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Neuropeptides in Amyotrophic Lateral Sclerosis with special focus on galanin

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1. Abstract

Amyotrophic lateral sclerosis (ALS), a disease characterized by motor neuron degeneration, inevitably leads to death predominantly of respiratory failure. Thus, tragically, treatment options are strongly limited as molecular mechanisms underlying ALS to a large extent are unknown. In this project a possible role of neuropeptides is explored, with the hope to obtain a novel perspective on this devastating disease. Neuropeptides represent the quantitatively largest family of messenger molecules in the nervous system and some of them are expressed in motor neurons. Galanin is a well-established neuropeptide, which not only can act as a neurotransmitter but also has been shown to exert neuroprotective and regenerative effects. In rat the synthesis of galanin in motor neurons is, in fact, upregulated after injury to the axons of these neurons.

In this study the plasticity in expression of various peptides following unilateral axotomy of the sciatic nerve in the adult mouse was examined. In situ hybridization and immunohistochemical techniques were used to visualize possible alterations in spinal cord motor neurons. Seven days after nerve transection, an ipsilateral upregulation of galanin and calcitonin gene-related peptide messenger ribonucleic acids (mRNAs) was detected in subpopulations of motor neurons. These results were confirmed with immunohistochemistry. In fact, of several other neuropeptides analyzed, neuropeptide Y expression was also upregulated in ipsilateral motor neurons. These data show that injury induces markedly altered peptide expression, as previously observed in other species such as rat. With regard to galanin receptors 1-3 (GalR1, GalR2, GalR3), they were only screened for by in situ hybridization, due to lack of specific antibodies. However, GalR1 was expressed bilaterally at similar levels after unilateral axotomy. Moreover, GalR2 and GalR3 could not be detected with the present methodology.

Finally, to build a basis for further experimental analysis of a role of galanin for motor neuron survival, we analysed stem-cell derived juvenile motor neurons in culture. Employing quantitative polymerase chain reaction (qPCR), the presence of transcripts for galanin and its three receptors could be demonstrated, partly confirmed for galanin with immunohistochemistry. Thus, this approach may represent an interesting system to further elucidate a possible impact of galanin on stressed or even degenerating cells.

2. Introduction

2.1 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease almost exclusively affecting motor neurons, whereas the underlying molecular mechanisms remain elusive. Exceptions have, however, been encountered, such as the famous physicist Stephen Hawking, who apparently suffers from an early-onset, slow-progression form of the disease. Symptoms for ALS include spasticity, pathologic hyperreflexia in upper motor neurons (UMN) and reduced tone, atrophy, fasciculations, as well as hyporeflexia in lower motor neurons (LMN), whereby patients predominantly die of respiratory failure[1]. Moreover, ALS occurs in approximately 2 out of 100,000 people per year in Europe[2], and as it remains incurable and leads to death after approximately 3 years after onset of symptoms[1], elucidating the fundamental processes leading to ALS, as well as finding options to interrupt disease progression are high priorities in research. Furthermore, 10% of cases appear to be hereditary[3,4] and are referred to as familial ALS (fALS). However, only some of the genes involved are known, and they are used in experimental research to generate ALS models by establishing transgenic mice and rats. One of these mutated genes, which was also the first to be discovered, is encoding for a gain-of-function super oxide dismutase-1 (SOD1), and accounts for 20% of fALS events[1]. Overexpression of pathogenic SOD1 in model organisms leads to neurodegeneration alike that seen in ALS[5]. Here, aggregates of misfolded proteins, as well as proteasome dysfunction have been detected, as well as higher sensitivity to cell death, all factors that may be involved in the pathogenesis of the disease[6]. Since ALS seems to have a tremendously complex and heterogeneous etiology, more research needs to be done to make progress, especially since most incidents of ALS are not familial but appear sporadic (sALS), and here causal connections

remain obscure. Nevertheless, also 10% of sALS (besides two-thirds of fALS) can be explained by genetics[1]. Moreover, correct and early diagnosis of ALS remain crucial, as it is easily confused with other neurodegenerative diseases sharing similar symptoms, such as multifocal motor neuropathy[1]. To that end, the so called El Escorial criteria were introduced to improve proper diagnosis of ALS. For a "definite" diagnosis, involvement of upper and lower motor neurons (UMN, LMN) in three out of four regions (lumbosacral, thoracic, cervical and bulbar) needs to be established, which has to be displayed by electromyographic (EMG) analysis[1]. If only two regions are engaged, "probable ALS" is considered. Furthermore, possible ALS is indicated if one region shows UMN as well as LMN signs. If only UMN or LMN findings appear, ALS is defined as "suspected"[1,7].

Because there is a lack of understanding of the molecular and cellular mechanisms underlying ALS, and since the picture of the disease has evolved from only involving motor neurons to an illness also engaging other cell types such as interneurons[8,9], research has expanded and now covers a broad range of approaches. For example, communication between motor neurons and immune cells at sites of neuronal injury appears to enhance production of cytokines and reactive oxygen species, further leading to degeneration of motor neurons[3,10]. This knowledge has led to the attempt to target neuroinflammation for intervention of disease progression[11]. For example, tetracycline antibiotics were found to have beneficial properties in animal models by limiting T cell contacts to microglia and therefore decreasing cytokine production[12–14]. The only drug proven to increase life expectancy of ALS patients thus far is riluzole. However, its mode of action is poorly understood and functional improvement is mostly not convincing. In fact, three month prolongation of survival remains a maximal effect[15]. Nonetheless, riluzole has shown to effectively increase glutamate uptake and therefore to reduce accumulation of excitotoxic, extracellular glutamate under pathological conditions such as found in ALS[16].

Astrocytes are key players in the regulation of neuronal excitability by controlling concentrations of neurotransmitters, such as glutamate. "Tripartite synapses" consisting of pre- and post-synaptic terminals of neurons besides astrocytes occur broadly in the CNS. In ALS, astrocytes are thought to change morphologically and physiologically, fail to take up glutamate in appropriate amounts and thus shift from a "neuroprotective" to a "neurodegenerative" role towards motor neurons (figure 1).[11]

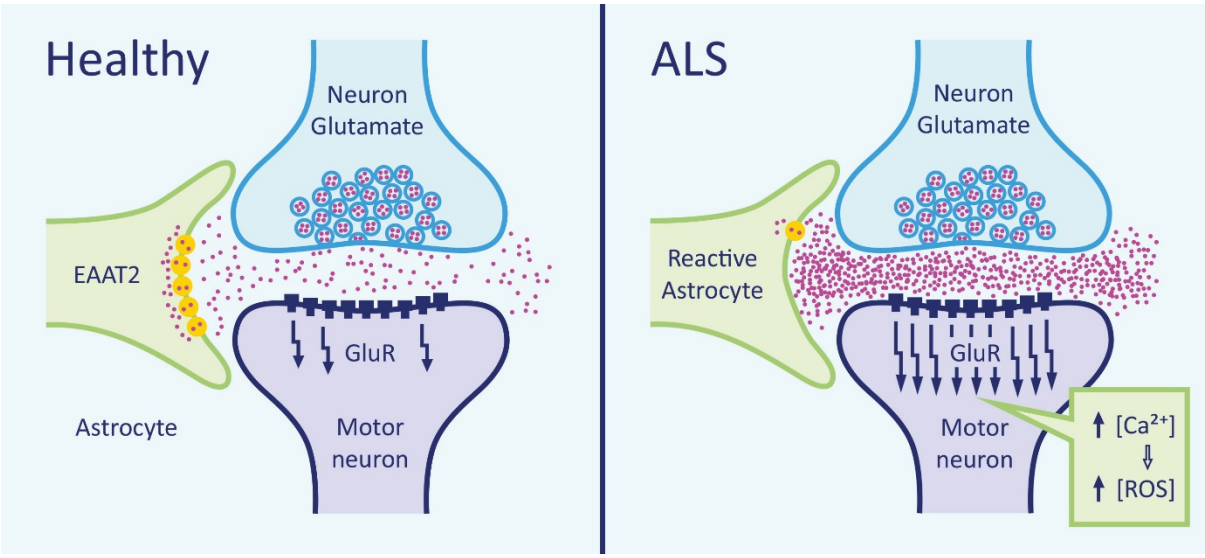


Figure 1 Glutamate occurrence and accumulation in a tripartite synapse in health and in ALS. *Left – healthy*: astrocytes take up glutamate which is released into the synaptic cleft. This is accomplished through sodium-dependent excitatory amino acid transporter1 (EEAT1) and -2 (EEAT2). *Right - ALS*: EEAT2 expression is reduced in astrocytes of the motor cortex and spinal cord in ALS patients and rodent fALS models which could cause accumulation of extracellular glutamate. Thereby, intracellular calcium concentration is elevated and cascades promoting motor neuron death are initiated. Figure modified from Rizzo et al[11].

Taken together, since there is no cure for ALS, treatment of symptoms is eminently important, and so far the only option is to improve the remaining time for the patients.

However, in more recent animal studies, growth factors have been given to transgenic mice carrying a $SOD1^{G83A}$ mutation with promising results, as have insulin-like growth factor-1

(IGF-1) and vascular endothelial growth factor (VEGF). These treatments delayed the onset of ALS as well as progression by protecting motor neurons from degeneration and thereby increasing survival of the animals tested[17]. One obvious drawback of this recent attempt is that no restoration of already lost functions (neurons) can be achieved; hence a new access would be to deliver growth factors together with stem cell treatment[17]. This approach has in principle a profound potential in the field of neurodegenerative research as it combines two strong strategies: neuroprotection and cellular replacement. However, it may also be worthwhile to consider neuroprotection based on neuropeptides, some of which have growth factor-like activity. One such neuropeptide is galanin.

2.2 Neuropeptides

More than three decades ago it was realized that neurons release multiple messenger molecules. Thus in addition to the 'main messengers', also known as "classic" transmitters, e.g. dopamine, noradrenaline, serotonin, and others, a neuron can also release a peptide molecule[18]. While those classic transmitters are released at any firing frequencies and under 'regular circumstances', neuropeptides are mainly secreted when neurons fire at high frequency or burst fire[19,20]. Subsequently the neuropeptides are degraded by extracellular peptidases[21], as there is no reuptake mechanism for neuropeptides such as that known for classic transmitters. When stored, neuropeptides are preserved in large dense-core vesicles (LDCVs)[22], classic transmitters on the other hand, are primarily stored in synaptic vesicles. Neuropeptides that are released need to be newly synthesized in the cell body, which can be witnessed by elevated transcription activity.[23]

Decrease or increase in expression of neuropeptides may occur in response to different physiologic and pathologic conditions, such as observed in nerve injury[24,25]. Cloning of

neuropeptide genes of interest made characterization of mRNA expression patterns via quantitative real-time polymerase chain reaction (qPCR) and in situ hybridization possible. Prior to that, mostly antibody-based technologies (radioimmunoassay, immunohistochemistry) were used, and still are, to monitor neuropeptides and peptide levels[23].

When Nakanishi and others succeeded with their attempt to clone the first neuropeptide receptor, they revealed a 7-transmembrane G protein-coupled receptor (GPCR)[26]. Subsequently, further research revealed that nearly all other neuropeptide receptors are GPCRs. However, reliable elucidation of the distribution of GPCRs is in most cases only possible at mRNA level, as specificity of available antibodies raised against these molecules, including neuropeptide receptors, remains an issue. Nonetheless, neuropeptide receptors could become important drug targets, particularly when keeping in mind that more than half of all drugs prescribed these days aim for GPCRs. Dopamine receptor agonists, which are used for treatment of Parkinson's disease, histamine H1 receptor antagonists which are applied in allergy and motion sickness, are ligands for GPCRs[27], to name only a few. As mentioned above, neuropeptides are mostly released if high frequent stimulation or bursting of neurons occur, and therefore are thought to be brought to action only under certain circumstances like stress, injury, drug abuse or neuropsychiatric disorders, supporting the assumption that drugs acting on neuropeptide systems might have less side effects. This hypothesis can be underscored by the supposedly more selective pharmacological actions that these drugs might have, due to more restricted distribution, compared to agents acting on glutamatergic, GABAergic or similar receptors that belong to more widely expressed systems.[28] Attention needs to be payed, however, when comparing functions of neuropeptides in various mammals, to the fact that neuropeptides can vary between species. In addition, when neuropeptides are supposed to operate within the brain,

there may be difficulties for these comparatively large molecules to pass the blood-brain barrier.[23]

However, as described above, a shift in expression can often be tracked following injury as observed after axotomy, which led to the early assumption that neuropeptides are involved in an "emergency response system" and act as trophic factors[29], enhance neuroregeneration, and regulate pain. The latter conclusion can be drawn when excitatory neuropeptides are downregulated (like for example CGRP, substance P) whereas inhibitory neuropeptides are upregulated (galanin, NPY), in dorsal root ganglia (DRG) following nerve transection in rats. This implicates that a decrease in neuronal excitability in the dorsal horn of the spinal cord might be leading to suppression of pain.[30]

2.2.1 Galanin

Galanin, a 29/30 amino acid long neuropeptide[31], was discovered by Kazuhiko Tatemoto and Viktor Mutt at Karolinska Institutet in the 1980s by its C-terminal amide structure[32] in porcine intestinal extracts[31]. The name "galanin" resulted from the attempt of Mutt to name peptides in simple and unbiased way; therefore galanin stands for its N-terminal **g**lycine and C-terminal **alanine**. A biologic activity was first discovered by MacDonald and collaborators, showing that the neuropeptide influences plasma glucose levels[23]. Galanin is a highly conserved peptide at its N-terminal end, as the first 19 amino acids show 90% identity from fish to humans. Keeping this fact in mind, much research regarding the development of galanin receptor-selective molecules has been done with N-terminal fragments like galanin 1-13 as the core sequence, since its high conservation is thought of as a site for biologic activity. The C-terminal sequence does not show high conservation and is amidated in all species except humans.[23]

Over the years, more and more research has been published on the distribution of galanin in the rat, followed by mouse and primate brain. One interesting finding was its presence in noradrenergic neurons in the locus coeruleus, in serotoninergic neurons within the dorsal and medullary raphe nuclei, as well as in cholinergic forebrain neurons[18]. However, soon also peripheral tissues were shown to express galanin. For example, the spinal cord and DRG, the genitourinary and respiratory tract, as well as the intestine, and endocrine tissues such as the adrenal medulla and anterior pituitary contain galanin to a higher or lower extent.

It has been shown that galanin has a wide spectrum of functions, both neuronal and nonneuronal, and it seems relevant to look upon this peptide as one of many regulatory peptides. Much of the knowledge collected on galanin over more than 30 years has been summarized in the review by Lang et al. (2015)[23]. To mention a few examples: with regard to regulation of galanin, its expression can be modulated by hormones, such as vasoactive intestinal polypeptide (VIP)[33], thyroid hormone[34], progesterone[35], as well as nerve growth factor and leukemia inhibitory factor[36–39]. Interestingly, colchicine that interferes with intracellular transport by acting on microtubules[40,41] has been shown to upregulate galanin synthesis[42].[23]

Modulation of gene expression and secretion in vivo occurs in cases of chronic stress[43], ischemic brain damage[44] , and axotomy, whereby expression may be many fold higher as compared to normal conditions[25,45–49]. Even if all these studies have been carried out on rodents, a study on ipsilateral axotomy of the sciatic nerve in Macaca mulatta, a monkey species, shows an increase in galanin in ipsilateral DRG and dorsal horn fibers[50]. This suggests that, with regard to galanin, similar mechanisms may operate in primates, perhaps even in human.

2.2.2 Galanin receptors

Alike many other receptors targeted by neuropeptides, also galanin receptors belong to the GPCR family. The first receptor to be involved in galanin signaling was galanin receptor 1 (GalR1), cloned from a human melanoma cell line[51]. Soon, two more receptors, receptor 2 and 3 (GalR2, GalR3) were discovered (see Ref. 24). Receptors show moderate homology to each other and high homology among different species. On a molecular level, GalR1 and GalR3 couple to $G_{i/o}$ opening potassium channels and inhibiting adenylyl cyclase.[51,52] One of the transduction pathways for GalR2 involves phospholipase C after coupling to $G_{q/11}$[53]. Trafficking of GalR1 and GalR2 receptors has been studied after tagging R1 and R2 in transfected cell lines[54–56].

