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The role of gamma-amino-butyric acid type B receptors in Schwann cell proliferation and differentiation

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Summary

This study is focused on the localisation and function of γ-amino-butyric acid type B ($GABA_B$) receptors in the peripheral nervous system. I studied spontaneously proliferating Schwann cells in a culture system with dorsal root ganglion cells from 17 days old rat embryos. I found that $GABA_B$ activation by the specific agonist baclofen gave no effect on proliferation, in opposite to what has earlier been shown in a model with artificial proliferation. I also found the initially uniform $GABA_B$ receptor expression of the Schwann cells getting increasingly restricted to the nodes as they were established by myelination in one-month-old cultures. Together with the fact that non-myelinating Schwann cells had a clear and uniform $GABA_B$ expression, this observation supported the suspicion that Schwann cell $GABA_B$ receptors would be negatively coupled to myelination. I did not find an effect on myelination in the dorsal root ganglion cultures by baclofen treatment, although more experiments are needed to verify this. In adult rat sciatic nerve, I could see that $GABA_B$ receptors seemed to be expressed more in and around the nodes than along the rest of the myelinating Schwann cells. Therefore, it looks like $GABA_B$ receptors could be involved in the control of node formation and maintenance.

Abbreviations

BrdU, 5-bromo-2-deoxyuridine
cAMP, cyclic adenosine monophosphate
Caspr1, contactin associated protein 1 (a paranodal marker)
CNS, central nervous system (brain and spinal cord)
DRG, dorsal root ganglion
E17, embryonic day 17
GABA, γ-amino-butyric acid
GFAP, glial fibrillary acidic protein, a cytoskeletal protein in glial cells
MBP, myelin basic protein
MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
P0 and P10, postnatal day zero (day of birth) and postnatal day 10
PNS, peripheral nervous system (all neural tissue outside the CNS)
RIP, receptor interacting protein (an early myelin marker)

Introduction

γ-amino-butyric acid – an amino acid involved in inhibition

γ-amino-butyric acid, GABA, is the major inhibitory neurotransmitter of the central nervous system (CNS). GABA is synthesized from glutamate by glutamic acid decarboxylase, GAD and exerts its action via both ionotropic ($GABA_A$, $GABA_C$) and metabotropic ($GABA_B$) receptors. One clinically interesting CNS function of GABA is modulating nociception, the perception of pain (Enna and McCarson, 2006). GABA and its receptors have been found also in the peripheral nervous system although their actions in the PNS are far from clear (Towers *et al.* 2000).

γ-amino-butyric acid B receptors control several inhibitory pathways

$GABA_B$ receptors exist in two isoforms, B1 and B2, which together make up a functional, membrane bound, heterodimer. Expression of the two kinds is not always temporally and spatially matched. When alone, the subunits are stored in the endoplasmatic reticulum (Bettler *et al.* 2004). The receptors are G-protein-coupled, and they largely exert their function through $G_{\alpha i}$ and $G_{\alpha 0}$ proteins, which inhibit adenylyl cyclase activity (Hill, 1985). Adenylyl cyclase transforms ATP into cyclic AMP (cAMP), a molecule that is central in cell metabolism. Inhibition of adenylyl cyclase thus lowers cAMP levels and thereby reduces the activity of protein kinase A, a cAMP-dependent protein that among other things modulates ion channels (Bettler *et al*. 2004). Meanwhile, the β- and γ-units of the $GABA_B$ G-protein inhibit voltage gated Ca^{2+} channels and hyperpolarize the cell membrane by stabilizing open K^+-channels (Bowery *et al*. 2002). $GABA_B$ receptors in synaptic areas thereby can have both slow and fast inhibitory effects on the release of neurotransmitters and on the signalling of postsynaptic neurons. $GABA_B$ receptors may also have long-term effects through interactions with transcription factors (Bettler et al. 2004).

The synthetic $GABA_B$-agonist baclofen can be used to mimic the actions of GABA. Baclofen is specific, and its inhibitory action in the brainstem is used in relieving muscle spasms (Purves *et al*. 2008, p. 124). There are many synthetic $GABA_B$ antagonists and one example of a specific competitive antagonist is CGP62349 (Urwyler *et al.* 2005).

Schwann cells – essential partners of peripheral nerve cells

All nerve cells depend on direct contact with glial cells for energy, protection and developmental guidance (Jessen and Mirsky, 2005). In the CNS, the main glial types are astrocytes and oligodendrocytes. Astrocytes are important for ensuring homeostasis for the energy demanding neurons, and oligodendrocytes insulate axons (Purves *et al*. 2008, p. 10). In the peripheral nerves, these different tasks are carried out by a group of glial cells called Schwann cells. They are divided into the myelinating and the non-myelinating Schwann cells. Thicker peripheral axons are insulated by myelination, where the Schwann cell winds itself around an axon in multiple layers. This creates insulation interrupted by evenly distributed gaps, or nodes of Ranvier, between every myelin sheath (Fig. 1). Electrical signals from the neural cell bodies jump from node to node, so that conduction in these axons becomes very fast. The thinner axons are not myelinated but embedded in non-myelinating Schwann cells (Fig. 2) (Jessen and Mirsky, 2005).

Schwann cells are present around all peripheral axons but are not the only peripheral glia; the enteric nervous system has its own glial cells, as does the olfactory nerve and other nervous structures (Jessen and Mirsky, 2005). Schwann cells in rat sciatic nerve have been shown to express $GABA_B$ receptors (Magnaghi *et al*. 2004).

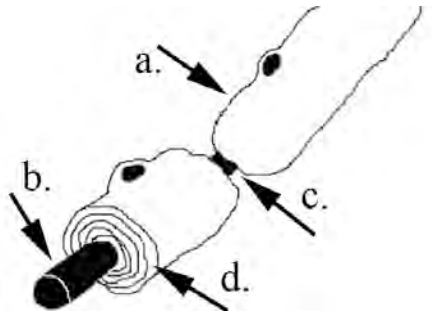

Figure 1. Myelinating Schwann cells around a thick calibre axon. **a.** Schwann cell. **b.** Axon. **c.** Node of Ranvier. **d.** Myelin layers.

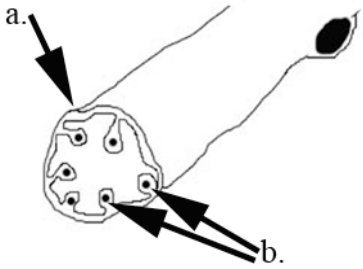

Figure 2. Non-myelinating Schwann cell around thin calibre axons. **a.** Schwann cell **b.** Axons

Proliferate or differentiate? – A developmental question

Forming functional organs and organisms out of a couple of stem cells is a huge task. It involves billions of "right choices" when every cell is to continue dividing or move into one of many new stages of maturation. Schwann cells originate from the neural crest; a developmental structure adjacent to what will become the brain and spinal cord. Neural crest cells are also the progenitors of a variety of other cell types such as melanocytes, neurons of the autonomic ganglia and facial cartilage cells (Purves *et al.* 2008, p. 547).

