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Depletion of endogenous noradrenaline does not prevent spinal cord plasticity following peripheral nerve injury

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Abstract

The present study examined the role of endogenous noradrenaline on glial and neuronal plasticity in the spinal cord in rats after peripheral nerve injury. An intrathecal injection of dopamine- β -hydroxylase antibody conjugated to saporin (D β H-saporin) completely depleted noradrenergic axons in the spinal cord and also reduced noradrenergic neurons in the locus coeruleus (A6) and A5 noradrenergic nucleus in the brainstem and noradrenergic axons in the paraventricular nucleus of the hypothalamus. D β H-saporin treatment itself did not alter mechanical withdrawal threshold, but enhanced mechanical hypersensitivity and intrathecal clonidine analgesia after L5–L6 spinal nerve ligation (SNL). In the spinal dorsal horn of SNL rats, D β H-saporin treatment increased choline acetyltransferase (ChAT) immunoreactivity as well as immunoreactivity in microglia of ionized calcium binding adaptor molecule 1 [IBA1] and in astrocytes of glial fibrillary acidic protein [GFAP], and brain derived nerve growth factor (BDNF) content. D β H-saporin treatment did not, however alter the fractional release of acetylcholine from terminals by dexmedetomidine after nerve injury. These results suggest that endogenous tone of noradrenergic fibers is not necessary for the plasticity of α 2-adrenoceptor analgesia and glial activation after nerve injury, but might play an inhibitory role on glial activation.

Perspective—This study demonstrates that endogenous noradrenaline modulates plasticity of glia and cholinergic neurons in the spinal cord after peripheral nerve injury and hence influences the pathophysiology of spinal cord changes associated with neuropathic pain.

Keywords

neuropathic pain; noradrenaline; acetylcholine; brain-derived neurotrophic factor; microglia; astrocytes

Introduction

Bulbospinal noradrenergic pathways have been shown to inhibit pain transmission³⁷. In both normal and neuropathic pain states, noradrenaline, released by descending noradrenergic axons activates α 2-adrenoceptors to produce acute antinociception via

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Disclosures

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reduction of neurotransmitter release from primary afferent terminals²⁷ and hyperpolarization of second order spinal dorsal horn neurons³⁵. Some of these effects are direct, but others reflect activation of cholinergic signaling^{30,31}. We previously demonstrated that α_2 -adrenoceptor agonists, clonidine and dexmedetomidine, inhibit KCl-evoked acetylcholine release in spinal cord slices and synaptosomes in normal rats^{15,28}, consistent with this classical inhibitory action of α_2 -adrenoceptors. In contrast, after peripheral nerve injury, activation of α_2 -adrenoceptors by dexmedetomidine results in Gs-protein mediated facilitation of acetylcholine release from the spinal dorsal horn synaptosomes¹⁵, consistent with increased cholinergic dependency of α_2 -adrenoceptor-mediated analgesia after nerve injury^{30,31}.

In normal animals, depletion of noradrenergic fibers in the spinal cord by the neurotoxins such as N-2-chloroethyl-N-ethyl-2-bromobenzylamine hydrochloride (DSP4) and 6-hydroxydopamine (6-OHDA) enhances clonidine analgesia, associated with denervation super-sensitivity of postsynaptic spinal α_2 -adrenoceptors^{32,33,39}. However, the role of these fibers, which release noradrenaline, ATP, and neuropeptide-Y on neuronal and glial plasticity associated with neuropathic pain states has not been fully tested. One goal of the current study was to test whether depletion of spinal noradrenergic axons by an intrathecal injection of dopamine- β -hydroxylase antibody conjugated to saporin (D β H-saporin) affects clonidine analgesia, ChAT immunoreactivity in the dorsal horn, and the facilitatory effect of dexmedetomidine on acetylcholine release from synaptosomes in rats after L5-L6 spinal nerve ligation (SNL). We hypothesized that denervation supersensitivity might result in an increased fractional release of acetylcholine from spinal cord synaptosomes after nerve injury in D β H-saporin treated animals.

Peripheral nerve injury increases brain-derived neurotrophic factor (BDNF) content in the spinal dorsal horn^{16,26} and the most likely sources of spinal BDNF after nerve injury are the central terminals of primary afferents and resident microglia^{4,11,16,34}. We recently reported that blockade of BDNF-tropomyosine receptor kinase B (trkB) signaling by spinal infusion of BDNF antibody or repeated intrathecal injection of trk inhibitor K252a reduces choline acetyltransferase (ChAT) immunoreactivity in the dorsal horn and also abolishes the shift from inhibition to facilitation by dexmedetomidine of acetylcholine release^{15,17}. These results suggest that BDNF-trkB signaling is essential for maintenance and functional change of cholinergic neurons in the spinal cord after nerve injury, and that this plasticity in cholinergic neurons is important for the α_2 -adrenoceptor-mediated analgesia in neuropathic pain.

