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Implication des astrocytes dans la modulation NMDA-dépendante de l'inhibition spinale

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List of abbreviations

ACSF: artificial cerebro-spinal fluid
ATP: adenosine triphosphate
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
BDNF: brain derived neurotrophic factor
CNQX: 6-cyano-7-nitroquinoxaline-2,3-dioneis
CCI: chronic constriction injury
DAAO: D-amino acid oxidase
DH: dorsal horn
EAAT: excitatory amino acid transporter
FC: fluorocitrate
GABA: γ-aminobutyric acid
$GABA_A$: GABA receptor type A
$GABA_B$: GABA receptor type B
GAD65 -67: Glutamate decarboxylase 65 -67
GAD65::EGFP: enhanced green fluorescent protein expressed under the promoter of the GAD65
GAT: GABA transporter
GluN: NMDA receptor subunit
KCC2: potassium and chloride co-transporter
Lamina IIi: Lamina II inner
Lamina IIo: Lamina II outer
LTD: long-term depression
LTP: long-term potentiation
IPSC: inhibitory post-synaptic current
mGlu receptor: metabotropic glutamate receptor
mIPSC: miniature inhibitory post-synaptic current
NMDA: N-méthyl D-aspartate
NMDAr: N-méthyl D-aspartate receptor
NO: nitric oxide
nNOS: neuronal nitric oxide synthase
PKA: protein kinase A
PKC: protein kinase C
sACSF: sucrose artificial cerebro-spinal fluid
sIPSC: spontaneous inhibitory post-synaptic current
SNI: spared nerve injury
SNL: spared nerve ligation
SST: somatostatin
TTX: tetrodotoxin
VGLUT: vesicular glutamate transporter
VIAAT: vesicular inhibitory amino acid transporter

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Foreword

To build a representation of the outside world in our minds, we need structures specialized in sensing our surroundings, be it for light, sound or touch. This information determines how we understand our surroundings, move in space and protect ourselves from harm. In the nervous system of vertebrates, this function is ensured by the somatosensory system, composed of neural structures, from those responsible for detecting stimuli to others responsible for integrating the information. One function of the somatosensory system is to detect potentially harmful stimuli, called nociceptive stimuli.

The dorsal horn (DH) of the spinal cord plays a key role in the reception and integration of nociceptive information received from the periphery and their transmission to supra-spinal centers. Free terminal in our tissues detect potentially harmful stimuli from the amount of pressure applied (mechanical), the temperature (thermal) and some molecules (chemical) on our skin. These stimuli are transduced into electrical signals conveyed by fibers to the spinal cord and transmitted to secondary neurons in the DH. There, a complex network of excitatory and inhibitory interneurons as well as glial cells integrate the information, before it is sent up to supra-spinal centers by projection neurons, where it might be consciously integrated as pain in the different cortices that form the pain matrix.

Pain serves a protective role for the organism, allowing for healing of the injured tissues as well as learning to avoid further harm. However, in some pathological cases such as nerve damage, pain loses its protective function and becomes maladaptive, lasting longer than the original injury and associated with spontaneous pain, allodynia and hyperalgesia. These clinical cases are often accompanied by depression, anxiety, and an overall reduction in quality of life. The therapeutic approaches to treat chronic pain are insufficient, and research must continue to decipher the mechanisms responsible for the development and maintenance of chronic pain. A lot still needs to be understood about network function in the DH.

The balance between excitation and inhibition, ensured by the interneuron populations in the DH, allows for normal processing of nociceptive information within the network. A proposed

mechanism for the development of chronic pain is a rupture in the excitation/inhibition balance, maintained in time. Many studies have focused on modulation and plasticity of excitatory or inhibitory synaptic transmissions, but few have focused on crosstalk between them. In other words, does excitation modulate inhibition within the network, and are these mechanisms altered in models of pathological pain? Could this cross-talk between excitation and inhibition be the switch responsible for the transition of a physiological network to a pathological one? An interesting candidate for study is the NMDA receptor (NMDAr). Expressed by virtually every neuron in the DH, some studies have shown that NMDAr are expressed at about a third of GABAergic terminals, but no study has focused on the type of NMDAr expressed or their potential function in modulation of GABAergic transmission. Interestingly, NMDAr were one of the first clinical targets for the treatment of chronic pain, however due to important secondary effects, clinical trials were quickly abandoned. With added knowledge on the types of NMDAr expressed in different localizations within the DH, we could get a clearer view on their varied role in synaptic modulation and plasticity.

An important actor in synaptic modulation and plasticity is astrocytes. Studies have shown that astrocytes can sense synaptic activity by transporters and receptors expression at their membranes, inducing intracellular cascades and the release of transmitters, such as glutamate and ATP. These gliotransmitters can then act on receptors on the presynaptic and postsynaptic elements, modulating synaptic transmission.

For all these reasons, my main PhD project was articulated around the study of the role of NMDAr in the modulation of GABAergic synaptic transmission in the DHSC, with a focal point on the potential role of astrocytes in this modulation, as well as a secondary project that I started during my PhD, the study of NMDAr expression in DH astrocytes.

The introduction of my thesis is organized in 3 parts: a first part is focused on the DH network, a second part on inhibitory transmission within the DH and its modulation, notably by astrocytes and the last part is on NMDAr in the DH.

As my PhD projects are the continuation of Benjamin Léonardon's doctoral work, the results section of my thesis will be shared between the publication "Modulation of GABAergic synaptic transmission by NMDA receptors in the DH of the spinal cord" that presents findings obtained

during Benjamin Léonardon's doctoral work where I am 3rd author, and the data I obtained during my PhD.

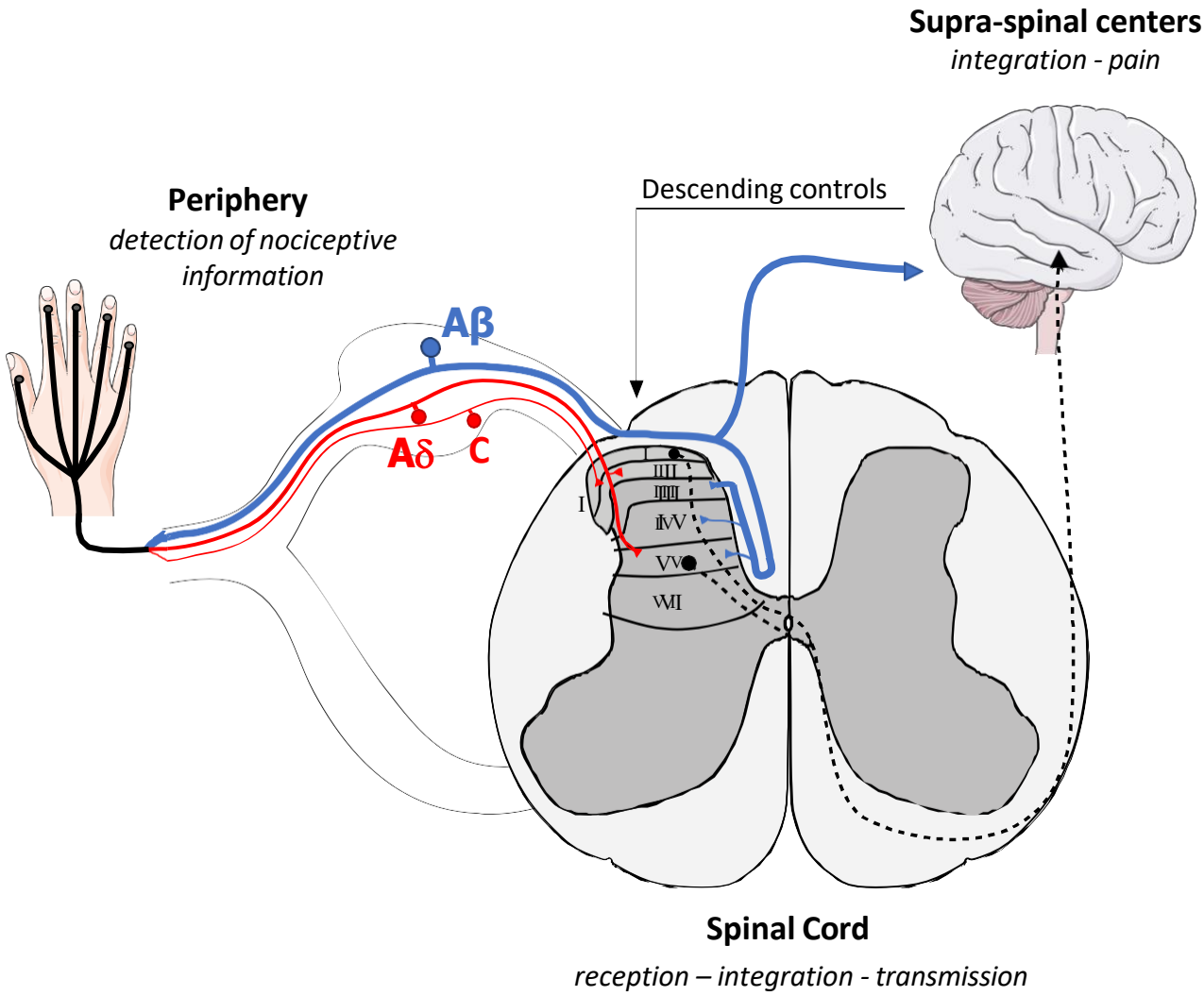


Figure 1: Schematic illustration of the somatosensorial system

Sensorial receptors detect stimuli in the periphery (skin, muscles, viscera). These information are transmitted to the DH of the spinal cord by the primary afferents, composed of Aβ, Aδ and C fibers. Most Aβ fibers ascend directly to supra-spinal centers without passing by the DH, even if some collaterals do form synapses with neurons of the DH. Aδ and C fibers mostly transmit nociceptive information. In the DH, a complex network of interneurons and glial cells integrate nociceptive information before its transmission to supra-spinal structures for more complex integration. Activity of DH cells is also regulated by descending controls, originating in supra-spinal centers.

Introduction

Part 1: Organization of the spinal nociceptive system

Nociceptive information is detected in peripheral tissues and conveyed to the DH of the spinal cord (DH) by the primary afferent fibers. Nociceptive information is conveyed mostly by Aδ and C fibers, smaller and less myelinated than Aβ fibers, which mostly transfer non-nociceptive information. In the DH, nociceptive information is integrated by a network of interneurons and glial cells, before being transmitted by projection neurons to supra-spinal centers. The DH network is composed of glutamatergic, glycinergic and GABAergic interneurons, as well as a plethora of glial cells, such as astrocytes and microglia. The excitation/inhibition balance in this network plays a key role in spinal processing of nociceptive information. The information will then be transmitted to supra-spinal structures for cortical integration, where the painful sensation associated with the nociceptive information can arise. In response, supra-spinal structures send descending controls to the DH, modulating its activity (figure 1).

In this part of the introduction, I will detail the components of the nociceptive pathway, from detection in the periphery to integration in the central nervous system. In my doctoral work, I focused mainly on lamina II within the DH, so I will further detail this structure during the introduction.

I. Organization of the dorsal horn of the spinal cord

The spinal cord extends caudally from the brainstem and can be divided in 4 segments in both humans and rodents: cervical, thoracic, lumbar and sacrum. On a transversal slice of spinal cord, the white matter is peripheral and is composed of fibers and oligodendrocytes, whereas the grey matter is central, composed of somas of cells. The dorsal part of the grey matter is mostly for

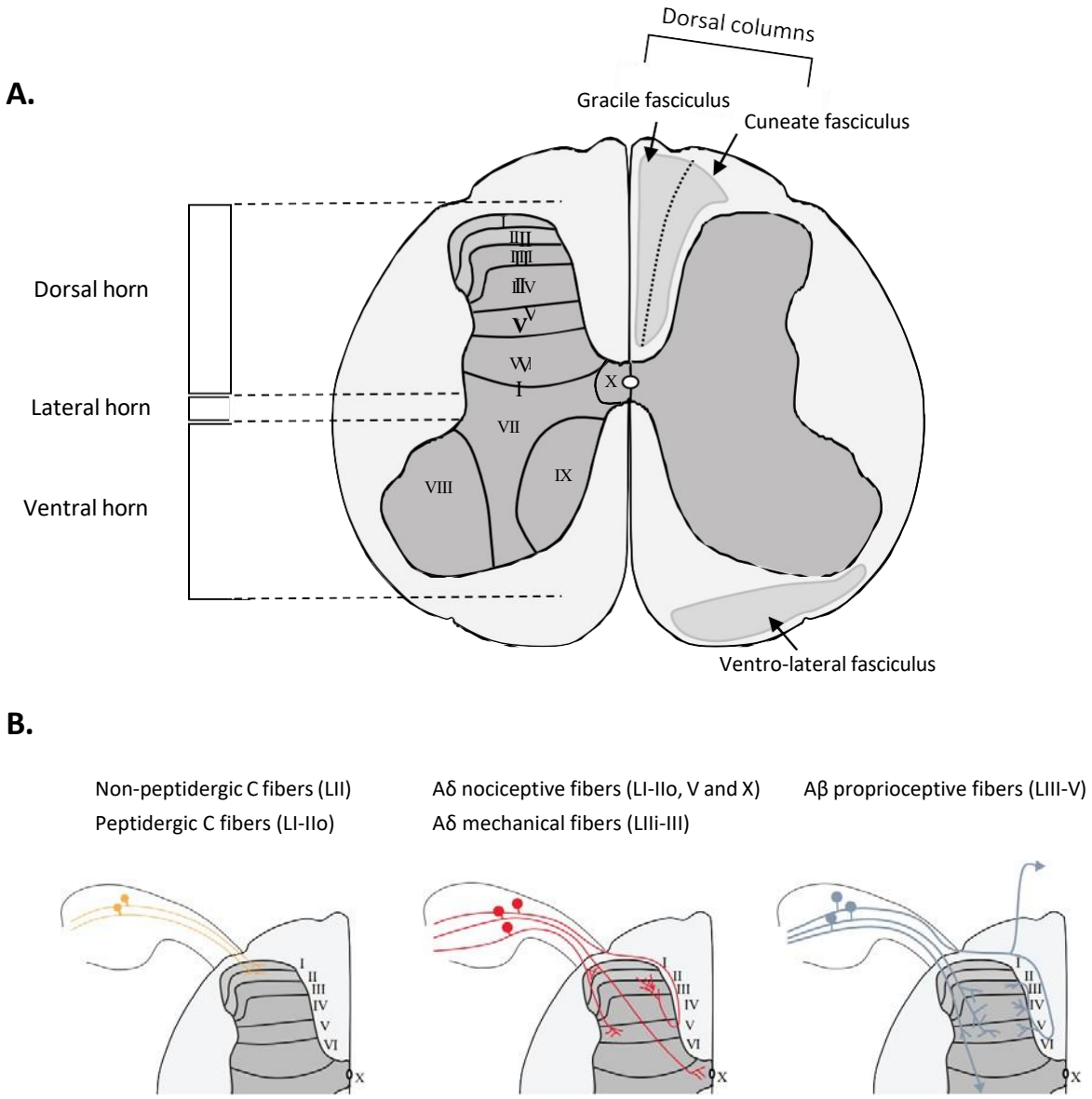


Figure 2: Organization of the spinal cord

A. The grey matter of the spinal cord is divided into 10 laminae. The lamina I-VI are the DH. The lateral lamina is constituted of the lamina VII. The ventral horn is formed by the laminae VIII-IX. Lamina X surrounds the central canal. The white matter of the spinal cord contains columns of fibers, of which the dorsal column, containing the gracile and cuneate fasciculi, that transmit information from the lower and upper limbs respectively. Of the ventral columns is represented the ventro-lateral fasciculus that transmits nociceptive information to supra-spinal structures.
B. C fibers can be divided into 2 sub-categories based on the expression of certain peptides (peptidergic and non peptidergic C fibers). They form synapses with neurons in the superficial laminae of the DH and transmit mostly nociceptive information. Aδ fibers can transmit nociceptive information and non-nociceptive mechanical information, that will form synapses with neurons from superficial and deeper laminae respectively. Aβ fibers transmit mostly non-nociceptive information to deeper laminae in the DH.

somatosensory information processing, whereas the ventral part is composed of motor neurons, whose axons innervate muscles.

Based on cytoarchitecture, the Swedish neuroanatomist Bror Rexed established 10 different laminae of spinal grey matter in 1952. The first 6 laminae are arranged one on top of the other, forming the DH. The laminae VII to IX form the ventral horn of the spinal cord. The last one, lamina X, can be found around the central canal, called the ependyme (Rexed, 1952) (figure 2A).

1. The laminae

The **lamina I**, also called the marginal zone, contains local interneurons and projection neurons. It is estimated that 90-95% of neurons in the lamina I are interneurons and the 5-10% remaining are glutamatergic projection neurons (Todd, 2017). Projection neurons have a bigger diameter, around 200 µm, than interneuron somas with a diameter smaller than 200 µm and a dendritic tree that spreads ventrally and enters the lamina II (Cordero-Erausquin et al., 2016).

The **lamina II,** also called gelatinous substance of Rolando, is less cell-dense than the lamina I, and is virtually composed of 100% interneurons, marking it as an essential zone for integration and modulation of nociceptive information coming from the periphery. When observed in a slice with an inversed microscope, the lamina II appears light compared to the rest of the slice, because it contains very little myelin. This lamina can be separated into 2 areas: the external (on the dorsal side, IIo) and internal (ventral side, IIi), depending on the type of cells that can be found there. In the rodent DH, 4 types of interneurons based on morphology have been identified, using Golgi's techniques for cell staining: islet, radial, central and vertical neurons. Islet cells are mainly found in the lamina IIi, and their dendritic tree spans rostro-caudally, for up to 400 µm in the rat DH. Central neurons are also found in the lamina IIi, and their dendritic tree spans rostro-caudally, but on a smaller area. Radial cells are present everywhere in the lamina II and their dendritic tree spans in a star-like shape. Finally, vertical neurons' somas are present in the lamina IIo, with a dendritic tree that spans dorsoventrally. However, it's important to note that these types based on morphology do not regroup all cells in the lamina II. Indeed, when using this morphological classification, ~30% of cells were "unclassified". And when making groups based on properties such as neurochemistry, marker expression or electrophysiology, they do not always overlap,

explaining why some studies have questioned whether they do form functional populations of neurons (Todd. 2010, 2017). As a lamina composed of only interneurons and thus, purely designed for integration, my doctoral work was focused on studying the network of the lamina II.

The **laminae III-IV** are less dense in cell somas compared to the more superficial laminae and contain a large number of myelin fibers. They are composed of a smaller proportion of projection neurons than in LI and interneurons that can be divided into 2 distinct populations: local axon cells and deep axon cells (Cordero-Erausquin et al., 2016). Both these neuron populations have a dendritic tree that extends dorsoventrally, in a limited way for the local axon cells and further for the deep axon cells that can reach the superficial and the deeper laminae. The axonal territory of the local axon cells extends rostro-caudally within the laminae III-IV, whereas the axonal territory of the deep axon cells spans ventrally and can innervate the laminae IV to VI (Schneider, 1992).

The **laminae V-VI** contain both interneurons and projection neurons. In lamina V, also called the neck of the DH, these neurons can be multimodal, called Wide Dynamic Range (WDR), receiving, and transmitting both nociceptive and non-nociceptive information. These WDR neurons have dendritic trees that can extend rostro-caudally and medio-laterally to lamina III and lamina VII. The lamina VI is mostly developed in the cervical and lumbar segments of the spinal cord.

2. White matter

Contrary to organization in the brain, white matter surrounds the grey matter in the spinal cord. It is composed of different columns: dorsal, lateral and ventral columns, composed themselves of ascending axons, bringing information towards supra-spinal centers from the periphery and descending axons coming from supra-spinal centers to modulate the activity of spinal cells, or initiate a muscle movement for example. I will briefly describe the main columns and their function in the paragraph on projection neurons and descending controls. For full review, see Millan, 1999.

The DH is composed of the first 6 laminae in the grey matter on the dorsal side of the spinal cord. White matter surrounds grey matter in the spinal cord.

II. Synaptic inputs to the dorsal horn of the spinal cord

Information about the surrounding environment, as well as that originating from our internal organs, also called sensory information, originates in the peripheral endings of primary somatosensory neurons, where they are transduced into electrical signals and conveyed to secondary neurons in the spinal cord or the brainstem. Information coming from the face and head are processed in the trigeminal network in the brainstem, homologous to the DH. As my work focused on the DH, I will focus on the DH network in the next paragraphs.

Sensory neurons are pseudo-unipolar with their somas in dorsal root ganglia, one axonal branch in the periphery and the other axonal branch in the spinal cord, where they are called primary afferents. All primary afferents are glutamatergic. The most common classification for primary afferents is based on the degree of myelination and resulting conduction velocities. Using this classification, different types of primary afferents have been distinguished: Aα and Aβ for the fast conduction (diameter >10 µm and velocity of 35-100 m/s), Aδ for the intermediate conduction fibers (2-6 µm and 5-35 m/s) and C for the slow conduction fibers (0.4-1.2 µm and <2 m/s) (Millan, 1999). The proportion of these fibers innervating the skin is 20% for Aβ fibers, 10% for Aδ fibers and 70% for C fibers. Aα fibers are proprioceptors that transmit information about the position of the body in space and will not be mentioned again in this manuscript.

Primary sensory neurons that detect nociceptive information are called nociceptors and can be activated by mechanical, thermal, or chemical stimuli. Some nociceptors are activated by only one modality and those are generally Aδ fibers. Nociceptors that can be activated by multiple modalities are called polymodal and comprise most of C-fibers and some Aδ fibers (for full review, see Millan, 1999). However, a limit to the categorization of nociceptive/non-nociceptive fibers is made clear when studying repeated stimulations of fibers. Indeed, responses by primary afferents are very plastic. For example, after repeated thermal stimulations, mechanical nociceptors become sensitive to nociceptive heat stimuli, and can respond to non-noxious thermal stimuli. Also, it should be noted that when noxious stimuli occur, they don't activate only nociceptive fibers, but also non-nociceptive low-threshold fibers (Cordero-Erausquin et al., 2016).

1. Aβ fibers

Most of Aβ fibers are activated by low threshold, non-nociceptive mechanical stimuli, even if some nociceptive Aβ fibers have been described. Aβ fibers are axons expressing a variety of tactile mechanoreceptors present in the skin (Djouhri & Lawson, 2004). In skin, Aβ represent about 20% of fibers (Millan, 1999).

2. Aδ fibers

Aδ fibers have free terminals in the skin. Two types of Aδ fibers have been described (Treede et al., 1998). Type 1 Aδ fibers are mechanical and thermal nociceptors that innervate hairy and hairless skin, with a moderate mechanical threshold and a high thermal threshold (>53°C). These fibers are implicated in hyperalgesia appearing after a long and high intensity thermal stimulus on hairless skin. Type 2 Aδ fibers have a high mechanical threshold and a low thermal threshold (>46°C) and participate in the sensation of pain after a thermal stimulation. Aδ fibers that innervate hairy skin are an isolated group. They are activated by mechanical stimuli and nociceptive cold stimuli (Cain et al., 2001). In the skin, Aδ fibers represent 10% of fibers (Millan, 1999).

3. C fibers

Like Aδ fibers, C fibers have free terminals in the periphery. Most C fibers are activated by nociceptive information, either thermal or mechanical, although some have been shown to participate in the transmission of non-nociceptive thermal information (hot or cold) and mechanical information. Some studies have also shown them to be implicated in the itch sensation, with a subpopulation of C fibers being characterized as pruriceptors (Cevikbas & Lerner, 2020). Interestingly some C fibers are also considered silent C fibers, as they are mechanically insensitive and heat sensitive, but transition to mechanically responding in the case of injury or insult to the organism. These C fibers represent about 25% of all C fibers. Another criterion for classification is on the presence or absence of peptides such as substance P and calcitonin-gene related peptide (CGRP) that can be released from dense vesicles at both terminals of the pseudo-unipolar neuron.

These fibers are called peptidergic fibers and those without peptides are called non-peptidergic fibers. In the skin, C fibers represent 70% of fibers (Millan, 1999).

After a nociceptive stimulus, Aδ fibers will rapidly transmit information, participating in the fast "sharp" pain response whereas C-fibers transmit the secondary slower "duller" painful response.

This is a simplified overview of the afferent fibers. In his extensive review on the subject, Millan, 1999, points out that multiple categories of Aδ and C fibers exist, but their classification has been historically complicated by differences in species, inconsistencies of nomenclature, variation of responsive fibers depending on the tissue (such as hairy and non-hairy skin) and the differences in methods used for their detection.

4. Projection sites within the dorsal horn

In each category of fibers, some are nociceptive, and some are non-nociceptive. However, a pattern emerges when studying in which area of the DH nociceptive and non-nociceptive fibers project (figure 2B).
Non-nociceptive information is directly sent up to supra-spinal structures through the dorsal cords pathway to form synapses in the dorsal columns nuclei, cuneate and gracile nuclei. However, some collaterals of Aβ fibers form synapses in the laminae III-VI and more rarely, in the external lamina II. Non-nociceptive Aδ fibers project in the laminae II-III and non-nociceptive C fibers terminate in lamina IIi.
Nociceptive afferents project more superficially in the DH. Indeed, Aβ fibers terminate in laminae III and IV, Aδ fibers in laminae I, IIo and V, and C fibers in laminae I-II, and more specifically, peptidergic c-fibers project in laminae I and IIo whereas non-peptidergic C fibers terminate in lamina IIi.

When looking at the types of cells targeted by the different afferent fibers, especially in lamina I and II, some connection motifs exist depending on the nature of the postsynaptic neuron. For

example, in the lamina I, a large proportion of projection neurons are directly contacted by C fibers (Ikeda et al., 2003; Lu & Perl, 2003). Most NK1-R expressing neurons receive direct contact from C fibers, about 30% receive contacts from Aδ fibers and about 15% receive contacts from both C fibers and Aδ fibers.

About 2 thirds of vertical and radial neurons receive direct monosynaptic contacts from Aδ fibers. C fibers contact the 4 main morphological types of cells, and islet cells exclusively get contacted by C fibers (Sandkühler et al., 1997; Yasaka et al., 2007; Lu & Perl, 2003). It should be noted that very little is known about connectivity between the primary afferent fibers and the cellular populations in the DH.

In conclusion, the DH is a structure specialized in receiving sensorial information coming from the periphery. Aβ fibers transmit mostly non-nociceptive information, and only a few collaterals transit in the DH, in deeper laminae. Aδ and C fibers transmit mostly nociceptive and thermal information and form synapses in the superficial DH.

III. Cellular populations in the dorsal horn of the spinal cord

In the DH, 90% of neurons are interneurons. In lamina II, interneurons represent 100% of the neuronal population. Interneurons of the DH can be divided into 2 functional groups: excitatory and inhibitory interneurons. Many classifications have been established, using neurochemistry, morphology, firing profile and marker expression. However, none of the populations using these criteria overlap with another. I will present here some of the criteria used.

1. Morphology

One of the first criterion used to distinguish different populations of cells in the DHSC was morphology. The most widely used morphological classification of interneurons in lamina II was developed by Grudt and Perl (Grudt & Perl, 2002) (figure 3A). They identified 4 major types of

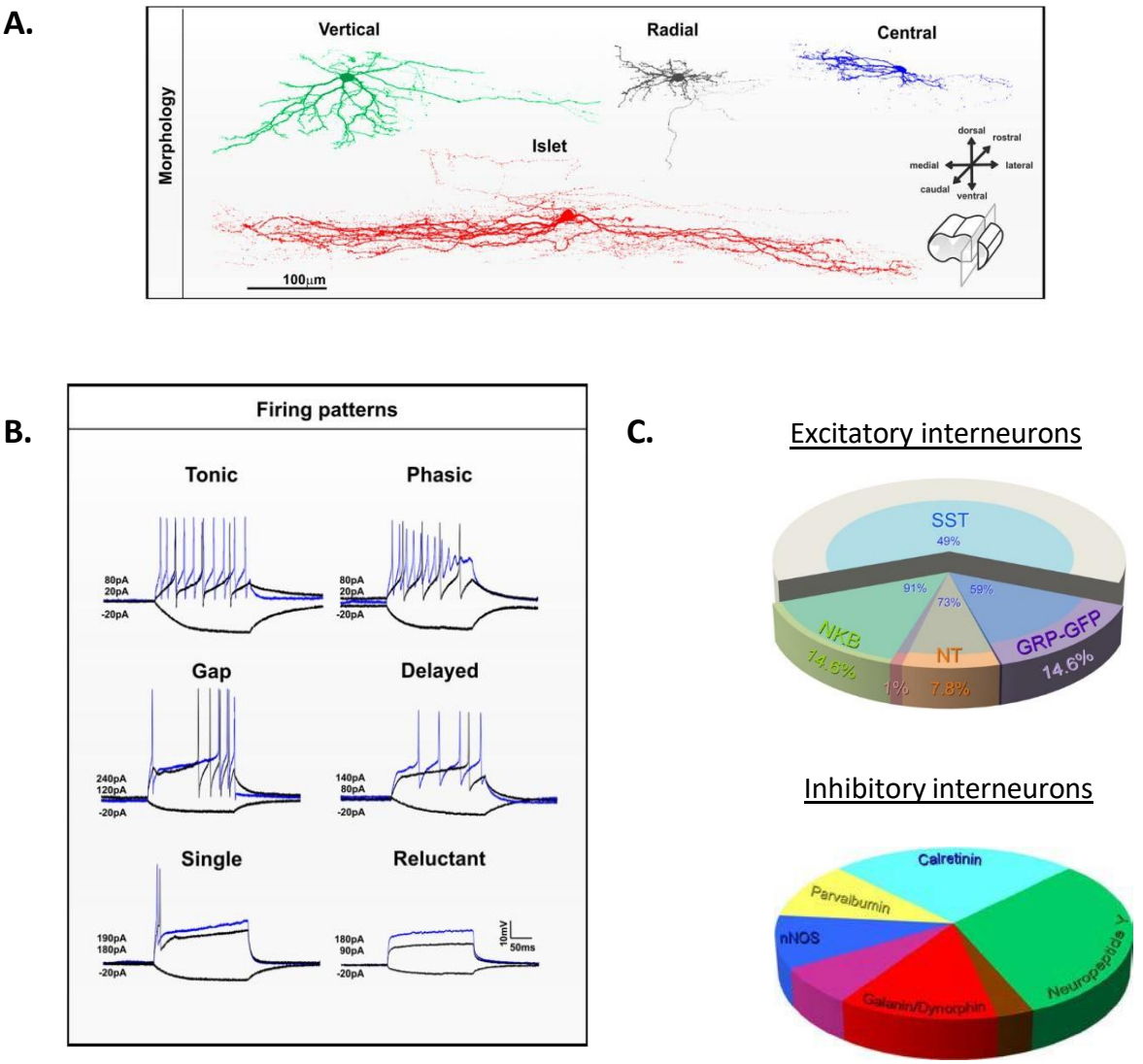


Figure 3: Morphology, firing patterns and neurochemistry of interneurons in the DH

A. Based on morphology, 4 categories of interneurons have been identified in the DH: vertical, radial, central and islet. From Peirs et al., 2020.
B. Based on action potential firing pattern, evoked by injecting a depolarizing current, 6 categories of interneurons have been identified: Tonic, Phasic, Gap, Delayed, Single and Reluctant firing. Peirs et al., 2020
C. On top: Identified populations of inhibitory interneurons based on the expression of single markers. The purple area represents neurons co-expressing Galanin/Dynorphin and neuronal nitric oxide synthase (nNos) and the brown area represents neurons co-expressing Galanin/Dynorphin and Neuropeptide Y. From Boyle et al., 2017. On the bottom: Identified populations of excitatory interneurons based on the expression of single markers. Somatostatin (SOM) is expressed by most neurons. In this representation, we can see for example that SOM is expressed in 59% of « gastrin-releasing peptide » (GRP). The pink area represents the neurons co-expressing Neurokinine B (NKB) and Neurotensine (NT). From Gutierrez-Mecinas et al., 2016.

neurons: **Islet cells**, with a highly elongated dendritic arbor along the rostrocaudal axis; **Radial cells**, with shorter dendrites that are along both the rostrocaudal and dorsoventral axis; **Vertical cells**, generally located in the lamina IIo, with dendrites that go ventrally from the soma in a cone-shape and often extend towards the lamina I, with some studies showing that they could innervate lamina I projection neurons; **Central cells**, that resemble islet cells, but with a smaller dendritic arbor. Based on morphology, vertical cells are the only neurons with axons leaving the lamina II (with somas in lamina II), they could be thus considered as the output of the lamina II interneuron network. Interestingly, it's been shown that cells in these 4 subgroups differ in their monosynaptic primary afferent inputs, the primary afferent-evoked inhibitory inputs as well as their connections in the network. An important limit to this assignation method is that 25% of the original sample used by Grudt and Perl could not be classified according to this scheme (Todd, 2017; Grudt & Perl, 2002).

2. Firing profile

In the lamina II, different action potential firing profiles were identified, induced by an injection of a depolarizing current: **tonic**, **phasic**, **delayed**, **single spike**, **gap-firing** and **no action potential** firing (figure 3B). Correlating to the morphology study, studies found that Islet cells and some of the vertical cells fired tonically. The rest of the vertical cells and the radial cells fired with a delay before the first action potential. The central cells were divided into 3 subgroups: the transient firing, with and without an A-type potassium current and the tonic firing. There has been no study on a possible correlation between firing pattern and function in the lamina II, whereas in lamina I, some have been described: tonic and delayed firing cells act more as integrators whereas phasic and single spike firing cells act more as coincidence detectors (Prescott & de Koninck, 2002).

3. Neurochemistry

In the DH, excitatory interneurons are glutamatergic and inhibitory interneurons are GABAergic and/or glycinergic. Studies using immunohistochemistry have shown that about 30-40% of interneurons contain GABA in the DH, and the rest are glutamatergic (Polgár et al., 2013). When looking more specifically at the different laminae, GABAergic interneurons represent around 30%

for the lamina I and II, and around 40% for lamina III. The highest glycine concentration is found in lamina III, and studies have shown that most glycine is largely restricted to GABAergic neurons, suggesting that glycine is always co-released with GABA. Electrophysiological studies did show the existence of pure glycinergic synapses in LI-II, from axons coming from deeper laminae, but the authors suggested that this was mostly due to the absence of $GABA_A$ receptors at these synapses (Todd, 2010).

Identification of excitatory and inhibitory neurons.

To visualize glutamatergic neurons, and more specifically glutamatergic axons, the vesicular glutamate transporter VGLUT can be labelled. 3 isoforms exist: VGLUT1-2-3. VGLUT1 is the most expressed in the laminae III and IV, VGLUT2 in the lamina I and II, whereas VGLUT3 is transiently expressed in one subpopulation of neurons in lamina IIi during development. However, these transporters are expressed at synaptic terminals and cannot be used as somatic markers (Cordero-Erausquin et al., 2016). In addition to this, primary afferent fiber terminals could not be differentiated to local glutamatergic interneurons. Transcription factors can also be used to distinguish neuronal populations: for example, neurons expressing T-cell leukemia homeobox protein 3 (TLX3) and LIM homeobox factor 1 beta (LMX1B) are always expressed in excitatory neurons in adults. Using these markers, it could be assumed that the unmarked neurons are inhibitory interneurons.

Another way, and the one used in our project, was to mark cells that express the enzyme that synthesizes GABA: glutamate decarboxylase 65 (GAD 65, the one we used) or 67 (GAD67). GABAergic neurons were stained, and unstained neurons were assumed to be excitatory glutamatergic neurons. The transcription factor *Pax2* is also specific to inhibitory neurons. To visualize glycinergic neurons, the glycine transporter can be used, GlyT2. To visualize both GABAergic and glycinergic neurons, the vesicular inhibitory amino acid transporter (VIAAT) can be used (Todd, 2017).

Using immunohistochemistry, it's been shown that GABAergic and glycinergic neurons are found across the grey matter of the spinal cord (Barber et al., 1982; Spike et al., 1997). GABAergic neurons are particularly accumulated within the laminae I-III, while glycinergic neurons are

relatively absent from those laminae and in more important numbers in deeper laminae (Todd & McKenzie, 1989; Todd, 2017).

Markers.

Markers, such as neuropeptides and calcium binding proteins, have also been used to try and regroup more sub-categories within excitatory and inhibitory interneurons.

In excitatory interneurons of the laminae I and II, exclusive markers include somatostatin (SST), neurokinin B (NKB), neurotensin (NT) and gastrin-releasing peptide (GRP), which are co-expressed with SST in varying degrees (figure 3C, top).

In the lamina I and II of rats, 5 groups within inhibitory interneurons have been identified: neurons expressing the neuropeptide Y (NPY) (Polgar et al., 2011), galanine (Simmons et al., 1995), parvalbumine (Boyle et al., 2017), the neuronal form of nitric noxide synthase (nNOS) (Sardella et al., 2011) and calretinine (Boyle et al., 2017) (figure 3C, bottom).

Using single-cell RNA sequencing, Russ et al. (2021) analyzed multiple marker expression in spinal neurons and proposed an atlas regrouping 69 populations of spinal cord neurons, providing laminae location, neurochemistry, combination of expressed markers and putative lineage. As they discuss in their paper however, this approach alone does not inform on cellular function, and will need to be associated with electrophysiological and behavioral data to obtain information on the different functions of these populations and a better understanding of circuit connectivity within the DH (Russ et al., 2021).

4. Connections within the lamina II

Neurons in the lamina II are densely interconnected. Studies have found that 76% of excitatory neurons in lamina II contact other lamina II neurons, higher than in the other laminae (14 and 25% for laminae I and III respectively) (Santos et al., 2009). Studies using paired recordings of lamina

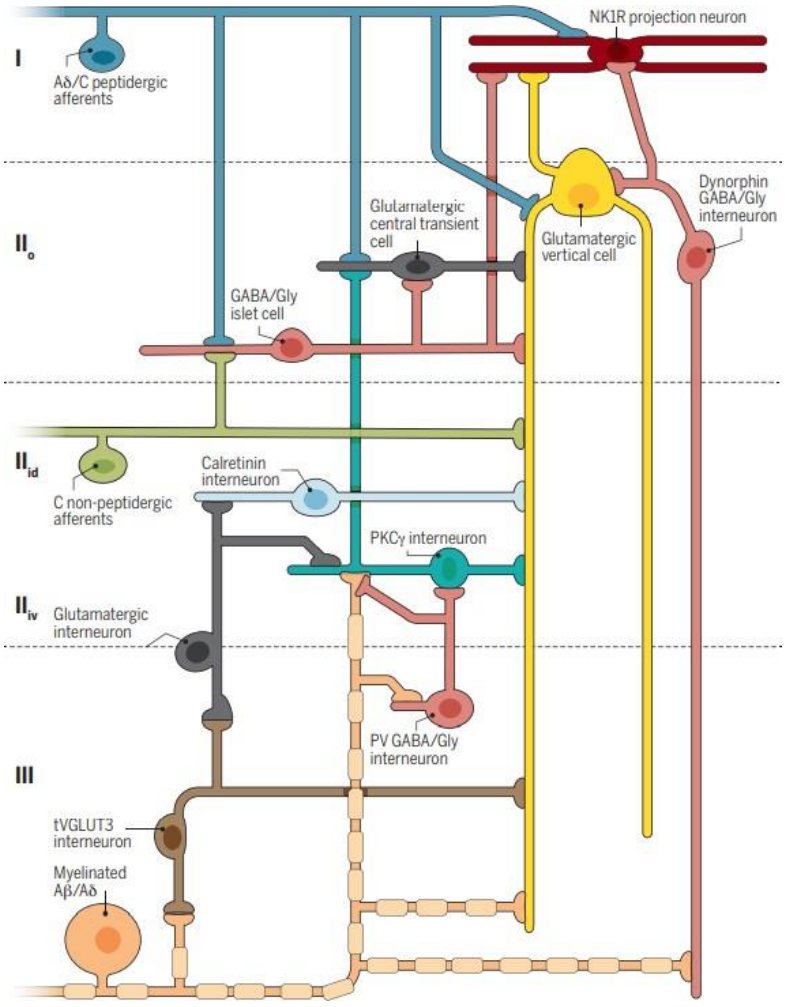


Figure 4: Organization of the neuronal network in the DH

The authors represent what we know about the neuronal connections in the superficial laminae of the DH. Lamina IIe corresponds to IIo on this graph. They divided the lamina IIi into 2 areas: IIi dorsal (LIIid) and IIi ventral (LIIiv). Nociceptive primary afferent fibers (Aδ and C fibers) are represented in blue. They project onto excitatory interneurons in lamina IIo, specifically central cells (in dark grey) and vertical cells (in yellow). They also project onto projection neurons, that express the neurokinin 1 receptor (NK1R, in red). In green are represented non-peptidergic C fibers that project to vertical cells in lamina IIid and inhibitory islet cells (in pink). Non-nociceptive Aβ and myelinated Aδ fibers (in orange) project onto excitatory interneurons expressing tVGLUT3 in LIII (in brown), interneurons expressing PKCγ in lamina IIiv (in teal blue), and vertical cells. They also project onto inhibitory radial interneurons expressing parvalbumin (PV, in pink) and inhibitory vertical cells expressing dynorphin (in pink). From Peirs and Seal., 2016.

As shown in this illustration, inhibitory interneurons block Aβ and myelinated Aδ transmission by inhibiting PKCγ interneurons, vertical cells and projection neurons, allowing for modality separation in information processing in the superficial laminae of the DH.

II interneurons and primary afferent stimulations have allowed to identify microcircuits in the DH. However, given the low connection probability between a pair of recorded neurons (10%) combined with the difficulty of the spinal slice preparation, these studies are rare and much remains to be understood about interneuron connectivity in the DH (Cordero-Erausquin et al., 2016).

Using a transgenic line of mice in which some GABAergic neurons are labeled with a fluorescent protein and simultaneous double cell recording, Zheng, Lu and Perl showed that 2 subgroups of inhibitory interneurons, probably islet cells and tonic firing central cells, share reciprocal connections (Zheng et al., 2010). Lu and Perl showed a connection between an inhibitory islet cell and a central excitatory neuron (Y. Lu & Perl, 2003) and later showed that excitatory central neurons synaptically contact vertical neurons located in lamina IIo, which themselves contact projection neurons in lamina I (Y. Lu & Perl, 2005). Glutamatergic interneurons also receive inhibitory synaptic inputs after C-fiber stimulation (Punnakkal et al., 2014) (figure 4).

Between laminae II and III, excitatory neurons expressing PKCγ/SST receive inputs from inhibitory neurons expressing PV and glycine. These PKCγ/SST^{+} neurons themselves contact vertical cells (Y. Lu et al., 2013). Interestingly, when these inhibitory inputs are blocked, non-nociceptive information transmitted by Aβ fibers is transmitted towards the superficial laminae of the DHSC, which is normally specialized in processing nociceptive information (Miracourt et al., 2007; Peirs & Seal, 2016; Petitjean et al., 2015) (figure 4). For further details on the established connections inside the lamina II network, see Cordero-Erausquin et al (2016) and Peirs and Seal (2016).

5. Plasticity within the dorsal horn of the spinal cord

Neuronal networks within the DH are highly plastic. Synaptic and morphological plasticities usually occur after repeated stimulations of primary afferents, like in the case of inflammation. After such intense activity, excitatory transmission is usually reinforced and inhibitory transmission is weakened, leading to states such as allodynia (painful interpretation of innocuous stimuli), hyperalgesia (amplification of the painful sensation resulting from nociceptive stimuli) and spontaneous pain. This state is called central sensitization, a state where activity within the

DH network is increased and changed (Latremoliere & Woolf, 2009). In the next chapter of the introduction, I will describe more in detail the modulation and plasticity of synaptic inhibitory transmission, and in chapter III, NMDA receptors (NMDAr), both involved in the development of central sensitization in the DH of the spinal cord. For a full review on central sensitization, see Latremoliere and Woolf. 2009.

When looking at morphology, firing patters, neurochemistry or marker expression, it becomes clear that the interneuron populations within the DH are varied. Using all these classifications, the emerging categories don't overlap with one another. New studies in the field are bringing new insights into how we could identify specific functional populations of interneurons in the DH. Using single-cell RNA-sequencing, Russ et al. proposed an atlas of neuronal populations in the spinal cord based on the expression of multiple markers (Russ et al., 2021). However more remains to be understood about the connections between the different populations of interneurons in the DH and their functions in nociceptive information processing.

IV. Output of the dorsal horn of the spinal cord

Projection neurons are the main output of DH networks and represent about 10% of DH neurons, present in the laminae I, III and V. Their synaptic targets are located in the thalamus, hypothalamus, periaqueductal gray, lateral parabrachial nucleus and various medullary nuclei (Millan, 1999).

Axons of projection neurons are located in white matter, as they join ascending tracts (for detailed review on ascending tracts, see Millan. 1999). Interestingly, most projection neurons seem to simultaneously project to different supra-spinal centers. Targeted structures are associated with different modalities and components in the processing of nociceptive information. These structures associate the individual's mood, attention, memory, emotions to the painful experience, allowing

to learn and remember the context that resulted in the lesion. I will briefly detail some of the main ascending tracts. This is not an exhaustive list.

The **dorsal column** pathway transmits tactile information from the skin and unconscious proprioceptive information from the muscles and the joints and is mostly formed by Aβ fibers. In the upper part of the body, the information transits via the cuneiform tract whereas lower body information transits via the gracile tract. This information will be directed towards the brainstem, the thalamus, and the primary sensorial cortices.

The **spino-cerebellar tract** transmits unconscious proprioceptive information from the muscles and the joints to the cerebellum, to allow for better postural control and fine tuning of movement. This tract is formed by axons of projection neurons located in lamina V of the DH (Peirs et al., 2020).

The **spino-thalamic** and **spino-reticulate tracts** transmit nociceptive and thermic information, from the spinal cord towards the thalamus and reticular formation respectively. Both these tracts play an important role in the sensory discrimination property of the nociceptive stimuli (Basbaum et al., 2009; Cordero-Erausquin et al., 2016). **The spino-thalamic** tract is formed by the axons of projection neurons located in the laminae I, IV, V, VI and VII. **The spino-reticulate tract** is formed by axons from projection neurons located in laminae I and V. These projections play an important role in sensory-discrimination of nociceptive stimuli, but are less precise than the spino-thalamic tract (Basbaum et al., 2009). Both these tracts ascend via the anterolateral tract.

The **spino-mesencephalic tract** transmits nociceptive information from the spinal cord to the mesencephalon, via the ventrolateral tract. This tract is composed only of axons from projection neurons in lamina I. The tract also projects in the brainstem: in the periaqueductal gray and parabrachial nucleus, from where the information will be transmitted to the amygdala and the hypothalamus (Millan., 1999; Basbaum et al., 2009). These structures are associated with the aversive, emotional aspects of pain, whereas the structures in the brainstem are responsible for activating descending controls.

The targeted structures are associated with different functions in the elaboration of pain perception: the primary and secondary somatosensory cortices, the ventral-posterior-lateral nucleus of the thalamus and the cerebellum process the sensory-discrimination component, which allows to detect the modality of the lesion, the localization, the intensity, and the length (Millan., 1999; Peirs & Seal, 2016). More emotional and cognitive components are integrated within the medial thalamic nuclei, the prefrontal cortex, the anterior cingulate cortex, the insula, the basal ganglia, the amygdala, the periaqueductal gray and the parabrachial nuclei (Millan., 1999; Peirs and Seal., 2016). All these structures form the pain matrix, associating a multi-dimensional experience to pain.

V. Descending controls

Cells in the DH also receive information from supra-spinal centers. Descending controls can be inhibitory or excitatory, with varied neurotransmitters, and it is generally admitted that, from a functional aspect, inhibitory controls are involved in analgesia whereas facilitatory controls are involved in secondary hyperalgesia development (Gebhart, 2004). These descending controls transit through the dorso-lateral and ventro-lateral tracts, to connect synaptically with neurons and glial cells in the DH, including the primary afferent terminals and local interneurons (Tsuda et al., 2017; Millan, 2002). Structures such as the prefrontal cortex, the hypothalamus, the amygdala, the locus coeruleus, the parabrachial nuclei, the raphe magnus nucleus and the periaqueductal gray (Bushnell et al., 2013; Millan., 1999, 2002) are involved in the descending controls. The periaqueductal gray controls the activity of the rostroventral medulla. Interestingly, descending inhibitory and excitatory neurons are located in the same structures, but the tract they take to descend is not the same (Gebhart, 2004). Descending controls can be recruited by ascending activity from the DH or from cognitive and attentional processes, arising from the surrounding context. For example, while being chased by a predator, pain would not be useful information, quite the contrary, and activity within the DH will be reduced by the descending inhibitory controls.

1. Inhibitory descending controls

Generally, serotoninergic descending controls from the reticular ventromedial formation and the raphe magnus nucleus are inhibitory. Noradrenergic descending controls from the locus coeruleus inhibit neurons from the laminae I, II, V and X.
Projection neurons in the rostroventral medulla, called "OFF neurons" can be GABAergic, glycinergic or serotoninergic, and inhibit nociceptive information transmission.

2. Facilitatory descending controls

The PAG is involved in facilitating nociceptive information transmission, via neuropeptides such as cholecystokinin and substance P.
Projection neurons in the rostroventral medulla, called "ON neurons" also facilitate nociceptive information.
For full review on descending controls, see Millan., 1999, 2002.

Supra-spinal centers implicated in integration of nociceptive information can control the activity in the DHSC, either facilitating or inhibiting nociceptive processing, depending on the context.

VI. Glial cells within the dorsal horn of the spinal cord

Glial cells represent more than half of cells in the CNS. Their role as support cells for neurons has long been established (Verkhratsky & Nedergaard, 2018). Studies in the recent decades have proposed a more complex functioning of glial cells within neuronal networks. In the CNS, glial cells are usually divided into astrocytes and oligodendrocytes that derive from the neuroepithelial, and microglia, which are resident macrophages that derive from progenitors in the embryonic yolk sac (Ginhoux et al., 2013). Astrocytes and microglia are in the grey matter of the spinal cord.

Oligodendrocytes are present in the white matter, as well as a specific subgroup of astrocytes called fibrous astrocytes (Verkhratsky & Nedergaard, 2018). During my doctoral work, I specifically studied astrocytes, so I will focus more on them in this section.

1. Microglia

In physiological conditions, microglia are usually present in a quiescent form. However, in some neuro-inflammation conditions, such as following a nerve injury, microglia are activated. This activated state is associated with a change in morphology and secretion patters (Tsuda et al., 2005; Ji et al., 2013). After a peripheral nerve injury, microglia proliferate in the DH (Inoue & Tsuda, 2018), a phenomenon called microgliosis. Studies have shown that interrupting DH microgliosis inhibits the development of pain hypersensitivity following peripheral nerve injury (Inoue & Tsuda., 2018). Mechanisms implicated in microgliosis and more broadly on the role of microglia in nociceptive information processing in the DH in physiological and pathological conditions have yet to be fully understood. For a full review on the implications of microglia in neuropathic pain, see Inoue & Tsuda., 2018.