A problem in the galanin receptor field is the lack of specific antibodies to these receptors[57], and therefore no exact information on the localization of the receptor protein has so far been obtained. However, mapping studies have been performed with in situ hybridization, and the localization of the three transcripts has been established[23,58–60]. These studies show a wide distribution of GalR1 and GalR2 in the rat brain, whereas GalR3 is more restricted. GalR1 mRNA was found in, for example, the olfactory structures, amygdala, hypothalamus, thalamus, pons, medulla and spinal cord. GalR2 was found in the hippocampus, hypothalamus, the dorsal vagal complex, and spinal cord. Within the spinal cord, GalR1 and GalR2 were detected using in situ hybridizations for mRNA mostly in dorsal horn neurons as well as outside the spinal cord in the DRG. Specifically, GalR1 mRNA positive neurons in the rat spinal cord were discovered in laminae I-III, within the ventral horn, and in laminae X. GalR2 mRNA expression in the rat was detected in primary sensory neurons of the DRG, while ventral horn motor neurons, as well as dispersed cells throughout the gray matter and laminae X show only moderate expression. Expression of GalR3 mRNA was found in laminae I-II, V and X by one

study, but is in general more abundant in peripheral tissues.[23,61–63] Peripheral nerve transection in rats resulted in a down-regulation of GalR1 and R2 expressional within DRG[64].

As mentioned above, drugs targeting GPCRs are widely appreciated in medicine and frequently prescribed, and also ligands for galanin receptors could be useful in the future for treating pain, mood disorders, and others[27]. However, a further problem is that only very few drugs (agonists, antagonists) have been developed which affect galanin signaling. Early on, in particular, Bartfai, Langel and collaborators generated a number of small peptides which do not pass the blood brain barrier[65]. The amino acid fragment (2-11) of galanin has been frequently used, as it has a 500-fold selectivity for GalR2 as compared to R1[66]. However, there are also some compounds that reach the brain after peripheral administration acting on GalR3[67] or GalR2[68,69].

2.2.3 Biological activity of galanin

There is increasing evidence that galanin can attenuate neuronal damage, increase neurogenesis, improve regeneration, and attenuate β-amyloid and glutamate toxicity and that these effects are primarily executed via GalR2[70–79]. Decreased neuronal survival in the hippocampus was found in galanin knockout (Gal-KO) animal models following peripheral kainic acid application, an excitotoxic amino acid. Hippocampal CA1 and CA3 regions of Gal-KO mice appeared to be significantly more affected with regard to extent of cell death than those of wildtype (WT) animals. Furthermore, galanin-overexpressing animals displayed resilience towards kainic acid-induced cell death.[70] However, since the upregulation of galanin expression is interpreted as an action to induce neuroprotection, efforts are made to elucidate potential roles of galanin in Alzheimer's disease (AD). For example, the effect of β-amyloid on neuronal survival has been analysed. This molecule is thought to play an important role in development and progression of AD. Thus, cultured rat basal forebrain neurons were found to be protected

against β-amyloid-induced cell death by application of galanin or Gal(2-11) acting via GalR1.[80] However, well established key responses for neuroprotective and neuroregenerative mechanisms seem GalR2 mediated, whereas this implies that a drug targeting GalR2 may be a useful tool to reduce progression of, and protect against, AD and other types of degeneration or nerve injury.

David Wynick's team, in particular, but also other groups have reported that galanin has trophic effects, such as stimulating neurite outgrowth of adult sensory neurons following axonal injury. For example, in Gal-KO animals nerve regeneration was shown to be reduced by 35% in comparison with wild type control animals following a crush injury to the sciatic nerve. Also in vitro galanin showed significant effects on neurite outgrowth. Thus in cultured dispersed adult DRG sensory neurons from Gal-KO mice, neurites only were half as long as neurites in WT cultures. Furthermore, the total number of neurons producing neurites in culture was reduced by one third. However, this deficit could be prevented by adding galanin or Gal(2-11) to the medium.[29]

A trophic effect of GalR2 is also supported by studies on GalR2-KO animals, which show a reduced neurite outgrowth from cultured adult DRG neurons, although this cannot be rescued by application of either Gal(2-11) or galanin itself[76].[74,81] To further characterize the receptor that mediates this trophic effect, GalR1-KO mice were screened after nerve crush injury and did not show any impairment of regeneration. Similarly, this result was also supported by studies in vitro[72]. Regarding the latter results, galanin's trophic impact is supposed to be mediated via activation of GalR2, which is supported by the finding that GalR2-KO animals show a reduction of neurite outgrowth by one-third in cultured adult DRG neurons, an effect that cannot be rescued by application of Gal(2-11) or galanin

Finally, galanin was shown to stimulate growth of neurospheres from adult hippocampal progenitor cells, showing an increased neurite outgrowth after galanin treatment.[77]

2.2.3.1 Galanin and ALS

As mentioned previously, galanin has a neuroprotective effect in several experimental models, including pathologic conditions such as neurodegeneration, and we hypothesize that this could be the case also in ALS. Glutamate excitotoxicity is a major factor in many neurodegenerative processes. In fact, a substantial number of studies implicate glutamatergic signaling in ALS, involving astrocytes[82–86]. Therefore, it is interesting that galanin, as already said, can inhibit glutamate release in various experimental models, both in vitro[78], as well as in vivo in the hippocampus, without altering GABA release, and thereby changing the excitatory tone without modifying inhibition[58].

2.2.4 Calcitonin gene-related peptide (CGRP)

CGRP is a neuropeptide with a length of 37 amino acids, which can be found in the peripheral and central nervous system and is produced by alternative splicing of the calcitonin gene RNA transcript[87]. The CGRP receptor consists of receptor activity modifying protein 1 (RAMP) and calcitonin-receptor-like receptor (CRLR). CRLR involves seven transmembrane domains and is presented to the cell surface by RAMP1 which is coupled to G-protein[88]. Two forms of the neuropeptide are known, α-CGRP and β-CGRP with a 90% structural identity, the former being the best studied variant. CGRP is, among others, localized in motor and DRG neurons, the latter belonging to the small and medium-sized populations[87]. These neurons innervate striated muscle cells and blood vessels, respectively. In fact, CGRP is a potent vasodilator with a very long duration of action, and plays an important role in the pathophysiology of migraine[59]. CGRP is known to be involved in many physiologic as well as pathophysiologic conditions,

including inflammatory bowel disease[89] and is also likely to transmit bidirectional signals within the gut-brain interactive system[90]. CGRP has also been shown to promote neural regeneration after injury to sensory neurons in rats. In agreement with this activity, siRNA for CGRP applied close to the transection site disrupts axonal outgrowth while in vitro isolated Schwann cells show enhanced proliferation after application of exogenous CGRP[91]. In the spinal cord, CGRP is upregulated within motor neurons after nerve injury[92,93], raising the possibility that CGRP may promote motor neuron regeneration and nerve regeneration in the ventral horn. On the other hand, CGRP is down-regulated after nerve injury in the DRG, as reported in monkeys[50,92,93], which points to a possible association with pain.

In this study, CGRP was used as a marker for motor neurons within the spinal cord, in which it is colocalized with acetylcholine (ACh). It is useful as a positive control, since it is upregulated after nerve injury, just as shown for galanin.

2.2.4.1 CGRP and ALS

In context with ALS, CGRP may attract some attention as it is thought to promote nerve regeneration after injury[94]. However, a study in 2012 involving ALS mouse models expressing pathogenic $SOD1^{G93A}$ revealed new insights regarding CGRP, as the basic CGRP expression rate within distinct motor neurons was shown to correlate with their vulnerability in ALS. In this study, it was revealed that 80% of motor neurons expressing high levels of CGRP were lost, whereas of the motor neurons expressing low levels of CGRP only 50% vanished and motor neurons lacking CGRP did not seem to be affected at all. This study therefore came to the conclusion that CGRP levels might predict vulnerability of motor neurons in ALS.[95]

In the present study, motor neurons in culture were screened for CGRP expression at the peptide level primarily to phenotypically characterize these murine cells.

2.2.5 Other peptides

In ALS, some motor neurons degenerate while others do not. In the past decades, research has focused on modes of action of neuropeptides, some of which were found to be involved in recovery and/or to mediate protective actions in neurons. Not only CGRP and galanin have been studied in motor neurons, also a number of other peptides have been examined especially after nerve injury, for example, in the rat. However, similar studies have not been carried out in mouse. Therefore, we screened murine spinal cords following sciatic nerve transection to reveal expression of seven further neuropeptides in motor neurons.

2.2.5.1 Cholecystokinin (CCK)

CCK was originally identified as a 33-amino acid peptide and gastro-intestinal hormone expressed mainly in the duodenum and jejunum[96]. It is secreted following intake of mostly lipids and proteins. It was then realized that a special form of CCK, a sulphated octapeptide, can also occur in the nervous system[97,98]. The peptide acts on two receptors: CCK1 and CCK2 which again are members of the GPCR superfamily[99]. Via its receptors, CCK is involved in the reduction of food intake and the control of pancreatic glucose homeostasis.[100] It may also be involved in pain and memory processes[101,102].

However, CCK is not only expressed within the gastrointestinal tract, it is also present in the central nervous system (CNS) where it is thought to be involved in various functions such as anxiety, analgesia and pain as well as in neuropsychiatric and neurodegenerative disorders[103]. For example, it was found to enhance visceral pain-related affective memory and might be involved in the phenomenon of improved memory retention observed in mice accessing food immediately following training sessions[104,105].

Within regard to motor neurons, CCK was found in the spinal cord on mRNA level in rat and monkeys. Here we explored to what extent CCK is expressed in the adult murine spinal motor neurons and if any effects can be seen after nerve transection, using immunohistochemistry.

2.2.5.2 Enkephalin (ENK) and dynorphin (DYN)

There are three families of endogenous opioid peptides which belong to the opioid system and are widely distributed within the CNS. They include enkephalins, dynorphins and endorphins[106], acting via three GPCRs: mu, delta and kappa (μ, δ, κ)[107]. These molecules have been associated, in particular, with pain-inhibitory mechanisms at the spinal level. A painful stimulus induced, for example, in the skin is forwarded by afferent, unmyelinated C-fibers, to second-order interneurons and then to projection neurons in the spinal cord, conveying information to the thalamus, and eventually to the cortex. Opioid peptides are expressed in a system descending from the brain to the spinal cord. Here, opioid peptides are released in the dorsal horn and via their receptors are involved in pain response management.[108,109]

Previously, following sciatic nerve transection, ENK was found to be downregulated in spinal motor neurons ipsilateral to injury, as observed at the mRNA level[110]. Interestingly, studies in rats revealed neurotoxic and degenerative effects of DYN following intrathecal injection of DYN and several other neuropeptides[111].

In this study, we aimed at studying expression patterns of ENK and DYN in motor neurons in mice following unilateral axotomy.

2.2.5.3 Somatostatin (SOM)

Somatostatin was discovered as the growth hormone release-inhibiting factor in the hypothalamus[112]. Soon afterwards, it was discovered that SOM also inhibits release of other hormones and is widely distributed in the brain and in peripheral nerves and endocrine

cells[113,114]. There are five SOM receptors, all of them belonging to the GPCRs, and all of them mediating inhibition[115].

Here, we focused on somatostatin expression in motor neurons in the murine spinal cord following transection of the sciatic nerve. Interestingly, in vitro studies suggest that SOM has growth-promoting effects on CNS and peripheral nervous system (PNS) neurons[116]. However, following intrathecal injection of SOM, neuronal damage has been observed in the spinal cord as reflected by a loss of motor neurons, hind limb paralysis and nociceptive effects due to neurotoxicity[111,117].

2.2.5.4 Substance P

Substance P was the first member of the tachykinin superfamily to be discovered[118]. This neuropeptide can be found within the brain, enteric and enteroendocrine cells and immune system, and has been shown to be involved in blood pressure regulation, gut-brain axis signaling, intestinal contractility, epithelial secretion, noviveption and other functions. Substance P has been implicated in a large number of pathological conditions such as pain, inflammation, and addictive disorders[89,119]. Tachykinins conduct information via three GPCRs, referred to as neurokinin receptors (NKRs)[78,120]. Despite the relatively broad knowledge about the participation of substance P in many medical conditions, antagonists have thus far failed to be efficacious in different clinical trials, except for one NKR subtype 1 antagonist which is used to blunt nausea and vomiting in chemotherapy and after surgery.[90]

However, substance P was also observed to promote proliferation of normal and ischemic adult neural progenitor cells[121]. Moreover, significant neurite outgrowth promoting effects of substance P were detected in vitro[116]. In this study, the focus was on possible expressional changes in murine motor neurons following axotomy.

2.2.5.5 Neuropeptide Y

Neuropeptide tyrosine (NPY)[122], which consists of 36 amino acids, is an abundant peptide in the central and peripheral nervous and endocrine systems[123] and interacts with five GPCRs; Y_1, Y_2, Y_4, Y_5, and y_6[124]. In the periphery, NPY induces vasoconstriction and inhibits colonic motility. Other effects include control of food-intake and regulation of stress resilience, affect and anxiety, which is relevant to mood disorders. Further roles of NPY include regulation of hormone secretion, circadian rhythm, reproduction and alcohol consumption. Therefore, much effort has been devoted to discovery and development of agonists and antagonists targeting NPY receptors. However, further studies need to be done to elucidate the clinical potential of antagonists acting at these receptors. Moreover, no efficacious non-peptide NPY agonist or antagonist has thus far been developed.[125]

Increased expression of NPY following nerve transection has previously been shown in ipsilateral motor neurons in the rat[110]. In this study, we set out to analyze whether this interesting shift of expression also occurs in mice.

2.2.5.6 Neuropeptide S (NPS)

NPS, which is named after its N-terminal serine residue and consists of only 20 amino acids in humans, rats, mice and others, acts via its GPCR receptor on the G_q protein to mobilize intracellular Ca^{2+} storage and is therefore thought to enhance neural excitability. Moreover, studies suggest functions of NPS in regulating feeding behavior, learning, memory, anxiety as well as arousal.[126,127]

Nevertheless, NPS expression patterns following nerve injury by sciatic nerve transection have not been studied thus far. In this study we focused on possible differences that might be observed following axotomy in mice, when comparing the ipsilateral site to the contralateral

site of the lumbar spinal cord. With this attempt, we aimed at making the first steps to a new approach in NPS research, addressing its potential within the field of neuroregeneration, and possible effects on injured motor neurons.

2.2.6 Aims of the study

The aim of this study was to shed light on expression patterns of galanin, GalR1, GalR2 and GalR3 as well as CGRP at the mRNA level within the murine spinal cord and to find out about a possible impact of unilateral sciatic nerve axotomy on expression. Furthermore, peptide expression patterns following axotomy were studied for galanin, CGRP, NPY, CCK, ENK, DYN, SOM, substance P, NPY and NPS.

The basic hypothesis that we wanted to test was that galanin acts as a pleiotropic factor to protect from early and progressive neurodegeneration in ALS and fosters regeneration of neurons and operates as a trophic agent. In order to find out whether galanin is involved in these beneficial mechanims, we performed axotomy on five male mice to elucidate whether upregulation of galanin expression occurs, as has been observed in rats. Furthermore, we received stem-cell derived juvenile motor neurons in cell culture from Eva Hedlund's group and examined if these cells express galanin themselves and could be relevant to follow-up studies. In this respect we considered to perform live-cell imaging and learn whether galanin could act as a rescue factor on murine motor neurons in culture.

