Activation of notch signaling mediates the induction and maintenance of mechanical allodynia in a rat model of neuropathic pain

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Received June 3, 2014; Accepted February 5, 2015

DOI: 10.3892/mmr.2015.3379

Abstract. Neuropathic pain is a major health problem caused by dysfunction or damage of the nerve fibers in the peripheral or central nervous system. Notch signaling is a highly conserved evolutionary pathway, which regulates the fate of cells in the developing nervous system and is important in synaptic plasticity and inflammation in the adult central nervous system. The aim of the present study was to investigate the potential roles of the notch signaling pathway in the induction and maintenance of mechanical allodynia in neuropathic pain. Neuropathic pain was induced through spared nerve injury (SNI) in rats. DAPT, a γ-secretase inhibitor of the notch signaling pathway, was intrathecally administered at different concentrations (5, 15, 50 and 150 μM), and time-points. In addition, Jagged-1 (JAG-1) peptide, a ligand of the notch signaling pathway, was administered intrathecally to normal rats. The mechanical allodynia was assessed using a von Frey test. The results demonstrated that administering DAPT prior to the appearance of pain sensitivity significantly prevented the decrease of mechanical paw withdrawal threshold (PWT) for >4 weeks (P<0.05 vs. SNI group). Administering DAPT following the appearance of pain sensitivity significantly reversed the decrease of mechanical PWT (P<0.05 vs. SNI group). Furthermore, early and late administration of DAPT resulted in dose-dependent antinociceptive effects. In addition, administration of JAG-1 induced a dose-dependent increase in the mechanical PWT of the normal rats. In conclusion, the results of the present study demonstrated that activation of notch signaling contributed to the induction and maintenance of mechanical allodynia in neuropathic pain, suggesting a potential novel therapeutic target for the treatment of neuropathic pain.

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Abbreviations: CNS, central nervous system; DMSO, dimethyl sulfoxide; DRG, dorsal root ganglion; i.t, intrathecal; JAG-1, Jagged-1; PWT, paw withdrawal threshold; SC-JAG-1, scrambled Jagged-1; SNI, spared nerve injury

Key words: neuropathic pain, notch signaling pathway, mechanical allodynia

Introduction

Neuropathic pain is a chronic and complex type of pain (1), which occurs due to dysfunction or damage of the nerve fibers in the peripheral nervous system (PNS) or central nervous system (CNS) (1). Neuropathic pain is characterized by increased sensitivity to painful stimuli (hyperalgesia), the perception of innocuous stimuli as painful (allodynia) and spontaneous pain. These symptoms are associated with: Hyperexcitability in the affected dorsal root ganglion (DRG) neurons (1); atrophic changes and a switch in neurotransmitter phenotype in the central afferent terminals (2); aberrant myelination (splitting, detachment and loss of myelin) (3,4); alterations in synaptic plasticity (5), and excitatory and inhibitory mechanisms in the spinal cord (6); loss of inhibitory interneurons and modifications of the brain stem input to the spinal cord (7). These changes mainly result from *de novo* gene transcription, post-translational modifications (8), alterations in ion channel conductivity and receptor function (9,10), neuroimmune interactions (11) and neuronal apoptosis (12). Treatments for neuropathic pain have improved, however, patients are often unresponsive to the entire range of therapeutic modalities, as the key mechanisms controlling the induction and maintenance of neuropathic pain remain to be elucidated (13).

Notch signaling is a highly conserved pathway in evolution, which regulates the determination of cell-fate during nervous system development (14,15), and is important in

synaptic plasticity (16) in the adult CNS. Numerous studies have demonstrated that the notch signaling pathway is crucial for several biological processes, including development (16), immunology (17), inflammation (18), vasculogenesis (19), tumor formation (20) and memory (21). Previous findings have suggested that the activation of notch signaling contributes to neuronal death (22,23), microglial cell (24) and astrocyte generation and activation (25), neurite growth inhibition (26), increased dendritic branching (26), oligodendrocyte progenitor cell differentiation (27) and demyelination (28) in the PNS and CNS (14). In addition, the notch signaling pathway controls the determination between excitatory and inhibitory cell-fates in the developing spinal cord, and activation of the notch signaling pathway promotes the generation of excitatory neurons from the sensory interneuron progenitors (29). Furthermore, the expression and activity levels of the notch signaling pathway are significantly increased following nerve injury (30).

