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THE SEA LAMPREY GABAB RECEPTOR. CHANGES AFTER SPINAL CORD INJURY AND ITS ROLE IN AXONAL REGENERATION

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D. Daniel Romaus Sanjurjo

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THE SEA LAMPREY GABAB RECEPTOR. CHANGES AFTER SPINAL CORD INJURY AND ITS ROLE IN AXONAL REGENERATION

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ABBREVIATIONS

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1 ABBREVIATIONS

B1	Bulbar Müller cell 1.
B2	Bulbar Müller cell 2.
B3	Bulbar Müller cell 3.
B4	Bulbar Müller cell 4.
B5	Bulbar Müller cell 5.
B6	Bulbar Müller cell 6.
dC	Dorsal cell.
DCN	Dorsal column nucleus.
DG	Dorsal grey.
DIG	Dorsal isthmic grey.
DMN	Dorsomedial neuropil.
DN	Dorsal nucleus of the octavolateral area.
eC	Edge cell.
GL	Glomerular layer.
Ha	Habenula.
HY	Hypothalamus.
I1	Isthmic Müller cell 1.
I3	Isthmic Müller cell 3.
I4	Isthmic Müller cell 4.
I5	Isthmic Müller cell 5.
IGL	Inner granular layer.
IIIi	Intermediate oculomotor subnucleus.
IIIl	Lateral oculomotor subnucleus.
IIIm	Medial oculomotor subnucleus.
IR	Infundibular recess.
IsRF	Isthmic reticular formation.
IVm	Trochlear motor nucleus.
LP	Lateral pallium.
LG	Lateral grey.
M1	Müller cell 1.
M2	Müller cell 2.

M3	Müller cell 3.
MA	Mature upstream migrating adult.
MCL	Mitral cell layer.
MLF	Medial longitudinal fasciculus.
MN	Medial nucleus of the octavolateral area.
Mn	Motor neurons.
MP	Medial pallium.
Mr	Mammilar region.
MRF	Middle rhombencephalic reticular formation.
MT	Mesencephalic tegmentum.
Mth	Mauthner cell.
nMLF	Nucleus of the medial longitudinal fasciculus.
NOMI	Intermediate octavomotor nucleus.
nTPOC	Nucleus of the tract of the postoptic commissure.
OB/P	Olfactory bulbs/pallium.
Ola	Octavolateral area.
OT	Optic tectum.
P	Pineal organ.
pc	Posterior commisure.
PCP	Paracommissural preoptic nucleus.
PL	Periventricular layer.
PM	Post-metamorphic sea lamprey.
PO	Preoptic nucleus.
POR	Preoptic recess.
PP	Parapineal organ.
PR	Postoptic recess.
PRF	Posterior rhombencephalic reticular formation.
PT	Pretectum.
PTh	Prethalamus.
PtN	Posterior tubercular nucleus.
PTu	Posterior tuberculum.
sa + gc	Stratum “album” et griseum centrale.
SCO	Subcommissural organ.
sfgs	Stratum fibrosum et griseum superficiale.
sgp	Stratum griseum periventriculare.

ShL	Subhippocampal lobe.
Sol	Nucleus of the solitary tract.
SP	Septum.
ST	Striatum.
Th	Thalamus.
TRF	Trigeminal reticular formation.
TS	Torus semicircularis.
Vm	Trigeminal motor nucleus.
VIIm	Facial motor nucleus.
VN	Ventral nucleus of the octavolateral area.
Xm	Vagal motor nucleus.

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SUMMARY

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2 SUMMARY

In humans, a traumatic spinal cord injury (SCI) causes permanent disability. In Spain, more than 30,000 people are affected by SCI and around 1,000 people suffer a traumatic SCI every year. SCI is a major health and social problem, but also an economical one.

In mammals, SCI causes irreversible loss of function. One of the major reasons for the failure of recovery following SCI is caused by the inability of axotomized neurons to regenerate their axons and reinnervate spinal cord regions below or above the site of injury. Traumatic SCI starts with the primary injury, which is caused by the mechanical forces of the traumatic event, followed by secondary damage that evolves over time. The primary injury results in the damage of the blood-brain barrier and local blood vessels, axons are axotomized and neuronal cell-membranes broken due to traction and compression forces. The second phase consists in a series of molecular events, being excitotoxicity the most destructive process. Glutamate is released from injured neurons causing overexcitement and massive Ca2+ influx in neighbouring healthy cells (neurons, astrocytes and oligodendrocytes) until they die. Retrograde damage to descending neurons is also likely due to the fact that Ca2+ ions gain access to the axoplasm of damaged axons. Prevention of retrograde degeneration is an obvious prerequisite for the occurrence of axonal regeneration.

Failure of proper axonal regeneration after a traumatic SCI in adult mammals, including humans, is known to occur due to extrinsic and intrinsic factors. Extrinsic factors include molecules, like chondroitin sulfate proteoglycans, that are present in the scar tissue that forms after the injury or in the myelin surrounding the damaged axon and that are inhibitory for axonal regrowth. Manipulations that eliminate or neutralize these extrinsic factors result in enhanced regrowth of some types of axons. However, in most cases, only a small number of injured CNS axons can regenerate, consistent with the idea that the lack of regeneration in the adult CNS of mammals is an intrinsic property of the injured neurons.

Lampreys belong to the most ancient group of vertebrates, the agnathans. Thanks to their phylogenetic position, at the base of vertebrate evolution, lampreys are an excellent animal model to study the origin and evolution of the nervous system of extant vertebrates in evolutionary and developmental studies (evo-devo). The lamprey CNS has a similar organization compared to that of jawed vertebrates, but it contains fewer neurons and glial cells. This, together with the fact the lamprey CNS can be maintained ex vivo for hours or even days, has facilitated their use as a model to study different neuronal circuits, such as those implicated in the control of locomotion.

In contrast to mammals, lampreys recover locomotion spontaneously after a complete SCI. This amazing recovery process involves the regeneration of descending axons and the formation of synaptic connections between the regenerated axons and neurons caudal to the lesion. Among the brain descending neurons of lampreys there are 36 identifiable giant descending neurons. These include the Mauthner neurons and several pairs of Müller cells. Interestingly, these identifiable descending neurons vary greatly in their regenerative abilities following SCI, even when their axons run in similar paths in a spinal cord that is permissive for axonal regrowth. Some of these neurons are considered “good regenerators” (i.e they regenerate their axon more than

55% of the times) and others are considered “bad regenerators” (i.e. they regenerate their axon less than 30% of the times). Thus, in lampreys, there is an opportunity to study both enhancement and inhibition of regeneration in the same preparation. An advantage of the lamprey model of SCI is that the identifiable descending neurons and their descending axons can be visualised in vivo and in CNS whole-mounts due to the transparency of the lamprey brain. Interestingly, recent work has shown that descending neurons known to be bad regenerators experiment a process of delayed cell death and are also “poor survivors” after a complete SCI.

In addition to regenerative events, lampreys also show a large degree of anatomical and functional plasticity, although different neurotransmitter systems show different responses to a SCI. For example, the intraspinal dopaminergic and GABAergic systems show a full anatomical recovery; in contrast to the serotonergic and glutamatergic systems that do not recover to a pre-injury state. Regarding functional plasticity, there is a significant decrease in the number of vesicle clusters and active zones in giant reticulospinal synapses distal to the lesion after SCI. Likewise, excitability is increased below the site of lesion in lampreys recovered from a complete SCI, and the cellular and synaptic modulatory effect of serotonin differ in lesioned and un-lesioned animals. Moreover, transected lampreys show a stronger tonic GABAergic inhibition during the recovery of locomotor function following SCI. All these results underline the importance of studying each neurotransmitter system in lesioned animals (below and above the site of injury) to understand the mechanisms that lead to functional recovery in lampreys.

GABA is the main inhibitory neurotransmitter in the CNS of vertebrates. GABA acting via metabotropic (GABAB) receptors addresses second messenger systems through the binding and activation of guanine nucleotide-binding proteins [G-protein-coupled receptors (GPCRs)], producing a slow and prolonged inhibition which results in reduced neuronal excitability. GABAB receptors have been identified on both pre- and postsynaptic terminals. Presynaptic GABAB receptors can exist as either auto- (those that control GABA release) or heteroreceptors (those activated by other neurons) to modulate neurotransmitter release. Currently, it is accepted that functional GABAB receptors are obligate heterodimers composed of a GABAB1 subunit, responsible for agonist binding; and a GABAB2 subunit, responsible for signal transduction following agonist activation through the activation of G-proteins.

GABAergic signalling is involved in the transmission of somatosensory information to higher centres, in the propriospinal coordination of fore and hind limb movements and in reflex activities. Also, descending GABAergic neurons are responsible for tonic inhibition affecting spinal motoneurons and premotor interneurons which leads to a reduced locomotion. In lampreys, GABA is not required for burst generation but it plays a powerful modulatory role during locomotion. For example, activation of GABAB receptors causes a reduction of burst frequency with a maintained well-coordinated locomotor activity. Moreover, GABAB receptors play a role at the level of dendrites and somas of interneurons and motoneurons modifying the intrinsic membrane properties. Due to the importance of GABA in the modulation of spinal circuitries, alterations of the GABAergic system lead to an impairment of function: genetic ablation of GABAergic interneurons results in motor deficits, and hypofunction of the GABAergic tone in the spinal dorsal horn is a key factor in central neuropathic pain (CNP) after SCI. However, no studies have looked at changes in the expression of GABAB receptor following a direct traumatic injury to the spinal cord in any vertebrate.

Some studies have suggested that GABA could have neuroprotective effects in some types of CNS injuries. Activation of pre-synaptic GABAB receptors causes inactivation of voltage-dependent Ca2+ channels, which could compensate the influx of Ca2+ ions due to glutamate release. In addition, GABA can modulate and promote neurite outgrowth in vitro or during development. Therefore, similar roles are possible during axonal regeneration after SCI. In fact, a recent study of our group showed that GABA accumulates in the form of "halos" around some axotomized axons of identifiable descending neurons and statistical analyses showed a significant correlation between GABA accumulation in the form of halos and a higher survival ability of the corresponding descending neurons. These results suggest a neuroprotective and pro-regenerative role of GABA following spinal cord injury in lampreys.

Here, we aimed to reveal changes in the expression of the GABAB receptor and the role of GABA acting through this receptor in the regeneration of descending neurons after a complete SCI in lampreys. The specific aims of this doctoral thesis were: (1) to clone and identify the cDNA sequences of the subunits of the GABAB receptor of the sea lamprey, (2) to obtain in situ probes for the gabab1 and gabab2 subunits, (3) To study the expression of gabab subunits in normal conditions and following SCI in the spinal cord of lampreys, and (4) to study the role of GABA and GABAB receptors in axon regeneration in identifiable descending neurons following SCI.

CLONING OF THE GABAB RECEPTOR SUBUNITS B1 AND B2 AND THEIR EXPRESSION IN THE CENTRAL NERVOUS SYSTEM OF THE ADULT SEA LAMPREY

We report for the first time the identification and characterization of the gabab1 and gabab2 cDNA sequences and the expression of both gabab transcripts in the central nervous system of the adult sea lamprey.

We used the BLAST tool to find sequences corresponding to gabab1 and gabab2 cDNAs in GenBank and the ensemble database and, after PCR confirmation, we cloned both cDNAs. The phylogenetic analyses of the corresponding aminoacidic sequences confirmed that the GABAB1 and GABAB2 partial amino acid sequences of the sea lamprey are located at the base of the vertebrate branches, as sister members, respectively, of the GABAB1 and GABAB2 sequences of gnathostomes. This location of sea lamprey GABAB sequences are in agreement with the phylogenetic position of lampreys and confirmed the GABAB identity of our sequences.

Then, in situ hybridization probes were generated based on the specific sequence of these subunits. It is widely accepted that to form a functional GABAB receptor both gabab subunits have to be present. In our study, we observed an overlapping pattern of expression of both gabab subunits throughout all CNS regions of the adult sea lamprey. A similar co-expression of both gabab subunits has been reported in invertebrate (D. melanogaster) and vertebrate (zebrafish, rat) species. Thus, it seems that a wide and highly overlapping expression of the gabab1 and gabab2 subunits is an ancestral and conserved character of vertebrates and invertebrates.

Some studies have shown an association between the GABAB2 subunit and M2 muscarinic receptors that enhances muscarinic signaling. In lampreys, pharmacological and electrophysiological approaches have shown that muscarinic receptors are involved in the modulation of the trigeminal-reticular pathway and in the activation of reticulospinal neurons. In our study, we report expression of the gabab2 transcript in the

region of the posterior rhombencephalic reticular nucleus, a region that contains cells immunoreactive for muscarinic receptors. It would be interesting to study whether there is a modulation of muscarinic receptors by the GABAB2 subunit in lampreys as in mammals, which would show us whether this is an ancestral characteristic of vertebrates.

Present results support previous pharmacological and electrophysiological reports studying GABAergic modulation through GABAB receptors in lampreys. The broad expression of both gabab cDNAs in the sea lamprey CNS reveal the importance of these receptors in the modulation of brain and spinal cord circuits. Gabab transcripts are present in non-GABAergic (descending neurons, spinal and rhombencephalic motor neurons, or spinal edge cells) and in GABAergic cells. Previous studies showed the GABAergic innervation and GABA modulation of the edge cells, and our present results suggest that GABA could act trough the GABAB receptor in these cells. Our expression results also are in agreement with other studies studying the role of GABAB receptors in the modulation of the lamprey respiratory network, or the modulatory action of GABA onto the pathway from lateral columns to reticulospinal neurons through GABAB receptors. Our study extends the number of neuronal populations known to be modulated through GABAB signaling and opens the opportunity to conduct functional studies on the role of GABA and the GABAB receptor in other circuits.

The presence of both gabab subunits in GABAergic cells present in brain and spinal cord suggest that the GABAB receptor can also modulate the activity of GABAergic cells in lampreys. The presence of both gabab transcripts in periglomerular cells of the olfactory bulbs, which are mainly GABAergic, or in the cerebrospinal fluid-contacting (CSF-c) cells of the spinal cord, which are mainly GABAergic as well, support this hypothesis. However, due to different fixative requirements, we could not perform double labeling (in situ hybridization and GABA immunohistochemistry) to confirm the presence of GABAB receptors in GABAergic cells of the sea lamprey. The presence of positive in situ signal in the dendrites of CSF-c cells of the spinal cord could indicate a role for GABAB receptors in the detection of GABA in the CSF. GABAB receptors could modulate CSF-c cells by acting as an autoreceptor and/or as a heteroreceptor with synapses from other GABAergic cells.

Present results show a lack of expression of both gabab transcripts in ependymocytes throughout the adult sea lamprey brain. In the brain of lampreys, ependymal cells are the main glial type and astrocytes are only associated to some nervous tracts. The spinal cord of lampreys contains both types of glial cells. Unlike jawed vertebrates, oligodendrocytes are not present in lampreys and the astrocytes of lampreys do not display immunoreactivity to glial fibrillary acid protein, but they express cytokeratins. Little is known about the expression of both gabab mRNAs and/or GABAB1 and GABAB2 subunits in the ependymal layer of vertebrates, only a pharmacological study has reported that GABAA, and not GABAB, receptor mediates the GABAergic responses in mammalian ependymal cells. Even if this study agrees with our results, more studies in other groups of vertebrates are necessary to determine the evolution of this character due to the large evolutionary distance between mammals and lampreys. A special type of ependymal cells, the cells of the subcommissural organ, did not show expression of gabab receptors in lampreys; supporting other results from studies in different vertebrates. Unlike rodent and human studies reporting the presence of GABAB subunits in astrocytes, no expression of the gabab transcripts was observed in astrocytes of the optic nerve of the sea lamprey. As mentioned above, more studies

are necessary in other groups of vertebrates to determine whether the lack of GABAB expression in astrocytes is the ancestral condition or a derived character.

EXPRESSION OF THE GABAB RECEPTOR AFTER A COMPLETE SCI IN LAMPREYS

Our results show a statistically significant decrease in the expression of both gabab subunits after a complete SCI in lampreys. This response of gabab receptors differs from that of other neurotransmitter receptors. For example, there is an acute increase in the expression of the serotonin 1a receptor, or a lack of changes in the expression of the dopamine d2 receptor after a complete SCI in lampreys. Lampreys also show plasticity at the functional level, as the occurrence of increased excitability below the site of lesion, changes in the cellular and synaptic modulatory effect of serotonin in lesioned animals compared to un-lesioned animals, or a stronger tonic GABAergic inhibition for a good recovery of locomotor function after SCI. All these studies underline the importance of studying each neurotransmitter system in lesioned animals (below and above the site of injury) to understand the mechanisms that lead to functional recovery in lampreys. It is also clear that the circuits underlying locomotion in lampreys are highly plastic after SCI, both at the anatomical and functional level.

We show that in animals that recovered normal appearing locomotion there are lower levels of expression of both gabab transcripts. A reduced expression of post-synaptic GABAB receptors could explain the higher inhibition via GABAA receptors reported in lesioned lampreys. First, this would mean that more GABA is available to activate GABAA receptors due to the presence of a completely recovered GABAergic system. In addition, lower levels of expression of gabab transcripts after injury could increase the levels of cAMP, since activation of GABAB receptors inhibits adenylyl cyclase. Higher levels of cAMP lead to an increase in protein kinase A (PKA) activity, which is a key factor to maintain a stable surface expression of GABAA receptors. Then, low levels of gabab transcripts could increase the number of GABAA receptors anchored to the cell surface in lesioned animals. Moreover, decreasing GABAB receptors in GABAergic cells might increase GABA release, given that presynaptic GABAB receptors suppress GABA release. All these facts could explain the higher inhibition through GABAA receptors in transected animals. It would be interesting to identify the GABAA subunits in the sea lamprey to carry out future studies looking at possible changes in their expression following a SCI in lampreys.

This is the first study demonstrating a reduction in the expression of the gabab receptor following a traumatic injury to the spinal cord in vertebrates. There are previous studies reporting changes in the expression of GABAB receptors in rodents and non-human primates after brain or non-traumatic spinal injuries. A decrease in the expression of GABAB receptors has been observed in the thalamus of rats suffering a traumatic brain injury, or in the cortex and cuneate nucleus of adult squirrel monkeys 1 to 5 years after median and ulnar nerve transection. Other studies also showed decreased levels of GABAB receptors in rats with a sciatic nerve ligation or with diabetic neuropathy. Present results together with all previous studies suggest that a decrease in the expression of the GABAB receptors is a common characteristic after nervous system injuries in vertebrates.

ROLE OF GABAB RECEPTORS IN AXONAL REGENERATION AFTER A COMPLETE SCI

A previous study of our group has showed that after a SCI, GABA accumulates extracellularly around the axons of identifiable descending neurons that show higher survival ability after spinal cord injury, strongly suggesting a role for GABA in protecting these neurons. Here, we first analysed changes in the expression of the gabab1 subunit in descending neurons of the sea lamprey. Our analyses revealed an increase in the expression of the gabab1 subunit in the soma of descending neurons 1 wpl. This further supported our hypothesis suggesting that GABA could be playing a neuroprotective and pro-regenerative role after SCI and indicated that it is acting through GABAB receptors.

We then performed gain and loss of function experiments to confirm this hypothesis. The gain of function experiment consisted of a GABOB treatment (a GABA analogue) aiming to enhance GABAergic signalling. Neuronal tract tracing revealed that GABOB significantly increased the number of descending neurons with regenerated axons. This result shows that GABAergic signalling promotes the regeneration of descending neurons after SCI. To determine whether endogenous GABA acting through GABAB receptors also plays a pro-regenerative role, loss of function treatments with gabab1 morpholinos were performed. First, we confirmed that the active gabab1 morpholino is able to reduce the expression of the gabab1 subunit using in situ hybridzation. Then, we performed long term treatments with gabab1 morpholinos. At 10 wpl, animals treated with the active gabab1 morpholino showed a significant decrease in axon regeneration of descending neurons after a complete SCI compared to control animals treated with a mismatch control morpholino. This result demonstrates that endogenous GABA acts as a pro-regenerative factor after SCI by activating GABAB receptors expressed in the descending neurons. Our results agree with previous in vitro and developmental studies showing that GABA signalling through GABAB receptors promotes neurite and axonal growth. But, as far as we are aware, our results are the first in vivo demonstration showing that GABA promotes axonal regrowth after a CNS injury by activating GABAB receptors.

RESUMO

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3 RESUMO

Nos humanos, unha lesión traumática na medula espiñal causa unha discapacidade permanente. En España, máis de 30.000 persoas sofren unha lesión na medula espiñal e ao redor de 1.000 persoas ao ano sofren unha lesión traumática. As lesións medulares supoñen un gran problema social, económico e de saúde.

Nos mamíferos, as lesións medulares causan unha perda funcional irreversible. Unha das maiores razóns para esta falta de recuperación despois da lesión é a incapacidade das neuronas afectadas de rexenerar os seus axóns e re-inervar rexións da medula espiñal por enriba ou por baixo do lugar da lesión. A lesión traumática da medula espiñal comeza ca lesión primaria, a cal é causada polas forzas mecánicas do propio evento traumático, e segue ca lesión secundaria que evoluciona co paso do tempo. A lesión primaria ten como resultado o dano na barreira hematoencefálica e nos vasos sanguíneos locais; así mesmo, a lesión primaria causa a sección de axóns e a ruptura das membranas celulares das neuronas debido ás forzas de tracción e compresión. A segunda fase consiste nunha serie de sucesos moleculares dos que a excitotoxicidade é o proceso máis destrutivo. O glutamato liberado dende as neuronas danadas causa unha entrada masiva de calcio nas células veciñas (neuronas, astrocitos e oligodendrocitos) o que provoca unha sobreexcitación e a posterior morte. O dano retrógrado nas neuronas descendentes débese probablemente ao feito de que os ións de calcio acceden a través do axoplasma dos axóns danados. A prevención da dexeneración retrógrada é un prerequisito obvio para lograr a rexeneración axonal.

Sábese que a falta dunha rexeneración axonal axeitada despois dunha lesión medular traumática nos mamíferos adultos (incluídos os humanos) é debida a factores extrínsecos e intrínsecos. Os factores extrínsecos inclúen moléculas, como os proteoglicáns de condroitín sulfato, que están presentes no tecido cicatrizado que se forma despois da lesión ou na mielina que rodea ao axón danado, e que inhiben o crecemento axonal. As accións que eliminan ou neutralizan estes factores extrínsecos resultan nun maior crecemento axonal dalgúns tipos de axóns. Con todo, na maioría dos casos, só un pequeno número de axóns danados presentes no sistema nervioso central (SNC) poden rexenerar, o cal coincide coa idea de que a falta de rexeneración no SNC de mamíferos adultos é unha propiedade intrínseca das neuronas afectadas.

As lampreas pertencen ao grupo máis antigo dos vertebrados, os agnatos. Grazas a súa posición filoxenética, na base da evolución dos vertebrados, as lampreas son un animal modelo excelente para estudar a orixe e evolución do sistema nervioso de vertebrados actuais en estudos de evolución e desenvolvemento (evo-devo). O SNC da lamprea ten unha organización similar ao mesmo dos vertebrados con mandíbula, pero contén menos neuronas e células de glía. Isto, xunto co feito de que o SNC da lamprea pode ser mantido ex vivo durante horas ou incluso días, facilitou o seu uso como un modelo para o estudo de diferentes circuítos neuronais, tales como aqueles implicados no control da locomoción.

A diferenza dos mamíferos, as lampreas recuperan a locomoción espontaneamente despois dunha lesión medular completa. Este incrible proceso de recuperación inclúe a rexeneración dos axóns descendentes e a formación de conexións sinápticas entre os axóns rexenerados e as neuronas caudais á lesión. Entre as neuronas descendentes situadas no cerebro hai 36 neuronas descendentes xigantes identificables. Estas inclúen

as neuronas de Mauthner e varios pares de células de Müller. Curiosamente, estas neuronas descendentes identificables varían nas súas capacidades de rexeneración despois dunha lesión medular, incluso cando os seus axóns corren en rutas similares nunha medula espiñal permisiva ao crecemento axonal. Algunhas destas neuronas considéranse "boas rexeneradoras" (rexeneran o seu axón máis do 55% das veces), e outras considéranse "malas rexeneradoras" (rexeneran o seu axón menos do 30% das veces). Deste xeito, nas lampreas, existe a posibilidade de estudar tanto a mellora como a inhibición da rexeneración na mesma preparación. Unha vantaxe do modelo da lamprea nas lesións medulares é que as neuronas descendentes identificables e os seus axóns poden ser visualizados in vivo e en preparacións in toto do SNC, grazas á transparencia do cerebro da lamprea. Curiosamente, un traballo recente mostrou que as neuronas descendentes coñecidas como "malas rexeneradoras" experimentan un proceso de morte celular retrasada, e que tamén son "malas superviventes" despois dunha lesión medular completa.

Ademais dos procesos rexenerativos, as lampreas mostran tamén un alto grao de plasticidade anatómica e funcional, aínda que os diferentes sistemas de neurotransmisores mostran respostas diferentes á lesión medular. Por exemplo, os sistemas intraespiñais dopaminérxico e GABAérxico mostran unha recuperación anatómica total; pola contra, os sistemas serotonérxico e glutamatérxico non non retornan a unha situación previa á lesión. Con respecto á plasticidade funcional, existe un descenso significativo do número de vesículas e zonas activas nas sinapses distais á lesión das células xigantes reticuloespiñais. Do mesmo xeito, a excitabilidade por debaixo da lesión está aumentada nas lampreas recuperadas dunha lesión medular completa, e o efecto modulatorio celular e sináptico da serotonina difire en animais lesionados e non lesionados. Ademais, as lampreas lesionadas mostran unha inhibición tónica GABAérxica más forte durante a recuperación da función locomotora despois dunha lesión medular. Todos estes resultados poñen de manifesto a importancia de estudar cada sistema de neurotransmisor en animais lesionados (por enriba e por baixo do lugar da lesión) para entender os mecanismos que levan a unha recuperación funcional nas lampreas.

GABA é o principal neurotransmisor inhibitorio no SNC dos vertebrados. GABA actúa a través de receptores metabotrópicos (GABAB) que, mediante a unión e activación de proteínas G, producen unha inhibición lenta e prolongada que ten como resultado unha excitabilidade neuronal reducida. Os receptores GABAB foron identificados tanto en terminais pre- coma postsinápticos. Os receptores GABAB presinápticos poden actuar como autoreceptores (controlando a liberación de GABA) ou como heteroreceptores (aqueles activados por outras neuronas) para modular a liberación dos neurotransmisores. Actualmente, acéptase que os receptores GABAB funcionais son heterodímeros obrigados compostos por unha subunidade GABAB1, responsable da unión do agonista; e unha subunidade GABAB2, responsable da transdución dos sinais a través da activación das proteínas G.