Activation of spinal glia also participates in neuropathic hypersensitivity⁵. Whether the products released by descending noradrenergic fibers alter this response is not known, but stimulation of α_2 -adrenoceptors reduces activation of microglia and astrocytes in the spinal cord after peripheral nerve injury or chronic inflammation^{10,41}. Peripheral nerve injury enhances spinal noradrenergic inhibition by increasing content and basal release of noradrenaline in the spinal dorsal horn^{14,16}. We therefore hypothesized that spinal noradrenergic fibers, perhaps by the release of noradrenaline, modulate glial activity and BDNF production in the spinal cord after nerve injury. A final goal of the current study was to test whether depletion of spinal noradrenergic fibers reduces expression of glial activation markers and BDNF content in the spinal cord in SNL rats and hence, as described above, the shift in direct effects of α_2 -adrenoceptor activation on cholinergic terminals from inhibition to excitation.

Materials and Methods

Animals

Male Sprague-Dawley rats (weighing 180–250 g) from Harlan Industries (Indianapolis, IN, USA), housed under a 12-h light-dark cycle with food and water *ad libitum*, were used. All experiments were approved by Animal Care and Use Committee at Wake Forest University (Winston Salem, NC, USA).

Surgical preparations and D β H-saporin treatment

Animals were anesthetized with 2% isoflurane in oxygen and intrathecal catheterization was performed as previously described⁴³. A small puncture was made in the atlanto-occipital membrane of the cisterna magnum and a polyethylene catheter (external diameter; 0.23 mm, internal volume; 6 μ L, ReCathCo LLC, Allison Park, PA, USA), 7.5 cm, was inserted so that the caudal tip reached the lumbar enlargement of the spinal cord. At least 5 days after intrathecal catheterization, animals received an intrathecal injection of mouse anti-D β H-saporin (Advanced Targeting Systems, San Diego, CA, USA, 5 μ g in 10 μ l of saline) or saporin (Advanced Targeting Systems, 5 μ g in 10 μ l of saline) followed by 10 μ l of saline. One week after injection, SNL was performed as previously described²³. Briefly, under anesthesia with 2% isoflurane, the right L6 transverse process was removed and the right L5 and L6 spinal nerves were tightly ligated using 5–0 silk suture. When animals were sacrificed to collect the spinal cord, the placement of catheter was verified. No catheter misplacement was observed.

Behavioral test

The person performing the behavioral test was blinded to drug and treatment. Paw withdrawal threshold in response to calibrated von Frey filaments (Stoelting, Wood Dale, IL, USA) was determined using an up–down statistical method³. Filaments were applied to the bending point for 5 s, and a brisk paw withdrawal was considered a positive response. At 12 days after SNL, saporin and D β H-saporin treated animals received an intrathecal injection of clonidine (Sigma Chemical CO., St. Louis, MO, USA, 15 μ g in 10 μ l of saline) with or without atropine (Sigma Chemical CO., 30 μ g in 10 μ l of saline) followed by 10 μ l of saline 1 h prior to the measurement. Doses of clonidine and atropine were determined from our previous studies^{30, 31}. At 14 days after SNL, spinal cord tissues from those animals were used for following experiments.

Immunohistochemistry

Immunostaining for ChAT and D β H was performed in the brain and/or spinal cord, as we previously described^{15, 17}. Briefly, brain and L4–L6 spinal cord sections were incubated at 4 °C with a goat anti-ChAT antibody (1:200, AB144P, Millipore, Billerica, MA, USA) or a mouse monoclonal anti-D β H antibody (1:500, MAB308, Chemicon International Inc., Temecula, CA, USA) followed by biotinylated donkey anti-goat or anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA, USA) in 1.5 % normal donkey serum (NDS, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), processed using Elite Vectastain ABC kit (Vector Laboratories), and then developed by the standard glucose oxidase-nickel method. For labeling of microglia and astrocytes, sections were incubated at 4°C with anti-ionized calcium binding adaptor molecule 1 (rabbit anti-IBA1, 1:2000, Wako Chemicals, Richmond, VA, USA) or anti-gial fibrillary acidic protein (rabbit anti-GFAP, 1:2000, Dako, Carpinteria, CA, USA) for 24 hr followed by CY3 conjugated donkey anti-rabbit IgG (1:600, Jackson Immunoresearch) for 2 hours in 1% NDS.

Images of the spinal cord were captured on a Nikon E600 epifluorescence microscope fitted with a CCD digital camera using a 10 \times objective at a resolution of 1,600 \times 1,200 pixels.

Quantification for each immunostaining was performed in 4–5 randomly selected L4–L6 spinal cord sections from each rat. Image analysis software (Image J; NIH Image, National Institutes of Health, Bethesda, MD) was used to quantify changes in immunofluorescence (GFAP and IBA1) or immunodensity (ChAT). In Image J, the upper and lower threshold optical densities were adjusted to encompass and match the immunoreactivity, providing an image with immunoreactive material appearing as red pixels. For quantification of ChAT immunoreactivity, the number of pixels of ChAT-immunoreactivity within the defined threshold and within the total area of the dorsal horn containing lamina I to IV were quantified per section. All sections for a given antibody reaction were reacted on the same day in the same reaction, using a custom designed plate. The image thresholds were determined and applied to all sections. Data are expressed as percentage of ChAT-immunoreactive pixels compared to the total number of pixels in the sampled area. For quantification of IBA1 and GFAP immunofluorescence, a fixed area ($250 \times 250 \mu\text{m}^2$) was positioned in the middle one third of the mediolateral extent of the spinal cord dorsal horn and the number of pixels of IBA1- or GFAP-immunoreactive cells within the threshold value was quantified. Data are expressed as number of pixels in the area. To limit variability in immunohistochemical measurements, all groups of rats were sacrificed and processed on the same day and the same threshold value was applied to all images for a given antibody. Images in figure 6 were pseudocolored with same adjustments to brightness or contrast to distinguish labeling of different proteins. The observer quantifying the sections was blind to the treatment. Negative controls were obtained for each antibody by omitting the primary antibody.

