Pain Related Inhibition by GABA and Glycine in the Rat Spinal Cord

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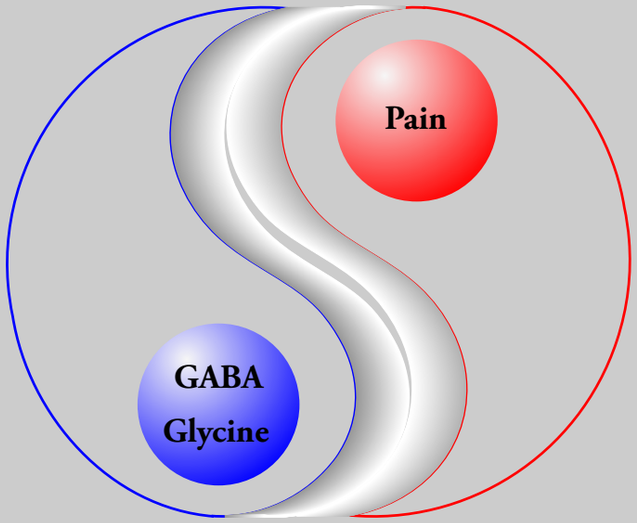

Mehdi Hossaini

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Pain related Inhibition by GABA and Glycine in the Rat Spinal Cord

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Table of Contents

Chapter I

General Introduction

1.1. The definition of pain

In the 17th century, René Descartes, who is generally considered as the 'father of modern philosophy', was one of the first in history to inquire into the feeling of pain on a rational (scientific) basis. Some of his philosophical reasoning, like his theory on the dualistic nature of humans with a clear separation between the mind and the body, are still found in our culture today. Several of his other statements, e.g. that animals do not have a mind and therefore are not able to feel pain, are now generally considered as outdated. Nevertheless, his view on the transmission of pain [6], represented as the pulling of a thread (Fig. 1), and on the functional importance of pain are not completely at odds with current views on pain transmission.

Fig. 1:*"For example, if the fire A is close to the foot B, the small particles of fire, which as you know move very swiftly, are able to move as well the part of the skin which they touch on the foot. In this way, by pulling at the little thread cc, which you see attached there, they at the same instant open e, which is the entry for the pore d, which is where this small thread terminates; just as, by pulling one end of a cord, you ring a bell which hangs at the other end.... Now when the entry of the pore, or the little tube, de, has thus been opened, the animal spirits flow into it from the cavity F, and through it they are carried partly into the muscles which serve to pull the foot back from the fire, partly into those which serve to turn the eyes and the head to look at it, and partly into those which serve to move the hands forward and to turn the whole body for its defense."*

Painful stimuli are indeed detected by specialized fibers in the skin and transmit their information to the spinal cord [56], and from there to higher centers of the central nervous system [23]. Furthermore, pain serves as a warning system leading to retraction of our body from the source of physical danger. Since Descartes' time and especially in the last century, there has been a tremendous increase in research on pain. The International Association for the study of Pain (IASP), founded in the 1973, describes pain as 'An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage'. This definition points out that pain, while serving as a warning system of our body, is in essence a feeling with a major emotional impact. This makes pain a prominent part of our daily live, especially for patients with chronic pain.

1.2. The pain system

The feeling of pain is generally initiated by the activation of specialized primary afferents, termed nociceptors, which innervate the skin [56] but also the majority of the internal organs [8, 74], with clinically notable exceptions like the liver, the lungs, and the brain. Nociceptors are activated by actual or potential damaging stimuli directed to our body and hence require our immediate attention. Activated nociceptors convey their information to the spinal cord, or, when our face is involved, to the brainstem [24, 80]. In the spinal cord and the brainstem, nociceptors establish the first synapse in the pain system by contacting second order nociceptive neurons [76]. There are two main types of nociceptive fibers, namely slow conducting unmyelinated C-fibers that are responsible for the dull aching pain, and fast conducting myelinated Aδ-fibers that are responsible for acute pin prick-like pain [22, 72]. C-fibers contact nociceptive neurons located in lamina I and especially lamina II, while Aδ-fibers project to secondary nociceptive neurons mainly in laminae I, IV and V [76]. Subsequently, via crossing axons of the second order neurons nociceptive information is conducted contralaterally to various nuclei in the brainstem, the midbrain and the thalamus by way of the antero-lateral system [23].

The antero-lateral system contains several important ascending pathways including projections to: 1) the thalamus, i.e. spinothalamic tract (STT) [23]; 2) the homeostatic control regions in the medulla and the brainstem, i.e. spinomedullary, spinopontine and spinomesencephalic pathways that project to the regions of catecholamine cell groups (A1-A7) [23, 68], the parabrachial nucleus (PB) [10, 14, 26], the periaqueductal grey (PAG) [82], and the reticular formation of the medulla and pons [9]; 3) the hypothalamus (the spinohypothalamic tract, SHT) and the ventral part of the forebrain [17]. In addition there are projections to the forebrain, originating in the brainstem, which include the anterior cingulate cortex (ACC), the insular cortex (IC) and the prefrontal cortex [23]. Further, there are also nociceptive projections to the SI and SII regions in the somato-sensory cortex, mainly through the thalamus [15]. While the projections to the somato-sensory cortex are primarily involved in identifying the location and intensity of the nociceptive stimulus [48, 49, 60], the projections to the limbic forebrain provide the basis for the emotional impact of the nociceptive stimulus [7, 31, 51, 79]. Collectively, these various supraspinal structures that are involved in pain processing are known as the pain matrix [11, 63]. The pain matrix, which includes sensory, motor, cognitive and emotional parts of the brain, emphasizes that the feeling of pain is a multidimensional percept, in line with the fact that pain is of essential importance in our daily lives.

1.3. Hyperalgesia and allodynia: increased sensitivity to pain stimuli

As experienced in daily life, an injury usually leads to a direct painful sensation. However, when the initial pain has subsided and the healing process has started, the area of injury often becomes more sensitive to both noxious and non-noxious stimuli. This condition, in which a non-noxious stimulus is perceived as painful (referred to as allodynia) and a noxious stimulus causes more pain than normal (referred to as hyperalgesia) [12, 45,

85] is important, since both allodynia and hyperalgesia will make a subject to protect the injured area, thus aiding the healing process. The increased sensitivity of the injured area to non-noxious and noxious stimuli is the result of an increased sensitivity of nociceptors in the periphery (peripheral or primary sensitization) as well as neurons in the spinal cord (central or secondary sensitization) [83, 86]. In addition, descending pathways originating in the brainstem further facilitate this process by modulating the sensitivity of the spinal nociceptive neurons [66, 81].

1.4. Chronic pain

The feeling of pain usually subsides with the disappearance of the nociceptive stimuli during the healing time. However, when an injury becomes chronic the associated pain, including allodynia and hyperalgesia, will persist. For example, during chronic arthritis the joints are affected by chronic inflammation [52], and the patients suffering from such a disease complain of a nagging pain in the affected joints, together with limitations of their movements due to their painfulness. Treatment is directed at eliminating or decreasing the inflammation process and suppressing the pain, using analgesics [75]. Another major cause of chronic pain is the so-called neuropathic pain, which develops after nerve injury, e.g. after transection by trauma or surgery, or after ischemic events [21]. Post-injury, there is ectopic firing in the damaged nerve, increased sensitivity of spinal nociceptive neurons, and altered descending control originating in the brainstem [5, 37, 59]. All these changes lead to spontaneous pain without the presence of a noxious stimulus at the injured area, often accompanied by allodynia and hyperalgesia [16, 84]. More so than chronic inflammatory pain, neuropathic pain is notoriously difficult to treat with even the strongest analgesics [5]. Both chronic inflammatory and neuropathic pain are seriously debilitating conditions that affects millions of people worldwide [19]. Thus, research dedicated to the understanding of the processes underlying chronic inflammatory and neuropathic pain is of essential importance for patients in need of pain relief.

1.5. Pain inhibition at the spinal cord level

1.5.1. Spinal inhibitory interneurons

Incoming nociceptive fibers not only contact projection neurons that relay the information to higher centers but also local excitatory and inhibitory interneurons [18, 27]. These interneurons are involved in controlling the spinal sensitivity to incoming nociceptive stimuli [41, 78, 90], and are therefore believed to contribute to the maintenance of chronic pain states [90]. Recently, it has been shown that glial cells, especially microglia, are also involved in gating the sensitivity for nociceptive stimuli, especially during neuropathic pain [13, 44, 69]. Thus, spinal projections neurons, local interneurons and glial cells form an intricate network that controls the nociceptive information that is conveyed to supraspinal sites, thereby strongly influencing the pain that is experienced.

Inhibitory neurons use the fast neurotransmitters glycine and/or GABA as their

inhibitory neurotransmitter(s) [2, 46]. The importance of GABA and glycine in pain transmission was shown by blocking glycinergic and/or GABAergic neurotransmission in naïve animals, which induced many behavioral signs of hypersensitivity as observed in inflammatory and neuropathic pain conditions [70, 71, 87]. Accordingly, decreased inhibition was found in the spinal cord during chronic inflammatory pain [58, 64], as glycinergic inhibition in the spinal cord was blocked by a pathway involving prostaglandin E2 (PGE2) [90]. During neuropathic pain there is loss of synaptic inhibition due to a shift in the neuronal chloride gradient, which reverses the inhibitory effect of GABA into a depolarizing one [13]. Further, it was recently shown that selective activation of GABA(A) receptors containing the α2 and/or α3 subunits leads to pronounced nociceptive inhibition in chronic pain states [50]. These findings underline the importance of glycine and GABA in modulating the spinal processing of nociceptive information, especially during chronic pain.

1.5.2. Rostral ventromedial medulla

Next to the influence exerted by spinal interneurons, the spinal nociceptive system is also under control of descending projections originating in the brainstem, especially from the locus coeruleus and subcoeruleus, and the rostral ventromedial medulla (RVM) [29, 36, 81]. The RVM, which is largely located in the pons, is predominantly under control of PAG that is located in the midbrain [36]. In turn, PAG receives projections from several cortical and subcortical structures such as the ACC, IC, hypothalamus and the amygdala (Amy) [3, 67, 89]. The first evidence that descending pathways are able to control pain transmission in the spinal cord was provided in 1976 [55] by producing analgesia with electrical stimulation of the PAG, without affecting the animal's response to most other environmental stimuli. Since the PAG has no direct projections to the dorsal horn [53], its effects are produced through its connections with the RVM, which has extensive projection to the spinal dorsal horn [30, 36]. Therefore, the PAG-RVM circuitry is of critical importance for the descending control of pain transmission in the spinal cord.

It has been shown that microinjection of morphine into the Amy, IC, PAG or RVM produces analgesia, while the analgesic effect of systemic administered opioids is abolished by microinjection of opioid antagonists into these sites [88]. With respect to the RVM, it has been shown that electrical stimulation of the RVM produces direct inhibition of spinal nociception [65], which is also produced by microinjection of excitatory amino acids into the RVM [29]. Further, there is enhancement of descending inhibition from the RVM in animals with chronic inflammatory pain induced by injection of complete Freund's adjuvant (CFA) [65]. Next to descending inhibition, RVM also facilitates spinal nociception, i.e. enhancing of nociceptive transmission, in inflammatory and neuropathic pain models resulting in hyperalgesia and allodynia [81]. Thus, the PAG-RVM system is capable of inhibiting and facilitating spinal nociception in certain pain models through RVM projections to the spinal dorsal horn.

The existence of a parallel inhibitory and facilitatory output from the RVM suggest that there are distinct neurons in the RVM that are involved in inhibiting or facilitating spi-

nal nociception. In the RVM there are indeed three different, physiologically characterized, neuronal cell types that project to the dorsal horn and have distinct noxious stimulus dependent activity patterns. The ON-cells, which enter a period of activity before execution of a withdrawal reflex from a noxious heat, and have a net facilitatory effect on nociception [29]. The OFF-cells, which enter a period of silence before execution of a withdrawal reflex and have a net inhibitory effect on nociception [35]. The third group, which was termed neutral cells, was classified by exclusion. These cells have no characteristic noxious stimulus dependent activity pattern and therefore are most likely not involved in the acute modulation of nociception [29, 54]. With respect to their neurochemical characteristics, there is still no convincing evidence which neurotransmitters the ON- and OFF-cells use to induce facilitation and inhibition, respectively [28, 57]. For a long time it was believed that serotonin was the neurotransmitter that induced pain inhibition at spinal level [33, 43]. However, later on other studies have shown the involvement of serotonin in facilitating spinal nociception [77], and that a subgroup of the neutral cells contain serotonin rather than the OFF-cells [32, 61]. Anterograde tracing from the RVM area, combined with GABA and glycine immunohistochemistry (IHC) at the ultrastructural level [20, 38, 39, 40] has shown that the terminals of the RVM fibers in the spinal dorsal horn contain glycine and GABA [1]. Up to now it is not clear whether these transmitters are present exclusively in the OFF-cells and whether the ON-cells, which have a facilitatory effect on pain transmission in the dorsal horn use glutamate as their neurotransmitter [28, 57].

1.6. Aim of this PhD project

Spinal neurons that use GABA and/or glycine as their inhibitory neurotransmitters (Gly/GABA neurons) play important roles in spinal nociception. Most likely, Gly/GABA neurons in the RVM that project to the spinal dorsal horn are also important in modulating spinal nociception, although up to now there is lack of evidence for their expression in OFF-cells and their involvement in spinal nociception. It has proven difficult to stain glycinergic and GABAergic neurons since using IHC to identify glycine, GABA, or their transmembrane transporters results in weak soma labeling and intense terminal labeling [4, 34, 42]. Therefore, the method of choice for identifying these inhibitory neurons is in situ hybridization (ISH), which will identify the mRNA of proteins that are specific for neurons that use glycine or GABA as their transmitter. Since mRNA is present in the cell soma and not in the terminals, ISH well label exclusively the cell somata of these neurons. In our studies we have used the glycine transporter 2 (GlyT2) [62, 73] to specifically identify the somata of glycinergic neurons, and glutamic acid decarboxylase (GAD) 67 [25, 47] to identify the somata of GABAergic neurons

In this PhD project, we have combined fluorescent ISH (FISH) for GlyT2 and GAD67 mRNA with fluorescent IHC, and with fluorescent tracing. Using these techniques we have investigated the activity patterns of Gly/GABA neurons in the spinal cord as well as the descending projections from the RVM. In *Chapter 2* we have described the distribution pattern of spinal glycinergic neuronal somata in the rat spinal cord. In *Chapter 3* the activa-

tion pattern of spinal inhibitory neurons in acute and chronic pain states was investigated. In *Chapter 4* we have determined the activation pattern of spinal inhibitory neurons after stimulation with capsaicin in rats with chronic inflammatory or neuropathic pain, and in *Chapter 5* the distribution pattern of RVM neurons that are inhibitory and project to the spinal cord was investigated. In addition, we have identified a new pathway that projects to the RVM that is inhibitory and originates in the spinal cord. In *Chapter 6* we have described the expression pattern of the immediate early gene Arc/Arg3.1 in the rat spinal cord in various acute and chronic pain models, with a focus on inhibitory Gly/GABA and enkephalinergic neurons, as well as the neurokinin-1 expressing nociceptive projection neurons. Further, we have determined the behavioral response of Arc/Arg3.1 knockout mice to acute and chronic pain stimuli. Finally, in the general discussion, *Chapter 7*, we discuss the various findings in a broader perspective.

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Chapter II

Distribution pattern of glycinergic neuronal somata in the rat spinal cord

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Research Report

Distribution of glycinergic neuronal somata in the rat spinal cord

Mehdi Hossaini[a], *Pim J. French*[b], *Jan C. Holstege*[a,*]

[a]*Department of Neuroscience, Erasmus MC, PO Box 2040, 3000 CA, Rotterdam, The Netherlands*
[b]*Department of Neurology, Josephine Nefkens Institute, Erasmus MC, PO Box 1838, 3000 DR Rotterdam, The Netherlands*

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Distribution of glycinergic neuronal somata in the rat spinal cord

Mehdi Hossaini[a], *Pim J. French*[b], *Jan C. Holstege*[a,*]

[a]*Department of Neuroscience, Erasmus MC, PO Box 2040, 3000 CA, Rotterdam, The Netherlands*
[b]*Department of Neurology, Josephine Nefkens Institute, Erasmus MC, PO Box 1838, 3000 DR Rotterdam, The Netherlands*

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ABSTRACT

Glycine transporter 2 (GlyT2) mRNA is exclusively expressed in glycinergic neurons, and is presently considered a reliable marker for glycinergic neuronal somata. In this study, we have performed non-radioactive in situ hybridization to localize GlyT2 mRNA in fixed free-floating sections of cervical (C2 and C6), thoracic (T5), lumbar (L2 and L5) and sacral (S1) segments of the rat spinal cord. The results showed that in all segments the majority of the GlyT2 mRNA labeled (glycinergic) neuronal somata was present in the deep dorsal horn and the intermediate zone (laminae III–VIII), with around 50% (range 43.7–70.9%) in laminae VII&VIII. In contrast, the superficial dorsal horn, the motoneuronal cell groups and the area around the central canal contained only few glycinergic neuronal somata. The density (number of glycinergic neuronal somata per mm^2) was also low in these areas, while the highest densities were found in laminae V to VIII. The lateral spinal nucleus and the lateral cervical nucleus also contained a limited number of glycinergic neurons.

Our findings showed that the distribution pattern of the glycinergic neuronal somata is similar in all the examined segments. The few differences that were found in the relative laminar distribution between some of the segments, are most likely due to technical reasons. We therefore conclude that the observed distribution pattern of glycinergic neuronal somata is present throughout the spinal cord. Our findings further showed that the non-radioactive in situ hybridization technique for identifying GlyT2 mRNA in fixed free-floating sections is a highly efficient tool for identifying glycinergic neurons in the spinal cord.

1. Introduction

Strong evidence that the amino acid glycine is acting as a neurotransmitter in the mammalian spinal cord was provided for the first time in 1965 (Aprison and Werman, 1965). In subsequent years glycine became established as the main inhibitory neurotransmitter in the caudal region of the central nervous system (CNS), next to the amino acid γ-aminobutyric acid (GABA). Glycinergic neurons are abundantly present in the spinal cord, brainstem and cerebellum (Borowsky et al., 1993; Luque et al., 1995), but scarce in rostral parts of the CNS, like the diencephalon and hippocampus (Rampon et al., 1996; Song et al., 2006).

The main strategies for identifying neurons that use an amino acid as neurotransmitter are based on the localization of the transmitter, its biosynthetic enzyme, or its transporter.

* *Corresponding author.*
E-mail address: j.holstege@erasmusmc.nl (J.C. Holstege).
Abbreviations: GlyT, glycine transporter; LSN, lateral spinal nucleus; LCN, lateral cervical nucleus

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Antibodies against glycine have been used most frequently to identify glycinergic neurons (Campistron et al., 1986; Rampon et al., 1996; Todd and Sullivan, 1990). In general, glycine antibodies preferentially stain glycinergic terminals because they contain a high concentration of glycine, while soma labeling is much weaker. However, in recent reports (Allain et al., 2006; Song et al., 2006; Zeilhofer et al., 2005) a glycine antibody was used that was directed against paraformaldehyde-fixed glycine (Pow et al., 1995), resulting in a much improved staining of neuronal somata in addition to axons and terminals. The use of antibodies against the biosynthetic enzyme that produces glycine in the brain, serine hydroxymethyltransferase, has never been reported, probably because the activity of this enzyme is correlated with the general pool of glycine (Daly and Aprison, 1974), rather than with the transmitter pool. Similarly the vesicle transporter that transports glycine also transports GABA, and therefore cannot be used for identifying glycinergic neurons only (Chaudhry et al., 1998). However, glycine transporter 2 (GlyT2), one of the GlyTs (Zafra et al., 1995a,b), is present exclusively in the plasma membrane of glycinergic neurons (Jursky and Nelson, 1995; Luque et al., 1995), where it is primarily involved in the reuptake of synaptically released glycine (Betz et al., 2006). GlyT1, on the other hand, is exclusively present in glial cells (Adams et al., 1995; Zafra et al., 1995a,b). Since GlyT2 is the only marker that is present exclusively in glycinergic neurons (Poyatos et al., 1997; Spike et al., 1997), and GlyT2 antibodies preferentially label glycinergic terminals rather than somata (Jursky and Nelson, 1995; Spike et al., 1997), GlyT2 in situ hybridization is the method of choice for identifying glycinergic neuronal somata. GlyT2 mRNA has been identified previously in the spinal cord by means of radioactive in situ hybridization (Zafra et al., 1995a). Since these studies described GlyT2 mRNA distribution throughout the CNS, the descriptions of the spinal cord were not very detailed. This study, which is the first step in our investigations on the role of glycinergic neurons in spinal sensory processing, was set up to obtain detailed knowledge on the distribution of spinal glycinergic neuronal somata at different levels of the spinal cord. For this purpose, we used non-radioactive in situ hybridization for GlyT2 mRNA on thick fixed free-floating sections.

2. Results

2.1. *General observations*

Light microscopic examination of the sections showed a bluish reaction product, representing the presence of GlyT2 mRNA, in a limited number of neuronal somata of various sizes. No staining was found in glial cells or fiber tracts. Staining was only present in neuronal somata, sometimes including their primary dendrites. Control sections hybridized with sense probes did not show any specific labeling.

The majority of the labeled neuronal somata were found in the deep dorsal horn and the adjoining intermediate zone (laminae VII&VIII) of the ventral horn (Fig. 1). In laminae I&II (Fig. 2A) the number of labeled neuronal somata observed never exceeded four neurons per section. The few labeled somata in lamina II were often located close to the border with lamina III. Laminae III to VI of the dorsal horn contained several labeled neurons, varying in size from 10 to 30 μm (Fig. 2B). In laminae IV, V&VI, labeled neuronal somata were more abundant medially than laterally (Figs. 1, 2B). The intermediate zone of the ventral horn also contained many labeled somata, including several of the largest labeled somata (Fig. 3). The large presumed motoneurons in lamina IX were never labeled. Occasionally a labeled neuron, considerably smaller than the presumed motoneurons, was present within the motoneuronal area (Figs. 1, 3A). The grey matter surrounding the central canal (lamina X) contained few labeled neurons. In all sections examined, a limited number of labeled neurons were found scattered in the white matter, close to either the dorsal or ventral horn (Figs. 1, 2A).

2.2. *GlyT2 mRNA labeled neuronal somata in specialized spinal nuclei*

We have examined several specialized nuclei, i.e., groups of cells that can be distinguished anatomically and usually subserve specialized functions (Holstege et al., 1996) for a more detailed description. These nuclei include the lateral spinal nucleus (LSN), the lateral cervical nucleus (LCN), the central cervical nucleus, the dorsal nucleus of Clarke, the intermediomedial nucleus, the intermediolateral nucleus, and, in the L6 segment, the dorsomedial and dorsolateral nuclei (Onuf's nucleus). In about 25% of the sections examined, one or two labeled neurons were identified in the LSN, which is located in the dorsolateral white matter (Fig. 4). In a few cases, labeled neurons were also observed in the LCN, which is present dorsal to the LSN at the C1–C3 level. In general, in both nuclei the intensity of the labeling was weak. The other specialized nuclei virtually never contained labeled neurons. However, several labeled somata were usually present around these nuclei.

2.3. *Quantitative aspects*

In all spinal segments examined, around 50% (range 43.7–70.9%) of the labeled neurons in that segment were located in the intermediate zone of the ventral horn (laminae VII&VIII), with the highest percentage (average 70.9%) at level L2 (Table 1). The superficial dorsal horn (laminae I&II), the motoneuronal cell groups (lamina IX) and lamina X contained the lowest percentage of glycinergic neurons (Table 1). A comparison between the segmental levels for each lamina separately (a total of 135 comparisons using the Bonferroni post hoc test) showed that in the large majority of the cases (116) there were no significant differences between the different segmental levels. The 19 cases that were significantly different (Table 1) were found mostly in L2 and S1. At the L2 segmental level, the average percentage in laminae V&VI (13.5%) was significantly lower than the percentage obtained for the same laminae in the other segments analyzed, while the average in laminae VII&VIII (70.9%) was significantly higher. In S1 lamina IV only contained 3.3% of the neurons at this level, significantly lower than most of the other segmental levels, while the average percentage (64.7%) obtained for laminae VII&VIII was significantly higher.

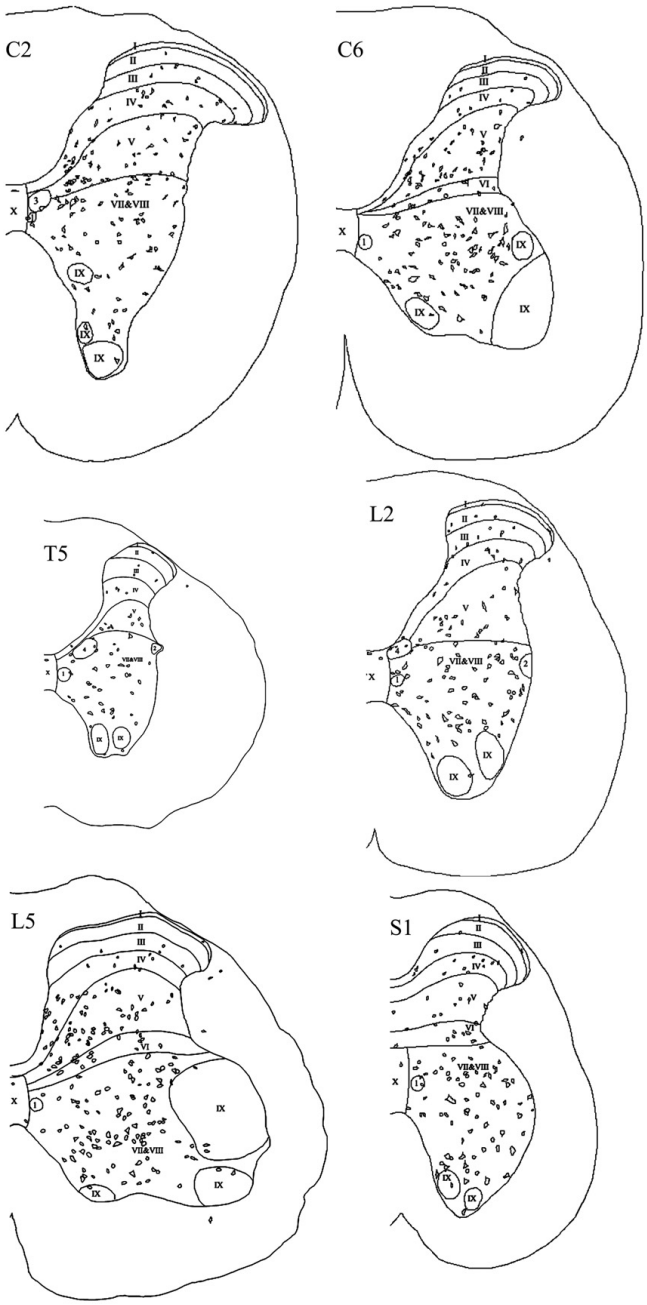

Fig. 1 – Schematic drawings illustrating the distribution of GlyT2 mRNA labeled (glycinergic) neuronal somata in analyzed single sections from rat spinal segments C2, C6, T5, L2, L5 and S1. Neurons in the dorsolateral funiculus are located in the lateral spinal nucleus. Other special nuclei are: intermediomedial nucleus (1), intermediolateral nucleus (2), central cervical nucleus (3), and dorsal nucleus of Clarke (4).

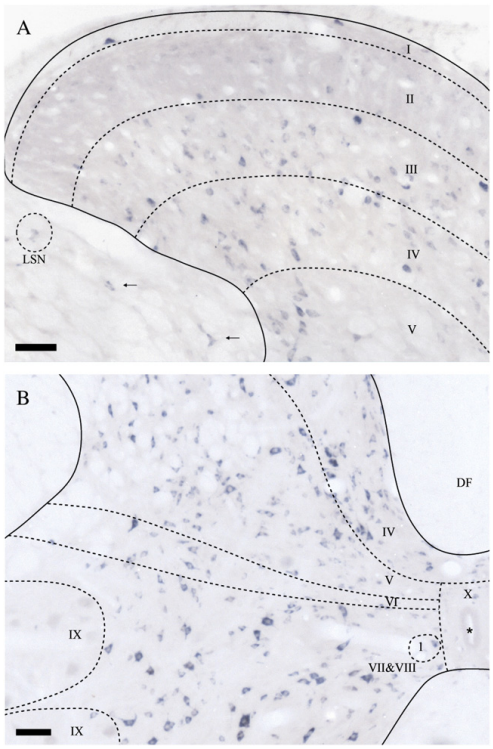

Fig. 2 – Light micrographs showing glycinergic neuronal somata as identified by GlyT2 mRNA labeling. (A) Few labeled somata are present in the superficial dorsal horn (segment C2), with increasing numbers of labeled somata in laminae III and IV. Note the labeled somata in the white matter (arrows) and the lateral spinal nucleus (LSN). Scale bar=50 μm. (B) Glycinergic neuronal somata of various sizes in the deep dorsal horn and the intermediate zone of the ventral horn. I: intermediomedial nucleus, DF: dorsal funiculus, *: central canal. Scale bare=50 μm.

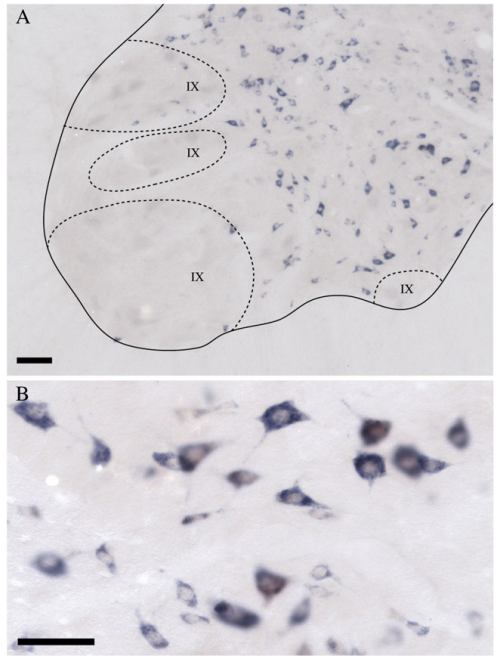

Fig. 3 – Light micrographs showing labeled neuronal somata in the lumbar ventral horn. (A) Overview of the L5 ventral horn. Few GlyT2 mRNA labeled neuronal somata are present in the motoneuronal cell groups (encircled areas). Scale bar=50 μm. (B) Labeled neuronal somata in lamina VII of the L5 spinal segment. Note the variability in size of the labeled neurons. Scale bar=50 μm.

When considering the density of the neurons in each lamina, i.e., the average number of neurons per mm^2, the highest densities are found in laminae V&VI and VII&VIII, and the lowest densities were found in laminae I, II, IX and X (Fig. 5). A comparison between the segmental levels for each lamina separately (a total of 120 comparisons using the Bonferroni post hoc test, excluding the specialized nuclei) showed that in the large majority of the cases (113) there were no significant differences between segmental levels. The exceptions were the density of lamina IV in the S1 segment, which was significantly different from segments C2, C6, and L2 ($p<0.05$); the density of laminae V&VI in S1, which was significantly different from segment C6 ($p<0.05$), and the density of laminae VII&VIII in L2, which was significantly different from segments T5, L5 and S1 ($p<0.05$). Since the large majority of the laminar densities in the different segmental

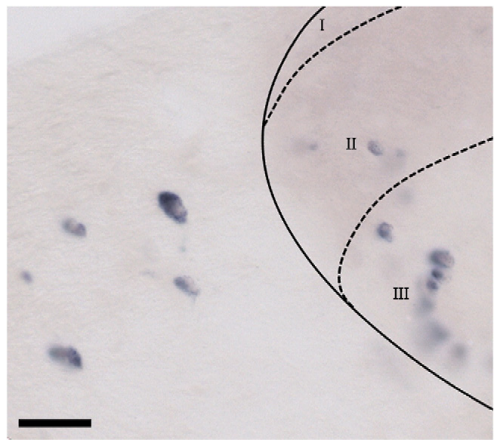

Fig. 4 – Light micrograph showing GlyT2 mRNA labeled (glycinergic) neuronal somata in the lateral spinal nucleus (LSN) at spinal segment T5. Scale bar=25 μm.

Table 1 – Relative laminar distribution of the glycinergic neuronal somata in different segments of the rat spinal cord

Lamina	C2	C6	T5	L2	L5	S1
	%±SD	%±SD	%±SD	%±SD	%±SD	%±SD
I	0.3±0.5	0.3±0.4	0.4±1.0	0.2±0.4	0.1±0.3	0.0
II	1.8±1.0	1.2±0.4	0.9±2.2	0.7±0.6	0.6±0.5	1.2±1.0
III	6.3±2.2	4.6±1.4	4.9±2.6	3.9±2.2	4.2±2.4	2.1±1.9
IV	14.1±3.5[a]	10.3±1.7	9.8±2.7	5.9±1.5	9.7±3.0	3.3±2.0[b]
V&VI	28.5±2.8	29.5±5.2	18.3±5.0[c]	13.5±3.4[d]	31.7±2.8	25.4±5.9
VII&VIII	43.7±6.4	49.0±8.7	54.8±7.9	70.9±2.1[e]	49.6±6.4	64.7±6.8[f]
IX	1.2±1.0	3.2±3.5	2.0±2.3	1.5±1.1	3.1±1.8	1.8±1.8
X	0.5±0.5	0.6±0.6	1.2±1.3	1.4±1.3	0.5±0.9	1.5±2.7
Special nuclei	3.5±1.8	1.3±0.9	7.6±5.8	2.0±1.9	0.3±0.5	0.0
n	606	760	255	720	788	356

For each lamina of the C2, C6, T5, L2, L5 and S1 segments the average percentage (±SD) of the total number of glycinergic neuronal somata is shown. *n*: Total number of labeled neuronal somata.
[a] Lamina IV in C2 is significantly different from the same lamina in L2 ($p<0.005$).
[b] Lamina IV in S1 is significantly different from C2, C6, T5, and L5 ($p<0.005$).
[c] Laminae V&VI in T5 is significantly different from C2, C6, and L5 ($p<0.005$).
[d] Laminae V&VI in L2 from C2, C6, L5, S1 ($p<0.005$).
[e] Laminae VII&VIII in L2 from C2, C6, T5, and L5 ($p<0.005$).
[f] Laminae VII&VIII in S1 from C2, C6, and L5 ($p<0.005$).

levels were not significantly different, the average density for each lamina(e) was calculated (Fig. 5). This showed that the densities of laminae I, II, IX and X are each significantly lower than the densities in the other laminae ($p<0.005$). The same holds true for lamina III ($p<0.05$), except that the density is not significantly different from lamina IV ($p>0.05$). The density of lamina IV is also not significantly different from the density of VII and VIII ($p>0.05$). Laminae V&VI have the highest average density, although not significantly different from laminae VII&VIII.

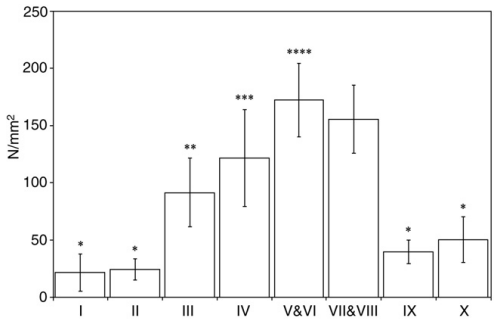

Fig. 5 – Average density of glycinergic neuronal somata per lamina in the analyzed segments. An increasing density is observed in the intermediate zone, while the superficial laminae, the motoneuronal cell groups (lamina IX) and lamina X have the lowest density. N/mm^2: calculated number of labeled glycinergic neuronal somata per mm^2. *Laminae I, II, IX and X are significantly different from laminae III, IV, V&VI, and VIII&VIII ($p<0.005$). **Lamina III is significantly different from all the other laminae ($p<0.05$), except lamina IV. ***Lamina IV is significantly different from all the other laminae ($p<0.005$), except laminae III and VII&VIII. ****Laminae V&VI are significantly different from all other laminae ($p<0.005$), except laminae VII&VIII. Error bars represent ±SD.

3. Discussion

The present study is the first detailed description of the distribution of GlyT2 mRNA in the spinal cord, using non-radioactive in situ hybridization on thick paraformaldehyde-fixed free-floating sections. Previous studies on the distribution of glycinergic (GlyT2 mRNA) and/or GABAergic (GAD67 mRNA) neuronal somata (Schreihofer et al., 1999; Tanaka and Ezure, 2004) in the brainstem have confirmed the sensitivity (Key et al., 2001) of the detection method used in the present study. In situ hybridization on free-floating 40-μm-thick sections has the advantage that the sections are easy to handle because the sections are fixed and much thicker than slide mounted non-fixed sections. Especially the washing steps in the procedure are more efficient on free-floating sections, which, in our hands, lead to a higher signal-to-noise ratio in comparison with slide mounted sections. The method used in this study is therefore the most reliable and sensitive approach presently available to identify spinal glycinergic neuronal somata. It has the advantage that it can be combined with other techniques like immunohistochemistry, and neuronal tracing (Stornetta et al., 2005).

Our results show that glycinergic neuronal somata are concentrated in the deep dorsal horn (laminae V&VI) and the intermediate zone of the ventral horn (laminae VII&VIII). In the superficial dorsal horn (laminae I&II), the area around the central canal (lamina X) and the motoneuronal cell groups (lamina IX) glycinergic neuronal somata were observed much less frequently, while laminae III and IV hold an intermediate position. This pattern is present in all the examined spinal segments (i.e., C2, C6, T5, L2, L5 and S1), strongly suggesting that this pattern is constant throughout the spinal cord. However, our analysis also showed some exceptions to this

rule, especially at spinal levels L2 (laminae V&VI, and VII&VIII) and S1 (laminae IV, and VII&VIII). The most simple explanation for this finding is that the laminar boundaries at the L2 and S1 level were identified incorrectly or that their location leads to relatively large differences in surface area as compared with the other segments. Especially at the L2 level, a ventral shift of the boundary between laminae V and VII, would increase the percentage of neurons in laminae V and decrease the percentage in laminae VII&VIII, likely resulting in the disappearance of the significant differences between the L2 and the other segments. Also in S1, which is a relatively small segment, small changes in boundary delineation may easily lead to significant changes in the percentages that were obtained. We therefore consider that the few significant differences in the laminar percentages that were found between segments are due to technical reasons, and do not signify any functional difference in the role of glycinergic neurons in these segments.

The bulk of the glycinergic neuronal somata was found in the intermediate zone (laminae VII&VIII). This finding is not surprising considering that this is the largest area as compared with the other spinal laminae. Thus even if the glycinergic neurons were evenly distributed over all the laminae in a section, the percentage of neurons in laminae VII&VIII would still be the highest. Therefore, we also calculated the average density of the glycinergic neurons in the different laminae. This showed that the superficial dorsal horn, the motoneuronal cell groups and lamina X have the lowest density. In case of the superficial dorsal horn, it may be expected that the density of glycinergic neurons would be relatively high considering the small surface area of these laminae. In contrast the density of the superficial laminae was very low, which confirms that these laminae are almost devoid of glycinergic neuronal somata.

The highest percentages of glycinergic neuronal somata in the various segments were found in laminae, V&VI and VII&VIII. These laminae also contained the highest densities of glycinergic neurons, which confirms the specific concentration of glycinergic neurons in these laminae.

In the analyzed specialized nuclei, only the LSN and LCN contained glycinergic neurons. This finding likely indicates that some of the widespread descending (Jansen and Loewy, 1997) and ascending (Ding et al., 1995; Keay et al., 1997) projections originating in the LSN are in part glycinergic.

Only one previous study (Todd and Sullivan, 1990) has provided a detailed description of glycinergic neuronal somata in the adult spinal cord with a focus on the superficial dorsal horn. This study used glycine antibodies in combination with pre-embedding immunohistochemistry on semithin plastic sections of rat lumbar segments. Their results are in general agreement with the results of the present study and show the same distribution pattern: a concentration of glycinergic neuronal somata in the deep dorsal horn and the intermediate zone of the ventral horn, while glycinergic neuronal somata are nearly absent from the superficial dorsal horn. Our findings further showed that this distribution pattern is similar in all the examined segments. Since the few differences that were found in the relative laminar distribution between some of the segments, are most likely due to technical reasons, we conclude that the observed distribution pattern of glycinergic neuronal somata is present throughout the spinal cord.

The functional significance of the laminar localization of glycinergic neuronal somata can only be appreciated in combination with many other data on these neurons, including the size of their dendritic tree, their axonal projection area, the parameters that determine the release of glycine and the properties of the postsynaptic glycine receptors. The dendritic tree of spinal neurons may extend into several neighboring laminae (Willis and Coggeshall, 2003). However, the morphology of the dendritic tree of glycinergic neurons is largely unknown. Similarly, little is known about the axonal arborization of glycinergic neurons. Areas that contain very few glycinergic neuronal somata, as we have shown here for the superficial dorsal horn (laminae I&II) and the motoneuronal cell groups (lamina IX), contain many glycinergic terminals (Rekling et al., 2000; Todd, 1990). It seems likely that the majority of these terminals is derived from spinal neurons located in other laminae, while some are derived from supraspinal sources (Antal et al., 1996; Holstege and Bongers, 1991). A lot more is known about the release properties and effects of glycine on other neurons. In this respect it is important to realize that both in the dorsal (Todd and Sullivan, 1990; Todd et al., 1996) and ventral horn (Taal and Holstege, 1994) glycine is often colocalized and co-released with GABA. However, there are also sets of neurons that contain glycine without GABA and vice versa, e.g., neurons in the intermediate zone that presynaptically inhibit Ia afferents on motoneurons only contain GABA and not glycine (Hughes et al., 2005; Mackie et al., 2003). In view of all these variables, it is likely that the glycinergic neurons in a specific lamina fulfill diverse functions. In laminae II–V the action of glycinergic neurons may be focused on the influencing sensory transmission (Willis and Coggeshall, 2003; Zeilhofer, 2005), while neurons in deeper laminae may be involved in affecting motor transmission (Rekling et al., 2000). In addition there are neurons in many of these laminae that project to supraspinal brain areas and some of these neurons may use glycine as a transmitter, although direct evidence for such a glycinergic projection is presently lacking.

Recently, glycinergic neurons in the spinal cord were also identified on the basis of the expression of GlyT2, but using a very different approach, namely by producing transgenic mice expressing enhanced green fluorescent protein (eGFP) under the control of the promotor of the GlyT2 gene (Zeilhofer et al., 2005). This study showed that the large majority of the eGFP producing neurons in the brain were also immunoreactive for glycine (in somata, dendrites and terminals) and GlyT2 (in terminals). However, in the dorsal horn, the area of the spinal cord examined in this paper, it was found that several glycine immunoreactive neuronal somata did not express eGFP and vice versa. In lamina II a subset of neurons was described as glycine immunoreactive, without expressing eGFP. In this case our findings in the rat dorsal horn would support the eGFP expression pattern, since we also found very few neurons in lamina II expressing GlyT2 mRNA. Whether this would indicate that there is a subset of neurons in lamina II that uses glycine as a transmitter without expressing GlyT2, or whether these cells show a high level of glycine, but do not use it as a transmitter, is unclear.

In this study we have identified glycinergic neurons throughout the spinal cord using GlyT2 mRNA in situ hybridization. We have shown that glycinergic neurons are concentrated in laminae III–VIII and provided evidence that this pattern is constant throughout the spinal cord. These findings provide a solid anatomical basis for further studies on the role of glycine in the spinal cord.

4. Experimental procedures

4.1. *Tissue preparation*

In this study we used 18 male Wistar rats, including six rats for quantitative analysis. Rats received an overdose of sodium pentobarbital and were transcardially perfused with 150 ml saline followed by 750 ml of 4% paraformaldehyde (PFA) in 0.12 M phosphate buffer (PB), pH 7.4. Subsequently, the entire spinal cord was dissected and left overnight in a RNAse-free solution of 4% PFA and 30% sucrose dissolved in 0.12 M PB at 4 °C. All the experiments have been approved by the Rotterdam Animal Ethics Committee.

4.2. *In situ hybridization*

All the solutions used in the following steps until the hybridization step were Diethylpyrocarbonat (DEPC)-treated. The regions of interest of the spinal cord were cut transversally in 40-μm sections on a freezing microtome and collected in 0.05 M PB. The sections were then treated with 0.2% glycine in phosphate-buffered saline (PBS, 5 min), rinsed in PBS, and then treated (10 min) in PBS containing 0.1 M triethanolamine (Merck, Germany) pH 8.0 and 0.0025% acetic anhydride (Sigma-Aldrich, Germany). Sections were then washed in 4× standard saline citrate (SSC, pH 4.5) and prehybridized for 1 h at 65 °C in hybridization mixture consisting of 50% formamide, 5× SSC (pH 4.5), 2% Blocking Reagent (Roche), 0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma-Aldrich), 1 μg/ml yeast tRNA (tRNA brewer's yeast, Sigma), 5 mM EDTA (pH 8.0), 50 μg/ml Heparin (Sigma-Aldrich), and 1× Denhardt's solution (Sigma-Aldrich).

The GlyT2 mRNA was obtained from a partial cDNA template (3.1 kb; a generous gift from Dr. N. Nelson, Tel Aviv University), encoding GlyT2. The riboprobes were obtained by linearizing the recombinant plasmids with restriction enzymes (*Xba*I for GlyT2 antisense, *Hin*dIII for GlyT2 sense) and transcribed with RNA polymerases (T7 for GlyT2 antisense, T3 for GlyT2 sense) in the presence of digoxigenin (DIG)-labeled 11-UTP (Roche).

The sections were hybridized for 16–24 h at 65 °C in hybridization mixture (minus Denhardt's) containing 500–600 pg/μl of GlyT2 anti-sense riboprobes. Some sections were hybridized with sense probe at a matched concentration to serve as control. After hybridization, the sections were washed in 2× SSC (pH 4.5), followed by three washes of 15 min each in 2× SSC (pH 4.5)/50% formamide at 65 °C, and finally washed in PBS. The sections were then pre-incubated (90 min, room temperature) in BSA-blocking solution consisting of PBS, 5% BSA (bovine serum albumin, Fraction V, Roche) and 0.5% Triton X-100. For detection of DIG, the sections were incubated in 2% BSA-blocking solution with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) diluted 1:4000 (overnight, 4 °C). Subsequently, the sections were washed in TBST (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris/HCl (pH 7.5), and 0.1% Tween-20), followed by NTM (100 mM NaCl, 100 mM Tris/HCl (pH 9.5), and 50 mM $MgCl_2$). The blue reaction product was produced by the reaction of alkaline phosphatase with levamisol, NBT (nitroblue tetrazolium; Roche) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt; Roche) for 1.5–2 h at room temperature in the dark. Usually, the exact reaction time was determined by assessing the staining in the light microscope. The sections were randomly mounted on slides, air dried overnight, dehydrated using absolute ethanol (<0.01% methanol), transferred to xylene and coverslipped with Permount (Fisher, Hampton, NH).

4.3. *Data analysis*

Light micrographs were made with a digital camera and processed using Adobe Photoshop. The images were not manipulated, except for brightness and contrast.

Analysis was carried out on cervical (C2 and C6), thoracic (T5), lumbar (L2 and L5), and sacral (S1) segments from six rats. Between 15 and 20 sections per segment were mounted on a slide in a random order. In the microscope, the slide was systematically examined, starting with the first section in the first row. The first section of the appropriate segmental level that was encountered was used for analysis. Occasionally this section appeared damaged during the procedure or incorrectly mounted, in which case the section was discarded. Thus a total of 36 sections were analyzed. Using a camera lucida microscope (Neurolucida, MicroBrightfield Inc., Williston, VT) the outline of the white and grey matter, the boundaries between the laminae and the contour of several spinal nuclei were drawn unilaterally (Molander et al., 1984, 1989), after which the labeled neurons were plotted in the drawing. A neuron was considered labeled only if the largest diameter was at least 10 μm, the cell soma contained a bluish reaction product and a non-stained nucleus was apparent. The number of plotted neurons per lamina was expressed as a percentage of the total number of neurons plotted in that section. The results obtained in the six rats were averaged for each lamina per segment. The density of glycinergic neurons per lamina is expressed as the number of plotted neurons in a lamina divided by the surface area, expressed in mm^2, of that lamina, as calculated by Neurolucida. The results obtained in the six rats were averaged for each laminar density per segment. The data for both the laminar distribution and the density of glycinergic neuronal somata were analyzed by performing the one-way ANOVA test followed by the Bonferroni post hoc test. $p<0.05$ was considered as significant.

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Chapter III

Differential distribution of activated spinal neurons containing glycine and/or GABA and expressing c-fos in acute and chronic pain models

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Differential distribution of activated spinal neurons containing glycine and/or GABA and expressing c-fos in acute and chronic pain models

Mehdi Hossaini [a], Liron S. Duraku [b], Çiğdem Saraç [a], Joost L.M. Jongen [a,c], Jan C. Holstege [a,*]

[a] Department of Neuroscience, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands
[b] Department of Plastic Reconstructive and Hand Surgery, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands
[c] Department of Neurology, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands

Abstract

The inhibitory transmitters GABA and glycine play an important role in modulating pain transmission, both in normal and pathological situations. In the present study we have combined in situ hybridization for identifying spinal neurons that use the transmitter(s) glycine and/or GABA (Gly/GABA neurons) with immunohistochemistry for c-fos, a marker for neuronal activation. This procedure was used with acute pain models induced by injection of capsaicin or formalin; and chronic pain models using Complete Freund's Adjuvant (CFA, chronic inflammation), and the spared nerve injury (SNI) model (neuropathic pain).

In all models Gly/GABA neurons were activated as indicated by their expression of c-fos. The pattern of Gly/GABA neuronal activation was different for every model, both anatomically and quantitatively. However, the averaged percentage of activated neurons that were Gly/GABA in the chronic phase (≥ 20 hrs survival, 46%) was significantly higher than in the acute phase (≤ 2 hrs survival, 34%). In addition, the total numbers of activated Gly/GABA neurons were similar in both phases, showing that the activation of non-Gly/GABA (presumed excitatory) neurons in the chronic phase decreased. Finally, morphine application equally decreased the total number of activated neurons and activated Gly/GABA neurons. This shows that morphine did not specifically activate Gly/GABA neurons to achieve nociceptive inhibition.

The present study shows an increased activity of Gly/GABA neurons in acute and chronic models. This mechanism, together with mechanisms that antagonize the effects of GABA and glycine at the receptor level, may determine the sensitivity of our pain system during health and disease.

1. Introduction

Nociceptors convey their information from the periphery to the spinal cord where they target secondary neurons located in the superficial (laminae I-II) and deep (laminae III-VI) dorsal horn [45]. While a subpopulation of these secondary neurons are projection neurons that relay the nociceptive information to higher centers [40], the majority are local interneurons [4]. A substantial number of these interneurons contain the fast inhibitory transmitters GABA and glycine, often colocalized in the same cell [47,48] and these neurons are directly innervated by primary afferent fibers [10,14]. The importance of GABA and glycine in pain transmission was shown by blocking glycinergic and/or GABAergic neurotransmission in naïve animals, which induced many behavioral signs of hypersensitivity as observed in inflammatory and neuropathic pain conditions [38,39,50].

Accordingly, decreased inhibition was found in the spinal cord during chronic inflammatory pain [33,37]. In this condition, glycinergic inhibition in the spinal cord is blocked by a pathway involving prostaglandin E2 (PGE2). When this PGE2 induced blockade was prevented (<24 hrs) the thermal and mechanical sensitization of inflammatory pain did not appear [52]. During neuropathic pain there is loss of synaptic inhibition due to a shift in the neuronal chloride gradient, which reverses the inhibitory effect of GABA into a depolarizing one [9]. Further, it was recently shown that selective activation of GABA(A) receptors containing the α2 and/or α3 subunits [26] leads to pronounced nociceptive inhibition.

These studies described above underline the importance of glycine and GABA in modulating the spinal processing of nociceptive information, especially during chronic pain. However, little is known about the activity of inhibitory neurons, and changes therein, during different pain states. For identifying neurons that were recently activated, the expression of c-fos protein has been used extensively in the nociceptive system [8], showing that spinal c-fos expression patterns are correlated with the type, intensity and duration of nociceptive stimuli [8]. However, data on the activation patterns of spinal inhibitory neurons during different pain states are scarce. So far, three studies have [46,54,55] identified activated glycinergic and/or GABAergic (Gly/GABA) neurons after acute nociceptive stimulation of the hind paw with capsaicin or formalin. These studies have used immunohistochemistry to identify the c-fos protein as well as Gly/GABA neurons. However, presently available antibodies preferentially label Gly/GABA terminals and have proven difficult for reliably labeling the somata of Gly/GABA neurons [2,17]. Therefore, in the present study we have used in situ hybridization with markers for Gly/GABA neurons. This technique will identify mRNA for GlyT2 (a marker for glycinergic neurons) [36,41] or GAD67 (a marker for GABAergic neurons) [12,24] with a high sensitivity, and can be combined with immunohistochemistry for c-fos [42]. Using this approach we set out to investigate the activation patterns of spinal Gly/GABA neurons in different acute and chronic pain states. For this purpose we determined the number and the percentage of c-fos labeled neurons that expressed GABA and/or glycine in acute (capsaicin, formalin) or chronic (inflammatory and neuropathic) pain conditions. In addition, we investigated the effect of morphine

application on c-fos expression in Gly/GABA neurons after stimulation with formalin.

2. Materials and methods

In this study we used a total of 67 male Wistar rats. All animal experiments were approved by the Rotterdam Animal Ethical Committee.

2.1. Pain models

Nociceptive stimuli for the capsaicin, formalin and CFA pain models were applied to the left foot sole under light anesthesia (2% isofluoran in 30%O_2/70%N_2O; 2-3 min). The left foot sole was injected with either sterile saline (50µl of 0.9% NaCl, Baxter; 90 min survival, n=5), capsaicin (50µl of 0.3% N-Vanillylnonanamide, Sigma-Aldrich, diluted in 80% saline, 10% Tween-80, and 10% ethanol-100%; 90 min survival, n=5), Complete Freund's Adjuvant (chronic inflammation model; CFA, 100µl; 90 min survival, n=5; 20 hrs survival, n=5; 4 days survival, n=5), or 4% paraformaldehyde (PFA diluted in 0.12 M phosphate buffer pH ≈ 7.5; 45 min survival, n=5; 90 min survival, n=5). For induction of neuropathic pain, the animals were kept under anesthesia during the whole procedure (20-30 minutes). We used the SNI model according to the protocol described in [11]. In short, the three branches of the sciatic nerve were exposed above the knee, and the tibial and common peroneal branches were ligated and cut 2 mm distal to the ligation, while the sural branch was left intact. In the sham model, the three sciatic branches were also exposed but then left intact. We used survival times of 2 hrs, 1 wk and 2 wks for the SNI model and their corresponding sham models (n=24). In the morphine experiment, the animals received 1.5 mg of morphine (morphine HCL-3H_2O) subcutaneously 20 min prior to injection of formalin (50µl of 4 % PFA) in the left foot sole (90 min survival, n=4). The control group received 1.5 ml of sterile saline instead of morphine (90 min survival, n=4).

2.2. Behavioral experiments

The mechanical thresholds of the hind paws were assessed using the Von Frey hair monofilaments (Stoelting) in the capsaicin, CFA, and SNI (1 wk and 2 wks) groups. Before the start of the experiments, all rats were habituated to the experimenter, the experiment room, and a transparent cage (15cm x 15cm; gridded floor) for 5 days. Thereafter, prior to each experiment the rats were habituated for 30 minutes to the experiment room, and for 10 minutes to the transparent cage. Each Von Frey hair was applied for 2 seconds at 5 seconds interval, and the threshold was set at 3 evoked responses in a maximum of 5 applications. The mechanical thresholds were assessed at 60 min after injection in the capsaicin group, at 20 hrs (CFA 20 hrs) or 4 days (CFA 4 days) in the CFA group, and at 1 wk (SNI 1 wk) and 2 wks (SNI 2 wks) in the SNI group. In the morphine experiment, the number of flinches and flutters and the time spent licking the injected paw was measured during 60 min after the formalin injection.

2.3. Tissue preparation

At the end of an experiment, the rats received an overdose of sodium pentobarbital, and were transcardially perfused with 150 ml saline followed by 750 ml of 4% PFA. Thereafter, the lumbar spinal cords were dissected and incubated overnight at 4 ºC in RNAse free solution consisting of phosphate buffer (PB), 4% PFA and 30% sucrose. Coronal sections were cut at 30 µm with a freezing microtome, collected in 9 separate jars and stored in glycerol at -20 ºC.