A sinalización GABAérxica está involucrada na transmisión da información somatosensorial aos centros cerebrais, na coordinación proprioespiñal dos movementos das extremidades anteriores e posteriores, e nos reflexos. As neuronas GABAérxicas descendentes son tamén responsables da inhibición tónica que afecta ás motoneuronas e interneuronas premotoras espiñais, o que leva a unha locomoción reducida. Nas lampreas, non se require GABA para comezar a locomoción, aínda que ten un papel moi importante na modulación da locomoción. Por exemplo, a activación dos receptores GABAB causa unha redución na frecuencia de disparo en presenza dunha actividade

locomotora coordinada. Ademais, os receptores GABAB teñen un papel nas dendritas e somas das motoneuronas e interneuronas, modificando as propiedades intrínsecas da membrana. Debido á gran importancia do GABA na modulación dos circuítos espiñais, as alteracións do sistema GABAérxico levan a unha deficiencia na función: a ablación xenética de interneuronas GABAérxicas resulta en erros motores, e a hipofunción do tono GABAérxico no hasta dorsal da medula espiñal é un factor clave na dor central neuropática despois dunha lesión medular. Con todo, ningún estudo mirou os cambios na expresión do receptor GABAB que se producen despois dun dano traumático directo na medula espiñal en vertebrados.

Algúns estudos suxiren que GABA podería ter efectos neuroprotectores nalgúns tipos de lesións do SNC. A activación de receptores GABAB presinápticos causa inactivación dos canais de calcio dependentes de voltaxe, o que podería compensar o fluxo de ións de calcio que se activa debido á liberación de glutamato. Ademais, GABA pode modular e promover a xeración de neuritas in vitro ou durante o desenrolo. Polo tanto, é posible que o GABA desempeñe papeis similares durante a rexeneración axonal despois dunha lesión medular. De feito, un estudo recente do noso grupo mostrou que o GABA se acumula en forma de "halos" ao redor dalgúns axons seccionados pertencentes ás neuronas descendentes identificables, e análises estatísticos mostraron unha correlación significativa entre a acumulación de GABA ao redor dos axons e unha maior habilidade de supervivencia das correspondentes neuronas descendentes. Estes resultados suxiren un papel neuroprotector e pro-rexenerador do GABA despois dunha lesión medular nas lampreas.

Neste estudo, propuxémonos revelar cambios na expresión do receptor de GABAB, e o papel do GABA, actuando a través deste receptor, na rexeneración de neuronas descendentes despois dunha lesión medular completa na lamprea. Os obxectivos específicos desta tese doutoral foron: (1) clonar e identificar as secuencias do ADNc das subunidades do receptor GABAB da lamprea de mar, (2) obter sondas de in situ das subunidades gabab1 e gabab2, (3) estudar a expresión das dúas subunidades en condicións normais e despois dunha lesión medular na medula espiñal das lampreas, and (4) estudar o papel do GABA e dos receptores GABAB na rexeneración axonal de neuronas descendentes identificables despois dunha lesión medular.

CLONACIÓN DAS SUBUNIDADES B1 E B2 DO RECEPTOR GABAB E A SÚA EXPRESIÓN NO SISTEMA NERVIOSO CENTRAL DA LAMPREA DE MAR ADULTA

Documentamos por primeira vez a identificación e caracterización das secuencias de ADNc das subunidades gabab1 e gabab2, e a súa expresión no sistema nervioso central da lamprea de mar adulta.

Usamos a ferramenta BLAST para atopar secuencias no "GenBank" e na base de datos "Ensembl" que se correspondesen cas secuencias de ADNc das subunidades gabab1 e gabab2. Despois da súa confirmación usando a técnica da PCR, clonamos ambos os dous ADNc. As análises filoxenéticas das secuencias proteicas correspondentes confirmaron que as secuencias parciais de aminoácidos, correspondentes ás subunidades GABAB1 e GABAB2 da lamprea de mar, estaban localizadas na base das ramas de vertebrados, como membros irmáns, respectivamente, das secuencias de GABAB1 e GABAB2 de gnatóstomos. Esta localización das secuencias das subunidades do receptor GABAB da lamprea de mar coinciden ca

posición filoxenética das lampreas e confirmaron que as nosas secuencias pertencen ao receptor GABAB.

Despois, xeráronse sondas para hibridación in situ baseándose nas secuencias específicas destas subunidades. Actualmente, é amplamente aceptado que se necesitan ambas as dúas subunidades do receptor de GABAB para formar un receptor funcional. No noso estudo, observamos un patrón de expresión solapado para ambas subunidades gabab ao longo de todas as rexión do SNC da lamprea de mar adulta. Un patrón similar de co-expresión foi visto en especies invertebradas (D. melanogaster) e vertebradas (peixe cebra, rata). Polo tanto, semella que un patrón de expresión solapado e amplo das dúas subunidades, gabab1 e gabab2, é un carácter ancestral e conservado de vertebrados e invertebrados.

Algúns estudos mostraron unha asociación entre a subunidade GABAB2 e os receptores muscarínicos M2, a cal potencia a sinalización muscarínica. Nas lampreas, técnicas farmacolóxicas e electrofisiolóxicas mostraron que os receptores muscarínicos están involucrados na modulación da ruta trixémino-reticular e na activación das neuronas reticuloespiñais. No noso estudo, presentamos a presenza de expresión do transcrito de gabab2 na rexión do núcleo reticular rombencefálico posterior, unha rexión que contén células inmunoreactivas para receptores muscarínicos. Sería interesante estudar se existe na lamprea unha modulación dos receptores muscarínicos por parte da subunidade GABAB2 semellante ao que ocorre nos mamíferos, o cal evidenciaría se isto é unha característica ancestral dos vertebrados.

Os resultados aquí presentados apoian estudos farmacolóxicos e electrofisiolóxicos previos acerca da modulación GABAérxica a través dos receptores GABAB nas lampreas. A ampla expresión de ámbolos dous ADNc das subunidades gabab no SNC da lamprea de mar revela a importancia destes receptores na modulación de circuítos cerebrais e espiñais. Os transcritos de gabab están presentes tanto en células GABAérxicas como non GABAérxicas (neuronas descendentes, neuronas espiñais e rombencefálicas, ou células do borde espiñais). Estudos previos mostraron a inervación GABAérxica e a modulación de GABA nas células do borde, e os nosos resultados suxiren que GABA podería actuar nestas células a través dos receptores GABAB. Os nosos resultados de expresión tamén concordan con outros estudos acerca do papel dos receptores de GABA na modulación da rede respiratoria da lamprea, ou a acción moduladora do GABA dentro da ruta dende a columna lateral ás neuronas reticuloespiñais a través dos receptores GABAB. O noso estudo amplia o número de poboacións neuronais que poden ser moduladas a través dunha sinalización mediada polos receptores GABAB, e abre a posibilidade de realizar estudos funcionais acerca do papel do GABA e o receptor GABAB en outros circuítos.

A presenza de ámbalas dúas subunidades gabab nas células GABAérxicas do cerebro e medula espiñal suxire que o receptor GABAB pode tamén modular nas lampreas a actividade das células GABAérxicas. A presenza de ámbalas dúas subunidades gabab nas células periglomerulares dos bulbos olfactivos, a maioría delas son GABAérxicas, ou nas células que contactan co líquido cerebroespiñal da medula espiñal, tamén GABAérxicas na súa maioría, apoia esta hipótese. Con todo, debido aos diferentes requisitos de fixación, fomos incapaces de realizar un dobre marcaxe (hibridación in situ e marcaxe inmunohistoquímico de GABA) para confirmar a presenza dos receptores GABAB en células GABAérxicas da lamprea de mar. A presenza de sinal de in situ positivo nas dendritas das células que contactan co líquido cerebroespiñal da medula espiñal podería indicar un papel dos receptores GABAB na detección de GABA no líquido cerebroespiñal. Os receptores GABAB poderían

modular a estas células actuando como un autoreceptor e/ou como un heteroreceptor con sinapses de outras células GABAérxicas.

Os nosos resultados mostran ausencia de expresión de ámbalas dúas subunidades gabab en ependimocitos ao longo do cerebro da lamprea de mar. No cerebro das lampreas, as células ependimarias son o principal tipo de células da glía, e os astrocitos están só asociados a algúns tractos nerviosos. A medula espiñal das lampreas contén ámbolos dous tipos de células gliais. A diferenza dos vertebrados con mandíbula, as lampreas non presentan oligodendrocitos e os seus astrocitos non expresan inmunoreactividade á proteína ácida fibrilar glial, se non que expresan citoqueratina. Coñécese pouco acerca da expresión de ámbolos dous ARNm das subunidades gabab e/ou das proteínas GABAB1 e GABAB2 na capa ependimaria dos vertebrados, só un estudo farmacolóxico documentou que é o receptor GABAA, e non o GABAB, o que media as respostas GABAérxicas nas células ependimarias dos mamíferos. Aínda que este estudo coincide cos nosos resultados, son necesarios máis estudos en outros grupos de vertebrados para determinar a evolución deste carácter debido á gran distancia evolutiva entre os mamíferos e as lampreas. Un tipo especial de célula ependimaria, as células do órgano subcomisural, non mostraron expresión das subunidades gabab en lamprea, apoiando outros resultados de estudos en diferentes vertebrados. A pesares de que estudos que mostran a presenza de subunidades GABAB en astrocitos de rato e humanos, non se observou expresión de transcritos das subunidades gabab en astrocitos do nervio óptico da lamprea de mar. Igual que o dito con anterioridade, máis estudos noutros grupos de vertebrados son necesarios para determinar se a falta de expresión do receptor GABAB en astrocitos é unha condición ancestral ou un carácter derivado.

EXPRESIÓN DO RECEPTOR GABAB DESPOIS DUNHA LESIÓN MEDULAR COMPLETA NA LAMPREA

Os nosos resultados mostran un descenso estatisticamente significativo na expresión de ámbalas dúas subunidades despois dunha lesión medular completa na lamprea. Esta resposta dos receptores gabab difire daquelas vistas noutros receptores de neurotransmisores. Por exemplo, prodúcese un incremento agudo na expresión do receptor 1a de serotonina, ou non se observa cambios na expresión do receptor d2 de dopamina despois dunha lesión medular completa na lamprea. As lampreas mostran tamén plasticidade a un nivel funcional, como o demostra a excitabilidade incrementada por baixo do lugar da lesión, os cambios no efecto modulador celular e sináptico da serotonina en animais lesionados, ou unha inhibición tónica GABAérxica máis forte cando ocorre boa recuperación da función locomotora despois da lesión. Todos estes estudos resaltan a importancia de estudar cada sistema neurotransmisor nos animais lesionados (por riba e por baixo da lugar da lesión) para entender os mecanismos que levan a unha recuperación funcional nas lampreas. Parece claro que os circuítos da lamprea encargados da locomoción son moi plásticos despois dunha lesión medular, tanto a un nivel anatómico como funcional.

Mostramos que en animais que recuperaron unha aparente locomoción normal hai niveles máis baixos de expresión de ámbolos dous transcritos de gabab. Un expresión diminuída dos receptores GABAB post-sinápticos podería explicar a alta inhibición a través dos receptores GABAA observada nas lampreas lesionadas. Primeiro, isto significaría que máis GABA está dispoñible para activar aos receptores GABAA debido á presenza dun sistema GABAérxico completamente recuperado. Ademais, niveles máis baixos de expresión dos transcritos gabab despois dunha lesión podería incrementar os

niveis de AMPc, posto que a activación dos receptores GABAB inhibe a adenilato ciclasa. Niveles máis elevados AMPc levan a un incremento da actividade da proteína quinasa A, que é un factor clave para manter unha expresión de superficie estable dos receptores GABAA. Entón, baixos niveis dos transcritos gabab podería incrementar o número de receptores GABAA unidos á superficie celular nos animais lesionados. Ademais, o descenso dos receptores GABAB nas células GABAérxicas aumentaría a liberación de GABA, dado que os receptores GABAB presinápticos suprimen a liberación de GABA. Todos estes factores poderían explicar a inhibición aumentada a través dos receptores GABAA nos animais afectados. Sería interesante identificar as subunidades do receptor GABAA na lamprea para levar a cabo estudos futuros acerca de posibles cambios na súa expresión despois dunha lesión medular na lamprea.

Este é o primeiro estudo que demostra a redución na expresión do receptor gabab despois dunha lesión traumática na medula espiñal en vertebrados. Existen estudos previos que documentan cambios na expresión do receptor GABAB despois de lesións espiñais non traumáticas ou lesións do cerebro en ratos e primates non humanos. Observouse un descenso na expresión do receptor GABAB no tálamo de ratas con dano cerebral traumático, ou no cortex e núcleo da columna dorsal de monos esquío entre 1 e 5 anos despois dunha sección dos nervios cubital e mediano. Outros estudos tamén mostraron niveis baixos de receptores GABAB en ratas cunha ligazón do nervio ciático ou cunha neuropatía diabética. Os resultados aquí presentados, xunto con tódolos estudos previos, suxiren que un descenso na expresión dos receptores GABAB é unha característica común despois de lesiones no sistema nervioso en vertebrados.

PAPEL DOS RECEPTORES GABAB NA REXENERACIÓN AXONAL DESPOIS DUNHA LESIÓN MEDULAR COMPLETA

Un estudo previo do noso grupo mostrou que despois dunha lesión medular, o GABA acumúlase extracelularmente ao redor dos axons de neuronas descendentes identificables que mostran unha maior habilidade de supervivencia despois dunha lesión medular, o que suxeriu un papel protector do GABA nestas neuronas. Neste estudo, analizamos primeiro os cambios na expresión da subunidade gabab1 nas neuronas descendentes da lamprea de mar. Os nosos análises revelaron un incremento na expresión da subunidade gabab1 no soma das neuronas descendentes unha semana despois da lesión. Este resultado reforza aínda máis a nosa hipótese de que GABA podería estar xogando un papel neuroprotector e pro-rexenerativo despois dunha lesión medular, así como indicou que está actuando a través de receptores GABAB.

Realizamos experimentos de ganancia e perda de función para confirmar esta hipótese. O experimento de ganancia de función consistiu nun tratamento con GABOB (un análogo do GABA) para potenciar a sinalización GABAérxica. Técnicas de trazado neuronal puxeron de manifesto que GABOB incrementou significativamente o número de neuronas con axons rexenerados. Este resultado mostra que a sinalización GABAérxica promove a rexeneración de neuronas descendentes despois dunha lesión medular. Para determinar se tamén o GABA endóxeno, actuando a través de receptores GABAB, xoga un papel pro-rexenerativo realizáronse tratamentos de perda de función con morfolinos de gabab1. Primeiro, usando hibridación in situ, confirmamos que o morfolino gabab1 activo é capaz de reducir a expresión da subunidade gabab1. Despois, realizamos tratamentos a longo prazo con morfolinos de gabab1. 10 semanas despois da lesión, os animais tratados co morfolino gabab1 activo mostraron un descenso significativo na rexeneración axonal das neuronas descendentes despois dunha lesión

medular completa, comparado con animais controis tratados cun morfolino control. Este resultado demostra que o GABA endóxeno actúa como un factor pro-rexenerativo activando receptores GABAB expresados nas neuronas descendentes despois dunha lesión medular. Os nosos resultados concordan con estudos previos in vitro e do desenrolo que mostran que a sinalización GABAérxica a través dos receptores GABAB promove crecemento axonal e de neuritas. Pero, ata onde podemos saber, os nosos resultados son a primeira demostración in vivo que mostra que o GABA promove a rexeneración axonal, activando receptores GABAB, despois dun dano no SNC.

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GENERAL INTRODUCTION

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4 GENERAL INTRODUCTION

4.1 SPINAL CORD INJURY (SCI)

In mammals, including humans, SCI leads to disability and an irreversible loss of sensory and motor function below the lesion site. In Spain, more than 30,000 people are affected by SCI and around 1,000 people suffer a traumatic SCI every year ("Ánalisis sobre la lesión medular en España", Aspaym, 2009). In developed countries, like Spain, SCI cases are increasing due to accidental falls in an aging population. SCI is not only a major health and social problem for our society, but also an economical one. For example, in the USA the estimated lifetime cost of a patient with SCI is between 1.1 and 4.6 million US dollars depending mainly on the age of patient and the severity of the injury (Facts at glance, Center NSCIS, 2016).

There are two types of SCI: traumatic, which is caused by mechanical forces, and non-traumatic (e.g. caused by tumours, ischemia or infections). Traumatic SCI starts with a first damage or primary injury caused by the mechanical lesion itself, and continues with secondary damage that evolves over time (Ahuja et al., 2017). The primary injury results in the damage of the blood-brain barrier and local blood vessels, axons are axotomized and neuronal cell-membranes broken due to traction and compression forces (Fig. 1). Then, extracellular release of neurotransmitters, like glutamate, and free radicals, as well as an ischemic process trigger the secondary injury cascade (for revision, see McDonald and Sadowsky, 2002). This second phase consists in a series of molecular changes that are activated after the primary injury and spread the initial damage, leading to inflammation, alterations in homeostasis, degeneration of neurofilaments, higher cell stress and apoptosis/necrosis (Yip and Malaspina, 2012) (Fig. 1). The most destructive process during the secondary injury is excitotoxicity, where glutamate is its key player. Glutamate is released from injured neurons, including their axons, and astrocytes, and causes overexcitement of neighbouring healthy cells (neurons, astrocytes and oligodendrocytes) until they die (McDonald and Sadowsky, 2002). In addition to massive cell death, reactive astrocytes up-regulate chondroitin sulfate proteoglycans (CSPGs) and form a glial scar rostral and caudal to the lesion site (Davies et al., 1999), which acts as a physical barrier for axonal regeneration (Fig. 1).

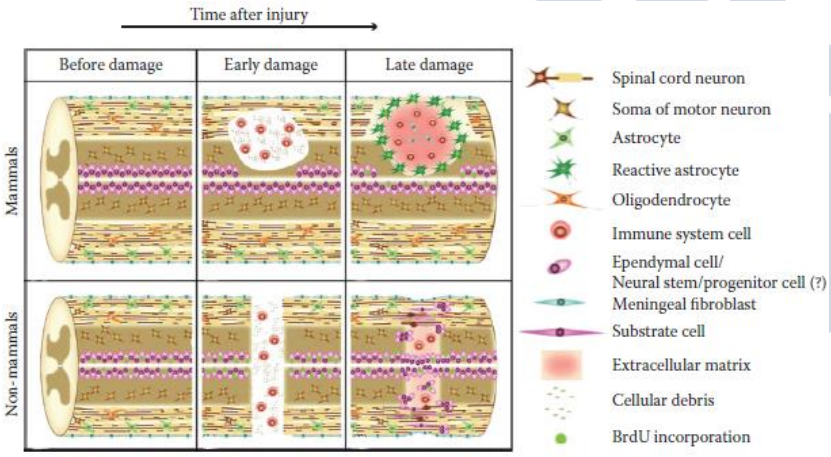

Figure 1. Damage progression after SCI in mammals versus non-mammals (Lee-Liu et al., 2013). With permission of John Wiley and Sons.

One of the major reasons for the failure of recovery following SCI is caused by the inability of axotomized neurons to regenerate their axons and reinnervate spinal cord regions below or above the site of injury. It is known that extrinsic and intrinsic factors are responsible for the failure of proper axonal regeneration after a traumatic SCI in adult mammals, including humans (He and Jin, 2016). Extrinsic factors include molecules that are present in the scar tissue which is formed after the injury, or in the myelin surrounding the damaged axon. These molecules are inhibiting axonal regrowth (Hagg and Oudega, 2006). Manipulations that eliminate or neutralize these factors result in enhanced regrowth of some types of axons. However, in most cases, only a small number of injured central nervous system (CNS) axons can regenerate, consistent with the idea that the lack of regeneration in the adult CNS of mammals is an intrinsic property of the injured neurons (He and Jin, 2016). It is generally accepted that several types of CNS neurons die or degenerate after axotomy. In fact, many studies in mammals have reported the death of at least some brain descending neurons after SCI (opossums: Fry et al., 2003; rats: Hains et al., 2003; Lee et al., 2004; Wu et al., 2003; humans: Holmes and May, 1909). The prevention of neuronal death is as a prerequisite for the occurrence of axonal regeneration in descending neurons after SCI.

Plasticity is defined as "the capacity for continuous alteration of the neural pathways and synapses of the living brain and nervous system in response to experience or injury" (http://medical.merriam-webster.com/). Although in mammals there is no robust functional regeneration and recovery after SCI, neural spontaneous plasticity actually occurs in surviving cells, which can help to restore a minimal degree of nervous function after SCI (Weidner et al., 2001; Bareyre et al., 2004; Ballermann et al., 2006; Rosenzweig et al., 2010). This reorganization is present in both rostral and caudal circuitry of the injured spinal cord as well as in the site of lesion and in supraspinal structures, and it affects in a behavioural, physiological, neuroanatomical, cellular and molecular way (Edgerton et al., 2004; Lynskey et al., 2008). For example, after a midthoracic hemisection in rats, a new intraspinal circuit forms between corticospinal tract collaterals and long propriospinal neurons situated in the cervical gray matter, creating a new intraspinal circuit relaying cortical inputs to their original spinal targets (Bareyre et al., 2004). After contusive SCI, there is cellular proliferation around the injury, replacing some of the lost oligodendrocytes and astrocytes and promoting remyelination of axons (Beattie et al., 1997; Zai et al., 2005). Cortical maps also suffer plasticity after SCI as it has been observed in both humans and rodents (Bareyre et al., 2004). However, spontaneous plasticity in mammals is limited and it never provides a complete recovery.

Some of the major challenges in the SCI field are to determine the cellular and molecular mechanisms that determine neuronal intrinsic regenerative ability, and to improve the little plasticity observed after injury. Lampreys recover spontaneously following a complete spinal cord injury (see below). This provides an interesting model to study regeneration and plasticity after SCI, which will help to understand the events leading to successful recovery.

4.2 THE SEA LAMPREY

Lampreys are cyclostomes or agnathans (jawless fish) belonging to the most ancient group of vertebrates (Hubbs and Potter, 1971). The fossil records have proven that lampreys have been existing since 460 million years ago, with no major changes in their evolution since 360 million years ago. This gives lampreys a key position in vertebrate evolution (Xu et al., 2016). Sea lampreys, *Petromyzon marinus* L., have a complex life cycle that starts with the spawning act and the adhesion of fertilized eggs to the river sediment (Hardisty and Potter, 1971) (Fig. 2). After several days of embryonic period, there is a hatching process that leads to a prolarval stage during which prolarvae use their yolk to feed and grow up until they enter the filter-feeding larval stage (Piavis, 1971). The larval stage is the longest period in the lamprey life cycle, with a duration of no less than 5 years and that can last up to 8 years (Beamish and Potter, 1975). Then, larval lampreys suffer a metamorphosis that implies a change from blind and filter-feeding larvae that live burrowed in the river sediment to active external parasite adult animals with a fully developed visual system. After metamorphosis, young adult lampreys migrate downstream to the sea where they grow feeding as parasites of teleost fishes. After 2 to 3 years, adult lampreys return to the river during the breeding season to build nest and die after spawning.

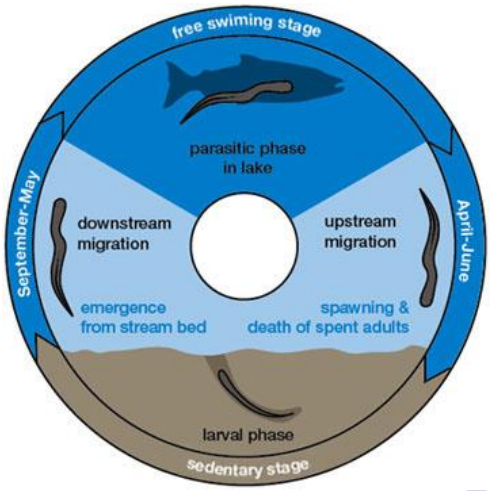

Figure 2. Life cycle of lamprey *Petromyzon marinus* L. Taken from http://www.seagrant.umn.edu/ais/sealamprey_battle

4.3 LAMPREYS AS AN ANIMAL MODEL IN NEUROBIOLOGY AND NEUROREGENERATION

The key phylogenetic position of lampreys, in the base of vertebrate evolution, makes them an interesting model to study the origin and evolution of the nervous system of current vertebrates in evolutionary and developmental studies (evo-devo) (Kuratani et al., 2002; Murakami and Watanabe, 2009; Xu et al., 2016). Lampreys possess the main structures of the typical vertebrate brain (Nieuwenhuys and Nicholson, 1998). In addition, the lamprey brainstem contains accessible giant neurons that have facilitated the application of electrophysiological methods (Rovainen, 1974; Dubuc et al., 1993). Many other experimental techniques have been also applied in lampreys like all types of histological methods, sequence analysis, studies of gene expression, pharmacological treatments or the use of genetic tools like morpholinos or CRISPR/Cas9 (Osório and Retáux, 2008; Square et al., 2015; Fogerson et al., 2016; Xu et al., 2016; Zu et al., 2016). Moreover, the recent sequencing of the sea lamprey genome has revealed that it contains approximately 25,000 genes and that most of them have mammalian orthologues, so now evolutionary studies, or other types of studies, are also being conducted from a genomic point of view (Smith et al., 2013). Recently, lampreys have been proposed as a model to study nervous system evolution from a regenerative point of view (evo-rego) (Barreiro-Iglesias, 2012). Also, lampreys have been used as a model to study the cytotoxic effects of proteins related to Alzheimer's and Parkinson's disease on individual neurons *in vivo* (Hall, 1999; Hall et al., 2000; Honson et al., 2009; Busch and Morgan, 2012; Busch et al., 2014).

The lamprey CNS has a similar organization compared to that of jawed vertebrates, including mammals (Suryanarayana et al., 2017). But on the other side, the simplicity of

its CNS (e.g. it contains fewer neurons) compared to mammals has been an advantage for studying the specific synaptic connectivity and the intrinsic properties of neurons controlling locomotion (Dubuc et al., 2008). Moreover, the whole CNS can be removed and maintained in physiological conditions for many hours, or even days, to perform electrophysiological experiments. Because of that, the lamprey model has been used to characterize the different circuits implicated in the control of locomotion. It is known that locomotion in lampreys can be triggered from external sensory cues or internal cues (Rossignol et al., 2006). In both cases, diencephalic and mesencephalic regions (DLR and MLR, respectively) as well as the pretectum (Capantini et al., 2017), excite reticulospinal cells situated in the brainstem. Then, reticulospinal neurons elicit locomotion by activating the central pattern regenerators (CPGs) in the spinal cord. Finally, the balance between excitatory inputs from glutamatergic spinal interneurons and inhibitory inputs from glycinergic interneurons, allows the alternative pattern for swimming (Rovainen, 1979; Dubuc and Grillner, 1989; Buchanan, 1999; Grillner, 2003; Grillner et al., 2007; for a revision see Dubuc et al., 2008; see also Grillner et al., 2008).