Therefore, it was hypothesized that activation of notch signaling may contribute to the induction and maintenance of neuropathic pain. The present study aimed to investigate the effects of the γ-secretase notch signaling inhibitor, DAPT, administered at different time-points and concentrations, on mechanical allodynia in a rat model of spared nerve injury (SNI)-induced neuropathic pain. In addition, the present study examined the effects of the notch signaling activator peptide, Jagged-1 (JAG-1), on mechanical allodynia in normal rats. The results of these investigations may provide a novel therapeutic target for the treatment of neuropathic pain.

Materials and methods

Animals. All experiments were performed on 180 (6 per group) adult male Sprague-Dawley rats, weighing 200-250 g (Laboratory Animal Center of the Fourth Military Medical University, Xi'an, China). The animals were housed in plastic boxes at 22-26˚C with access to food and water *ad libitum*. A 12-h light/dark cycle, with lights on at 08:00, was maintained, and assessments were performed between 09:00 and 18:00. Prior to experimental manipulation, the animals were allowed to acclimate to the housing facilities and were handled daily for at least 3 days. All experimental and animal handling procedures were approved by the Institutional Animal Care and Use Committee of General Hospital of Tianjin Medical University (Tianjin, China), and were performed in accordance with the guidelines for the ethical treatment of animals established by the International Association for the Study of Pain. All efforts were made to minimize animal suffering and to reduce the number of animals used. The study was approved by the ethics committee of General Hospital of Tianjin Medical University (Tianjin, China).

Intrathecal (i.t.) catheterization and drug delivery. A permanent i.t. catheter (PE-10 polyethylene tube; BD Biosciences, Franklin Lakes, NJ, USA) was inserted into each animal through the space between the T_3 and T_4 vertebrae and extended slowly into the subarachnoid space of the lumbar enlargement (L_4 and L_5) following intraperitoneal administration of pentobarbital sodium anesthesia (40 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) (31). The catheter was filled with sterile saline (~4 μl; Otsuka Pharmaceutical Co., Ltd., Tianjin, China) and the outer end was plugged. The cephalic portion of the catheter was externalized through the dorsal skin, where it was relatively inaccessible to the paws of the animal. All the animals appeared to be free of infection on gross inspection. The cannulated animals were housed separately, then allowed to recover for 4 days. Motoric integrity was assessed in all animals using a righting reflex and inclined plane test (32). Animals exhibiting any neurological deficits (~4.4% rats) were excluded from the subsequent experiments.

Subsequently, drugs were injected over a period of 1 min via the catheter at a volume of 10 μl, followed by 5 μl sterile saline for flushing. DAPT (Sigma-Aldrich, St. Louis, MO, USA) and JAG-1 peptide (CDDYYYGFGCNKFCRPR; AnaSpec, Inc., San Jose, CA, USA), as a potent inhibitor and activator of notch signaling pathway, respectively, were freshly dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) (23) at a concentration of DAPT (5, 15, 50 or 150 μM) and at a concentration of JAG-1 peptide (1, 10 or 100 μM). Drugs were injected over a period of 1 min via the catheter at a volume of 10 µl.. The DMSO or scrambled peptide (SC)-JAG-1 (RCGPDCFDNYGRYKYCF; AnaSpec, Inc.) were used as a control. The location of the distal end of this catheter was verified at the end of each experiment by injection of pontamine sky blue (Sigma-Aldrich) into the catheter. The location of the distal end of this catheter was verified at the end of each experiment by injection of 2% pontamine sky blue solution (1 μl) via the i.t. catheter.

