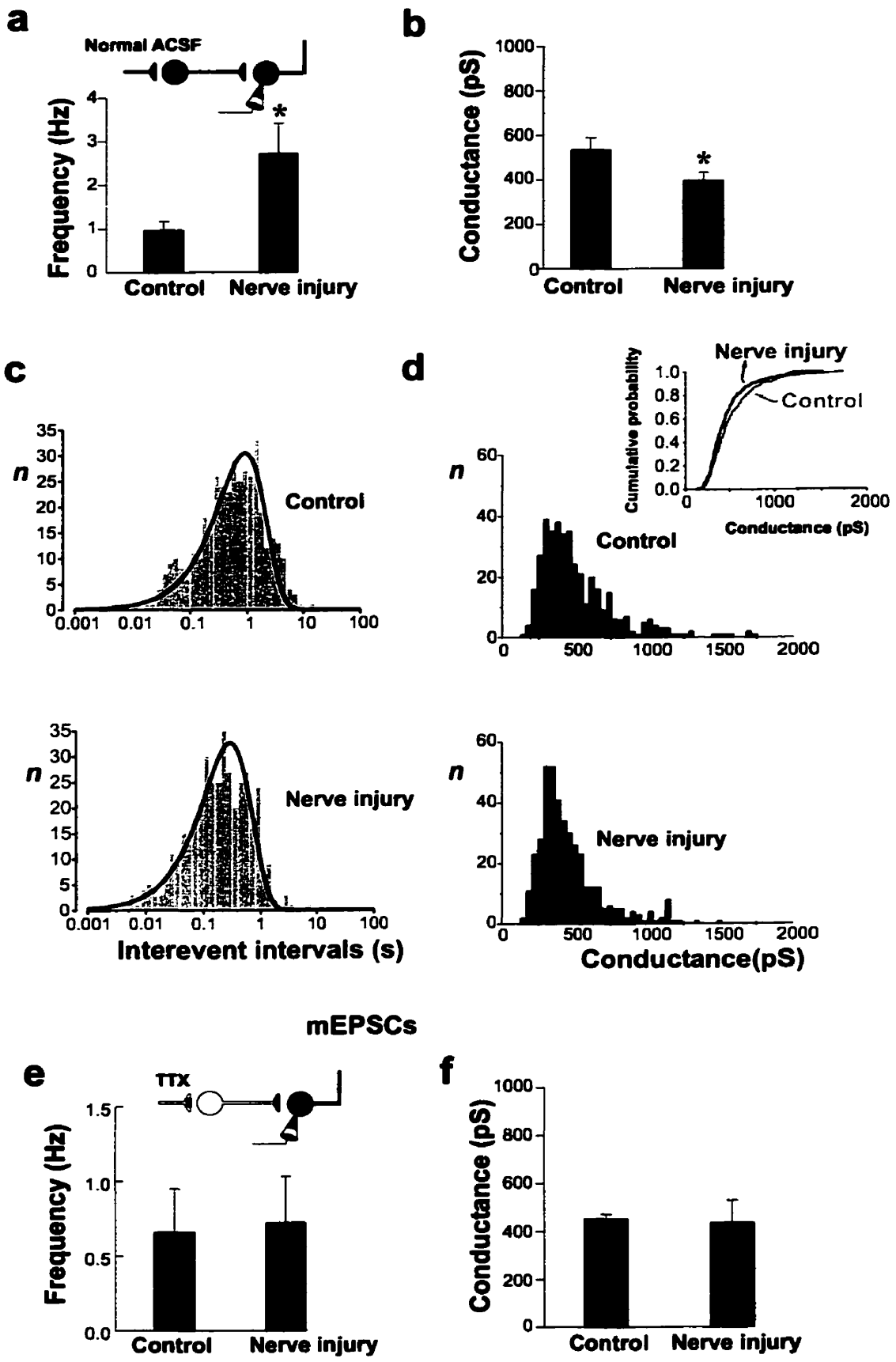

sEPSCs
a
Normal ACSF
Frequency (Hz)
Control
Nerve injury
b
Conductance (pS)
Control
Nerve injury
c
n
Control
Nerve injury
Interevent intervals (s)
d
Nerve injury
Control
Cumulative probability
Conductance (pS)
n
Control
Nerve injury
Conductance(pS)
mEPSCs
e
TTX
Frequency (Hz)
Control
Nerve injury
f
Conductance (pS)
Control
Nerve injury

Figure 4. Recruitment of a $GABA_AR$ component to mIPSCs following nerve injury. Strychnine (100 nM) completely blocked mIPSCs in neurons from control slices (**a**), but only partially affected mIPSCs from lamina I neurons of rats with nerve injury (**b**). The remaining mIPSCs were abolished upon further application of bicuculline (10 μM), indicating a recruitment of $GABA_AR$-mediated component to the mIPSCs in nerve injured animals. **c**, Superimposed (4-5) traces of pure GlyR-mediated mIPSCs in a control lamina I cell, illustrating their fast kinetics. **d**, The GlyR-mediated mIPSCs in nerve injured animals (*top*) had a smaller amplitude; the mIPSCs in control condition and the GlyR mIPSCs component in nerve injured animals had comparable decay kinetics (**e**, *top*; **h**). The mixed GlyR/$GABA_AR$ mIPSCs (***middle trace in* d**) had mean amplitudes comparable to the control condition with a slower decay due to the additional $GABA_AR$ component. The additional $GABA_AR$ mIPSCs (***bottom trace in* d**) display slower decay time constants than GlyR mIPSCs, but comparable rising kinetics (**e**, *top*; **g**). This additional $GABA_AR$ component of mIPSCs had a significantly faster rising phase than $GABA_AR$ mIPSCs unveiled in control animals by addition of flunitrazepam (**e**, *bottom*; **g**) (Chéry, De Koninck, 1999). **f**, This graph represents the distribution of pure GlyR (36.3±9.9%), mixed GlyR/$GABA_AR$ (40.0±7.5%) and pure $GABA_AR$ mIPSCs (24.1±4.3%).

