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THE ROLE OF GALANIN
IN CHRONIC PAIN MECHANISMS

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For Scott

and Samuel

Cecilia hon dansar på en lina
mellan masterna på briggen Albertina
När det blåser på Atlanten
tar hon tag i kjortelkanten
och så balanserar hon för hela slanten.

-Lennart Hellsing

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Abstract

The general aim of these studies is to further elucidate the role of galanin in pain mechanisms. We have utilized normal and genetically modified animals and a variety of techniques. In order to use genetically modified mice to study chronic pain, we adapted a photochemically-induced, peripheral nerve ischemia model, originally developed in rats, to mice. Both morphological and behavioral studies were conducted to determine the optimal irradiation time for producing hypersensitivity after partial nerve injury. This model was then used to study galanin over-expressing mice and mice lacking the galanin receptor 1. The normal basal response of these mice to sensory testing was also determined. During basal conditions, the over-expressing mice showed less sensitivity to thermal stimulation than the normal controls. After the photochemically-induced peripheral nerve ischemia, the over-expressing mice showed reduced development of heat and mechanical hyperalgesia as compared to wild-type mice. In contrast, the mice lacking the galanin receptor 1 displayed hypersensitivity to cold and heat in the hot-plate test under basal condition. After photochemically-induced nerve injury these mice exhibited a longer lasting hypersensitivity than wild-type controls, and this was not due to a slower nerve regeneration.

A microdialysis technique to measure the release of galanin in the dorsal horn of the spinal cord in rat was developed. Using this method it could be demonstrated that electrical stimulation of primary afferent C-fibers causes release of galanin in the dorsal horn of normal rats.

The data presented in this thesis suggest that galanin primarily plays an inhibitory role in nociception under basal conditions. This role is further strengthened after peripheral nerve ischemia, where endogenous galanin appears to reduce the severity and duration of pain-like behaviors via activation of galanin receptor 1.

List of papers

I. Hao, J.-X., **Hygge Blakeman, K.**, Yu, W., Hultenby, K., Xu, X.-J., and Wiesenfeld-Hallin, Z. (2000).Development of a mouse model of neuropathic pain following photochemically induced ischemia in the sciatic nerve. *Experimental Neurology* 163, 231-238.

II. Hygge Blakeman, K., Holmberg, K., Hao, J.-X., Xu, X.-J., Kahl, U., Lendahl, U., Bartfai, T., Wiesenfeld-Hallin, Z., and Hökfelt, T. (2001). Mice over-expressing galanin have elevated heat nociceptive threshold. *NeuroReport* 12, 423-425.

III. Hygge Blakeman, K., Hao, J.-X., Xu, X.-J, Brumovsky, P., Hökfelt, T., Crawley, J. N., and Wiesenfeld-Hallin, Z. (2002). Galanin over-expression decreases the development of neuropathic pain-like behaviors in mice after partial sciatic nerve injury. (manuscript).

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V. Hygge Blakeman, K., Wiesenfeld-Hallin, Z., and Alster, P. (2001). Microdialysis of galanin in rat spinal cord: in vitro and in vivo studies. *Experimental Brain Research* 139(3): 354-358.

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CONTENTS

ABBREVIATIONS

AC	Adenylate cyclase
ATP	Adenosine tri-phosphate
cAMP	Cyclic adenosine mono-phosphate
CCI	Chronic constriction injury (Bennett model)
CNS	Central nervous system
DAG	Diacylglycerol
DBH	Dopamine β-hydroxylase
DRG	Dorsal root ganglion
EGFR	Epidermal growth factor receptor
ES	Embryonic stem
GALKO	Galanin knock-out
GALOE	Galanin over-expressing
Galr1$^{-/-}$	Galanin receptor 1 knock-out
Galr1$^{+/-}$	Galanin receptor 1 heterozygote
Galr1$^{+/+}$	Galanin receptor 1 wild-type
GALR1, 2 or 3	Galanin receptor 1, 2 or 3
GMAP	Galanin message-associated peptide
hDBH	Human dopamine β-hydroxylase
IASP	International association for the study of pain
IP_3	Inositol (1, 4, 5)-trisphosphate
kDa	Kilo Dalton
Lamina II_i	Lamina II inner zone
Lamina II_o	Lamina II outer zone
M-35	Putative galanin receptor antagonist, Galanin-(1-12)-pro-bradykinin-(2-9)
MAPK	Mitogen-activated protein kinase
neo^r	Neomycin resistance gene
PAG	Periaqueductal gray
PDGF-B	Platelet-derived growth factor-B
PINI	Photochemically-induced nerve ischemia (Gazelius model)
PIP_2	Phosphatidylinositol (4, 5)-diphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PNL	Partial nerve ligation (Seltzer model)
RER	Rough endoplasmatic reticulum
SMT	Spinomesencephalic tract
SNI	Spared nerve injury (Decosterd model)
SNL	Spinal nerve ligation (Chung model)
SRT	Spinoreticular tract
STT	Spinothalamic tract
tk	Thymidine kinase gene
VAS	Visual analogue scale

ADDITIONAL TERMINOLOGY

Allodynia	Pain due to a stimulus that does not normally evoke pain.
Anesthesia dolorosa	Pain in an area or region that is anesthetic.
Autotomy	The act of casting off or mutilating a body part following axotomy.
Axotomy	Transection of an axon.
Chimera	An animal that contains cells from two independent sources (In Greek mythology, the Chimera was a monster with a lions' head, goats' middle, and serpents' back).
Dialysis area	The area of the cylinder formed by the membrane length $\times$ membrane diameter $\times \pi$.
Flexor reflex model	A model based on the measurement of the hamstring muscle response after noxious stimulation to the foot.
Genome	The total set of genes carried by an individual or a cell.
Germ line gametes	Specialized reproductive cells that can pass on genetic material to offspring.
Hyperalgesia	An increased response to a stimulus that is normally painful.
Hypoalgesia	Diminished pain in response to a normally painful stimulus.
Modality/Sensory modality	A class of sensations connected by a qualitative continuum.
Nociceptors	A receptor preferentially sensitive to a noxious stimulus or to a stimulus that would be noxious if prolonged.
Noxious stimulus	A stimulus that is potentially damaging to normal tissues.
Referred pain	Pain from deep structures perceived as arising from a surface area remote from its actual origin.
Sensory detection threshold	Usually, the intensity of a sensory stimulation that a can be detected 50 percent of the time.
Speed congenics	Breeding an inbreed strain in short time.
Visceral pain	Pain originating from any of the large interior organs in any body cavity, especially the abdomen.

INTRODUCTION

Pain

Pain definitions and classification

According to the International Association for the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey and Bogduk, 1994). By this definition, pain is not by necessity associated with, or in proportion to, tissue damage. In fact many patients report pain without a pathophysiological cause (Merskey and Bogduk, 1994). Because pain is not the injury per se but an experience, the psychological component of pain is very strong. The psychology can work to aggravate pain, as mentioned above, or alleviate pain as in placebo pain relief.
Pain is always a sensory experience according to this definition. Sensory input follows the laws of psychophysics (Stevens, 1959 and 1971) and displays an exponential relationship to stimulation. This is used for evaluation of human pain, where one method for rating pain is the visual analogue scale (VAS). In animal research the estimation of pain is based mostly on testing of sensory thresholds, as described below.

Pain can be defined by the components that describe it. The experience of pain depends on, for example, the organ of origin, the duration and the homeostasis of the body when the pain develops. These components can be divided into categories often used in combination to describe pain. Classification according to the organ where the pain originates can be used to divide pain into, for example skin pain, muscle pain, visceral pain, joint pain etc. For pain originating from the same organ, some clinical characteristics are common, such as pain quality, localization and tendency to be referred (Hansson, 1997). This classification is convenient in a clinical setting.

Duration is another defining component of pain. Categories are acute pain, transient pain, persistent pain and chronic pain. Because of the large psychological component of pain, duration is an important factor in the pain experience. In a social context, chronic pain is usually far more debilitating to the patient than acute pain. Also, different molecules may be involved in mediating pain of short and long duration, respectively (Hökfelt *et al.*, 1997).

The state of the system is a determining factor for the development of pain. This homeostatically oriented classification divides pain into three categories depending on the condition of the body, that is physiological, inflammatory, and neuropathic pain (Woolf and Salter, 2000). A normal response to a painful stimulus is called physiological pain, and such an ability to react to a noxious stimulus is an essential

warning system that is necessary for survival. Pain can be initiated by inflammation, which contributes to the general sensitivity of the system. The pain persists as long as there is an ongoing inflammation (Vikman, 2002). In neuropathic pain there is an injury or dysfunction in the nervous system (Merskey and Bogduk, 1994), with symptoms such as allodynia and loss of sensation. In allodynia a non-painful stimulus is experienced as painful. Neuropathic pain is chronic in nature and usually difficult to treat (Suzuki and Dickenson, 2000).

Pain is primarily a sensory emotion that can be defined by the components that describe it. The pain sensation depends on the condition of the body, the origin and duration of the pain.

Carrying pain to the brain

Reception

Pain is initiated by a noxious stimulation in the periphery. Nociceptors respond to the stimulation and dispatch the information via the primary afferent to the spinal cord. Because nociceptors do not have a receptor structure but rather terminate as free nerve endings, the nociceptor is usually classified by the size of its axon and the modality it transmits. In the periphery, the axons are divided into three types depending on size and myelination, Aβ -, Aδ -, and C-fibers. Only the smallest fibers, myelinated Aδ -, and unmyelinated C-fibers, transmit noxious stimuli. The most common nociceptors are Aδ mechanical nociceptors and C polymodal nociceptors (Willis and Coggeshall, 1991).