2.4. Fluorescent in situ hybridization combined with fluorescent immunohistochemistry

For fluorescent in situ hybridization (FISH), the partial cDNA templates encoding GlyT2 (3.1 kb; a generous gift from Dr. N. Nelson, Tel Aviv University), or GAD67 (3.2 kb; a generous gift from Dr. A.J. Tobin and N. Tillakaratne, PhD, UCLA) were used. The recombinant plasmids were linearized, and subsequently riboprobes were transcribed using the appropriate RNA polymerases in the presence of fluorescein-labeled 11-UTP (Roche). For FISH the protocol described in [19] was applied with the following modifications. Sections from a jar were incubated with a mixture of GAD67 and GlyT2 probes in order to identify spinal inhibitory neurons (GABAergic and/or glycinergic). After riboprobe hybridization, the sections were incubated (48 hours at 4 ºC) with mouse monoclonal anti-fluorescein antibody (Roche; 1:500) and rabbit anti-c-fos (1: 4000; Oncogene Research Products, La Jolla, CA) in a cocktail of phosphate buffered saline (PBS), 2% milk powder (Profitar Plus, Nutricia) and 0.5% Triton X-100. Thereafter, sections were rinsed in PBS and incubated with biotinylated goat-anti-mouse (Vector; for detection of fluorescein), and donkey-anti-rabbit tagged with Cy3 (Jackson) for detection of c-fos antibody in a 2% milk powder cocktail for 90 minutes at RT. Subsequently, sections were rinsed in PBS and incubated with Avidin-Biotin-Complex (ABC, Vector) tagged with horseradish peroxidase (HRP) for 90 min at RT. After rinsing in PBS, a tyramide amplification procedure was performed by reacting HRP with H_2O_2 and a self prepared FITC tyramide solution as described previously [18]. Thereafter, the sections were washed in PB, mounted on slides and cover slipped with Vectashield (Vector).

2.5. Analyzing labeled neurons

Analysis was carried out on sections from the L4 and L5 lumbar spinal segments. Sections for analysis were chosen by starting in the first row of the randomly mounted sections and searching for sections from the appropriate segmental level, i.e. from rostral L4 to caudal L5. Per rat, the first 5 or 6 sections were analyzed in a Leica fluorescent microscope with a FITC and/or Cy3 filter. Labeling for GlyT2/GAD67 mRNAs (FITC) was considered as neuronal labeling if the staining was present in the cell soma and the shortest diameter was at least 10µm. In order to investigate colocalization of c-fos (Cy3) with GlyT2/GAD67 mRNAs (FITC), first c-fos labeling was assessed in a 40x objective. Thereafter, in the same focus field, we assessed whether there was labeling for GlyT2/GAD67 mRNA pres-

ent in the cytoplasm. If in the same focus field, c-fos labeling was surrounded by somatic GlyT2/GAD67 mRNA staining, the neuron was counted as a double labeled neuron. Single c-fos labeled and c-fos/GlyT2/GAD67 double labeled neurons were plotted by hand in an illustrated representation of the appropriate segmental level. In this illustration, the grey matter was divided in 10 laminae according to the laminar distribution in the rat [31]. In order to correct cell counts for double counting error, we measured the shortest and largest diameter of the nuclei of c-fos labeled neurons and c-fos labeled Gly/GABA neurons in laminae I-II, III-VI and VIII-X. The average diameter of the nuclei was calculated by averaging the sum of the shortest and largest diameters, and we then performed corrections for sampling bias related to cell size using Abercrombie's formula as described previously [15].Per rat, the average numbers of c-fos and c-fos/Gly/GABA double labeled neurons, and the average percentages of double labeled neurons were calculated. Per group, the results were averaged and compared with the average results in the other groups. Errors in the variations were assessed as standard error of the mean (SEM). The unpaired *t*-test or one-way ANOVA with a Bonferroni post-hoc test was performed for statistical comparison between groups. $p<0.05$ was taken as significant.

3. Results

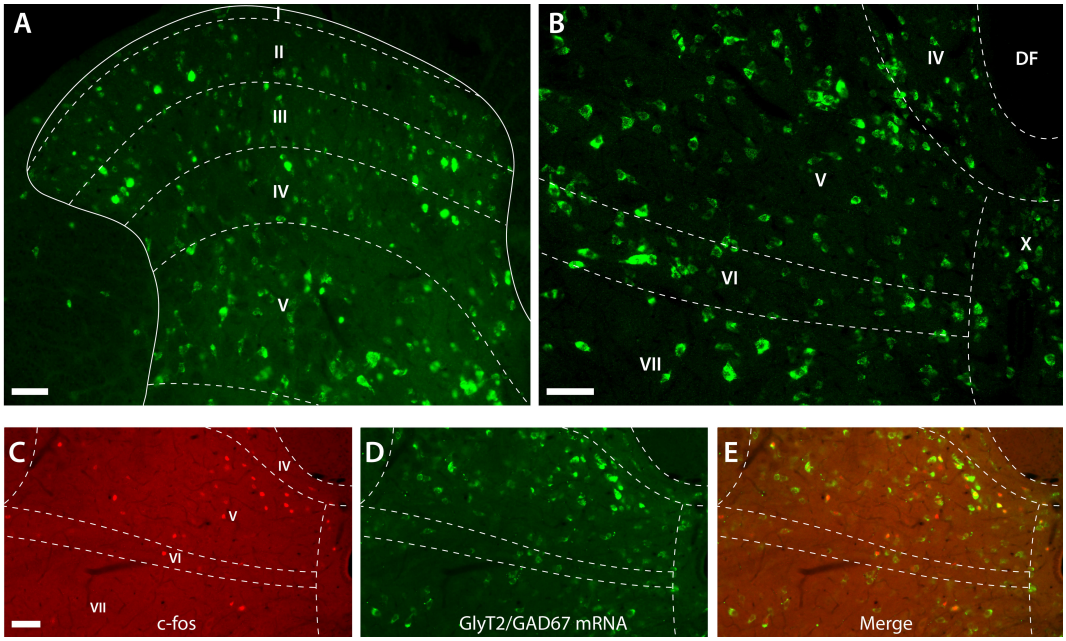
3.1. General observations

Fluorescent in situ hybridization (FISH) with a mixture of GlyT2/GAD67 probes resulted in cytoplasmic labeling of neurons that contained either GlyT2 mRNA, GAD67 mRNA or both mRNAs, thus representing neurons that use glycine and/or GABA as neurotransmitter(s) (Gly/GABA neurons) (Fig. 1A, B). In the superficial dorsal horn we observed labeled neurons that were relatively small in size. These neurons contain most likely GABA and not glycine since glycinergic neurons are scarce in laminae I and II [19,46]. Labeled neurons in the deep dorsal and ventral horn were mostly medium to large in size, representing neurons using glycine and/or GABA as neurotransmitters [47].

Fluorescent immunohistochemistry (IHC) for c-fos resulted in labeling of nuclei with various intensities (Fig. 1C). In the spinal cords of naïve rats c-fos labeled neurons were rare, while many were present in the spinal cords of rats that received a nociceptive stimulus. Most c-fos labeled neurons were located ipsilateral to the stimulus in the superficial (laminae I-II) and deep (III-VI) dorsal horn and much less in the ventral horn (VII-X). When FISH was combined with IHC, double labeled neurons were observed in which the labeling for c-fos (reddish nucleus) was surrounded by the labeling for GlyT2/GAD67 mRNA (greenish cytoplasm) (Fig. 1C, D, and E).

Fig. 1. Fluorescence micrographs of lumbar spinal sections showing the distribution of neurons labeled for GlyT2 and/or GAD67 mRNAs, and/or neurons labeled for c-fos protein after stimulation with formalin (45 min). (A and B) Note that labeled neurons in the superficial layers, which are mostly small and somewhat difficult to see at this magnification, are predominantly GABAergic neurons, while small and large labeled neurons

in the deeper laminae are glycinergic and/or GABAergic. (C, D and E) Labeling for c-fos protein in nuclei of neurons in the deep dorsal horn after stimulation with formalin (45 min) combined with labeling for GlyT2 and/or GAD67 mRNAs. Note that double labeled neurons are yellow. Roman figures indicate laminae; DF: dorsal funiculus; Scale bar: 50 µm.



3.2. Capsaicin, formalin, CFA and SNI pain models

The effects of nociceptive stimulations were assessed by behavioral analysis using Von Frey hair monofilaments. As expected, we found that the mechanical threshold (grams) was significantly decreased in the capsaicin (33 g before treatment, 3 g after capsaicin, $p<0.001$), chronic inflammation (CFA; 37 g before treatment, 5 g after CFA 1.5 hrs, $p<0.005$; 7 g after CFA 20 hrs, $p<0.005$; 10 g after CFA 4 days, $p<0.01$) and neuropathic pain (SNI; 27 g before treatment, 2 g after SNI 1 wk, $p<0.005$; 1 g after SNI 2 wks, $p<0.005$) groups as compared to the mechanical threshold of the same paw before treatment. In the sham-SNI groups we did not find a significant decrease in the mechanical threshold (27g before treatment, 20 g after sham-SNI 1wk, $p>0.05$; 19 g after sham-SNI 2 wks, $p>0.05$)

3.3. c-fos labeling pattern

In naïve unstimulated rats we found on average a total 0.5 ± 0.2 c-fos labeled neurons per section. In view of this low number, we did not further investigate the colocalization of c-fos in Gly/GABA neurons in naïve rats. Stimulation with capsaicin or formalin induced significantly higher average numbers of c-fos labeled neurons per section than stimulation with saline (control) (Fig. 2). In the CFA pain model, there were no significant differences between the numbers of c-fos labeled neurons obtained at different survival times (ANOVA). In the SNI pain model there were no significant differences between the

number of c-fos labeled neurons at 1 wk (18 ± 2.8) or 2 wks (14 ± 1.9) after nerve injury, and they were significantly lower (p<0.001, ANOVA) than at 2 hrs after nerve injury (Fig. 2). When considering all pain models (Fig. 2), the highest numbers of c-fos labeled neurons were found 90 minutes after stimulation wit formalin (53 ± 4) and at 2 hrs after spared nerve injury (61 ± 3.6).

With respect to the laminar distribution (Table 1A), c-fos labeled neurons were approximately equally abundant in laminae I&II and in laminae III-VI in the acute pain models (after saline, capsaicin, formalin) and in the acute phases of inflammation and neuropathic pain. However, in the chronic phases of inflammation (CFA 20 hrs and 4 days) and neuropathic pain (1 wk and 2 wks) the bulk of c-fos labeled neurons was present in laminae III-VI (Table 1A). In all pain models, laminae VII-X contained the lowest numbers of c-fos labeled neurons.

3.4. The number of c-fos labeled Gly/GABA neurons

In the saline, capsaicin and formalin models, the average number of c-fos labeled Gly/GABA neurons per section was significantly higher after stimulation with capsaicin (11 ± 1.1), 45 and 90 minutes after formalin injection (15 ± 0.8 and 21 ± 1.7, respectively) when compared to saline (4 ± 0.3, control) (Fig. 3, Fig. 4 and Fig. 5). These findings indicate that a saline injection, which served as control, also induced c-fos expression in spinal Gly/GABA neurons.

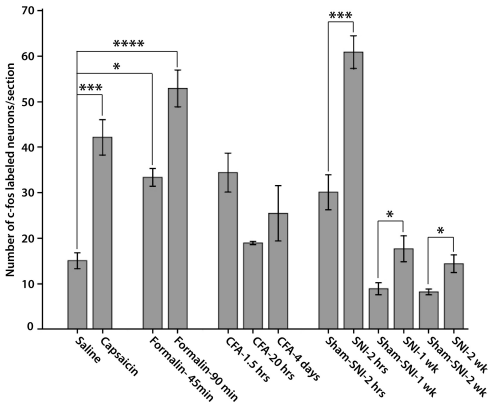


Fig. 2. Histogram showing the average number of c-fos labeled neurons per section in lumbar spinal cord in various rat pain models and their controls. Capsaicin, 45 and 90 minutes after formalin injection induced significantly higher numbers of c-fos labeled neurons than the control stimulation with saline (ANOVA). The SNI models induced significantly higher numbers of c-fos labeled neurons than their corresponding sham models (unpaired t-test). * = p<0.05; ** = p<0.01; *** = p<0.005; **** = p<0.001.

In the CFA inflammation model there were no significant differences between the numbers of c-fos labeled Gly/GABA neurons at 1.5 hrs (10 ± 2.7), 20 hrs (10 ± 0.4) and 4 days (10 ± 2.3) after injection of CFA (Fig. 4 and Fig. 5). In the SNI model, the numbers of c-fos labeled Gly/GABA neurons were higher in nerve injured than in the corresponding sham-SNI operated animals (controls) at all time points (Fig. 4). Further, a significantly (p<0.001, ANOVA) higher number of c-fos labeled Gly/GABA neurons was found at 2 hrs after nerve injury (25 ± 3.1) than at 1 wk (8 ± 1.2) or 2 wks (8 ± 1.4). Note that the higher

number at 2 hrs after nerve injury is partly explained by the contribution of the operation procedure, as shown by the relatively high number of activated Gly/GABA neurons in the sham operated animals (Fig. 4).

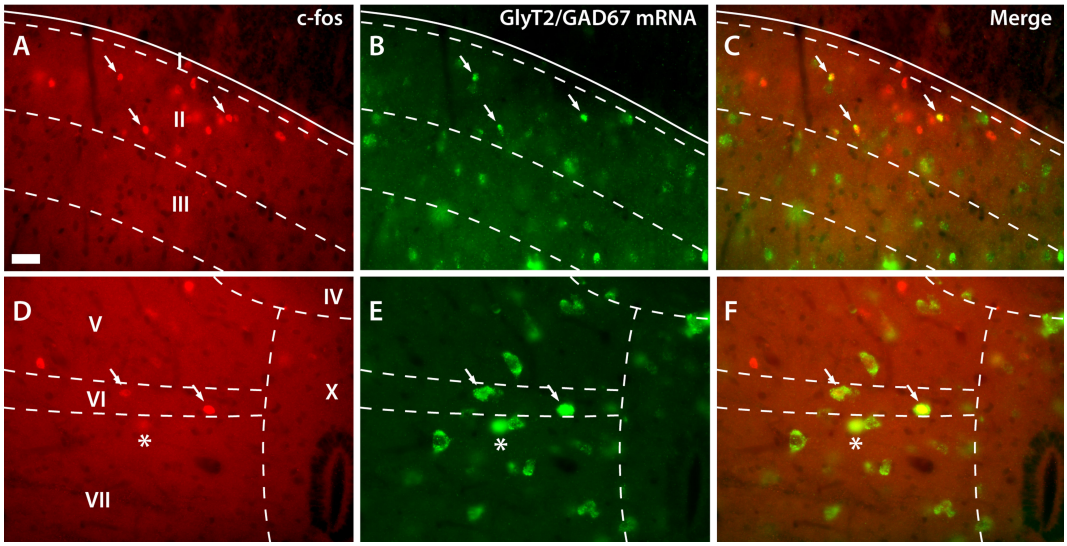


Fig. 3. Fluorescence micrographs from lumbar spinal sections showing labeling for c-fos protein and GlyT2/GAD67 mRNAs. Arrows indicate c-fos labeled neurons that also contained GlyT2 and/or GAD67 mRNAs. (A, B and C) Superficial dorsal horn at caudal L4 level of a rat stimulated with capsaicin. (D, E and F) Deep dorsal horn at caudal L5 level of a rat with chronic inflammation (CFA 4 days). Asterisk indicates a c-fos labeled neuron that was out of focus but also contained GlyT2 and/or GAD67 mRNA. Scale bar: 50 µm.

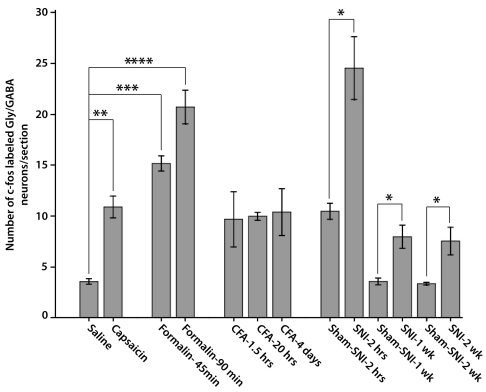


Fig. 4. Histograms showing the average number of c-fos labeled neurons per section that also contained GlyT2 and/or GAD67 mRNAs (Gly/GABA neurons) in different rat pain models. Capsaicin, 45 and 90 minutes after formalin injection induced significantly higher numbers of c-fos labeled Gly/GABA neurons than the control stimulation with saline (ANOVA). The SNI models induced significantly higher numbers of c-fos labeled neurons than their corresponding sham models (unpaired t-test). * = p<0.05; ** = p<0.01; *** = p<0.005; **** = p<0.001.

When comparing the number of c-fos labeled Gly/GABA neurons in all the pain models, there were no significant differences (p>0.05, ANOVA) between the capsaicin (11 ± 1.1) , CFA 1.5 hrs (10 ± 2.7), CFA 20 hrs (10 ± 0.4), CFA 4 days (10 ± 2.3), SNI 1 wk (8 ± 1.2) and SNI 2 wks (8 ± 1.2) pain models (Fig. 4, Fig. 5). Further, SNI 2 hrs (25 ± 3.1) induced significantly the highest number of c-fos labeled Gly/GABA neurons as compared to all other pain models (p<0.05, ANOVA), except for 90 minutes after formalin injection. When considering the distribution pattern of c-fos labeled Gly/GABA neurons, in most pain models the majority of the labeled neurons were located in the deep dorsal horn (III-VI), and with

lower percentages in the superficial dorsal horn and in the ventral horn (Table 1B).

	A			B		
	Laminae I-II	Laminae III-VI	Laminae VII-X	Laminae I-II	Laminae III-VI	Laminae VII-X
Saline	39 ± 8	47 ± 7	14 ± 2	20 ± 10	57 ± 12	23 ± 6
Capsaicin	45 ± 4	43 ± 3	12 ± 2	14 ± 4	57 ± 5	29 ± 7
Formalin-45	36 ± 3	54 ± 4	10 ± 1	27 ± 3	59 ± 5	13 ± 2
Formalin-90	35 ± 2	51 ± 1	14 ± 1	23 ± 2	56 ± 1	22 ± 2
CFA-1.5 hrs	52 ± 11	32 ± 4	16 ± 7	30 ± 8	40 ± 4	30 ± 10
CFA-20 hrs	10 ± 4	61 ± 1	29 ± 3	2 ± 1	59 ± 1	39 ± 2
CFA-4 days	14 ± 4	61 ± 2	25 ± 2	10 ± 2	65 ± 3	25 ± 4
Sham-SNI 2 hrs	49 ± 3	38 ± 1	13 ± 3	37 ± 4	41 ± 6	22 ± 6
SNI-2 hrs	37 ± 1	42 ± 2	21 ± 1	34 ± 2	40 ± 4	26 ± 3
Sham-SNI 1 wk	19 ± 2	64 ± 4	17 ± 4	16 ± 2	61 ± 4	23 ± 5
SNI-1 wk	17 ± 4	70 ± 4	13 ± 1	13 ± 5	74 ± 6	13 ± 1
Sham-SNI 2 wks	19 ± 3	63 ± 3	18 ± 4	13 ± 2	60 ± 3	27 ± 5
SNI-2 wks	14 ± 2	71 ± 2	15 ± 4	7 ± 0.4	80 ± 4	13 ± 5

Table 1. Laminar distribution of c-fos labeled and c-fos labeled Gly/GABA neurons in the various pain models. (A, B) Percentages (± SEM) of the total number of c-fos labeled (A) or c-fos labeled Gly/GABA (B) neurons that were located in laminae I-II, III-VI or VII-X are shown for each pain model. Note that the majority of c-fos labeled and c-fos labeled Gly/GABA neurons were located in the deep dorsal horn (laminae III-VI), especially in the chronic pain models.

3.5. The percentage of c-fos labeled neurons that were Gly/GABA

After calculating the average percentages of c-fos labeled neurons that were double labeled with Gly/GABA in all the pain models, we found that in the chronic phase of the CFA, sham-SNI and SNI models, there were no significant differences (p>0.05, ANOVA) between the percentages obtained for the two chronic survival times in each model. Since we were interested in possible differences between the acute and chronic phases, we

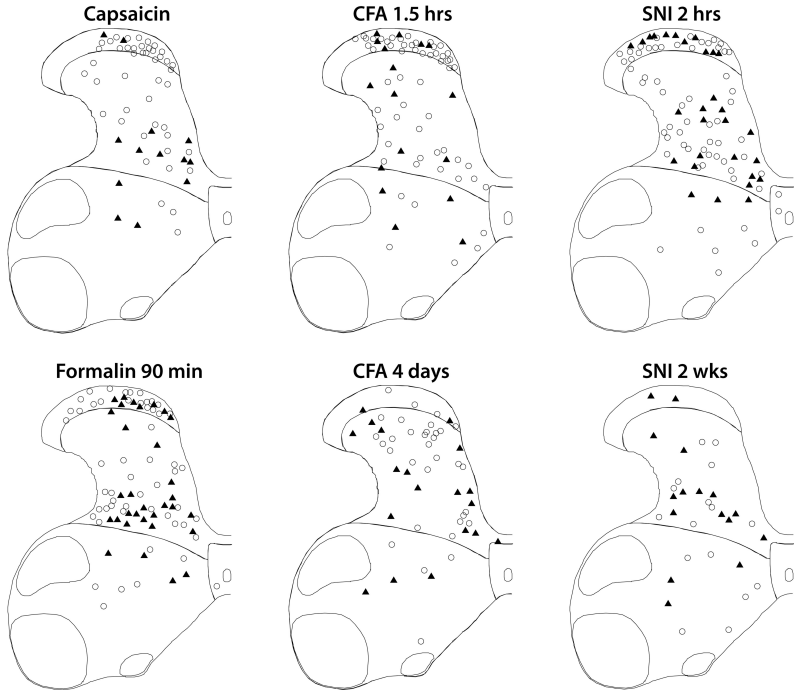


Fig. 5. Drawings showing the spinal distribution of c-fos labeled and c-fos labeled Gly/GABA neurons in randomly chosen single sections from the lumbar spinal cord, each from a different pain model. An open circle represents a single c-fos labeled neuron, and each filled triangle represents a single c-fos labeled neuron that also contained GlyT2/GAD67 mRNAs (Gly/GABA). 90 min after formalin injection and 2 hrs after nerve injury induced the highest numbers of c-fos labeled Gly/GABA neurons. Note that in most pain models, the bulk of c-fos labeled Gly/GABA neurons were located in the deep dorsal horn (laminae III-VI; see also table 1B). In most pain models, few c-fos labeled Gly/GABA neurons were located in the ventral horn (laminae VII-X).

combined in each group the chronic phases (i.e. 20 hrs + 4 days in the CFA model; 1wk + 2wks in the sham-SNI and SNI models). We then compared the result for the chronic phase with that of the acute phase in the different models (Fig. 6). This showed that in the CFA and SNI models the percentages of c-fos labeled neurons that were Gly/GABA were significantly higher in the chronic phases as compared to their acute phases. Also in the formalin model there was no significant difference between the two survival times. We therefore combined the two results and found that the percentage of c-fos labeled neurons that were Gly/GABA was significantly higher in the formalin model as compared to the saline and capsaicin models (Fig. 6).

We then averaged the percentages of all the chronic phases in the various models (i.e. CFA 20 hrs and 4 days; sham-SNI and SNI 1 wk and 2 wks), and compared it with the average percentages of all the acute phases (saline, capsaicin, formalin, CFA 1.5 hrs, sham-SNI and SNI 2 hrs). As a result we found that in the chronic phase the overall average percentage of the c-fos labeled neurons that were Gly/GABA was 46% (± 1.5), which is significantly higher (p<0.0005; unpaired t-test) than 34% (± 2), the overall percentage obtained for the acute phase.

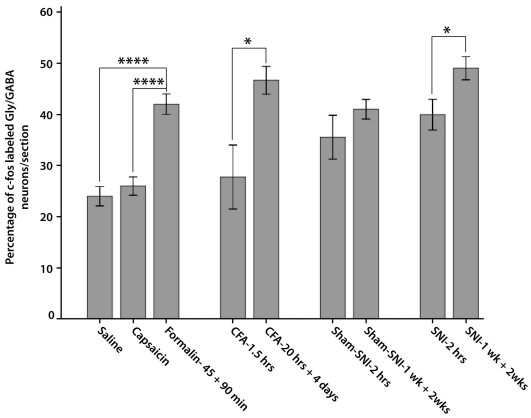


Fig. 6. Histogram showing the average percentages of c-fos labeled neurons per section that also contained GlyT2/GAD67 mRNA (Gly/GABA). A significantly higher percentage was found in the combined formalin (45 + 90 min) models as compared to the saline and capsaicin models (ANOVA). Note that the percentages of c-fos labeled neurons that were Gly/GABA in the chronic phases of the CFA (20 hrs + 4 days), and SNI (1 wk + 2 wks) models were significantly higher than the corresponding acute phases (unpaired t-test). * = p<0.05; **** = p<0.001.

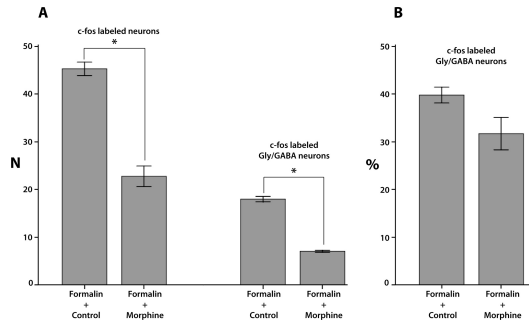


Fig. 7. Histograms showing the average numbers (A) or percentages (B) of c-fos labeled neurons after formalin stimulation in rats pretreated with saline (control, 20 min) or morphine (20 min). (A) The average number per section of c-fos labeled neurons or the average number of c-fos labeled neurons containing GlyT2/GAD67 mRNA (Gly/GABA) after formalin stimulation. (B) The percentage of c-fos labeled neurons that were Gly/GABA. Note that despite the decrease in the total number of c-fos labeled Gly/GABA neurons (A), the percentage of c-fos labeled neurons that were Gly/GABA did not change significantly when the rats were pretreated with morphine. *: p<0.05

3.6. Effects of morphine application in the formalin model

In order to determine the effect of morphine on the number of c-fos labeled Gly/GABA neurons we used the formalin pain model. The behavioral analysis confirmed the notion that morphine significantly reduced the licking time (saline: 630 sec ± 77, morphine: 71 s ± 12, $p<0.005$, unpaired *t*-test) and the number of fluttering and flinches (saline: 676 ± 61, morphine: 16 ± 5, $p<0.005$, unpaired *t*-test) of the injected paw. After formalin stimulation, the total number of c-fos labeled neurons and the number of c-fos labeled Gly/GABA neurons decreased by subcutaneous morphine application when compared to control (Fig. 7A). When considering the percentage of c-fos labeled neurons that were Gly/GABA, we found no difference between the percentages of the control and the morphine treated groups (Fig. 7B).

4. Discussion

In the present study we have shown that Gly/GABA neurons are activated (i.e. expressing c-fos protein) in all the pain models that we have investigated. Our data further showed that the percentage of activated neurons that are Gly/GABA is higher in the chronic phase (46%) than in the acute phase (34%). Furthermore, the systemic application of morphine, preceding formalin injection, reduced the activation of Gly/GABA neurons and non-Gly/GABA (presumed excitatory) neurons in an equal manner. These findings show that active recruitment of Gly/GABA neurons is inherent to the normal and pathological processing of nociceptive stimuli in the spinal cord.

4.1. Technical aspects

FISH was used to identify glycinergic neurons by their expression of glycine transporter 2 (GlyT2) mRNA [23,28], and GABAergic neurons were identified by their expression of the mRNA for the GABA synthesizing enzyme (GAD) [13]. The GAD67 isoform of this enzyme is found in the large majority of spinal GABAergic neurons, often together with the GAD65 isoform [30]. A few neurons in the ventral horn, involved in presynaptic inhibition of 1A afferents only express the GAD65 isoform [20]. These neurons have not been identified in the present study, but it is highly unlikely that this has significantly affected our results. GABA and glycine are often colocalized in neurons [43,47] and co-released at synapses [22]. Therefore, we did not attempt to identify glycinergic and GABAergic neurons separately but rather aimed at labeling them simultaneously.

For identifying spinal neurons that were activated in various pain models we have used c-fos, an immediate early gene (IEG) that is widely used as a marker for neuronal activation in pain research [8]. Our results on the expression of c-fos in various pain models are in general agreement with other studies [6,21,29]. Application of GABA and glycine antagonists in naïve animals leads to a hypersensitive spinal cord [38,39]. This indicates the presence of a continuous inhibitory tone, which is most likely due to activity in nearby Gly/GABA neurons [1]. Apparently, the activity of these neurons does not induce significant

amounts of c-fos protein, since labeled c-fos neurons are very low in naïve animals. Thus, c-fos expression in spinal neurons signals phasic activity associated with active nociceptive processing rather than tonic activity associated with the presumed ongoing inhibition in the naïve spinal cord. Therefore, the results obtained in the present study are associated with the processing of phasic nociceptive stimuli in the different pain models.

4.2. c-fos expressing Gly/GABA neurons

In all our pain models, we found that a substantial proportion (ranging between 24 and 53%) of the activated neurons expressed glycine and/or GABA. It seems likely that this activation was induced by nociceptive afferents, which are known to contact Gly/GABA neurons [5,10,14,16], although indirect activation through interneurons cannot be excluded. Previous studies [46,54,55] using different techniques have also shown the activation of spinal inhibitory neurons by means of c-fos expression after capsaicin or formalin stimulation. Our data are in general agreement with the study by Todd et al. [46], however we found a lower percentage of activated GABAergic neurons in the superficial dorsal horn after capsaicin stimulation when compared to the studies by Zou et al [54,55].

In our study, capsaicin induced a significant increase in the number of Gly/GABA activated neurons, when compared to saline. However, the percentages of activated Gly/GABA neurons after saline (24%) and capsaicin (26%) stimulation were similar. This shows that in this paradigm, irrespective of the stimulus intensity (i.e. low after saline and high after capsaicin), the number of activated Gly/GABA neurons is proportional to the total number of activated neurons. In the formalin model, which typically shows a first and second phase in the behavioral response [44], the total number of activated neurons is higher at 90 min after formalin injection than at 45 min. Also in this case the percentages of activated Gly/GABA neurons were not significantly different at the two time points, although, when combined, it was higher than after saline or capsaicin injections. These results suggest that in a given pain model the proportion of activated neurons that are Gly/GABA remains stable, irrespective of the stimulus intensity. However, when we examined the CFA experiments we found the reverse situation: the total number of activated Gly/GABA neurons was similar at the different time points (i.e. 1.5 hrs, 20 hrs and 4 days), while the percentage of activated Gly/GABA neurons was higher in the chronic phase than at 1.5 hrs. In the SNI experiments, the total number of activated Gly/GABA neurons declined significantly in the chronic phase, while there was a significant increase in the percentage. In the sham-SNI group, in which the operation procedure by itself resulted in a significant number of c-fos expressing neurons, the percentage did not increase in the chronic phase as compared to the acute phase. We therefore concluded that there was no consistent pattern of Gly/GABA neuronal activation when comparing the different pain models. However, within a particular pain model there were consistencies in the numbers or percentages of activated Gly/GABA neurons.

We then examined the data from another viewpoint by subdividing all pain models in acute (≤ 2 hrs) and chronic (≥ 20 hrs) phases. Our data showed that in the chronic phase the percentage of activated Gly/GABA neurons (46%) was higher than in the acute phase (34%). This increase in the percentage in the chronic phases was due to a decrease in the total number of activated neurons while the number of activated Gly/GABA neurons remained stable. Assuming that the majority of the non-Gly/GABA activated neurons were excitatory, our data show a decline in the number of activated excitatory neurons in the chronic phase.

Hypersensitivity in chronic pain conditions has been shown to result from loss of spinal inhibition due to blockade of glycinergic and GABAergic receptors [53]. At the same time, our data indicate an increased activity of inhibitory neurons in chronic pain conditions. Whether the balance between these apparently opposing mechanisms determines the sensitivity of spinal neurons for incoming nociceptive stimuli, or whether they act on different aspects of pain transmission, is presently unclear.

4.3. Laminar distribution of c-fos labeled Gly/GABA neurons

Activated Gly/GABA neurons in the superficial dorsal horn are mainly GABAergic [19,46], while activated Gly/GABA neurons in the deep dorsal horn are glycinergic and/or GABAergic [47]. Deeper located Gly/GABA neurons have been suggested as a source of inhibitory input to the superficial dorsal horn [51], which may be lost in chronic pain states, leading to touch evoked allodynia. There is also loss of GABAergic inhibition in the superficial dorsal horn in chronic neuropathic pain states, which may be due to loss of GABAergic interneurons [32,34,35]. Our data showed that the number of activated Gly/GABA neurons in the deep dorsal horn remained stable over time in the chronic phase, while the numbers of presumed excitatory neurons were declining. Thus, our results on activated Gly/GABA neurons do not indicate loss of inhibition in chronic pain states due to decreased activation of spinal Gly/GABA neurons.

It has been suggested that GABAergic neurons in the superficial dorsal horn receive C-fiber input [16], and are important for regulating the spinal transmission of innocuous and nociceptive information, especially during acute pain [10]. Our data confirm the presence of activated Gly/GABA neurons in the superficial dorsal horn, but their numbers are low when compared to the number of activated Gly/GABA neurons in the deep dorsal horn.

4.4. The morphine experiment

After a systemic morphine injection preceding a formalin injection to the hind paw, there was a significant reduction in the total number of c-fos labeled neurons and a proportional decrease in the number of c-fos labeled Gly/GABA neurons. It seems most

likely that most inhibition induced by morphine is due to presynaptic inhibition of primary afferents expressing mu-opioid receptors [3]. Postsynaptic mu-opioid receptors in the superficial dorsal horn are expressed preferentially by neurons, mainly in laminae II, that are not GABAergic or glycinergic [25]. This would suggest that inhibitory neurons escape postsynaptic inhibition by morphine. However, our finding of an equal reduction of c-fos expression in Gly/GABA and non-Gly/GABA (presumed excitatory) neurons, indicates that the overall effect of the pre- and postsynaptic inhibition induced by morphine is about equally strong on inhibitory and excitatory neurons. Similarly, the suggestion [7] that morphine would activate Gly/GABA neurons, especially GABAergic neurons in lamina II, is not supported by our findings. Thus, the proportional decrease in the number of c-fos labeled Gly/GABA neurons after morphine injection shows that morphine did not specifically recruit spinal Gly/GABA neurons for the inhibition of nociceptive inputs.

4.5. Conclusions

A characteristic feature of the pain models that we have examined is the development of hypersensitivity for (in)nocuous stimuli. This phenomenon is due to glutamate induced sensitization in spinal neurons [27,49], along with a blockade [53] or reversal [9] of inhibitory impulses. We now show that similar numbers of Gly/GABA neurons are activated by nociceptive stimuli during acute and chronic pain states, while in chronic pain states the activation of presumed excitatory neurons is declining. Therefore it seems most likely that the balance between all these mechanisms, and the disturbances therein during pathological pain states, will determine the sensitivity of our pain system during health and disease.

Conflict of Interest Statement:

The authors have no conflict of interest that could compromise the conduct of this study or the reporting of the results.

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Chapter IV

C-fos activation of spinal inhibitory neurons after contralateral hindpaw stimulation of rats with inflammatory or neuropathic pain

Hossaini M[1], Saraç C[1], Jongen JL[1,2], Holstege JC[1]

[1]Dep. of Neuroscience, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands; [2]Dep. of Neurology, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands

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Chapter V

Distribution of GABA and glycine containing RVM neurons that project to the spinal cord and vice versa

Hossaini M[1], Goos JAC[1], Kohli S[1], Holstege JC[1]

[1]Dep. of Neuroscience, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands

Manuscript submitted

Chapter VI

Nociceptive stimulation induces expression of Arc/Arg3.1 in the spinal cord with a preference for neurons containing enkephalin

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Nociceptive stimulation induces expression of Arc/Arg3.1 in the spinal cord with a preference for neurons containing enkephalin

Mehdi Hossaini[1], Joost LM Jongen[2,1], Karla Biesheuvel[1], Dietmar Kuhl[3], Jan C Holstege[1*]

[1]Dep. of Neuroscience, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands; [2]Dep. of Neurology, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands; [3]Institute for Molecular and Cellular Cognition, Center for Molecular Neurobiology (ZMNH), University Medical Center Hamburg-Eppendorf, Falkenried 94, 20251 Hamburg

Hossaini *et al. Molecular Pain* 2010, **6**:43
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Nociceptive stimulation induces expression of Arc/Arg3.1 in the spinal cord with a preference for neurons containing enkephalin

Mehdi Hossaini[1], Joost LM Jongen[2,1], Karla Biesheuvel[1], Dietmar Kuhl[3], Jan C Holstege[1*]

Abstract

Background: In pain processing, long term synaptic changes play an important role, especially during chronic pain. The immediate early gene Arc/Arg3.1 has been widely implicated in mediating long-term plasticity in telencephalic regions, such as the hippocampus and cortex. Accordingly, Arc/Arg3.1 knockout (KO) mice show a deficit in long-term memory consolidation. Here, we identify expression of Arc/Arg3.1 in the rat spinal cord using immunohistochemistry and in situ hybridization following pain stimuli.

Results: We found that Arc/Arg3.1 is not present in naïve or vehicle treated animals, and is *de novo* expressed in dorsal horn neurons after nociceptive stimulation. Expression of Arc/Arg3.1 was induced in an intensity dependent manner in neurons that were located in laminae I (14%) and II (85%) of the spinal dorsal horn. Intrathecal injection of brain derived neurotrophic factor (BDNF) also induced expression of Arc/Arg3.1. Furthermore, 90% of Arc/Arg3.1 expressing neurons also contained the activity marker c-Fos, which was expressed more abundantly. Preproenkephalin mRNA was found in the majority (68%) of the Arc/Arg3.1 expressing neurons, while NK-1 was found in only 19% and GAD67 mRNA in 3.6%. Finally, pain behavior in Arc/Arg3.1 KO mice was not significantly different from their wild type littermates after application of formalin or after induction of chronic inflammatory pain.

Conclusions: We conclude that Arc/Arg3.1 is preferentially expressed in spinal enkephalinergic neurons after nociceptive stimulation. Therefore, our data suggest that Arc/Arg3.1 dependent long term synaptic changes in spinal pain transmission are a feature of anti-nociceptive, i.e. enkephalinergic, rather than pro-nociceptive neurons.

Background

The experience of pain is usually initiated by the activation of nociceptors, which are the peripheral terminations of nociceptive ganglion neurons. The central projections of these neurons enter the dorsal horn of the spinal cord to terminate on second order neurons [1]. After strong nociceptive stimulation these neurons may show an enhanced responsiveness to afferent inputs, which may last for several hours [2-4]. The mechanism underlying this enhanced responsiveness is similar to that of long-term potentiation (LTP) [5], which is a form of activity dependent plasticity that has been investigated extensively in other parts of the CNS, especially in the hippocampus [6]. Another form of activity dependent plasticity is long-term depression (LTD), a state of decreased sensitivity of neurons. Whether LTP or LTD is produced in the spinal nociceptive system depends on many variables, including the type of activity in nociceptive afferents [2]. For long term changes to become persistent it is essential that activity regulated genes, including immediate early genes (IEG), orchestrate a cascade of transcriptions and subsequent protein synthesis [7]. The first IEG that was found to be strongly increased in spinal neurons after a nociceptive stimulus is c-Fos [8]. This IEG is now widely used for the identification of activated nociceptive neurons [9]. Other IEGs that have been implicated in plastic changes are c-Jun, Jun-d, Krox 24 and Homer 1a [10,11]. Recently it has become clear that in cortex, hippocampus and other higher brain centers, the IEG named Arc/Arg3.1 (activity regulated

* Correspondence: j.holstege@erasmusmc.nl
[1]Dept. of Neuroscience, Erasmus University Medical Center, PO Box 2040, 3000 CA, Rotterdam, The Netherlands

Hossaini *et al. Molecular Pain* 2010, **6**:43
http://www.molecularpain.com/content/6/1/43

cytoskeleton-associated protein/activity regulated gene 3.1) plays a crucial role in activity dependent synaptic plasticity [12]. Moreover, Arc/Arg3.1 is critically involved in processes essential for synaptic structural rearrangement such as LTP, LTD and homeostatic scaling of AMPA receptors [13,14]. These mechanisms are also essential in spinal processing [15], and dysfunctional forms of activity dependent plasticity such as LTP and LTD that lead to persistent changes in neuronal sensitivity, may underlie chronic pain disorders [16]. Therefore, in this study we set out to investigate the role of Arc/Arg3.1 in nociceptive processing in the spinal cord.

Our findings show that Arc/Arg3.1 is not expressed at detectable levels in naïve spinal cord. However, after peripheral nociceptive stimulation we found *de novo* expression of Arc/Arg3.1 in a limited number of neurons in the superficial dorsal horn, depending on the type of stimulus. Further, Arc/Arg3.1 is predominantly expressed in spinal interneurons located in lamina II and many of these neurons also contain the opioid neurotransmitter enkephalin. Finally, we found that the pain behavior in Arc/Arg3.1 knockout (KO) mice after nociceptive stimuli was not significantly different from their wild type (WT) littermates.

Results

General observations

In the spinal cord of naïve rats and mice there was no detectable expression of Arc/Arg3.1 mRNA or protein when using in situ hybridization (ISH) and immunohistochemistry (IHC), respectively. However, after application of a peripheral nociceptive stimulus to the hind paw, Arc/Arg3.1 was expressed in a limited number of cells in the superficial layers of the lumbar dorsal horn. ISH using the alkaline phosphatase (AP) reaction produced a bluish/brownish reaction product in the cytoplasm and in some occasions in the nucleus and primary dendrites of Arc/Arg3.1 mRNA expressing neurons (Fig. 1A,B). Arc/Arg3.1 protein, visualized by bright field IHC, was present primarily in the cytoplasm, occasionally combined with nuclear labeling or labeling in proximal dendrites (Fig. 1C). Applying fluorescent IHC for Arc/Arg3.1 protein produced very similar labeling characteristics. In order to ascertain that Arc/Arg3.1 is expressed in neurons rather than in glial cells, we combined FISH for Arc/Arg3.1 mRNA with fluorescent IHC for NeuN, which is a specific marker for neuronal cells (Fig. 1D). It was found that 95% ± 1.3 (SEM) of the cells expressing Arc/Arg3.1 mRNA also expressed NeuN (99% ± 0.4 for

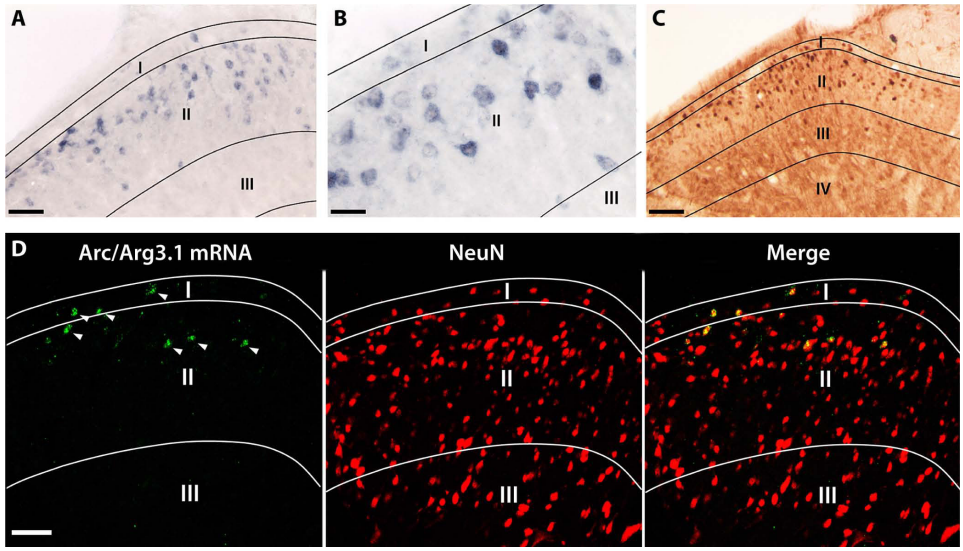

Figure 1 Arc/Arg3.1 is only expressed in the superficial dorsal horn. Light micrographs showing the distribution of neurons expressing Arc/Arg3.1 mRNA (***A, B***) or protein (***C***) in the rat spinal dorsal horn after peripheral stimulation with 25% mustard oil gauze wrapped around one hind paw for 2 hours. Note that many of the Arc/Arg3.1 labeled neurons are located in laminae I&II, and very few labeled neurons are present below lamina II. ***D,*** Fluorescent micrograph showing the expression of Arc/Arg3.1 mRNA and its colocalization with the neuronal marker NeuN. Arrowheads indicate Arc/Arg3.1 mRNA and NeuN double labeled neurons. Scale bar: 50 μm (***A*** and ***D***); 25 μm (***B***); 100 μm (***C***).

25% MO/1 h, n = 4; 95% ± 3.3 for 25% MO/2 hrs, n = 5; 94% ± 2.8 for CFA for 1.5 hrs, n = 4).

For both ISH and IHC, we observed that the intensity of the labeled neurons varied in a single section. We did not observe any labeling indicative of localization of Arc/Arg3.1 in intermediate or distal dendrites. Although labeling patterns obtained with ISH and IHC were identical, labeling efficiency was higher for ISH than for IHC. Therefore, ISH was used for the quantification of neurons expressing Arc/Arg3.1. The specificity of our detection techniques was assessed by omitting the probes/primary antibodies in the ISH and IHC procedures, respectively, and by applying ISH and IHC on spinal tissue of Arc/Arg3.1 KO mice. These experiments did not show any labeling in the spinal cord. ISH performed on cortex of naïve rats showed Arc/Arg3.1 mRNA labeling in the cortex and the hippocampus as previously reported [17].

Distribution and quantification of Arc/Arg3.1 mRNA expressing neurons in the spinal cord following nociceptive stimulation

Several types of nociceptive stimuli applied to the hind paw induced Arc/Arg3.1 mRNA expressing neurons on the ipsilateral side (Fig. 2) but not on the contralateral side of the lumbar superficial dorsal horn. A single subcutaneous injection of capsaicin resulted in the lowest average number of labeled neurons per section (2.6 ± 0.6 SEM, n = 6), while wrapping the paw in a gauze soaked with 25% mustard oil (MO) for 2 hrs induced the highest number of neurons (50 ± 3.5 SEM, n = 5). On average, lamina II accounted for 85% ± 3.5 of the labeled neurons, while lamina I (14% ± 3.2) and III (0.6% ± 0.4) contained the remaining labeled neurons. The other laminae very rarely contained labeled neurons.

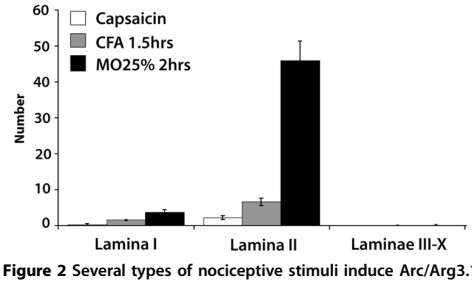

Figure 2 Several types of nociceptive stimuli induce Arc/Arg3.1 mRNA in the ipsilateral superficial dorsal horn. Histogram showing the number of Arc/Arg3.1 mRNA labeled neurons after each specific nociceptive stimulus. Error bars represent ± SEM.

Expression of Arc/Arg3.1 mRNA following nociceptive stimulation occurs in a subset of c-Fos labeled neurons and is intensity dependent

The number of neurons expressing the neuronal activation marker c-Fos or Arc/Arg3.1 mRNA was counted in separate sections treated with IHC or ISH, respectively. c-Fos labeled neurons outnumbered Arc/Arg3.1 mRNA labeled neurons (Fig. 3A), except after 2 hrs mustard oil stimulation when about equal number of neurons were labeled. FISH and fluorescent IHC were applied to simultaneously visualize Arc/Arg3.1 mRNA and c-Fos protein, respectively (Fig. 3B). When data from the 25% mustard oil and the CFA groups were taken together (Fig. 3C), 90% ± 6.8 of the Arc/Arg3.1 mRNA expressing neurons also contained c-Fos protein. In order to determine whether the number of Arc/Arg3.1 expressing neurons was stimulus intensity dependent, rats received a single application (by brush) of either 10% (n = 5) or 50% (n = 5) mustard oil on one hind paw. It was found that 50% MO induced significantly higher numbers of Arc/Arg3.1 mRNA positive neurons that 10% mustard oil (Fig. 4A). The number of c-Fos labeled neurons showed a similar significant increase.

Temporal expression of Arc/Arg3.1 mRNA in an acute and a chronic pain model

As a model for acute pain, 25% MO soaked gauze was wrapped around one hind paw, with survival times ranging from 25 min to 8 hrs. The number of Arc/Arg3.1 mRNA expressing neurons increased over time, reached a peak at 4 hours and then declined (Fig. 4B). The distribution of labeled neurons remained unchanged over time. As a model for chronic pain CFA was injected in the hind paw, with survival times ranging from 1.5 hrs to 60 hrs. Temporal expression of Arc/Arg3.1 mRNA was highest at 1.5 hrs post injection and then gradually declined (Fig. 4C). No expression of Arc/Arg3.1 mRNA was found at survival times of 10 hrs and longer. The number of c-Fos expressing neurons was increased at all survival times. In the spared nerve injury (SNI) model for neuropathic pain, expression of Arc/Arg3.1 mRNA was only observed at two hours after the operation. Arc/Arg3.1 mRNA was not expressed 1 week or 2 weeks after the operation (not shown) when the neuropathic pain symptoms, i.e. mechanical and thermal hyperalgesia and allodynia, had developed. There was no significant difference in the number of Arc/Arg3.1 mRNA labeled neurons between the SNI and sham operated group ($p>0.05$, unpaired t-test) (Fig. 4D).

Hossaini *et al. Molecular Pain* 2010, **6**:43
http://www.molecularpain.com/content/6/1/43

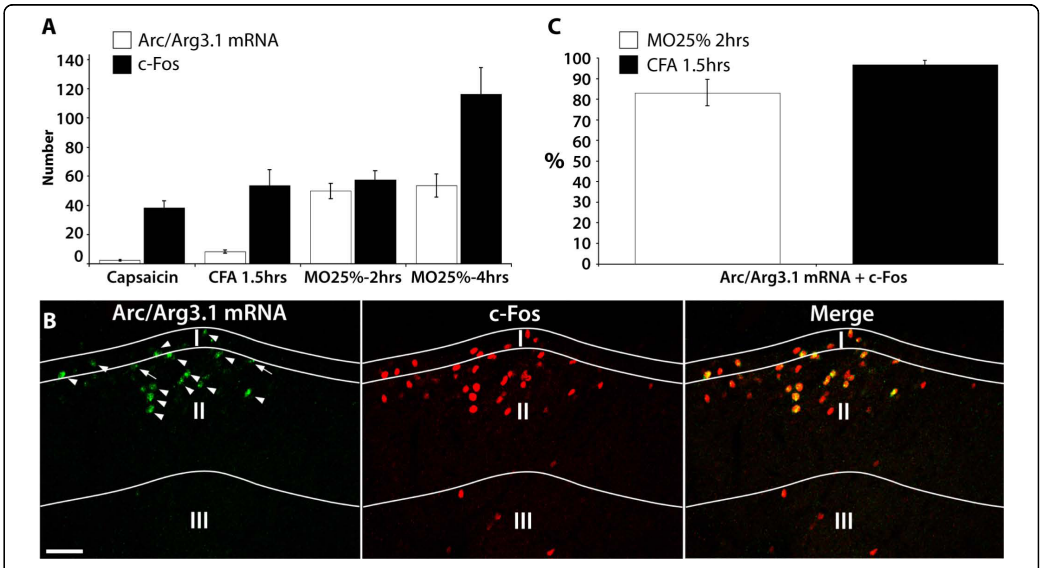

Figure 3 The number of neurons expressing c-Fos outnumber Arc/Arg3.1 mRNA labeled neurons. ***A***, Histogram showing the number of neurons labeled for c-Fos or Arc/Arg3.1 mRNA following various nociceptive stimuli. capsaicin, n = 6; CFA1.5 hrs, n = 4; MO25%-2 hrs, n = 4; MO25%-4 hrs, n = 4. ***B***, Fluorescent micrograph showing Arc/Arg3.1 mRNA and/or c-Fos protein labeled neurons in spinal dorsal horn after stimulation with 25% mustard oil for 2 hours. Arrows indicate Arc/Arg3.1 mRNA labeled neurons that were not in focus and therefore not included in the analysis. Arrow heads indicate Arc/Arg3.1 mRNA and c-Fos protein labeled neurons. ***C***, Histogram showing the percentage of Arc/Arg3.1 mRNA labeled neurons that also express the neuronal activation marker c-Fos. Scale bar: 50 μm. Error bars represent ± SEM.

Arc/Arg3.1 mRNA is expressed in specific subpopulations of dorsal horn neurons

In this experiment, the colocalization of Arc/Arg3.1 with various neuronal markers was investigated (Fig. 5A-E). We found that about a fifth of Arc/Arg3.1 mRNA positive neurons also express the NK-1 receptor (CFA 1.5 hrs: 21.7% ± 7.6, n = 4; MO25%/2 hrs: 17% ± 3.4, n = 5) (Fig. 6). Less than 10% of Arc/Arg3.1 mRNA expressing neurons also expressed PKC-γ protein (CFA 1.5 hrs: 7.7% ± 3.7, n = 4; MO25%/1h: 9.3% ± 3.8, n = 4). Further, Arc/Arg3.1 mRNA expressing neurons showed a low level of co-existence with calbindin (CFA 1.5 hrs: 9.7% ± 1.4, n = 4; MO25%/1h: 10.5% ± 2.6, n = 4).

In order to identify Arc/Arg3.1 in inhibitory neurons, FISH for GAD67 mRNA, the specific marker for GABAergic neurons, and fluorescent IHC for Arc/Arg3.1 protein were combined. Very few of the Arc/Arg3.1 labeled neurons were GABAergic (CFA 3 hrs: 1.7% ± 0.8, n = 4; MO25%/2 hrs: 4.5% ± 0.8, n = 5; MO25%/4 hrs: 4.5% ± 1.5, n = 4) (Fig. 6). Preproenkephalin mRNA is a marker for the subpopulation of enkephalinergic neurons in the spinal cord. Interestingly, a large majority of the Arc/Arg3.1 positive neurons also expressed preproenkephalin mRNA (CFA 3 hrs: 74.2% ± 9.2, n = 4; MO25%/2 hrs: 61.5% ± 2.6, n = 4; MO25%/4 hrs: 68.1% ± 3, n = 4) (Fig. 6).