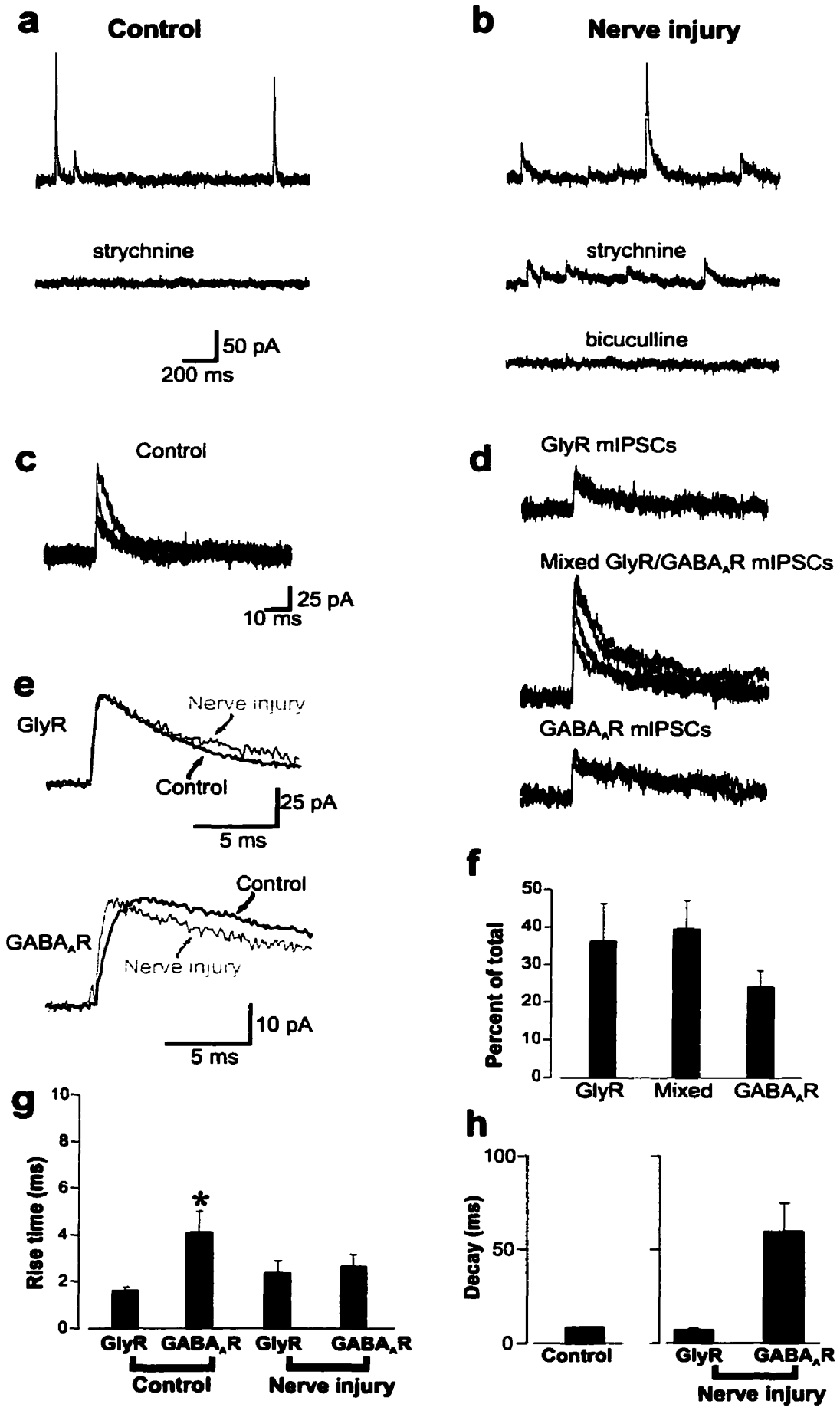
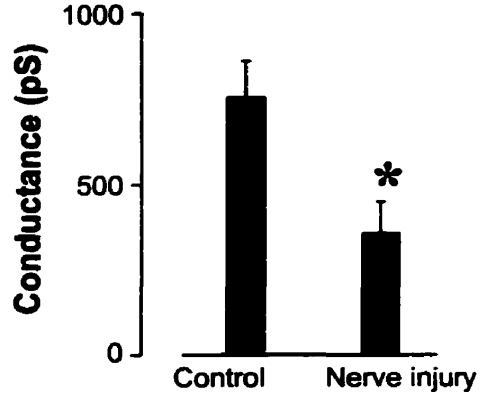

a
Control
strychnine
50 pA
200 ms
b
Nerve injury
strychnine
bicuculline
c
Control
25 pA
10 ms
d
GlyR mIPSCs
Mixed GlyR/GABA$_A$R mIPSCs
GABA$_A$R mIPSCs
e
GlyR
Nerve injury
Control
25 pA
5 ms
Control
GABA$_A$R
Nerve injury
10 pA
5 ms
f
Percent of total
50
40
30
20
10
0
GlyR
Mixed
GABA$_A$R
g
Rise time (ms)
10
8
6
4
2
0
*
GlyR
GABA$_A$R
GlyR
GABA$_A$R
Control
Nerve injury
h
Decay (ms)
100
50
0
Control
GlyR
GABA$_A$R
Nerve injury

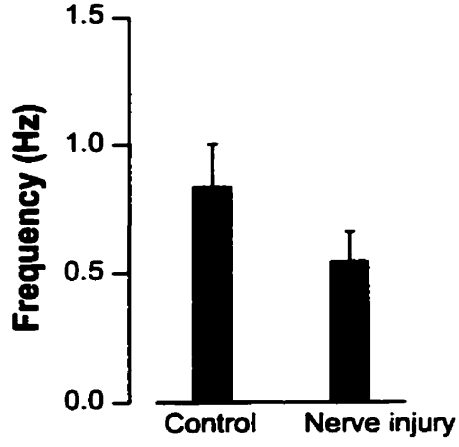
Figure 5. Decrease in amplitude of GlyR-mediated mIPSCs in lamina I neurons. **a**, The average conductance of GlyR mIPSCs was significantly reduced in nerve injured animals (356±82 pS) compared to that in control animals (753±81 pS; $p<0.05$). **b**, The frequency of pure GlyR mIPSCs in nerve injured animals was also reduced, however not significantly.