For a Schwann cell aspirant, the next step after being neural crest is to migrate to associate with axons and become a Schwann cell precursor. These precursors are flexible and may turn in to fibroblasts (extracellular matrix-producing cells) *in vivo*, and *in vitro* even to melanocytes (pigment-producing cells). Schwann cell precursors become immature Schwann cells through signals from neighbouring axons and through the establishment of autocrine survival factor expression. They can be identified by expression of glial fibrillary acidic protein (GFAP) and S100, another glial marker. In rat, this transition takes place around embryonic days 15 to 17. During the same period, the peripheral nerves start to get their blood supply and their structure is finalized (Jessen and Mirsky, 2005).

The ultimate decision to differentiate rather than proliferate is made when the immature Schwann cell becomes a myelinating or non-myelinating Schwann cell, entirely depending on whether it receives signals from a thick or thin calibre axon. Exactly what these signals are, or which signals are responsible for the nodal gaps, is not known (Jessen and Mirsky, 2005). Interestingly, Schwann cell development does not end here. When a nerve is cut, its myelinating Schwann cells can dedifferentiate back to the immature state. As the axon distal to the injury degenerates, the myelin sheaths are fragmented and phagocytosed by macrophages and by the Schwann cells themselves. The rejuvenated Schwann cells serve as scaffolds for the regenerating axons to grow in and also help the axons grow by upregulating neural growth factor expression. Eventually new myelin sheaths are formed and the nerve may heal to near pre-injury capacity (Scherer and Salzer, 2001).

Axon lesions in the CNS are not healed in a similar fashion. The myelin of the afflicted nerve is not as effectively removed and the CNS glia act to form a compact scar instead of promoting regrowth. The mechanisms behind the difference in healing potential are of course of great interest for all scientists aiming to develop therapies for patients with CNS injuries (Filbin *et al.* 2001).

Culturing cells from the dorsal ganglion

Dorsal root ganglions (DRGs) are capsules of sensory neurons, glial cells and connective tissue that lie outside the spinal cord. Cultures from DRGs therefore are composed of neurons, satellite cells, Schwann cells and fibroblasts (Fig. 3). All types of cell cultures have specific demands on their media. By co-culturing neurons and glia, fewer synthetic factors have to be added in order for the cells to proliferate. Both Schwann cell precursors and immature Schwann cells provide survival factors for neurons *in vivo*. Neurons return this favour by supplying mitogens and survival factors (Jessen and Mirsky, 2005). The Schwann cells in DRG cultures do not spontaneously myelinate the growing axons. Ascorbic acid can be added as a differentiation factor. This helps the Schwann cells to establish their basal lamina and make myelin (Eldridge *et al.* 1987).

In a model with cultures of only Schwann cells, Valerio Magnaghi and colleagues showed that $GABA_B$ receptor activation by baclofen reduces Schwann cell proliferation *and* decreases myelin protein expression (Magnaghi *et al*, 2004). In this model, the proliferation is induced with forskolin, which increases adenylate cyclase activity and thereby cAMP production. It has long been known that baclofen does not affect basal levels of cAMP but lowers forskolin-induced levels of this compound (Hill, 1985). *In vivo*, nerve cells are likely providing the proliferation cues. Since Schwann cells proliferate without forskolin in DRG cultures, the mechanisms controlling proliferation *in vivo* may still be intact (Svenningsen *et al*. 2003).

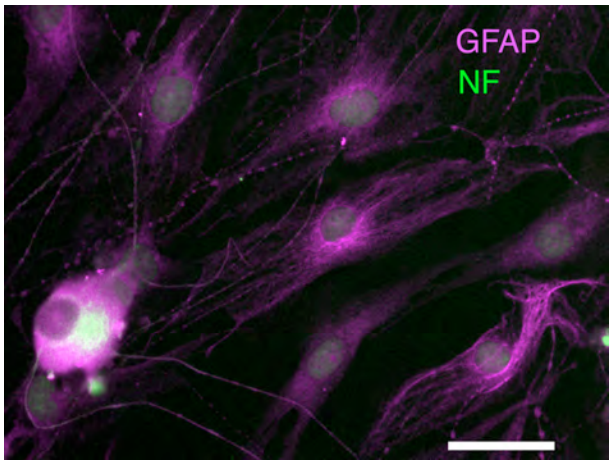

Figure 3. A neuron and several Schwann cells in a DRG-culture with Schwann cell marker GFAP labelled in magenta and neurofilament (NF) in green. Co-localisation is white. NF and GFAP are both filamentous cytoskeleton proteins and some unspecific antibody binding has occurred in this picture. Scalebar: 30 μm

One way of visualizing proliferation in a cell culture is to add 5-bromo-2-deoxyuridine (BrdU) some hours before fixation. BrdU is a thymidine analogue that is incorporated into DNA in the replication process. Immunostaining against BrdU therefore visualizes all dividing cells.

Immunohistochemistry – highlighting proteins of interest
Immunohistochemistry is the art of using the biological specificity of antibodies to visualize the distribution of a given protein in cell and tissue samples. Antibodies against a certain protein are produced by B-lymphocytes in animals injected with that protein. The antibodies can then be purified from the animal's serum. When added to, for instance, a tissue section, the antibodies bind to their target protein. These primary antibodies can have a fluorescent dye attached, which makes their binding sites visible in a fluorescence microscope. For flexibility, it is more common to use secondary antibodies on top of the primary ones. They are made to bind all antibodies made in a species and are labelled with a fluorophore. A fluorescence microscope sends out light of the different wavelengths that excites the fluorophores and makes them visible (Alberts *et al*. 2002, p. 287).

A test that makes proliferation visible
The MTT-test uses the compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, or MTT, to measure the metabolic activity of cell cultures. MTT is water soluble and yellow until it is taken up by functional mitochondria, where its tetrazolium ring is cleaved by dehydrogenases. The cleaved substance is insoluble in water and forms purple crystals. When dissolved in isopropanol, these crystals give the solution a purple colour. The intensity of this colour reflects the metabolic activity and can be measured spectrophotometrically (Edmondson *et al*. 1988).

Aims
The aim of this degree project was to study the localisation of $GABA_B$ receptors in embryonic rat DRG cell cultures and rat sciatic nerve. I also looked at the effect of the specific $GABA_B$ agonist baclofen on Schwann cell proliferation and myelination in DRG cultures to see if the $GABA_B$ receptors were functional in these processes in the model.

Results

Localisation of γ-amino-butyric acid B receptors in immunolabelled cells and tissue samples

$GABA_B$ receptor expression was studied by immunofluorescent labelling of sciatic nerve preparations and cultures from dorsal root ganglia (DRG).

In newly plated Schwann cells, the $GABA_{B1}$ and $GABA_{B2}$ receptors were uniformly expressed with higher concentration in the cell edges and endoplasmatic reticulum (ER) (Fig. 4). There was also presumed artificial $GABA_B$ immunoreactivity in the nuclei. In the older cultures, $GABA_B$ receptor expression was redistributed to the forming nodal areas (Fig. 5).