Acetylcholine release from synaptosomes

Preparation of crude synaptosomes from the L4–L6 spinal dorsal horn and measurement of [^3H]-Acetylcholine release were performed as previously described¹⁵. Each synaptosome preparation contained 3 unilateral dorsal quadrants of spinal cord from 3 SNL rats. Briefly, after loading with 1 μM choline chloride (combination of both tritiated and unlabeled choline) for 20 min at 37°C, synaptosomes were perfused in temperature controlled perfusion chambers (SF-12, Brandel, Gaithersburg, MD) with Krebs buffer (in mM: 124 mM NaCl, 3 mM KCl, 2 mM MgSO_4 , 2 mM CaCl_2 , 1.25 mM KH_2PO_4 , 25 mM NaHCO_3 , and 10 mM glucose, pH 7.35, 0.67 ml/min) for 25 min to remove free radioactivity, and then fractions were collected every 5 min for 20 min. After a 10 min baseline collection, synaptosomes were perfused with 10 nM dexmedetomidine alone for 2 min, then stimulated with 12 mM KCl-Krebs buffer (in mM: 115 NaCl, 12 KCl, 2 MgSO_4 , 2 CaCl_2 , 1.25 KH_2PO_4 , 25 NaHCO_3 , and 10 glucose, pH 7.4) containing dexmedetomidine for 3 min. Concentrations of KCl and dexmedetomidine were determined from our previous studies^{15,17}. An inhibitor of the acetylcholine transporter, hemicholinium-3 (10 μM), was present during perfusion to prevent reuptake of acetylcholine. [^3H]-Acetylcholine release from synaptosomes in each fraction was measured by a liquid scintillation counter (LS6500, Beckman Coulter Inc., Fullerton, CA, USA). All chemicals were purchased from Sigma Chemical Co. except [^3H]-choline (Perkin Elmer, Boston, MA, USA).

BDNF assay

The dorsal quadrants of the spinal cord ipsi- and contralateral to SNL were collected from saporin and D β H-saporin treated animals. Tissues were weighed and homogenized in ice cold homogenization buffer (mammalian cell lysis kit, Sigma Chemical CO) containing 1 mM phenylmethanesulfonyl fluoride (Sigma Chemical CO) and 1 % glycerol. The homogenates were centrifuged at 14,000 \times G for 30 min and the resulting supernatants were used to measure BDNF concentration using ChemiKine BDNF ELISA kit (Millipore) according to the manufacturer's instructions.

Statistical analyses

Data were normally distributed and are presented as mean \pm SE. Differences among groups were determined using one or two-way ANOVA as appropriate. $P < 0.05$ was considered significant.

Results

Effects of spinal noradrenaline depletion on hypersensitivity and clonidine analgesia after nerve injury

Consistent with previously reports^{22, 25}, intrathecal injection of D β H-saporin but not saporin alone completely depleted D β H-immunoreactive axons in the spinal dorsal horn (Fig 1 A, B) and also reduced D β H-immunoreactive neurons in the locus coeruleus (Fig 1 C, D) and A5 noradrenergic nuclei (Fig 1 E, F) as well as D β H-immunoreactive axons in the paraventricular nucleus (Fig 1 G, H). Similar results were observed in 3 saporin and 3 D β H-saporin treated rats (Images not shown). Animals tolerated both saporin and D β H-saporin treatments with no significant change in withdrawal threshold values in the right paw at 7 days after injection (Table 1). At 12 days after SNL, withdrawal threshold values were significantly lower than baseline in both saporin and D β H-saporin treated animals ($p < 0.01$). D β H-saporin treated animals showed slightly but significantly lower withdrawal threshold values compared to saporin treated animals ($p < 0.05$), as previously reported²². In both saporin and D β H-saporin treated animals, intrathecal injection of clonidine (15 μ g) significantly increased withdrawal threshold in the paw ipsilateral to SNL compared to pre-clonidine values ($p < 0.01$) and co-administration of intrathecal atropine (30 μ g) completely blocked the analgesia from clonidine (Fig. 2). D β H-saporin treated animals showed enhancement of peak analgesic efficacy of clonidine compared to saporin treated animals ($p < 0.05$).

ChAT immunoreactivity and acetylcholine release from synaptosomes in the spinal dorsal horn

Figure 3 A and B depict ChAT immunoreactivity in the lumbar spinal dorsal horn in SNL rats treated with saporin or D β H-saporin. The vast majority of ChAT immunoreactivity in the spinal dorsal horn in all treatment groups was found in axons but a few ChAT-positive cells were also observed. D β H-saporin treated animals showed a trend towards higher ChAT immunoreactivity in the spinal dorsal horn ipsilateral ($p = 0.051$) and contralateral ($p < 0.05$) to SNL compared to saporin treated animals (Fig. 3C).