3. Materials

3.1 Solutions, media and hardware

Table M1 Solutions, media and hardware listed, referring to company and usage in the experiments

Solution/ medium/ hardware	Company	Country	Used for
Agar	Merck	Darmstadt, Germany	Bacteria culture
AL4 fixative	Kodak, Sigma	Steinheim, Germany	Radioactive ISH
Amplification diluent	Perkin Elmer	Boston, USA	Immunohistochemistry, fluorescent ISH
Autoradiographic emulsion	Kodak, Sigma	Steinheim, Germany	Radioactive ISH
Bed resin	Bio-Rad	California, USA	Deionized formamide
Blocking reagent	PerkinElmer	Boston, USA	TNB buffer
BSA	Sigma Aldrich	Steinheim, Germany	ISH
Conc. HCl	Honeywell	Seelze, Germany	ISH
CryoStar™ NX70	Thermo Scientific	Walldorf, Germany	Sectioning tissue
DABCO	Sigma Aldrich	Steinheim, Germany	Mounting slides
Denhart's solution 50x	Sigma Aldrich	Steinheim, Germany	ISH
Diethyl pyrocarbonate (DEPC)	Sigma Aldrich	Steinheim, Germany	Fixing tissue, ISH
EDTA pH 8.0	Ambion	California, USA	ISH
Ethanol 99.5%	Kemetyl	Haninge, Sweden	ISH
FITC	Perkin Elmer	Waltheim, USA	Fluorescent ISH

Glycerol	Merck	Darmstadt, Germany	Glycerol in PBS
HRP DIG-AB (POD)	Roche	Mannheim	Fluorescent ISH
H_2O_2 30%	Merck	Darmstadt, Germany	Quenching
Ilford hypam rapid fixer	Harman Technologies	Cheshire, UK	Radioactive ISH
Imaging plate falcon 96 well	BD Falcon	Stockkholm, Sweden	Cell Culture
Isoflurane	Baxter	Kista, Sweden	Animal surgery
Kanamycine	Sigma Aldrich	Steinheim, Germany	Bacterial work
KCl	Merck	Darmstadt, Germany	ISH
KH_2PO_4	Merck	Darmstadt, Germany	ISH
Kodak BioMax MR film	Kodak (VWR International)	Rochester, NY (Stockholm, Sweden)	Radioactive ISH
Kodak professional developer D-19	Kodak, Sigma	Steinheim, Germany	Radioactive ISH
LSM700 meta confocal microscope, helium-neon lasers	Carl Zeiss	Jena, Germany	Fluorescent ISH, immunohistochemistry
NaCl	Merck	Darmstadt, Germany	ISH
$NaH_2PO_4.2H2O$	Merck	Darmstadt, Germany	Lana's Fix
$Na_2HPO_4.2H_2O$	Merck	Darmstadt, Germany	ISH, lana's Fix
NaOH 5M	BDH, VWR	Radnor, USA	ISH

Nikon DXM 1200	Nikon	Düsseldorf, Germany	Radioactive ISH
Nikon Eclipse E600	Nikon	Düsseldorf, Germany	Radioactive ISH
Normal donkey serum	Jackson Immunoresearch	Baltimore, USA	Immunohistochemistry
O.C.T Cryomount	HistoLab	Göteborg, Sweden	Sectioning tissue
Paraformaldehyde	Sigma Aldrich	Steinheim, Germany	ISH
Picric acid	Sigma Aldrich	Steinheim, Germany	Immunohistochemistry
PureLink quick plasmid miniprep kit	Invitrogen	Carlsbad, California	Plasmid preparation
QiaQuick gel purification Kit	Qiagen	Stockholm, Sweden	Gel purification
RNaseA	Roche	Mannheim, Germany	ISH
SSC 20x	Ambion	California, USA	ISH
SuperFrost Plus	Thermo Scientific	Walldorf, Germany	Coverslip sides, tissue preparation
Triethanolamine	Sigma	Steinheim, Germany	ISH
Tris-HCl	Ambion	California, USA	ISH
Triton-X-100	Sigma Aldrich	Steinheim, Germany	Immunohistochemistry
Tween 20	Sigma Aldrich	Steinheim, Germany	TNT buffer
Wallac 1409 DSA liquid scintillation counter	PerkinElmer	Boston, USA	Radioactive ISH

Yeast t-RNA (50mg/ml)	Ambion	California, USA	ISH prehybridization
[^{35}S]-UTP	PerkinElmer	Boston, USA	Radioactive ISH
Enzymes, molecular reagents, cells			
BamHI (10 U/µl)	Promega	Stockholm, Sweden	Restriction of TOPO II vector + Insert
DIG RNA labeling mix 10x	Sigma Aldrich	Steinheim, Germany	Labeling fluorescent ISH
[DIG]-UTP (25 µL [10 mM])	Sigma Aldrich	Steinheim, Germany	Labeling radioactive ISH
DNAse I (10 U/µl)	Roche (Sigma Aldrich)	Steinheim, Germany	Transcription
dNTP mix	Sigma Aldrich	Steinheim, Germany	PCR
EDTA (0.2 M)	Sigma Aldrich	Steinheim, Germany	Stop reactions
iScript select cDNA synthesis	BioRad	California, USA	Reverse transcription
Multicore buffer 10x	Promega	Stockholm, Sweden	Digestions, cloning
Nitrocellulose membrane	Sigma Aldrich	Steinheim, Germany	Spotting of labeled probes
One shot chemically competent cells TOP 10 (50 µl each)	invitrogen	Carlsbad, California	Cloning
PCR buffer 10x	Invitrogen	Carlsbad, California	PCR
pCRII-TOPO vector (10ng/µl plasmid DNA)	Invitrogen	Carlsbad, California	Cloning

Quick spin RNA columns	Roche (Sigma Aldrich)	Steinheim, Germany	Purification of riboprobes
RNAse free water	Sigma Aldrich	Steinheim, Germany	RNA work
RNA SP6 polymerase (20 U/µl)	invitrogen	Carlsbad, Canada	Transcription of wanted sequence in TOPO II vector
RNA T7 polymerase (20 U/µl)	invitrogen	Carlsbad, Canada	Transcription of wanted sequence in TOPO II vector
RNeasy kit	Qiagen	Stockholm, Sweden	mRNA extraction
Salt solution: (1.2 M NaCl, 0.06 M $MgCl_2$)	Invitrogen	Carlsbad, California	Cloning
S.O.C. medium	Invitrogen	Carlsbad, California	Cloning
Taq DNA polymerase (5 U/µL)	Sigma Aldrich	Steinheim, Germany	PCR
Transcription buffer 10x	Sigma Aldrich	Steinheim, Germany	Transcription
XbaI (10 U/µl)	Promega	Stockholm, Sweden	Restriction of TOPO II vector + insert

3.2 Buffers and reagents

Table M2 Recipes of buffers and reagents listed, as well as usage in the experiments.

Buffer/ reagent	Ingredients	Amount	Used for
Blocking buffer	Normal donkey serum Triton X-100	10% (vol/vol) 0.1% (vol/vol)	Immunohistochemistry

	1xPBS		
Box buffer	Formamide 20x SSC DEPC-water	50% (vol/vol) To 1x	ISH prehybridization
Buffer 1 (pH 7.5)	Maleic acid, NaCl,	100 mM 150 mM	Riboprobe synthesis (fluorescent ISH)
Buffer 2	Blocking reagent stock solution: Buffer 1	1:10	Riboprobe synthesis (fluorescent ISH)
Buffer 3 (pH 9.5)	Tris-HCl, NaCl	100 mM 100mM	Riboprobe synthesis (fluorescent ISH)
DABCO in glycine	DABCO Glycine	2.5% (vol/vol)	Mounting slides
Deionized formamide (stired at 4°C overnight; pH 5.5, filtered, stored at -20°C)	Bed resin Formamide	33.3% of formamide volume	ISH prehybridization
Dextran sulphate (dissolved at 37°C in waterbath, stored at -20°C)	Dextran Sulphate DEPC-water	50% (vol/vol)	ISH hybridization
Dithiothreitol (DTT) 2M filter through sterile 0.45µm syringe filter	DTT DEPC-water	30% (vol/vol)	ISH hybridization
Glycerol in PBS	Glycerol PBS	87% (vol/vol)	Mounting slides radioactive ISH
HCl stock 1M and 0.5M	Conc. HCL DEPC-water	 Dilute	Tissue fixation
Hybridization mix	NaCl DTT	0.3M 20mM	ISH hybridization

	Dextran sulfate 50x Denharts solution Yeast t-RNA (50mg/ml) Poly-A-RNA (10mg/ml) DEPC-water	10% (vol/vol) To 1x 0.5mg/ml 0.1mg/ml Fill up	
Paraformaldehyde in PBS 4% (pH 7.5)	Paraformaldehyde 10xPBS 5M NaOH Conc. HCl DEPC-water	4% (vol/vol) To 1x until clear To adjust pH Fill up	Tissue fixation
PBS 10x	NaCl Na2HPO4.2H2O KH_2PO_4 KCl	8% (vol/vol) App. 3% (vol/vol) 0.2% (vol/vol) 0.2% (vol/vol)	Tissue fixation
Prehybridization mix (stored at -20°)	Tris-HCl (pH 7.6) EDTA (pH 8.0) 50x Denharts solution Yeast t-RNA NaCl DEPC-water	50mM 25mM To 2.5x 0.25mg/ml 20mM Fill up	ISH prehybridization
Quenching solution	30% H_2O_2 TNT buffer	To final conc. of 0.3% (vol/vol)	ISH hybridization
TAE buffer	Tris acetic acid EDTA.	40 mM 20 mM 1 mM	Electrophoresis
TNB buffer	Tris NaCl Buffer Blocking reagent	0.5% (vol/vol)	ISH hybridization
TNT buffer	Tween 20 Tris NaCl buffer	0.05% (vol/vol)	ISH hybridization

Triethanolamine 0.1M	Triethanolamine Conc. HCl DEPC-water Acetic Acid	 To adjust pH 0.25% (vol/vol)	Tissue fixation
Tris NaCl buffer (pH 7.5)	TRIS NaCl Conc. HCl	50mM 150mM To adjust pH	Buffer preparation
Yeast t-RNA (50mg/ml)	Solved t-RNA in DEPC-water		ISH prehybridization

3.3 Primers

Table M3 Primer sequences and companies listed.

Gene	Primer description	Company	Country	Sequence
CGRP	Forward	Sigma Aldrich	Sweden	5'-GTGCAGAACTATATGCAGATGAAAG-3'
	Rev	Sigma Aldrich	Sweden	3'-GGACTAGATTTGCTACCAGATAAGC -5'
Gal	Forward	Sigma Aldrich	Sweden	5'-GACAACCACAGATCATTTAGCGAC-5'
	Rev	Sigma Aldrich	Sweden	3'-CAAGAGACGAAACTTCGACACAATA-5'
GalR1	Forward	Sigma Aldrich	Sweden	5'-ACAAGCTCCACAAGAAGGCTTA-5'
	Rev	Sigma Aldrich	Sweden	3'-CGCATGTTCGTTCACAAGTTCA-5'
GalR2	Forward	Sigma Aldrich	Sweden	5'-GTACTCTTCTGCCTCTGTTGGATG-5'
	Rev	Sigma Aldrich	Sweden	3'-ACAATTTCCTGGTTTCCCGTAGAT-5'
GalR3	Forward	Sigma Aldrich	Sweden	5'-GGCTGACATCCAGAACATTTCG-3'
	Rev	Sigma Aldrich	Sweden	3'-GATGGATTCGATGATGCCGTGC-5'

	Primer T7 promoter (for Sequencing of TOPO-II-vector)	Invitrogen	Carlsbad, Canada	5´-TAATACGACTCACTATAGGG-3´

3.4 Temperature profile for PCR

Table M4 PCR temperature profile.

Temperature	Time	Cycles
95°C	2 minutes	-
95°C	30 seconds	37x
55°C	30 seconds	
72°C	1 minute	
72°C	10 minutes	-

3.5 Temperature profile reverse transcription

Table M5 Reverse transcription temperature profile.

Temperature	Time
4°C	1 minute
25°C	5 minutes
42°C	30 minutes
85°C	5 minutes

3.6 Cell culture medium

Table M6 Media used for cell culture experiments and companies listed.

Medium Ingredients	Amount	Company	Country
BDNF	5-10ng/ml	R&D Systems	Minneapolis, USA
B27 supplement	2%	Invitrogen	Carlsbad, California
β-Mercaptoethanol (55mM)	0.5%	Invitrogen	Carlsbad, California
CNTF	5-10ng/ml	R&D Systems	Minneapolis, USA
DMEM/F12	48%	Life Technologies	Carlsbad, California
GDNF	5-10ng/ml	R&D Systems	Minneapolis, USA
L-glutamine (200mM)	0.5%	Invitrogen	Carlsbad, California
Neurobasal	48%	Invitrogen	Carlsbad, California
NT3	5-10ng/ml	R&D Systems	Minneapolis, USA
Penicillin-streptomycin	1%	Invitrogen	Carlsbad, California

3.7 Cell culture fixation and immunohistochemistry

Table M7 Media and solutions used for fixation and immunohistochemistry listed.

Medium, Solution	Ingredients	Amount	Usage
Lana's fix pH 7.4	Paraformaldehyde 5M NaOH	4% Clear solution	Cell culture fixation

	$Na_2HPO_4.2H2O$ $NaH_2PO_4.2H2O$ Picric acid (saturated)	28.8% 11.2% + to adjust pH 14%	
Blocking solution	0.3% Triton-X-100 in PBS Normal donkey serum 1xPBS	0.1% 10%	Immunohistochemistry

3.8 Primary antibodies

Table M8 Primary antibodies for immunohistochemistry, used dilutions and experiments listed.

Antibody	Host species, mono- vs. polyclonal	Dilution used	Used for	Manufacturer data (received from)
CCK mouse	Rabbit, polyclonal	1:8000	Tissue sections (TSA)	Prof. P. Frey (Frey, 1983, code R-1983)
CGRP mouse	Rabbit, polyclonal	1:5000 1:10000	Cell culture Tissue sections	Sakurada et al., 1991
ChAT mouse	Goat, polyclonal	1:1000	Tissue sections (TSA)	Merck Millipore, polyclonal
DYN mouse	Rabbit, polyclonal	1:2000	Tissue sections (TSA)	Bergström et al., 1983
ENK mouse	Rabbit, polyclonal	1:8000	Tissue sections (TSA)	Schultzberg et al., 1978
GAL mouse	Rabbit, polyclonal	1:400 – 1:800 1:4000	Cell Culture Tissue sections (TSA)	Theodorsson and Rugarn, 2000
NPS mouse	Rabbit	1:4000	Tissue sections (TSA)	Abcam Biochemicals,

				polyclonal
NPY mouse	Rabbit, polyclonal	1:3000	Tissue sections (TSA)	Theodorsson-Norheim et al., 1985
SOM mouse	Rabbit, polyclonal	1:8000	Tissue sections (TSA)	Benoit et al., 1980
Substance P mouse	Rabbit, polyclonal	1:4000	Tissue sections (TSA)	Bergstro¨m et al., 1983 (code 497)

3.9 Secondary antibodies and fluorophores

Table M9 Secondary antibodies and fluorophores used for immunohistochemistry listed.

Fluorophor, primary antibody	Host species	Dilution used	Company
Cy3, goat	Donkey	1:40 (in triton)	Jackson, Sweden
Cy3, rabbit	Donkey	1:100 (in 10% serum and 1x PBS)	Jackson, Sweden
Cy5, mouse	Donkey	1:40 (in 10% serum and 1x PBS)	Jackson, Sweden
HRP, rabbit	Swine	1:200 (in TNB)	Dako, Denmark

4. Methods

4.1 Sciatic nerve axotomy

Five male C57BI/6 mice (12-14 weeks of age, derived from SCANBUR AB) were kept under standard conditions with a 12/12 hours light and dark cycle and free access to food and water. All experiments were approved by a local ethical committee (Stockholms Norra djurföröksetiska nämnd, N134/12) and procedures were performed in accordance with Swedish policy for the use of research animals, whereas the number of mice used was kept as low as possible, as well as their suffering throughout the experiments.[128]

The animals were deeply anaesthetized with 1.7–2.0% (vol/vol) isoflurane, and the left sciatic nerve was exposed at mid-thigh level. The sciatic nerve was ligated and unilaterally transected distally to the ligation, with a 5 mm portion of the nerve being resected. The animals were allowed to survive for another 7 days after surgery until decapitation and dissection of the spinal cords were performed. The tissue was frozen immediately and stored at -80°C. The levels L4 and L5 were cut out of the spinal cords and embedded in O.C.T Cryomount using liquid carbon dioxide on 10% sucrose soaked paper, with the ventral part of the spinal cord pieces facing downside. The “left” sides of the tissue pieces were referred to as ipsilateral, whereas the right sides were labeled contralateral.

For fluorescence immunohistochemistry of spinal cords, 5 male mice were treated as described above, whereas animals were perfused this time using picric acid before the spinal cords were removed from the bodies.

4.2 Tissue preparation and fixation

Spinal cord tissue was sectioned using a cryostat to obtain 20 µm slices, which were collected on thermofrost glass slides and stored at -80°C until being fixed for in situ hybridization.

For fixation, all solutions were prepared using DEPC water. DEPC water was prepared with DEPC and deionized water in a ratio of 1:1000 respectively, followed by vigorous shaking and overnight incubation at RT. The DEPC water was then autoclaved and ready to use. Slides were fixed in 4% paraformaldehyde (PFA) for ten minutes at 4°C under an air-flow hood, followed by washing steps at room temperature (RT): 5 minutes each in 1xPBS, DEPC-water, 0.1M HCl, 3 minutes in 1xPBS twice, 20 minutes in 0.1M triethanolamine, followed by 3 minutes in 1xPBS twice. The sections were then dehydrated in graded alcohol series with 70%, 80% and 99.5% for 2 minutes each. Slides were dried for approximately 30 minutes and stored at -20°C until further use.

4.3 Riboprobe synthesis

Riboprobes were synthesized for mouse galanin, GalR1, GalR2, GalR3 and CGRP transcripts. Primers were designed using NCBI's primer-BLAST tool and synthesized by Sigma Aldrich. The primers were reconstituted as per instructions using nuclease free water and stored at +4°C.