Spared nerve injury (SNI) model. The neuropathic pain was induced using a left SNI model, as previously described (33). In brief, under 1% pentobarbital sodium anesthesia (40 mg/kg, i.p.), an incision was made on the lateral thigh and the underlying muscle was separated to expose the sciatic nerve. The three terminal branches of the sciatic nerve (tibial, common peroneal and sural nerves) were then carefully separated. Following separation, the tibial and common peroneal nerves were tightly ligated with 5.0 silk (Ethicon, Somerville, NJ, USA), and 2-3 mm of the nerves distal to the ligation was removed. The muscle and skin incisions were then closed separately. A sham group was included, in which identical surgery was performed, without the passage of ligatures or transection of the nerves.

Assessment of mechanical allodynia. Mechanical allodynia was assessed using a von Frey test, as previous described (34). In brief, the mechanical allodynia of rats was determined by measuring the paw withdrawal threshold (PWT) of the rats in response to mechanical stimuli, produced by a calibrated series of von Frey filaments (Stoelting Co., Chicago, IL, USA). The rats were adapted to the assessment situation for at least 30 min prior to the initiation of stimulation. During the assessment, the rats were placed on a metal mesh floor, covered with the same plastic box used for housing, and von Frey filaments were applied from underneath the metal mesh floor to the lateral plantar surface of the paw, which is the area innervated by the sural nerve. Each filament was presented perpendicularly against the paw, with sufficient force to cause slight bending, and held for 2-3 sec. The filament was applied only when the rats were stationary and standing on all four paws. A withdrawal response was considered valid only if the hindpaw was completely removed from the customized platform; lifting of the paw due to normal locomotor behavior was ignored. The monofilaments were applied with