Intrathecal injection of BDNF induces Arc/Arg3.1 mRNA expression

Intrathecal injection of brain-derived neurotrophic factor (BDNF) induced Arc/Arg3.1 mRNA expression in the superficial dorsal horn neurons (10 ± 1.7/section, n = 6). We found that 45% ± 8 of Arc/Arg3.1 mRNA labeled neurons were located in lamina I and 55% ± 8 in lamina II. 93.6% ± 2.5 of Arc/Arg3.1 mRNA labeled neurons expressed NeuN, 55.6% ± 9.1 expressed c-Fos, and 16.8% ± 6.4% expressed NK-1. Since it has been shown [18] that administration of BDNF together with NBQX, which is an AMPA receptor blocker, increases Arc/Arg3.1 mRNA expression in cortical neurons, we injected BDNF intrathecally together with NBQX. This combination resulted in 13.8 ± 2.9 Arc/Arg3.1 mRNA labeled neurons/section (n = 6) (Fig. 7A), which was not significantly different from intrathecal BDNF injection alone (unpaired *t*-test). c-Fos expression after BDNF + NBQX injection was also not significantly different from BDNF injection alone (p= 0.08 for lamina II) (Fig. 7B). Intrathecal injection of vehicle (n = 2) or NBQX (n = 2) alone did not induce Arc/Arg3.1 mRNA expression in

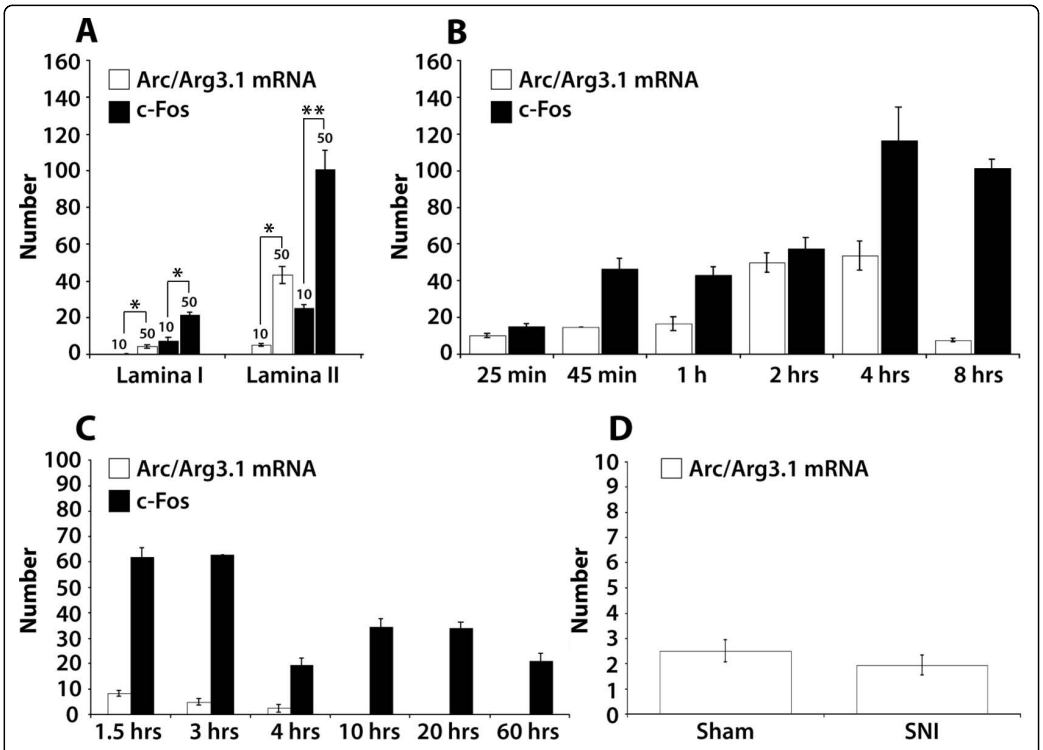

Figure 4 Arc/Arg3.1 expression is stimulus intensity dependent and is present only in the acute phase of chronic inflammatory and neuropathic pain. ***A,*** Histogram showing the numbers of Arc/Arg3.1 mRNA or c-Fos protein labeled neurons at 2 hours after a brush applied stimulation with either 10% (n = 5) or 50% (n = 5) mustard oil. * = $p < 0.05$; ** = $p < 0.005$ (unpaired *t*-test). ***B***, Time course of Arc/Arg3.1 mRNA and c-Fos protein expression after stimulation with 25% mustard oil gauze wrapped around one hind paw for different survival times. 25 min, n = 4; 45 min, n = 4; 1 h, n = 4; 2 hrs, n = 5; 4 hrs, n = 4; 8 hrs, n = 4. ***C***, Time course of Arc/Arg3.1 mRNA and c-Fos protein expression after CFA injection in the hind paw. 1.5 hrs, n = 4; 3 hrs, n = 4; 4 hrs, n = 4; 10 hrs, n = 4; 20 hrs, n = 4; 60 hrs, n = 4. ***D***, The number of Arc/Arg3.1 mRNA labeled neurons 2 hours after sham operation (n = 3) or after cutting the common peroneal and tibial nerves in the SNI model (n = 3). Error bars represent ± SEM.

the spinal cord. Furthermore, we found that intrathecal injection with NMDA (n = 2), which served as a positive control, also induced Arc/Arg3.1 expression in the dorsal horn (not shown).

Pain behavior in the Arc/Arg3.1 KO mice

Mechanical and thermal thresholds

Freely moving Arc/Arg3.1 knockout (KO) mice did not display any overt behavioral abnormalities in comparison with their wild type (WT) littermates, as reported previously [13]. With respect to pain behavior, the mechanical thresholds and hot plate withdrawal latencies were tested. We found that the mechanical thresholds in Arc/Arg3.1 KO mice were not significantly different from their WT littermates (Fig. 8A). However, in the hotplate test Arc/Arg3.1 KO mice showed significantly longer withdrawal latencies than WT mice (Fig. 8B).

Acute pain: formalin test

Subcutaneous injection of formalin in the hind paw induced a two-phased pain behavior in both WT and Arc/Arg3.1 KO mice, consisting of licking and fluttering of the injected paw. In both groups, the first phase was apparent in the first 10 minutes after injection, and the second phase began 25 minutes after injection with licking as the prominent behavior. No significant difference (repeated-measures ANOVA, $p>0.05$) was found between the WT and Arc/Arg3.1 KO mice in licking or fluttering behavior (Fig. 9A,B). Also the total licking time (WT: 200 sec. ± 34 (SEM); KO: 275 sec. ± 49 (SEM); $p>0.05$, unpaired *t*-test) nor the total numbers of flutters (WT 100 ± 21 (SEM); KO 128 ± 32 (SEM); $p>0.05$, unpaired *t*-test) were

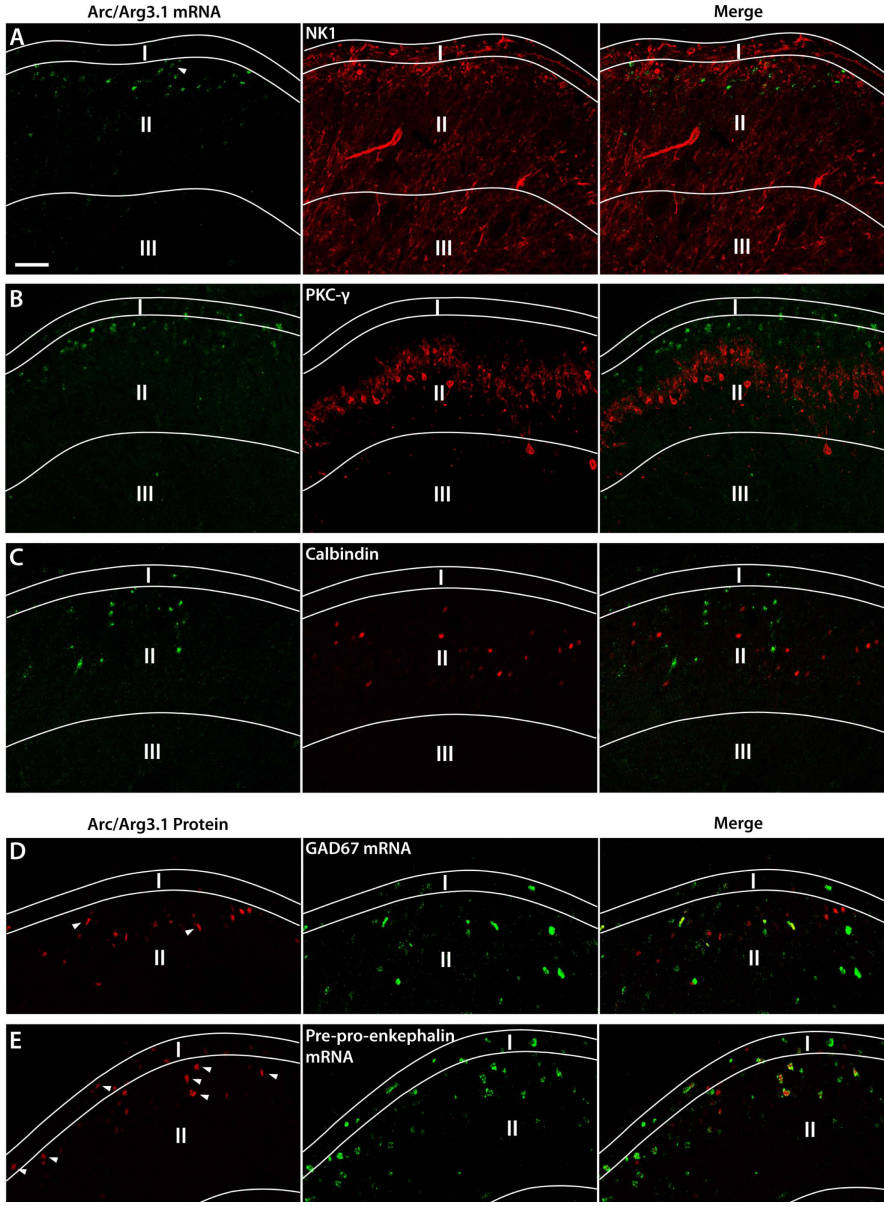

Figure 5 Arc/Arg3.1 is expressed in a subpopulation of superficial dorsal horn neurons with a preference for neurons containing enkephalin. ***A-E***, Fluorescent micrographs showing neurons in the superficial dorsal horn that express Arc/Arg3.1 mRNA (***A-C***) or protein (***D and E***) and markers that identify neurons expressing the neurokinin-1 receptor (NK1), protein kinase C gamma (PKC-γ), Calbindin, GAD67 mRNA (GABAergic neurons), or preproenkephalin mRNA (enkephalinergic neurons) respectively. The following nociceptive stimuli were used. ***A***, CFA, survival time 1.5 hrs, ***B and C***: Mustard oil 25% gauze wrapped, survival time 1 h, ***D and E***, Mustard oil 25% gauze wrapped, survival time 2 h. Arrow heads indicate Arc/Arg3.1 labeled neurons that also express one of the markers mentioned above. Scale bar: 50 μm.

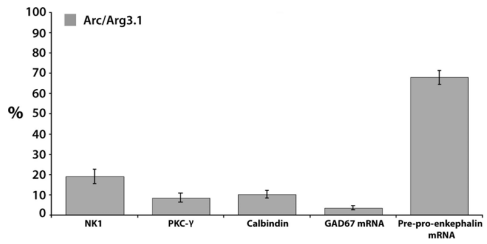

Figure 6 Histogram showing the averaged percentages of neurons expressing Arc/Arg3.1(induced after various nociceptive stimuli, as in fig. 5), that co-express either NK1, PKC-γ, Calbindin, GAD67 mRNA or preproenkephalin mRNA. Error bars represent ± SEM. For details, see text.

significantly different. In addition, c-Fos expression due to the formalin injection did not appear different from the c-Fos expression in the WT mice.

Chronic pain: inflammation

Induction of chronic inflammation by CFA injection in the hind paw resulted in decreased mechanical thresholds of the injected paw (Fig. 10A). A repeated measures ANOVA did not reveal any significant differences between WT and Arc/Arg3.1 KO mice regarding the mechanical or thermal thresholds at any time point (Fig. 10A,B).

Discussion

In this study we have used in situ hybridization (ISH) and immunohistochemistry (IHC) to show that nociceptive stimulation induced Arc/Arg3.1 mRNA and protein in the superficial dorsal horn of the spinal cord. Both techniques specifically identified Arc/Arg3.1 since standard controls, most notably nociceptively stimulated spinal cord of Arc/Arg3.1 knockout (KO) mice, did not show any specific labeling. In naïve or vehicle treated animals expression of Arc/Arg3.1 mRNA and protein was absent in the spinal cord, in agreement with a study using RT-PCR [19]. This strongly indicates that in the spinal cord a nociceptive stimulus induces *de novo* expression of Arc/Arg3.1, in contrast with other areas of the nervous system, like hippocampus [17] and cortex [20].

Arc/Arg3.1 mRNA and protein were induced in the superficial dorsal horn in the acute phases of all pain models that we tested, i.e. after nociceptive stimulation with capsaicin, CFA, formalin and mustard oil. Injection of CFA induces an inflammatory process [21] that leads to the release of cytokines and other local messengers, all of which may activate different types of receptors on nociceptive fibers. Capsaicin, however, specifically activates nociceptive fibers expressing the transient receptor potential vanilloid-1 (TRPV1) [22]. Further, mustard oil and formalin both specifically activate the TRPA1 receptor, although formalin may exert TRPA1-independent effects at higher concentrations [23,24]. The number of neurons producing Arc/Arg3.1 mRNA varied in the different pain models, and increasing the intensity of the pain stimulus resulted in an increased number of neurons expressing Arc/Arg3.1 as shown in the mustard oil experiments. Therefore, our data indicate that the number of neurons expressing Arc/Arg3.1 depends on the intensity of the stimulus, but is not limited to the activation of one specific receptor on peripheral nerves.

Neurons expressing Arc/Arg3.1 in the spinal cord are most likely driven by direct input from afferent

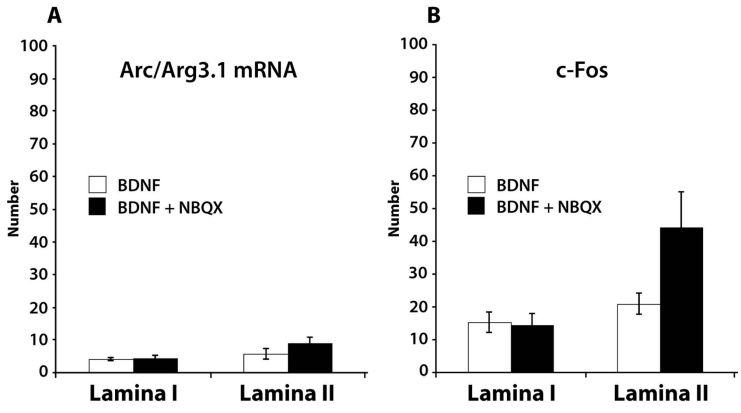

Figure 7 Intrathecal injection of BDNF induces Arc/Arg3.1 mRNA expression in spinal dorsal horn. ***A and B***, The averaged number of neurons that express Arc/Arg3.1 mRNA (***A***) or c-Fos protein (***B***) after intrathecal injection with BDNF or BDNF together with NBQX. Error bars represent ± SEM.

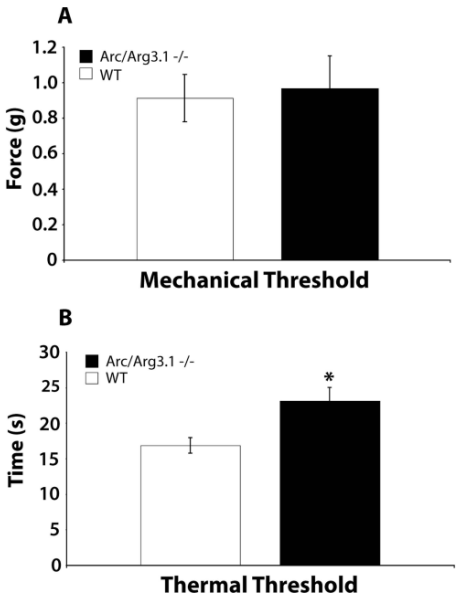

Figure 8 Naïve Arc/Arg3.1 knockout (KO) mice showed significantly longer response times on the hotplate than their naïve wild type (WT) littermates. ***A and B***, Histogram showing the mechanical threshold (***A***) and thermal withdrawal latency (***B***) of Arc/Arg3.1 KO and WT mice, assessed with the Von Frey and the hotplate test, respectively. Error bars represent ± SEM. *: $p < 0.05$, unpaired *t*-test; n = 4 for A and for B.

nociceptive fibers that use glutamate as their main neurotransmitter [25]. Apart from glutamate and various neuropeptides, these fibers may also contain growth factors like BDNF [26] or GDNF [27]. We found that intrathecal injection of NMDA or BDNF induced Arc/Arg3.1 mRNA in spinal dorsal horn neurons. This is in line with Arc/Arg3.1 expression in cultured neurons following BDNF application [18]. The same study showed a significantly enhanced expression of Arc/Arg3.1 mRNA when NBQX, a potent AMPA receptor blocker, was applied together with BDNF. However, in the present study a significant increase in the number of Arc/Arg3.1 mRNA expressing neurons could not be confirmed after intrathecal injection of BDNF and NBQX together. Taken together, our findings are in line with the idea that release of glutamate and/or BDNF from activated nociceptive fibers are at least partly responsible for Arc/Arg3.1 induction in the spinal dorsal horn.

Following nociceptive stimulation, Arc/Arg3.1 was often expressed in activated neurons as identified by c-Fos. Especially after nociceptive stimulation with capsaicin, and after chronic inflammatory pain, the number of neurons expressing Arc/Arg3.1 is low as compared to those showing c-Fos expression. This finding may be interpreted to indicate that Arc/Arg3.1 is only expressed in activated neurons that received the strongest input from nociceptive fibers. This assumption is in line with our finding that Arc/Arg3.1 expression is intensity dependent. On the other hand, there may be specific subpopulations of spinal nociceptive neurons that are capable of producing Arc/Arg3.1, while others are not. In search of such a neuronal subpopulation that specifically expressed Arc/Arg3.1, we focused on neurons that were characterized by the expression of the neurokinin-1 (NK-1) receptor, Protein Kinase C gamma (PKC-γ), calbindin, GAD67 or preproenkephalin. We found a high percentage of Arc/Arg3.1 expressing neurons (68%) to contain preproenkephalin, while percentages of colocalization with other markers were less prominent (19% for NK-1; 8.5% for PKC-γ; 3.6% for GAD67; 10% for calbindin). NK-1 expressing neurons project to supraspinal sites [28] and are essential for the initiation and maintenance of chronic neuropathic and inflammatory pain [29], and neurons expressing PKC-γ are considered critically important for the development of neuropathic pain after peripheral nerve injury [30]. The finding that only a small number of Arc/Arg3.1 positive neurons also expressed NK-1 or PKC-γ indicates that Arc/Arg3.1 is

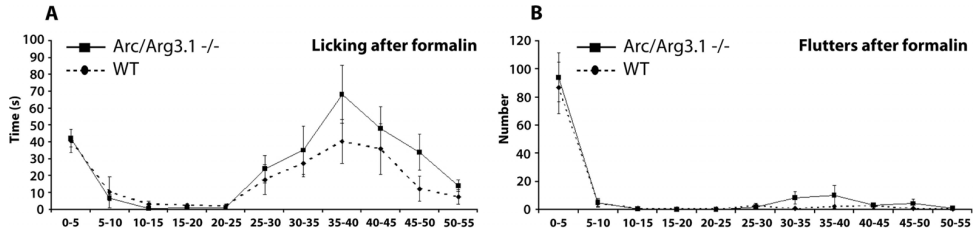

Figure 9 Arc/Arg3.1 KO mice do not differ from WT mice concerning licking or fluttering of the paw injected with formalin. ***A and B***, Graphs showing the time spent licking (***A***) and the number of flutters (***B***) after formalin injection in the hind paw during an observation period of 55 minutes. Error bars represent ± SEM. Differences were not significant (repeated-measures ANOVA, $p>0.05$). n = 4 for A and for B.

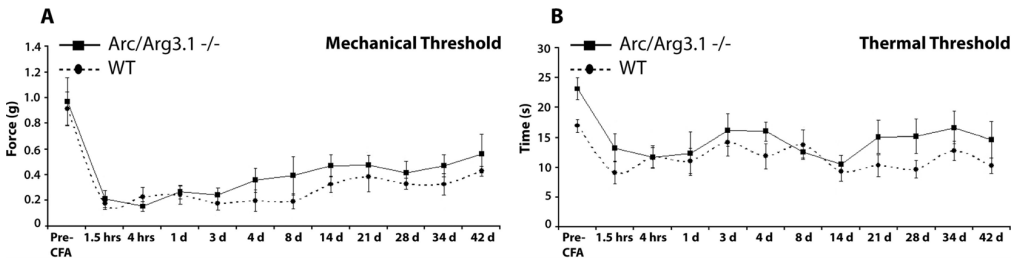

Figure 10 No differences in mechanical and thermal thresholds between the Arc/Arg3.1 KO and WT mice during the time course of inflammatory pain. ***A and B***, Graphs showing mechanical (***A***) and thermal (***B***) thresholds after CFA injection in one hind paw of Arc/Arg3.1 KO and WT mice. Differences were not significant at any time point (repeated-measures ANOVA, $p>0.05$). n = 4 for A and for B.

not strongly involved in pain processing by the NK-1 or PKC-γ subpopulations of dorsal horn neurons. This is remarkable since especially the NK-1 expressing neurons projecting to the parabrachial area or periaqueductal grey show LTP formation after high or low frequency stimulation, respectively [31]. Our finding indicates that Arc/Arg3.1 dependent long term changes may occur preferentially in local interneurons rather than in projection neurons. Further, we found low colocalization with GAD67, the marker for GABAergic neurons, indicating that the expression of Arc/Arg3.1 is low in the total subpopulation of dorsal horn inhibitory neurons since glycinergic neurons are virtually absent in the superficial dorsal horn [32-34], and, if present, also contain GABA [35]. In the hippocampal and neocortical neurons expression of Arc/Arg3.1 in GABAergic positive neurons is also low but this is not the case in the dorsal striatum [20]. Together, NK-1, PKC-γ and/or preproenkephalin constitute more than 90% of the Arc/Arg3.1 expressing neurons. Since to date there is no evidence for the colocalization of these substances with each other, we conclude that Arc/Arg3.1 is preferentially expressed in the subpopulation of enkephalinergic neurons. Preproenkephalin mRNA is the precursor of both Met- and Leu-enkephalin, which are both expressed by neurons in the spinal cord and mainly exert their effect on the δ-opioid receptor (DOR) [36]. Also, preproenkephalin mRNA in the spinal cord is increased after peripheral inflammation and is also present in neurons that express c-Fos after nociceptive stimuli [37]. Further, using VgluT2 immunohistochemistry for identifying glutamatergic terminals, it was shown [38] that 85% of the enkephalin containing terminals in the dorsal horn use glutamate as transmitter. However, a study [39] using cultured dorsal horn neurons showed 42% colocalization of immunohistochemically identified GAD and enkephalin. A more recent study [40] using preproenkephalin green fluorescent protein transgenic mice, showed that 43% of the fluorescent enkephalin neurons also expressed immunohistochemically identified GABA. Colocalization of enkephalin with VgluT2 was not explored in these studies. Since we found a low level of colocalization of Arc/Arg3.1 with GABAergic neurons, it is not unlikely that several of the enkephalinergic neurons in the spinal cord that express Arc/Arg3.1 also use glutamate as a transmitter. The functional role of glutamate in these fibers is unclear, since it is not known whether they activate inhibitory or excitatory (i.e. anti- or pro-nociceptive) circuits in the spinal cord, nor is it known under which circumstances enkephalin and/or glutamate is released from these fibers. Since the activation of the delta opioid receptor (DOR), through which enkephalin exerts its effect, decreases pain behavior during chronic peripheral inflammation [41], we tend to conclude that the overall effect of Arc/Arg3.1 expressing enkephalinergic neurons is anti-nociceptive.

In order to understand the functional role of Arc/Arg3.1 in enkephalinergic neurons at the behavioral level, we employed Arc/Arg3.1 KO mice and their WT littermates. The only significant difference between these mice was that in the hotplate test the thermal threshold of naïve Arc/Arg3.1 KO mice was significantly higher as compared to naïve WT mice. This finding is difficult to interpret since naïve WT mice, like their KO littermates, do not show Arc/Arg3.1 expression in the spinal cord. One explanation may be that there is a very low basal expression of Arc/Arg3.1 that we and others [19] were not able to detect, and that the permanent lack of Arc/Arg3.1 in the KO mice may have altered spinal processing of nociceptive thermal stimuli over time. Alternatively there may be supraspinal changes in nociceptive processing. After nociceptive stimuli, we did not find any difference in the pain behavior between the KO and WT mice in the formalin test and chronic inflammatory pain model. We therefore conclude that Arc/Arg3.1 KO mice do not show a clear phenotypic change that can be attributed to pain transmission in the spinal cord.

Several studies have shown that in hippocampus knockdown of Arc/Arg3.1 leads to enhanced LTP in the early phase but impaired consolidation of LTP and long term depression (LTD) in the late phase [13]. In the spinal cord, LTP is one of the major components of central sensitization [16], especially in lamina I projecting neurons [31]. LTP leads to enhanced responsiveness of spinal nociceptive neurons, which is important for maintenance of hyperalgesia and allodynia during acute and chronic pain. Our finding that Arc/Arg3.1 KO mice develop hypersensitivity in acute and chronic pain models in the same way as their WT littermates, suggests that the LTP formation that contributes to central sensitization and subsequent developing hyperalgesia is unaffected by the lack of Arc/Arg3.1. It seems therefore that Arc/Arg3.1 is not critically involved in LTP as occurring in the dorsal horn projection neurons, which in line with our result that few NK-1 positive neurons express Arc/Arg3.1.

The low number of spinal projection neurons that express Arc/Arg3.1 may be explained by the fact that, in contrast to other areas of the brain, structural long-term changes in the excitability of these spinal neurons are counterproductive if they persist after the healing process has been completed. Our finding that Arc/Arg3.1 is expressed predominantly in enkephalinergic neurons may suggest that in these neurons long term changes are actually consolidated. However, Arc/Arg3.1 KO mice that lack consolidation of long term changes show normal pain behavior. This would not exclude that enkephalinergic neurons, which have an inhibitory effect on pain transmission, may serve as an anti-nociceptive mechanism against strong nociceptive inputs that may occur in the future.

Conclusions

Our data show that Arc/Arg3.1, which is critically involved in consolidating long term structural changes in the forebrain, is preferentially induced in spinal enkephalinergic neurons after nociceptive stimulation. This finding suggests that Arc/Arg3.1 dependent memory formation in spinal pain transmission is a predominant feature of neurons, which are anti-nociceptive rather than pro-nociceptive.

Methods

Animal experiments

In this study we used 99 male Wistar rats and 16 Arc/Arg3.1 KO mice and their wild type littermates.

Rats

50 μl of 0.3% capsaicin (Sigma-Aldrich) solution consisting of 80% saline, 10% Tween-80, and 10% ethanol 100% (n: 6; survival: 1.5 hrs) or 100 μl of Complete Freund's Adjuvant (CFA, Sigma-Aldrich; n: 24; survival: 1.5 hrs, 3 hrs, 4 hrs, 10 hrs, 20 hrs, 60 hrs) was injected in a hind paw under anesthesia with 2% isofluorane in 30%O_2/70% N_2O. In experiments applying mustard oil (MO) (Allylisothiocyanat, Merck) the animals were kept under anesthesia during entire survival time and subsequent perfusion. For 25% MO application (n: 25; survival: 25 min, 45 min, 1 h, 2 hrs, 4 hrs, 8 hrs) the left paw was shaved and wrapped in a gauze soaked with MO and then covered with foil. For application of 10% (n: 5; survival: 2 hrs) and 50% (n: 5, survival: 2 hrs) MO, the left paw was shaved and MO was applied once at the beginning of the experiment using a brush. For the experiments using intrathecal injections, the same protocol was used as described in [42]. Brain-derived neurotrophic factor (BDNF, 10 μg, Tocris) was injected intrathecally in a total injection volume of 40 μl (n: 6; survival: 75 min). In another experiment, 5 μg of 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[*f*]quinoxaline-7-sulfonamide (NBQX, Tocris) was injected concomitantly with BDNF (n: 6; survival: 75 min). For control intrathecal experiments, 25 nmol N-Methyl-d-asparate (NMDA; Sigma, St. Louis, MO; n: 2; survival: 75 min), or only vehicle (1% bovine serum albumin in 0.025 M phosphate buffer; n: 2; survival: 75 min) or only NBQX (n: 2; survival: 75 min) was injected intrathecally. After the injections, the rats were placed back in their cages. For induction of neuropathic pain, the spared nerve injury (SNI) model and a control operation were used [43]. In short, the sciatic nerve was exposed and the three branches were isolated. The tibial and the common peroneal branches were ligated and then cut while the sural nerve was left intact (n: 9; survival: 2 hrs, 7 days, 14 days). As a control, the sciatic nerve was only exposed and isolated (n:7; survival: 2 hrs, 7 days, 14 days).

Arc/Arg3.1 KO and WT mice

All mice were habituated for 5 days to the experimenter, the experiment room, and the transparent cage that was used for the Von Frey measurements. Thereafter, prior to each experiment the mice were habituated for 30 minutes to the room in which the behavioral experiments took place.

Von Frey experiment before each Von Frey measurement, the mice were allowed to habituate to a transparent cage (15 cm × 15 cm with a gridded floor) for 10 minutes. We used calibrated von Frey filaments, which were applied for 2 seconds at 5 seconds interval, and the threshold was set at 3 evoked responses in a maximum of 5 applications.

Hotplate test the thermal thresholds were assessed by measuring the time a mouse spent on the hotplate (51° C) before showing a response like fluttering or licking of the hind paw, or jumping. Immediately after a response or after maximally 45 seconds, the mouse was taken off the hotplate.

The formalin pain model the mice were restrained by the experimenter and 15 μl of formalin, i.e. a freshly made solution of 4% paraformaldehyde (PFA) in phosphate buffer (PB), was injected subcutaneously in the left hind paw. The number of flutters and the time spent licking of the injected paw were measured during 55 minutes post-injection. After 90 minutes the mice were perfused and the tissue was processed as described below. n = 4 for Arc/Arg3.1 KO mice; n = 4 for WT littermates.

The CFA pain model 25 μl of CFA was injected in a hind paw of restrained mice and thereafter the mechanical and thermal thresholds were assessed at 1.5 h, 4 hrs, 1 d, 3 d, 4 d, 8 d, 14 d, 21 d, 28 d, 34 d, and 42 d post-injection. n = 4 for Arc/Arg3.1 KO mice; n = 4 for WT littermates.

Statistical analysis An unpaired *t*-test or a repeated measures ANOVA was performed, $p < 0.05$ was taken as significant.

Examination of the Arc/Arg3.1 KO mice spinal tissue After experiments the mice were sacrificed and further processed for immunohistochemistry (IHC) or in situ hybridization (ISH). Histological examination of Arc/Arg3.1 KO mice spinal cord did not reveal any morphological abnormalities in comparison with their WT littermates.

Tissue preparation

At the end of the survival times the animals received an overdose of sodium pentobarbital and were transcardially perfused with 100 ml saline (rats) or 10 ml (mice) followed by 750 ml of 4% PFA (rats) or 50 ml (mice) dissolved in 0.12 M phosphate buffer (PB), pH 7.4. The spinal cord was dissected and left overnight in a solution of 4% PFA and 30% sucrose at 4°C. Subsequently, sections were cut (30 μm) on a freezing microtome and collected in RNAse-free PB. Serial sections were cut and collected in 9 separate jars, and therefore sections in one jar were at least 270 μm apart. The sections were kept in a solution of 40% glycerol, 40% ethyleenglycol and 20% RNAse-free PB for long-term storage at -20°C.

In situ hybridization and immunohistochemistry

The partial cDNA templates encoding the following mRNAs were used: Arc/Arg3.1 (3.5 kb, full length probe encoding the mus musculus Arc/Arg3.1 gene, GeneID: 11838; Image Clone number: 3498057), GAD67 (3.2 kb; a generous gift from Dr. A.J. Tobin, UCLA), preproenkephalin (0.95 kb, a generous gift from Dr. S.L. Sabol, NIH). The riboprobes were obtained by linearizing the recombinant plasmids with the appropriate restriction enzymes and RNA polymerases. The transcription was performed in the presence of digoxigenin (DIG)- or fluorescein-labeled 11-UTP (Roche). ISH based on alkalic phosphatase (AP) reaction was performed following the protocol described previously [32]. For fluorescent in situ hybridization (FISH) the following modifications were applied to the protocol. After riboprobe hybridization, the detection of DIG or fluorescein was achieved with sheep polyclonal anti-Dig antibody (Roche) or mouse monoclonal anti-fluorescein antibody (Roche), respectively (1:500; 48 hours at 4°C in phosphate buffer saline (PBS), 2% milk powder and 0.5% Triton X-100). Thereafter, the anti-DIG or anti-fluorescein primary antibodies were detected using biotinylated rabbit-anti-goat (Vector) or goat-anti-mouse (Vector), respectively. Subsequently, the sections were incubated with Avidin-Biotin-Complex (ABC, Vector) tagged with horseradish peroxidase (HRP). A tyramide amplification procedure was performed by reacting HRP with H_2O_2 and a self made FITC tyramide according to protocol described in [44]. Thereafter, the sections were washed in PBS and processed for fluorescent IHC using the following antibodies diluted in 2% milk power solution: rabbit anti-Arc (1/3000; a generous gift from Dr. D. Kuhl), rabbit anti-c-Fos (1: 40.000; Oncogene Research Products, La Jolla, CA), rabbit anti-neurokinin-1 (NK1; 1:5000; Advanced Targeting System, CA, USA), rabbit anti-calbindin (1:7000; Swant, Switzerland), rabbit anti-PKC-γ (1/750; Santa Cruz), and mouse anti-neuronal nuclei (NeuN) monoclonal antibody (1:5000, Chemicon). These primary antibodies were detected with Cy3 tagged fluorescent secondary antibodies donkey-anti-rabbit or donkey-anti-mouse (1:200). Thereafter, the section were washed in PB and mounted on slides and coverslipped with Vectashield (Vector).

Data analysis

Analysis was carried out on L4 and L5 segments of the spinal cord, except for the BDNF experiments, in which also S1 and S2 segments were included in the analysis. Slides were systematically examined starting from the first section in the first row for the appropriate segmental level. The first 5 to 6 sections that were encountered and were not damaged during the procedure were included in the analysis [42]. For illustrations, light micrographs were made with a digital camera and confocal images with a Zeis LSM 510 confocal laser scanning microscope and a 20× objective. The images were processed using Adobe Photoshop and were not manipulated, except for brightness and contrast. Quantitative analysis of Arc/Arg3.1 mRNA positive neurons based on AP-ISH was achieved using a camera lucida microscope (Neurolucida, Microbrightfield Inc., Williston, VT). The grey and white matter and the boundaries between the laminae were drawn according to [45] and labeled neurons were identified only if the largest diameter was at least 10 μm, and the cell soma contained a

bluish/brownish product. Labeled neurons were expressed as the average number of labeled neurons per section.

For double labeling based on FISH combined with fluorescent IHC, confocal images were analyzed using the Zeis LSM image browser. For each section, the dorsal horn showing Arc/Arg3.1 labeled neurons was analyzed in a vertical plane consisting of 9 slices with an optical thickness of 2.46-2.76 μm. Every fifth section was analyzed for double labeled neurons. For markers that label the cytoplasm, the criterion was that the diameter of a profile was at least 10 μm to be counted as a neuron. For statistical analysis, an unpaired *t*-test was performed, and $p < 0.05$ considered significant.

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Author details
[1]Dept. of Neuroscience, Erasmus University Medical Center, PO Box 2040, 3000 CA, Rotterdam, The Netherlands. [2]Dept. of Neurology, Erasmus University Medical Center, PO Box 2040, 3000 CA, Rotterdam, The Netherlands. [3]Institute for Molecular and Cellular Cognition, Center for Molecular Neurobiology (ZMNH), University Medical Center Hamburg-Eppendorf, Falkenried 94, 20251 Hamburg.

Authors' contributions
MH performed or contributed to all experiments, analyzed data and drafted the paper. JLMJ contributed to experiments and analysis. KB contributed to experiments. DK provided KO mice and gave advice. JCH conceived and supervised the project and edited the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests

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Chapter VII

General Discussion

In this thesis we have identified the location of glycinergic neurons in the spinal cord and investigated the activation pattern of spinal glycinergic and GABAergic (Gly/GABA) neurons in various acute and chronic pain states. Further, we have identified spinal Gly/GABA neurons in the area around the central canal that project to the RVM, and we have investigated the expression pattern of Arc/Arg3.1 in naive spinal cord and after stimulation with nociceptive stimuli. Finally, with respect to descending pathways we have investigated the distribution pattern of Gly/GABA neurons in the RVM that project to the spinal cord. The following general discussion will first focus on the role of spinal Gly/GABA neurons in the naive spinal cord, and during spinal nociception. Thereafter, we will focus on the expression of Arc/Arg3.1 in the spinal cord and long-term memory consolidation in nociceptive pathways. Finally, we will discuss the role of the descending projection of Gly/GABA neurons in the RVM and their role in the inhibitory control of spinal nociception.

1. The role of spinal glycinergic and GABAergic neurons in pain inhibition

In the 1960's it was shown for the first time that glycine and GABA act as inhibitory neurotransmitters in the central nervous system [3, 44]. Glycine is an amino acid produced from serine by the enzyme serine hydroxymethyltransferase, which is not specific for glycinergic neurons. Further, glycine is an important transmitter in the spinal cord and the brainstem, but is not found in higher levels of the nervous system, where GABA is the main inhibitory transmitter [6, 39, 69]. The inhibitory effect of glycine is produced by the glycine receptor, a ligand gated chloride channel, opening of which leads to membrane hyperpolarization [41]. GABA is an amino acid that is produced from glutamic acid by the enzyme glutamic acid decarboxylase (GAD) [81]. GAD is only present in neurons that use GABA as their transmitter and can therefore be used for the identification of GABAergic neurons [14, 40]. GABA acts on GABA receptors of which there are two main types: GABA-A and GABA-B receptors. GABA-A receptors are ligand gated chloride channels, the opening of which leads to membrane hyperpolarization. GABA-B receptors, on the other hand, are G-protein coupled receptors, which are indirectly linked to a potassium channel, that also has a hyperpolarizing effect on the membrane [53]. Glycine and GABA are often colocalized in spinal neurons [48], both in the dorsal [86, 87] and ventral horn [82]. In accordance, physiological studies have shown that glycine and GABA and are co-released at synapses [36]. Only few populations of spinal neurons use only glycine or only GABA as their inhibitory neurotransmitter [24, 87]. They include neurons in the superficial dorsal horn, which are predominantly GABAergic since there are few glycinergic neurons in these laminae, as we have shown in *chapter 2*. Furthermore, in the ventral horn there are specific GABAergic neurons that express GAD65, which is one of the isoforms of GAD and often colocalized with the other isoform GAD67, that are involved in the presynaptic inhibition of muscle spindle afferents on motoneurons [31]. Next to the inhibitory input provided by these Gly/GABA interneurons, there is also inhibitory input from descending glycinergic and GABAergic terminals originating from Gly/GABA neurons in the RVM [2, 5, 49, 56]. The terminals of these Gly/GABA projections contact projection neurons in the superficial layers [2],

interneurons in the deeper layers (laminae III-VI), and also neurons located in the ventral horn [28, 29]. Taken together, glycinergic and GABAergic neurotransmission in the spinal cord is either produced by spinal Gly/GABA interneurons or by Gly/GABA neurons in the RVM with projections to the spinal cord.

1.1. The naive spinal cord

Abolishing glycinergic or GABAergic neurotransmission in the naive spinal cord results in many behavioral signs of hypersensitivity as observed in neuropathic and inflammatory pain conditions [75, 78, 97]. This finding indicates that nociceptive spinal neurons, including projections neurons, are active in the absence of a nociceptive stimulus, and there is the necessity for a continuous glycine and GABA induced inhibition of nociceptive neurons in order to block their activity in the naive animal. It is unclear which Gly/GABA neurons, i.e. spinal interneurons and/or RVM projection neurons, are the source of this inhibitory control. Although there is evidence of descending Gly/GABA terminals contacting spinal dorsal horn neurons [2], and for inhibitory effects after RVM stimulation [50], it remains unclear whether Gly/GABA projection neurons in the RVM are involved in the tonic inhibition of the naive spinal cord. Similarly, the role of spinal inhibitory neurons in this tonic inhibition of nociceptive neurons in the naive spinal cord is not clear. C-fos protein, which is an immediate early gene that is widely used as a marker for neuronal activation [32], is expressed by spinal neurons that are activated by a nociceptive stimulus [22]. In the naive spinal cord, c-fos is only expressed by very few neurons [9], suggesting the absence of active inhibitory neurons in the naive spinal cord. Thus, based on c-fos activation, it seems that spinal Gly/GABA neurons do not play a role in inhibiting nociceptive spinal neurons in the naive animal. This apparent contradiction may be resolved when it is assumed that c-fos expression in spinal neurons is induced by phasic activity rather than tonic activity. Therefore, the lack of c-fos expression in the naive spinal cord would not necessarily imply the absence of active Gly/GABA neurons. Apparently, these neurons only start to express c-fos when they become activated after a nociceptive stimulus, which leads to a sudden strong increase in their activity. In conclusion, spinal inhibitory neurons, whether or not in combination with descending inhibitory neurons, probably play a role in inhibiting nociceptive neurons in the naive spinal cord but the mechanisms underlying this inhibition are still unclear.

1.2. Nociceptive activated spinal cord

1.2.1. Ipsilateral pain stimulation

In the past decade, is has become increasingly clear that glycine and GABA play an important role in controlling spinal nociceptive processing. For example, during chronic inflammatory pain glycinergic inhibition is blocked in the spinal cord by a pathway that involves prostaglandin E2 (PGE2) [58, 72], a process which underlies the development of thermal and mechanical hyperalgesia [98]. Further, activation of selective subunits of

the GABA receptor, i.e. the α2 and/or α3, result in pronounced pain inhibition in chronic pain states [42]. During neuropathic pain states, there is evidence for reduced GABAergic inhibition in the superficial dorsal, which may [57] or may not [65, 66] be due to loss of GABAergic interneurons. At the same time there is loss of synaptic inhibition due to a shift in the chloride gradient, which reverses the inhibitory effect of GABA into a depolarizing one [11].

Despite the knowledge on the involvement of inhibitory neurotransmission in spinal nociceptive processing, there are few data about the role of Gly/GABA interneurons in the loss of spinal inhibition and their activation pattern in acute and chronic pain states [85, 101, 102]. In *chapter* 3 of this thesis, we have investigated the activation pattern of spinal Gly/GABA neurons in various acute and chronic pain states affecting one hindpaw by determining the number and the percentage of c-fos activated neurons that were Gly/GABA on the ipsilateral side. We found that the percentage of c-fos activated neurons that was inhibitory was higher (46%) in chronic (≥ 20hrs) pain states as compared to acute (≤ 2hrs) pain states (34%). This increase in percentage was caused by a reduction in the number of c-fos expressing non-Gly/GABA neurons in chronic pain states while the number of c-fos expressing Gly/GABA neurons remained stable. This finding indicates that in chronic pain states there is a relatively increased activation of Gly/GABA neurons. However, as mentioned earlier, several studies have shown a loss of inhibitory transmissions in the spinal cord during chronic inflammatory and neuropathic pain [99]. A possible explanation may be that the activated Gly/GABA neurons that we found are not functional, because the effects of the glycine and GABA they release is blocked at the synaptic level or that there effect is reversed (see above). Another explanation may be that the activated Gly/GABA neurons serve as a compensation, albeit insufficient, of the apparent loss of inhibitory neurotransmissions in other pathways during chronic pain states. Yet another view would be that the expression of c-fos occurs in Gly/GABA neurons involved in phasic activity, while the loss of inhibition occurs in tonically active Gly/GABA neurons, which are not identified by the expression of c-fos. Finally it may be argued, that the activated Gly/GABA neurons are involved in another role, unrelated to the inhibition of pain transmission in the dorsal horn, e.g. related to inhibition of motoneurons. Next to the activity of spinal interneurons, there are also glycinergic and GABAergic inputs by descending pathways from the RVM to the spinal cord [2, 50]. Therefore, it is possible that the activity in these inhibitory descending pathways is decreased, counteracting the effect of the activated Gly/GABA neurons that we have indentified during chronic pain states.

In conclusion, in chronic pain states there is loss of synaptic inhibition in the spinal cord while at the same time there are c-fos activated spinal Gly/GABA neurons, and possibly inhibitory input by descending pathways from the RVM. Whether all these inhibitory inputs are blocked, resulting in the observed loss of spinal inhibition in chronic pain states, or whether their activation is induced by the loss of spinal inhibition but fail to compensate for that loss is currently unclear.

1.2.2. Contralateral pain stimulation

In *chapter IV* of this thesis we have shown that the number of activated Gly/GABA neurons is increased after a capsaicin injection in the hindpaw of rats which have chronic inflammatory or neuropathic pain in the other hindpaw. This indicates that a unilateral chronic pain state increases the excitability of Gly/GABA neurons on the contralateral side of the spinal cord. As a result a subsequent pain stimulus on that side will activate a larger number of Gly/GABA neurons, relative to non-Gly/GABA neurons. Previous studies have shown that primary afferent fibers not only result in ipsilateral activation of the spinal cord, but also in contralateral activation by polysynaptic mechanisms [27]. Further, it has been shown that an one sided noxious stimulation induces c-fos activation of spinal neurons on the contralateral side [9], and that the number of c-fos expressing neurons is increased after a second stimulus on that contralateral side [45, 46]. In accordance, it was shown recently by means of autofluorescent flavoprotein imaging that there is activity on the contralateral side of the spinal cord immediately after an ipsilateral nociceptive stimulation [37]. The following question now arises: what is the functional meaning of the activation and the increased excitability that develops on the contralateral side? One possible explanation may be that it is important for survival: when a body part is injured on one side, the same body part on the contralateral side must remain functional despite the injuries and possible new injuries to that body part. In this situation, an enhancement of the local inhibition of nociception would be beneficial for a proper function of that body part [63, 70]. However, in these situations it is likely that the PAG-RVM system, that controls spinal pain transmission through its descending projections to the dorsal horn [26], will also become involved. Therefore it is likely that changes that we have observed on the contralateral side of an injury, are the result of changes in the activity and excitability of local spinal neurons as well as neurons in the RVM. Taken together our findings show that the excitability and subsequent activation pattern of Gly/GABA neurons on one side of the spinal cord are affected by painful events on the contralateral side, while the underlying mechanisms need further investigation.

2. Long-term memory consolidation in spinal nociceptive pathways

Central sensitization plays a important role in the development and the maintenance of hyperalgesia and allodynia after nociceptive stimuli. There are three mechanisms that underlie the central sensitization [94, 95]. In the first place there is wind-up, a form of activity dependant plasticity, resulting in the increase of action potential output of dorsal horn neurons. Wind-up is induced by and only manifest during a train of repeated low-frequency C-fiber or nociceptor stimuli [12, 52, 55, 83] and occurs only at the active synapse (homosynaptic). For example, when a noxious thermal or mechanical stimulus with a constant intensity is repeatedly applied to the skin, wind up of spinal neurons induces an increase in pain with each successive stimulus, while the intensity of the noxious stimuli is constant [68]. 2) A second mechanism is heterosynaptic central sensitization. In this case the increased excitability of spinal nociceptive dorsal horn neurons, elicited by

a brief and intense nociceptive stimulus, results in the activation of dorsal horn neurons by primary afferent inputs that are normally subthreshold [10, 51, 96]. This form of central sensitization is heterosynaptic, meaning that that the potentiation of synaptic output not only applies to the synapses that were activated by the initiating stimulus, but also to other synapses not activated by the initiating stimulus [76]. At the behavioral level, this heterosynaptic potentiation is the underlying cause of secondary hyperalgesia and allodynia in the area around the primary injury site [77]. A third mechanism is known as long-term potentiation (LTP), a process at the homosynaptic level which results in an increased efficacy of excitatory primary afferent input [34, 92]. It has been shown that this LTP induced enhancement of monosynaptic excitatory synaptic responses lasts for days to weeks, is NMDA receptor dependent, and leads to the phosphorylation of AMPA receptors and the recruitment of new AMPA receptors into the cell membrane [71, 90]. It is generally assumed that most processes inherent to LTP formation in cortical and hippocampal neurons are probably similar to the LTP-related processes occurring in spinal nociceptive neurons [35]. Taken together, there is evidence for nociception induced homosynaptic and heterosynaptic enhancement of spinal nociceptive neuronal excitability, and LTP based long-term consolidation of synaptic changes in spinal nociceptive pathways, and all these mechanisms contribute to central sensitization.

In this thesis we have investigated the expression pattern of Arc/Arc3.1 mRNA and protein in the rat spinal cord after nociceptive stimuli, and determined the pain behavior in Arc/Arg3.1 knockout (KO) mice. Arc/Arg3.1, an immediate early gene (IEG), was first identified and extensively investigated in cortex and hippocampus [8, 21, 64, 73, 74], where it plays an essential role in long-term memory consolidation by regulating AMPA receptor trafficking [7]. Consequently, knockdown of Arc/Arg3.1 results in the loss of LTP and long term depression (LTD), and the loss of long-term memory while short term memory is unchanged [64]. In *chapter 6* we showed that Arc/Arg3.1 is *de novo* expressed after a nociceptive stimulus and that the expression of Arc/Arg3.1 is intensity dependent, i.e. a stronger nociceptive stimulus induces a higher number of Arc/Arg3.1 expressing neurons. We further found that Arc/Arg3.1 is predominantly expressed in enkephalinergic interneurons, and not in NK1 receptor expressing projection neurons, that relay nociceptive signals to supraspinal levels, nor in Gly/GABA interneurons that are likely involved in the local inhibition of spinal pain transmission. In line with this lack of expression in the most important neurons of the spinal nociceptive system, we found that the Arc/Arg3.1 KO mice showed no changes in their pain behavior to acute (formalin) and chronic (inflammation) pain stimuli as compared to their wild type littermates. This strongly suggests that the important “memory molecule” Arc/Arg3.1 is not crucially involved in the long-term consolidation of “pain memory”, i.e. the changes that occur during inflammatory and neuropathic pain states. This finding may reflect a special property of pain memory in the spinal cord, which is that it should be reversible and not lead to permanent changes. In functional terms: if an injured part of our body remained sensitized to non-noxious (allodynia) and noxious (hyperalgesia) stimuli, we would be forced to maintain the protection of an injured area long after the healing process of that area had been completed. Instead,

central sensitization in the spinal cord that develops post injury slowly subsides with tissue healing, resulting in the disappearance of hyperalgesia and allodynia in and around the area of tissue injury. As a consequence the functional organization of the spinal cord, and the sensitivity of the healed area of injury, will return to the normal pre-injury situation. Since Arc/Arg3.1 is involved in establishing permanent changes in synaptic strength, its absence from spinal nociceptive projection neurons, as well as Gly/GABA expressing spinal neurons, is in line with the idea that permanent changes in the spinal pain system are unfavorable for its proper function. However, there is one group of neurons that did express Arc/Arg3.1 after nociceptive activation: the enkephalin expressing neurons. This indicates that these neurons will develop permanent changes after nociceptive activation and become more sensitive for incoming nociceptive stimuli for a long period of time. As a consequence, these enkephalinergic neurons, which are a minority of the c-fos activated neurons, may become more easily activated when the injured area would be injured a second time. Since enkephalin exerts an inhibitory effect on pain transmission in the dorsal horn [17, 61], this would mean that a subsequent injury would be less painful then the previous one. If this effect is strong enough, it would be possible to test this hypothesis in Arc/Arg3.1 KO mice. However, due to a limited availability of these mice, were not able to put this to the test.

In conclusion, our results indicate that Arc/Arg3.1 in the spinal cord is not involved in the development of pain behavior in the formalin and chronic inflammation models. This indicates that mechanisms that produce central sensitization in the spinal cord do not lead to long-term changes that outlast the healing period. In that sense, LTP in the spinal cord is different from other areas of the central nervous system (CNS), where long term consolidation of synaptic changes is a prerequisite for memory formation.

3. Inhibitory projections from the RVM to the spinal cord

Next to the spinal interneurons, descending projections from the RVM are also important in modulating spinal nociception [16], as activation of these projections leads to facilitation or inhibition of spinal nociceptive transmission [15]. The facilitating effect of the RVM on spinal nociception is induced by the ON cells [4, 15, 25], while inhibition of spinal nociception by descending RVM projections is mainly achieved by OFF cell activation [15, 26], with simultaneous inhibition of the ON cells. Many studies have focused on the transmitters involved in producing the effects of the descending RVM projections and most of the neuropeptides that were identified showed a facilitating effect, including cholecystokinin [43, 89] and neurotensin [60, 79, 88, 89]. With respect to the inhibitory projections from the RVM, it was believed for a long time that the transmitter involved was serotonin [23, 33, 38, 47]. However, more recently it became clear that serotonin may also induce facilitation of spinal pain transmission [62, 84, 100] and that serotonin was not localized in ON or OFF cells, but in Neutral cells, which show an activity pattern unrelated to nociceptive stimulation [18, 67]. Thus, if serotonin is not present in the OFF cells, which

is then the main transmitter, producing direct inhibition on spinal nociception? There is evidence that glycine and GABA are released in the dorsal horn upon RVM stimulation [49, 50, 80], but is has not been established from which terminals, i.e. descending projections or local spinal interneurons. In fact there are several mechanisms that may result in the inhibition of spinal nociception, including direct inhibition of projection neurons in the dorsal horn [91, 93], opioid dependent inhibition of transmitter release by primary nociceptive afferents [19], direct inhibition of nociceptive neurons in the dorsal horn [13, 20] and the activation of inhibitory interneurons in the dorsal horn [1, 54, 80]. In an electron microscopical study [2], which combined anterograde tracing from the RVM with glycine and GABA immunohistochemistry, it was shown that in the dorsal horn glycine and GABA were present in terminals that were labeled from the RVM. In *chapter 6* we have shown, using retrograde tracing from the spinal cord with fluorescent in situ hybridization for GABA and glycine, that 44% of the neurons projecting to the spinal dorsal horn are indeed Gly/GABA. Thus, based on this anatomical evidence, it seems very likely that Gly/GABA neurons represent the OFF cells. Whether this means that glutamate is the likely fast excitatory transmitter in the ON cells (next to various neuropeptides) remains unclear.

In *chapter 6* we have also shown that there are Gly/GABA neurons in the spinal cord, located in the area around the central canal (CC), that project to the RVM. This pathway is the only projection from the spinal cord to a supraspinal structure (i.e. the RVM) that contains the fast inhibitory transmitters GABA and glycine. This finding shows that the spinal cord is not merely "passing on" information to higher structures, but is also involved in the inhibition of specific supraspinal nuclei, like the RVM. Within the same spinal area around the CC there are also neurons that use opioids such as enkephalin and dynorphin [59] that project to supraspinal sites including the RVM. Enkephalin is frequently colocalized with GABA in the spinal cord [30], therefore it is not excluded that the opioids in these neurons act as modulators that are colocalized with fast transmitters like GABA and glycine, and thus represent the same neurons that we have identified as Gly/GABA. It is presently unclear which type(s) of neurons in the RVM are inhibited by these connections and whether or not they act as feedback loops involved in the control of the nociceptive transmission in the spinal cord. Taken together, our finding of glycinergic and GABAergic ascending pathways from the spinal cord to the RVM, shows that the idea of the spinal cord as a relay station for sensory information, should be adapted to include the existence of inhibitory connections, controlling the activity of supraspinal structures, like RVM.

4. Final conclusions

In this thesis we have shown that spinal Gly/GABA neurons have specific activation pattern in acute and chronic pain states. Whether these activated Gly/GABA neurons can compensate for the loss of inhibitory neurotransmission during chronic pain, or whether they are involved in another process inherent to chronic pain states is unclear. Our investigation on the spinal expression of Arc/Arg3.1 indicates that Arc/Arg3.1 based long-term memory consolidation is not predominant in the spinal cord. This finding is in line with

our suggestion that long-term consolidation of synaptic activity in the spinal cord is unfavorable as it would lead to a hypersensitive spinal cord with permanent hyperalgesia and allodynia even after tissue healing. Further, our findings provide strong evidence for the involvement of RVM Gly/GABA projection neurons in spinal processing. However, whether Gly/GABA projection neurons are the OFF cells that have been characterized to inhibit spinal nociception, or that they are a specific subset of neurons, next to OFF cell population remains to be determined. Finally, the existence of reciprocal inhibitory connections between the RVM and the spinal cord suggest that the spinal cord may, via a feed-back loop, be able to modulate RVM neurons in order to alter its own neurons that are under control of descending RVM pathways. Taken together our findings further underline the importance of Gly/GABA neurons in the spinal cord and the brainstem for controlling the feeling we all know: pain

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Summary

Samenvatting

Summary

In this thesis we have employed fluorescent in situ hybridization (FISH) in order to identify neurons in the spinal cord and the brainstem that use glycine or gamma-amino butyric acid (GABA) or both transmitters (Gly/GABA neurons). With this technique we have made a detailed analysis of the localization of glycinergic cell somata in the spinal cord (*Chapter 2*). In the subsequent experiments we have combined FISH with the retrograde tracing technique, using fluorescent microspheres, or with the fluorescent immunohistochemistry technique for identifying various proteins, including c-fos, a marker for neuronal activation.