GlyR mIPSCs

a



b



CHAPTER 6

DISCUSSION

The studies presented in this thesis addressed inhibitory transmission mediated by GABA and glycine in the marginal zone of the spinal cord. In this chapter, I will comment on some important findings and on additional issues they might raise. I will also discuss approaches that could be taken to address some of these issues.

6.1 Distinct inhibitory role of GABA and glycine in lamina I

The issue of the respective role of GABA and glycine in lamina I is one of the most important questions that prompted these studies. Because they both exert an inhibitory control of neuronal activity in the adult CNS (Sivilotti, Nistri, 1991; Betz et al., 1994), their extensive co-localization in the superficial dorsal horn (Todd, Sullivan, 1990), suggested that GABA and glycine may have a synergistic inhibitory function in the spinal cord (Bohlhalter et al., 1994; Todd et al., 1996b). The results of my studies provided functional evidence that GABA and glycine are contained in the same synaptic vesicles and therefore are co-released at lamina I inhibitory synapses (see chapters 3 and 4).

While GABA and glycine may act together at the same synaptic junctions in lamina I following their co-release, they also appear to have separate inhibitory roles in this area. I have shown that glycine mediates miniature inhibitory transmission, acting on GlyRs clustered at synaptic junctions, while $GABA_A$Rs are likely located at extrasynaptic sites on lamina I postsynaptic membranes (Figure 1c). Thus, at least to a certain extent, the subcellular postsynaptic localization may determine the nature of inhibition mediated by GABA and glycine, respectively, in lamina I neurons. Indeed, our interpretation of an extrasynaptic localization of $GABA_A$Rs is supported by several observations (presented in chapter 3). One important argument was the slow rising kinetics of the $GABA_A$R mIPSCs recorded in lamina I (~10 times slower than the rising kinetics of GlyR mIPSCs in this spinal area). One could still argue, however, that the $GABA_A$R subunit combination expressed in lamina I may have distinct on-rate kinetic properties, compared

to other $GABA_AR$s in the CNS. Indeed, the subunit composition of $GABA_AR$s is responsible in large part for the gating of the channels (Draetta et al., 1988). Thus, one possible interpretations was that the $GABA_AR$s in lamina I had kinetic properties particularly distinct from those of deeper laminae. In fact, studies of recombinantly expressed $GABA_AR$s with different subunit combinations [comprising the $\alpha 2$, $\beta 2/\beta 3$ or $\gamma 2$ subunits, which appear to be abundant in the superficial spinal layers (Bohlhalter et al., 1996; Todd et al., 1996b; Alvarez et al., 1996)] show that the different combinations confer distinct pharmacological and kinetic properties to the receptor (Verdoorn et al., 1990; Angelotti, Macdonald, 1993; Ducic et al., 1993; Puia et al., 1994; Chen et al., 1989; Lavoie et al., 1997). However, none of those various combinations of subunits produced $GABA_AR$s with on-rate kinetics comparable to that of mIPSCs in our study (chapter 3). Indeed, on the one hand, at the concentrations of GABA thought to be released into the synaptic cleft, the rise time of endogenous $GABA_AR$ activation should be fast [in the sub-millisecond range; (Maconochie et al., 1994)], and comparable to that of GlyR mIPSCs in lamina I or $GABA_AR$ mIPSCs in lamina II, in our study. On the other hand, even if the concentration of GABA was low, the short time course of transmitters in the cleft is not expected to generate longer decay times (Maconochie et al., 1994; Miller, 1988; Galarreta, Hestrin, 1997). Thus, we favored the hypothesis that $GABA_AR$s are located at extrasynaptic sites on lamina I postsynaptic membranes.

These results can be interpreted as having several consequences. One possible interpretation is that extrasynaptic $GABA_AR$s play mainly a role in tonic inhibition, which may involve receptor activation by ambient (background) level of transmitter, as described by some investigators (Kaneda et al., 1995; Brickley et al., 1996; Rossi, Hamann, 1998; Beushausen et al., 1988). However, our data argue against this possibility. Indeed, the presence of ambient levels of transmitter can usually be measured in the form of a background noise in the recording signal (*e.g.* in between synaptic events). A change in the characteristics of this noise following the application of antagonist, is usually

interpreted as an indication of the presence of background levels of transmitter (LoTurco et al., 1990; Rossi, Hamann, 1998). In our study (chapter 3) we did not observe any change in the baseline noise level, following the application of bicuculline during the recordings, suggesting that ambient levels of GABA are unlikely to account for tonic inhibition in lamina I of the spinal cord. Tonic inhibition, on the other hand, can also be described as that which results from basal levels of synaptic activity; for example, on-going bombardment by spontaneous miniature IPSCs (Otis et al., 1991; Otis, Mody, 1992a; Mody et al., 1994; Soltesz et al., 1995). Thus, from my results it can be inferred that glycine (GlyRs) appears to mediate tonic inhibition in lamina I, whereas the contribution of GABA ($GABA_ARs$) may be more important during intense synaptic activity.

These respective properties of GlyRs and $GABA_ARs$ may have distinct implications for the processing of sensory information in lamina I, under normal and pathological conditions, as discussed below (also see figure 1).

The rapid kinetics displayed by GlyR currents suggest that they may be important in limiting the firing of neurons during low-frequency input in lamina I. Fast rising IPSCs may curtail the amplitude of incoming EPSCs more effectively, thereby decreasing the probability of action potential generation (Kandel et al., 1991).

On the other hand, $GABA_AR$-mediated IPSCs with their slower decay, may be more effective in preventing long lasting repetitive firing of the neurons. Our observation that $GABA_AR$ activation was not detected upon minimal-intensity stimuli, but only following large-intensity stimuli (chapters 3 and 4), which results in the recruitment of a larger number of inhibitory fibers, supports the hypothesis that an important role for GABA could be to control repetitive neuronal firing, during intense synaptic activity in lamina I.