2. Astrocytes

Astrocytes are a diversified class of non-neuronal cells. Astrocytes have many functions in the central nervous system, such as controlling molecular homeostasis, by transporting ions and protons between the extracellular and intracellular spaces, removing neurotransmitters, releasing gliotransmitters and metabolic precursors, such as glutamine. They supply neurons with energy, by releasing energy substrates. They can also, through their vascular end-feet, control blood-flow to the central nervous system. They are categorized, with microglia, as the main defensive system in the central nervous system (Verkhratsky & Nedergaard, 2018). Astrocytes surround synapses, forming what is known as the tripartite synapse (Araque et al., 1999): it is estimated that one astrocyte can be in contact with 100,000 synapses in the rodent brain, and 1,000,000 in the human brain. In addition to supporting neurotransmission, more recent studies have shown that they are implicated in modulation of synaptic transmission by releasing transmitters that can act on receptors expressed by the presynaptic or postsynaptic element, or other astrocytes (Kang et al.,

1998; Lalo et al., 2006; Araque et al., 1999; Panatier et al., 2006; Panatier & Robitaille, 2016; Dallérac et al., 2013; Diamond, 2006; Verkhratsky & Nedergaard, 2018).

Visualization of astrocytes

To visualize astrocytes, markers can be used. However, there exists no universal marker, illustrating the much-diversified group of cells that exist under this nomenclature. The most common marker used is glial fibrillary acidic protein (GFAP), that can be found in many cells outside the central nervous system but seems to be specific to subgroups of astrocytes. GFAP is part of the extended family of intermediate filaments and form the astrocytic cytoskeleton. Interestingly, when staining for GFAP in cultured astrocytes, almost all cells are positive for GFAP, but *in situ* or *in vivo* staining, the positive population is substantially smaller. The proportion of GFAP immunoreactive cells also varies between structures: for example, Bergmann glia in the cerebellum are all GFAP positive. It should also be noted that GFAP immunoreactivity increases in reactive astrocytes (Verkhratsky & Nedergaard, 2018).

Other markers can be used, such as: **glutamine synthetase** (GS), a specific astroglial metabolic enzyme that converts ammonia and glutamate into glutamine, the marking of which produces a cytosolic labeling in almost all astrocytes; certain transporters, such as the **excitatory amino acid transporter**s (EAAT) and more specifically the isoforms EAAT1 and EAAT2, are expressed by astrocytes; **ALDH1L1**, a key enzyme in folate metabolism, whose expression changes with age, and can also be found in a subgroup of oligodendrocytes; **S100B protein**, a calcium binding protein, that stains more cells than GFAP. For more information on markers used and their limits, see Verkhratsky & Nedergaard, 2018.

Using sulforhodamine 101 (SR 101), a fluorescent dye that will be preferentially taken up by live astrocytes, can also allow for astrocyte visualization until the dye is washed out.

Other parameters can be observed to study astrocytes. Interestingly, despite a prominent morphological and functional diversity, astrocytes show very similar electrophysiological parameters, such as more hyperpolarized membrane potential than neurons (around -80mV) and low input resistance, due to an important resting membrane permeability to K^+. Astrocytes also exhibit very passive electrophysiological properties: depolarizing astrocytes will not lead to action potential firing (Verkhratsky & Nedergaard, 2018).

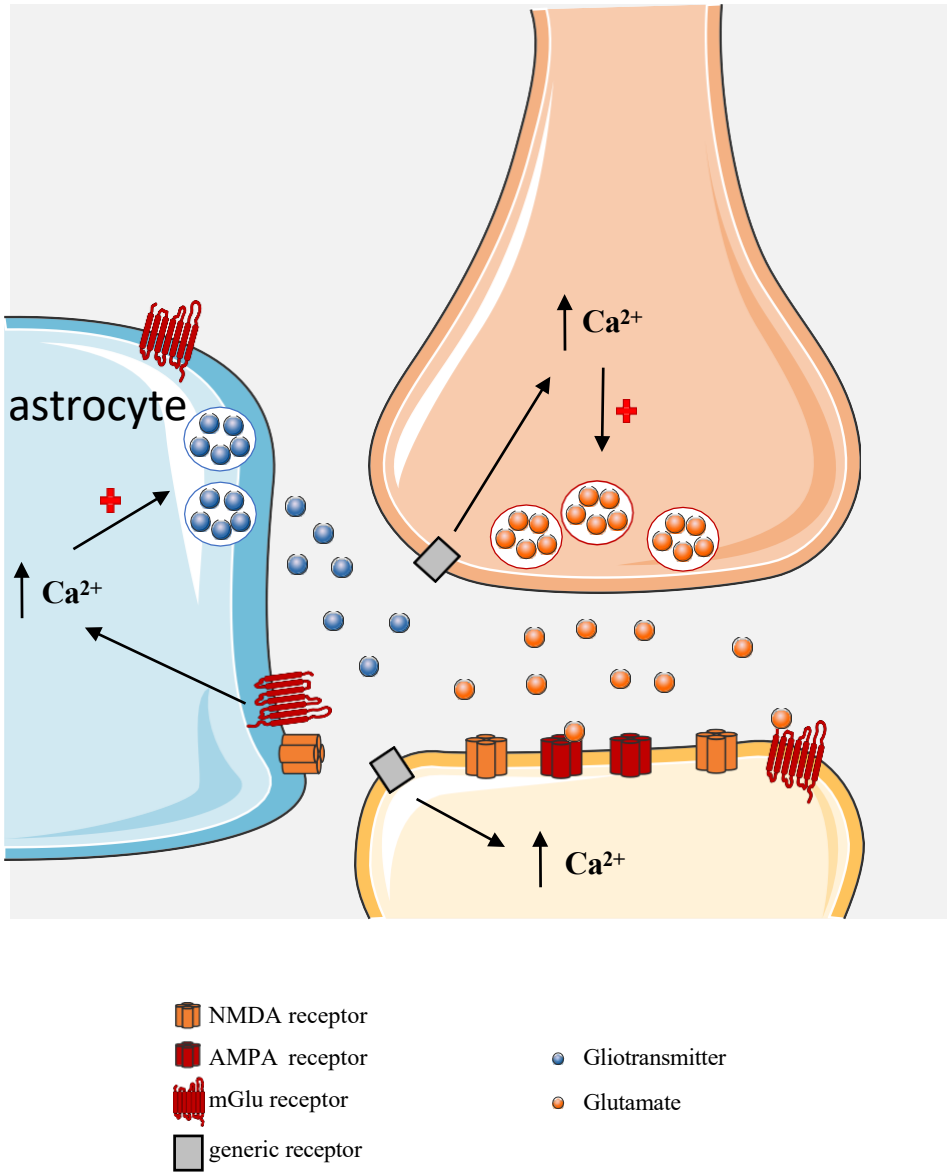


Figure 5: Astrocyte dependent-modulation of synaptic transmission

Studies have shown that astrocytic processes surrounding synapses can express receptors that can be activated by neurotransmitters, inducing an influx or a release of intracellular stores of calcium. This increase in the $[Ca^{2+}]_i$ can in turn induce the release of transmitters by the astrocytes. Astrocytes have been shown to release glutamate and ATP for example. These gliotransmitters can act on receptors expressed on the presynaptic or postsynaptic éléments, or other astrocytes, and modulate synaptic transmission. Simplified from Bazargani and Attwell., 2016.

Calcium signaling and Gliotransmission

Studies in primary astrocyte cultures found that when chemically or mechanically stimulating astrocytes, it almost systematically induced changes in intracellular free calcium concentrations ($[Ca^{2+}]_i$), introducing the concept of calcium excitability in astrocytes. Another, more debated concept was brought forward: in response to an increase in $[Ca^{2+}]i$, astrocytes can release gliotransmitters, such as glutamate, GABA and ATP, modulating synaptic and extrasynaptic transmission. The release of these gliotransmitters is called gliotransmission and remains debated in the literature (Hamilton & Attwell, 2010; Fiacco & McCarthy, 2018). In their 2016 review, Bazargani & Attwell discuss the controversies around gliotransmission and their partial resolution, mostly brought on by technological and methodological advances in the study of astrocytes and their microdomains (Bazargani & Attwell, 2016) (see also Savtchouk & Volterra, 2018) (figure 5).

Interestingly, astrocytes form a syncytium with surrounding astrocytes, connected by connexins (Cx, notably Cx30 and Cx43) through which small molecules can pass and spread to the syncytium. When inducing an increase in $[Ca^{2+}]_i$ in a group of astrocytes, Ca^{2+} can spread to the surrounding, connected astrocytes. This phenomenon is called a calcium wave and could potentially affect vascular smooth muscle control and thus, blood flow to a region, as well as gliotransmission. This syncytium would allow for large scale network modulation, where the activity of one region can be affected by activity in another distant region (Verkhratsky & Nedergaard, 2018).

In the Dorsal Horn of the spinal cord

In the DHSC, astrocytes express neurotransmitter receptors, such as P2Y and P2X (Watkins et al., 2005) and NMDAr in neonatal rats (Verkhratsky & Chvátal, 2020).

Astrocytes in the DH modulate baseline mechanical nociceptive processing. In a study from 2011, Foley et al. used a transgenic mouse in which SNARE-mediated gliotransmission was selectively attenuated. Using Von Frey filaments, the authors show that these transgenic mice exhibited a reduced sensitivity threshold to mechanical stimulation compared to WT mice. Interestingly, after a nerve injury (spared nerve injury model), sensitivity thresholds were comparable between controls and transgenic mice. They concluded that gliotransmission contributes to basal

mechanical nociception but not in pathological conditions (Foley et al., 2011).

A study published in 2020 by Kohro et al. identified a specific population of astrocytes in the superficial DH genetically defined by the expression of *Hes5* (hairy and enhancer of split 5, a transcription factor) that are the target of facilitating noradrenergic descending controls from the locus coeruleus, gating mechanical hypersensitivity (Kohro et al., 2020). To my knowledge, it's the only study that identifies a functional subpopulation of astrocytes in the DH, in agreement with Verkhratsky's proposed model that functional astrocytic subgroups exist, resembling functional neuronal categories (Verkhratsky & Nedergaard, 2018).

A study published in 2021 by Xu et al showed that astrocytes could gate nociceptive signals transmission from NK1R-positive projection neurons. Indeed, stimulation of Aβ fibers activated astrocytes, which in turn induced long-term depression in NK1R-positive projection neurons, a proposed model resembling gate-control theory, but involving a glial actor (Q. Xu et al., 2021).

Astrocytes and microglia have also been studied in conditions of pathological pain. It's been proposed that astrocytes play a more important role in maintaining central sensitization whereas microglia are more important for the development of such conditions (Scholz & Woolf, 2007; Ji et al., 2013). In their 2013 review, Ji et al. present how painful syndromes are associated with **reactive** astrocytes and microglia, including an increase in the expression of some proteins and a change in morphology; **phosphorylation** of mitogen-activated protein kinase pathways; **increase in expression** of ATP and chemokine receptors as well as a downregulation of glutamate transporters; **gliotransmission** and release of glial mediators such as cytokines, which have been shown to impact synaptic transmission. All these observations have led to the concept that chronic pain could result from a gliopathy, which is to say the malfunctioning of glial function within the central nervous system and the periphery. This concept has been discussed in other reviews, such as Kronschläger et al., 2016.

Astrocytes are involved in a variety of functions, such as ionic homeostasis and immune responses. In more recent studies, they have been shown to be also important actors in synaptic transmission modulation: they are able to detect surrounding synaptic activity, inducing changes in their $[Ca^{2+}]_i$ which in turn can trigger the release of gliotransmitters, such as ATP and glutamate, acting on surrounding receptors.

Conclusion of part 1

The lamina II of the DH is a structure involved in integration of nociceptive information from the periphery, transmitted by Aδ and C fibers. It is composed of a complex network of inhibitory and excitatory interneurons that remains to be fully understood. In parallel to the neuronal network exists a plethora of glial cells, of which astrocytes, that maintain a functional and healthy environment for neurons. In addition, more recent studies have shown that astrocytes can directly modulate synaptic transmission. After transmission of nociceptive information to supra-spinal structures involved in its integration, descending controls can modulate activity within the DH.

Part 2: GABAergic synaptic transmission in the dorsal horn

Synaptic inhibitory transmission plays an essential role in nociceptive information processing in the DH. Two types of inhibitory transmission have been described: phasic and tonic inhibition. Phasic inhibition depends on the activation of synaptic $GABA_A$ receptors, whereas tonic inhibition is mediated by the sustained activation of non-synaptic $GABA_A$ receptors by ambient GABA (Joo et al., 2014). Recent studies estimate that about 30-40% of interneurons in the superficial laminae of the DH are inhibitory (Polgár et al., 2013). Pharmacological blockade of synaptic inhibitory transmission induces abnormal pain sensations, such as allodynia, hyperalgesia, and spontaneous pain. Interestingly, these symptoms can be found in pathological pain conditions as well.

Synaptic inhibitory transmission acts via 2 neurotransmitters in the DH: glycine and GABA. GABA is an amino acid neurotransmitter, widespread in the nervous system. Virtually undetectable in non-nervous tissue, GABA accounts for about 1mg per gram of mammalian nervous tissue (experiments of Eugene Roberts and Jorge Awapara, 1950) (Jorgensen, 2005). GABA mediates its effects via the ionotropic $GABA_A$ receptor for fast transmission and via the metabotropic $GABA_B$ receptor for slower transmission. Glycine acts via ionotropic glycine receptors. For my doctoral work, I focused on fast GABAergic synaptic transmission mediated by ionotropic $GABA_A$ receptors. $GABA_A$ receptors are pentameric receptors, permeable to Cl^- and HCO^{3-} ions. In the adult central nervous system, their activation usually induces an entry of Cl^-, hyperpolarizing the membrane potential and thus reducing neuronal excitability.

This second part of the introduction presents GABAergic synaptic transmission in the DH, its mechanisms, modulation, and plasticity with the last section focusing on what we know about the implication of astrocytes in GABAergic synaptic transmission modulation. Classic GABAergic transmission is presented with postsynaptic GABA receptors, but it should be noted that GABA receptors can be expressed at the presynaptic element, astrocytes, and extra-synaptic sites. I will focus mostly on canonical GABAergic inhibitory synaptic transmission.

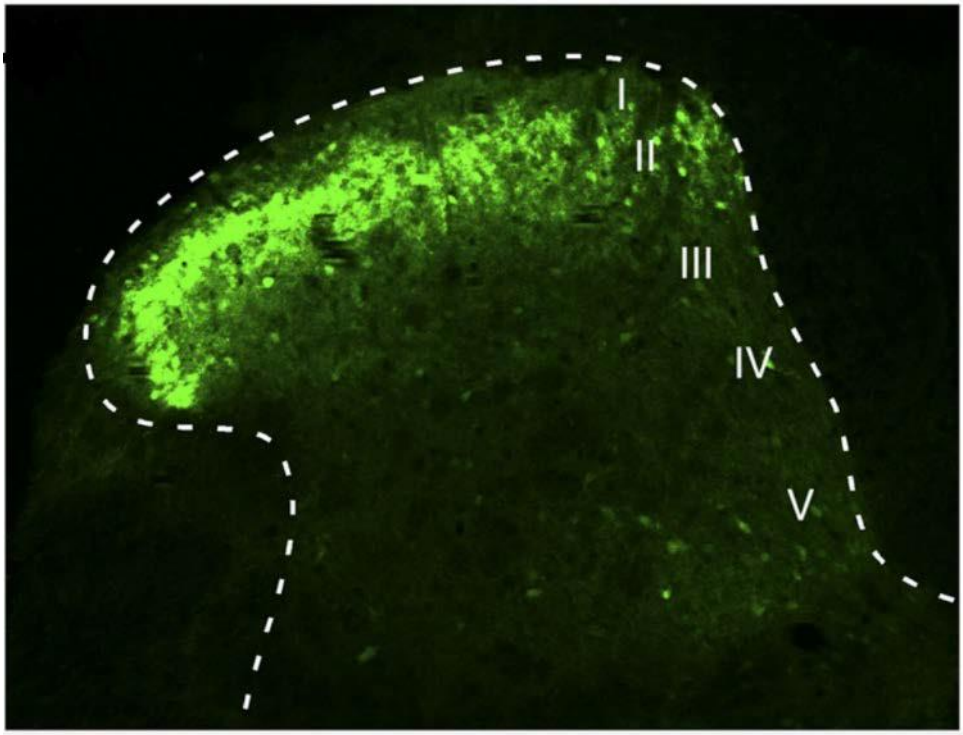


Figure 6: GAD67 distribution in the DH

Distribution of GABAergic neurons, by visualization of eGFP expression under the promoter of GAD67, an enzyme responsible for the synthesis of GABA. From Zeilhoffer et al., 2012

In our project, we used GAD65-eGFP mice, and the fluorescence distribution in the DH is the same as seen here.

I. GABA synthesis, storage, release, and reuptake

GABA is synthesized from L-glutamate by the glutamate decarboxylase (GAD) enzyme, which exists in 2 isoforms, depending on its molecular weight: GAD65 and GAD67. Studies have shown that both isoforms are present in the DH. Mackie and collaborators showed in 2003 that when labelling GAD-immunoreactive synaptic boutons, virtually all were labelled with both isoforms of GAD, with clusters of varying intensities for either GAD65 or GAD67 (Mackie et al., 2003). GAD65, encoded by the *Gad2* gene is more present in the axonal terminals, while GAD67, encoded by *Gad1* gene, is more present in the somato-dentritic compartment of the cell. Once synthesized, GABA is transported and stocked in clear vesicles by vesicular inhibitory amino acid transporter (VIAAT). After fusion of the vesicles and release of GABA in the extracellular space, clearance of GABA is ensured by GABA transporters (GAT), expressed by pre-synaptic cell, post-synaptic cell or astrocytes. GATs belong to the family of neurotransmitter sodium symporters, SLC6, with a stoichiometry of $2Na^+/1Cl^-$ and 1 GABA. Four types of GATs have been described: GAT1 (*slc6a1*), GAT2 (*slc6a13*), GAT3 (*slc6a11*) and BGT (betaine-GABA transporter, *slc6a12*) (Zeilhofer et al., 2012). It has been shown that distribution of GAT across membranes is very dynamic and can be influenced by surrounding activity, allowing for fine-tune regulation of GABA clearance and thus GABAergic transmission (Scimemi, 2014). GAT-3 is thought to be specific to astrocytes, whereas GAT-1 can be found in both astrocytes and neurons (Verkhratsky & Nedergaard, 2018).

When visualizing GAD-eGFP on a spinal slice of mouse, the superficial laminae of the DH appears the most fluorescent compared to the rest au the slice (figure 6).

Recent studies have shown that GABA can also be synthesized in a GAD-independent manner, in astrocytes. Yoon et al studied GABA synthesis in Bergmann glia, a type of cerebellar astrocyte. After quantifying GABA concentrations in the cells, they found that this astrocytic GABA was synthesized by monoamine oxidase B (MOAB) from a polyamine, putrescine. Using gene silencing in Bergmann glia in the cerebellum and astrocytes in the striatum, they show that astrocyte-synthesized GABA is released to mediate tonic inhibition (Yoon et al., 2014).

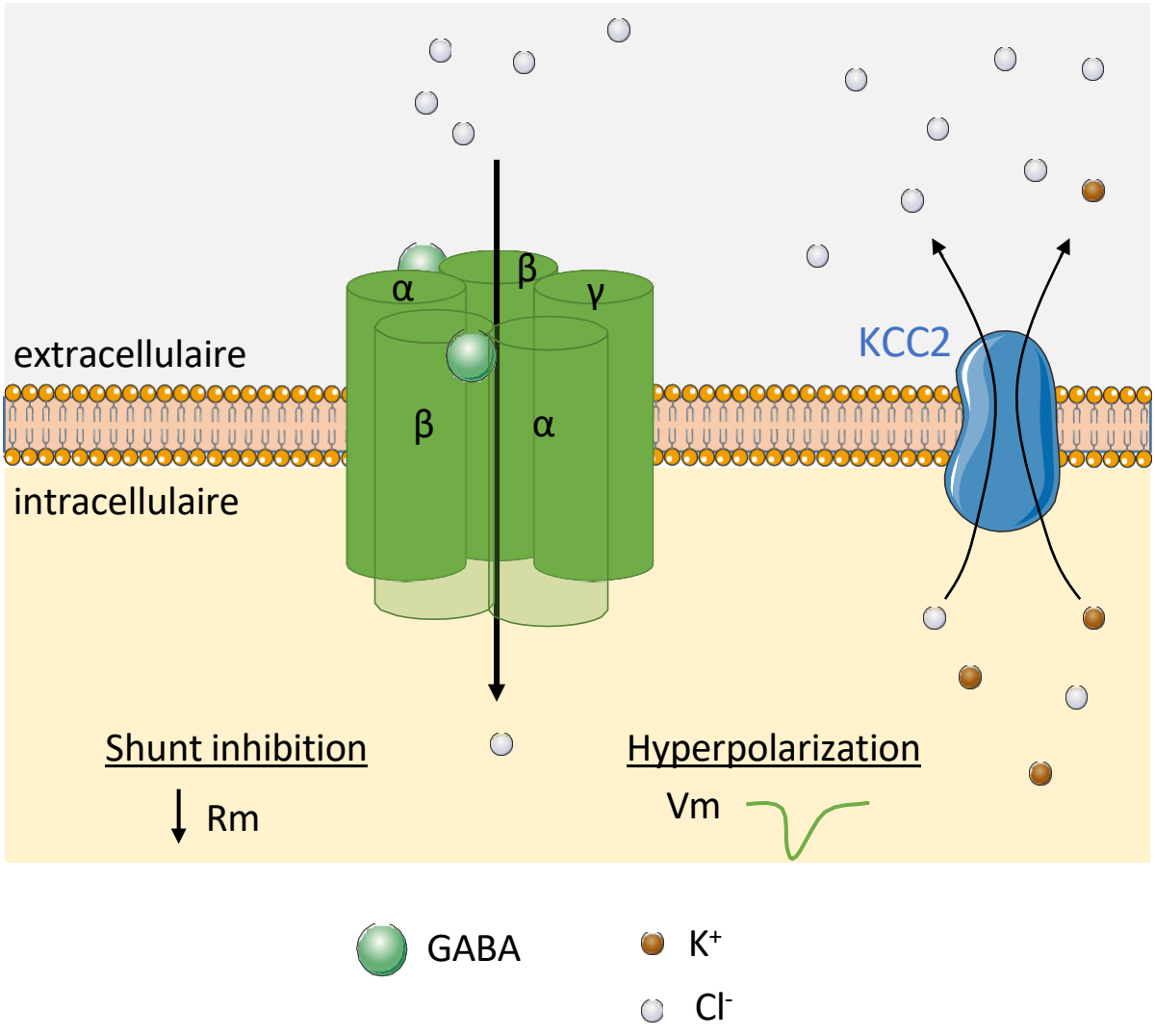


Figure 7: $GABA_A$ receptor

The $GABA_A$ receptor is a heteropentameric transmembrane ionic channel receptor activated by GABA. It is permeable to chloride (Cl^-). The chloride gradient is maintained by 2 enzymes : NKCC1 (not represented) and KCC2. KCC2 is a K^+ /Cl^- co-transporter that extrudes Cl^-. At resting membrane potential (~60 mV), $GABA_A$ activation induces an influx of Cl^-, inducing membrane hyperpolarization. Another form of inhibition induced by the opening of $GABA_A$ receptor is a drastic decrease in membrane resistance, called shunt inhibition.

II. $GABA_A$ receptors

1. Structure and subunits

The $GABA_A$ receptor is a GABA-gated chloride channel receptor, composed of 5 sub-units. It belongs to the Cys loop ligand-gated ion channel superfamily. All members of this superfamily share a motif: 2 cysteine residues separated by 13 amino acids in the extracellular domain, as well as 4 conserved transmembrane domains (TMs) and a large intracellular loop domain between TM3 and TM4 (Comenencia-Ortiz et al., 2014). For human $GABA_A$ receptors, there are 19 known genes coding for the subunits. The subunits are α (1-6), β (1-3), γ (1-3), δ, ε, θ, ρ (1-3), π. It should be noted that ρ is the subunit making up $GABA_C$ receptors, which are now thought to be a subtype of $GABA_A$ receptors. The most common assembly found in the nervous system is 2α, 2β and 1γ and even though a very large number of assemblies should be possible, studies have shown that only a limited number of subunit assemblies can form and reach the cell membrane (Comenencia-Ortiz et al., 2014). These subunits are assembled around the central channel and can be mostly found across from GABAergic synaptic boutons (figure 7). $GABA_A$ receptors contain many cavities in the intramembrane space, conferring them with a rich pharmacology. For full review, consult Sigel & Steinmann, 2012.
Interestingly, it's the intracellular domain that presents the most diversity between the different subtypes, which contains multiple sites for post-translational modifications. For a full review on post-translational modifications and receptor trafficking, see Comenencia-Ortiz et al., 2014.

In the adult rat spinal cord, a specific laminar motif of expression of subunits has been described. Using immunohistochemistry, Bohlhalter et al. found that staining for α2-3, β2-3 and γ2 was particularly intense across the superficial laminae (I-III) of the DH, whereas α1 and α5 were restricted to layer III (Bohlhalter et al., 1996) (figure 8). Knowing that the subtypes of $GABA_A$ receptors present different pharmacological sensitivities, different assemblies of receptors across the laminae might be modulated differently, allowing for fine tuning of GABAergic transmission across the DH. More recently, the same pattern of distribution of subunits has been described in the mouse (Zeilhofer et al., 2012) (figure 8).

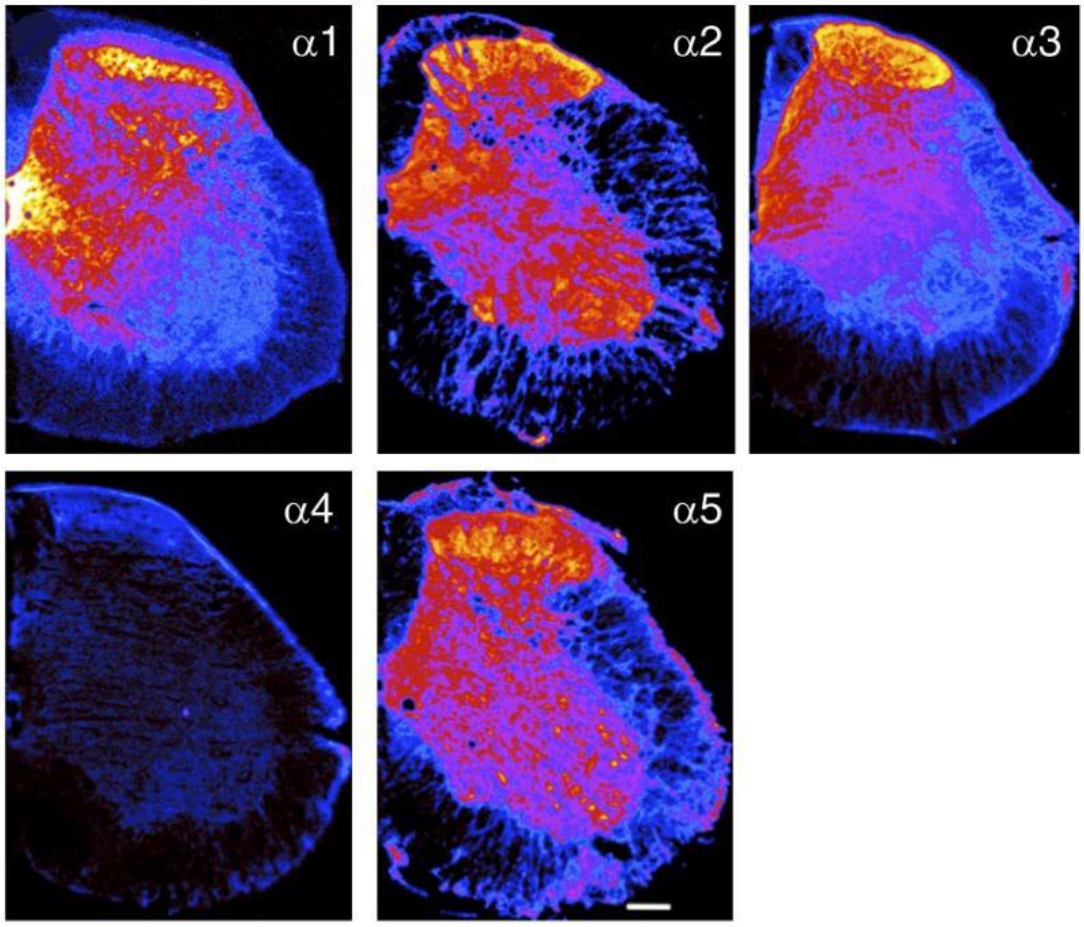


Figure 8: $GABA_A$ receptor distribution in the spinal cord

Distribution of α subunits of $GABA_A$ receptors in the spinal cord, illustrated in pseudocolor images: yellow represents the highest density and blue represents the lowest density of expression of $GABA_A$ receptors. From Zeilhoffer et al., 2012

2. Effects of $GABA_A$ receptor activation

Two types of inhibition can be described: hyperpolarizing inhibition, by which the inhibited neuron's membrane potential is polarized further from the rheobase, and shunt inhibition, which is the sudden decrease of membrane resistance, blocking the propagation of currents or the genesis of action potentials.

Hyperpolarizing inhibition

When GABA activates its receptor $GABA_A$, a change of conformation will occur and the inner channel will open, allowing Cl^- influx (at resting membrane potential, ~60 mV). This negative current induces membrane hyperpolarization, reducing neuronal excitability. This is called hyperpolarizing inhibition. Indeed, in adult vertebrate nervous systems, Cl^- is pumped out of the cell, maintaining a trans-membrane gradient with a higher concentration of Cl^- outside the cell. The chloride gradient is controlled by 2 co-transporters: KCC2, a K^+/Cl^- co-transporter that Cl^- and NKCC1, a $Na^+/K^+/Cl^-$ co-transporter that transports Cl^- into the cell (figure 7). In the developing nervous system, NKCC1 is more expressed than KCC2, resulting in a higher concentration of Cl^- ions inside the cell and depolarizing $GABA_A$ receptor activity (Ben-Ari et al., 2012; Payne et al., 2003). At about a week post-natal, KCC2 is more expressed than NKCC1, inverting the Cl^- gradient and establishing hyperpolarizing inhibition.

An interesting point about hyperpolarization of a neuron's membrane potential is that even if it does polarize the neuron's membrane further from the rheobase, it also allows for more voltage-gated sodium channels to be excitable, thus increasing neuronal excitability. It could be proposed that the inhibitory efficiency lies in the equilibrium between the distance between the rheobase and the neuron's membrane potential, and the population of excitable voltage-gated sodium channels.

Shunt inhibition

Interestingly, in the adult central nervous system, some cells maintain an inversed chloride gradient and $GABA_A$ activation induces an efflux of chloride ions, depolarizing the membrane. This is the case for the primary afferent neurons, whose somas are in the dorsal root ganglia. Studies have found that the equilibrium potential for Cl^- (ECl^-) is around -40 mV. Thus, GABA depolarizes

their peripheral and central terminals, a phenomenon called primary afferent depolarization (PAD) (Rocha-González et al., 2008), while still inducing pre-synaptic inhibition of the primary afferents. It could also be that the opening of large GABA channels in the membrane when GABA activates its receptor drastically decreases the membrane resistance, thus blocking the propagation of currents in the membrane, as Ohm's law states U=RI. This is called shunt inhibition. In 1955, in a study on the effects of GABA on muscle contraction, the authors also hypothesized that the drastic increase in Cl^- conductance induced by the opening of the $GABA_A$ channel was enough to "shunt" excitatory currents propagated by the primary afferents: the membrane potential would be clamped at the chloride equilibrium potential and no further depolarizations could be possible (Kuffler & Eyzaguirre, 1955). Another study also proposed that the small depolarization induced by the efflux of chloride would be enough to inactivate voltage-dependent Na channels, thus blocking the genesis of action potentials. This sort of inhibition is called "depolarizing inhibition" (Edwards et al., 1999).

We can note that within physiological conditions in the central nervous system, the equilibrium potential of chloride is very close to the resting membrane potential (around -70mV), so GABA-induced hyperpolarizing currents are quite small, and a mixture of both hyperpolarizing and shunt inhibition mechanisms might contribute to inhibitory synaptic transmission.

3. Pharmacology

GABA's binding site is located at the junction between α and β subunits: on a classic $GABA_A$ receptor composed of 2α, 2β and a γ subunit, there are 2 activation sites for GABA (figure 7). Apart from GABA, other agonists can act at these sites, such as muscimol and isoguvacine, as well as competitive antagonists like bicuculline and gabazine.
Phosphorylation can impact the activity of the $GABA_A$ receptor, as they can influence receptor assembly, membrane trafficking and stability, and even neurosteroid-dependent modulation of the receptor (Comenencia-Ortiz et al., 2014).

$GABA_A$ receptors present a rich pharmacology. Drugs that act as agonists at GABA receptors are usually used in clinical work as anticonvulsants, sedatives, and anxiolytics, to increase efficacy of

GABAergic inhibition, thus reducing convulsions and anxiety. $GABA_A$ receptor agonists include ethanol, barbiturate, and benzodiazepine (M. J. Allen et al., 2021). They act as allosteric modulators, inducing changes in the efficacy of the receptor's activation without binding to the orthosteric site. For example, benzodiazepine bind to a site between α and γ subunits and it potentiates agonist-induced currents by increasing the affinity of the receptor for its agonist.

Endogenous allosteric modulators also exist within the nervous system, such as the endogenous 3α–5α-reduced neurosteroids: metabolites of progesterone and deoxycorticosterone bind directly to the $GABA_A$ receptor and enhance the actions of GABA at concentrations <100 nM and can even act as agonists at higher concentrations. These molecules originating from the peripheral glands can pass the blood-brain barrier, but they can also be synthesized in neural cells (neurons and glia), acting in a paracrine and autocrine manner (Lambert et al., 2009). Interestingly, extra-synaptic $GABA_A$ receptors, believed to be implicated in sensing low ambient GABA concentrations and inducing a form of tonic inhibition (Brickley & Mody, 2012), might be the preferred target for neurosteroids. In the DH, it has been shown that neurosteroids positively modulate $GABA_A$ receptors in the superficial laminae during the post-natal phase and after an inflammation (Poisbeau et al., 2005; Inquimbert et al., 2007; Inquimbert et al., 2008).

Inhibitory transmission resulting from $GABA_A$ receptors depends on multiple parameters, such as the quantity of GABA molecules and GABA receptors, the sub-units composing the GABA receptors, the chloride gradient, and the presence of neuromodulators. As any of these parameters might change, so might inhibitory synaptic transmission.

III. GABAergic transmission in the DH

In the DH, GABAergic neurons represent about 30-40% of all neurons in the superficial laminae of the DH (Polgar et al., 2013). In laminae I and IIo, most synaptic inhibitory transmission is GABAergic (Takazawa & MacDermott, 2010). As within many structures, the balance between excitation and inhibition within the DH network is key for physiological processing of somatosensory stimuli. One of the main causes for the maintenance of chronic pain is thought to

be a diminished efficacy of inhibitory transmission in the DH. It is of great importance to dissect the mechanisms underlying GABAergic transmission in the DH in physiological and pathological conditions, to understand its role in the development and maintenance of chronic pain.

In 1965, Melzack and Wall proposed a model for nociceptive information processing in the DH network, called the "gate control theory of pain" where synaptic inhibition plays a key role. With this model, the authors were trying to conciliate the 2 mutually exclusive theories that were proposed at the time, which were that nociceptive information was either processed in a specific independent sensory system (specific system theory) or that nociception resulted from a specific type of activity within a system that also processed non-nociceptive information (pattern theory). Indeed, in their new model, non-nociceptive and nociceptive information modulates different inhibitory interneuron populations, with different outcomes on information transmitted from the DH network through projection neurons (Melzack & Wall, 1965): simply put, pain- and touch-sensing neurons antagonize each other, allowing for the network to be nociceptive or non-nociceptive depending on the stimulus. However, studies on the network since then have shown that composition and organization are a lot more complex than what they originally thought, with very diverse neuronal populations described (Woolf, 2022). Research is still ongoing today to study their interactions and organization, allowing for processing of nociceptive and non-nociceptive information within the same network.

Some studies have taken a closer look at the general role of GABAergic transmission in processing of nociceptive information, using specific antagonists such as bicuculline by intrathecal administration on live and behaving animals. Intrathecal injections of GABA receptor antagonists, such as picrotoxine or bicuculline, induces painful hypersensitization with behaviors such as vocalizations after non-nociceptive mechanical stimuli (interpreted as allodynia) and a reduction of the threshold for tail withdrawal after electrical stimulations (interpreted as hyperalgesia) (Roberts et al., 1986; Yaksh, 1989). These phenomena, appearing after injection of antagonists of inhibitory transmission, show how important it is in modality gatekeeping and regulation of synaptic activity in the DH network. Interestingly, these painful sensations are some of the main symptoms associated with pathological pain conditions. The role of reduced inhibitory efficacy has also been studied by electrophysiological approaches: in their 2003 study, Baba et al. found

Function of inhibition	Mechanism	Effect	Consequence when compromised
Attenuate nociceptive information	Inhibition of spinal excitatory neurons after a stimulus	Appropriate response to a nociceptive stimulation	Hyperalgesia
Silence spontaneous activity	Inhibition of spinal excitatory neurons in the absence of a stimulus	Avoid spontaneous activity of nociceptive neurons	Spontaneous pain
Separate sensorial modalities	Inhibition of Aβ synaptic entries (for example)	Keeping a defined processing of different modalities	Allodynia
Limit the diffusion of excitation in the network	Inhibition of neurons across somatotopic frontiers	Keeping a defined spatial processing of nociceptive information	Radiating, projected pain

Figure 9: Role of inhibitory transmission in the DH

Developped in Sandkühler., 2009

that perfusion of the $GABA_A$ receptor antagonist bicuculline induced the appearance of long-lasting polysynaptic responses after primary afferents stimulation, indicative of over-excitation in the network (Baba et al., 2003). Interestingly bicuculline has less effect in a mouse model of neuropathic pain, suggesting that inhibitory transmission is downregulated in these conditions. Studies have also shown that injecting agonists of GABA receptors, transplanting GABA synthesizing stem cells or facilitating GABAergic transmission with drugs such as benzodiazepines reduces allodynia and hyperalgesia in models of pathological pain such as neuropathic pain (Kontinen & Dickenson, 2000; Knabl et al., 2008; Novak et al., 2001).

In his 2009 review, Sandkühler organized these findings and presented the 4 functions of inhibition within the DH network (including both GABAergic and glycinergic transmissions) for nociceptive information processing: attenuation of activity of nociceptive neurons, silencing neuronal activity in the absence of noxious stimuli, separating nociceptive and non-nociceptive pathways, and limiting the spread of nociceptive information transmission within the network, to maintain somatotopy of the incoming nociceptive information (Sandkühler, 2009) (figure 9).

1. Attenuation of nociceptive information

Inhibitory synapses (both GABAergic and glycinergic) on the central terminals of the primary afferents as well as on the projection neurons regulate both the information input and output of the DH network. Studies have shown that $GABA_A$ and glycinergic receptor blockade in vivo induces an increase in neuronal responses to nociceptive stimuli (Zieglgänsberger & Sutor, 1983; Saadé et al., 1985).

2. Silencing neuronal activity in the nociceptive network

As in most somatosensory information processing networks, it is important to maintain an efficient signal/noise ratio, which means maintaining low activity in the network in the absence of nociceptive stimuli. Pharmacological blockade of $GABA_A$ and glycinergic receptors induces an increase in spontaneous action potential firing in the network, that can synchronize (Ruscheweyh

& Sandkühler, 2003). When studying behavior, blockade of $GABA_A$ and glycinergic receptors induce spontaneous pain behaviors in awake rats (Loomis et al., 2001).

3. Separating sensorial modalities

As seen previously, gate control theory was proposed as a model to explain how one network of interconnected neurons can integrate both nociceptive and non-nociceptive information, attributing a key focus on inhibitory transmission as the gate. A malfunction in this gate mechanism could lead to the integration of non-nociceptive information in the nociceptive subnetwork, resulting in allodynia. A few studies have focused on identifying these populations of neurons that allow for separation of nociceptive and non-nociceptive information processing (Baba et al., 2003; Miracourt et al., 2007; Peirs et al., 2015). For example, more recently, it was shown that ablation of specific inhibitory neurons in the deeper laminae (between III-V), that express tyrosine kinase receptor (RET) neonatally, induced mechanical allodynia and mechanical, thermal, and cold hyperalgesia in mice. It was shown by the authors that in physiological conditions, this population of inhibitory neurons inhibited post-synaptically a population of excitatory neurons positive for PKCγ and SST, as well as inhibited pre-synaptically afferent fibers. Their activation induced a reduction of basal pain perception. The authors propose that this population of cells acts as an inhibitory switch between pain and touch pathways (Cui et al., 2016).

4. Limit the diffusion of excitation in the DH network

As presented in the first part of the introduction, primary afferent fibers project in specific regions of the DH, which indicates organization in how the DH processes nociceptive information coming from the periphery. Pharmacological blockade of $GABA_A$ and/or glycinergic receptors induces calcium waves in all the neurons in a rat spinal slice, indicating the propagation of excitation although the network and the potential loss of organized processing of nociceptive information in the DH which could result in radiating pain (Ruscheweyh & Sandkühler, 2003).

A dysfunction in any of these functions may lead to hyperalgesia, spontaneous pain, allodynia and radiating pain respectively (Sandkühler, 2009). Given the diversity of interneuron subpopulations, the question remains whether these functions are taken on by different subpopulations within the DH and ongoing research is being led to try and identify such subpopulations of inhibitory interneurons. For a full review on inhibitory synaptic transmission in the DH, see (Zeilhofer et al., 2012).

These results show how important inhibitory synaptic transmission is for "normal" nociceptive information processing. Inhibitory interneurons decrease synaptic activity coming from the afferent fibers as well as in the dorsal interneuron network, limit the spread of nociceptive information within the network and separate the different modalities (Sandkühler et al., 2009).

IV. Modulation and plasticity of GABAergic synaptic transmission

Artificially modulating synaptic inhibitory transmission can induce the development of painful symptoms such as hyperalgesia, allodynia and spontaneous pain. It is known that in pathological pain conditions, there is a decrease in inhibitory synaptic efficacy (Sandkühler., 2009), associated with the development of such symptoms. The different changes in inhibitory transmission show that the inhibitory network is not static, but can change depending on previous activity, increasing (potentiation) or decreasing (depression) inhibitory synaptic efficacy, for short-term or long-term modulation.

An increase in primary afferents' activity, as is the case following peripheral nerve injury (X. Liu et al., 2000; Wall et al., 1974; Wu et al., 2001), leads to an increase in spinal network activity, due to plastic changes of synaptic transmission. Mechanisms underlying excitatory synaptic plasticity have been widely studied. For example, many studies have shown long-term potentiation (LTP) of synapses between nociceptive afferent fibers and lamina I projecting neurons, involving presynaptic and/or postsynaptic mechanisms (X. G. Liu & Sandkühler, 1995; Ikeda et al., 2003; Ikeda et al., 2006). It's also been shown that these plastic changes underlie the development and

the maintenance of chronic pain (Pockett, 1995; Lynskey et al., 2008) and depend on activation of NMDAr, AMPA receptors, mGlu receptors and/or TrkB. For more information on plasticity of excitatory synaptic transmission in the DH, see Latremoliere & Woolf, (2009) and Kuner, (2015).

Modulation and plasticity of GABAergic synaptic transmission in the DH has been very little studied. I will present the few cases of described GABAergic synapse modulation and plasticity in the DH, as well as what is known in other structures, as we can propose that some mechanisms might be similar in the DH. As I mostly worked on presynaptic modulation of GABAergic transmission, I will focus more on that part. For a full review on plasticity of GABAergic synaptic transmission in all structures, see Castillo et al. (2011) and Kullmann et al. (2012). For a full review on plastic changes of inhibitory synaptic transmission, see Gradwell et al. (2020) and Zeilhofer et al. (2012).

1. Changes in the Cl^- gradient

A decrease in KCC2 expression in the lamina I was described following peripheral nerve injury (Coull et al., 2003). This induced an increase in the intracellular concentration of Cl^-, and a decrease in hyperpolarizing inhibitory transmission efficiency (van den Pol et al., 1996). When deciphering this, studies have shown that activated microglia release BDNF and ATP, responsible for the decrease in KCC2 expression (Coull et al., 2005; Rivera et al., 2004; Thacker et al., 2009). When artificially increasing KCC2 expression after peripheral nerve injury, a physiological ECl^- was restored (Li et al., 2016). Some studies have also shown that NMDAr activity is linked to the changes in KCC2 expression, and I will develop this part in the 3rd chapter of the introduction.

2. Presynaptic modulation of GABAergic transmission

Presynaptic modulation and plasticity of GABAergic transmission usually depends on retrograde messengers produced by the postsynaptic element that will act at the presynaptic terminal and

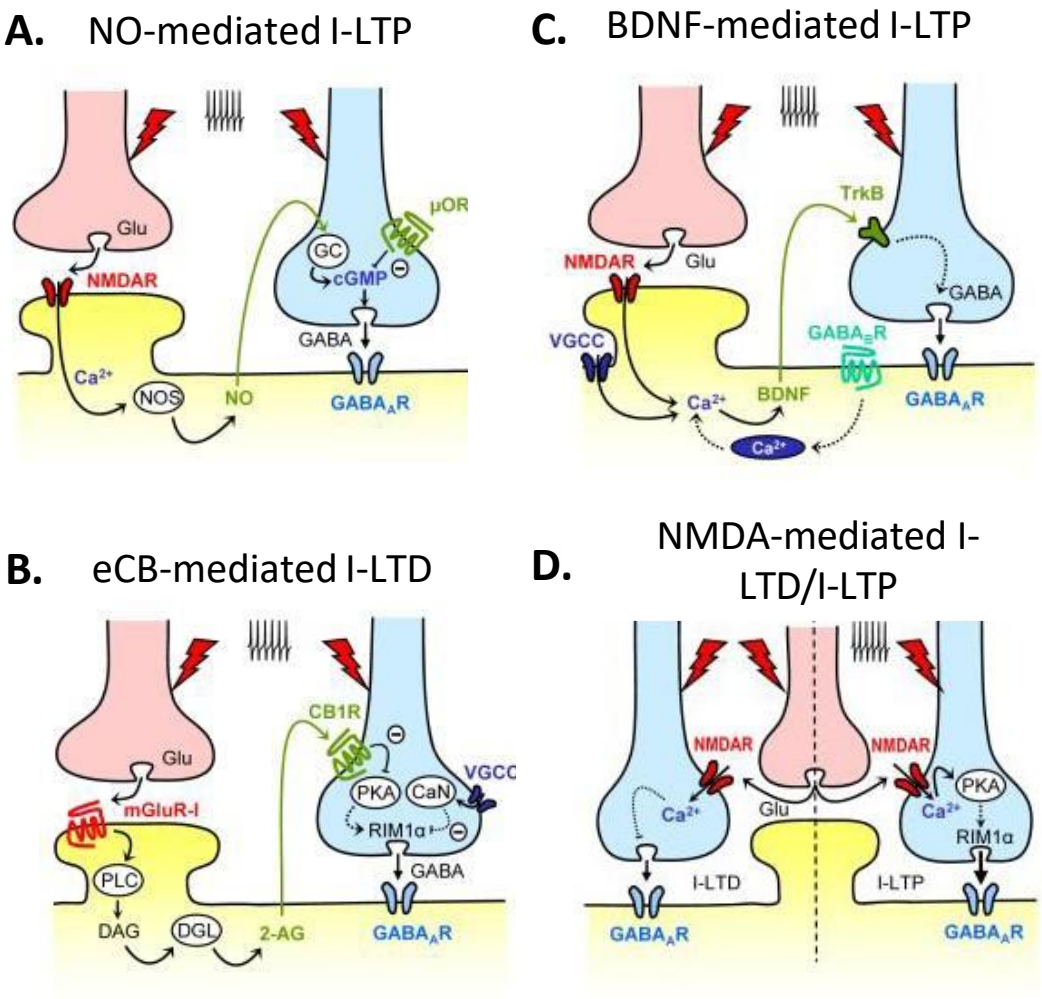


Figure 10: Presynaptic plasticity of GABAergic synaptic transmission. From Castillo et al., 2011

A. NO-mediated I-LTP depends on the activation of NMDAr expressed at the postynaptic element in the ventral tegmental area (Nugent et al., 2007), which induces the activation of the nNOS and the production of NO in the postysnaptic element. NO will act on the presynaptic element, activating guanylate cyclase, increasing cGMP and thus, the release of GABA. A similar mechanism was discovered in the lamina I of the DH, but the activation of nNOS depends on the activation of group I mGLuR (Fenselau et al., 2011).

B. eCB-mediated I-LTD depends on the activation of group I mGluR, which induces the production of diaglycerol (DAG) via activation of the phospholipase C. DAG is cleaved into 2-AG, which will cross the plasmic membrane and activate CB1 receptors expressed at the presynaptic element. The activation of these receptors reduces the activity of the PKC and results in the I-LTD of the GABAergic synapse.

C. BDNF-mediated I-LTP depends on an increase in $[Ca^{2+}]_i$ induced by the activation of NMDAr, voltage-dependent calcium channels (VGCC) or intracellular stores of calcium. BDNF is synthesized in the postsynaptic element and activates the TrkB receptor expressed at the presynaptic element, inducing LTP of the GABAergic synapse.

D. Glutamate from surrounding glutamatergic synapses can activate NMDAr expressed at the presynaptic element of the GABAergic synapse and induce either I-LTP or I-LTD.

modulate the release of neurotransmitters. Transmitters can also act presynaptically, such as glutamate released from nearby excitatory synapses (figure 10).

Nitric oxide

In their 2011 study, Fenselau et al. observed that the protocol inducing LTP at excitatory synapses between C-fibers and lamina I neurons also induced heterosynaptic LTP at GABAergic synapses that depended on the activation of group I mGlu receptors. This GABAergic LTP was expressed presynaptically. The authors found that this presynaptic effect was due to the release of nitric oxide that increased the release of GABA by inducing the activation of guanylate cyclase. They propose that this mechanism to increase GABAergic synaptic transmission in situations of increased primary afferent activity serves to maintain the relative balance between excitation and inhibition within the network, allowing for the 4 main functions of inhibition to be ensured properly. It should be noted however, that the physiological consequences of GABAergic synaptic transmission potentiation depends on the nature of the postsynaptic neuron and/or how it modulates network activity, parameters that were not observed in this study (Fenselau et al., 2011). Interestingly, it's also been shown in vivo that GABAergic transmission potentiation blocks potentiation at C-fibers (Hu et al., 2006). Then, an additional role could be that GABAergic LTP serves as a control mechanism to reduce LTP at excitatory synapses.

A very similar form of plasticity was described in the ventral tegmental area, except that the synthesis and release of nitric oxide depended on NMDAr activation (Nugent et al., 2007) (figure 9A).

Endocannabinoids and glutamate

There have been no studies showing a mechanism of inhibitory synaptic plasticity in the DH dependent on endocannabinoids. However, type I endocannabinoid receptors are expressed in the superficial laminae of the DH and endocannabinoids are present in the spinal cord (Farquhar-Smith et al., 2000).