In contrast to mammals, lampreys recover normal appearing locomotion spontaneously after a complete SCI (Rovainen, 1976; Selzer, 1978, for a revision see Rodicio and Barreiro-Iglesias, 2012). In 1980, the National Institute of Health of the United States (NIH), proposed five criteria that should be achieved to demonstrate functional regeneration in the spinal cord (Guth et al., 1980). Lampreys meet all these five criteria during spontaneous recovery following SCI, which are: (1) The experimental lesion must cause disconnection of nerve processes, (2) Processes of CNS neurons must bridge the level of injury, (3) The regenerated fibres must make junctional contacts, (4) The regenerated fibres must generate post-junctional responses and (5) Changes in function must derive from regenerated connections (for a revision see Shifman et al., 2007). This amazing recovery process involves the regeneration of descending axons (Jacobs et al., 1997), the formation of synaptic connections between the regenerated axons and neurons caudal to the lesion (Rovainen, 1976; Selzer, 1978), and the generation of new neurons after the injury (Zhang et al., 2014). Descending neurons of lampreys have a complex cytoarchitecture, with 36 identifiable giant reticulospinal neurons (Jacobs et al., 1997). These reticulospinal neurons vary greatly in their regenerative abilities (Jacobs et al., 1997), even when their axons run in a spinal cord environment that is permissive for axonal growth. Some identifiable descending neurons are considered “good regenerators” (i.e they regenerate their axon more than 55% of the times) and others “bad regenerators” (i.e. they regenerate their axon less than 30% of the times) (Fig. 3). Thus, in lampreys, there is an opportunity to study both

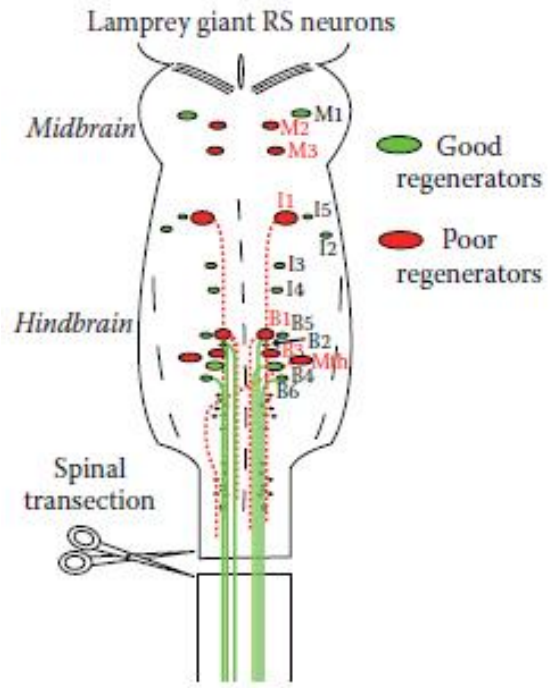

Figure 3. Variable regeneration capacities among identifiable reticulospinal neurons (Fogerson et al., 2016). With permission of Elsevier.

enhancement and inhibition of regeneration in the same preparation. An additional advantage of the lamprey model of SCI is that the identifiable descending neurons and their descending axons can be visualised *in vivo* and in CNS whole-mounts due to the transparency of the lamprey brain.

Recently, it was reported that a complete SCI induces the delayed death of lamprey descending neurons that, at earlier times post-injury, had been identified as bad regenerators (Shifman et al., 2008). Evidence for cell death included the disappearance of Nissl staining, the loss of neurofilament expression, the absence of labelling when using retrograde tracers (Shifman et al., 2008), and the earlier staining of these neurons with Fluoro-Jade C (Busch and Morgan, 2012), a marker for degenerating neurons. The appearance of TUNEL staining (Shifman et al., 2008; Hu et al., 2013) and activated caspases (Barreiro-Iglesias and Shifman, 2012, 2015; Hu et al., 2013; Barreiro-Iglesias et al., 2017) in the axotomized perikarya suggested that the death of bad regenerating neurons in lampreys is apoptotic. Recent reports (Barreiro-Iglesias and Shifman, 2015; Barreiro-Iglesias et al., 2017) have shown that caspase activation in the cell perykarya of bad regenerating descending neurons of lampreys is preceded by the initial activation of caspase-8 in the axotomized axons at the lesion site followed by its later appearance at the cell soma within the first hours after the injury. This suggests that the degenerative process is initiated at spinal levels. Transport of injury signals by microtubules appears to play a key role in the activation of caspase-8 at the cell body of axotomized neurons (Barreiro-Iglesias et al., 2017). These data indicate that the identifiable descending neurons known to be "bad regenerators" suffer a process of slow and delayed degeneration after SCI and, therefore, are also "poor survivors". Thus, lampreys are a convenient vertebrate model for the *in vivo* study of the mechanisms underlying the death/survival of spinal-projecting neurons after SCI and of how this relates to the activation of axonal regeneration.

Lampreys show an amazing anatomical and functional plasticity after a complete SCI (for a revision see Parker, 2017), although different responses exist in different neurotransmitter systems. The intraspinal dopaminergic and GABAergic systems show an anatomical full recovery after a complete SCI in lampreys (Fernández-López et al., 2015; Romaus-Sanjurjo et al., 2018). However, while the dopaminergic system is not fully restored until approximately 4 wpl, the GABAergic system is completely recovered at 1wpl. On the other hand, the intraspinal glutamatergic (Fernández-López et al., 2016) and serotonergic (Cohen et al., 2005) systems show a good but incomplete restoration of their anatomical organization even in the presence of functional recovery. Regarding changes in the expression of neurotransmitter receptors, different responses have been also observed for different receptors following a complete SCI in the sea lamprey. For example, there is an acute increase in the expression of the serotonin 1a receptor (Cornide-Petronio et al., 2014) and there are no changes in the expression of the dopamine d2 receptor (Fernández-López et al., 2015). Plasticity is also seen at the functional level in lampreys. For example, there is a significant decrease in the number of vesicle clusters and active zones in giant reticulospinal synapses distal to the lesion after SCI (Oliphint et al., 2010). Excitability is increased below the site of lesion in lampreys recovered from a complete SCI (Cooke and Parker, 2009; Hoffman and Parker, 2011) and the cellular and synaptic modulatory effect of serotonin differs in lesioned and un-lesioned animals (Becker and Parker, 2015). Moreover transected lampreys show a stronger tonic GABAergic inhibition, during the recovery of locomotor function following SCI (Svensson et al., 2013). Interestingly, this stronger GABAergic inhibition is only modulated by somatostatin after the injury (Svensson et

al., 2013). These studies show that the circuits underlying locomotion in lampreys are highly plastic after SCI, both at the anatomical and functional level. In addition, they stress the importance of studying each neurotransmitter system in lesioned animals (below and above the site of injury) to understand the mechanisms that lead to functional recovery in lampreys.

4.4 STRUCTURE OF THE LAMPREY BRAIN

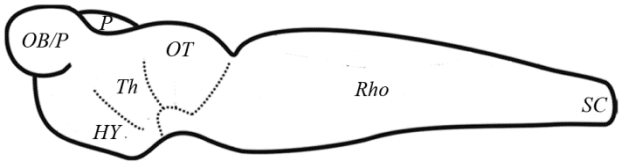

Figure 4. Schematic drawing of a lateral view of the lamprey brain.

4.4.1 Prosencephalon

The prosencephalon of the lamprey is divided in secondary prosencephalon and diencephalon.

4.4.1.1 Secondary prosencephalon

The secondary prosencephalon is composed by telencephalon and hypothalamus. The telencephalon is an alar derivative, while the hypothalamus contains both alar and basal derivatives (Martínez de la Torre et al., 2011).

4.4.1.1.1 Telencephalon

The telencephalon of lampreys comprises three parts: the olfactory bulbs, the pallium, and the subpallium.

In the olfactory bulbs, the fibres of the olfactory nerve surround the entire surface of the bulb forming the layer of fibres of the olfactory nerve. Their end branches, together with dendrites of the mitral cells, contribute to the second layer of the bulb, the glomeruli. The mitral cells lie between and directly central to the glomeruli and their axons course in the olfactory tracts. The deeper zone of the olfactory bulb is occupied by granule cells, which have two to four long dendrites that diverge into the glomerular layer (Nieuwenhuys and Nicholson, 1998). Olfaction is an important primary sensory modality in lampreys.

The pallium comprises three principal parts, the lateral pallium, the subhippocampal lobe and the medial pallium (Northcutt and Puzdrowski, 1988; Northcutt and Wicht, 1997). The lateral pallium comprises two zones: an inner zone consisting of diffusely arranged cells and an outer zone composed by smaller and larger cell clusters. The subhippocampal lobe is a strip-like zone that separates the medial pallium from the evaginated part of the pallium and striatum. Its neurons are arranged in a wide and rather diffuse zone of central grey. The medial pallium has a narrow, compact layer of periventricular grey in which the majority of its neurons are situated, but some have migrated outward to the lateral part, which is formed mainly by fibres (Nieuwenhuys and Nicholson, 1998).

In the subpallium three areas can be distinguished: the corpus striatum, the septum and the preoptic nucleus, (Heier 1948; Schober 1964). The striatal grey is arranged in some layers of closely packed cells, separated from the ventricular surface by a wide

stratum periventricular. The septum is represented by a narrow zone of closely packed cells around the rostral part of the unpaired ventricle. The preoptic nucleus, consists of some compact laminae of cells, which are situated at some distance from the ventricular surface (Nieuwenhuys and Nicholson, 1998). Some of the cells of the preoptic nucleus are secretory (Goossens et al., 1977).

In general, pallium and subpallium are involved in the control of eye movements, locomotion, posture and other patterns of behaviour (Grillner et al., 2005; for revision see Grillner and Robertson, 2016).

4.4.1.1.2 Hypothalamus

It surrounds the ventral part of the third ventricle which widens ventrally into the infundibular recess. It extends rostrally forming the anterior recess and caudally forming the posterior recess. There are 3 differentiated regions in the hypothalamus: postoptic, tuberal and mammilar. The postoptic region contains the paraventricular nucleus, the paracommissural preoptic nucleus and the postoptic commissure nucleus. The tuberal region is composed of the dorsal (caudal) and ventral (rostral) hypothalamic nuclei. The mammillary region surrounds the posterior recess (Nieuwenhuys and Nicholson, 1998).

Throughout the hypothalamus, small neurons are concentrated in a compact layer of periventricular grey. Many of these neurons have a central process that extends between the ependymal cells toward the ventricular surface (Nieuwenhuys and Nicholson, 1998). It has been proposed that the alar plate of the hypothalamus acts as a processing centre which receives olfactory impulses from the cerebral peduncle and projects to rhombencephalic visceral centres (Heier, 1948; Puelles and Rubenstein, 2015), and the basal plate is related to hypophysis and hormone delivery (Puelles and Rubenstein, 2015).

4.4.1.2 Diencephalon

The diencephalon is composed of an alar plate, subdivided in prethalamus, epithalamus, thalamus, subcommissural organ and pretectum; and basal plate or diencephalic tegmentum.

Prethalamus: The prethalamus is a narrow strip of central grey. Most of the neurons in the prethalamus are larger than those in the other diencephalic regions. The prethalamus is a locomotor coordinating centre that receives projections from telencephalon and sensory regions and projects to lower coordinating centres, to motor centres and to the hypothalamus (Heier, 1948).

Epithalamus: The epithalamus is composed of the pineal complex and the habenula. The pineal complex in lampreys is a photosensory organ (Pu and Dowling, 1981). It is formed by a pineal organ and a parapineal organ (Hardisty 1979). The pineal retina comprises an apical layer made up of photoreceptor cells of different types and supporting cells, and a basal layer composed of pineal ganglion cells. The parapineal organ shows a similar overall structure. The habenula are prominent and asymmetric structures, where the right habenula is bigger than left habenula. In larvae, the habenula receives projections from the subhippocampal lobe, the rostral thalamus and the parapineal organ (Yañez and Anadón, 1994; Yañez et al., 1999); and in adults also from sensory regions and the striatum (Stephenson-Jones et al., 2012). It acts as an integrative structure, receiving olfactory, visual and general sensory information and projecting to the interpeduncular nucleus and the trigeminal motor nucleus through the fasciculus retroflexus (Yañez and Anadón, 1994).

Thalamus: The thalamus contains a wide, compact plate of central grey and, external to that, a zone of diffusely arranged cells. The thalamus is relatively well developed in the lamprey and its periventricular grey (Schober, 1964), as well as its zone of migrated cells (Heier, 1948), have been subdivided into several moieties. The thalamus is also a station to relay sensorial information towards the telencephalon (Butler and Hodos, 2005).

Subcommissural organ: The subcommissural organ contains ependymal and subependymal cells that secrete the Reissner fibre (Rodríguez et al., 1992; Barreiro-Iglesias et al., 2009).

Pretectum: Two different regions can be distinguished, a periventricular region with high density of cells; and a lateral region where there is a neuropil, fibre tracts and some disperse neurons (Cornide-Petronio et al., 2011). Its main function is to process information from the retina.

Diencephalic tegmentum: The diencephalic ventral/basal region is small. It comprises the posterior tuberculum nucleus, whose cells are dopaminergic, the posterior tuberculum commissure and the pretectal tegmentum. The pretectal tegmentum contains the first pair of Müller cells, and the medial longitudinal fascicle nucleus (nMLF) (Pombal et al., 2009).

4.4.2 Mesencephalon

The mesencephalon of lampreys is divisible into two principal regions, an alar plate, comprising the optic tectum and the torus semicircularis, and a basal plate, which is termed the mesencephalic tegmentum.

4.4.2.1 The optic tectum

The optic tectum is a laminated structure that occupies the dorsal part of the midbrain and is the main visual centre in lampreys. The structure of the optic tectum has been extensively described by Johnston (1902), Ariens Kappers et al. (1936) and Heier (1948). Its layers are, from external to internal:

- *Stratum fibrosum et griseum superficial*, containing fine optic tract fibres and terminals, and some neurons.
- *Stratum opticum*, through which optic fibres pass. It is in contact with the scattered intrinsic neurons.
- *Stratum "album" et griseum central*, a layer that also receives afferents from other sources and whose constituent neurons contribute to the efferent tracts of the tectum.
- *Stratum griseum periventricular*, consisting of densely packed cells, which are arranged in several sublayers and send their dendrites towards the surface.
- *Stratum "album" periventricular*, made up by afferent fibres from diencephalic areas as well as from the torus semicircularis. It also contains efferent fibres to the torus semicircularis.

4.4.2.2 Torus semicircularis

The torus semicircularis consists of a zone of grey that is a direct ventral continuation of the stratum griseum periventricular of the optic tectum. In basis to their connections, it has been proposed to be a multisensory center that integrates head cutaneous sensitivity with both mechano- and electrosensory information from the octavolateral area and visual information (González et al., 1999).

4.4.2.3 The tegmentum mesencephalic

The tegmentum mesencephalic contains the reticular mesencephalic nucleus, the oculomotor nucleus and the rostral part of the interpeduncular nucleus. The mesencephalic reticular nucleus (MLR) contains about 90 reticulospinal cells, including the third pair of Müller neurons, which project to the ipsilateral spinal cord (Swain et al., 1993; Dubuc et al., 2008; for revision see Brodin et al., 1988). The M5 region is also present (Schober, 1964) as well as cells that project to the retina. The oculomotor neurons constitute the most rostral somatic efferent centre of the brain.

4.4.3 Rhombencephalon

The rhombencephalon is well developed and includes a bilaterally, vertically oriented alar plate and a horizontal basal plate. The alar plate comprises 3 sensorial regions: viscerosensory zone, general somatosensory system and special somatosensory zone. Likewise, the rhombencephalic basal plate is clearly divisible into a medial or somatomotor zone and a lateral or visceromotor zone. It also contains the caudal part of the interpeduncular nucleus.

4.4.3.1 The viscerosensory zone

The viscerosensory zone receives sensory fibres from facial (VII), glossopharyngeal (IX) and vagus (X) nerves. It is considered as solitary tract nucleus (Pombal et al., 2001, 2006; Barreiro-Iglesias et al., 2010; Villar-Cerviño et al., 2013).

4.4.3.2 The general somatosensory system

The general somatosensory system can be divided into dorsal column nucleus, trigeminal tractus descendens nucleus and the primary nucleus of the trigeminus, which is composed of the dorsal rhombencephalic cells (Anadón et al., 1989).

4.4.3.3 The special somatosensory zone

The special somatosensory zone occupies almost the entire rostral part of the alar plate. This area contains three longitudinally arranged cell masses which are the dorsal, medial and ventral nuclei of the octavo-lateral area. The ventral nucleus can also be divided into three nuclei: anterior, intermediate and posterior octavomotor nuclei.

4.4.3.4 The somatomotor zone

The somatomotor zone contains the somatomotor and reticular nuclei. The somatomotor nuclei include the trochlear nucleus, the abducens nucleus, and the rostral part of the spinal column, which occupies the caudomedial part of the basal plate.

There is some controversy in the establishment of the reticular rhombencephalic divisions. Some authors describe 4 different regions: isthmic reticular formation (IsRF), trigeminal reticular formation (TRF), medial reticular rhombencephalic nucleus (MRRN), and posterior reticular rhombencephalic nucleus (PRRN) (Koyama et al., 1989; Ronan and Northcutt, 1990; Pombal et al., 1997; Villar-Cerviño et al., 2008a). On the other hand, other authors claim that there are 3 reticular nuclei: anterior rhombencephalic reticular nuclei (ARRN) which comprises the IsRF and the TRF, the middle and the posterior rhombencephalic reticular nuclei (MRRN and PRRN, respectively) (Nieuwenhuys, 1977; Ronan, 1989; Davis and McClellan, 1994). The IsRF contains Müller cells (I1-I6), while the TRF contains a few reticulospinal cells. The MRRN has about 330 RS cells, including the Mauthner cells and Müller cells B1-B6 (Dubuc et al., 2008). Finally, the PRRN contains a large number of RS neurons, approximately 730 neurons (Dubuc et al., 2008).

4.4.3.5 The visceromotor zone

The visceromotor zone extends from the isthmic region caudally to the level of the obex, where it passes gradually into the spinal grey matter. The visceromotor zone constitutes a series of five distinct cell masses, the efferent nuclei of trigeminus (V), facial (VII), glossopharyngeus (IX), and the rostral and caudal nucleus of vagus (X). The motor nucleus of V is extraordinarily large.

4.4.3.6 The caudal part of interpeduncular nucleus (IP)

The caudal part of IP is located in the isthmic medioventral region and receives afferents from the habenula (Bianco and Wilson, 2009).

4.5 THE SPINAL CORD OF LAMPREYS

Although the spinal cord of lampreys exhibits primitive features as it lacks myelin (Bullock et al., 1984), and the blood vessels do not enter in its parenchyma, the organization of the lamprey spinal cord follows the basic vertebrate plan and is well suited for histological and electrophysiological analyses because it is flat and transparent. The grey matter has a lateral distribution. There is an embryonic origin shared between the medial region and the dorsal horn of jawed vertebrates, as well as between the lateral region and the ventral horn (Meléndez-Ferro et al., 2003). The white matter surrounds the grey matter. Most of the spinal neurons are situated in the grey matter, with some dispersed neurons in the white matter (Nieuwenhuys and Nicholson, 1998).

Several classes of neurons in the spinal cord of lampreys can be recognized on the basis of morphological and physiological features such as axonal projection, soma size/location, and other visual cues (Rovainen, 1974). The motor neurons constitute a laterally situated column on either side of the cord. Most of them have a medium size (for revision see Nieuwenhuys and Nicholson, 1998). Dorsal cells are placed in the dorsomedial part of the spinal grey, forming two distinct longitudinal rows on either side of the midline. They are first-order sensory neurons with a process leaving the spinal cord via the dorsal roots and ending as mechanoreceptor in the skin (Rovainen, 1967; Martin and Wickelgren, 1971). The spinal cord of lampreys has numerous intrinsic neurons whose dendrites and axons do not leave the CNS. The most important types of intrinsic neurons are: giant interneurons, lateral interneurons, edge cells, excitatory interneurons (EINs), crossed caudal interneurons (CCINs), small ipsilateral inhibitory interneurons (IINs) and cerebrospinal fluid-contacting cells (CSF-c) (Rovainen, 1967; Tang and Selzer, 1979; Buchanan, 1982; Buchanan and Grillner, 1987; Buchanan, 1999; Einum and Buchanan, 2006). Briefly, giant interneurons are located in the caudal half to two thirds of the cord and act as secondary mechanosensory cells. Lateral interneurons have a lateral position in the spinal grey, with a large lateral dendrite reaching the edge of the spinal cord. They are inhibitory neurons contacting with CCINs, promoting wavy postures via relaxation of the ipsilateral side of the cord. The edge cells are situated outside the grey matter, in the lateral fascicle. They are capable of sensing the lateral bending of the cord during locomotion, acting as

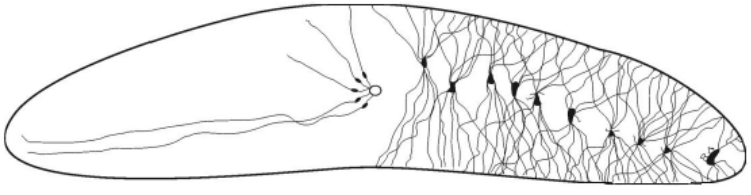

Figure 5. Schematic drawing of transverse section of the lamprey spinal cord showing the distribution of glial cells.

stretch receptor neurons. EINs, CCINs and IINs are interneurons involved in the lamprey network underlying locomotion. CSF-c cells are small cells surrounding the central canal, and projecting to the lateral marginal plexus, making contacts with the edge cells (Christenson et al., 1991). They also possess a dendritic process that protrudes into the central canal. Spinal cord interneurons can also be classified in different types according to their neurotransmitter content (Harris-Warrick et al., 1985; Brodin et al., 1990; Schotland et al., 1996¸ Villar-Cerviño et al., 2008b; Mahmood et al., 2009; Fernández-López et al., 2012)

Retzius (1893) studied the glial cells in the spinal cord of lampreys (Fig. 5). Two types of glial cells are present, astrocyte-like and ependymoglia. While astrocyte-like cells have their somas in the narrow strip of spinal grey matter, the ependymoglia is situated around the central canal (Fig. 5). In contrast to astrocyte-like cells, which extend large processes radially, the ependymoglia have a short central process and a long peripheral process (Fig. 5).

The white matter of the lamprey spinal cord contains fibres of diverse size, and it can be subdivided into dorsal, ventral and lateral fascicles. The dorsal root is situated between the dorsal and lateral fascicles, while the ventral root is situated between the ventral and lateral fascicles. In lampreys, there are two descending systems from the brain to the spinal cord, the reticulospinal and the vestibulospinal (VS). Moreover, there also are two ascending systems, the dorsal column (DC) and the spinal lemniscus. In the DC is where the axons from primary afferents and from dorsal cells are located (Dubuc et al., 1993). Spinal lemniscus fibres convey tactile, pain, temperature stimuli to the brain and information about the motor pattern to the brainstem (Nieuwenhuys and Nicholson, 1998).

4.6 THE GABAERGIC SYSTEM OF THE SEA LAMPREY

γ-aminobutyric acid (GABA) (Fig. 6) is the main inhibitory transmitter in the central nervous system of vertebrates. The GABAergic system appears early during the development of the CNS in lampreys and is composed by neurons and their fibres (Meléndez-Ferro et al., 2002, 2003). In general, GABA-immunoreactive (-ir) neurons are small and medium size cells that are widely distributed along the lamprey brain: different types in the olfactory bulbs, pallium, in two populations of the striatum, septum, preoptic nucleus, hypothalamus, prethalamus, pineal complex, thalamus, nucleus of the posterior commissure, nucleus of the medial longitudinal fascicle, optic tectum, torus semicircularis, M5 region, MLR, internuclear part of the oculomotor nucleus, octavolateral area, somatomotor nuclei and rhombencephalic reticular nuclei (Pombal et al., 1999; Meléndez-Ferro et al., 2000, 2001; Rodicio et al., 2005; Robertson et al., 2007; Valle-Maroto et al., 2011). Throughout the lamprey brain, GABA-ir neurons are implicated in many functions. For example, some brain GABA-ir neurons are main players in the selection of motor programs for locomotion (Grillner et al., 2005). Likewise, they also have important roles in neuroendocrine signalling (Robertson et al., 2007), the visual system (Meléndez-Ferro et al., 2002; Robertson et al., 2007) or in steering and posture modulation (Robertson et al., 2007; Grillner et al., 2008). The spinal cord of lampreys contains an intrinsic GABAergic component with 3 different types of GABA-ir cells in the grey matter: dorsal, lateral

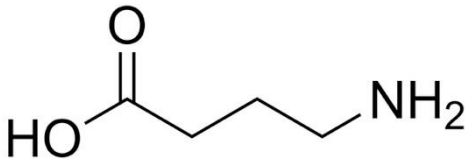

Figure 6. Chemical structure of GABA. Taken from https://commons.wikimedia.org/wiki/File:GABA.png

and CSF-c neurons (Batueva et al., 1990; Brodin et al., 1990; Meléndez-Ferro et al., 2003; Ruiz et al., 2004; Rodicio et al., 2008; Fernández-López et al., 2014; Jalalvand et al., 2014; Villar-Cerviño et al., 2014), and an extrinsic component formed by the axons of GABAergic reticulospinal cells of the anterior and posterior rhombencephalic nuclei (Valle-Maroto et al., 2011). Dorsal and lateral GABA-ir interneurons as well as GABA-ir reticulospinal neurons have modulatory roles in the spinal CPGs during locomotion (Schmitt et al., 2004). Recently, it has been discovered that spinal CSF-c cells act both as mechanoreceptors and chemoreceptors, detecting movements and pH-changes in the cerebrospinal fluid and responding to them (Jalalvand et al., 2016).