Transmission and Modulation

The cell body of the primary afferent lies in the dorsal root ganglion (DRG). The DRG neurons are different from other neurons in that they have two axons and no dendrites. One branch projects to the periphery and one enters the spinal cord dorsal horn via the dorsal root (Zigmond *et al.*, 1999). In the dorsal horn, the primary afferent projects to different layers mainly depending on the type of information it conveys (Willis and Coggeshall, 1991; Zigmond *et al.*, 1999). The spinal cord was divided into laminae based on their morphological appearance by the Swedish scientist Bror Rexed in 1952 (Rexed, 1952). The laminar divisions correspond roughly to function, and laminae I-VI make up the dorsal horn. Lamina I neurons respond primarily to noxious stimuli and project in the spinothalamic tract (STT), described below. Lamina II contains mostly interneurons, which modulate lamina I and V (Basbaum and Bushnell, 2002). There is a further subdivision of lamina II into II_o and II_i, whereof II_o contains more densely packed cells (Molander and Grant, 1995). Laminae III and IV cells respond to innocuous stimuli and lamina V cells respond to both noxious and innocuous stimuli (Basbaum and Bushnell, 2002). At the base of the dorsal horn,

lamina V neurons respond to cutaneous and proprioceptive input of both noxious and innocuous stimuli (Basbaum and Bushnell, 2002; Molander and Grant, 1995).

Ascending Pathways

There are three ascending nociceptive pathways, the STT, the spinomesencephalic tract (SMT), and the spinoreticular tract (SRT). The ascending nociceptive pathways are involved in transmitting different aspects of the pain sensation to the brain. The STT is the main projection pathway and signals information about localization and character of the nociceptive input to higher centers (Willis *et al.*, 1995). In the spinal cord, STT cells are located primarily in lamina I and V and send their axons across the midline to ascend in the contralateral funicle to the thalamus (Willis *et al.*, 1995). One STT pathway projects to the ventroposterior thalamic nucleus, from where neurons send their axons on to the somatosensory cortex (Basbaum and Bushnell, 2002; Craig, 1999; Willis *et al.*, 1995). Another projection pathway for the STT neurons is to the posterior thalamus, which in turn projects to the insular cortex (Basbaum and Bushnell, 2002; Craig, 1999; Willis *et al.*, 1995). In a third STT projection, neurons send their axons to the medial thalamus (Basbaum and Bushnell, 2002; Craig, 1999; Willis *et al.*, 1995). Firing in the medial thalamus is influenced by the behavioral state, with less firing when the animal is distracted (Basbaum and Bushnell, 2002). From the medial thalamus, STT neurons project to many cortical and subcortical regions reflecting the diversity in the pain experience (Basbaum and Bushnell, 2002). SRT cells originate in laminae V, VII, VIII, and X (Willis *et al.*, 1995). Some SRT neurons are involved in pain modulation and some terminate in the thalamus along with STT neurons (Basbaum and Bushnell, 2002). The SMT contributes to signaling the motivational-affective aspects of pain, as well as triggering the activity in descending control systems (Willis *et al.*, 1995). Cells in the SMT originate in laminae I, V, and X (Willis *et al.*, 1995) and project mainly to the superior colliculus and the periaqueductal gray (PAG) (Basbaum and Bushnell, 2002). Processing in the superior colliculus involves behavioral reactions to, and integration of painful stimuli. The PAG projection activates pain control systems (Basbaum and Bushnell, 2002).

Descending Pathways

The descending pathways are the body's endogenous system to modulate pain whereby higher centers modulate nociception at several locations along the neural axis. Descending control can be either inhibitory or facilitory and implements several neurotransmitters (Millan, 2002).

Sensitization

Sensitization is a process that renders the system more sensitive to stimulation and can be of either peripheral or central origin. Peripheral sensitization is an increased excitability of the sensory receptors. During acute pain, numerous inflammatory agents

and transmitter substances are released into the tissue in response to injury (Basbaum and Bushnell, 2002). They interact with the primary afferents and increase the probability of neuronal firing by depolarizing the membrane and by modifying the voltage-gated Na^+- and K^+-channels (Bevan, 1999). The threshold for firing of the Aδ and C nociceptive afferents is lowered into the non-noxious range. During central sensitization, there is an increase in excitability of the neurons in the spinal cord (Woolf, 1983). Central sensitization is produced by prolonged or repeated activation of C-fibers. With central sensitization, low-threshold Aβ -mechanoreceptors become capable of generating pain (Fields *et al.*, 1999).

Neuropathic pain

IASP has defined neuropathic pain as "pain initiated or caused by a primary lesion or dysfunction in the nervous system"(Merskey and Bogduk, 1994). If a mixed nerve with a sensory branch is involved, neuropathic pain usually manifests itself as an area of abnormal sensation in the innervation area of the nerve (Fields *et al.*, 1999). Abnormal sensations can include allodynia, hyperalgesia, and anesthesia dolorosa (Fields *et al.*, 1999; Levitt, 1985; Scadding, 1999; Wall *et al.*, 1979). It should be emphasized that, although patients display similarities in clinical features, there is considerable symptom variation between different individuals (Scadding, 1999). The variation in symptoms suggests that there are multiple mechanisms involved in generating neuropathic pain (Fields and Rowbotham, 1993).

Animal models of neuropathic pain

Animal models for pain have been developed as a tool to study painful human conditions in the animal. It is therefore desirable to employ an animal model that mimics some of the relevant human symptoms. Such animal models are aimed at understanding the mechanisms underlying abnormal sensations such as allodynia, hyperalgesia, and anesthesia dolorosa (Bennett and Xie, 1988; Decosterd and Woolf, 2000; Gazelius *et al.*, 1996; Kim and Chung, 1992; Kupers *et al.*, 1998; Seltzer *et al.*, 1990). There are, of course, considerable limitations in working with pain in animal models. Because pain is a personal emotional experience and animals cannot communicate verbally, pain cannot be assessed directly in animals. However, there *are* ways to assess pain in animals, and veterinarians have defined a number of behavioral signs that are valuable in that evaluation (Levitt, 1985). Animals can be observed, either under normal conditions or after a stimulus has been applied, and these pain-related behaviors are scored when assessing pain (i.e. cold score and spontaneous behavior). Furthermore, sensory detection thresholds can be established. A stimulus is applied, and the animal's response is rated as a withdrawal or not (i.e.hot-plate, tail-flick, and Hargreaves). Most animal models for neuropathic pain involve damage to the branches, roots or trunk of the sciatic nerve. In all models (except for

total nerve transection), the animals develop a reversible hypersensitivity to several types of stimuli (Bennett and Xie, 1988; Decosterd and Woolf, 2000; Kim and Chung, 1992; Kupers *et al.*, 1998; Seltzer *et al.*, 1990). The chronic constriction injury (CCI) model is performed by applying three or four loose ligatures around the trunk of the sciatic nerve (Bennett and Xie, 1988). In the partial nerve ligation (PNL) model, a tight ligature is tied around about a third of the sciatic nerve trunk (Seltzer *et al.*, 1990). Tight ligatures are also used in the spinal nerve ligation (SNL) model where the L4 and L5 spinal root are tied (Kim and Chung, 1992). For the spared nerve injury (SNI) model, two branches of the sciatic nerve are sectioned, the tibial and common peroneal nerves, leaving the remaining sural nerve intact (Decosterd and Woolf, 2000). The photochemically-induced nerve ischemia (PINI) model involves an ischemic injury of the sciatic nerve (Gazelius *et al.*, 1996; Kupers *et al.*, 1998), and is described in more detail under Materials and Methods.

After complete transection of the sciatic nerve trunk, the animals do not develop hypersensitivity but rather complete sensory loss in the sciatic nerve innervation area. In some instances the animals display autotomy behavior after nerve transection (Wall *et al.*, 1979). Autotomy also occasionally occurs after CCI (Bennett and Xie, 1988), probably due to excessive nerve damage. The percentage of animals that display autotomy varies greatly among both species and strains (Mogil *et al.*, 1999; Seltzer *et al.*, 2001; Wiesenfeld and Hallin, 1981).

Galanin and its Receptors

The relevance of galanin in pain

Galanin is widely distributed throughout the nervous system and is present in a small population of small DRG neurons in rodents (Ch'ng *et al.*, 1985; Ju *et al.*, 1987; Skofitsch and Jacobowitz, 1985). After axotomy, the expression of galanin is upregulated in about 40% of the DRG neurons, both small and large (Hökfelt *et al.*, 1987; Villar *et al.*, 1989). This suggests that galanin may be involved in neuronal function especially after nerve injury. Galanin is also expressed at various levels during embryogenesis in the DRG (Marti *et al.*, 1987; Xu *et al.*, 1996a) suggesting a developmental role of galanin.

Administering galanin intrathecally to normal animals has yielded mixed results, with both excitatory and inhibitory effects. Rats receiving chronic intrathecal galanin developed persistent mechanical and/or thermal hypersensitivity (Kerr *et al.*, 2000; Liu *et al.*, 2001). In the dorsal horn, inhibitory effects of galanin have been well established (Xu *et al.*, 2000; Yanagisawa *et al.*, 1986). High doses of galanin given to mice show an inhibitory effect on pain-related behaviors (Post *et al.*, 1988), and in galanin knock-out (GALKO) mice the sensitivity to noxious stimuli is increased (Kerr

et al., 2000). The seemingly dual action of galanin was investigated in a flexor reflex model (Wiesenfeld-Hallin *et al.*, 1989), and it was shown that galanin produces a biphasic dose-dependent response. At low doses, intrathecal galanin was facilitatory, whereas it was inhibitory at high doses, and at moderate doses galanin produced a brief facilitation followed by inhibition.

After nerve injury galanin seems to attenuate pain-related behaviors. It has been shown that galanin is released in the dorsal horn after nerve injury (Colvin *et al.*, 1997). Furthermore, galanin is expressed to a higher extent in non-allodynic CCI model rats than in allodynic CCI-rats (Shi *et al.*, 1999). However, GALKO mice showed opposite effects with less thermal and mechanical hypersensitivity after nerve injury compared to wild-type controls (Kerr *et al.*, 2000). When evaluating these data it should be considered that GALKO animals have 15% fewer neurons in the DRGs and that after injury the damaged axons of the DRG neurons regenerate more slowly in the transgenic than in wild-type mice (Holmes *et al.*, 2000).