In other structures of the CNS like the hippocampus and the cerebellum, plasticity depending on an endocannabinoid retrograde messenger has been demonstrated: the depolarization of pyramidal neurons/-Purkinje cells induces a transitory depression of the synaptic release of GABA. This form of plasticity is called "Depolarization-induced suppression of inhibition" (DSI) (Llano et al., 1991;

Pitler & Alger, 1992). DSI usually depends on the synthesis and release of 2-arachidonoylglycerol (2-AG) that will act on presynaptic CB1 receptors and transiently decrease GABA release (Kano et al., 2009). This mechanism depends on activation of postsynaptic NMDAr or mGluR (figure 9B). It's also been shown in the cerebellum that an intense stimulation of Purkinje cells induces the release of endocannabinoids as well as glutamate, that can activate presynaptic NMDAr on GABAergic synaptic terminals, inducing an increase in intracellular calcium concentrations and facilitating the release of GABA, illustrating that different forms of plasticity can co-exist, and be differently activated depending on the intensity of the stimulation. DSI has now been described in many other structures of the central nervous system, as well as in glycinergic synaptic transmission in the hypoglossal nucelus (Kullmann et al., 2012).

BDNF

During the early stages of embryonic development, BDNF is released by dendrites of dissociated hippocampal cultured neurons in an activity-dependent manner to strengthen GABAergic synapses (Kullman., 2012). In the adult central nervous system, studies have shown that BDNF's retrograde actions can lead to potentiation of GABAergic synaptic transmission in the hippocampus, visual cortex, and optical tectum (Gubellini et al., 2005; Inagaki et al., 2008; Sivakumaran et al., 2009). This LTP depends on TrkB receptor activation. It was demonstrated that BDNF synthesis and release requires an increase in intracellular calcium concentrations, which can be exogenous, from NMDAr for example (Kullman., 2012) (figure 9C).

In the spinal cord, both BDNF and TrkB receptor are expressed. We can imagine that similar forms of modulation and plasticity occur.

Other transmitters

Implication of other modulators of GABA synaptic transmission, released by surrounding neurons and/or glial cells may be possible in the DH, as demonstrated in other structures of the central nervous system. For example, activation of presynaptic NMDAr by glutamate can induce long-term depression (LTD) as described in the optic nerve of the xenopus (Lien et al., 2006; Y. Liu et al., 2007) or LTP of GABAergic transmission, as observed in the cerebellum (S. J. Liu & Lachamp, 2006; Lachamp et al., 2009) (figure 9D).

In the DH, ATP has been shown to induce an increase in spontaneous and miniature GABAergic IPSC, as well as increase evoked GABAergic IPSC, suggesting a presynaptic expression of ATP-dependent modulation of GABAergic transmission. Interestingly, this mechanism was preferentially observed in neurons co-releasing GABA and ATP (Hugel & Schlichter, 2000). A more recent study from our team has shown that GABAergic synapses are differentially modulated during high and low frequency stimulations depending on the neurochemistry of the postsynaptic neuron, with GABAergic inputs in non-GABAergic neurons modulated by presynaptic A1 adenosine receptors and GABAergic input in GABAergic neurons modulated by $GABA_B$ autoreceptors, allowing for fine-tuning of GABAergic transmission in the network (Cathenaut et al., 2022).

3. Postsynaptic modulation and plasticity of GABAergic transmission

To my knowledge, no study has focused on postsynaptic plasticity of GABAergic transmission in the adult DH. However, in other structures, postsynaptic mechanisms of GABAergic plasticity have been described. In cultured hippocampus neurons for example, activation of postsynaptic NMDAr at GABAergic synapses induced an influx of calcium, which in turn induced the activation of intracellular cascades including the calcium/calmoduline dependent kinase 2, the protein kinase A (PKA) and protein kinase C (PKC). These proteins will influence the levels of phosphorylation, the mobility, and the sub-unit composition of the GABA receptors (Marsden et al., 2007; Vithlani et al., 2011). These modifications can lead to a potentiation of GABAergic transmission, for example in Purkinje cells (Kano et al., 1992) or their inhibition, for example in the hippocampus (Wanaverbecq et al., 2007).

4. Spike timing dependent plasticity

Another type of plasticity, called spike timing dependent plasticity is expressed depending on the precise spike sequence of pre- and post-synaptic neurons. This type of plasticity was described in the ventral tegmental area. Using patch-clamp recordings, Kodangattil et al. found that LTP and LTD can be induced at GABAergic synapses onto dopaminergic neurons, depending on the precise sequence of spike stimulation of the pre-and postsynaptic neurons. They found that this type of

plasticity is heterosynaptic in nature and depends on NMDAr activation. They proposed that this type of plasticity is thus triggered by a correlated activity of the presynaptic glutamatergic neurons and the postsynaptic dopaminergic neurons and plays an important role in addiction behaviors (Kodangattil et al., 2013).

5. Astrocytes and modulation of inhibitory synaptic transmission

As developed in the first section of the introduction, we know astrocytes are essential actors in synaptic transmission, not only for maintaining conditions allowing for "normal" synaptic transmission (ionic gradients, neurotransmitter clearance for example), but they can also directly modulate synaptic activity by releasing transmitters such as ATP and glutamate, that will act on receptors expressed on the presynaptic or postsynaptic element, or on other astrocytes. Most studies have focused on the implication of astrocytes in modulation of excitatory transmission. I will present here what we know about modulation of GABAergic synaptic transmission by astrocytes, in the DH and other structures.

Astrocytes express GABA transporters (GAT), and more specifically GAT1 and GAT3 (Mederos & Perea, 2019), allowing them to sense surrounding GABAergic transmission. It's been shown that GABA uptake by GAT1 and GAT3 can modulate $GABA_A$-mediated synaptic transmission (Moldavan et al., 2017; Song et al., 2013). In hippocampal astrocytes, GABA uptake by GAT3 induced the release of ATP/adenosine by astrocytes, participating in the downregulation of excitatory transmission by activation of presynaptic adenosine receptors (Boddum et al., 2016) and contributing to the enhancement of inhibitory synaptic transmission by activation of postsynaptic adenosine receptors (Matos et al., 2018). It's also been shown that activity of GAT3 in cerebellar and hippocampal astrocytes induces intracellular calcium signaling in astrocytes (Doengi et al., 2009; Matos et al., 2018). Interestingly, when specifically downregulating calcium signaling in astrocytes, Yu et al. described an up-regulation of GAT3 expression at the membrane in astrocytes (Yu et al., 2018). Taken together, these data show GATs not only allow for regulation of extracellular GABA concentration, but their activity can induce astrocytic calcium signaling, which can lead to further modulation of synaptic transmission (Mederos & Perea, 2019).

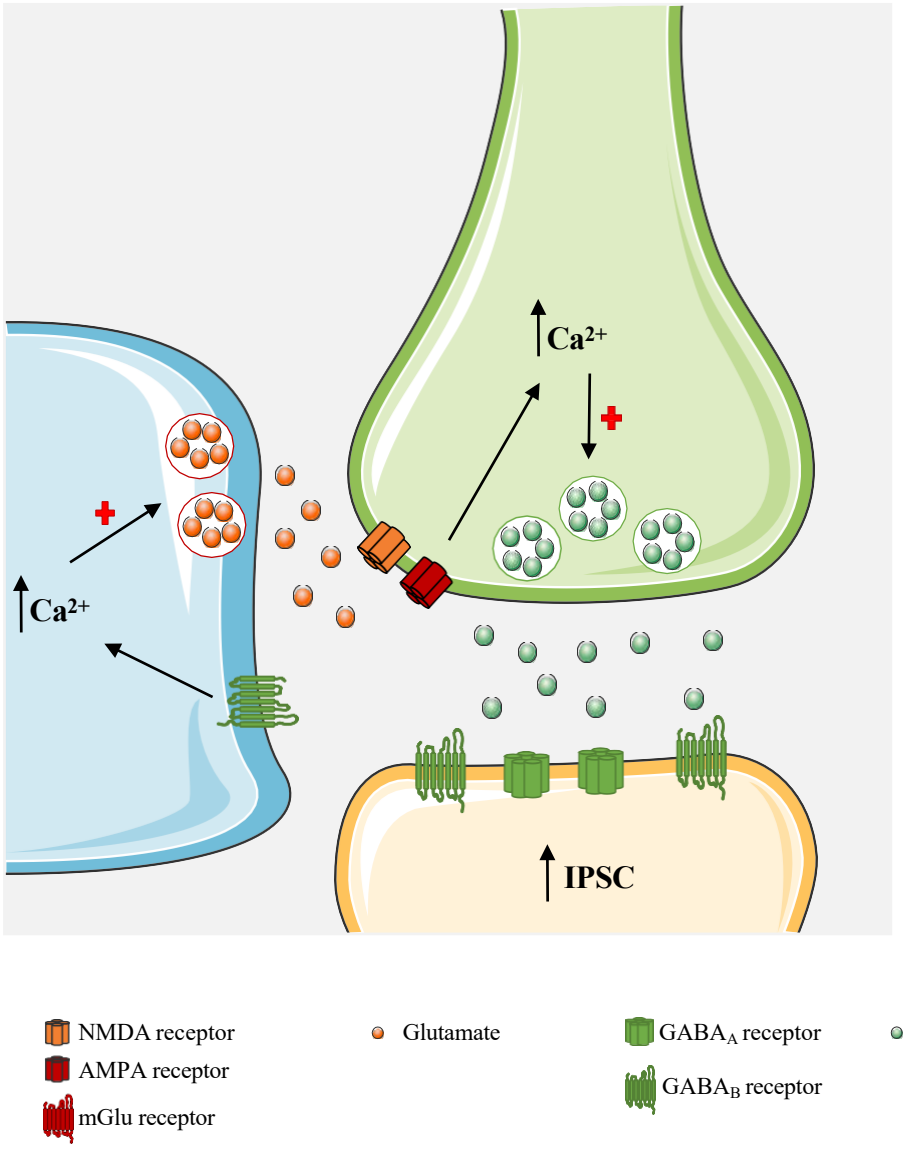


Figure 11: Example of astrocyte-mediated modulation of GABAergic synaptic transmission

In hippocampal slices, Kang et al. observed that repetitive firing of an interneuron decreased the probability of synaptic failures in evoked IPSC in CA1 neurons (facilitation of inhibitory transmission). When stimulating astrocytes or activating $GABA_B$ receptors, the authors observed a potentiation of mIPSC in pyramidal neurons. These effects were locked by injecting the calcium chelator BAPTA and antagonists of ionotropic glutamate receptors. This illustration shows the mechanisms proposed by the authors: GABA released at the inhibitory synapse activates $GABA_B$ receptors located in the surrounding astrocytes, inducing an increase in $[Ca^{2+}]_i$. In response, the astrocytes release glutamate that activates NMDA and AMPA receptors located on the presynaptic element, inducing an influx of calcium into the synaptic terminal, and thus an increase of GABA released (Kang et al., 1998).

Astrocytes also express ionotropic and metabotropic receptors, at the fine processes surrounding synapses, but also at the level of the soma and the endfeet in contact with blood vessels (Mederos et al., 2019). Studies have shown that activation of ionotropic $GABA_A$ receptors induce depolarizing currents in cultured astrocytes as well as in slices, which in turn induces calcium signaling in astrocytes (Mederos et al., 2019). Activation of metabotropic $GABA_B$ in astrocytes can also induce an increase in intracellular calcium signaling implicating $G_{i/o}$ proteins and release of intracellular calcium stores. This intracellular calcium signaling can lead to the release of transmitters, such as ATP and glutamate. In 1998, Kang et al. used double patch-clamp to record paired interneuron and CA1 pyramidal neurons in hippocampal slices. They observed that repetitive firing of afferent interneurons induced a decrease in probability of synaptic failures (potentiation) in CA1 pyramidal neurons. Dissecting this phenomenon, the authors found that interneuron firing induces a $GABA_B$ receptor-mediated increase in calcium signaling in astrocytes, which in turn induces a release of glutamate from astrocytes, which acts on NMDAr/AMPA receptors expressed on the presynaptic GABAergic terminal. The authors conclude that astrocytes play an important role in activity-dependent modulation of inhibitory synaptic transmission in the hippocampus (Kang et al., 1998) (figure 11).

An important parameter of astrocytic networks is that they can contact up to 100,00 synapses in the mouse central nervous system, allowing for large scale modulation of synaptic activity, depending on activity coming from elsewhere in the network for example. Some studies have focused on a more integrative role of astrocytes and found that astrocytic networks have been shown to regulate excitation/inhibition ratios and to participate in temporal integration of neuronal action potential firing (Mederos et al., 2019). GABAergic interneuron populations are very diverse, and much remains to be understood about differences in the involvement of different sub-populations of astrocytes. For a complete review on astrocyte-GABAergic interneuron interactions, see Mederos et al., 2019.

In the DH, very little is known about modulation of GABAergic synaptic transmission by astrocytes. One study from 2016 focused on the role of astrocytes in pain gating in the DH, using an optogenetic approach. They expressed channelrhodopsine 2, a photoactivable cation channel, under the control of the GFAP promoter and proceeded with behavioral and electrophysiological studies. They found that optogenetic stimulation of astrocytes induced pain hypersensitivity

behavior in mice. They then recorded inhibitory interneurons and observed that photostimulation induced a reduction of action potential firing in these cells, as well as a hyperpolarization of their membrane potential. To complete this experiment, the authors observed an increase in c-fos expression in projection neurons, concluding that photostimulation of astrocytes induced a decrease in inhibitory interneuron activity in parallel to an increase in projection neuron activity (Nam et al., 2016). It should be noted that channelrhodopsine 2 is mostly permeable to sodium. A large influx of sodium in astrocytes could influence the sodium gradient, and the functioning of neurotransmitter transporters such as EAAT, potentially reversing them and inducing a massive release of glutamate from astrocytes. More generally, sodium signaling plays an important role in astrocytic functioning (Verkhratsky & Nedergaard, 2018).

A study from 2018 in turtles showed that spinal astrocytes can release GABA after being stimulated by glutamate (Christensen et al., 2018), suggesting a compensatory mechanism by astrocytes in upscaling inhibitory transmission when stimulated with glutamate to maintain excitatory/inhibition balance within the DH network. However, the authors did not investigate the network consequences of this release of GABA, which could lead to pro or anti-nociceptive pathways depending on the surrounding cells.

GABAergic synaptic transmission can be modulated presynaptically and postsynaptically, potentially leading to plastic changes that can last either a few seconds or up to a few weeks. Astrocytes have been shown to play an important role in modulation GABAergic synaptic transmission in some structures, but little is known about their potential implication in modulation of GABAergic transmission within the DH network.

Conclusion of part 2

GABAergic synaptic transmission plays an important role in "normal" integration of nociceptive information, as it allows to decrease activity within the DH network and separate

modalities. GABAergic synapses are plastic, allowing for activity-dependent modulation of inhibitory transmission in the DH. These changes can increase or decrease GABAergic transmission. Finally, we know that astrocytes can be important actors in modulation of GABAergic synaptic transmission.

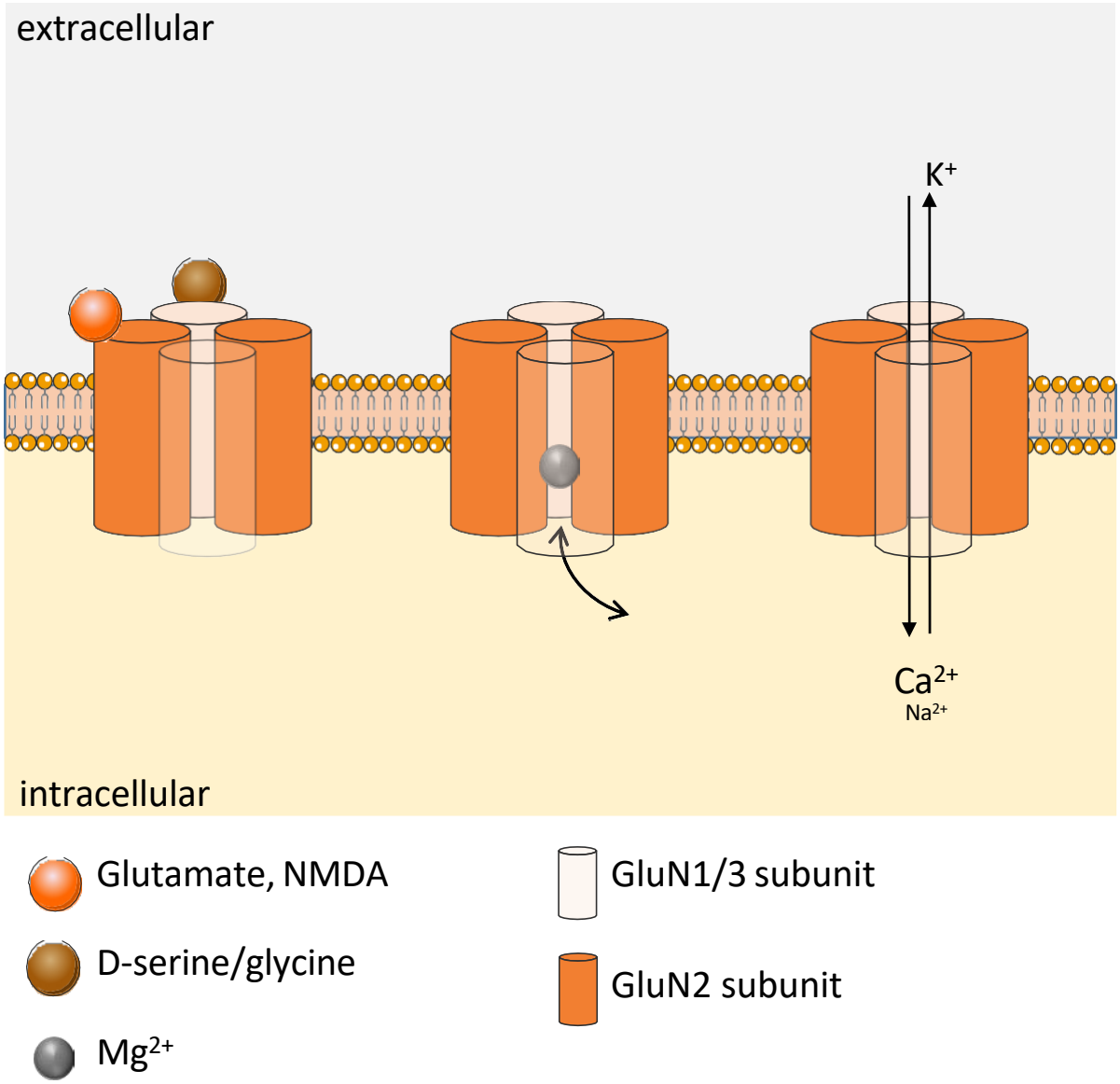


Figure 12: NMDAr activation

To activate NMDAr, the agonist (glutamate, NMDA) is needed, as well as a co-agonist (D-serine, glycine). Depending on the subunit composition of the receptor, a voltage-dependent clearing of the Mg^{2+} block is also needed to allow ion flux through the channel. Once open, NMDAr are permeable to cations, and especially Ca^{2+}.

Part 3: NMDA receptors in the dorsal horn

Glutamate is the main excitatory neurotransmitter in the central nervous system. In the DH, it plays a central role in the transmission of the nociceptive message, from the primary afferents to the spinal neuron, in the spinal network and from projection neurons to the supra-spinal centers.

NMDAr, ionotropic glutamate receptors, are expressed by virtually all neurons in the superficial layers of the DH. Canonically known as postsynaptic receptors at glutamatergic synapses, their involvement in plasticity of excitatory synaptic transmission has been well established. In conditions of sustained activity of the primary afferent fibers, in the case of an inflammation or of a peripheral nerve injury for example, plastic changes in the DH can occur. These plastic changes can lead to central sensitization, a state defined as an increase and change of responsiveness of spinal neurons to nociceptive and non-nociceptive stimuli. Central sensitization leads to abnormal pain sensations, such as allodynia and hyperalgesia. When these phenomena are acute and associated to a lesion, pain is considered physiological, as it serves a function for the organism, to facilitate healing and prevent future injuries. However, when pain no longer serves a protective function, it becomes pathological. As central sensitization contributes to many situations of pathological pain, it is important to dissect its mechanisms. For a complete review on central sensitization, see Latremoliere & Woolf., 2009.

Two mechanisms participating in the development and maintenance of central sensitization have been identified: an increase in excitation induced by NMDAr recruitment and a decrease in efficiency of inhibition (disinhibition) (Latrémolière and Woolf., 2009). NMDAr have been clinically targeted in the treatment of pathological pain, but due to severe secondary effects, clinical trials were abandoned and research on NMDAr in the DH has slowed down in the last years. However, new data from recent studies are showing a more complex implication of NMDAr in the normal and pathological function of the DH network.

This third and final part of the introduction will present a brief overview of NMDAr properties; what we know about NMDAr subunit expression and function in different populations of cells in

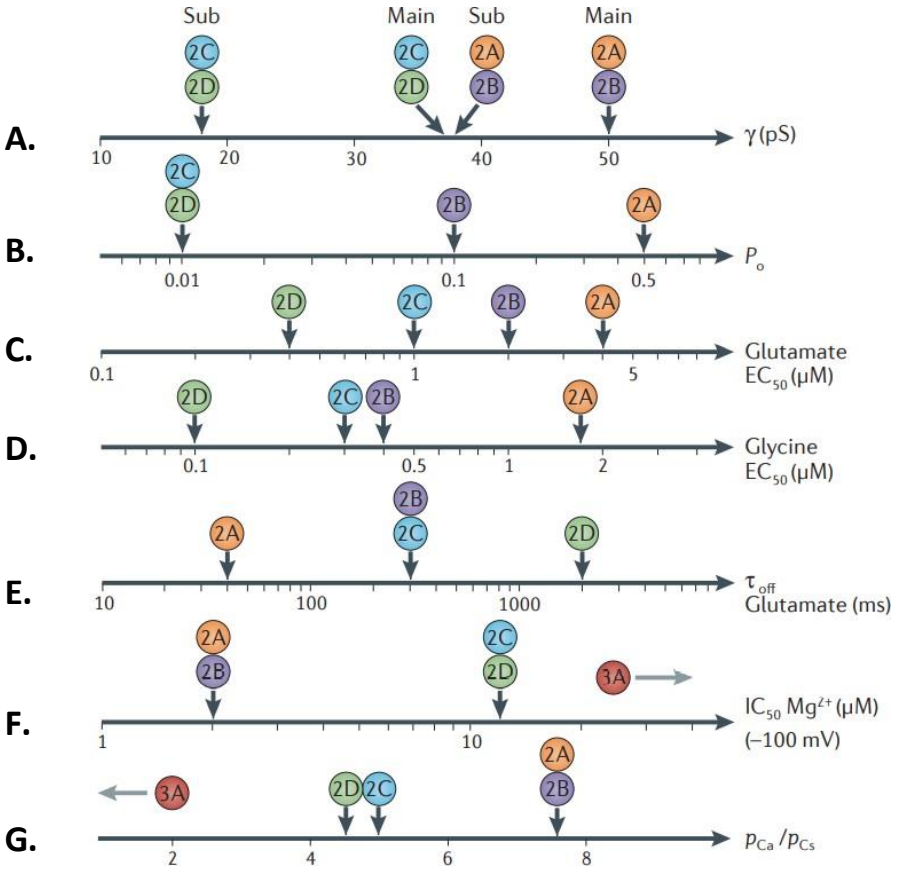


Figure 13: NMDAr subunit properties

Different subunits confer different properties to the receptor. In this illustration: (A) single channel conductance; (B) maximum opening probability; (C) glutamate affinity; (D) glycine affinity; (E) glutamate deactivation time constant; (F) the Mg^{2+} sensitivity; (G) calcium permeability. From Paoletti et al., 2013.

the DH, with a specific segment on astrocytes; and their involvement in network function and plasticity of excitatory and inhibitory transmission in the DH.

I. Properties of NMDAr

NMDAr are tetrameric glutamate-gated ion channels, composed of 4 subunits. Binding of an agonist (glutamate, NMDA) and a co-agonist (glycine, D-serine) is required to activate NMDAr. At polarized membrane potentials, most of the NMDAr are blocked by an Mg^{2+}. These NMDAr thus require the coincidence of multiple conditions to be activated: binding of the agonist and the co-agonist as well as the depolarization of the membrane to unblock the magnesium ion from the channel (figure 12). NMDAr are permeable to cations and more specifically, calcium. The coincidence detection property as well as calcium permeability make NMDAr essential players in synaptic plasticity mechanisms. The subunit composition impacts these channel properties.

1. NMDAr subunits

Cloning studies have identified 7 subunits, subsequently categorized into 3 subfamilies based on sequence homology: GluN1; GluN2A, GluN2B, GluN2C and GluN2D, encoded by 4 different genes; GluN3A and GluN3B encoded by 2 different genes (figure 13).

The **GluN1** subunit is mandatory for the formation of a functional channel. It has a co-agonist binding site. It is present as 8 different isoforms due to alternative splicing, affecting the receptor's pharmacological gating and intracellular subunit trafficking properties. It is still unclear how the different isoforms affect receptor function today.

The **GluN2A** subunit is the most expressed subunit in the adult CNS. The presence of GluN2A confers the highest probability of channel opening (0.5), the lowest glutamate and glycine affinity, the highest sensitivity to the magnesium blockade and the highest calcium permeability to the receptor. Usually, synaptic NMDAr are enriched in GluN2A subunits (Paoletti et al., 2013).

The **GluN2B** subunit is mostly found in the cerebellum and the olfactory bulb. The presence of GluN2B confers a lesser probability of channel opening (0.1), a higher affinity to glutamate and glycine than GluN2A, the highest sensitivity to magnesium blockade and the highest calcium permeability to the receptor. Studies have found that peri-and extrasynaptic NMDAr are enriched in GluN2B. However, synaptic NMDAr also contain GluN2B subunits (Paoletti et al., 2013).
The **GluN2C** subunit is mostly found in the cerebellum and the olfactory bulb (Watanabe et al., 1993) in the adult CNS. The presence of GluN2C confers the lowest probability of channel opening (0,01), a high affinity to glutamate and glycine, and low sensitivity to magnesium blockade and low calcium permeability to the receptor.
The **GluN2D** subunit is mostly found in the diencephalon and mesencephalon. The presence of GluN2D confers the lowest probability of channel opening (0.01), the highest affinity to glycine and glutamate, the lowest sensitivity to the magnesium blockade and the lowest calcium permeability to the receptor.
The **GluN3A** subunit is mostly present in the olfactory bulb (Sucher et al., 1995) in the adult CNS. Little is known about the influence of the presence of the GluN3A subunit on channel opening probability, glutamate and glycine affinity, but studies have shown that it lowers sensitivity to the magnesium blockade and lowers calcium permeability.
The **GluN3B** subunit is expressed in the adult central nervous system but very little is known about the influence of the subunit in NMDAr properties.

For a complete review on NMDAr, see Paoletti et al., 2013.

A few unconventional NMDAr have been described: an ion flux independent NMDAr (also called metabotropic NMDAr) (Dore et al., 2017) and assemblies of GluN1 and GluN3, forming excitatory glycine receptors (Pachernegg et al., 2012). Not much is known about these receptors in the spinal cord.

2. Receptor desensitization, internalization and dephosphorylation

A few mechanisms allow for the end of receptor-mediated cellular signaling to regulate synaptic transmission and inhibit excessive receptor activity, of which receptor desensitization, internalization and dephosphorylation.

Receptor **desensitization** can be defined as a decrease in response after a stimulus in a short interval. NMDAr can be desensitized after a prolonged exposure to glutamate or glycine (Hansen et al., 2018). Desensitization results on structural changes, however the mechanisms remain little understood. It seems that NMDAr containing GluN2A subunits are more sensitive to intracellular calcium concentrations increases than NMDAr containing GluN2B and GluN2C subunits (Medina et al., 1995; Traynelis et al., 2010; Carroll & Zukin, 2002).

Receptor **internalization** results from activation of specific sequences in the C-terminal domain, which can interact with endocytic proteins.

Receptor **dephosphorylation** can also end NMDAr activity. Most of the observations of NMDAr dephosphorylation were in heterologous expression models (Carroll & Zukin, 2002).

3. Pharmacology of NMDAr

Competitive antagonists of NMDA include: **APV**, that works especially well in NMDAr containing GluN2A subunits and less well for other GluN2 subunits; 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (**DCPP**) that works also more especially in NMDAr containing GluN2A subunits; (2R,3S)-1-[(phenanthrene-3-yl)piperazine-2,3-dicarboxylic) acid (**UBP141**) which works well for NMDAr containing GluN2C-D.

Non-competitive antagonists include (4-((1H-Indol-7yl)carbamoyl)phenyl diethylcarbamate) (**NAB14**) that targets NMDAr containing GluN2C/D subunits and **ifenprodil** that targets NMDAr containing GluN2B subunits.

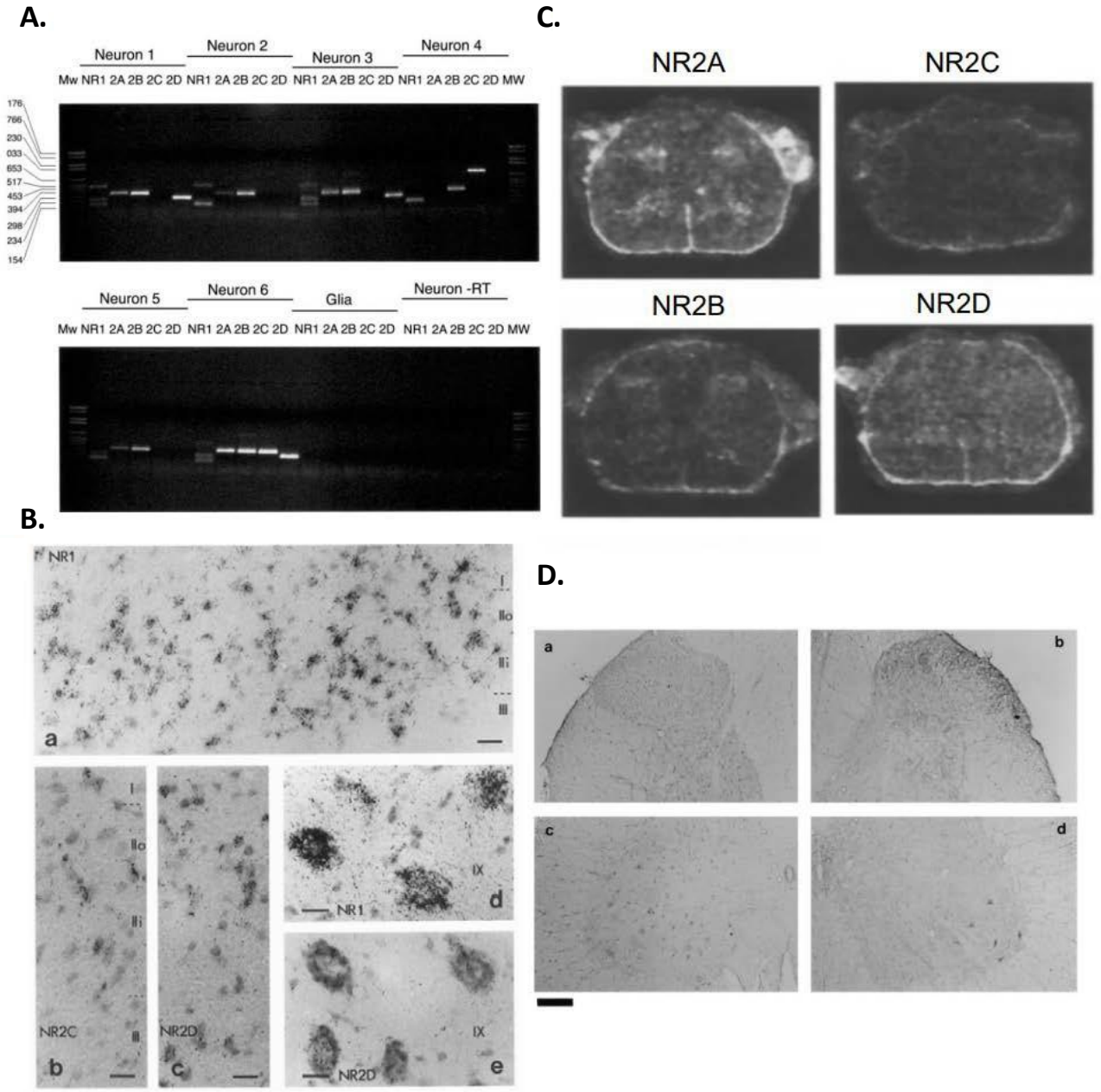


Figure 14: NMDAr subunit expression in the spinal cord

A. Detection of mRNA of different subunits of NMDAr by RT-PCR. This was done on 6 neurons, 1 glial cell and a neuron without reverse transcriptase (neuron-RT). NR1,2,3… are the old nomenclature for GluN1,2,3…From Karlsson et al., 2002.

B. Detection of mRNA of different subunits of NMDAr by radiography on a lumbar segment of the spinal cord. (a-b-c) in the LII; (d) in a motoneuron (e) in a motoneuron in the IX. Scale bar: (a) 30 µm; (b-e) 20 µm. From Tolle et al., 1993.

C. Detection of mRNA of GluN2 in transversal slices of the spinal cord. From Cumberbatch et al., 2002.

D. Detection of GluN2A in the dorsal horn (a) and the ventral horn (c) in rat transversal spinal slices. Detection of GluN2B in the dorsal horn (b) and the ventral horn (d) in rat transversal spinal slices. Scale bar: 10 µm. From Boyce et al., 1999.

Channel blockers inhibit the passage of ions through the channel (and thus require for the channel to be opened to act) and include **MK-801**, **memantine, ketamine** and **magnesium**. Interestingly, NMDAr containing GluN2C-D and GluN3A-B are less sensitive to these blockers (Henson et al., 2010).

Allosteric modulators, which act to increase or decrease the activation of the receptor, include Zn^{2+}, which can be synaptically released with glutamate and GABA, and preferentially inhibits NMDAr containing GluN2A (Paoletti et al., 2013).

NMDAr are ionotropic glutamate receptors, which need an agonist and a co-agonist to be activated. Depending on the subunit composition, NMDAr can present different properties. NMDAr activation can be modulated by a multitude of substances.

II. NMDAr expression in the DH

1. Subunit expression in the spinal cord

NMDAr are widely distributed in the spinal cord but the subunits are not all expressed homogeneously, and efforts have been made to specify their pattern of expression (figure 14). GluN1 subunits are essential for the formation of a functional NMDAr channel and are therefore ubiquitously expressed (figure 14). Regarding mRNA level, in situ hybridization experiments performed on rats indicate as expected a strong expression of GluN1 as well as for GluN2A mRNA (Häring et al., 2018; Karlsson et al., 2002; Luque et al., 1994; Monyer et al., 1994; Tolle et al., 1993) (figure 13C). In line with mRNA level, protein expression of GluN1 and GluN2A investigated by immunochemistry and electronic microscopy show a high expression in the DH, particularly important in the lamina III – IV (Boyce et al., 1999; Nagy et al., 2004; Yung, 1998) (figure 13D).

Similarly, to GluN1-2A subunits, mRNA levels of GluN2B and GluN2D subunits are found throughout the spinal cord with a lower detection for GluN2D (Karlsson et al., 2002; Luque et al., 1994; Tolle et al., 1993) (figure 13C). These results are in accordance with the expression level of these subunits, GluN2B being widely expressed, particularly concentrated within the lamina I and II while GluN2D has a lower expression (Boyce et al., 1999; Yung, 1998).

The GluN2C subunit has the lowest level of mRNA found in the spinal cord as well as the lowest expression (Tolle et al., 1993) (figure 13C). However, contradictory results have been described regarding mRNA levels of GluN2B, GluN2C and GluN2D which can be due to inefficient probes (Luque et al., 1994; Tolle et al., 1993; Yung, 1998).

Although GluN2C and GluN2D subunits exhibit the lowest expression among GluN2 subunits, functional studies support the expression of these subunits preferentially at extra-synaptic sites (Green & Gibb, 2001; Hildebrand et al., 2014; Mahmoud et al., 2020; Momiyama, 2000; Shiokawa et al., 2010). It is also important to note that they appear to be mainly found in inhibitory interneurons (Häring et al., 2018; Shiokawa et al., 2010). It could be that NMDAr containing GluN2C or GluN2D subunits, less sensitive to the magnesium block and located on inhibitory interneurons, are implicated in a specific function in the processing of nociceptive information in the DH.

Finally, a recent single cell sequencing study indicates a large distribution of GluN3A mRNA in DH neurons while GluN3B mRNA is only found in the ventral horn (Haring et al., 2018). In the DH, information on the expression or function of these subunits is almost nonexistent. In theory, one GluN3 subunit could be associated with 2 GluN1 and 1 GluN2 subunits to form a triheterotrimeric receptor with specific properties (Paoletti et al., 2013).

Subunit expression in conditions of chronic pain

Several studies have attempted to identify the type of NMDAr involved in neuropathic pain, resulting from nerve injury. This might allow the targeting of specific subtypes of NMDAr, and

maybe reduce the secondary effects of targeting all NMDAr (Lipton, 2004). At 7 and 14 days after nerve injury in rats, Karlsson et al described a reduced expression of mRNAs encoding the GluN2A subunit. In addition, their results obtained by electrophysiology in the DH indicate that the expression of GluN2B/D subunits increases (Karlsson et al., 2002).
The intrathecal injection of GluNB containing NMDAr antagonists has also been shown to reduce pain symptoms in several models of chronic pain. For example, traxoprodil, an antagonist of NMDAr containing the GluN2B subunit, reduces mechanical allodynia 7 days after spinal nerve ligation (SNL, model of neuropathic pain) (Boyce et al., 1999). Finally, in an SNL model, the injection of ifenprodil or Ro25-6981, another specific antagonist of NMDAr containing the GluN2B subunit, before nerve injury also prevents the development of mechanical allodynia (Qu et al., 2009).

To date, no antagonists of NMDAr containing the GluN2C/D subunits have been tested on pain symptoms in a pathological pain model.

2. Cellular and subcellular location

The exact cellular and subcellular location of NMDAr subunits remains to be established in the DH. Nevertheless, there are some distinctions in the expression of NMDAr depending on cell identity.

In neurons

Within the spinal cord, NMDAr are expressed by all excitatory interneurons and are mainly composed of GluN1 and GluN2A/B subunits. Most NMDAr are expressed at the postsynaptic site (Nagy et al., 2004) in the DH where they exert their classical role of coincidence detector and play a key role in plasticity mechanisms.

Interestingly, a subset of NMDAr is also expressed at the presynaptic site of excitatory neurons. This atypical expression has been shown by electronic microscopy (H. Liu et al., 1994; Zeng et al., 2006). Activation of these presynaptic NMDAr can facilitate or inhibit synaptic release through calcium or sodium increase (M. D. Glitsch, 2008; Kunz et al., 2013; McGuinness et al., 2010).

In the spinal cord, NMDAr located on excitatory neuron terminals seems to act as autoreceptors and contribute to modulate the excitatory synaptic transmission. Presynaptic NMDAr are notably expressed by 30% of type C afferent fiber and their activation increases the release of glutamate and substance P (Bardoni et al., 2004; Yan et al., 2013; Zeng et al., 2006). For a full review on presynaptic NMDAr in the DH, see Deng et al., 2019.

NMDAr are also found at the extrasynaptic site in the lamina II (Momiyama, 2000) but their role remains unclear. These receptors might be involved in the development of pain symptoms in the DH through glycine spill-over after sustained activity, glycine being necessary for the activation of NMDAr (Ahmadi et al., 2003).

Finally, NMDAr expression is almost undocumented in inhibitory interneurons within the spinal cord. Only one study using electronic microscopy has brought proof of NMDAr expression, in the adult rat DH (C. R. Lu et al., 2005). In this study, the authors showed a postsynaptic expression, as well as a presynaptic expression of the GluN1 subunit in 37% of GABAergic terminals. However, the role of these NMDAr has not been studied yet. In other regions of the CNS, such as cerebellum, neocortex, prefrontal cortex and visual cortex, immunocytochemical and functional studies have documented the presence of presynaptic NMDAr and their involvement in modulation of GABA release (Abrahamsson et al., 2017; M. Glitsch & Marty, 1999; Mathew & Hablitz, 2011; Pafundo et al., 2018).

In astrocytes

In other structures, studies have shown the presence of mRNA and protein expression of all the NMDAr subunits, as well as expression of functional NMDAr (For full review, see Verkhratsky & Chvátal, 2020). Results vary between studies, depending on how astrocytic NMDAr are studied, whether in culture, *in vivo* or in freshly dissociated cells (Balderas & Hernández, 2018). Results point towards NMDAr containing more GluN2C/D and GluN3A/B, indicating an expression of NMDAr with low magnesium sensitivity, allowing their activation with limited membrane depolarization. These subunits composition also confer to NMDAr a low calcium permeability (Lalo et al., 2011), suggesting a very localized calcium signaling. This low calcium permeability could explain the contradictory results obtained in calcium imaging in studies focusing on

functional NMDAr in astrocytes. For a full review of the history of contradictory results regarding NMDAr studies in astrocytes, see Balderas & Hernández, 2018.

In the spinal cord, no study has described the expression of NMDAr subunits in astrocytes by gene or protein expression. However, functional data indicates the presence of NMDAr in DH astrocytes. Ziak et al. showed that a perfusion of NMDA induces an inward current in astrocytes in spinal cord slices from postnatal rats (5 to 13 days old). This NMDA-induced current is reproducible and blocked by APV but is not altered by a 3 mM magnesium solution, nor by a pre-incubation in a calcium-free solution, in agreement with glial NMDAr containing GluN2C/D and GluN3A/B (Žiak et al., 1998). No study has shown the precise localization of these receptors, nor studied their function in the DH network.

NMDAr are expressed by virtually every neuron in the DH. Almost all the subunits are found in the superficial laminae of the DH, indicating the presence of NMDAr with many different biophysical properties, indicative of varied roles of the receptor in processing of nociceptive information. However, a lot remains to be understood about NMDAr expression by different cellular populations in the DH, and the different functions they perform.

III. Sources of agonist and co-agonist in the DH

As seen previously, to activate NMDAr, the fixation of two molecules of agonists, most often glutamate, as well as two molecules of co-agonist: D-serine or glycine are required.

1. Sources of agonist

Glutamate in the DH might be released by the primary afferent fibers or by local excitatory interneurons. It is also conceivable that glial cells could release glutamate in the DH and therefore represent a third source of glutamate as it has been shown in other structures (Jourdain et al., 2007; Navarrete & Araque, 2008). Interestingly, in conditions of intense primary afferents activity,

saturation of glutamate transporters may occur, leading to spill-over of glutamate in the extrasynaptic space and activating extrasynaptic NMDAr (Nie & Weng, 2010; Weng et al., 2006).

2. Sources of co-agonist

NMDAr activation also requires the fixation of two molecules of co-agonist on the GluN1 subunits. Glycine was assumed to be the main endogenous co-agonist for NMDAr in the DH, given the presence of glycinergic synaptic transmission. Indeed, glycine concentration in the DH extracellular medium is about 2.6 µM (Ahmadi et al., 2003; Whitehead et al., 2001). Furthermore, blockade of GlyT1/2 in the rat lamina X increases NMDAr activation (Bradaïa et al., 2004) and a spillover of synaptically released glycine facilitates NMDAr currents (Ahmadi et al., 2003) suggesting that glycine is an important co-agonist for NMDAr in the spinal cord.

However, other co-agonists exist such as D-serine, synthesized by serine racemase from L-serine. The localization of serine racemase was long thought to be exclusively astrocytic in the whole central nervous system. However, Miya et al, using serine racemase KO mice as negative controls and highly specific monoclonal antibodies, found that serine racemase is mainly expressed in neurons in the forebrain (Miya et al., 2008). Similar results were found in the cerebellum and in many structures since (for full review, see Wolosker et al., 2016). However, a recent study in the DH has shown that 70% of GFAP-positive cells, assumed to be astrocytes, express serine racemase. That expression is upregulated in neuropathic rats compared to sham (Lefèvre et al., 2015), suggesting an increased synthesis of D-serine in the DH in conditions of increased activity of the primary afferents. More studies are necessary to determine the different cellular localization of serine racemase.

A study from 2016 focused on availability of NMDAr co-agonists in synaptic and extra-synaptic NMDAr. By oxidating D-serine or glycine, Papouin et al. found that D-serine acts preferentially at synaptic NMDAr and glycine at extrasynaptic NMDAr in the CA1 region of the hippocampus (Papouin et al., 2012). They proposed that this preference matches the availability of coagonists in the synaptic cleft and extracellular media. It should be noted that glycine has been shown to be a negative regulator of serine racemase (Neame et al., 2019), so attention must be given to the

timeframe of experiments using glycine oxidase, as this might artificially increase the concentration of D-serine and mask any effect of the loss of glycine. Such data on co-agonist availability at different synapses is lacking in the DH.

There are different sources of agonist and co-agonist in the DH, however little is known about the specificity of a co-agonist over the other to certain NMDAr depending on the localization of the receptor, or in which cell it is expressed for example.

IV. NMDAr function in the DH

In conditions of low activity in the DH, such as no or low intensity stimulation of primary afferent fibers, most NMDAr are not activated and do not participate to the processing of nociceptive information. Indeed, since the most common form of NMDAr are composed of GluN2A/B subunits and are postsynaptic, sustained activity is needed to unblock the channel from Mg^{2+} and recruit NMDAr. It is for instance shown that NMDAr antagonist injection in healthy animals does not modify synaptic transmission or impact nociceptive sensitivity baseline levels (Woolf & Salter, 2000).

The DH is a plastic network that can go through structural and functional changes under specific circumstances such as intense or repeated stimulation of primary afferent fibers. NMDAr play a crucial role in these plasticity mechanisms due to their high permeability to calcium. They have been shown to induce various changes in the excitatory and inhibitory synaptic transmission in the DH.

In conditions of physiological pain, such as inflammation, when primary afferents activity is increased, NMDAr have been shown to enable facilitation of nociceptive information processing (Cheng et al., 2008; Ren & Dubner, 1993; South et al., 2003; Weyerbacher et al., 2010). Indeed, using NMDAr antagonists suppresses pain hypersensitivity and development of hyperalgesia and spontaneous pain in models of acute inflammation (Petrenko et al., 2003).

NMDAr blockers are also effective in reducing pain symptoms in several models of neuropathic pain. For example, intrathecal injection of memantine, an ion channel blocker of NMDAr, reduces mechanical allodynia in mice seven days after SNL (Carlton & Hargett, 1995). Similarly, intrathecal injection of MK-801, another channel blocker, or the NMDAr antagonist APV, decreases thermal hyperalgesia and reduces hyperesthesia following chronic constriction injury (CCI, a model of nerve injury) in rats without affecting the sensitivity of the contralateral paw (Seltzer et al., 1991; Tal & Bennett, 1994; Yamamoto & Yaksh, 1992). Finally, in a CCI model, intrathecal injection of APV reduces mechanical allodynia (Bennett et al., 2000). In all the studies, except the one of Seltzer et al. (1991), intrathecal injections were performed between 7 and 24 days following the lesion or constriction of the sciatic nerve, a time when neuropathic pain is already well established in the animal. The effectiveness of these antagonist injections in reducing pain symptoms seems to indicate that NMDAr play an important role in maintaining neuropathic pain long-term. However, it should be noted that the intrathecal injection of memantine and APV just before the CCI significantly decreases self-injury in rats (Seltzer et al., 1991). Likewise, injecting ifenprodil before performing SNL prevents the development of mechanical allodynia (Qu et al., 2009). It therefore seems that the activation of NMDAr also has an important role in the establishment of neuropathic pain. The mechanisms by which NMDAr are implicated in the development of chronic pain are still being studied.

1. NMDAr-dependent plasticity of excitatory transmission

NMDAr and wind-up

NMDAr are also responsible for the "Wind-up" phenomenon. This is an activity-dependent plasticity observed within the lamina V. This homosynaptic plasticity is characterized by a progressive increase of neuronal activity following stimulation of C fibers (Mendell, 1984). An intrathecal injection of NMDA in the rat facilitates the wind up (Chapman et al., 1994). Furthermore, the wind up is strongly reduced by APV, an antagonist of NMDAr (Thompson et al., 1990).

NMDAr-dependent LTP and LTD at glutamatergic synapses

NMDAr-dependent long-term potentiation (LTP) of the glutamatergic synapse is one of the best

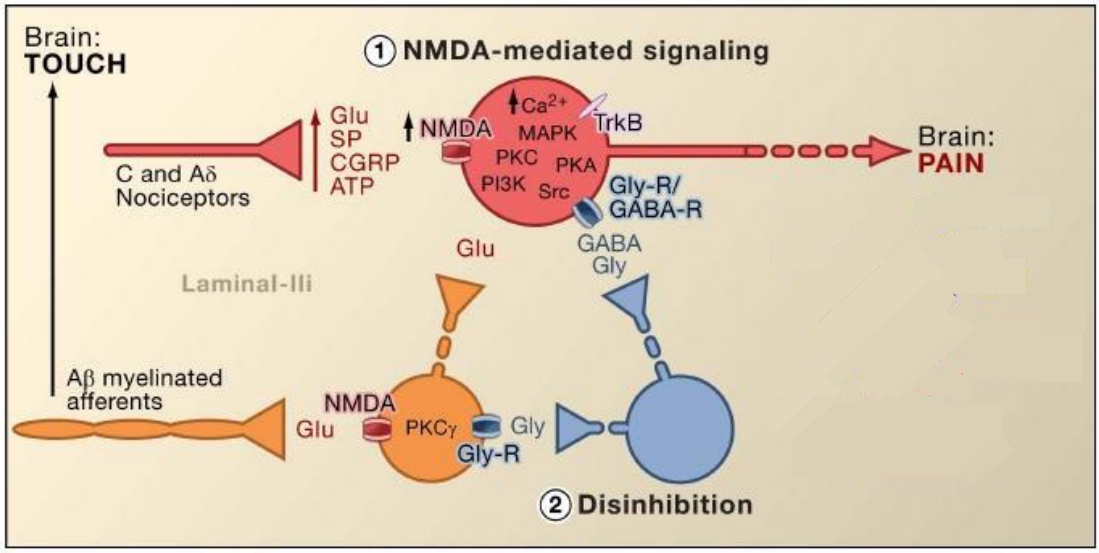


Figure 15: Central sensitization

In this illustration are represented 2 of the mechanisms leading to central sensitization:

1. NMDAr-dependent sensitization: After a sustained and intense activity of the primary afferents, like in conditions of nerve lesion, NMDAr are recruited in the DH network, leading to plastic changes of transmission and an increase in neuronal excitability and nociceptive information transmission.

2. Disinhibition: represented in blue, inhibitory interneurons play a very important role in normal processing of nociceptive information in the DH (Sandkühler., 2009). In certain pathological conditions, inhibitory transmission is reduced in the network, leading to the development of abnormal pain perceptions, such as allodynia, by the processing of innocuous information in the nociceptive network, and hyperalgesia. The mechnisms leading to this disinhibition are still the key focus of many studies today.