Repetitive firing of neurons is likely to occur following peripheral nerve injury (Kajander, Bennett, 1992; Laird, Bennett, 1993; Bennett, 1994; Goslin et al., 1988), a

condition mimicked by the experimental neuropathy model (Mosconi, Kruger, 1996) that we used (chapter 5). Dorsal horn neurons have been shown to display hyperexcitability in animal models of nerve injury (Laird, Bennett, 1993) and such a hyperexcitability may be linked to the significant increase in the frequency of excitatory events (EPSCs) found in lamina I neurons from nerve injured rats (see chapter 5, and also section **6.3** below). The additional $GABA_AR$ component of the IPSCs that was revealed from the recordings in lamina I neurons of nerve injured animals (chapter 5) would be consistent with a role for $GABA_AR$-mediated events in controlling repetitive neuronal firing (this would imply that the participation of $GABA_ARs$ to spontaneous IPSCs in lamina I represents a compensatory mechanisms from these cells to counterbalance the increased frequency of excitatory input).

Thus, both GlyRs and $GABA_ARs$ may have important functions in controlling lamina I excitability during low and/or intense synaptic activity in this area. This is consistent with reports that impairment of spinal GlyR and/or $GABA_AR$ function produce symptoms similar to those observed in experimental neuropathy (see below).

The observations that intrathecal strychnine induces hypersensitivity to both innocuous and noxious stimuli [allodynia, (Yaksh, 1989; Yamamoto, Yaksh, 1993; Sivilotti, Woolf, 1994; Sherman, Loomis, 1995; Sorkin, Puig, 1996; Sherman, Loomis, 1996; Sherman et al., 1997a)] suggest that glycine plays a critical role in the control of sensory input in the dorsal horn. This is also supported by the evidence that nociceptive-specific neurons in the thalamus [a critical CNS area for the central representation of pain and temperature (Willis, Coggeshall, 1991; Light, 1992; Craig et al., 1994; Lipski et al., 1988)] could become activated by innocuous stimulus, following intrathecal treatment of rats with strychnine (Sherman et al., 1997a). Thus, impairment of GlyR function in the spinal cord may unmask innocuous input to (previously) nociceptive-specific dorsal horn neurons (many of which are present in lamina I (Willis, Coggeshall, 1991)), to cause such input to be interpreted as painful. Such impairment of GlyR function may therefore

underlie the hypersensitivity to light tactile stimuli (for example light brushing of the skin) displayed by patients with neuropathic pain [for review, see (Bennett, 1994; Woolf, Mannion, 1999)]. This also suggests an important role for glycine in the control of nociception at the spinal level.

Like GlyRs, $GABA_A$Rs may also play an important role in lamina I processing of sensory inputs. The demonstrations that intrathecal administration of bicuculline (like strychnine) causes an enhanced response to both non-noxious and noxious stimuli (Yamamoto, Yaksh, 1993; Sivilotti, Woolf, 1994; Cucchiaro et al., 1988) indicates that a reduction in inhibitory function normally mediated by GABA in the dorsal horn could have important consequences on the processing of non-nociceptive and nociceptive input, and may explain the abnormal sensations (such as allodynia, or hyperalgesia) induced by nerve injury, as observed, for example, in clinical practice (Goslin et al., 1988). The evidence that spinal cord stimulation-induced GABA release reverses allodynia following sciatic nerve lesions (Stiller et al., 1996) also indicates a role for GABA in controlling the excitability of dorsal horn neurons (although this may be less specific, because it may simply reflect a generalized decrease in neuronal excitability).

In summary, my results indicate that both GABA and glycine play a role in inhibitory control of neuronal function in lamina I. While they appear to be co-released and act together at the same synaptic junctions in this area of the spinal cord, they are not equivalent and appear to mediate distinct forms of inhibition. Thus, they may modulate different aspects of nociceptive and non-nociceptive processing. For example, it appears that glycinergic and GABAergic inhibitory events counteract distinct types of excitatory responses following specific glutamate receptor activation. Indeed, it was shown that strychnine-induced allodynia can be inhibited by non-NMDA and NMDA receptor antagonists (Yaksh, 1989; Onaka et al., 1996), whereas bicuculline-induced allodynia was inhibited only by a kainate receptor antagonist and metabotropic receptor antagonists

(Onaka et al., 1996). This suggest that GABA and glycine may have a selective control of excitatory transmission in lamina I.

6.2 Spillover of GABA at lamina I inhibitory synapses

We have suggested that in lamina I, $GABA_A$Rs were probably activated only by stimulus conditions that allow enough GABA accumulation to spillover the synapses (see section **6.1** and chapters 3 and 4). Transmitter spillover has been suggested to occur at other CNS synapses and may have important implications for the mechanisms of synaptic plasticity (Isaacson et al., 1993; Kullmann et al., 1996; Barbour, Hausser, 1997; Asztely et al., 1997). For example, it has been suggested that spillover of GABA could make a crucial contribution to the regulation of cerebellar granule cell excitability, by increasing the number of Golgi neurons that may exert a feedback inhibitory control of granule cells (Rossi, Hamann, 1998) This corresponds to the concept of volume transmission, in which release from one nerve terminal affects several targets (Zoli et al., 1999). What may therefore be the role of GABA spillover in lamina I? Under conditions where $GABA_A$Rs activation is detectable in lamina I (*i.e.* following enough GABA release to possibly create a "build-up" of GABA), GABA may be able to diffuse far enough from its release site to also activate $GABA_A$Rs and $GABA_B$ receptors present on neighboring excitatory profiles in the dorsal horn (Todd et al., 1996b; Price et al., 1987). Thus, spillover of GABA may be a means to provide an inhibitory signal that may last longer than the normally faster IPSCs in other synapses of the CNS (Sivilotti, Nistri, 1991; Otis, Mody, 1992b; Isaacson et al., 1993; Rossi, Hamann, 1998). In this way, spillover of GABA in lamina I may have important physiological implications, particularly under circumstances of dorsal horn hyperexcitability, which may involve long lasting repetitive neuronal firing (Bennett, 1994; Goslin et al., 1988).

6.3 Plastic changes in GABAergic and glycinergic transmission in lamina I in experimental neuropathy

The results presented in chapter 5 indicate that the hypersensitivity to touch (allodynia) caused by peripheral nerve injury (Mosconi, Kruger, 1996) may be the consequence of a possible disinhibition of lamina I neurons (Woolf, Mannion, 1999) and/or other excitatory fibers in the dorsal horn (*e.g.* from the periphery or other dorsal horn neurons; see Figure 1). Our results suggest that such disinhibition involves a reduced activity of inhibitory interneurons that normally control lamina I cells (see Figure 1).