In *Chapter 3* it is demonstrated that spinal Gly/GABA neurons have specific activation patterns in acute, chronic inflammatory and neuropathic pain. Furthermore, the averaged percentage of activated neurons that were Gly/GABA in the chronic phase (≥20h survival, 46%) was significantly higher than in the acute phase (≤2h survival, 34%). Morphine application equally decreased the total number of activated neurons and activated Gly/GABA neurons. This showed that morphine did not specifically activate Gly/GABA neurons to achieve nociceptive inhibition. Since there is evidence for an overall loss of spinal inhibitory neurotransmissions in chronic pain states, we conclude that the effect of the activated Gly/GABA neurons is insufficient to produce an overall increase in inhibition during these chronic pain states.

In *Chapter 4* we have shown that a chronic pain state in the hind paw on one side leads to increased excitability of Gly/GABA neurons located on the contralateral side of the spinal cord, since a pain stimulus on that contralateral side resulted in an increased number of activated Gly/GABA neurons.

In *Chapter 5* we have used FISH combined with fluorescent retrograde tracing. The results showed that about 40% of the neurons in the rostral ventromedial medulla (RVM) that project to the cervical spinal cord were Gly/GABA, i.e. containing either glycine or GABA or both transmitters. From the projections of the RVM to the lumbar dorsal horn 43% were Gly/GABA and this percentage was 35% for the projections to the lumbar ventral horn. In the caudal medulla, relatively few neurons projecting to the lumbar dorsal horn were Gly/GABA (5%), while this percentage was much higher (19%) for projections to the ventral horn. The percentages obtained for GABA and glycine separately were very similar to those obtained for Gly/GABA. These findings show that virtually all of the Gly/GABA projections to the spinal cord contain both transmitters and that the projections to the dorsal horn originate preferentially in the RVM. In this chapter we have also shown the presence of spinal Gly/GABA neurons located in the area around the central canal that project to the RVM. This inhibitory ascending pathway, which is the only inhibitory (Gly/GABA) pathway from the spinal cord to the brainstem identified up to now, might allow the spinal cord to modulate the RVM, thereby controlling the descending control of the RVM on spinal pain processing.

In *Chapter 6* we have investigated in the spinal cord the role of Arc/Arg3.1, an immediate early gene that is essential for long-term memory consolidation in cortical and hippocampal neurons. Arc/Arg3.1 is not present in the naive spinal cord, but is *de novo* expressed by nociceptive stimuli. The majority (68%) of the Arc/Arg3.1 expressing neurons contained enkephalin, while only 19% were neurokinin-1 expressing projection neurons and 3.6% were inhibitory (GABA) neurons. These findings showed that Arc/Arg3.1 is expressed in only a few projection and inhibitory neurons, which belong to the most important neurons of the spinal pain system. Accordingly Arc/Arg3.1 knockout mice did not show any changes in their pain behavior after formalin injection and after induction of chronic inflammation. These findings suggest that long term memory consolidation is not required and may even hamper normal functioning of the nociceptive system.-

Taken together, this thesis gives a detailed analysis of the distribution pattern of activated Gly/GABA neurons in the spinal cord during different pain states as well as the distribution of Gly/GABA neurons in the RVM that project to the spinal cord. The expression pattern of Arc/Arg3.1 during different pain states substantiates the idea that long term synaptic changes in the spinal pain system, including the Gly/GABA neurons, are unfavorable for the normal functioning of spinal pain transmission. Thus our findings further underline the importance of Gly/GABA neurons in the spinal cord and the brainstem for controlling the feeling we all know: pain.

Samenvatting

In dit proefschrift hebben we gebruik gemaakt van de fluorescerende in situ hybridizatie (FISH) techniek om de neuronen in het ruggenmerg en de hersenstam te identificeren die glycine of gamma-amino boterzuur (GABA) of beide transmitters bevatten (Gly/GABA neuronen). Met deze techniek hebben we een gedetailleerde analyse gemaakt van de localisatie van de glycinerge neuronen in het ruggenmerg (*Hoofdstuk 2*). In de volgende experimenten hebben we de FISH techniek gecombineerd met de retrograde neuronale opsporingstechniek met behulp van fluorescerende micro-bolletjes of met de fluorescende immunohistochemie techniek om verschillende eiwitten te identificeren, waaronder c-fos, het eiwit dat geactiveerde neuronen labelt.

In *Hoofdstuk 3* wordt aangetoond dat Gly/GABA neuronen in het ruggenmerg volgens een specifiek patroon geactiveerd worden gedurende acute pijn, chronische ontstekingspijn en neuropathische pijn. Het gemiddelde percentage geactiveerde Gly/GABA neuronen was significant hoger in de chronische fase (≥20h overleving, 46%) vergeleken met de acute phase (≤2h overleving, 34%). De toediening van morfine verminderde het totale aantal geactiveerde neuronen en het totaal aantal geactiveerde Gly/GABA neuronen in dezelfde mate. Dit laat zien dat de pijnonderdrukking door morfine niet tot stand kwam door het activeren van Gly/GABA neuronen. Op grond van aanwijzingen dat de totale mate van pijnonderdrukking in het ruggenmerg minder wordt gedurende chronische pijn, kan worden geconcludeerd dat het effect van de geactiveerde Gly/GABA neuronen onvoldoende is om gedurende chronische pijn een toename van pijnonderdrukking te bewerkstelligen.

In *Hoofdstuk 4* laten we zien dat chronische pijn aan de achterpoot aan één kant, leidt tot een verhoogde prikkelbaarheid van Gly/GABA neuronen aan de andere kant van het ruggenmerg, aangezien een pijnprikkel aan die andere kant resulteerde in een verhoogd aantal geactiveerde Gly/GABA neuronen.

In *Hoofdstuk 5* hebben we de FISH techniek gecombineerd met de retrograde neuronale opsporingstechniek. De resultaten lieten zien dat ongeveer 40% van de neuronen in de rostral ventromediale medulla (RVM) die naar het cervicale ruggenmerg projecteerden Gly/GABA bevatten, ofwel dat deze neuronen of glycine of GABA of beide transmitters bevatten. Van de projecties van de RVM naar de lumbale dorsale hoorn waren 43% Gly/GABA en wat betreft de projecties naar de lumbale ventrale hoorn was dit percentage 35%. De ventrale medulla, caudaal van de RVM, bevatte relatief weinig neuronen met Gly/GABA (5%), die naar de lumbale dorsale hoorn projecteerden, terwijl dit percentage veel hoger lag (19%) voor de neuronen met projecties naar de lumbale ventrale hoorn. De percentages die werden verkregen voor GABA en glycine apart waren vrijwel gelijk aan de bovengenoemde percentages voor Gly/GABA. Samengevat kan gesteld worden dat nagenoeg alle projecties van de RVM en caudale medulla naar het ruggenmerg zowel glycine als GABA bevatten en dat de projecties naar de dorsale hoorn voornamelijk afkomstig zijn van de RVM. In dit hoofdstuk hebben we ook laten zien dat er Gly/GABA neuronen

aanwezig zijn rond het centrale kanaal van het ruggenmerg die naar de RVM projecteren. Deze baan van het ruggenmerg naar de hersenstam is de enige opstijgende baan, met een remmend (Gly/GABA) effect, die tot nu toe bekend is. Waarschijnlijk kan deze baan de RVM beïnvloeden en daarmee de afdalende banen van de RVM controleren, die op hun beurt de pijnverwerking in het ruggenmerg controleren.

Hoofdstuk 6 laat in het ruggenmerg het onderzoek zien aangaande Arc/Arg3.1, een snel afgeschreven genproduct dat in de cortex en de hippocampus neuronen essentieel is voor het vastleggen van het lange termijn herinneringen. Arc/Arg3.1 is normaliter niet aanwezig in het ruggenmerg, maar wordt pas tot expressie gebracht als er een pijnprikkel wordt toegediend. De meeste neuronen die Arc/Arg3.1 tot expressie brengen bevatten enkephaline (68%), terwijl slechts 19% de neurokinine-1 receptor (kenmerkend voor projectie neuronen) tot expressie brengen en 3.6% waren GABA neuronen, die een remmende werking hebben. Deze bevindingen tonen aan dat Arc/Arg3.1 slechts in enkele projectie-neuronen en remmende neuronen tot expressie komt, terwijl die neuronen behoren tot de belangijkste in het pijnsysteem van het ruggenmerg. In overeenstemming hiermee lieten Arc/Arg3.1 knockout muizen dan ook geen veranderingen zien in hun pijngedrag na injectie van formaline en gedurende chronische infectie. Deze bevindingen geven aan dat het vastleggen van “pijnherinneringen” voor de lange termijn niet noodzakelijk of zelfs hinderlijk zijn voor het normaal functionerende pijn systeem.

Samengevat kan gesteld worden dat dit proefschrift een gedetailleerde analyse geeft van zowel de organisatie patronen van geactiveerde Gly/GABA neuronen in het ruggenmerg bij verschillende oorzaken van pijn, als ook de verdeling van de organisatie van de Gly/GABA neuronen in de RVM en caudale medulla, die naar het ruggenmerg projecteren. Het expressie patroon van Arc/Arg3.1 gedurende de verschillende oorzaken van pijn verschaft een basis aan het idee dat het vastleggen van synaptische veranderingen voor de lange termijn in het pijnsysteem, daarbij inbegrepen de Gly/GABA neuronen, ongunstig zijn voor het normaal functionerende pijnsysteem in het ruggenmerg. Deze bevindingen benadrukken nogmaals het belang van Gly/GABA neuronen in het ruggenmerg en de hersenstam voor het controleren van het gevoel dat we allemaal kennen: pijn.

CURRICULUM VITAE

Personal

Name	Seyed Mehdi (Aram) Hossaini
Date of birth	November 11, 1981
Place of birth	Shiraz , Iran

Education

September 1994 - June 2001	Havo and VWO Walburg College, Zwijndrecht
September 2001 - April 2006	Medical school Erasmus University in Rotterdam
February 2004 - August 2006	Master of Neuroscience Department of Neuroscience, Erasmus MC, Rotterdam
November 2006 - May 2010	Mozaïek Laureate 2006 (grant nr. 017.003.030) PhD student, Department of Neuroscience, Erasmus MC, Rotterdam
June 2010 - present	Medical internships

List of publications

Differential distribution of activated spinal neurons containing glycine and/or GABA and expressing c-fos in acute and chronic pain models

Hossaini M, Duraku LS, Saraç C, Jongen JL, Holstege JC

Pain 2010;151;356-365

Nociceptive stimulation induces expression of Arc/Arg3.1 in the spinal cord with a preference for neurons containing enkephalin

Hossaini M, Jongen JL, Biesheuvel K, Kuhl D, Holstege JC

Mol Pain 2010;23;6:43

Loss of Hoxb8 alters spinal dorsal laminae and sensory responses in mice

Holstege JC, de Graaff W, **Hossaini M**, Cano SC, Jaarsma D, van den Akker E, Deschamps J

Proc Natl Acad Sci U S A 2008;105:6338-43

Distribution of glycinergic neuronal somata in the rat spinal cord

Hossaini M, French PJ, Holstege JC

Brain Res. 2007;1142:61-9

Distribution of RET immunoreactivity in the rodent spinal cord and changes after nerve injury

Jongen JL, Jaarsma D, **Hossaini M**, Natarajan D, Haasdijk ED, Holstege JC

J Comp Neurol. 2007;500:1136-53

Distribution of GABA and glycine containing RVM neurons that project to the spinal cord and vice versa

Hossaini M, Goos JAC., Kohli S, Holstege JC.

Manuscript submitted (this thesis)

C-fos activation of spinal inhibitory neurons after contralateral hind paw stimulation of rats with inflammatory or neuropathic pain

Hossaini M, Saraç C, Jongen JL, Holstege JC

Manuscript submitted (this thesis)

Degeneration of spinal inhibitory interneurons in SOD1-ALS mice does not require glial mutant SOD1 expression.

Hossaini M, Cano SC, van Dis V, Haasdijk ED, Hoogenraad CC, Holstege JC, Jaarsma D

Manuscript submitted

Spinal enkephalinergic neurons: A study on c-fos activation pattern and ascending enkephalinergic pathways to the medulla oblangata

Hossaini M, Kohli S, Holstege JC

Manuscript in preparation

Glycinergic, GABAergic and enkephalinergic projections from the spinal cord to the lateral reticular nucleus

Hossaini M, Kohli S, Holstege JC

Manuscript in preparation

Dankwoord

Het is 22.56 uur op een donderdagavond in een stilstaande trein op het centraal station in Breda. Het is zoals gewoonlijk een regelrechte chaos op de Nederlandse spoorwegen en de ergernis van de reizigers trekt als een dampige mist over de spoorwegen. Moedig had ik besloten om vanavond na mijn avonddienst op de spoedeisende hulp in het Amphiaziekenhuis toch naar huis terug te reizen, maar mijn moed is nergens meer te bekennen en de stilstaande trein maakt me onrustig. Dan maar aan mijn dankwoord beginnen:

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Het is 22.37 uur op een zondagavond in Zwijndrecht. Het heeft maar vier dagen geduurd om het af te schrijven.

Pain related Inhibition by GABA and Glycine in the Rat Spinal Cord

Mehdi Hossaini

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Table of Contents

Chapter I

General Introduction

1.1. The definition of pain

In the 17th century, René Descartes, who is generally considered as the 'father of modern philosophy', was one of the first in history to inquire into the feeling of pain on a rational (scientific) basis. Some of his philosophical reasoning, like his theory on the dualistic nature of humans with a clear separation between the mind and the body, are still found in our culture today. Several of his other statements, e.g. that animals do not have a mind and therefore are not able to feel pain, are now generally considered as outdated. Nevertheless, his view on the transmission of pain [6], represented as the pulling of a thread (Fig. 1), and on the functional importance of pain are not completely at odds with current views on pain transmission.

Fig. 1:*"For example, if the fire A is close to the foot B, the small particles of fire, which as you know move very swiftly, are able to move as well the part of the skin which they touch on the foot. In this way, by pulling at the little thread cc, which you see attached there, they at the same instant open e, which is the entry for the pore d, which is where this small thread terminates; just as, by pulling one end of a cord, you ring a bell which hangs at the other end.... Now when the entry of the pore, or the little tube, de, has thus been opened, the animal spirits flow into it from the cavity F, and through it they are carried partly into the muscles which serve to pull the foot back from the fire, partly into those which serve to turn the eyes and the head to look at it, and partly into those which serve to move the hands forward and to turn the whole body for its defense."*

Painful stimuli are indeed detected by specialized fibers in the skin and transmit their information to the spinal cord [56], and from there to higher centers of the central nervous system [23]. Furthermore, pain serves as a warning system leading to retraction of our body from the source of physical danger. Since Descartes' time and especially in the last century, there has been a tremendous increase in research on pain. The International Association for the study of Pain (IASP), founded in the 1973, describes pain as 'An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage'. This definition points out that pain, while serving as a warning system of our body, is in essence a feeling with a major emotional impact. This makes pain a prominent part of our daily live, especially for patients with chronic pain.

1.2. The pain system

The feeling of pain is generally initiated by the activation of specialized primary afferents, termed nociceptors, which innervate the skin [56] but also the majority of the internal organs [8, 74], with clinically notable exceptions like the liver, the lungs, and the brain. Nociceptors are activated by actual or potential damaging stimuli directed to our body and hence require our immediate attention. Activated nociceptors convey their information to the spinal cord, or, when our face is involved, to the brainstem [24, 80]. In the spinal cord and the brainstem, nociceptors establish the first synapse in the pain system by contacting second order nociceptive neurons [76]. There are two main types of nociceptive fibers, namely slow conducting unmyelinated C-fibers that are responsible for the dull aching pain, and fast conducting myelinated Aδ-fibers that are responsible for acute pin prick-like pain [22, 72]. C-fibers contact nociceptive neurons located in lamina I and especially lamina II, while Aδ-fibers project to secondary nociceptive neurons mainly in laminae I, IV and V [76]. Subsequently, via crossing axons of the second order neurons nociceptive information is conducted contralaterally to various nuclei in the brainstem, the midbrain and the thalamus by way of the antero-lateral system [23].

The antero-lateral system contains several important ascending pathways including projections to: 1) the thalamus, i.e. spinothalamic tract (STT) [23]; 2) the homeostatic control regions in the medulla and the brainstem, i.e. spinomedullary, spinopontine and spinomesencephalic pathways that project to the regions of catecholamine cell groups (A1-A7) [23, 68], the parabrachial nucleus (PB) [10, 14, 26], the periaqueductal grey (PAG) [82], and the reticular formation of the medulla and pons [9]; 3) the hypothalamus (the spinohypothalamic tract, SHT) and the ventral part of the forebrain [17]. In addition there are projections to the forebrain, originating in the brainstem, which include the anterior cingulate cortex (ACC), the insular cortex (IC) and the prefrontal cortex [23]. Further, there are also nociceptive projections to the SI and SII regions in the somato-sensory cortex, mainly through the thalamus [15]. While the projections to the somato-sensory cortex are primarily involved in identifying the location and intensity of the nociceptive stimulus [48, 49, 60], the projections to the limbic forebrain provide the basis for the emotional impact of the nociceptive stimulus [7, 31, 51, 79]. Collectively, these various supraspinal structures that are involved in pain processing are known as the pain matrix [11, 63]. The pain matrix, which includes sensory, motor, cognitive and emotional parts of the brain, emphasizes that the feeling of pain is a multidimensional percept, in line with the fact that pain is of essential importance in our daily lives.

1.3. Hyperalgesia and allodynia: increased sensitivity to pain stimuli

As experienced in daily life, an injury usually leads to a direct painful sensation. However, when the initial pain has subsided and the healing process has started, the area of injury often becomes more sensitive to both noxious and non-noxious stimuli. This condition, in which a non-noxious stimulus is perceived as painful (referred to as allodynia) and a noxious stimulus causes more pain than normal (referred to as hyperalgesia) [12, 45,

85] is important, since both allodynia and hyperalgesia will make a subject to protect the injured area, thus aiding the healing process. The increased sensitivity of the injured area to non-noxious and noxious stimuli is the result of an increased sensitivity of nociceptors in the periphery (peripheral or primary sensitization) as well as neurons in the spinal cord (central or secondary sensitization) [83, 86]. In addition, descending pathways originating in the brainstem further facilitate this process by modulating the sensitivity of the spinal nociceptive neurons [66, 81].

1.4. Chronic pain

The feeling of pain usually subsides with the disappearance of the nociceptive stimuli during the healing time. However, when an injury becomes chronic the associated pain, including allodynia and hyperalgesia, will persist. For example, during chronic arthritis the joints are affected by chronic inflammation [52], and the patients suffering from such a disease complain of a nagging pain in the affected joints, together with limitations of their movements due to their painfulness. Treatment is directed at eliminating or decreasing the inflammation process and suppressing the pain, using analgesics [75]. Another major cause of chronic pain is the so-called neuropathic pain, which develops after nerve injury, e.g. after transection by trauma or surgery, or after ischemic events [21]. Post-injury, there is ectopic firing in the damaged nerve, increased sensitivity of spinal nociceptive neurons, and altered descending control originating in the brainstem [5, 37, 59]. All these changes lead to spontaneous pain without the presence of a noxious stimulus at the injured area, often accompanied by allodynia and hyperalgesia [16, 84]. More so than chronic inflammatory pain, neuropathic pain is notoriously difficult to treat with even the strongest analgesics [5]. Both chronic inflammatory and neuropathic pain are seriously debilitating conditions that affects millions of people worldwide [19]. Thus, research dedicated to the understanding of the processes underlying chronic inflammatory and neuropathic pain is of essential importance for patients in need of pain relief.

1.5. Pain inhibition at the spinal cord level

1.5.1. Spinal inhibitory interneurons

Incoming nociceptive fibers not only contact projection neurons that relay the information to higher centers but also local excitatory and inhibitory interneurons [18, 27]. These interneurons are involved in controlling the spinal sensitivity to incoming nociceptive stimuli [41, 78, 90], and are therefore believed to contribute to the maintenance of chronic pain states [90]. Recently, it has been shown that glial cells, especially microglia, are also involved in gating the sensitivity for nociceptive stimuli, especially during neuropathic pain [13, 44, 69]. Thus, spinal projections neurons, local interneurons and glial cells form an intricate network that controls the nociceptive information that is conveyed to supraspinal sites, thereby strongly influencing the pain that is experienced.

Inhibitory neurons use the fast neurotransmitters glycine and/or GABA as their

inhibitory neurotransmitter(s) [2, 46]. The importance of GABA and glycine in pain transmission was shown by blocking glycinergic and/or GABAergic neurotransmission in naïve animals, which induced many behavioral signs of hypersensitivity as observed in inflammatory and neuropathic pain conditions [70, 71, 87]. Accordingly, decreased inhibition was found in the spinal cord during chronic inflammatory pain [58, 64], as glycinergic inhibition in the spinal cord was blocked by a pathway involving prostaglandin E2 (PGE2) [90]. During neuropathic pain there is loss of synaptic inhibition due to a shift in the neuronal chloride gradient, which reverses the inhibitory effect of GABA into a depolarizing one [13]. Further, it was recently shown that selective activation of GABA(A) receptors containing the α2 and/or α3 subunits leads to pronounced nociceptive inhibition in chronic pain states [50]. These findings underline the importance of glycine and GABA in modulating the spinal processing of nociceptive information, especially during chronic pain.

1.5.2. Rostral ventromedial medulla

Next to the influence exerted by spinal interneurons, the spinal nociceptive system is also under control of descending projections originating in the brainstem, especially from the locus coeruleus and subcoeruleus, and the rostral ventromedial medulla (RVM) [29, 36, 81]. The RVM, which is largely located in the pons, is predominantly under control of PAG that is located in the midbrain [36]. In turn, PAG receives projections from several cortical and subcortical structures such as the ACC, IC, hypothalamus and the amygdala (Amy) [3, 67, 89]. The first evidence that descending pathways are able to control pain transmission in the spinal cord was provided in 1976 [55] by producing analgesia with electrical stimulation of the PAG, without affecting the animal's response to most other environmental stimuli. Since the PAG has no direct projections to the dorsal horn [53], its effects are produced through its connections with the RVM, which has extensive projection to the spinal dorsal horn [30, 36]. Therefore, the PAG-RVM circuitry is of critical importance for the descending control of pain transmission in the spinal cord.

It has been shown that microinjection of morphine into the Amy, IC, PAG or RVM produces analgesia, while the analgesic effect of systemic administered opioids is abolished by microinjection of opioid antagonists into these sites [88]. With respect to the RVM, it has been shown that electrical stimulation of the RVM produces direct inhibition of spinal nociception [65], which is also produced by microinjection of excitatory amino acids into the RVM [29]. Further, there is enhancement of descending inhibition from the RVM in animals with chronic inflammatory pain induced by injection of complete Freund's adjuvant (CFA) [65]. Next to descending inhibition, RVM also facilitates spinal nociception, i.e. enhancing of nociceptive transmission, in inflammatory and neuropathic pain models resulting in hyperalgesia and allodynia [81]. Thus, the PAG-RVM system is capable of inhibiting and facilitating spinal nociception in certain pain models through RVM projections to the spinal dorsal horn.

The existence of a parallel inhibitory and facilitatory output from the RVM suggest that there are distinct neurons in the RVM that are involved in inhibiting or facilitating spi-

nal nociception. In the RVM there are indeed three different, physiologically characterized, neuronal cell types that project to the dorsal horn and have distinct noxious stimulus dependent activity patterns. The ON-cells, which enter a period of activity before execution of a withdrawal reflex from a noxious heat, and have a net facilitatory effect on nociception [29]. The OFF-cells, which enter a period of silence before execution of a withdrawal reflex and have a net inhibitory effect on nociception [35]. The third group, which was termed neutral cells, was classified by exclusion. These cells have no characteristic noxious stimulus dependent activity pattern and therefore are most likely not involved in the acute modulation of nociception [29, 54]. With respect to their neurochemical characteristics, there is still no convincing evidence which neurotransmitters the ON- and OFF-cells use to induce facilitation and inhibition, respectively [28, 57]. For a long time it was believed that serotonin was the neurotransmitter that induced pain inhibition at spinal level [33, 43]. However, later on other studies have shown the involvement of serotonin in facilitating spinal nociception [77], and that a subgroup of the neutral cells contain serotonin rather than the OFF-cells [32, 61]. Anterograde tracing from the RVM area, combined with GABA and glycine immunohistochemistry (IHC) at the ultrastructural level [20, 38, 39, 40] has shown that the terminals of the RVM fibers in the spinal dorsal horn contain glycine and GABA [1]. Up to now it is not clear whether these transmitters are present exclusively in the OFF-cells and whether the ON-cells, which have a facilitatory effect on pain transmission in the dorsal horn use glutamate as their neurotransmitter [28, 57].

1.6. Aim of this PhD project

Spinal neurons that use GABA and/or glycine as their inhibitory neurotransmitters (Gly/GABA neurons) play important roles in spinal nociception. Most likely, Gly/GABA neurons in the RVM that project to the spinal dorsal horn are also important in modulating spinal nociception, although up to now there is lack of evidence for their expression in OFF-cells and their involvement in spinal nociception. It has proven difficult to stain glycinergic and GABAergic neurons since using IHC to identify glycine, GABA, or their transmembrane transporters results in weak soma labeling and intense terminal labeling [4, 34, 42]. Therefore, the method of choice for identifying these inhibitory neurons is in situ hybridization (ISH), which will identify the mRNA of proteins that are specific for neurons that use glycine or GABA as their transmitter. Since mRNA is present in the cell soma and not in the terminals, ISH well label exclusively the cell somata of these neurons. In our studies we have used the glycine transporter 2 (GlyT2) [62, 73] to specifically identify the somata of glycinergic neurons, and glutamic acid decarboxylase (GAD) 67 [25, 47] to identify the somata of GABAergic neurons

In this PhD project, we have combined fluorescent ISH (FISH) for GlyT2 and GAD67 mRNA with fluorescent IHC, and with fluorescent tracing. Using these techniques we have investigated the activity patterns of Gly/GABA neurons in the spinal cord as well as the descending projections from the RVM. In *Chapter 2* we have described the distribution pattern of spinal glycinergic neuronal somata in the rat spinal cord. In *Chapter 3* the activa-

tion pattern of spinal inhibitory neurons in acute and chronic pain states was investigated. In *Chapter 4* we have determined the activation pattern of spinal inhibitory neurons after stimulation with capsaicin in rats with chronic inflammatory or neuropathic pain, and in *Chapter 5* the distribution pattern of RVM neurons that are inhibitory and project to the spinal cord was investigated. In addition, we have identified a new pathway that projects to the RVM that is inhibitory and originates in the spinal cord. In *Chapter 6* we have described the expression pattern of the immediate early gene Arc/Arg3.1 in the rat spinal cord in various acute and chronic pain models, with a focus on inhibitory Gly/GABA and enkephalinergic neurons, as well as the neurokinin-1 expressing nociceptive projection neurons. Further, we have determined the behavioral response of Arc/Arg3.1 knockout mice to acute and chronic pain stimuli. Finally, in the general discussion, *Chapter 7*, we discuss the various findings in a broader perspective.

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Chapter II

Distribution pattern of glycinergic neuronal somata in the rat spinal cord

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Research Report

Distribution of glycinergic neuronal somata in the rat spinal cord

Mehdi Hossaini[a], *Pim J. French*[b], *Jan C. Holstege*[a,*]

[a]*Department of Neuroscience, Erasmus MC, PO Box 2040, 3000 CA, Rotterdam, The Netherlands*
[b]*Department of Neurology, Josephine Nefkens Institute, Erasmus MC, PO Box 1838, 3000 DR Rotterdam, The Netherlands*

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Research Report

Distribution of glycinergic neuronal somata in the rat spinal cord

Mehdi Hossaini[a], *Pim J. French*[b], *Jan C. Holstege*[a,*]

[a]*Department of Neuroscience, Erasmus MC, PO Box 2040, 3000 CA, Rotterdam, The Netherlands*
[b]*Department of Neurology, Josephine Nefkens Institute, Erasmus MC, PO Box 1838, 3000 DR Rotterdam, The Netherlands*

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ABSTRACT

Glycine transporter 2 (GlyT2) mRNA is exclusively expressed in glycinergic neurons, and is presently considered a reliable marker for glycinergic neuronal somata. In this study, we have performed non-radioactive in situ hybridization to localize GlyT2 mRNA in fixed free-floating sections of cervical (C2 and C6), thoracic (T5), lumbar (L2 and L5) and sacral (S1) segments of the rat spinal cord. The results showed that in all segments the majority of the GlyT2 mRNA labeled (glycinergic) neuronal somata was present in the deep dorsal horn and the intermediate zone (laminae III–VIII), with around 50% (range 43.7–70.9%) in laminae VII&VIII. In contrast, the superficial dorsal horn, the motoneuronal cell groups and the area around the central canal contained only few glycinergic neuronal somata. The density (number of glycinergic neuronal somata per mm^2) was also low in these areas, while the highest densities were found in laminae V to VIII. The lateral spinal nucleus and the lateral cervical nucleus also contained a limited number of glycinergic neurons.

Our findings showed that the distribution pattern of the glycinergic neuronal somata is similar in all the examined segments. The few differences that were found in the relative laminar distribution between some of the segments, are most likely due to technical reasons. We therefore conclude that the observed distribution pattern of glycinergic neuronal somata is present throughout the spinal cord. Our findings further showed that the non-radioactive in situ hybridization technique for identifying GlyT2 mRNA in fixed free-floating sections is a highly efficient tool for identifying glycinergic neurons in the spinal cord.

1. Introduction

Strong evidence that the amino acid glycine is acting as a neurotransmitter in the mammalian spinal cord was provided for the first time in 1965 (Aprison and Werman, 1965). In subsequent years glycine became established as the main inhibitory neurotransmitter in the caudal region of the central nervous system (CNS), next to the amino acid γ-aminobutyric acid (GABA). Glycinergic neurons are abundantly present in the spinal cord, brainstem and cerebellum (Borowsky et al., 1993; Luque et al., 1995), but scarce in rostral parts of the CNS, like the diencephalon and hippocampus (Rampon et al., 1996; Song et al., 2006).

The main strategies for identifying neurons that use an amino acid as neurotransmitter are based on the localization of the transmitter, its biosynthetic enzyme, or its transporter.

* *Corresponding author.*
E-mail address: j.holstege@erasmusmc.nl (J.C. Holstege).
Abbreviations: GlyT, glycine transporter; LSN, lateral spinal nucleus; LCN, lateral cervical nucleus

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Antibodies against glycine have been used most frequently to identify glycinergic neurons (Campistron et al., 1986; Rampon et al., 1996; Todd and Sullivan, 1990). In general, glycine antibodies preferentially stain glycinergic terminals because they contain a high concentration of glycine, while soma labeling is much weaker. However, in recent reports (Allain et al., 2006; Song et al., 2006; Zeilhofer et al., 2005) a glycine antibody was used that was directed against paraformaldehyde-fixed glycine (Pow et al., 1995), resulting in a much improved staining of neuronal somata in addition to axons and terminals. The use of antibodies against the biosynthetic enzyme that produces glycine in the brain, serine hydroxymethyltransferase, has never been reported, probably because the activity of this enzyme is correlated with the general pool of glycine (Daly and Aprison, 1974), rather than with the transmitter pool. Similarly the vesicle transporter that transports glycine also transports GABA, and therefore cannot be used for identifying glycinergic neurons only (Chaudhry et al., 1998). However, glycine transporter 2 (GlyT2), one of the GlyTs (Zafra et al., 1995a,b), is present exclusively in the plasma membrane of glycinergic neurons (Jursky and Nelson, 1995; Luque et al., 1995), where it is primarily involved in the reuptake of synaptically released glycine (Betz et al., 2006). GlyT1, on the other hand, is exclusively present in glial cells (Adams et al., 1995; Zafra et al., 1995a,b). Since GlyT2 is the only marker that is present exclusively in glycinergic neurons (Poyatos et al., 1997; Spike et al., 1997), and GlyT2 antibodies preferentially label glycinergic terminals rather than somata (Jursky and Nelson, 1995; Spike et al., 1997), GlyT2 in situ hybridization is the method of choice for identifying glycinergic neuronal somata. GlyT2 mRNA has been identified previously in the spinal cord by means of radioactive in situ hybridization (Zafra et al., 1995a). Since these studies described GlyT2 mRNA distribution throughout the CNS, the descriptions of the spinal cord were not very detailed. This study, which is the first step in our investigations on the role of glycinergic neurons in spinal sensory processing, was set up to obtain detailed knowledge on the distribution of spinal glycinergic neuronal somata at different levels of the spinal cord. For this purpose, we used non-radioactive in situ hybridization for GlyT2 mRNA on thick fixed free-floating sections.

2. Results

2.1. *General observations*

Light microscopic examination of the sections showed a bluish reaction product, representing the presence of GlyT2 mRNA, in a limited number of neuronal somata of various sizes. No staining was found in glial cells or fiber tracts. Staining was only present in neuronal somata, sometimes including their primary dendrites. Control sections hybridized with sense probes did not show any specific labeling.

The majority of the labeled neuronal somata were found in the deep dorsal horn and the adjoining intermediate zone (laminae VII&VIII) of the ventral horn (Fig. 1). In laminae I&II (Fig. 2A) the number of labeled neuronal somata observed never exceeded four neurons per section. The few labeled somata in lamina II were often located close to the border with lamina III. Laminae III to VI of the dorsal horn contained several labeled neurons, varying in size from 10 to 30 μm (Fig. 2B). In laminae IV, V&VI, labeled neuronal somata were more abundant medially than laterally (Figs. 1, 2B). The intermediate zone of the ventral horn also contained many labeled somata, including several of the largest labeled somata (Fig. 3). The large presumed motoneurons in lamina IX were never labeled. Occasionally a labeled neuron, considerably smaller than the presumed motoneurons, was present within the motoneuronal area (Figs. 1, 3A). The grey matter surrounding the central canal (lamina X) contained few labeled neurons. In all sections examined, a limited number of labeled neurons were found scattered in the white matter, close to either the dorsal or ventral horn (Figs. 1, 2A).

2.2. *GlyT2 mRNA labeled neuronal somata in specialized spinal nuclei*

We have examined several specialized nuclei, i.e., groups of cells that can be distinguished anatomically and usually subserve specialized functions (Holstege et al., 1996) for a more detailed description. These nuclei include the lateral spinal nucleus (LSN), the lateral cervical nucleus (LCN), the central cervical nucleus, the dorsal nucleus of Clarke, the intermediomedial nucleus, the intermediolateral nucleus, and, in the L6 segment, the dorsomedial and dorsolateral nuclei (Onuf's nucleus). In about 25% of the sections examined, one or two labeled neurons were identified in the LSN, which is located in the dorsolateral white matter (Fig. 4). In a few cases, labeled neurons were also observed in the LCN, which is present dorsal to the LSN at the C1–C3 level. In general, in both nuclei the intensity of the labeling was weak. The other specialized nuclei virtually never contained labeled neurons. However, several labeled somata were usually present around these nuclei.

2.3. *Quantitative aspects*

In all spinal segments examined, around 50% (range 43.7–70.9%) of the labeled neurons in that segment were located in the intermediate zone of the ventral horn (laminae VII&VIII), with the highest percentage (average 70.9%) at level L2 (Table 1). The superficial dorsal horn (laminae I&II), the motoneuronal cell groups (lamina IX) and lamina X contained the lowest percentage of glycinergic neurons (Table 1). A comparison between the segmental levels for each lamina separately (a total of 135 comparisons using the Bonferroni post hoc test) showed that in the large majority of the cases (116) there were no significant differences between the different segmental levels. The 19 cases that were significantly different (Table 1) were found mostly in L2 and S1. At the L2 segmental level, the average percentage in laminae V&VI (13.5%) was significantly lower than the percentage obtained for the same laminae in the other segments analyzed, while the average in laminae VII&VIII (70.9%) was significantly higher. In S1 lamina IV only contained 3.3% of the neurons at this level, significantly lower than most of the other segmental levels, while the average percentage (64.7%) obtained for laminae VII&VIII was significantly higher.

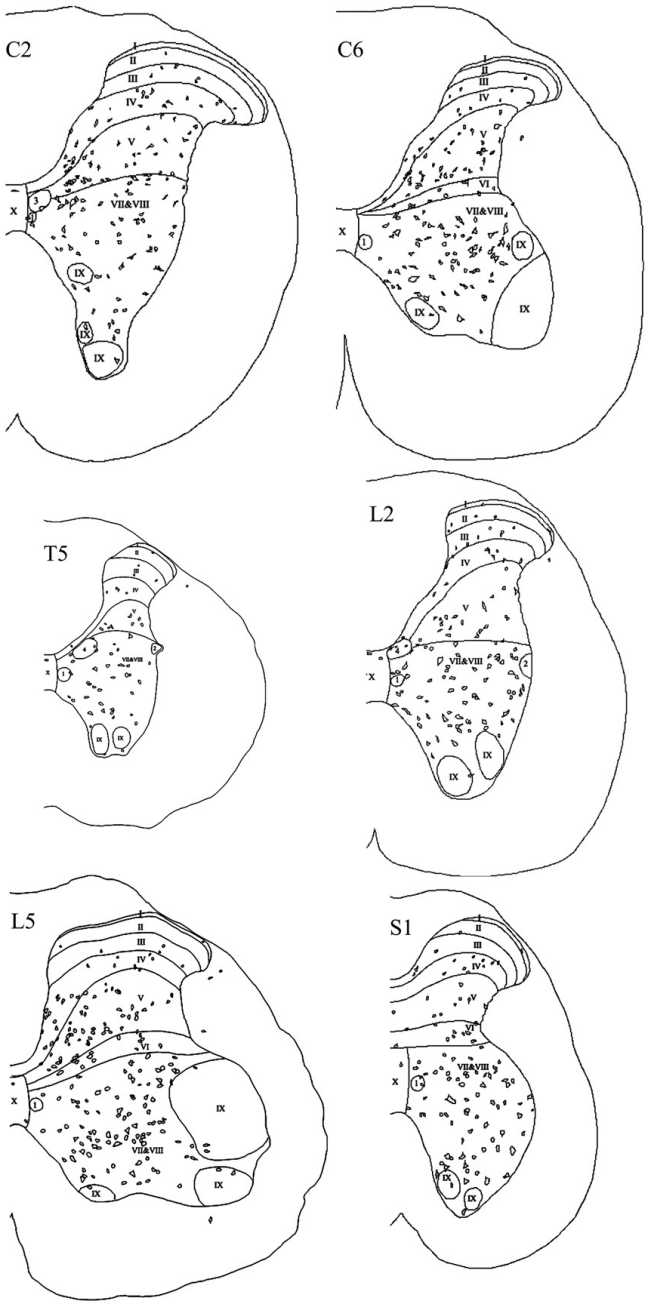

Fig. 1 – Schematic drawings illustrating the distribution of GlyT2 mRNA labeled (glycinergic) neuronal somata in analyzed single sections from rat spinal segments C2, C6, T5, L2, L5 and S1. Neurons in the dorsolateral funiculus are located in the lateral spinal nucleus. Other special nuclei are: intermediomedial nucleus (1), intermediolateral nucleus (2), central cervical nucleus (3), and dorsal nucleus of Clarke (4).

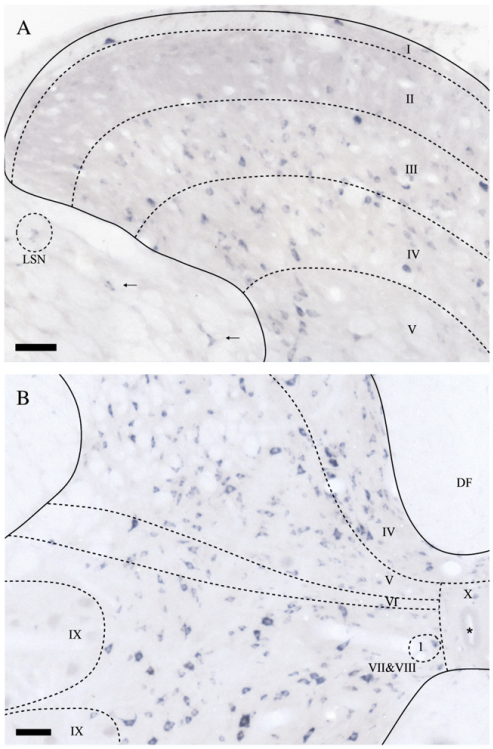

Fig. 2 – Light micrographs showing glycinergic neuronal somata as identified by GlyT2 mRNA labeling. (A) Few labeled somata are present in the superficial dorsal horn (segment C2), with increasing numbers of labeled somata in laminae III and IV. Note the labeled somata in the white matter (arrows) and the lateral spinal nucleus (LSN). Scale bar=50 μm. (B) Glycinergic neuronal somata of various sizes in the deep dorsal horn and the intermediate zone of the ventral horn. I: intermediomedial nucleus, DF: dorsal funiculus, *: central canal. Scale bare=50 μm.

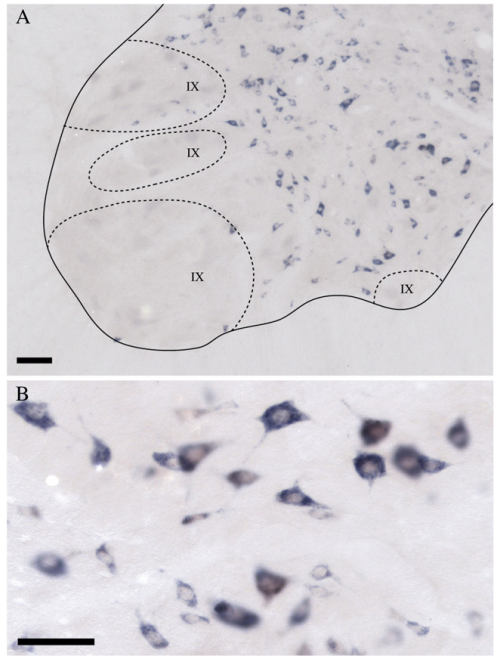

Fig. 3 – Light micrographs showing labeled neuronal somata in the lumbar ventral horn. (A) Overview of the L5 ventral horn. Few GlyT2 mRNA labeled neuronal somata are present in the motoneuronal cell groups (encircled areas). Scale bar=50 μm. (B) Labeled neuronal somata in lamina VII of the L5 spinal segment. Note the variability in size of the labeled neurons. Scale bar=50 μm.

When considering the density of the neurons in each lamina, i.e., the average number of neurons per mm^2, the highest densities are found in laminae V&VI and VII&VIII, and the lowest densities were found in laminae I, II, IX and X (Fig. 5). A comparison between the segmental levels for each lamina separately (a total of 120 comparisons using the Bonferroni post hoc test, excluding the specialized nuclei) showed that in the large majority of the cases (113) there were no significant differences between segmental levels. The exceptions were the density of lamina IV in the S1 segment, which was significantly different from segments C2, C6, and L2 ($p<0.05$); the density of laminae V&VI in S1, which was significantly different from segment C6 ($p<0.05$), and the density of laminae VII&VIII in L2, which was significantly different from segments T5, L5 and S1 ($p<0.05$). Since the large majority of the laminar densities in the different segmental

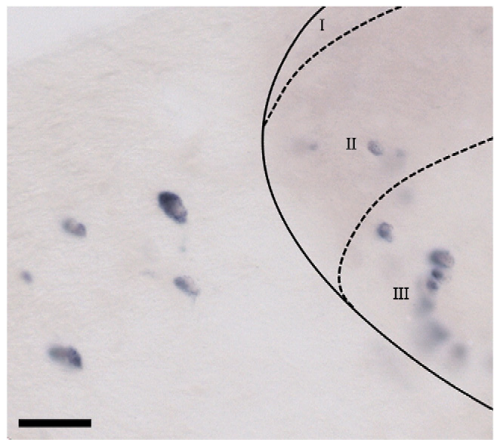

Fig. 4 – Light micrograph showing GlyT2 mRNA labeled (glycinergic) neuronal somata in the lateral spinal nucleus (LSN) at spinal segment T5. Scale bar=25 μm.

Table 1 – Relative laminar distribution of the glycinergic neuronal somata in different segments of the rat spinal cord

Lamina	C2	C6	T5	L2	L5	S1
	%±SD	%±SD	%±SD	%±SD	%±SD	%±SD
I	0.3±0.5	0.3±0.4	0.4±1.0	0.2±0.4	0.1±0.3	0.0
II	1.8±1.0	1.2±0.4	0.9±2.2	0.7±0.6	0.6±0.5	1.2±1.0
III	6.3±2.2	4.6±1.4	4.9±2.6	3.9±2.2	4.2±2.4	2.1±1.9
IV	14.1±3.5[a]	10.3±1.7	9.8±2.7	5.9±1.5	9.7±3.0	3.3±2.0[b]
V&VI	28.5±2.8	29.5±5.2	18.3±5.0[c]	13.5±3.4[d]	31.7±2.8	25.4±5.9
VII&VIII	43.7±6.4	49.0±8.7	54.8±7.9	70.9±2.1[e]	49.6±6.4	64.7±6.8[f]
IX	1.2±1.0	3.2±3.5	2.0±2.3	1.5±1.1	3.1±1.8	1.8±1.8
X	0.5±0.5	0.6±0.6	1.2±1.3	1.4±1.3	0.5±0.9	1.5±2.7
Special nuclei	3.5±1.8	1.3±0.9	7.6±5.8	2.0±1.9	0.3±0.5	0.0
n	606	760	255	720	788	356

For each lamina of the C2, C6, T5, L2, L5 and S1 segments the average percentage (±SD) of the total number of glycinergic neuronal somata is shown. *n*: Total number of labeled neuronal somata.
[a] Lamina IV in C2 is significantly different from the same lamina in L2 ($p<0.005$).
[b] Lamina IV in S1 is significantly different from C2, C6, T5, and L5 ($p<0.005$).
[c] Laminae V&VI in T5 is significantly different from C2, C6, and L5 ($p<0.005$).
[d] Laminae V&VI in L2 from C2, C6, L5, S1 ($p<0.005$).
[e] Laminae VII&VIII in L2 from C2, C6, T5, and L5 ($p<0.005$).
[f] Laminae VII&VIII in S1 from C2, C6, and L5 ($p<0.005$).

levels were not significantly different, the average density for each lamina(e) was calculated (Fig. 5). This showed that the densities of laminae I, II, IX and X are each significantly lower than the densities in the other laminae ($p<0.005$). The same holds true for lamina III ($p<0.05$), except that the density is not significantly different from lamina IV ($p>0.05$). The density of lamina IV is also not significantly different from the density of VII and VIII ($p>0.05$). Laminae V&VI have the highest average density, although not significantly different from laminae VII&VIII.

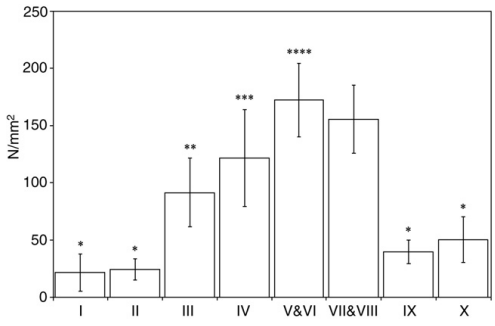

Fig. 5 – Average density of glycinergic neuronal somata per lamina in the analyzed segments. An increasing density is observed in the intermediate zone, while the superficial laminae, the motoneuronal cell groups (lamina IX) and lamina X have the lowest density. N/mm^2: calculated number of labeled glycinergic neuronal somata per mm^2. *Laminae I, II, IX and X are significantly different from laminae III, IV, V&VI, and VIII&VIII ($p<0.005$). **Lamina III is significantly different from all the other laminae ($p<0.05$), except lamina IV. ***Lamina IV is significantly different from all the other laminae ($p<0.005$), except laminae III and VII&VIII. ****Laminae V&VI are significantly different from all other laminae ($p<0.005$), except laminae VII&VIII. Error bars represent ±SD.

3. Discussion

The present study is the first detailed description of the distribution of GlyT2 mRNA in the spinal cord, using non-radioactive in situ hybridization on thick paraformaldehyde-fixed free-floating sections. Previous studies on the distribution of glycinergic (GlyT2 mRNA) and/or GABAergic (GAD67 mRNA) neuronal somata (Schreihofer et al., 1999; Tanaka and Ezure, 2004) in the brainstem have confirmed the sensitivity (Key et al., 2001) of the detection method used in the present study. In situ hybridization on free-floating 40-μm-thick sections has the advantage that the sections are easy to handle because the sections are fixed and much thicker than slide mounted non-fixed sections. Especially the washing steps in the procedure are more efficient on free-floating sections, which, in our hands, lead to a higher signal-to-noise ratio in comparison with slide mounted sections. The method used in this study is therefore the most reliable and sensitive approach presently available to identify spinal glycinergic neuronal somata. It has the advantage that it can be combined with other techniques like immunohistochemistry, and neuronal tracing (Stornetta et al., 2005).

Our results show that glycinergic neuronal somata are concentrated in the deep dorsal horn (laminae V&VI) and the intermediate zone of the ventral horn (laminae VII&VIII). In the superficial dorsal horn (laminae I&II), the area around the central canal (lamina X) and the motoneuronal cell groups (lamina IX) glycinergic neuronal somata were observed much less frequently, while laminae III and IV hold an intermediate position. This pattern is present in all the examined spinal segments (i.e., C2, C6, T5, L2, L5 and S1), strongly suggesting that this pattern is constant throughout the spinal cord. However, our analysis also showed some exceptions to this

rule, especially at spinal levels L2 (laminae V&VI, and VII&VIII) and S1 (laminae IV, and VII&VIII). The most simple explanation for this finding is that the laminar boundaries at the L2 and S1 level were identified incorrectly or that their location leads to relatively large differences in surface area as compared with the other segments. Especially at the L2 level, a ventral shift of the boundary between laminae V and VII, would increase the percentage of neurons in laminae V and decrease the percentage in laminae VII&VIII, likely resulting in the disappearance of the significant differences between the L2 and the other segments. Also in S1, which is a relatively small segment, small changes in boundary delineation may easily lead to significant changes in the percentages that were obtained. We therefore consider that the few significant differences in the laminar percentages that were found between segments are due to technical reasons, and do not signify any functional difference in the role of glycinergic neurons in these segments.

The bulk of the glycinergic neuronal somata was found in the intermediate zone (laminae VII&VIII). This finding is not surprising considering that this is the largest area as compared with the other spinal laminae. Thus even if the glycinergic neurons were evenly distributed over all the laminae in a section, the percentage of neurons in laminae VII&VIII would still be the highest. Therefore, we also calculated the average density of the glycinergic neurons in the different laminae. This showed that the superficial dorsal horn, the motoneuronal cell groups and lamina X have the lowest density. In case of the superficial dorsal horn, it may be expected that the density of glycinergic neurons would be relatively high considering the small surface area of these laminae. In contrast the density of the superficial laminae was very low, which confirms that these laminae are almost devoid of glycinergic neuronal somata.

The highest percentages of glycinergic neuronal somata in the various segments were found in laminae, V&VI and VII&VIII. These laminae also contained the highest densities of glycinergic neurons, which confirms the specific concentration of glycinergic neurons in these laminae.

In the analyzed specialized nuclei, only the LSN and LCN contained glycinergic neurons. This finding likely indicates that some of the widespread descending (Jansen and Loewy, 1997) and ascending (Ding et al., 1995; Keay et al., 1997) projections originating in the LSN are in part glycinergic.

Only one previous study (Todd and Sullivan, 1990) has provided a detailed description of glycinergic neuronal somata in the adult spinal cord with a focus on the superficial dorsal horn. This study used glycine antibodies in combination with pre-embedding immunohistochemistry on semithin plastic sections of rat lumbar segments. Their results are in general agreement with the results of the present study and show the same distribution pattern: a concentration of glycinergic neuronal somata in the deep dorsal horn and the intermediate zone of the ventral horn, while glycinergic neuronal somata are nearly absent from the superficial dorsal horn. Our findings further showed that this distribution pattern is similar in all the examined segments. Since the few differences that were found in the relative laminar distribution between some of the segments, are most likely due to technical reasons, we conclude that the observed distribution pattern of glycinergic neuronal somata is present throughout the spinal cord.

The functional significance of the laminar localization of glycinergic neuronal somata can only be appreciated in combination with many other data on these neurons, including the size of their dendritic tree, their axonal projection area, the parameters that determine the release of glycine and the properties of the postsynaptic glycine receptors. The dendritic tree of spinal neurons may extend into several neighboring laminae (Willis and Coggeshall, 2003). However, the morphology of the dendritic tree of glycinergic neurons is largely unknown. Similarly, little is known about the axonal arborization of glycinergic neurons. Areas that contain very few glycinergic neuronal somata, as we have shown here for the superficial dorsal horn (laminae I&II) and the motoneuronal cell groups (lamina IX), contain many glycinergic terminals (Rekling et al., 2000; Todd, 1990). It seems likely that the majority of these terminals is derived from spinal neurons located in other laminae, while some are derived from supraspinal sources (Antal et al., 1996; Holstege and Bongers, 1991). A lot more is known about the release properties and effects of glycine on other neurons. In this respect it is important to realize that both in the dorsal (Todd and Sullivan, 1990; Todd et al., 1996) and ventral horn (Taal and Holstege, 1994) glycine is often colocalized and co-released with GABA. However, there are also sets of neurons that contain glycine without GABA and vice versa, e.g., neurons in the intermediate zone that presynaptically inhibit Ia afferents on motoneurons only contain GABA and not glycine (Hughes et al., 2005; Mackie et al., 2003). In view of all these variables, it is likely that the glycinergic neurons in a specific lamina fulfill diverse functions. In laminae II–V the action of glycinergic neurons may be focused on the influencing sensory transmission (Willis and Coggeshall, 2003; Zeilhofer, 2005), while neurons in deeper laminae may be involved in affecting motor transmission (Rekling et al., 2000). In addition there are neurons in many of these laminae that project to supraspinal brain areas and some of these neurons may use glycine as a transmitter, although direct evidence for such a glycinergic projection is presently lacking.

Recently, glycinergic neurons in the spinal cord were also identified on the basis of the expression of GlyT2, but using a very different approach, namely by producing transgenic mice expressing enhanced green fluorescent protein (eGFP) under the control of the promotor of the GlyT2 gene (Zeilhofer et al., 2005). This study showed that the large majority of the eGFP producing neurons in the brain were also immunoreactive for glycine (in somata, dendrites and terminals) and GlyT2 (in terminals). However, in the dorsal horn, the area of the spinal cord examined in this paper, it was found that several glycine immunoreactive neuronal somata did not express eGFP and vice versa. In lamina II a subset of neurons was described as glycine immunoreactive, without expressing eGFP. In this case our findings in the rat dorsal horn would support the eGFP expression pattern, since we also found very few neurons in lamina II expressing GlyT2 mRNA. Whether this would indicate that there is a subset of neurons in lamina II that uses glycine as a transmitter without expressing GlyT2, or whether these cells show a high level of glycine, but do not use it as a transmitter, is unclear.

In this study we have identified glycinergic neurons throughout the spinal cord using GlyT2 mRNA in situ hybridization. We have shown that glycinergic neurons are concentrated in laminae III–VIII and provided evidence that this pattern is constant throughout the spinal cord. These findings provide a solid anatomical basis for further studies on the role of glycine in the spinal cord.

4. Experimental procedures

4.1. *Tissue preparation*

In this study we used 18 male Wistar rats, including six rats for quantitative analysis. Rats received an overdose of sodium pentobarbital and were transcardially perfused with 150 ml saline followed by 750 ml of 4% paraformaldehyde (PFA) in 0.12 M phosphate buffer (PB), pH 7.4. Subsequently, the entire spinal cord was dissected and left overnight in a RNAse-free solution of 4% PFA and 30% sucrose dissolved in 0.12 M PB at 4 °C. All the experiments have been approved by the Rotterdam Animal Ethics Committee.

4.2. *In situ hybridization*

All the solutions used in the following steps until the hybridization step were Diethylpyrocarbonat (DEPC)-treated. The regions of interest of the spinal cord were cut transversally in 40-μm sections on a freezing microtome and collected in 0.05 M PB. The sections were then treated with 0.2% glycine in phosphate-buffered saline (PBS, 5 min), rinsed in PBS, and then treated (10 min) in PBS containing 0.1 M triethanolamine (Merck, Germany) pH 8.0 and 0.0025% acetic anhydride (Sigma-Aldrich, Germany). Sections were then washed in 4× standard saline citrate (SSC, pH 4.5) and prehybridized for 1 h at 65 °C in hybridization mixture consisting of 50% formamide, 5× SSC (pH 4.5), 2% Blocking Reagent (Roche), 0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma-Aldrich), 1 μg/ml yeast tRNA (tRNA brewer's yeast, Sigma), 5 mM EDTA (pH 8.0), 50 μg/ml Heparin (Sigma-Aldrich), and 1× Denhardt's solution (Sigma-Aldrich).