Modified from Basbaum et al., 2009

described plasticity mechanisms in the central nervous system. It is characterized by an increase of the synaptic transmission between two neurons following an intense activity of the primary afferents. This plasticity occurs in the DH between nociceptive fibers (C and Aδ fibers) and DH projection neurons after applying a high frequency stimulation protocol (Ikeda et al., 2003; Ranclic et al., 1993; Svendsen et al., 1999; Zeng et al., 2006). In the DH, NMDAr-dependent LTP is expressed at the postsynaptic element and requires an influx of Ca^{2+} through the NMDAr (Ikeda et al., 2006). This leads to activation of signaling pathways such as CamKII, PKA, PLC, IP3 and ERK, expression of neuronal proteins (AMPA receptors for example), phosphorylation of receptors (AMPA/NMDA), etc. Sandkuhler and Liu showed in 1998 that this LTP is blocked by NMDA antagonists (Sandkühler & Liu, 1998).

Few studies have shown NMDAr-dependent LTD of glutamatergic synapses in the DH. One study showed NMDA-induced LTD at synapses between the primary afferents and the DH interneurons (Ranclic et al., 1993). There are also more examples of NMDAr-dependent LTD in the SNC. These mechanisms often consist of AMPA receptors internalization following a low frequency stimulation protocol of the presynaptic neuron, leading to a decrease of the synaptic transmission. More details about NMDAr-dependent LTP and LTD can be found in this review: Lüscher & Malenka, 2012.

NMDAr-dependent central sensitization

The intrathecal injection of NMDA or iontophoretic application to the DH of naive rats is sufficient to induce central sensitization (Aanonsen et al., 1990; Aanonsen & Wilcox, 1987). This is a reversible process like NMDAr-dependent LTP, which requires an increase of free intracellular Ca^{2+}, mainly coming from NMDAr activation but can also take place through AMPA receptor activation (Larsson & Broman, 2008) or voltage dependent calcium and intracellular Ca^{2+} stores (Woolf & Salter, 2000). The increase of intracellular Ca^{2+} activates CaMKII (Lisman et al., 2002) as well as PKC which are two major effectors of central sensitization. The subsequent activation of PKA and ERK allow PKC and CaMKII to phosphorylate AMPA and NMDA receptors, modifying their properties. At the same time, the insertion of new AMPA receptors into the membrane and transcriptional modifications occurs. These cellular processes can lead to central

sensitization and to the development of symptoms such as allodynia or hyperalgesia (figure 15). For full review of these mechanisms, see (Latremoliere & Woolf, 2009).

In addition to this increase in excitatory transmission, central sensitization in the DH is associated with network disinhibition.

2. NMDAr-dependent plasticity of inhibitory transmission

Following a nerve injury, the primary fibers develop an exacerbated spontaneous activity leading to the release of glutamate in the DH and the recruitment of NMDAr (Latremoliere & Woolf, 2009; X. Liu et al., 2000; Wall et al., 1974; Wu et al., 2001). This can eventually lead to the development of central sensitization, and an increase in excitation in the network. Associated to this is a decrease in efficiency of inhibition in the network (figure 14).

NMDAr-dependent modulation and plasticity of GABAergic inhibitory synaptic transmission

In many areas of the CNS, NMDAr are involved in the plasticity of inhibitory synaptic transmission (Castillo et al., 2011; Kullmann et al., 2012). Yet only one study in the DH shows that NMDAr could play a role in the plasticity of glycinergic synaptic transmission (Kloc et al., 2019). In this study, NMDAr activation increases glycinergic synaptic transmission onto GABAergic interneurons in the lamina II. This LTP mechanism requires calcium influx in the postsynaptic neurons following NMDAr activation and is expressed at the postsynaptic element, shown by the absence of paired pulse ratio modification.

To my knowledge, no study has reported plasticity of GABAergic synaptic transmission induced by NMDAr in the DH. However, mechanisms of plasticity occurring in other structures could also take place in the DH. It is for instance known that NMDAr plays a crucial role in plasticity of GABAergic synaptic transmission in the VTA, cerebellum, and other structures, notably by the synthesis of retrograde messengers such as nitric oxide, as presented in the second part of this

introduction (part II-chapter IV) (Nugent et al., 2007). And as mentioned previously, a study from 2005 shows that 37% of GABAergic terminals express NMDAr (C. R. Lu et al., 2005).

NMDAr-dependent modification of KCC2

In 2003, it was shown that following chronic constriction injury (CCI) in rats, the expression of KCC2 is decreased in lamina I neurons (Coull et al., 2003). By artificially increasing expression of KCC2, Li et al. restored the function of the co-transporter following a spared nerve ligation and re-established a physiological chloride gradient. In addition, the increased expression of KCC2 restores physiological activity of NMDAr (Li et al., 2016). Similarly, the pharmacological blockade of NMDAr after SNL restores physiological chloride gradient and synaptic inhibition (Lee et al., 2011; Zhou et al., 2012). NMDAr probably play an important role in the regulation of the KCC2 transporter and thus, inhibitory transmission regulation through modulation of the chloride gradient in the DH.

NMDAr-dependent loss of inhibitory interneurons

A prolonged exposure to glutamate can induce excitotoxicity leading to neuronal death by calcium-activated caspases (Choi, 1994). Several teams have therefore studied whether a loss of neurons and more particularly inhibitory neurons takes place in the DH following a nerve injury, which are conditions of heightened glutamate synaptic transmission, to explain the decrease in efficiency of inhibition in the DH following a nerve injury. The results obtained have been the subject of controversy. Using the TUNEL (Terminal Transferase dUTP Nick End Labeling) to detect cells undergoing apoptosis, several studies have reported apoptosis in rat and mouse DH that starts as early as one hour after the injury and peaks at seven days (Inquimbert et al., 2018; Moore et al., 2002; Polgár et al., 2005; Scholz et al., 2005). Detection of the activated form of caspase 3 has also been used as an apoptosis marker (Scholz et al., 2005). Moore et al. (2002) report neuronal apoptosis via the use of the NeuN marker just like Scholz et al. (2005) who observe a co-localization between NeuN and the active form of caspase 3 in a spared nerve injury model (SNI) (Scholz et al., 2005).

Conversely Polgár et al. (2005) find that TUNEL positive cells are labeled with the microglial marker Iba-1 following an SNI and conclude that the observed cell death corresponds to a loss of

glial cells (Polgár et al., 2005). By stereological methods, Scholz et al. estimate neuronal loss at 20% in laminae I to III four weeks after SNI while Polgar et al. found no loss of neurons in these regions two and four weeks after an SNI or CCI (Polgár et al., 2005; Scholz et al., 2005). These contradictory results may be partly explained by the analytical approaches and techniques used, which differ between studies. However, several studies have confirmed the results in favor of neuronal loss under neuropathic conditions and more specifically the loss of inhibitory interneurons (Inquimbert et al., 2018; Scholz et al., 2005; Yowtak et al., 2013).

Regarding inhibitory interneurons, two studies in the 1990s report almost a total loss of neurons by labeling the neurotransmitter GABA between 3 days and 2 weeks later CCI in rats (Eaton et al., 1998; Ibuki et al., 1996). More recent studies report a more moderate loss: a 25% loss of mRNA for GAD67 4 weeks after SNI in the rat (Scholz et al., 2005) and about 10% loss of GABAergic neurons in lamina II one and two weeks after SNL or SNI in mice (Inquimbert et al., 2018; Yowtak et al., 2013). Recently, following an SNI, it has been demonstrated that there is an irreversible loss of inhibitory neurons dependent on NMDAr activation, contributing to the maintenance of neuropathic pain (Inquimbert et al., 2018). The authors also show that NMDAr play an essential role in this phenomenon of excitotoxicity. Indeed, the conditional deletion of the Grin1 gene encoding the GluN1 subunit before SNI prevents the loss of inhibitory neurons. Interestingly, the deletion of the Grin1 gene did not prevent the development of mechanical allodynia but prevents maintenance of neuropathic pain.

For a full review on compromised inhibition in conditions of pathological pain, see Gradwell et al., 2020.

3. Astrocytes in NMDAr-dependent modulation of transmission

In their 2010 study, Bardoni et al. use biphotonic calcium imaging to visualize intracellular calcium signaling in rat DH astrocytes (Bardoni et al., 2010). The authors used BzATP, an agonist of purinergic receptors expressed in atrocytes, or low extracellular calcium to trigger Ca^{2+} elevations in astrocytes. They recorded slow inward currents (SICs) in neurons as a result of these Ca^{2+} elevations. These SICs can be blocked by APV. The authors proposed that the astrocytes

released glutamate, inducing synchronous activity of the neurons in LII. In a model of peripheral inflammation, more spontaneous SICs were recorded, correlated with the development of thermal hyperalgesia and mechanical allodynia. As mentioned previously, an interesting property of astrocytes, is that a single astrocyte can contact up to 100,000 synapses in the mouse central nervous system, allowing for large scale modulation of neuronal networks (N. J. Allen & Eroglu, 2017). Given the importance of astrocytes in physiological nociceptive information processing (Foley et al., 2011) and their emerging role in the development and maintenance of pathological pain (Ji et al., 2013; Kronschläger et al., 2016), more studies focusing on NMDAr, astrocytes and plasticity of synaptic transmission in the DH are needed.

Most NMDAr are not activated in conditions of low intensity stimulation of the primary afferents. Their activation is associated with the development of central sensitization, which can occur after intense and sustained stimulation of the primary afferents, leading to abnormal pain sensations. The exact mechanisms leading to central sensitization are still being dissected.

Conclusion of part 3

NMDAr are glutamate gated-ionic channels. They are expressed by virtually every neuron in the superficial laminae of the DH. In conditions of low intensity activity of the primary afferents, most NMDAr are not activated. However, in conditions of sustained and intense activity of the primary afferents, NMDAr are recruited, leading to plastic changes in the DH network. NMDAr-dependent increase in excitatory transmission and disinhibition are 2 of the mechanisms that lead to central sensitization in the DH, a state resulting in abnormal pain perceptions. However, questions remain as to the role of "atypical" subunit containing NMDAr, such as GluN2C-D containing NMDAr, in the physiological and pathological processing of nociceptive information. Very little is known on the potential role of NMDAr in modulation of inhibitory transmission in the DH, when this could allow for better

understanding of crosstalk between excitation and inhibition in the DH, and its malfunction in conditions of pathological pain.

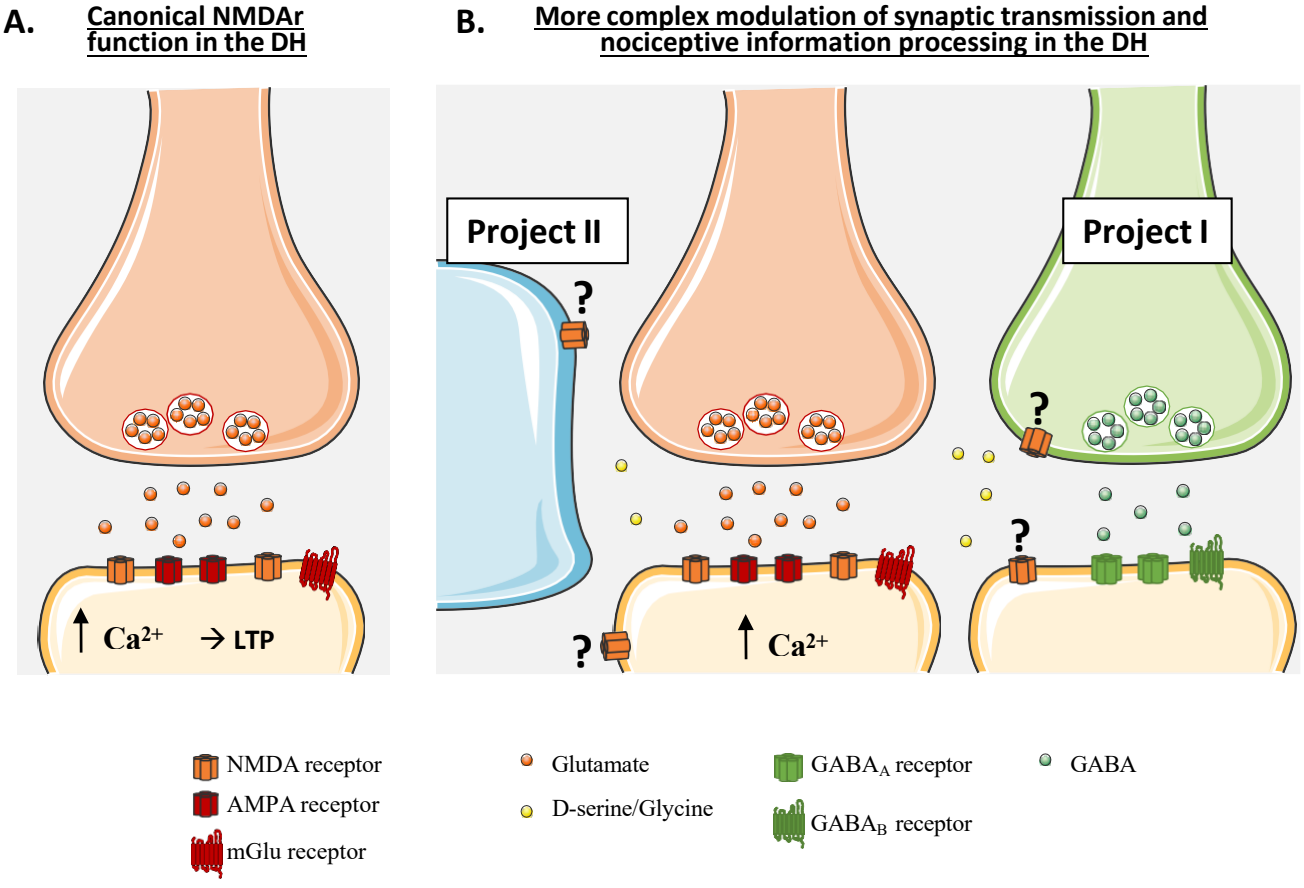


Figure 16: My PhD projects

A. Illustration of the canonical function of NMDAr as post-synaptic coincidence detectors with a high permeability to calcium. These receptors usually contain GluN2A/B subunits and play an important role in post-synaptic plastic changes of synaptic transmission.
B. Recent studies focusing on subunit expression, co-agonist, subcellular and cellular localizations describe a more complex view of NMDAr function in the DH. Many questions remain, indicated on the illustration by a question mark, and my PhD project is articulated around them.

PhD project objectives

We have seen that lamina II is essential for the processing of nociceptive information. Indeed, a complex network of interneurons and glial cells integrate nociceptive information before it is transmitted to supra-spinal structures for further integration. GABAergic synaptic transmission plays a key role in nociceptive integration, allowing for normal processing of nociceptive information in the DH. In conditions of intense and sustained activity of afferent fibers, plastic changes can occur, leading to central sensitization. Among the mechanisms leading to central sensitization, NMDAr recruitment and network disinhibition have been identified. However, no studies have focused on the role of NMDAr on modulation and plasticity of GABAergic synaptic transmission in the DH.

The objective of my doctoral work was to study the implication of NMDAr on modulation of GABAergic synaptic transmission in the lamina II of the DH, with a focus on the role of astrocytes.

My work was divided into 2 projects:

Project I was focused on characterizing NMDA-dependent modulation of GABAergic synaptic transmission, trying to isolate a potential target specificity of such a modulation by recording both putative GABAergic and glutamatergic neurons and on the implication of astrocytes in this modulation (figure 16).

Project II was focused on the characterization of NMDAr in astrocytes of the DH. Many studies have described such receptors in astrocytes of other structures, but very little is known about NMDAr in spinal astrocytes (figure 16).

Material and Methods

During my PhD thesis, I was involved in 2 main projects: (1) the implication of astrocytes in NMDA-dependent modulation of spinal inhibition and (2) the study of the potential expression of NMDAr by DH astrocytes. For the project (1), results were obtained with electrophysiological recordings on acute slices of adult mouse spinal cord. Results for the project (2) were obtained by calcium imaging and electrophysiological recordings on cultured astrocytes and electrophysiological recordings of astrocytes in acute slices of adult mouse spinal cord. The next paragraphs will detail the experimental approaches for each project.

I. Ethics

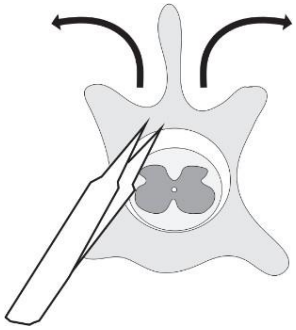
All experiments were conducted in accordance with the guidelines of the Ministère de l'Education Supérieure et de la Recherche (authorization #2015030911301894) and the local ethical committee, the Comité Régional en Matières d'Expérimentation Animale de Strasbourg (CREMEAS). All animals used were born and bred in the "Chronobiotron" animal housing facility (UMS3415), with food and water ad libitum and a day/night photoperiod of 12 hours/12 hours.

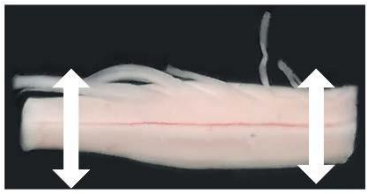
II. Implication of astrocytes in NMDA-dependent modulation of spinal inhibition

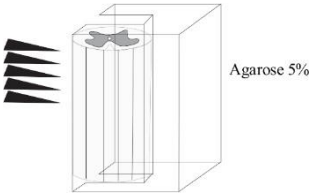
1. Animals

Our study was realized on C57bl/6J and heterozygous GAD65::eGFP adult male mice (characterized in Cui et al., 2011) , aged between 6 and 10 weeks.

A. Laminectomy **B. Selecting the lumbar segments** **C. Slicing with the vibratome**







- Under deep anethesia (uréthane 1,9 mg/kg)

- slices of 300 µm thickness
- sACSF at 0-4°C

Figure 17: Laminectomy and slices of spinal cord

2. Surgery and slices

The next steps were all performed using an artificial cerebro-spinal fluid (ACSF) enriched with sucrose (sACSF). This solution was kept at 4°C and saturated with carbogen (95% O_2; 5% CO_2), allowing to oxygenate the tissue and buffer the pH at 7.3. Both ACSF and sACSF were prepared from concentrated stock solutions (table 1).

In this solution of sACSF, the sucrose replaces the NaCl, limiting the genesis of action potential by neurons, and thus protecting the cells from an excessive liberation of glutamate and the resulting excitotoxicity during the section of the tissue. With this same objective, an antagonist of glutamate ionotropic receptors, kynurenic acid (2mM), is added to the sACSF.

Laminectomy

To deeply anesthetize mice, urethane (1.9 g/kg) was injected into the peritonea. Urethane leads to a deep and stable anesthesia, controlled by testing the nociceptive and oculi-palpebral reflexes. The skin of the back of the animal was shaved and cut along the spine. The paravertebral muscles were removed, to see the vertebrae. With laminectomy forceps (FST by Dumont n°11223-20), the dorsal part of the vertebrae was delicately broken and pulled off, starting in the sacred zone and moving towards the cervical zone. The meninges were carefully removed with fine forceps and micro-scissors (FST by Dumont n°15018-10). The spinal cord was then quickly levied out of the spine and placed in a petri dish containing sACSF. The animal was killed by decapitation.

Once the spinal cord is in the petri dish, the remaining meninges, and the dorsal roots as well as the ventral roots were removed with iridectomy scissors (figure 17).

Slices

After isolating the lumbar section of the spinal cord, it was placed in a 5% agarose cube, to support the tissue during the slicing. The cube was then glued onto the metallic stage of the vibratome (Leica VT1200S) and placed in the slicing chamber, filled with sACSF. Transversal slices of 300 µm thickness were obtained, with a slicing speed of 0.05 mm/second and with a slicing amplitude of 2mm (figure 17). The slices were then kept in ACSF at room temperature for at least 1 hour before the start of recordings.

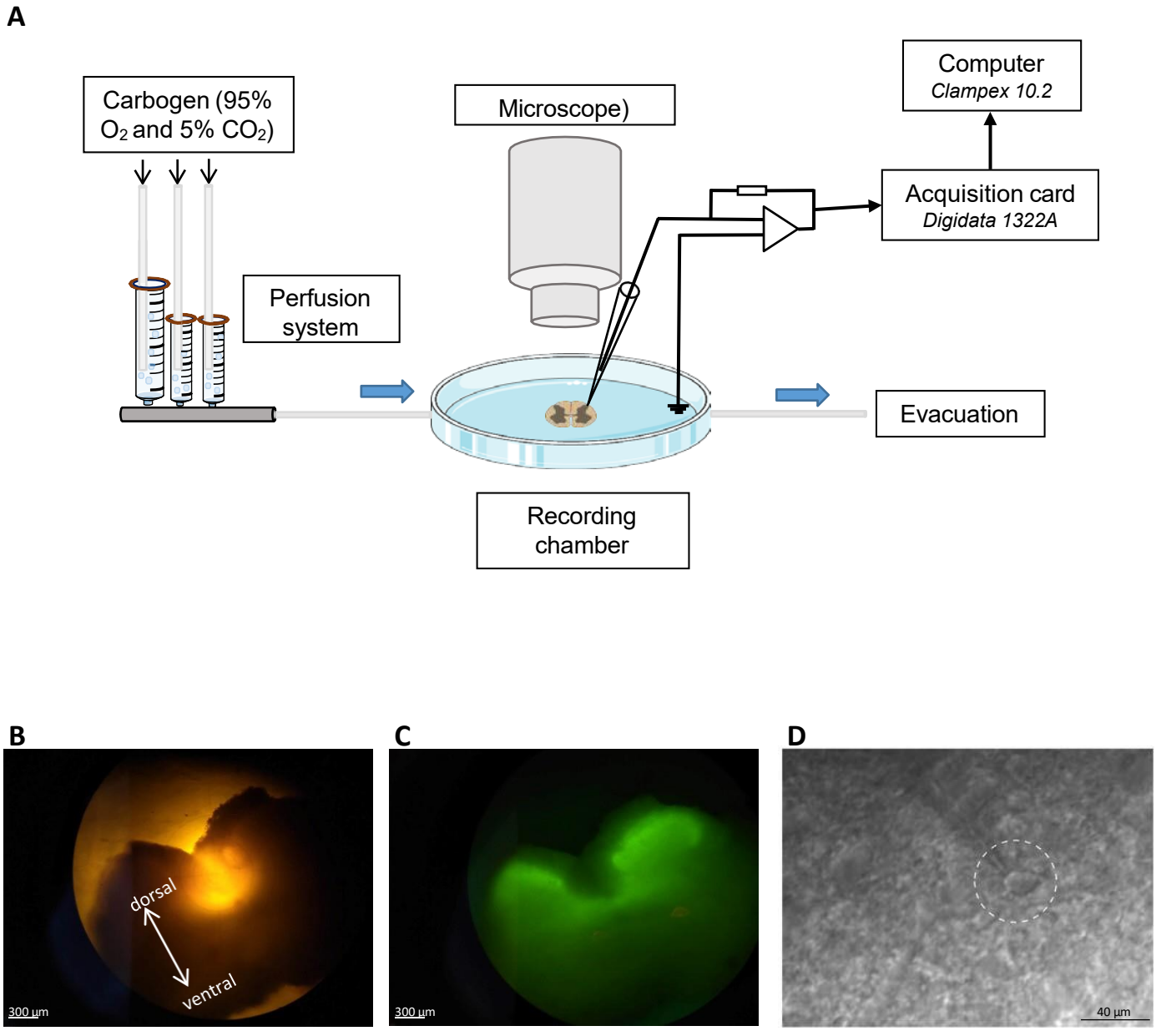


Figure 18: Patch-clamp electrophysiology set-up and observations of the DH of the spinal cord, with and without fluorescence, and an example of a recorded neuron.

A. Patch-clamp electrophysiology set-up
B. Picture of a spinal slice under the microscope at 10x.
C. Visualization of eGFP in a spinal slice, obtained from a GAD65::eGFP mouse, at 10x.
D. Picture of a recorded neuron, at 40x.

3. Electrophysiological recordings

A spinal slice was placed in the recording chamber of the microscope and immobilized by a U-shaped platine harp. The recording chamber was continuously perfused with ACSF, saturated with carbogen, at room temperature (20-22°C). The perfusion was performed with a gravity-driven system at a flow rate of 1mL/min. The liquid level in the recording chamber was controlled by aspiration of excess liquid using a peristaltic pump (Minipuls 2, Gilson), allowing for maintaining a constant ACSF level in the recording chamber (2 mL) (figure 18A).

The reference electrode was a silver chloride filament placed in the recording chamber. The slices were visualized with an upright microscope (Axiscop 2, Zeiss) mounted on a Gibraltar X-Y table. Lamina II was visualized at the smallest magnification (x10), as a translucent crescent in the DH. The x40 immersion magnification (Zeiss) and the infrared camera (IR-DIC, Till Photonics Gmbh, Germany) were used to visualize and target the neurons of interest. We used GAD65-eGFP transgenic to identify GABAergic neurons. Indeed, in these mice, the expression of eGFP is driven by the promoter of GAD65, one of the 2 neuronal enzymes responsible for the synthesis of GABA in neurons. Thus, GABAergic neurons were characterized by the presence of a green fluorescence (Cui et al, 2011) (figure 19). Neurons were chosen depending on their morphological appearance and presence/absence of fluorescence. In this manuscript, $eGFP^+$ neurons are referred to as GAD^+ neurons and $eGFP^-$ cells referred to as GAD^- neurons (figure 18B, C, D).

Pipettes and intracellular solutions

The recording pipettes were made from borosilicate glass capillaries (CC120F-10 model; external diameter = 1.2 mm; internal diameter = 0.69 mm; Warner Instruments, Harvard Apparatus), pulled with a horizontal puller (P1000; Sutter Instruments Co, Novato, California, USA). The micropipettes were filled with the intra-pipette solution (table 2), set at a pH of 7,3±0,1 with cesium hydroxide and an osmolarity of 300±10 Mosml with sucrose.

The pipette filled with the intracellular solution was placed on the electrode holder of the preamplifier linked to the amplifier (Axopatch 200B, Axon Instruments). Using a micromanipulator (MPC-200, Sutter Instrument), the tip of the pipette was carefully placed in contact with the targeted cell.

Table 1: composition of extracellular solutions (in mM)

Substances	sACSF	ACSF	ACSF 0 Mg2+	HEPES
Sucrose	252	0	0	0
NaCl	0	126	126	135
KCl	2,5	2,5	2,5	5
CaCl2	2	2	2	2,5
MgCl2	2	2	0	1
Glucose	10	10	10	10
NaHCO3	26	26	26	0
NaH2Po4	1,25	1,25	1,25	0
HEPES	0	0	0	10

Table 2: composition of intrapipette solutions (in mM)

Substances	Neuron recording solution	Astrocyte recording solution
CsCl	130	0
HEPES	10	10
MgCl2	2	0
Biocytine	10	10
Potassium gluconate	0	135
KCl	0	7,5
Sodium phosphocreatine	0	10
MgATP	0	2
NaGTP	0	0,3

Types of recording

To isolate GABAergic synaptic transmission, 6-cyano-7-nitroquinoxaline -2,3-dione (CNQX, 10 µM, Sigma) was used to block fast glutamatergic transmission through AMPA/Kaïnate receptors and strychnine (1 µM, Sigma) was used to block glycine receptors. We studied the effect of the activation of NMDAr on the modulation of GABAergic synaptic transmission in the lamina II. We recorded spontaneous inhibitory postsynaptic currents (sIPSC) with the patch-clamp technique, in the whole cell configuration and with a holding potential of -60 mV (figure 19). The reversal potential of Cl^- in our experimental conditions was 0 mV. Therefore, at the holding potential of -60mV and the IPSC appeared as inward currents.

Miniature IPSC (mIPSC) were recorded in the presence of tetrodotoxin (TTX 0.5 µM, Latoxan) in the extracellular media. TTX blocks voltage dependent Na^{2+} channels, thus blocking genesis of action potentials. Under these conditions, the probability of synchronization of multiple synaptic release sites is very low, allowing to estimate the properties of single release sites. The study of mIPSC gives information on the probability of release, the filling of the synaptic vesicles and the state of the postsynaptic receptors. A change in the frequency of mIPSC without a change in mIPSC amplitude, serves as an indicator of a presynaptic modulation, whereas a change in kinetics and/or amplitude indicates a postsynaptic modulation of synaptic transmission.

Recording

The recording pipette was placed in the recording chamber filled with ACSF, with positive pressure inside. The pipette was then approached to the cell. The depression caused by the release of the positive pressure allows for sealing of the membrane to the glass pipette. When the resistance of the seal equals at least 1GOhm, the configuration obtained is called “cell attached” (figure 19). The capacitive currents of the pipette were compensated with the amplifier. To access the “whole cell” configuration, the holding potential was fixed at -60 mV and the piece of membrane in the pipette is ruptured by a short suction (figure 19). The series capacitance and resistance were manually compensated. The series resistance is equal to the pipette resistance (indicated when the pipette is put into the solution filled recording chamber) and the access resistance, which reflects the quality of the access to the intracellular space. Only cells with a series resistance below 30 MOhm were kept for recording and analysis. The series resistance was taken again at the end of the recording. If a variation of at least 20% or more was observed between the series resistance at

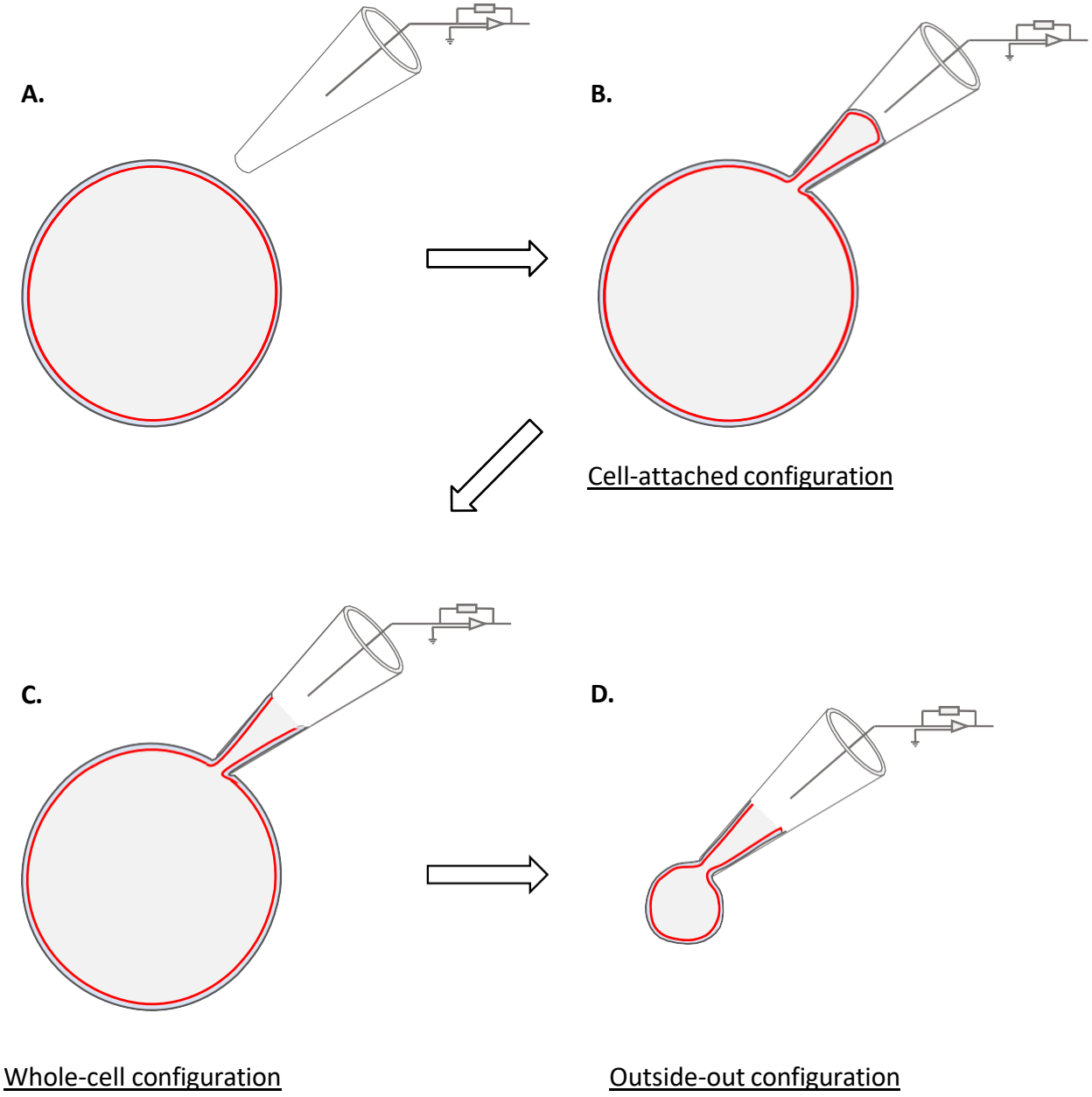


Figure 19: Patch-clamp configurations used in my PhD projects.

the beginning and at the end of the recording, the cell was not kept for further analysis. The pipette offset was also checked at the end of each recording: when the offset was larger than +/- 5 mV, the cell was not kept for analysis.

Acquisition and storage of data

The recordings were done with an Axopatch 200B amplifier (Axon Instruments, USA). The values of the steady holding membrane potential were set at -60mV (neuron recording) or -80mV (astrocyte recording). The electrical signal obtained from these recordings was filtered with a low-pass filter of 5 kHz and digitized with an analogic/digital interface (digidata 1322A) at a sampling frequency of 10 kHz before being stored on the computer's hard drive of the computer. Recordings were observed in real time with the software Clampex 10.2.

4. Pharmacology

Drugs were prepared as stock solutions 1000 to 10000x concentrated (table 3).

Fluorocitrate (FC) was used to study the implication of astrocytes. FC is preferentially taken up by astrocytes in the nervous system and inhibits the enzyme aconitase and the oxidation of citric acid, a step in the Krebs cycle, thus blocking ATP production in astrocytes. Fluorocitrate is available as barium salts, which blocks barium-sensitive potassium channels. To avoid this effect, barium ions were removed by using the following protocol: 8 mg of the barium salt, add 1 mL of hydrochloric acid 0.1M (HCl) and vortex until the powder is dissolved. Then add a few drops of sodium sulfate 0.1M (NaSO4) and 2 mL of sodium hydrogenophosphate 0.1M (Na2HPO4), then centrifuge for 10 min at 3000 rpm. The supernatant was then harvested and diluted in ACSF (Paulsen et al., 1987). The slices were incubated in the solution of ACSF containing 100 µM of FC for 30 min before being recorded. The incubation period and the concentration were chosen to block astrocytes with minimal damage to neurons (Swanson & Graham, 1994). Recording of cells from the incubated slices must start within 15 min of the end of the incubation, otherwise the effect of FC is washed out.

Table 3: Pharmacological drugs and their properties

Name	Pharmacological properties	Solvant	Stock concentration	Use concentration	Supplier
Kynurenic acid	Ionotropic glutamate receptors antagonist	sACSF	/	2 µM	Sigma
Strychnine	Ionotropic glycine receptor antagonist	DMSO	1 mM	1 µM	Sigma
CNQX	AMPA receptor antagonist	DMSO	100 mM	10 µM	Tocris
TTX	Na+ channel antagonist	H2O	1 mM	0.5 µM	Latoxan
NMDA	NMDA receptor agonist	H2O	100 mM	100 µM	Tocris
APV	NMDA receptor contaning GluN2 antagonist	H2O	50 mM	50 µM	Abcam
TBOA	EAAT antagonist	DMSO	50 mM	50 µM	Tocris
NAB14	NMDA receptor containing GluN2C/D antagonist	DMSO	10 mM	10 µM	Aobious
D-serine	NMDA receptor co-agonist	H2O	50 mM	100 µM	Sigma
Fluorocitrate	Aconitase inhibitor (Krebs cycle blocker)	ACSF	/	100 µM	Sigma
DAAO	D amino acid oxidizer	H2O	50 mM	50 µM	Abcam

5. Data analysis

To optimize analysis and the results, files were anonymized to allow for blind analysis.

Detection of postsynaptic currents

Analysis of synaptic events/currents was performed with WinEDR (V3.8.6, Strathclyde Electophysiology Software, John Dempster, University of Strathclyde, Glasgow, UK). The software can be programmed for threshold detection of post-synaptic currents, setting a minimum value for amplitude of -3mV during at least 1ms: the software will then detect all inflections towards the bottom of at least 3mV during at least 1ms. It is then necessary to visually validate all events detected by the software, to keep only the synaptic currents: events with a fast opening (around 1 ms) an exponential decrease (the closing of channels).

Analysis of the frequency of CPS

To evaluate whether a substance influenced the frequency of post-synaptic currents, the cumulative distribution of events was plotted as a function of time during the control period and during an application of a drug using Kyplot (2.0, Koichi Yoshioka) (figure 20). This distribution was fitted by the sum of 2 linear components:

$$\mathbf{N = f_o t + a} \qquad \mathbf{for\ t < t_c}$$

and

$$\mathbf{N = f_c t + (f_o - f_c)t_c + a} \qquad \mathbf{for\ t \geq t_c}$$

Where f_o represents the frequency during baseline and f_c the frequency during an application of the studied substance. Tc is the value of time corresponding to the break between the curve of the baseline and the curve of the application of NMDA, so the moment where the frequency is changed. “a” is a constant in the equation of the curve, where it intersects with the Y axis. To make sure the change in frequency is due to the applied substance, t_c must be within 100 seconds of the

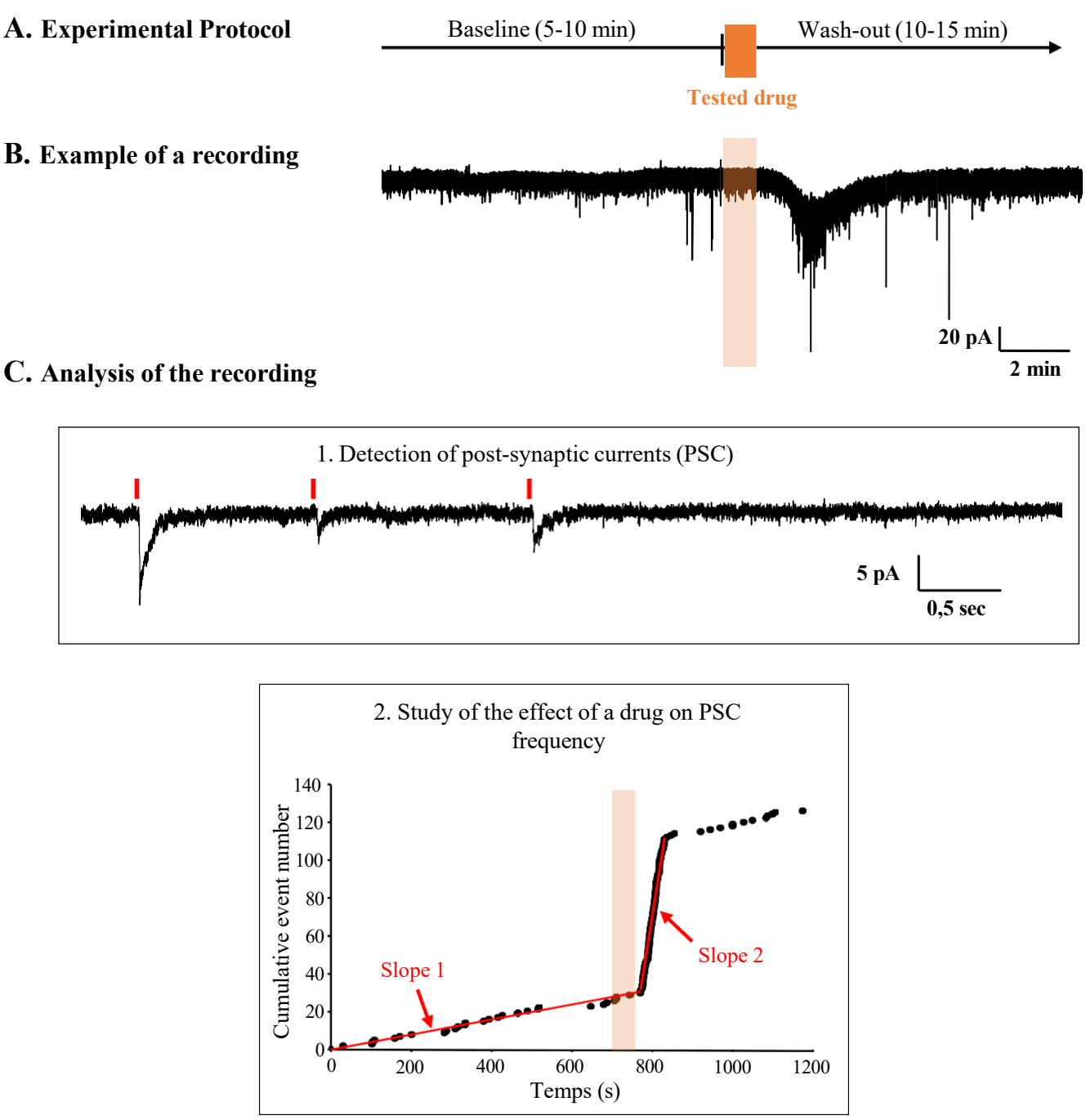


Figure 20: Experimental protocol to test the effect of drugs on post-synaptic current frequency

A. The protocol can be separated into 3 parts: baseline recording of the spontaneous activity of the cell, followed by an application of a tested drug and the washout period.
B. Example of a raw trace obtained with the experimental protocol in A (the drug tested is NMDA, 100µM)
C. 1. The analysis software WinEDR detects all events of more than -3pA. All detected events must be visually confirmed so that only PCS are selected. 2. With KyPlot, the cumulative event number can be plotted as a function of time, and 2 slopes can be analyzed: the baseline slope (slope 1) and the application slope (slope 2). In this example, NMDA (100 µM) was applied and we can see a break at around 800 seconds. A fit function on KyPlot can quantify the break in the curve, as well as the 2 slopes. To standardize the results, only breaks in the curve superior to 20% were interpreted as an increase in frequency induced by the tested drug.

beginning of the application. This length was also adapted to the speed of the gravity-dependent perfusion system, as well as the depth of the cell in the slice: if the cell was very deep, the break in the curves could appear a bit delayed. To standardize the effect of a drug on the frequency of PSC, only when the variation between the control curve and the drug curve was superior to 20% was the cell categorized as having an effect of NMDA.

The change in frequency of PSC was calculated like this:

$$\textbf{Frequency change (\%)} = ((f\text{c} - f\text{o}) / f\text{o}) * 100$$

The frequency of PSC during the 200 seconds before the application of NMDA was called control frequency and served as a control to compare the frequency during the application of a studied drug, obtained by the frequency for 200 seconds starting when there's a change in the slope of the distribution. To check whether the effects of the drug were reversible, the frequency of the last 200 seconds of the recording was also kept and called the wash-out frequency. Some cells were not recorded until the wash-out, being too unstable after the application of the studied drug, however if the recordings covered the whole period of the application, the cells were kept.
In addition to comparing an application period to the control and wash-out periods, control and drug application periods of different cells were compared to each other.

Analysis of the amplitude of PSC

Once the frequency of cells was analyzed, the amplitudes could be obtained with WinWCP (V5.4.5), using the waveform measurement protocol which calculates the exact value of the peak compared to the baseline. Once plotted, PSC amplitudes from the baseline, drug application and wash-out periods were compared.
In addition to comparing PSC amplitudes from an application period to the control and wash-out periods, PDC amplitudes of the control and drug application periods of different cells were compared to each other.

III. Study of the expression of NMDAr by dorsal horn astrocytes

In the second project of my PhD, we studied the possible expression of NMDAr by spinal astrocytes, as it has been described in other structures of the central nervous system (Verkhratsky & Chvátal, 2020). Part of the results of this project were obtained using primary astrocyte cultures (calcium imaging and electrophysiological recordings) and other results were obtained using acute slices of adult mouse spinal cord (electrophysiological recordings).

1. Animals

C57bl/6J mice pup between 3 and 5 days post-natal were used for the primary astrocyte cultures. Spinal slices were obtained from adult C57bl/6J mice (as described in the paragraphs II-2).

2. Preparation of primary astrocyte cultures

All the steps were performed in sterile conditions, under a laminar flow hood, wearing gloves and a gown.

Preparation of the glass slides

The astrocyte cultures were prepared on either glass cover slips or calcium imaging culture plates, depending on which experiment was being performed. The culture plates used are plastic petri dishes with a diameter of 35mm. A circle of 16mm of diameter was cut out from the bottom of the dish and a glass disc of 30mm of diameter was glued onto the external side, forming a central well of a 200µL volume. Before being used, the dishes were washed 3 times with ethanol. They were then exposed to UVs under the laminary flow for an hour. The glass cover slips are 12mm in diameter and were put into a Poupinel oven at 160°C for an hour, then kept in sterile conditions. As the rest of the preparation is the same for the glass cover slips and culture plates, I will not differentiate them and refer to them as glass slides.

A solution of poly-L-lysine at 0.01 mg/mL prepared in borate buffer (boric acid 0.1 mM, pH adjusted at 8.4 with NaOH) was deposited on the glass slides, then placed in the incubator at 37°C for an hour. The poly-L-lysine 0.01 mg/mL was rinsed 3 times with sterile distilled water.

Dissection, retrieval, and culturing of astrocytes for the primary cultures

Mice pups were killed by decapitation. After opening the skin of the back, a laminectomy was performed by a bilateral incision of the vertebrae to expose the spinal cord. Meninges were carefully removed and using a curved razor blade to cut along the longitudinal axis, the dorsal half of the spinal cord was removed and placed in a falcon tube filled with Dulbecco's Modified Eagles's medium (DMEM) F/12 +15 mM of 4-(2-hysroxyethyl)-1 piperazine ethane sulfonique acid (HEPES); (Gibco). A mechanical dissociation was performed by vigorous pipetting tissues with glass Pasteur pipettes (with decreasing tip diameter obtained by flame polishing). After centrifugation (800 tr/min, 6.5 min), the supernatant was carefully removed. The pellet was resuspended in an adequate volume of culture medium composed of DMEM, fetal veal serum (10% v/v), penicillin/streptomycin (penicillin: 50 units/mL and streptomycin: 50 µg/mL; Gibco). Dissociated cells were plated on the glass slides and incubated at 37°C in a CO2 incubator (5%). After 30 min, 2mL of culture medium was added to the cells. The culture medium was completely changed every 2 days.

3. Calcium imaging

To study the effect of an application of NMDA on spinal astrocytes, we used calcium imaging, as it's been shown in other studies to induce variations in astrocytic $[Ca^{2+}]_i$ (Palygin et al., 2010, 2011). The variations of $[Ca^{2+}]_i$ were observed with the ratiometric probe fura-2. The probe's fluorescence emission is measured at 510 nm. When the $[Ca^{2+}]_i$ increases and the calcium ions link to the Fura-2 probe, the fluorescence increases when excited at 350 nm and decreases when excited at 380 nm. The fluorescence ratio (F350/F380) is calculated from the measurements of the fluorescence after excitation at 350 and 380 nm. F350/F380 informs of the variations of the $[Ca^{2+}]_i$ independently of the concentration of probe in the cells, freeing the measure from potential differences in cell loading with the dye.

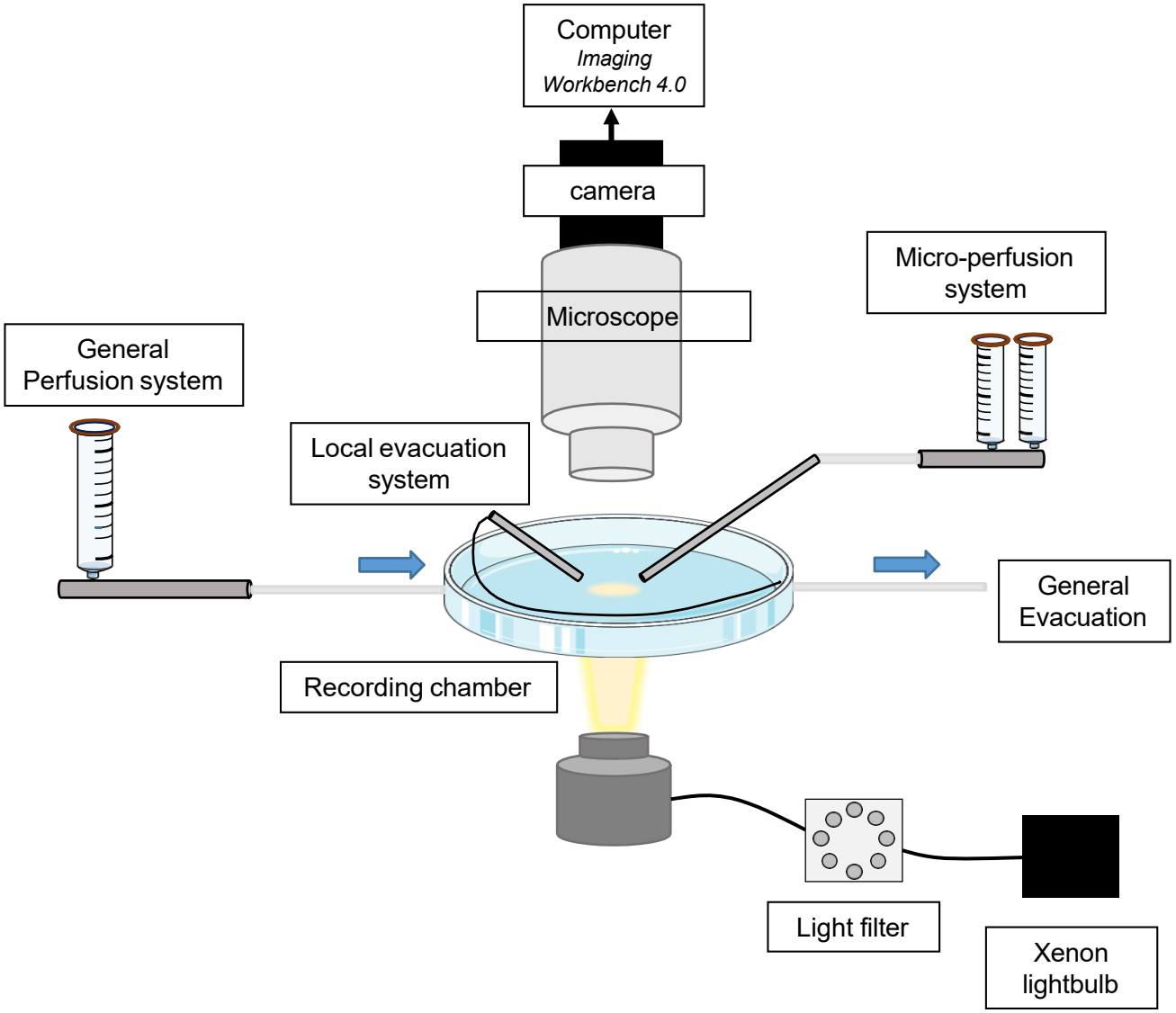


Figure 21: Calcium imaging experimental set-up.