Galanin biochemistry

Galanin was isolated from porcine intestine in 1983 by Tatemoto, Mutt and colleagues and has since been characterized in detail. In this 29 amino acid neuropeptide (30 in human), the N-teminal 1-14 amino acids are fully conserved across species (Langel and Bartfai, 1998), suggesting a functional significance of this portion. It has been confirmed biochemically and behaviorally that the N-terminal fragment 1-16 to a large extent carries the same actions as full-length galanin (Fisone *et al.*, 1989; Xu *et al.*, 1990).

Like other peptides, galanin is synthesized on ribosomes in the neuronal cell bodies as a large precursor molecule, preprogalanin (Andell-Jonsson, 1997; Zigmond *et al.*, 1999). When preprogalanin enters the rough endoplasmatic reticulum (RER) the signal peptide is cleaved off, and the remaining progalanin moves to the Golgi apparatus where it is packed into large dense core vesicles. During axonal transport to the nerve terminal, the propeptide is cleaved and two neuropeptides, galanin and galanin message-associated peptide (GMAP), are formed (Figure 1) (Andell-Jonsson, 1997). Peptidergic signaling is different from classical neurotransmission. Several peptides can be stored inside the large core dense vesicles and usually co-exist in the same neuron with classical neurotransmitters (Andell-Jonsson, 1997; Saar, 2001). The vesicle release is not restricted to the active zone, requires higher frequencies to be initiated, and mediates slow synaptic signaling (Andell-Jonsson, 1997). There is no reuptake after signaling as for classical transmitters. The action of neuropeptides is terminated by diffusion from the receptor or by inactivation by cleavage (Andell-Jonsson, 1997; Bedecs, 1995). The half-life of full-length galanin varies between 100 and 120 min, while that of galanin (1-16) ranges between 28 and 60 min (Bedecs *et al.*, 1995; Land

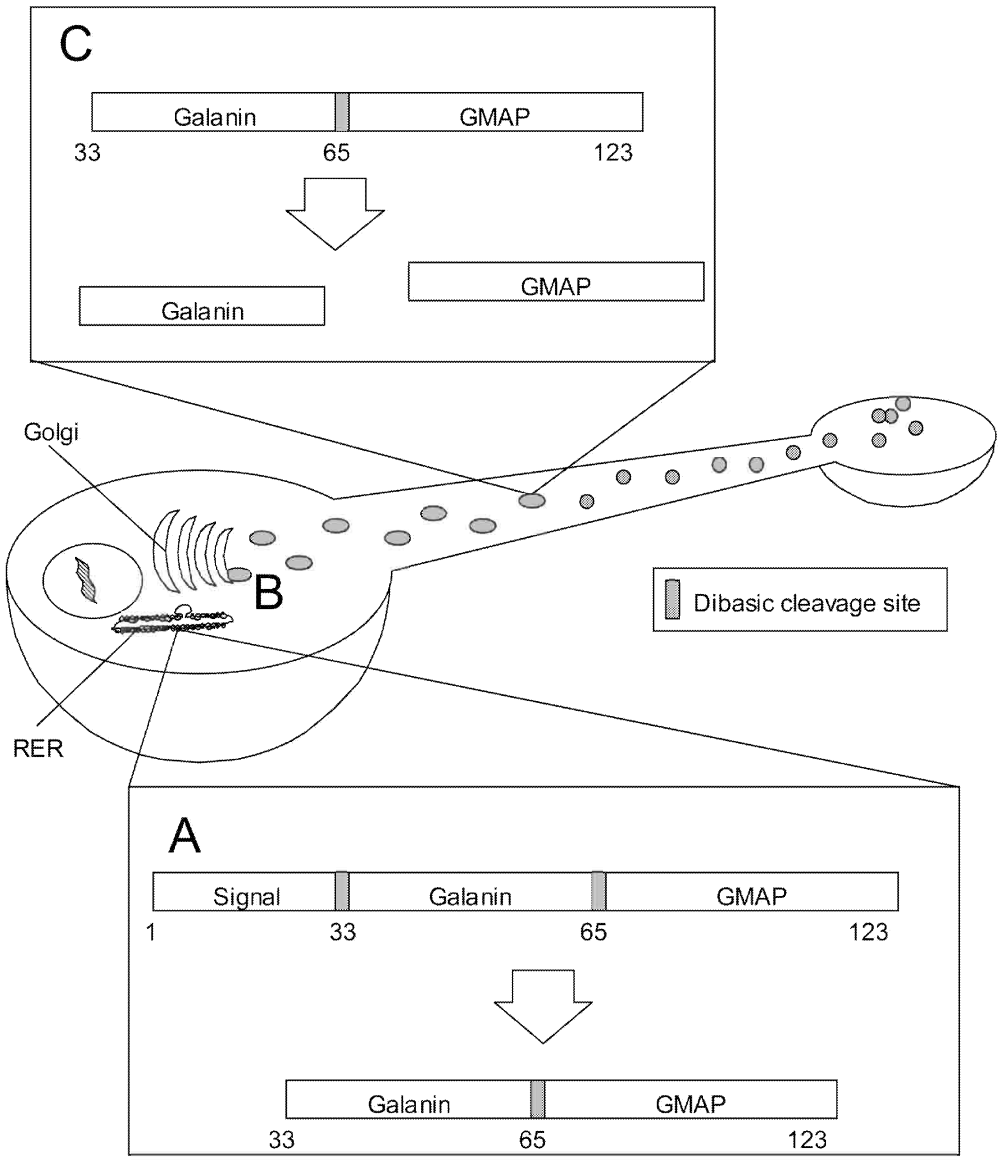

Figure 1: Schematic picture of a neuron illustrating the processing of galanin. Preprogalanin is cleaved in the RER into progalanin/GMAP (A). This fragment is then transported to the Golgi apparatus and packed into large dense core vesicles (B). During axonal transport, the propeptide in the large dense core vesicles is cleaved into two functional peptides (C). Adapted from Andell-Jonsson 1997.

et al., 1991). The endopeptidase enzymes responsible for the degradation of galanin can be inhibited by bacitracin (Bedecs *et al.*, 1995; Land *et al.*, 1991).

Galanin receptors

Three galanin receptors have been identified, GALR1, GALR2, and GALR3. All three receptors are predicted to have the structure of a G-protein coupled receptor with seven transmembrane regions (Branchek *et al.*, 2000).

GALR1

GALR1 expression is mainly confined to the nervous system. The distribution of GALR1 in the CNS is in good agreement with galanin binding and expression. High levels of GALR1 mRNA are detected in DRGs, dorsal horn of the spinal cord, brainstem, thalamus, ventral hippocampus, amygdala, and hypothalamus (Branchek *et al.*, 2000; Burgevin *et al.*, 1995; O'Donnell *et al.*, 1999; Parker *et al.*, 1995; Waters and Krause, 2000). In the spinal cord, the highest levels can be seen in the dorsal horn in laminae I and II (Table 1) (O'Donnell *et al.*, 1999). However, this can vary with different probes (see Xu *et al.*, 1996b). GALR1 expression in the DRG is highest in large neurons (Table 1) (O'Donnell *et al.*, 1999).

Table 1: Distribution of rat galanin receptor mRNA in the spinal cord and DRG. The level of expression is approximate and denoted by plus sign(s); "-" denotes absence of signal. Adapted fromO'Donnell 1999 (studying number and intensity of cells expressing GALR1 and GALR2 mRNA) and Waters and Krause 2000 (studying tissue mRNA for all three receptors).

Area		GALR3	GALR2	GALR1
Spinal Cord	Dorsal horn, lamina I & II		+	+++
	Dorsal horn, lamina III -VII	+	+	++
	Ventral horn, lamina IX		+	-
DRG	Small cells	+	++++	++
	Medium cells		+++	++
	Large cells		+	++++

Galanin signaling via GALR1 appears to be inhibitory in nature. GALR1 intracellular signaling via $G_{i\beta\gamma}$ reduces the cAMP concentration and stimulates MAPK activity. Also, GALR1 is coupled to $G_{i/o\alpha}$ and hyperpolarizes the cell via inwardly rectifying K^+-channels (Figure 2) (Branchek *et al.*, 1998; Branchek *et al.*, 2000; Iismaa and Shine, 1999; Liu and Hökfelt, 2002). The inhibitory effect of galanin in allodynic CCI model animals (Liu *et al.*, 2001), and on C-fiber stimulation-induced facilitation of the rat flexor reflex (Pooga *et al.*, 1998; Rezaei *et al.*, 2001) has been shown to be

GALR1 mediated. After axotomy, GALR1 down-regulates in the DRG (Xu *et al.*, 1996b), suggesting a decreased importance of the GALR1 after nerve injury.

GALR2

GALR2 is widely distributed in all tissues, but the highest levels are expressed in the small and medium sized DRG neurons (O'Donnell *et al.*, 1999). In the spinal cord, there are low levels of GALR2 throughout the dorsal and ventral horn (Table 1) (O'Donnell *et al.*, 1999). Activation of GALR2 leads to several intracellular events (Figure 2). The proposed main pathway involves activation of PLC via $G_{q/11}$. Activated PLC hydrolyses PIP_2 into IP_3 and DAG. An increase in intracellular IP_3 mobilises calcium from intracellular stores. Both calcium and DAG can activate PKC (Figure 2) (Branchek *et al.*, 2000; Iismaa and Shine, 1999). PKC is an important factor in the development of central sensitization (Coderre *et al.*, 1993) and mice lacking PKC_γ show reduced development of neuropathic pain (Malmberg *et al.*, 1997). PKC_γ displays a restricted expression pattern in lamina II_i (Malmberg *et al.*, 1997) and increases significantly in animals treated with exogenous galanin (Kerr *et al.*, 2000), suggesting an excitatory, pain-promoting role for GALR2. In accord with this, the excitatory effect elicited by low doses of galanin (Wiesenfeld-Hallin *et al.*, 1989) appears to be mediated by the GALR2 in normal animals (Liu *et al.*, 2001). In CCI animals however, a GALR2 agonist had no effect (Liu *et al.*, 2001). This could be explained in part by the down-regulation of GALR2 in the DRG after nerve injury (Shi *et al.*, 1997). Further intracellular signaling of the GALR2 is mediated via G_o to activate MAPK, and through the $G_{i\alpha}$ to inhibit AC activity, which reduces cAMP levels (Figure 2) (Branchek *et al.*, 2000; Iismaa and Shine, 1999). The *c-fos* gene, generally used as a marker for neural activity, is stimulated by MAPK activity and inhibited by reduction in cAMP concentration (Zigmond *et al.*, 1999). Chronic intrathecal administration of galanin in the normal rat showed a strong upregulation of *c-fos* activity mainly in the deep lamina and to a lesser extent in the superficial lamina (Kerr *et al.*, 2000). Because *c-fos* regulation is not receptor specific, a global pattern of galanin activation through all receptors is demonstrated.