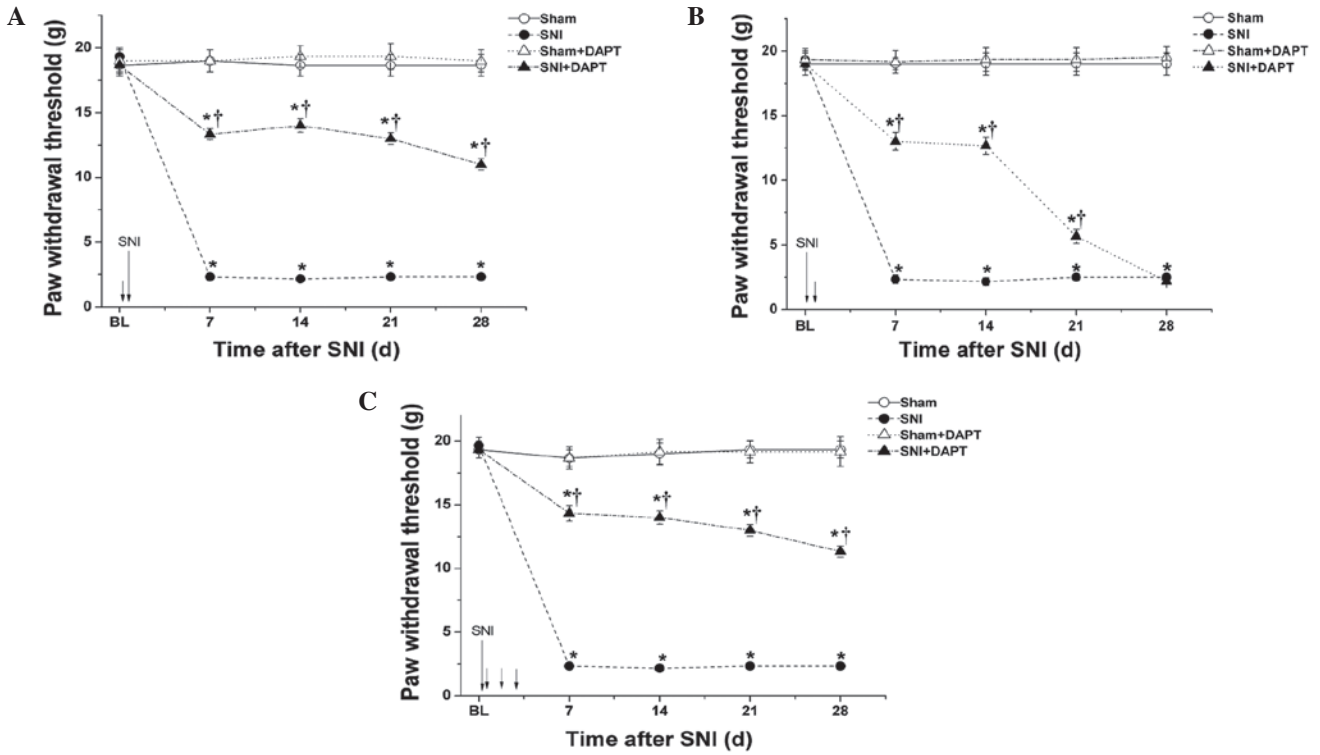

Figure 1. DAPT, a notch signaling pathway inhibitor, administered prior to appearance of pain sensitivity prevents the development of mechanical allodynia in SNI-induced neuropathic pain. (A) Single administration of DAPT (50 μM;10 μl) 0.5 h prior to SNI surgery. (B) Single administration of DAPT (50 μM; 10 μl) 0.5 h following SNI surgery. (C) Once-daily administration of DAPT (50 μM; 10 μl) for three consecutive days, beginning 0.5 h after SNI surgery. The mechanical paw withdrawal threshold was measured at 24 h prior to (BL) and 7, 14, 21 and 28 days following SNI or sham surgery. The short arrows indicate points of DAPT administration. Data are presented as the mean ± standard error of the mean (n=6 per group). *P<0.05, vs. Sham group; †P<0.05, vs. SNI group. SNI, spared nerve injury; BL, baseline; d, days.

increasing force until the rat withdrew the paw. Each filament was applied 10 times at 5 sec intervals. The bending force value of the von Frey filament, which led to a paw withdrawal reflex at an occurrence of 50% on stimulation 10 times was expressed as the PWT. Following determination of the threshold for the left hindpaw, the same procedure was repeated on the right hindpaw at 5 min intervals. The assessment trial was run a second and third time for the two hindpaws and, if the PWT in the second or third trials did not match that of the previous trial in a given hindpaw, the next large filament in the series was used. This was repeated until the PWT in the three successive trials matched. In order to avoid inter-experimental differences and subjective bias, all behavioral observations were observed by one assessor, in a blinded-manner.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Statistical comparisons between the groups were performed using univariate or repeated measures analysis of variance. All statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Administration of DAPT prior to appearance of pain sensitivity prevents the development of mechanical allodynia in SNI-induced neuropathic pain. The effects of pretreatment with DAPT, a notch signaling pathway inhibitor, on mechanical allodynia were investigated in a rat model of SNI-induced neuropathic pain. Mechanical allodynia in all animals was measured 24 h prior to SNI surgery (baseline) and 7, 14, 21 and 28 days following SNI or sham surgery. The results demonstrated that the SNI-induced animals developed a marked hypersensitivity to innocuous mechanical stimulation of the lateral surface of the hindpaw (sural nerve skin area) compared with the sham group (Fig. 1). The hindpaw contralateral to the surgery was assessed over the entire period, and no statistically significant changes in mechanical PWT were observed from the baseline (data not shown). In addition, the mechanical PWT between 7 and 28 days after SNI decreased significantly compared with the corresponding time-point in the sham group (P<0.05; Fig. 1). A single administration of DAPT (50 μM; 10 μl) 0.5 h prior to SNI surgery significantly improved the mechanical PWT for >28 days following surgery, compared with the SNI group (P<0.05;Fig. 1A). In addition, a single administration of DAPT (50 μM; 10 μl) 0.5 h following SNI surgery significantly improved the mechanical PWT for ~21 days following surgery, compared with the SNI group (P<0.05;Fig. 1B). Furthermore, a once-daily administration of DAPT (50 μM; 10 μl) for three consecutive days, beginning 0.5 h following SNI surgery, significantly improved the mechanical PWT for >28 days following surgery compared with the SNI group (P<0.05; Fig. 1C). These results suggested that early inhibition of the notch signaling pathway may prevent the induction of mechanical allodynia in neuropathic pain.