4.7 GABA AND GABAB RECEPTORS AND THEIR POSSIBLE ROLE IN RECOVERY AFTER SCI

4.7.1 GABA and GABAB receptors

GABA acts via ionotropic (GABAA) and metabotropic (GABAB) receptors (Pinard et al., 2010; Chalifoux and Carter, 2011). Unlike GABAA receptors that form ion channels, GABAB receptors address second messenger systems through the binding and activation of guanine nucleotide-binding proteins [G-protein-coupled receptors (GPCRs)], producing a slower and more prolonged inhibition than ionotropic GABAA receptors, which results in reduced neuronal excitability (Blein et al., 2000; Bettler et al., 2004; Pinard et al., 2010). GABAB receptors have been identified on both pre- and postsynaptic terminals (Curtis et al., 1968, 1977; Alford and Grillner, 1991). Presynaptic GABAB receptors can exist as either auto- (those that control GABA release) or heteroreceptors (those activated by other neurons) to modulate neurotransmitter release (Bettler et al., 2004). Depending on whether GABAB receptors are activated on excitatory or inhibitory terminals, their effects on the postsynaptic neuron are inhibitory or dis-inhibitory, respectively. Currently, it is accepted that functional GABAB receptors are obligate heterodimers (Blein et al., 2000; Kammerer et al., 1999; Kuner et al., 1999; Geng et al., 2013; Villemure et al., 2005) composed of two receptor subunits, GABAB1 (Kaupmann et al., 1997; Padgett et al., 2010) and GABAB2 (Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999) (Fig. 7). The GABAB1 receptor subunit is responsible for agonist binding (Gálvez et al., 2001), whereas the GABAB2 receptor subunit is essential for trafficking of the heterodimer to the cell surface and for signal transduction following agonist activation (Calver et al., 2002) through the activation of G-proteins (Villemure et al., 2005) (Fig. 7). Interestingly, a study of Maurel et al., (2008) indicates a possible formation of GABAB oligomers, which show decreased G-protein coupling efficiency. Thus, formation of GABAB oligomers may regulate receptor efficacy, making their formation potentially critical to cellular function.

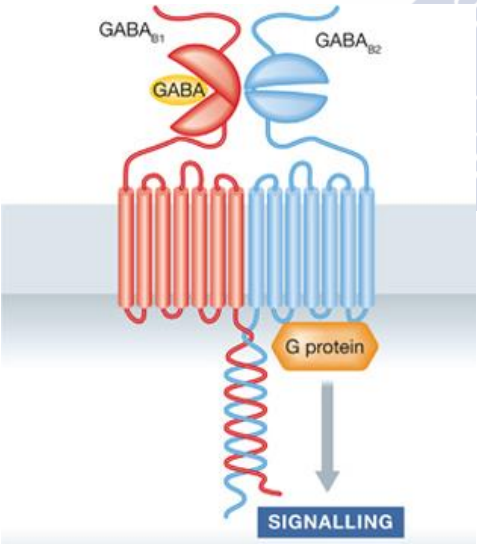

Figure 7. Structure of GABAB receptors. Modified from Benke and Zeilhofer (2012). With permission of John Wiley and Sons.

The presence of GABAB receptors in a single-cell species of *Paramecium* has been demonstrated by immunohistochemical methods (Ramoino et al., 2006), which indicates that the GABAB receptor appeared early during evolution. Only a few studies

have shown the presence and/or expression of GABAB receptors in invertebrate species. An optogenetic study revealed GABAB receptor expression in motor neurons of *Caenorhabditis elegans* (Schultheis et al., 2011). Ramoino and colleagues (2007) demonstrated a GABAergic-like system as well as the expression of GABAB1 and GABAB2 subunits in a marine demosponge, *Chondrilla nucula*. Expression of GABAB receptors has been reported in olfactory sensory neurons of moths (Pregitzer et al., 2013) and in the entire CNS of cockroaches (Blankenburg et al., 2015), spiders (Panek et al., 2003) and *Drosophila melanogaster* (Mezler et al., 2001). The expression of gabab transcripts (gabab1 and gabab2) has been also reported in a few jawed vertebrate species (zebrafish: Tabor et al., 2008; frogs: Kaeser et al., 2011; rats: Bischoff et al., 1999; Fritschy et al., 1999; non-human primates: Muñoz et al., 1998, 2001; and humans: Calver et al., 2000; Berthele et al., 2001). These studies reveal a wide distribution of this receptor in the entire CNS of invertebrate and vertebrate species. So far, the expression of the GABAB subunits has not been reported in any jawless vertebrate.

4.7.2 GABAergic system in normal and injured animals

In mammals, GABA plays important roles in both pre- and post-synaptic sites. GABAergic cells are involved in the transmission of somatosensory information to higher centres, in the propriospinal coordination of fore and hind limb movements and in reflex activities (Barber et al., 1982). GABAergic neurons produce synaptic inhibition via both GABAA and GABAB receptors in the spinal cord (Cazalets et al., 1994; Castro et al., 2011; Marchenko et al., 2015). In turtles, transmitter release by motoneurons is modulated by GABAB receptors (Castro et al., 2007). In *Xenopus laevis*, descending GABAergic neurons are responsible for tonic inhibition affecting spinal motoneurons and premotor interneurons which led to a reduced locomotion (Lambert et al., 2004). In the spinal locomotor network of lampreys, GABA is not required for burst generation but it plays a powerful modulatory role during locomotion (Homma and Rovainen, 1978; Tegner et al., 1993; Grillner, 2003; Schmitt et al., 2004). Activation of GABAB receptors causes a reduction of burst frequency with a maintained well-coordinated locomotor activity (Tegnér et al., 1993; Schmitt et al., 2004). GABAB receptors play a role at the level of dendrites and somas of interneurons and motoneurons modifying the intrinsic membrane properties (Matsushima et al. 1993; El Manira and Bussieres 1997; Wikstrom and El Manira 1998). Moreover, there is an inhibitory pre-synaptic GABAergic modulation through GABAB receptors in afferent fibres and axons of interneurons (Alford et al. 1991; Alford and Grillner 1991).

Due to the importance of GABA in the modulation of spinal circuitries, alterations of the GABAergic system lead to an impairment of function. In mice, genetic ablation of GABAergic interneurons responsible for the phasic modulation results in motor deficits (Fink et al., 2014). In rodents, spinal injuries cause a reduction in the number of GABAergic interneurons (Zhang et al., 1994; Gwak et al., 2008; Meisner et al., 2010). In cats, there is an increase in the expression of glutamate decarboxylase 67 (GAD67; the enzyme that catalyses the decarboxylation of glutamate to GABA), but not in the expression of GAD65 (a second isoform whose molecular weight is 65 kDa), below the site of injury several months after a complete thoracic SCI (Tillakaratne et al., 2000). The hypofunction of the GABAergic tone in the spinal dorsal horn is a key factor in central neuropathic pain (CNP) after SCI (Zhang et al., 1994; Drew et al., 2004; Liu et al., 2004; Gwak et al., 2006). In contrast, spinal GABAergic system of lampreys suffers an acute decrease 1 hour after a complete spinal cord transection and a fast recovery of this immunoreactivity (within the first week after the injury) (Fernández-López et al.,

2014; Romaus-Sanjurjo et al., 2018). Regards to GABAB receptor, no studies have looked at changes in the expression of GABAB receptor following a direct traumatic injury to the spinal cord. In rodents, other nerve injuries decrease the expression of GABAB receptors or gabab mRNA in the spinal cord (Castro-Lopes et al., 1995; Wang et al., 2011).

4.7.3 Possible role of GABA and GABAB in neuronal survival and axonal regeneration after SCI

After SCI, cell death associated with a delayed, progressive, and extended damage after the primary injury opens the door for possible intervention, so that the number of cells lost can be reduced and axonal degeneration might be reduced or inhibited (e.g. Blight 1989; Blight et al. 1991), which in turn will favour axonal regeneration.

One of the main molecular players in the initiation and progression of secondary injury is glutamate. In mammals, there is a massive glutamate release after SCI, which causes glutamate excitotoxicity (Liu et al., 1991) and neuronal death (Liu et al., 1999; Xu et al., 2004). High extracellular glutamate levels result in excessive activation of glutamate receptors, triggering massive Ca^{2+} influx into cells and activating calcium-dependent proteases which lead to neuronal death (Choi, 1988; Schane et al., 1979). The phenomenon of excitotoxicity has been mainly studied in intrinsic spinal cells. However, retrograde damage to descending neurons is also likely due to the fact that Ca^{2+} ions also gain access to the axoplasm of the damaged axons (Schlaepfer, 1974). An increase in extracellular levels of other aminoacidergic neurotranmistters, like glycine and GABA, has been also reported in mammals after spinal cord injury (Demediuk et al., 1989; Panter et al., 1990). Extracellular glycine could contribute to glutamate excitotoxicity (Panter et al., 1990), since it is a co-agonist of the N-methyl-D- aspartate (NMDA) glutamate receptor (Ransom and Stec, 1988). In contrast, it has been suggested that GABA could have neuroprotective effects, at least in other types of CNS injuries (Han et al., 2008; Llorente et al., 2013). For example, activation of pre-synaptic GABAB receptors causes inactivation of voltage-dependent Ca^{2+} channels (Gaiarsa and Porcher, 2013), which could compensate the influx of Ca^{2+} ions due to glutamate release. In addition, GABA can modulate and promote neurite outgrowth *in vitro* or during development (for reviews, see Sernagor et al., 2010; Gaiarsa and Porcher, 2013). Therefore, similar roles are possible during axonal regeneration after SCI.

Recent studies of our group demonstrated that in lampreys, as in mammals, there is a massive release of glutamate and GABA after a complete spinal injury (Fernández-López et al., 2014; Fernández-López et al., 2016; Romaus-Sanjurjo et al., 2018). Our results showed that immediately after SCI in lampreys, there is a massive release of glutamate, glycine and GABA from most of the spinal neurons close to the lesion site as it happens in mammals (see above). Interestingly, between 1 and 3 days after the injury we observed the accumulation of GABA in the form of “halos” around some axotomized axons of identifiable descending neurons. Statistical analyses showed a significant correlation between GABA accumulation in the form of halos and a higher survival ability of the corresponding descending neurons (Fernández-López et al., 2014). Also, another study has found a correlation between the presence of increased GABA inhibition and a better recovery of function in spinal lesioned lampreys (Svensson et al., 2013). These results could indicate a possible neuroprotective role of GABA following spinal cord injury in lampreys.

JUSTIFICATION AND AIMS

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5 JUSTIFICATION AND AIMS OF THE THESIS

The present results are part of the studies that are being developed by our group to reveal a possible neuroprotective and pro-regenerative role of GABA after a complete SCI in lampreys.

The specific aims of the thesis are:

- To clone and identify the cDNA sequence of the subunits of the GABAB receptor of the sea lamprey. This will allow us to compare the homology between the sea lamprey GABAB receptors and other vertebrate GABAB receptors.
- To obtain *in situ* probes for the gabab1 and gabab2 subunits. This will allow us to carry out a comparative analysis of the expression of GABAB receptors between jawless and jawed vertebrates. In addition, it will be the tool to study changes in the expression of GABAB receptors in reticulospinal neurons and spinal cord of the sea lamprey following SCI.
- To study the expression of GABAB subunits in normal conditions and following SCI in the spinal cord of lampreys. This will provide new information about the relationship between the changes in the GABAergic system and the regeneration and recovery of the animals.
- To study the role of GABA and GABAB receptors in axon regeneration in identifiable descending neurons following SCI. Loss and gain of function experiments will allow us to determine the role of GABA acting through GABAB receptors in the regeneration of identifiable reticulospinal neurons after a complete SCI.

The results of this thesis have been organized in three parts:

1. Cloning of the gabab receptor subunits b1 and b2 and their expression in the central nervous system of the adult sea lamprey. We report the identification and characterization, by means of phylogenetic and sequence analyses, of sea lamprey sequences corresponding to gabab1 and gabab2 cDNAs. We also report the pattern of expression of these transcripts in the brain and spinal cord of young (post-metamorphic) and mature (upstream migrating) adults of the sea lamprey. Moreover, we compared the expression pattern of the two gabab transcripts in the sea lamprey with those of other species. This study provides an anatomical and genetic basis to further understand the roles that this pre- and post-synaptic receptor may have in mediating inhibitory neurotransmission in the CNS.
2. Changes in the expression of the gabab receptor in the spinal cord after a complete SCI in lampreys. We performed gabab1 and gabab2 *in situ* hybridization and semiautomatic quantifications of gabab1 and gabab2 positive profiles to compare their expression in control and lesioned animals. This is the first study reporting the quantitative changes in the expression of gabab receptors at different times of recovery in the spinal cord of any vertebrate following a traumatic SCI.

3. Role of GABA and GABAB receptors in the regeneration of identifiable neurons after SCI. We carried out gabab1 *in situ* hybridization in identifiable descending giant neurons of lampreys to quantify the changes in the expression of this receptor after a complete SCI. Pharmacological treatments using GABOB (a GABA analogue) and genetic manipulations using morpholinos against gabab1 were used to test whether GABA signalling promotes axon regeneration in identifiable neurons, and if this is mediated through GABAB receptors expressed in identifiable descending neurons.

MATERIAL AND METHODS

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6 MATERIAL AND METHODS

6.1 ANIMALS AND ETHICAL STATEMENT

Large, developmentally stable larvae (80 - 160 mm in body length) (n=139), downstream migrating young adult (n=4) and upstream migrating adult sea lampreys (n=4), *Petromyzon marinus*, were used in the present study. Larvae and young adult lampreys were collected from the river Ulla (Galicia, NW, Spain), with permission from the Xunta of Galicia (AUPES 004/2014), and from the Lake Michigan (MI, US). Upstream migrating adults were bought to a local supplier in Galicia. Larvae were maintained in aerated fresh water aquaria until their use for experimental procedures, whereas adults were used immediately after their arrival to the laboratory.

All experiments were approved by the Bioethics Committee at the University of Santiago de Compostela and the *Consellería do Medio Rural e do Mar* of the *Xunta de Galicia* (project code JLPV/IId; Spain) and were performed in accordance to European Union and Spanish guidelines on animal care and experimentation. During the experimental procedures, special effort was taken to minimize animal suffering and to reduce the use of animals.

Table 1. Number of larvae used in each experimental group.

		Number of animals used in each experiment
RNA extraction		10
Changes in the expression of gabab1 in the spinal cord	Control	10
	1 wpl	7
	4 wpl	7
	10 wpl	5
Changes in the expression of gabab2 in the spinal cord	Control	6
	1 wpl	6
	4 wpl	5
	10 wpl	5
Changes in the expression of gabab1 in reticulospinal neurons	Control	7
	1 wpl	7
	4 wpl	6
GABOB treatment (12 wpl)	Control	15
	Treated	14
gabab1 morpholino (ISH) (2 wpl)	Control	3
	Active	4
gabab1 morpholino (10 wpl)	Control	9
	Active	13
	TOTAL	139

6.2 SCI SURGICAL PROCEDURES

Before the experiments, all animals were deeply anaesthetized with 0.1% tricaine methanesulfonate (MS-222; Sigma, St. Louis, MO) in Ringer solution (pH 7.4; NaCl 137 mM, KCl 2.9 mM, CaCl2 2.1 mM, HEPES 2 mM). Larval sea lampreys were randomly assigned to different experimental groups: control un-lesioned animals, animals with a complete spinal cord transection analysed 1 week post-lesion (wpl), 2

wpl, 4 wpl, 10 wpl and 12 wpl. Some of the animals analysed 10 wpl were assigned to a GABAB1 morpholino (see below, morpholino treatment) or control morpholino treated groups, and the 12 wpl animals were assigned to GABOB (see below, GABOB treatment) or vehicle treated groups (see Table 1). Until 2 wpl the animals were only able to move the body and the head above the site of injury. At 4 wpl, the animals had some locomotor activity below the injury site showing a few caudally propagating flexion waves. The animals recovered normal appearing locomotion at 10 wpl (see below, behavioural analyses). The complete spinal cord transection was performed as usual in our research group (see Barreiro-Iglesias et al., 2014). The spinal cord was exposed from the dorsal midline at the level of the 5th gill, a complete spinal cord transection was performed with Castroviejo scissors and the spinal cord cut ends were visualized under the stereomicroscope. Then, the animals were kept on ice for 1 h to allow the wound to air dry. After this hour, the animals were returned to fresh water tanks and each transected animal was examined 24 hours after surgery to confirm that there was no movement caudal to the lesion site. Then the animals recovered in fresh water tanks at 19.5ºC.

6.3 BEHAVIOURAL ANALYSES

The behavioural recovery of non-treated animals was analysed at 10 wpl based on the study of Ayers (1989) and following the protocol of Hoffman and Parker (2011). This qualitative assessment of locomotor function was made from video recordings of 5 minutes (camera: Panasonic Full-HD HC-V110). The animals were placed in a plastic aquarium (36 x 23 x 10.5 cm) and swimming activity was initiated by lightly pinching the tail of the animal using a pair of forceps. The locomotor recovery of the animals was categorized in a scale of 1 to 6 (Ayers, 1989; Hoffman and Parker, 2011). Animals in stage 5 or 6 correspond to animals in which regeneration of axons through the site of injury has occurred based on activity evoked by stimulation across the lesion site in the isolated spinal cord (Hoffman and Parker, 2011). Two blinded experimenters (I and one of my co-workers) independently analysed each 10 wpl animal. Based on both analyses, a mean stage value of locomotor recovery was obtained for each animal.

6.4 CLONING AND SEQUENCING OF SEA LAMPREY GABAB1 AND GABAB2 PARTIAL CDNAS

Normal larvae were deeply anaesthetized with 0.1% MS-222 in Ringer solution (as above) and killed by decapitation. Fresh brains and spinal cords were removed under sterile condition, and total RNA was isolated from these tissues using the TriPure reagent (Roche, Mannhein, Germany). The first-strand cDNA synthesis reaction from total RNA was catalysed with Superscript III reverse transcriptase (Invitrogen, Massachusetts, USA) using random primers (hexamers; Invitrogen). For polymerase chain reaction (PCR) cloning, specific oligonucleotide primers, 5'-TGGCACTGGCCCTGAACAAG-3' forward and 5'-GTTGAGGTTGGGCTGCGAGT-3' reverse; and 5'-GACAAATCTTGCTCGACGCC-3' forward and 5'-AAACGTTGCTGAGGACACCA-3' reverse, were designed based on the sea lamprey gabab1 and gabab2 sequences, respectively, annotated in the sea lamprey genome and deposited in the Ensembl database (Smith et al., 2013; http://www.ensembl.org). The amplified fragments were cloned into pGEM-T vectors (Promega, Madison, WI) and sequenced by GATC Biotech (Cologne, Germany) using Sanger sequencing. Sequence analysis and comparison was done through Basic Local Alignment Search Tool (BLAST) on NCBI and in the SMART website (Schultz et al., 1998; Letunic et al., 2015; http://smart.embl-heidelberg.de/).

6.5 PHYLOGENETIC ANALYSIS

For this analysis, GABAB1 (protein GenBank ID: *Canis lupus familiaris*: XP_005640226.1; *Drosophila melanogaster*: AAK13420.1; *Danio rerio*: CAP09588.1; *Bos taurus*: AAI46242.1; *Homo sapiens*: AAH50532.2; *Mus musculus*: AAH54735.1; *Rattus norvegicus*: CAE84069.1; *Columba livia*: XP_013227121.1; *Xenopus laevis*: ADQ43745.1; *Pan troglodytes*: XP_009449053.1) and GABAB2 (protein GenBank ID: *Canis lupus familiaris*: XP_538749.2; *Danio rerio*: NP_001137515.1; *Drosophila melanogaster*: AAK13421.1; *Columba livia*: XP_013227167.1; *Homo sapiens*: AAH35071.2; *Mus musculus*: NP_001074610.1; *Rattus norvegicus*: EDL98850.1; *Xenopus laevis*: ADQ43746.1; *Pan troglodytes*: XP_009455264.1; *Bos taurus*: XP_002689780.1) sequences of representative species were aligned with the sea lamprey GABAB1 and GABAB2 partial deduced protein sequences deposited in the Ensembl database. All sequences were aligned using CLUSTALW and a phylogenetic tree was constructed using the neighbour-joining method with Poisson corrected distances on amino acids and bootstrap analysis (1,000 replications) using MEGA 6 software (Tamura et al., 2013). An alternative phylogenetic tree was constructed with the minimum-evolution method with Poisson corrected distances on amino acids and bootstrap analysis (1,000 replications) using also the MEGA 6 software.

6.6 *IN SITU* HYBRIDIZATION

Templates for *in vitro* transcription were prepared by PCR amplification as follows. Two 459 base pairs (bp) and 446 bp fragments corresponding to gabab1 and gabab2 sequences, respectively, were obtained using the primers mentioned above. In this case, the reverse primers include the sequence of the universal T7 promoter (TAAGCTTTAATACGACTCACTATAGGGAGA). For the generation of sense probes, the sequence of the T7 promoter was included in the forward primers. The identity of the amplified fragments was confirmed by direct sequencing. Digoxigenin (DIG)-labelled riboprobes were synthesized using the amplified fragments as templates and following standard protocols using T7 polymerase (Roche Diagnostics, Germany).

In situ hybridization experiments were performed as previously described for riboprobes against the serotonin 1a receptor (Cornide-Petronio et al., 2013). Brains/rostral spinal cords of young and mature adults as well as brains and spinal cords of larvae (either control larvae of lesioned larvae), were fixed by immersion for 12 h in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) at 4ºC. After fixation, the tissue was washed with PBS and cryoprotected with sucrose 30%. Then, it was embedded in Neg 50TM (Microm International GmbH, Walldorf, Germany), frozen in liquid nitrogen-cooled isopentane, sectioned on a cryostat in the transverse plane (14 µm thick) and mounted on Superfrost Plus glass slides (Menzel, Braunschweig, Germany). Unlike brains of larvae used for quantification of gabab1 expression in reticulospinal neurons, two parallel series of sections were obtained from brains/rostral spinal cords of young and mature adults as well as spinal cords of larvae. The sections of each series were incubated with the gabab1 or gabab2 DIG-labelled probes, respectively, at 70ºC overnight in hybridization mix and treated with RNAse A (Invitrogen) in the post-hybridization washes. Then, the sections were incubated with a sheep anti-DIG antibody conjugated to alkaline phosphatase (1:2,000; Roche) overnight. Staining was conducted in BM Purple (Roche) at 37ºC until the signal was clearly visible. Finally, the sections were mounted in Mowiol® (Calbiochem; Temecula, CA).

6.7 QUANTIFICATION OF THE CHANGES IN THE EXPRESSION OF GABAB1 AND GABAB2

6.7.1 Quantification of gabab1 and gabab2 expression in the spinal cord

We quantified the number of gabab1 and gabab2 positive profiles per spinal cord section in the 400 µm between 150 µm and 550 µm rostral and caudal to the lesion site. 1 out of each 4 consecutive sections was analysed. In control un-lesioned larvae, 4 sections rostral and 4 sections caudal to the level of the 5th gill were analysed. The average number of positive profiles per half section was then calculated for each animal (rostral and caudal). The semiautomatic quantification of the number of positive gabab1 and gabab2 profiles per spinal cord half section was done as previously described for the quantification of 5-ht1a positive *in situ* profiles (Cornide-Petronio et al., 2014). The dorsomedial midline of the central canal was used as a landmark, and positive gabab1 and gabab2 profiles were only quantified in the grey matter. To decrease background and increase edge and shape detection of the small positive profiles, the threshold was adjusted for each image at the initial border of the histogram and the Feature J Hessian plug-in for Image J was used (smallest eigenvalue Hessian tensor; smoothing scale to 1.0) according to an establish protocol (Grider et al., 2006). Upon conversion to binary images, positive profiles of the grey matter were quantified by using the "analyse particle" function of Image J with particle size from 1 to infinity and circularity from 0.00 to 1.00. All images were blinded previously to quantification.

6.7.2 Quantification of gabab1 expression in identifiable reticulospinal neurons

For the quantification of the level of gabab1 positive signal in reticulospinal cells, first we stablished the intensity rank of positive colorimetric *in situ* signal. For this, we analysed 10 random images from different reticulospinal cells of control and lesioned animals. The "histogram" function in Image J indicates the number of pixels in each image in a range of intensity from 0 to 255. With these images, we compared the intensity values in regions with clear *in situ* signal and the intensity values in regions without signal. Based on this, we established a value of 179 as the lower limit to consider the colorimetric *in situ* signal as positive. Then the number of pixels of positive *in situ* signal was quantified for each section of each identifiable reticulospinal neuron. Only the cells that were identified in at least two different sections were included in the quantifications (the M1, M2, M3, I1, I3, I4, I5, B1, B3, B4, B6 and Mth neurons). Then, we calculated the average number of pixels per section for each individual neuron and this data was used for statistical analyses. All images were blinded previously to quantification.

6.8 GABOB TREATMENT

After the complete SCI, some animals were left in tanks with 4-Amino-3-hydroxybutyric acid (GABOB; Sigma, Cat# A56655, MW 119.12 g/mol) dissolved in water (concentration of 50 µM). Larvae were treated with GABOB 4 days a week, changing the drug and the water each of these days, during 12 weeks following the SCI. Since this drug is water soluble, control lesioned animals were left in tanks with fresh water only.

GABOB is a GABA analogue with a greater capacity to cross the blood-brain barrier than GABA (Hayashi, 1959). For this reason, we decided to use GABOB instead of GABA for this treatment.

6.9 MORPHOLINO APPLICATION

Morpholino application was performed as previously described by Fogerson et al., (2016). The spinal cord was transected at the level of the 5th gill (see surgical

procedures), and morpholinos (20 µg; GeneTools, LLC; Philomath, OR) were added at the time and site of spinal injury via a small piece of Gelfoam (Pfizer; New York, NY). These included a splicing-blocking gabab1 morpholino (5'-ACGTCTGCAACGGAGAGTCATGAGA-3') generated against the boundary between the second intron and the second exon in the partial sea lamprey GABAB1 sequence (GenBank Accession KX655780) (see below in Results, Figure 21), and a five-base pair mismatch gabab1 control morpholino (5'-ACcTCTcCAACcGAGAcTCATcAGA-3'). The morpholinos are then retrogradely transported to the cell soma of descending neurons (Fogerson et al., 2016). Animals were allowed to recover until 10 wpl. *In situ* hybridization was used to control the specificity of the gabab1 morpholino knockdown in animals processed at 2 wpl.

6.10 RETROGRADE TRACT TRACING

After GABOB or morpholino treatment, at 12 or 10 wpl, respectively, the spinal cord was exposed from the dorsal midline at the level of the 6th gill (caudal to the original lesion site at the level of the 5th gill) and a complete spinal cord transection was performed. Crystals of Neurobiotin (NB; 322.8 Da; Vector Laboratories, Burlingame, CA) were applied with a minute pin (00) in the rostral stump. Then, the animals were kept on ice for 1 h to allow the wound to air dry. After this hour, the animals were returned to fresh water tanks and recovered during 6 days to allow transport of NB from the spinal cord to the reticulospinal neurons (Barreiro-Iglesias et al., 2008).