To generate the probes, total RNA was extracted from mouse olfactory bulb and dorsal raphe nucleus samples using RNeasy Mini Kit. RNA purity and yield was checked using a Nanodrop. 1µg of RNA was reverse transcribed using the iScript Select cDNA Synthesis Kit and random primers as per manufacturer's suggestion. Target sequences for all the transcripts mentioned above were amplified by PCR and using the primers designed (see table M3, M4 in Materials). The PCR products were confirmed by gel electrophoresis (PCR product size, see table 1) and

purified using QIAquick gel purification kit. The purified PCR product was then cloned into TOPOII vector and transformed to One Shot TOP10 chemically competent E. coli cells (for TOPO II map, see supplementary information). Bacteria were spread-plated on LB Agar plates with kanamycine (0.05mg/ml) and incubated overnight at 37°C. Colony PCR was performed to check for the positive clones. Four well-spaced colonies per plate were picked, transferred to 50µl sterile water and incubated in boiling water for 10 minutes, followed by centrifugation at 16000g for 5 minutes. The supernatant was used to perform a PCR (same primers and temperature profile were used as for fragment preparation). The PCR products were then screened via agarose gel electrophoresis, and the 'positive' clones were picked, transferred to terrific broth with ampicillin (0.05mg/ml) and incubated overnight at 37°C in a shaker at 220 rpm. Plasmids were purified using PureLink Quick Plasmid miniprep kit. Homology and orientation of the probes was confirmed by sequencing at KI Gene (Stockholm) (see table 1). Restriction site analysis was performed using CLCSequence viewer. All three plasmids were linearized using XbaI and BamHI to generate sense (S) and antisense (AS) riboprobes by incubating plasmids with the Enzyme, 10x Multicore Buffer (final concentration 1x) and Nuclease free water for 2 hours at 37°C.

Table 1 Direction of sequence of interest (galanin, GalR1, GalR2, CGRP) in Topo II vector. Restriction enzymes used to generate AS or S probe.

Gene	Antisense prromoter	Restriction Enzyme for AS probe	Restriction Enzyme for S probe	PCR Product size (bp)
CGRP	T7	BamHI	XbaI	500
Galanin	T7	BamHI	XbaI	350
GalR1	SP6	XbaI	BamHI	381
GalR2	T7	BamHI	XbaI	398

4.4 In situ hybridization

In situ hybridization was performed with two different approaches: radioactive in situ hybridization (ISH) and non-radioactive fluorescent in situ hybridization (FISH). Irrespective of the chemistry used, ISH was performed by labeling the probes with either [^{35}S]-UTP (for radioactive ISH) or DIG (for FISH), followed by prehybridization, hybridization, washes and detection. The details for each approach are mentioned below.

4.4.1 Radioactive in situ hybridization

4.4.1.1 Labeling of riboprobes

Plasmid DNA was linearized using the enzymes mentioned in table 1. After linearization of the plasmid DNA, S and AS riboprobes were transcribed by SP6 or T7 polymerase in the presence of [^{35}S]-UTP as per instructions in the manual (MaxiScript® *In vitro Transcription Kit*)[129]. Briefly, 1µg of the plasmid DNA was incubated with 10x transcription buffer (final concentration 1x), 10mM of ATP, CTP, GTP, [^{35}S]-UTP and the enzyme mix for 1 hour at 37°C followed by digestion using TURBO DNAse I for 15 minutes at 37°C and subsequently all reactions were stopped by adding 1µl of 0.2 M EDTA. Unincorporated nucleotides were purified using mini Quick Spin RNA columns. Radioactivity was measured by liquid scintillation counting (for results see supplementary information). An average of two counts per probe was taken to achieve the demanded probe activity of $1*10^6$ counts per million (cpm), to be added to the hybridization solution.

4.4.1.2 Prehybridization, hybridization and washes

Slides were removed from the -20°C freezer to dry at room temperature (RT) for half an hour. Sections were pre-hybridized using the prehybridization mix and deionized formamide in a 1:1

ratio for about 2 hours at 65°C followed by hybridization in a humidified chamber overnight (14-16 hours) at 65°C. Labeled probes were diluted to a final concentration of 1.0x106 cpm/200µl in a solution containing hybridization mix and 50% (vol/vol) deionized formamide. After hybridization, sections were washed twice in 1x SSC, an RNAse free buffer, for 30 minutes at 55°C, 1 hour in 50% (vol/vol) formamide/0.5x SSC at 55°C, for 15 minutes in 1x SSC at 55°C, for 1 hour in RNase A buffer at 37°C, in 1x SSC for 15 minutes at 55°C twice, and finally sections were dehydrated in an ascending series of ethanol (70%, 90%, 95%, 99.5%; 2 minutes each) and air-dried.

4.4.1.3 Detection

Sections were first exposed to Kodak BioMax MR film in a film cassette (in dark room) for 5 days and developed for 2 minutes using Kodak professional D19 developer, rinsed in water and fixed in Ilford Hypam rapid fixer for 15 minutes. See figure 4 for results of exposing the slides to the film. Hybridization can be seen as black dots on the tissue sections. The slides were then dipped in autoradiographic emulsion diluted to a 1:1 ratio with distilled water. After 'individual' exposure times (5 days for galanin and CGRP, 4 weeks for GalR1, 7 weeks for GalR2 and GalR3), slides were developed using Kodak professional D19 developer for 3 minutes and AL4 fixative for 7 minutes, dried at RT, and then mounted with a medium containing 90% glycerol and 10% PBS (vol/vol)

4.4.1.4 Microscopic analysis

Sections were analyzed using a Nikon Eclipse E600 microscope equipped with a bright- and dark-field condenser and epi-polarization with side entrance illumination and epi-fluorescence with appropriate filter combinations connected to Nikon DXM 1200 digital camera. Digital images were imported into GIMP 2 (GNU Image Manipulation Program) and optimized for brightness, contrast, and sharpness.

4.4.2 Fluorescent in situ hybridization

4.4.2.1 Labeling of riboprobes

Linearized plasmid (1µg) was in vitro transcribed using 10x DIG RNA labeling mix, [DIG-UTP] (final concentration 1x), 10x transcription buffer (diluted to 1x), RNAse free water, RNA polymerase and incubated for 2 hours at 37°C, followed by DNAse I treatment at 37°C for 15 minutes. The reaction was stopped by adding 0.2M EDTA to the mixture. Unincorporated nucleotides were purified using Quick Spin RNA Columns. A dot blot assay was performed to check for the efficacy of the transcription and to estimate the concentration of the labeled probes. A 10 fold serial dilution of the transcribed probes and control RNA (20µg/ml) was prepared in RNA dilution buffer (formamide:SSC:water 5:3:2). 1µl of the dilutions (probes and control RNA) were spotted on a nitrocellulose membrane and cross-linked using UV light for about 10 minutes. The membrane was then washed in buffer 1 twice for about 15 minutes and incubated in buffer 2 for 30 minutes. Anti-DIG-alkaline phosphatase was diluted 1:5000 in buffer 2 and the membrane was incubated overnight at 4°C. On the next day, the membrane was washed in buffer 1 twice for 15 minutes. To detect the labeled probes and confirm labeling, NBT/BCIP stock solution was prepared in buffer 3 as suggested by the manufacturer and the membrane was incubated in the dark for about 20 minutes until spots were visible (figure 2 and 3). Procedures were performed as suggested in Roche 'DIG Application Manual'[130].

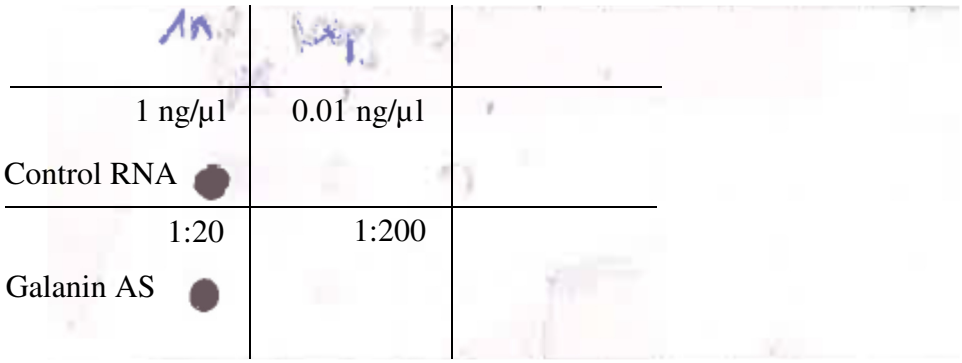


Figure 2 Confirmation of probe labeling. Control RNA was spotted with a concentration of 1 ng/µl (left upper spot) and 0.01 ng/µl (very light upper spot to the right). Galanin AS probe was spotted 1:20 diluted (left lower spot) as well as 1:200 diluted (very light right lower spot) and showed similar strength, suggesting comparable concentration. Concentrations noted in the figure refer to control RNA spotted below.

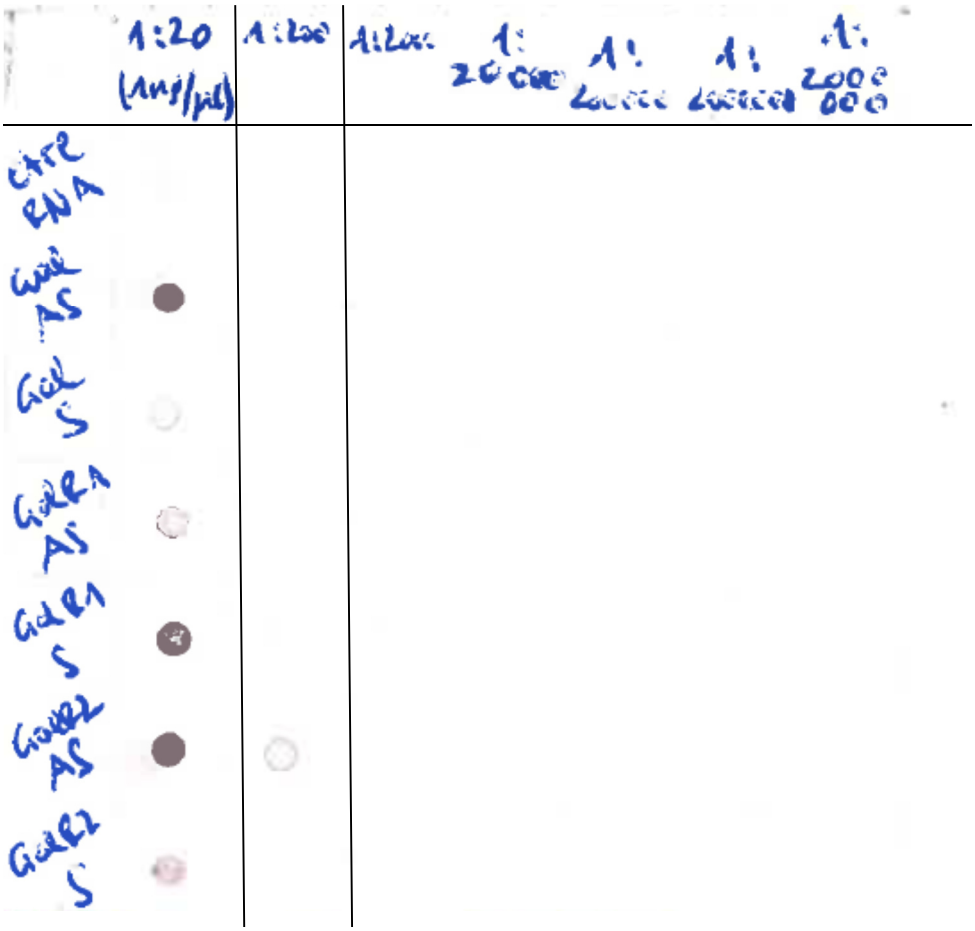


Figure 3 Confirmation of probe labeling. Control RNA was spotted with concentration of 1 ng/µl (see figure 2) and 0.01 ng/µl (figure 2). Galanin AS probe was spotted 1:20 diluted (Gal-AS) as well as 1:200 diluted. Gal-S, GalR1-AS, GalR1-S, GalR2-AS, GalR2-S probes were spotted in the same dilutions below Gal-AS and showed similar strength, confirming successful labeling. Concentrations noted in the figure refer to control RNA (ctrl RNA).

4.4.2.2 Prehybridization and hybridization

Prehybridization and hybridization were performed as described above for the radioactive ISH.

4.4.2.3 Washing and detection

Slides were washed in 2x SSC for 1 hour at RT followed by incubation in 50% (vol/vol) formamide/0.5x SSC for 1 hour at 55°C, and 0.1x SSC (twice) at 60°C for 30 minutes. The slides were then incubated in TNT buffer + 0.3% H_2O_2 to quench intracellular peroxidases for 10 minutes at RT in a humid chamber. Slides were then washed three times in TNT buffer at RT for 5 minutes followed by TNB blocking solution for 30 minutes at RT. The slides were incubated overnight at 4°C in 1:100 Anti-DIG HRP.

The next day, tissue sections were washed in TNT three times for 5 minutes followed by incubation for two hours at RT in amplification diluent and 1:100 fluorescein isothiocyanate (FITC) in a humid chamber. Eventually, slides were washed in TNT buffer three times at RT and mounted and cover slipped using DABCO in glycine.

4.5 Statistics

The software ImageJ was used to quantify the radioactive signal intensity on contralateral vs ipsilateral sides of 60 spinal cord sections, that are represented as arbitrary units (AU). Signals of 101 areas on both sides of the spinal cord sections were collected for galanin, and 157 areas on both sides of the sections were analyzed for CGRP. Statistical analysis was performed using Graphpad Prism5 to compare AU by Mann-Whitney-U-test (p-value selection criteria <0.05).

4.6 Fluorescent immunohistochemical staining

Spinal cord sections of perfused mice were collected as previously described, although they were stored at -20°C until used.

To perform immunostaining using tyramide signal amplification (TSA) technology, following procedure was undertaken: slides were removed from freezer and air-dried for 2 hours at RT. Using a DAKO-pen, tissue sections were surrounded and incubated in 1x PBS for about 15 minutes at RT. Sections were incubated in primary antibodies in 0.3% Triton and PBS at 4°C overnight. For details of the antibodies and dilutions, see table M8 in Materials part. The next day, tissue sections were rinsed in TNT buffer for 15 minutes, blocked in TNB buffer for 30 minutes, incubated in HRP labeled secondary antibody (table M9) for yet another 30 minutes and rinsed again in TNT buffer for 15 minutes (all at RT). To label secondary antibodies with a fluorophore, sections were incubated in FITC 1:100 diluted in amplification diluent for 10 minutes at RT in the dark. Sections were then rinsed in TNT buffer for another 15 minutes at RT and mounted with DABCO medium and cover slipped for fluorescent microscopy.

4.6.1 Ranking of staining intensity

Fluorescent microscopy was used to visually rank expression of neuropeptides in spinal cord sections by staining intensity. Staining was objectively ranked between (-) for absence of staining in described part of spinal cord section and (+++) for very intense staining.

4.7 Double staining

To determine whether the staining for galanin appeared in motor neurons, double staining with ChAT in addition to galanin was performed. Following the last TNT buffer wash-step in the

regular TSA staining for galanin (as described above), spinal cord sections were washed three times for 15 minutes in PBS and blocked in TNB buffer. Before adding the primary antibody for ChAT diluted in Triton, sections were washed in PBS again and after incubating overnight at +4°C, sections were washed in TNT and TNB buffer. Eventually, sections were incubated with secondary antibody diluted in TNB buffer (Cy3, anti-goat) for one hour at RT and subsequently washed in PBS for 10 minutes 3 times.

4.8 Cell culture

4.8.1 Motor neuron differentiation and cell culture procedures

Motor neuron cell cultures were provided by Eva Hedlund's group (Karolinska Institutet, Stockholm). They were gained by inducing stem cells following published protocols[17]. Cells differentiated to motor neurons can be differentiated from others by green fluorescent protein (GFP)-tagged motor neuron marker Hb-9 expression.

Cells were maintained at 37°C and 5% CO_2. Half of the medium was exchanged every alternate day to keep factors produced by the cells in culture. Cells were fixed at 5 different time points: 2 days after showing Embryonic Bodies (EBs), as well as 1 week, 2 weeks, 3 weeks, and 4 weeks after exhibiting EBs. Fixation was performed using picric acid (Lana's Fix). First a 1:1 solution with Lana's Fix and 1x PBS was applied for 10 minutes at RT under the hood, followed by pure Lana's Fix for another 10 minutes under the hood. Subsequently, cells were washed 3 times with 1x PBS until yellow color was no more visible and then stored at 4°C in 1x PBS.

4.8.2 Immunohistochemistry procedures

Coverslips with motor neurons were incubated with blocking buffer for 40 minutes at RT followed by the primary antibody mix at 4°C overnight, the mix containing diluted CGRP primary antibody and β-Tubulin primary antibody in blocking solution (see table number M8 in Materials part for dilutions). The next day, cells were washed with 1xPBS with 10% normal donkey serum (vol/vol) for an hour at RT to subsequently be incubated with fluorophore-labeled secondary antibodies Cy3 and Cy5 (table M9) for 1 hour at RT. After 3 washing steps using 1x PBS, the coverslips were mounted onto slides with DABCO in PBS. On selected coverslips, the primary antibody was omitted to verify specificity of staining.