In transections of adult sciatic nerve, the non-myelinating Schwann cells and outer surfaces of the myelin sheaths were clearly $GABA_{B1}$ and $GABA_{B2}$ positive (Fig. 6). Some inner rings of $GABA_B$ were also visible in the myelin annuli or "donuts" (Fig. 7). The inner $GABA_B$ rings partly co-localized with the axonal proteins Caspr1 (contactin associated protein 1) and neurofilament. This suggests that the $GABA_B$ distribution may be present at the Schwann cell and axon or, if on the Schwann cell only, in close connection to the axonal structures of the node.

The annular structure was much less clear in transections of sciatic nerve from P0 rats, pups of postnatal day zero. Here $GABA_B$ expression looked more uniform (not shown). Still, at P1, one day after birth, the myelin sheath or the nodes were not completely developed.

In the adult rat sciatic nerves where the fibres had been separated (teased) and dried onto glass slides, the non-myelinating Schwann cells showed strong $GABA_B$ expression (Figs. 9 and 10) while the myelin sheaths had a generally much fainter expression. Stronger $GABA_{B1}$ and $GABA_{B2}$ staining could be observed in and around the nodes (Fig. 8). In some pictures, the $GABA_B$ staining of the outer myelin sheaths continued inside the node, forming a funnel shape that implied the receptors were in the Schwann cell membrane (Figs. 11 and 12). In other cases, the axons showed clear $GABA_B$ receptor expression in the node (Fig. 13). Three-dimensional confocal microscope images also showed axonal $GABA_B$ receptor expression in the node (not shown).

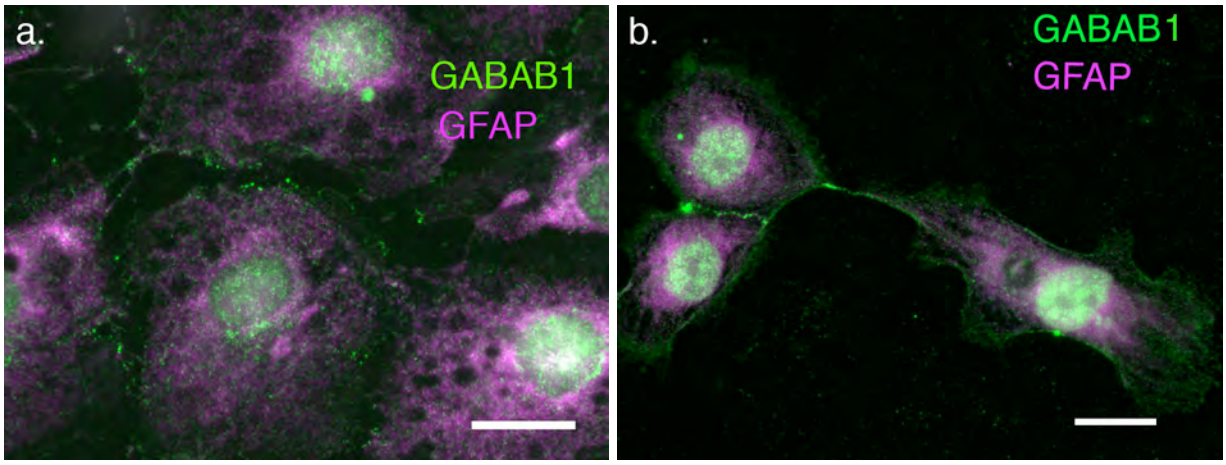

Figure 4. Schwann cells in 4-day-old DRG cultures. $GABA_{B1}$ expression is marked in green and Schwann cell marker GFAP is labelled in magenta. In **a**. the cells are in a lawn structure and **b.** shows cells from a less dense culture. The strong nuclear expression is an artefact. Scale bar = 16 μm

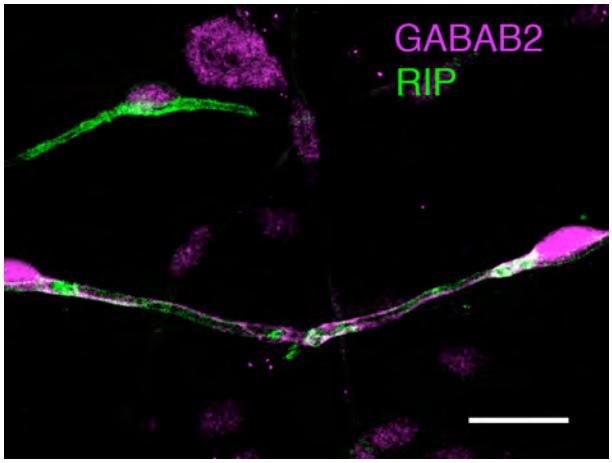

Figure 5. Confocal microscope picture from a six weeks old DRG-culture with myelinating Schwann cells, identified with early myelin marker RIP (green). $GABA_{B2}$ receptors in magenta. The strong nuclear $GABA_{B2}$ staining is an artefact. Scale bar = 20 μm

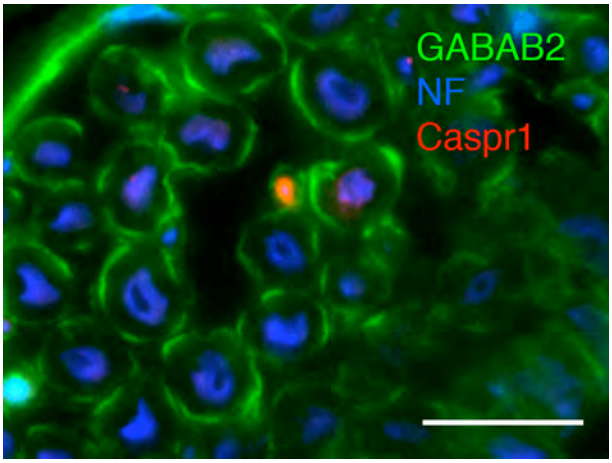

Figure 6. Fluorescence microscope picture of a transection of an adult rat sciatic nerve. $GABA_{B2}$ receptor staining is green and axons are labelled with neurofilament (NF, blue). Axonal paranode protein Caspr1 can be seen in red, and is also shown in magenta in fig. 7. Scale bar = 16 μm