After 7 days, larvae were deeply anesthetized and the brains were dissected out and then fixated by immersion in 4% paraformaldehyde in 0.05M Tris buffered saline (TBS; pH 7.4) for 2 hours. After fixation, the posterior and cerebrotectal commissures of the brain were cut along the dorsal midline, and the alar plates were deflected laterally and pinned flat to a small strip of Sylgard (Dow Corning Co., USA). Brains were re-fixed in 4% paraformaldehyde in TBS for 2 hours. Then, the brains were washed with TBS. For detection of NB, the brain whole-mounts were incubated at 4ºC with fluorescein isothiocyanate (FITC)-labelled avidin D (Vector Laboratories, Burlingame, CA) diluted 1:500 in TBS containing 0.3% Triton X-100, for 2 days. The brains were washed with distilled water and mounted on Superfrost Plus glass slides (Menzel, Braunschweig, Germany) using Mowiol in TBS.

6.10.1 Quantification of identifiable regenerated reticulospinal neurons

The percentage of neurons with regenerated axons (labelled by the tracer) respect to the total number of analyzed neurons was calculated for each type of identifiable reticulospinal neuron.

6.11 IMAGE ACQUISITION

An Olympus photomicroscope (AX-70; Provis) with a 20x Apochromatic 0.75 lens and equipped with a colour digital camera (Olympus DP70, Tokyo, Japan) was used to acquire images of the sections of the brain and spinal cord from the *in situ* hybridization experiments. Identifiable regenerated reticulospinal neurons from the GABOB and morpholino experiments were photographed and analysed with an Olympus microscope or by spectral confocal microscopy (model TCS-SP2; Leica, Wetzlar, Germany).

After the quantifications, contrast and brightness were minimally adjusted and figure plates were generated and lettering was added using Adobe Photoshop CS4 (Adobe Systems, San José, CA, USA). Schematic drawings were generated using Corel Suite 7 (Corel, Ottawa, Canada).

6.12 STATISTICAL ANALYSES

Statistical analysis was carried out using Prism 6 (GraphPad software, La Jolla, CA). Data were presented as mean ± S.E.M. Normality of the data was determined by the Kolmogorov-Smirnov test or the D'Agostino-Pearson omnibus test and the homoscedasticity was determined by the Brown-Forsythe test. The *in situ* hybridization data that were normally distributed and homoscedastic were analysed by a one-way ANOVA. Post-hoc Dunnett´s multiple comparison tests were used to compare pairs of data. *In situ* hybridization data that were not normally distributed were analysed by a Kruskal-Wallis test and post-hoc Dunn's multiple comparisons test. The results of control vs treatment groups were analysed by a paired t-test. The *in situ* hybridization data after morpholino application was analysed by a Student's t-test. The significance level was set at 0.05. In the figures, significance values were represented by a different number of asterisks: 1 asterisk (p value between 0.01 and 0.05), 2 asterisks (p value between 0.001 and 0.01), 3 asterisks (p value between 0.0001 and 0.001) and 4 asterisks (p value < 0.0001).

RESULTS

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7 RESULTS

7.1 CLONING OF THE GABAB RECEPTOR SUBUNITS B1 AND B2 AND THEIR EXPRESSION IN THE CENTRAL NERVOUS SYSTEM OF THE ADULT SEA LAMPREY

7.1.1 Characterization and phylogenetic analysis of the sea lamprey GABAB subunits

The cloned gabab1 and gabab2 cDNA sequences corresponded to the nucleotides 302 to 760 for gabab1 (Fig. 8A), and 729 to 1162 for gabab2 (Fig. 8A') of the partial cDNA sequences of these receptors annotated in sea lamprey genome of the Ensembl database. The cloned sequences had a 100% similarity with the putative gabab1 and gabab2 sequences of the Ensemble database, respectively. The cloned sequences were deposited in the GenBank database (gabab1: KX655780; gabab2: KX655781). Due to a high level of similarity between the cloned sequences and those annotated in the Ensembl database, we used the partial, but longer, GABAB1 and GABAB2 protein sequences (Fig. 8B, 8B', respectively) deduced from the sea lamprey genome sequences ENSPMAG00000006844 (corresponds to GABAB1), which is located in the scaffold GL479777, and ENSPMAG00000004383 (corresponds to GABAB2), which is located in the scaffold GL478877, for the subsequent analyses. Both partial deduced protein sequences contained the "ligand-binding domain of GABAB receptors" and the "seven transmembrane sweet-taste receptor of 3 GCPR" as revealed by the SMART online tool (Fig. 8C-8C').

Percentages of similarity between the sea lamprey GABAB1 and GABAB2 partial deduced protein sequences and those of other species are shown in Table 2. We observed a percentage of similarity of 68% to 73 % and 66% to 69% between the sea lamprey partial GABAB1 and GABAB2 deduced protein sequences, respectively, and the corresponding deduced protein sequences of other vertebrate species. As expected, the percentage of similarity of the partial sea lamprey GABAB1 and GABAB2 deduced amino acids sequences with those of the protostome invertebrate *D. melanogaster* is low (less than 50%). The similarity between the sea lamprey partial GABAB1 and GABAB2 deduced protein sequences is only 33%.

Table 2. Percentage of homology between the partial amino acid sequences of the sea lamprey GABAB subunits and the amino acid sequences of GABAB subunits of different vertebrate and invertebrate species. Romaus-Sanjurjo et al., 2016; permitted by Frontiers.

GABAB1	*Petromyzon marinus* (%)
Xenopus laevis (ADQ43745.1)	73
Homo sapiens (AAH50532.2)	72
Mus musculus (AAH54735.1)	72
Pan troglodytes (XP_009449053.1)	72
Bos taurus (AAI46242.1)	71
Canis lupus (XP_005640226.1)	71
Columba livia (XP_013227121.1)	71
Danio rerio (CAP09588.1)	71
Rattus norvegicus (CAE84069.1)	68
Drosophila melanogaster (AAK13420.1)	48
GABAB2	*Petromyzon marinus* (%)
Canis lupus (XP_538749.2)	69
Columba livia (XP_013227167.1)	69
Homo sapiens (AAH35071.2)	69

Pan troglodytes (XP_009455264.1)	69
Mus musculus (NP_001074610.1)	68
Rattus norvegicus (EDL98850.1)	68
Bos taurus (XP_002689780.1)	67
Xenopus laevis (ADQ43746.1)	67
Danio rerio (NP_001137515.1)	66
Drosophila melanogaster (AAK13421.1)	41

The GABAB1 and GABAB2 deduced protein sequences annotated in the Ensembl database were aligned with GABAB1 and GABAB2 deduced protein sequences of representative vertebrate and invertebrate species and this alignment was used to construct a phylogenetic tree using the neighbour-joining and the minimum-evolution methods. All vertebrate GABAB1 and GABAB2 sequences, including the sea lamprey sequences, were clustered together in their respective groups in the resulting trees and the sea lamprey sequences were found to emerge as an outgroup to the corresponding gnathostome sequences (Fig. 9A-B).

7.1.2 Expression of gabab1 and gabab2 subunits in the adult sea lamprey brain

We observed a widespread expression of the gabab1 and gabab2 transcripts in neuronal populations in the brain of the adult sea lamprey. No clear and obvious differences were observed in the expression of these transcripts between young and mature adult sea lampreys. *In situ* hybridization signal appeared as a dotted labeling in the tissue sections.

No clear differences were noted in the expression of the transcripts of the two gabab subunits (Fig. 10). Accordingly, only 1 set of schematic drawings of transverse sections taken from a young post-metamorphic adult showing the expression pattern of both transcripts is presented in Figure 11. Photomicrographs of representative transverse sections of the brain are shown in Figures 10, 12 and 13. These photomicrographs were taken from two young postmetamorphic and three upstreaming migrating adults.

In the telencephalon, the olfactory bulbs showed numerous dots of positive gabab1 and gabab2 expression (Fig. 11A, 12A). Most of the expression was observed in cells of the mitral and inner cellular layers. Some expression was also observed in cells localized between the glomeruli. In the medial pallium, gabab1 and gabab2 expression was very scarce in cells of the periventricular layer and no expression was observed in migrated cells (Fig. 11C, 12C). We observed expression of both transcripts in neurons surrounding the dorsomedial neuropil (Fig. 11C). The lateral pallium showed more gabab1 and gabab2 expression in the cells of the periventricular layer than in migrated cells (Fig. 11B-C, 12C). In the subhippocampal lobe, most of the gabab1 and gabab2 expression was observed in the cells of the periventricular layer (Fig. 11C). In the subpallium, the cells of the septum showed gabab1 and gabab2 positive dots (Fig. 11B, 12D). In the striatum, gabab1 and gabab2 expression was observed mainly in migrated cells, with less expression being observed in the periventricular layer (Fig. 10A-A', 11C). In contrast, the preoptic nucleus showed wide gabab1 and gabab2 expression in periventricular cells, mainly in the intermediate layer of the preoptic nucleus (Fig. 10A-A', 11C).

The hypothalamus showed wide expression of the gabab1 and gabab2 transcripts in cells of the periventricular layers, while the migrated cells showed very scarce expression (Fig. 12E). Many gabab1 and gabab2 positive dots were observed in the nucleus of the postoptic commissure, the paracommissural preoptic nucleus, the dorsal

and ventral hypothalamus and the mammillary nucleus (Fig. 11D-F, 12E). The pattern of expression observed in the hypothalamus was also observed in the prethalamus and thalamus (Fig. 11D-F, 12F-G). Gabab1 and gabab2 expression was also observed in the pineal and parapineal organs (Fig. 11C). The habenula showed gabab1 and gabab2 expression, with some differences between the left and right sides (Fig. 10B-B',11 D-E). In the right habenula, which is bigger than the left one, gabab1 and gabab2 positive dots were more numerous than in the left habenula, although positive dots in cells of the left habenula were larger. The pretectum showed expression of both transcripts in cells of the periventricular layer and scarce expression in migrated neurons (Fig. 11F). In the paratubercular nucleus, very few cells showed expression of the transcripts (Fig. 11F). In contrast, a large number of gabab1 and gabab2 positive dots were observed in the nucleus of the medial longitudinal fascicle including the first and second Müller cells (Fig. 11G, 12I).

In the mesencephalon, the optic tectum showed gabab1 and gabab2 positive dots with most of them distributed throughout the cells of the stratum griseum periventriculare, but also in cells of the stratum "album" et griseum central (Fig. 10C-C', 11H). The torus semicircularis showed numerous positive dots. The positive dots in cells of the torus were larger than those in the optic tectum (Fig. 11H). The migrated neurons of both areas showed very little expression of the transcripts. There was strong gabab1 and gabab2 expression in the tegmentum and the mesencephalic reticular area (including the third Müller cell) and the motor neurons of the oculomotor nuclei showed a large amount of gabab1 and gabab2 expression in their somas (Fig. 11H).

In the alar plate of the rhombencephalon, the dorsal isthmic gray showed expression of both transcripts (Fig. 11I, 13A). In the octavolateral nuclei, there was gabab1 and gabab2 expression, mainly in cells of the periventricular layer (Fig. 11J-K, 13B-C). The octavomotor nuclei showed gabab1 and gabab2 positive dots (Fig. 11K, 13C). Numerous gabab1 and gabab2 positive dots were observed in cells of the periventricular layer of the solitary tract (Fig. 11L, 13D) and dorsal column nuclei. Dorsal cells of the caudal rhombencephalon (primary medullary and spinal nucleus of the trigeminus) also showed gabab1 and gabab2 expression (Fig. 13B-C). In the basal plate of the rhombencephalon, the soma of the cells of the visceromotor nuclei (V, VII, IX and X) showed many gabab1 and gabab2 positive dots (Fig. 11J-L, 13E). In addition, wide gabab1 and gabab2 expression was observed in all rhombencephalic reticular nuclei (isthmic, trigeminal, middle and posterior reticular nuclei), including the giant Müller and Mauthner cells (Fig. 11I-L, 13F).

7.1.3 Expression of the gabab1 and gabab2 subunits in the spinal cord

In the rostral spinal cord of the adult sea lamprey, most neuronal types showed gabab1 and gabab2 expression (Fig. 11M, 13G-H). Dorsal cells showed large amounts of gabab1 and gabab2 positive dots in their soma (Fig. 11M, 13G). We also observed some expression of the transcripts in the edge cells (Fig. 11M). Gabab1 and gabab2 expression was observed in interneurons of different sizes located in the dorsal and lateral regions (Fig. 11M, 13G). Expression of both transcripts was observed in the somas of motor neurons (Fig. 13G). Cerebrospinal fluid-contacting (CSF-c) cells showed numerous gabab1 and gabab2 positive dots, even in their apical dendrites (Fig. 13H).

7.1.4 Expression of gabab1 and gabab2 subunits in glial cells

The glial cells of the brain of the sea lamprey are almost exclusively ependymal cells, although astrocytes could be identified in the optic nerve. No expression of any of

the two transcripts was observed in brain ependymocytes (examples in Fig. 12B-C) or in the subcommissural organ (Fig. 11F, 12H). The astrocytes of the optic nerve, which are easily identified because of the absence of neurons in the optic nerve, did not show expression of the transcripts (Fig 10D-D').

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7.1.5 Figures

Figure 8. Partial cDNA sequences of the gabab1 (**A**) and gabab2 (**A'**) subunits from the Ensembl database, with the cloned sequences marked in red. Sequences of the deduced GABAB1 (**B**) and GABAB2 (**B'**) proteins with the sequence corresponding to the cloned cDNA sequences highlighted in red. Analyses of the predicted GABAB1 (**C**) and GABAB2 (**C'**) proteins in SMART and BLAST. From Romaus-Sanjurjo et al., 2016; permitted by Frontiers.

A

5'_1_gtctacaaggagaagctctacggcaagaagcacgtgtggttcatcatcggctggtacgccgacaagtggtacctcaagcccgacctggccatcaactgcacggccgagcagatgcaggaggctgtcgagggtcaggtcacc
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B

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WIGGSPPPDRTKVVIQFRYLSQKLFISLSVLAGIGIIFGCVCLVFNIYNRNVRYIQNSQPNLNNLTALGCILTLAVVLPLGLDGLHISESQFPFICQVRV
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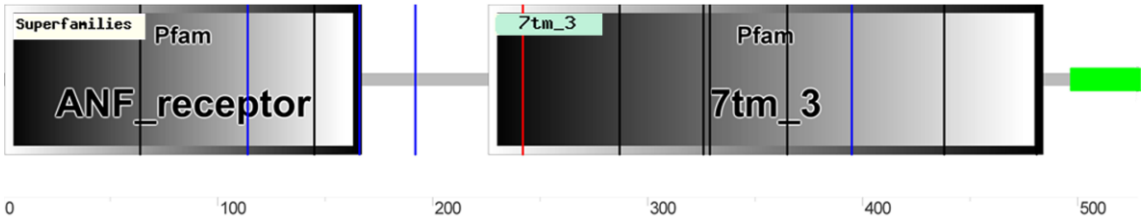
C



A'

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a_1206_3'

B'

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GVSEVAPHSKFHGYAYDGIWVIAQVLNRTIELLEADKSLIASIESFSYTNQRIGQILLDALNETNFLGVTGQVLFRNGERLGTIEFMQFQSTERVKVG
EYNAVPDTLELINSTMRFQGPDPPWDRTIVQSKLREVYLPLYSILSVLTCLGMFMASAFLFFNIKNRNQKLIKMSSPYMNNLIILGGMLSYMTIFLFG
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C'

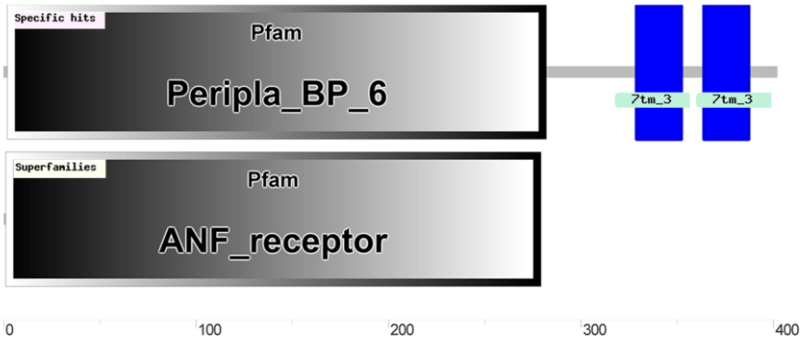
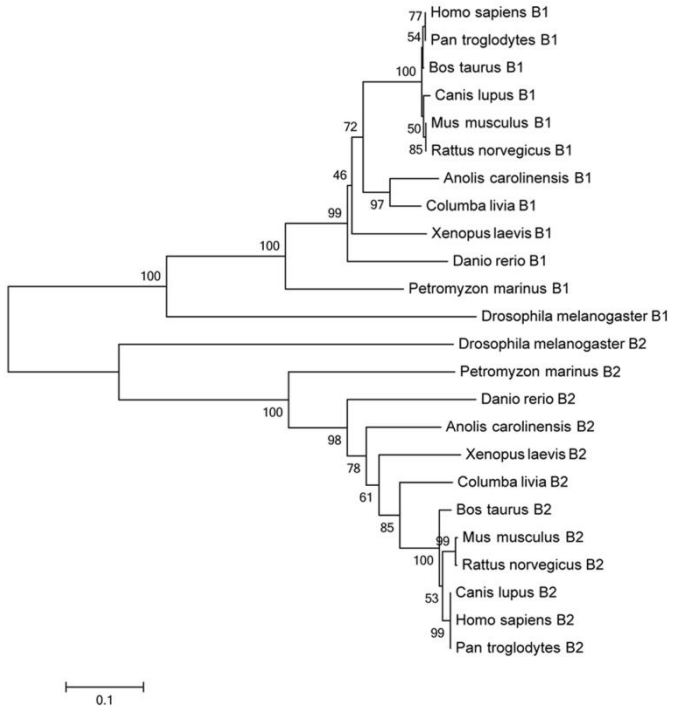


Figure 9. Phylogenetic trees of GABAB protein sequences of different species according the neighbor-joining (**A**) and the minimum-evolution methods (**B**). The bootstraps are indicated on a scale of 100 based on 1000 replications. Scale indicates 0.1 amino acids substitutions per locus. From Romaus-Sanjurjo et al., 2016; permitted by Frontiers.

A


Homo sapiens B1
Pan troglodytes B1
Bos taurus B1
Canis lupus B1
Mus musculus B1
Rattus norvegicus B1
Anolis carolinensis B1
Columba livia B1
Xenopus laevis B1
Danio rerio B1
Petromyzon marinus B1
Drosophila melanogaster B1
Drosophila melanogaster B2
Petromyzon marinus B2
Danio rerio B2
Anolis carolinensis B2
Xenopus laevis B2
Columba livia B2
Bos taurus B2
Mus musculus B2
Rattus norvegicus B2
Canis lupus B2
Homo sapiens B2
Pan troglodytes B2
77
54
100
72
50
85
46
97
99
100
100
100
98
78
61
85
99
100
53
99
0.1

B

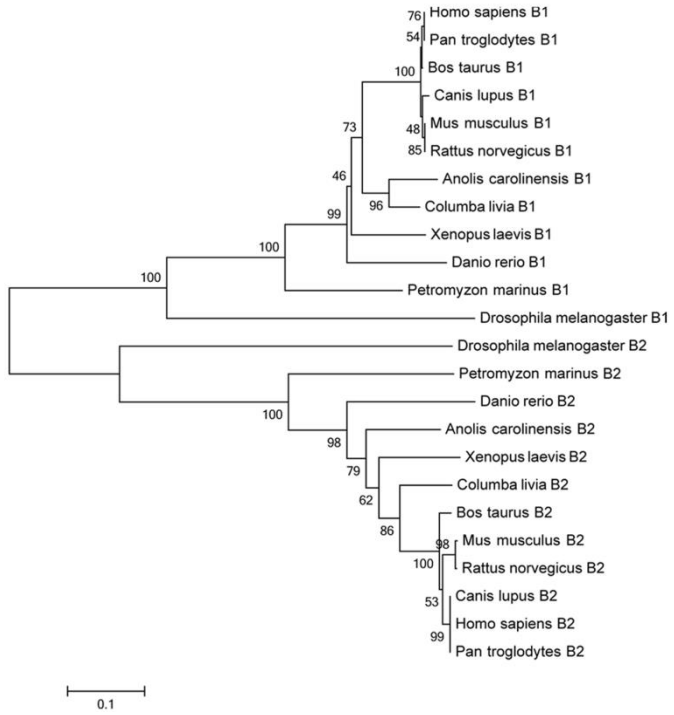

Homo sapiens B1
Pan troglodytes B1
Bos taurus B1
Canis lupus B1
Mus musculus B1
Rattus norvegicus B1
Anolis carolinensis B1
Columba livia B1
Xenopus laevis B1
Danio rerio B1
Petromyzon marinus B1
Drosophila melanogaster B1
Drosophila melanogaster B2
Petromyzon marinus B2
Danio rerio B2
Anolis carolinensis B2
Xenopus laevis B2
Columba livia B2
Bos taurus B2
Mus musculus B2
Rattus norvegicus B2
Canis lupus B2
Homo sapiens B2
Pan troglodytes B2
76
54
100
73
48
85
46
96
99
100
100
100
98
79
62
86
98
100
53
99
0.1

Figure 10. Photomicrographs from the sea lamprey brain showing the expression of both gabab subunits in the same brain regions. **A-D** corresponds to gabab1 expression and **A'-D'** corresponds to gabab2 expression. The arrows indicate some small positive dots. Please note in **D-D'** that the astrocytes are located in the central part of the optic nerve. Asterisks indicate the ventricles. Scale bars = 50µm. From Romaus-Sanjurjo et al., 2016; permitted by Frontiers.

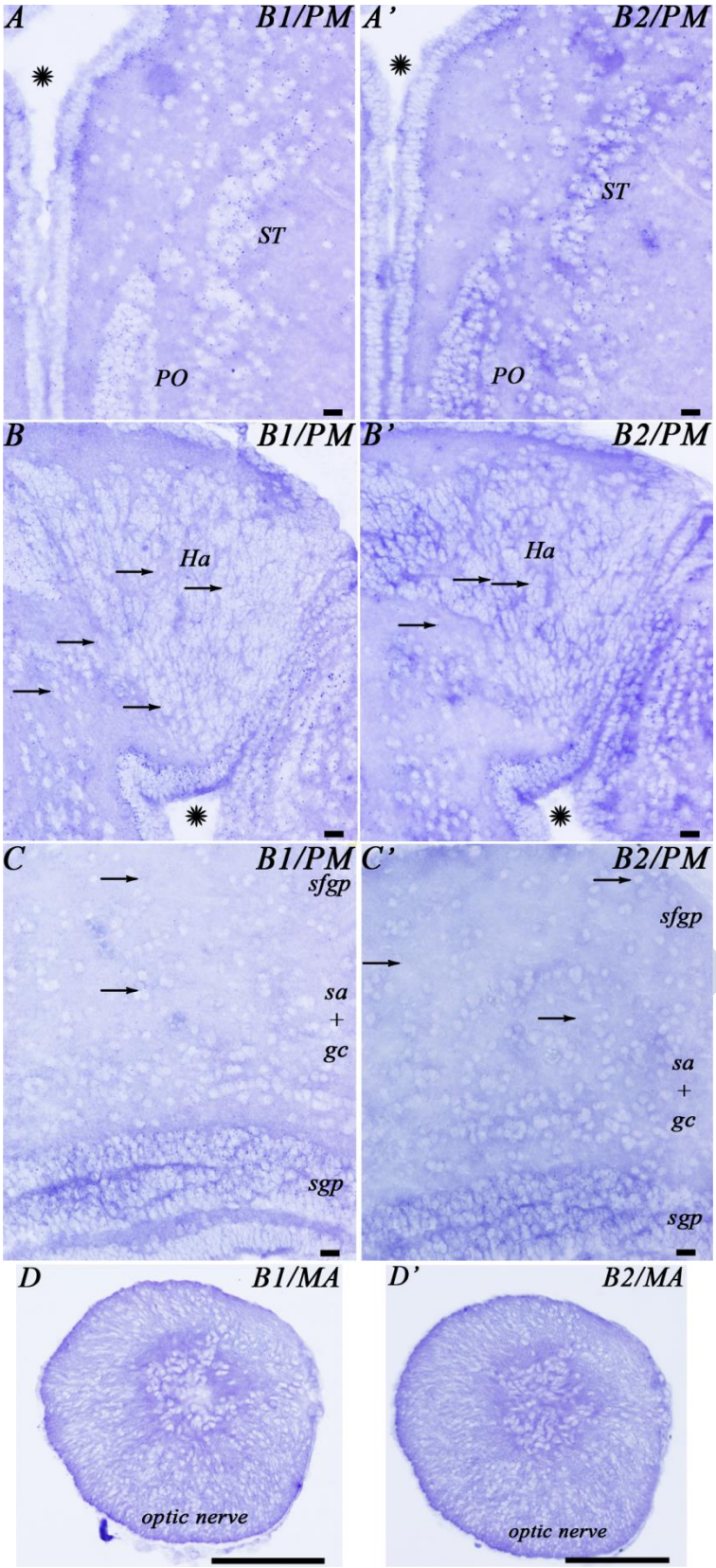

A
B1/PM
ST
PO
A'
B2/PM
ST
PO
B
B1/PM
Ha
B'
B2/PM
Ha
C
B1/PM
sfgp
sa
+
gc
sgp
C'
B2/PM
sfgp
sa
+
gc
sgp
D
B1/MA
optic nerve
D'
B2/MA
optic nerve

Figure 11. Schematic drawings of transverse sections of the adult sea lamprey brain and spinal cord showing the distribution of gabab transcripts (on the right side), and the main brain and spinal cord regions (on the left side). The level of sections is indicated in the upper left figure showing a lateral view of the brain. Dots represent the location and an estimate of the relative density of both gabab transcripts. Correspondence with photomicrographs in other figures is indicated by squared areas. Scale bars = 50µm. From Romaus-Sanjurjo et al., 2016; permitted by Frontiers.