To determine specific binding of the primary antibody for galanin, absorptions were performed. A total of 1.1 mg rat galanin was dissolved in 1x PBS to gain a 10^{-3} M stock solution. Subsequently, galanin antibody and peptide were mixed to gain a 10^{-5} or 10^{-4} M peptide concentration as well as a 1:400 or in case of TSA staining 1:4000 diluted antibody. This mixture was incubated overnight at 4°C on a shaker to allow binding.

4.9 Fluorescent microscopy

Cells were examined using an LSM700 Meta confocal microscope equipped with ultraviolet, argon, and helium-neon lasers, and LSM software ZEN 2011 (September 2011).

5. Results

5.1 Fluorescent and radioactive ISH on spinal cord sections

Riboprobes were confirmed to hybridize to galanin and CGRP mRNA sequences by exposing spinal cord sections to the Kodak BioMax MR film (figure 4). However, galanin receptors GalR1, GalR2 and GalR3 did not show any signal on slides hybridized with antisense (AS) or sense (S) riboprobes. Therefore, the time of exposure of the GalR1-R3 slides to the emulsion was increased.

5.1.1 Galanin and CGRP mRNA in the spinal cord

Numerous galanin$^+$ mRNA neurons were observed in the dorsal horn of the lumbar spinal cord (figure 5a-c), whereas their distribution varied between different layers, with the highest density of galanin$^+$ neurons in laminae I-III (figure 5a). CGRP$^+$ mRNA neurons were observed in the ventral horn of the spinal cord, with no detectable signal observed in the dorsal horn of the spinal cord (figure 6a-c).

Unilateral axotomy altered expression patterns of peptide mRNAs for galanin (figure 5a-c) and CGRP (figure 6a-c) in the ventral horn. Galanin mRNA was undetectable on the contralateral side (figure 5a1, b1, c1) but a dramatic upregulation was observed on the ipsilateral side (figure 5a2, b2, c2). CGRP positive neurons were observed on both the contralateral and ipsilateral sides of the spinal cord, but the ipsilateral side (figure 6a2, b2, c2) showed a marked increase in expression when compared to the contralateral side (figure 6a1, b1, c1). The quantitative results in terms of the grain density obtained for galanin and CGRP mRNA are shown in figure

7, in which a significant increase is seen in galanin (figure 7a) and CGRP (figure 7b) mRNA in the ipsilateral ventral horn compared to the contralateral ventral horn.

These results are confirmed by the results obtained from fluorescent ISH for galanin and CGRP AS riboprobes (figure 6d,e).

5.1.1.1 Statistics of radioactive ISH slides

The Mann-Whitney-U-test was used to compare expression signals of the contralateral vs. ipsilateral sides of the ventral spinal cord sections on Graphpad Prism5. The expression signals (AU) differed significantly between the two sides (figure 7). For galanin, the signal density on the side contralateral to the injury was 15.82 ± 0.41 AU (mean ± standard error of the mean) compared to 95.58 ± 5.34 AU on the ipsilateral side. For CGRP the signal density was 38.41 ± 2.44 AU (mean ± standard error of the mean) on the contralateral side, relative to 54.04± 2.59 on the ipsilateral side. These results are illustrated in figure 7a and b.

5.1.2 Galanin receptors in the spinal cord

Radioactive probes for the galanin receptors were also hybridized to elucidate effects of axotomy on the expression patterns of the receptor mRNAs. Several GalR1+ neurons were observed in the dorsal horn of the lumbar spinal cord (figure 8a). Their distribution varied within the different layers but the highest density was observed in laminae I-III and GalR1+ neurons were also detected in area X, usually dorsal to the central canal (figure 8a). Additionally, very few GalR1+ neurons were observed in the ventral horn but were not quantified since the number of positive neurons were sparse and no obvious effect of axotomy could be detected by comparing ipsilateral to contralateral sides of the spinal cord sections (figure 8a1, a2). GalR2+ and GalR3+ neurons were not observed in any sections of the lumbar spinal cord (8b, c). This

could account for the absence of the transcript at a detectable level or that the probe did not hybridize efficiently under the experimental conditions.

Hybridization using S probes (as controls) complementary to the AS probes for the transcripts of galanin, CGRP and GalR1 showed a complete absence of signal (figure 8d, e, f).

An anatomic scheme from Allan Brain Atlas[131,132] was adapted and applied in figure 5a for spinal cord sections L4-L5 to visualize laminae I-X in these levels of the spinal cord. The areas "Crural extensor motor neurons" (Cex9), "Crural flexor motor neurons" (CFl9), "Gluteal motor neurons" (Gl9), "Hamstring motor neurons" (Hm9), and "Axial muscle motor neurons" (Ax9) were expected to contain motor neurons, highlighted with black dotted lines in the anatomic scheme.

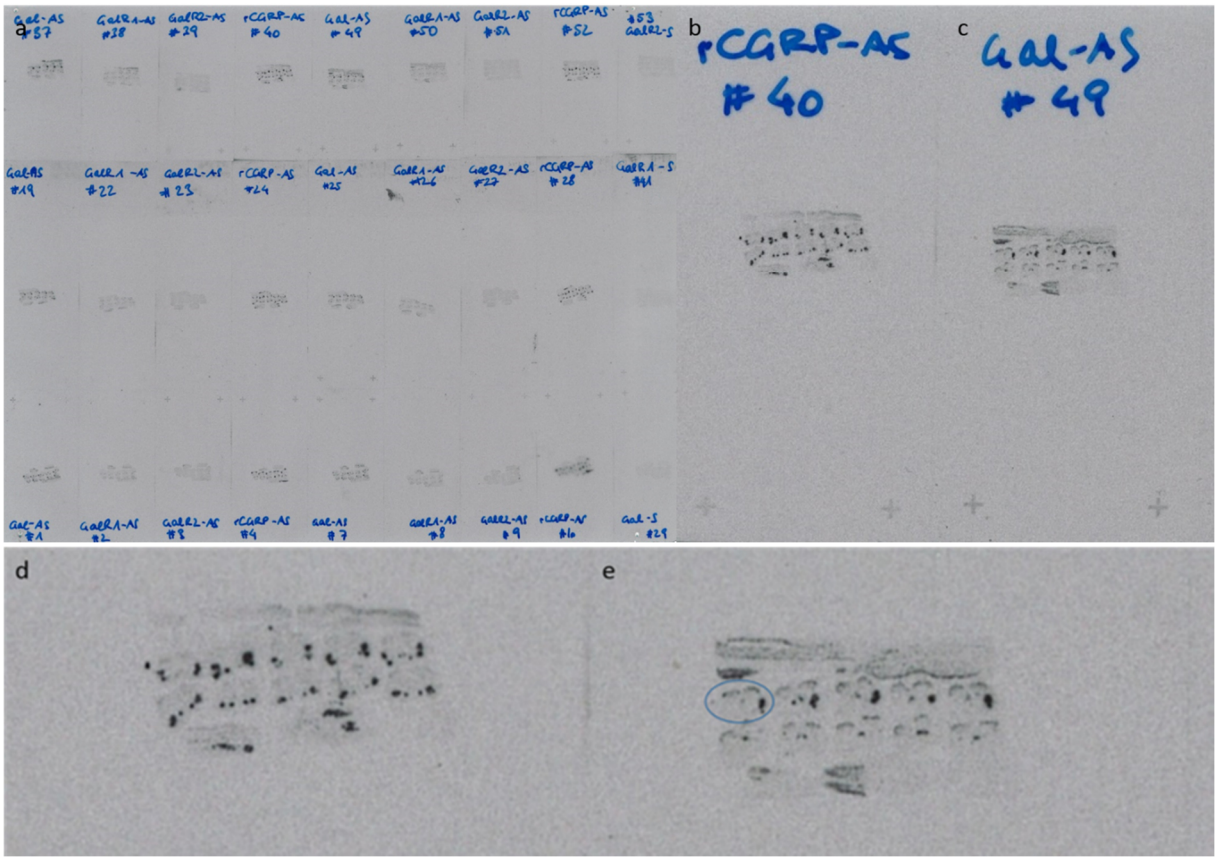


Figure 4 Autoradiographic film (exposure time 5 days) showing the ISH data (after hybridizations with 35S labeled antisense (AS) and sense (S, control) probes) for galanin, GalR1, GalR2, GalR3 and CGRP transcripts in the murine spinal cord. (a) Overview of developed film shows galanin, GalR1, R2, R3 and CGRP mRNA expression in spinal cord sections after unilateral axotomy of the sciatic nerve, detected by

hybridization with 35S labeled AS and S riboprobes, rostro-caudally (the numbers of sections are given). (b) Slides of section #40 (800 µm caudal from beginning of sectioning) for CGRP. (c) Slide #49 (980 µm caudal from beginning of sectioning) for galanin. The film confirms efficient hybridization of the probes and was also used to determine exposure time for slides when dipped in a photographic emulsion for microscopic and anatomical characterization of the positive neurons. (d), (e) Enlarged images of (b) and (c), showing five spinal cord sections in the upper row and five spinal cord sections below. In (e) the left upper spinal cord section is circled.

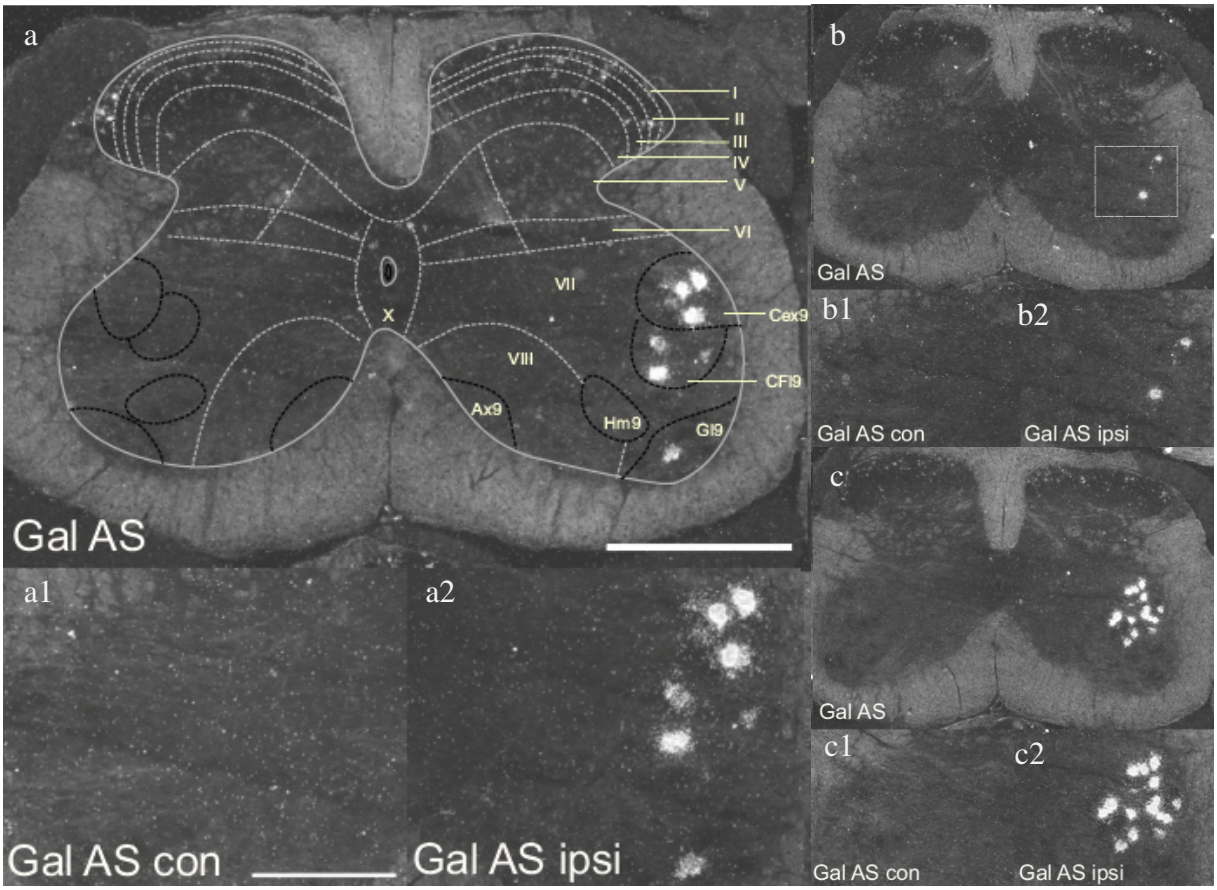


Figure 5 Radioactive ISH for galanin mRNA in murine spinal cord sections. Dark-field micrographs of galanin (a-c) mRNA expression in motor neurons throughout one spinal cord (three sections L4-L5) after unilateral axotomy of the sciatic nerve, detected by hybridization with 35S labeled AS riboprobes. In (a), an anatomic scheme from Allan Brain Atlas is applied to display Rexed laminae I-X of the gray matter. Lamina 9 in the ventral horn, highlighted with black dotted lines (area Cex9, CFl9, Gl9, Hm9, Ax9), is where motor neurons of interest are expected to appear. The white dotted zone in (b) marks the magnified area of the spinal cord sections shown in the figures below. An L5 section is exhibited in (c), to point out the strong signal on the ipsilateral side throughout L4-L5. Ipsilateral sides (a2, b2, c2; ipsi) of the spinal cords contain galanin mRNA+ motor neurons, whereas contralateral sides (a1, b1, c1; con) do not reveal any signal. In dorsal horn of spinal cord sections (a, b, c), galanin mRNA+ signal appears throughout the sections, with no difference between ipsilateral and contralateral sides. Scale bar (a): 500 µm, (a1): 250 µm.

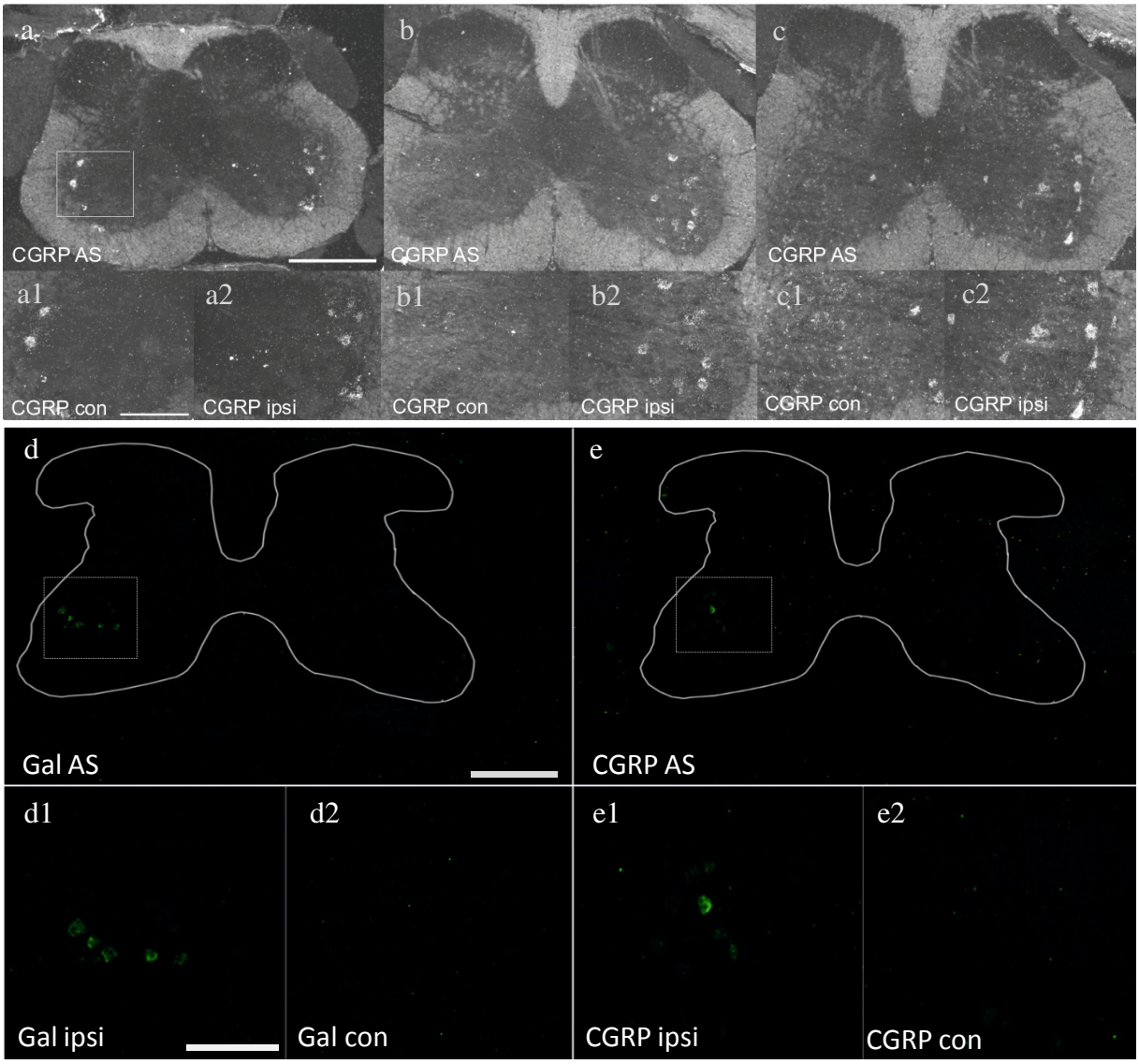
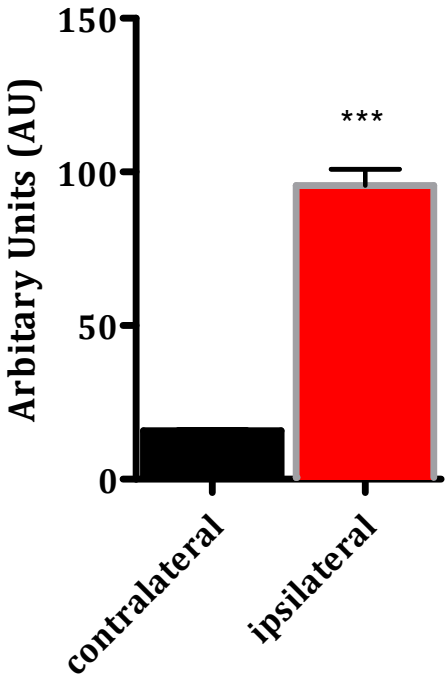