Several studies indicate that alterations in the number and activity of dorsal horn inhibitory interneurons may occur following injury to a peripheral nerve. This pathological condition appears to cause a reduction in the number of GABAergic dorsal horn neurons (Castro-Lopes et al., 1993; Dumoulin et al., 1996; Ibuki et al., 1997) and possibly of glycinergic neurons, based on studies of the effect of strychnine on the occurrence of "dark" neurons in the dorsal horn, interpreted as indicating a transynaptic degeneration (loss) of inhibitory neurons (Sugimoto et al., 1990; Nachemson, Bennett, 1993). The number of "dark" neurons is increased following administration of strychnine, and these changes in GABAergic and glycinergic interneurons appear to occur primarily in the superficial layers of the dorsal horn (Sugimoto et al., 1990; Castro-Lopes et al., 1993; Dumoulin et al., 1996; Ibuki et al., 1997). What may cause a decrease in inhibitory interneuron function? A reduction or loss of inhibitory activity in the dorsal horn has been proposed to be due to an enhanced activation of NMDA and non-NMDA receptors by endogenous excitatory transmitter. Indeed, there is evidence that pretreatment with NMDA receptor antagonists can prevent dorsal horn neurons hyperexcitability and allodynia (Woolf, Chong, 1993; Satoh, Omote, 1996). On the other hand, a down-regulation (and possibly loss) of NMDA receptors on inhibitory interneurons appears to also occur following nerve injury, as suggested by a study (Coggeshall, Carlton, 1997), and this could underlie a possible decrease in excitatory drive onto inhibitory interneurons

in the dorsal horn, under neuropathic conditions, as suggested by the results presented in chapter 5. In this case, the synaptic connection of excitatory fibers with inhibitory interneurons (see figure 1a, b) could be inactive, thus resulting in an uncoupling of inhibitory interneurons from excitatory input (Sloviter, 1991). Therefore, they could be dormant, *i.e.* still present, but lacking appropriate activation (Sloviter, 1991), as it has been proposed for a population of hippocampal inhibitory interneurons, in certain animal models of epilepsy (Sloviter, 1991). This uncoupling of inhibitory interneurons from excitatory influences could be a common mechanism of altered inhibition under pathological conditions in the CNS.

The possible disinhibition of lamina I neurons following nerve injury, may simply result from a decreased intrinsic activity of inhibitory interneurons alone. This reduction in inhibitory interneuron activity could be due, for example, to a change in the intrinsic conductances in these cells (North, 1986), for a change in membrane conductance may affect the excitability of neurons (Frank, 1985).

In fact, the long-term recovery of GABAergic profiles in the dorsal horn, following their significant reduction by nerve injury (Ibuki et al., 1997) indicates that the inhibitory interneurons might not degenerate, but rather alter their activity.

In this study (chapter 5), the animals with sciatic nerve injury displayed hypersensitivity to innocuous mechanical stimuli. Since the type of primary afferents that normally terminate in lamina I are the Aδ and C fibers (Light et al., 1979; Brown, 1981a; Gobel et al., 1981; Woolf, Fitzgerald, 1983; Willis, Coggeshall, 1991), the first possible explanation is that Aδ and C fiber nociceptors now respond to innocuous input. However, it was shown that neonatal treatment of primary afferents with capsaicin (which destroys Aδ and C fibers) did not affect strychnine-induced allodynia (Sherman, Loomis, 1996). Nevertheless, an involvement of Aδ and C fibers in the hypersensitive behavior to innocuous tactile stimuli displayed by nerve injured animals cannot be excluded, as it appears that nociceptive-specific neurons in the superficial dorsal horn (that normally

respond to high-threshold (Aδ/C fiber) input), may begin to respond to low-threshold stimulation following the sensitization of peripheral Aδ/C fiber chemoreceptors (Woolf et al., 1994).

Another possibility is that Aβ fibers, which are thought to normally carry innocuous sensory information (Willis, Coggeshall, 1991), may be involved in this aberrant sensitivity displayed by rats with nerve injury. However, low-threshold myelinated Aβ fibers do not appear to normally project directly to lamina I, but rather to laminae III-VI (Brown, 1981a; Woolf, Fitzgerald, 1983; Woolf, 1987; Willis, Coggeshall, 1991; Light, 1992; Woolf, Doubell, 1994). Yet, reports of activation of lamina I neurons in the normal situation, following stimuli of Aβ afferents, exist (Light et al., 1979; Woolf, Fitzgerald, 1983), and the mechanisms for such activation was assumed to be via polysynaptic pathways. Nevertheless, some direct (monosynaptic) Aβ fiber input could still be possible, considering that some lamina I neurons have dendrites reaching deeper laminae (Lima, Coimbra, 1986), although the above-cited electrophysiological studies showed no evidence of Aβ monosynaptic input to lamina I.

It appears that nerve injury induces a reduction in the inhibitory effect of Aβ stimulation on the activity of these primary afferent fibers and dorsal horn neurons (Woolf, Wall, 1982) (Wall, Devor, 1981; Laird, Bennett, 1992). This suggests that, under normal circumstances, Aβ may stimulate inhibitory interneurons in deep dorsal horn layers which in turn would inhibit the activity of lamina I neurons. A removal of Aβ fiber input to inhibitory interneurons and a branching of these fibers in the superficial laminae, following nerve injury, represents a likely mechanism for the suggested disinhibition of lamina I neurons. This hypothesis is supported by the observations of a significant reduction in the number of Aβ fibers in animals with experimental nerve injury (Mosconi, Kruger, 1996; Coggeshall et al., 1997), and by the apparent a sprouting of some Aβ fibers into the superficial laminae (Woolf et al., 1995). Thus, inhibitory interneurons in deeper laminae that are normally activated by Aβ fiber input may lose some of this excitatory

drive. In turn, lamina I neurons may begin to receive direct input from Aβ fibers following peripheral nerve injury. This rearrangement of connections may account for the generation of exaggerated response to innocuous stimuli (Woolf et al., 1992), but is not necessarily an exclusive substrate of the nerve injury-induced hypersensitivity, since direct Aβ input to lamina I has not been demonstrated (Woolf et al., 1992).