The GlyT2 mRNA was obtained from a partial cDNA template (3.1 kb; a generous gift from Dr. N. Nelson, Tel Aviv University), encoding GlyT2. The riboprobes were obtained by linearizing the recombinant plasmids with restriction enzymes (*XbaI* for GlyT2 antisense, *HindIII* for GlyT2 sense) and transcribed with RNA polymerases (T7 for GlyT2 antisense, T3 for GlyT2 sense) in the presence of digoxigenin (DIG)-labeled 11-UTP (Roche).

The sections were hybridized for 16–24 h at 65 °C in hybridization mixture (minus Denhardt's) containing 500–600 pg/μl of GlyT2 anti-sense riboprobes. Some sections were hybridized with sense probe at a matched concentration to serve as control. After hybridization, the sections were washed in 2× SSC (pH 4.5), followed by three washes of 15 min each in 2× SSC (pH 4.5)/50% formamide at 65 °C, and finally washed in PBS. The sections were then pre-incubated (90 min, room temperature) in BSA-blocking solution consisting of PBS, 5% BSA (bovine serum albumin, Fraction V, Roche) and 0.5% Triton X-100. For detection of DIG, the sections were incubated in 2% BSA-blocking solution with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) diluted 1:4000 (overnight, 4 °C). Subsequently, the sections were washed in TBST (0.14 M NaCl, 2.7 mM KCl, 25 mM Tris/HCl (pH 7.5), and 0.1% Tween-20), followed by NTM (100 mM NaCl, 100 mM Tris/HCl (pH 9.5), and 50 mM $MgCl_2$). The blue reaction product was produced by the reaction of alkaline phosphatase with levamisol, NBT (nitroblue tetrazolium; Roche) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt; Roche) for 1.5–2 h at room temperature in the dark. Usually, the exact reaction time was determined by assessing the staining in the light microscope. The sections were randomly mounted on slides, air dried overnight, dehydrated using absolute ethanol (<0.01% methanol), transferred to xylene and coverslipped with Permount (Fisher, Hampton, NH).

4.3. *Data analysis*

Light micrographs were made with a digital camera and processed using Adobe Photoshop. The images were not manipulated, except for brightness and contrast.

Analysis was carried out on cervical (C2 and C6), thoracic (T5), lumbar (L2 and L5), and sacral (S1) segments from six rats. Between 15 and 20 sections per segment were mounted on a slide in a random order. In the microscope, the slide was systematically examined, starting with the first section in the first row. The first section of the appropriate segmental level that was encountered was used for analysis. Occasionally this section appeared damaged during the procedure or incorrectly mounted, in which case the section was discarded. Thus a total of 36 sections were analyzed. Using a camera lucida microscope (Neurolucida, MicroBrightfield Inc., Williston, VT) the outline of the white and grey matter, the boundaries between the laminae and the contour of several spinal nuclei were drawn unilaterally (Molander et al., 1984, 1989), after which the labeled neurons were plotted in the drawing. A neuron was considered labeled only if the largest diameter was at least 10 μm, the cell soma contained a bluish reaction product and a non-stained nucleus was apparent. The number of plotted neurons per lamina was expressed as a percentage of the total number of neurons plotted in that section. The results obtained in the six rats were averaged for each lamina per segment. The density of glycinergic neurons per lamina is expressed as the number of plotted neurons in a lamina divided by the surface area, expressed in mm^2, of that lamina, as calculated by Neurolucida. The results obtained in the six rats were averaged for each laminar density per segment. The data for both the laminar distribution and the density of glycinergic neuronal somata were analyzed by performing the one-way ANOVA test followed by the Bonferroni post hoc test. $p<0.05$ was considered as significant.

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Chapter III

Differential distribution of activated spinal neurons containing glycine and/or GABA and expressing c-fos in acute and chronic pain models

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Differential distribution of activated spinal neurons containing glycine and/or GABA and expressing c-fos in acute and chronic pain models

Mehdi Hossaini [a], Liron S. Duraku [b], Çiğdem Saraç [a], Joost L.M. Jongen [a,c], Jan C. Holstege [a,*]

[a] Department of Neuroscience, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands
[b] Department of Plastic Reconstructive and Hand Surgery, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands
[c] Department of Neurology, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands

Abstract

The inhibitory transmitters GABA and glycine play an important role in modulating pain transmission, both in normal and pathological situations. In the present study we have combined in situ hybridization for identifying spinal neurons that use the transmitter(s) glycine and/or GABA (Gly/GABA neurons) with immunohistochemistry for c-fos, a marker for neuronal activation. This procedure was used with acute pain models induced by injection of capsaicin or formalin; and chronic pain models using Complete Freund's Adjuvant (CFA, chronic inflammation), and the spared nerve injury (SNI) model (neuropathic pain).

In all models Gly/GABA neurons were activated as indicated by their expression of c-fos. The pattern of Gly/GABA neuronal activation was different for every model, both anatomically and quantitatively. However, the averaged percentage of activated neurons that were Gly/GABA in the chronic phase (≥ 20 hrs survival, 46%) was significantly higher than in the acute phase (≤ 2 hrs survival, 34%). In addition, the total numbers of activated Gly/GABA neurons were similar in both phases, showing that the activation of non-Gly/GABA (presumed excitatory) neurons in the chronic phase decreased. Finally, morphine application equally decreased the total number of activated neurons and activated Gly/GABA neurons. This shows that morphine did not specifically activate Gly/GABA neurons to achieve nociceptive inhibition.

The present study shows an increased activity of Gly/GABA neurons in acute and chronic models. This mechanism, together with mechanisms that antagonize the effects of GABA and glycine at the receptor level, may determine the sensitivity of our pain system during health and disease.

1. Introduction

Nociceptors convey their information from the periphery to the spinal cord where they target secondary neurons located in the superficial (laminae I-II) and deep (laminae III-VI) dorsal horn [45]. While a subpopulation of these secondary neurons are projection neurons that relay the nociceptive information to higher centers [40], the majority are local interneurons [4]. A substantial number of these interneurons contain the fast inhibitory transmitters GABA and glycine, often colocalized in the same cell [47,48] and these neurons are directly innervated by primary afferent fibers [10,14]. The importance of GABA and glycine in pain transmission was shown by blocking glycinergic and/or GABAergic neurotransmission in naïve animals, which induced many behavioral signs of hypersensitivity as observed in inflammatory and neuropathic pain conditions [38,39,50].

Accordingly, decreased inhibition was found in the spinal cord during chronic inflammatory pain [33,37]. In this condition, glycinergic inhibition in the spinal cord is blocked by a pathway involving prostaglandin E2 (PGE2). When this PGE2 induced blockade was prevented (<24 hrs) the thermal and mechanical sensitization of inflammatory pain did not appear [52]. During neuropathic pain there is loss of synaptic inhibition due to a shift in the neuronal chloride gradient, which reverses the inhibitory effect of GABA into a depolarizing one [9]. Further, it was recently shown that selective activation of GABA(A) receptors containing the α2 and/or α3 subunits [26] leads to pronounced nociceptive inhibition.

These studies described above underline the importance of glycine and GABA in modulating the spinal processing of nociceptive information, especially during chronic pain. However, little is known about the activity of inhibitory neurons, and changes therein, during different pain states. For identifying neurons that were recently activated, the expression of c-fos protein has been used extensively in the nociceptive system [8], showing that spinal c-fos expression patterns are correlated with the type, intensity and duration of nociceptive stimuli [8]. However, data on the activation patterns of spinal inhibitory neurons during different pain states are scarce. So far, three studies have [46,54,55] identified activated glycinergic and/or GABAergic (Gly/GABA) neurons after acute nociceptive stimulation of the hind paw with capsaicin or formalin. These studies have used immunohistochemistry to identify the c-fos protein as well as Gly/GABA neurons. However, presently available antibodies preferentially label Gly/GABA terminals and have proven difficult for reliably labeling the somata of Gly/GABA neurons [2,17]. Therefore, in the present study we have used in situ hybridization with markers for Gly/GABA neurons. This technique will identify mRNA for GlyT2 (a marker for glycinergic neurons) [36,41] or GAD67 (a marker for GABAergic neurons) [12,24] with a high sensitivity, and can be combined with immunohistochemistry for c-fos [42]. Using this approach we set out to investigate the activation patterns of spinal Gly/GABA neurons in different acute and chronic pain states. For this purpose we determined the number and the percentage of c-fos labeled neurons that expressed GABA and/or glycine in acute (capsaicin, formalin) or chronic (inflammatory and neuropathic) pain conditions. In addition, we investigated the effect of morphine

application on c-fos expression in Gly/GABA neurons after stimulation with formalin.

2. Materials and methods

In this study we used a total of 67 male Wistar rats. All animal experiments were approved by the Rotterdam Animal Ethical Committee.

2.1. Pain models

Nociceptive stimuli for the capsaicin, formalin and CFA pain models were applied to the left foot sole under light anesthesia (2% isofluoran in 30%O_2/70%N_2O; 2-3 min). The left foot sole was injected with either sterile saline (50µl of 0.9% NaCl, Baxter; 90 min survival, n=5), capsaicin (50µl of 0.3% N-Vanillylnonanamide, Sigma-Aldrich, diluted in 80% saline, 10% Tween-80, and 10% ethanol-100%; 90 min survival, n=5), Complete Freund's Adjuvant (chronic inflammation model; CFA, 100µl; 90 min survival, n=5; 20 hrs survival, n=5; 4 days survival, n=5), or 4% paraformaldehyde (PFA diluted in 0.12 M phosphate buffer pH ≈ 7.5; 45 min survival, n=5; 90 min survival, n=5). For induction of neuropathic pain, the animals were kept under anesthesia during the whole procedure (20-30 minutes). We used the SNI model according to the protocol described in [11]. In short, the three branches of the sciatic nerve were exposed above the knee, and the tibial and common peroneal branches were ligated and cut 2 mm distal to the ligation, while the sural branch was left intact. In the sham model, the three sciatic branches were also exposed but then left intact. We used survival times of 2 hrs, 1 wk and 2 wks for the SNI model and their corresponding sham models (n=24). In the morphine experiment, the animals received 1.5 mg of morphine (morphine HCL-3H_2O) subcutaneously 20 min prior to injection of formalin (50µl of 4 % PFA) in the left foot sole (90 min survival, n=4). The control group received 1.5 ml of sterile saline instead of morphine (90 min survival, n=4).

2.2. Behavioral experiments

The mechanical thresholds of the hind paws were assessed using the Von Frey hair monofilaments (Stoelting) in the capsaicin, CFA, and SNI (1 wk and 2 wks) groups. Before the start of the experiments, all rats were habituated to the experimenter, the experiment room, and a transparent cage (15cm x 15cm; gridded floor) for 5 days. Thereafter, prior to each experiment the rats were habituated for 30 minutes to the experiment room, and for 10 minutes to the transparent cage. Each Von Frey hair was applied for 2 seconds at 5 seconds interval, and the threshold was set at 3 evoked responses in a maximum of 5 applications. The mechanical thresholds were assessed at 60 min after injection in the capsaicin group, at 20 hrs (CFA 20 hrs) or 4 days (CFA 4 days) in the CFA group, and at 1 wk (SNI 1 wk) and 2 wks (SNI 2 wks) in the SNI group. In the morphine experiment, the number of flinches and flutters and the time spent licking the injected paw was measured during 60 min after the formalin injection.

2.3. Tissue preparation

At the end of an experiment, the rats received an overdose of sodium pentobarbital, and were transcardially perfused with 150 ml saline followed by 750 ml of 4% PFA. Thereafter, the lumbar spinal cords were dissected and incubated overnight at 4 ºC in RNAse free solution consisting of phosphate buffer (PB), 4% PFA and 30% sucrose. Coronal sections were cut at 30 µm with a freezing microtome, collected in 9 separate jars and stored in glycerol at -20 ºC.

2.4. Fluorescent in situ hybridization combined with fluorescent immunohistochemistry

For fluorescent in situ hybridization (FISH), the partial cDNA templates encoding GlyT2 (3.1 kb; a generous gift from Dr. N. Nelson, Tel Aviv University), or GAD67 (3.2 kb; a generous gift from Dr. A.J. Tobin and N. Tillakaratne, PhD, UCLA) were used. The recombinant plasmids were linearized, and subsequently riboprobes were transcribed using the appropriate RNA polymerases in the presence of fluorescein-labeled 11-UTP (Roche). For FISH the protocol described in [19] was applied with the following modifications. Sections from a jar were incubated with a mixture of GAD67 and GlyT2 probes in order to identify spinal inhibitory neurons (GABAergic and/or glycinergic). After riboprobe hybridization, the sections were incubated (48 hours at 4 ºC) with mouse monoclonal anti-fluorescein antibody (Roche; 1:500) and rabbit anti-c-fos (1: 4000; Oncogene Research Products, La Jolla, CA) in a cocktail of phosphate buffered saline (PBS), 2% milk powder (Profitar Plus, Nutricia) and 0.5% Triton X-100. Thereafter, sections were rinsed in PBS and incubated with biotinylated goat-anti-mouse (Vector; for detection of fluorescein), and donkey-anti-rabbit tagged with Cy3 (Jackson) for detection of c-fos antibody in a 2% milk powder cocktail for 90 minutes at RT. Subsequently, sections were rinsed in PBS and incubated with Avidin-Biotin-Complex (ABC, Vector) tagged with horseradish peroxidase (HRP) for 90 min at RT. After rinsing in PBS, a tyramide amplification procedure was performed by reacting HRP with H_2O_2 and a self prepared FITC tyramide solution as described previously [18]. Thereafter, the sections were washed in PB, mounted on slides and cover slipped with Vectashield (Vector).

2.5. Analyzing labeled neurons

Analysis was carried out on sections from the L4 and L5 lumbar spinal segments. Sections for analysis were chosen by starting in the first row of the randomly mounted sections and searching for sections from the appropriate segmental level, i.e. from rostral L4 to caudal L5. Per rat, the first 5 or 6 sections were analyzed in a Leica fluorescent microscope with a FITC and/or Cy3 filter. Labeling for GlyT2/GAD67 mRNAs (FITC) was considered as neuronal labeling if the staining was present in the cell soma and the shortest diameter was at least 10µm. In order to investigate colocalization of c-fos (Cy3) with GlyT2/GAD67 mRNAs (FITC), first c-fos labeling was assessed in a 40x objective. Thereafter, in the same focus field, we assessed whether there was labeling for GlyT2/GAD67 mRNA pres-

ent in the cytoplasm. If in the same focus field, c-fos labeling was surrounded by somatic GlyT2/GAD67 mRNA staining, the neuron was counted as a double labeled neuron. Single c-fos labeled and c-fos/GlyT2/GAD67 double labeled neurons were plotted by hand in an illustrated representation of the appropriate segmental level. In this illustration, the grey matter was divided in 10 laminae according to the laminar distribution in the rat [31]. In order to correct cell counts for double counting error, we measured the shortest and largest diameter of the nuclei of c-fos labeled neurons and c-fos labeled Gly/GABA neurons in laminae I-II, III-VI and VIII-X. The average diameter of the nuclei was calculated by averaging the sum of the shortest and largest diameters, and we then performed corrections for sampling bias related to cell size using Abercrombie's formula as described previously [15].Per rat, the average numbers of c-fos and c-fos/Gly/GABA double labeled neurons, and the average percentages of double labeled neurons were calculated. Per group, the results were averaged and compared with the average results in the other groups. Errors in the variations were assessed as standard error of the mean (SEM). The unpaired *t*-test or one-way ANOVA with a Bonferroni post-hoc test was performed for statistical comparison between groups. $p<0.05$ was taken as significant.

3. Results

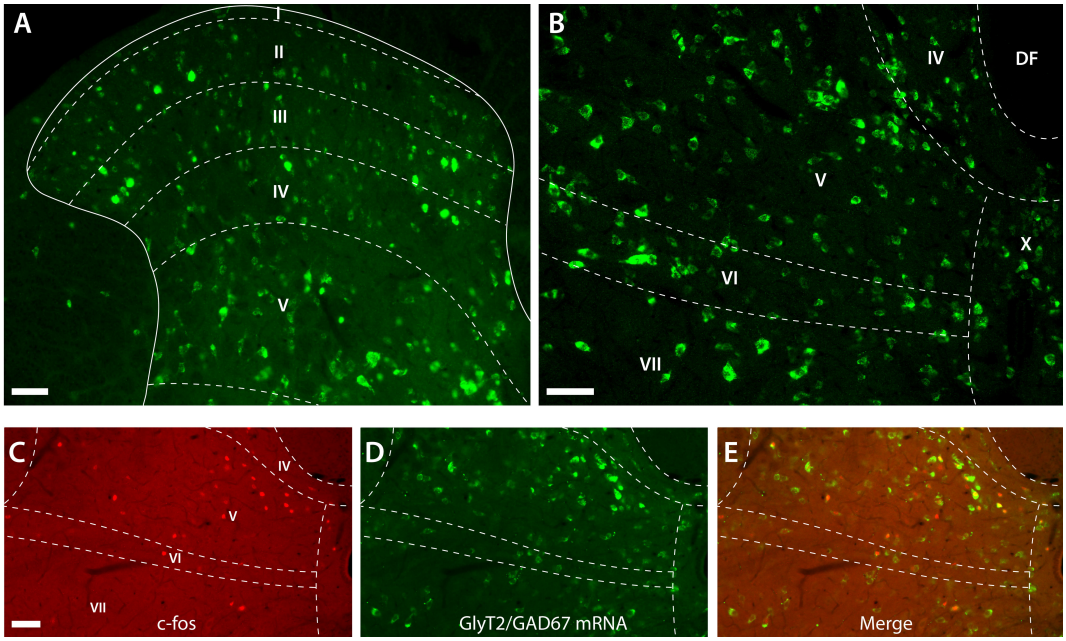
3.1. General observations

Fluorescent in situ hybridization (FISH) with a mixture of GlyT2/GAD67 probes resulted in cytoplasmic labeling of neurons that contained either GlyT2 mRNA, GAD67 mRNA or both mRNAs, thus representing neurons that use glycine and/or GABA as neurotransmitter(s) (Gly/GABA neurons) (Fig. 1A, B). In the superficial dorsal horn we observed labeled neurons that were relatively small in size. These neurons contain most likely GABA and not glycine since glycinergic neurons are scarce in laminae I and II [19,46]. Labeled neurons in the deep dorsal and ventral horn were mostly medium to large in size, representing neurons using glycine and/or GABA as neurotransmitters [47].

Fluorescent immunohistochemistry (IHC) for c-fos resulted in labeling of nuclei with various intensities (Fig. 1C). In the spinal cords of naïve rats c-fos labeled neurons were rare, while many were present in the spinal cords of rats that received a nociceptive stimulus. Most c-fos labeled neurons were located ipsilateral to the stimulus in the superficial (laminae I-II) and deep (III-VI) dorsal horn and much less in the ventral horn (VII-X). When FISH was combined with IHC, double labeled neurons were observed in which the labeling for c-fos (reddish nucleus) was surrounded by the labeling for GlyT2/GAD67 mRNA (greenish cytoplasm) (Fig. 1C, D, and E).

Fig. 1. Fluorescence micrographs of lumbar spinal sections showing the distribution of neurons labeled for GlyT2 and/or GAD67 mRNAs, and/or neurons labeled for c-fos protein after stimulation with formalin (45 min). (A and B) Note that labeled neurons in the superficial layers, which are mostly small and somewhat difficult to see at this magnification, are predominantly GABAergic neurons, while small and large labeled neurons

in the deeper laminae are glycinergic and/or GABAergic. (C, D and E) Labeling for c-fos protein in nuclei of neurons in the deep dorsal horn after stimulation with formalin (45 min) combined with labeling for GlyT2 and/or GAD67 mRNAs. Note that double labeled neurons are yellow. Roman figures indicate laminae; DF: dorsal funiculus; Scale bar: 50 µm.



3.2. Capsaicin, formalin, CFA and SNI pain models

The effects of nociceptive stimulations were assessed by behavioral analysis using Von Frey hair monofilaments. As expected, we found that the mechanical threshold (grams) was significantly decreased in the capsaicin (33 g before treatment, 3 g after capsaicin, $p<0.001$), chronic inflammation (CFA; 37 g before treatment, 5 g after CFA 1.5 hrs, $p<0.005$; 7 g after CFA 20 hrs, $p<0.005$; 10 g after CFA 4 days, $p<0.01$) and neuropathic pain (SNI; 27 g before treatment, 2 g after SNI 1 wk, $p<0.005$; 1 g after SNI 2 wks, $p<0.005$) groups as compared to the mechanical threshold of the same paw before treatment. In the sham-SNI groups we did not find a significant decrease in the mechanical threshold (27g before treatment, 20 g after sham-SNI 1wk, $p>0.05$; 19 g after sham-SNI 2 wks, $p>0.05$)

3.3. c-fos labeling pattern

In naïve unstimulated rats we found on average a total 0.5 ± 0.2 c-fos labeled neurons per section. In view of this low number, we did not further investigate the colocalization of c-fos in Gly/GABA neurons in naïve rats. Stimulation with capsaicin or formalin induced significantly higher average numbers of c-fos labeled neurons per section than stimulation with saline (control) (Fig. 2). In the CFA pain model, there were no significant differences between the numbers of c-fos labeled neurons obtained at different survival times (ANOVA). In the SNI pain model there were no significant differences between the

number of c-fos labeled neurons at 1 wk (18 ± 2.8) or 2 wks (14 ± 1.9) after nerve injury, and they were significantly lower (p<0.001, ANOVA) than at 2 hrs after nerve injury (Fig. 2). When considering all pain models (Fig. 2), the highest numbers of c-fos labeled neurons were found 90 minutes after stimulation wit formalin (53 ± 4) and at 2 hrs after spared nerve injury (61 ± 3.6).

With respect to the laminar distribution (Table 1A), c-fos labeled neurons were approximately equally abundant in laminae I&II and in laminae III-VI in the acute pain models (after saline, capsaicin, formalin) and in the acute phases of inflammation and neuropathic pain. However, in the chronic phases of inflammation (CFA 20 hrs and 4 days) and neuropathic pain (1 wk and 2 wks) the bulk of c-fos labeled neurons was present in laminae III-VI (Table 1A). In all pain models, laminae VII-X contained the lowest numbers of c-fos labeled neurons.

3.4. The number of c-fos labeled Gly/GABA neurons

In the saline, capsaicin and formalin models, the average number of c-fos labeled Gly/GABA neurons per section was significantly higher after stimulation with capsaicin (11 ± 1.1), 45 and 90 minutes after formalin injection (15 ± 0.8 and 21 ± 1.7, respectively) when compared to saline (4 ± 0.3, control) (Fig. 3, Fig. 4 and Fig. 5). These findings indicate that a saline injection, which served as control, also induced c-fos expression in spinal Gly/GABA neurons.

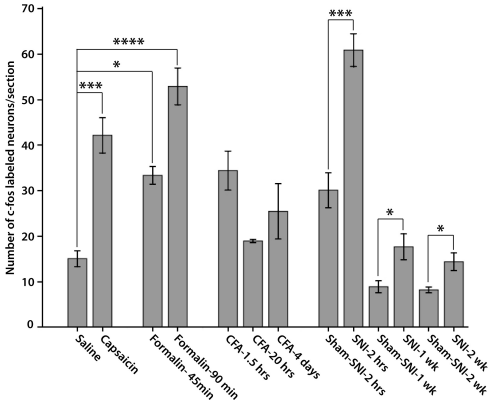


Fig. 2. Histogram showing the average number of c-fos labeled neurons per section in lumbar spinal cord in various rat pain models and their controls. Capsaicin, 45 and 90 minutes after formalin injection induced significantly higher numbers of c-fos labeled neurons than the control stimulation with saline (ANOVA). The SNI models induced significantly higher numbers of c-fos labeled neurons than their corresponding sham models (unpaired t-test). * = p<0.05; ** = p<0.01; *** = p<0.005; **** = p<0.001.

In the CFA inflammation model there were no significant differences between the numbers of c-fos labeled Gly/GABA neurons at 1.5 hrs (10 ± 2.7), 20 hrs (10 ± 0.4) and 4 days (10 ± 2.3) after injection of CFA (Fig. 4 and Fig. 5). In the SNI model, the numbers of c-fos labeled Gly/GABA neurons were higher in nerve injured than in the corresponding sham-SNI operated animals (controls) at all time points (Fig. 4). Further, a significantly (p<0.001, ANOVA) higher number of c-fos labeled Gly/GABA neurons was found at 2 hrs after nerve injury (25 ± 3.1) than at 1 wk (8 ± 1.2) or 2 wks (8 ± 1.4). Note that the higher

number at 2 hrs after nerve injury is partly explained by the contribution of the operation procedure, as shown by the relatively high number of activated Gly/GABA neurons in the sham operated animals (Fig. 4).

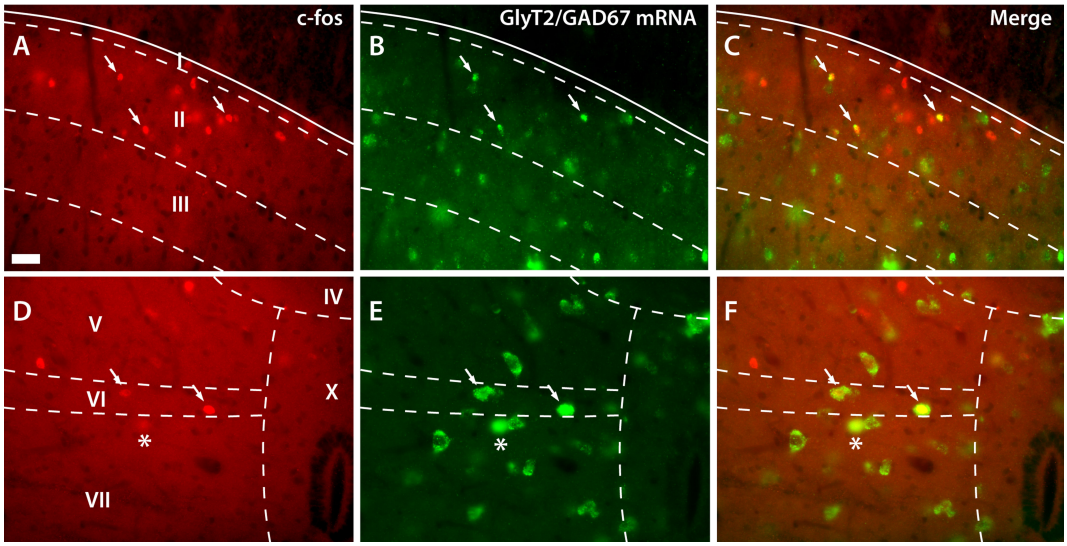


Fig. 3. Fluorescence micrographs from lumbar spinal sections showing labeling for c-fos protein and GlyT2/GAD67 mRNAs. Arrows indicate c-fos labeled neurons that also contained GlyT2 and/or GAD67 mRNAs. (A, B and C) Superficial dorsal horn at caudal L4 level of a rat stimulated with capsaicin. (D, E and F) Deep dorsal horn at caudal L5 level of a rat with chronic inflammation (CFA 4 days). Asterisk indicates a c-fos labeled neuron that was out of focus but also contained GlyT2 and/or GAD67 mRNA. Scale bar: 50 µm.

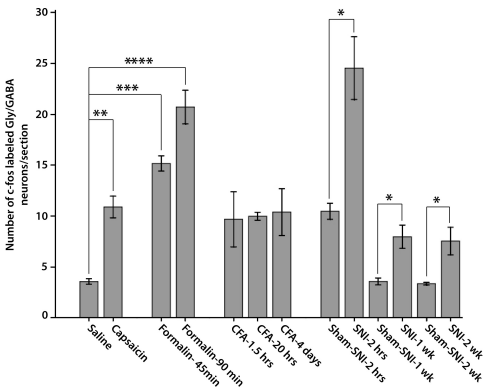


Fig. 4. Histograms showing the average number of c-fos labeled neurons per section that also contained GlyT2 and/or GAD67 mRNAs (Gly/GABA neurons) in different rat pain models. Capsaicin, 45 and 90 minutes after formalin injection induced significantly higher numbers of c-fos labeled Gly/GABA neurons than the control stimulation with saline (ANOVA). The SNI models induced significantly higher numbers of c-fos labeled neurons than their corresponding sham models (unpaired t-test). * = p<0.05; ** = p<0.01; *** = p<0.005; **** = p<0.001.

When comparing the number of c-fos labeled Gly/GABA neurons in all the pain models, there were no significant differences (p>0.05, ANOVA) between the capsaicin (11 ± 1.1) , CFA 1.5 hrs (10 ± 2.7), CFA 20 hrs (10 ± 0.4), CFA 4 days (10 ± 2.3), SNI 1 wk (8 ± 1.2) and SNI 2 wks (8 ± 1.2) pain models (Fig. 4, Fig. 5). Further, SNI 2 hrs (25 ± 3.1) induced significantly the highest number of c-fos labeled Gly/GABA neurons as compared to all other pain models (p<0.05, ANOVA), except for 90 minutes after formalin injection. When considering the distribution pattern of c-fos labeled Gly/GABA neurons, in most pain models the majority of the labeled neurons were located in the deep dorsal horn (III-VI), and with

lower percentages in the superficial dorsal horn and in the ventral horn (Table 1B).

	A			B		
	Laminae I-II	Laminae III-VI	Laminae VII-X	Laminae I-II	Laminae III-VI	Laminae VII-X
Saline	39 ± 8	47 ± 7	14 ± 2	20 ± 10	57 ± 12	23 ± 6
Capsaicin	45 ± 4	43 ± 3	12 ± 2	14 ± 4	57 ± 5	29 ± 7
Formalin-45	36 ± 3	54 ± 4	10 ± 1	27 ± 3	59 ± 5	13 ± 2
Formalin-90	35 ± 2	51 ± 1	14 ± 1	23 ± 2	56 ± 1	22 ± 2
CFA-1.5 hrs	52 ± 11	32 ± 4	16 ± 7	30 ± 8	40 ± 4	30 ± 10
CFA-20 hrs	10 ± 4	61 ± 1	29 ± 3	2 ± 1	59 ± 1	39 ± 2
CFA-4 days	14 ± 4	61 ± 2	25 ± 2	10 ± 2	65 ± 3	25 ± 4
Sham-SNI 2 hrs	49 ± 3	38 ± 1	13 ± 3	37 ± 4	41 ± 6	22 ± 6
SNI-2 hrs	37 ± 1	42 ± 2	21 ± 1	34 ± 2	40 ± 4	26 ± 3
Sham-SNI 1 wk	19 ± 2	64 ± 4	17 ± 4	16 ± 2	61 ± 4	23 ± 5
SNI-1 wk	17 ± 4	70 ± 4	13 ± 1	13 ± 5	74 ± 6	13 ± 1
Sham-SNI 2 wks	19 ± 3	63 ± 3	18 ± 4	13 ± 2	60 ± 3	27 ± 5
SNI-2 wks	14 ± 2	71 ± 2	15 ± 4	7 ± 0.4	80 ± 4	13 ± 5

Table 1. Laminar distribution of c-fos labeled and c-fos labeled Gly/GABA neurons in the various pain models. (A, B) Percentages (± SEM) of the total number of c-fos labeled (A) or c-fos labeled Gly/GABA (B) neurons that were located in laminae I-II, III-VI or VII-X are shown for each pain model. Note that the majority of c-fos labeled and c-fos labeled Gly/GABA neurons were located in the deep dorsal horn (laminae III-VI), especially in the chronic pain models.

3.5. The percentage of c-fos labeled neurons that were Gly/GABA

After calculating the average percentages of c-fos labeled neurons that were double labeled with Gly/GABA in all the pain models, we found that in the chronic phase of the CFA, sham-SNI and SNI models, there were no significant differences (p>0.05, ANOVA) between the percentages obtained for the two chronic survival times in each model. Since we were interested in possible differences between the acute and chronic phases, we

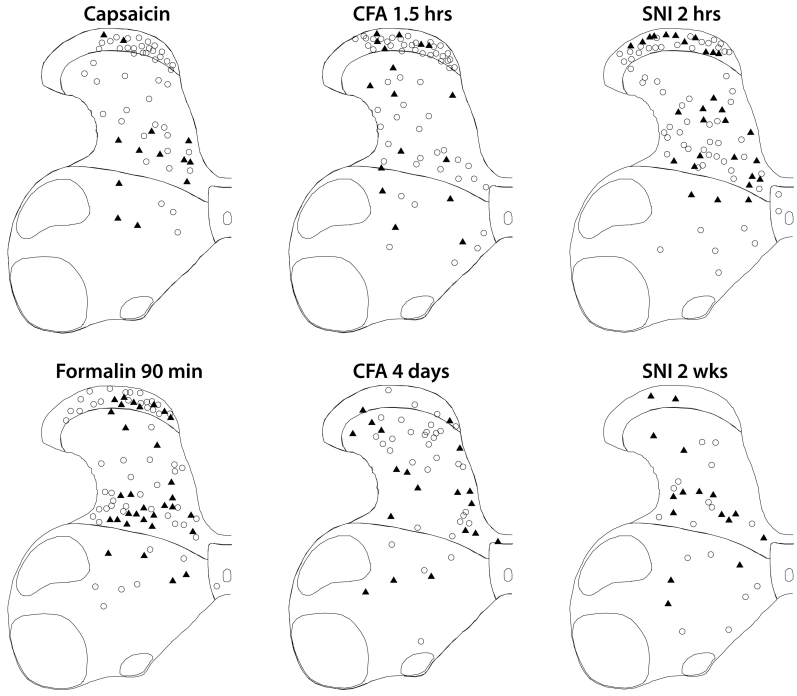


Fig. 5. Drawings showing the spinal distribution of c-fos labeled and c-fos labeled Gly/GABA neurons in randomly chosen single sections from the lumbar spinal cord, each from a different pain model. An open circle represents a single c-fos labeled neuron, and each filled triangle represents a single c-fos labeled neuron that also contained GlyT2/GAD67 mRNAs (Gly/GABA). 90 min after formalin injection and 2 hrs after nerve injury induced the highest numbers of c-fos labeled Gly/GABA neurons. Note that in most pain models, the bulk of c-fos labeled Gly/GABA neurons were located in the deep dorsal horn (laminae III-VI; see also table 1B). In most pain models, few c-fos labeled Gly/GABA neurons were located in the ventral horn (laminae VII-X).

combined in each group the chronic phases (i.e. 20 hrs + 4 days in the CFA model; 1wk + 2wks in the sham-SNI and SNI models). We then compared the result for the chronic phase with that of the acute phase in the different models (Fig. 6). This showed that in the CFA and SNI models the percentages of c-fos labeled neurons that were Gly/GABA were significantly higher in the chronic phases as compared to their acute phases. Also in the formalin model there was no significant difference between the two survival times. We therefore combined the two results and found that the percentage of c-fos labeled neurons that were Gly/GABA was significantly higher in the formalin model as compared to the saline and capsaicin models (Fig. 6).

We then averaged the percentages of all the chronic phases in the various models (i.e. CFA 20 hrs and 4 days; sham-SNI and SNI 1 wk and 2 wks), and compared it with the average percentages of all the acute phases (saline, capsaicin, formalin, CFA 1.5 hrs, sham-SNI and SNI 2 hrs). As a result we found that in the chronic phase the overall average percentage of the c-fos labeled neurons that were Gly/GABA was 46% (± 1.5), which is significantly higher (p<0.0005; unpaired t-test) than 34% (± 2), the overall percentage obtained for the acute phase.

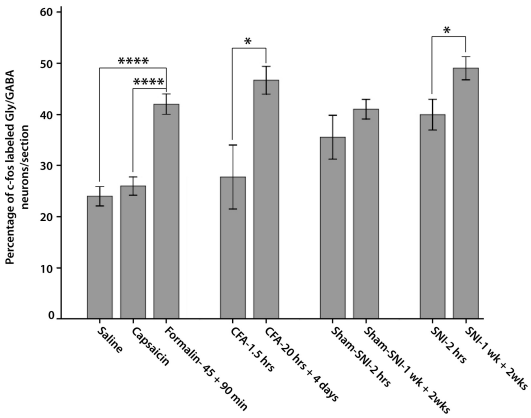


Fig. 6. Histogram showing the average percentages of c-fos labeled neurons per section that also contained GlyT2/GAD67 mRNA (Gly/GABA). A significantly higher percentage was found in the combined formalin (45 + 90 min) models as compared to the saline and capsaicin models (ANOVA). Note that the percentages of c-fos labeled neurons that were Gly/GABA in the chronic phases of the CFA (20 hrs + 4 days), and SNI (1 wk + 2 wks) models were significantly higher than the corresponding acute phases (unpaired t-test). * = p<0.05; **** = p<0.001.

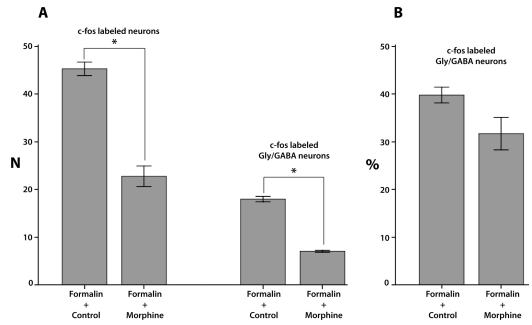


Fig. 7. Histograms showing the average numbers (A) or percentages (B) of c-fos labeled neurons after formalin stimulation in rats pretreated with saline (control, 20 min) or morphine (20 min). (A) The average number per section of c-fos labeled neurons or the average number of c-fos labeled neurons containing GlyT2/GAD67 mRNA (Gly/GABA) after formalin stimulation. (B) The percentage of c-fos labeled neurons that were Gly/GABA. Note that despite the decrease in the total number of c-fos labeled Gly/GABA neurons (A), the percentage of c-fos labeled neurons that were Gly/GABA did not change significantly when the rats were pretreated with morphine. *: p<0.05

3.6. Effects of morphine application in the formalin model

In order to determine the effect of morphine on the number of c-fos labeled Gly/GABA neurons we used the formalin pain model. The behavioral analysis confirmed the notion that morphine significantly reduced the licking time (saline: 630 sec ± 77, morphine: 71 s ± 12, $p<0.005$, unpaired *t*-test) and the number of fluttering and flinches (saline: 676 ± 61, morphine: 16 ± 5, $p<0.005$, unpaired *t*-test) of the injected paw. After formalin stimulation, the total number of c-fos labeled neurons and the number of c-fos labeled Gly/GABA neurons decreased by subcutaneous morphine application when compared to control (Fig. 7A). When considering the percentage of c-fos labeled neurons that were Gly/GABA, we found no difference between the percentages of the control and the morphine treated groups (Fig. 7B).

4. Discussion

In the present study we have shown that Gly/GABA neurons are activated (i.e. expressing c-fos protein) in all the pain models that we have investigated. Our data further showed that the percentage of activated neurons that are Gly/GABA is higher in the chronic phase (46%) than in the acute phase (34%). Furthermore, the systemic application of morphine, preceding formalin injection, reduced the activation of Gly/GABA neurons and non-Gly/GABA (presumed excitatory) neurons in an equal manner. These findings show that active recruitment of Gly/GABA neurons is inherent to the normal and pathological processing of nociceptive stimuli in the spinal cord.

4.1. Technical aspects

FISH was used to identify glycinergic neurons by their expression of glycine transporter 2 (GlyT2) mRNA [23,28], and GABAergic neurons were identified by their expression of the mRNA for the GABA synthesizing enzyme (GAD) [13]. The GAD67 isoform of this enzyme is found in the large majority of spinal GABAergic neurons, often together with the GAD65 isoform [30]. A few neurons in the ventral horn, involved in presynaptic inhibition of 1A afferents only express the GAD65 isoform [20]. These neurons have not been identified in the present study, but it is highly unlikely that this has significantly affected our results. GABA and glycine are often colocalized in neurons [43,47] and co-released at synapses [22]. Therefore, we did not attempt to identify glycinergic and GABAergic neurons separately but rather aimed at labeling them simultaneously.

For identifying spinal neurons that were activated in various pain models we have used c-fos, an immediate early gene (IEG) that is widely used as a marker for neuronal activation in pain research [8]. Our results on the expression of c-fos in various pain models are in general agreement with other studies [6,21,29]. Application of GABA and glycine antagonists in naïve animals leads to a hypersensitive spinal cord [38,39]. This indicates the presence of a continuous inhibitory tone, which is most likely due to activity in nearby Gly/GABA neurons [1]. Apparently, the activity of these neurons does not induce significant

amounts of c-fos protein, since labeled c-fos neurons are very low in naïve animals. Thus, c-fos expression in spinal neurons signals phasic activity associated with active nociceptive processing rather than tonic activity associated with the presumed ongoing inhibition in the naïve spinal cord. Therefore, the results obtained in the present study are associated with the processing of phasic nociceptive stimuli in the different pain models.

4.2. c-fos expressing Gly/GABA neurons

In all our pain models, we found that a substantial proportion (ranging between 24 and 53%) of the activated neurons expressed glycine and/or GABA. It seems likely that this activation was induced by nociceptive afferents, which are known to contact Gly/GABA neurons [5,10,14,16], although indirect activation through interneurons cannot be excluded. Previous studies [46,54,55] using different techniques have also shown the activation of spinal inhibitory neurons by means of c-fos expression after capsaicin or formalin stimulation. Our data are in general agreement with the study by Todd et al. [46], however we found a lower percentage of activated GABAergic neurons in the superficial dorsal horn after capsaicin stimulation when compared to the studies by Zou et al [54,55].

In our study, capsaicin induced a significant increase in the number of Gly/GABA activated neurons, when compared to saline. However, the percentages of activated Gly/GABA neurons after saline (24%) and capsaicin (26%) stimulation were similar. This shows that in this paradigm, irrespective of the stimulus intensity (i.e. low after saline and high after capsaicin), the number of activated Gly/GABA neurons is proportional to the total number of activated neurons. In the formalin model, which typically shows a first and second phase in the behavioral response [44], the total number of activated neurons is higher at 90 min after formalin injection than at 45 min. Also in this case the percentages of activated Gly/GABA neurons were not significantly different at the two time points, although, when combined, it was higher than after saline or capsaicin injections. These results suggest that in a given pain model the proportion of activated neurons that are Gly/GABA remains stable, irrespective of the stimulus intensity. However, when we examined the CFA experiments we found the reverse situation: the total number of activated Gly/GABA neurons was similar at the different time points (i.e. 1.5 hrs, 20 hrs and 4 days), while the percentage of activated Gly/GABA neurons was higher in the chronic phase than at 1.5 hrs. In the SNI experiments, the total number of activated Gly/GABA neurons declined significantly in the chronic phase, while there was a significant increase in the percentage. In the sham-SNI group, in which the operation procedure by itself resulted in a significant number of c-fos expressing neurons, the percentage did not increase in the chronic phase as compared to the acute phase. We therefore concluded that there was no consistent pattern of Gly/GABA neuronal activation when comparing the different pain models. However, within a particular pain model there were consistencies in the numbers or percentages of activated Gly/GABA neurons.

We then examined the data from another viewpoint by subdividing all pain models in acute (≤ 2 hrs) and chronic (≥ 20 hrs) phases. Our data showed that in the chronic phase the percentage of activated Gly/GABA neurons (46%) was higher than in the acute phase (34%). This increase in the percentage in the chronic phases was due to a decrease in the total number of activated neurons while the number of activated Gly/GABA neurons remained stable. Assuming that the majority of the non-Gly/GABA activated neurons were excitatory, our data show a decline in the number of activated excitatory neurons in the chronic phase.

Hypersensitivity in chronic pain conditions has been shown to result from loss of spinal inhibition due to blockade of glycinergic and GABAergic receptors [53]. At the same time, our data indicate an increased activity of inhibitory neurons in chronic pain conditions. Whether the balance between these apparently opposing mechanisms determines the sensitivity of spinal neurons for incoming nociceptive stimuli, or whether they act on different aspects of pain transmission, is presently unclear.

4.3. Laminar distribution of c-fos labeled Gly/GABA neurons

Activated Gly/GABA neurons in the superficial dorsal horn are mainly GABAergic [19,46], while activated Gly/GABA neurons in the deep dorsal horn are glycinergic and/or GABAergic [47]. Deeper located Gly/GABA neurons have been suggested as a source of inhibitory input to the superficial dorsal horn [51], which may be lost in chronic pain states, leading to touch evoked allodynia. There is also loss of GABAergic inhibition in the superficial dorsal horn in chronic neuropathic pain states, which may be due to loss of GABAergic interneurons [32,34,35]. Our data showed that the number of activated Gly/GABA neurons in the deep dorsal horn remained stable over time in the chronic phase, while the numbers of presumed excitatory neurons were declining. Thus, our results on activated Gly/GABA neurons do not indicate loss of inhibition in chronic pain states due to decreased activation of spinal Gly/GABA neurons.

It has been suggested that GABAergic neurons in the superficial dorsal horn receive C-fiber input [16], and are important for regulating the spinal transmission of innocuous and nociceptive information, especially during acute pain [10]. Our data confirm the presence of activated Gly/GABA neurons in the superficial dorsal horn, but their numbers are low when compared to the number of activated Gly/GABA neurons in the deep dorsal horn.

4.4. The morphine experiment

After a systemic morphine injection preceding a formalin injection to the hind paw, there was a significant reduction in the total number of c-fos labeled neurons and a proportional decrease in the number of c-fos labeled Gly/GABA neurons. It seems most

likely that most inhibition induced by morphine is due to presynaptic inhibition of primary afferents expressing mu-opioid receptors [3]. Postsynaptic mu-opioid receptors in the superficial dorsal horn are expressed preferentially by neurons, mainly in laminae II, that are not GABAergic or glycinergic [25]. This would suggest that inhibitory neurons escape postsynaptic inhibition by morphine. However, our finding of an equal reduction of c-fos expression in Gly/GABA and non-Gly/GABA (presumed excitatory) neurons, indicates that the overall effect of the pre- and postsynaptic inhibition induced by morphine is about equally strong on inhibitory and excitatory neurons. Similarly, the suggestion [7] that morphine would activate Gly/GABA neurons, especially GABAergic neurons in lamina II, is not supported by our findings. Thus, the proportional decrease in the number of c-fos labeled Gly/GABA neurons after morphine injection shows that morphine did not specifically recruit spinal Gly/GABA neurons for the inhibition of nociceptive inputs.

4.5. Conclusions

A characteristic feature of the pain models that we have examined is the development of hypersensitivity for (in)nocuous stimuli. This phenomenon is due to glutamate induced sensitization in spinal neurons [27,49], along with a blockade [53] or reversal [9] of inhibitory impulses. We now show that similar numbers of Gly/GABA neurons are activated by nociceptive stimuli during acute and chronic pain states, while in chronic pain states the activation of presumed excitatory neurons is declining. Therefore it seems most likely that the balance between all these mechanisms, and the disturbances therein during pathological pain states, will determine the sensitivity of our pain system during health and disease.

Conflict of Interest Statement:

The authors have no conflict of interest that could compromise the conduct of this study or the reporting of the results.

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Chapter IV

C-fos activation of spinal inhibitory neurons after contralateral hindpaw stimulation of rats with inflammatory or neuropathic pain

Hossaini M[1], Saraç C[1], Jongen JL[1,2], Holstege JC[1]

[1]Dep. of Neuroscience, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands; [2]Dep. of Neurology, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands

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Abstract

In the present study we have investigated the c-fos activation pattern of glycinergic and/or GABAergic (Gly/GABA) neurons in the spinal cord after capsaicin injection in rats with acute, chronic inflammatory (i.e. complete freund's adjuvant (CFA) induced inflammation) or neuropathic pain (i.e spared nerve injury (SNI)). For this purpose we employed fluorescent in situ hybridization (FISH) in order to identify glycinergic (GlyT2 mRNA) and GABAergic (GAD67 mRNA) neurons, and fluorescent immunohistochemistry (IHC) for identifying c-fos, a marker for neuronal activation. In rats in which capsaicin, CFA (1.5 hrs or 20 hrs), or saline was injected in the hind paw, stimulation of the contralateral hind paw with capsaicin did not result in a significantly different number or percentage of c-fos labeled neurons that were Gly/GABA as compared to capsaicin injection alone. However, we found a higher number and percentage of c-fos labeled neurons that were Gly/GABA after contralateral capsaicin injection in rats with chronic inflammatory (4 days), sham-SNI or SNI (2 weeks) as compared to capsaicin injection alone. In these rats, the mechanical threshold after capsaicin injection, as determined by Von Frey hairs, did not differ from the mechanical threshold in rats that only received capsaicin injection. Our data show that chronic inflammatory and neuropathic pain in a hindpaw alters the activation pattern of Gly/GABA neurons on the contralateral side of the spinal cord, indicating that not only the ipsilateral, but also the contralateral side of the spinal cord is affected by a nociceptive stimulation on one side.

1. Introduction

Nociceptors are primary afferent nerve fibers that are activated by stimuli that are potentially damaging to our body. These nociceptive signals are conveyed to the spinal cord, where nociceptors establish synapses with secondary nociceptive specific or wide-dynamic range neurons in various laminae of the dorsal horn [21]. Many of these neurons also receive inputs from local interneurons and neurons from supraspinal sites. Apart from excitatory inputs, a substantial number of these inputs are inhibitory in nature, since they use GABA and/or glycine as their neurotransmitter [23]. This network of inputs forms a system that plays a major role in setting the responsiveness of the spinal nociceptive system to incoming nociceptive signals [1, 9, 22].

At the spinal level, incoming nociceptive stimuli activate neurons on the ipsilateral side of the spinal cord, which are nociceptive-specific neurons located in the superficial (laminae I-II) and wide-dynamic range neurons in the deep dorsal horn (laminae III-VI) [3]. In many studies, activated spinal nociceptive neurons are identified by the expression of the c-fos protein [3, 6], which is an immediate early gene [12]. After peripheral nociceptive stimulation, spinal neurons expressing c-fos are predominantly located on the ipsilateral side, but c-fos is also expressed on the contralateral side [3], especially during chronic pain states. This suggests that nociceptive neurons on both sides of the spinal cord are activated by a unilateral stimulus. In animal models, when noxious stimulation (formalin or complete Freund's adjuvant) of a hind paw is followed by a second noxious stimulation of the contralateral paw, c-fos expression induced by the second stimulus was stronger when compared to a single stimulus [15]. Further, it has been shown that peripheral Transcutaneous Electrical Nerve Stimulation (TENS) of the side contralateral to the nerve injured side reduces mechanical allodynia on the injured side [20]. These studies suggest that activated spinal neurons on the contralateral side of the stimulus may play a functional role in nociceptive processing.

In the present study, we investigated the number and the percentage of c-fos expressing neurons that use glycine and/or GABA (Gly/GABA neurons) as their neurotransmitter after capsaicin stimulation, when a prior noxious stimulus was applied to the contralateral hind paw. This study is a continuation to a previous study in which we investigated the expression of c-fos in spinal Gly/GABA neurons after a single noxious stimulus [9]. The objective of the present study was to investigate whether the activation pattern of spinal Gly/GABA neurons in an acute pain model is modulated by prior noxious stimulation of the contralateral hind paw. We show here that the number and the percentage of c-fos expressing Gly/GABA neurons is increased when chronic inflammation or neuropathic pain is induced previously in the contralateral hind paw.

2. Methods

All animal experiments were approved by the Rotterdam Animal Ethical Committee and were in line with ethical guidelines of International Association for the study of

pain [26].

2.1. Pain models

All noxious stimulations were applied under anesthesia with 2% isofluoran in 30%O_2/70%N_2O

to the left paw (the first noxious stimulation) or the right paw (the second noxious stimulation, or capsaicin injection in the control group). The following stimulants were applied to either the right and/or the left hind paw (see also Table): *Group A-K*: Capsaicin (50µl of 0.3%; N-Vanillylnonanamide, Sigma-Aldrich, diluted in 80% saline, 10% Tween-80, and 10% ethanol-100%); *Group C-E*: Complete Freund's Adjuvant (chronic inflammatory pain; CFA, 100µl); *Group F and G*: Spared Nerve Injury (SNI) model (neuropathic pain) was applied following the protocol described in [4]. In short, the three branches of the sciatic nerve are exposed, and the tibial and common peroneal branches are ligated and cut 2-3 mm distal to the ligation. The sural branch is left intact. As control operation (sham) the three branches of sciatic are only exposed; *Group H-K*: Injection of an empty syringe or sterile saline (100µl; 0.9% sodium chloride, Baxter).

Group	Left paw	Right paw	*n*
A (Control)	-	Capsaicin (90 min)	5
B	Capsaicin (90 min)	Capsaicin (90 min)	4
C	CFA (1.5 hrs)	Capsaicin (90 min)	4
D	CFA (20 hrs)	Capsaicin (90 min)	4
E	CFA (4 days)	Capsaicin (90 min)	5
F	Sham (14 days)	Capsaicin (90 min)	4
G	SNI (14 days)	Capsaicin (90 min)	4
H	Syringe (20 hrs)	Capsaicin (90 min)	3
I	Syringe (4 days)	Capsaicin (90 min)	3
J	Saline (20 hrs)	Capsaicin (90 min)	4
K	Saline (4 days)	Capsaicin (90 min)	5

2.2. Mechanical threshold

The mechanical threshold was assessed using the Von Frey hair monofilaments (Stoelting). Before commencement of experiments, all rats were habituated to the experimenter, the experiment room, and a transparent cage (15cm x 15cm; gridded floor) for 5 days. Thereafter, prior to each experiment the rats were habituated for 30 minutes to the experiment room, and for 10 minutes to the transparent cage. Each Von Frey hair was applied for 2 seconds at 5 seconds interval, and the threshold was set at 3 evoked responses in a maximum of 5 applications. In all the groups, the mechanical threshold after capsaicin stimulation was assessed 60 minutes after injection. Further, in the groups with bilateral stimulation, the mechanical threshold of the left hind paw (first stimulation) was assessed 30 minutes prior to the capsaicin injection in the right hind paw (second stimulation).

2.3. Tissue preparation

All rats were received an overdose of sodium pentobarbital 90 minutes after capsaicin injection, and were transcardially perfused with 150 ml saline followed by 750 ml of 4% PFA. Thereafter, the lumbar spinal cord was dissected and incubated overnight at 4°C in a RNAse free solution of 4% PFA and 30% sucrose dissolved in phosphate buffer (PB). Coronal sections were cut at 30 µm in a freezing microtome and stored in glycerol at -20°C.

2.4. Fluorescent in situ hybridization combined with fluorescent immunohistochemistry

For fluorescent in situ hybridization (FISH) the following partial cDNA templates encoding the following mRNAs were used: GlyT2 (3.1kb; a generous gift from Dr. N. Nelson, Tel Aviv University), GAD67 (3.2kb; a generous gift from Dr. A.J. Tobin, UCLA). The riboprobes were obtained by linearizing the recombinant plasmids with the appropriate restriction enzymes and RNA polymerases. The transcription was performed in the presence of fluorescein-labeled 11-UTP (Roche). For (FISH) the following modifications were applied to the protocol described in [10]: sections were incubated with GAD67 mRNA together GlyT2 mRNA in order to identify GABAergic and/or glycinergic (inhibitory) neurons. After riboprobe hybridization, the sections were incubated (48 hours at 4 ºC) with mouse monoclonal anti-fluorescein antibody (Roche; 1:500) and rabbit anti-c-fos (1: 4000; Oncogene Research Products, La Jolla, CA) in a cocktail of phosphate buffered saline (PBS), 2% milk powder (Profitar Plus, Nutricia) and 0.5% Triton X-100. Thereafter, sections were rinsed in PBS and incubated with biotinylated goat-anti-mouse (GAM, Vector) and donkey-anti-rabbit tagged with Cy3 in a 2% milk powder cocktail for 90 minutes at RT. Subsequently, after rinsing in PBS sections were incubated (overnight at 4°C) with Avidin-Biotin-Complex (ABC, Vector) tagged with horseradish peroxidase (HRP). After rinsing in PBS, a tyramide amplification procedure was performed by reacting HRP with H_2O_2 and a self made FITC tyramide according to protocol described in [8]. Thereafter, the sections were washed in PB and mounted on slides and coverslipped with Vectashield (Vector).

