● PERSPECTIVE

Gamma-aminobutyric acid (GABA) promotes recovery from spinal cord injury in lampreys: role of GABA receptors and perspective on the translation to mammals

In mammals, spinal cord injury (SCI) is a devastating event that can lead to a permanent loss of motor, sensory and autonomic functions below the site of injury. In the last years, the role of different neurotransmitter systems on regeneration and recovery from SCI has been deciphered. For example, studies in lampreys have shown that neurotransmitters play a key role in modulating the survival and regeneration of brainstem descending neurons after SCI (Romaus-Sanjurjo et al., 2018; Sobrido-Cameán et al., 2018). Glutamate is known to play a prominent role after SCI since it causes excitotoxicity to non-injured neurons during the secondary phase after a traumatic injury both in mammals and lampreys (Fernández-López et al., 2014). In contrast, recent work in lampreys has shown that γ-aminobutyric acid (GABA) can play a neuroprotective and pro-regenerative role after SCI (Romaus-Sanjurjo et al., 2018). GABA is the main inhibitory neurotransmitter in the central nervous system (CNS) of vertebrates, and acts through both metabotropic G-protein-coupled GABAB receptors, and ionotropic ligand-gated chloride channel GABAA receptors. Here, we discuss recent work from our group and others on the possible role of different GABA receptors in neuronal survival and regeneration after SCI and provide a perspective on future work in this field.

Endogenous GABA promotes neuronal survival and axon regeneration after SCI in lampreys: In contrast to mammals, lampreys spontaneously recover normal appearing locomotion after a complete SCI. Functional recovery occurs in part because, approximately, 50% of lamprey descending neurons regenerate their axons after a complete SCI. The brainstem of lampreys contains 36 descending giant neurons that can be identified individually. Interestingly, these neurons show different survival and regenerative abilities after SCI, even when their axons run in the same spinal cord environment. This offers an interesting vertebrate model in which to inhibit or promote neuronal survival and regeneration in the same *in vivo* preparation after SCI.

In lampreys, GABA is massively released after a complete SCI and it accumulates around some of the axotomized axons of reticulospinal neurons (Fernández-López et al., 2014). Moreover, we found a significant correlation between GABA accumulation and a high survival ability of the corresponding neurons (Fernández-López et al., 2014). These initial results led us to study the possible role of GABA as a neuroprotectant and/or pro-regenerative factor after SCI in lampreys. In a recent article, we showed that endogenous GABA promotes the survival and axon regeneration of identifiable descending neurons of lampreys after a complete SCI (Romaus-Sanjurjo et al., 2018). Treatments with GABA [or GABOB (a GABA analogue)] and baclofen (a GABAB agonist) inhibited caspase activation and promoted axonal regeneration in identifiable reticulospinal neurons after a complete SCI in lampreys. Moreover, a morpholino treatment showed that endogenous GABA acts as a pro-regenerative factor by activating GABAB receptors expressed in descending neurons after a complete SCI in lampreys (Romaus-Sanjurjo et al., 2018). Now, it would be of interest to understand the contribution of different GABA receptors in this process and the secondary pathways activated by these receptors in injured neurons.

Role of GABAB receptors after CNS injuries: GABAB receptors are obliged heterodimeric G protein-coupled receptors composed of two receptor subunits, GABAB1 and GABAB2. The GABAB1 subunit is responsible for GABA binding, while the GABAB2 subunit mediates the G-protein response. Once GABA binds to a GABAB receptor, the Gαi protein is activated, which inhibits adenylyl cyclase and inactivates voltage-dependent Ca^{2+} channels (VDCCs) (Mele et al., 2016).