GALR3

GALR3 shows a widespread distribution throughout the body, with low levels in DRGs and spinal cord (Table 1) (Branchek *et al.*, 2000; Iismaa and Shine, 1999; Waters and Krause, 2000). Like GALR1, GALR3 signals via G_i to reduce cAMP concentrations and is linked to $G_{i/o}$ -protein coupled to inwardly rectifying K^+-channels causing hyperpolarization (Figure 2) (Branchek *et al.*, 1998; Branchek *et al.*, 2000; Iismaa and Shine, 1999). No behavioral studies have been performed focusing on the GALR3; however, based on the similarity with regard to GALR1 intracellular signaling, it might play an inhibitory role (Branchek *et al.*, 2000; Iismaa and Shine, 1999).

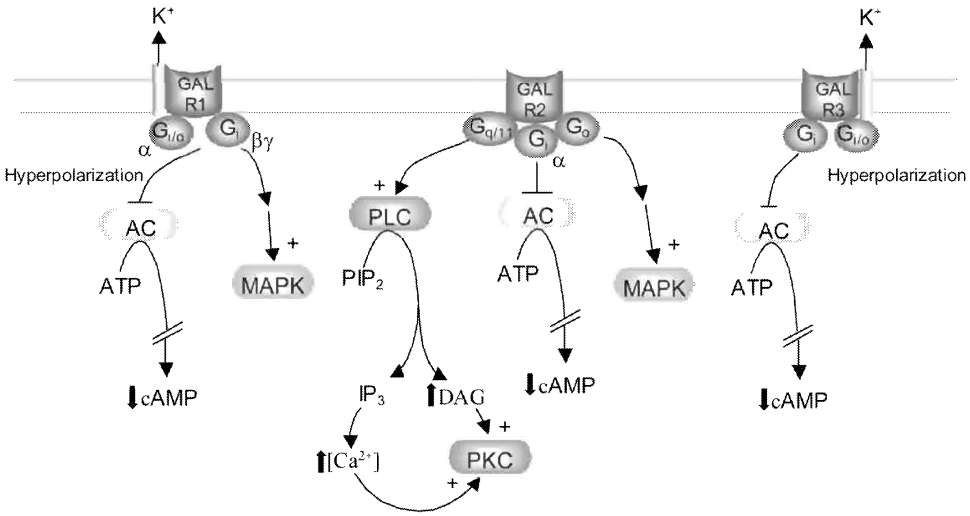

Figure 2: Intracellular signaling throughthe three galanin receptors, GALR1, GALR2, and GALR3. GALR1 signaling reduces cAMP concentrations and stimulates MAPK activity. Also, G-protein coupled inwardly rectifying K^+- channels are opened by GALR1 activation, hyperpolarizing the cell.

Genetically Modified Mice

There are two types of genetically modified mice: transgenic mice and mice with a targeted gene mutation of which knock-out mice are one example (for review, see Crawley, 2000).

Knock-out mice

Knock-out mice lack a specific gene or have an inactivated gene. A mutation is introduced in a critical part of the gene of interest. The mutation is usually a selective deletion that not only makes the gene incomplete, but also shifts the reading frame to make sure that any possible transcription would render a non-functional product. In addition to a deletion, a neomycin marker, neo^r, is inserted into the coding region to prevent gene translation. Furthermore, a thymidine kinase gene, *tk*, that makes the cells sensitive to gancyclovir, is added to the construct. The construct is introduced into embryonic stem (ES) cells and a few cells will incorporate the construct into the genome via homologous recombination. Only the cells that have incorporated the construct properly will be resistant to both neomycin and gancyclovir. The selected cells are injected into the inner cell mass of a mouse blastocyst that is then implanted into the pseudopregnant female mouse. If the injected cells are incorporated into the developing pup, the resulting animal will become a chimera, displaying cells originating

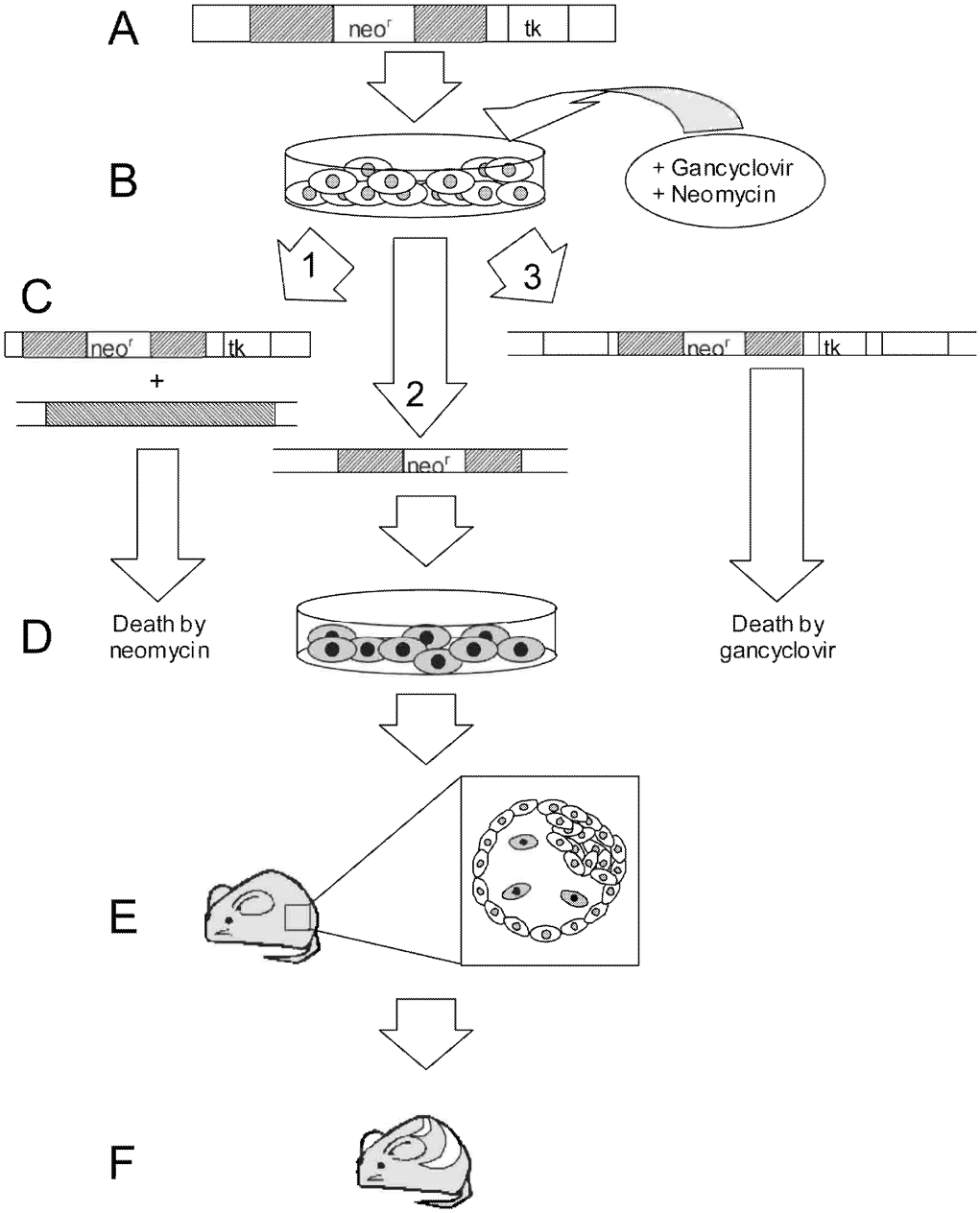

Figure 3: The generation of a knock-out mouse strain begins with the generation of a construct (A). The construct is added to ES cells with the addition of gancyclovir and neomycin (B). In most cases, the construct is not taken up by the cell (1). Some cells incorporate the cell randomly (3), but a few cells integrate the construct into the genome by homologous recombination (2) (C). The cells that have integrated the construct correctly will be resistant to both gancyclovir and neomycin, all others will be killed (D). Surviving cells are injected into a blastula that is reentered into a pseudopregnant female (E). The surrogate mother will give birth to chimeric mice (F).

from both blastula and ES cells. Only when the injected ES cells result in germ line gametes can the mutation be transmitted to the next generation.
Unfortunately for behavioral neuroscientists, the mouse is the only species in which any gene has successfully been knocked out. This has posed a challenge in adapting models optimized for rats to mice.

Transgenic mice

In transgenic mice, the aim is to add a foreign gene or copies of an existing gene to the mouse genome. A crucial portion of the desired gene is fused with a promoter to create the transgene. Using microinjection, the transgene is transferred into the pronucleus of a fertilized mouse oozyte. The promoter presents a way to control both the amount and location of the gene expression once the transgene is in place in the genome. However, it is difficult to control where the gene is integrated and how many copies of it will be incorporated. After microinjection of the gene construct, the oozytes are implanted into the oviducts of pseudopregnant females. The mouse that develops from this microinjected egg, carrying the transgene, is called a founder. If the gene construct is integrated into the genome before cell division, all cells will contain the transgene. However, if the construct is integrated later, the mice will develop into a genetic mosaic, carrying cells both with and without the transgene. As with the knock-out mice, it is only when the gene construct contributes to the germ line gametes that the transgene can be transmitted to the next generation.