Thus, different mechanisms may underlie a decreased on-going activity in inhibitory interneurons that normally control lamina I cells. In turn, a reduction in GABA and/or glycine function in lamina I is likely to cause a disinhibition of lamina I neurons and a facilitation of excitatory input to the marginal zone (see Figure 1b). The increase in the frequency of EPSCs found in this study (see chapter 5), the significant decrease in GlyR conductance in lamina I neurons (see also figure 1d), and the increase in spontaneous and evoked discharges from injured nerves and from dorsal horn neurons after nerve injury (Kajander, Bennett, 1992; Laird, Bennett, 1993; Bennett, 1994; Goslin et al., 1988) suggest a possible facilitation of excitatory input to lamina I. All of these findings suggest a possible substrate of the abnormal sensitivity to innocuous mechanical input displayed by the animals in this study (chapter 5) and other studies (Bennett, 1994), and accordingly, of the abnormal pain sensation to light tactile stimuli displayed by patients with nerve injury (Bennett, 1994; Goslin et al., 1988).

6.3.1 Changes in the GlyR component of mIPSCs

The reduced amplitude of GlyR-mediated mIPSCs may involve a negative modulation or a decreased (or repressed) expression of GlyRs in lamina I neurons, in this experimental condition, consistent with a previous immunohistochemical report (Dubuc et al., 1988). A reduction of GlyR currents may be explained by several factors. Glycine receptors can be modulated by various kinases (Uhl et al., 1988), including PKA and PKC. PKC, for example, was shown to play an important role in the hyperexcitability of dorsal horn neurons following nerve injury (Coderre et al., 1993; Malmberg et al., 1997).

Although the effect of phosphorylation of GlyRs by PKA and PKC appears to depend on the subunit composition of GlyRs (Vaello et al., 1994; Xu et al., 1996), it is possible that the enhanced activity of these kinases produces a negative modulation of GlyRs after nerve injury, one that would result in either a decreased expression of GlyRs, or a decrease in the affinity of the receptors for glycine. This hypothesis remains to be tested. These enhanced kinase activity appear to be often due to an increase in the intracellular calcium concentration (Coderre et al., 1993). Since calcium is able to activate transcription factors and indirectly induce or repress the expression of genes (Kandel et al., 1991), it may have a role in the modulation of GlyRs during nerve injury. This possibility has not been explored.

6.3.2 A switch to $GABA_A$Rs mediated inhibition in experimental neuropathy

The other important plastic alteration found in lamina I neurons of rats with nerve injury was the participation of $GABA_A$Rs to mIPSCs.

The additional $GABA_A$R component revealed may arise from different mechanisms. One possibility is a recruitment of $GABA_A$Rs at synapses, and this may involve phosphorylation systems (Wan et al., 1997). The clustering of, either existing extrasynaptic $GABA_A$Rs, and/or the translocation of additional $GABA_A$Rs from the intracellular compartment may have occurred. Immunocytochemistry with specific antibodies to $GABA_A$R subunits expressed in lamina I may help resolve this issue. For instance, quantitative electron-microscopic analysis of labeled $GABA_A$Rs in lamina I could be performed, using antibodies against specific subtypes expressed in the marginal layer such as $\alpha 2$, $\alpha 3$ and/or $\beta 2$, $\beta 3$ (Bohlhalter et al., 1996). This would allow to compare the distribution and density of $GABA_A$Rs receptors in neuropathic conditions to the normal situation. A translocation and clustering of the receptors could have significant effects on sensory processing in lamina I. For example, additional receptors may be an effective way to potentiate inhibitory currents (Wan et al., 1997), thereby enhancing the

inhibitory control of sensory input to lamina I. Alternatively, the addition of $GABA_ARs$ could have a non-inhibitory role. For example, GABA is thought to play a neurotrophic role in the developing CNS by acting on $GABA_ARs$ (Sivilotti, Nistri, 1991). Thus $GABA_ARs$ may be involved in the modulation of trophic processes in lamina I, and perhaps in the reorganization of input from the central projection of afferent fibers in the superficial dorsal horn (Dumoulin et al., 1996).

The rising kinetics of the additional $GABA_A$ component can lead to distinct interpretations. One possibility is that they may be recruited closer to lamina I synaptic junctions (see Figure 1d). Indeed the rise time of the $GABA_AR$ component of mIPSCs revealed in experimental neuropathy was significantly faster (chapter 5) than that of $GABA_AR$ mIPSCs unmasked by a benzodiazepine in control conditions (see chapter 3). And we also observed that the average rise time of this $GABA_AR$ component was not significantly slower than the rise time of GlyR mIPSCs in control and neuropathy conditions. It is possible that a different subcellular location of the receptors leads to a different function. Another explanation for the more rapid rising kinetics of the $GABA_AR$ mIPSCs following nerve injury, is a possible change in $GABA_AR$-subunit combination or expression. A different subunit composition of $GABA_ARs$ may have important consequences on the affinity of the receptors for GABA (Draetta et al., 1988). An increase in the concentration of GABA in the vesicles in also a likely explanation, however, this would be hard to reconcile with the reported significant decrease in GABAergic profiles in the superficial dorsal horn in animals with nerve injury (Ibuki et al., 1997). Nevertheless, the remaining GABAergic neurons may begin to synthesize more GABA, under pathological conditions (Castro-Lopes et al., 1992).

With the slow decay of the $GABA_AR$ mIPSCs, inhibition of long-lasting repetitive firing in lamina I might be better achieved than with the GlyRs which display fast kinetics (see section **6.1**). And long-lasting repetitive firing is likely to occur following nerve injury (Kajander, Bennett, 1992; Laird, Bennett, 1993; Bennett, 1994; Goslin et al.,

1988). Thus, an additional $GABA_AR$ inhibition may represent a compensatory response from lamina I neurons to the heightened excitatory input.