2.5. Analyzing c-Fos labeled neurons

Analysis was carried out on sections from lumbar (L4 and L5) segment of the spinal cord. Sections for analysis were chosen by starting in the first row of mounted sections and searching for undamaged sections of appropriate segmental level, ranging from rostral L4 to caudal L5. Per rat, 5 to 6 sections were analyzed, and were analyzed in a Leica fluorescent microscope with FITC and/or Cy3 filter. Fluorescent labeling for GAD67+GlyT2 mRNA was accounted as neuronal labeling if the staining was present in the cell soma and an empty nucleus was apparent. However, in the superficial dorsal horn some neurons labeled for GAD67+GlyT2 mRNA are intensely stained, and an empty nucleus was not always visible. These neurons were accounted as labeled neurons if the shortest diameter was at least 10µm.

In order to investigate the colocalization of c-fos with GAD67/GlyT2 mRNA, first c-fos labeling was assessed in a 40x objective. Thereafter, in the same focus field, the c-fos labeled neuron was analyzed for cytoplasmatic labeling of a mRNA. If the same focus field, c-fos labeling was surrounded by somatic mRNA staining, then the neuron was accounted as a double labeled neuron. Single or double labeled neurons were plotted by hand in an illustrated representation of the appropriate segmental level. The grey matter was divided in three main parts (laminae I-II, laminae III-VI, and laminae VII-X) according to the laminae distribution described [16]. The unpaired *t*-test was performed for statistical compari-

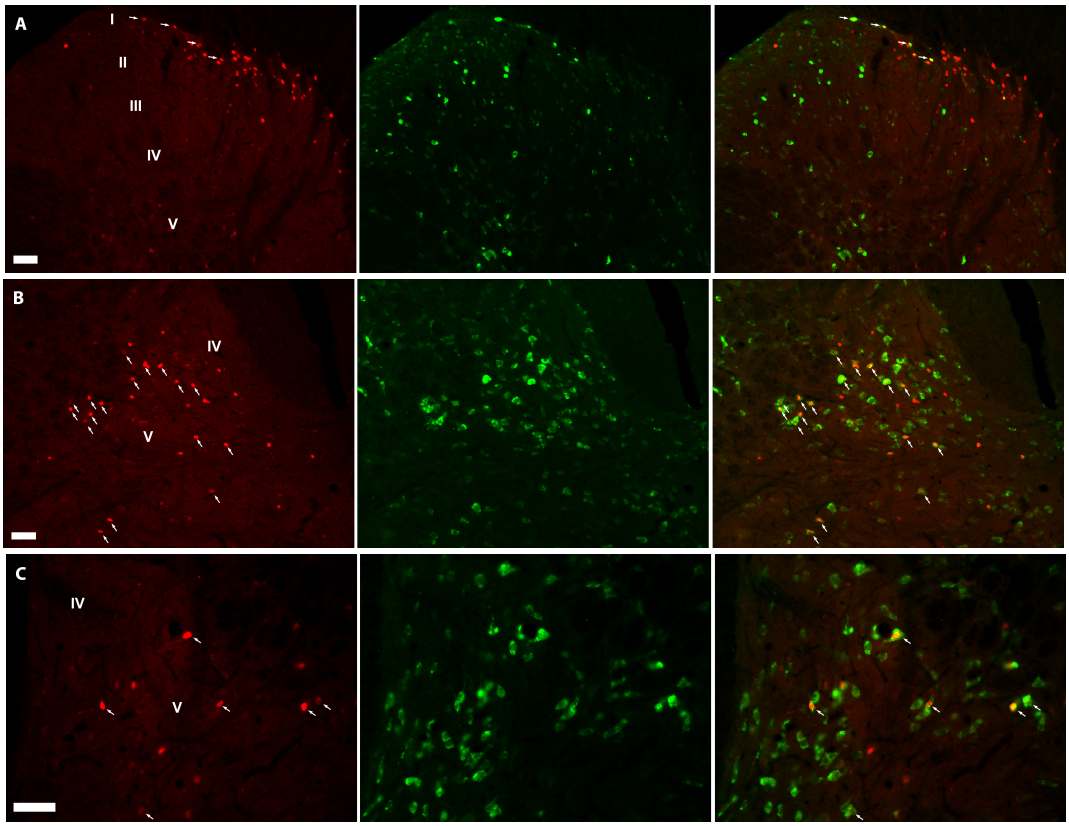
son between the groups, and $P<0.05$ was taken as significant.

3. Results

3.1. General observation

Applying FISH with GlyT2 mRNA together with GAD67 mRNA resulted in cytoplasmatic labeling of spinal inhibitory, i.e. Gly/GABA neurons (Fig. 1). Labeled neurons were located in all the laminae of the grey matter and were of various sizes, and in most cases cytoplasmatic labeling was apparent. By using GlyT2 together with GAD67 mRNA, spinal inhibitory neurons were identified as one group without distinction between GABAergic and/or glycinergic neurons. Our labeling pattern was consistent with previous findings [9, 23, 24]. In order to identify activated spinal neurons, the neuronal activation marker c-fos was detected by applying fluorescent immunohistochemistry (IHC), which resulted in nuclear labeling of neurons (Fig. 1). Depending on the type of peripheral stimulus, c-Fos labeled neurons were located solely in the superficial dorsal or in the superficial and deep dorsal horn, and sometimes also in the ventral horn of the spinal cord.

Fig. 1: Fluorescence micrographs showing labeling for GlyT2/GAD67 mRNAs (Gly/GABA neurons) and for c-fos protein. A, B, C: c-fos expression in spinal dorsal horn neurons after stimulation with capsaicin in rats with inflammatory pain (A, C), and neuropathic pain (B). Arrows indicate Gly/GABA neurons that also expressed c-fos protein. Scale bar:50µm.



3.2. Capsaicin injection (control)

After a single capsaicin injection (90 minutes), which served as control, we found 51 ± 3.7 c-fos labeled neurons on the ipsilateral side of the spinal cord. 47% ± 2 (mean ± SEM) of these neurons were located in laminae I-II and 45% ± 2 were located in laminae III-VI. Furthermore, when c-fos was colocalized with GAD67/GlyT2 mRNAs, 13 ± 1 c-fos labeled neurons also contained GAD67/GlyT2 mRNA, indicating that these neurons were inhibitory. This accounted for 26% ± 3 of the total c-fos labeled neurons, and these double labeled neurons were mainly located in laminae III-VI.

3.3. Bilateral paw stimulation

In the following groups, a first noxious stimulation was applied to the left hind paw followed by a second stimulation with capsaicin of the right hind paw. Results on c-fos expression and colocalization pattern after subsequent capsaicin stimulation were compared to the results in the control group in which only single capsaicin stimulation (see above) was applied.

3.3.1. Capsaicin-capsaicin

Number of c-fos cells

When capsaicin injection in the left hind paw was followed by a capsaicin injection in the right hind paw, the second capsaicin injection induced 48 ± 4 c-Fos labeled neurons (Fig. 2), which is not significantly different from a single capsaicin injection (51 ± 4, see above) (p>0.05, unpaired t-test). Also, the distribution of c-fos labeled neurons in laminae I&II and laminae III-VI was not different between the two groups (Table 1A).

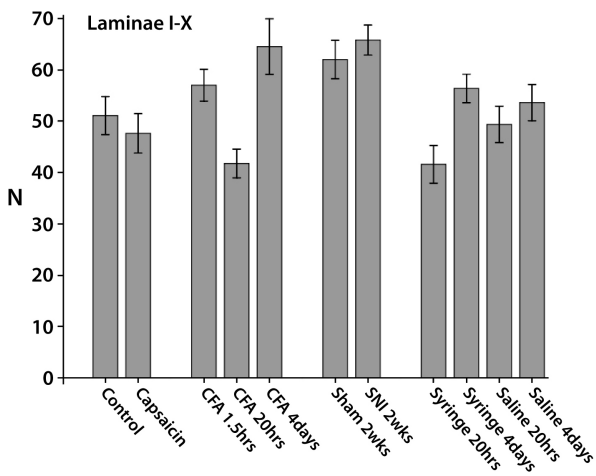


Fig. 2: Histogram showing the total number of c-fos labeled neurons in laminae I to X of the grey matter after capsaicin injection in naive (control) rats, and rats in pretreated with capsaicin, CFA (1.5hrs, 20hrs, or 4 days), sham-SNI (1 wk, or 2 wks), SNI (1 wk, or 2 wks) or saline (20hrs, or 4 days). We found no significant differences in the number of c-fos labeled neurons between the control stimulation (single capsaicin injection) as compared to other pain models.

Percentage and number of c-Fos and GAD67/GlyT2 mRNA double labeled cells

When the second capsaicin injection was applied to the right hind paw after a prior left sided capsaicin injection, 28% ± 2 of c-fos positive neurons were inhibitory (Fig. 3A), and this was not significantly different from single capsaicin stimulation (26% ± 3) (p>0.05, unpaired t-test). Also, the number of c-fos double labeled neurons that were inhibitory neurons was not different after second capsaicin stimulation (Fig. 3B). Further, the distribution of c-fos neurons containing GlyT2/GAD67 in laminae I&II and laminae III-VI was not different between the two groups (Table 1B).

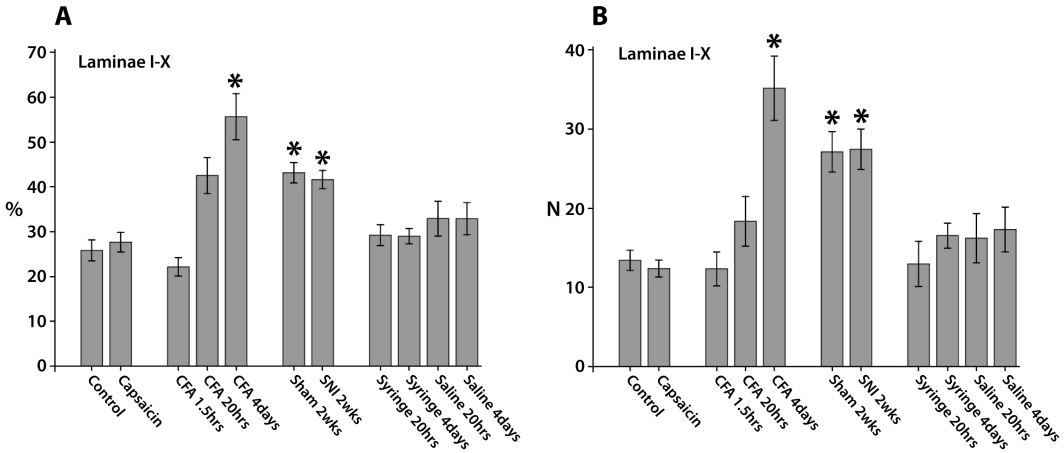


Fig. 3: Histogram showing the percentage (A) and the number (B) of c-fos labeled neurons that also contained GlyT2/GAD67 mRNA in various pain models. * = p<0.05, as compared to control.

3.3.2. CFA-capsaicin

Number of c-Fos cells

When CFA was injected in the left hind paw, followed by right hind paw stimulation with capsaicin at different time points (1.5 hrs, 20 hrs, and 4 days), the number of c-fos labeled neurons induced by the second stimulation was not significantly different from a single capsaicin injection (Fig. 2) (p>0.1, ANOVA). There was also no difference in the laminar distribution of c-fos labeled neurons between CFA-capsaicin treated groups and capsaicin injection alone (Table 1A).

Percentage and number of c-Fos and GAD67/GlyT2 mRNA double labeled cells

The number of c-fos labeled neurons that were inhibitory after subsequent capsaicin stimulation (left paw) was significantly higher (35 ± 4) after 4 days of CFA induced inflammation in the contralateral hind paw (right paw) (Fig. 3B) (p<0.05, ANOVA), in comparison to 1.5hrs (12 ± 2) or 20hrs (18 ± 3) of inflammation in the right hind paw, or single capsaicin stimulation (control). Furthermore, the percentage of c-fos labeled neurons that was inhibitory after subsequent capsaicin stimulation significantly increased at 4 days of

inflammation in the contralateral paw when compared to single capsaicin injection (Fig. 3A) (p<0.05, ANOVA). Thus, our data indicate that a relatively decreased threshold for capsaicin induced c-fos expression is achieved in spinal inhibitory neurons when inflammation (4 days) is induced in the contralateral paw.

		Control	Capsaicin	CFA 1.5hrs	CFA 20hrs	CFA 4 days	Sham 2wks	SNI 2wks	Saline 20hrs	Saline 4days
A	Laminae I-II	47 ± 2	48 ± 2	49 ± 1	43 ± 3	42 ± 2	35 ± 1	36 ± 1	41 ± 2	48 ± 2
	Laminae III-VI	45 ± 2	43 ± 1	42 ± 1	47 ± 2	49 ± 1	51 ± 1	51 ± 1	51 ± 2	43 ± 2
B	Laminae I-II	15 ± 3	24 ± 5	26 ± 0.3	35 ± 4	42 ± 4	27 ± 5	37 ± 5	33 ± 2	33 ± 3
	Laminae III-VI	61 ± 4	54 ± 5	46 ± 4	53 ± 2	46 ± 2	50 ± 1	48 ± 5	54 ± 3	49 ± 4
C	Capsaicin	2 ± 0.6	2 ± 0.8	3 ± 0.7	3 ± 0.7	1 ± 0.3	2 ± 0.2	3 ± 0.7	2 ± 0.8	4 ± 1

Table 1: The percentages of c-fos labeled (A), and c-fos labeled neurons that also contained GlyT2/GAD67 mRNAs (B) that were located in laminae I-II and in laminae III-VI of the spinal cord. C: The mechanical threshold (g) in the hind paw after capsaicin injection.

3.3.3. SNI-capsaicin

Number of c-Fos cells

When compared to a single capsaicin injection, the number of c-fos labeled neurons is not different after a subsequent capsaicin stimulation if sham or SNI is applied two weeks before to the contralateral (left) paw (Fig. 2).

Percentage and number of c-Fos and GAD67/GlyT2 mRNA double labeled cells

We found that the number of c-fos positive inhibitory neurons is increased after subsequent capsaicin stimulation (compared to single capsaicin stimulation), when SNI or sham operation is previously applied to the contralateral paw (Fig. 3B) (p<0.05, ANOVA). Also the percentage of c-fos positive inhibitory neurons after subsequent capsaicin is increased when sham or SNI is previously applied to the contralateral hind paw (Fig. 3A) (p<0.05, ANOVA). There was no difference in the number or percentage of c-fos double labeled neurons after capsaicin stimulation between sham or SNI is applied to the contralateral hind paw (p>0.05, unpaired t-test). Also, the distribution of c-Fos neurons containing GlyT2/GAD67 in laminae I&II and laminae III-VI was not different between the two groups (Table 1B).

3.3.4. Saline and capsaicin

Saline (20hrs or 4days) stimulation of the hind paw contralateral to the capsaicin stimulated hind paw did not affect the number of c-fos labeled neurons induced by capsaicin, when compared to single capsaicin stimulation (Fig. 2) (p>0.05, ANOVA). Also, neither the percentage nor number of c-fos labeled neurons that is inhibitory (GAD67/GlyT2 mRNA) is changed (Fig. 3) (p>0.05, ANOVA).

3.4. Mechanical thresholds

After capsaicin injection, the mechanical threshold of the stimulated hind paw decreased from 33 ± 3 grams to 3 ± 2. In all the other groups, the mechanical threshold was determind after subsequent capsaicin stimulation, and we found no difference in the threshold when compared to single capsaicin stimulation (Table 1C).

4. Discussion

In the present study we have investigated the expression of c-fos in spinal inhibitory, Gly/GABA neurons after hind paw stimulation with capsaicin when a prior noxious stimulus was applied to the contralateral paw. We found that after capsaicin stimulation, the number of Gly/GABA neurons that expressed c-fos was significantly increased if chronic inflammation (CFA 4 days), postoperative pain (sham operations) or neuropathic pain (2 wks) was induced priory in the contralateral paw, when compared to capsaicin stimulation in naïve rats. Furthermore, in most cases the increase in the number of c-fos expressing inhibitory neurons was observed in laminae I and II. Thus, our data suggest that application of peripheral noxious stimuli, especially chronic pain states, increases c-fos expression in superficial dorsal horn inhibitory neurons on the contralateral side of the spinal cord upon capsaicin stimulation.

We used GAD67 together with GlyT2 mRNAs to identify somas of spinal GABAergic and/or glycinergic neuronal somatas. In spinal cord, glycine and GABA are the main inhibitory neurotransmitters, which are involved in anti-nociception [18, 19, 25]. Since glycine and GABA are often colocalized in the spinal cord [24], we identified glycinergic and/or GABAergic neurons as one group, representing spinal inhibitory neurons. Previous studies have shown that c-fos is expressed in spinal inhibitory neurons after a variety of noxious stimuli and nerve injury of a hind paw [9, 22]. Here, we used capsaicin as a noxious stimulus, which induces a transient burning pain and leads to hyperalgesia for heat and mechanical stimuli [2]. c-fos was strongly expressed in spinal inhibitory neurons on the ipsilateral side after capsaicin stimulation in naïve animals, which is in accordance with our previous findings [9].

As a step further, in the present study we have shown that inducing a chronic pain condition in one hind paw increases c-fos expression in spinal inhibitory neurons on the contralateral side after stimulation with capsaicin. This finding suggests that induction of a chronic pain state, i.e. inflammation or neuropathic, reduces the threshold for c-fos expression in spinal inhibitory neurons on the contralateral side. Previous studies have shown increased number of c-fos expressing neurons on the contralateral side of the spinal cord after a unilateral stimulus [11, 13] and after a stimulation of one hind paw (mechanical or chemical) when a prior stimulus was applied to the contralateral hind paw [14, 15]. The general belief is that application of the first noxious stimulus sensitizes spinal nociceptive neurons located on the contralateral side of the spinal cord, which results in

a lower activation threshold and hence c-fos expression in these neurons. We found an increase in the total number of c-fos labeled Gly/GABA neurons after capsaicin stimulation only if chronic inflammation (CFA 4 days), postoperative pain or neuropathic pain (SNI 2wks) was induced in the contralateral paw. Thus, only chronic pain is able to induce contralateral sensitization of spinal inhibitory neurons.

Our data showing a preference for increased c-fos expression in spinal inhibitory neurons, suggest an increase in the number of activated neurons that have an inhibitory input in spinal nociceptive processing. However, the rats developed normal pain behavior as indicated by no significant changes in the mechanical threshold compared to a single unilateral capsaicin injection. Thus, there is an increase in the number of activated, i.e. c-fos expressing, spinal inhibitory neurons without any changes in pain behavior. The ensuing question would be whether activation of spinal inhibitory neurons during a painful event is actually involved in inhibiting spinal pain transmission.

It has been shown that in inflammatory and neuropathic conditions there is a loss of synaptic inhibition in spinal cord [15]. Many behavioral signs of inflammatory and neuropathic conditions are mimicked in naïve animals with reduced GABAergic and/or glycinergic neurotransmission in the dorsal horn [19]. Therefore we expected that increased GABAergic and/or glycinergic neurotransmission would decrease pain behavior. Since we did not find any changes in the behavior response might suggest that the Von Frey hairs are not sensitive enough to detect possible changes in the mechanical threshold. On the other hand, it is also possible that the increased activation of Gly/GABA neurons does not result in an increase of spinal inhibition, and thus explaining the absence of any changes in the pain behavior. C-fos is a proto-oncogene that links neuronal activity to protein synthesis by regulating the downstream expression of target genes [6]. In the past two decades, many studies on spinal nociception have used c-fos as a marker for neurons that are activated in response to noxious stimulation. Further, it has been shown that spinal c-fos expression pattern is linked to the type of noxious stimuli, and that c-fos is expressed in spinal nociceptive neurons that project to higher centers, and also in neurons that use GABA and/or glycine, dynorphin or enkephalin as neurotransmitter(s) [3]. However, conflicting evidence dispute the functional role of c-fos expressing in spinal cord, i.e. inhibiting or facilitating nociception, and also to what extent c-fos expression correlates with spinal neuronal activity [6]. Also, the threshold for induction of c-fos is different between the subpopulation of spinal neurons [6]. Thus, it is necessary to be cautious when drawing inferences about spinal nociceptive activity based on the number or type of neurons expressing c-fos. Therefore, our finding that after subsequent capsaicin stimulation there is increased c-fos expression in spinal inhibitory neurons is not necessarily linked with increased activation of these neurons. And this suggestion is further substantiated by the lack of finding any changes in mechanical hyperalgesia. However, we did not measure the thermal threshold in order to assess the development of thermal hyperalgesia, which might have changed by the increased inhibition. Further, an alternative suggestion would be that c-fos expression is indeed linked with neuronal activity, and hence an increase in inhibition of spinal

nociception. However, this inhibitory effect might rather serve as a counterpart to the activation of spinal excitatory neurons, most likely the pain transmitting neurons, which are also activated after a noxious stimulation. This would suggest that activation of inhibitory neurons does not result in active inhibition of pain transmission but rather serving as a balancing factor in spinal nociceptive processing. Therefore, it remains elusive whether increased inhibitory input by spinal inhibitory neurons is indeed altering the output of spinal nociceptive processing, and subsequently modulating the pain behavior. All the alternative suggestions made above are based on the assumption that applying a noxious stimulus alters the activation threshold of neurons located on the contralateral side of the spinal cord. This contralateral effect might be the result of commissural pathways, which are especially prominent in the dorsal horn [17], or by descending pathways that modulate the spinal nociceptive processing [5, 7].

In conclusion, our data show an increase in c-fos expression in inhibitory neurons after capsaicin stimulation, if a chronic pain state is induced on the contralateral paw. Whether this enhancement in c-fos activation pattern of inhibitory interneurons leads to an increase in spinal nociception is currently unclear.

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Chapter V

Distribution of GABA and glycine containing RVM neurons that project to the spinal cord and vice versa

Hossaini M[1], Goos JAC[1], Kohli S[1], Holstege JC[1]

[1]Dep. of Neuroscience, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands

Manuscript submitted

Abstract

Descending projections from rostral ventromedial medulla (RVM) on spinal dorsal horn neurons are important modulators of spinal nociception by facilitating and inhibiting spinal nociception. In the present study we have investigated the distribution pattern of glycinergic and/or GABAergic (Gly/GABA) neurons in the RVM that project to the lumbar dorsal or ventral horn and the cervical spinal cord. For this purpose we used fluorescent retrograde tracing for identifying projection neurons and combined this with fluorescent in situ hybridization (FISH) for identifying glycinergic (GlyT2 mRNA) and GABAergic (GAD67 mRNA) neurons. Our data showed that in the RVM 40% of neurons projecting to the cervical spinal cord were Gly/GABA, while this was 43% for neurons projecting to the lumbar dorsal horn, and 35% for neurons projecting to the lumbar ventral horn. We found no differences in the percentages for projection neurons containing GlyT2, GAD67 or Gly2/GAD67 mRNA(s), indicating that these descending inhibitory projections use both glycine and GABA as their neurotransmitters. In addition, we have found a novel ascending pathway in the spinal cord to the RVM originating predominantly from Gly/GABA neurons located in the area around the central canal. In the cervical and lumbar segments of the spinal cord, 22% and 21% of the neurons projecting to the RVM were Gly/GABA, respectively. Further, these ascending projections likely use both glycine and GABA as their neurotransmitters. Taken together, this is the first study that describes the distribution pattern of glycinergic and GABAergic neurons in the RVM that project to the spinal cord, and has identified a novel ascending Gly/GABA projection from the spinal cord to the RVM, indicating that the spinal cord might be involved in modulating the RVM.

1. Introduction

Nociceptive information from the periphery has to go through several synaptic relay stations before it becomes a conscious pain experience. For the whole body, except the head, the first relay station is the spinal dorsal horn [53]. Here, the synaptic transmission of the nociceptive information is influenced by local interneurons [70], glial cells [35, 59], and also by descending projections from the brainstem [22]. One important source of descending control from the brainstem is the rostral ventromedial medulla (RVM) [14, 76], which includes the midline nucleus raphe magnus (RM) and the adjacent reticular formation. The RVM is mainly under control of the periaqueductal grey (PAG) [22], which in turn receives projections from limbic cortical and subcortical areas [4, 66, 81]. The importance of the PAG and the RVM for the inhibition of pain transmission in the spinal cord was shown in 1976 as analgesia was produced by electrical stimulation of the PAG [48]. Further studies showed that analgesia was also produced by electrical stimulation of the RVM, and by microinjection of morphine in the PAG or the RVM [79]. Since the RVM has extensive projection to the spinal dorsal horn [16, 22], unlike the PAG [45], the effects of the PAG on spinal nociception are produced through the RVM.

The RVM not only produces inhibition but also facilitation of spinal nociception [77], resulting in hyperalgesia and allodynia as observed in inflammatory and neuropathic pain models [76]. The existence of both inhibitory and facilitatory descending control from the RVM suggest that there are specific subset of RVM projection neurons that are involved in inhibiting and facilitating spinal nociception. Indeed, electrophysiological studies have shown that there are so-called ON-cells in the RVM that have a net facilitatory effect on nociception [15], while a second group, designated as OFF-cells, have a net inhibitory effect on nociception [21]. Another group of cells, designated as neutral cells, did not show any characteristics of the ON- or OFF-cells activity pattern, and are most likely are not involved in modulating nociception [15, 47]. While the neutral cells were shown to contain serotonin [19, 62], it is not certain which neurotransmitters are used by the ON- and OFF-cells to induce facilitation and inhibition respectively. An electron microscopical study, combining anterograde tracing from the RVM to the spinal dorsal horn with immunohistochemistry for GABA and glycine has shown that there are projections from the brainstem that contain GABA and/or glycine (Gly/GABA) and likely originate in the RVM [3]. However, up to now the distribution pattern of these spinally projecting glycinergic and GABAergic neuronal somata in the RVM has not been identified.

In order for the RVM to change its activation pattern in response to nociceptive stimuli, it must receive information from the spinal cord [11, 2]. However, direct projections from the spinal cord to the RVM are limited [78], and it is assumed that the RVM receives most information from the spinal cord indirectly by projections from the PAG, or adjacent to the RVM located LPGi [6, 7, 74, 75]. However, a retrograde tracing study from the brainstem has shown that a spinal neurons in the area around the central canal [78] project to the RVM area, and that some of these neurons contain the neuropeptide enkephalin, which may have an inhibitory effect [55].

In the present study we have combined retrograde tracing with in situ hybridization for GABA and glycine to identify the location of the Gly/GABA neurons in the RVM that project to the cervical and lumbar spinal cord. In addition, we have investigated whether the RVM received a projection from Gly/GABA neurons in the spinal cord. We found that Gly/GABA neurons in the area around the central canal project to the RVM, showing that there is a reciprocal inhibitory connection between the RVM and the spinal cord.

2. Materials and methods

In this study we used a total of 43 male Wistar rats. All animal experiments were approved by the Rotterdam Animal Ethical Committee.

2.1. Tracer injections

We used FluoSpheres (0.04 µm; molecular Probes, Eugene, OR), consisting of fluorescent polystyrene micro spheres for retrograde tracing. For tracer injections in the spinal cord and the brainstem the rats were kept under general anesthesia with 2% isofluoran. For injections, a glass micropipette was used, and with each injections between 80 to 100 nl of FluoSpheres tracer was injected. Further, after each injection the micropipette was left untouched for a period of 3 minutes in order to avoid regurgitation of the injected volume. All injections were made in the left side of the spinal grey matter or in the left side of the brainstem. After the operation the animals were returned to standard housing for a period of 25-28 days.

2.2. Spinal cord injections

For identifying neurons in the RVM that project to the spinal cord, FluoSpheres was injected in the enlargement area of the lumbar segment or the cervical segment. First, laminectomy was performed at L4-L6 levels (lumbar injections) or at C4-C6 levels (cervical injections) of the spinal cord, producing an open area of approximately 3 mm with a visible dura mater. Next, the animal was fixed using stereotaxic instruments, and the posterior spinal vein was taken as the reference for the midline. In the lumbar spinal cord, injections were made between 0.7 and 0.9 mm lateral to the midline, and in the cervical spinal cord injections were made between 0.5 and 1.5 mm lateral to the midline. For injections in the lumbar dorsal horn (n = 7), the spinal cord was approached dorsally at an angle of 45 °, and the injections were made at of 0.5 and 0.9 mm deep into the spinal cord. For the ventral horn (n = 5), the spinal cord was approached in the lateral axis at an angle of ± 30 °, and the injections were at depths of 1.2 and 1.7 mm into the spinal cord. In the cervical spinal cord (n = 5), we did not differentiate between the dorsal and the ventral horn, and therefore the injections were made at different depths ranging between 0.5 and 1.5 mm deep into the spinal cord at different lateral distances from the midline.

2.3. Brainstem injections

The injections were made in the left rostral ventromedial medulla (RVM), encompassing the raphe magnus (RM), gigantocellular reticular nucleus (Gi) and lateral paragigantocellular reticular nuclei (LPGi) (n = 6). The rostral part of the cervical vertebrae and the caudal part of the skull was revealed and laminectomy was performed. The sulcus in the medulla oblangata was taken as the reference for the midline. The injections were made at ± 1.6 mm rostral to the obex, between 0.4 and 1.0 mm lateral to the midline, and between 4.5 and 5.0 mm deep into the tissue.

2.4. Tissue preparation

After survival periods of 25-28 days, the rats received an overdose of sodium pentobarbital, and were then transcardially perfused with 150 ml saline followed by 750 ml of 4% paraformaldehyde (PFA). Thereafter, the spinal cord and the brainstem were dissected and incubated overnight at 4 °C in RNAse free solution consisting of phosphate buffer (PB), 4% PFA and 30% sucrose. Coronal sections were cut at 30 µm with a freezing microtome, collected in 9 separate jars and stored in a solution consisting of PB/formamide (50%/50%) at -20 °C. We did not use glycerol, since we had experienced that glycerol reduced the fluorescence intensity of the beads over time. This was not observed when sections were stored in PB/formamide solution.

2.5. Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) was performed using the protocol described in Hossaini et al. [29, 31] , using the same cDNA templates (GlyT2 cDNA: 3.1 kb; a generous gift from Dr. N. Nelson, Tel Aviv University; GAD67 cDNA: 3.2 kb; a generous gift from Dr. A.J. Tobin and N. Tillakaratne, PhD, UCLA) to produce the GlyT2 and GAD67 mRNA probes. After riboprobe hybridization, as primary antibody we used sheep anti-digoxigenin poly antibody (Roche) to identify digoxigenin. Subsequently, on the last day of the experiment the primary antibody was identified with biotinylated rabbit-anti-goat antibody (Vector). The subsequent steps in the procedure were performed as described in the previous protocol [29, 31].

2.6. Analyzing labeled neurons

In rats with tracer injections in the RVM, we analyzed sections from the cervical, upper and lower parts of the thoracic, lumbar and sacral segments. Sections for analysis were chosen by starting in the first row of the randomly mounted sections and searching for sections from the appropriate segmental level (C4 to T5 for cervical and upper thoracic segments; T10 to S4 for lower thoracic, lumbar and sacral segments). Per rat, 10-12 sections for cervical and upper thoracic segments, and 10-12 sections for lower thoracic,

lumbar and sacral segments were analyzed in a Leica fluorescent microscope with a FITC and/or Cy3 filter. In rats with tracer injections in the cervical or lumbar spinal cord, sections from the caudal medulla at three levels that included the inferior olive nucleus (OI), sections from the pons at three levels ranging from caudal to rostral RVM were analyzed.

Labeling for GlyT2 and/or GAD67 mRNAs (FITC; greenish) was determined as neuronal labeling if the staining was present in the cell soma and the shortest diameter was at least 10μm. In order to investigate colocalization of the FluoSpheres beads (apparent as red dots using a Cy3 filter) with GlyT2/GAD67 mRNAs (FITC) staining, first labeling for the tracer (red dots) was determined in a 40x objective. A neuron was counted as positive for tracer labeling if at least ten bright red dots were present in the soma. Then, in the same focus field, we determined whether there was labeling for GlyT2/GAD67 mRNA present in the cytoplasm. If in the same focus field, tracer labeling was present together with somatic GlyT2/GAD67 mRNA staining, the neuron was counted as a double labeled neuron. Single tracer positive neurons, and tracer positive neurons that also contained GlyT2/GAD67 were plotted by hand in an illustrated representation of the appropriate segmental level. In the illustration used for spinal cord sections, the grey matter was divided in 10 laminae according to the laminar distribution in the rat [51]. For brainstem sections, we used sections from the rat brain illustrated in [61]. Per rat, the average numbers of tracer positive and tracer/Gly/GABA double labeled neurons was determined. Thereafter, we calculated the percentage of tracer positive neurons that also contained GlyT2 and/or GAD67 mRNA. Per group, the results on the numbers and the percentages were averaged and compared with the average results in the other groups. Errors in the variations were assessed as standard error of the mean (SEM). The unpaired *t*-test or one-way ANOVA with a Bonferroni post-hoc test was performed for statistical comparison between groups. $p<0.05$ was taken as significant.

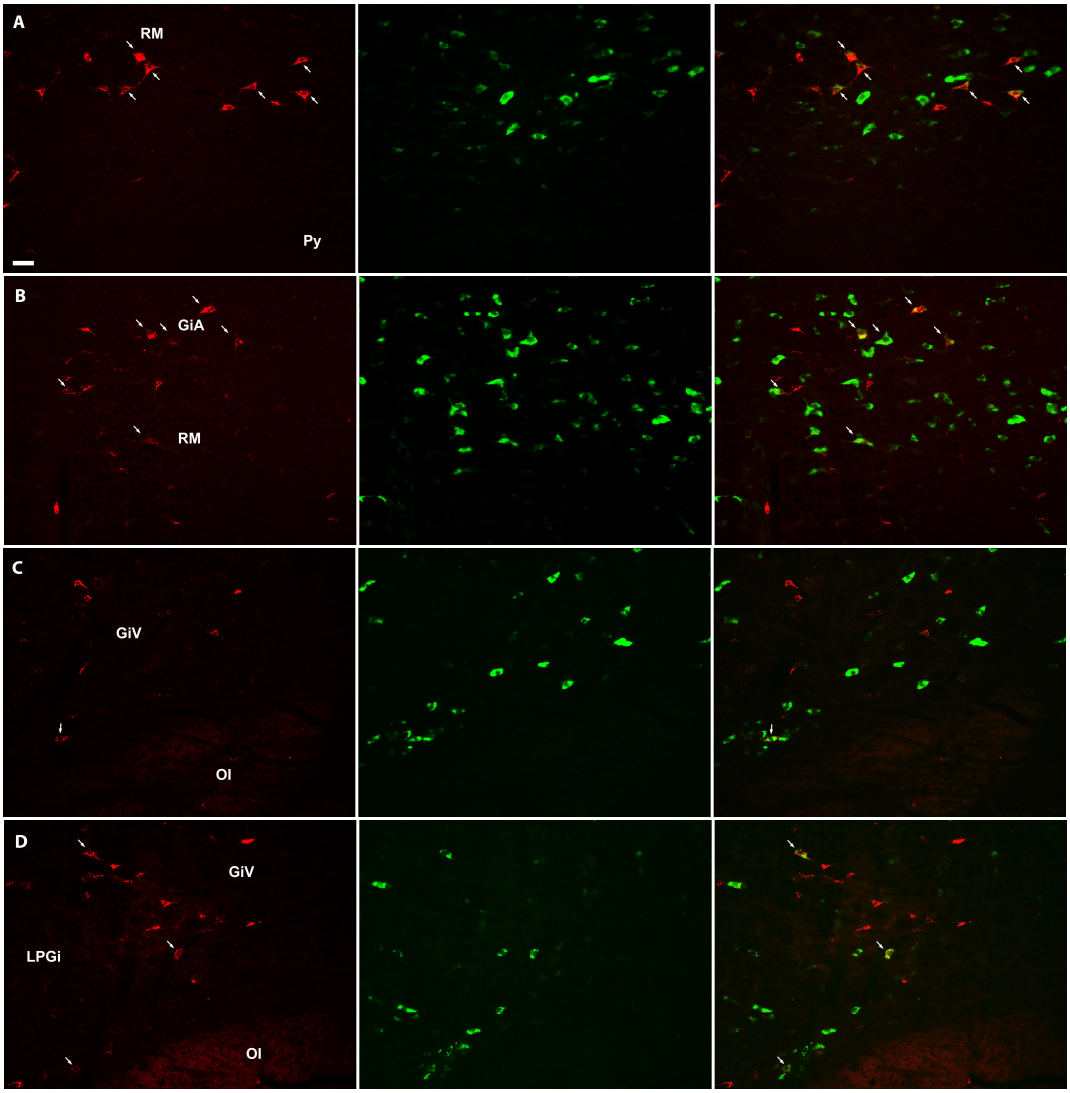
3. Results

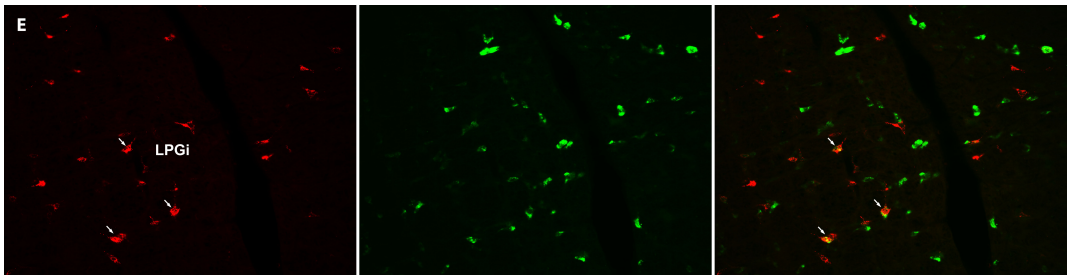
3.1 General observations

Inhibitory neurons, i.e. neurons that use either glycine or GABA or both transmitters, were labeled by applying fluorescent in situ hybridization (FISH) with a mixture of GlyT2 mRNA and GAD67 mRNA (GlyT2/GAD67) probes, resulting in labeling of glycinergic and/or GABAergic (Gly/GABA) neurons (Fig. 1 and Fig. 6). We also applied FISH with only GlyT2 (for identifying glycinergic neurons) and with only GAD67 (for identifying GABAergic neurons) probes, in order to reveal any differences between these two population of inhibitory neurons. FISH was applied on sections from the spinal cord and the brainstem, and as a result we found greenish labeled neuronal somata of various sizes. The distribution pattern of the labeled neurons, i.e. glycinergic and/or GABAergic, in the spinal cord and the brainstem was in accordance with previous findings [30, 44, 69, 82].

After injection of fluorescent microspheres in different parts of the spinal cord, we found retrogradely labeled neurons of various sizes in the brainstem (Fig. 1). Bright red dots, representing the retrogradely transported microspheres, were present in the somata and primary dendrites of the labeled neurons. The number of red dots varied between the labeled neurons. Similar labeling characteristics were found for retrogradely labeled neurons in the spinal cord after injection of fluorescent microspheres in the brainstem (Fig. 6).

Fig. 1: Fluorescence micrographs showing retrogradely (red) labeled neurons, and neurons that contain GlyT2/GAD67 mRNA. **A:** Gly/GABA (GlyT2+GAD67 mRNA) neurons in the rostral RVM projecting to the lumbar dorsal horn. **B:** Gly/GABA neurons in the caudal RVM projecting to the ventral lumbar horn. **C:** Gly/GABA neurons at the caudal Olive Inferior level projecting to the lumbar ventral horn. **D:** Gly/GABA neurons at the rostral Olive Inferior level projecting to the cervical spinal cord (dorsal+ventral). **E:** Glycinergic (GlyT2 mRNA) neurons in the caudal RVM projecting to the cervical spinal cord (dorsal+ventral). Arrows indicate projections neurons that also contained GlyT2 and/or GAD67 mRNA. Scale bar = 50µm





3.2. Projections to the cervical spinal cord

3.2.1. Description of injection sites

For this experiment we injected fluorescent microspheres in the cervical spinal enlargement, i.e. C5 and C6. Several injection were made unilaterally in the dorsal as well as the ventral horn, encompassing both the medial and lateral parts of the horn (Fig. 2A). In addition, we found also fluorescent microspheres present in the laterodorsal and ventral white matter.

3.2.2. Projections from the caudal medulla to the cervical spinal cord

In the caudal medulla we analyzed retrogradely labeled neurons that were located in the following nuclei: raphe pallidus (RP), raphe obscures (ROB), medial reticular formation (MRF), gigantocellular reticular nucleus (Gi), the ventral part of Gi (GiV), and the lateral paragigantocellular nucleus (LPGi), ranging from the caudal to the rostral part of the olive nucleus inferior (OI). We found that 76% of the total number of retrogradely labeled neurons were located on the side ipsilateral to the injection sites in the spinal cord. Further, our data showed that 19% ± 3 of the retrogradely labeled neurons on the ipsilateral side also contained GAD67 mRNA, 15% ± 5 contained GlyT2 mRNA, and 18% ± 4 contained GlyT2/GAD67 mRNA. Similar results were found for neurons located on the contralateral side. There were no significant differences in the percentages obtained for the different mRNA probes ($p > 0.1$, ANOVA).

3.2.3. Projections from the RVM to the cervical spinal cord

We analyzed sections from the pons ranging from caudal to rostral parts of the RVM area, which included the midline nucleus raphe magnus (RM), RP, part alpha of the Gi nucleus (GiA), part alpha of the LPGi nucleus (LPGiA), and the external part of LPGi (LPGiE). Our data showed that 87% ± 3 of the total retrogradely labeled neurons in the RVM were located on side ipsilateral to the injections. Further, about 60% of the retrogradely labeled neurons were located in the RM + GiA, while the remaining 40% was found in the LPGiA and LPGiE. We found on the ipsilateral side that 40% ± 6 of the neurons projecting to the spinal cord contained also GlyT2/GAD67 mRNA. However, the percentage of retrogradely

labeled neurons on the ipsilateral side that also contained GAD67, GlyT2, or GlyT2/GAD67 mRNA was significantly higher in the RM and GiA as compared to the LPGiA and LPGiE ($p < 0.05$, unpaired *t*-test) (Fig. 2B). This difference between these subgroups of nuclei was not found for retrogradely labeled neurons that were Gly/GABA located on the contralateral side (Table 1A).

Fig. 2A

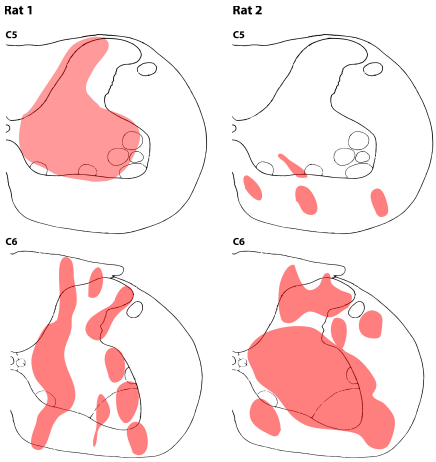


Fig. 2B

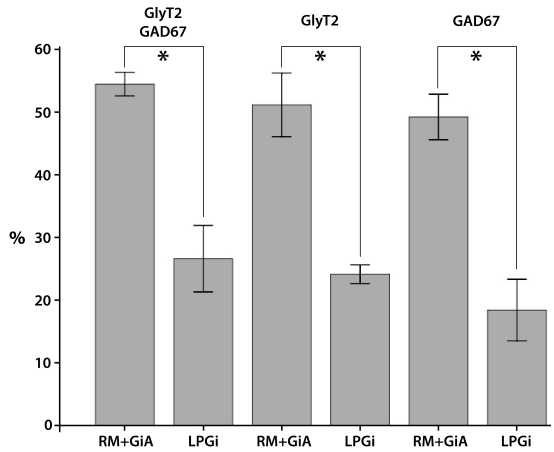


Fig. 2: A: Illustrations showing the injection sites of fluorescent microspheres in the cervical sections of two different rats. **B:** Histogram showing the percentage of RVM neurons projecting to the cervical spinal cord that also contained GlyT2 and/or GAD67 mRNA. RM: nucleus Raphe Magnus; LPGi: lateral paragigantocellular reticular nuclei; GiA: gigantocellular reticular nucleus pars Alpha. *=*P*<0.05.

3.3. Projections to the lumbar spinal cord

In the analysis of the results, we found that the majority of the retrogradely labeled neurons (>77%) were located in the RM and the GiA (RM + GiA) when compared to LPGiA + LPGiE. This was true for the ipsilateral and contralateral side of the RVM. We then analyzed the percentages of retrogradely labeled neurons that also contained GlyT2 and/or GAD67 mRNA, and found no differences in the percentages between RM + GiA nuclei and the LPGiA + LPGiE nuclei in contrast to the cervical spinal cord. Therefore, in the description of the results below we combined the results on these nuclei (RM + GiA + LPGiA + LPGiE) on each side, and refer them as the RVM.

3.3.1. Description of injection sites

For this experiment we injected fluorescent microspheres either in the dorsal or ventral horn of the lumbar spinal cord, ranging from L4 to L6 segments (Fig. 3). With respect to the dorsal horn, injection sites included laminae I to VI, and in some cases the dorsal and/or lateral white matter. In the ventral horn, the injections were found in laminae VII to IX, and in the ventrolateral and lateral parts of the white matter.

3.3.2. Projections from the caudal medulla to the lumbar dorsal horn and the ventral horn

For projections to the dorsal horn we found the highest number of tracer labeled and tracer/Gly/GAD labeled neurons in the rostral IO sections (Table 1B), while for projections to the ventral horn the highest numbers were found in the mid and rostral IO sections (Table 1C). We found that 8% ± 1 of the retrogradely labeled neurons projecting to the dorsal horn also contained GAD67, 7% ± 2 contained GlyT2, and 5% ± 1 contained GlyT2/GAD67 (Table 1B). With respect to the neurons projecting to the ventral horn of the spinal cord, we found that 8% ± 1 of the retrogradely labeled neurons also contained GAD67, while significantly higher percentages were found for colocalization with GlyT2 (19% ± 3) and GlyT2/GAD67 (19% ± 3) (p<0.05, ANOVA) (Table 1C). These percentages were significantly higher than the percentages for projections to the dorsal horn (p<0.05, ANOVA)

Table 1: A: numbers represent the percentage of neurons on the contralateral side of the RVM that projected to spinal cord that also contained GlyT2 and/or GAD67 mRNA. **B:** The number and the percentage of neurons on the ipsi- and contralateral side of the caudal medulla that projected to the *dorsal* lumbar spinal cord and also contained Gly2 and/or GAD67 mRNA. **C:** The number and the percentage of neurons on the ipsi- and contralateral side of the caudal medulla that projected to the *ventral* lumbar spinal cord and also contained Gly2 and/or GAD67 mRNA**.**

Table 1A

	Lumbar dorsal horn	Lumbar ventral horn	Cervical spinal cord
GlyT2/GAD67	54 ± 9	31 ± 4	26 ± 4
GlyT2	45 ± 9	32 ± 9	33 ± 3
GAD67	46 ± 4	32 ± 5	22 ± 5

Table 1B

	Ipsilateral			Contralateral		
	GlyT2/GAD67	GlyT2	GAD67	GlyT2/GAD67	GlyT2	GAD67
Caudal IO	27 (1)	26 (3)	23 (2)	14 (1)	17 (2)	12 (1)
Mid IO	60 (2)	52 (3)	28 (3)	15 (3)	27 (3)	26 (3)
Rostral IO	132 (10)	96 (5)	86 (7)	70 (19)	42 (9)	52 (11)
percentage	5 ± 1	7 ± 2	8 ± 1	15 ± 4	13 ± 2	12 ± 3

Table 1C

	Ipsilateral			Contralateral		
	GlyT2/GAD67	GlyT2	GAD67	GlyT2/GAD67	GlyT2	GAD67
Caudal IO	37 (7)	33 (5)	29 (3)	43 (2)	35 (1)	32 (2)
Mid IO	117 (17)	35 (11)	97 (4)	118 (10)	125 (19)	138 (14)
Rostral IO	136 (45)	128 (28)	119 (11)	206 (49)	165 (50)	111 (30)
percentage	18 ± 4	18 ± 3	7 ± 2	11 ± 4	13 ± 6	12 ± 5

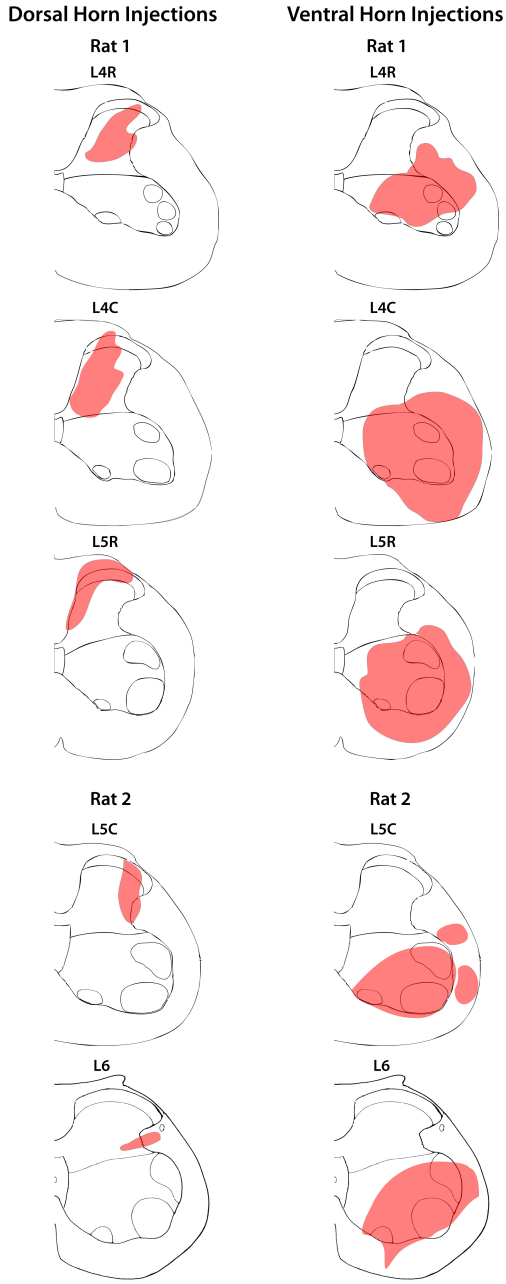


Fig. 3: Illustrations showing the injection sites of fluorescent microspheres in the lumbar dorsal horn, and in the lumbar ventral horn of two different rats for each horn.

3.3.3. Projections from the RVM to the lumbar dorsal horn

In total, we counted 1082 retrogradely labeled neurons for colocalization with GlyT2 (n=340), GAD67 (n=338), and GlyT2/GAD67 (n=404) mRNA. Further, 77% ± 2 of the RVM neurons that were retrogradely labeled from the lumbar dorsal horn were located on side ipsilateral to the injection. Applying FISH with GlyT2/GAD67 mRNA probes showed that 49% ± 3 of the retrogradely labeled neurons in the ipsilateral RVM also contained GAD67 mRNA, 39% ± 6 contained GlyT2 mRNA, and 43% ± 4 contained GlyT2/GAD67 mRNA (Fig. 4A). These percentages were not significantly different from each other, indicating that the neurons projecting from the RVM to the lumbar dorsal horn use both glycine and GABA as their neurotransmitters.

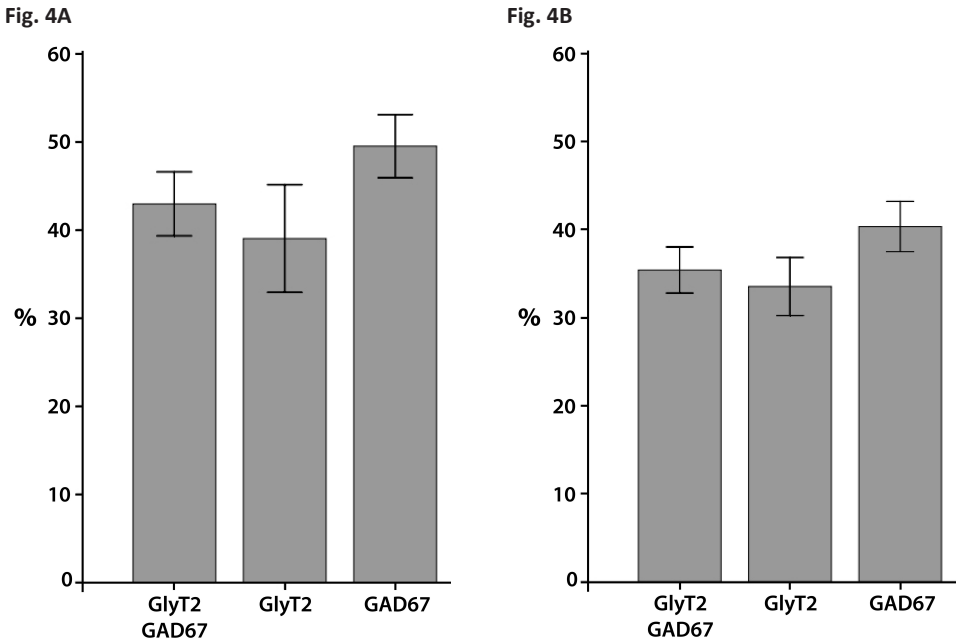


Fig. 4: Histograms showing the percentage of RVM neurons that project to the lumbar dorsal (**A**) or the ventral (**B**) horn and also contained Gly2 and/or GAD67 mRNA. Note that there were no significant differences in the percentages of projections neurons that contained GlyT2, GAD67, or GlyT2/GAD67 mRNA for projections to the dorsal and the ventral horn.

Therefore, when combining the three percentages, our data showed that 44% ± 3 of the neurons projecting to the dorsal horn are inhibitory. Similar percentages for double labeling were found for projection neurons located on the contralateral side of the RVM (Table 1A).

3.3.4. Projections from the RVM to the lumbar ventral horn

A total 1443 retrogradely labeled neurons were counted for colocalization with GlyT2 (n=308), GAD67 (n=631) and GlyT2/GAD67 (n=504). Similar to the projections to the dorsal horn, we found that 76% ± 2 of the RVM neurons that were retrogradely labeled

with tracer were located on side ipsilateral to the injections in the spinal cord. Our data showed that on the ipsilateral side 40% ± 3 of the retrogradely labeled neurons contained GAD67 mRNA, 34% ± 3 contained only GlyT2 mRNA, and 35% ± 3 contained only GlyT2/GAD67 mRNA (Fig. 4B). Similar to the projections to the lumbar dorsal horn, we found no significant differences between the percentages indicating that the neurons projecting to the lumbar dorsal horn use both glycine and GABA as their neurotransmitters. Therefore, when combining the three percentages, our data showed that 36% ± 0.8 of the neurons projecting to the ventral horn are inhibitory. Similar results were found for projection neurons on the contralateral side of the RVM (Table 1A).

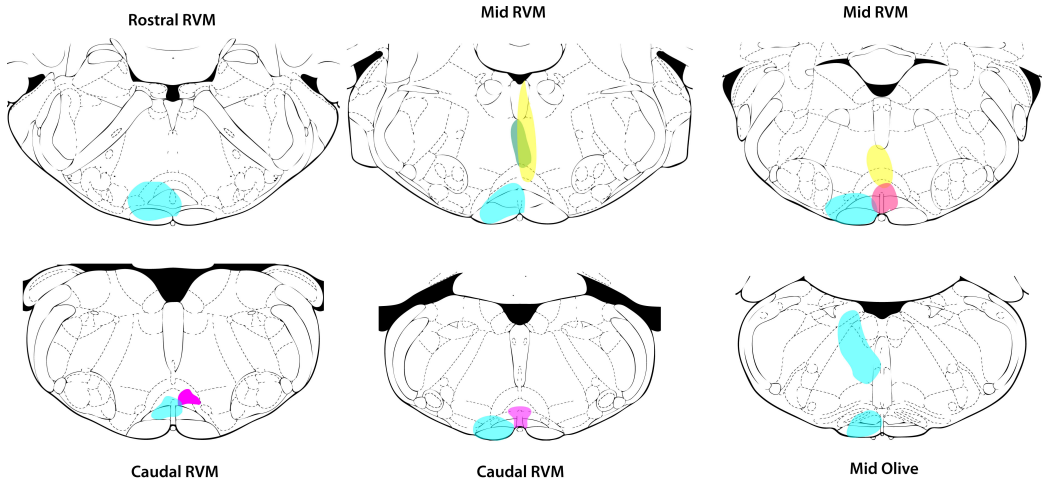
When comparing the percentages of RVM neurons that also contained GAD67, GlyT2, GlyT2/GAD67 between the projections to the dorsal horn and the ventral horn, we found no differences in the percentages ($p > 0.1$, unpaired *t*-test for each mRNA) between them.