The micro-perfusion system allows for very local application of drugs.

Loading with the cells with Fura-2 probe

To load the cells with the probe, Fura-2 was linked to an acetoxy-methyl ester group, which allows it to move across the plasmic membrane. Once in the cytoplasm, esterases cleave the ester links and the probe can no longer leave the cell.

Cultured cells were rinsed 3 times with the recording medium HEPES (table 1), before being incubated for an hour at room temperature with Fura-2-AM (4µM, Molecular Probes) and pluronic acid (F-127, Molecular Probes), a non-ionic detergent that facilitates the passage of Fura-2-AM into the cells. At the end of the incubation, the cells are rinsed 3 times with the HEPES recording medium maintained at 34°C, and the dish is placed in the calcium imaging apparatus.

Drugs used

All solutions were prepared in advance. To study the response in astrocytes to an application of NMDA, a solution of NMDA (100µM) was prepared, in presence or not of D-serine (100µM), in presence or not of Mg^{2+} (2mM). Uridine Triphosphate (UTP) (30 µM), an agonist of certain P2Y receptors, which are expressed by virtually all cells in the nervous system and induce an increase in $[Ca^{2+}]_i$,was used at the beginning and end of recordings of a field of cells, as a positive control to make sure that cells could have variations in their $[Ca^{2+}]_i$.

Experimental set-up

An inverted epi-fluorescence microscope (Axiovert 35, Zeiss) was used, with an oil immersion objective (Fluor 40 / 1.30 oil, Nikon). The light source was a lamp (Müller Elektronik-optik, Germany) with a xenon lightbulb of 150W (XBO 150 W OFR, OSRAM). Light rays are filtered through a specific filter to select excitation light waves for Fura-2 (350 nm and 380 nm), mounted on a filter-holder wheel controlled by a lambda 10-2 device (Sutter Instruments, USA). The light rays are then directed towards the cells, with a dichroic mirror that reflects all light waves below 510 nm. Fura-2 emits fluorescence above 510 nm that passes through a dichroic mirror and is detected by a CCD camera (Cool SNAP HQ, Photometrics). The camera has a 1392 x 1038 pixels resolution and digitizing is done over 12 bits, which is to say 4096 gray levels (figure 21).

Recording

Basal $[Ca^{2+}]_i$ was recorded for 300 seconds before drug application (10 to 30 seconds, depending on the drug). After drug application, the drugs were washed out for 100 to 500 seconds. During recording, the cells are continuously perfused with the HEPES recording solution maintained at 34°C.

Data acquisition

Calcium signals were recorded with a cooled CCD camera (CoolSNAP HQ; Roper Scientific, Tucson, AZ, USA) and an image analysis software package (Imaging workbench 4.0; Axon Instruments, Molecular Devices). Excitation at wavelengths of 350 and 380 nm was achieved with a lambda-10 filter wheel (Sutter instruments, Molecular Devices), and emitted light was collected at 520 nm. This system allows for acquisition of pairs of images every 1.1 second. In the results section, intracellular calcium levels will be expressed as the F350/380 ratio described previously (paragraph III.3) and is obtained after background signal subtraction.

Analysis

For analysis of calcium signals and their variations, the image analysis software package (Imaging workbench 4.0; axon instruments, Molecular Devices) was used. Regions of interest (ROIs) were manually delimited in a visual field, corresponding to individual cells. ROIs were chosen depending on the response to UTP (30µM) (a positive control). All cells that responded to UTP were kept for analysis, regardless of the fact that they responded or not to any other substance applied.

4. Electrophysiological recordings

To study the expression of functional NMDAr in DH astrocytes, we used patch-clamp to record unitary receptor currents in cultured astrocytes. We also used patch-clamp to record currents in astrocytes, induced by an application of NMDA in spinal slices. I will detail both procedures in the next paragraphs.

Unitary channel current recording in primary astrocyte cultures

To characterize NMDAr in cultured astrocytes, we used the patch-clamp technique in 2 single-channel configurations: attached-cell and outside-out in cultured astrocytes (figure 19). The outside-out configuration is obtained from whole-cell configuration: once the whole-cell configuration is established, the pipette is gently pulled back until the membrane breaks. This can be monitored by watching the capacitive currents suddenly shrink, indicative of a reduction in membrane surface. These configurations are used to study unitary channels. Agonists, antagonists, and modulators can be applied in the recording medium, to study the composition in subunits and properties, such as conductance of the receptors.

NMDA-induced currents in DH astrocytes in spinal slices

Spinal cord slices were obtained as described in the paragraph II.2. To visualize the astrocytes, sulforhodamine 101 (SR 101) was used. Slices were incubated in a solution containing 1 µM of SR 101 during 20 min at 34°C prior to recording. SR 101 is a red fluorescent dye, preferentially taken up by astrocytes. The peak of excitation is 586 nm, and the peak of emission is 605 nm (Verkhratsky & Nedergaard, 2018). The choice of cell, approach and seal with the pipette is similar to neurons (see paragraph II.3).

As a complementary step to SR 101, to make sure we were recording an astrocyte, we looked at the resting membrane potential after passing to the whole cell configuration. After that, we did voltage steps from -120 mV to -30 mV by increments of 10 mV, to determine passive properties of the recorded cell (figure 7), as astrocytes are non-excitable cells and cannot spike. This last step was performed during TTX-free recordings. Once an astrocyte was validated, the membrane potential was maintained at -80 mV, closest to physiological astrocyte membrane potential. NMDA was then applied to the slice and responses in astrocytes were assessed and analyzed.

Analysis

Single-channel recordings: Analysis was performed using Clampfit (10.2) software. Recordings were filtered with a cut off frequency of 1 kHz. The threshold for detecting channel opening and closing transition states was set at 50% of the open level of each event. All events shorter than 0.5 ms were rejected. Open times, closed times, and amplitude of channel currents were extracted from the recordings. These parameters allowed to determine the channel opening probability and

amplitude distributions of the channel. By fitting a linear regression to the mean unitary amplitudes obtained at a minimum of 4 different potentials, the conductance and reversal potential of the channel were obtained.

Astrocytes in spinal slices recordings : Recordings obtained from astrocytes after an NMDA application on spinal slices were analyzed with clampfit (10.2) and WinEDR (V3.8.6, Strathclyde Electophysiology Software, John Dempster, University of Strathclyde, Glasgow, UK). After visually inspecting the time-locked area following an NMDA application, 2 types of responses were observed: a slow inward current, and fast, channel-like responses. Cells were categorized according to which response was observed.

IV. Statistics and representation of data

Statistical analysis was realized using GraphPad Prism. Normal distribution was tested with the d'Agositino and Pearson test, unless the n was smaller than 8, in which case the Shapiro Wilk test was used. To compare parameters with a normal distribution, we used the paired Student's test to compare control and drug application periods of the same cell, and for non-normal distribution of the data, we used the Wilcoxon Signed Rank test. Fisher's exact test was used to compare the proportions of cells presenting a specific effect in different conditions. To compare baseline frequencies, amplitudes, and slopes of GAD^- and GAD^+ neurons, in control conditions or after a pre-incubation with FC, we used a two-way ANOVA.

Data is presented as mean ± standard error of the mean (SEM). Significance threshold (p) is fixed at 0.05. The symbols used in the figures to indicate p value are: *<0.05; **<0.01; ***<0.001.

Results part I

Published article

"Modulation of GABAergic synaptic transmission by NMDA receptors in the dorsal horn of the spinal cord"

Subject context:

Our first aim was to characterize the effect of NMDAr activation on GABAergic synaptic transmission in naïve mice by recording neurons in the lamina II.

In the lamina II, NMDAr activation specifically modulates GABAergic synaptic transmission, and has no effect on glycinergic synaptic transmission was observed. In this article, we describe NMDA-dependent facilitation of spontaneous and miniature GABAergic synaptic transmission, observed as an increase in sIPSC and mIPSC frequency, with no change in the amplitude of sIPSC and mIPSC. Interestingly, this NMDA-dependent facilitation depends on NMDAr containing GluN2C/D subunits.

When recording specifically in putative GABAergic and glutamatergic neurons, we observed a target-specificity of NMDA-dependent facilitation. Indeed, 100% of putative GABAergic neurons and 85% of putative glutamatergic neurons displayed NMDA-dependent facilitation of GABAergic synaptic transmission. When recording miniature synaptic transmission, we observed NMDA-dependent facilitation in 91% of putative GABAergic neurons and only 22% of putative glutamatergic neurons. We can hypothesize that facilitation of GABAergic synaptic transmission in putative GABAergic neurons could be a mechanism of network disinhibition. We also showed that NMDAr inhibits evoked sIPSC in putative GABAergic neurons, consistent with NMDAr-mediated shunt of spike-evoked GABA release. As we observed a change in paired-pulse ratio, we can conclude that NMDAr activation presynaptically modulated GABAergic synaptic transmission.

Our results suggest differential modulation of evoked and spontaneous GABAergic synaptic transmission in the lamina II of the DH. This mechanism could be implicated in fine-tuning of the excitation/inhibition balance in the DH neuronal network, which plays a key role in nociceptive information processing.

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ORIGINAL RESEARCH
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Modulation of GABAergic Synaptic Transmission by NMDA Receptors in the Dorsal Horn of the Spinal Cord

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The dorsal horn (DH) of the spinal cord is an important structure involved in the integration of nociceptive messages. Plastic changes in the properties of neuronal networks in the DH underlie the development of analgesia as well as of hyperalgesia and allodynia in acute and chronic pain states. Two key mechanisms are involved in these chronic pain states: increased electrical activities and glutamate release leading to the recruitment of NMDAr and plastic changes in the synaptic inhibition. Although: (1) the balance between excitation and inhibition is known to play a critical role in the spinal network; and (2) plastic changes in spinal excitation and inhibition have been studied separately, the relationship between these two mechanisms has not been investigated in detail. In the present work, we addressed the role of NMDA receptors in the modulation of GABAergic synaptic transmission in the DH network. Using tight-seal whole-cell recordings on adult mice DH neurons, we characterized the effect of NMDAr activation on inhibitory synaptic transmission and more especially on the GABAergic one. Our results show that, in a subset of neurons recorded in lamina II, NMDAr activation facilitates spontaneous and miniature GABAergic synaptic transmission with a target specificity on GABAergic interneurons. In contrast, NMDA reduced the mean amplitude of evoked GABAergic IPSCs. These results show that NMDAr modulate GABAergic transmission by a presynaptic mechanism of action. Using a pharmacological approach, we investigated the composition of NMDAr involved in this modulation of GABAergic synaptic transmission. We found that the NMDA-induced facilitation was mediated by the activation of NMDAr containing GluN2C/D subunits. Altogether, our results bring new insights on nociceptive information processing in the spinal cord network and plastic changes in synaptic inhibition that could underlie the development and maintenance of chronic pain.

Keywords: dorsal horn, synaptic inhibition, NMDA receptors, nociception, pain

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INTRODUCTION

The dorsal horn (DH) of the spinal cord is an important structure involved in the integration and transmission of nociceptive messages from the inner and outer environment. Spinal integration of this information relies on the interplay between different DH neurons forming a complex and functionally plastic neuronal network (Cordero-Erausquin et al., 2016).

The DH neuronal network is composed of inhibitory and excitatory interneurons. A fine-tuning of the excitatory/inhibitory balance is crucial in the control of the transmission of nociceptive messages from the DH to the supraspinal structures where it may lead to pain perception. Imbalance between excitation and inhibition in DH networks is known to be one of the mechanisms leading to enhanced pain sensation and underlying the development and maintenance of pathological pain, such as neuropathic pain. Impairment of DH inhibitory synaptic transmission plays a pivotal role in this disruption of the excitation/inhibition balance (Sivilotti and Woolf, 1994; Coull et al., 2003; Harvey et al., 2004; Torsney and MacDermott, 2006). Indeed, pharmacological blockade of ionotropic $GABA_A$ and glycine receptors induces thermal hyperalgesia and mechanical allodynia (Beyer et al., 1985; Roberts et al., 1986).

The major excitatory transmitter released by primary afferent fibers is glutamate. Under physiological conditions, fast glutamatergic transmission in the DH is mediated by postsynaptic AMPA receptors (AMPAr). Sustained or repeated afferent fiber stimulation leads to an increased release of glutamate in the DH and consequently to the recruitment of NMDA receptors (NMDAr; Woolf and Thompson, 1991). In the DH, NMDAr activation is critically involved in long-term potentiation of excitatory synapses (Woolf and Salter, 2000; Sandkuhler, 2007; Latremoliere and Woolf, 2009). Moreover activation of presynaptic NMDAr expressed on afferent fiber terminals facilitates glutamate release in the DH (Liu et al., 1994, 1997; Bardoni, 2013) and modulates transmission of nociceptive messages in the spinal cord (Bardoni, 2013; Deng et al., 2019). Interestingly, one study demonstrated that 37% of GABAergic synaptic terminals in the DH of the rat spinal cord expressed NMDAr (Lu et al., 2005), but the subunit composition as well as the role of these presynaptic receptors has not been investigated so far.

Plastic changes in the strength of inhibitory synaptic transmissions play a crucial role in information processing and tuning of neural activity. In several regions of the CNS, activity-dependent long-term plasticities (LTP and LTD) of inhibitory synapses have been described (Kullmann et al., 2012). In the DH, one study has described a heterosynaptic plasticity at GABAergic synapses in lamina I neurons, after activation of metabotropic glutamate receptors (Fenselau et al., 2011) and one recent study described an NMDAr-dependent potentiation of glycinergic synapses (Kloc et al., 2019). In other structures, postsynaptic and presynaptic NMDAr have been shown to be key players in the modulation of GABA release (Glitsch and Marty, 1999; Duguid and Smart, 2004; Crabtree et al., 2013) and in the functional plasticity of inhibitory synapses (Nugent et al., 2007; Mapelli et al., 2016). It is known that changes in spinal inhibition are crucial in the processing of nociceptive information and that NMDAr are activated by an increased activity in the DH network. However, nothing is known about the possibility that activity-dependent modulation at inhibitory synapses engaging NMDAr could take place in the DH.

In the present work, we addressed the role of NMDAr in the modulation of GABAergic synaptic transmission in the DH network. Using patch-clamp recordings on adult mice DH neurons, we characterized the effect of NMDAr activation on inhibitory synaptic transmission and more especially on the GABAergic one. Our results indicate that NMDAr activation differentially modulates spontaneous and electrically-evoked GABA release and that this effect targets preferentially GABAergic synapses established with GABAergic interneurons.

MATERIALS AND METHODS

Animals

All procedures were performed in accordance with European directives and were approved by the regional ethics committee and the French Ministry of Agriculture (license No. 2015030911301894). We used C57BL/6j (n = 118) and GAD65-eGFP mice (n = 28). GAD65-eGFP were obtained from Ferenc Erdelyi and Gabor Szabo (Institute of Experimental Medicine, Budapest) and have been described previously (Cui et al., 2011). The mice were interbred, born and housed in the animal house of the laboratory (Chronobiotron, agreement No. A67-2018-38) at room temperature with a 12 h light/dark cycle and with free access to food and water. Experiments were performed with male adult mice (5–10 weeks old).

Slice Preparation

Mice were anesthetized by intraperitoneal injection of urethane (1.9 g/kg body weight) prior to realizing a laminectomy. The lumbar spinal cord was removed and immediately immersed in ice-cold sucrose-based artificial cerebrospinal fluid (sACSF) containing in mM: sucrose (252), KCl (2.5), $NaCl_2$ (2), $MgCl_2$ (2), glucose (10), $NaHCO_3$ (26), and NaH_2PO_4 (1.25) continuously gassed with carbogen (5% CO_2 and 95% O_2). The spinal cord was embedded in agarose (5%) and 300 μm-thick transverse slices were cut through lumbar 3–5 segments using a Leica VT1200S vibratome (Leica Microsystems Inc.). Slices were stored at room temperature (22°C–24°C) in a chamber filled with ACSF containing (in mM): NaCl (126), $NaHCO_3$ (26), $NaCl_2$ (2), KCl (2.5), NaH_2PO_4 (1.25), $MgCl_2$ (2), glucose (10), and continuously gassed with carbogen.

Electrophysiology

Slices were transferred to the recording chamber and continuously perfused with oxygenated ACSF. Recordings were performed from lamina II neurons.

Patch pipettes were pulled from borosilicate glass capillaries (1.2 mm o.d. 0.69 mm i.d.; Harvard Apparatus) using a P-1000 puller (Sutter Instruments, Novato, CA, USA) and had final tip resistances between 3 and 6 MΩ. Pipettes were filled with an intracellular solution containing (in mM): CsCl (130), HEPES (10), and $MgCl_2$ (2). The intracellular solution had a pH of 7.3 adjusted with CsOH and an osmolarity of 300 mOsm adjusted with sucrose. In these conditions, the theoretical equilibrium potential for Cl^- anions was 0 mV.

Whole-cell patch-clamp recordings were performed from neurons identified under visual control using an infrared differential interface contrast optics. Gad65-eGFP neurons were identified using epifluorescent illumination.

Voltage-clamp recordings were performed with an Axopatch 200 B amplifier (Molecular Devices, San Jose, CA) at a holding potential fixed at −60 mV allowing visualization of excitatory postsynaptic currents and inhibitory postsynaptic currents (EPSCs; IPSCs) as inward currents. Recordings were low-pass filtered (5 kHz) and acquired with Clampex software (Molecular Devices, San Jose, USA). Current traces were digitized (10 kHz) and stored on the hard drive of a personal computer. All experiments were performed at room temperature (22°C–24°C).

Paired-pulse ratio experiments were performed by stimulation of a presynaptic neuron with a 0.25 mA current injection with an extracellular electrode filled with ACSF.

The stimulation was performed as described in Cathenaut et al by applying current steps (0.25 ms; 0.10–0.40 mA) through a patch pipette filled with ACSF (Cathenaut et al., 2022). This stimulation electrode was placed at 20–150 μm from the cell body of the recorded neuron. For each recorded neuron, the lowest amplitude of stimulation evoking IPSCs was determined and was increased by 0.05 mA to evoke IPSCs for each stimulation applied. Synaptic contacts were identified as monosynaptic unitary connections when the following criteria were satisfied: (1) all-or-none eIPSCs appearance; (2) absence of increase in eIPSC amplitude when minimal stimulation amplitude was increased by 0.05 mA; (3) disappearance of eIPSCs when stimulation polarity was inverted; and (4) constant latency of the eIPSCs.

Three different interstimulus intervals of stimulation were realized: 20, 50, and 100 ms and were repeated every 3 s. Paired-pulse ratio was determined as the amplitude ratio of the second to the first IPSC evoked in the postsynaptic neuron.

Pharmacological Substances

Different drugs were used in order to study NMDA effects on inhibitory synaptic transmission. We recorded spontaneous IPSCs in the presence of antagonist of AMPA and Kainate receptors, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM, Tocris). To isolate GABAergic or glycinergic currents, we used strychnine (1 μM, Sigma) or bicuculline methiodine (10 μM, Sigma), respectively. Miniature IPSCs were recorded in the presence of tetrodotoxin (TTX; 0.5 μM, Latoxan) to block voltage gated sodium channels.

To assess the role of NMDAr, we used the specific agonist N-Methyl-D-aspartic acid (NMDA, 100 μM, Tocris). The specific antagonist of NMDAr, D-2-amino-5-phosphonovalerate (APV 50 μM; Abcam), was used to block all NMDAr. Two specific antagonists of NMDAr containing GluN2C/D subunits were used, (2R,3S)-1-[(phenanthren-3-yl)carbonyl]piperazine-2,3-dicarboxylic acid) (UBP141 10 μM and 25 μM) and (4-((1H-Indol-7yl)carbamoyl)phenyl diethylcarbamate) NAB14 (10 μM, from Abcam and Aobious respectively). Ifenprodil (10 μM, Sigma) was used to block NMDAr containing GluN2B subunits. Zinc (100 nM, Sigma) was used to inhibit NMDAr containing GluN2A subunits. Finally, we also used a channel blocker of NMDAr, dizocilpine (MK-801; 2 mM in the intracellular solution, Tocris). Drugs were prepared as 1,000× or 10,000× concentrated stock solutions in dimethylsulphoxide (DMSO), water or ethanol in accordance with indications from the manufacturer. The stocks were stored at −20°C. All substances were diluted to their final concentration in ACSF at the beginning of each experiment and applied by general bath perfusion (flow rate: 1.0 ml/min; total chamber volume: 2 ml).

Data Analysis

Synaptic events were detected using WinEDR with an amplitude threshold detection algorithm and visually inspected for validity. The analysis of frequency has been performed as described in a previous study of our lab (Petitjean et al., 2012). For each neuron analyzed, the cumulative number, N, of synaptic events was plotted as a function of time. A two-linear-segment curve was fitted by non-linear regression (KyPlot 2.15; KyensLab, Tokyo, Japan) using the following equation:

$$N = fo \times t \times a$$

for $t < tc$ and

$$N = fc \times t + (fo - fc) \times tc + a$$

for $t > tc$

Where the slope "fo" represents an estimation of the average frequency under control conditions and "fc" provides an estimation of the mean frequency following the application of the drug. "tc" is the time at which the change in frequency occurs. Drugs were considered to have an effect when "tc" occurred around 100 s following the beginning of the application of the substance and when the change in frequency exceeded 20% of the recorded basal frequency.

The percentage of increase in frequency was calculated as follows:

$$\text{Change in frequency}\,(\%) = \frac{(fc - fo)}{fo} \times 100$$

Peak amplitude was determined by using WinWCP (waveform measurements) to determine the exact value of the peak. Tau rise and Tau decay were determined by fitting the trace with an exponential decay (EPC endplate current-fitting function of WinWCP) using the following equation:

$$y(t) = 0.5 * A * (1 + \text{erf}(x - \tau\text{rise})) * \exp\frac{-x}{\tau\text{decay}}$$

Where A is the amplitude (in pA), τrise is the rise time (in ms) and τdecay is the decay time (in ms). Following these analyses, each neuron was classified either as displaying a change in EPSCs or IPSCs frequency, amplitude or kinetics in response to the application of the substance or as being non-responsive to this substance.

Statistics

Data are represented as mean ± Standard Error to the Mean (SEM). Statistical analyses were performed with GraphPad Prism (GraphPad Software 6.07, La Jolla, CA, USA). To compare proportions of neurons, Fisher's exact test was used. For the

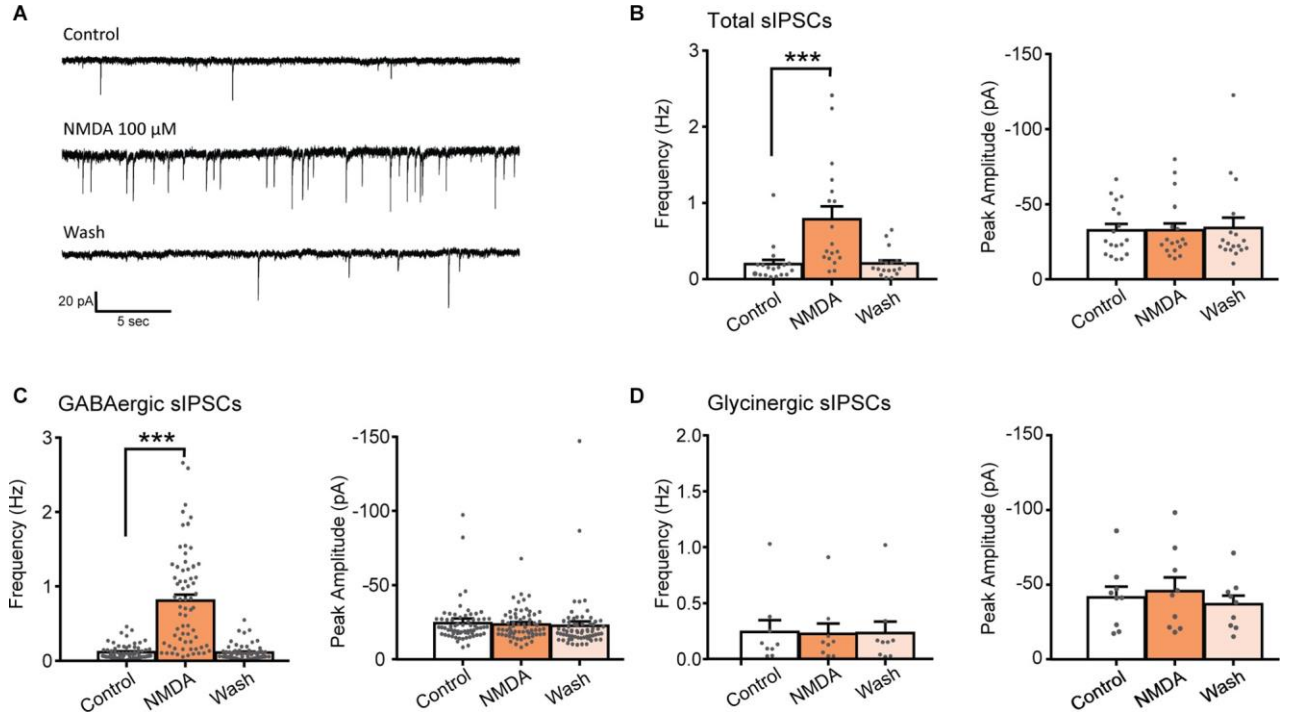

FIGURE 1 | NMDA increases GABA sIPSC frequency in lamina II neurons. (A) Representative traces of total sIPSCs recorded from lamina II neuron in spinal cord slice from C57Bl6 mouse before (Control) and during NMDA application (NMDA; 100 s after the beginning of application) and after a washout (wash; 360 s after the beginning of NMDA washout). (B) Bar graph showing total sIPSC frequency and peak amplitude before (Control) and during NMDA application (NMDA) and after a washout (wash; $N = 18$). NMDA application resulted in a reversible increase in sIPSC frequency without change in sIPSC amplitude. (C,D) Frequency and peak amplitude of GABAergic sIPSCs (C) and glycinergic sIPSCs (D) isolated pharmacologically. NMDA application enhances GABAergic sIPSC (recorded in presence of strychnine) frequency ($N = 67$) but did not affect glycinergic sIPSCs, recorded in presence of bicuculline (D) ($N = 9$). ***$p < 0.001$. Paired t-test was used. Error bars indicate SEM.

effect of drug application, we used the paired t-Test or the Wilcoxon test, and the unpaired t-Test or the Mann-Whitney test were used for unpaired comparisons, depending on the data distribution previously tested with the D'Agostino and Pearson normality test.

RESULTS

NMDA Receptor Activation Leads to an Increase in Spontaneous GABAergic Synaptic Transmission

Bath application of NMDA (100 µM, 60 s) caused a significant increase in the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in 75% of the recorded neurons ($N = 18/24$). This facilitation was always followed by a full recovery after a washout (**Figures 1A,B**). In neurons displaying a significant increase in sIPSCs frequency, the increase was of 415% (control: 0.19 ± 0.06 Hz, NMDA: 0.79 ± 0.17 Hz; $N = 18$; $t = 4.147$, $p = 0.0007$, paired t-test). In these neurons, increase in sIPSCs frequency occurred without changes in sIPSCs amplitude (control: −32.5 ± 4.0 pA, NMDA: −32.5 ± 4.7 pA; $N = 18$; $t = 0.003$, $p = 0.9975$, paired t-test; **Figure 1B**).

Fast synaptic inhibition in the dorsal horn of the spinal cord is mediated by GABA and glycine. Effects of NMDA on GABAergic sIPSCs were examined in presence of 1 µM strychnine, a glycine receptor antagonist and effects of NMDA on Glycinergic sIPSCs were examined in presence of 10 µM bicuculline, a $GABA_A$ receptor antagonist. NMDA application induced a reversible increase of GABAergic sIPSCs in 80.7% of the recorded neurons ($N = 67/83$). In neurons displaying a significant effect, NMDA increased GABAergic sIPSCs frequency by 736% (control: 0.11 ± 0.01 Hz, NMDA: 0.81 ± 0.08 Hz; $N = 67$; $t = 4.147$, $p = 0.0007$, paired t-test) without changing sIPCSs amplitude (control: −24.2 ± 1.6 pA, during NMDA: −23.5 ± 1.2 pA; $N = 67$; $t = 0.9188$, paired t-test; **Figure 1C**). NMDA application neither changed glycinergic sIPSCs frequency nor amplitude ($N = 9$) indicating that NMDA modulates specifically GABAergic synaptic transmission (**Figure 1D**).

These results indicate that activation of NMDAr increases the frequency of GABAergic and not glycinergic spontaneous IPSCs.

In neurons in which NMDA facilitated GABAergic sIPSCs, a second application of NMDA yielded a similar effect (relative increase in frequency: NMDA = 0.93 ± 0.29, $N = 6$; **Figure 2A**). Taking advantage of the reproductibility of NMDA effect, we examined the effect of NMDAr antagonists in NMDA-responsive neurons. To this end, a second application of NMDA was

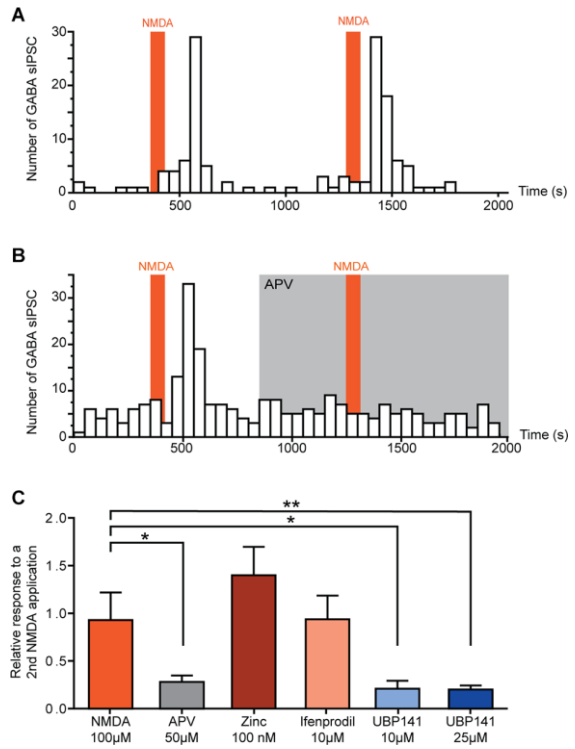

FIGURE 2 | Activation of GluN 2C/D containing NMDA receptors induces increase of spontaneous GABAergic transmission. (A,B) Example of the effect of two successive NMDA applications on the number of GABAergic sIPCSs (50 s bin). NMDA effect is reproducible (A) and completely blocked in presence of APV (50 μM) (B). (C) Effect of a second application of NMDA in presence of different NMDAr selective antagonists. Histogram showing the effect of a second NMDA application normalized to a first application performed in absence of antagonists. NMDA-induced increase in GABA sIPSC frequency was inhibited in presence of APV (50 μM, $N = 8$) and UBP 141 (10 μM, $N = 4$; 25 μM, $N = 9$). Presence of Zinc (100 nM, $N = 4$) or Ifenprodil (10 μM, $N = 8$) did not affect NMDA effect. *$p < 0.05$ and **$p < 0.01$. Mann-Whitney test was used. Error bars indicate SEM.

performed in presence of NMDAr antagonists or modulators, and effects of both applications were compared.

In the presence of 50 μM APV, a selective NMDAr antagonist, NMDA-induced increase in GABAergic sIPSCs frequency was reduced by 72% (relative increase in frequency: NMDA = 0.93 ± 0.29, NMDA+APV = 0.28 ± 0.07, $N = 8$, $p = 0.02$, Mann-Whitney test; **Figure 2B**).

NMDAr is a tetrameric ionotropic glutamate receptor with a most frequent configuration consisting of two mandatory GluN1 and two GluN2 subunits (GluN2 A-D). To assess the composition of NMDAr contributing to the increase in GABAergic sIPSCs frequency, we tested the effect of NMDAr subunit-selective antagonists.

Zinc (100 nM), which blocks specifically NMDAr containing GluN2A subunit, had no effect on NMDA-induced effect (relative increase in frequency: Zinc = 1.40 ± 0.30, $N = 4$), neither did ifenprodil (10 μM), a selective antagonist of GluN2B-containing NMDAr (relative increase in frequency: ifenprodil = 0.94 ± 0.25, $N = 8$). However, NMDA-induced increase in GABAergic sIPSCs frequency was reduced by 79% in presence of UBP 141 (UBP 10 μM: relative increase in frequency = 0.21 ± 0.08, $N = 4$, $p = 0.019$, Mann-Whitney test; UBP 25 μM: relative increase in frequency = 0.20 ± 0.04, $N = 9$, $p = 0.0048$, Mann-Whitney test) a specific antagonist of GluN2C/D-containing NMDAr (**Figure 2C**), indicating the involvement of NMDAr containing these subunits in the effects of NMDA on GABAergic transmission.

NMDA Receptor Activation Selectively Increases GABA Release

We next examined whether NMDA-induced facilitation of spontaneous GABAergic transmission involved a direct effect on presynaptic GABAergic terminals or whether it required action potential firing and propagation in presynaptic interneurons. To this end, we recorded miniature IPSCs (mIPSCs) in the presence of 0.5 μM TTX. In 58% of recorded-neurons ($N = 14/24$), application of NMDA induced a significant increase in mIPSCs frequency (control: 0.04 ± 0.01 Hz, NMDA: 0.21 ± 0.05 Hz; $N = 14$; $t = 3.664$, $p = 0.0029$, paired t-test), without change in mIPSCs amplitude. We next recorded pharmacologically isolated GABAergic mIPSCs in the presence of the channel blocker TTX and the glycine receptor antagonist Strychnine (STR). In 54% of recorded neurons ($N = 35/65$), NMDA induced a significant increase in GABAergic mIPSCs frequency (control: 0.07 ± 0.01 Hz, NMDA: 0.26 ± 0.04 Hz; $N = 36$; $t = 5.364$, $p < 0.0001$, paired t-test) without changes in GABAergic mIPSCs amplitude (**Figures 3A,B**). Moreover, NMDA never induced significant changes neither in amplitude, nor in mIPSCs activation or deactivation kinetics, suggesting that NMDA increased GABA release probability *via* a presynaptic action.

To examine the specificity of the NMDA effect on GABAergic synapses, we examined the effect of NMDA application on mEPSCs or glycinergic mIPSCs. NMDA applications neither changed mEPSCs (recorded without CNQX and in presence of bicuculline and strychnine), nor glycinergic mIPSCs (recorded in absence of Strychnine and in presence of CNQX and bicuculline; data not shown).

As previously performed for spontaneous GABAergic transmission, we observed the effects of NMDAr antagonists on the NMDA-induced increase in mIPSCs frequency.

APV (50 μM) reduced by 72% the NMDA-induced increase in GABAergic mIPSCs frequency (relative response to the first application: NMDA = 0.59 ± 0.11, APV = 0.17 ± 0.04, $N = 6$, $p = 0.0087$, Mann-Whitney test; **Figure 2B**). These results confirm that NMDA acted on NMDAr to increase the frequency of GABAergic mIPSCs.

NMDA-induced increase in GABAergic mIPSCs frequency was reduced by 77% in presence of NAB14 (10 μM) a highly selective antagonist of GluN2C/D-containing NMDAr (relative response: NAB14 = 0.23 ± 0.05, $N = 5$, $p = 0.0173$, Mann-Whitney test) confirming the involvement of GluN2C/D-containing NMDAr in NMDA-induced facilitation of GABAergic synaptic transmission (**Figure 3C**).

The voltage-dependent block of NMDAr by Mg^{2+} is stronger for NMDAr containing GluN2A or GluN2B subunits than for those containing GluN2C/D subunit (Paoletti et al., 2013).

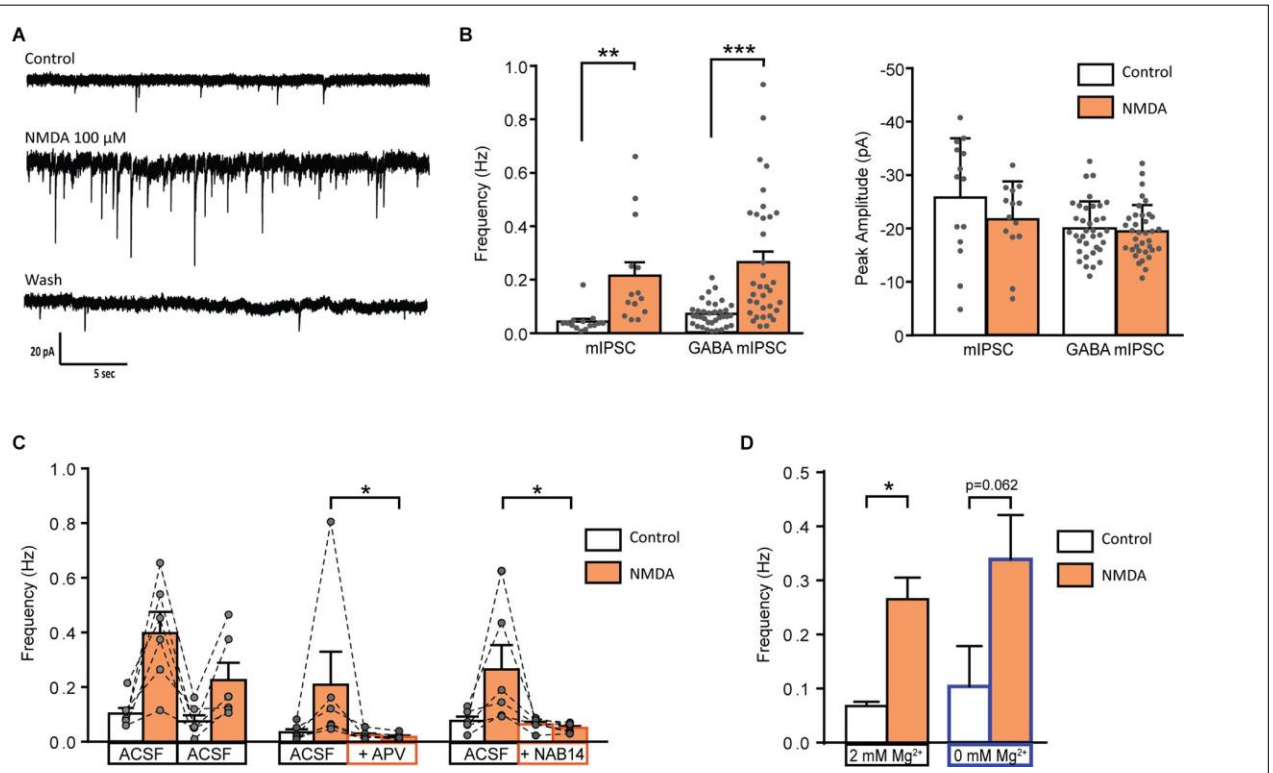

FIGURE 3 | Activation of GluN2C/D containing NMDA receptor increase miniature GABAergic IPSCs. (A) Representative traces of mIPSCs recorded from a lamina II neuron in spinal cord slice from C57Bl6 mouse before (Control) during NMDA application (NMDA; 100 s after the beginning of application) and after a washout (wash; 400 s after the beginning of NMDA washout). (B) Bar graph showing total mIPSC ($N = 14$) and GABA mIPSC ($N = 36$) frequency and peak amplitude before (Control) and during NMDA application (NMDA). (C) Effect of APV (50 μM, $N = 6$) and NAB 14 (10 μM, $N = 5$) on NMDA-induced increase in GABA mIPSC frequency. Both antagonists blocked effect of NMDA application. (D) NMDA-induced increase in GABA mIPSC frequency in presence (2 mM Mg^{2+}, $N = 36$) or in absence of external Mg^{2+} (0 mM Mg^{2+}, $N = 5$) is comparable. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Paired t-test (A), Mann-Whitney test (B), and Wilcoxon test (C) were used. Error bars indicate SEM.

The experiments described so far were performed in the presence of 2 mM Mg^{2+}. To assess whether the contribution of GluN2A- or GluN2B-containing NMDAr was masked by a Mg^{2+}-dependent block, we assessed the effect of NMDA on GABAergic mIPSCs recorded in absence of extracellular Mg^{2+}. These recordings were performed in presence of MK801 (2 mM) in the recording pipette in order to prevent activation of NMDAr present on the recorded neuron. The stimulatory effect of NMDA on GABAergic mIPSCs was unchanged under these conditions. In 50% of the recorded cells ($N = 5/10$) NMDA application induced an increase in frequency of GABAergic mIPSCs (control: 0.10 ± 0.07 Hz, during NMDA: 0.34 ± 0.08 Hz; $N = 5$; $p = 0.0625$, Wilcoxon test) without any change in mIPSCs amplitude (data not shown; **Figure 3D**).

NMDAr-Dependent Modulation of GABAergic Transmission Depends on the Neurochemical Identity of the Postsynaptic Neuron

The NMDA-induced increase in GABAergic IPSCs frequency was only observed in 80.7% (spontaneous IPSC) and 54.0% (miniature IPSC) of recorded neurons. We therefore wondered whether NMDA-induced facilitation of GABAergic transmission would depend on the excitatory or inhibitory phenotype of the postsynaptic neurons (from which we recorded). To this end, we perfrormed recordings using slices prepared from GAD65-eGFP mice.

NMDA increased the frequency of spontaneous GABAergic IPSCs recorded both in eGFP+ and eGFP-, in similar proportions (eGFP+: 100%, $N = 10/10$; eGFP-: 85% $N = 6/7$). These results were similar to those obtained from C57Bl6 mice (**Figure 4A**).

A large majority of eGFP+ neurons displayed a facilitatory effect of NMDA on GABAergic mIPSCs (91%, $N = 20/22$). However, a significantly lower proportion of eGFP- neurons (22%, $N = 2/9$, $p = 0.00042$, Fischer's exact test) showed an effect of NMDA on GABAergic mIPSCs (**Figure 4B**). As previously observed in "unidentified" neurons, NMDA application increased GABAergic mIPSCs frequency (control: 0.07 ± 0.02 Hz, during NMDA: 0.25 ± 0.05 Hz; $N = 20$; $t = 4.880$, $p = 0.0001$, paired t-test) but neither change in mIPSC amplitude (control: −17.5 ± 1.3 pA, during NMDA: −16.8 ± 1.4 pA; $N = 20$; $t = 0.5017$, paired t-test) nor kinetics (data not shown; **Figures 4C,D**). In eGFP- neurons displaying an effect, NMDA application also increased GABAergic mIPSCs frequency

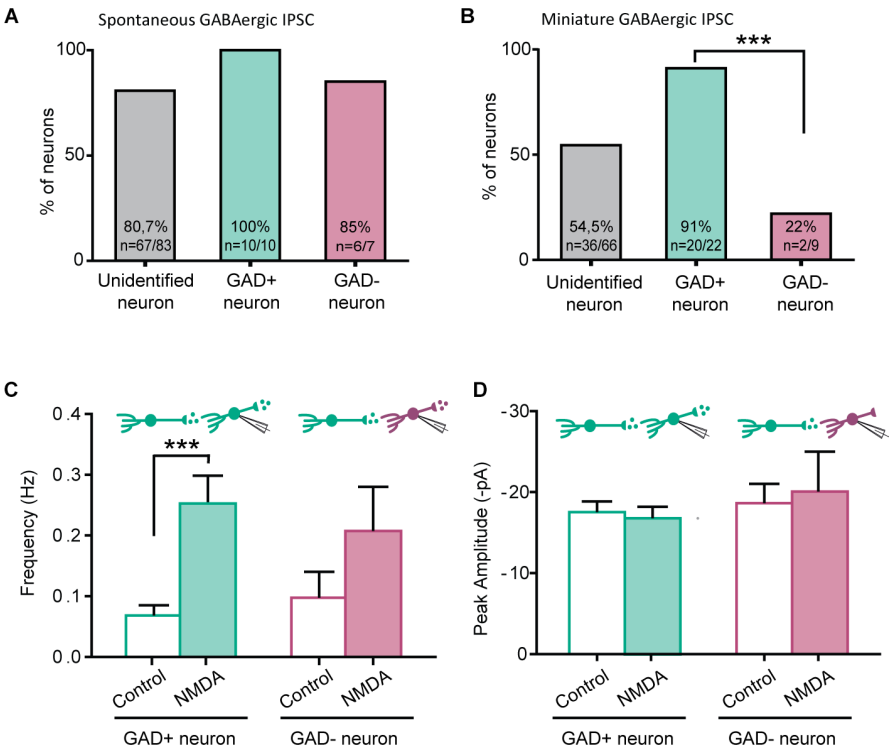

FIGURE 4 | NMDA enhancement of mIPSC frequency target specifically GABAergic interneurons. (A) Proportion of recorded neurons showing an increase of GABA sIPCS frequency by NMDA application, in unidentified neurons from C57bl6 mice and GAD positive (GAD+) or GAD negative (GAD-) neurons from GAD65-eGFP mice. (B) Proportion of recorded neurons recorded showing an increase of GABA mIPCS frequency by NMDA application is significantly higher in GAD positive (GAD+) neurons than for GAD negative (GAD-). (C,D) Frequency (C) and peak amplitude (D) of GABAergic mIPSCs recorded in GAD+ and GAD- neurons from GAD65-eGFP mice. Effect of NMDA on mIPSC frequency is independent of the neuron type. ***$p < 0.001$. Fischer's exact test (B) and Paired t-test (C) were used. Error bars indicate SEM.

(control: 0.10 ± 0.04 Hz, during NMDA: 0.21 ± 0.07 Hz; $N = 2$; **Figure 4C**). These results indicate that the facilitation of GABAergic synaptic transmission by presynaptic NMDAr depends on the postsynaptic target: it preferentially occurs in GABAergic connections onto eGFP+ neurons.

NMDA Changes the Paired-Pulse Ratio and Inhibits Evoked GABAergic IPSCs

An increase in mIPSCs frequency with no change in amplitude suggested a presynaptic mechanism of action of NMDA. We further tested this possibility by determining the effect of NMDA on the paired-pulse ratio (PPR) of electrically-evoked IPSCs (eIPSCs) in eGFP+ neurons (**Figure 5A**). The relative change in absolute PPR was consistently different from 0 at all interpusle interval tested: 20 ms interpulse (ΔPPR = 0.19 ± 0.07, $N = 8$, $t = 2.751$, $p = 0.0285$, one sample t-test), 50 ms interpulse (ΔPPR = 0.19 ± 0.06, $N = 9$, $t = 3$, $p = 0.0112$, one sample t-test) or 100 ms interpulse (ΔPPR = 0.19 ± 0.07, $N = 9$, $t = 0.1944$, $p = 0.0205$, one sample t-test; **Figure 5B**).

In these neurons, NMDA reduced the mean amplitude of eIPSCs (control: −37.5 ± 3.9 pA, during NMDA: −24.7 ± 2.6 pA; $N = 17$; $t = 6.029$, $p < 0.0001$, paired t-test). This inhibition remains significant at least during 5 min following NMDA washout (Wash −24.4 ± 2.6 pA, $N = 17$, compared to control $t = 2.440$, $p = 0.0267$, paired t-test; **Figures 5C,D**). These results are consistent with a NMDAr-mediated shunt of action potential dependent release of GABA and confirm that NMDAr modulate GABAergic transmission by a presynaptic mechanism of action.

DISCUSSION

Our results show that activation of NMDAr containing GluN2C/D subunits increased the frequency of GABAergic spontaneous and miniature IPSCs in lamina II neurons. Furthermore, this facilitation of spontaneous and miniature GABAergic IPSCs by NMDAr was preferentially observed for GABAergic connections onto eGFP+ neurons. Interestingly, NMDAr activation reduced the amplitude of electrically-evoked IPSCs recorded.

NMDAr Subunit Composition and Mg^{2+} Sensitivity

NMDAr are abundant in the dorsal horn with GluN1 and GluN2A/B receptors subunits expressed virtually in all lamina I and II neurons (Nagy et al., 2004). Concerning GluN2C/D

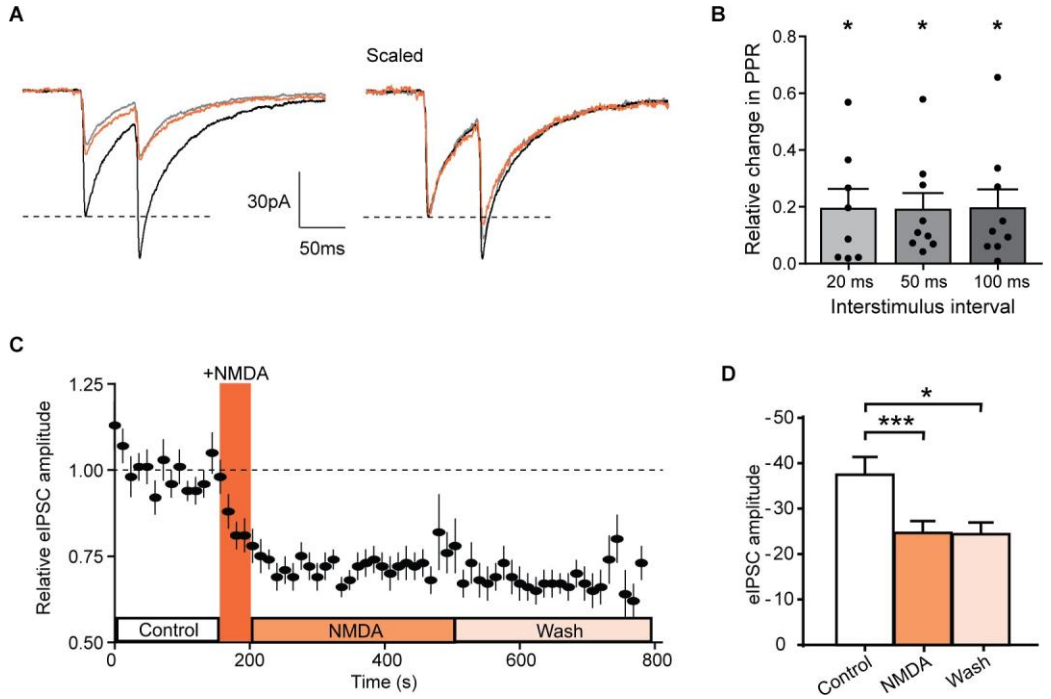

FIGURE 5 | NMDA inhibits evoked IPSCs in GAD-eGFP positive neurons. (A) Average of 10 traces representing the response to two successive stimulations separated by 50 ms before (black, control) and during NMDA application (orange, NMDA) and after a washout (gray, wash). (B) Histograms showing the relative change in PPR measured in GAD positive cells, for three different paired stimulation interval: 20 ms ($N = 8$), 50 ms ($N = 9$), and 100 ms ($N = 9$). (C) Time course of normalized GABAergic eIPSC amplitude before and after NMDA application ($N = 17$). (D) Histogram showing average eIPSC amplitude before (control) NMDA application, after NMDA application (NMDA) and after a washout (wash; $N = 17$). *$p < 0.05$ and ***$p < 0.001$. One sample t-test (B) and paired t-test (D) were used. Error bars indicate SEM.

subunits, their level of expression is lower in the dorsal horn compared to other GluN2 subunits. But it has been shown that GABAergic interneurons also express NMDAr containing GluN2C/D subunits. These receptors may play a role in modulating the activity of inhibitory interneurons in the dorsal horn of the spinal cord (Shiokawa et al., 2010). Recently, a sex-specific expression pattern of GluN2D subunit has been reported in young rats (P21), with a larger expression of this subunit in male compared to female in superficial regions of the dorsal horn (Temi et al., 2021). We used only male mice in our study; therefore, the modulation of the GABAergic synaptic transmission by NMDAr activation could be different in female mice and would require further investigation.