[³H]-acetylcholine release from synaptosomes evoked by 12 mM KCl with or without dexmedetomidine did not differ between saporin and D β H-saporin treated animals. In both saporin and D β H-saporin treated animals, [³H]-acetylcholine release increased about 25–35 % from the baseline by 12 mM KCl and dexmedetomidine significantly enhanced KCl-evoked [³H]-acetylcholine release compared to KCl alone in synaptosomes both ipsilateral and contralateral to SNL surgery (Fig. 4; $p < 0.05$). Consistent with our recent observations^{15, 17}, there was a significant difference in the facilitatory effect of dexmedetomidine on KCl-evoked [³H]-acetylcholine release between synaptosomes ipsilateral and contralateral to SNL in both saporin and D β H-saporin treated animals. We only examined fractional release in these experiments, and did not examine quantitative uptake or release.

BDNF content and glial markers in the lumbar spinal dorsal horn

In spinal dorsal horn in saporin treated animals, BDNF content bilaterally increased at 7 days after SNL surgery compared to normal animals (Fig. 5; $p < 0.05$), and then decreased to normal levels by day 14, consistent with our previous observation¹⁶. D β H-saporin treated

animals showed more BDNF content in both sides of spinal dorsal horn at 3 and 7 days after surgery than normal and saporin treated animals ($p < 0.05$). Based on this result, we chose a time point at 3 days after SNL to examine glial activation in the spinal dorsal horn after surgery. Figure 6 A–F depict GFAP (an astrocyte marker) and IBA1 (a microglial marker) immunoreactivity in the spinal dorsal horn in normal and SNL animals treated with saporin and D β H-saporin. At 3 days after SNL, saporin treated animals showed more GFAP and IBA1 immunoreactivity in the spinal cord ipsilateral to surgery compared to the normal (Fig. 6G and H; $p < 0.05$). In the spinal dorsal horn of D β H-saporin treated animals, GFAP immunoreactivity was higher bilaterally and IBA1 immunoreactivity was higher on the ipsilateral side compared to normal and saporin treated animals ($p < 0.05$).

Discussion

Bulbospinal noradrenergic fibers contain and release NA, ATP, and neuropeptide Y, factors which regulate neuronal and glial activation state and responses. The key finding of the current study is that presence of these fibers is not necessary for NA plasticity in analgesia after nerve injury and a suggestion that they may be important in braking glial activation. Spinal NA regulation of nociception has been studied for 3 decades, but many investigators consider it of minor importance in the pathophysiology of neuropathic pain. This conclusion is derived from the moderate effect of destruction of these noradrenergic pathways on hypersensitivity after nerve injury,²² (as reproduced in the current study) and from the lack of effect of intrathecally injected α 2-adrenoceptor antagonists on this hypersensitivity.^{14, 24} These observations are, however, weakened by the inability to see exacerbated hypersensitivity in an already hypersensitive state after NA destruction and by the assumption that NA release in the spinal cord produces an effect which can be immediately reversed with an antagonist. If NA release, as we postulate, alters glial and neuronal plasticity which require hours or days to achieve, one would not necessarily anticipate an acute inhibition of this effect within minutes of administration of a NA antagonist.

In the current study, intrathecal injection of D β H-saporin completely depleted noradrenergic axons in the spinal cord. We also observed partial reduction of noradrenergic neurons and axons in the noradrenergic nuclei (LC and A5) and paraventricular nucleus by this D β H-saporin treatment, consistent with previous observations^{22, 25}. These results indicate that intrathecal injection of D β H-saporin not only depletes spinal noradrenaline but also reduces supra-spinal noradrenergic innervation to some extent. Clearly, extensive supraspinal noradrenergic denervation has profound effects on various physiologic states, and such extensive denervation or silencing, by intracerebroventricular injection of D β H-saporin or intra-LC injection of lidocaine, reduces hypersensitivity after nerve injury². In contrast, more selective ablation of noradrenergic innervation to the spinal cord by intrathecal injection of D β H-saporin as in the current and previous studies²², results in a small increase rather than decrease in hypersensitivity in rats after spinal nerve ligation. This protective effect at a time of extreme hypersensitivity after nerve injury is consistent with laboratory and clinical observations that activation of LC or spinal noradrenergic pathway reduces hypersensitivity in rats and humans with chronic pain^{8, 9, 12–14, 29, 30}. Nonetheless, a clear limitation of this study is the partial reduction in supraspinal NA innervation induced by intrathecal D β H-saporin injected, which could have affected behavior and descending influences on glial and neuronal plasticity.

In rodents, activation of microglia and astrocytes in the spinal cord contributes to the development and maintenance of hypersensitivity after peripheral nerve injury³⁸. Consistent with behavioral observations, depletion of spinal NA in the current study did not affect immunohistochemical markers of activation of microglia and astrocytes in the normal spinal cord²², but increased expression of these markers after nerve injury. We recognize that

static examination of IBA-1 and GFAP do not adequately characterize activation state of glia or their anti- or pro-nociceptive or –inflammatory effects. Nonetheless, the approach used in the current study is widely applied to provide a rough gauge of glial activation. Previous studies demonstrated that α 2-adrenoceptor agonists inhibit activation of microglia and astrocytes in the spinal cord in rats after peripheral nerve injury or inflammation^{10,41}. We have recently demonstrated that peripheral nerve injury enhances spinal noradrenergic inhibition by increasing content and basal release of noradrenaline in the spinal dorsal horn^{14,16}. These results suggest that peripheral nerve injury enhances endogenous spinal noradrenergic tone which, over time, directly or indirectly modulates the degree of glial activation and hypersensitivity, although this degree of endogenous noradrenergic tone itself is not sufficient to relieve neuropathic pain. The current study cannot determine whether increased glial activation in noradrenaline-depleted animals reflects reduced inhibition of primary afferent release or a direct effect on glia.