As far as we are aware, there are no functional reports studying the role of GABAB receptors after a SCI in mammals. Only a few articles have reported studies on the expression of GABAB receptors after different types of brain injuries. These studies reveal that, in the brain of mammals, the expression of GABAB subunits decreases after injury (Huang et al., 2017). Interestingly, this study also reported the neuroprotective effect of elevating GABAB expression after ischemic damage in rats (Huang et al., 2017). In lampreys, the expression of the gabab1 subunit increases in identifiable descending neurons after a complete SCI (Romaus-Sanjurjo et al., 2018). Giving the regenerating nature of lampreys, this observation agrees with the results of Huang et al. (2017) and supports the idea that GABA signaling through GABAB receptors is beneficial for neuronal survival after injury. In our recent work in lampreys, we showed that a treatment with baclofen decreased caspase activation and promoted axonal regeneration in reticulospinal neurons after a complete SCI (Romaus-Sanjurjo et al., 2018). The question that remains to be answered is: how are GABAB receptors promoting neuronal survival and axon regeneration? We have proposed that GABA acting through GABAB receptors could compensate the increase in intracellular Ca^{2+}, which is mainly caused by massive glutamate release after injury. This would protect neurons against excitotoxicity, which in turn would allow for the activation of axonal regeneration mechanisms after SCI (Romaus-Sanjurjo et al., 2018). Future work should analyze changes in gene expression elicited by baclofen in injured neurons to find new genes involved in neuronal survival and axon regeneration in these animals. A recent data article from our group has also shown that a muscimol (a GABAA agonist) treatment also decreased caspase activation in identifiable descending neurons after a complete SCI in lampreys (Sobrido-Cameán et al, 2018).

Roles of GABAA receptors and cation-chloride co-transporters after injury: are they a possible target to promote neuroprotection and/or regeneration after SCI? The GABAA receptor is a pentamer forming a chloride ion channel. GABA binding opens the chloride ion channel, hyperpolarizing mature neurons. In mammals, 19 distinct genes encode GABAA receptor subunits (α1–6, β1–3, γ1–3, δ, ε, π, ρ1–3, τ). This allows for many different combinations, with different pharmacologic and ion channel properties, which are expressed in different neuronal populations, and even with different membrane locations in a given cell (Mele et al., 2016).

In the CNS, the maintenance of the intracellular concentration of chloride ($[Cl^-]_i$) depends mainly on cation-chloride co-transporters. The cation-chloride co-transporters family is composed of three groups: Na^+-Cl^- co-transporters, Na^+-K^+-$2Cl^-$ co-transporters (NKCCs), and K^+-Cl^- co-transporters (KCCs). Na^+-Cl^- co-transporters are not expressed in the nervous system. Na^+-K^+-$2Cl^-$ co-transporters have two isoforms, NKCC1 and NKCC2, of which only NKCC1 is expressed in the CNS (neurons, glial cells, choroid plexus and vascular endothelial cells). While the function of NKCC1 is to increase the $[Cl^-]_i$, the different isoforms of KCCs (KCC1–4) are responsible for decreasing the $[Cl^-]_i$. Of these KCCs isoforms, KCC2 plays a key role in mature neurons, where it is responsible for the low $[Cl^-]_i$ of these cells (**Figure 1A**).

A study by Naik et al. (2012) showed that the application of muscimol to the dorsal root ganglion of rats restored protein expression of peripheral myelin protein 22, which is a protein of the basal lamina that plays a key role in peripheral myelin formation and nerve regeneration. Our recent results in lampreys have shown that a treatment with muscimol decreased caspase activation in identifiable reticulospinal neurons after a complete SCI (Sobrido-Cameán et al., 2018). Together, these results suggest that activation of GABAA receptors has a beneficial effect after a nervous system injury.