In the transgenic animal, the constructed gene is always added on top of the existing genome. An endogenous transmitter can be counteracted by, for example, adding a non-functional receptor, but without deleting the original function. However, there is always a risk of creating an "accidental knock-out". If the gene-construct recombinates into the exon of a transcribed gene, it can render the recipient gene non-functional, thus knocking it out. For this reason, it is wise to limit testing of transgenic animals to heterozygotes, thereby avoiding "accidental knock-outs".

Uses and considerations

The benefit of studying genetically modified animals is that the function of the endogenous gene product can be investigated. However, there are certain factors that have to be considered when interpreting the data from these animals, such as compensation, influence of background, the role of the promoter and several other factors. Many of these aspects will be discussed further under Technical Considerations.

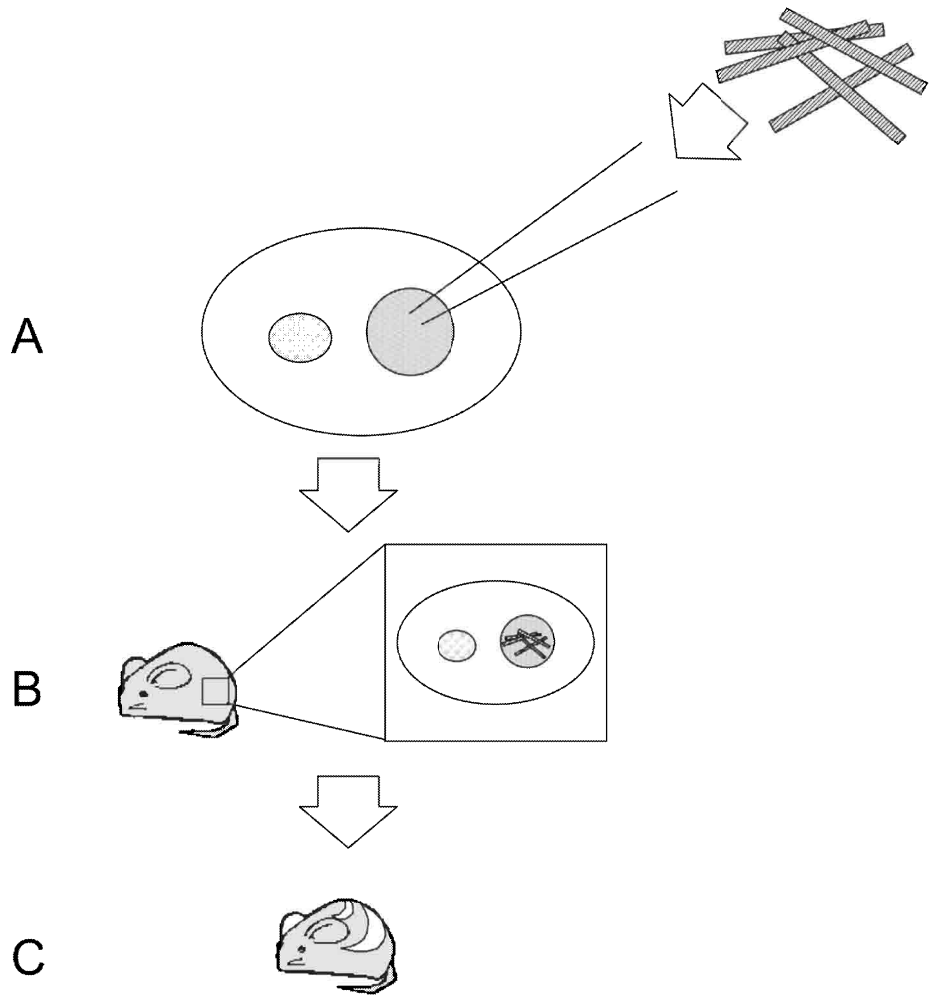

Figure 4: The transgene is microinjected into the pronucleus of a fertilized mouse oozyte (A). The oozytes are then implanted into the oviducts of pseudopregnant females (B). The chimeric mouse that develops from the microinjected egg is called a founder (C).

AIMS

The general aim of this thesis project is to study the role of galanin in pain mechanisms, particularly after nerve injury. The specific aims are as follows:

- Develop a mouse model of partial nerve injury after sciatic nerve ischemia and relate displayed behavioral abnormalities to morphological data.

- Investigate the role of galanin in pain generation by studying two different strains of mice over-expressing galanin.Examine behavior at baseline and use the model of partial nerve injury to study behavior after nerve injury.

- Investigate the role of galanin 1 receptor signaling in pain generation by studying a strain of mice lacking the galanin receptor 1.Examine behavior at baseline and use the model of partial nerve injury to study behavior after nerve injury.

- Develop a microdialysis method to study release of galanin from the spinal cord of rats in response to peripheral nerve stimulation.

MATERIALS AND METHODS

Animals

All animals were housed at constant room temperature (20°C) with free access to food and water. They were kept on a 12:12 h light-dark cycle (lights on at 0600 h) with all experiments performed during the light period. The experiments were carried out according to the Ethical Guidelines of the International Association for the Study of Pain, and the regional ethical committee for experiments on laboratory animals approved the experimental protocol.

Rats

Rats were used as experimental animals in study V. These experiments were performed on male Sprague-Dawley rats weighing 300-380 g (B&K Universal, Sollentuna, Sweden). The rats were anaesthetized with urethane (ethylcarbamate, Merck, Germany, 1.5-1.7 g/kg body weight, intraperitoneally) before surgery.

Mice

In study I, experiments were performed on normal C57BL6 males weighing 20-25 g (B&K Universal, Sollentuna, Sweden). In studies II-IV, the experiments were performed using genetically modified mice that were backcrossed to C57BL6 for several generations, creating mice with a predominantly C57BL6 background.

GALOE/PDGF-B

A PDGF-B promoter/galanin gene construct was injected into oocytes (C57BL6 x CBA) resulting in three founders. The three GALOE lines showed only minor differences, and all experiments were performed on the B3 line. Twenty copies of the construct had been inserted, and the line showed high galanin expression in cell bodies in superior cervical ganglions and DRGs (Holmberg *et al.*, 2000). The B3 founder was backcrossed with C57BL6 for approximately 15 generations (Holmberg, personal communication). Littermates were used as wild-type controls. Only heterozygote over-expressors were used. This strain of GALOE mice was created, bred, and genotyped at Karolinska Institutet, Stockholm, Sweden.

GALOE/hDBH

GALOE mice were constructed by linking a section of hDBH to the entire galanin coding sequence. The targeting vector was injected into oozytes (SJL x C57BL6) forming four founders. Three lines were generated, and all experiments were performed on line 1923. The founder was backcrossed into the C57BL6/J strain for at least six

generations (Mazarati *et al.*, 2000) to generate the GALOE (hDBH) mice. All animals used were male mice and weighed 20-30 g. Littermates were used as wild-type controls. This strain of mice was created at University of California Los Angeles, CA, USA. All mice used in the present study were bred and genotyped at Jackson Laboratories, Maine, USA. Both homozygote and heterozygote over-expressors were used.

Galr1$^{-/-}$

Embryonic stem cells from 129Sv carrying one mutant allele of the gene encoding GALR1 (*Galr1*) were injected into C57BL/6J blastocysts. The resultant chimeric was mated to C57BL/6J mice to produce heterozygous (*Galr1*$^{+/-}$) mice. These F1 mice that carried the *Galr1* knock-out (*Galr1*$^{-/-}$) allele in their germ line were backcrossed to C57BL/6J mice to generate *Galr1*$^{+/-}$ for mating to produce *Galr1*$^{-/-}$ and galanin receptor 1 wild-type (*Galr1*$^{+/+}$) littermates for analysis (Jacoby *et al.*, 2002). Current experiments were conducted on mice that had been backcrossed to C57BL/6J for at least 7 generations. All animals used were male mice and weighed 20-30 g. All mice used in the present study were bred and genotyped at Jackson Laboratories, Maine, USA.

Surgical Procedures

PINI in mice

The mice were anaesthetized with chloral hydrate (300 mg/kg, i.p.) and the sciatic nerve was exposed. Erythrosin B, a photosensitive dye that forms singlet oxygen when irradiated with a wavelength of 514 nm (Cameron, 1988), was injected i.v. and the exposed nerve was irradiated with the knife-edge beam of an argon laser at a that wavelength. The singlet oxygen reacts with the lining of the local blood vessels of the sciatic nerve and, starts the coagulation cascade. The resulting clot produces localized focal ischemia that leads to the nerve injury (Gazelius *et al.*, 1996; Hao *et al.*, 2000; Kupers *et al.*, 1998).

Sciatic nerve crush and toe-spread measurement

The sciatic nerve was exposed under chloral hydrate anesthesia (300 mg/kg, i.p.). Just above trifurcation, the nerve was crushed for 10 s with a no. 5 jeweler's forceps. Toe spread was measured before and 2, 4, 7, 9, 11 and 14 days after the crush. The mouse's hind paw was immersed in ink and the mouse was placed on a white absorbent surface. The distance in mm, between the first and fifth toe from the three clearest impressions, was measured and averaged (Holmes *et al.*, 2000; Hoogeveen *et al.*, 1993)

Intrathecal injections in mice

All intrathecal injections were done via a lumbar puncture in awake animals according to the method of (Hylden and Wilcox, 1980). It has been shown that lumbar injection allows the injected substance to diffuse up to the lumbar spinal cord segments (Hylden and Wilcox, 1981).