The most likely sources of BDNF in the spinal cord after peripheral nerve injury include the terminals of primary afferents and microglia.^{4,11,16,34} In normal and injured spinal cord, astrocytes can also produce BDNF^{6,7,19}. We and others have shown that peripheral nerve injury increases BDNF content in the primary sensory neurons and the spinal dorsal horn^{11,16,26,34}. Previous studies demonstrated that depletion of noradrenaline itself does not alter BDNF content or expression in the brain^{18,40}, consistent with the lack of effect of noradrenaline depletion on glial activity in the brain in the absence of nerve injury²². Although the current study did not test whether NA depletion alters BDNF content in the spinal dorsal horn in the absence of injury, these previous observations in the brain suggest that NA depletion itself is unlikely to affect spinal BDNF content. In the current study, D β H-saporin treated SNL animals showed higher BDNF content in the spinal dorsal horn compared to saporin treated SNL animals, consistent with enhanced glial activation in D β H-saporin treated spinal dorsal horn. Although the contribution of primary sensory afferents to the increase in BDNF content in the spinal dorsal horn after nerve injury is possible, these results suggest that depletion of endogenous spinal noradrenaline induces more BDNF production in the spinal dorsal horn likely via enhancement of glial activation after nerve injury.

BDNF plays important roles in survival and axonal growth in cholinergic neurons and also shifts action of α 2-adrenoceptors with those neurons from Gi/o-protein mediated inhibition to Gs-protein-mediated facilitation of acetylcholine release¹⁵. Previous studies demonstrated that spinal infusion of BDNF or over-expression of BDNF by gene-transfer increases survival and axonal growth of cholinergic neurons in rats after spinal cord injury^{20,42}, and that intracerebroventricular infusion of BDNF prevents loss of cholinergic neurons in the brainstem after hypoglossal nerve transection³⁶. We have recently demonstrated that blockade of BDNF-trkB signaling by spinal infusion of BDNF antibody or repeated intrathecal injection of K252a reduced ChAT-immunoreactivity in the spinal dorsal horn and blocked the shift in α 2-adrenoceptor-mediated facilitatory effect on acetylcholine release in the spinal cord^{15,17}. The current study confirmed the α 2-adrenoceptor-mediated facilitation of acetylcholine release after nerve injury and further demonstrated that D β H-saporin treatment increased cholinergic immunoreactivity in the spinal dorsal horn, consistent with enhancement of BDNF up-regulation after nerve injury by D β H-saporin treatment. Interestingly, in the current study, spinal BDNF level in D β H-saporin treated animals was higher than the saporin treated and normal animals at 3–7 days and returned to normal level at 14 days after surgery, suggesting that initial enhancement of BDNF production is important to increase cholinergic immunoreactivity in the spinal dorsal horn. These results should, however, be qualified, since the methodology employed did not allow us to determine whether increased ChAT-immunoreactivity was associated with increased quantitative release of acetylcholine release, as only fractional release was studied.

As such, other interpretations of increased ChAT-immunoreactivity, such as increased antigen availability to the antibody without change in fiber density, are possible.

It may appear paradoxical that clonidine's behavioral effect was enhanced after nerve injury in animals receiving D β H-saporin, consistent with denervation supersensitivity or increased receptor expression, but α 2-adrenoceptor facilitated release of acetylcholine in synaptosomes was not increased. Previous studies showed that depletion of spinal noradrenaline increases α 2-adrenoceptor number in the spinal cord or affinity to clonidine in normal animals^{21, 32}, while peripheral nerve injury itself does not alter neither number nor affinity of α 2-adrenoceptors¹. Although the current study does not address whether expression or affinity of α 2-adrenoceptors alters in cholinergic neurons by D β H-saporin treatment, this plasticity in α 2-adrenoceptors by depletion of spinal noradrenaline may contribute to enhanced antihypersensitive effect of clonidine after nerve injury. As regards acetylcholine release, if one assumes that the increased ChAT immunostaining reflects increased capacity for acetylcholine release, then the same fractional release of acetylcholine release observed with dexmedetomidine in synaptosomal preparations after D β H-saporin as control would quantitatively release more acetylcholine. Neither the relationship between ChAT immunostaining and acetylcholine content nor the quantitative release of acetylcholine were examined in the current study.

In summary, depletion of spinal noradrenergic fibers does not prevent α 2-adrenoceptor analgesia and associated plasticity of its interaction with cholinergic terminals, suggesting that tonic activity of these fibers does not drive these plastic responses. NA fiber depletion is associated with increased expression of a markers of glial activation, consistent with acute pharmacologic studies demonstrated glial inhibition by NA receptor activation.