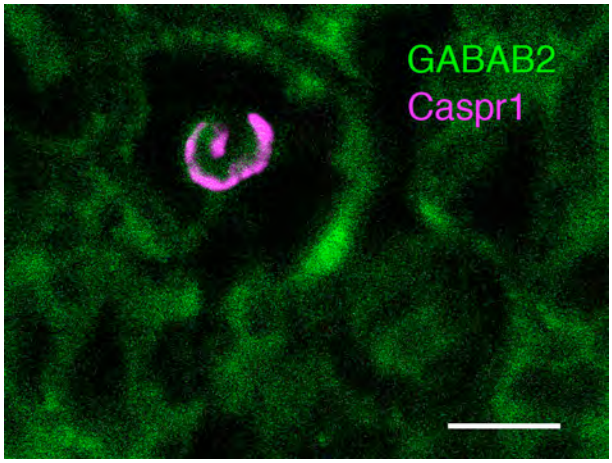

Figure 7. Confocal microscope image of myelin sheaths in a transection of a sciatic nerve from adult rat. The outer surface of the myelin sheaths are visible with $GABA_B$ labelling (green). The paranodal axon protein Caspr1 (magenta). Scale bar = 10 μm

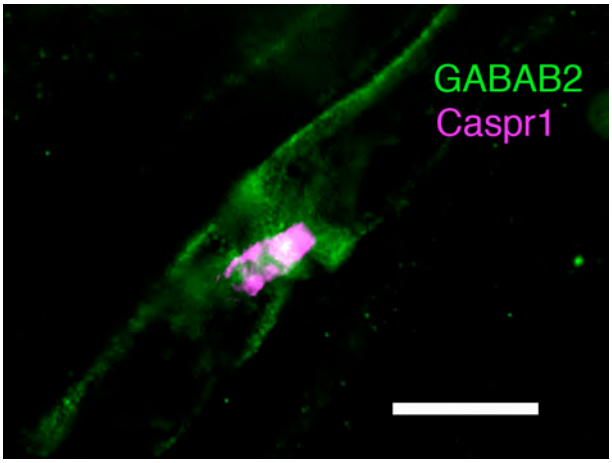

Figure 8. A node in a teased sciatic nerve from an adult rat. $GABA_{B2}$ expression shown in green and axon node marker Caspr1 is in magenta. Scale bar = 16 μm

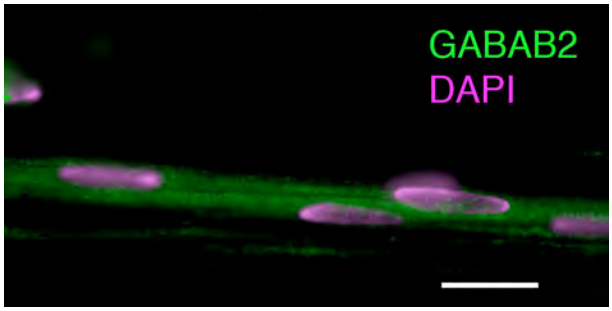

Figure 9. Non-myelinating Schwann cells together enclose thin axons in a Remak bundle in teased adult rat sciatic nerve. $GABA_{B2}$ expression in green, nuclei are shown in magenta (DAPI). Scale bar = 16 μm

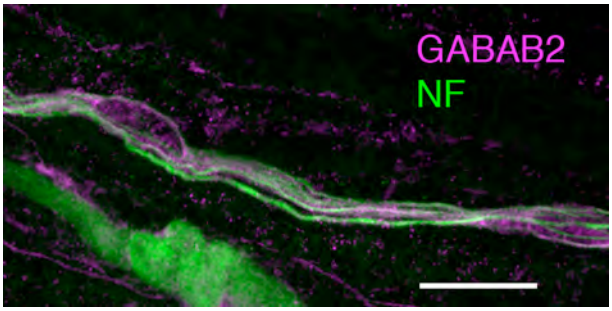

Figure 10. Thin green axons (neurofilament labelling) in association with two $GABA_{B2}$ positive (magenta) non-myelinating Schwann cells from a teased sciatic nerve. Scale bar = 10 μm

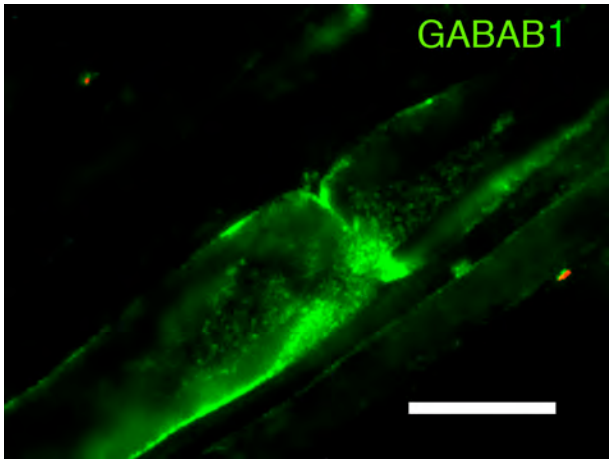

Figure 11. A $GABA_{B1}$ positive node from a teased sciatic nerve. Scale bar = 16 μm

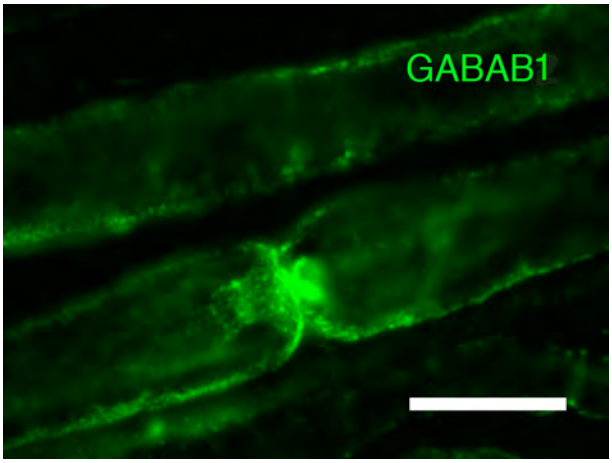

Figure 12. A $GABA_{B1}$ positive node from adult sciatic nerve. Scale bar = 16 μm

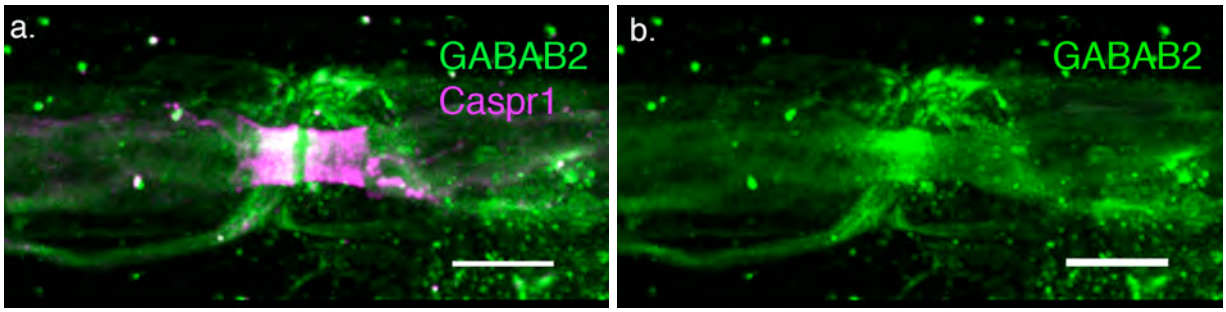

Figure 13. Confocal microscope image of a node from a teased sciatic nerve. **a.** $GABA_{B2}$ expression is labelled green and axon paranode protein Caspr1 labelled in magenta. **b.** $GABA_{B2}$ (green). Scale bar = 10 μm

γ-amino-butyric acid B receptors and Schwann cell proliferation

Differences in proliferation rate are best seen when the cells divide the most. In order to see when the cell cultures reached their proliferation peak, a proliferation assay was performed. 24, 48 and 72 hours old DRG-cultures were treated with 5-bromo-2-deoxyuridine (BrdU), a thymidine analogue. The number of cells that were positive for the non-myelinating Schwann cell marker glial fibrillary acidic protein (GFAP) and also BrdU-positive were counted. After calculating the percentage, it could be concluded that the Schwann cells show the highest proliferation rate 48 hours after plating (Fig. 14).