6.4 Possible therapeutic implication and strategy

Although there was a clear correlation between the abnormal sensitivity to innocuous input of the nerve injured animals and the increase in the frequency of excitatory events to lamina I (which may result from a disinhibition of these cells), this does not necessarily imply that these cellular changes underlie the allodynia observed. Nevertheless, since this specific symptom can be replicated following impairment of spinal GlyR and $GABA_AR$ function (Yamamoto, Yaksh, 1993; Sivilotti, Woolf, 1994; Cucchiaro et al., 1988), and since our study suggested a decrease in GlyR-mediated inhibition, specific agents aiming at restoring or enhancing these inhibitory receptors function may constitute potential therapeutic strategies. Agents that can selectively modulate GlyR function, however, are unfortunately lacking. Alternatively, modulation of $GABA_ARs$ may prove useful in the control of dorsal horn neurons, under neuropathy conditions. For example, agents that enhance $GABA_AR$ currents, such as benzodiazepines appear to exert analgesic actions, although their antinociceptive properties are difficult to assess behaviorally, because of their superimposed sedative and myorelaxant effects when administered via the systemic circulation (García-Moncó, Berciano, 1988; Schreier, 1989). The diversity of $GABA_AR$ subunits in the dorsal horn (Bohlhalter et al., 1996) may provide an important tool for the targeting of specific subpopulations of $GABA_ARs$.

6.5 Summary and Conclusion

The main objective of this thesis was to gain an understanding of the respective inhibitory roles of GABA and glycine in the marginal zone (lamina I) of the rat spinal cord. To address specific questions regarding synaptic events mediated by these inhibitory transmitters, I have developed a spinal cord slice preparation in which synaptic

contacts are kept intact and that allowed to delineate the marginal layer in live spinal cord tissue for electrophysiological recordings. Several findings have emerged from this study. I have found that lamina I neurons are exclusively bombarded by GlyR-mediated mIPSCs, although I have also shown that GABA and glycine are contained in the same synaptic vesicles and co-released at lamina I synapses, indicating that glycine is responsible for tonic inhibition of neurons in this spinal layer. I have demonstrated that $GABA_A$Rs present on the postsynaptic membrane of lamina I cells are likely located extrasynaptically, that their activation is detectable under conditions that allow GABA spillover from synapses, and that their inhibitory role may be more important during large synchronous input to lamina I neurons. I have provided evidence that the amount of GABA released during basal synaptic activity first activatesy presynaptic $GABA_B$ autoreceptors, possibly as a regulation mechanism of inhibitory transmitter release in the marginal zone. Further testing of the plasticity of inhibitory transmission in lamina I in experimental neuropathy (Mosconi, Kruger, 1996) revealed an alteration in the balance of excitatory and inhibitory input to lamina I neurons, which might explain the hyperexcitability of dorsal horn cells in such pathological condition (Lipski et al., 1988). I have shown that activity in inhibitory interneurons is significantly reduced, which may underlie the substantial increase in the frequency of spontaneous EPSCs that I have found. Finally, I have also provided evidence of an additional participation of $GABA_A$Rs to inhibitory currents, possibly as a partial compensation for the increase in excitatory input to lamina I neurons in this experimental mode of neuropathy.

Figure 1 summarizes into a schematic diagram the possible substrate for the inhibitory mechanism mediated by GABA and glycine in lamina I during normal and pathological conditions. This diagram is not intended to represent the actual substrates of inhibition in such conditions, but is used to illustrate the likely inhibitory mechanisms in lamina I, as suggested from previous studies and from those presented in this thesis.

Figure 1. Summary of the findings presented in this thesis.

This figure illustrates a proposed mechanism of inhibitory control mediated by GABA and glycine in lamina I.

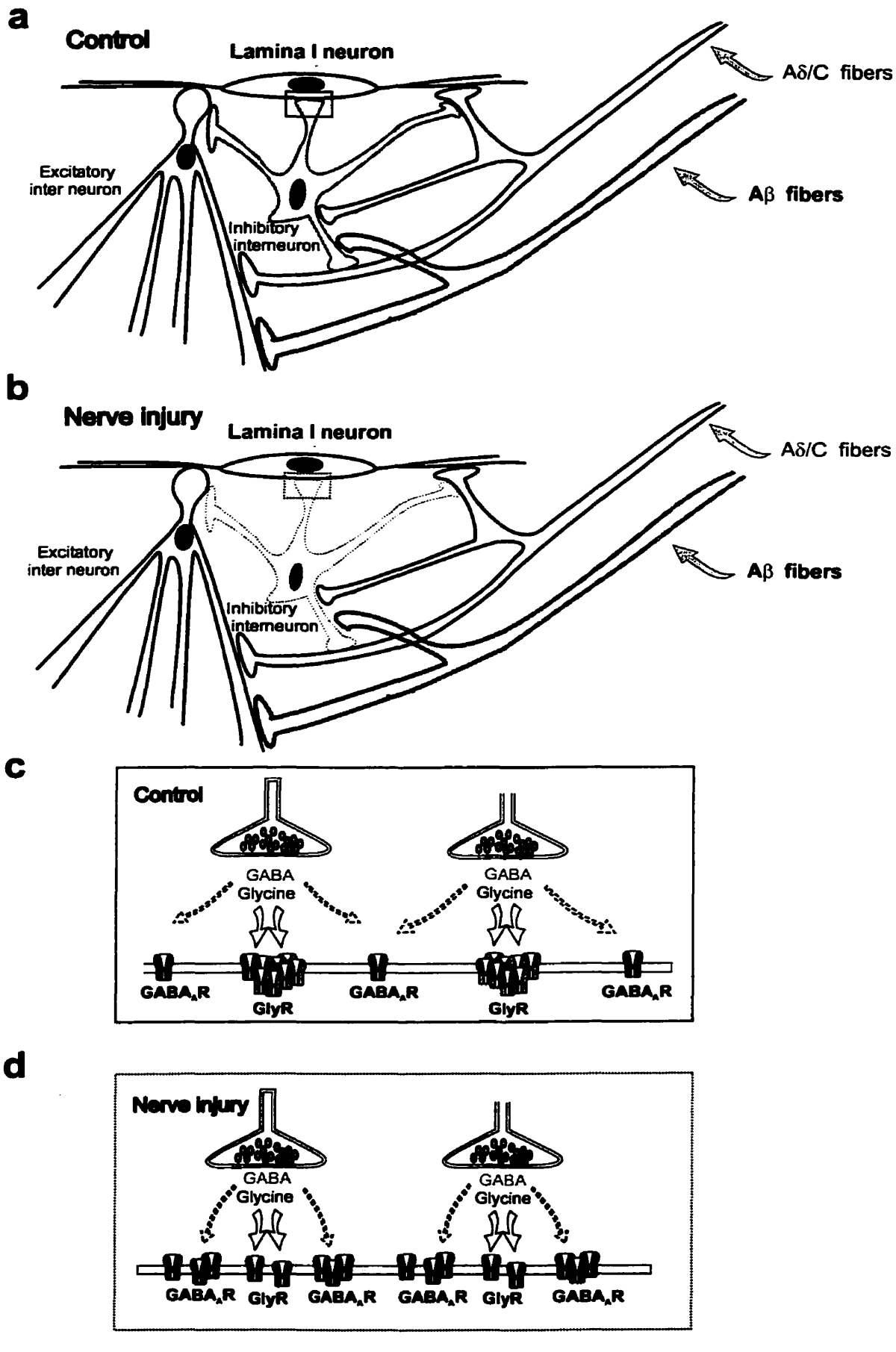
a, Under normal conditions, lamina I neurons receives direct input from primary afferent fibers predominantly of the high-threshold Aδ type (Light, Perl, 1979b; Light et al., 1979; Beal, Bicknell, 1981; Woolf, Fitzgerald, 1983; Willis, Coggeshall, 1991), but also from the high-threshold C-fiber type (Christensen, Perl, 1970; Light et al., 1979; Gobel et al., 1981; Woolf, Fitzgerald, 1983; Fitzgerald, Wall, 1980).