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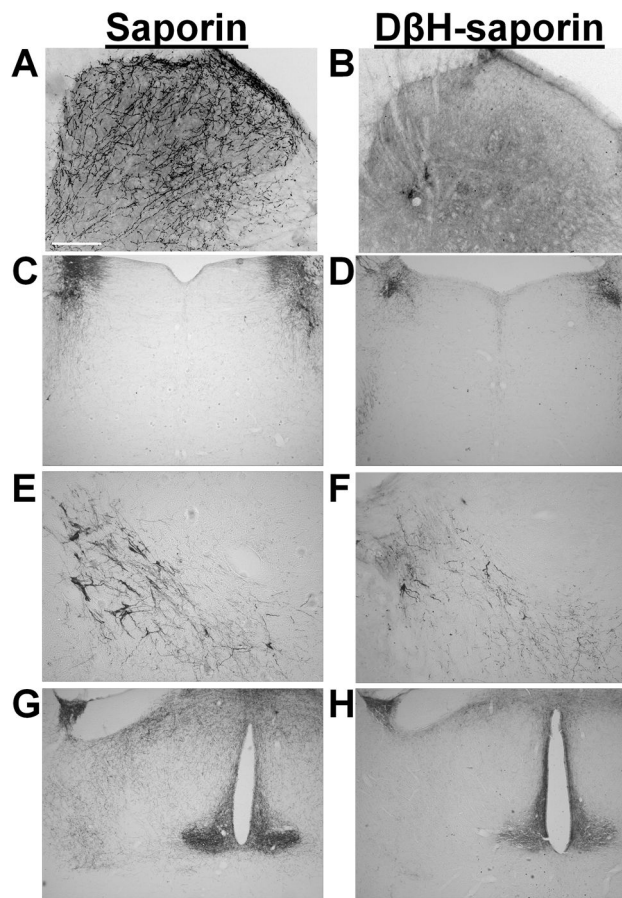


Figure 1. Photomicrographs of D β H immunoreactive axons and neurons in the ipsilateral spinal dorsal horn (A, B), locus coeruleus (C, D), A5 noradrenergic nucleus (E, F), and paraventricular nucleus (G, H) of saporin and D β H-saporin treated SNL rats at 14 days after surgery. Scale bar in A, B, E, and F = 100 μ m; in C, D, G, and H = 250 μ m.

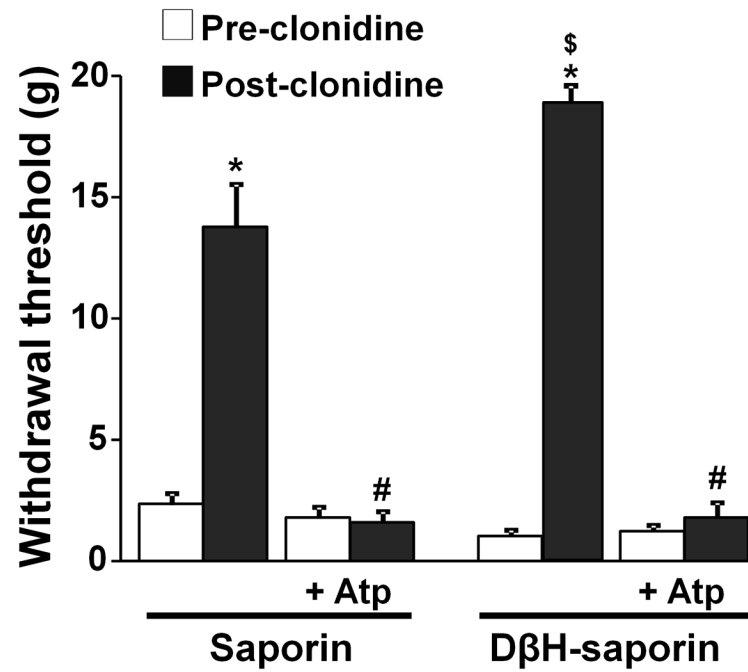


Figure 2. DβH-saporin treatment enhanced the antihypersensitivity effect of clonidine in SNL rats. Effect of intrathecal clonidine (15 μg) with or without atropine (30 μg) was tested in saporin and DβH-saporin treated SNL rats at 12 days after surgery (values are mean ± S.E of n=8 in each group). Post-clonidine withdrawal threshold values were measured 1 h following clonidine administration. *p<0.01 vs. pre-clonidine. #p<0.05 vs. clonidine alone. \$p<0.05 vs. saporin.

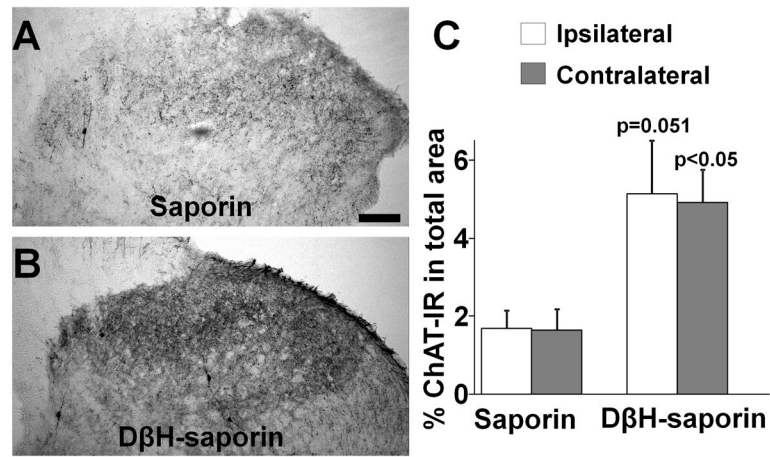


Figure 3.