The same method was used to investigate whether $GABA_B$ activity has an effect on Schwann cell proliferation. The proliferation in 48 hours old, baclofen (a $GABA_B$ specific agonist) -treated and untreated DRG-cultures was measured. No significant difference could be seen, neither in the cultures treated with 200 μM baclofen (data not shown) nor in the ones treated with 500 μM (Fig. 15). The procedure was also repeated with the specific $GABA_B$ antagonist CGP62349, which did not influence proliferation (data not shown). The reason for the difference in proliferation rates between figure 14 and 15 is that cell densities differed in the cultures. Dense cultures grow faster due to more growth factor release.

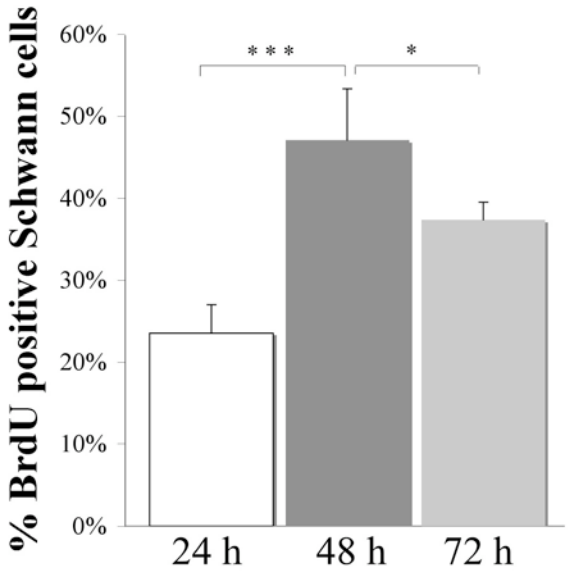

Figure 14. Percentage of Schwann cells which proliferated in DRG cultures at 24, 48 and 72 hours after plating.
n = 4 cultures per category. The error bars show standard deviation. P-values: 9,1 x 10^{-6} and 0,03.

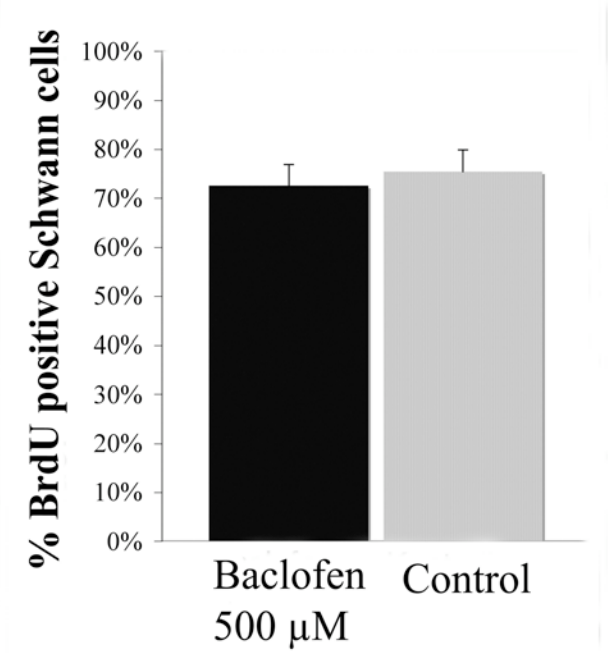

Figure 15. Percentage of Schwann cells that proliferated in 48 hours old DRG cultures treated with baclofen and control cultures.
n = 8 treated cultures and 7 control cultures.

Proliferation with and without baclofen was also measured with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) -based tests on pure Schwann cell cultures and DRG-cultures. Living cells are stained purple in this test. Unfortunately the measurements from the MTT-tests were inconsistent and dubious. First of all, the spectrophotometric values did not correlate with the visible colour intensities. The values were also extremely scattered. Even in the blanks, which contained only solvent and MTT, the values diverged. To check if the remaining cell debris contributed to the absorbance, wells without MTT were also measured. They turned out also to have high and dispersed absorbance values. The results of two MTT-tests on 48 and 72 hours old sister cultures (8 wells per category) are shown in Fig. 16.