Behavioral Testing

Mechanical stimuli

To determine paw withdrawal-threshold to mechanical stimulation, the mice were placed in glass jars with a metal mesh floor. The plantar surface of the hind paws were stimulated with a set of calibrated nylon monofilaments (von Frey hairs, Stoelting, USA) with increasing force until the mouse withdrew the limb. Each monofilament was applied 5 times. The withdrawal threshold was taken as the force, at which the mouse withdraws the paw to at least 3 out of 5 consecutive stimuli.

Heat stimuli

Tail flick

Tail flick is a test of lower level pain-like response, involving only the spinal reflex. The time required to obtain a tail reflex in response to heat stimulation is measured (D'Amour and Smith, 1941). The mice were held gently and a radiant heat source was focused 1-2 cm from the tip of the tail. The latency to withdraw the tail from the heat source was recorded automatically (IITC, Woodland Hills CA, USA).

Hot-plate

The hot-plate test was first used in mice in 1943 (Woolfe and MacDonald, 1943) and produces a more complicated response than the tail-flick, involving higher brain centers. The animal was placed on a metal plate maintained at a set temperature of 54 ± 0.2 °C and prevented from escaping by a Plexiglas hollow cylinder. For mice, the criterion for removal from the plate is display of discomfort to the hindpaw. The response can be either picking up the hindpaw and licking it, jumping (i.e. trying to escape over the Plexiglas border) or vocalization. When the mouse displayed one of these behaviors it was removed from the hot-plate, and the response time was recorded.

Hargreaves method

Unlike the hot-plate method, the Hargreaves method (Hargreaves *et al.*, 1988) does not depend on full contact with the floor and is therefore useful in situations when full weight is not placed on the paw. After sciatic nerve injury, there is a tendency to shift weight away from the injured paw (Matsuura *et al.*, 2001), rendering the Hargreaves method useful in these situations. We used a modified Hargreaves method,

where the mouse was placed in a box with a Plexiglas floor. The mice can also be held lightly, keeping both hind paws on the floor. The plantar surface of the hind paw is directly stimulated with a radiant heat source through the floor and the latency to withdrawal of the stimulated paw is measured automatically (Ugo Basile, Varese, Italy).

Cold response

The response of the mice to cold stimulation is tested by gently contacting the plantar skin of the hind paw with an acetone bubble formed at the tip of a 1 ml syringe (Choi *et al.*, 1994; Hao *et al.*, 2000). The responses are classified according to the following scale (Hao *et al.*, 2000; Hygge Blakeman *et al.*, 2001):

0 No response.
1 Startle response without paw withdrawal.
2 Brief withdrawal of the paw.
3 Withdrawal of the paw often combined with flinching and licking the paw.
4 Prolonged or repeated withdrawal combined with licking the paw and/or vocalization.

Spontaneous behavior

Paw lifting is used as a measure of spontaneous pain-like behaviors. The behavior is observed at day 0, before irradiation and days 1, 3, 10, 14, 21 and 28 after irradiation. Each mouse was observed for five minutes and the number of spontaneous paw lifts recorded. We also examined if autotomy was present after irradiation.

Microdialysis Setup

Probes

The probe used in all experiments is a BR-2200 (BAS, Bioanalytical Systems Inc, West Lafayetta, USA) with a semi-permeable polyacrylonitrile membrane. The membrane has an outer diameter of 0.30 mm and is 2 mm long with a molecular cutoff of 30 kDa.

Microdialysis in vitro

The probe was placed in Eppendorf tubes kept at 37°C containing Krebs-Ringer and perfused with Krebs-Ringer at 3.5 µl/min. The perfusate was collected for 30 min, and these fractions were stored at -20°C until they were analyzed for galanin content. Several Eppendorf tubes were used, containing galanin in various concentrations. The sampling sequence was as follows: 1100 pM, Krebs-Ringer, 2100 pM, Krebs-Ringer, 4300 pM. Each solution was sampled for two fractions.

Microdialysis in vivo

Before surgery, the animals were anaesthetized, intubated and ventilated. During the entire procedure, the EKG and body temperature of the rat was monitored. A stereotaxic spinal unit was used to fix the vertebral column of the rat without interfering with rib cage movement. A small laminectomy at spinal level T13 was performed. The dura mater was removed, and the probe was inserted into the dorsal horn of the lumbar spinal cord through a small hole in the pia mater. For sciatic nerve stimulation the nerve was carefully dissected free rostral to the level of the poplietal fossa. A well around the exposed sciatic nerve was formed and filled with paraffin oil. A bipolar silver hook electrode was placed under the nerve and the electrode was isolated from the surrounding tissue with a piece of parafilm placed under the nerve. The probe was perfused with Krebs-Ringer at 3.5 µl/min, and the perfusate was collected for 30 min. The fractions were stored at -20°C until analyzed for galanin content.

Radioimmunoassay

Analysis of galanin content was done by radioimmunoassay. Rat galanin (Peninsula Laboratories, Merseyside, UK) at nine concentrations (1.2, 2.5, 4.9, 9.9, 19.7, 39.5, 79.0, 158.0 316.0 pM) were used as standards. Twenty-five µl of the primary antibody (Rabbit-Anti-Galanin, Peninsula Laboratories, Merseyside, UK) was added to the 100 µl of standards and samples and the tubes were mixed and incubated for 24 h at 4°C. After the addition of 25 µl ^{125}I-galanin (Rat, Peninsula Laboratories, Merseyside, UK) another incubation followed at 4°C for 48 h. Bound galanin was precipitated by the addition of 250 µl sheep anti-rabbit antibody-coated sepharose suspension (Decanting suspension 3, Pharmacia and Upjohn, Uppsala, Sweden) per tube and incubated for 30 min at ambient temperature. After centrifugation, separation was performed by aspiration of the supernatant. The radioactivity of the remaining pellet was determined in a γ-counter. The detection limit of the *in vitro* assay was 6.2 pM and 7.9 pM in the *in vivo* assay (at 90% binding).

Histology

Study I

One week after irradiation, the mice were deeply anaesthetized and a 2 mm section of the sciatic nerve including the site of irradiation was removed. The nerve section was immediately immersion fixed in 2% glutaraldehyde with 3 nM $CaCl_2$ and 0.1 M cacodylate buffer (pH 7.2) over night and then rinsed in buffer. The tissue was osmicated in 2% OsO_4 for 2 h at 4°C, dehydrated in a graded series of alcohol and acetone, and embedded in LX-112 plastic (Ladd Research Industries, Williston, VT, USA). For light microscopy, transverse semi-thin sections (1 µm) were cut, air-dried, and stained

with toluidine blue. For electron microscopy, consecutive ultra-thin sections were cut and placed on formvar-coated copper grids. The sections were contrast stained with 2% uranyl acetate followed by lead citrate and examined in an 80-kV transmission electron microscope (EM, Phillips 420).

Study V

At the end or the experiment, the probe was infused with cresyl violet to mark the probe placement. The rats were transcardially perfused with saline containing heparin followed by 4% paraformaldehyde. The spinal segment marked by cresyl violet was dissected out, postfixed in 4% paraformaldehyde over night, and washed with phosphate buffer containing 20% glucose. After freezing, the tissue was cut in a cryostat in 14 µ m sections, mounted, and stained with cresyl violet.

RESULTS

Study I

A peripheral nerve ischemia model (PINI or the Gazelius model) that has been used in rats (Gazelius *et al.*, 1996; Kupers *et al.*, 1998) was adapted to mice. The irradiation time was investigated and the model validated behaviorally and morphologically. Of the three irradiation-times tested (30 sec, 1 min, and 2 min) the 1 min irradiation produced a partial ischemic injury of the sciatic nerve as could be observed both with light and electron microscopy (Paper I, Figs. 5 and 6). One-minute irradiation also produced the most robust allodynia-like behaviors for mechanical, heat and cold stimulation with an onset of 1-2 days and maximum effect at approximately 10 days after irradiation (Paper I, Figs. 2A, 3A, and 4A). The increased sensitivity to stimulation was observed bilaterally, even on the unoperated side (Paper I, Figs. 2B, 3B, and 4B). Less allodynia was seen in mice irradiated for 2 min. This apparent 'normalization' of behavior probably stems from excessive injury to the nerve. This differs from the 30 s irradiation, where minor allodynia was associated with minor morphological damage.

Study II

Differences in baseline sensitivity between galanin over-expressing (GALOE/PDGF-B) mice and wild-type controls were investigated. GALOE/PDGF-B animals showed decreased heat sensitivity in both the Hargreaves and tail-flick tests compared to wild-type controls (Paper II, Figs. 1c and d). There was no difference in their mechanical and cold sensitivity (Paper II, Figs. 1a and b). To verify that the difference in heat sensitivity was mediated via a galanin dependent process, the mice were injected intrathecally with the non-specific galanin antagonist M-35. M-35 increased the level of heat sensitivity of the GALOE/PDGF-B animals to the heat sensitivity of the wild-type controls, but had no significant effect on the thresholds of the wild-type controls (Paper II, Fig. 2). These results strongly suggest that the increased galanin levels in these animals mediated hyposensitivity to heat.

Study III

GALOE/hDBH mice, like the GALOE/PDGF-B in Study II, exhibited decreased heat sensitivity in the Hargreaves and tail-flick tests with no change in mechanical sensitivity compared to wild-type controls (Paper III, Fig. 1). The GALOE/hDBH mice were subjected to ischemia of the sciatic nerve and tested for mechanical and

heat allodynia for seven weeks after injury. The GALOE/hDBH were less allodynic to heat than wild-types (Paper III, Fig. 3). In the mechanical sensitivity test, the GALOE/hDBH showed less allodynia and a faster recovery to baseline (Paper III, Fig. 2). Toe spread deficits after nerve injury did not differ between GALOE/hDBH and wild-types (Paper III, Fig.4), suggesting that the faster recovery from mechanical hypersensitivity is not a result of improved nerve regeneration in GALOE/hDBH mice compared to controls. These results support a role of galanin in attenuating hypersensitivity to heat. Further, they suggest a role for galanin in reducing intensity and duration of allodynia-like behaviors after nerve injury.