DβH-saporin treatment increased ChAT immunoreactivity in the spinal dorsal horn 14 days following SNL. (A, B) Photomicrographs depict ChAT immunoreactivity in the ipsilateral spinal dorsal horn from saporin (A) and DβH-saporin treated (B) rats. Scale bar =100 μm. (C) Quantification of ChAT immunoreactivity in the ipsilateral and contralateral spinal dorsal horn from saporin and DβH-saporin treated rats (values are mean ± S.E of n=4 in each group).

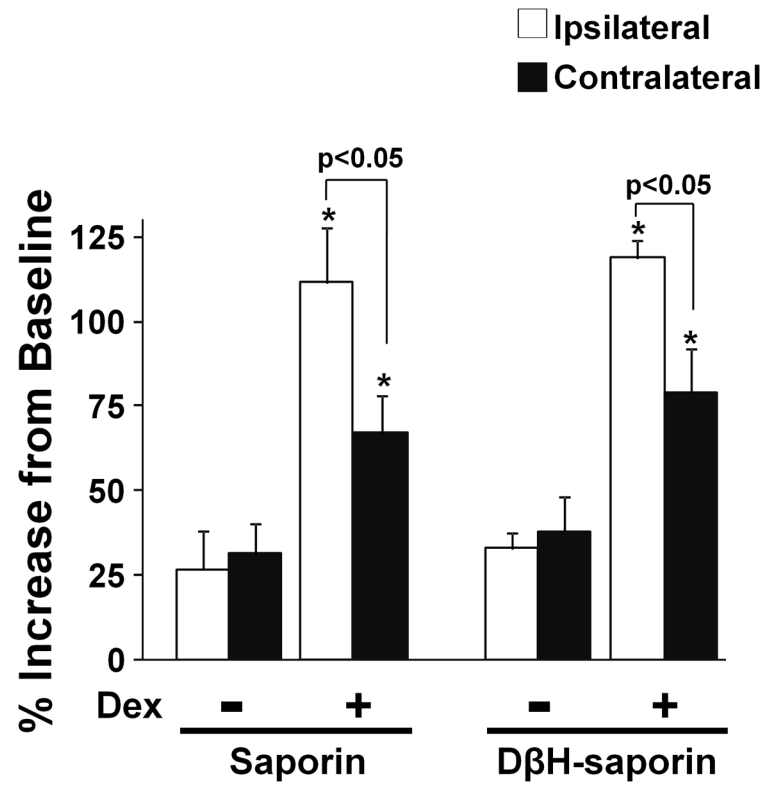


Figure 4.

DβH-saporin treatment did not alter the facilitatory effect of dexmedetomidine on KCl-evoked acetylcholine release in spinal dorsal horn synaptosomes from rats after SNL. SNL synaptosomes were prepared from saporin and DβH-saporin treated animals at 14 days after surgery, and treated with 12mM KCl or a combination of 12 mM KCl with 10 nM dexmedetomidine (Dex). Values are mean \pm S.E of n=6 in each group. *p<0.05 vs. KCl alone.