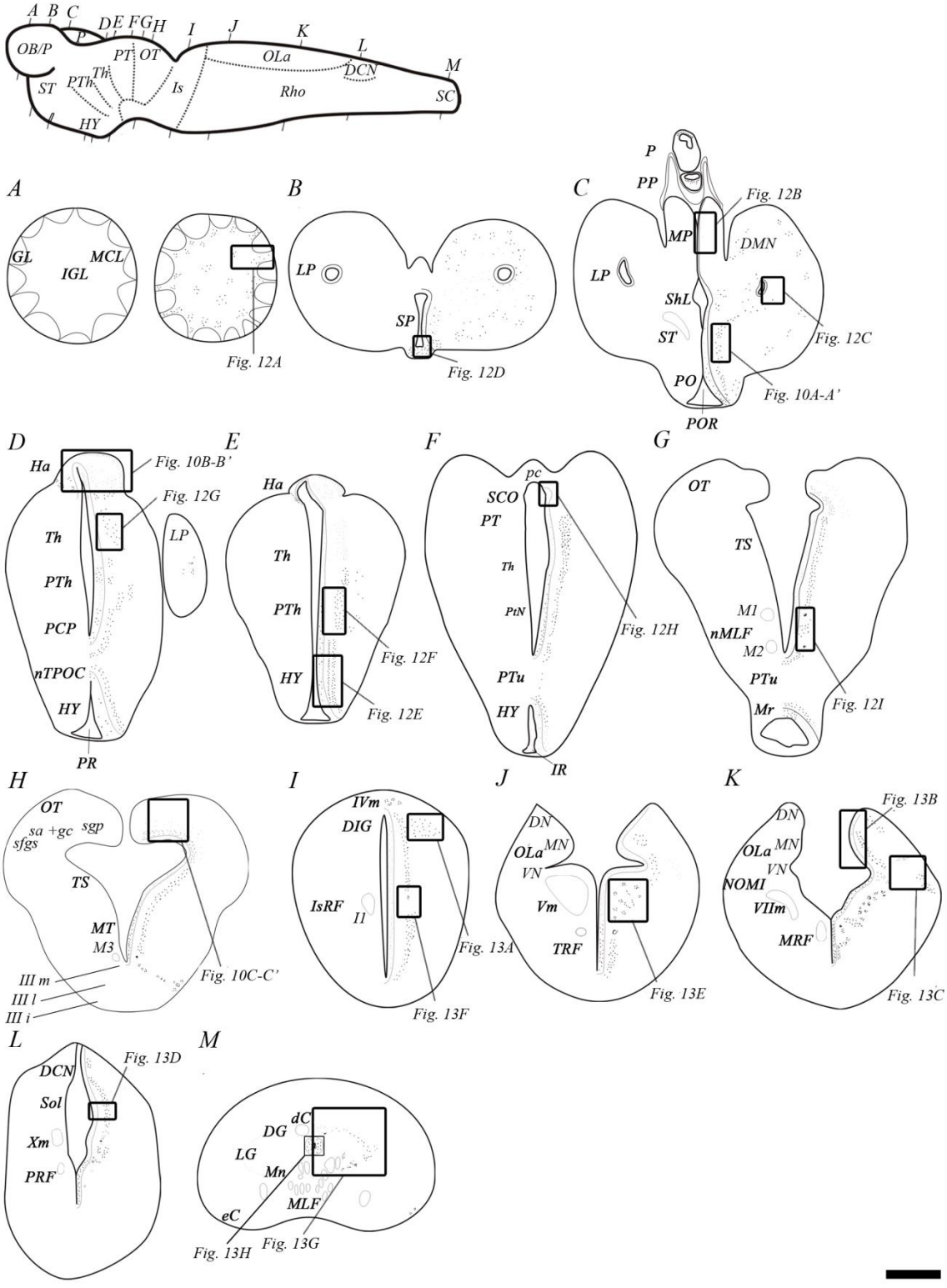

A B C DE FG H I J K L M
OB/P P PT OT OLa DCN
ST PTh Th Is Rho SC
HY
A
GL MCL IGL
Fig. 12A
B
LP SP
Fig. 12D
C
P PP Fig. 12B MP DMN
LP ShL ST Fig. 12C PO Fig. 10A-A'
POR
D
Ha Fig. 10B-B' Fig. 12G LP
Th PTh PCP nTPOC HY
PR
E
Ha Th PTh HY
Fig. 12F Fig. 12E
F
pc SCO PT Th PtN Fig. 12H PTu HY
IR
G
OT TS M1 nMLF M2 PTu Mr Fig. 12I
H
OT sa +gc sgp sfgs TS MT M3
III m III l III i Fig. 10C-C'
I
IVm DIG IsRF II Fig. 13A
Fig. 13F
J
DN OLa MN VN Vm TRF Fig. 13E
K
Fig. 13B
DN OLa MN VN NOMI VIIm MRF
Fig. 13C
L
Fig. 13D DCN Sol Xm PRF
M
dC DG LG Mn MLF eC
Fig. 13H Fig. 13G

Figure 12. Photomicrographs from different regions of the prosencephalon showing expression of both gabab transcripts. Arrows indicate small positive dots. Arrowheads indicate accumulations of transcript expression in giant cells. Asterisks indicate the ventricle. Scale bars = 50μm. From Romaus-Sanjurjo et al., 2016; permitted by Frontiers.

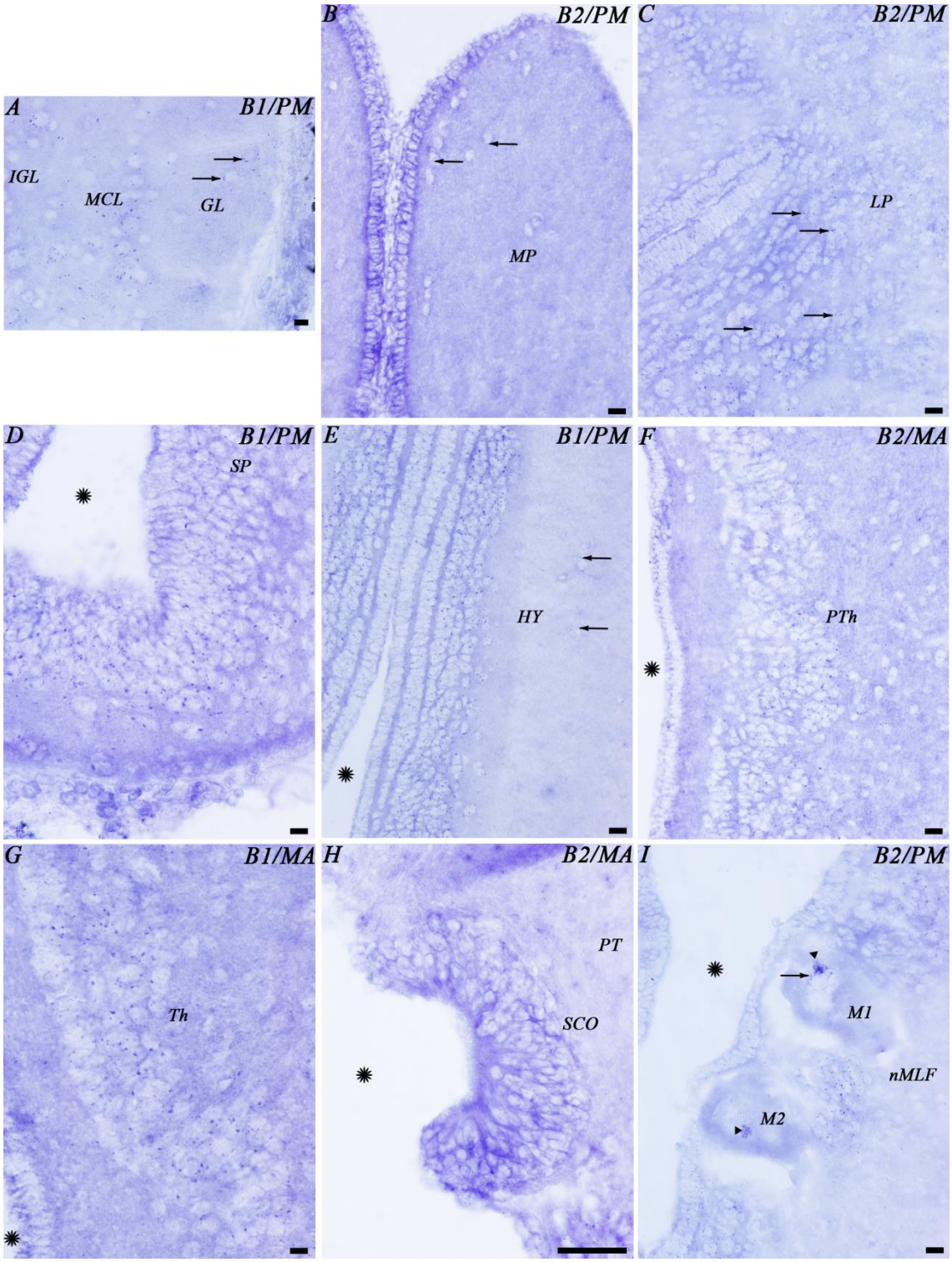
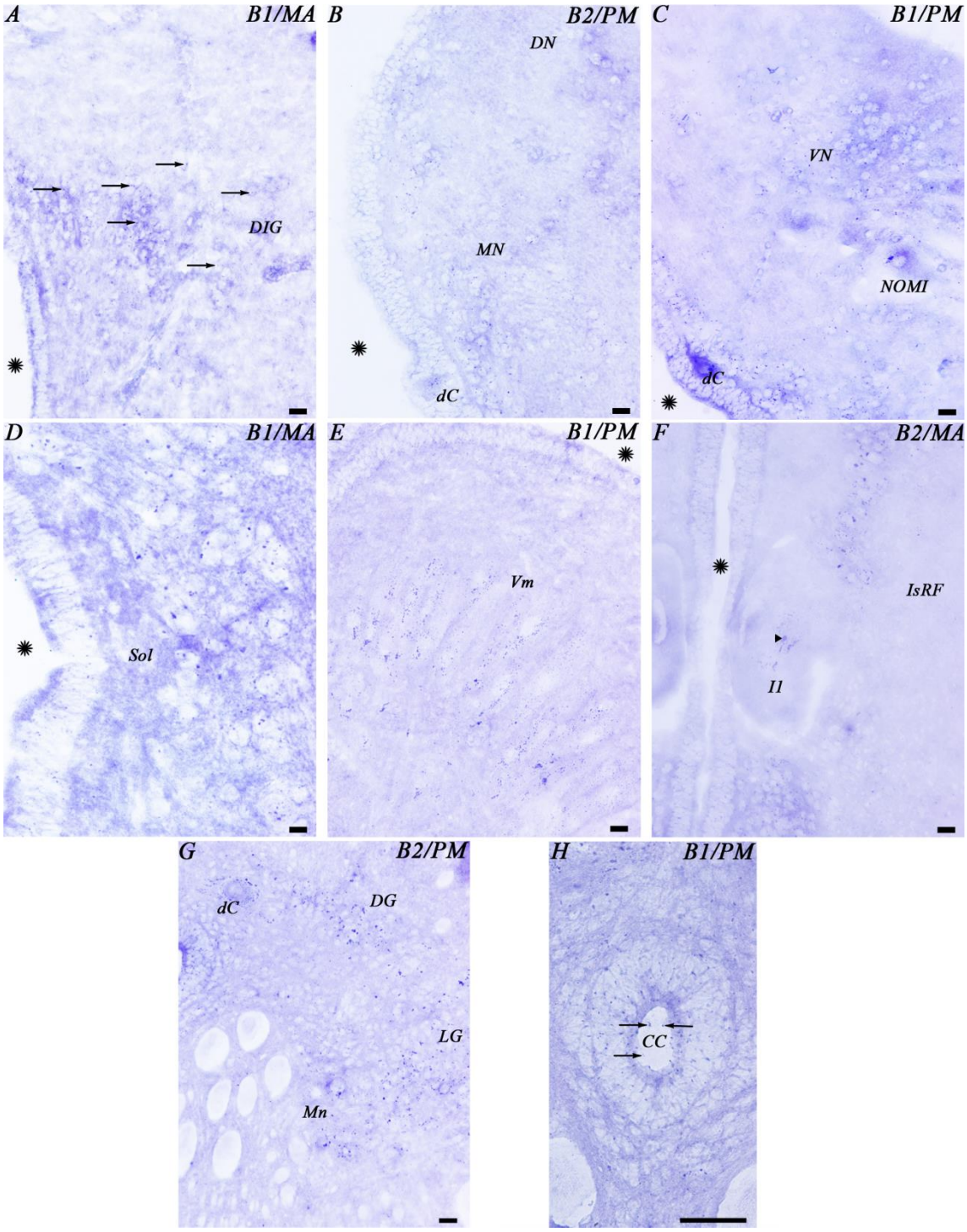

A
B1/PM
IGL
MCL
GL
B
B2/PM
MP
C
B2/PM
LP
D
B1/PM
SP
E
B1/PM
HY
F
B2/MA
PTh
G
B1/MA
Th
H
B2/MA
PT
SCO
I
B2/PM
M1
nMLF
M2

Figure 13. Photomicrographs from different regions of the mesencephalon (**A-B**), rhombencephalon (**C-F**) and spinal cord (**G-H**) showing expression of both gabab transcripts. Arrows indicate small positive dots. The arrowhead indicates accumulations of transcript expression in a giant cell. Asterisks indicate the ventricle. Scale bars = 50µm. From Romaus-Sanjurjo et al., 2016; permitted by Frontiers.


A
B1/MA
DIG
B
B2/PM
DN
MN
dC
C
B1/PM
VN
NOMI
dC
D
B1/MA
Sol
E
B1/PM
Vm
F
B2/MA
IsRF
II
G
B2/PM
dC
DG
LG
Mn
H
B1/PM
CC

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7.2 EXPRESSION OF THE GABAB RECEPTOR AFTER A COMPLETE SCI IN LAMPREYS

7.2.1 Behavioural recovery of the animals

At 1 wpl the animals were only able to move the head and the body above the site of injury. At 4 wpl, the animals had some locomotor activity below the site of injury showing a few caudally propagating flexion waves. All animals recovered normal appearing locomotion at 10 wpl with all of them reaching stage 6 in the Ayers' scale of locomotor recovery (as an example see Suppl. Videos 1 to 3).

7.2.2 Expression of the gabab1 and gabab2 subunits in the spinal cord of larval sea lamprey

As previously described for adult lampreys (see above in Results, section 5.1.3), we observed expression of both gabab transcripts in the grey matter of control spinal cords and the colorimetric *in situ* signal appeared as a dotted labelling (Fig. 14). The pattern of expression of the gabab subunits observed in larval lampreys at the level of the 5th gill was the same as the one observed in our previous study in adult lampreys at more rostral levels (see above in Results, 5.1.3). Expression of gabab transcripts was observed in motor neurons and interneurons, which can be identified based on their different morphology and location in the grey matter [motor neurons are large or medium size cells that are only present in the ventrolateral region of the grey matter (Nieuwenhuys and Nicholson, 1998) (Fig. 14)]. Dorsal cells showed expression of the gabab transcripts (not shown) as well. Expression of both transcripts was also observed in a band of cells lateral to the grey matter, where only a few neurons are present and most of the cells are astrocytes (Retzius, 1893; see also Fig 1D in Fernández-López et al., 2014) (Fig. 14). Expression of both gabab transcripts was also observed around the central canal, which contains CSF-c and ependymoglial cells (Fig. 14).

7.2.3 Changes in gabab1 and gabab2 expression following a complete spinal cord transection

Quantification of the number of gabab1 positive *in situ* profiles per section showed significant differences between control animals and each of the experimental groups (Fig. 15; Table 3). Rostral to the site of injury, we observed a 48.3%, 37.2% and 45% reduction in the number of positive profiles at 1, 4 and 10 wpl, respectively, as compared to control un-lesioned animals (ANOVA, $p = 0.001$; Fig. 5A-E; Table 3). Caudal to the site of injury, we observed a 72.2%, 48.3% and 47.1% reduction in the number of positive profiles at 1, 4 and 10 wpl, respectively, as compared to control un-lesioned animals (ANOVA, $p = 0.0009$; Fig. 15B, F-I; Table 3).

Quantification of the number of gabab2 positive *in situ* profiles per section showed significant differences between control animals and each of the experimental groups (Fig. 16; Table 3). Rostral to the site of injury, we observed a 68.9%, 57.1% and 76.6% reduction in the number of positive profiles at 1, 4 and 10 wpl, respectively, as compared to control un-lesioned animals (ANOVA, $p < 0.0001$; Fig. 6A-E; Table 3). Caudal to the site of injury, we observed a 60.8%, 54.9% and 65.1% reduction in the number of positive profiles at 1, 4 and 10 wpl, respectively, as compared to control un-lesioned animals (ANOVA, $p = 0.0006$; Fig. 16, F-I; Table 3).

Table 3. Mean ± SEM values of the number of gabab positive profiles/section in the spinal cord of control animals and injured animals (rostral and caudal). Refers to Figures 15 and 16. From Romaus-Sanjurjo et al., 2018; permitted by Elsevier.

gabab profiles/section		Control	1 wpl	4 wpl	10 wpl
gabab1	Rostral	500.3 ± 42.89	258.7 ± 38.1	314.1 ± 44.2	275.3 ± 50.6
	Caudal	554.8 ± 85.77	154.4 ± 22.6	287.1 ± 37.9	293.4 ± 64.9
gabab2	Rostral	1324 ± 176.2	411.6 ± 97.4	568.2 ± 110.5	309.2 ± 74.5
	Caudal	1047 ± 119.6	410.2 ± 75.8	472.3 ± 149.9	364.9 ± 58.7

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7.2.4 Figures

Figure 14. A-D: Photomicrographs (A, C) and schematic drawings (B, D) of a transverse section of the spinal cord of a sea lamprey larva showing the expression of the gabab1 (A, B) and gabab2 (C, D) transcripts. Please note in panels **B** and **D** the presence of both gabab transcripts in a band of cells lateral and outside the gray matter in a region where mainly glial cells are present (asterisks) (Retzius, 1893). Stars indicate the central canal. Arrows indicate examples of positive gabab *in situ* profiles. Dorsal is to the top. Scale bars: 50 µm. From Romaus-Sanjurjo et al., 2018; permitted by Elsevier.

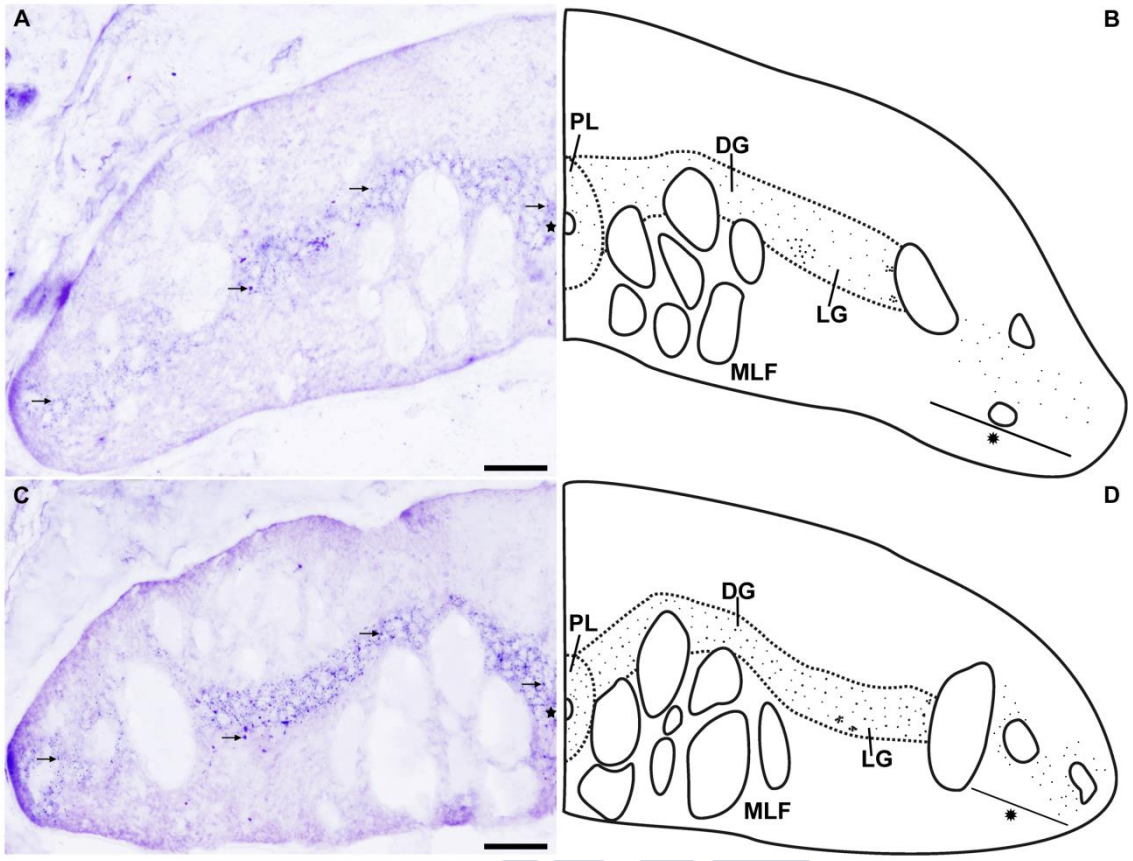


Figure 15. Quantitative changes in the expression of the gabab1 subunit rostral (R) and caudal (C) to the site of injury after a complete spinal cord transection. **A**: Graph showing significant changes (*asterisks*) in the number of gabab1 positive profiles in the spinal cord rostral to the lesion site. The mean ± SEM values are provided in table 3. **B:** Photomicrograph of a transverse section of the spinal cord showing the high level of expression of the gabab1 transcript in the spinal cord at the level of the fifth gill in control animals. **C-E**: Photomicrographs of transverse sections of the spinal cord rostral to the site of injury showing the decreased expression of the gabab1 transcript in lesioned animals at 1 wpl (C), 4 wpl (D) and 10 wpl (E). **F**: Graph showing significant changes (*asterisks*) in the number of gabab1 positive profiles in the spinal cord caudal to the lesion site. The mean ± SEM values are provided in table 3. **G-I**: Photomicrographs of transverse sections of the spinal cord caudal to the site of injury showing the decreased expression of the gabab1 transcript in lesioned animals at 1 wpl (G), 4 wpl (H) and 10 wpl (I). Stars indicate the central canal. Arrows indicate examples of positive gabab1 *in situ* profiles. Dorsal is to the top and the midline to the right. Scale bars: 50 µm. From Romaus-Sanjurjo et al., 2018; permitted by Elsevier.

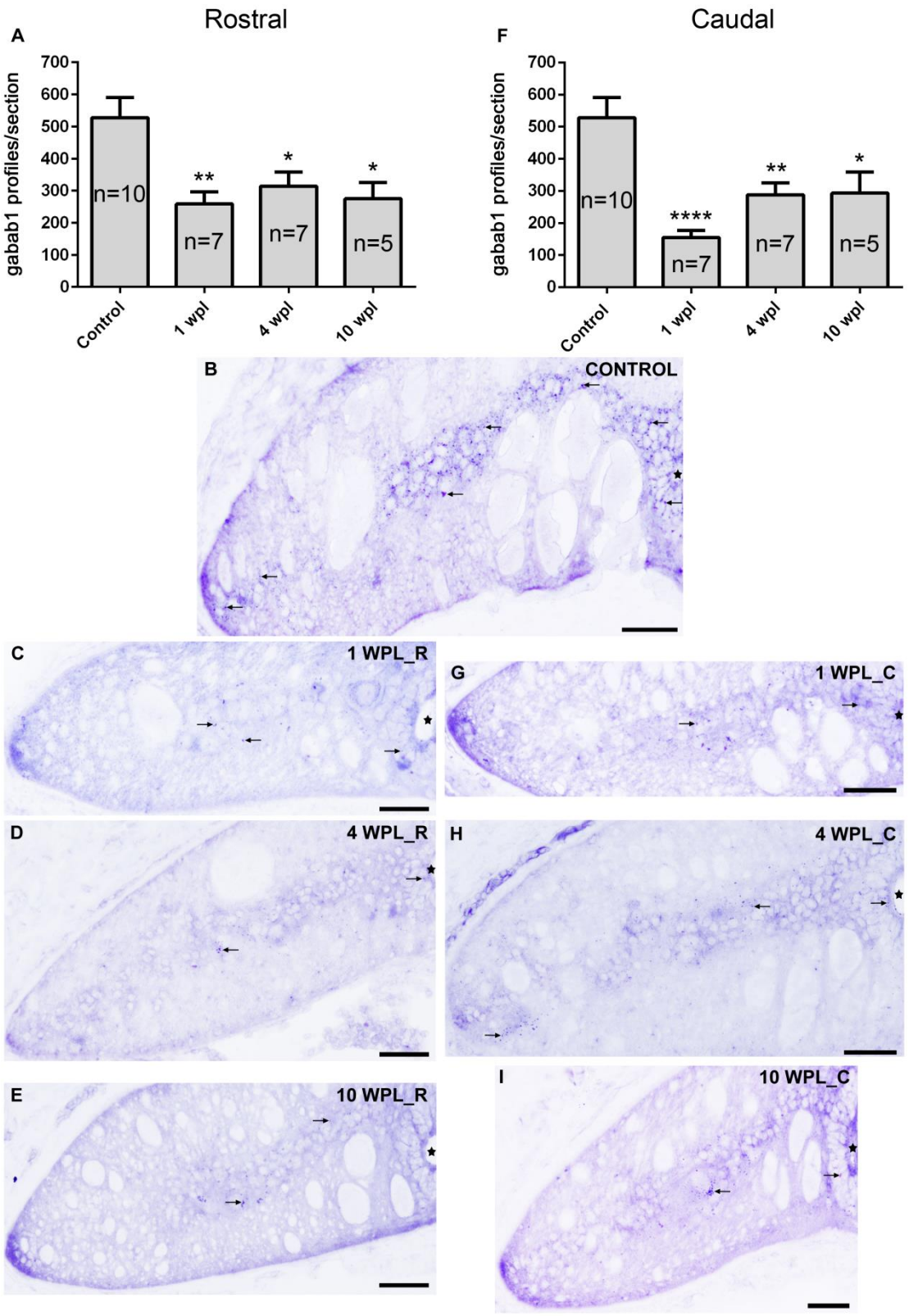
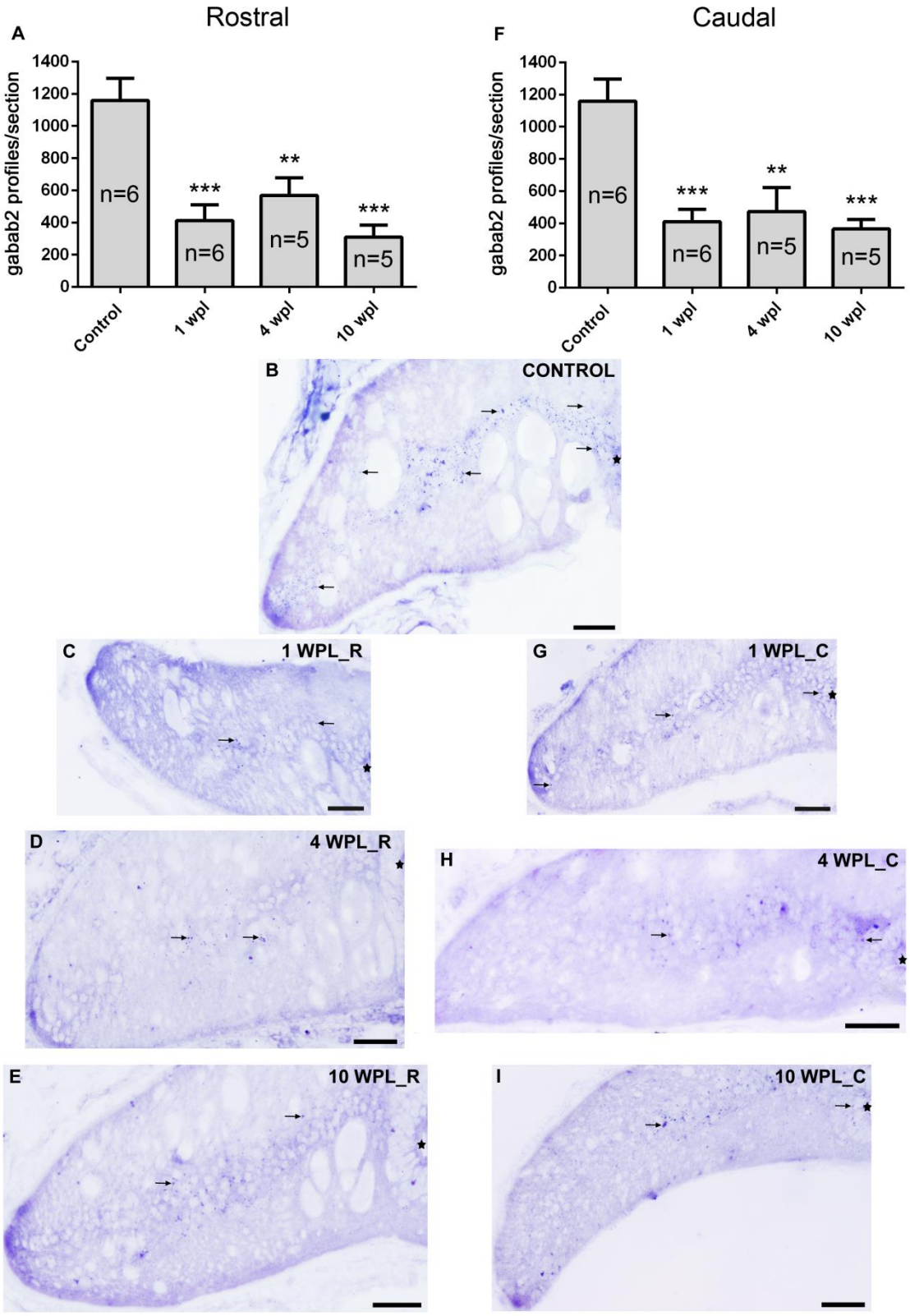