Figure 6 Radioactive ISH for CGRP and fluorescent ISH for galanin and CGRP mRNA in murine spinal cord sections. Dark-field micrographs of CGRP (a-c) mRNA expression in motor neurons throughout one spinal cord (three sections L4-L5) after unilateral axotomy of the sciatic nerve, detected by hybridization with 35S labeled AS riboprobes. The white dotted zone in (a,d,e) marks the magnified area of the spinal cord sections shown in the figures below. For CGRP displayed in (a-c), a strong signal on the ipsilateral sides (a2, b2, c2; ipsi) of the spinal cord sections as well as a slightly weaker signal on the contralateral sides (a1, b1, c1; con) is observed. Scale bar (a): 500 µm, (a1): 250 µm. Fluorescent ISH for galanin (d) as well as for CGRP (e) reveal upregulation on the injury side, whereas mRNA+ can be observed by green signal (d1, e1; ipsi). Both in (d) and (e), an outline of the gray matter is applied from Allan Brain Atlas in white. In (d2, e2; con) no hybridization of AS riboprobe on contralateral sides of spinal cord sections is visible. Scale bar (d): 400 µm, (d1) 200 µm.

(a) Galanin



(b) CGRP

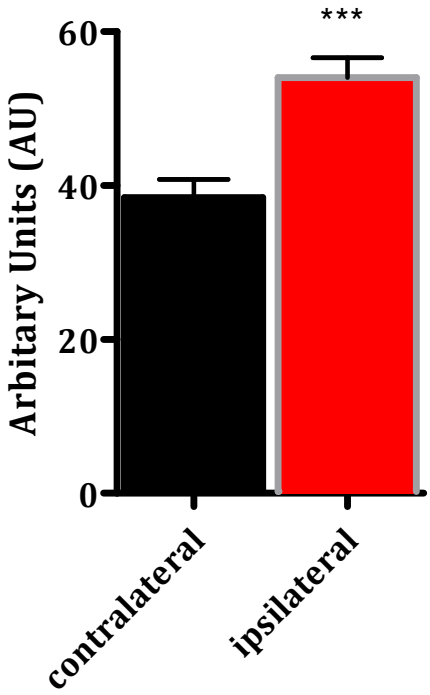


Figure 7 Quantitative evaluation of ISH signals for galanin and CGRP in the ventral half of the spinal cord, ipsilateral as well as contralateral to the injury side. The mean gray densities were measured on both sides with ImageJ, and statistical analysis was performed with the Mann-Whitney-U test. The bars show means standard errors of the mean; ***p<0.0001 versus contralateral side.

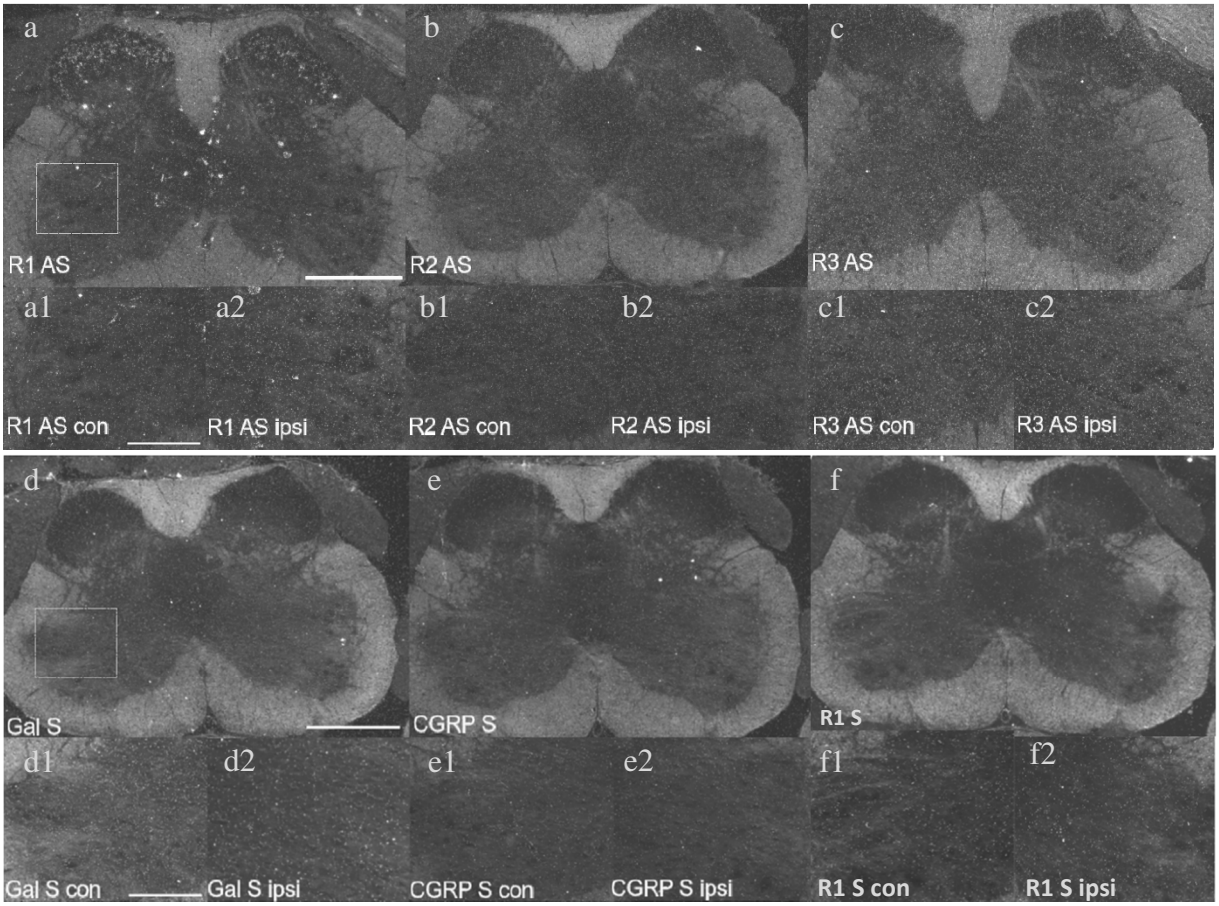


Figure 8 Radioactive ISH with AS riboprobes for GalR1, GalR2 and GalR3 and S riboprobes for galanin, CGRP and GalR1 on spinal cord sections. Dark-field micrographs of GalR1 (a), GalR2 (b), and GalR3 (c) mRNA expression in spinal cord motor neurons after unilateral axotomy of the sciatic nerve, detected by hybridization with 35S labeled AS riboprobes. Hybridization with AS probe for GalR1 reveals a signal in the dorsal horn, as well as around the central canal of the gray matter. For R2 and R3, no signal is obtained after 8 weeks of exposure. In (a, d), the white dotted zone marks the area magnified in the figures below each spinal cord section. Scale bar (a, d): 500 µm, (a1, d1): 250 µm. Hybridization with 35S labeled S riboprobes for all transcripts mentioned above used as negative controls showed no signal.

5.2 Immunostaining on spinal cord sections

Immunohistochemical analysis confirmed the in situ hybridization results for galanin and CGRP. To expand the study, additional peptides were studied including CCK, ENK, SOM, NPS, DYN and substance P.

5.2.1 Galanin and CGRP

Galanin staining was observed equally on both sides of spinal cord sections in neuron branches of the dorsal horn, with strongest intensity observed in laminae I-II, and area X around the central canal. The number of galanin$^+$ neurons in the ventral horn of the ipsilateral side was strikingly higher than on the contralateral side (figure 9a, b). Double staining with ChAT suggested that the galanin$^+$ neurons represent a subpopulation of the ChAT$^+$ neurons in the spinal cord (figure 9c). Immunostaining for CGRP revealed expression on both the ipsilateral as well as the contralateral side in the ventral horn after axotomy, as observed previously at mRNA level, however with a slightly stronger staining noticeable on the injury side of the spinal cord (figure 10a-c). CGRP also emerged in the dorsal horn and area X around the central canal.

5.2.2 Other peptides

For NPY, similar effects as those observed for galanin were obtained. NPY expression was strongly upregulated ipsilateral to the lesion with intense staining within some cell bodies. Staining patterns were also observed within the dorsal horn and around the central canal, but not in motor neuron cell bodies in the contralateral ventral horn (Fig 10 d-f).

Immunohistochemical staining for SOM, CCK, ENK, DYN, and substance P revealed no differences in ipsilateral compared to contralateral sides after unilateral axotomy, and none of the peptides were observed within cell bodies at spinal cord levels (L4-L5). Stainings for the above peptides emerged within neuron branches in the dorsal horn and around the central canal (figure 11). Immunohistochemical stainings for NPS did not reveal any signal within the spinal cord white and gray matter. Outside the white matter, dots mark an interaction of the antibody with tissues surrounding the spinal cord (figure 11d).

Ranking of staining intensity for all neuropeptides is summarized in Table 2.

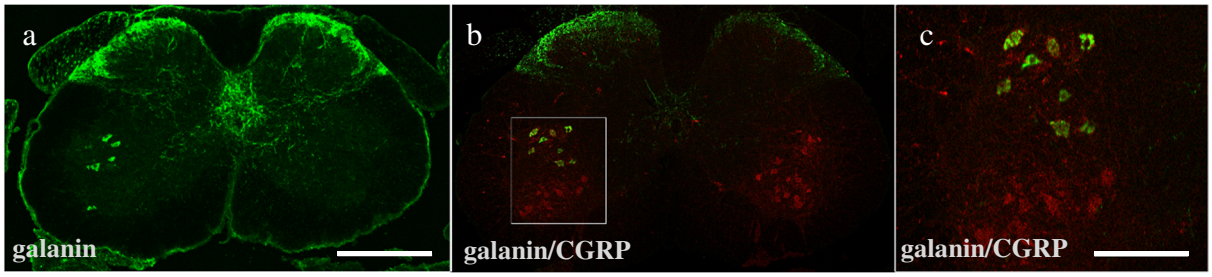


Figure 9 Immunohistochemical staining for (a) galanin and (b) double staining for galanin (green) and ChAT (red) 7 days post axotomy. Left side of spinal cord sections represents injury side. (a) Galanin expression is upregulated on the ipsilateral side in motor neurons (green) and in dorsal horn fibers as well around the central canal, but there is no expression on the contralateral side in the ventral horn (scale bar = 400 µm). (b) Double staining for galanin and ChAT reveals that stained cells positive for galanin in the spinal cord sections represent motor neurons. (c) Magnification of marked zone in (b) (scale bar = 200 µm).

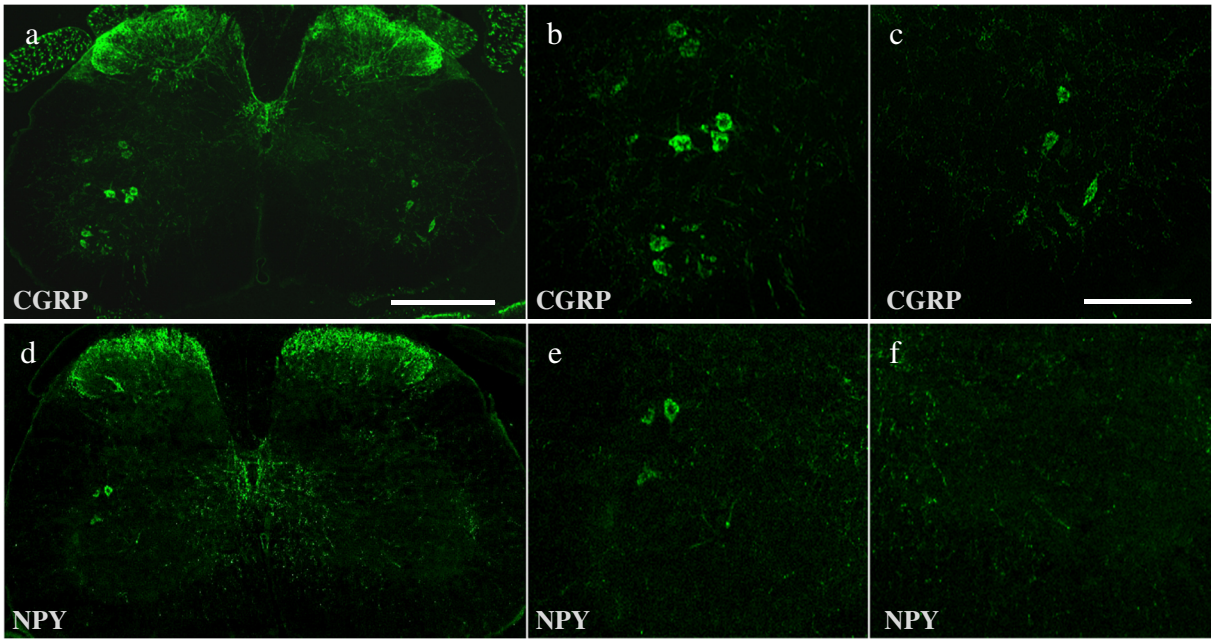


Figure 10 Immunohistochemical staining for CGRP (a-c) and NPY (d-f) 7 days after axotomy. (a) CGRP is expressed on both sides of the spinal cord, whereas a remarkable upregulation can be observed on the ipsilateral (left) side in the ventral horn (scale bar = 400 µm). Panel (b) shows the magnified ipsilateral side, and (c) the contralateral side of the spinal cord section stained for CGRP (scale bar = 200 µm). NPY staining can be observed ipsilateral to axotomy (d, and magnified e) in motor neurons, whereas contralateral no staining in cell bodies can be spotted (f). CGRP and NPY are shown to be present in dorsal horn fibers on both sides and around the central canal.

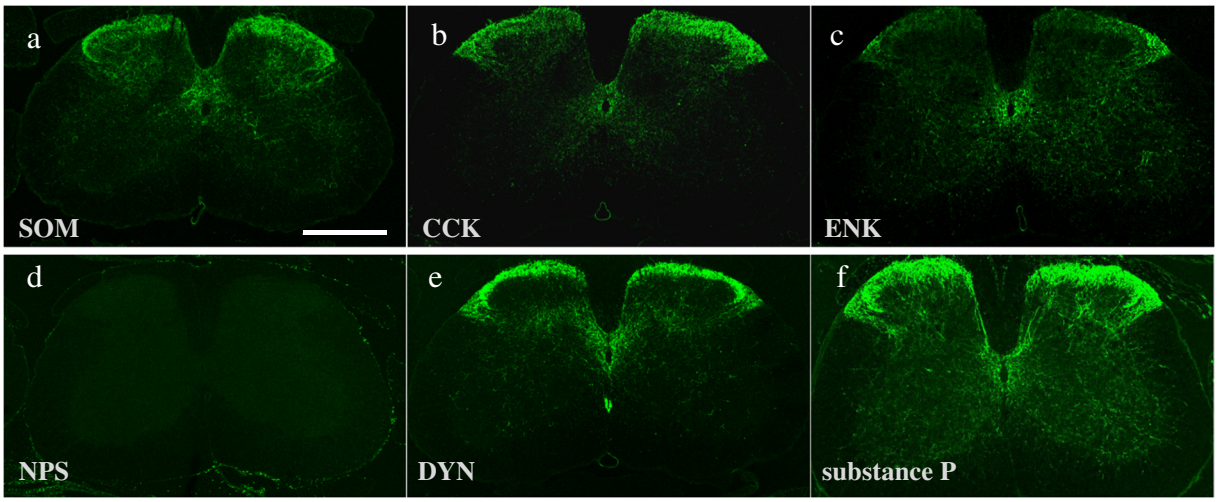


Figure 11 Immunohistochemical staining for SOM (a), CCK (b), ENK (c), NPS (d), DYN (e), and substance P (f) 7 days after axotomy. SOM, CCK, ENK, DYN and substance P show no differences in expression between ipsilateral and contralateral side and are present bilaterally in dorsal horn fibers as well as around the central canal (a-c, e, f). Staining for NPS revealed dotty signal around the white matter of the spinal cord, but not within the spinal cord white or gray matter (d). (a) Scale bar = 400 µm.