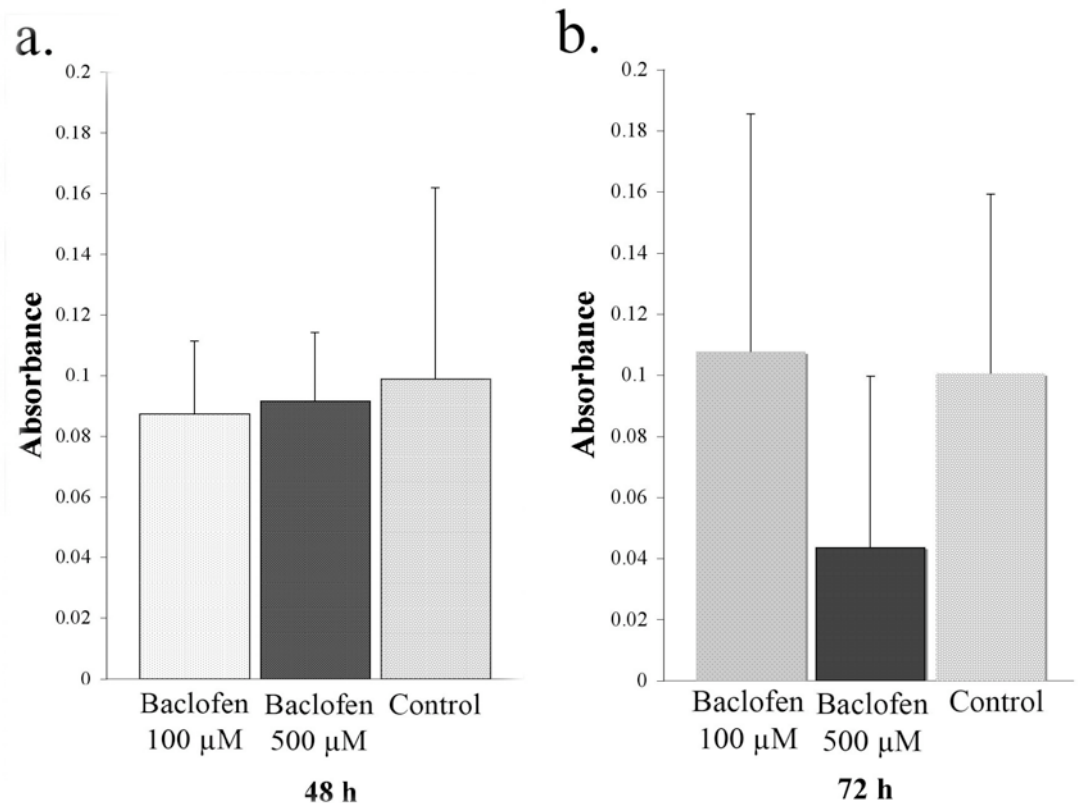

Figure 16. MTT-tests in DRG cultures. The absorbance, at 570 nm minus background absorbance at 690 nm, is related to the relative metabolic activity of the cells. The effects of 100 µM and 500 µM baclofen were measured in 48 hours old (**a.**) and 72 hours old (**b.**) cultures. The error bars show the standard deviation.
n = 8 ELISA-plate wells per category (48 samples in total)

γ-amino-butyric acid B receptors in Schwann cell myelination

DRG cultures were grown with and without daily addition of baclofen (200 µM and 500 µM). Ascorbic acid was added as a differentiation factor to promote myelination in the cultures. In an attempt to visualize and count the myelin sheaths, one-month-old DRG cultures were labelled with antibodies against the early myelin marker RIP (receptor interacting protein) and the later myelin marker MBP (myelin binding protein). Myelination had begun to the same extent in both treated and control cultures. The RIP expression was rather blurry (Fig. 17). It was impossible to perform immunochemistry on a few of the cultures, since they detached from the chamber slide floor after fixation. This prevented the planned quantification of possible effects of baclofen on myelination. No obvious myelination difference between baclofen treated cultures and controls could be observed.

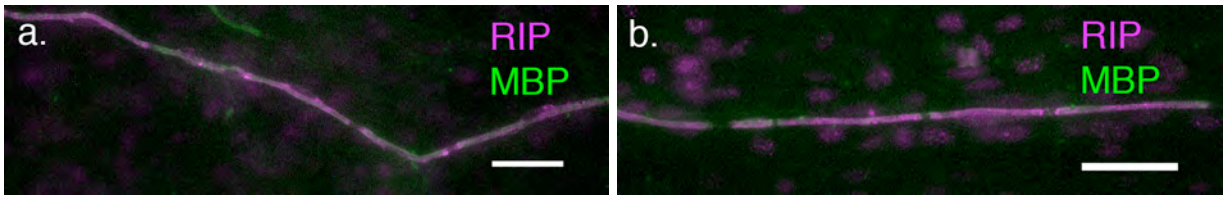

Figure 17. Schwann cells in one-month old DRG cultures. The early myelin marker RIP is shown in magenta and the later myelin marker MBP in green.
a. Untreated culture, **b.** Culture treated daily with 500 µM baclofen. Scale bars = 33 µm

Discussion

Are γ-amino-butyric acid B receptors involved in Schwann cell proliferation *in vivo*?
In this study, I show that Schwann cell proliferation in DRG cultures is not affected by the $GABA_B$ agonist baclofen. Important differences exist between the model used in this study and the model in which Magnaghi *et al*., in 2004 found Schwann cell proliferation to be decreased by baclofen. First of all, dissociated DRG cultures were used instead of purified Schwann cells. The DRG cultures contain neurons and endogenous Schwann cells and the Schwann cells proliferate spontaneously through interactions with neurons and fibroblasts, mimicking the situation *in vivo*. In cultures of purified Schwann cells, proliferation can only be obtained if forskolin is added. Forskolin pushes adenylate cyclase to elevate the levels of cAMP. In culture, a cAMP increase makes undifferentiated non-proliferating Schwann cells wake up and pick one of the two fates depending on the presence of growth factors (Mirsky and Jessen, 2001) Baclofen agonism at the $GABA_B$ receptor does not affect the basal levels of cAMP but decreases forskolin induced cAMP production (Hill, 1985).

The peak of the spontaneous Schwann cell proliferation in the DRG cultures was after 48 hours. However, upon treating new 48-hour cultures with 200 or 500 μM of baclofen, no change in the rate of proliferation could be seen. Neither 100 (previous work in this laboratory), 200 nor 500 μM of baclofen decreased proliferation in the system I used, while Magnaghi *et al.* (2004) saw a decreased proliferation in response to 100 μM baclofen. The differences between cell culturing systems may be the reason for this discrepancy. I therefore conclude that baclofen does not have an anti-proliferative effect *in vivo*, although this has to be further investigated.

During my experiments the group also observed the DRG cultures produced endogenous GABA. If its concentration is high enough for maximal activation of the $GABA_B$ receptors, baclofen cannot affect the system. This explanation is contradicted by my finding that the competitive $GABA_B$ antagonist CGP62349 did not affect the Schwann cell proliferation rate in DRG cultures.

Perhaps the proliferation cue in the DRG culture model is not mediated through adenylate cyclase activation and cAMP. Another possibility is that the proliferation signal does work through the cAMP pathway, but that it is so forceful that it overrides $GABA_B$ activation. To further study this, a future experiment could be to treat cultures with adenylate cyclase inhibitors.

Another difference is the source of the cells. Magnaghi *et al.* (2004) used Schwann cells from the sciatic nerve of rats from post-natal day 3 (P3) while I used DRG-cells from E17 rat embryos. All Schwann cell precursors in the rat sciatic nerve have differentiated to immature Schwann cells by E17 and around birth the Schwann cells start to myelinate (Jessen and Mirsky, 2005). It is not clear at what stage DRG Schwann cells are at E17 but one can guess that they are less differentiated, and/or more heterogeneous, than in P3 sciatic nerve. I did see similar expression of $GABA_{B1}$ and $GABA_{B2}$ as Magnaghi *et al*. (2004) showed, but potentially other parts of the receptor pathway could differ with the developmental stage.

γ-amino-butyric acid B receptor localisation

In immunolabelled 4 day-old cultures from E17 rat dorsal root ganglia, I found that the $GABA_{B1}$ and $GABA_{B2}$ receptors were uniformly expressed with a concentration towards the outer edges of the Schwann cells. The cells in this stage were forming a so-called lawn structure of rounded undifferentiated cells side by side. If the receptors are used in cell-cell interaction it is reasonable that they are concentrated towards the edges in a monolayer culture. *In vivo*, the entire cell membrane of course is involved in cell-cell interactions.