3.4. Inhibitory spinal neurons projecting to the RVM

3.4.1. Description of injection sites

For this experiment, fluorescent microspheres were injected in the midline in the RVM area. We found that many of the injections included the pyramid tract, the RM nucleus, the RP nucleus, and the medial part of the LPGi nucleus (Fig. 5).

Fig. 5: illustrations showing the injection sites of fluorescent microspheres in the rostral ventromedial medulla (RVM) area for three different rats (yellow, blue, red). The injection areas include the raphe magnus nucleus, the lateral paragigantocellular reticular nucleus**,** and the raphe pallidus and raphe obscurus nuclei.



3.4.2. Lumbo-sacral spinal cord

Our data showed that a significantly higher percentage of retrogradely labeled neurons was located on the side contralateral (59% ± 2) to the injections in the RVM in comparison with the ipsilateral side (41% ± 2) ($p < 0.005$, unpaired *t*-test). Further, the

majority of the retrogradely labeled neurons were located in the area around the central canal (91% ± 2 on the ipsilateral side; 90% ± 1 on the contralateral side), which included lamina X and the medial parts of laminae VI, V, VI, and VII. With respect to this population of retrogradely labeled neurons, when FISH was applied (Fig. 6) we found that on the contralateral side 22% ± 3 of these neurons also contained GlyT2/GAD67 (Fig. 7A). This percentage was not different from the percentages of retrogradely labeled neurons that also contained only GlyT2, or GAD67, or from the percentages obtained for neurons located on the ipsilateral side (Fig. 7A). Further, very few double labeled retrogradely labeled neurons were found in the lateral spinal nucleus (LSN), and in the white matter lateral to laminae III, IV and V. Our findings indicate that the inhibitory spinal neurons that project to the RVM are predominantly located in the area around the central canal, and use both glycine and GABA as their neurotransmitters.

3.4.3. Cervico-thoracic spinal cord

We found that the neurons projecting to the RVM were equally distributed on the ipsilateral (45% ± 3) and contralateral (55% ± 3) side to the tracer injections in the RVM. Similar to the projection neurons in the lumbar spinal cord, the majority of neurons projecting to the RVM were located in the area around the central canal (95% ± 1 for ipsilateral; 93% ± 2 for contralateral). With respect to this population of retrogradely labeled neurons, our data showed on the ipsilateral side 21% ± 4 of these neurons also contained GlyT2/GAD67 (Fig. 7B). Similar percentages were found for colocalization with only GlyT2, or GAD67, and they were all not different from the percentages obtained for neurons located on the contralateral side (Fig. 7B). Further, very few double labeled retrogradely labeled neurons were found in the lateral spinal nucleus (LSN), and in the white matter lateral to laminae III, IV and V.

3.4.4. Cervico-thoracic vs. Lumbo-sacral spinal cord

Finally, when we compared the results on the lower spinal cord with the results on the upper spinal cord, we found no significant differences between these segments with respect to the percentages of retrogradely labeled neurons that also contained GlyT2 and/or GAD67. Thus, the RVM receives projections from inhibitory neurons that are located in the cervical, thoracic, and lumbar spinal cord, and use both glycine and GABA as their neurotransmitters.

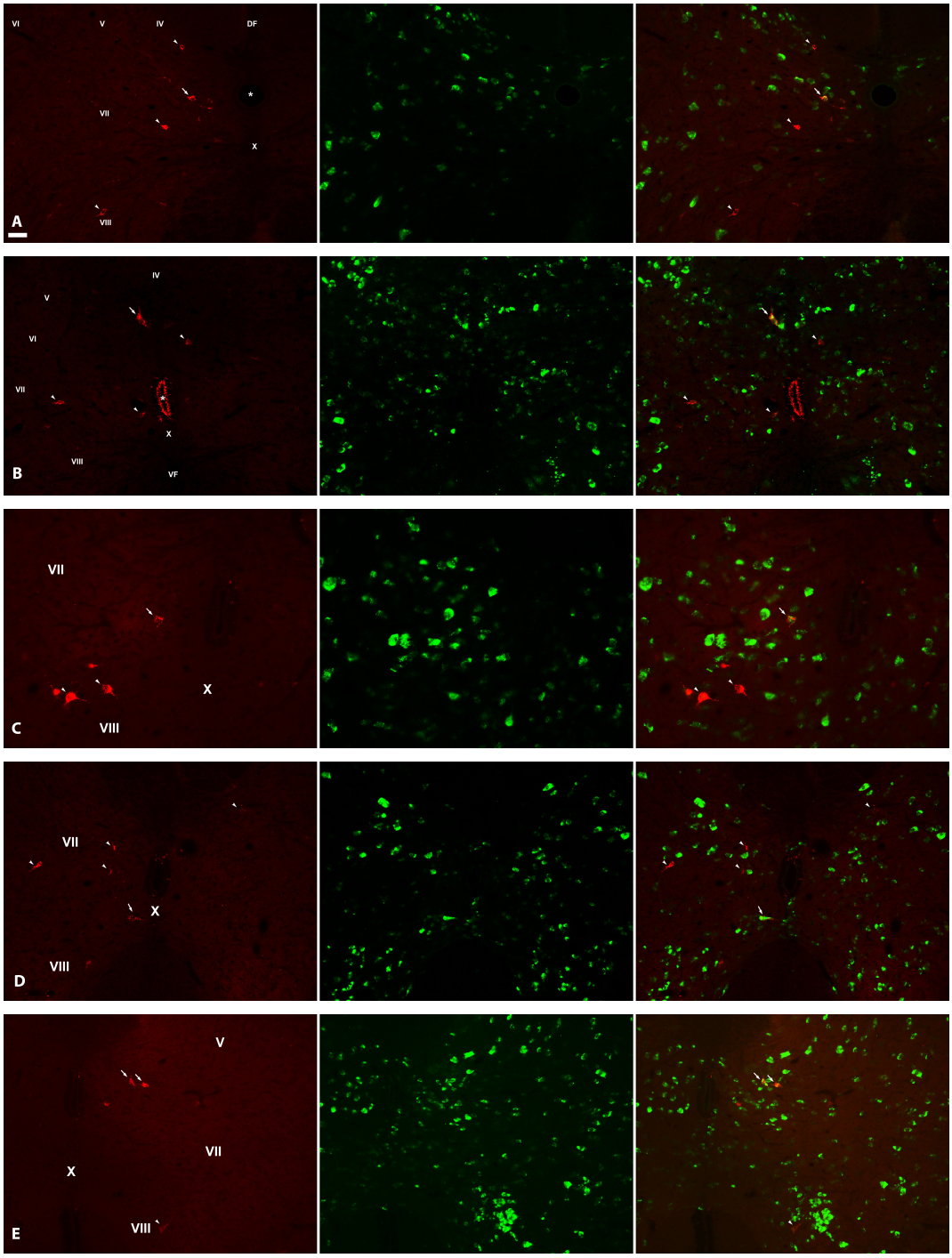


Fig. 6: Fluorescence micrographs showing retrogradely labeled neurons that project to the RVM area, and neurons that are labeled for GlyT2 and/or GAD67 mRNA (green). Arrows indicate neurons that project to the RVM area and also contain GlyT2 and/or GAD67 mRNA. **A:** FISH with GlyT2 mRNA at cervical C5 level. **B + C:** FISH with GlyT2+GAD67 mRNA at lumbar L6 level. **D:** FISH with GlyT2+GAD67 mRNA at lumbar L4 level. **E:** FISH with GlyT2+GAD67 mRNA at lumbar L5 level. Scale bar = 50µm.

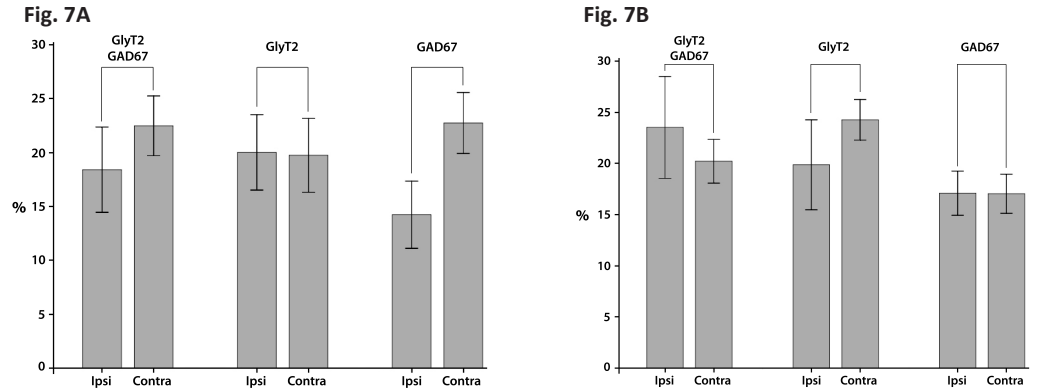


Fig. 7: Histograms showing the percentage of retrogradely labeled spinal neurons in the area around the central canal that project to the RVM and also contain GlyT2 and/or GAD67 mRNA (Gly/GABA). **A:** Percentage of projection neurons that are Gly/GABA in the *lower thoracic*, *lumbar and sacral* spinal cord. **B:** Percentage of projection neurons that are Gly/GABA in the *cervical and upper thoracic* spinal cord. Note that there were no significant differences were found in the percentages of GlyT2, GAD67, and GlyT2/GAD67 mRNA containing projections neurons in the sacral, lumbar, thoracic and cervical spinal segments.

4. Discussion

In the present study we have used a combination of retrograde tracing with fluorescent microspheres and ISH for GlyT2 and GAD65 to identify glycinergic and GABAergic neurons, respectively. Using this technique we investigated the distribution pattern of neuronal somata that project to the spinal cord and contain glycine and/or GABA (Gly/GABA) in the ventral part of the caudal medulla at the levels of inferior olive (OI), and in the rostrally adjoining RVM area. Our results showed that the percentage of neurons that were Gly/GABA was higher for spinal projections from the RVM as compared to the projections from the OI level. RVM neurons that projected to the cervical spinal cord were on average Gly/GABA in 40% of the cases, while this percentage was 43% for projections to the lumbar dorsal and 35% for the projections to the lumbar ventral horn. The percentages obtained for GABA and glycine separately were very similar to those obtained for Gly/GABA, indicating that virtually all inhibitory RVM neurons projecting to the spinal cord use both glycine and GABA as their neurotransmitters. In addition, we have shown the existence of spinal Gly/GABA neurons in the area around the central canal that have ascending projections to the RVM.

For the identification of neurons in de caudal medulla and RVM projecting to the spinal cord and vice versa, we used fluorescent microspheres as a retrograde tracer. This technique has been shown to be highly efficient for retrograde tracing and is taken up by terminals rather than by intact passing fibers [40]. In the spinal cord our injection sites also encompassed parts of the white matter. However it is unlikely that passing fibers have taken up tracer, since damage in the white matter was minimal. The same holds true for injections in the RVM. In this case damaged passing fibers in the injection site may have been present, although the major tracks run outside the injection site, except for the pyra-

midal tract, but this tract will not label neurons in the spinal cord. The injection sites in the spinal cord varied in size, but always included the major part of the dorsal and ventral spinal gray matter for the cervical injections. The lumbar injections in the dorsal horn never included the ventral horn, and the lumbar injections in the ventral horn, which were made under an angle, never included the dorsal horn. Therefore the dorsal and ventral horn injections were completely separated. There were differences in the number of retrogradely labeled cells that were obtained for the different injections, which may be explained by differences in the rostro-caudal extent of the injections. The distribution pattern was in line with previous studies [5, 23, 24, 26, 36, 80] and the number of neurons that we found were comparable to a similar retrograde tracing study, using different tracers [43]. The injections in the RVM area always included the RVM bilaterally, but varied with respect to the medio-lateral, dorso-ventral and rostro-caudal extent. However the pattern of the retrogradely labeled cells in the spinal cord was always similar and in line with previous studies using retrograde tracing from the RVM [10].

For identifying Gly/GABA neurons we applied FISH with GlyT2 and GAD67 mRNA probes, a previously described approach [29, 30] that results in reliable labeling of glycinergic (i.e. containing GlyT2 mRNA) [44, 63] and GABAergic (i.e. containing GAD67 mRNA) neuronal somatas [12, 41]. Previous studies showed that labeling glycinergic neuronal somata by means of IHC is difficult as it results in weak soma labeling and intense terminal labeling [9, 64, 71]. We have overcome these problems by using ISH for GlyT2 for the labeling of glycinergic neuronal somata [30].

Previous studies [3, 38, 65] have shown that the descending projections from the RVM to the spinal cord contain GABA. In the present study we have confirmed this finding and further showed that the percentages of RVM neurons projecting to the spinal cord that were only glycinergic, only GABAergic or contained GABA and/or glycine were not statistically different, indicating that in the RVM projections to the spinal cord that contain GABA, also contain glycine. The Gly/GABA projections to the dorsal horn and to a lesser extent those to the ventral horn showed some differences in the percentages of the RVM neurons that were Gly/GABA when comparing projections to the lumbar dorsal horn (43%), lumbar ventral horn (35%) , and the cervical spinal cord (40%). However these differences were not statistically significant. This finding is in line with the non-topographic organization of the RVM projections to the spinal cord, since it has been shown that there is a high level of intersegmental and dorsoventral collateralization of bulbospinal projection neurons [43]. In addition we did not find any differences in the percentages of Gly/GABA neurons when comparing the ipsilateral and contralateral side, although the number of retrogradely labeled neurons was higher on the ipsilateral than on the contralateral side. This finding is also in line with the diffuse character of the organization of the descending projections from the RVM to the spinal cord [43].

With respect to the functional meaning of the RVM projection to the dorsal horn, most studies have focused on the effects on the dorsal horn. The RVM descending control on the spinal cord is a parallel system that facilitates and inhibits spinal nociception de-

pending on the type of nociceptive stimulus [22, 76, 77]. The facilitatory effect is exerted by the physiologically characterized ON cells [17, 22, 58], and the same effect is achieved by the neuropeptide transmitters cholecystokinin [42, 73], neurotensin [58, 67, 73, 72], which may therefore be contained within the ON cells. The inhibitory effect of the RVM is mainly produced by the physiologically characterized OFF cells [14, 17, 22]. For a long time it was believed that serotonin was the transmitter in the RVM descending pathways that inhibited spinal nociception [20, 34, 37, 46]. However, serotonin also facilitates nociception [60], and that all serotonergic cells recorded in vivo in the RVM are characterized as neutral cells [19, 62]. Further, it has been shown that the activity of the RVM neurons, especially the serotonergic neurons, are determined by the animal's state, i.e. level of arousal or stage of sleep [18], and that the activity of serotonergic neurons might modulate the effect of the OFF and ON cells on spinal nociception [18, 46]. However, it is still unclear which neurotransmitters the ON and OFF cells use to exert their effect. In the present study, we have shown that 43% of the neurons projecting to the spinal dorsal horn are inhibitory, suggesting that glycine and GABA are at least partly responsible for descending inhibition on spinal nociception. Indeed, several studies have shown the relation of RVM stimulation with antinociception exerted by GABAergic and glycinergic neurotransmission in the dorsal horn [49, 50, 68]. Further, there is evidence that serotonin and GABA are co-localized in RVM projection neurons [8, 25], and recently a subpopulations of GABAergic OFF and ON cells have been identified [52]. Also in vivo patch-clamping of the substantia gelatinosa of the spinal cord showed the existence of monosynaptic inhibitory potentials by GABA and glycine after electrical stimulation of the RVM [39]. Taken together, there is strong evidence for the presence of Gly/GABA in the descending RVM pathways as represented by the physiologically characterized OFF cells.

In the present study we have shown that 90% of the neurons projecting to the RVM are located in the area around the central canal (CC), and that about 20% of these projections neurons contain glycine as well as GABA. This pathway is the only projection from the spinal cord to a supraspinal structure (i.e. the RVM area) that contains the fast inhibitory transmitters GABA and glycine. Using anterograde tracing, neurons located in the CC area, have been shown to have direct projections, albeit limited, to the RVM and the area adjacent to the RVM, via the ventrolateral funiculus in the spinal cord [78]. Since the injections that we made in the RVM also encompassed the area immediately adjacent to the RVM, the retrogradely labeled neurons in the CC area may also terminate in this area. Within the same spinal area around the CC there are also neurons that use enkephalin and dynorphin [55] that project to supraspinal sites including the RVM. It is therefore not excluded that enkephalin is also present in the Gly/GABA neurons that project to the RVM [33]. Many neurons in the CC area have been identified to respond to innocuous and noxious stimuli from the periphery and viscera [27, 28, 54, 56, 57]. This strongly suggests that the neurons in the CC area are involved in the processing and transmission of sensory and especially visceral nociceptive information [1, 13, 32]. It is not clear whether the inhibitory neurons in the CC area respond to peripheral and visceral noxious stimuli and whether the ascending inhibitory projections are also involved in inhibiting the OFF, ON or neutral cells

in the RVM.

In conclusion, we have shown a many neurons in the RVM and the caudal medulla that contain both GABA and glycine and project to the dorsal and ventral horn of the spinal cord. It seems likely that these inhibitory projections to the dorsal horn are involved in the inhibition of pain transmission in the spinal cord and thus represent the physiologically characterized OFF cells. We also have shown the existence of a more limited projection that contains GABA and glycine, originating from the CC area and terminating in the RVM area. Whether these neurons are involved in regulating the activity of the (inhibitory) descending RVM neurons, thereby indirectly modulating spinal nociception remains to be determined.

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Chapter VI

Nociceptive stimulation induces expression of Arc/Arg3.1 in the spinal cord with a preference for neurons containing enkephalin

Hossaini *et al. Molecular Pain* 2010, **6**:43
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Nociceptive stimulation induces expression of Arc/Arg3.1 in the spinal cord with a preference for neurons containing enkephalin

Mehdi Hossaini[1], Joost LM Jongen[2,1], Karla Biesheuvel[1], Dietmar Kuhl[3], Jan C Holstege[1*]

[1]Dep. of Neuroscience, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands; [2]Dep. of Neurology, Erasmus University Medical Center, P.O. Box 2040, 3000 CA, Rotterdam, The Netherlands; [3]Institute for Molecular and Cellular Cognition, Center for Molecular Neurobiology (ZMNH), University Medical Center Hamburg-Eppendorf, Falkenried 94, 20251 Hamburg

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Nociceptive stimulation induces expression of Arc/Arg3.1 in the spinal cord with a preference for neurons containing enkephalin

Mehdi Hossaini[1], Joost LM Jongen[2,1], Karla Biesheuvel[1], Dietmar Kuhl[3], Jan C Holstege[1*]

Abstract

Background: In pain processing, long term synaptic changes play an important role, especially during chronic pain. The immediate early gene Arc/Arg3.1 has been widely implicated in mediating long-term plasticity in telencephalic regions, such as the hippocampus and cortex. Accordingly, Arc/Arg3.1 knockout (KO) mice show a deficit in long-term memory consolidation. Here, we identify expression of Arc/Arg3.1 in the rat spinal cord using immunohistochemistry and in situ hybridization following pain stimuli.

Results: We found that Arc/Arg3.1 is not present in naïve or vehicle treated animals, and is *de novo* expressed in dorsal horn neurons after nociceptive stimulation. Expression of Arc/Arg3.1 was induced in an intensity dependent manner in neurons that were located in laminae I (14%) and II (85%) of the spinal dorsal horn. Intrathecal injection of brain derived neurotrophic factor (BDNF) also induced expression of Arc/Arg3.1. Furthermore, 90% of Arc/Arg3.1 expressing neurons also contained the activity marker c-Fos, which was expressed more abundantly. Preproenkephalin mRNA was found in the majority (68%) of the Arc/Arg3.1 expressing neurons, while NK-1 was found in only 19% and GAD67 mRNA in 3.6%. Finally, pain behavior in Arc/Arg3.1 KO mice was not significantly different from their wild type littermates after application of formalin or after induction of chronic inflammatory pain.

Conclusions: We conclude that Arc/Arg3.1 is preferentially expressed in spinal enkephalinergic neurons after nociceptive stimulation. Therefore, our data suggest that Arc/Arg3.1 dependent long term synaptic changes in spinal pain transmission are a feature of anti-nociceptive, i.e. enkephalinergic, rather than pro-nociceptive neurons.

Background

The experience of pain is usually initiated by the activation of nociceptors, which are the peripheral terminations of nociceptive ganglion neurons. The central projections of these neurons enter the dorsal horn of the spinal cord to terminate on second order neurons [1]. After strong nociceptive stimulation these neurons may show an enhanced responsiveness to afferent inputs, which may last for several hours [2-4]. The mechanism underlying this enhanced responsiveness is similar to that of long-term potentiation (LTP) [5], which is a form of activity dependent plasticity that has been investigated extensively in other parts of the CNS, especially in the hippocampus [6]. Another form of activity dependent plasticity is long-term depression (LTD), a state of decreased sensitivity of neurons. Whether LTP or LTD is produced in the spinal nociceptive system depends on many variables, including the type of activity in nociceptive afferents [2]. For long term changes to become persistent it is essential that activity regulated genes, including immediate early genes (IEG), orchestrate a cascade of transcriptions and subsequent protein synthesis [7]. The first IEG that was found to be strongly increased in spinal neurons after a nociceptive stimulus is c-Fos [8]. This IEG is now widely used for the identification of activated nociceptive neurons [9]. Other IEGs that have been implicated in plastic changes are c-Jun, Jun-d, Krox 24 and Homer 1a [10,11]. Recently it has become clear that in cortex, hippocampus and other higher brain centers, the IEG named Arc/Arg3.1 (activity regulated

* Correspondence: j.holstege@erasmusmc.nl
[1]Dept. of Neuroscience, Erasmus University Medical Center, PO Box 2040, 3000 CA, Rotterdam, The Netherlands

Hossaini *et al. Molecular Pain* 2010, **6**:43
http://www.molecularpain.com/content/6/1/43

cytoskeleton-associated protein/activity regulated gene 3.1) plays a crucial role in activity dependent synaptic plasticity [12]. Moreover, Arc/Arg3.1 is critically involved in processes essential for synaptic structural rearrangement such as LTP, LTD and homeostatic scaling of AMPA receptors [13,14]. These mechanisms are also essential in spinal processing [15], and dysfunctional forms of activity dependent plasticity such as LTP and LTD that lead to persistent changes in neuronal sensitivity, may underlie chronic pain disorders [16]. Therefore, in this study we set out to investigate the role of Arc/Arg3.1 in nociceptive processing in the spinal cord.

Our findings show that Arc/Arg3.1 is not expressed at detectable levels in naïve spinal cord. However, after peripheral nociceptive stimulation we found *de novo* expression of Arc/Arg3.1 in a limited number of neurons in the superficial dorsal horn, depending on the type of stimulus. Further, Arc/Arg3.1 is predominantly expressed in spinal interneurons located in lamina II and many of these neurons also contain the opioid neurotransmitter enkephalin. Finally, we found that the pain behavior in Arc/Arg3.1 knockout (KO) mice after nociceptive stimuli was not significantly different from their wild type (WT) littermates.

Results

General observations

In the spinal cord of naïve rats and mice there was no detectable expression of Arc/Arg3.1 mRNA or protein when using in situ hybridization (ISH) and immunohistochemistry (IHC), respectively. However, after application of a peripheral nociceptive stimulus to the hind paw, Arc/Arg3.1 was expressed in a limited number of cells in the superficial layers of the lumbar dorsal horn. ISH using the alkaline phosphatase (AP) reaction produced a bluish/brownish reaction product in the cytoplasm and in some occasions in the nucleus and primary dendrites of Arc/Arg3.1 mRNA expressing neurons (Fig. 1A,B). Arc/Arg3.1 protein, visualized by bright field IHC, was present primarily in the cytoplasm, occasionally combined with nuclear labeling or labeling in proximal dendrites (Fig. 1C). Applying fluorescent IHC for Arc/Arg3.1 protein produced very similar labeling characteristics. In order to ascertain that Arc/Arg3.1 is expressed in neurons rather than in glial cells, we combined FISH for Arc/Arg3.1 mRNA with fluorescent IHC for NeuN, which is a specific marker for neuronal cells (Fig. 1D). It was found that 95% ± 1.3 (SEM) of the cells expressing Arc/Arg3.1 mRNA also expressed NeuN (99% ± 0.4 for

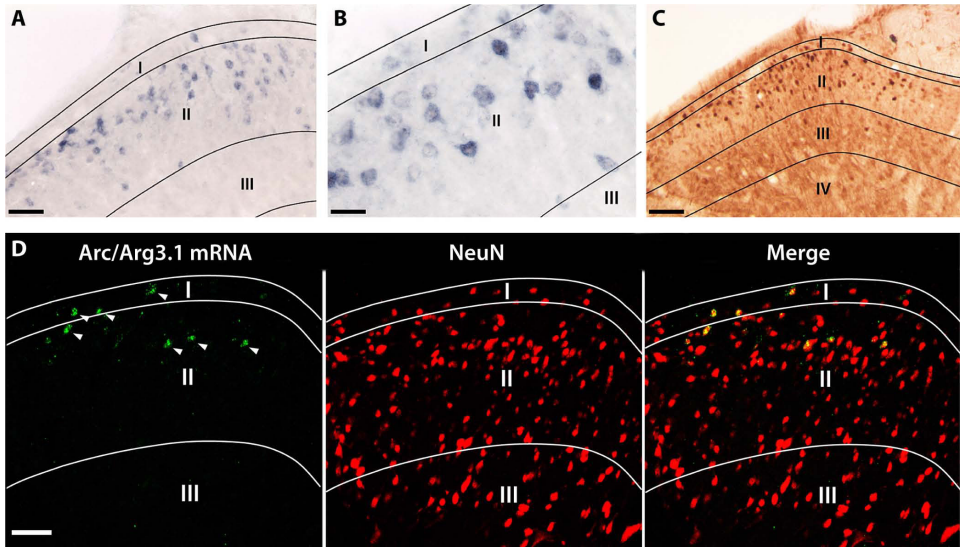

Figure 1 Arc/Arg3.1 is only expressed in the superficial dorsal horn. Light micrographs showing the distribution of neurons expressing Arc/Arg3.1 mRNA (***A, B***) or protein (***C***) in the rat spinal dorsal horn after peripheral stimulation with 25% mustard oil gauze wrapped around one hind paw for 2 hours. Note that many of the Arc/Arg3.1 labeled neurons are located in laminae I&II, and very few labeled neurons are present below lamina II. ***D,*** Fluorescent micrograph showing the expression of Arc/Arg3.1 mRNA and its colocalization with the neuronal marker NeuN. Arrowheads indicate Arc/Arg3.1 mRNA and NeuN double labeled neurons. Scale bar: 50 μm (***A*** and ***D***); 25 μm (***B***); 100 μm (***C***).

25% MO/1 h, n = 4; 95% ± 3.3 for 25% MO/2 hrs, n = 5; 94% ± 2.8 for CFA for 1.5 hrs, n = 4).

For both ISH and IHC, we observed that the intensity of the labeled neurons varied in a single section. We did not observe any labeling indicative of localization of Arc/Arg3.1 in intermediate or distal dendrites. Although labeling patterns obtained with ISH and IHC were identical, labeling efficiency was higher for ISH than for IHC. Therefore, ISH was used for the quantification of neurons expressing Arc/Arg3.1. The specificity of our detection techniques was assessed by omitting the probes/primary antibodies in the ISH and IHC procedures, respectively, and by applying ISH and IHC on spinal tissue of Arc/Arg3.1 KO mice. These experiments did not show any labeling in the spinal cord. ISH performed on cortex of naïve rats showed Arc/Arg3.1 mRNA labeling in the cortex and the hippocampus as previously reported [17].

Distribution and quantification of Arc/Arg3.1 mRNA expressing neurons in the spinal cord following nociceptive stimulation

Several types of nociceptive stimuli applied to the hind paw induced Arc/Arg3.1 mRNA expressing neurons on the ipsilateral side (Fig. 2) but not on the contralateral side of the lumbar superficial dorsal horn. A single subcutaneous injection of capsaicin resulted in the lowest average number of labeled neurons per section (2.6 ± 0.6 SEM, n = 6), while wrapping the paw in a gauze soaked with 25% mustard oil (MO) for 2 hrs induced the highest number of neurons (50 ± 3.5 SEM, n = 5). On average, lamina II accounted for 85% ± 3.5 of the labeled neurons, while lamina I (14% ± 3.2) and III (0.6% ± 0.4) contained the remaining labeled neurons. The other laminae very rarely contained labeled neurons.

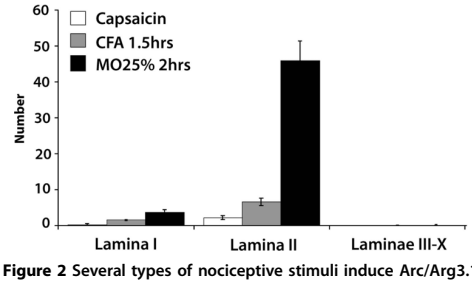

Figure 2 Several types of nociceptive stimuli induce Arc/Arg3.1 mRNA in the ipsilateral superficial dorsal horn. Histogram showing the number of Arc/Arg3.1 mRNA labeled neurons after each specific nociceptive stimulus. Error bars represent ± SEM.

Expression of Arc/Arg3.1 mRNA following nociceptive stimulation occurs in a subset of c-Fos labeled neurons and is intensity dependent

The number of neurons expressing the neuronal activation marker c-Fos or Arc/Arg3.1 mRNA was counted in separate sections treated with IHC or ISH, respectively. c-Fos labeled neurons outnumbered Arc/Arg3.1 mRNA labeled neurons (Fig. 3A), except after 2 hrs mustard oil stimulation when about equal number of neurons were labeled. FISH and fluorescent IHC were applied to simultaneously visualize Arc/Arg3.1 mRNA and c-Fos protein, respectively (Fig. 3B). When data from the 25% mustard oil and the CFA groups were taken together (Fig. 3C), 90% ± 6.8 of the Arc/Arg3.1 mRNA expressing neurons also contained c-Fos protein. In order to determine whether the number of Arc/Arg3.1 expressing neurons was stimulus intensity dependent, rats received a single application (by brush) of either 10% (n = 5) or 50% (n = 5) mustard oil on one hind paw. It was found that 50% MO induced significantly higher numbers of Arc/Arg3.1 mRNA positive neurons that 10% mustard oil (Fig. 4A). The number of c-Fos labeled neurons showed a similar significant increase.

Temporal expression of Arc/Arg3.1 mRNA in an acute and a chronic pain model

As a model for acute pain, 25% MO soaked gauze was wrapped around one hind paw, with survival times ranging from 25 min to 8 hrs. The number of Arc/Arg3.1 mRNA expressing neurons increased over time, reached a peak at 4 hours and then declined (Fig. 4B). The distribution of labeled neurons remained unchanged over time. As a model for chronic pain CFA was injected in the hind paw, with survival times ranging from 1.5 hrs to 60 hrs. Temporal expression of Arc/Arg3.1 mRNA was highest at 1.5 hrs post injection and then gradually declined (Fig. 4C). No expression of Arc/Arg3.1 mRNA was found at survival times of 10 hrs and longer. The number of c-Fos expressing neurons was increased at all survival times. In the spared nerve injury (SNI) model for neuropathic pain, expression of Arc/Arg3.1 mRNA was only observed at two hours after the operation. Arc/Arg3.1 mRNA was not expressed 1 week or 2 weeks after the operation (not shown) when the neuropathic pain symptoms, i.e. mechanical and thermal hyperalgesia and allodynia, had developed. There was no significant difference in the number of Arc/Arg3.1 mRNA labeled neurons between the SNI and sham operated group ($p>0.05$, unpaired t-test) (Fig. 4D).

Hossaini *et al. Molecular Pain* 2010, **6**:43
http://www.molecularpain.com/content/6/1/43

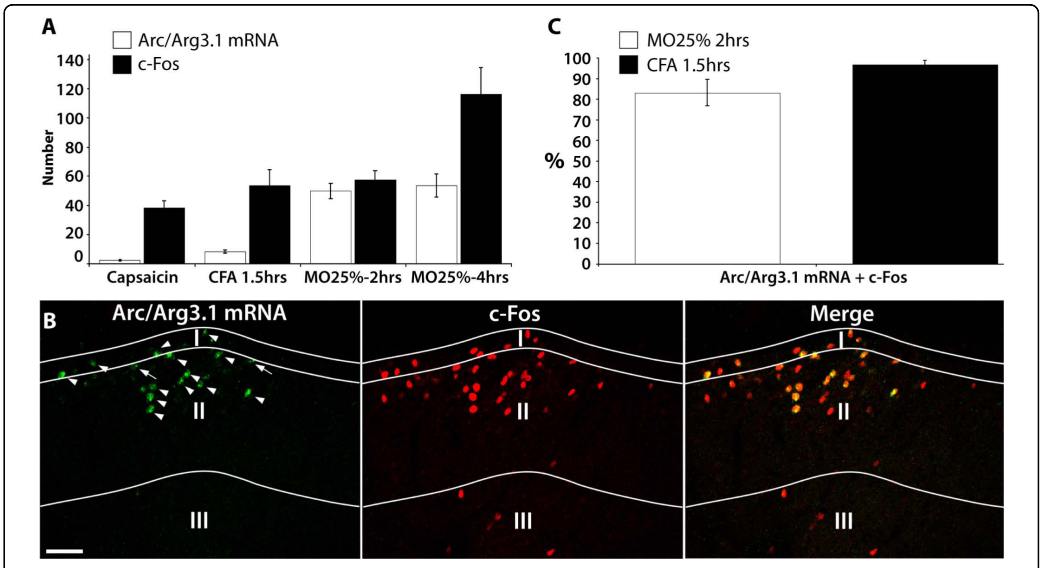

Figure 3 The number of neurons expressing c-Fos outnumber Arc/Arg3.1 mRNA labeled neurons. ***A***, Histogram showing the number of neurons labeled for c-Fos or Arc/Arg3.1 mRNA following various nociceptive stimuli. capsaicin, n = 6; CFA1.5 hrs, n = 4; MO25%-2 hrs, n = 4; MO25%-4 hrs, n = 4. ***B***, Fluorescent micrograph showing Arc/Arg3.1 mRNA and/or c-Fos protein labeled neurons in spinal dorsal horn after stimulation with 25% mustard oil for 2 hours. Arrows indicate Arc/Arg3.1 mRNA labeled neurons that were not in focus and therefore not included in the analysis. Arrow heads indicate Arc/Arg3.1 mRNA and c-Fos protein labeled neurons. ***C***, Histogram showing the percentage of Arc/Arg3.1 mRNA labeled neurons that also express the neuronal activation marker c-Fos. Scale bar: 50 μm. Error bars represent ± SEM.

Arc/Arg3.1 mRNA is expressed in specific subpopulations of dorsal horn neurons

In this experiment, the colocalization of Arc/Arg3.1 with various neuronal markers was investigated (Fig. 5A-E). We found that about a fifth of Arc/Arg3.1 mRNA positive neurons also express the NK-1 receptor (CFA 1.5 hrs: 21.7% ± 7.6, n = 4; MO25%/2 hrs: 17% ± 3.4, n = 5) (Fig. 6). Less than 10% of Arc/Arg3.1 mRNA expressing neurons also expressed PKC-γ protein (CFA 1.5 hrs: 7.7% ± 3.7, n = 4; MO25%/1h: 9.3% ± 3.8, n = 4). Further, Arc/Arg3.1 mRNA expressing neurons showed a low level of co-existence with calbindin (CFA 1.5 hrs: 9.7% ± 1.4, n = 4; MO25%/1h: 10.5% ± 2.6, n = 4).

In order to identify Arc/Arg3.1 in inhibitory neurons, FISH for GAD67 mRNA, the specific marker for GABAergic neurons, and fluorescent IHC for Arc/Arg3.1 protein were combined. Very few of the Arc/Arg3.1 labeled neurons were GABAergic (CFA 3 hrs: 1.7% ± 0.8, n = 4; MO25%/2 hrs: 4.5% ± 0.8, n = 5; MO25%/4 hrs: 4.5% ± 1.5, n = 4) (Fig. 6). Preproenkephalin mRNA is a marker for the subpopulation of enkephalinergic neurons in the spinal cord. Interestingly, a large majority of the Arc/Arg3.1 positive neurons also expressed preproenkephalin mRNA (CFA 3 hrs: 74.2% ± 9.2, n = 4; MO25%/2 hrs: 61.5% ± 2.6, n = 4; MO25%/4 hrs: 68.1% ± 3, n = 4) (Fig. 6).

Intrathecal injection of BDNF induces Arc/Arg3.1 mRNA expression

Intrathecal injection of brain-derived neurotrophic factor (BDNF) induced Arc/Arg3.1 mRNA expression in the superficial dorsal horn neurons (10 ± 1.7/section, n = 6). We found that 45% ± 8 of Arc/Arg3.1 mRNA labeled neurons were located in lamina I and 55% ± 8 in lamina II. 93.6% ± 2.5 of Arc/Arg3.1 mRNA labeled neurons expressed NeuN, 55.6% ± 9.1 expressed c-Fos, and 16.8% ± 6.4% expressed NK-1. Since it has been shown [18] that administration of BDNF together with NBQX, which is an AMPA receptor blocker, increases Arc/Arg3.1 mRNA expression in cortical neurons, we injected BDNF intrathecally together with NBQX. This combination resulted in 13.8 ± 2.9 Arc/Arg3.1 mRNA labeled neurons/section (n = 6) (Fig. 7A), which was not significantly different from intrathecal BDNF injection alone (unpaired *t*-test). c-Fos expression after BDNF + NBQX injection was also not significantly different from BDNF injection alone (p= 0.08 for lamina II) (Fig. 7B). Intrathecal injection of vehicle (n = 2) or NBQX (n = 2) alone did not induce Arc/Arg3.1 mRNA expression in

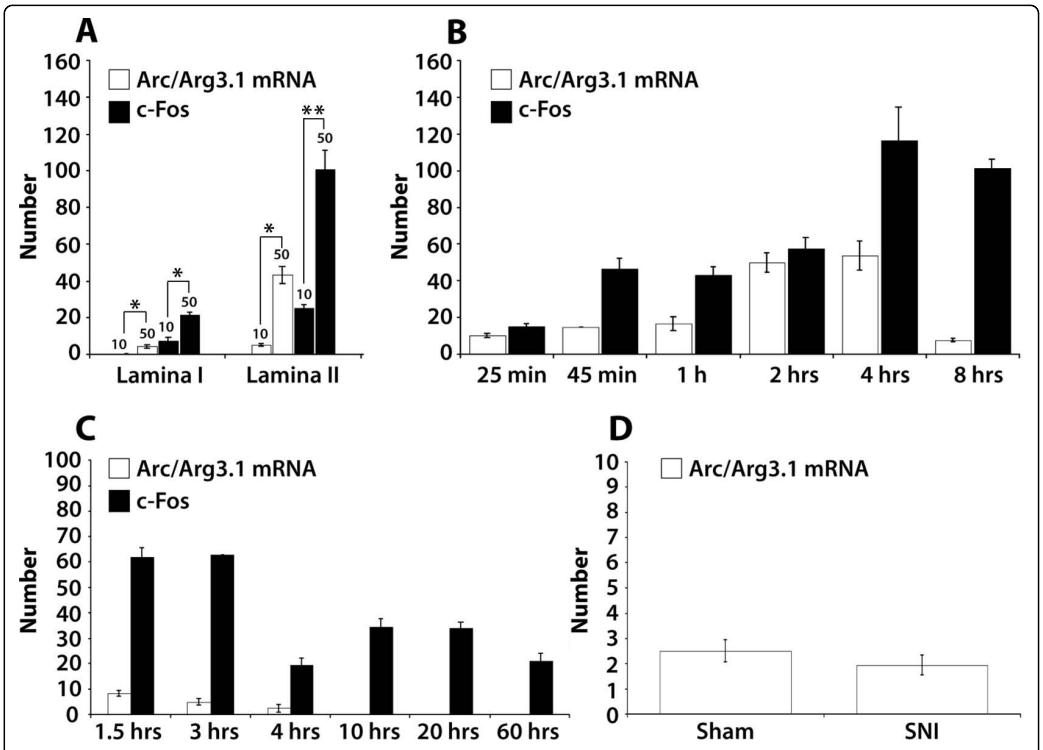

Figure 4 Arc/Arg3.1 expression is stimulus intensity dependent and is present only in the acute phase of chronic inflammatory and neuropathic pain. ***A,*** Histogram showing the numbers of Arc/Arg3.1 mRNA or c-Fos protein labeled neurons at 2 hours after a brush applied stimulation with either 10% (n = 5) or 50% (n = 5) mustard oil. * = $p < 0.05$; ** = $p < 0.005$ (unpaired *t*-test). ***B***, Time course of Arc/Arg3.1 mRNA and c-Fos protein expression after stimulation with 25% mustard oil gauze wrapped around one hind paw for different survival times. 25 min, n = 4; 45 min, n = 4; 1 h, n = 4; 2 hrs, n = 5; 4 hrs, n = 4; 8 hrs, n = 4. ***C***, Time course of Arc/Arg3.1 mRNA and c-Fos protein expression after CFA injection in the hind paw. 1.5 hrs, n = 4; 3 hrs, n = 4; 4 hrs, n = 4; 10 hrs, n = 4; 20 hrs, n = 4; 60 hrs, n = 4. ***D***, The number of Arc/Arg3.1 mRNA labeled neurons 2 hours after sham operation (n = 3) or after cutting the common peroneal and tibial nerves in the SNI model (n = 3). Error bars represent ± SEM.

the spinal cord. Furthermore, we found that intrathecal injection with NMDA (n = 2), which served as a positive control, also induced Arc/Arg3.1 expression in the dorsal horn (not shown).

Pain behavior in the Arc/Arg3.1 KO mice

Mechanical and thermal thresholds

Freely moving Arc/Arg3.1 knockout (KO) mice did not display any overt behavioral abnormalities in comparison with their wild type (WT) littermates, as reported previously [13]. With respect to pain behavior, the mechanical thresholds and hot plate withdrawal latencies were tested. We found that the mechanical thresholds in Arc/Arg3.1 KO mice were not significantly different from their WT littermates (Fig. 8A). However, in the hotplate test Arc/Arg3.1 KO mice showed significantly longer withdrawal latencies than WT mice (Fig. 8B).

Acute pain: formalin test

Subcutaneous injection of formalin in the hind paw induced a two-phased pain behavior in both WT and Arc/Arg3.1 KO mice, consisting of licking and fluttering of the injected paw. In both groups, the first phase was apparent in the first 10 minutes after injection, and the second phase began 25 minutes after injection with licking as the prominent behavior. No significant difference (repeated-measures ANOVA, $p>0.05$) was found between the WT and Arc/Arg3.1 KO mice in licking or fluttering behavior (Fig. 9A,B). Also the total licking time (WT: 200 sec. ± 34 (SEM); KO: 275 sec. ± 49 (SEM); $p>0.05$, unpaired *t*-test) nor the total numbers of flutters (WT 100 ± 21 (SEM); KO 128 ± 32 (SEM); $p>0.05$, unpaired *t*-test) were

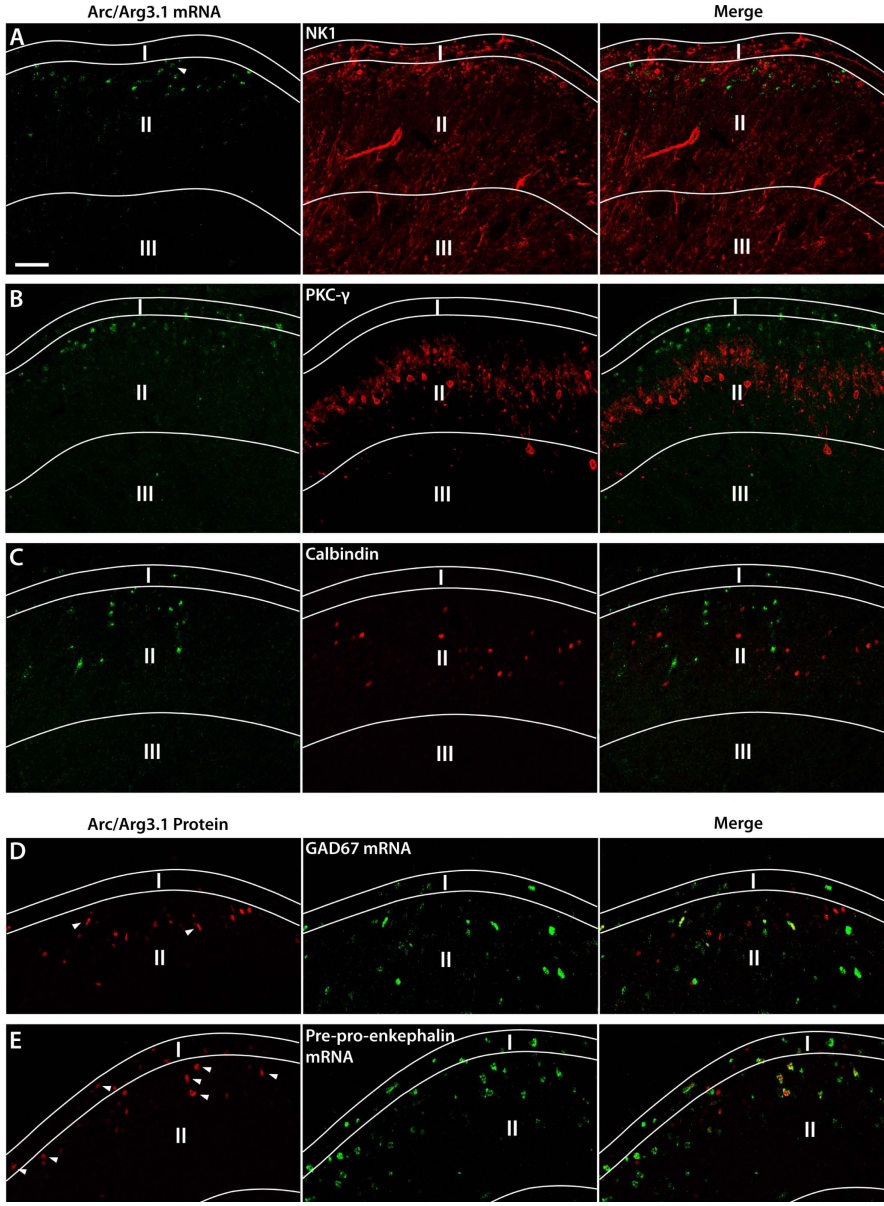

Figure 5 Arc/Arg3.1 is expressed in a subpopulation of superficial dorsal horn neurons with a preference for neurons containing enkephalin. ***A-E***, Fluorescent micrographs showing neurons in the superficial dorsal horn that express Arc/Arg3.1 mRNA (***A-C***) or protein (***D and E***) and markers that identify neurons expressing the neurokinin-1 receptor (NK1), protein kinase C gamma (PKC-γ), Calbindin, GAD67 mRNA (GABAergic neurons), or preproenkephalin mRNA (enkephalinergic neurons) respectively. The following nociceptive stimuli were used. ***A***, CFA, survival time 1.5 hrs, ***B and C***: Mustard oil 25% gauze wrapped, survival time 1 h, ***D and E***, Mustard oil 25% gauze wrapped, survival time 2 h. Arrow heads indicate Arc/Arg3.1 labeled neurons that also express one of the markers mentioned above. Scale bar: 50 μm.

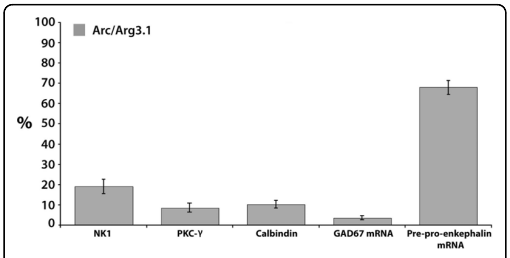

Figure 6 Histogram showing the averaged percentages of neurons expressing Arc/Arg3.1(induced after various nociceptive stimuli, as in fig. 5), that co-express either NK1, PKC-γ, Calbindin, GAD67 mRNA or preproenkephalin mRNA. Error bars represent ± SEM. For details, see text.

significantly different. In addition, c-Fos expression due to the formalin injection did not appear different from the c-Fos expression in the WT mice.

Chronic pain: inflammation

Induction of chronic inflammation by CFA injection in the hind paw resulted in decreased mechanical thresholds of the injected paw (Fig. 10A). A repeated measures ANOVA did not reveal any significant differences between WT and Arc/Arg3.1 KO mice regarding the mechanical or thermal thresholds at any time point (Fig. 10A,B).

Discussion

In this study we have used in situ hybridization (ISH) and immunohistochemistry (IHC) to show that nociceptive stimulation induced Arc/Arg3.1 mRNA and protein in the superficial dorsal horn of the spinal cord. Both techniques specifically identified Arc/Arg3.1 since standard controls, most notably nociceptively stimulated spinal cord of Arc/Arg3.1 knockout (KO) mice, did not show any specific labeling. In naïve or vehicle treated animals expression of Arc/Arg3.1 mRNA and protein was absent in the spinal cord, in agreement with a study using RT-PCR [19]. This strongly indicates that in the spinal cord a nociceptive stimulus induces *de novo* expression of Arc/Arg3.1, in contrast with other areas of the nervous system, like hippocampus [17] and cortex [20].

Arc/Arg3.1 mRNA and protein were induced in the superficial dorsal horn in the acute phases of all pain models that we tested, i.e. after nociceptive stimulation with capsaicin, CFA, formalin and mustard oil. Injection of CFA induces an inflammatory process [21] that leads to the release of cytokines and other local messengers, all of which may activate different types of receptors on nociceptive fibers. Capsaicin, however, specifically activates nociceptive fibers expressing the transient receptor potential vanilloid-1 (TRPV1) [22]. Further, mustard oil and formalin both specifically activate the TRPA1 receptor, although formalin may exert TRPA1-independent effects at higher concentrations [23,24]. The number of neurons producing Arc/Arg3.1 mRNA varied in the different pain models, and increasing the intensity of the pain stimulus resulted in an increased number of neurons expressing Arc/Arg3.1 as shown in the mustard oil experiments. Therefore, our data indicate that the number of neurons expressing Arc/Arg3.1 depends on the intensity of the stimulus, but is not limited to the activation of one specific receptor on peripheral nerves.

Neurons expressing Arc/Arg3.1 in the spinal cord are most likely driven by direct input from afferent

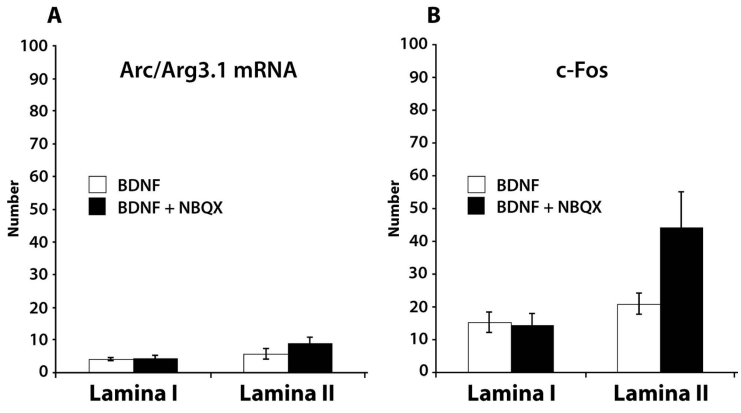

Figure 7 Intrathecal injection of BDNF induces Arc/Arg3.1 mRNA expression in spinal dorsal horn. ***A and B***, The averaged number of neurons that express Arc/Arg3.1 mRNA (***A***) or c-Fos protein (***B***) after intrathecal injection with BDNF or BDNF together with NBQX. Error bars represent ± SEM.

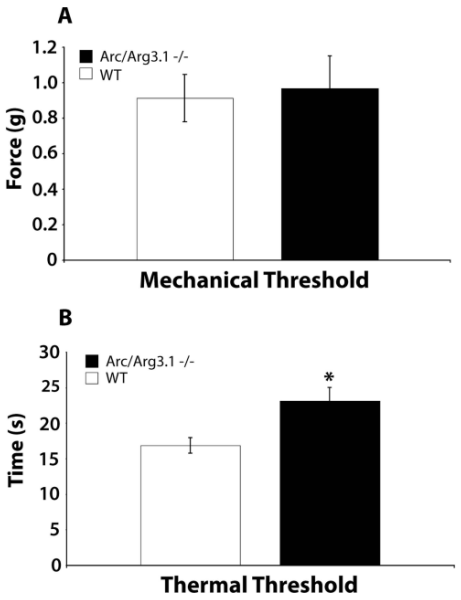

Figure 8 Naïve Arc/Arg3.1 knockout (KO) mice showed significantly longer response times on the hotplate than their naïve wild type (WT) littermates. ***A and B***, Histogram showing the mechanical threshold (***A***) and thermal withdrawal latency (***B***) of Arc/Arg3.1 KO and WT mice, assessed with the Von Frey and the hotplate test, respectively. Error bars represent ± SEM. *: $p < 0.05$, unpaired *t*-test; n = 4 for A and for B.

nociceptive fibers that use glutamate as their main neurotransmitter [25]. Apart from glutamate and various neuropeptides, these fibers may also contain growth factors like BDNF [26] or GDNF [27]. We found that intrathecal injection of NMDA or BDNF induced Arc/Arg3.1 mRNA in spinal dorsal horn neurons. This is in line with Arc/Arg3.1 expression in cultured neurons following BDNF application [18]. The same study showed a significantly enhanced expression of Arc/Arg3.1 mRNA when NBQX, a potent AMPA receptor blocker, was applied together with BDNF. However, in the present study a significant increase in the number of Arc/Arg3.1 mRNA expressing neurons could not be confirmed after intrathecal injection of BDNF and NBQX together. Taken together, our findings are in line with the idea that release of glutamate and/or BDNF from activated nociceptive fibers are at least partly responsible for Arc/Arg3.1 induction in the spinal dorsal horn.

Following nociceptive stimulation, Arc/Arg3.1 was often expressed in activated neurons as identified by c-Fos. Especially after nociceptive stimulation with capsaicin, and after chronic inflammatory pain, the number of neurons expressing Arc/Arg3.1 is low as compared to those showing c-Fos expression. This finding may be interpreted to indicate that Arc/Arg3.1 is only expressed in activated neurons that received the strongest input from nociceptive fibers. This assumption is in line with our finding that Arc/Arg3.1 expression is intensity dependent. On the other hand, there may be specific subpopulations of spinal nociceptive neurons that are capable of producing Arc/Arg3.1, while others are not. In search of such a neuronal subpopulation that specifically expressed Arc/Arg3.1, we focused on neurons that were characterized by the expression of the neurokinin-1 (NK-1) receptor, Protein Kinase C gamma (PKC-γ), calbindin, GAD67 or preproenkephalin. We found a high percentage of Arc/Arg3.1 expressing neurons (68%) to contain preproenkephalin, while percentages of colocalization with other markers were less prominent (19% for NK-1; 8.5% for PKC-γ; 3.6% for GAD67; 10% for calbindin). NK-1 expressing neurons project to supraspinal sites [28] and are essential for the initiation and maintenance of chronic neuropathic and inflammatory pain [29], and neurons expressing PKC-γ are considered critically important for the development of neuropathic pain after peripheral nerve injury [30]. The finding that only a small number of Arc/Arg3.1 positive neurons also expressed NK-1 or PKC-γ indicates that Arc/Arg3.1 is

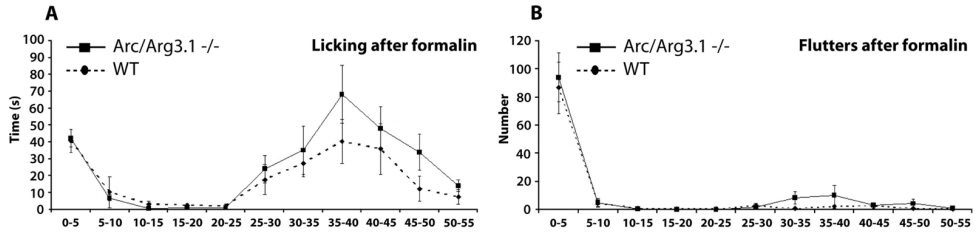

Figure 9 Arc/Arg3.1 KO mice do not differ from WT mice concerning licking or fluttering of the paw injected with formalin. ***A and B***, Graphs showing the time spent licking (***A***) and the number of flutters (***B***) after formalin injection in the hind paw during an observation period of 55 minutes. Error bars represent ± SEM. Differences were not significant (repeated-measures ANOVA, $p>0.05$). n = 4 for A and for B.