It has been well documented that, under certain circumstances, as during development or after CNS injury, GABAA receptor activation causes neuronal depolarization (Payne et al., 2003). In immature neurons, there is a high expression of NKCC1 and a low expression of KCC2, which leads to a higher $[Cl^-]_i$ compared to the extracellular concentration of chloride ($[Cl^-]_e$), resulting in a net efflux of this ion and neuronal depolarization after the activation of the GABAA receptor-coupled channel. This depolarization also leads to an activation of VDCCs, which increases $[Ca^{2+}]_i$. This is needed for neuronal differentiation, growth and maturation. After CNS injuries, there is a downregulation of the KCC2 protein and no change in the expression of NKCC1 (**Figure 1B**), which increases the $[Cl^-]_i$ leading to depolarizing GABAA receptor-mediated responses, like during development (Payne et al., 2003) (**Figure 1B**). It has been shown that the GABAA-induced $[Ca^{2+}]_i$ increase is required for neurons to become dependent on brain-derived neurotrophic factor (BDNF) for survival (Shulga et al., 2008). BDNF acting through the TrkB receptor is essential for the *in vivo* survival of lesioned corticospinal neurons. Subsequently, BDNF also causes an upregulation in KCC2 expression of lesioned corticospinal neurons to prevent depolarizing GABAA-mediated responses (Shulga et al., 2008). This suggests that injured neurons need to reactivate developmental programs to promote neuronal survival and regeneration after injury. We propose a scenario in which there is a balance and timing in the influence of the different GABA receptors. Immediately after SCI, GABAB receptors would protect against the toxic excess of Ca^{2+} ions by inactivating VDCCs. Then, depolarizing GABAA responses would activate VDCCs triggering BDNF release (**Figure 1B**), which activates TrkB receptors and promotes neuronal survival (**Figure 1B'**). BDNF would also lead to the upregulation of KCC2 expression to return to "normal" hyperpolarizing GABAA-mediated responses (**Figure 1B'**).

β Subunits, μ2 subunit, and δ subunit have a critical motif for GABAA receptor interaction with the adaptor protein 2 (AP2). When this motif is dephosphorylated, AP2 binds to GABAA receptors and triggers internalization. Phosphorylation by protein kinase A, protein kinase C, and calcium/calmodulin-dependent kinase II increases surface levels of β subunit containing GABAA receptors. It is known that in developing neurons BDNF release is triggered by depolarizing GABAA receptors, and this BDNF activates the