Study IV

The nociceptive sensitivity in mice lacking the galanin R1 receptor (*Galr1*$^{-/-}$) was assessed. The *Galr1*$^{-/-}$ mice displayed increased heat sensitivity in the hot-plate, but not Hargreaves and tail-flick, tests compared to wild-types (Paper IV, Figs.2A, B and E). They were also hypersensitive to cold stimuli (Paper IV, Fig. 2D). However, no difference between groups was seen in mechanical sensitivity (Paper IV, Fig. 2C). The sciatic nerve in *Galr1*$^{-/-}$, *Galr1*$^{+/-}$, and *Galr1*$^{+/+}$ mice was irradiated and the mice were tested for mechanical and heat sensitivity for seven weeks after injury. There was no difference between groups in the magnitude of change in sensitivity. However, *Galr1*$^{-/-}$ mice showed a more delayed recovery time from heat and mechanical hyperalgesia compared to *Galr1*$^{+/+}$ mice (Paper IV, Figs. 3 and 4). There was no difference in toe spread deficits after nerve injury between *Galr1*$^{-/-}$ mice and controls (Paper IV, Fig. 5), suggesting that delayed recovery is not due to slower regeneration. These data suggest that galanin signaling through GALR1 plays a role in duration of hypersensitivity after injury. The GALR1 seems to play a part in basal heat and cold sensitivity.

Study V

Microdialysis combined with a RIA was developed in order to study the release of galanin in the dorsal horn of the spinal cord in rat. The microdialysis probe was first tested *in vitro* to verify that it could detect varying concentrations of galanin in the external medium (Paper V, Fig. 1). After the *in vitro* testing of the probe, *in vivo* release in normal rats was measured. During the experiment the sciatic nerve was stimulated electrically, and the galanin release from the lumbar spinal cord was measured in 30 min fractions. Significant galanin release during stimulation of the sciatic nerve was detected with this method (Paper V, Fig. 2).

Discussion

PINI Model in Mice

Animal models do not mimic every aspect of the human pathology that they are intended to model. In fact, it has been pointed out that the symptoms modeled in animals are very few compared to the plethora of symptoms that can be exhibited in the patient. However, the underlying principle of an animal model is not its similarity to the human pathology but rather its usefulness in leading to strategies alleviating the symptoms in humans. All animal models of neuropathic pain have been useful in helping to alleviate clinical neuropathic pain in two broad ways (Bennett *et al.*, 2002). They have made it possible to scientifically address the mechanisms for development of neuropathic pain. Furthermore, they have played a key role in the discovery of new drugs. To counteract the potential selectivity of the models, several of them combined can be implemented to cover the spectra of human pain syndromes.

In all models of partial sciatic nerve injury described above, the rats develop a reversible hypersensitivity to one or more types of stimuli (Bennett and Xie, 1988; Decosterd and Woolf, 2000; Kim and Chung, 1992; Kupers *et al.*, 1998; Seltzer *et al.*, 1990). To take advantage of genetically modified mice, it is important to demonstrate that the models developed in rat also can be used in the mouse. In Study I, we combined behavioral and histological methods to characterize the pathophysiology of the PINI model in mice. Similarly to the rat (Kupers *et al.*, 1998), partial nerve injury contributed to the development of hypersensitivity to heat, cold, and mechanical stimulation. However, mice required shorter irradiation times and displayed no spontaneous pain-like behavior after irradiation (Kupers *et al.*, 1998). We have chosen the PINI model, because it is standardized and in the rat yields a high percentage allodynic animals compared to many of the ligation models of neuropathic pain (Shi *et al.*, 1999). The PNL model has been adapted to mice (Malmberg and Basbaum, 1998) presenting results similar to ours and similar to those produced in rats employing the same model (Seltzer *et al.*, 1990). It is more difficult to specify the amount of injury in the partial mechanical injury models (CCI and PNL) that rely on nerve ligation. Reproducibility of results is simplified in PINI, because it relies on factors that are easier to quantify (injection volume and concentration of Erythrosin B and effect and wavelength of the argon laser). Because mice are smaller than rats, a small difference in procedure will produce greater effects. Hence, with the genetically modified mice, reproducibility is of great importance. The total nerve transection model is also easy to perform and quantify (Wall *et al.*, 1979). Because there is a complete sensory loss, no sensory thresholds can be determined, and the only ratable behavior, autotomy, is spontaneous.

The interpretation of the autotomy behavior displayed by these animals is still under intense debate (Kauppila, 1998) and contributes to a certain hesitation in using this model.

Genetically Modified Animals

There are some obvious benefits in using genetically modified animals as a scientific tool. The endogenous actions of a gene can be studied directly without a potentially unspecific intermediary tool. The role of a certain molecule, e.g. messenger or receptor, can be studied without the existence of a pharmacological tool. Effects on development, which have previously been very difficult to target pharmacologically, can also be analyzed (Capecchi, 1994).

Knock-outs and transgenics have the potential to provide new insight into development and behavior. However the methods have some disadvantages. In fact, it may be impossible to study a gene with a crucial role in development since the deletion may be lethal. Also, it may be very difficult to understand the role of the gene in the adult animal due to severe developmental abnormalities (Nelson, 1997; Steele *et al.*, 1998). There is great redundancy in biological systems, and deleting a gene many times leads to compensation by other systems (Nelson, 1997; Steele *et al.*, 1998). Redundancy is paramount to survival, but a confounding factor when working with genetically modified animals. Both developmental disturbances and compensation mechanisms have been addressed in creating conditional knock-out mice, in which both time and place of gene-expression can be specified (Nelson, 1997). The background of the genetically modified animal affects the whole system in which the gene is expressed and will be discussed further below.

Background

The background of genetically modified mice refers to all the genetic material that is not the transgene or the targeted gene mutation. In the transgenic mouse, the background of the founder comes from the oozyte donor, not the pseudopregnant female. The chimeric knock-out mouse has a mosaic background of a combination of the ES cells and the blastula donor. However, because the knock-out strain will be developed by breeding the chimera, only the genetics of the gametes is important. The gametes are either of a pure ES cell or blastula origin. When the chimera is crossed to an inbred strain, the resulting heterozygotes will be on mixed background of the inbred strain and the ES cell strain.

It is important to keep track of the background in the strain one is working with. The individual strains differ in their response to measures of nociception (Mogil *et al.*, 1999). Most ES cells used to carry out targeted mutations are derived from various substrains of the 129 strain (Simpson *et al.*, 1997). Standard breeding techniques involve

crossing the chimera with C57BL6 mice, resulting in a mixed 129 × C57BL6 background. Unfortunately, the two strains differ significantly in their response to noxious stimuli. Among the twelve nociceptive dependent measures that Mogil *et al.* investigated, 129J and C57BL6 displayed differential phenotypes in eight. In the tail flick test, the latencies for the two strains were at opposite ends of the spectrum (Mogil *et al.*, 1999).

The background can also interact with the transgene/mutated gene to produce a new synergistic effect. This has been demonstrated in the epidermal growth factor receptor (EGFR) knock-out where, on three different backgrounds, the knock-outs display very different phenotypes (Threadgill *et al.*, 1995). On a CF-1 background, homozygous embryos died before embryonic day 7.5, homozygosity on an inbred 129/Sv genetic background produced a mid-gestation lethality and on a CD-1 genetic background homozygous pups survived as long as postnatal day 18. Also in the EGFR knock-out, the importance of backcrossing is shown (Sibilia and Wagner, 1995). When the 129/Sv were crossed with C57BL6 a small number of mutant fetuses developed to term. On the 129/Sv × C57BL6 × MF1 background, a few mutant mice survived until postnatal day 20 (Sibilia and Wagner, 1995). There might be modifier genes in the background that alter the gene expression of the transgene/mutated gene (Allen *et al.*, 1990; MacPhee *et al.*, 1995). Because the background contributes to such a great extent to the phenotype, it is important to study null-mutants on different backgrounds (Gerlai, 1996). If the phenotype persists on different backgrounds, the change was most likely due to the mutation.

The summary presented above emphasizes the importance of a controlled background. The simplest way to achieve this is by crossing to an inbred stain (Gerlai, 1996) until the mutation is on a pure background. With classic breeding strategies, this takes at least 10 generations (Wakeland *et al.*, 1997; Visscher, 1999; Wong, 2002). That amounts to roughly two years of breeding before the strain is ready for testing. With the assistance of marker-aided breeding techniques, speed congenics, the breeding time can be reduced to about half (Wakeland *et al.*, 1997; Visscher, 1999; Wong, 2002).

Considerations for genetically modified mice

GALOE/ PDGF-B

After peripheral nerve ischemia , galanin is down-regulated in the DRG of the GALOE/PDGF-B mice (Holmberg, 2001). This galanin regulation is the opposite of what happens in normal mice, where nerve injury produces an intense galanin upregulation in the DRG. However, after downregulation in GALOE/PDGF-B and upregulation in normal controls, positive galanin neuron profiles and grain density were comparable in the DRGs of both strains (Holmberg, 2001).

GALOE/ hDBH

DBH is the enzyme that catalyses the hydroxylation of dopamine to norepinephrine. It only exists in cells containing norepinephrine or epinephrine (Goodman Gilman *et al.*, 1985). Theoretically, using an hDBH promoter would yield a specific expression pattern in cells expressing DBH. However, in mice expressing *lacZ* under the DBH promoter, the DBH-*lacZ* transgene was expressed in DBH-immunonegative sites (Mercer *et al.*, 1991). This misregulation is called ectopic expression and can be both temporal and spatial (Mercer *et al.*, 1991). In the DBH-*lacZ* transgenic mice 10%-49% of the animals showed staining in 10% or less of the DRG neurons (Mercer *et al.*, 1991). No expression was seen in the spinal cord (Mercer *et al.*, 1991). This suggests that the behavioral effect of galanin in the GALOE/hDBH mice is through DRG neuron, and not dorsal horn interneuron, signaling.