Table 2 Neuropeptide expression in murine spinal cord following unilateral axotomy, visually ranked by staining intensity. The symbol (+) stands for occurrence in described part of spinal cord, (-) for absence of staining. Intensity was ranked between + and +++ objectively.

Peptide	Ventral (ipsilateral)	Dorsal (ipsilateral)	Ventral (contralateral)	Dorsal (contralateral)	Central Canal	
CCK	-	-	-	-	-	Cell bodies
	-	+++	-	+++	++	Fibers
CGRP	+++	+	++	-	-	Cell bodies
	-	++	-	++	++	Fibers
DYN	-	-	-	-	-	Cell bodies
	-	+++	-	+++	+++	Fibers
ENK	-	-	-	-	-	Cell bodies
	-	+	-	+	++	Fibers
Galanin	+++	-	-	-	-	Cell bodies
		+++	-	+++	+++	Fibers
NPS	-	-	-	-	-	Cell bodies
	-	-	-	-	-	Fibers
NPY	++	-	-	-	-	Cell bodies
	-	++	-	++	++	Fibers
SOM	-	-	-	-	-	Cell bodies
	-	++	-	++	++	Fibers
Substance P	-	-	-	-	-	Cell bodies
	-	+++	-	+++	+++	Fibers

5.3 Immunostaining of cell cultures

Previous qPCR studies done on mouse embryonic stem cells (mESC), embryonic bodies (EBs), mature embryonic bodies and 2-4 day motor neuron (MN) cell cultures revealed high levels of transcript expression for galanin and its receptors in mature EBs relative to mESC. Furthermore, when mature EBs were sorted using FACS, galanin and its receptors were expressed in sorted (GFP positive) as well as unsorted cells (unpublished data). Hence, in this present study, we primarily tried to phenotypically characterize these cells using the immunohistochemical approach.

Preliminary experiments using murine embryonic stem cell (mESC) derived motor neurons revealed expression of galanin and CGRP via immunohistochemical staining on day 2 as well as in already decaying cells on day 7 (figures 12 and 13).

Because motor neurons were fading after one week, cultures older than 7 days are not shown. GFP tagged motor neuron marker (Hb-9) was used to identify the motor neurons, which are shown in green in figure 12a, e and 13a, e. Galanin positive (red) neurons were observed in the cell culture at day 2 and day 7 (figure 12f, 13f). CGRP staining (red) was observed clearly in numerous motor neurons (figure 12b, 13b), although staining was weaker compared to galanin staining which can be explained by the comparatively lower concentration of primary antibodies used (table M8).

The galanin and CGRP signals co-localized with GFP suggesting the peptides are expressed in the mESC derived motor neurons. β-Tubulin was used as a cytoskeletal marker for eukaryotic cells in culture.

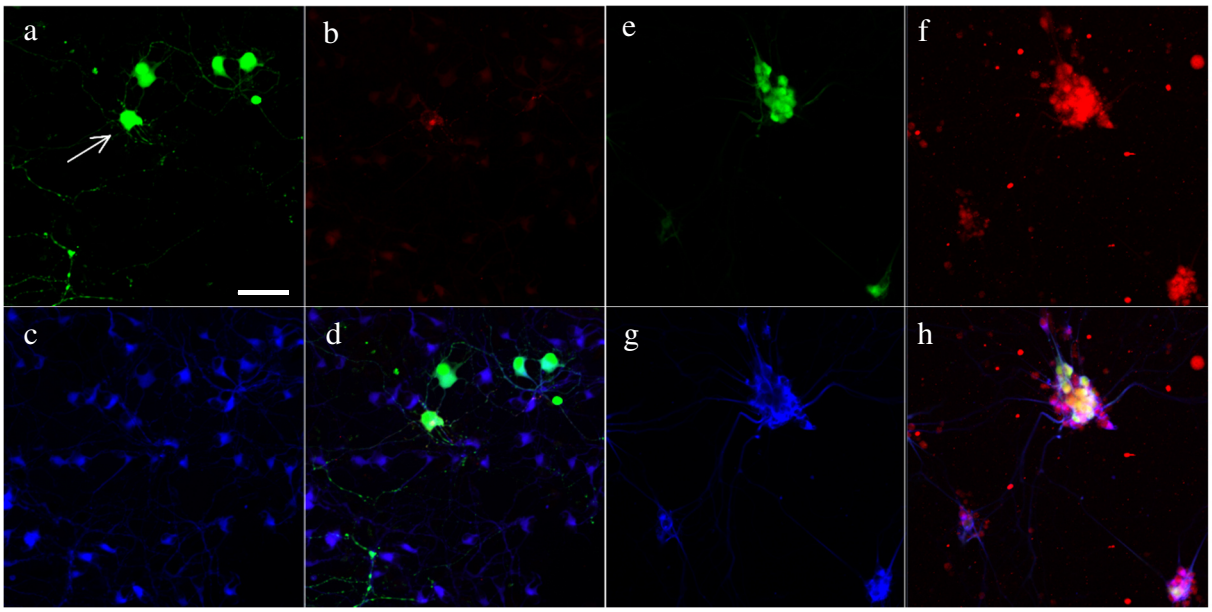


Figure 12 CGRP (a-d) and galanin (e-h) expression in motor neuron cell cultures derived from mEBs after 2 days of cultivation. Motor neurons are tagged with GFP at Hb-9 (green). Arrow in (a) points to motor neuron which is also positive for CGRP (red, b and d) and β-Tubulin, the positive control for immunohistochemical staining (blue, c and d). Panel (d) is an overlay of (a-c) and shows a triple stained cell (in white). Galanin expression is shown in red (f, h) which appears in motor neurons (green, a and h) as well as in other cells present in this culture, stained in blue (g and h). Panel (h) shows an overlay for motor neurons, with galanin and β-Tubulin staining. (a) Scale bar = 50 µm.

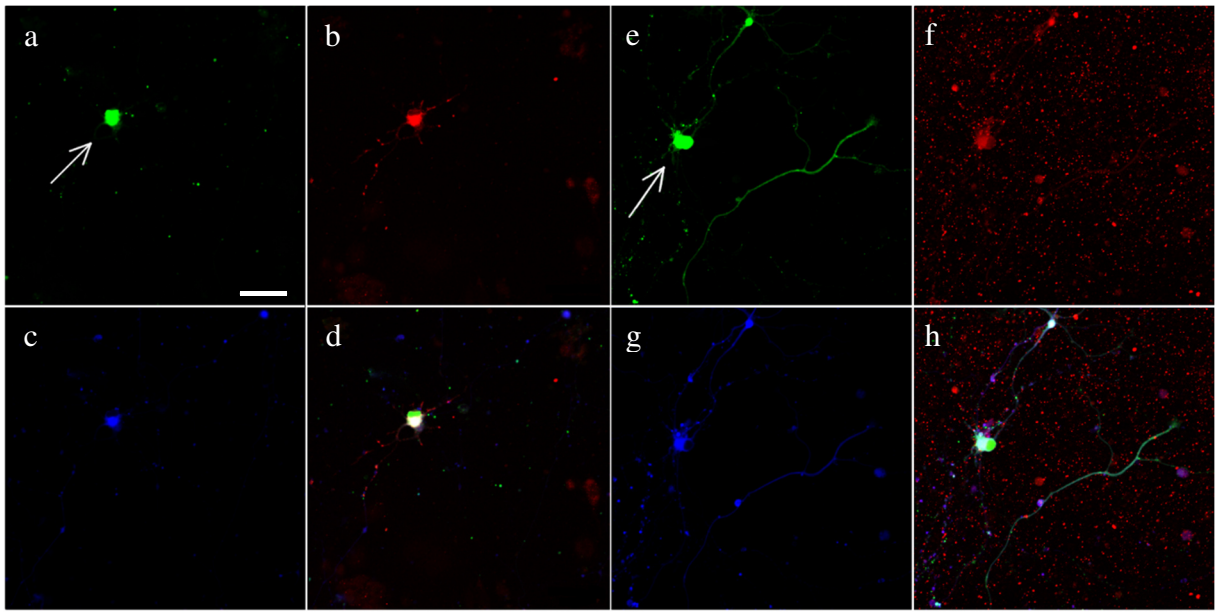


Figure 13 CGRP (a-d) and galanin (e-h) expression in motor neuron cell cultures derived from mEBs after one week of cultivation. (a) shows GFP-tagged motor neurons in green, whereas the arrow points to a cell which is also positive for CGRP (red, b and d) and β-Tubulin, (blue, c and d). (d) is an overlay of (a-c) and shows a triple stained cell (in white). Galanin expressing motor neurons are shown in red (f,h). The arrow (e) points at a motor neuron expressing galanin as well as β-tubulin (blue g and h). An overlay picture is shown in (h) with a triple staining (in white). (a) Scale bar = 50 µm.

6. Discussion

6.1 Peptides and receptors in spinal neurons

Much effort has thus far been put into neuropeptide research by monitoring expression changes induced by nerve damage. These changes are assumed to represent an adaptation to injury, such as prevention of loss of neurons and initiation of regeneration. Here certain neuropeptides may play a role as evidenced by studies with pain models which encompass various types of lesions and examined by the effects on DRG and/or motor neurons mainly in rodents. The present study focused on spinal motor neurons, that are large neurons in the ventral horns of the spinal cord. A marker of those neurons, used extensively, is CGRP which can distinctly be seen in motor neurons under physiologic circumstances[133] and is upregulated after injury. However, in rat several other neuropeptides including GAL, SOM, NPY and substance P[110] are also upregulated in a similar manner in motor neurons of the spinal cord, at least at mRNA level. In the present study on the mouse we have studied several neuropeptides in L4-L5 spinal motor neurons following sciatic nerve transection.

The role of most investigated peptides in regenerative processes in motor neurons is still incompletely understood. Nevertheless, based on studies of DRG neurons, that is a sensory system, galanin is strongly upregulated following nerve transection48, which may serve two functions: a trophic effect and involvement in pain signaling. In the latter function, galanin has been proposed to act as an endogenous analgesic compound, thus representing a way for the body to manage emergency situations. The mechanisms underlying these observed alterations in neuropeptide expression are not well known, but lack of a retrogradely transported molecule such as nerve growth factor (NGF) may play a role.110 Observations made in the present study

reinforce the assumption that certain peptides are involved in survival and regenerative processes.

6.1.1 Neuropeptides in ventral horn motor neurons

Our results show that in the mouse the expression of some neuropeptides in motor neurons within the ventral horn is also regulated by nerve injury. Using immunohistochemistry, galanin, CGRP and NPY were all upregulated in motor neurons ipsilateral to the transection of the sciatic nerve. These results broadly agree with earlier studies in the rat[110].

Galanin-like immunoreactivity (LI) was seen both in neurons of varying size in the ventral horn, presumably α-motor neurons. The staining either filled out the entire cytoplasm or was more patchy, extending into dendritic processes. These findings agree with previous studies in the rat and will be discussed more closely in the following section. CGRP-LI was also observed to be highly upregulated, although immunoreactivity appeared both ipsilateral and contralateral to injury.

With regard to NPY previous results on the rat have shown that following sciatic nerve transection, NPY could be detected ipsilaterally at the mRNA, but not peptide level[110]. However, NPY expression was observed to be upregulated during development and therefore, NPY is thought to exert different trophic-differentiating and/or neuromodulatory roles[134]. Furthermore, NPY was reported to act as angiogenic factor[135]. In our study, we analyzed expression only at peptide level and found an ipsilateral upregulation of NPY in motor cell bodies, which could be taken to suggest a trophic function of this neuropeptide in ALS research on mouse models.

In the rat spinal cord SOM mRNA-positive neurons were encountered ipsilaterally within 3 days after axotomy, with an increasing number of neurons in the following course of time until

a peak after 4 weeks. Immunohistochemically stained cells were observed as well, confirming results from in situ hybridization.[110] In contrast to these results we could not detect this peptide in mouse ventral horn cell bodies, neither ipsi- nor contralaterally. Considering this, it might be worthwhile to expand the time course ofobservation following axotomy in mice. Also in situ hybridization should be employed. The effects of SOM in the motor neuron system remain unclear: SOM was previously observed to hold growth promoting effects on CNS and PNS neurons[116], although following intrathecal injection of SOM, neuronal damage occurred in the spinal cord as reflected by a loss of motor neurons, among others.[111,117]

Expression of CCK within motor neurons in the spinal cord was observed at mRNA level in the rat and monkey. Furthermore, alterations in expression as a response to axotomy differ widely between species. In monkeys, no alteration in CCK mRNA expression was noted in spinal cord motor neurons after axotomy of the sciatic nerve. Interestingly, CCK was observed to exert trophic effects towards ventral spinal cord cells explanted from rat embryo[136].[137] Therefore this peptide might have a regenerative influence on motor neurons. However, in the present study, we could not observe any alterations following axotomy in mice using immunohistochemistry. Nontheless, further efforts in this direction may be appropriate, for example studying a more complete time course following injury.

Also peptides belonging to the opioid family have been investigated. In rat, ENK mRNA expression has been shown to be downregulated in spinal ventral motor neurons ipsilaterally to injury following sciatic nerve transection110, albeit these results could not be confirmed at peptide level, as immunohistochemically no staining was observed. In contrast, no effect on DYN expression patterns was observed in the monkey spinal cord following axotomy50. Interestingly, studies on rats have revealed neurotoxic and degenerative effects of DYN following intrathecal injection111. In the present study, we could not observe any

immunohistochemical staining for ENK or DYN in murine ventral motor neurons, neither on the ipsilateral nor on the contralateral side following sciatic nerve transection. Still it may be worthwhile to conduct more studies including longer time courses to exclude a role of opioid peptides in murine motor neurons under physiologic conditions and in response to nerve injury, for example in recovery and protection.

In rat motor neurons, sciatic nerve axotomy induced substance P expression at mRNA level in the ipsilateral ventral horn; however, immunohistochemical analysis did not confirm this result at peptide level[110]. In the present study of murine spinal cord sections no staining for substance P-LI could be observed in ventral motor neurons by immunohistochemistry. Nevertheless, it would be worthwhile to conduct more studies including in situ hybridization for substance P mRNA to elucidate the expression pattern of this peptide in mice.

Scarcely any research has been conducted with regard to the effect of injury on NPS expression in murine spinal motor neurons. In the current study the expression pattern of NPS in the murine spinal cord remained unchanged following sciatic nerve transection. Thus a role of NPS in motor neuron injury remains obscure. The signal around the white matter of spinal cord sections could reflect expression of NPS by blood vessels surrounding the spinal cord.

Taken together, we think that some of the discussed neuropeptides might represent interesting debutants in ALS research. Since CGRP, galanin, NPY, CCK and substance P are generally thought to have proliferation promoting effects, they might as well have a regenerative influence on motor neurons and could act as plasticity inducing factors post injury. Therefore, it would be interesting to conduct more studies in murine ALS models and elucidate whether these neuropeptides could be involved in murine neuronal recovery and/or protective mechanisms.

To track the path of neuropeptide expression, it would be of interest to conduct qPCR, in situ hybridization, as well as blotting techniques at nucleotide and peptide level. Nevertheless, even if mRNA expression appears upregulated, translation control mechanisms and mRNA degradation, for example, might interfere with peptide expression after all. Even if mRNA levels do not appear to be rate-limiting for protein synthesis in most of the observed cases, mRNAs could be sequestered in stress granules or specific regions of the cytoplasm, and therefore, accessibility for translation might be limited. Degradation of mRNAs through the action of microRNAs (miRNA) could be another reason for absence of elevated peptide occurrence. Even during the translation process, specific control mechanisms could inhibit peptide expression.[138] In addition, changes at the mRNA but not peptide level can be the result of increased activity of the neuron, releasing an enhanced number of peptide molecules which stimulates transcription, whereas increased translation is obscured by the increased release of the peptide.