An input from the low-threshold Aβ fibers also exists (Light et al., 1979; Woolf, Fitzgerald, 1983), but might be via polysynaptic pathways, since Aβ fibers appear to terminate in laminae III-VI (Brown, 1981a). The primary afferent fibers may also excite inhibitory interneurons (*cell in the middle*) in the dorsal horn (Willis, Coggeshall, 1991). The diagram shows that an inhibitory interneuron (*cell in the middle*) exerts a control on primary afferent input (to lamina I neurons and other excitatory interneurons in the dorsal horn) through axo-axonic synapses (Willis, Coggeshall, 1991), and may also directly controls lamina I output neurons and excitatory interneurons that may further activate lamina I cells.

Following nerve injury (**b**), the level of activity in inhibitory interneurons appears to be substantially reduced (Woolf, Wall, 1982; Castro-Lopes et al., 1993; Dumoulin et al., 1996; Ibuki et al., 1997; Sugimoto et al., 1990; Coggeshall, Carlton, 1997), possibly because of a decrease in excitatory input to these inhibitory interneurons, or a change in their intrinsic activity. Because of this loss or reduction of activity of inhibitory interneurons, excitatory fibers and lamina I neurons may be disinhibited (Woolf, Mannion, 1999), and this may lead to a facilitation of excitatory transmission in the dorsal horn, which might be reflected by the increase in the frequency of excitatory events recorded in lamina I neurons in this study (chapter 5).

c, This is an enlargement of the synapse (box) in **a**, showing that in the control situation, GABA and glycine are co-released from synaptic vesicles at lamina I inhibitory synapses; while glycine mediates tonic inhibition by acting at GlyRs clustered at synaptic junctions, GABA diffuses to reach $GABA_A$Rs located at extrasynaptic sites (Chéry, De Koninck, 1999).

d, This enlargement of the synapse (box) in **b** illustrates the possible reorganization of GlyR and $GABA_A$Rs following nerve injury. In such situation, while there appears to be a reduction in GlyRs [(Simpson, Huang, 1998) and see chapter 5], a recruitment of $GABA_A$Rs (which would be located closer to synaptic junctions) may occur (chapter 5), and could serve as a partial compensatory mechanism to counteract better the increase in excitatory input to lamina I cells (chapter 5).


a
Control
Lamina I neuron
Aδ/C fibers
Excitatory inter neuron
Aβ fibers
Inhibitory interneuron
b
Nerve injury
Lamina I neuron
Aδ/C fibers
Excitatory inter neuron
Aβ fibers
Inhibitory interneuron
c
Control
GABA
Glycine
GABA
Glycine
GABA$_A$R
GlyR
GABA$_A$R
GlyR
GABA$_A$R
d
Nerve injury
GABA
Glycine
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ORIGINAL CONTRIBUTIONS

- I have developed a method for a clear delineation of lamina I from deeper dorsal horn layers in live rat spinal cord tissue, using gradient contrast videomicroscopy. This approach offers optimal conditions for electrophysiological recordings of visually identified lamina I neurons.

- I have provided evidence that glycine and GABA play distinct inhibitory roles in lamina 1 of the spinal cord. My data indicate that glycine mediates tonic inhibition in lamina I neurons by acting on GlyRs likely clustered at postsynaptic membranes, while the inhibitory role of GABA appears to be more important during intense synaptic activity. Moreover, my findings indicate that $GABA_A$Rs in lamina I are likely located at extrasynaptic sites.

- I have demonstrated that GABA and glycine could be co-localized in the same synaptic vesicles, and therefore co-released at the same synaptic junction at inhibitory synapses in lamina I, yet they mediate separate inhibitory responses in this spinal layer.

- I have shown that presynaptic $GABA_B$ receptors are the first to be activated by GABA released from inhibitory synapses in lamina I, and that these $GABA_B$ autoreceptors would play an important role in regulating the release of both GABA and glycine in the marginal zone.

- I have provided evidence of an altered balance between excitation and inhibition in lamina I of the dorsal horn in experimental neuropathy condition.

- My results indicate that the excitatory drive onto inhibitory interneurons is reduced in experimental neuropathy. This is likely to result in a disinhibition of lamina I neurons.

- I have shown that there is a three-fold increase in the frequency of spontaneous excitatory events in lamina I neurons from rats with sciatic nerve injury.

- I have demonstrated a switch in the relative contribution of GlyRs and $GABA_AR$ components to the mIPSCs in lamina I from rats with experimental nerve injury.

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