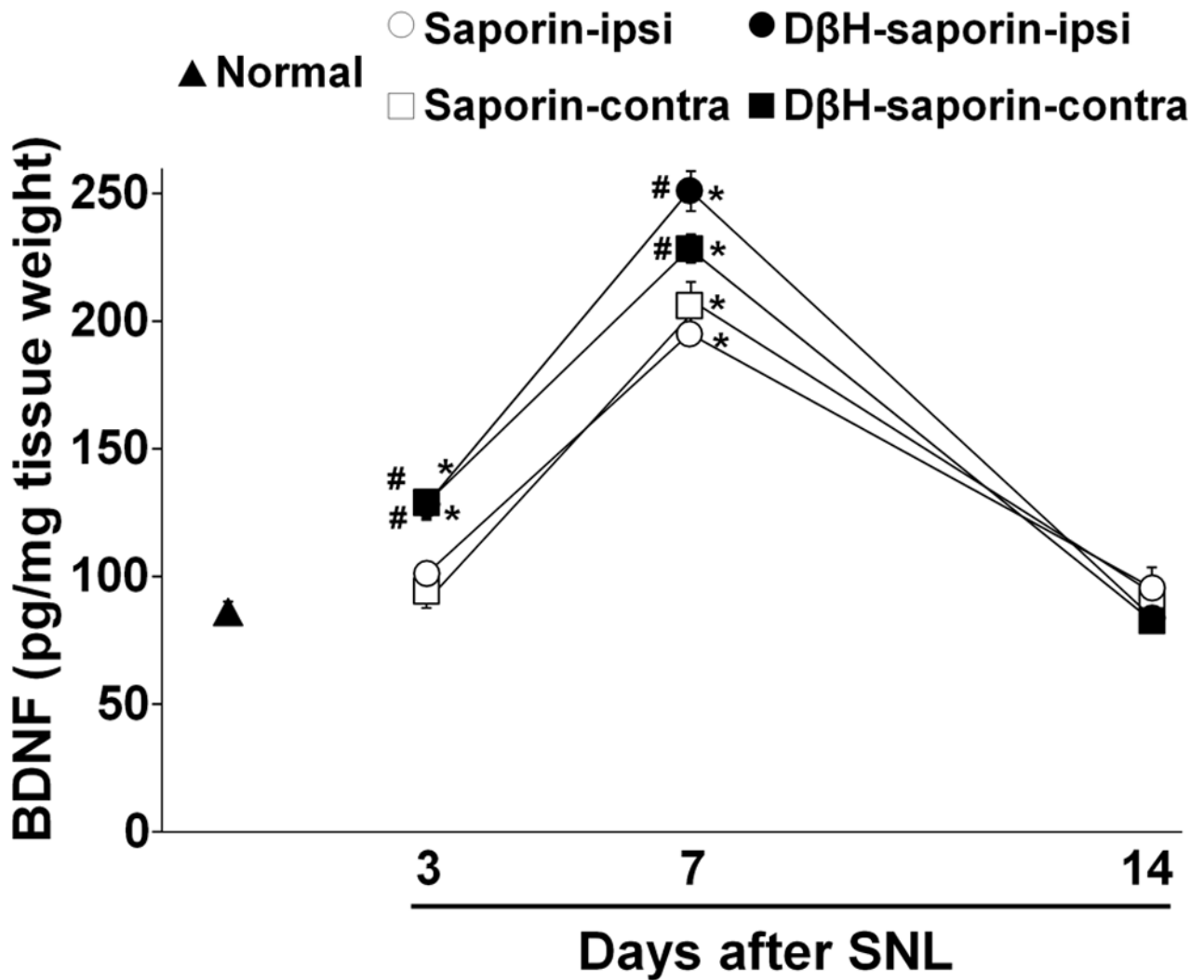


Figure 5. DβH-saporin treatment enhanced upregulation of BDNF in the spinal dorsal horn following SNL. BDNF content in L4–L6 level of ipsilateral (ipsi) and contralateral (contra) spinal dorsal horn was measured from normal, saporin treated SNL (3, 7, 14 days after surgery), and DβH-saporin treated SNL (3, 7, 14 days after surgery) animals. Values are mean ± S.E of n=7–9 in each group. *p<0.01 vs. normal. #p<0.05 vs. saporin.

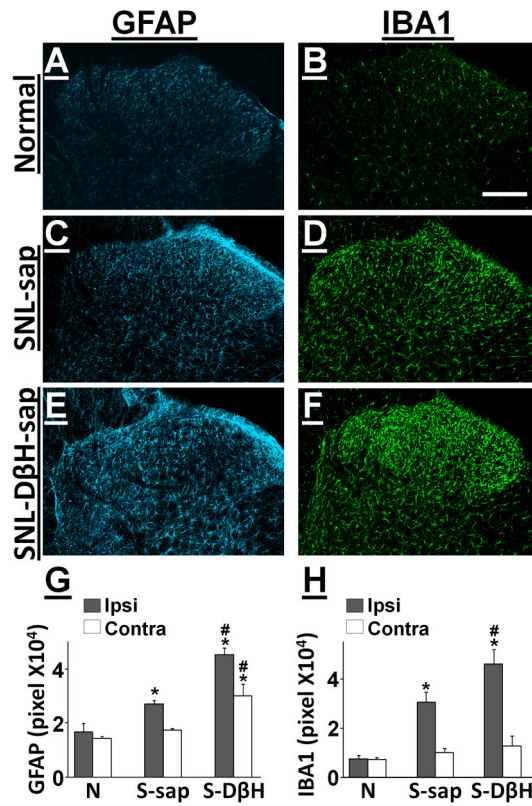


Figure 6. DβH-saporin treatment increased glial markers in the spinal dorsal horn at 3 days following SNL. Photomicrographs depict GFAP and IBA1 immunoreactivity in the ipsilateral spinal dorsal horn from normal (A, B), SNL-saporin (C, D), and SNL-DβH-saporin treated (E, F) rats. Scale bar =100 μm. Quantification of GFAP (G) and IBA1 (H) immunoreactivity in the ipsilateral and contralateral spinal dorsal horn from normal (N), SNL-saporin(S-sap), and SNL-DβH-saporin (S-DβH) treated rats. Values are mean ± S.E of n=4-6 in each group.*p<0.01 vs. normal. #p<0.05 vs. saporin.

Table 1Effect of D β H-saporin on hypersensitivity after SNL

| | Baseline | 7 days after injection | 12 days after SNL |
|----------------------------|----------------|------------------------|-------------------|
| Saporin (n=16) | 18.0 \pm 0.9 | 17.4 \pm 1.0 | 2.0 \pm 0.3* |
| D β H-saporin (n=16) | 18.0 \pm 0.9 | 16.3 \pm 1.0 | 1.1 \pm 0.2*# |

Mean values of right paw withdrawal threshold (g) \pm SE.

* p<0.01 vs baseline.

p<0.05 vs saporin.