6.1.1.1 Galanin, GalR1-3 and CGRP mRNA expression in the ventral horn

Using radioactive in situ hybridization, expression of CGRP, galanin, GalR1, GalR2 and GalR3 at mRNA level was screened throughout the spinal cord. The two neuropeptides were observed to be upregulated ipsilaterally to axotomy in the ventral horn, which not only confirms immunohistochemical results of this study, but also demonstrates that the pattern of changes is similar to that documented beforehand in the rat[110]. However, the remarkably high CGRP expression on the contralateral side of injury might be in part a technical artefact reflecting overexposure that could have occurred after 5 days already.

In naïve rat lumbar spinal cord, GalR1 mRNA positive neurons in laminae I-III, X, as well as ventral horn and lateral spinal nucleus have been described[63]. The same study of rat lumbar spinal cord found comparatively low signals for GalR2 mRNA, which were more restricted to

the ventral horn and area X, although a rare signal was also seen in dorsal horn and intermediate lateral cell column neurons. Transection of the sciatic nerve led to enhanced expression of GalR2 mRNA in ipsilateral motor neurons, but no difference regarding GalR1 expression was observed.[63] These results agree with earlier studies, in which facial nerve crush in rats induced a significant increase in galanin and a modestly increased GalR2 expression at mRNA level[46]. In the present study, GalR1 was found expressed within the entire lumbar spinal cord at mRNA level. Therefore the expression was presumably not in motor neurons, as no difference between ipsilateral to contralateral side was observed after axotomy. These results point to a consistency of rat and mouse spinal GalR1 expression patterns. Surprisingly, riboprobe hybridization of GalR2 mRNA was negative in the current study. As this outcome was unexpected, spinal cords treated with anti-GalR2 mRNA riboprobe were exposed to emulsion, and checked at various time points for up to 7 weeks, with no apparent alteration over time. However, GalR2 upregulation in rat motor neurons following injury suggests an impact of galanin in neuronal regeneration mechanisms implemented via this receptor subtype, and similar observations in mice would be required to conclude a comparable effect. Nevertheless, no GalR2 mRNA signal was seen in spinal cord sections whatsoever, which is at variance with this receptor subtype being expected to be expressed in this location. The apparent absence of GalR2 casts doubt on of galanin's possible applicability in ALS treatment, as trophic effects are thought to be mediated by GalR2. Reasons for this negative result remain elusive, nonetheless the riboprobe itself represents an essential factor for in situ hybridization to succeed. Therefore it would be of high interest to perform a positive control. Moreover, qPCR analyses might be worthwhile to confirm results. Additionally, development of specifically GalR2 recognizing antibodies or high-affinity ligands would help clarifying whether increased mRNA levels directly result in receptor protein expression.

GalR3 thus far remains to be the least studied receptor. Also, during this study, corresponding mRNA was not found expressed and could not be spotted throughout all spinal cord sections screened. As has been mentioned for GalR2, qPCR data as well as positive controls could be valuable approachess to confirm or disprove its presence.

As found in motor neurons of many different species, galanin expression is upregulated in mice following sciatic nerve axotomy, and therefore the possibility of autoreceptor interactions is raised. This is thought to be exerted via somatodendritic release of galanin as has been suggested for CGRP which is also upregulated in motor neurons following peripheral nerve injury[139]. It is broadly known that GalR1 and GalR2 expression as well as alterations in response to pathophysiologic conditions differ between neuronal subtypes, given that in sensory DRG neurons both receptor subtypes are downregulated, which cannot be observed in cholinergic motor neurons. Therefore, vastly complex adaptive mechanisms are thought to be involved in the regulation of the galanin system.

Since the first attempts to elucidate galanin, GalR1, GalR2 and GalR3 expression changes following peripheral nerve injury in mice have been conducted in this study, it would certainly be of high relevance to continue these lines of investigations. However, it has become clear, as a consequence of this study, that possible effects of the neuropeptide galanin on ALS can be studied within the mouse.

When comparing effects of nerve transection on neuropeptide and receptor expression in mice, as illustrated in this study, with results in rats as mentioned above, differences as well as similarities emerge. In order to understand any species differences in the response to injury, comparative studies with a standardized protocol concerning type, anatomic location and time course of injury are needed. Since in the rat nerve transection has been reported to cause peak changes in galanin mRNA expression 7 days following injury[110], our study was modeled

according to this finding. Despite some differences in the experimental protocol, rather similar results regarding upregulation of galanin, CGRP and NPY in response to nerve injury have been found in mice (this study), rats and other species[50,110]. Therefore it is possible to generalize some assumptions about the impact of these neuropeptides on neuronal regeneration, trophic effects and related processes. Furthermore, knowing that mice upregulate galanin expression within motor neurons following nerve injury makes them appropriate model organisms to study possible beneficial effects of galanin and CGRP on ALS.

If in future studies GalR2 can be found expressed in murine motor neurons, it might be advisable to perform experiments using GalR2 agonists in ALS models of this species, since neuroprotection as well as neurogenesis are thought to be promoted via this receptor subtype.

6.1.2 Neuropeptides in the dorsal horn

Lumbar dorsal horn neuropeptide expression patterns, visualized by immunohistochemistry in murine injury models, have been in the focus of previous studies and have led to the conclusion that 14 days after sciatic nerve transection, CGRP and substance P peptide expression is decreased within laminae I-II afferent fibers. On the other hand, galanin-, DYN- and NPY-LI was increased in the same area as well as in deeper laminae of the murine spinal cord.[140]

In the current study in mice, rather dissimilar results were observed 7 days following axotomy, as galanin-LI was not explicitly upregulated ipsilaterally to axotomy throughout L4 – L5 spinal cord sections and no change in expression could be seen at mRNA level via in situ hybridization. In understanding this discrepancy it need be kept in mind that strong variations over the post-injury course of time110. CGRP, in contrast, seemed to be slightly upregulated ipsilaterally as immunoreactivity could be observed in some lamina III-V neurons of the dorsal horn. However,

these patterns were not spotted at mRNA level, because no in situ hybridization signal was obtained on either side of the spinal cord within the dorsal horn.

The other peptides examined NPY, DYN, CCK, ENK, SOM, NPS and substance P, did not undergo any distinct expression changes within the dorsal horn following transection of the sciatic nerve.

Previous studies on monkeys, revealed that unilateral axotomy of the sciatic nerve failed to change the expression of NPY within dorsal horn fibers, although NPY-positive neurons were seen after immunohistochemical staining of dorsal root ganglia neurons that were not observed in control animals[50]. In rat studies, NPY mRNA-positive cells occurred bilaterally in the dorsal horns of axotomized as well as control animals[110]. Following unilateral axotomy of the sciatic nerve in monkeys, a marked decrease of substance P immunostaining and mRNA signal ipsilaterally to the injury was encountered in dorsal horn fibers as well as in sensory neurons[50]. Axotomy effects on the spinal CCK system were mostly studied in the rat thus far, and ipsilateral down-regulation was detected in the dorsal horn[141,142]. ENK-positive fibers within the dorsal horn of the monkey spinal cord did not seem to change in appearance following nerve ligation and remained alike those observed in the untreated animals[50]. In the rat, ENK mRNA expression did not appear altered following injury, an observation that was not confirmed at peptide level, as ENK-LI appeared to be absent after axotomy. SOM positive neurons were encountered at mRNA as well as peptide level in the rat dorsal horn and did not appear to differ between rats subjected to sciatic nerve axotomy and control animals. This observation is comparable to the immunochemical results in this study, since immunoreactivity for SOM occurred only in fibers of the dorsal horn and was not altered by sciatic nerve transection.[110] However, in monkeys an axotomy-induced decrease of SOM expression in ipsilateral fibers of the dorsal horn as well as in DRG neurons was observed[50], which marks another possible

interspecies difference in injury response. As previously mentioned, hardly any data is available at this point regarding axotomy and NPS, especially within mice. We therefore conclude at the current point, that no changes within spinal NPS expression can be observed following sciatic nerve transection and that any possible impact of NPS on injury remains elusive.

6.2 Cell culture

Uncertainties about the molecular causes of ALS persist and call for urgent research into the pathogenesis of the disease. One prevailing attempt to develop drugs preventing progression of motor neuron degeneration is to promote protection and neuroregeneration. In a general perspective, galanin was shown to foster both processes, as well as to mediate trophic effects[70–74,77].

Excitotoxicity is supposedly involved in many acute and chronic neurodegenerative disorders, such as stroke, traumatic spinal cord injury, as well as AD, Huntington's disease, Parkinson's disease and ALS. Initiation of excitotoxic cascades seems to occur in postsynaptic dendrites, which ultimately leads to neuronal apoptosis.[143]

Glutamate mediates excitotoxicity and is therefore a pathophysiological factor when occurring at high concentrations. Processes triggered by binding of glutamate at postsynaptic dendrite receptors involve, for example, an overload of Ca^{2+} by influx and intracellular release, oxyradical production, as well as activation of cascades leading to apoptosis. One of the established molecular mechanisms resulting in cell death is activation of cysteine proteases, so called calpains, by excessive intracellular calcium concentrations, which as soon as active, degrade MAP-2, which is vital for maintaining microtubule structure. Therefore, impairment of MAP-2 causes morphological damage, impairs transport mechanisms and cellular function, triggering cell death. Studies using primary hippocampal neuron cultures from the rat, aiming

at elucidating a possible interference of the galanin agonist AR-M1896 with glutamate-induced molecular events were performed by exposing cultured cells to glutamate and AR-M1896 in varying concentrations. AR-M1896, which preferably binds GalR2 and probably also GalR3 to a lesser extent, was thereby shown to promote neuron survival in case of glutamate induced excitotoxicity. This was shown to occur not only at the level of cytoskeleton preservation, but also by an effect of AR-M1896, to downregulate c-fos expression which is thought to promote apoptosis, and to reduce DNA condensation. However, the precise pathways underlying the action of the galanin agonist to mediate protection against excitotoxicity remain elusive.[78]

In this study, we aimed to make the first steps towards a murine motor neuron cell culture, that conceivably could be used for life-cell imaging experiments to reveal any impact of galanin on glutamatergic excitotoxicity in this specific cell type. Thereby, it would be possible to generate some hypotheses about galanin's involvement in ALS affected motor neurons. To further elucidate signal transduction pathways relevant in this respect it would be necessary to operate with agonistic and antagonistic molecules to disclose which galanin receptor subtypes play a role. To address the question whether GalR 2 which is thought to mediate the neuroprotective role of galanin is expressed in the cultured neurons, experiments involving qPCR as well as blotting techniques would be required.

It would be interesting to apply glutamate and other cellular stressors in combination with galanin and selective galanin receptor agonists to dissect the protective effect of the galanin system against glutamate-induced excitotoxicity. Similar studies have been performed on primary hippocampal neurons, the results of these studies pointing to a reduction of glutamate toxicity in consequence of supplementary galanin application[78].

Here, we distinguished cells differentiated to motor neurons from others by GFP-tagging of the marker Hb-9. To confirm the applicability of immunohistochemical methods, cells in culture

were indirectly stained for CGRP as well, which was found to be broadly expressed throughout the culture within GFP-tagged motor neurons, but no other cells. In contrast, galanin immunostaining proved to be difficult, as relatively high concentrations of the primary antibody were needed to obtain signals. Nevertheless, identified motor neurons exhibited galanin-LI already two days after the appearance of EBs and seemed to still do so seven days later, which is a first indication for this culture to be interesting for ALS research focusing on galanin. One possible cause for the need of higher concentrated primary antibody might be the comparably low expression of galanin. This could be triggered by the NGF, which was added to the medium to facilitate the growth of the cells, but on the other hand is thought to have inhibitory effects on galanin expression[144]. Nonetheless, leukemia inhibitory factor (LIF) was also supplemented and is known to positively regulate galanin expression[145].

Motor neurons started to perish after approximately seven days in culture, therefore it could be worthwhile to substitute the medium with higher concentrated nutrients and growth factors to maintain galanin production over a longer time span. However, it also would be valuable to study galanin expression between two to seven days following the appearance of EBs, to evaluate expression patterns over a defined time course.

Taken together, galanin could act as a pleiotropic factor against early and progredient neurodegeneration in ALS, as well as foster regeneration of neurons and operate as a trophic agent. Therefore, it would be especially interesting to intensify research in this regard.

7. References

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8. Supplementary information

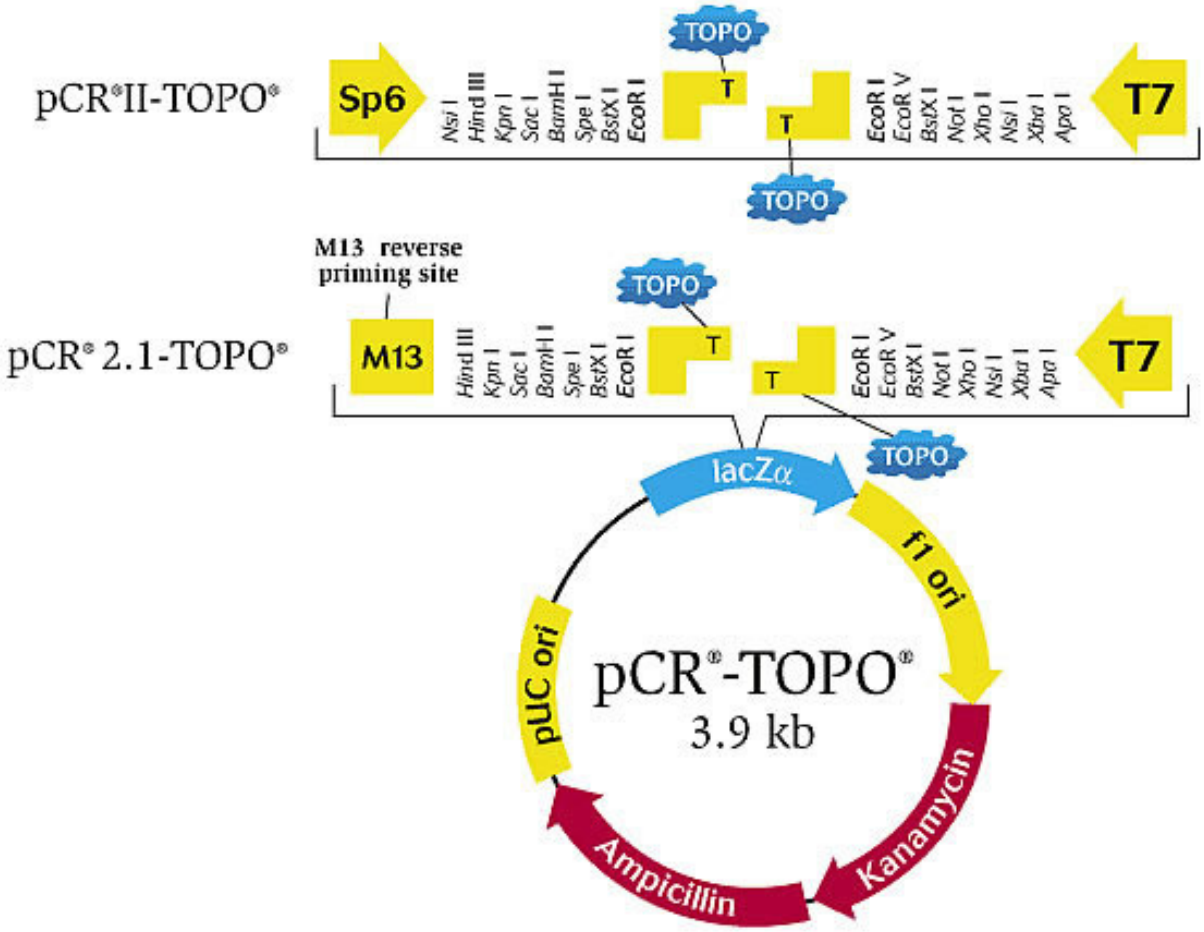

Figure 14 Topo II vector map, invitrogen.[147]

Table 3 Radioactivity measurments by liquid scintillation counting. An average of two counts per probe was taken (demanded probe activity of $1*10^6$ counts per million (CPM)) of S^{35} labeled sense (S) and antisense (AS) riboprobes for galanin, GalR1, GalR2, CGRP.

Gene	Promoter	S35_CPM (mean probe activity $1*10^6$)
CGRP	T7 (AS)	3.92
Galanin	T7 (AS)	2.49
GalR1	Sp6 (AS)	1.45
GalR2	T7 (AS)	3.6
CGRP	Sp6 (S)	0.44

Galanin	Sp6 (S)	0.54
GalR1	T7 (S)	4.55
GalR2	Sp6 (S)	0.36