As the cultures started receiving ascorbic acid in the medium, the Schwann cells differentiated. They became elongated and some eventually found axons to myelinate. They also seemed to become less $GABA_B$-positive, with the exception of the regions were nodes were forming. This suggests that $GABA_B$ receptors are involved in the placing of the nodes, i.e. the lengths of the myelin sheaths. That $GABA_B$ receptors may mediate a stop signal for myelination is an alternative that fits with this observation. I also found that the $GABA_B$ expression is kept in the nodal regions of the Schwann cells in adult rat sciatic nerve, which implicated that they have a function not only in the establishment but also in the maintenance of the node. Also an observation previously made in the laboratory, that the non-myelinating Schwann cells have a much stronger $GABA_B$ expression than the myelinating ones, supports this hypothesis.

In transections of adult sciatic nerve, the outsides of the myelin sheaths show clear $GABA_{B1}$ and $GABA_{B2}$ expression. Also inner rings are visible that may show expression of $GABA_B$ receptors either on the insides of the myelin sheath or on axons, or even both. These inner rings are not only found in the paranodal area, indicated by Caspr1 expression, but also in many more of the circles. This matches the observations in teased nerves that inner $GABA_B$ expression sometimes continues quite far into the myelin sheath. Right by the node, axonal GABA has some space to diffuse to the $GABA_B$ receptors at the very ends of the Schwann cells. To respond to GABA from the rest of the axon it is reasonable that the Schwann cells need $GABA_B$ receptors on the inside.

In the confocal microscope, three-dimensional pictures of my immunostainings of teased sciatic nerve showed the $GABA_B$ receptors in the node. This means that the funnel-shaped inner $GABA_B$ expression might not only be on the Schwann cell, but also on the axonal side. To verify whether $GABA_B$ is expressed in the actual axon and node, my group is going to use electron microscope imaging. If it turns out to be the case, what is their function? Can they be involved in an autocrine feedback control, regulating the production and release of GABA from the axon?

It has been shown that the specific $GABA_B$ agonist baclofen reduces Schwann cell production of myelin proteins (Magnaghi *et al*. 2004). Proliferation and differentiation towards myelination are opposite cell fates for Schwann cells (Mirsky and Jessen, 2001). It is therefore very unlikely that the same signal molecule (GABA) via the same receptor ($GABA_B$) would regulate both the proliferation and the myelination *in vivo*. Since I could not find any effect of GABA on Schwann cell proliferation, this made it very interesting to investigate how baclofen affects the immature Schwann cell differentiation towards myelin-producing Schwann cells.

Effect of γ-amino-butyric acid B receptor activation on Schwann cell myelination

I cultured DRG cultures with and without 500 μM baclofen for a month. Immunofluorescent labelling of the early myelin protein RIP revealed that myelination had begun and some myelin sheaths stained positive for the later myelin basic protein (MBP), in control as well as in treated cultures. Some cultures were lost as they detached after fixation and could not be used for immunocytochemistry. The overall blurry RIP expression and the lost cultures stopped me from quantifying the effect of baclofen on myelination. However, if GABA from the axons acts to halt myelination, cultures treated for a month with 500 μM baclofen should not appear to have the same degree of myelination as untreated controls. Still, GABA is endogenously present in the cultures as well as in the intact sciatic nerve. In experiments performed by my supervisor Mikael Corell, he demonstrated that the level of the enzyme GAD 65/67 (glutamic acid decarboxylase) that transforms glutamate into GABA decreases with age in the rat sciatic nerve (unpublished data). To further test if GABA is involved in myelination, a good future experiment would be treating long term DRG cultures with a $GABA_B$ antagonist.

Conclusion

Since the $GABA_{B1}$ and $GABA_{B2}$ receptors clearly are present in Schwann cells at different developmental stages and in various contexts, they surely have a function. As for now, I conclude that they are more likely to be involved in the establishment and maintenance of the node than in proliferation or differentiation towards myelination. Maybe the receptors have a completely different function in the Schwann cells. This study could not provide evidence for a $GABA_B$ involvement in proliferation or myelination and the question is still open.

Materials and methods

Dorsal root ganglion cultures

Pregnant (embryonic day 17 - E17) Sprague-Dawley rats were killed by an overdose of CO_2 and the embryos were collected. The embryonic dorsal root ganglia were isolated in 1 ml of Leibovitz's L-15 (Invitrogen, Stockholm, Sweden) and treated with 1 ml 0.25% trypsin (Invitrogen) in L-15 at 37 °C, and mechanically dissipated until individual cells were free of connective tissue (10-20 min). To halt the trypsin activity, 1 ml of newborn calf serum (NCS, Sigma-Aldrich, Stockholm, Sweden) was added to the cell suspension. The cells were then washed by centrifugation twice, for five minutes in L-15 at 160 g. The cell culture medium Nb++ was used to dissolve the pellet. This medium consists of neurobasal medium (Invitrogen) supplemented with 2% (v/v) B27 (a supplement for neural cultures with vitamins, fatty acids and growth factors, Invitrogen), 0.6 mM glutamine (Invitrogen) and 100 ng/ml of neural growth factor, NGF (Millipore, Stockholm, Sweden). The cells were plated at a density of 100 000 – 500 000 cells/ ml in poly-L-lysine (Sigma-Aldrich) -coated Lab-Tek 4-well chamber slides (Invitrogen). In the first cultures I used 1% (v/v) penicillin-streptomycin (Sigma-Aldrich) in the medium but as antibiotics may interfere with myelination, most of the cultures were made without. The slides were kept in a sterile incubator at 37° C with 5% CO_2. Some cultures were treated daily with 200 or 500 μM baclofen (Sigma-Aldrich). Sister cultures were kept untreated as controls. After four days all cultures were given new Nb++ media, supplemented with 50 μg/ml of ascorbic acid (Sigma-Aldrich). Media was changed every three to four days.

Cultures were fixed in Stefanini's fixative; 2% (w/v) formaldehyde (Sigma-Aldrich), 15% (v/v) saturated picric acid (Sigma-Aldrich) in PBS (phosphate buffered saline, 137 mM NaCl (Sigma-Aldrich), 2.7 mM KCl (Sigma-Aldrich), 10 mM Na_2HPO_4 (Fischer Scientific, Stockholm), 1.5 mM KH_2PO_4 (Sigma Aldrich), pH 7.3), for 15 minutes. After rinsing thoroughly with PBS, cultures were either short-term (days) stored in PBS in 4° C or soaked over night in cryoprotective solution (PBS with 10% w/v sucrose) and long-term (weeks) stored at -20° C until immunostained.

Tissue preparations

Stefanini-fixed sciatic nerve from rats of ages P0 (post natal day 0), P10 and adult were embedded in Optimum Cutting Temperature compound (Histolab, Gothenburg, Sweden). 10, 12 and 20 μm thick sections of the tissue were cut in a Reichert-Jung Cryocut 18 000 cryostat cutter (Leica Microsystems, Stockholm, Sweden). The sections were left to dry for at least an hour before -20 °C storage or immunolabelling.

Teased fibres were prepared from fresh adult sciatic nerve by freeing it from connective tissue and separating the nerve fibres directly on Thermo Scientific Super Frost Plus glass slides (Histolab). The samples were allowed to dry for a minimum of two hours and then fixed with Stefanini's fixative and stored soaked in cryoprotective solution at -20 °C.