Functional Role of Galanin and its Receptors

Tonic actions of galanin

In both the GALOE/hDBH and GALOE/ PDGF-B mice, the over-expressing mice showed a hyposensitivity to heat under normal conditions. This suggests that in the naïve state, galanin would appear to have a tonic inhibitory role in these mice. Data from galanin knock-out mice supports the role of galanin acting as a tonic inhibitory peptide under basal conditions (Kerr *et al.*, 2000). Reversal of the hyposensitivity in the GALOE/ PDGF-B by administration of the putative galanin receptor antagonist M-35, confirmed that this was a galanin receptor mediated effect. In study IV, *Galr1*$^{-/-}$ mice show an increased sensitivity to cold and heat (in one behavioral test), suggesting that the tonic inhibition is partially mediated by the GALR1. The thermal hypersensitivity seen in the hot-plate test for *Galr1*$^{-/-}$ mice was small but significant. In the two other thermal tests, Hargreaves and tail-flick, the withdrawal latencies were shorter, which would make it more difficult to identify small differences between groups, because of a high within group variation. During basal conditions, galanin seems to exert a tonic inhibitory effect on thermal nociception that is partially mediated by the GALR1. This is supported in a previous study (Wiesenfeld-Hallin *et al.*, 1993) where galanin in normal rats causes a more pronounced hypoalgesia for noxious heat than noxious mechanical stimulation.

Galanin after partial sciatic nerve injury

GALOE/hDBH mice showed less development of both heat and mechanical hyperalgesia after PINI compared to wild-type controls. For heat hypersensitivity especially, the persistence of the behavioral changes was significantly shorter in these mice. These results indicate that galanin both reduces the magnitude and shortens the

duration of hypersensitivity. However, work done with the GALKO mice tells a different story (Kerr *et al.*, 2000). After partial or complete nerve injury, the GALKO mice have reduced development of neuropathic-like behaviors (Kerr *et al.*, 2000), suggesting that galanin aggravates pain after nerve injury. Especially for knock-out mice, the role of galanin in development and regeneration (Holmes *et al.*, 2000; Marti *et al.*, 1987; Xu *et al.*, 1996a) is of particular interest because of compensation mechanisms. In the GALKO, damaged axons of the DRG neurons regenerate slower after injury, and there are 15% fewer neurons in the DRGs than in control animals (Holmes *et al.*, 2000). The difference in regeneration properties might contribute to the discrepancy in neuropathic-like behaviors after nerve injury. We detected no difference in regenerative properties in the *Galr1*$^{-/-}$ mice or GALOE/hDBH mice compared to wild-type controls.

The *Galr1*$^{-/-}$ mice displayed a significantly longer hypersensitivity to both heat and mechanical stimulation than *Galr1*$^{+/+}$ mice, suggesting a role for GALR1 in the persistence of heat and mechanical hypersensitivity. There was no difference in the magnitude of hypersensitivity between *Galr1*$^{-/-}$ mice and *Galr1*$^{+/+}$ mice after injury, which might be explained by the downregulation of GALR1 after nerve injury (Xu *et al.*, 1996b). When GALR1 is downregulated, there is less difference between GALR1 expression in *Galr1*$^{-/-}$ mice and *Galr1*$^{+/+}$ mice. When GALR1 expression returns, difference in sensitivity can be detected as shorter hypersensitivity in the *Galr1*$^{+/+}$ mice. There is a remaining inhibitory effect of galanin in uninjured *Galr1*$^{-/-}$ mice (Grass et al., unpublished observations), suggesting that the inhibitory effect of galanin does not require GALR1 signaling but can be compensated for. A compensatory inhibitory mechanism of this sort might also occur in the *Galr1*$^{+/-}$ mice, explaining why they fail to develop mechanical hypersensitivity after PINI. The antinociceptive effect of galanin in the second phase of the formalin test has been shown to be largely mediated by GALR1 (Hua *et al.*, 2002). Taken together, it seems that GALR1 plays an inhibitory role in pain-like states

Microdialysis in the Spinal Cord

Choice of method

There are several benefits with employing microdialysis as a tool to study galanin release. Each animal can be used as its own control, and the response to a stimulus can be measured individually. Furthermore, repeated measures across time address the temporal component of galanin release. Another method that has been used to measure galanin release in the spinal cord of rats is the antibody-coated microprobe technique (Colvin and Duggan, 1998; Colvin *et al.*, 1997; Duggan and Riley, 1996; Hope *et al.*, 1994). Duggan and collaborators have been able to quantify and localize both basal

and evoked release of galanin in the spinal cord in normal rats, as well as those with peripheral inflammation and nerve injury (Colvin and Duggan, 1998; Colvin *et al.*, 1997; Duggan and Riley, 1996; Hope *et al.*, 1994). The thin glass microprobes, coated with immobilized antibodies, are inserted into the dorsal horn, where they provide an excellent tool to investigate the spatial distribution of galanin release. However, the method is only semi-quantitative and has limited temporal resolution.

Optimizing galanin microdialysis

Several factors influence the success of microdialysis, such as length and diameter of the probe membrane, stability of the peptide under experimental conditions, and binding of the peptide to the dialysis membrane or outlet tubing (Connelly, 1999; Kendrick, 1990). For galanin, we tried to overcome the generally low relative recovery of neuropeptides (Kendrick, 1990) by keeping the dialysis area large without excessively damaging the dorsal horn in the process. We used a 2 mm probe with a diameter of 0.30 mm inserted into the spinal cord at a 45° angle to the vertical (Gustafsson *et al.*, 1999), to maximize relative recovery, restrict dialysis to the dorsal horn and minimize tissue damage. Previous studies demonstrated that probes of similar size produced an intact receptor-mediated release of cholecystokinin by morphine (Lucas *et al.*, 1998), indicating that tissue damage was not extensive. Because galanin is a substrate for bacitracin sensitive neutral endopeptidase 24.11 (Bedecs *et al.*, 1995; Land *et al.*, 1991), bacitracin was added to the perfusion solution to prevent degradation of galanin before the RIA was performed. Galanin has a tendency to adsorb to surfaces in the probe and to the outlet tubing (Kendrick, 1990); therefore, we added 0.2% BSA to the perfusion solution to minimize adsorption to surfaces.

Galanin release

With our microdialysis method, the basal release of galanin was balancing on the detection level. This is in contrast to the antibody microprobe technique, where a basal release of galanin could be detected in normal rats (Hope *et al.*, 1994). Because basal levels were close to the detection limit with our microdialysis setup, we were limited to measuring acute release or increased basal release of galanin. We did observe galanin release induced after electrical stimulation of the sciatic nerve at intensities that activate A- and C-fibers. (Klein *et al.*, 1992) showed that electrical stimulation of C-fibers reduces immunostaining of galanin in the spinal cord, suggesting stimulus-induced release similar to our results. Galanin release after electrical stimulation in normal animals has not been seen with the microprobe technique (Colvin and Duggan, 1998; Morton and Hutchison, 1989). The source of the released galanin is unknown, but may include primary afferents and/or interneurons containing galanin.

SVENSK SAMMANFATTNING

Smärta har vanligtvis en livsviktig och skyddande funktion. Kroppen använder smärta för att tala om för oss vad vi ska undvika, som varma spisplattor och frätande syra. Ibland skadas nervsystemet som förmedlar smärtsignalen till hjärnan och ett kroniskt smärttillstånd uppstår. Den smärtan kallas för neuropatisk smärta och har inte längre en skyddande funktion. Signaleringen av smärta från en kroppsdel till hjärnan sker i flera steg där signalsubstanser i nervsystemet binder till sina receptorer. En av de signalsubstanser som är inblandade i denna signalering är ett litet protein som heter galanin. Vid kroniska smärttillstånd som neuropatisk smärta finns mer galanin än normalt i de delar av nervsystemet som är inblandade i smärtreglering. Galanin tros ha en hämmande effekt på signaleringen till hjärnan och i denna avhandling presenteras flera studier som gjorts för att undersöka detta.

För att undersöka närmare hur galanin-signalering går till utvecklade vi en dialysmetod (mikrodialys) för att titta på frisättning an galanin i ryggmärgen hos råtta. Ryggmärgen är en väldigt viktig omkopplingsstation för signalering till hjärnan. Vid mikrodialys inför man en mycket tunn kateter i ryggmärgen på en sövd råtta. Genom katetern kan man sedan samla upp de signalsubstanser som frisätts i ryggmärgen. Vi stimulerade nerven hos den sövda råttan och såg att detta ökade frisättningen av galanin. Att galaninfrisättningen sedan avtog efter avslutad stimulering talade om för oss att galaninfrisättningen är direkt kopplad till stimulering av nerven.

Genetiskt modifierade djur används ofta inom neurovetenskap för att testa en hypotes. Vi har använt oss av möss som har mer galanin än normalt (överuttryckare) och möss som saknar en av receptorerna för galanin, receptorn GALR1. För att testa vikten av galanin vid kronisk smärta i dessa möss har vi utvecklat och använt oss av en musmodell för neuropatisk smärta. I modellen ges sövda möss en kort syrebrist (ischemi) i ischiasnerven. Detta leder till övergående överkänslighet för beröring, värme och kyla. Galanin-överuttryckarna visar minskad känslighet för värme. Efter ischias ischemi visar de en minskad benägenhet att utveckla överkänslighet för beröring och i viss mån, värme. Mössen som saknar GALR1 (GALR1-knockarna) visar något större känslighet för värme än normala möss. Efter ischias ischemi tar det lite längre tid för GALR1-knockarna än för normala möss att återvända till normal känslighet. Galanin-överuttryckarna visar att galanin spelar en skyddande roll både i normala fall och vid neuropatisk smärta. Det verkar som att en viss del av denna effekt signaleras genom GALR1 eftersom avsaknaden av denna receptor leder till fler smärtliknande symptom.

Frisättningen av galanin är stimulus inducerad och påverkar känsligheten för sensoriska stimuli. I normala fall minskar galanin känsligheten för värme. Efter en nervskada mildrar galanin främst utvecklandet av överkänslighet för beröring. Båda dessa effekter av galanin signaleras delvis genom GALR1.

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