A
Rostral
gabab1 profiles/section
700
600
500
400
300
200
100
0
n=10
n=7
n=7
n=5
**
*
*
Control
1 wpl
4 wpl
10 wpl
F
Caudal
gabab1 profiles/section
n=10
n=7
n=7
n=5
****
**
*
Control
1 wpl
4 wpl
10 wpl
B
CONTROL
C
1 WPL_R
G
1 WPL_C
D
4 WPL_R
H
4 WPL_C
E
10 WPL_R
I
10 WPL_C

Figure 16. Quantitative changes in the expression of the gabab2 subunit rostral (R) and caudal (C) to the lesion site after a complete spinal cord transection. **A**: Graph showing significant changes (*asterisks*) in the number of gabab2 positive profiles in the spinal cord rostral to the lesion site. The mean ± SEM values are provided in table 3. **B:** Photomicrograph of a transverse section of the spinal cord showing the high level of expression of the gabab2 transcript in the spinal cord at the level of the fifth gill in control animals. **C-E**: Photomicrographs of transverse sections of the spinal cord rostral to the lesion site showing the decreased expression of the gabab2 transcript in lesioned animals at 1 wpl (C), 4 wpl (D) and 10 wpl (E). **F**: Graph showing significant changes (*asterisks*) in the number of gabab2 positive profiles in the spinal cord caudal to the site of injury. The mean ± SEM values are provided in table 3. **G-I**: Photomicrographs of transverse sections of the spinal cord caudal to the lesion site showing the decreased expression of the gabab2 transcript in lesioned animals at 1 wpl (G), 4 wpl (H) and 10 wpl (I). Stars indicate the central canal. Arrows indicate examples of positive gabab2 *in situ* profiles. Dorsal is to the top and the midline to the right. Scale bars: 50 µm. From Romaus-Sanjurjo et al., 2018; permitted by Elsevier.


A
Rostral
gabab2 profiles/section
1400
1200
1000
800
600
400
200
0
n=6
n=6
n=5
n=5
***
**
***
Control
1 wpl
4 wpl
10 wpl
F
Caudal
gabab2 profiles/section
n=6
n=6
n=5
n=5
***
**
***
Control
1 wpl
4 wpl
10 wpl
B
CONTROL
C
1 WPL_R
G
1 WPL_C
D
4 WPL_R
H
4 WPL_C
E
10 WPL_R
I
10 WPL_C

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7.3 Role of GABAB receptors in axonal regeneration after a complete SCI

7.3.1 Increased expression of the gabab1 subunit in identifiable descending neurons after SCI

GABAB receptors are obligate heterodimers formed by gabab1 and gabab2 subunits (Kammerer et al., 1999). We reported the expression of the gabab1 and gabab2 receptor subunits in identifiable reticulospinal neurons of adult sea lampreys under normal conditions (see above in Results, section 5.1.2). Here, we used gabab1 *in situ* hybridization first to confirm that this receptor is also expressed in identifiable descending neurons of mature larval sea lampreys (Fig. 17B, D, F) and then to quantify changes in its expression after SCI (Fig. 17A). In transverse sections of the brain the M1, M2, M3, I1, I3, I4, I5, B1, B3, B4, B6 and Mth neurons were included in the analyses. This revealed a significant increase in the expression of the gabab1 subunit in the M2 (ANOVA, p = 0.0049), M3 (ANOVA, p = 0.002), I1 (Kruskal-Wallis, p = 0.0022), I3 (Kruskal-Wallis, p = 0.0097), B1 (ANOVA, p = 0.0095) and B3 (Kruskal-Wallis, p = 0.0178) neurons (Fig. 17A; B-G; Table 4) and a non-significant increase in the expression of the gabab1 subunit in the M1, I4, I5, B4, B6 and Mth neurons (Fig. 18) in 1 wpl animals as compared to control un-lesioned animals. At 4 wpl, the expression of the gabab1 subunit was not significantly different to control un-lesioned animals in all the studied neurons (Fig.17A; Fig. 18). This shows that a complete SCI induced an increase in the expression of the gabab1 subunit in identifiable descending neurons.

Table 4. Mean ± SEM values of the number of gabab1 positive pixeles/section in identifiable descending neurons of control animals and injured animals. Refers to Figures 17 and 18.

gabab1 pixels/section	Control	1 wpl	4 wpl
M1	258,170±86,006	320,350±78,104	148,657±43,920
M2	237,552±61,602	618,158±176,397	131,367±19,089
M3	144,448±56,162	423,439±93,059	85,245±16,702
I1	165,195±127,550	627,179±137,246	176,392±74,810
I3	39,783±17,599	204,914±49,099	61,945±34,492
I4	68,678±11,645	147,613±44,903	45,723±7,610
I5	37,027±21,463	62,221±13,380	54,634±23,587
B1	198,893±57,286	429,418±43,503	202,694±75,156
B3	184,112±51,392	488,947±86,189	161,864±70,596
B4	183,470±49,454	317,944±79,980	274,012±88,414
B6	222,515±105,034	456,303±87,734	273,466±101,442
Mth	201,230±84,794	395,530±90,345	139,834±31,750

7.3.2 GABOB long-term treatment promotes axonal regeneration in descending neurons after SCI

Then, we studied the long-term effect of increasing GABAergic signalling in axonal regeneration after SCI. Neuronal trac-tracing revealed that a treatment with GABOB during 12 weeks significantly promoted axonal regeneration in identifiable neurons after a complete SCI as compared to control animals (Paired t-test, p = 0.0006; Fig. 19). This shows that an increase in GABAergic signalling promotes axonal regeneration after a complete SCI in lampreys.

7.3.3 Endogenous gaba signalling through gabab receptors promotes axonal regeneration after SCI

We should take into account that GABOB could be exerting its effects by activating GABA receptors expressed in the descending neurons and/or indirectly by activating GABA receptors expressed in other cells. So, we decided to used morpholinos to specifically knockdown the expression of the gabab1 subunit in descending neurons after a complete SCI (Fig. 20A, B-E; Fig. 21). First, we used *in situ* hybridization to confirm that the active gabab1 morpholino is able to knockdown the expression of the gabab1 mRNA in identifiable neurons of 2 wpl animals (M1: unpaired t-test, p = 0.0264; I1: unpaired t-test, p = 0.0066; Fig. 20A). Neuronal tract-tracing revealed that the treatment with the active gabab1 morpholino significantly inhibited axonal regeneration of identifiable descending neurons after a complete SCI as compared to the animals treated with the gabab1 mismatch control morpholino (Paired t-test, p = 0.0394; Fig. 20F-L). This results show that endogenous GABA promotes axonal regeneration of descending neurons after a complete SCI by activating GABAB receptors expressed in the descending neurons.

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7.3.4 Figures

Figure 17. Quantitative changes in the expression of the gabab1 subunit in identifiable reticulospinal neurons after a complete spinal cord transection. **A**: Graphs showing significant changes (*asterisks*) in the number of gabab1 positive pixels in the soma of identifiable descending neurons. The mean ± SEM values are provided in table 4. **B, F:** Photomicrographs of transverse sections of reticulospinal neurons showing the expression of the gabab1 transcript in their soma in control animals. **C, G**: Photomicrographs of transverse sections of reticulospinal neurons showing the increased expression of the gabab1 transcript in lesioned animals at 1 wpl. Scale bars: 20 µm.

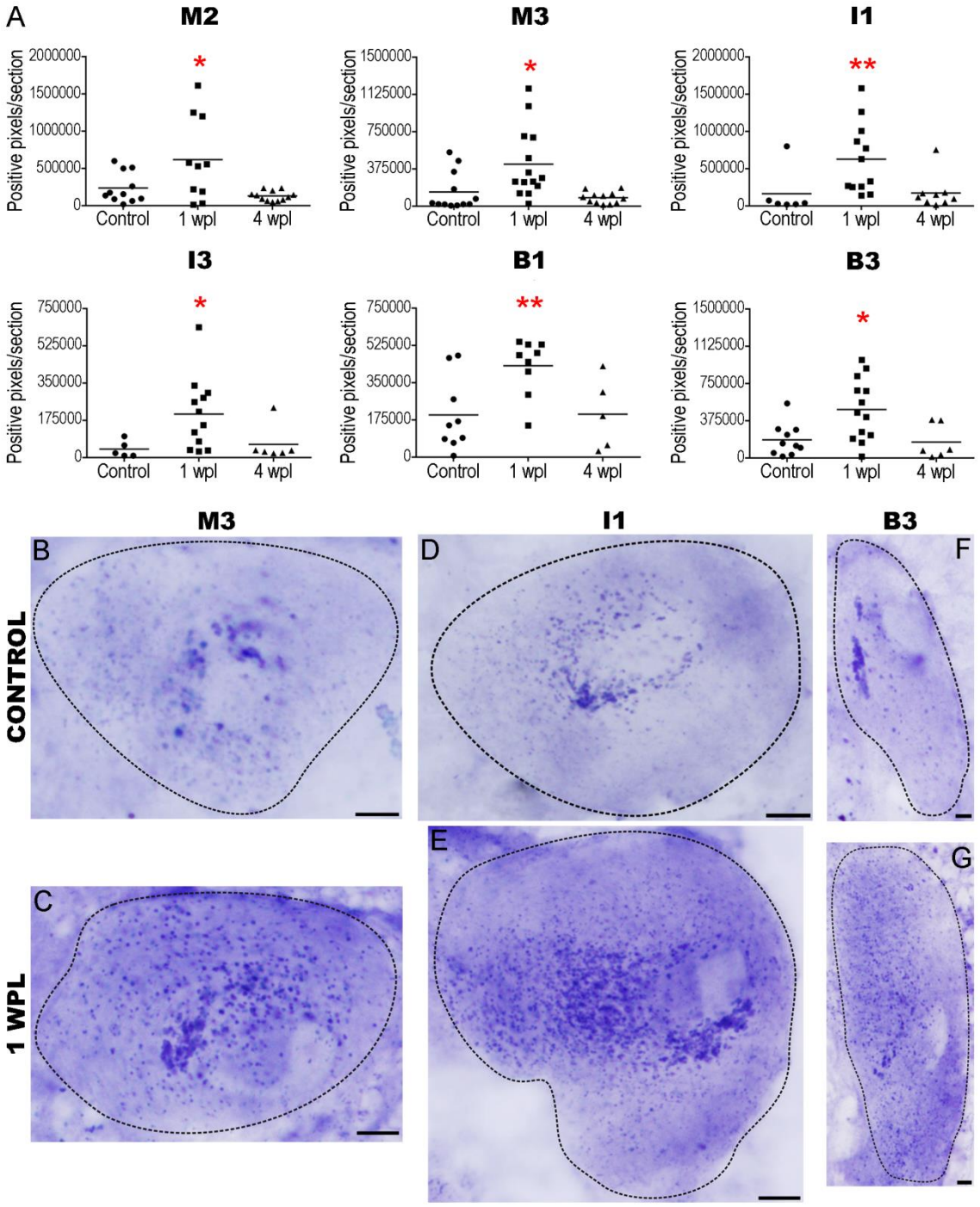
A
M2
M3
I1
I3
B1
B3
Positive pixels/section
Control
1 wpl
4 wpl
CONTROL
1 WPL
B
C
D
E
F
G

Figure 18. Graphs of identifiable neurons in which the expression of the gabab1 subunit did not change significantly after a complete spinal cord transection. The mean ± SEM values are provided in table 4.

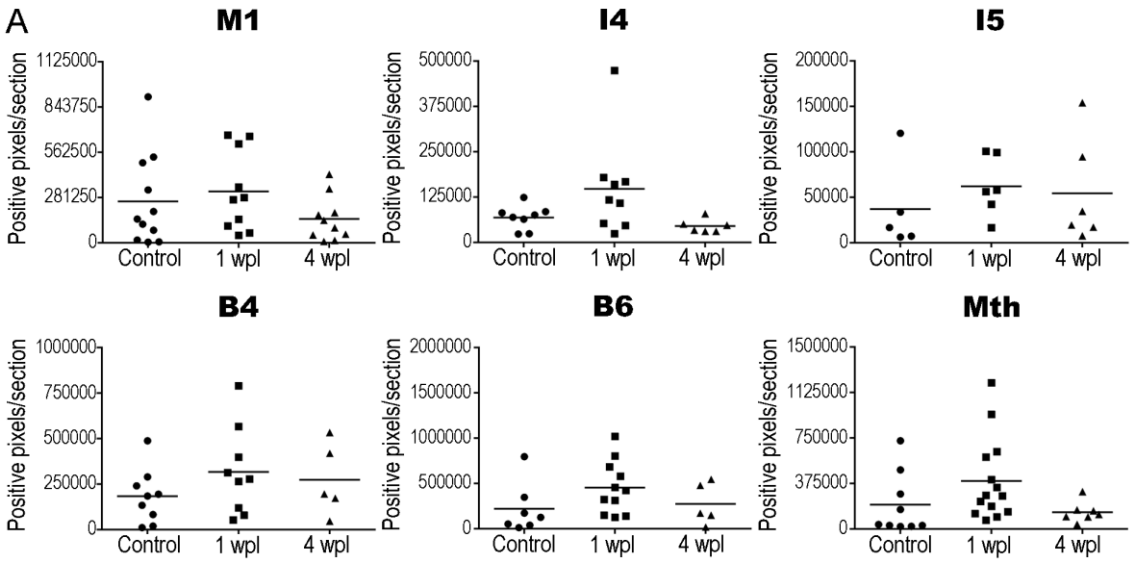

Figure 19. GABOB treatment promoted axonal regeneration 12 weeks after a complete SCI. **A, C, F:** Photomicrographs of whole-mounted brains showing different reticulospinal populations with a few regenerated identifiable neurons in control animals. **B, D, G:** Photomicrographs of whole-mounted brains showing different reticulospinal populations with an increase in the number of regenerated identifiable neurons in treated animals. **H:** Graph showing significant changes (*asterisks*) in the percentage of descending neurons with regenerated axons after GABOB treatment. Arrows indicate descending neurons that regenerated in treated animals but not in controls after the GABOB treatment. Scale bars: 50 µm.

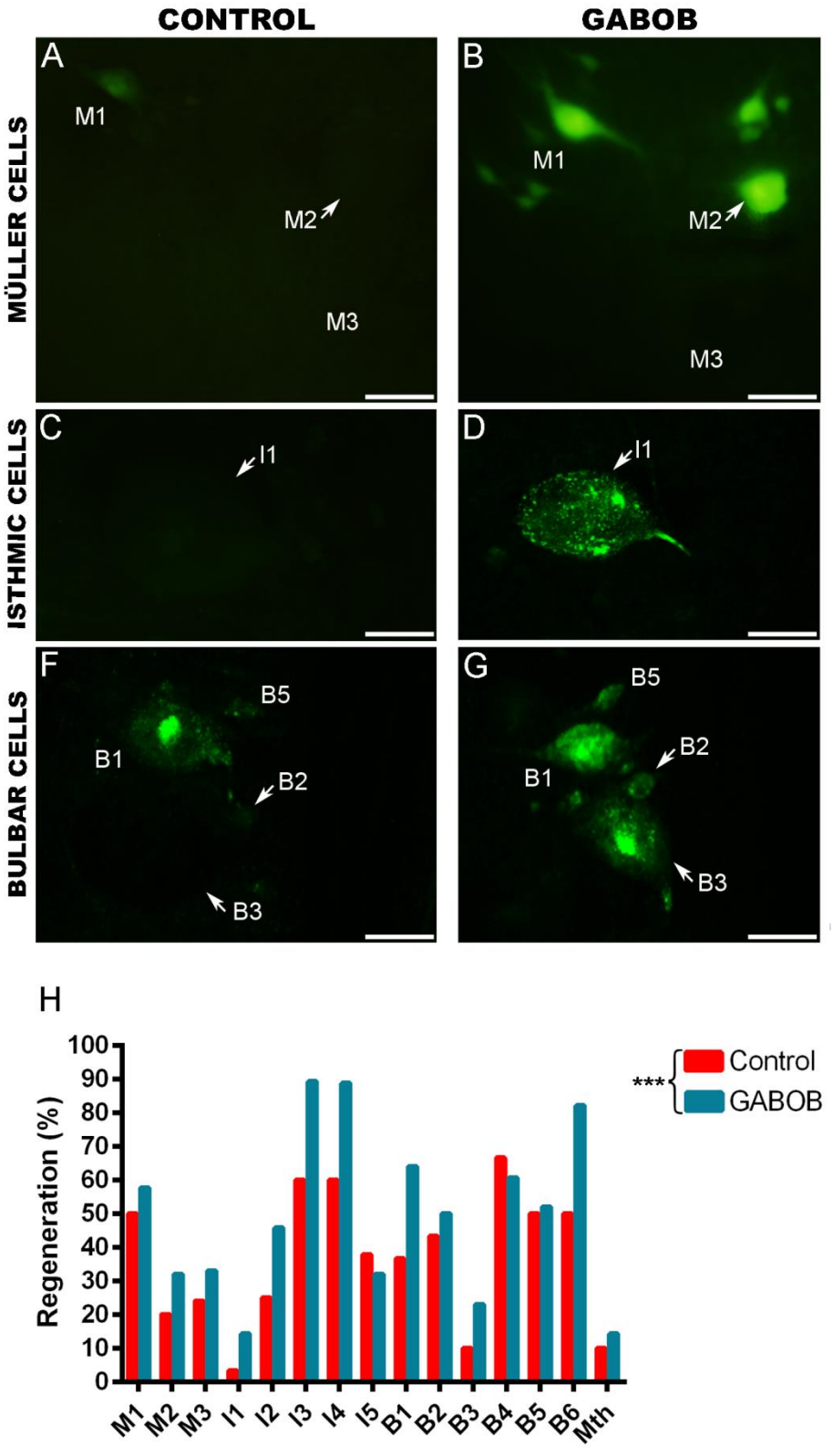
CONTROL
GABOB
MÜLLER CELLS
ISTHMIC CELLS
BULBAR CELLS
A
B
C
D
F
G
M1
M2
M3
I1
B1
B2
B3
B5
H
Regeneration (%)
100
90
80
70
60
50
40
30
20
10
0
M1 M2 M3 I1 I2 I3 I4 I5 B1 B2 B3 B4 B5 B6 Mth
***
Control
GABOB

Figure 20. Morpholino knockdown treatment and its effect on axonal regeneration. **A:** Graphs showing significant changes (*asterisks*) in the number of gabab1 positive pixels in the soma of M1 and I1 reticulospinal neurons after the morpholino treatment. **B, D:** Photomicrographs of transverse sections of M1 and I1 reticulospinal neurons showing the expression of the gabab1 transcript in control animals. **C, E:** Photomicrographs of transverse sections of M1 and I1 reticulospinal neurons showing the decreased expression of the gabab1 transcript in morpholino-treated animals. **F, H, J:** Photomicrographs of whole-mounted brains showing different reticulospinal populations with regenerated identifiable neurons in animals treated with the control morpholino. **G, I, K:** Photomicrographs of whole-mounted brains showing different reticulospinal populations with a decrease in the number of regenerated identifiable neurons in animals treated with active morpholino. **L:** Graph showing significant changes (*asterisks*) in the percentage of descending neurons with regenerated axons after morpholino knockdown treatment. Arrows indicate descending neurons that regenerated in control animals but not in animals treated with active morpholino. Scale bars: black, 20 µm; white, 50 µm.

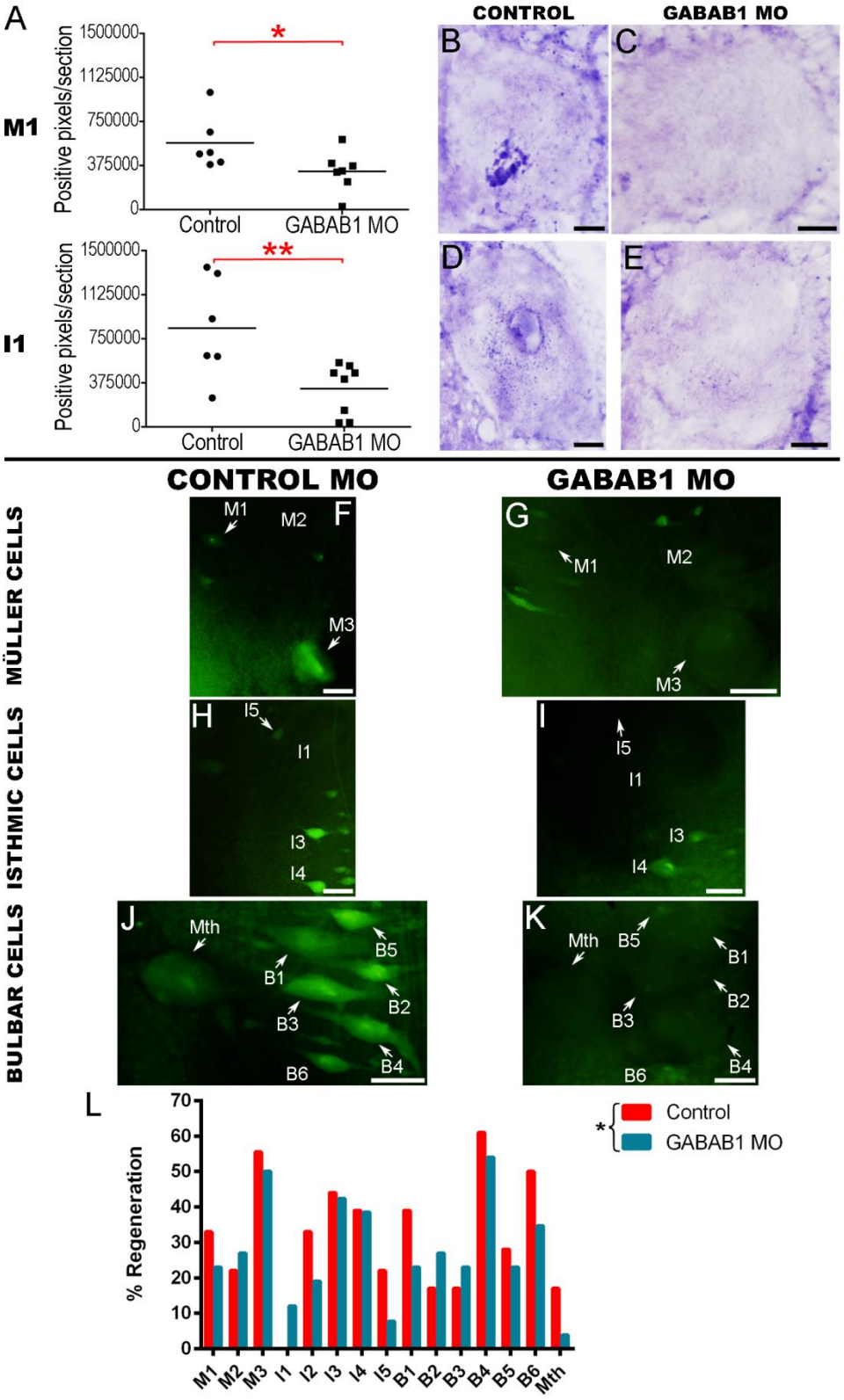
A
M1
I1
Positive pixels/section
1500000
1125000
750000
375000
0
Control
GABAB1 MO
*
**
CONTROL
GABAB1 MO
B
C
D
E
CONTROL MO
GABAB1 MO
MÜLLER CELLS
ISTHMIC CELLS
BULBAR CELLS
F
G
H
I
J
K
M1
M2
M3
I5
I1
I3
I4
Mth
B5
B1
B2
B3
B4
B6
L
% Regeneration
70
60
50
40
30
20
10
0
Control
GABAB1 MO
M1 M2 M3 I1 I2 I3 I4 I5 B1 B2 B3 B4 B5 B6 Mth

Figure 20. Partial sequence of the gabab1 subunit, with exons in red, and introns in black. Target sequence of the gabab1 probe is highlighted in green. The target sequence for gabab1 morpholino is highlighted in yellow (boundary of the second intron and second exon of the partial sequence).