Using a pharmacological approach, we identify GluN2C/D containing NMDAr as responsible for the NMDA-induced increase of spontaneous GABA release. The increase in mIPSC frequency was comparable in the presence of 2 mM Mg^{2+} or in the absence of Mg2+ indicating low sensitivity to Mg^{2+} block of the NMDAr. This is in agreement with the fact that the presence of GluN2C/D subunits reduces the voltage-sensitive Mg^{2+} block of NMDAr and therefore allows an activation by endogenous glutamate even without depolarization to relieve the Mg^{2+} block (Paoletti, 2011). These properties of NMDAr are well suited for a presynaptic NMDAr function because it may allow receptors to sense level of ambient glutamate and to be activated in the absence of depolarization, and thus under resting conditions. Inhibition may be thus finely regulated according to the overall level of glutamate and to the excitatory activity in the network (Bouvier et al., 2015).

NMDA Receptor Localization

In the superficial laminae of the dorsal horn, NMDAr are present in virtually every excitatory synapse and therefore in every interneuron (Nagy et al., 2004). NMDA receptors have also been immunocytochemically detected on presynaptic terminals of primary afferents (Liu et al., 1994; Lu et al., 2003) where they regulate neurotransmitter release from the terminals of primary afferent neurons (Bardoni, 2013). Presynaptic localization of NMDAr on GABAergic terminals is less documented. Only one immunohistochemical study has reported that, NMDArs are present on a subset of GABAergic terminals (37%) in rat superficial dorsal horn (Lu et al., 2005); however, the role of these receptors has not been investigated so far.

In other regions of the CNS, such as cerebellum, neocortex, prefrontal cortex, and visual cortex, immunocytochemical and functional studies have documented the presence of presynaptic NMDAr involved in modulation of GABA release (Glitsch and Marty, 1999; Mathew and Hablitz, 2011; Abrahamsson et al., 2017; Pafundo et al., 2018). Some of these studies reported a differential regulation of evoked and spontaneous release by

presynaptic NMDAr (Glitsch and Marty, 1999; Abrahamsson et al., 2017) comparable with our results. Indeed, we showed that NMDA increased the frequency of GABAergic mIPSCs but depressed evoked GABAergic IPSCs recorded in lamina II neurons. Considering a presynaptic localization, NMDAr activation could induce a calcium influx through the NMDAr channel, and thereby facilitate synaptic vesicle exocytosis and spontaneous GABA release. The same presynaptic NMDAr could depress evoked GABA release by decreasing the input resistance, thereby shunting the propagation of incoming action potentials. Presynaptic NMDAr could also depolarize synaptic boutons, inactivate voltage gated Na^+ channels and elevate the threshold for action potential generation. Both mechanisms would result in an increase in transmission failure and a reduction of the probability of evoked GABA release. Finally, presynaptic NMDAr could act *via* distinct and independent pathways to control evoked and spontaneous release separately as recently shown in the visual cortex (Abrahamsson et al., 2017).

However, our results provide indirect evidence for a presynaptic localization of NMDAr with important limitations. We cannot exclude that NMDAr are expressed near the GABAergic synapse and that NMDA might act by an indirect effect implicating a second messenger. Indeed, postsynaptic NMDAr activation could be the source of calcium needed to trigger and/or release a retrograde messenger such as NO. For example, NO could act retrogradely on the presynaptic terminal and facilitate GABA release. Such a mechanism has been reported in the dorsal horn were NO release was triggered by metabotropic glutamate receptor (mGluR1) activation and induced a heterosynaptic LTP of GABAeric synapses (Fenselau et al., 2011). Interestingly, in the dorsal horn, NO synthase is expressed in 17% of GABAergic interneurons (Boyle et al., 2017) and we found that NMDAr-dependent modulation of GABAergic transmission targeted preferentially inputs to GABAergic neurons. However, this mechanism could only explain the facilitatory effect observed on spontaneous transmission but not the depression of evoked IPSCs.

Finally, recent investigations have shown that functional NMDAr were present on astrocytes (Ziak et al., 1998; Lalo et al., 2006; Palygin et al., 2011), and activation of these astroglial NMDAr is involved in neuron-to-glia communication, and in the modulation of inhibitory synaptic transmission (Lalo et al., 2006, 2014). Such astroglial receptors could be involved in our results but once again could not completely explain the differential modulation of spontaneous and evoked GABA release.

However implication of different NMDAr with different localizations can drive bidirectional plasticities at GABAergic synapses (Mapelli et al., 2016) and thus could explain the differential regulation of GABAergic transmission that we observed.

In our experiments, the frequency of glycinergic mIPSCs was unaffected, indicating that presynaptic modulation of inhibitory synaptic transmission by NMDAr activation was exclusively observed at GABAergic nerve terminals and selectively controlled spontaneous GABAergic synaptic transmission. These results are in accordance with a recent study by Kloc and collaborators who showed that NMDAr activation induced LTP at glycinergic synapses that depended upon an increase in the number and/or the properties of Glycinergic receptors but was independent of glycine release (Kloc et al., 2019). In the DH, most dorsal horn neurons receive both GABAergic and glycinergic inputs, but these inputs may arise from neurons having their cell body localized in distinct laminae. Inhibitory interneurons in lamina II are virtually only GABAergic since glycinergic cell bodies were almost absent in this lamina (Zeilhofer et al., 2005; Punnakkal et al., 2014). A selective modulation of GABAergic transmission by NMDAr might therefore correspond to a restricted modulation of inhibition within lamina II processing nociceptive information.

Physiologically, glutamate required to activate NMDAr responsible for GABAergic synapse modulation may originate from neighboring excitatory synapses. In this case, NMDAr activation would depend on glutamate diffusion from neighboring synapses as previously described in the cerebellum (Huang and Bordey, 2004; Duguid and Smart, 2009). In the dorsal neuronal horn network ambient levels of glutamate are tightly regulated by glutamate transporters. These transporters (mainly expressed by glial cells) have a crucial role in limiting glutamate diffusion and crosstalk between neighboring synapses. Interestingly, in pathological states such as during neuropathic pain induced by a nerve injury, disruption of glutamate homeostasis in the DH led to an increase in extracellular levels of glutamate and subsequent spillover (Inquimbert et al., 2012). Therefore the modulation of GABAergic synaptic transmission by NMDA receptors we described in this study could be a new spinal mechanism involved in the development of neuropathic pain. Moreover, subunit composition and properties of NMDAr are altered by inflammation and peripheral nerve injury (Guo and Huang, 2001; Iwata et al., 2007) and consequently the modulation of GABAergic transmission by NMDAr activation could be modified.

Altogether our results strongly suggest that glutamate can directly activate GluN2C/D containing NMDAr which differentially regulate GABA release. This crosstalk between excitation and inhibition could control the excitation/inhibition balance in the spinal neuronal network. It will be critical in future work to clarify the localization of NMDAr involved in the regulation of GABAergic transmission targeting GABAergic neurons and to define their role in the processing of nociceptive information in spinal neuronal network in physiological, inflammatory and neuropathic pain conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Regional ethics committee and the French Ministry of Agriculture.

AUTHOR CONTRIBUTIONS

BL, LC, and LV-M performed acquisition and analysis of data. This study was designed by PI, SH, and RS. The manuscript was written by PI, SH, RS, and BL. All authors contributed to the article and approved the submitted version.

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Results part II

Results project I

Implication of astrocytes in NMDA-dependent modulation of synaptic GABAergic transmission

Experiments were performed in the presence of CNQX (10µM) and strychnine (1µM) to isolate fast ionotropic GABAergic synaptic transmission. TTX (0.5 µM) was added to the extracellular medium in some experiments, to study miniature synaptic transmission.

I. Effect of FC on NMDA-dependent facilitation of GABAergic synaptic transmission

1. Study of spontaneous synaptic transmission

To study the implication of astrocytes in NMDA-dependent modulation of GABAergic synaptic transmission, we used FC. The slices were incubated in a solution of FC (100 µM) for 30 minutes before recording. The same protocol as described in the article was used: 5 min of baseline activity recording followed by a perfusion of 60 seconds of NMDA perfusion and 10 min of washout out. Neurons recorded without an FC pre-incubation were used as a control to the FC group. To simplify the following paragraphs, "NMDA-dependent facilitation of GABAergic synaptic transmission" will be referred to as "NMDA-dependent facilitation".

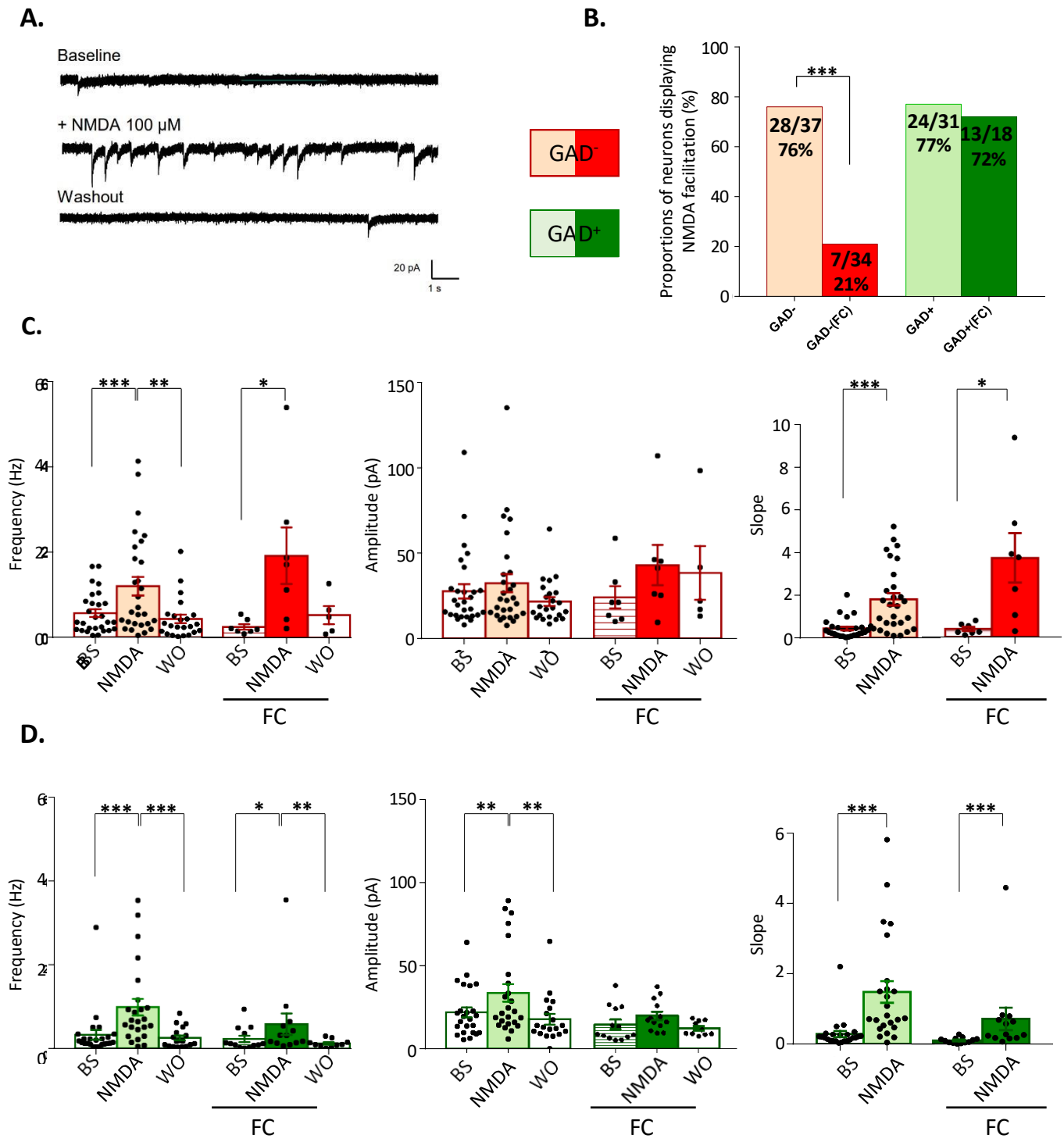

Figure 22: Effect of NMDA perfusion on GABAergic synaptic transmission in GAD⁻ and GAD⁺ neurons, in control conditions and after FC incubation

A. Illustration of 3 periods of recording: baseline (top), during NMDA perfusion (middle) and washout (bottom).
B. Histogram representing proportions of neurons displaying NMDA-dependent facilitation in control conditions (GAD⁻ : pink; GAD⁺ : light green) and after a pre-incubation with FC (GAD⁻ : red; GAD⁺ : dark green). Fisher's exact test ****p<0.0001
C-D. Histograms representing frequencies, amplitudes and distribution slopes of GABAergic sIPSC in GAD⁻ neurons (**C.**) and GAD⁺ neurons (**D.**), in control conditions and after a pre-incubation with FC. Paired T-test, *p<0.05, **p< 0.01, ***p<0.001; Wilcoxon test, *p<0.05, **p<0.01, ***p<0.001

1.1. Effect of NMDA perfusion on GABAergic synaptic transmission.

NMDA perfusion induced facilitation of GABAergic synaptic transmission in GAD^- and GAD^+ neurons (figure 22A). To assess the effect of FC on NMDA-dependent modulation of GABAergic synaptic transmission, we first compared the proportions of recorded neurons displaying NMDA-dependent facilitation of GABAergic synaptic transmission between control and FC incubated neurons.

In GAD^- neurons, FC incubation induced a significant reduction in the proportion of neurons displaying NMDA-dependent facilitation (control: 76%, n=28/37 neurons, 26 mice; FC: 21%, n=7/34 neurons, 26 mice; $p<0.0001$, Fisher's exact test) (figure 22B).
In GAD^+ neurons, FC incubation did not affect the proportion of neurons displaying NMDA-dependent facilitation (control: 77%, n=24/31 neurons, 25 mice; FC: 72%, n=13/18 neurons, 16 mice; p= 0.74, Fisher's exact test) (figure 22B).

In GAD^- neurons

In control conditions, facilitation of GABAergic synaptic transmission was reversible and always followed by a full recovery after a washout. In neurons displaying NMDA-dependent facilitation, sIPSC frequency increase was of |211|% (baseline: 0.57 ± 0.09 Hz; NMDA: 1.20 ± 0.22 Hz; p= 0.0004 Wilcoxon test; n=28 neurons, 22 mice). The sIPSC distribution slopes were obtained from the cumulative event plots, plotted for each cell individually (see Material and Methods, paragraph II-5). The slope increase was |414|% (baseline: 0.43 ± 0.08; NMDA: 1.81 ± 0.29; $p<0.0001$, Wilcoxon test; n=28 neurons, 22 mice). The increase in sIPSC frequency was not associated with a change in sIPSC amplitude (baseline: -27.67 ± 4.17 pA; NMDA: -32.46 ± 5.32 pA; p= 0.9019, Wilcoxon test; n=28 neurons, 22 mice) (figure 22C).

In FC incubated neurons displaying NMDA-dependent facilitation, sIPSC frequency increase was of |750| % (baseline + FC: 0.26±0.06 Hz; NMDA + FC: 1.93±0.67 Hz; p= 0.0359 paired t-test; n=7 neurons, 7 mice). The slope increase was of |985|% (baseline + FC: 0.38 ±0.10; NMDA + FC: 3.70 ± 01.15; p=0.0302, paired t-test; n=7 neurons, 7 mice). sIPSC frequency increase was not

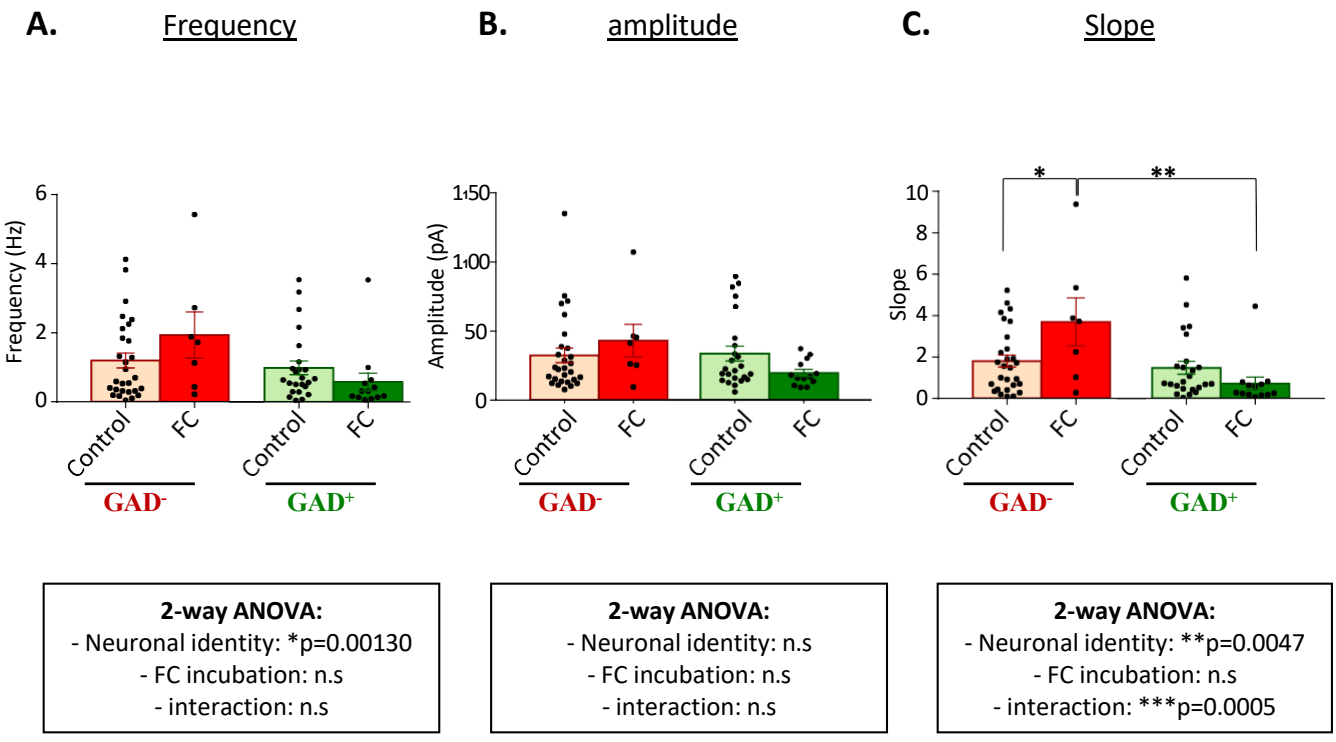

Figure 23: GABAergic sIPSC frequency, amplitude, and distribution slope in GAD$^-$ and GAD$^+$ neurons during NMDA perfusion, in control conditions and after FC incubation

Histograms representing frequencies (**A.**), amplitudes (**B.**) and distribution slopes (**C.**) of GABAergic sIPSC during NMDA perfusion in control conditions (GAD$^-$: pink; GAD$^+$: light green) and after FC incubation (GAD$^-$: red; GAD$^+$: dark green). *p<0.05, ***p<0.001 2-way ANOVA (1st factor: neuronal identity, 2nd factor: FC incubation); *p<0.05, **p<0.01 Tuckey's multiple comparisons test

associated with a change in sIPSC amplitude (baseline + FC: -24.21 ± 6.50 pA; NMDA + FC: -43.04 ± 11.80 pA; p = 0.1772, paired t-test; n=7 neurons, 7 mice) (figure 22C).

In GAD^+ neurons

In control conditions, NMDA perfusion induced an increase in sIPSC frequency of |301|% (baseline: 0.33 ± 0.12 Hz; NMDA: 0.99 ± 0.20 Hz; p<0.0001, Wilcoxon test; n=24 neurons, 20 mice). The slope increase was |519|% (baseline: 0.29 ± 0.09; NMDA: 1.48 ± 0.31; p<0.0001, Wilcoxon test; n=24 neurons, 20 mice). sIPSC frequency increase was associated with a significant increase in sIPSC amplitude, of |171|% (baseline: -21.94 ± 3.02 pA; NMDA: -33.60 ± 5.30 pA; p=0.0096, Wilcoxon test; n=24 neurons, 20 mice) (Figure 22D).

In FC incubated neurons displaying NMDA-dependent facilitation, sIPSC frequency increase was of |252|% (baseline + FC: 0.23 ± 0.08 Hz; NMDA + FC: 0.58 ± 0.26 Hz; p=0.0217, Wilcoxon test; n=13 neurons, 11 mice). The slope increase was of |720|% (baseline + FC: 0.10 ± 0.02; NMDA + FC: 0.72 ± 0.32; p=0.0002, Wilcoxon test; n=13 neurons, 11 mice). There was no change in sIPSC amplitude (baseline + FC: -14.45 ± 2.91 pA; NMDA + FC: -19.60 ± 2.54 pA; p=0.1795, paired t-test; n=13 neurons, 11 mice) (figure 22D).

1.2.Effect of neuronal neurochemical identity and FC on NMDA-dependent facilitation

To assess a different intensity of NMDA-dependent facilitation depending on neurochemical neuronal identity and/or FC incubation, we compared sIPSC frequency, amplitude, and distribution slope during the perfusion of NMDA, obtained from neurons displaying NMDA-dependent facilitation.

We found a significant effect of neuronal identity on sIPSC frequency during NMDA perfusion and a tendency at an interaction effect of neuronal identity and FC incubation (GAD^- NMDA: 1.20 ± 0.22 Hz; GAD^+ NMDA: 0.99 ± 0.20 Hz; GAD^- NMDA + FC: 1.93 ± 0.67 Hz; GAD^+ NMDA + FC: 0.58 ± 0.26 Hz; 2-way ANOVA; neuronal identity, p=0.0130; FC incubation, p=0.5922; interaction p=0.0670; n=72 neurons, 60 mice) (figure 23A).

We found no effect of neuronal identity or FC incubation on sIPSC amplitude (figure 23B).

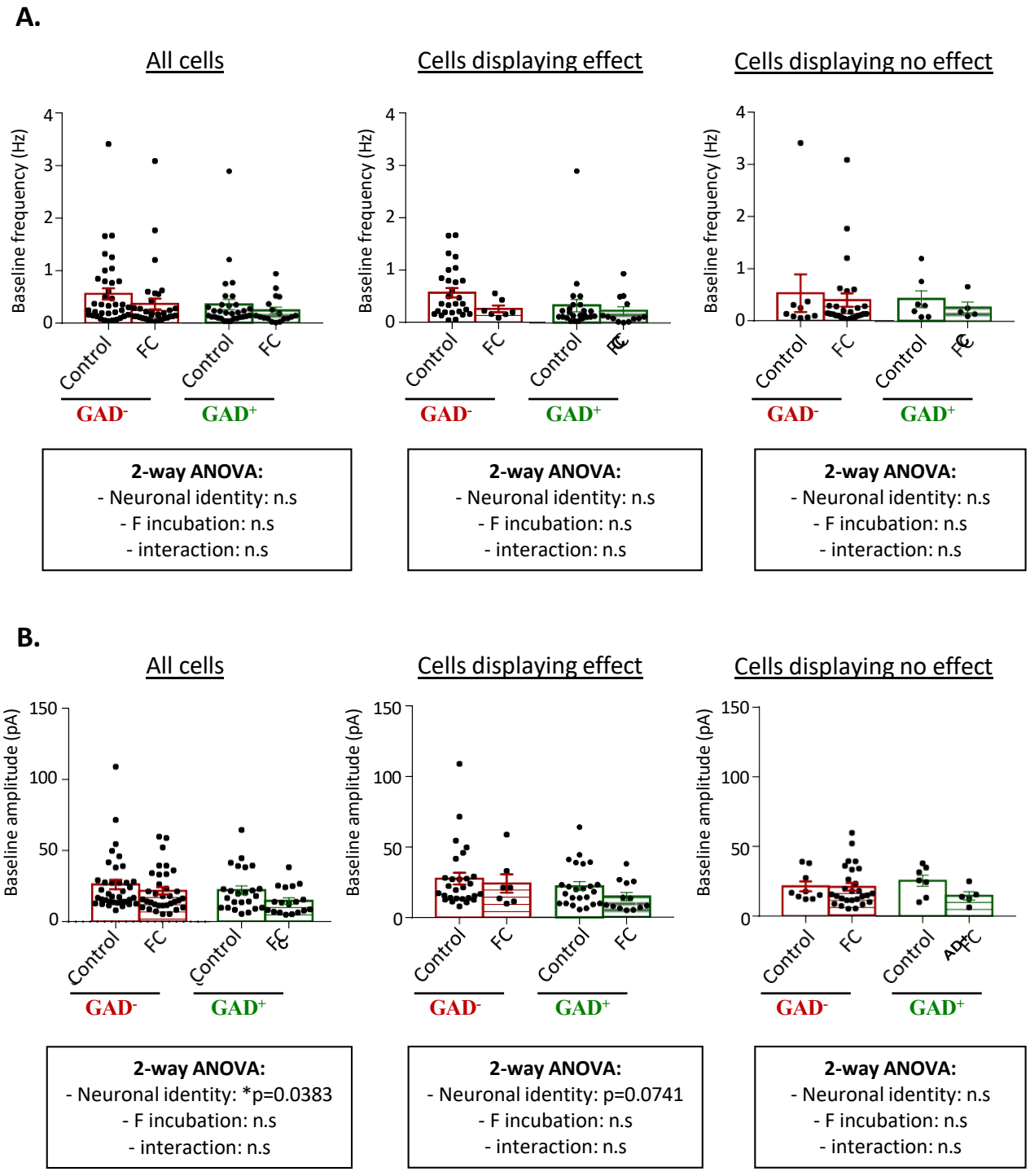

Figure 24: Baseline GABAergic sIPSC frequency and amplitude in GAD$^-$ and GAD$^+$ neurons, in control conditions and after FC incubation

Histograms representing baseline GABAergic sIPSC frequencies (**A.**) and amplitudes (**B.**) in GAD$^-$ and GAD$^+$ neurons, in control conditions and after FC incubation. The 1st histogram is all neurons, the 2nd histogram is neurons displaying NMDA-dependent facilitation and the 3rd histogram is neurons not displaying NMDA-dependent facilitation. *p<0.05, 2-way ANOVA (1st factor: neuronal identity, 2nd factor: FC incubation)

We found a significant effect of neuronal identity, and interaction between FC incubation and neuronal identity on the sIPSC distribution slopes during NMDA perfusion (GAD^- NMDA: 1.81 ± 0.29; GAD^+ NMDA: 1.48 ± 0.31; GAD^- NMDA+FC: 3.70 ± 1.15; GAD^+ NMDA + FC: 0.72 ± 0.32; 2-way ANOVA; neuronal identity p= 0.0047; FC incubation p=0.2162; interaction p= 0.0005; n=72 neurons, 60 mice) (figure 23C). As the interaction between neuronal identity and FC incubation has a significant effect on sIPSC distribution slope, we proceeded with post-hoc multiple comparisons analysis. We found a significantly higher sIPSC distribution slope during NMDA-perfusion after FC incubation in GAD^- neurons compared to GAD^+ neurons (GAD^- NMDA + FC: 3.7 ± 1.15; GAD^+ NMDA + FC: 0.72 ± 0.32; p=0.0016, Tukey's test; n= 20 neurons, 18 mice), and compared to GAD^- neurons in control conditions (GAD^- NMDA: 1.81 ± 0.29; GAD^- NMDA + FC: 3.7 ± 1.15; p= 0.0432, Tukey's test; n=35 neurons, 29 mice) (figure 23C).

1.3.Effect of neuronal identity and FC on basal GABAergic synaptic transmission

To control for the differences that we observed in the intensity of NMDA-dependent facilitation, we compared baseline sIPSC frequency and amplitude in GAD^- and GAD^+ neurons in control conditions and after FC incubation.

We found no influence of neurochemical neuronal identity, FC incubation or interaction between the 2, on GABAergic sIPSC frequency in GAD^- and GAD^+ neurons (GAD^- baseline: 0.56 ± 0.11 Hz; GAD^+ baseline: 0.35 ± 0.10 Hz; GAD^- baseline + FC: 0.37 ± 0.10 Hz; GAD^+ baseline + FC: 0.24 ± 0.06 Hz; 2-way ANOVA; neuronal identity, p=0.1201; FC incubation, p=0.1656; interaction, p=0.7063, n=120 neurons, 60 mice). When dividing neurons based on the presence or absence of NMDA-dependent facilitation, we found no effect of neuronal identity, FC incubation or interaction between the 2 on GABAergic sIPSC frequency in either group (figure 24A).

We found a significant effect of neurochemical neuronal identity on sIPSC amplitude (GAD^- baseline: -26.15 ± 3.28 pA; GAD^+ baseline: -20.15 ± 3.13 pA; GAD^- baseline + FC: -21.69 ± 2.53 pA; GAD^+ baseline + FC: -14.45 ± 2.21 pA; 2-way ANOVA, factor neuronal identity p= 0.0383; factor FC incubation p=0.1107; factor interaction p=0.8444; n=120 neurons, 60 mice). When dividing neurons based on the presence or absence of NMDA-dependent facilitation, we found a

non-significant tendency at an effect of neuronal identity on sIPSC amplitude in neurons displaying NMDA-dependent facilitation (GAD^- baseline: -27.66 ± 4.17 pA; GAD^+ baseline: -18.69 ± 3.86 pA; GAD^- baseline + FC: -24.21 ± 6.50 pA; GAD^+ baseline + FC: -14.45 ± 2.90 pA; 2-way ANOVA; neuronal identity p= 0.0741; FC incubation p= 0.4587; interaction p= 0.9399; n=72 neurons, 52 mice) (figure 24B).

In summary, FC incubation induced a significant reduction in the proportion of neurons displaying NMDA-dependent facilitation in GAD^- neurons, but not GAD^+ neurons. When comparing the degree of NMDA-dependent facilitation, we found a significant influence of neurochemical neuronal identity on sIPSC frequency during NMDA perfusion. The sIPSC distribution slope during NMDA perfusion after FC incubation in GAD^- was steeper compared to GAD^+ neurons, as well as compared to GAD^- in control conditions. We observed a significant effect of neurochemical neuronal identity on baseline amplitudes of sIPSC in GAD^- and GAD^+ cells, in control conditions and after FC incubation.

2. Study of miniature GABAergic synaptic transmission

The same protocol as for the study of spontaneous GABAergic synaptic transmission was used. All recordings were performed in the presence of TTX (0.5 μM).

2.1 Study of the effect of NMDA perfusion on miniature GABAergic synaptic transmission

NMDA perfusion induced facilitation of miniature GABAergic synaptic transmission in GAD^- and GAD+ neurons (figure 25A). To assess the effect of FC on NMDA-dependent modulation of miniature GABAergic synaptic transmission, we first compared the proportions of recorded neurons displaying NMDA-dependent facilitation.

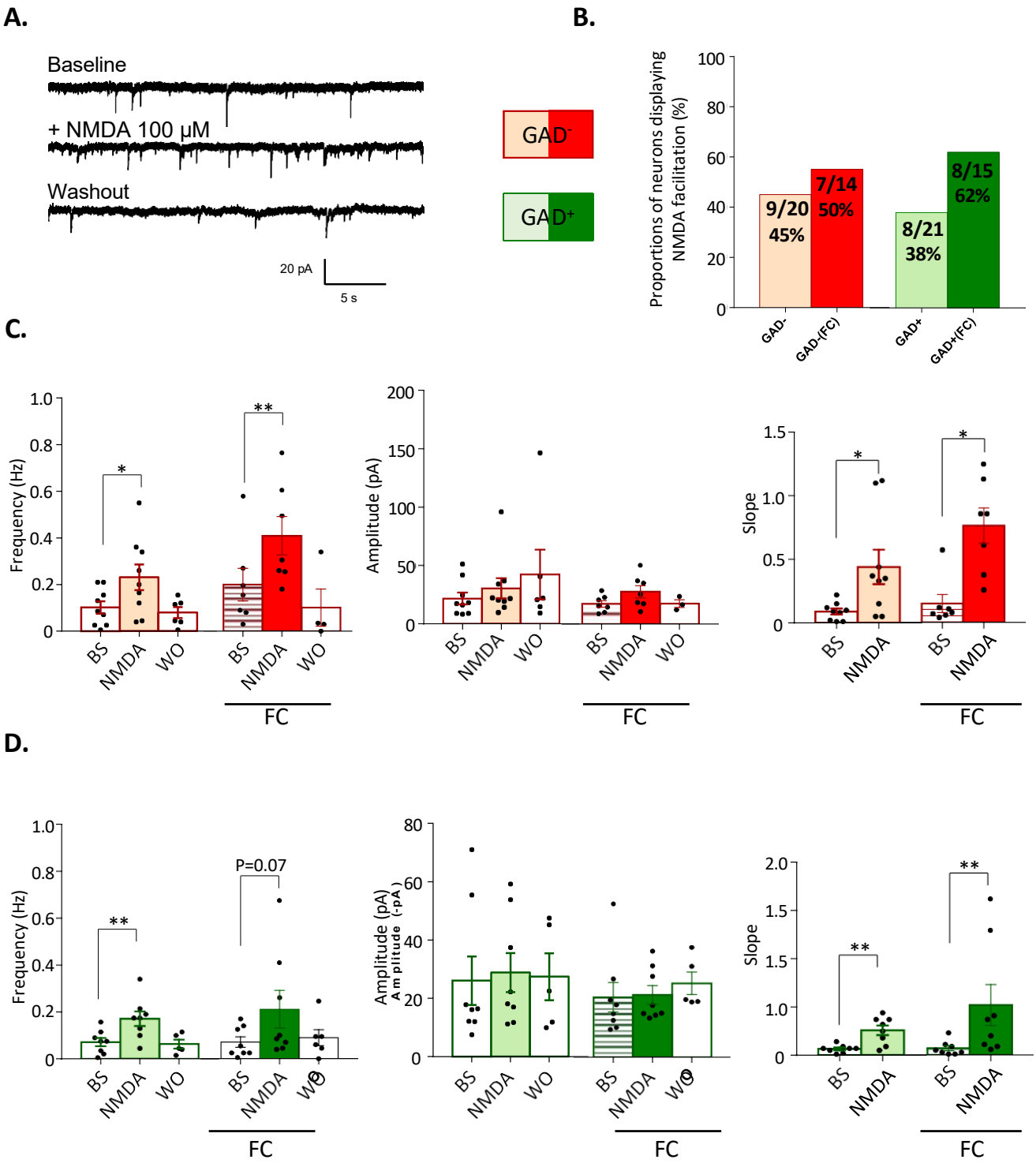

Figure 25: Effect of NMDA perfusion on miniature GABAergic synaptic transmission in GAD⁻ and GAD⁺ neurons, in control conditions and after FC incubation

A. Illustration of 3 periods of recording: baseline (top), during NMDA perfusion (middle) and washout (bottom).
B. Histogram representing proportions of neurons displaying NMDA-dependent facilitation of miniature GABAergic synaptic transmission in control conditions (GAD⁻ : pink; GAD⁺ : light green) and after a pre-incubation with FC (GAD⁻ : red; GAD⁺ : dark green).
C-D. Histograms representing frequencies, amplitudes and distribution slopes of GABAergic mIPSC in GAD⁻ neurons (**C.**) and GAD⁺ neurons (**D.**), in control conditions and after a pre-incubation with FC. Paired T-test, *p<0.05, **p< 0.01; Wilcoxon test, *p<0.05, **p<0.01

In GAD$^-$ neurons, FC incubation did not change the proportion of neurons displaying NMDA-dependent facilitation (control: 45%, n=9/20 neurons, 15 mice; FC: 50%, n=7/14 neurons, 12 mice; p=1, Fisher's exact test) (figure 25B).

In control conditions, the proportion of neurons displaying NMDA-dependent facilitation of miniature GABAergic synaptic transmission is lower than the proportion of neurons displaying NMDA-dependent facilitation of spontaneous GABAergic synaptic transmission (control spontaneous: 76%, n=28/37 neurons, 26 mice; control miniature: 45%, n=9/20 neurons, 15 mice; p= 0.0401, Fisher's exact test).

In GAD$^+$ neurons, FC incubation induced no significant change in the proportion of neurons displaying NMDA-dependent facilitation (control: 38%, n=8/21 neurons, 20 mice; FC: 56%, n=8/15 neurons, 13 mice; p=0.4996, Fisher's exact test) (figure 25B).

In control conditions, the proportion of neurons displaying NMDA-dependent facilitation of miniature GABAergic synaptic transmission is significantly lower than the proportion of neurons displaying NMDA-dependent facilitation of spontaneous GABAergic synaptic transmission (control spontaneous: 77%, n=24/31 neurons, 25 mice; control miniature: 38%, n=8/21 neurons, 20 mice; p= 0.0082, Fisher's exact test).

In GAD$^-$ neurons

In control conditions, facilitation of GABAergic synaptic transmission was reversible and always followed by a full recovery after a washout. In neurons displaying NMDA-dependent facilitation, the increase in frequency was of |230|% (baseline: 0.10 ± 0.03 Hz; NMDA: 0.23 ± 0.06 Hz; p=0.038, paired t-test; n=9 neurons, 7 mice). The mIPSC distribution slope increase was |489|% (baseline: 0.09 ± 0.0.02; NMDA: 0.44 ± 0.13; p=0.0271, paired t-test; n=9 neurons, 7 mice). The increase in mIPSC frequency was not associated with a change in mIPSC amplitude (baseline: -21.70 ± 5.07; NMDA: -30.49 ± 8.67; p=0.6523, Wilcoxon test; n=9 neurons, 7 mice) (figure 25C).

In FC incubated neurons displaying NMDA-dependent facilitation, the increase in mIPSC frequency was of |205| % (baseline + FC: 0.20 ±0.07 Hz; NMDA + FC: 0.41 ± 0.08 Hz; p=0.0015, paired t-test; n=7 neurons, 7 mice). The slope increase was of |511|% (baseline + FC: 0.15 ±0.07; NMDA + FC: 0.77 ± 0.14; p=0.0156, Wilcoxon test; n=7 neurons, 7 mice). The mIPSC frequency increase was associated with a tendency at an increase in mIPSC amplitude (baseline +FC: -17.23

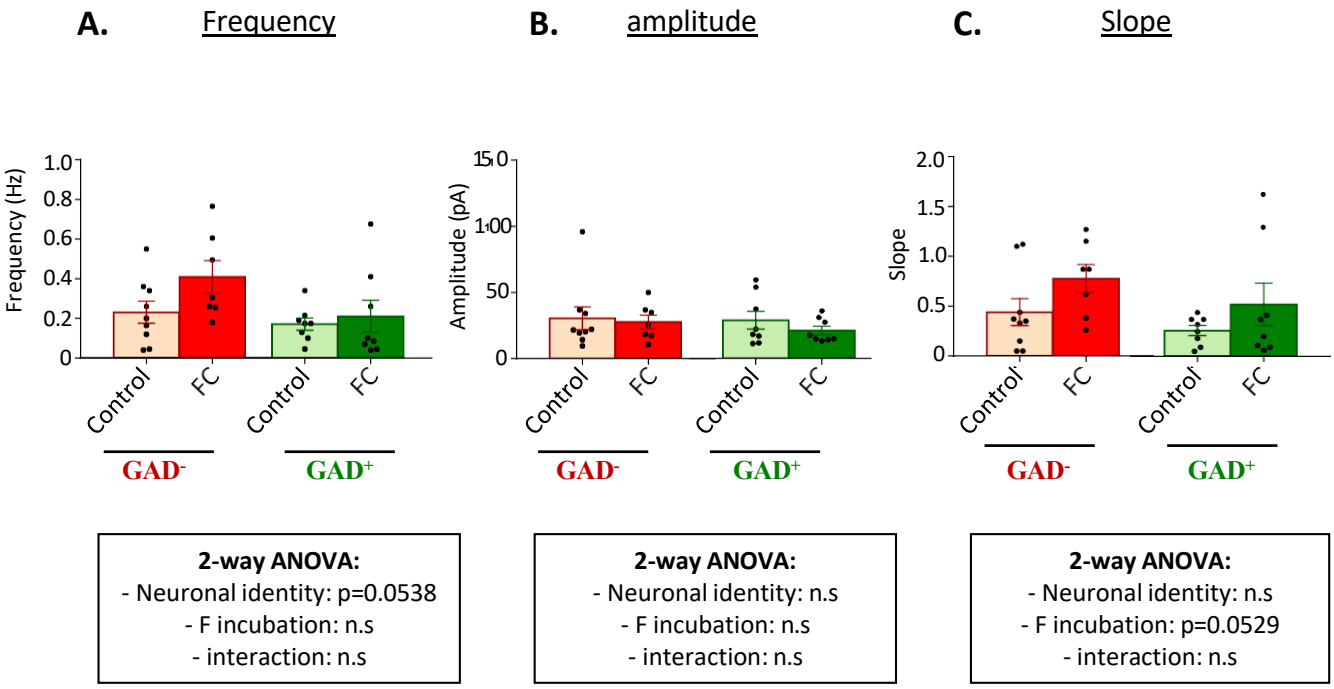

Figure 26: GABAergic mIPSC frequency, amplitude, and distribution slope in GAD$^-$ and GAD$^+$ neurons during NMDA perfusion, in control conditions and after FC incubation

Histograms representing frequencies (**A.**), amplitudes (**B.**) and distribution slopes (**C.**) of GABAergic sIPSC during NMDA perfusion in control conditions (GAD$^-$: pink; GAD$^+$: light green) and after FC incubation (GAD$^-$: red; GAD$^+$: dark green).

± 2.71 pA; NMDA + FC: -27.70 ±5.16 pA; p = 0.0638, paired t-test; n=7 neurons, 7 mice) (figure 25C).

In GAD^+ neurons

In control conditions, NMDA perfusion induced an increase in mIPSC frequency of |243|% (baseline: 0.07 ± 0.02 Hz; NMDA: 0.17 ± 0.03 Hz; p=0.0035, paired t-test; n=8 neurons, 8 mice). The slope increase was of |371|% (baseline miniature: 0.07 ± 0.01; NMDA miniature: 0.26± 0.05; p=0.0041, paired t-test; n=8 neurons, 8 mice). The increase in mIPSC frequency was not associated with an increase in mIPSC amplitude (baseline miniature: -26.09 ± 8.32; NMDA miniature: -28.85 ± 6.71; p=0.7933, paired t-test; n=8 neurons, 8 mice) (figure 25D).

In FC incubated neurons displaying NMDA-dependent facilitation, there was a tendency at an increase in mIPSC frequency (baseline + FC: 0.07 ± 0.02 Hz; NMDA + FC: 0.21 ± 0.08 Hz; p=0.0674 paired t-test; n=8 neurons, 7 mice). The increase in mIPSC distribution slope was of |743|% (baseline + FC miniature: 0.07 ± 0.03; NMDA + FC miniature: 0.52 ± 0.21; p=0.0078 Wilcoxon test; n=8 neurons, 7 mice). There was no change in mIPSC amplitude (baseline + FC: -20.32 ± 4.98 pA; NMDA + FC: -21.15 ± 3.15 pA; p=0.6406, Wilcoxon test; n=8 neurons, 7 mice) (figure 25D). It should be noted that each neuron in this group displays NMDA-dependent facilitation (as is presented in figure 21), however due to variability and a low n, the neurons pooled together do not present a significant change in either mIPSC frequency or amplitude.

2.2 Effect of neuronal identity and FC on NMDA-dependent facilitation

To assess a different intensity of NMDA-dependent facilitation depending on neuronal identity and/or FC incubation, we compared mIPSC frequency, amplitude, and distribution slope during NMDA perfusion.

We found a tendency of an effect of neuronal identity on mIPSC frequency during NMDA perfusion (GAD^- NMDA: 0.23 ± 2.27 Hz; GAD^+ NMDA: 0.17 ± 0.03 Hz; GAD^- NMDA+FC:

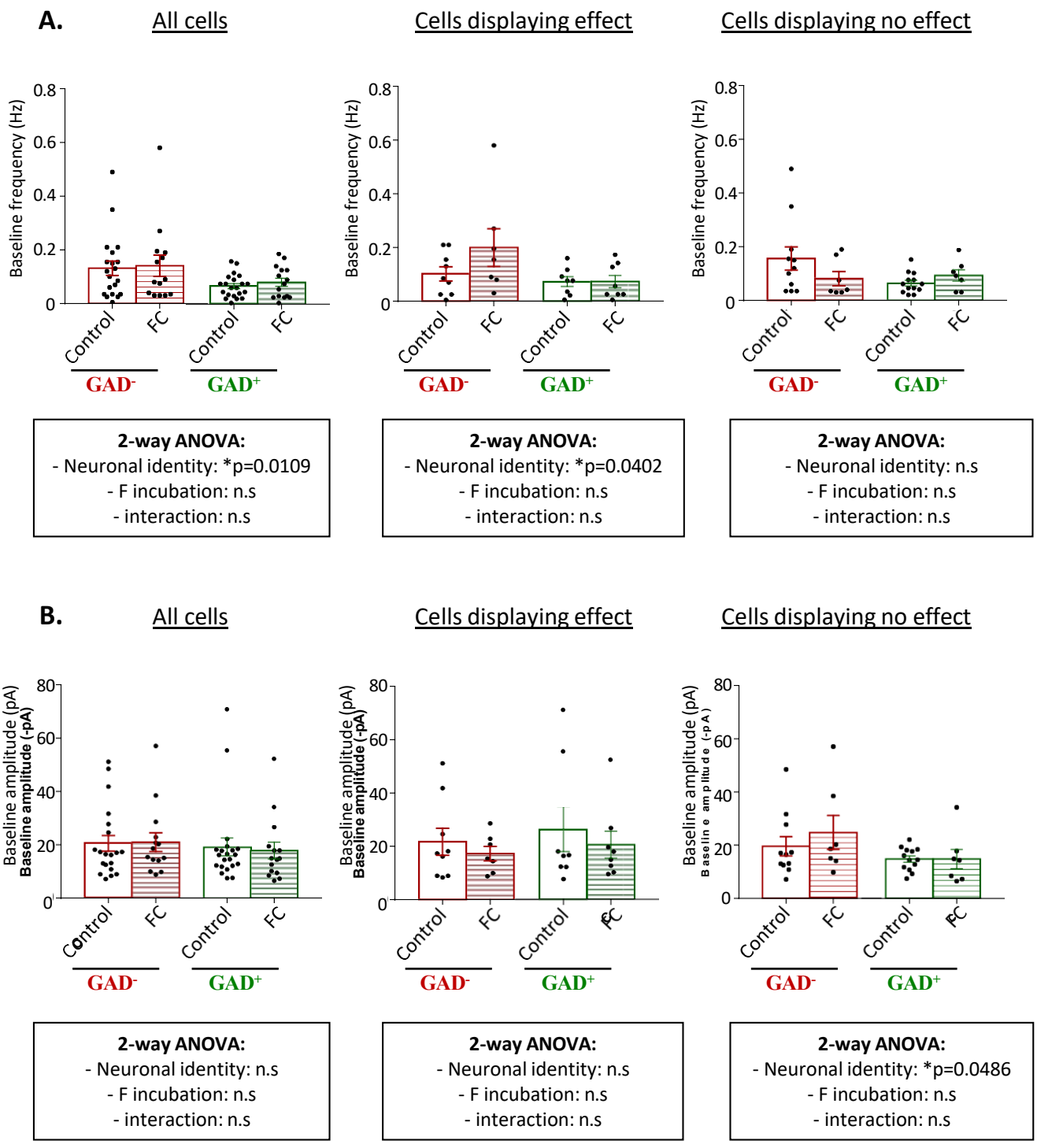

Figure 27: Baseline GABAergic mIPSC fequency and amplitude in GAD$^-$ and GAD$^+$ neurons, in control conditions and after FC incubation

Histograms representing baseline GABAergic mIPSC frequencies (**A.**) and amplitudes (**B.**) in GAD$^-$ and GAD$^+$ neurons, in control conditions and after FC incubation. The 1st histogram is all neurons, the 2nd histogram is neurons displaying NMDA-dependent facilitation and the 3rd histogram is neurons not displaying NMDA-dependent facilitation. *p<0.05, 2-way ANOVA (1st factor: neuronal identity, 2nd factor: FC incubation)

0.41 ± 0.08 Hz; GAD^+ NMDA+FC: 0.21 ± 0.08 Hz; 2-way ANOVA: neuronal identity p= 0.0538, FC incubation p= 0.1014, interaction p=2895; n=32 neurons, 26 mice) (figure 26A).

We found no effect of neuronal identity or FC incubation on mIPSC amplitude during NMDA perfusion (figure 26B).

We found a tendency at an effect of FC incubation on mIPSC distribution slope during NMDA perfusion (GAD^- NMDA: 0.44 ± 0.13; GAD^+ NMDA: 0.26 ± 0.05; GAD^- NMDA + FC: 0.77 ± 0.14; GAD^+ NMDA + FC: 0.52 ± 0.21; 2-way ANOVA: neuronal identity: 0.1524, FC incubation p= 0.0529, interaction p= 0.8097; n=32 neurons, 26 mice) (figure 26C).

2.3 Effect of FC on basal miniature GABAergic synaptic transmission

To assess an effect of neuronal identity and/or FC incubation on basal miniature GABAergic synaptic transmission, we compared baseline mIPSC frequency in GAD^- and GAD^+ neurons in control conditions and after FC incubation.

We found an influence of neuronal identity on baseline frequency of mIPSC (GAD^- baseline: 0.13 ± 0.03 Hz; GAD+ baseline: 0.07 ± 0.01 Hz; GAD^- baseline + FC: 0.14 ± 0.04 Hz; GAD^+ baseline + FC: 0.08 ± 0.02 Hz; 2-way ANOVA: neuronal identity p= 0.0109, FC incubation p= 0.6313, interaction p= 0.9150; n=70 neurons, 48 mice). When dividing neurons based on the presence or absence of NMDA-dependent facilitation, we found a significant effect of neuronal identity on mIPSC baseline frequency in neurons displaying NMDA-dependent facilitation (GAD^- baseline: 0.10 ± 0.03 Hz; GAD^+ baseline: 0.07 ± 0.02 Hz; GAD^- baseline + FC: 0.2 ± 0.07 Hz; GAD^+ baseline + FC: 0.07 ± 0.02 Hz; 2-way ANOVA: neuronal identity p=0.0402, FC incubation p= 1952, interaction p= 0.1917; n=70 neurons, 48 mice) (figure 27A).