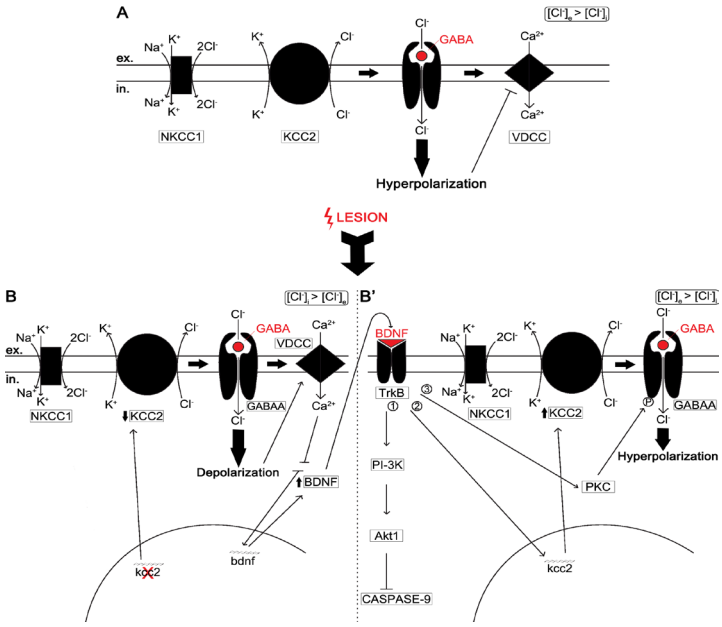

Figure 1 Role of NKCC1 and KCC2 cotransporters in the effect of Cl^- flow through GABAA receptor.
(A) In a normal situation, the expression of both cotransporters leads to a higher $[Cl^-]_e$ compared to the $[Cl^-]_i$. This results in neuronal hyperpolarization when GABAA receptors are activated, which inactivates VDCC channels. (B) After injury, the expression of KCC2 cotransporter decreases, leading to a higher $[Cl^-]_i$ compared to the $[Cl^-]_e$, which results in depolarizing GABAA receptor-mediated responses. This depolarization activates VDCC channels, which increases $[Ca^{2+}]_i$ promoting the synthesis and release of BDNF. (B') BDNF acts through TrkB receptors and triggers different signaling pathways for neuronal survival and reverts GABA signaling to the "normal" situation: (1) BDNF decreases caspase-9 activation, promoting cell survival; (2) BDNF upregulates the expression of KCC2, restoring the "normal" $[Cl^-]_i$ that leads to hyperpolarizing GABAA receptor-mediated responses; (3) the binding of BDNF with TrkB receptor can also result in the activation of protein kinase C, which phosphorylates GABAA receptors increasing their presence in the cell surface. PI-3K: Phosphoinositide 3-kinase; Akt1: RAC-alpha serine/threonine-protein kinase (protein kinase-B); NKCC1: Na^+-K^+-$2Cl^-$ co-transporter 1; KCC2: K^+-Cl^- co-transporter 2; GABA: γ-aminobutyric acid; VDCC: voltage-dependent Ca^{2+} channel; BDNF: brain-derived neurotrophic factor; PKC: protein kinase C.

TrkB pathway resulting in a higher phosphorylation of GABAA receptors *via* protein kinase C (Mele et al., 2016). Interestingly, a study in lampreys revealed the existence of stronger tonic GABAA receptor-mediated currents after SCI (Svensson et al., 2013). This could be due to a higher surface presence of GABAA receptors caused by the activation of the BDNF/TrkB pathway and a subsequent increased phosphorylation of GABAA receptors (**Figure 1B**'). The increased membrane presence of GABAA receptors could favor the recovery process after SCI. Future studies should experimentally test these hypotheses in the SCI situation.

Future directions: As shown here, recent studies in lampreys suggest a beneficial role for GABAergic signaling through both GABAA and GABAB receptors after SCI. Now, it would be interesting to test if a combinatorial treatment with baclofen and muscimol can improve neuronal survival, regeneration and functional recovery after SCI in mammals. As proposed here, an acute treatment with baclofen could protect against excitotoxicity after SCI. Then, a treatment with muscimol could activate BDNF release and further promote neuronal survival. The study of possible interactions between GABAA and GABAB receptor signaling will reveal molecular pathways with important implications for SCI patients.

The study of the cation-chloride co-transporters NKCC1 and KCC2 as therapeutic targets also offers a new way to find treatments for SCI patients. In fact, recent results have shown that a treatment with a KCC2 agonist was able to facilitate the relay of brain inputs towards the lumbar spinal cord after a SCI in mice (Chen et al., 2018). So, the pre-clinical development of pharmacological treatments targeting cation-chloride co-transporters might be a promising approach in SCI research.

We would like to thank the following individual donors of the crowdfunding campaign in Precipita: Blanca Fernández, Emilio Río, Guillermo Vívar, Pablo Peŕez, Jorge Fernández, Ignacio Valiño, Pago de los Centenarios, Eva Candal, María del Pilar Balsa, Jorge Faraldo, Isabel Rodríguez-Moldes, José Manuel López, Juan José Pita, María E. Cameán, Jesús Torres, José Pumares, Verónica Rodríguez, Sara López, Tania Villares Balsa, Rocío Lizcano, José García, Ana M. Cereijo, María Pardo, Nerea Santamaría, Carolina Hernández, Jesús López and María Maneiro.

This work was supported by grants from Spanish Ministry of Economy and Competitiveness and the European Regional Development Fund 2007–2013 (BFU2014-56300-P and BFU-2017-87079-P); a fellowship from EMBO (7010, to DRS); a grant from the Xunta de Galicia (2016-PG008, to ABI) and a grant from the crowdfunding platform Precipita (FECYT; Spanish Ministry of Economy and Competitiveness; 2017-CP081).

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***Received:** January 17, 2019*
***Accepted:** March 13, 2019*

***doi:** 10.4103/1673-5374.257515*

***Plagiarism check:** Checked twice by iThenticate.*
***Peer review:** Externally peer reviewed.*

***Open peer reviewer:** Armin Blesch, Indiana University School of Medicine, USA.*
***Additional file:** Open peer review report 1.*

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P-Reviewer: Blesch A; C-Editors: Zhao M, Li JY; T-Editor: Jia Y