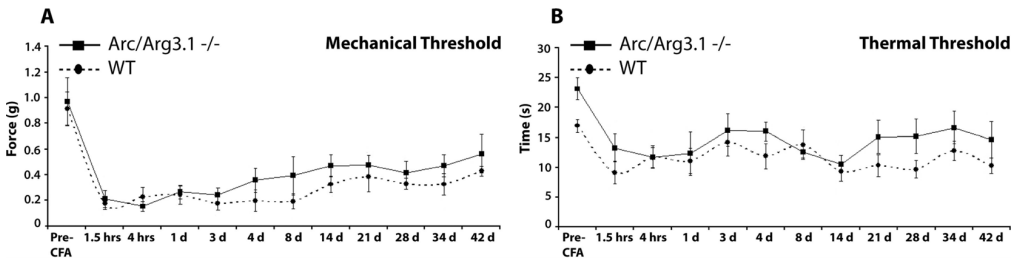

Figure 10 No differences in mechanical and thermal thresholds between the Arc/Arg3.1 KO and WT mice during the time course of inflammatory pain. ***A and B***, Graphs showing mechanical (***A***) and thermal (***B***) thresholds after CFA injection in one hind paw of Arc/Arg3.1 KO and WT mice. Differences were not significant at any time point (repeated-measures ANOVA, $p>0.05$). n = 4 for A and for B.

not strongly involved in pain processing by the NK-1 or PKC-γ subpopulations of dorsal horn neurons. This is remarkable since especially the NK-1 expressing neurons projecting to the parabrachial area or periaqueductal grey show LTP formation after high or low frequency stimulation, respectively [31]. Our finding indicates that Arc/Arg3.1 dependent long term changes may occur preferentially in local interneurons rather than in projection neurons. Further, we found low colocalization with GAD67, the marker for GABAergic neurons, indicating that the expression of Arc/Arg3.1 is low in the total subpopulation of dorsal horn inhibitory neurons since glycinergic neurons are virtually absent in the superficial dorsal horn [32-34], and, if present, also contain GABA [35]. In the hippocampal and neocortical neurons expression of Arc/Arg3.1 in GABAergic positive neurons is also low but this is not the case in the dorsal striatum [20]. Together, NK-1, PKC-γ and/or preproenkephalin constitute more than 90% of the Arc/Arg3.1 expressing neurons. Since to date there is no evidence for the colocalization of these substances with each other, we conclude that Arc/Arg3.1 is preferentially expressed in the subpopulation of enkephalinergic neurons. Preproenkephalin mRNA is the precursor of both Met- and Leu-enkephalin, which are both expressed by neurons in the spinal cord and mainly exert their effect on the δ-opioid receptor (DOR) [36]. Also, preproenkephalin mRNA in the spinal cord is increased after peripheral inflammation and is also present in neurons that express c-Fos after nociceptive stimuli [37]. Further, using VgluT2 immunohistochemistry for identifying glutamatergic terminals, it was shown [38] that 85% of the enkephalin containing terminals in the dorsal horn use glutamate as transmitter. However, a study [39] using cultured dorsal horn neurons showed 42% colocalization of immunohistochemically identified GAD and enkephalin. A more recent study [40] using preproenkephalin green fluorescent protein transgenic mice, showed that 43% of the fluorescent enkephalin neurons also expressed immunohistochemically identified GABA. Colocalization of enkephalin with VgluT2 was not explored in these studies. Since we found a low level of colocalization of Arc/Arg3.1 with GABAergic neurons, it is not unlikely that several of the enkephalinergic neurons in the spinal cord that express Arc/Arg3.1 also use glutamate as a transmitter. The functional role of glutamate in these fibers is unclear, since it is not known whether they activate inhibitory or excitatory (i.e. anti- or pro-nociceptive) circuits in the spinal cord, nor is it known under which circumstances enkephalin and/or glutamate is released from these fibers. Since the activation of the delta opioid receptor (DOR), through which enkephalin exerts its effect, decreases pain behavior during chronic peripheral inflammation [41], we tend to conclude that the overall effect of Arc/Arg3.1 expressing enkephalinergic neurons is anti-nociceptive.

In order to understand the functional role of Arc/Arg3.1 in enkephalinergic neurons at the behavioral level, we employed Arc/Arg3.1 KO mice and their WT littermates. The only significant difference between these mice was that in the hotplate test the thermal threshold of naïve Arc/Arg3.1 KO mice was significantly higher as compared to naïve WT mice. This finding is difficult to interpret since naïve WT mice, like their KO littermates, do not show Arc/Arg3.1 expression in the spinal cord. One explanation may be that there is a very low basal expression of Arc/Arg3.1 that we and others [19] were not able to detect, and that the permanent lack of Arc/Arg3.1 in the KO mice may have altered spinal processing of nociceptive thermal stimuli over time. Alternatively there may be supraspinal changes in nociceptive processing. After nociceptive stimuli, we did not find any difference in the pain behavior between the KO and WT mice in the formalin test and chronic inflammatory pain model. We therefore conclude that Arc/Arg3.1 KO mice do not show a clear phenotypic change that can be attributed to pain transmission in the spinal cord.

Several studies have shown that in hippocampus knockdown of Arc/Arg3.1 leads to enhanced LTP in the early phase but impaired consolidation of LTP and long term depression (LTD) in the late phase [13]. In the spinal cord, LTP is one of the major components of central sensitization [16], especially in lamina I projecting neurons [31]. LTP leads to enhanced responsiveness of spinal nociceptive neurons, which is important for maintenance of hyperalgesia and allodynia during acute and chronic pain. Our finding that Arc/Arg3.1 KO mice develop hypersensitivity in acute and chronic pain models in the same way as their WT littermates, suggests that the LTP formation that contributes to central sensitization and subsequent developing hyperalgesia is unaffected by the lack of Arc/Arg3.1. It seems therefore that Arc/Arg3.1 is not critically involved in LTP as occurring in the dorsal horn projection neurons, which in line with our result that few NK-1 positive neurons express Arc/Arg3.1.

The low number of spinal projection neurons that express Arc/Arg3.1 may be explained by the fact that, in contrast to other areas of the brain, structural long-term changes in the excitability of these spinal neurons are counterproductive if they persist after the healing process has been completed. Our finding that Arc/Arg3.1 is expressed predominantly in enkephalinergic neurons may suggest that in these neurons long term changes are actually consolidated. However, Arc/Arg3.1 KO mice that lack consolidation of long term changes show normal pain behavior. This would not exclude that enkephalinergic neurons, which have an inhibitory effect on pain transmission, may serve as an anti-nociceptive mechanism against strong nociceptive inputs that may occur in the future.

Conclusions

Our data show that Arc/Arg3.1, which is critically involved in consolidating long term structural changes in the forebrain, is preferentially induced in spinal enkephalinergic neurons after nociceptive stimulation. This finding suggests that Arc/Arg3.1 dependent memory formation in spinal pain transmission is a predominant feature of neurons, which are anti-nociceptive rather than pro-nociceptive.

Methods

Animal experiments

In this study we used 99 male Wistar rats and 16 Arc/Arg3.1 KO mice and their wild type littermates.

Rats

50 μl of 0.3% capsaicin (Sigma-Aldrich) solution consisting of 80% saline, 10% Tween-80, and 10% ethanol 100% (n: 6; survival: 1.5 hrs) or 100 μl of Complete Freund's Adjuvant (CFA, Sigma-Aldrich; n: 24; survival: 1.5 hrs, 3 hrs, 4 hrs, 10 hrs, 20 hrs, 60 hrs) was injected in a hind paw under anesthesia with 2% isofluorane in 30%O_2/70% N_2O. In experiments applying mustard oil (MO) (Allylisothiocyanat, Merck) the animals were kept under anesthesia during entire survival time and subsequent perfusion. For 25% MO application (n: 25; survival: 25 min, 45 min, 1 h, 2 hrs, 4 hrs, 8 hrs) the left paw was shaved and wrapped in a gauze soaked with MO and then covered with foil. For application of 10% (n: 5; survival: 2 hrs) and 50% (n: 5, survival: 2 hrs) MO, the left paw was shaved and MO was applied once at the beginning of the experiment using a brush. For the experiments using intrathecal injections, the same protocol was used as described in [42]. Brain-derived neurotrophic factor (BDNF, 10 μg, Tocris) was injected intrathecally in a total injection volume of 40 μl (n: 6; survival: 75 min). In another experiment, 5 μg of 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[*f*]quinoxaline-7-sulfonamide (NBQX, Tocris) was injected concomitantly with BDNF (n: 6; survival: 75 min). For control intrathecal experiments, 25 nmol N-Methyl-d-asparate (NMDA; Sigma, St. Louis, MO; n: 2; survival: 75 min), or only vehicle (1% bovine serum albumin in 0.025 M phosphate buffer; n: 2; survival: 75 min) or only NBQX (n: 2; survival: 75 min) was injected intrathecally. After the injections, the rats were placed back in their cages. For induction of neuropathic pain, the spared nerve injury (SNI) model and a control operation were used [43]. In short, the sciatic nerve was exposed and the three branches were isolated. The tibial and the common peroneal branches were ligated and then cut while the sural nerve was left intact (n: 9; survival: 2 hrs, 7 days, 14 days). As a control, the sciatic nerve was only exposed and isolated (n:7; survival: 2 hrs, 7 days, 14 days).

Arc/Arg3.1 KO and WT mice

All mice were habituated for 5 days to the experimenter, the experiment room, and the transparent cage that was used for the Von Frey measurements. Thereafter, prior to each experiment the mice were habituated for 30 minutes to the room in which the behavioral experiments took place.

Von Frey experiment before each Von Frey measurement, the mice were allowed to habituate to a transparent cage (15 cm × 15 cm with a gridded floor) for 10 minutes. We used calibrated von Frey filaments, which were applied for 2 seconds at 5 seconds interval, and the threshold was set at 3 evoked responses in a maximum of 5 applications.

Hotplate test the thermal thresholds were assessed by measuring the time a mouse spent on the hotplate (51° C) before showing a response like fluttering or licking of the hind paw, or jumping. Immediately after a response or after maximally 45 seconds, the mouse was taken off the hotplate.

The formalin pain model the mice were restrained by the experimenter and 15 μl of formalin, i.e. a freshly made solution of 4% paraformaldehyde (PFA) in phosphate buffer (PB), was injected subcutaneously in the left hind paw. The number of flutters and the time spent licking of the injected paw were measured during 55 minutes post-injection. After 90 minutes the mice were perfused and the tissue was processed as described below. n = 4 for Arc/Arg3.1 KO mice; n = 4 for WT littermates.

The CFA pain model 25 μl of CFA was injected in a hind paw of restrained mice and thereafter the mechanical and thermal thresholds were assessed at 1.5 h, 4 hrs, 1 d, 3 d, 4 d, 8 d, 14 d, 21 d, 28 d, 34 d, and 42 d post-injection. n = 4 for Arc/Arg3.1 KO mice; n = 4 for WT littermates.

Statistical analysis An unpaired *t*-test or a repeated measures ANOVA was performed, $p < 0.05$ was taken as significant.

Examination of the Arc/Arg3.1 KO mice spinal tissue After experiments the mice were sacrificed and further processed for immunohistochemistry (IHC) or in situ hybridization (ISH). Histological examination of Arc/Arg3.1 KO mice spinal cord did not reveal any morphological abnormalities in comparison with their WT littermates.

Tissue preparation

At the end of the survival times the animals received an overdose of sodium pentobarbital and were transcardially perfused with 100 ml saline (rats) or 10 ml (mice) followed by 750 ml of 4% PFA (rats) or 50 ml (mice) dissolved in 0.12 M phosphate buffer (PB), pH 7.4. The spinal cord was dissected and left overnight in a solution of 4% PFA and 30% sucrose at 4°C. Subsequently, sections were cut (30 μm) on a freezing microtome and collected in RNAse-free PB. Serial sections were cut and collected in 9 separate jars, and therefore sections in one jar were at least 270 μm apart. The sections were kept in a solution of 40% glycerol, 40% ethyleenglycol and 20% RNAse-free PB for long-term storage at -20°C.

In situ hybridization and immunohistochemistry

The partial cDNA templates encoding the following mRNAs were used: Arc/Arg3.1 (3.5 kb, full length probe encoding the mus musculus Arc/Arg3.1 gene, GeneID: 11838; Image Clone number: 3498057), GAD67 (3.2 kb; a generous gift from Dr. A.J. Tobin, UCLA), preproenkephalin (0.95 kb, a generous gift from Dr. S.L. Sabol, NIH). The riboprobes were obtained by linearizing the recombinant plasmids with the appropriate restriction enzymes and RNA polymerases. The transcription was performed in the presence of digoxigenin (DIG)- or fluorescein-labeled 11-UTP (Roche). ISH based on alkalic phosphatase (AP) reaction was performed following the protocol described previously [32]. For fluorescent in situ hybridization (FISH) the following modifications were applied to the protocol. After riboprobe hybridization, the detection of DIG or fluorescein was achieved with sheep polyclonal anti-Dig antibody (Roche) or mouse monoclonal anti-fluorescein antibody (Roche), respectively (1:500; 48 hours at 4°C in phosphate buffer saline (PBS), 2% milk powder and 0.5% Triton X-100). Thereafter, the anti-DIG or anti-fluorescein primary antibodies were detected using biotinylated rabbit-anti-goat (Vector) or goat-anti-mouse (Vector), respectively. Subsequently, the sections were incubated with Avidin-Biotin-Complex (ABC, Vector) tagged with horseradish peroxidase (HRP). A tyramide amplification procedure was performed by reacting HRP with H_2O_2 and a self made FITC tyramide according to protocol described in [44]. Thereafter, the sections were washed in PBS and processed for fluorescent IHC using the following antibodies diluted in 2% milk power solution: rabbit anti-Arc (1/3000; a generous gift from Dr. D. Kuhl), rabbit anti-c-Fos (1: 40.000; Oncogene Research Products, La Jolla, CA), rabbit anti-neurokinin-1 (NK1; 1:5000; Advanced Targeting System, CA, USA), rabbit anti-calbindin (1:7000; Swant, Switzerland), rabbit anti-PKC-γ (1/750; Santa Cruz), and mouse anti-neuronal nuclei (NeuN) monoclonal antibody (1:5000, Chemicon). These primary antibodies were detected with Cy3 tagged fluorescent secondary antibodies donkey-anti-rabbit or donkey-anti-mouse (1:200). Thereafter, the section were washed in PB and mounted on slides and coverslipped with Vectashield (Vector).

Data analysis

Analysis was carried out on L4 and L5 segments of the spinal cord, except for the BDNF experiments, in which also S1 and S2 segments were included in the analysis. Slides were systematically examined starting from the first section in the first row for the appropriate segmental level. The first 5 to 6 sections that were encountered and were not damaged during the procedure were included in the analysis [42]. For illustrations, light micrographs were made with a digital camera and confocal images with a Zeis LSM 510 confocal laser scanning microscope and a 20× objective. The images were processed using Adobe Photoshop and were not manipulated, except for brightness and contrast. Quantitative analysis of Arc/Arg3.1 mRNA positive neurons based on AP-ISH was achieved using a camera lucida microscope (Neurolucida, Microbrightfield Inc., Williston, VT). The grey and white matter and the boundaries between the laminae were drawn according to [45] and labeled neurons were identified only if the largest diameter was at least 10 μm, and the cell soma contained a

bluish/brownish product. Labeled neurons were expressed as the average number of labeled neurons per section.

For double labeling based on FISH combined with fluorescent IHC, confocal images were analyzed using the Zeis LSM image browser. For each section, the dorsal horn showing Arc/Arg3.1 labeled neurons was analyzed in a vertical plane consisting of 9 slices with an optical thickness of 2.46-2.76 μm. Every fifth section was analyzed for double labeled neurons. For markers that label the cytoplasm, the criterion was that the diameter of a profile was at least 10 μm to be counted as a neuron. For statistical analysis, an unpaired *t*-test was performed, and $p < 0.05$ considered significant.

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Author details
[1]Dept. of Neuroscience, Erasmus University Medical Center, PO Box 2040, 3000 CA, Rotterdam, The Netherlands. [2]Dept. of Neurology, Erasmus University Medical Center, PO Box 2040, 3000 CA, Rotterdam, The Netherlands. [3]Institute for Molecular and Cellular Cognition, Center for Molecular Neurobiology (ZMNH), University Medical Center Hamburg-Eppendorf, Falkenried 94, 20251 Hamburg.

Authors' contributions
MH performed or contributed to all experiments, analyzed data and drafted the paper. JLMJ contributed to experiments and analysis. KB contributed to experiments. DK provided KO mice and gave advice. JCH conceived and supervised the project and edited the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests

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Chapter VII

General Discussion

In this thesis we have identified the location of glycinergic neurons in the spinal cord and investigated the activation pattern of spinal glycinergic and GABAergic (Gly/GABA) neurons in various acute and chronic pain states. Further, we have identified spinal Gly/GABA neurons in the area around the central canal that project to the RVM, and we have investigated the expression pattern of Arc/Arg3.1 in naive spinal cord and after stimulation with nociceptive stimuli. Finally, with respect to descending pathways we have investigated the distribution pattern of Gly/GABA neurons in the RVM that project to the spinal cord. The following general discussion will first focus on the role of spinal Gly/GABA neurons in the naive spinal cord, and during spinal nociception. Thereafter, we will focus on the expression of Arc/Arg3.1 in the spinal cord and long-term memory consolidation in nociceptive pathways. Finally, we will discuss the role of the descending projection of Gly/GABA neurons in the RVM and their role in the inhibitory control of spinal nociception.

1. The role of spinal glycinergic and GABAergic neurons in pain inhibition

In the 1960's it was shown for the first time that glycine and GABA act as inhibitory neurotransmitters in the central nervous system [3, 44]. Glycine is an amino acid produced from serine by the enzyme serine hydroxymethyltransferase, which is not specific for glycinergic neurons. Further, glycine is an important transmitter in the spinal cord and the brainstem, but is not found in higher levels of the nervous system, where GABA is the main inhibitory transmitter [6, 39, 69]. The inhibitory effect of glycine is produced by the glycine receptor, a ligand gated chloride channel, opening of which leads to membrane hyperpolarization [41]. GABA is an amino acid that is produced from glutamic acid by the enzyme glutamic acid decarboxylase (GAD) [81]. GAD is only present in neurons that use GABA as their transmitter and can therefore be used for the identification of GABAergic neurons [14, 40]. GABA acts on GABA receptors of which there are two main types: GABA-A and GABA-B receptors. GABA-A receptors are ligand gated chloride channels, the opening of which leads to membrane hyperpolarization. GABA-B receptors, on the other hand, are G-protein coupled receptors, which are indirectly linked to a potassium channel, that also has a hyperpolarizing effect on the membrane [53]. Glycine and GABA are often colocalized in spinal neurons [48], both in the dorsal [86, 87] and ventral horn [82]. In accordance, physiological studies have shown that glycine and GABA and are co-released at synapses [36]. Only few populations of spinal neurons use only glycine or only GABA as their inhibitory neurotransmitter [24, 87]. They include neurons in the superficial dorsal horn, which are predominantly GABAergic since there are few glycinergic neurons in these laminae, as we have shown in *chapter 2*. Furthermore, in the ventral horn there are specific GABAergic neurons that express GAD65, which is one of the isoforms of GAD and often colocalized with the other isoform GAD67, that are involved in the presynaptic inhibition of muscle spindle afferents on motoneurons [31]. Next to the inhibitory input provided by these Gly/GABA interneurons, there is also inhibitory input from descending glycinergic and GABAergic terminals originating from Gly/GABA neurons in the RVM [2, 5, 49, 56]. The terminals of these Gly/GABA projections contact projection neurons in the superficial layers [2],

interneurons in the deeper layers (laminae III-VI), and also neurons located in the ventral horn [28, 29]. Taken together, glycinergic and GABAergic neurotransmission in the spinal cord is either produced by spinal Gly/GABA interneurons or by Gly/GABA neurons in the RVM with projections to the spinal cord.

1.1. The naive spinal cord

Abolishing glycinergic or GABAergic neurotransmission in the naive spinal cord results in many behavioral signs of hypersensitivity as observed in neuropathic and inflammatory pain conditions [75, 78, 97]. This finding indicates that nociceptive spinal neurons, including projections neurons, are active in the absence of a nociceptive stimulus, and there is the necessity for a continuous glycine and GABA induced inhibition of nociceptive neurons in order to block their activity in the naive animal. It is unclear which Gly/GABA neurons, i.e. spinal interneurons and/or RVM projection neurons, are the source of this inhibitory control. Although there is evidence of descending Gly/GABA terminals contacting spinal dorsal horn neurons [2], and for inhibitory effects after RVM stimulation [50], it remains unclear whether Gly/GABA projection neurons in the RVM are involved in the tonic inhibition of the naive spinal cord. Similarly, the role of spinal inhibitory neurons in this tonic inhibition of nociceptive neurons in the naive spinal cord is not clear. C-fos protein, which is an immediate early gene that is widely used as a marker for neuronal activation [32], is expressed by spinal neurons that are activated by a nociceptive stimulus [22]. In the naive spinal cord, c-fos is only expressed by very few neurons [9], suggesting the absence of active inhibitory neurons in the naive spinal cord. Thus, based on c-fos activation, it seems that spinal Gly/GABA neurons do not play a role in inhibiting nociceptive spinal neurons in the naive animal. This apparent contradiction may be resolved when it is assumed that c-fos expression in spinal neurons is induced by phasic activity rather than tonic activity. Therefore, the lack of c-fos expression in the naive spinal cord would not necessarily imply the absence of active Gly/GABA neurons. Apparently, these neurons only start to express c-fos when they become activated after a nociceptive stimulus, which leads to a sudden strong increase in their activity. In conclusion, spinal inhibitory neurons, whether or not in combination with descending inhibitory neurons, probably play a role in inhibiting nociceptive neurons in the naive spinal cord but the mechanisms underlying this inhibition are still unclear.

1.2. Nociceptive activated spinal cord

1.2.1. Ipsilateral pain stimulation

In the past decade, is has become increasingly clear that glycine and GABA play an important role in controlling spinal nociceptive processing. For example, during chronic inflammatory pain glycinergic inhibition is blocked in the spinal cord by a pathway that involves prostaglandin E2 (PGE2) [58, 72], a process which underlies the development of thermal and mechanical hyperalgesia [98]. Further, activation of selective subunits of

the GABA receptor, i.e. the α2 and/or α3, result in pronounced pain inhibition in chronic pain states [42]. During neuropathic pain states, there is evidence for reduced GABAergic inhibition in the superficial dorsal, which may [57] or may not [65, 66] be due to loss of GABAergic interneurons. At the same time there is loss of synaptic inhibition due to a shift in the chloride gradient, which reverses the inhibitory effect of GABA into a depolarizing one [11].

Despite the knowledge on the involvement of inhibitory neurotransmission in spinal nociceptive processing, there are few data about the role of Gly/GABA interneurons in the loss of spinal inhibition and their activation pattern in acute and chronic pain states [85, 101, 102]. In *chapter* 3 of this thesis, we have investigated the activation pattern of spinal Gly/GABA neurons in various acute and chronic pain states affecting one hindpaw by determining the number and the percentage of c-fos activated neurons that were Gly/GABA on the ipsilateral side. We found that the percentage of c-fos activated neurons that was inhibitory was higher (46%) in chronic (≥ 20hrs) pain states as compared to acute (≤ 2hrs) pain states (34%). This increase in percentage was caused by a reduction in the number of c-fos expressing non-Gly/GABA neurons in chronic pain states while the number of c-fos expressing Gly/GABA neurons remained stable. This finding indicates that in chronic pain states there is a relatively increased activation of Gly/GABA neurons. However, as mentioned earlier, several studies have shown a loss of inhibitory transmissions in the spinal cord during chronic inflammatory and neuropathic pain [99]. A possible explanation may be that the activated Gly/GABA neurons that we found are not functional, because the effects of the glycine and GABA they release is blocked at the synaptic level or that there effect is reversed (see above). Another explanation may be that the activated Gly/GABA neurons serve as a compensation, albeit insufficient, of the apparent loss of inhibitory neurotransmissions in other pathways during chronic pain states. Yet another view would be that the expression of c-fos occurs in Gly/GABA neurons involved in phasic activity, while the loss of inhibition occurs in tonically active Gly/GABA neurons, which are not identified by the expression of c-fos. Finally it may be argued, that the activated Gly/GABA neurons are involved in another role, unrelated to the inhibition of pain transmission in the dorsal horn, e.g. related to inhibition of motoneurons. Next to the activity of spinal interneurons, there are also glycinergic and GABAergic inputs by descending pathways from the RVM to the spinal cord [2, 50]. Therefore, it is possible that the activity in these inhibitory descending pathways is decreased, counteracting the effect of the activated Gly/GABA neurons that we have indentified during chronic pain states.

In conclusion, in chronic pain states there is loss of synaptic inhibition in the spinal cord while at the same time there are c-fos activated spinal Gly/GABA neurons, and possibly inhibitory input by descending pathways from the RVM. Whether all these inhibitory inputs are blocked, resulting in the observed loss of spinal inhibition in chronic pain states, or whether their activation is induced by the loss of spinal inhibition but fail to compensate for that loss is currently unclear.

1.2.2. Contralateral pain stimulation

In *chapter IV* of this thesis we have shown that the number of activated Gly/GABA neurons is increased after a capsaicin injection in the hindpaw of rats which have chronic inflammatory or neuropathic pain in the other hindpaw. This indicates that a unilateral chronic pain state increases the excitability of Gly/GABA neurons on the contralateral side of the spinal cord. As a result a subsequent pain stimulus on that side will activate a larger number of Gly/GABA neurons, relative to non-Gly/GABA neurons. Previous studies have shown that primary afferent fibers not only result in ipsilateral activation of the spinal cord, but also in contralateral activation by polysynaptic mechanisms [27]. Further, it has been shown that an one sided noxious stimulation induces c-fos activation of spinal neurons on the contralateral side [9], and that the number of c-fos expressing neurons is increased after a second stimulus on that contralateral side [45, 46]. In accordance, it was shown recently by means of autofluorescent flavoprotein imaging that there is activity on the contralateral side of the spinal cord immediately after an ipsilateral nociceptive stimulation [37]. The following question now arises: what is the functional meaning of the activation and the increased excitability that develops on the contralateral side? One possible explanation may be that it is important for survival: when a body part is injured on one side, the same body part on the contralateral side must remain functional despite the injuries and possible new injuries to that body part. In this situation, an enhancement of the local inhibition of nociception would be beneficial for a proper function of that body part [63, 70]. However, in these situations it is likely that the PAG-RVM system, that controls spinal pain transmission through its descending projections to the dorsal horn [26], will also become involved. Therefore it is likely that changes that we have observed on the contralateral side of an injury, are the result of changes in the activity and excitability of local spinal neurons as well as neurons in the RVM. Taken together our findings show that the excitability and subsequent activation pattern of Gly/GABA neurons on one side of the spinal cord are affected by painful events on the contralateral side, while the underlying mechanisms need further investigation.

2. Long-term memory consolidation in spinal nociceptive pathways

Central sensitization plays a important role in the development and the maintenance of hyperalgesia and allodynia after nociceptive stimuli. There are three mechanisms that underlie the central sensitization [94, 95]. In the first place there is wind-up, a form of activity dependant plasticity, resulting in the increase of action potential output of dorsal horn neurons. Wind-up is induced by and only manifest during a train of repeated low-frequency C-fiber or nociceptor stimuli [12, 52, 55, 83] and occurs only at the active synapse (homosynaptic). For example, when a noxious thermal or mechanical stimulus with a constant intensity is repeatedly applied to the skin, wind up of spinal neurons induces an increase in pain with each successive stimulus, while the intensity of the noxious stimuli is constant [68]. 2) A second mechanism is heterosynaptic central sensitization. In this case the increased excitability of spinal nociceptive dorsal horn neurons, elicited by

a brief and intense nociceptive stimulus, results in the activation of dorsal horn neurons by primary afferent inputs that are normally subthreshold [10, 51, 96]. This form of central sensitization is heterosynaptic, meaning that that the potentiation of synaptic output not only applies to the synapses that were activated by the initiating stimulus, but also to other synapses not activated by the initiating stimulus [76]. At the behavioral level, this heterosynaptic potentiation is the underlying cause of secondary hyperalgesia and allodynia in the area around the primary injury site [77]. A third mechanism is known as long-term potentiation (LTP), a process at the homosynaptic level which results in an increased efficacy of excitatory primary afferent input [34, 92]. It has been shown that this LTP induced enhancement of monosynaptic excitatory synaptic responses lasts for days to weeks, is NMDA receptor dependent, and leads to the phosphorylation of AMPA receptors and the recruitment of new AMPA receptors into the cell membrane [71, 90]. It is generally assumed that most processes inherent to LTP formation in cortical and hippocampal neurons are probably similar to the LTP-related processes occurring in spinal nociceptive neurons [35]. Taken together, there is evidence for nociception induced homosynaptic and heterosynaptic enhancement of spinal nociceptive neuronal excitability, and LTP based long-term consolidation of synaptic changes in spinal nociceptive pathways, and all these mechanisms contribute to central sensitization.

In this thesis we have investigated the expression pattern of Arc/Arc3.1 mRNA and protein in the rat spinal cord after nociceptive stimuli, and determined the pain behavior in Arc/Arg3.1 knockout (KO) mice. Arc/Arg3.1, an immediate early gene (IEG), was first identified and extensively investigated in cortex and hippocampus [8, 21, 64, 73, 74], where it plays an essential role in long-term memory consolidation by regulating AMPA receptor trafficking [7]. Consequently, knockdown of Arc/Arg3.1 results in the loss of LTP and long term depression (LTD), and the loss of long-term memory while short term memory is unchanged [64]. In *chapter 6* we showed that Arc/Arg3.1 is *de novo* expressed after a nociceptive stimulus and that the expression of Arc/Arg3.1 is intensity dependent, i.e. a stronger nociceptive stimulus induces a higher number of Arc/Arg3.1 expressing neurons. We further found that Arc/Arg3.1 is predominantly expressed in enkephalinergic interneurons, and not in NK1 receptor expressing projection neurons, that relay nociceptive signals to supraspinal levels, nor in Gly/GABA interneurons that are likely involved in the local inhibition of spinal pain transmission. In line with this lack of expression in the most important neurons of the spinal nociceptive system, we found that the Arc/Arg3.1 KO mice showed no changes in their pain behavior to acute (formalin) and chronic (inflammation) pain stimuli as compared to their wild type littermates. This strongly suggests that the important "memory molecule" Arc/Arg3.1 is not crucially involved in the long-term consolidation of "pain memory", i.e. the changes that occur during inflammatory and neuropathic pain states. This finding may reflect a special property of pain memory in the spinal cord, which is that it should be reversible and not lead to permanent changes. In functional terms: if an injured part of our body remained sensitized to non-noxious (allodynia) and noxious (hyperalgesia) stimuli, we would be forced to maintain the protection of an injured area long after the healing process of that area had been completed. Instead,

central sensitization in the spinal cord that develops post injury slowly subsides with tissue healing, resulting in the disappearance of hyperalgesia and allodynia in and around the area of tissue injury. As a consequence the functional organization of the spinal cord, and the sensitivity of the healed area of injury, will return to the normal pre-injury situation. Since Arc/Arg3.1 is involved in establishing permanent changes in synaptic strength, its absence from spinal nociceptive projection neurons, as well as Gly/GABA expressing spinal neurons, is in line with the idea that permanent changes in the spinal pain system are unfavorable for its proper function. However, there is one group of neurons that did express Arc/Arg3.1 after nociceptive activation: the enkephalin expressing neurons. This indicates that these neurons will develop permanent changes after nociceptive activation and become more sensitive for incoming nociceptive stimuli for a long period of time. As a consequence, these enkephalinergic neurons, which are a minority of the c-fos activated neurons, may become more easily activated when the injured area would be injured a second time. Since enkephalin exerts an inhibitory effect on pain transmission in the dorsal horn [17, 61], this would mean that a subsequent injury would be less painful then the previous one. If this effect is strong enough, it would be possible to test this hypothesis in Arc/Arg3.1 KO mice. However, due to a limited availability of these mice, were not able to put this to the test.

In conclusion, our results indicate that Arc/Arg3.1 in the spinal cord is not involved in the development of pain behavior in the formalin and chronic inflammation models. This indicates that mechanisms that produce central sensitization in the spinal cord do not lead to long-term changes that outlast the healing period. In that sense, LTP in the spinal cord is different from other areas of the central nervous system (CNS), where long term consolidation of synaptic changes is a prerequisite for memory formation.

3. Inhibitory projections from the RVM to the spinal cord

Next to the spinal interneurons, descending projections from the RVM are also important in modulating spinal nociception [16], as activation of these projections leads to facilitation or inhibition of spinal nociceptive transmission [15]. The facilitating effect of the RVM on spinal nociception is induced by the ON cells [4, 15, 25], while inhibition of spinal nociception by descending RVM projections is mainly achieved by OFF cell activation [15, 26], with simultaneous inhibition of the ON cells. Many studies have focused on the transmitters involved in producing the effects of the descending RVM projections and most of the neuropeptides that were identified showed a facilitating effect, including cholecystokinin [43, 89] and neurotensin [60, 79, 88, 89]. With respect to the inhibitory projections from the RVM, it was believed for a long time that the transmitter involved was serotonin [23, 33, 38, 47]. However, more recently it became clear that serotonin may also induce facilitation of spinal pain transmission [62, 84, 100] and that serotonin was not localized in ON or OFF cells, but in Neutral cells, which show an activity pattern unrelated to nociceptive stimulation [18, 67]. Thus, if serotonin is not present in the OFF cells, which

is then the main transmitter, producing direct inhibition on spinal nociception? There is evidence that glycine and GABA are released in the dorsal horn upon RVM stimulation [49, 50, 80], but is has not been established from which terminals, i.e. descending projections or local spinal interneurons. In fact there are several mechanisms that may result in the inhibition of spinal nociception, including direct inhibition of projection neurons in the dorsal horn [91, 93], opioid dependent inhibition of transmitter release by primary nociceptive afferents [19], direct inhibition of nociceptive neurons in the dorsal horn [13, 20] and the activation of inhibitory interneurons in the dorsal horn [1, 54, 80]. In an electron microscopical study [2], which combined anterograde tracing from the RVM with glycine and GABA immunohistochemistry, it was shown that in the dorsal horn glycine and GABA were present in terminals that were labeled from the RVM. In *chapter 6* we have shown, using retrograde tracing from the spinal cord with fluorescent in situ hybridization for GABA and glycine, that 44% of the neurons projecting to the spinal dorsal horn are indeed Gly/GABA. Thus, based on this anatomical evidence, it seems very likely that Gly/GABA neurons represent the OFF cells. Whether this means that glutamate is the likely fast excitatory transmitter in the ON cells (next to various neuropeptides) remains unclear.

In *chapter 6* we have also shown that there are Gly/GABA neurons in the spinal cord, located in the area around the central canal (CC), that project to the RVM. This pathway is the only projection from the spinal cord to a supraspinal structure (i.e. the RVM) that contains the fast inhibitory transmitters GABA and glycine. This finding shows that the spinal cord is not merely "passing on" information to higher structures, but is also involved in the inhibition of specific supraspinal nuclei, like the RVM. Within the same spinal area around the CC there are also neurons that use opioids such as enkephalin and dynorphin [59] that project to supraspinal sites including the RVM. Enkephalin is frequently colocalized with GABA in the spinal cord [30], therefore it is not excluded that the opioids in these neurons act as modulators that are colocalized with fast transmitters like GABA and glycine, and thus represent the same neurons that we have identified as Gly/GABA. It is presently unclear which type(s) of neurons in the RVM are inhibited by these connections and whether or not they act as feedback loops involved in the control of the nociceptive transmission in the spinal cord. Taken together, our finding of glycinergic and GABAergic ascending pathways from the spinal cord to the RVM, shows that the idea of the spinal cord as a relay station for sensory information, should be adapted to include the existence of inhibitory connections, controlling the activity of supraspinal structures, like RVM.

4. Final conclusions

In this thesis we have shown that spinal Gly/GABA neurons have specific activation pattern in acute and chronic pain states. Whether these activated Gly/GABA neurons can compensate for the loss of inhibitory neurotransmission during chronic pain, or whether they are involved in another process inherent to chronic pain states is unclear. Our investigation on the spinal expression of Arc/Arg3.1 indicates that Arc/Arg3.1 based long-term memory consolidation is not predominant in the spinal cord. This finding is in line with

our suggestion that long-term consolidation of synaptic activity in the spinal cord is unfavorable as it would lead to a hypersensitive spinal cord with permanent hyperalgesia and allodynia even after tissue healing. Further, our findings provide strong evidence for the involvement of RVM Gly/GABA projection neurons in spinal processing. However, whether Gly/GABA projection neurons are the OFF cells that have been characterized to inhibit spinal nociception, or that they are a specific subset of neurons, next to OFF cell population remains to be determined. Finally, the existence of reciprocal inhibitory connections between the RVM and the spinal cord suggest that the spinal cord may, via a feed-back loop, be able to modulate RVM neurons in order to alter its own neurons that are under control of descending RVM pathways. Taken together our findings further underline the importance of Gly/GABA neurons in the spinal cord and the brainstem for controlling the feeling we all know: pain

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Summary

Samenvatting

Summary

In this thesis we have employed fluorescent in situ hybridization (FISH) in order to identify neurons in the spinal cord and the brainstem that use glycine or gamma-amino butyric acid (GABA) or both transmitters (Gly/GABA neurons). With this technique we have made a detailed analysis of the localization of glycinergic cell somata in the spinal cord (*Chapter 2*). In the subsequent experiments we have combined FISH with the retrograde tracing technique, using fluorescent microspheres, or with the fluorescent immunohistochemistry technique for identifying various proteins, including c-fos, a marker for neuronal activation.

In *Chapter 3* it is demonstrated that spinal Gly/GABA neurons have specific activation patterns in acute, chronic inflammatory and neuropathic pain. Furthermore, the averaged percentage of activated neurons that were Gly/GABA in the chronic phase (≥20h survival, 46%) was significantly higher than in the acute phase (≤2h survival, 34%). Morphine application equally decreased the total number of activated neurons and activated Gly/GABA neurons. This showed that morphine did not specifically activate Gly/GABA neurons to achieve nociceptive inhibition. Since there is evidence for an overall loss of spinal inhibitory neurotransmissions in chronic pain states, we conclude that the effect of the activated Gly/GABA neurons is insufficient to produce an overall increase in inhibition during these chronic pain states.

In *Chapter 4* we have shown that a chronic pain state in the hind paw on one side leads to increased excitability of Gly/GABA neurons located on the contralateral side of the spinal cord, since a pain stimulus on that contralateral side resulted in an increased number of activated Gly/GABA neurons.

In *Chapter 5* we have used FISH combined with fluorescent retrograde tracing. The results showed that about 40% of the neurons in the rostral ventromedial medulla (RVM) that project to the cervical spinal cord were Gly/GABA, i.e. containing either glycine or GABA or both transmitters. From the projections of the RVM to the lumbar dorsal horn 43% were Gly/GABA and this percentage was 35% for the projections to the lumbar ventral horn. In the caudal medulla, relatively few neurons projecting to the lumbar dorsal horn were Gly/GABA (5%), while this percentage was much higher (19%) for projections to the ventral horn. The percentages obtained for GABA and glycine separately were very similar to those obtained for Gly/GABA. These findings show that virtually all of the Gly/GABA projections to the spinal cord contain both transmitters and that the projections to the dorsal horn originate preferentially in the RVM. In this chapter we have also shown the presence of spinal Gly/GABA neurons located in the area around the central canal that project to the RVM. This inhibitory ascending pathway, which is the only inhibitory (Gly/GABA) pathway from the spinal cord to the brainstem identified up to now, might allow the spinal cord to modulate the RVM, thereby controlling the descending control of the RVM on spinal pain processing.

In *Chapter 6* we have investigated in the spinal cord the role of Arc/Arg3.1, an immediate early gene that is essential for long-term memory consolidation in cortical and hippocampal neurons. Arc/Arg3.1 is not present in the naive spinal cord, but is *de novo* expressed by nociceptive stimuli. The majority (68%) of the Arc/Arg3.1 expressing neurons contained enkephalin, while only 19% were neurokinin-1 expressing projection neurons and 3.6% were inhibitory (GABA) neurons. These findings showed that Arc/Arg3.1 is expressed in only a few projection and inhibitory neurons, which belong to the most important neurons of the spinal pain system. Accordingly Arc/Arg3.1 knockout mice did not show any changes in their pain behavior after formalin injection and after induction of chronic inflammation. These findings suggest that long term memory consolidation is not required and may even hamper normal functioning of the nociceptive system.-

Taken together, this thesis gives a detailed analysis of the distribution pattern of activated Gly/GABA neurons in the spinal cord during different pain states as well as the distribution of Gly/GABA neurons in the RVM that project to the spinal cord. The expression pattern of Arc/Arg3.1 during different pain states substantiates the idea that long term synaptic changes in the spinal pain system, including the Gly/GABA neurons, are unfavorable for the normal functioning of spinal pain transmission. Thus our findings further underline the importance of Gly/GABA neurons in the spinal cord and the brainstem for controlling the feeling we all know: pain.

Samenvatting

In dit proefschrift hebben we gebruik gemaakt van de fluorescerende in situ hybridizatie (FISH) techniek om de neuronen in het ruggenmerg en de hersenstam te identificeren die glycine of gamma-amino boterzuur (GABA) of beide transmitters bevatten (Gly/GABA neuronen). Met deze techniek hebben we een gedetailleerde analyse gemaakt van de localisatie van de glycinerge neuronen in het ruggenmerg (*Hoofdstuk 2*). In de volgende experimenten hebben we de FISH techniek gecombineerd met de retrograde neuronale opsporingstechniek met behulp van fluorescerende micro-bolletjes of met de fluorescende immunohistochemie techniek om verschillende eiwitten te identificeren, waaronder c-fos, het eiwit dat geactiveerde neuronen labelt.

In *Hoofdstuk 3* wordt aangetoond dat Gly/GABA neuronen in het ruggenmerg volgens een specifiek patroon geactiveerd worden gedurende acute pijn, chronische ontstekingspijn en neuropathische pijn. Het gemiddelde percentage geactiveerde Gly/GABA neuronen was significant hoger in de chronische fase (≥20h overleving, 46%) vergeleken met de acute phase (≤2h overleving, 34%). De toediening van morfine verminderde het totale aantal geactiveerde neuronen en het totaal aantal geactiveerde Gly/GABA neuronen in dezelfde mate. Dit laat zien dat de pijnonderdrukking door morfine niet tot stand kwam door het activeren van Gly/GABA neuronen. Op grond van aanwijzingen dat de totale mate van pijnonderdrukking in het ruggenmerg minder wordt gedurende chronische pijn, kan worden geconcludeerd dat het effect van de geactiveerde Gly/GABA neuronen onvoldoende is om gedurende chronische pijn een toename van pijnonderdrukking te bewerkstelligen.

In *Hoofdstuk 4* laten we zien dat chronische pijn aan de achterpoot aan één kant, leidt tot een verhoogde prikkelbaarheid van Gly/GABA neuronen aan de andere kant van het ruggenmerg, aangezien een pijnprikkel aan die andere kant resulteerde in een verhoogd aantal geactiveerde Gly/GABA neuronen.

In *Hoofdstuk 5* hebben we de FISH techniek gecombineerd met de retrograde neuronale opsporingstechniek. De resultaten lieten zien dat ongeveer 40% van de neuronen in de rostral ventromediale medulla (RVM) die naar het cervicale ruggenmerg projecteerden Gly/GABA bevatten, ofwel dat deze neuronen of glycine of GABA of beide transmitters bevatten. Van de projecties van de RVM naar de lumbale dorsale hoorn waren 43% Gly/GABA en wat betreft de projecties naar de lumbale ventrale hoorn was dit percentage 35%. De ventrale medulla, caudaal van de RVM, bevatte relatief weinig neuronen met Gly/GABA (5%), die naar de lumbale dorsale hoorn projecteerden, terwijl dit percentage veel hoger lag (19%) voor de neuronen met projecties naar de lumbale ventrale hoorn. De percentages die werden verkregen voor GABA en glycine apart waren vrijwel gelijk aan de bovengenoemde percentages voor Gly/GABA. Samengevat kan gesteld worden dat nagenoeg alle projecties van de RVM en caudale medulla naar het ruggenmerg zowel glycine als GABA bevatten en dat de projecties naar de dorsale hoorn voornamelijk afkomstig zijn van de RVM. In dit hoofdstuk hebben we ook laten zien dat er Gly/GABA neuronen

aanwezig zijn rond het centrale kanaal van het ruggenmerg die naar de RVM projecteren. Deze baan van het ruggenmerg naar de hersenstam is de enige opstijgende baan, met een remmend (Gly/GABA) effect, die tot nu toe bekend is. Waarschijnlijk kan deze baan de RVM beïnvloeden en daarmee de afdalende banen van de RVM controleren, die op hun beurt de pijnverwerking in het ruggenmerg controleren.

Hoofdstuk 6 laat in het ruggenmerg het onderzoek zien aangaande Arc/Arg3.1, een snel afgeschreven genproduct dat in de cortex en de hippocampus neuronen essentieel is voor het vastleggen van het lange termijn herinneringen. Arc/Arg3.1 is normaliter niet aanwezig in het ruggenmerg, maar wordt pas tot expressie gebracht als er een pijnprikkel wordt toegediend. De meeste neuronen die Arc/Arg3.1 tot expressie brengen bevatten enkephaline (68%), terwijl slechts 19% de neurokinine-1 receptor (kenmerkend voor projectie neuronen) tot expressie brengen en 3.6% waren GABA neuronen, die een remmende werking hebben. Deze bevindingen tonen aan dat Arc/Arg3.1 slechts in enkele projectie-neuronen en remmende neuronen tot expressie komt, terwijl die neuronen behoren tot de belangijkste in het pijnsysteem van het ruggenmerg. In overeenstemming hiermee lieten Arc/Arg3.1 knockout muizen dan ook geen veranderingen zien in hun pijngedrag na injectie van formaline en gedurende chronische infectie. Deze bevindingen geven aan dat het vastleggen van “pijnherinneringen” voor de lange termijn niet noodzakelijk of zelfs hinderlijk zijn voor het normaal functionerende pijn systeem.

Samengevat kan gesteld worden dat dit proefschrift een gedetailleerde analyse geeft van zowel de organisatie patronen van geactiveerde Gly/GABA neuronen in het ruggenmerg bij verschillende oorzaken van pijn, als ook de verdeling van de organisatie van de Gly/GABA neuronen in de RVM en caudale medulla, die naar het ruggenmerg projecteren. Het expressie patroon van Arc/Arg3.1 gedurende de verschillende oorzaken van pijn verschaft een basis aan het idee dat het vastleggen van synaptische veranderingen voor de lange termijn in het pijnsysteem, daarbij inbegrepen de Gly/GABA neuronen, ongunstig zijn voor het normaal functionerende pijnsysteem in het ruggenmerg. Deze bevindingen benadrukken nogmaals het belang van Gly/GABA neuronen in het ruggenmerg en de hersenstam voor het controleren van het gevoel dat we allemaal kennen: pijn.

CURRICULUM VITAE

Personal

Name	Seyed Mehdi (Aram) Hossaini
Date of birth	November 11, 1981
Place of birth	Shiraz , Iran

Education

September 1994 - June 2001	Havo and VWO Walburg College, Zwijndrecht
September 2001 - April 2006	Medical school Erasmus University in Rotterdam
February 2004 - August 2006	Master of Neuroscience Department of Neuroscience, Erasmus MC, Rotterdam
November 2006 - May 2010	Mozaïek Laureate 2006 (grant nr. 017.003.030) PhD student, Department of Neuroscience, Erasmus MC, Rotterdam
June 2010 - present	Medical internships

List of publications

Differential distribution of activated spinal neurons containing glycine and/or GABA and expressing c-fos in acute and chronic pain models

Hossaini M, Duraku LS, Saraç C, Jongen JL, Holstege JC

Pain 2010;151;356-365

Nociceptive stimulation induces expression of Arc/Arg3.1 in the spinal cord with a preference for neurons containing enkephalin

Hossaini M, Jongen JL, Biesheuvel K, Kuhl D, Holstege JC

Mol Pain 2010;23;6:43

Loss of Hoxb8 alters spinal dorsal laminae and sensory responses in mice

Holstege JC, de Graaff W, **Hossaini M**, Cano SC, Jaarsma D, van den Akker E, Deschamps J

Proc Natl Acad Sci U S A 2008;105:6338-43

Distribution of glycinergic neuronal somata in the rat spinal cord

Hossaini M, French PJ, Holstege JC

Brain Res. 2007;1142:61-9

Distribution of RET immunoreactivity in the rodent spinal cord and changes after nerve injury

Jongen JL, Jaarsma D, **Hossaini M**, Natarajan D, Haasdijk ED, Holstege JC

J Comp Neurol. 2007;500:1136-53

Distribution of GABA and glycine containing RVM neurons that project to the spinal cord and vice versa

Hossaini M, Goos JAC., Kohli S, Holstege JC.

Manuscript submitted (this thesis)

C-fos activation of spinal inhibitory neurons after contralateral hind paw stimulation of rats with inflammatory or neuropathic pain

Hossaini M, Saraç C, Jongen JL, Holstege JC

Manuscript submitted (this thesis)

Degeneration of spinal inhibitory interneurons in SOD1-ALS mice does not require glial mutant SOD1 expression.

Hossaini M, Cano SC, van Dis V, Haasdijk ED, Hoogenraad CC, Holstege JC, Jaarsma D

Manuscript submitted

Spinal enkephalinergic neurons: A study on c-fos activation pattern and ascending enkephalinergic pathways to the medulla oblangata

Hossaini M, Kohli S, Holstege JC

Manuscript in preparation

Glycinergic, GABAergic and enkephalinergic projections from the spinal cord to the lateral reticular nucleus

Hossaini M, Kohli S, Holstege JC

Manuscript in preparation

Dankwoord

Het is 22.56 uur op een donderdagavond in een stilstaande trein op het centraal station in Breda. Het is zoals gewoonlijk een regelrechte chaos op de Nederlandse spoorwegen en de ergernis van de reizigers trekt als een dampige mist over de spoorwegen. Moedig had ik besloten om vanavond na mijn avonddienst op de spoedeisende hulp in het Amphiaziekenhuis toch naar huis terug te reizen, maar mijn moed is nergens meer te bekennen en de stilstaande trein maakt me onrustig. Dan maar aan mijn dankwoord beginnen:

Allereerst wil ik mijn promotor Prof. Chris de Zeeuw bedanken. Alhoewel wij maar een paar minuten met elkaar hebben gesproken in de afgelopen zeven jaar en de meeste zaken tussen ons via Joan zijn verlopen wil ik je hierbij bedanken voor de steun op de achtergrond en meedelen dat je ambities mij aan het denken hebben gezet. Hierbij wil ik ook graag de rest van de leden van de promotiecommissie bedanken voor de tijd die ze hebben genomen om mijn proefschrift te lezen en vragen te formuleren.

Mijn copromotor en begeleider Dr. J.C. Holstege. Beste Joan, we hebben wonderlijk genoeg meer dan zeven jaar met elkaar samengewerkt en evenredig aan het aantal jaren, evenzoveel artikelen geschreven. Ik ben in de afgelopen jaren veel veranderd en jij bent een van de weinigen geweest die daar invloed op heeft gehad. Je ongezouten mening, dat vaak de waarheid weerspiegelde, heb ik altijd op prijs gesteld. Vaak had je aan begeleiding je handen vol aan mij, en in de wetenschap was mijn enthousiasme vaak ongericht, maar gelukkig was jij er om me de goede richting in te sturen. Je kennis over het pijnsysteem heeft me vaak verrast, en je manier van aanpak om een vraagstuk te beantwoorden heeft mij veel bijgeleerd. Verder, het feit dat een artikel minstens 25x hergeschreven moest worden laat zien hoe perfectionistisch jij bent of hoe slecht ik ben. De wetenschappelijke en niet-wetenschappelijke gebeurtenissen die wij in de afgelopen jaren hebben meegemaakt is een unieke en leerzame ervaring geweest. Het is niet makkelijk om volledig te zijn in mijn bewoordingen maar het komt er op neer dat ik een geweldige tijd heb gehad en ik wil je hierbij bedanken voor je hulp en geduld. Aangezien we vaak filosofische gesprekken hebben gevoerd, zal ik mijn dank maar eindigen met het volgende: ‘Perceptie maakt wetenschap mogelijk, en wetenschap is een feitelijke handgreep om te sublimeren. Maar gelukkig is sublimatie een droom binnen dromen.’ Ps. Ik moet wel toegeven dat het nog steeds jeukt dat we geen paper over ‘Jeuk’ hebben gepubliceerd.

Beste Elize! In de afgelopen jaren heb ik ontelbare keren ‘Elize’ geroepen en altijd werd dat met een vriendelijke lach beantwoord. Jij bent zeker de onmisbare schakel in het histologielab, en zonder jouw kennis had ik het op bepaalde momenten niet kunnen redden. Hierbij wens ik je in de komende jaren veel plezier op het histolab, en geniet van je jaarlijkse reizen naar de meest exotische plekken op de aarde.

Erika, bedankt voor de vele leuke gesprekken. Jouw gevoel voor humor komt redelijk dicht in de buurt van die van Joan en ergens denk ik dat jouw vele jaren op het lab, en je samenwerking met Joan aan het begin van je carrière, wel degelijk van invloed zijn geweest. Als je de Masterstudenten ooit zat wordt, geef me dan een seintje, want dan kunnen we ons toneelstuk uitproberen. Mandy, Loes, en Edith, jullie ook bedankt voor jullie hulp in de afgelopen jaren, en nog veel plezier op de afdeling in de aankomende jaren.

Liron, als jij niet op de afdeling was verschenen zouden de afgelopen twee jaar voor mij heel moeilijk zijn geweest. Jij bent een chirurg die wetenschappenlijk is ingesteld, een Kosovaar van top tot teen en gekleed volgens de regels van de Italiaanse mode. Jouw Balkanese mentaliteit gecombineerd met mijn Perzische mentaliteit heeft veel leuke momenten opgeleverd in de late avonduren op het lab; onze manier om de wetenschappelijke tegenslagen van de dag te verteren. Dit was vooral het geval in het eerste jaar toen we alle immunotechnieken aan de gang moesten krijgen en hierdoor hebben wij ook een sterke vriendschap opgebouwd. Je experimenten lopen nu goed en voor je het weet begin jij ook aan het dankwoord van jouw proefschrift! En bedankt dat je mijn paranimf wilt zijn!

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Beste Sebastian, wij rolden het Master of Neuroscience programma binnen in het tweede jaar nadat het gestart was. We hebben drie soorten knockout muizen 'gepijnigd' maar uiteindelijk hebben zij óns gepijnigd. Wat waren we toch gemotiveerd aan het begin en wat waren wij toch gedemotiveerd aan het eind! Wat wij waren toch naïeve Masterstudenten aan het begin en verbitterde Masterstudenten aan het eind. Maar het blijven leuke tijden! Vooral de memorabele 'formaline test' in het kleine donkere kamertje waarbij de slapeloosheid van ons evenredig was aan die van de muizen. Veel succes met je opleiding tot Psychiater en hopelijk zal ik in de toekomst niet tegenover jou komen te zitten, want dan is er iets goed misgegaan!

Querido John! Yo tu prometido que yo dar gracias en Español, et yo cumplir de promesa usanta Van Dale diccionario. Muchas gracias por leccionas de Español, entretenids y algunas veces locos tiempos en laboratorio. Nosotros tenemos abiertas mentas, y esporo que encontrara en futuro en zona differente del medicina, y estoy curisio que serás. Yo me parece Español un bello lengua, y en cuanto más tiempos en mi vida apprendé Español. Hasta luego amigo!

Verder wil ik Tom Ruigrok bedanken voor de nuttige discussies en je hulp met de plotmicroscoop in de afgelopen jaren. Het is wel een ouderwetse ding, maar je kan er ongenaakbare anatomiedata mee genereren. Veel succes in de komende jaren met je onderzoek.

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maakt en op andere momenten ben je gewoon Dick. Heerlijk! Als laatste: Sebastian en ik wachten nog steeds…., nog steeds…., nog steeds…., nog steeds…., nog steeds…… op ons artikel!

Kenneth, bedankt voor de soms diepgaande en de altijd gezellige gesprekken! Als ik later in mijn mid-veertig er zo jong uitzie als jij, dan word ik acteur!

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Het is 22.37 uur op een zondagavond in Zwijndrecht. Het heeft maar vier dagen geduurd om het af te schrijven.