Immunochemistry

Sections, teased fibres and fixed cultures were rinsed in PBS and blocked from unspecific binding with blocking solution, containing PBS with 0.25% Triton X-100 (Sigma-Aldrich) and 0.25% bovine serum albumin (BSA, Sigma-Aldrich). The samples were incubated over night at

4°C with primary antibodies (table 1) in blocking solution. The slides were then rinsed in washing solution, PBS containing 0.25% (v/v) of Triton X-100, and incubated for one hour at room temperature with secondary antibodies (see table 2). The samples were again rinsed with washing solution and mounted with mounting medium; glycerol with 2.5% (w/v) DABCO (1,4-diazabicyclo[2.2.2]octane, Sigma-Aldrich) and 10% (w/v) 0.5 M Tris-HCl pH 8.6 (Sigma-Aldrich). 0.000415% (w/v) of DAPI (4', 6-diamidino-2-phenylindole, Sigma-Aldrich) was added in the mounting medium. The slides were analyzed using an Olympus BX61WI fluorescence microscope and photos were taken with Volocity 4.0 (Improvision). Higher magnification pictures were taken in a Zeiss LSM 510 confocal microscope. Colours, contrast and brightness were adjusted in Photoshop 7.0 (Adobe). All changes were made homogenously over the entire picture.

Immunochemistry on 5-bromo-2-deoxyuridine-incorporating cultures

To label proliferating cells, 20 μM 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich), a thymidine analogue, was added to the cultures 24 hours before fixation. Fixed cultures were incubated for three hours at room temperature in 2 M hydrochloric acid (Fischer Scientific) and then washed 4 times 5 minutes in PBS. Anti-GFAP antibodies were added in blocking solution at 4° C over night. The primary antibody was washed away with wash solution, 2 times 15 minutes. The secondary antibody, RRx anti-mouse IgG (table 2), was added in blocking solution together with the FITC-conjugated anti-BrdU antibody. After four hours of dark incubation at room temperature, the cultures were washed 2 times 15 minutes in washing solution and 15 minutes in PBS. Mounting was done with DAPI in the mounting medium.

Table 1. Primary antibodies

Antibody specificity	Host	Dilution	Manufacturer
Contactin associated protein (Caspr1)	Rabbit	1:1000	Gift from Colman lab, New York, USA
$GABA_{B1}$-R	Rabbit	1:200	AbCam, Cambridge, UK
$GABA_{B2}$-R	Guinea pig	1:200	Millipore, Stockholm, Sweden
Glial Fibrillary Acidic Protein (GFAP)	Mouse	1:1000	Millipore
Glial Fibrillary Acidic Protein (GFAP)	Rabbit	1:1000	Sigma-Aldrich, Stockholm, Sweden
Glial Fibrillary Acidic Protein (GFAP)	Chicken	1:1000	AbCam
Myelin Basic Protein (MBP)	Rabbit	1:1000	Gift from Colman lab
Neurofilament (NF)	Mouse	1:1000	Sigma-Aldrich
Neurofilament (NF)	Rabbit	1:1000	Sigma-Aldrich
Neurofilament (NF)	Chicken	1:1000	AbCam
Receptor Interacting Protein (RIP)	Mouse	1:1000	Millipore
BrdU-FITC (pre-conjugated)		1:75	BioSite, Stockholm, Sweden

Table 2. Secondary antibodies

Fluorophore	Binding	Dilution	Supplier
FITC	Mouse IgG	1:200	Fischer Scientific, Stockholm, Sweden
	Rabbit IgG	1:200	Fischer Scientific
	Guinea pig IgG	1:200	Fischer Scientific
Alexa 488	Mouse IgG	1:400	Invitrogen, Stockholm, Sweden
	Rabbit IgG	1:400	Invitrogen
	Guinea pig IgG	1:400	Invitrogen
RRx	Mouse IgG	1:400	Fischer Scientific
	Rabbit IgG	1:400	Fischer Scientific
	Guinea pig IgG	1:400	Fischer Scientific
Alexa 647	Mouse IgG	1:200	Invitrogen
	Rabbit IgG	1:200	Invitrogen
	Chicken IgG	1:200	Invitrogen
	Guinea pig IgG	1:200	Invitrogen

Quantifying proliferation and myelination

To establish the potential differences in proliferation caused by the baclofen treatment, the cells had to be counted. The cultures were photographed with an Olympus BX61WI fluorescence microscope, five frames in each of the four wells on the chamber slides. The pictures were acquired in the Volocity 4.0 software (Improvision) and the percentage of GFAP positive cells with incorporated BrdU was counted manually using the same program. The data were entered in Excel (Microsoft) and Student's t-tests were also performed in Excel.

To attempt to measure the effect of baclofen on myelination, DRG cultures were cultured for 1 month and labelled with antibodies against the early myelin protein RIP (receptor interacting protein) and MBP (myelin basic protein) in order to make the myelin sheaths countable.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide-test of cell viability
DRG-cultures were prepared as described above. 96-well Cellstar ELISA-plates (VWR, Stockholm, Sweden) were coated with poly-L-lysine. The cell density was adjusted to between 500 000-600 000 cells/ml by counting cells in a Bürker chamber and then dissolving and diluting the pellet in Nb++-medium. 100 µl cellsuspension was added per well, so that each well contained 50 000 – 60 000 cells. Baclofen was added at 100 or 500 µM to the treated wells every 24 hours and wells with only Nb++ served as blanks.

Schwann cell cultures were dissociated by 1-3 minutes incubation with 0.25% trypsin, and washed two times, at 160 g, in DMEM-F10 (Dulbecco's Modified Eagles Medium, Sigma-Aldrich with 10% (v/v) fetal bovine serum (Sigma-Aldrich), 1% (v/v) penicillin-streptomycin (Sigma-Aldrich), 0.6 mM L-glutamine (Invitrogen) and plated with 2 µM forskolin (Sigma-Aldrich) in poly-L-lysine coated 96-well plates. 100 µl of cell suspension containing 600 000 cells/ml was added to each well, that is about 60 000 cells/well. Baclofen was added to a concentration of 100 µM, in half of the wells, every 24 hours.) Coated wells with medium and supplements, but no cells, served as blank samples.

The cultures were kept in an incubator at 37° C. 10 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) from the MTT-based proliferation-kit from Sigma-Aldrich was added 20 hours after plating and cultures were then incubated at 37° C for 4 hours. The medium was then removed and the MTT formazan crystals that were formed in living cells during the incubation were dissolved in 100 µl of the kit's MTT solvent (propanol with 0.2 M HCl, Sigma-Aldrich) in each well as the plate was put on a shaker for 20 minutes. The absorbance of the dissolved crystals was then measured spectrophotometrically at 570 nm and the background absorbance was measured at 690 nm in a Multiskan MS spectrophotometer (Labsystems, Stockholm, Sweden).

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