We found no effect of neuronal identity or FC incubation on mIPSC amplitude in all cells. When dividing neurons based on the presence or absence of NMDA-dependent facilitation, we found a significant effect of neuronal identity on mIPSC amplitude in neurons not displaying NMDA-dependent facilitation (GAD^- baseline: -19.56 ± 3.61 pA; GAD^+ baseline: -21.83 ± 6.03 pA; GAD^- baseline+NMDA: -18.56 ± 3.62 pA; GAD^+ baseline+NMDA: -16.29 ± 1.90 pA; 2-way ANOVA

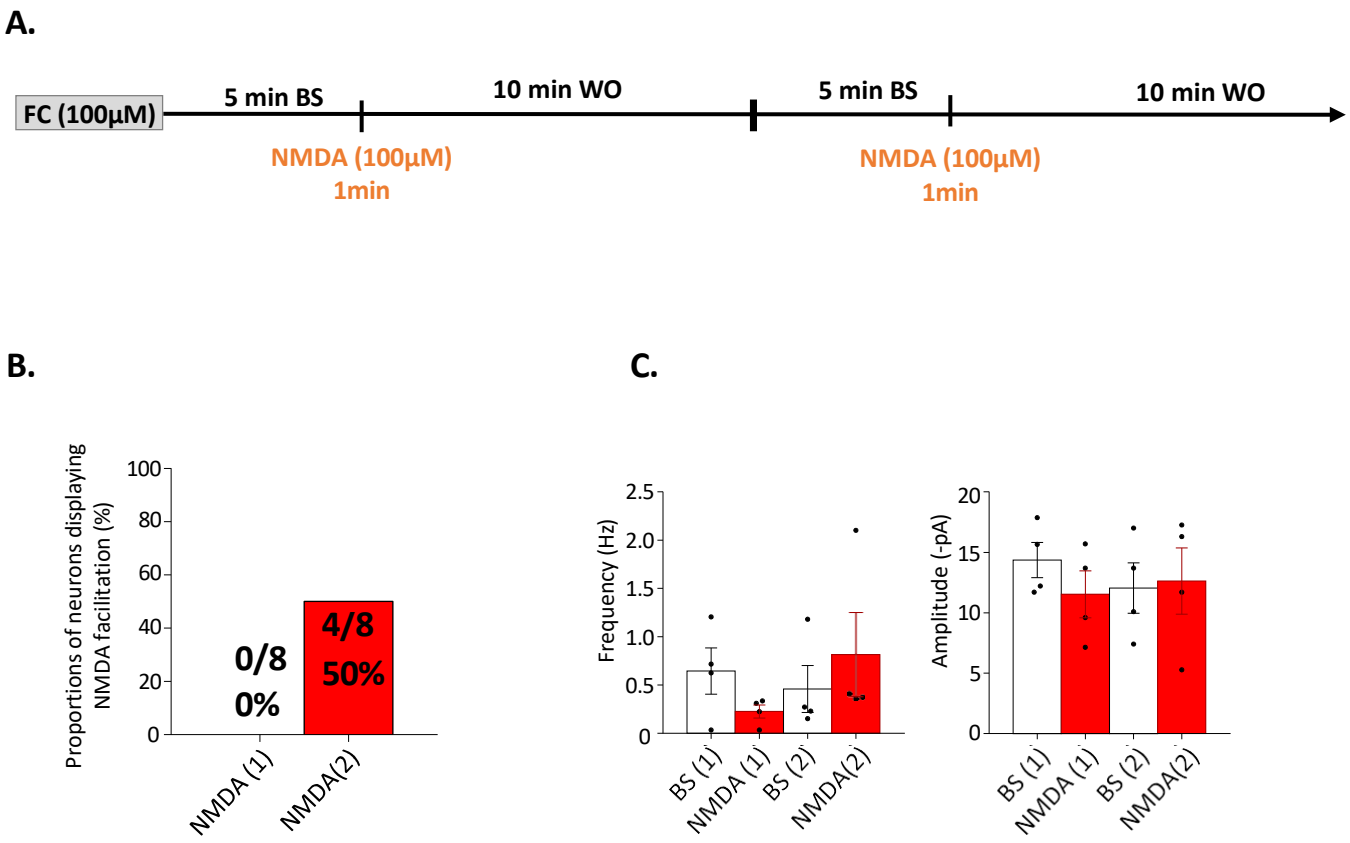

Figure 28: Double NMDA perfusion after FC incubation in GAD- neurons

A. Protocol used to assess the effect of double NMDA perfusion after FC incubation.
B. Proportion of neurons displaying NMDA-dependent facilitation of GABAergic synaptic transmission during a 2nd NMDA perfusion.
C. The histogram on the left shows GABAergic sIPSC frequency during baseline (white) and NMDA perfusion (red). The histogram on the right shows GABAergic sIPSC amplitude during baseline (white) and NMDA perfusion (red).

neuronal identity p= 0.0486, FC incubation p= 4803, interaction p= 0.4760; n=70 neurons, 48 mice) (figure 27B).

In summary, FC incubation did not induce any change in the proportion of neurons displaying NMDA-dependent facilitation of miniature GABAergic synaptic transmission. Neuronal identity had an influence on mIPSC baseline frequency in GAD^- and GAD^+ neurons, in control conditions and after a pre-incubation with FC. When dividing neurons on the presence or absence of NMDA-dependent facilitation, neuronal identity had an influence on mIPSC baseline frequency in neurons displaying NMDA-dependent facilitation and on amplitudes of mIPSC of neurons not displaying NMDA-dependent facilitation.

FC induced a reduction in the proportion of neurons displaying NMDA-dependent facilitation of spontaneous GABAergic synaptic transmission when recording GAD^- neurons but has no effect proportion of neurons displaying NMDA-dependent facilitation when recording miniature GABAergic synaptic transmission.

II. Effect of two consecutive applications of NMDA after FC incubation

To control whether we could perform sequential perfusions after FC incubation for pharmacological experiments, we performed 2 perfusions of NMDA in a row after FC incubation (figure 28A). We selected GAD^- neurons that did not display NMDA-dependent facilitation of spontaneous GABAergic synaptic transmission after a 1^{st} NMDA perfusion and performed a 2^{nd} NMDA perfusion.

NMDA-dependent facilitation following a 2^{nd} NMDA perfusion was observed in 50% of recorded neurons (N=4/8 neurons, 8 mice) (figure 28B). As the number of neurons recorded is low, no

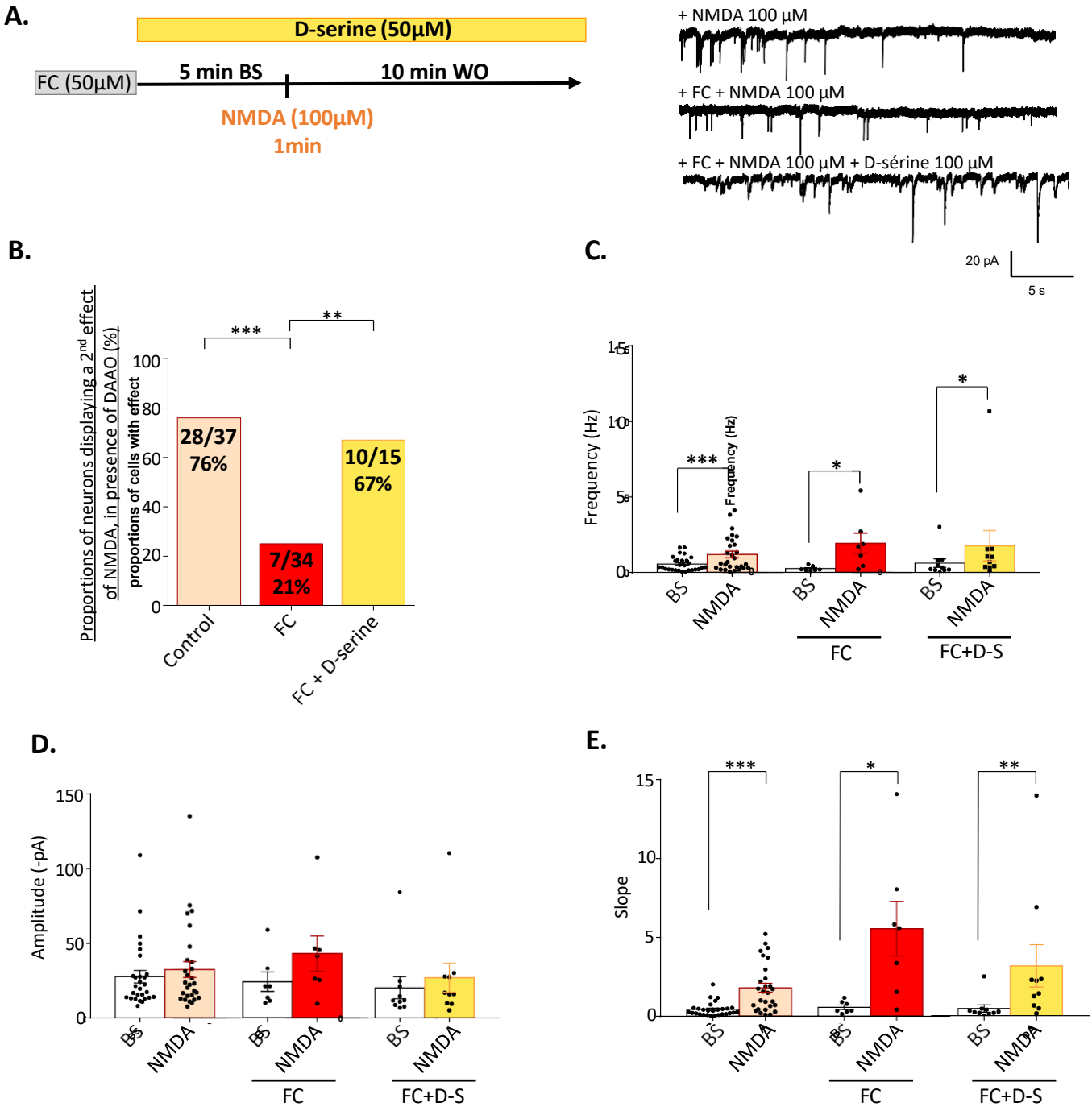

Figure 29: Effect of D-serine perfusion after FC incubation

A. Protocol used to study the effect of a D-serine perfusion on NMDA-dependent facilitation of GABAergic synaptic transmission after FC incubation.
B. Proportion of neurons displaying NMDA-dependent facilitation of GABAergic synaptic transmission during NMDA perfusion in control conditions (pink), after FC incubation (red) and after FC incubation and a co-perfusion with D-serine (blue). Fisher's exact test **p<0.05, ***p<0.001
C-D-E. Histograms representing GABAergic sIPSC frequency (**C.**), amplitude (**D.**) and distribution slope (**E.**) in control conditions (2 left bars on the histograms), after FC incubation (2 middle bars on the histograms) and after FC incubation and a co-perfusion with D-serine (2 right bars on the histograms). Paired T-test, *p<0.05, Wilcoxon test, *p<0.05, **p< 0.01, ***p<0.001

statistical analysis was performed. The values of sIPSC frequency were: 2nd baseline + FC: 0.46 ± 0.24 Hz; 2nd NMDA + FC: 0.86 ± 0.43 Hz (n=4 neurons, 4 mice). The values of sIPSC distribution sIPSC slope were: 2nd baseline + FC: 0.32 ± 0.10; 2nd NMDA + FC: 1.07 ± 0.66 (n=4 neurons, 4 mice) (not represented in graph). The values of sIPSC amplitude were 2nd baseline + FC: -12.21 ± 2.10 pA; 2nd NMDA + FC: -12.60 ± 2.74 pA (n=4 neurons, 4 mice) (figure 28C). It should be noted that each neuron in this group displays NMDA-dependent facilitation (as is presented in figure 20).

The effect of FC lessens as FC is washed out during the recording, allowing for recovery of NMDA-dependent facilitation of spontaneous GABAergic synaptic transmission in some recorded GAD^- neurons.

III. Study of D-serine in NMDA-dependent facilitation

Astrocytes are involved in the synthesis of D-serine, a co-agonist of NMDAr. We hypothesized that the effect of FC on NMDA-dependent facilitation of spontaneous GABAergic synaptic transmission in GAD^- neurons was mediated by blocking D-serine availability, thus preventing NMDAr activation. As FC incubation alters the proportion of neurons displaying NMDA-dependent facilitation when recording spontaneous synaptic transmission, we studied spontaneous transmission.

1. Effect of D-serine perfusion after FC incubation on NMDA-dependent facilitation

To test our hypothesis, we first recorded GAD^- neurons after FC incubation, and perfused D-serine (50 µM) during the whole recording. We then proceeded with the same protocol: 5 min of baseline activity recording, followed by NMDA perfusion (100 µM, 60 sec) (figure 29A).

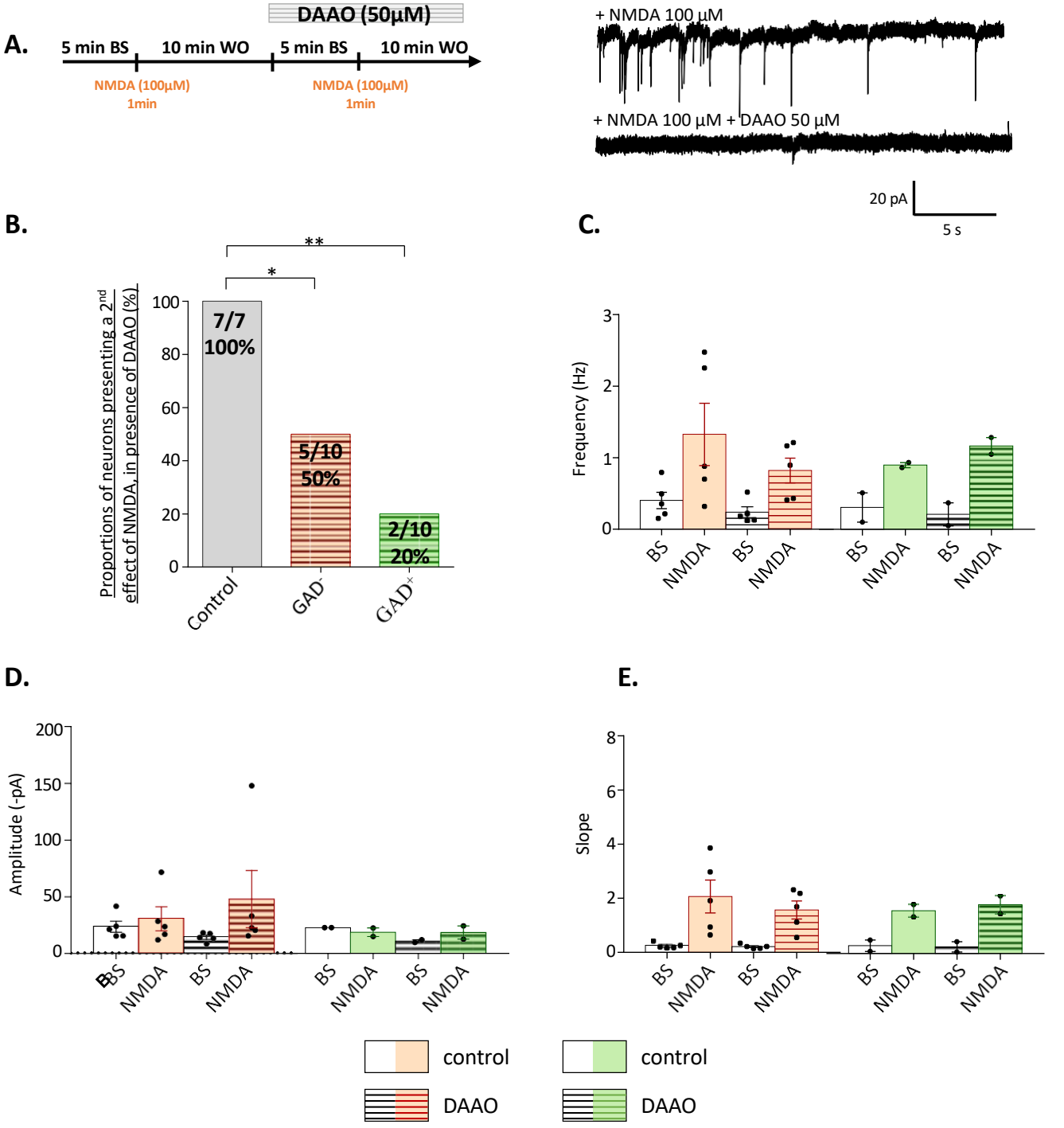

Figure 30: Effect of DAAO on a 2nd NMDA perfusion

A. Protocol used to study the effect of DAAO on NMDA-dependent facilitation of GABAergic synaptic transmission.

B. Histogram showing the proportion of neurons displaying NMDA-dependent facilitation of GABAergic synaptic transmission during a 2nd perfusion of NMDA, in the presence of DAAO: control neurons (grey), GAD^- neurons (pink) and GAD^+ neurons (green). Fisher's exact test *$p<0.05$, **$p<0.01$

C-D-E. Histograms showing frequencies (**C.**), amplitudes (**D.**) and distribution slopes (**E.**) of GABAergic sIPSC in control conditions and during a co-perfusion of DAAO.

1.1.Effect of D-serine perfusion on NMDA-dependent facilitation

To assess the effect of D-serine perfusion after FC incubation, we first compared the proportions of recorded neurons displaying NMDA-dependent facilitation during D-serine perfusion, with the proportions of cells displaying NMDA-dependent facilitation in control neurons and FC incubation. The perfusion of D-serine after FC incubation induced a significant increase in the proportion of neurons displaying NMDA-dependent facilitation compared to the group of neurons recorded after FC incubation (FC: 21%, n=7/34 neurons, 7 mice; FC + D-serine group: 67%, N=10/15 neurons, 7 mice; p=0.0031, Fisher's exact test). The proportion of neurons displaying NMDA-dependent facilitation in the control neurons and the neurons perfused with D-serine after FC incubation was comparable (figure 29B). Interestingly, D-serine perfusion after FC incubation seems to restore the proportion of neurons displaying NMDA-dependent facilitation in GAD- neurons.

In neurons FC incubated and perfused with D-serine, displaying NMDA-dependent facilitation, the increase in sIPSC frequency was of |284|% (baseline + D-serine: 0.63 ± 0.28 Hz; NMDA + FC + D-serine: 1.79 ± 1.01 Hz; p=0.0195 Wilcoxon test; n=10 neurons, 6 mice) (figure 29C). The increase in sIPSC distribution slope was of |618|% (baseline + FC + D-serine: 0.46 ± 0.23; NMDA + FC + D-serine: 3.17 ± 1.35; p=0.0020 Wilcoxon test; n=10 neurons, 6 mice) (figure 29E). There was no change in sIPSC amplitude (baseline + FC + D-serine: -20.23 ± 7.36 pA; NMDA + FC + D-serine: -26.93 ± 9.69 pA; p=0.1602 Wilcoxon test; n=10 neurons, 6 mice) (figure 29D).

1.2.Effect of DAAO on NMDA-dependent facilitation

To confirm the implication of D-serine in NMDA-dependent facilitation of spontaneous GABAergic synaptic transmission, we selected GAD^- and GAD^+ neurons displaying NMDA-dependent facilitation and performed a 2^{nd} NMDA perfusion in the presence of D-amino-acid-oxidase (DAAO), an enzyme that oxidizes and thus degrades D-serine (figure 30A). As a control group, we used neurons recorded during 2 consecutive perfusions of NMDA, displaying 2 NMDA-dependent facilitation of spontaneous GABAergic synaptic transmission (n=7 neurons, 3 mice).

To assess the effect of DAAO on NMDA-dependent facilitation of GABAergic synaptic transmission, we first compared the proportion of neurons displaying NMDA-dependent facilitation following a second perfusion of NMDA.

In GAD^- neurons, perfusion of DAAO during the second perfusion of NMDA induced a significant reduction in the proportion of neurons displaying NMDA-dependent facilitation (control group: 100%, n=7/7 neurons, 3 mice; DAAO group: 50%, n=5/10 neurons, 6 mice; p=0.04412, Fisher's exact test) (figure 30B, C, D, E).

In GAD^+ neurons, Perfusion of DAAO during the second perfusion of NMDA induced a significant reduction in the proportion of neurons displaying NMDA-dependent facilitation (control group 100%, n=7/7 neurons, 3 mice; DAAO group: 20%, n=2/10 neurons, 9 mice; p=0.00226, Fisher's exact test) (figure 30B, C, D, E).

It should be noted that each neuron in this group displays NMDA-dependent facilitation (as is presented in figure 20), but the number of neurons is too low for statistical analysis, and more neurons must be added to complete this analysis.

D-serine perfusion, following FC incubation, restores a proportion of neurons displaying NMDA-dependent facilitation similar to the one in control conditions when recording GAD^- neurons. Oxidation of D-serine reduces the proportion of neurons displaying NMDA-dependent facilitation, for both GAD^- and GAD^+ neurons.

IV. Study of the endogenous source of glutamate

Studies have shown that in conditions of intense prolonged activity of primary afferents, glutamate transporters in the DH can get saturated, resulting in glutamate diffusing in the extracellular space. This phenomenon is called spill-over. We hypothesized that glutamate originating from glutamate spill-over is the main agonist of the NMDAr involved in NMDA-dependent facilitation of

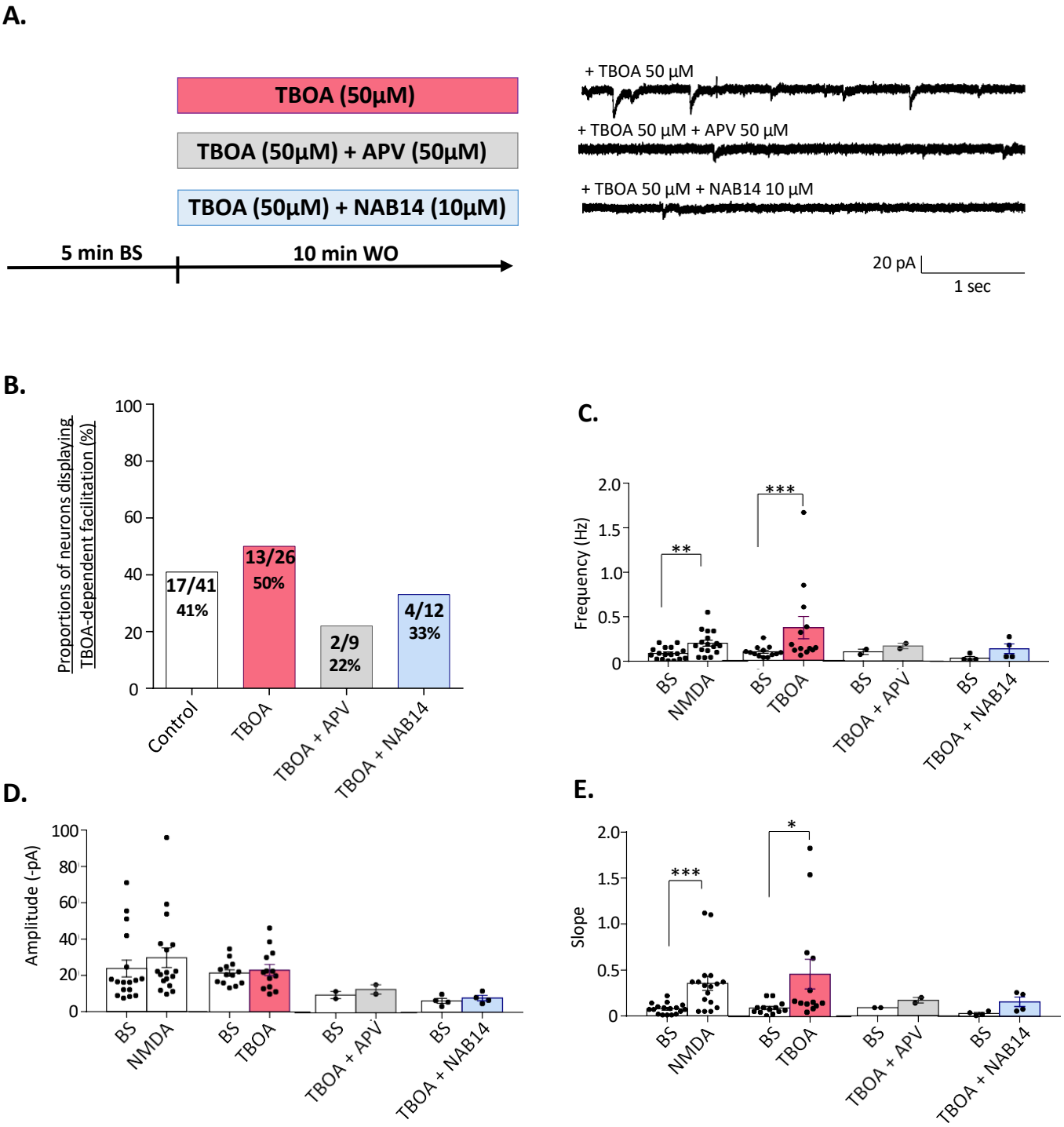

Figure 31: Effect of TBOA on GABAergic synaptic transmission

A. Protocol used to study the effect of TBOA on miniature GABAergic synaptic transmission.
B. Proportion of neurons displaying TBOA-dependent facilitation of miniature GABAergic synaptic transmission.
C-D-E. Histograms showing frequencies (**C.**), amplitudes (**D.**) and distribution slopes (**E.**) of GABAergic mISPC in control conditions (2 bars on the far left), during TBOA perfusion (2 bars middle left), during TBOA and APV co-perfusion (2 bars middle right) and during TBOA and NAB14 co-perfusion (2 bars far right). Paired T-test, **p< 0.01, Wilcoxon test *p<0.05, ***p<0.001

GABAergic synaptic transmission. To test this hypothesis, we used TBOA, an inhibitor of EAAT, responsible for glutamate clearance from the extracellular space, to increase the extracellular concentration of glutamate figure 31A). This study was achieved by recording miniature GABAergic synaptic transmission in C57bl/6J mice, so neurons were not identified as GAD^- or GAD^+.

1. Effect of TBOA on miniature GABAergic synaptic transmission

To assess the effect of TBOA (50 µM, 15min) on miniature GABAergic synaptic transmission, we first compared the proportion of neurons displaying TBOA-dependent facilitation to neurons displaying NMDA-dependent facilitation. Perfusion of TBOA induced a facilitation of miniature GABAergic synaptic transmission in 50% of recorded cells (n=13/26 neurons, 14 mice). This proportion of neurons displaying TBOA-dependent facilitation is not different than the proportion of neurons displaying NMDA-dependent facilitation (miniature control: 55%, n=36/66 neurons; TBOA: 50%, n=13/26 neurons, 14 mice; p=0.81720, Fisher's exact test) (figure 31B).

In neurons displaying TBOA-induced facilitation of miniature GABAergic synaptic transmission, TBOA perfusion induced an increase in mIPSC frequency of |370|% (baseline TBOA: 0.10 ± 0.02 Hz; TBOA: 0.37 ± 0.13 Hz; p=0.0002 Wilcoxon test; n=13 neurons, 11 mice) (figure 33C). The significant slope increase was of |470|% (baseline TBOA: 0.10 ± 0.02; TBOA: 0.47 ± 0.16; p=0.0002, Wilcoxon test, n=13 neurons, 11 mice) (figure 32E). This increase in mIPSC frequency was not associated with an increase in mIPSC amplitude (baseline TBOA: -21.52 ± 1.80 pA; TBOA: -23.14 ± 3.10 pA; p=0.4077, paired t-test, n=13 neurons, 11 mice) (figure 31D).

2. Effect of APV on TBOA-dependent facilitation of GABAergic synaptic transmission

To determine if the effect of TBOA was mediated by NMDAr activity, we co-applied TBOA with APV (50 µM) a competitive antagonist of NMDAr containing GluN2 subunits (figure 31A).

Co-perfusion of TBOA and APV induced a TBOA-dependent facilitation in only 22% of recorded neurons (n=2/9 neurons, 5 mice). There is no significant reduction in the proportion of neurons displaying TBOA-dependent facilitation in the presence of APV, as the number of cells is still low (TBOA: 50%, n=13/26 neurons, 14 mice; TBOA + APV: 22%, n=2/9 neurons, 5 mice; p= 0.2444, Fisher's exact test) (figure 31B, C, D, E).

3. Effect of NAB14 on TBOA-dependent facilitation of GABAergic synaptic transmission

To determine if the effect of TBOA was mediated by activity of NMDAr containing GluN2C/D, we co-applied TBOA with NAB14 (10 µM), an antagonist of NMDAr containing GluN2C/D subunits (figure 31A).

Co-perfusion of TBOA and NAB14 induced TBOA-dependent facilitation in 33% of recorded neurons (n=4/12 neurons, 6 mice). There is no significant reduction in the proportion of neurons displaying TBOA-dependent facilitation in the presence of NAB14 (33%, n=4/12 neurons, 6 mice; p=0.4862, Fisher's exact test) (figure 31B, C, D, E).

Inhibiting EAAT induces a facilitation of miniature GABAergic synaptic transmission.

<u>Conclusion</u>: We have shown that NMDAr activation induces a facilitation of spontaneous and miniature GABAergic synaptic transmission in GAD^- and GAD^+ neurons of the DH. Blocking astrocytic metabolism with FC causes a reduction in the proportion of neurons displaying NMDA-dependent facilitation of spontaneous GABAergic transmission in GAD^- neurons, but no effect of FC was observed on miniature GABAergic synaptic transmission. A perfusion of D-serine after a pre-incubation with FC induces an increase in the proportion of neurons displaying NMDA-dependent facilitation, reaching a similar proportion to the control group. Oxidation of D-serine causes a reduction in the proportion of neurons

displaying NMDA-dependent facilitation in both GAD- and GAD+ neurons. Finally, inhibiting EAAT function induces facilitation of miniature GABAergic synaptic transmission.

Results project 2

Expression of NMDAr in spinal astrocytes

NMDAr have been shown to be expressed by astrocytes in many structures (Lalo et al., 2006; Palygin et al., 2011; Verkhratsky & Chvátal, 2020). These NMDAr often contain GluN2C/D subunits, conferring to them Mg^{2+} insensitivity and lower Ca^{2+} permeability compared to NMDAr containing GluN2A/B subunits. We hypothesized that some astrocytes in the DH express functional NMDAr, that when activated, induce the release of transmitters. These transmitters might act on other receptors such as presynaptic receptors, potentially implicated in facilitation of GABAergic synaptic transmission. To study the possible expression of NMDAr in astrocytes, we used 3 approaches. We first recorded NMDA-induced responses in DH astrocytes by whole-cell recordings in slices of spinal cord (I). We then studied the effect of NMDAr activation in cultured astrocytes, using calcium imaging (II) and single channel currents with patch-clamp (III). I will present the results obtained for these 3 subprojects in the following paragraphs. It should be noted that these results are preliminary, and this project is still ongoing in our team.

I. Effect of NMDA application in DH astrocytes

To study the effect of NMDAr activation in DH astrocytes, we used the whole-cell configuration of patch-clamp (figure 32).

To visualize astrocytes, we used SR 101 (see Material and Methods, II-4) (figure 32A). Despite a prominent morphological and functional diversity, astrocytes show very similar electrophysiological parameters, such as more hyperpolarized membrane potential than neurons. The resting membrane potential of recorded cells was of -73.52 ± 1.19 mV (n=20 cells, 11 mice). Astrocytes also exhibit very passive electrophysiological properties: depolarizing astrocytes will not lead to action potential firing (Verkhratsky & Nedergaard, 2018). When performing voltage steps in the cells, we observed 2 types of profiles: one rectifying profile (50%) and one purely passive

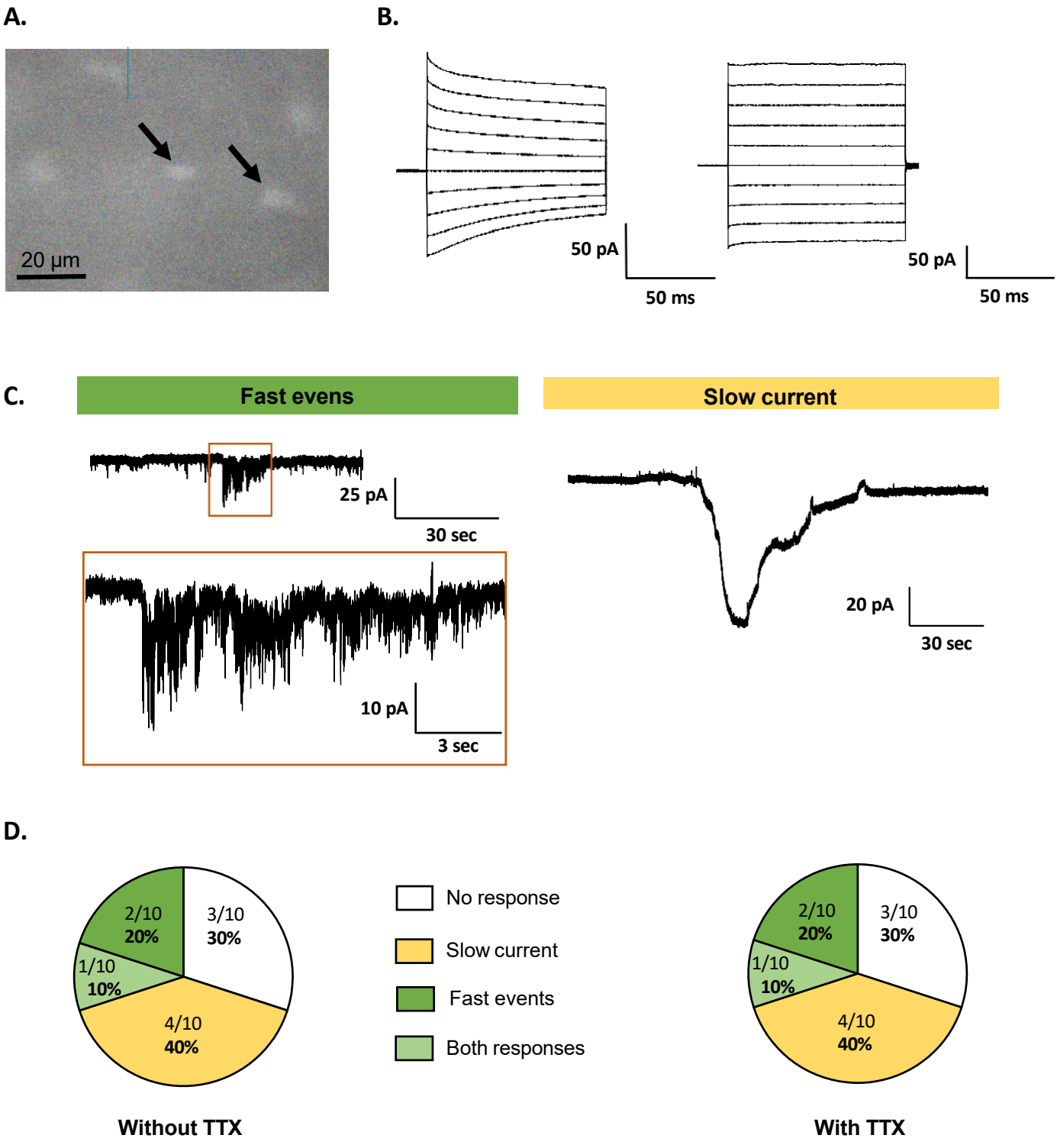

Figure 32: Effect of NMDA perfusion in DH astrocytes in spinal slices

A. Illustration of SR101 staining on a spinal slice.
B. 2 examples of passive properties recorded in $SR101^{+}$ cells.
C. 2 types of responses recorded in putative astrocytes in spinal slices.
D. Proportions of responses recorded in putative astrocytes in spinal slices, without TTX (left) and in the presence of TTX (right).

profile (50%) (figure 32B). Fluorescent cells displaying a more hyperpolarized membrane potential compared to neurons and that did not spike when depolarized were considered astrocytes.

We tested the effect of an NMDA-perfusion on 20 putative astrocytes. We observed a response to NMDA perfusion in 70% of recorded cells (n=7/10, 5 mice). We distinguished 2 types of responses: the first that we called “fast events” and the second that we called “slow current”. We observed that 20% of cells responded by the “fast events” response (n=2/10), 40% of cells responded by the “slow current” response (n=4/10) and 10% responded by a mixture of the 2 responses (n=1/10) (figure 32C, D). To determine whether these responses were due to neuronal network activity, we perfused NMDA in the presence of TTX. We observed the same responses as in the absence of TTX, and in the same proportions (n=6 mice) (figure 32C, D).

When applied twice on 1 cell presenting a “slow current” response, the effect of an NMDA perfusion was reproducible (n=1) and a co-perfusion of NMDA with APV blocked the second response (n=2). However, given the very low n, these results are preliminary and must be completed.

Passive properties profile of the recorded cells, the resting membrane potential and the presence and type of response induced by a perfusion of NMDA were not correlated.

These data are preliminary, and discussion on the analysis of such data is still ongoing in our team.

A perfusion of NMDA induced an inward current in some astrocytes of the DH, independently of neuronal network activity.

II. Study of calcium signals induced by NMDA in spinal cultured astrocytes.

To assess the effect of NMDAr activation on $[Ca^{2+}]_i$ in cultured spinal astrocytes, we used Ca^{2+} imaging (see Material and Methods, III-3 for a detailed explanation of the experimental set-up and preparation of the cells).

We tested different conditions for the age of the cultures, the presence or absence of the co-agonist D-serine and magnesium in the recording medium.

We observed an increase in $[Ca^{2+}]_i$ in 1.62% of recorded cultured astrocytes (n=10/618). Of these astrocytes responding to NMDA, 50% were in the presence of NMDA without D-serine and the presence of magnesium in the recording medium and the other 50% were obtained in the presence of NMDA and D-serine, and the absence of magnesium in the recording medium.

As a positive control, we used astrocytes obtained from cortical tissues as it has been described in some studies that cortical astrocytes express functional NMDAr (Verkhratsky & Chvátal, 2020). We failed to observe any response induced by NMDA perfusion in these cells.

As suggested in the review by Balderas & Hernández (2018) and as described in Palygin et al. (2010, 2011) we used freshly dissociated cells (used the same day they were obtained) from pups and adult mice but we failed to observe any response following NMDA perfusion in these cells.

A perfusion of NMDA induced a $[Ca2^{+}]_i$ in a very small subset of cultured astrocytes.

III. Study of NMDAr properties by unitary channel recordings

As we failed to observe any variations in $[Ca^{2+}]_i$ following NMDA perfusion, we used unitary channel recordings with patch-clamp. To study the single-channel currents induced by NMDA perfusion in cultured astrocytes, we used patch-clamp in 2 different configurations: cell-attached

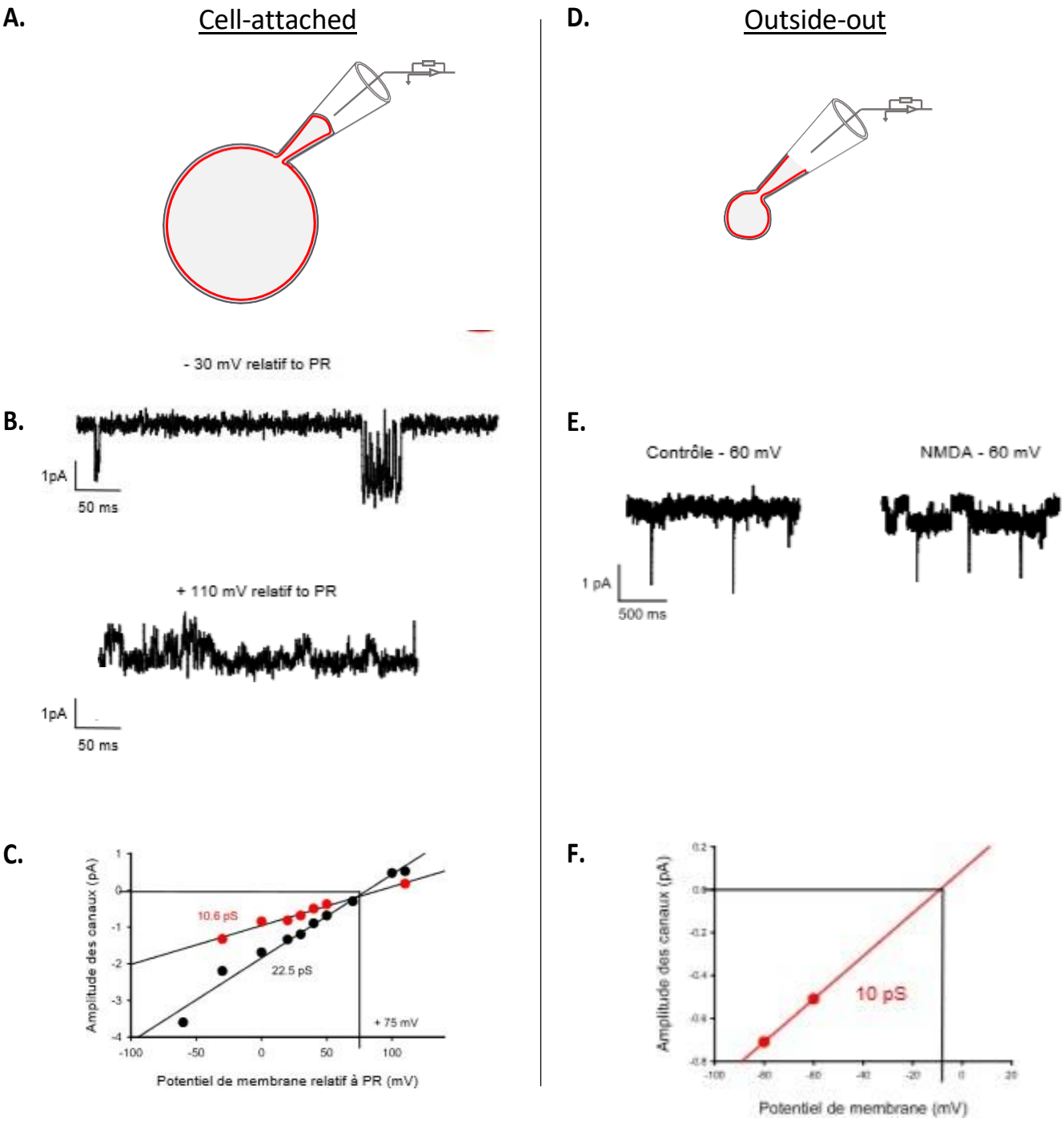

Figure 33: Effect of NMDA on unitary channel currents

A. Schematic representation of cell-attached configuration.
B. Illustration of recordings obtained in cultures astrocytes in the cell attached configuration at 2 imposed potentials.
C. Unitary current-voltage relationship of the channel, allowing to find the conductance of the channel.
D. Schematic representation of outside-out configuration.
E. Illustration of recordings obtained in outside-out configuration, during baseline (left) and during NMDA perfusion (right).
F. Example of a unitary current-voltage relationship of the channel recorded in outside-out in one cell.

and outside out (see Material and Methods, III-4 and figure 20) (figure 33A, D). In cell-attached, the pipette was filled with recording medium containing NMDA (100 µM).

We recorded unitary channel currents that inversed at -75 mV in the pipette (n=2), which equals 0 mV if we estimate the resting membrane potential of astrocytes around -75 mV. A reversal of current sign at 0mV corresponds to cation permeability. Conductance for these channels was calculated at 10.6 and 22.2 pS (figure 33C), which corresponds to the conductance associated to NMDAr containing GluN2C/D subunits (Paoletti et al., 2013; Scheppach, 2016). However, similar currents were observed without NMDA in the pipette, in all cells. To better control the moment of NMDAr activation, we decided to record unitary channel currents in the outside-out configuration.

In the outside-out configuration, we recorded unitary channel currents evoked by the perfusion of NMDA (100 µM) (n=4). The recorded currents changed polarity at 0mV. Conductance for these channels was estimated at 10 pS (figure 33D, E, F).

About 40 recordings were obtained during the 3rd year of my PhD with co-perfusions of NMDA and APV and are still being analyzed in our team.

Channels with similar properties to NMDAr containing GluN2C/D were electrophysiologically detected in cultured spinal astrocytes.

<u>Conclusion</u>: We have shown that NMDAr activation induces responses in DH astrocytes in spinal slices in presence or absence of TTX. However, we also found that NMDAr activation induces $[Ca^{2+}]_i$ signaling in a very small subset of cultured spinal astrocytes, and these results were not reproducible. Finally, we observed unitary channel currents induced by the perfusion of NMDA in cultured spinal astrocytes displaying properties similar to NMDAr containing GluN2C/D subunits. These results are preliminary and need to be completed.

Discussion and perspectives

During my doctoral work, we characterized the NMDA-dependent facilitation of GABAergic synaptic transmission in GAD^- (putative glutamatergic) and GAD^+ (putative GABAergic) neurons, by recording neurons from the lamina II. The principal results from this first project are presented here:

1. We observed NMDA-dependent facilitation in both GAD^- and GAD^+ neurons when recording miniature and spontaneous transmission. The NMDA-dependent facilitation was observed in the same proportion of GAD^- and GAD+ neurons, with a similar intensity (frequency and amplitude of IPSC, slope).

2. Blocking astrocyte function with an FC pre-incubation induced a decrease in the proportion of neurons displaying NMDA-dependent facilitation of spontaneous GABAergic synaptic transmission, but only in GAD^- neurons. However, FC had no effect on the proportion of neurons displaying NMDA-dependent facilitation of miniature GABAergic synaptic transmission in either GAD^- or GAD+ neurons.

3. Perfusion of D-serine rescued the FC-induced decrease in proportion of GAD^- neurons displaying NMDA-dependent facilitation of GABAergic synaptic transmission. When oxidating D-serine, we saw that a proportion of both GAD^+ and GAD^- neurons lost NMDA-dependent facilitation, showing that D-serine is the necessary co-agonist for both these populations of neurons.

4. Blocking EAAT function in the DH induces facilitation of miniature GABAergic synaptic transmission.

I also generated preliminary data for a secondary project focusing on the potential expression of NMDAr in spinal astrocytes.

1. By using whole-cell patch-clamp recording of astrocytes in spinal slices, I showed NMDA perfusion induced inward currents in recorded astrocytes.

2. By using calcium imaging in cultured astrocytes, we failed to observe any variation in $[Ca^{2+}]_i$ following NMDA perfusion.

3. By using unitary channel recordings in cultured spinal astrocytes, I detected channels displaying similar properties to NMDAr.

I. Methodological limits

1. GAD65::EGFP model

The transgenic mouse strain GAD65-eGFP that we used in the project I, was obtained from Ferenc Erdelyi and Gabor Szabo (Institute of Experimental Medicine, Budapest). This line was characterized in Cui et al., in which they found that in the lamina II, ~79% of fluorescent cells were GABAergic and ~60% of GABAergic neurons were eGFP+ (averaged over 3 mice) (Cui et al., 2011). This might have led us to include a significant proportion of GABAergic neurons in our GAD^- population. An important detail for Cui et al. is that they used an anti-GABA antibody, measuring for GABA-immunoreactivity, and not GAD65 or GAD67 immunoreactivity. They did not mark for NeuN, to confirm the neuronal identity of the $eGFP^+$ cells. As we have seen previously, astrocytes express GATs and can also synthesize GABA in a GAD-independent manner, resulting in GABA levels potentially detected in astrocytes. It might be interesting to measure GAD65 immunoreactivity in addition to GABA. However, the proportions obtained in Cui et al. are similar to ones published in GAD67-eGFP mice, where they described that 86% of $eGFP^+$ neurons were GABAergic and 54% of GABAergic neurons were $eGFP^+$ in the hippocampus (Oliva et al., 2000). In this study, they marked for NeuN, confirming GABA-immunoreactivity in neurons. It would be necessary to check if expression of eGFP is limited to subpopulations of GABAergic interneurons in the DH.

Data from the litterature has shown that most GABAergic neurons in the lamina II present a tonic firing pattern (Cui et al., 2011; Daniele & MacDermott, 2009; Grudt & Perl, 2002; Heinke et al., 2004; Kloc et al., 2019; Yasaka et al., 2010). Cathenaut et al. recorded the firing pattern of $eGFP^+$ cells and observed that most of them displayed a tonic firing pattern (Cathenaut et al., 2022). In our study, we did not perform systematic firing pattern characterization of recorded neurons, which could add to identity verification.

GAD65-eGFP mice are a heterozygous fusion model, meaning that one GAD65 isoform is fused with the eGFP. This could potentially lead to a reduced production of GABA, and changes in DH network function for example. Asada et al. studied the effect of the absence of one isoform of GAD65 and found no differences in GABA content in the central nervous system wild-type mice

and mice lacking one isoform of GAD65. The authors did find that mice lacking one isoform of GAD65 were more prone to seizures (Asada et al., 1996). Stork et al. observed an increase in GABA levels in the amygdala, hypothalamus, and parietal cortex around the 2nd month postnatal in wild-type mice which was delayed by 2 months in mice lacking one isoform of GAD65. This was associated with decreased aggressivity in mice lacking one isoform of GAD65 (Stork et al., 2000). Unpublished results obtained with the Von Frey test in our team have shown that there is no significant difference in baseline nociceptive sensitivity between naïve C57Bl6 and GAD65-eGFP mice. However, no study has investigated the potential effects in a pathological pain model, such as SNI, in the GAD65-eGFP mice, where inhibition efficiency is already compromised (Gradwell et al., 2020). It will be a necessary control before moving on to a model of pathological pain.

2. Identity of the recorded cell

Apart from the expression of GAD65, we did not investigate the identity of the recorded neurons. As mentioned in the first part of the introduction, interneurons in the DH are a heterogenous population. As this was a characterization study of the effect of NMDA-dependent facilitation of GABAergic synaptic transmission in the whole DH network, the exact identity of recorded neurons (neurochemistry, firing patter, morphology, marker expression) was not fundamental. However, neither NMDA-dependent facilitation nor FC-induced inhibition of NMDA-dependent facilitation was displayed in all recorded cells. We can thus hypothesize that specific subpopulations of interneurons are affected by NMDA-dependent facilitation. So, in future studies regarding NMDA-dependent facilitation, it could be interesting to investigate the expression of NMDA-dependent facilitation in different subpopulations of interneurons, allowing us to better understand the role of this effect in nociceptive information processing. Indeed, in Matos et al., the authors described the role of astrocytes in upregulating synaptic transmission specifically in SST-expressing interneurons in the hippocampus (Matos et al., 2018). It has been hypothesized that astrocytes can regulate the activity of a single synapse, through fast and local Ca^{2+} signaling (Arizono et al., 2020), adding to fine-tuning of information processing in networks composed of varied populations of interneurons, as is the case in the DH network. Many studies are focused on

the precise function of each subpopulation of interneurons in the DH, and NMDA-dependent facilitation of GABAergic transmission might display different effects on information processing depending on which subpopulations of interneurons are concerned.

One approach to identify the recorded neuron would be to look at the firing profiles, as is already performed in our team. There is no perfect correlation between firing profile, morphology, neurochemistry, and marker identity (Cordero-Erausquin et al., 2016; Todd, 2017). We could also look at the cellular morphology in real-time by using luciferin in the pipette. Another approach used in almost all the central nervous system is including biocytin in the patch-clamp recording pipette, to allow for immunostaining of the slice after electrophysiological recordings. Trials using this approach have been performed in the team, but unsuccessfully as the neurons are almost invariably destroyed when the pipette is pulled away. Once a specific interneuronal population implicated in NMDA-dependent facilitation will be identified, we could use a transgenic mouse line.