CTACTA[…]GCGTAGGTCTACAAGGAGAAGCTCTACGGCAAGAAGCACGTG
TGGTTCATCATCGGCTGGTACGCCGACAAGTGGTACCTCAAGCCCGACCTG
GCCATCAACTGCACGGCCGAGCAGATGCAGGAGGCTGTCGAGGGTCAGGTC
ACCACGGAGATCCTCATGGTCGACCTGGCCAACACCCGCGGCGTCTCGAAC
ATGGTAACA[…]TAAGTCTCTCATGACTCTCCGTTGCAGACGTCGGACGAGT
TCATCAGCCGCCTGGTGAAGAACCTCACCAAGACTCCGGAGGAGACGGGAG
GCTTCCAGGAGGCCCCCTTGGCCTACGACGCCATCTGGGCGTTGGCACTGGC
CCTGAACAAGACGGCTCAGGAGCTCGCGAAGAAAGGTGTGC[…]CTGCAGG
GATGCGGCTGGAGGATTTCAATTACTCCGACAACACGATCACCAACGAGAT
CTACAAGGCGCTCAACTCGTCCTCGTTCGACGGCGTTTCGGTAAAC[…]ACG
CAGGGCCACGTGGTGTTTGACGCAAGCGGCGCGAGAATGGCCTGGACTCTC
ATAGAGCAGCTTCAAGGTGAGC[…]TTCTAGATGGCAAGTATGTGAAGATTG
GGTACTACGACAGCAACAACAATAATCTCTCCTGGCTCAACACGGACCGAT
GGATCGGTGTGTGT[…]CCTCCAGGAGGCTCGCCTCCACCCGACCGAACCAAGG
TGGTCATCCAGTTCCGCTACCTCTCGCAGAAGCTCTTCATCTCCCTCTCCGTC
CTCGCCGGAATCGGCATCATCTTCGGATGTGTGTGCCTCGTCTTCAACATCT
ACAACCGGAACGTCAGGTGAGC[…]CTGCAGATACATCCAGAACTCGCAGC
CCAACCTCAACAACTTGACGGCGCTGGGATGCATCCTCACGCTGGCCGTGG
TGCTACCACTTGGTCTGGACGGCCTCCACATCAGCGAGAGTCAGTTCCCGTT
CATCTGCCAGGTAATA[…]GTGCAGGTGCGCGTGTGGCTCCTGAGCATTGGA
TTCGGCATGGGCTACGGCAGCATGTTCACCAAGATCTGGTGGGTGCACACC
CTGTTCACCAAGAAGGACGACAAGAAGGAGATGAGACAGGTACGG[…]CCA
CCGTTAAACCCGCCGGTG[…]CCGCAGCAACTGGAGCAATGGAAGCTCTACG
TCACCGCGGCAGTTCTCATCTTCATTGATGCCGTCACCATCCTCATCTGGCA
GCTCGTCGACCCCTTGCAGAGAACCGTCGAGGTAATCCGT[…]ACGCAGGGC
TTCGGCAAGGAGAATCTGGTGGGCGACGACGTTGAGATACTCCCGCAGCTG
GAGCACTGCAGCTCGCGCAAGATGACCACGTGGCTGGGTGAGA[…]GCACA
GGCATCGTGTACACGTACAAGGGCCTCCTGCTGCTGCTGGGCATTTTCCTGG
CGTACGAGACCAAGAGCGTTTCCACGGAGAAAATCAACGACCACCGCTCCG
TGGGGATGGCGATTTACAACGTGTCGGTAATG[…]GTGCAGGTTCTGTGCAT
GATCACGGCGCCCGTGGCCATGATCGTGAGCAAGCAGCAGGATGCTTCCTT
TGCTTTCGCCGCGCTGGCCGTCATCTTCTCCTCCTACATCACCCTCATCGTCC
TCTTTGTGCCCAAGGTAAGC[…]CTGCAGATGCGGAGGCTGATCACGCGCGG
CGAGTGGCAGACGGAGCAGCACGACGCCAACAAGACCATCTCGACGACGC
TCAACAACGAGGAGGAGAAGTCGCGGCAGCTGGAGCGTGAGAACCGCGAG
CTGGAGCGCATCATCGCCGAGGTG[…]TGCGAA

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DISCUSSION

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8 DISCUSSION

8.1 CLONING OF THE GABAB RECEPTOR SUBUNITS B1 AND B2 AND THEIR EXPRESSION IN THE CENTRAL NERVOUS SYSTEM OF THE ADULT SEA LAMPREY

8.1.1 Relationships and phylogeny of the sea lamprey gabab genes and GABAB protein sequences

This thesis shows for the first time the identification and characterization of the gabab1 and gabab2 cDNA sequences and the distribution of the gabab transcripts in the central nervous system of the adult sea lamprey.

In our BLAST searches of the sea lamprey genome database, we found 1 gabab1 gene and 1 gabab2 gene, whose sequences were confirmed after PCR amplification and cloning of the respective cDNAs. The presence of only a single gene of each gabab subunit is also the case in mammals, birds and amphibians. Two paralogous copies of the gabab1 subunit gene have been found in zebrafish (Klee et al., 2012), which is probably due to the additional whole-genome duplication that occurred in the actinopterygian lineage (Taylor et al., 2001). In the phylogenetic trees, the GABAB1 and GABAB2 partial amino acid sequences of the sea lamprey were located at the base of the vertebrate branches clustering the GABAB1 and GABAB2 sequences, appearing as sister members, respectively, of the GABAB1 and GABAB2 sequences of gnathostomes. Each of the subunits is grouped in an independent cluster (Fig. 9). The location of the sea lamprey GABAB1 and GABAB2 sequences at the base of the vertebrate branches of the phylogenetic tree and its higher similarity with the vertebrate sequences than with those of *Drosophila* are in agreement with the phylogenetic position of lampreys and confirmed the GABAB identity of the sea lamprey sequences identified in our study.

8.1.2 Heterodimerization of the GABAB receptor

It is currently accepted that a functional GABAB receptor consists of a heterodimer of GABAB1 and GABAB2 subunits (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Bettler et al., 2004). GABAB1 binds to GABA, while GABAB2 is needed to transmit the signal, because G-protein coupling is mediated via GABAB2 (Margeta-Mitrovic et al., 2000; Calver et al., 2002; Gálvez et al., 2001; Geng et al., 2013; Pagano et al., 2001). Because of this, it seems clear that the co-expression of gabab1 and gabab2 mRNAs is necessary to form a functional GABAB receptor. In our study, we observed an overlapping expression of the gabab1 and gabab2 mRNAs in all brain regions and in the spinal cord of the adult sea lamprey when using consecutive brain sections. Co-localization of both subunits in the same single cells of lampreys is clear in the giant individually identifiable reticulospinal neurons like the Mauthner and Müller neurons. This is also the case in *D. melanogaster*, where no differences were observed between the expressions of gabab subunits in *in situ* hybridization assays (Mezler et al., 2001). In zebrafish, a recent study using qPCR methods has shown that the two b1 and the b2 subunits are all expressed in the same brain regions, with the b1b and b2 being more represented than the b1a in some regions and in the brain as a whole (Cocco et al., 2016). Kuner and colleagues (1999) observed by analyzing serial rat brain sections, that gabab1 and gabab2 transcripts are widely expressed and that they also show considerable overlap in most regions of the brain, although in some regions the

expression of the gabab1 transcript was enriched. This suggests that a wide and highly overlapping expression of the GABAB1 and GABAB2 subunits is an ancestral and conserved character of vertebrates and invertebrates.

In the mouse brain, an association between the GABAB2 subunit and M2 muscarinic receptors has been shown which appears to enhance muscarinic signaling (Boyer et al., 2009). In lampreys, pharmacological treatments combined with electrophysiological studies have shown a role for muscarinic receptors in the modulation of the trigeminal-reticular pathway (Le Ray et al., 2004) and in the activation of reticulospinal neurons (Smetana et al., 2007). Moreover, the presence of cells immunoreactive for muscarinic receptors has been shown in the region of the posterior rhombencephalic reticular nucleus of lampreys (Smetana et al., 2007), a region that shows expression of the gabab2 transcript (present results). Whether the modulation of muscarinic receptors by the GABAB2 subunit also occurs in lampreys needs further investigation. This would show us whether this is an ancestral characteristic of vertebrates.

8.1.3 Analysis of the functional significance of the gabab1 and gabab2 expression observed in the central nervous system of the sea lamprey

All brain regions and the spinal cord showed a broad expression of both gabab transcripts in the adult sea lamprey, which is in concordance with previous reports in invertebrates [e.g. *D. melanogaster* (Mezler et al., 2001), cockroaches (Blankenburg et al., 2015) or spiders (Panek et al., 2003)] and in jawed vertebrates [e.g. humans (Calver et al., 2000; Berthele et al., 2001), non-human primates (Muñoz et al., 1998, 2001), rats (Bowery et al., 1987; Bischoff et al., 1999; Clark et al., 2000), birds (Veenman et al., 1994), frogs (Kaeser et al., 2011) and zebrafish (Tabor et al., 2008; Cocco et al., 2016)]. Positive *in situ* signal in sea lamprey brain sections had a granular appearance probably due to low expression of these mRNAs in each single cell of the sea lamprey. Previous studies looking at the expression of different neurotransmitter receptors in lampreys have shown that the *in situ* hybridization signals appeared as a dotted labeling in sections of the CNS: serotonin receptor 1A (Cornide-Petronio et al., 2013, 2014), dopamine receptor D2 (Robertson et al., 2012; Fernández-López et al., 2015), and dopamine receptor D4 (Pérez-Fernández et al., 2016); suggesting that low expression levels are a common feature of different metabotropic neurotransmitter receptors.

The broad expression of these transcripts in the CNS suggests that this receptor is extensively used in the modulation of brain circuits in lampreys. The expression of both gabab transcripts in non-GABAergic cells, which can be identified by their size and/or location, such as the mitral cells of the olfactory bulbs, the giant reticulospinal neurons, the spinal motoneurons, the motoneurons of the visceromotor rhombencephalic nuclei, the primary sensory cells of the rhombencephalon and spinal cord or the edge cells of the spinal cord indicate that the GABAB receptor plays a role in the modulation of the activity of non-GABAergic cells in the sea lamprey brain. The expression of gabab transcripts observed here is in agreement with the GABAergic modulation of spinal motoneurons and interneurons mediated by the GABAB receptor as reported in previous pharmacological and electrophysiological studies (Alford and Grillner, 1991; Alford et al., 1991; Matsushima et al., 1993; Schmitt et al., 2004). Edge cells in the spinal cord are richly innervated by GABAergic fibers (Fernández-López et al., 2012) and recent work has shown that they are modulated by GABA (Svensson et al., 2013). Our results suggest that GABA could act trough the GABAB receptor in the edge cells. The present results also support the idea of the role of the GABAB receptor in the modulation of the lamprey respiratory network (Bongianni et al., 2006; Cinelli et al.,

2014). Also, in agreement with our expression results, a modulatory action of GABA onto the pathway from lateral columns to reticulospinal neurons has been suggested to be mediated by the GABAB receptor (Vinay et al., 1998). These reticulospinal inputs are also regulated by peptidergic transmitters (Parker, 2000). Our study extends the number of neuronal populations known to be modulated through GABAB signaling and opens the opportunity to conduct functional studies on the role of GABA and the GABAB receptor in other circuits.

The broad expression of the gabab transcripts together with the broad distribution of GABAergic cells in the brain and spinal cord (Meléndez-Ferro et al., 2001; Robertson et al., 2007) suggests that the sea lamprey GABAB receptor could modulate the activity of GABAergic cells. The expression of the gabab transcripts in periglomerular cells of the olfactory bulbs, which are mainly GABAergic (Meléndez-Ferro et al., 2001), or in the cerebrospinal fluid-contacting (CSF-c) cells of the spinal cord, which are mainly GABAergic as well (Villar-Cerviño et al., 2008b; Rodicio et al., 2008; Fernández-López et al., 2012; Jalalvand et al., 2014), could potentially support this hypothesis. Results from pharmacological and electrophysiological studies have shown that inhibitory premotor interneurons respond to the application of GABAB agonists (Alford and Grillner, 1991; Alford et al., 1991; Matsushima et al., 1993). Unfortunately, the different requirements of fixative for *in situ* hybridization and GABA immunohistochemistry precluded us from providing definitive demonstration of the presence of GABAB receptors in GABAergic cells of the sea lamprey. The present results show expression of the gabab transcripts in the dendrites of CSF-c cells of the spinal cord. Currently, some functions have been proposed for CSF-c neurons in controlling the composition of the CSF and releasing substances into the ventricular system (Vígh et al., 2004; Jalalvand et al., 2016). The GABAB receptor in these cells could play a role for detecting GABA in the CSF. The detection of serotonin from the CSF has been also proposed for the 5-HT1A receptor due its expression in CSF-c dendrites (Cornide-Petronio et al., 2013). As stated above, the GABAB receptor could modulate CSF-c cells acting as an autoreceptor and/or as a heteroreceptor trough synapses from other GABAergic cells.

8.1.4 Gabab1 and gabab2 expression in glial cells

Our results show a lack of expression of the gabab transcripts in ependymocytes along the lamprey brain. The ependymal cells are the main glial type present in the brain of the sea lamprey, while astrocytes are only associated to some nervous tracts. However, the spinal cord shows both types of glial cells (Retzius, 1893). In contrast to jawed vertebrates, oligodendrocytes are not present in lampreys. The glial cells of lampreys do not display immunoreactivity to glial fibrillary acid protein (GFAP), but they express cytokeratins (Merrick et al., 1995). Few works have been done to study the expression of gabab1 and gabab2 mRNAs and/or GABAB1 and GABAB2 subunits in the ependymal layer of vertebrates. A pharmacological study of Corns and colleagues, (2013) reported that only the GABAA, and not GABAB, receptor mediates the GABAergic responses in mammalian ependymal cells surrounding the central canal. More studies in other groups of vertebrates are necessary to determine the evolution of this character due to the large evolution distance between mammals and lampreys. In addition, the cells of the subcommissural organ, a special type of ependymal cells, also lack the GABAB receptor both in lampreys, and in other vertebrates like teleosts, frogs and mammals (Jiménez et al., 2000; Saha et al., 2000; Nürnberger and Schöniger, 2001). Studies in these vertebrates have shown that the GABAergic responses in the subcommissural organ are also mediated by GABAA receptors.

No expression of the gabab transcripts was observed in astrocytes of the optic nerve although previous studies have reported the presence of GABAB subunits in astrocytes, of rodents (Charles et al., 2003; Luyt et al., 2007; Oka et al., 2006; Beenhakker and Huguenard, 2010), and the expression of gabab1 and gabab2 mRNAs in human astrocytes (Lee et al., 2011). Again, more studies are necessary in other groups of vertebrates to determine whether the lack of GABAB expression in astrocytes is the ancestral condition or a derived character.

8.2 EXPRESSION OF THE GABAB RECEPTOR AFTER A COMPLETE SCI IN LAMPREYS

8.2.1 Functional relevance of gabab subunits downregulation after injury

Here, we observed a decrease in the expression of the gabab subunits after the complete SCI, which differs with the response of other neurotransmitter receptors. Following a complete SCI in lampreys, there is an acute increase in the expression of the serotonin 1a receptor (Cornide-Petronio et al., 2014) and there are no changes in the expression of the dopamine d2 receptor (Fernández-López et al., 2015). Plasticity is also seen at the functional level in lampreys. Excitability is increased below the site of lesion in lampreys recovered from a complete SCI (Cooke and Parker, 2009; Hoffman and Parker, 2011) and the cellular and synaptic modulatory effect of serotonin differs in lesioned and un-lesioned animals (Becker and Parker, 2015). Also, good recovery of locomotor function in lampreys following SCI is associated with stronger tonic GABAergic inhibition (Svensson et al., 2013). These studies stress the importance of studying each neurotransmitter system in lesioned animals (below and above the site of injury) to understand the mechanisms that lead to functional recovery in lampreys. This shows that the circuits underlying locomotion in lampreys are highly plastic after SCI, both at the anatomical and functional level.

8.2.2 Physiological relevance of gabab subunits downregulation after injury

The fast and full recovery of the number of GABAergic cells and processes after the initial loss of GABA immunoreactivity agrees with a previous physiological study in lampreys looking at the proprioceptive system, which showed that good recovery of function is associated with raised endogenous GABA levels (Svensson et al., 2013). Bicuculline (a GABAA receptor antagonist) only potentiates the bending-evoked responses in lesioned animals, which reflects that there is a stronger tonic GABAergic inhibition in lesioned animals through the GABAA receptor (Svennson et al., 2013). These results suggest that there is a need of high GABA levels for recovery following SCI in lampreys. Our study also shows that functional recovery is associated with lower levels of expression of gabab transcripts. The decrease in the expression of GABAB receptors could explain the increased inhibition through GABAA receptors in lesioned animals. First, a lower expression of post-synaptic GABAB receptors in the presence of a completely recovered GABAergic system means that more GABA is available to act on GABAA receptors. Second, GABAB receptors address second messenger systems through the binding and activation of guanine nucleotide-binding proteins (G-protein-coupled receptors), which inhibit adenylyl cyclase decreasing cAMP levels (Padgett and Slesinger, 2010). Lower levels of expression of gabab transcripts following SCI could lead to an increased availability of cAMP, which subsequently leads to increased protein kinase A (PKA) activity. Phosphorylation by PKA is a key factor to maintain a stable surface expression of GABAA receptors (Mele et al., 2016). So, decreased levels of gabab transcripts could lead to the anchoring of GABAA receptors in the cell surface in lesioned animals. Finally, a decrease in GABAB receptor expression in GABAergic cells could also potentiate GABA release, since GABAB receptors acting pre-synaptically are known to suppress GABA release (Bowery et al., 2002; Kobayashi et al., 2012). It would be of interest to analyse the possible changes in the expression of GABAA receptors in response to injury in future studies. This would require first to identify the GABAA subunits in the sea lamprey, which have not been properly annotated in the sea lamprey genome yet.

8.2.3 Comparison to other animal models

This is the first study demonstrating a reduction in the expression of the gabab receptor following a traumatic injury to the spinal cord in vertebrates. Previous studies have reported changes in rodent GABAB receptors after other types of spinal or brain injuries. The expression of GABAB receptors is decreased in the thalamus of rats after a traumatic brain injury (Drexel et al., 2015). The expression of post-synaptic GABAB receptors is also decreased in area 3b of the cortex and in the cuneate nucleus of the adult squirrel monkeys 1 to 5 years after median and ulnar nerve transection (Mowery et al., 2015). Castro-Lopes and colleagues (1995) observed a significant reduction in the expression of GABAB receptors 2-4 weeks after sciatic nerve ligation and in rats with chronic peripheral inflammation induced by injections of complete Freund´s adjuvant. Diabetic neuropathy also decreases protein and mRNA levels of GABAB receptors in the spinal dorsal horn of rats (Wang et al., 2011). It seems that a decrease in the expression of the GABAB receptor is a common characteristic after nervous system injuries in different vertebrate species.

8.3 ROLE OF GABAB RECEPTORS IN AXONAL REGENERATION AFTER A COMPLETE SCI

The analysis of the changes of expression of the gabab1 subunit in response to a complete SCI revealed a significant increase in the expression of this subunit in some identifiable descending neurons. As stated in the introduction, massive glutamate release and subsequent activation of glutamate receptors lead to an increase in Ca^{2+} influx into cells which causes excitotoxicity and neuronal death after SCI (Berdichevsky et al., 1983; Choi, 1988; Liu et al., 1991, 1999; see Mehta et al., 2013). GABAB receptors can cause the inactivation of voltage-dependent Ca^{2+} channels (Gaiarsa and Porcher, 2013). Therefore, an increase in the expression of GABAB receptors could compensate the influx of Ca^{2+} into axotomized descending neurons due to massive glutamate release. This might promote neuronal survival. So, in lampreys, this acute increase in the expression of the gabab1 subunit in descending neurons could be one of the mechanisms favouring neuronal survival and axonal regeneration after injury. A few studies have analysed the expression of both gabab subunits after brain injury in mammals, although none after SCI (traumatic brain injury: Drexel et al., 2015; cerebral ischemia: Huang et al., 2017). In contrast to lampreys, these studies showed that the expression of GABAB receptors decreases after the injury in different regions of the brain (Drexel et al., 2015; Huang et al., 2017). This could be an expected difference between regenerating and non-regenerating animals, since axons of the later do not show good regenerative abilities after CNS injuries. Interestingly, Huang and colleagues (2017) reported that an elevation in the protein expression of GABAB receptors in the cerebral cortex promotes neuroprotection after ischemic damage.

Encouraged by previous (Fernández-López et al., 2014) and present data, we performed gain and loss of function experiments, using pharmacological and genetic treatments, that support the role of GABA as a molecule that promotes true axonal regeneration of descending neurons through the site of injury. In fact, our experiments using a gabab1 morpholino demonstrated that endogenous GABA acts as a pro-regenerative factor after SCI by activating GABAB receptors expressed in the descending neurons. Our data agrees with previous *in vitro*, *in vivo* and developmental studies (Ferguson and McFarlane, 2002; López-Bendito et al., 2003). López-Bendito and coworkers (2003) showed that the GABAB antagonist CGP52432 decreases the length of the leading process in migrating inhibitory neurons in brain slice cultures of mice. Also, both GABA and baclofen stimulate retinal ganglion neurite outgrowth in *Xenopus* cultures and the GABAB antagonist CGP54262 shortened the developing optic projection *in vivo* (Ferguson and McFarlane, 2002). But, as far as we are aware, our results are the first *in vivo* demonstration showing that GABA promotes axonal regrowth after a CNS injury by activating GABAB receptors. Present and previous (Fernández-López et al., 2014) results indicate that the GABAergic system of lampreys responds successfully to a SCI to prevent retrograde degeneration and promote the regeneration of descending pathways.

We have revealed a major role for GABA and GABAB receptors in promoting the regeneration of individually identifiable descending neurons of lampreys following a complete SCI. Now, it would be of interest to decipher the underlying mechanisms behind the pro-regenerative effect of GABA. Based on previous results in lampreys showing a negative effect of Ca^{2+} in neurite outgrowth (Ryan et al., 2007; McClellan et al., 2008); a decrease in Ca^{2+} levels due to the activation of GABAB receptors could be one of the key events in the activation of axonal regeneration by GABA. In future studies it might be also interesting to analyse changes in gene expression elicited by

GABA to reveal new pathways involved in axonal regeneration in lampreys. Our results provide a strong basis to translate this knowledge to mammalian models of SCI for the development of new therapies for patients with SCI. A recent large observational cohort study has found that the early administration of gabapentinoids (which are administered as anticonvulsants for SCI patients) improves motor recovery following SCI (Warner et al., 2017). Interestingly, baclofen is already in use in the clinic, even for the treatment of SCI patients with neuropathic pain (Lee et al., 2013), which could facilitate the clinical translation of similar results in pre-clinical models of SCI.

CONCLUSIONS

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9 CONCLUSIONS

The analyses of the results obtained in the present thesis led us to the following conclusions:

1. The location of the sea lamprey GABAB1 and GABAB2 sequences in the phylogenetic trees, their similarity with those of other species and the analyses of their putative protein domains are in agreement with the phylogenetic position of lamprey and confirms their identity.
2. The extensive and overlapping expression of both gabab transcripts in neuronal populations of the CNS of the adult sea lamprey reveals a conserved pattern of expression as compared with other vertebrate and invertebrate species.
3. No expression of gabab transcripts was observed in the ependymal layer of the sea lamprey, which suggests that these cells might respond to GABA through GABAA receptors as in mammals.
4. The pattern of expression of both gabab subunits in the larval spinal cord is the same as the one observed in the adult spinal cord, indicating that this is established before the metamorphosis.
5. The decrease in the expression of both gabab subunits in the larval spinal cord, even in 10 wpl animals that had recovered normal appearing locomotion, indicates that plastic changes in the GABAergic system help to achieve functional recovery after SCI in lampreys.
6. The sustained decrease in the expression of the GABAB receptor following a CNS injury appears to be a conserved feature between lampreys and mammals.
7. The acute increase in the expression of the gabab1 subunit in descending neurons after a complete SCI supports the role of endogenous GABA as a pro-regenerative signal following SCI in lampreys.
8. The results of GABOB treatments show that GABAergic signalling promotes axonal regeneration of descending neurons after a complete SCI.
9. The results of the morpholino treatments confirm that endogenous GABA acting through GABAB receptors expressed in descending neurons promotes axonal regeneration after a complete SCI.

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PUBLICATIONS

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11 PUBLICATIONS

The results corresponding to “cloning of the GABAB receptor subunits B1 and B2 and their expression in the central nervous system of the adult sea lamprey” have been published in the article:

Romaus-Sanjurjo D, Fernández-López B, Sobrido-Cameán D, Barreiro-Iglesias A, Rodicio MC (2016) Cloning of the GABA(B) Receptor Subunits B1 and B2 and their Expression in the Central Nervous System of the Adult Sea Lamprey. *Front Neuroanat* 10:118.

The results corresponding to “expression of the gabab receptor after a complete SCI in lampreys” have been published together with the “changes in the GABAergic system during regeneration after SCI”, belonging to the doctoral thesis of Silvia Valle Maroto, in the article:

Romaus-Sanjurjo D, Valle-Maroto SM, Barreiro-Iglesias A, Fernández-López B, Rodicio MC (2018) Anatomical recovery of the GABAergic system after a complete spinal cord injury in lampreys. *Neuropharmacology* 131:389-402. Romaus-Sanjurjo D and Valle-Maroto SM are equal contributors.

The results corresponding to “role of GABAB receptors in axonal regeneration after a complete SCI”, together with the “role of GABA and baclofen in neuronal survival after a complete SCI”, results from in progress of the doctoral thesis of Rocío Ledo García, will be submitted to *Cell Death and Differentiation*:

Romaus-Sanjurjo D, Ledo R, Fernández-López B, Hanslik K, Morgan JR, Barreiro-Iglesias A, Rodicio MC (2018) GABA promotes survival and axonal regeneration in identifiable descending neurons after spinal cord injury in lampreys.

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