To identify astrocytes in slices, we used SR 101. However, we did find that dead neurons were fluorescent after SR 101 incubation. We added supplementary steps to identify astrocytes: voltage steps and resting membrane potential. SR 101 is washed out after about 2 hours. It could be interesting to use a transgenic mouse, expressing a fluorescent probe under the control of a specific promoter. However, in astrocytes, many promoters have been identified, and none offers 100% cover of the astrocytic population (Verkhratsky & Nedergaard, 2018). The most used in astrocytes is the promoter of the glial fibrillary acid protein (GFAP) (Lefèvre et al., 2015; Nam et al., 2016; Verkhratsky & Nedergaard, 2018), which has also been used for optogenetic manipulation of $GFAP^+$ astrocytes (Nam et al., 2016; Wahis et al., 2021). It should be noted that GFAP is an imperfect marker for astrocytes, however, there are no perfect alternatives (Verkhratsky & Nedergaard, 2018; J. Xu, 2018).

3. Use of FC:

The use of FC is considered standard practice to study astrocytes (Hassel et al., 1992; Paulsen et al., 1987). FC acts as a false substrate of aconitase in the Krebs cycle, thus blocking it and the production of ATP in the cell. Preferentially taken up by astrocytes with the protocol used in our study, FC induces astrocytic metabolism blockade. Theoretically, all ATP-dependent functions in the astrocytes are blocked, including gradient maintenance for example. As seen previously, astrocytes are implicated in many functions, such as the maintenance of ionic gradients and neurotransmitter clearance. Thus, FC provides a very general way of looking at astrocytic involvement, as many functions are affected. It has been used to study the implication of astrocytes in modulation of inhibitory transmission (Christian & Huguenard, 2013; Yang & Cox, 2011).

As seen in the introduction, astrocytes can respond to stimuli by calcium signaling, leading to the concept of calcium excitability (Bazargani & Attwell, 2016b). A question about the use of FC is whether it alters calcium signaling in astrocytes. In one study looking at calcium signaling induced by activation of PAR-1 and P2Y receptors in astrocytes, the authors saw no PAR-1 and P2Y-evoked $[Ca^{2+}]_i$ transients after FC incubation or the use of the Ca^{2+} chelator BAPTA (Shigetomi et al., 2008). This suggests that FC blocks Ca^{2+} signaling in astrocytes.

To assess how long we could wait after the pre-incubation with FC before the effect of it was washed out, we controlled for its activity by performing 2 sequential perfusions of NMDA. In GAD- neurons that did not display NMDA-dependent facilitation of GABAergic transmission after the first perfusion (recorded within 15 minutes of the end of FC incubation), we performed the same protocol. We observed NMDA-dependent facilitation displayed in 50% of the neurons after the second perfusion. The effect of fluorocitrate is reversible, as it's washed out over time after the incubation.

However, as many mechanisms are affected by FC, interpretation of results obtained with FC can be limited. These results obtained in this study are preliminary in dissecting the mechanisms of implication in astrocyte-mediated NMDA-dependent facilitation of GABAergic synaptic transmission. Astrocytes could be implicated via Ca^{2+} dependent or independent mechanisms for example. We could use BAPTA to chelate Ca^{2+} signaling in astrocytes, as is described in Baudon

et al. (2022), or target SNARE specific to gliotransmission (Foley et al., 2011). Further study is necessary to identify the exact mechanisms at play.

4. Recording temperature

We performed all of our electrophysiology recordings at room temperature. However, astrocytes express temperature sensitive receptors (Ahmadi et al., 2003). To approach physiological conditions, it is necessary to set the recording temperature close to 37°C.

One study focused on the disparity between the frequency in spontaneous Ca^{2+} transients in astrocytes, observed in vivo and in vitro. Using cultured astrocytes and acute cortical slices and recording at 20 and 37°C, they found a significantly lower frequency of Ca^{2+} transients at 37°C, indicating that temperature plays a key role in Ca^{2+} signaling in astrocytes (Schipke et al., 2008). When measuring Ca^{2+} in cultured astrocytes in our project, we recorded at 35°C. However, we performed all electrophysiological recordings at room temperature. This may have impacted astrocyte functionning in our study and should be taken into consideration in the interpretation of the results. For future studies, a comparison between results obtained from room temperature recordings and recordings obtained at 37°C is necessary.

II. Baseline GABAergic synaptic transmission in GAD^- and GAD^+ neurons, and the effect of FC

We found no significant effect of neuronal identity or FC incubation on baseline GABAergic sIPSC frequency, indicating that in baseline conditions, GABAergic sIPSC frequency is similar in GAD^- and GAD^+ neurons, and astrocytes are not implicated in regulation of GABAergic sIPSC frequency. We did however find a significant effect of neuronal identity on GABAergic sIPSC amplitude. When looking at neurons displaying NMDA-dependent facilitation, we found a tendency at an effect of neuronal identity. A difference in amplitude of sIPSC could lead to differences in inhibitory efficiency and message integration in the post-synaptic neuron.

Surprisingly, when recording miniature GABAergic transmission, we found a significant effect of neuronal identity on mIPSC frequency. As we observed no differences in frequency of sIPSC, we can hypothesize that an active neuronal network plays a role in maintaining similar frequencies of GABAergic sIPSC in GAD^- and GAD^+ neurons.

A difference miniature currents frequency indicates a difference in release probability of vesicles. To my knowledge, no study has described differences in release probability between different populations of interneurons in the DH. Differences in release probability could be due to many mechanisms, including expression of voltage-sensitive calcium channels or other receptors at the presynaptic element. In Cathenaut et al., the authors described differential modulation by presynaptic $GABA_B$ and adenosine receptors at GABAergic synapses on GAD^- or GAD^+ neurons (Cathenaut et al., 2021). However, they did not investigate if this regulation was due to the prevalence of expression of these receptors or the preferential presence of the agonists at one or another synapse. We could hypothesize that a difference in probability release between GABAergic transmission in GAD^- and GAD^+ neurons is due to the expression of different receptors on the presynaptic or post-synaptic, or the presence of different agonists, at these synapses. Using the preferential agonist at each synapse (GABA and adenosine), this question could be answered. However, these results confirm differential functional regulation of GABAergic synapses dependent on the neurochemical identity of the post-synaptic neuron. Target specificity has been recognized as playing a very important role in network information processing in many structures, as reviewed here in the cerebellum and the hippocampus (Park et al., 2018). The mechanisms responsible for the establishment of specific synaptic circuitry during development have been described (Williams et al., 2010). However, little is known about the factors responsible for synaptic target specificity in the adult central nervous system (Park et al., 2018).

We did not find an effect of FC incubation on either frequency of amplitude of basal GABAergic sIPSC and mIPSC. First, these results confirm that FC incubation did not directly affect neurons. These results also suggest that astrocytes are not implicated in tonic regulation of baseline GABAergic synaptic transmission in the DH, as it's been shown in other structures of the central nervous system (Panatier et al., 2011). An important aspect to take into consideration in this

situation, is that Ca^{2+} signaling in astrocytes is slow, compared to fast synaptic transmission. Spontaneous synaptic activity in the DH is low in the absence of primary afferents stimulation. In our experimental conditions, we blocked excitatory synaptic transmission, artificially lowering even more synaptic activity in the network. Baseline sIPSC frequencies could potentially be too low to trigger responses in astrocytes.

III. NMDA-dependent facilitation in GAD^- and GAD^+ neurons

1. Proportions of neurons displaying NMDA-dependent facilitation of GABAergic synaptic transmission in the DH

We observed NMDA-dependent facilitation when recording both spontaneous and miniature GABAergic synaptic transmission in about 80% of GAD^- and GAD^+ neurons. These results are similar to what we observed in Léonardon et al., where NMDA-dependent facilitation was observed in 100 % of recorded GAD^+ neurons and 85% of recorded GAD- neurons (Leonardon et al., 2022). However, when recording miniature synaptic transmission, we obtained very different results compared to what is described in Léonardon et al.: we found that 45% of recorded GAD^- neurons and 38% of recorded GAD^+ neurons displayed NMDA-dependent facilitation. In Léonardon et al., we found that 22% of recorded GAD^- and 91% of recorded GAD^+ neurons display NMDA-dependent facilitation. In Léonardon et al., we interpreted it as a target specificity of NMDA-dependent facilitation. In our study, no such target specificity appears. This could be due to a difference in neuron sampling between experimenters. As mentioned previously, the interneuron populations are very diverse in the DH (Cordero-Erausquin et al., 2016; Polgár et al., 2013), and maybe some are more represented than others in Léonardon et al. and this study. Marking the precise localization of the recorded neurons for each experimenter and identifying specific populations in future studies might help focus on a population of interneurons and reduce variability in results between projects.

After FC incubation, we observed a significant reduction in the proportion of neurons displaying NMDA-dependent facilitation in GAD^- neurons, but no change in the proportion in GAD^+ neurons when recording spontaneous GABAergic synaptic transmission. As mentioned previously, other published work from our team has described differences in regulation of synapses depending on the neurochemical identify of the post-synaptic neuron (Cathenaut et al., 2021). We could hypothesize that astrocytes either release a transmitter specifically at these synapses, or that they express a specific receptor at these synapses. Studies have already shown that astrocytes can specifically modulate synaptic transmission at a single synapse (Panatier et al., 2011). In Kang et al., the authors describe an astrocyte dependent facilitation of inhibitory transmission via the activation of $GABA_B$ receptors on the astrocytes. The activation of astrocytic $GABA_B$ receptors induces the release of glutamate, acting on presynaptic NMDA/AMPA receptors on the presynaptic GABAergic synaptic terminal (Kang et al., 1998) (figure 11). We could hypothesize that a similar mechanisms specifically regulated NMDA-dependent facilitation of GABAergic synaptic transmission in GAD^- neurons.

In this study, we found a significant difference in the proportion of neurons displaying NMDA-dependent facilitation between spontaneous and miniature in both GAD^- and GAD^+ neurons, indicating that an active neuronal network is necessary to mediate NMDA-dependent facilitation for a proportion of recorded neurons. It is also of note that when recording miniature synaptic transmission, we did not find a reduction in the proportion of neurons displaying NMDA-dependent facilitation after a pre-incubation with FC. This indicates that astrocytes do not mediate NMDA-dependent facilitation when the neuronal network is inactive. Astrocytes mediate NMDA-dependent facilitation of GABAergic synaptic transmission in GAD^- via an intact neuronal network. Studies have shown that activation of neurotransmitter transporters in astrocytes can induce Ca^{2+} responses in astrocytes. In Matos et al., the authors describe how astrocytes in the hippocampus “sense” GABAergic synaptic transmission via activation of GAT3 and $GABA_B$ receptors, inducing Ca^{2+} signaling and the release of ATP, thus modulating synaptic inhibition of pyramidal cells by SST-interneurons specifically (Matos et al., 2018). We could hypothesize that astrocytes mediate NMDA-dependent facilitation of GABAergic synaptic transmission via “sufficient” activity of GABAergic synaptic transmission, thus needing an active neuronal network to induce sufficient levels of extracellular GABA to activate GAT and GABA receptors in astrocytes.

2. Effect of FC on intensity of NMDA-dependent facilitation

We found a significant effect of neuronal identity on GABAergic sIPSC frequency during NMDA-dependent facilitation in neurons displaying NMDA-dependent facilitation. Linked to this difference in sIPSC frequency, we also found a higher sIPSC slope distribution after FC incubation in GAD^- neurons compared to GAD^+ neurons, as well as compared to GAD^- in control conditions, indicating a stronger NMDA-dependent facilitation in GAD^- neurons when astrocytes are blocked. Looking at GABAergic sIPSC distribution slope is indicative of the "strength" of NMDA-dependent facilitation. A higher slope value in GAD^- neurons after FC incubation compared to control GAD^- neurons suggests that astrocytes are implicated in regulating NMDA-dependent intensity. The GAD^- neurons displaying NMDA-dependent facilitation after FC incubation could also be a specific subpopulation of neurons, as NMDA-dependent facilitation is not mediated by astrocytes in these neurons, whereas it is in most recorded GAD^- neurons. We found a tendency of an effect of neuronal identity on GABAergic mIPSC frequency when recording miniature GABAergic synaptic transmission, indicating a potential difference of release probability, which we had previously seen in baseline recording. However, the number of recorded neurons is still low, and should be increased to confirm this difference.

3. Localization of the NMDAr implicated in NMDA-dependent facilitation of GABAergic synaptic transmission.

Our results show that NMDA induces presynaptic facilitation of GABAergic synaptic transmission in the DH. However, the localization of the receptors implicated in this effect remain to be determined (figure 34). This is also discussed in Léonardon et al., 2022.

Expression of NMDAr at GABAergic synaptic terminals

In their 2005 immunohistochemistry study, Lu et al. reported having found that 37% of GABAergic synaptic terminals expressed GluN1 in the rat DH (C. R. Lu et al., 2005). No study

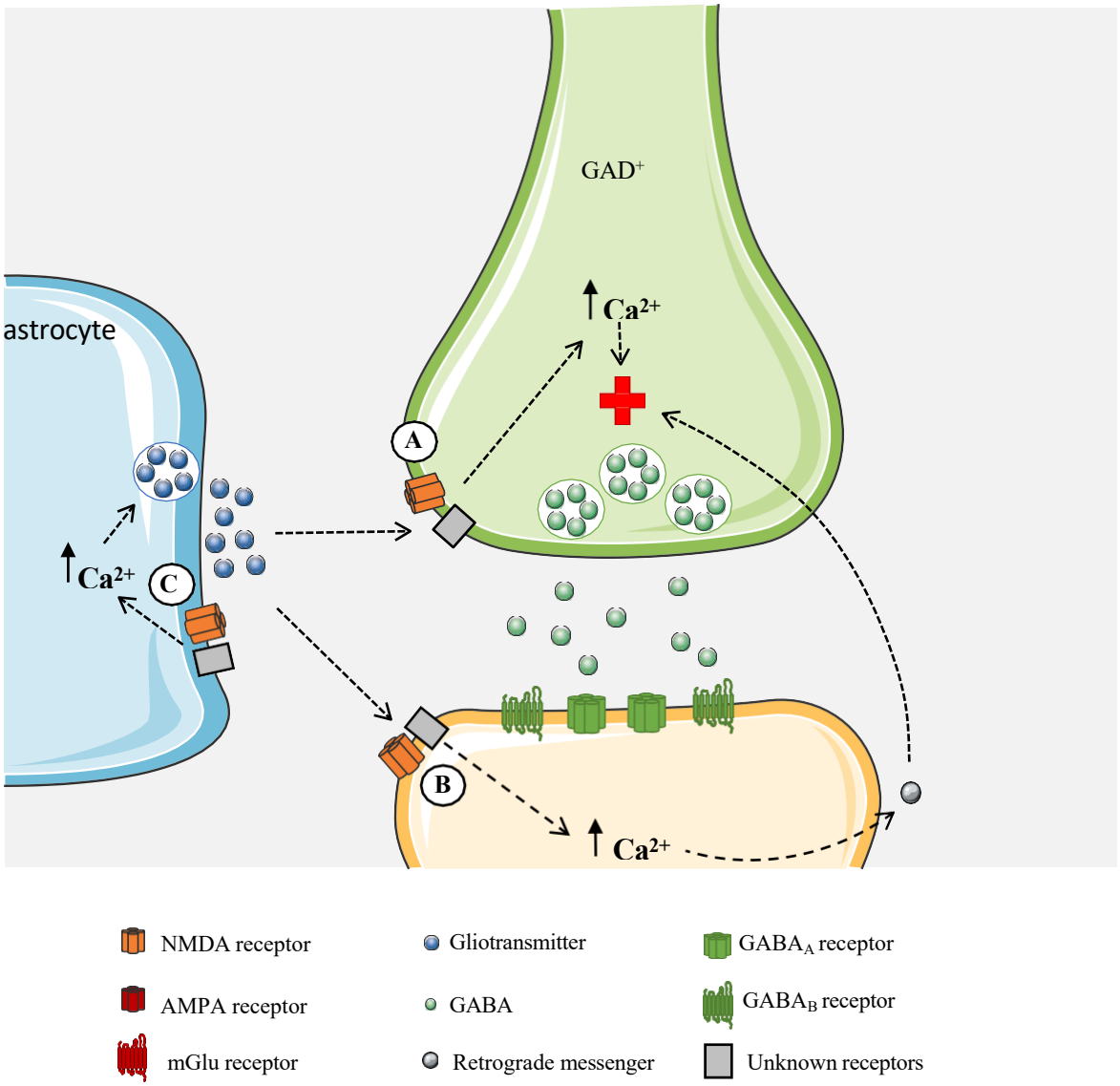

Figure 34: Hypothesized localizations of NMDAr and potentiel astrocyte implication in NMDA-dependent facilitation of GABAergic synaptic transmission

Ⓐ **Presynaptic expression.** Presynaptic NMDAr activation could lead to an increase in [Ca^{2+}] in the presynaptic element, resulting in an increase in GABA release.
Ⓑ **Postsynaptic expression.** Postsynaptic NMDAr activation could lead to an increase in [Ca^{2+}] in the postsynaptic element, leading to the release of a retrograde messenger, such as NO, increasing GABA release. Similar mechanisms have been shown in the modulation of GABAergic synaptic transmission in the DH and the ventral tegmental area (Fenselau et al., 2011; Nugent et al., 2007).
Ⓒ **Astrocytic expression.** Functional NMDAr could be expressed in astrocytes and their activation would lead to an increase in [Ca^{2+}], resulting in the release of gliotransmitters, like glutamate (Kang et al., 1998).

Our results show that astrocytes are not implicated in NMDA-dependent facilitation when recording putative GABAergic neurons. However, we do not know if the absence of astrocyte implication is due to the absence of gliotransmitter release, or the absence of receptors expressed at these synapses.

has since then confirmed these results or looked into their function. Our first hypothesis is that these presynaptic receptors expressed at GABAergic terminals in the DH are the ones implicated in NMDA-dependent facilitation. The data we published in Léonardon et al. shows that the NMDAr implicated in NMDA-dependent facilitation contain GluN2C-D subunits (Leonardon et al., 2022). These subunits would confer interesting pharmacological properties to these presynaptic NMDAr as they would not require a depolarization to be activated (Paoletti et al., 2013) (figure 13). They also present a higher affinity to glutamate, making them more sensitive to ambient glutamate released from a nearby synapse or glial cells for example. It could be interesting to confirm these results in the mouse DH, as well as precisely identify the presynaptic and the postsynaptic neuron.

Postsynaptic expression of NMDAr

As presented in the second chapter of the introduction, activation of a postsynaptic receptor such as NMDA or mGlu receptors can induce, via an increase in $[Ca^{2+}]_i$, the release of a retrograde messenger such as nitric oxide that can act on the presynaptic terminal (Fenselau et al., 2011; Nugent et al., 2007). However, in Léonardon et al., MK-801, a NMDAr channel blocker was used in the recording pipette to block post-synaptic NMDAr and it did not alter NMDA-dependent facilitation of GABAergic synaptic transmission, suggesting that postsynaptic NMDAr are not implicated in this effect. An additional control is necessary, as MK-801 requires activation of NMDAr to block the channel to confirm these results. Interestingly, neuronal nitric oxide synthase is mainly expressed in inhibitory neurons in the DH (Sardella et al., 2011).

Glial expression of NMDAr

This was the focus of the secondary project of my doctoral work, since only one study has shown the expression of functional NMDAr in spinal astrocytes (Verkhratsky & Chvátal, 2020; Žiak et al., 1998). As FC induced a reduction of the proportion of neurons displaying NMDA-dependent facilitation of GABAergic synaptic transmission, we hypothesized that for that proportion of neurons, NMDA acts via astrocytic NMDAr, inducing the release of a transmitter which acts on the presynaptic GABAergic terminal at synapses onto GAD^- neurons.

Our preliminary data on NMDAr expression in spinal astrocytes remains to be completed. We failed to obtain reproducible data in Ca^{2+} imaging with cultured astrocytes. In their review, Balderas & Hernández, discuss the history of contradictory data obtained in the study of NMDAr

in astrocytes, with a specific part on cultures. Many studies have shown that NMDAr activation in cultured astrocytes do not induce large transmembrane currents, leading to difficulties in data interpretation and contradictions between studies (Balderas & Hernández, 2018). It should also be noted that many studies have shown that astrocytes closely surround synapses and most Ca^{2+} signaling and gliotransmission occurs in the small astrocytic processes surrounding synapses. NMDAr subunits mostly found in astrocytes, such as GluN2C-D and GluN3A-B, are less permeable to calcium as well, potentially explaining the negative results we obtained in calcium imaging (Paoletti et al., 2013; Verkhratsky and Chvàtal., 2019). The influx of calcium could be very localized and hard to measure on our set-up, as our time resolution is quite low, indicating that NMDAr in astrocytes act in a very localized way. We tried many different configurations, regarding co-agonist co-perfusion and presence/absence of Mg^{2+} in the recording solution, in freshly dissociated cells, like in Palygin et al., (2010), as well as in older cultures, and the few responses we obtained were not reproducible across the sessions and cultures. Another hypothesis is that the expression of NMDAr in cultured astrocytes is low, and the few responses we managed to obtain were cells with unusually high expression of NMDAr. Calcium imaging to study $[Ca^{2+}]_i$ signaling in astrocytes in slices is used in other structures, such as the amygdala (Baudon et al., 2022; Wahis et al., 2021) Using SR 101 and Oregon green as a Ca^{2+} probe for example, it could be possible to characterize the Ca^{2+} responses induced by NMDA perfusion in the slice. We can also use genetically encoded ca^{2+} probes, such as GCaMP (Verkhratsky & Nedergaard, 2018).

As we failed to obtain any responses in Ca^{2+} imaging, we performed unitary channel recordings using 2 configurations of patch-clamp (cell-attached and outside-out) to characterize NMDAr in cultured astrocytes. We used the same cultures that were used for Ca^{2+} imaging, and we did record channels that resembled NMDAr. However, we did not apply antagonists, such as APV and NAB14 to confirm the identity of these channels. This project is still ongoing in the lab, as further experiments and analysis are needed to determine if we recorded NMDAr. It could also be interesting to characterize these channels in astrocytes in slices.

The next step in this part of the project will use an RNA-scope technique, to determine the expression of NMDAr subunits in astrocytes of the DH.

Interestingly, in slices, we did observe currents in putative astrocytes, induced by NMDA perfusion, in the presence and the absence of TTX. As we observed the same responses when the

neuronal network was blocked, we could hypothesize that the currents could be the result of activation of NMDAr expressed in the astrocytes, activation of another receptor or currents resulting from neurotransmitter transporter activity.

Facilitation of GABAergic synaptic transmission could have a very different physiological consequence depending on the nature of the postsynaptic neuron, and even further, depending on the chain of neurons activated downstream. It would be interesting to study the impact of this NMDAr-dependent facilitation by recording the projection neurons in the DH when these NMDAr are activated, as this could be a pro- or anti-nociceptive mechanism in the DH and could potentially be altered in conditions of pathological pain.

4. Role of D-serine in NMDA-dependent facilitation

Our first hypothesis to explain the implication of astrocytes in NMDA-dependent facilitation was that the synthesis of D-serine was altered after a pre-incubation with FC, inducing a decrease in the proportion of GAD^- neurons displaying NMDA-dependent facilitation. To study this, we perfused D-serine and NMDA after FC incubation and compared proportions of GAD^- neurons displaying NMDA-dependent facilitation of GABAergic synaptic transmission. We found that D-serine rescues FC-dependent decrease of the proportion of neurons displaying NMDA-dependent facilitation of GABAergic synaptic transmission. To confirm these results, we used DAAO to specifically oxidate D-serine levels in the DH. We selected neurons displaying NMDA-dependent facilitation and proceeded with a 2^{nd} perfusion of NMDA in the presence of DAAO. We found that D-serine is implicated in both types of synapses, as more than half of GAD^+ neurons and half of GAD^- neurons lose NMDA-dependent facilitation when D-serine is oxidized. There is no significant difference in the proportion of GAD^- and GAD^+ neurons that didn't display NMDA-dependent facilitation in the absence of DAAO as the number of neurons per group is quite low. It might be interesting to increase the number of neurons, to assess if there is a difference between GAD^- and GAD^+ neurons in which D-serine as the necessary co-agonist in NMDA-dependent facilitation of GABAergic synaptic transmission.

It was long thought that only astrocytes expressed serine racemase. Recent data obtained from the use of more selective antibodies and KO mice, has shown that astrocytes are the source of L-serine and neurons, expressing serine racemase, synthesize D-serine in many structures of the central nervous system (Wolosker et al., 2016). However, these findings are discussed in Sherwood et al., where the authors discuss the existence of multiple isoforms of serine racemase in the central nervous system, suggesting different functions depending on the localization and explain the contradictory data when using more specific antibodies (Sherwood et al., 2021). One recent study in the DH has shown serine racemase expression is mostly concentrated in astrocytes of the superficial laminae (Lefèvre et al., 2015). More studies are necessary in the DH to confirm the expression of serine racemase in astrocytes and/or neurons. One step further would be to assess which populations of interneurons in the DH express serine racemase. One study in the neocortex and hippocampus has shown that the majority of serine racemase in these structures is expressed in glutamatergic neurons, and the remaining 25-40% of serine racemase being expressed in interneurons. However, they also showed that only a small fraction of neurons staining positive for D-serine also expressed serine racemase. Interestingly, more than half the neurons positive for D-serine were GABAergic. It might be interesting to stain for D-serine in the DH, to assess the stores of D-serine and see if there is a difference between GAD^- and GAD^+ neurons. Incubating cultured astrocytes in FC for 60 min at 1mM and injecting it via micro-dialysis in the pFC or rats, Tanahashi et al. observed a reduction in D-serine contents (Tanahashi et al., 2012). We can question if FC incubation alters D-serine synthesis and storage in different populations of cells in the DH.

A perfusion of D-serine restores the proportion of neurons displaying NMDA-dependent facilitation of GABAergic synaptic transmission, suggesting that the implication of astrocytes in mediating NMDA-dependent facilitation depends on the availability of the co-agonist, as a perfusion of D-serine could potentially hide a loss of glycine. It was long thought that glycine was the main co-agonist in the DH, as glycine is co-released with GABA at some synapses in the DH (Cordero-Erausquin et al., 2016). However, studies suggest that glycine extracellular concentrations are tightly regulated. In Ahmadi et al., the authors induce a spill-over of synaptically released glycine by altering temperature-sensitive glycine transporter activity, which are expressed on astrocytes. This spill-over of glycine induces facilitation of NMDAr-mediated currents (Ahmadi et al., 2003). Using glycine oxidase in parallel to DAAO, it could be interesting

to complete the study of the prevalence of one or other co-agonist at GABAergic synaptic transmission in GAD^- and GAD^+ neurons, as co-agonist availability seems to be in tight relationship with astrocytic functioning which has been shown to be altered in conditions of pathological pain (Ji et al., 2013).

5. Endogenous source of glutamate

A remaining question concerning NMDA-dependent facilitation of GABAergic synaptic transmission was the source of glutamate. During our study, we perfused 100 µM of NMDA on spinal slices, which is quite a high concentration, probably activating many NMDAr in the slice. It was necessary to understand if these NMDAr implicated in NMDA-dependent facilitation could be activated physiologically. To assess this, we used TBOA, a blocker of EAAT. Blocking EAAT induces an increase in glutamate concentrations in the extracellular space, as it continues to be released synaptically but not transported into cells, inducing a spill-over of glutamate. Using TBOA, we observed TBOA-dependent facilitation of miniature GABAergic synaptic transmission. As glutamate acts on many types of receptors, we used APV to confirm that TBOA-dependent facilitation of GABAergic synaptic transmission was mediated via NMDAr activation. The results obtained in this part are not statistically significant, as the groups have not yet been completed. We will increase the number of neurons in each group to confirm the preliminary data presented in the results part of this manuscript. We can however already discuss the few neurons displaying TBOA-dependent facilitation of GABAergic synaptic transmission in the presence of APV, suggesting that other receptors, such as mGlu receptors, might be implicated in GABAergic synaptic facilitation. In the DH, almost all mGlu receptors are expressed (except for mGlu receptors 6 and 8), specifically mGlu receptors 1, 5, 2 and 3 in the superficial laminae of the DH (Alvarez et al., 2000; Aronica et al., 2001). mGlu receptors 1 and 5 are coupled with a Gq protein, inducing the release of intracellular Ca^{2+} stores, whereas mGlu receptors 2 and 3 are coupled with a $G_{i/o}$ protein. As we observe a facilitation of GABAergic synaptic transmission, we could hypothesize that mGlu receptors 1 and 5 could be potentially implicated in facilitation of GABAergic synaptic transmission. As presented in the introduction, mGlu receptors are important

actors in modulation and plasticity mechanisms of inhibitory synaptic transmission (Castillo et al., 2011; Fenselau et al., 2011; Gandolfi et al., 2020).

It should be noted that we do not know the concentration of glutamate induced by TBOA perfusion. We do not know if the concentrations of competitive antagonists that we used are sufficient to completely block NMDAr activation. We may have to try higher concentrations of antagonists to assess if NMDAr are effectively blocked.

The recordings performed for this part of the project were done on spinal slices from C57 mice, so neurons were not identified as GAD^- or GAD^+. It could be interesting to see if we would obtain the same proportions of neurons displaying TBOA-dependent facilitation of GABAergic synaptic transmission in GAD^- and GAD^+ neurons as we observed when perfusing NMDA, which could be indicative of a non-targeted facilitation of GABAergic synaptic transmission in conditions of glutamate spill-over.

Blocking EAAT to induce glutamate spill-over imitates what has been observed in conditions of intense and sustained stimulation of the primary afferents. This can be the case in conditions of pathological pain. Nerve injury can induce a transitory up-regulation followed by a sustained down-regulation of the expression of EAAT in the DH (Sung et al., 2003; Xin et al., 2009), leading to an increased concentration of glutamate in the extracellular space and the development of spontaneous pain (Liaw et al., 2005; Weng et al., 2006).

As a glutamate spill-over induces NMDA-dependent facilitation of GABAergic synaptic transmission, we can propose that this mechanism is a compensatory mechanism activated by the increased activity in the network, to induce an increase GABAergic transmission efficiency in the network. It could also be an amplifier of excitatory transmission in the network, as maybe it reduces inhibitory transmission. To answer these questions, it is necessary to identity the interneuron populations implicated in these mechanisms, as well as record the projection neurons. This mechanism could be altered in conditions of pathological pain.

Conclusion

To conclude, our results showed that NMDAr activation facilitates GABAergic synaptic transmission in the DH.

When recording putative GABAergic and glutamatergic neurons, NMDA-dependent facilitation was observed in the same proportions. However, astrocytes are necessary to mediate NMDA-dependent facilitation of GABAergic synaptic transmission only in putative glutamatergic neurons. However, we do not yet know the role of NMDA-dependent facilitation in network processing of nociceptive information.

When perfusing D-serine, we observed a restoration of the proportion of neurons displaying NMDA-dependent facilitation, suggesting that the implication of astrocytes depends on the availability of the co-agonist. When oxidizing D-serine, we found that it is a necessary co-agonist for NMDA-dependent facilitation in both putative GABAergic and glutamatergic neurons.

Blocking EAAT function to increase the extracellular concentration of glutamate, induces facilitation of GABAergic synaptic transmission. Additional experiments are needed to confirm that TBOA-dependent facilitation is mediated by NMDAr. As glutamate spill-over can be observed in conditions of intense and sustained activity of the primary afferents, we can hypothesize that NMDA-dependent facilitation occurs in conditions of intense nociceptive stimulation.

Finally, preliminary data from our secondary project suggests the expression of NMDAr in DH astrocytes. However, additional data is necessary to confirm these results.

Over the past decades, studies have shown that the main mechanisms implicated in central sensitization of the DH are an NMDAr-dependent increase in excitatory transmission and network disinhibition. However very few studies have focused on the cross-talk between excitatory and inhibitory transmission in the DH. As was shown in 2005, 37% of GABAergic terminals express NMDAr. However nothing is known about the NMDAr subunit expression, the function of these receptors at GABAergic terminals or the potential interneuron populations implicated. Astrocytes have also been shown to play important roles in modulation synaptic transmission and plasticity.

More recently, astrocytes have been shown to gate nociceptive information processing, making them important players in network function. Studies have shown that astrocyte function is altered in conditions of pathological pain. More studies focused on NMDAr function, GABAergic synaptic transmission and its modulation, and astrocytes are needed to decipher the mechanisms implicated in DH network function and its alteration in conditions of pathological pain.

Our results have allowed further understanding of modulation of GABAergic synaptic transmission in the DH, specifically by astrocytes and NMDAr, and open new perspectives for the study of DH network information processing.

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Résumé étendu en français

Implication des astrocytes dans la modulation NMDA-dépendante de l'inhibition spinale

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Contexte scientifique

Des récepteurs spécialisés, appelés nocicepteurs, détectent les informations potentiellement dommageables pour l'organisme, appelées informations nociceptives, qui sont acheminées vers le système nerveux central. Les fibres afférentes forment des synapses dans les différentes laminae composant les cornes dorsales de la moelle épinière (CDME). Un réseau complexe d'interneurones excitateurs et inhibiteurs intègrent les informations nociceptives et modulent l'activité des neurones de projection qui représentent la principale sortie des réseaux des CDME. Ces informations seront transmises aux centres supra-spinaux responsables de l'intégration des différentes composantes de l'information nociceptive, notamment la composante sensori-discriminative et la composante cognitive, associant la stimulation nociceptive à l'expérience cognitive et émotionnelle de la douleur.

L'équilibre entre l'excitation et l'inhibition dans le réseau de la corne dorsale définit l'intensité de l'information qui sera transmise par les neurones de projection pour être intégrée par les centres supra-spinaux et est donc essentiel pour un traitement « normal » des informations nociceptives. Dans la corne dorsale, la transmission synaptique excitatrice est médiée par le glutamate et la

transmission synaptique inhibitrice met en jeu l'acide g-aminobutyrique (GABA) et la glycine. Des études ont montré l'importance de la transmission inhibitrice dans la corne dorsale : en effet, en utilisant des antagonistes spécifiques des récepteurs GABAergiques et/ou glycinergiques, des études ont montré le développement d'allodynie (une interprétation douloureuse d'une stimulation non-nociceptive) et d'hyperalgésie (une amplification de la sensation douloureuse à la suite d'une stimulation nociceptive). De façon intéressante, ce sont les symptômes que l'on observe chez des personnes atteintes de douleurs pathologiques, comme la douleur chronique qui perdure bien après la guérison de la lésion. Les phénomènes de plasticité observés dans ces conditions induisent une diminution de l'efficacité de l'inhibition dans le réseau et donc, une surexcitation du neurone de projection.

Virtuellement tous les neurones de la corne dorsale expriment des récepteurs N-méthyl-D-aspartate (NMDA - NMDAr), des récepteurs ionotropes du glutamate. Dans leur composition la plus classiquement retrouvée, les NMDAr nécessitent la coïncidence de plusieurs évènements pour être activés : l'arrivée de l'agoniste (le glutamate, le NMDA), du co-agoniste (glycine, D-sérine) et une levée du bloc magnésium qui obstrue le canal par une dépolarisation de la membrane. Une fois activés et ouverts, les NMDAr sont perméables aux cations, et plus particulièrement au calcium. Une entrée de calcium dans la cellule peut induire des modifications plastiques de la cellule, et modifier la transmission synaptique.
Les NMDAr peuvent être recrutés à la suite d'une forte activité dans le réseau de la CDME. Leur implication dans les modifications plastiques de la transmission synaptique excitatrice a été largement étudiée. Une étude d'immunohistochimie de 2005 a montré l'expression de NMDAr dans des terminaisons GABAergiques. Cependant, très peu est connu de leur potentiel rôle dans la modulation de la transmission inhibitrice.

Un acteur important dans la modulation de la transmission synaptique est l'astrocyte. Longtemps considéré comme un simple support des neurones, responsables d'établir et maintenir l'homéostasie ionique et fournissant des substrats énergétiques aux neurones, des études plus récentes ont montré que les astrocytes peuvent exprimer des récepteurs aux neurotransmetteurs. Leur activation induit des changements de la concentration intracellulaire de calcium libre dans les astrocytes, qui peuvent à leur tour induire la libération de transmetteurs, tels que le glutamate

et l'ATP. Ces gliotransmetteurs peuvent agir sur des récepteurs exprimés par l'élément présynaptique, postsynaptique ou par d'autres astrocytes et ainsi moduler la transmission synaptique.

Objectifs de la thèse

Des données publiées par l'équipe ont montré que l'activation spécifique des NMDAr dans la CDME induit une facilitation de la transmission synaptique GABAergique. Notre objectif était de caractériser cet effet du NMDA sur la transmission synaptique GABAergique, en s'intéressant particulièrement à un potentiel rôle des astrocytes dans cet effet. Mon travail de thèse s'est donc divisé en 2 projets : un 1er portant sur l'implication des astrocytes dans la modulation NMDA-dépendante de l'inhibition spinale et un 2ème sur l'expression potentielle de NMDAr par les astrocytes de la CDME.

Approches méthodologiques

Nous avons utilisé des approches d'électrophysiologie sur tranches aiguës de moelle épinière de souris adultes. Afin de différencier les neurones inhibiteurs des neurones excitateurs, nous avons utilisé une lignée de souris transgéniques qui expriment une protéine verte fluorescente (GFP) sous le contrôle du promoteur de l'enzyme glutamate décarboxylase 65 (GAD65), une enzyme de synthèse du GABA. De ce fait, les neurones GABAergiques apparaissent en fluorescent. Les neurones non fluorescents sont considérés comme des neurones glutamatergiques putatifs. Lors de mes enregistrements, j'ai donc fait la différence entre les neurones GAD^+ et les neurones GAD^-. Afin d'évaluer l'effet de l'activation des NMDAr par son agoniste spécifique NMDA (100µM) sur la transmission synaptique GABAergique, j'ai utilisé la technique du patch clamp, en configuration cellule entière, afin d'analyser les courants post-synaptiques inhibiteurs spontanés (CPSIs) et miniatures (CPSIm). Les enregistrements de la transmission synaptique spontanée nous

renseignent d'un potentiel effet du réseau dans les effets observés. Les enregistrements de la transmission synaptique miniature ont été réalisés en présence de tétrodotoxine (TTX, 0,5µM), un bloqueur des canaux sodiques voltage-dépendants (inhibant donc la genèse des potentiels d'action), afin d'isoler l'activité des synapses contactant la cellule enregistrée. Nous avons analysé la fréquence d'apparition des CPSIs et CPSIm, leur amplitude et leur cinétique. Un changement de fréquence d'apparition des CPSIs et CPSIm renseigne d'un changement de la probabilité de libération (effet présynaptique).

I. Effet du NMDA sur la transmission synaptique GABAergique

1. Effet du NMDA sur la transmission synaptique GABAergique spontanée

Dans un premier temps, nous avons étudié l'effet d'une application de NMDA (100µM) sur la transmission synaptique GABAergique dans la lamina II. Nos résultats montrent que l'activation des NMDAr par l'agoniste NMDA (100µM) induit une facilitation de la transmission synaptique GABAergique spontanée dans 77% des neurones GAD^+ enregistrés (n=24 neurones sur 31) et dans 76% des neurones GAD^- enregistrés (n=28 neurones sur 37). Cet effet facilitateur se traduit par une augmentation de la fréquence d'apparition des CPSIs dans les neurones GAD^- enregistrés après l'application de NMDA (période contrôle : 0.57±0.09 Hz ; période NMDA : 1.2±0.22 Hz ; $p<0.001$) sans changement de l'amplitude (période contrôle : -27.67±4.17 pA ; période NMDA : -32.46±5.32 pA). Dans les neurones GAD^+, cet effet se traduit par une augmentation de la fréquence d'apparition des CPSIs (période contrôle : 0,33±0,11 Hz ; période NMDA : 0,99±0,20 Hz ; $p<0,0001$) et une augmentation de l'amplitude des CPSIs (période contrôle : -18,70±3,86 pA ; période NMDA : -32,04±5,71 pA ; $p<0.01$).

2. Effet du NMDA sur la transmission synaptique GABAergique miniature

En étudiant la transmission synaptique GABAergique miniature, nous avons observé un effet facilitateur du NMDA de la transmission synaptique GABAergique miniature dans 48% des neurones GAD^- enregistrés (n=10 neurones sur 21) et 38% des neurones GAD^+ enregistrés (n=8 neurones sur 20). Cet effet facilitateur se traduit par une augmentation de la fréquence d'apparition des CPSIm dans les neurones GAD^- (période contrôle : 0,10±0.03 Hz ; période NMDA : 0,23±0,06 Hz, $p<0.05$) et les neurones GAD^+ (période contrôle : 0,07±0,02 Hz ; période NMDA : 0,17±0,03 Hz, $p<0.01$) sans changement de l'amplitude (**GAD^-** : période contrôle : -21.70±5.07 pA ; période NMDA : -30.49±8.67 pA et **GAD^+** : période contrôle : -20.32±4.98 pA ; période NMDA : -21.15±3.15 pA).

Nos résultats montrent que l'activation des NMDAr induit une facilitation de la transmission synaptique GABAergique spontanée et miniature, de façon similaire dans les neurones GAD^- et GAD^+, mais les proportions diffèrent entre la transmission spontanée et miniature, indiquant une implication du réseau de la corne dorsale pour la modulation NMDA-dépendante de la transmission synaptique GABAergique.

II. Rôle des astrocytes dans la modulation NMDA-dépendante de la transmission synaptique GABAergique

1. Implication des astrocytes dans la modulation NMDA-dépendante de la transmission synaptique GABAergique

Afin d'évaluer une potentielle implication des astrocytes dans cet effet, nous avons utilisé du fluorocitrate. Il s'agit d'un faux substrat de l'aconitase, préférentiellement repris par les astrocytes, qui va bloquer le cycle de Krebs et la production d'ATP dans ces cellules. Théoriquement, toutes les fonctions ATP-dépendantes seront impactées, telles que le maintien des gradients ioniques et l'activité des transporteurs de neurotransmetteurs. Après incubation des tranches avec le fluorocitrate, nous avons procédé avec le même protocole que précédemment. Cette fois, l'effet facilitateur du NMDA a été observé dans 21% des neurones GAD^- enregistrés (n=7 neurones sur

34) et 72% des neurones GAD^+ enregistrés (n=13 neurones sur 18). Il y a donc une diminution de la proportion de neurones GAD^- présentant l'effet facilitateur du NMDA sur la transmission synaptique spontanée GABAergique ($p<0,05$), mais pas d'effet pour les neurones GAD^+ enregistrés. De façon intéressante, il n'y a pas de changement de proportions quand on enregistre la transmission synaptique GABAergique miniature après une incubation avec du fluorocitrate, indiquant l'absence d'un maillon glial dans l'effet facilitateur du NMDA dépendant de l'activité du réseau pour les neurones GAD^-.

2. Implication de la D-sérine dans la modulation NMDA-dépendante de la transmission synaptique GABAergique

Nous savons que les astrocytes sont nécessaires pour la synthèse de D-sérine, un co-agoniste des NMDAr, en libérant de la L-sérine qui va être reprise par les neurones, selon le modèle actuel. Nous savons que les astrocytes sont nécessaires pour induire l'effet facilitateur du NMDA dans les neurones GAD^-. Nous avons émis l'hypothèse que l'implication astrocytaire dans l'effet du NMDA reposait sur la libération de L-sérine par les astrocytes qui serait ensuite convertie en D-sérine. Afin d'étudier cette hypothèse, des neurones GAD^- ont été enregistrés après une incubation avec du fluorocitrate et le même protocole que précédemment a été réalisé, mais en présence de D-sérine (50µM). Nous avons observé un effet facilitateur du NMDA sur la transmission synaptique GABAergique dans 67% des neurones enregistrés (n=10 neurones sur 15), une proportion différente de celle obtenue après une incubation avec du fluorocitrate sans D-sérine ($p<0,05$) et non différente de la proportion contrôle sans incubation avec du fluorocitrate. Nous avons donc récupéré la proportion de neurones GAD^- qui présentent l'effet facilitateur du NMDA en présence de D-sérine après une incubation avec du fluorocitrate.

Afin de confirmer ces résultats, nous avons sélectionné les cellules présentant un effet du NMDA à la suite d'une $1^{ère}$ application de NMDA et avons procédé à une $2^{ème}$ application de NMDA en présence de D-amino acid oxydase (DAAO, 50µM), une enzyme dégradant la D-sérine. Nous avons observé un $2^{ème}$ effet dans 50% des neurones GAD^- enregistrés (n=5 sur 10 neurones ; $p<0,05$) et 20% des neurones GAD^+ enregistrés (n=2 neurones sur 10 ; $p<0,05$), soit des

proportions significativement réduites par rapport au contrôle (2 applications à la suite de NMDA 100µM, sans DAAO).

Pris ensemble, ces résultats suggèrent que la diminution de la proportion de neurones GAD^- présentant l'effet facilitateur du NMDA à la suite d'une incubation avec du fluorocitrate est due à un manque de co-agoniste, fourni directement ou indirectement par les astrocytes. Cependant, nous observons une diminution de la proportion de cellules présentant l'effet du NMDA en enregistrant les neurones GAD^+, qui ne sont pas affectés par l'incubation avec du fluorocitrate. Les astrocytes seraient la source du co-agoniste seulement pour les neurones GAD^-. Il serait intéressant, afin de compléter cette observation, d'étudier l'expression de la sérine racémase dans les différentes populations d'interneurones de la lamina II. Il pourrait également être intéressant de reproduire ces expériences en présence de glycine oxydase, une enzyme de dégradation de la glycine, qui est un autre co-agoniste des NMDAr.

III. L'origine du glutamate dans la modulation NMDA-dépendante de la transmission synaptique GABAergique

Nous avons montré que l'activation des NMDAr induit une facilitation de la transmission synaptique GABAergique dans la lamina II de la CDME. Il a été montré que dans des conditions d'activité intense, à la suite de stimulations répétées des fibres afférentes par exemple, les transporteurs de glutamate (Excitatory Amino Acid Transporter, EAAT) sont saturés et le glutamate se répand dans le milieu extracellulaire, un phénomène appelé « spill-over ». Ce spill-over de glutamate pourrait être la source d'agoniste activant les NMDAr impliqués dans la modulation de la transmission synaptique GABAergique. Afin d'étudier cette hypothèse, nous avons enregistré les CPSIm GABAergiques et utilisé le DL-threo-β-Benzyloxyaspartic acid (TBOA, 50µM), un bloqueur des EAAT. Nous avons observé une facilitation de la transmission synaptique GABAergique miniature dans 50% des cellules enregistrées (n=13 cellules sur 26). Afin de contrôler qu'il s'agissait bien des NMDAr impliqués dans cette facilitation TBOA-dépendante, nous avons enregistré les CPSIm GABAergiques en présence de TBOA et de D-2-

amino-5-phosphonevolerate (APV, 50µM), un antagoniste spécifique des NMDAr contenant les sous-unités GluN2 et nous avons observé un effet facilitateur du TBOA dans seulement 22% des cellules enregistrées (n=2 cellules sur 9). Des résultats publiés dans l'équipe ont montré que les NMDAr impliqués dans l'effet facilitateur du NMDA contiennent les sous-unités GluN2C et GluN2D. Afin de confirmer qu'il s'agissait de la même population de NMDAr impliquée dans l'effet facilitateur du TBOA, nous avons utilisé un antagoniste spécifique des NMDAr contenant les sous-unités GluN2C-D (NAB14, 10µM) et avons observé un effet facilitateur du TBOA dans 33% des cellules enregistrées (n=4 cellules sur 12). Il faudrait par la suite augmenter le nombre de cellules enregistrées dans ces 2 derniers groupes, afin de s'assurer que la diminution de la proportion de cellules présentant un effet facilitateur du TBOA en présence d'APV et de NAB14 soit significative. On peut également se questionner sur les cellules qui présentent un effet facilitateur du TBOA en présence d'APV et de NAB14, suggérant que d'autres récepteurs soient activés par le spill-over de glutamate, tels que les récepteurs métabotropes du glutamate. Il pourrait être intéressant d'ajouter un antagoniste de ces récepteurs et observer un potentiel changement de la proportion de cellules présentant un effet facilitateur du TBOA.

Conclusion

Pour conclure, la réalisation de cette thèse a permis d'améliorer la compréhension des mécanismes de modulation de la transmission synaptique GABAergique dans le réseau de la CDME par les NMDAr et les astrocytes. Il a permis de mieux comprendre les interactions entre les transmissions excitatrices et inhibitrices et l'implication d'un maillon astrocytaire dans ce cross-talk. Parmi les mécanismes impliqués dans le développement et le maintien des douleurs pathologiques, le recrutement des NMDAr, une diminution de l'efficacité de l'inhibition et une activation des cellules gliales sont parmi les plus importants. Il est très important de comprendre comment ces mécanismes sont liés afin de potentiellement développer des stratégies thérapeutiques pour améliorer le quotidien des personnes atteintes de douleurs chroniques.

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Implication des astrocytes dans la modulation NMDA-dépendante de l'inhibition spinale

Résumé

La corne dorsale de la moelle épinière joue un rôle essentiel dans la réception et l'intégration des informations nociceptives provenant de la périphérie. Ces informations seront ensuite transmises aux centres supra-spinaux où elles seront consciemment interprétées comme douloureuses. Un réseau complexe d'interneurones excitateurs et inhibiteurs, ainsi que des cellules gliales, intègrent les informations nociceptives et modulent l'activité des neurones de projection. L'équilibre entre l'excitation et l'inhibition au sein du réseau définit l'intensité du message transmis. La rupture de l'équilibre entre l'excitation et l'inhibition peut mener à des conditions de douleurs anormales, qui peuvent se maintenir dans le temps. Le recrutement des récepteurs NMDA et une diminution de l'efficacité de l'inhibition dans le réseau jouent un rôle critique dans le développement et la maintenance de la sensibilisation centrale. Cependant, ces mécanismes ont principalement été étudiés séparément. Un acteur important dans la modulation de la transmission synaptique est l'astrocyte. Ce projet apporte de nouvelles pistes quant au rôle des récepteurs NMDA et des astrocytes dans la modulation de la transmission synaptique GABAergique dans la corne dorsale de la moelle épinière.

Mots-clefs : corne dorsale, astrocytes, récepteurs NMDA, transmission synaptique GABAergique

Abstract

The dorsal horn of the spinal cord plays a key role in reception and integration of nociceptive information from the periphery. This information will be transmitted to supra-spinal centers, where it might be consciously interpreted as painful. A complex network of excitatory and inhibitory interneurons, as well as glial cells, integrate nociceptive information and modulate the activity of the projection neurons. The excitation/inhibition balance defines the intensity of the transmitted message. A break in the excitation/inhibition balance can lead to abnormal pain sensations, which can last in time. NMDA receptor recruitment and a decrease in inhibitory efficiency play a crucial role in the development and maintenance of central sensitization. However, these mechanisms have mostly been studied separately. Astrocytes play an important role in modulation of synaptic transmission. This project provides new insights into the role of NMDA receptors and astrocytes in modulation of GABAergic synaptic transmission in the dorsal horn of the spinal cord.

Keywords : dorsal horn, astrocytes, NMDA receptors, GABAergic synaptic transmission