Administration of DAPT following the appearance of pain sensitivity significantly reverses the mechanical allodynia in

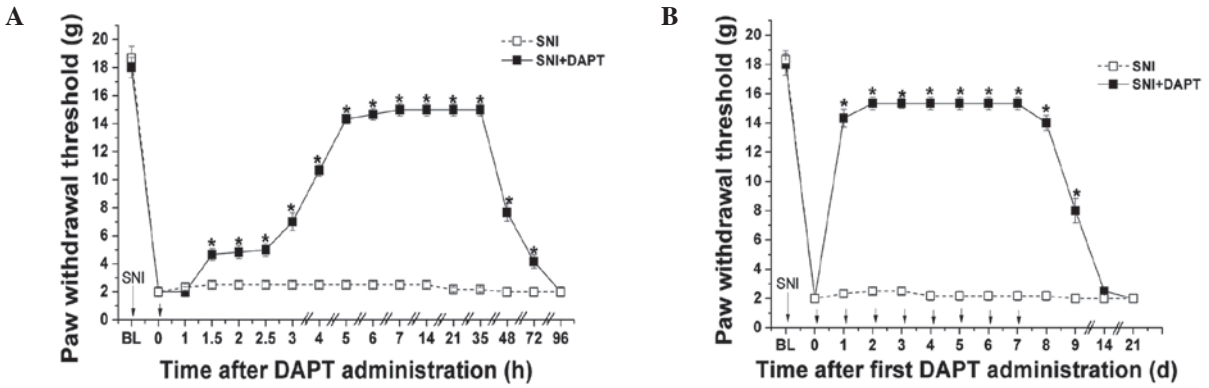

Figure 2. Administration of DAPT following observation of pain sensitivity significantly reverses mechanical allodynia in SNI-induced neuropathic pain. (A) DAPT (50 μM; 10 μl) or DMSO were administrated once following the appearance of SNI-induced mechanical allodynia. Mechanical PWT was measured prior to SNI surgery (BL), prior to administration (0 h) and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 14, 21, 35, 48, 72 and 96 h following DAPT or DMSO administration. (B) DAPT (50 μM; 10 μl) or DMSO were administrated once a day for seven consecutive days following the appearance of SNI-induced mechanical allodynia. Mechanical PWT was measured prior to SNI surgery, prior to administration (0 h) and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14 and 21 days following DAPT or DMSO administration. Short arrows indicate points of administration. Data are presented as the mean ± standard error of the mean (n=6 per group). *P<0.05, vs. SNI group. SNI, spared nerve injury; PWT, paw withdrawal threshold; DMSO, dimethyl sulfoxide; BL, baseline; d, days.

SNI-induced neuropathic pain. The effects of post-treatment administration of the notch signaling pathway inhibitor, DAPT on mechanical allodynia in SNI-induced neuropathic pain were examined. In a preliminary experiment, the animals developed significant mechanical allodynia 3 days after SNI surgery (data not shown). A single administration of DAPT (50 μM; 10 μl) following the appearance of pain sensitivity significantly increased the mechanical PWT between 1.5 and 72 h after DAPT administration compared with the SNI group (P<0.05; Fig. 2A). In addition, a once-daily administration of DAPT (50 μM; 10 μl) following the appearance of pain sensitivity for 7 days consecutively significantly increased the mechanical PWT compared with the SNI group (P<0.05); however, the duration of its antinociceptive action was only ~3 days following the final DAPT administration (Fig. 2B). These results suggested that late inhibition of the notch signaling pathway may reverse the mechanical allodynia of neuropathic pain.

Administration of DAPT dose-dependently attenuates mechanical allodynia in SNI-induced neuropathic pain. The antinociceptive effects of different concetrations of DAPT on mechanical allodynia were determined in SNI-induced neuropathic pain. Different doses of DAPT (5, 15, 50 and 150 μM) were administered 0.5 h prior to SNI surgery, and the mechanical PWT was evaluated 7 days after surgery. As shown in Fig. 3A, administration of DAPT prior to SNI surgery dose-dependently increased the mechanical PWT of the rats (P<0.05). Furthermore, different concentrations of DAPT (5, 15, 50 and 150 μM) were administered following the appearance of pain sensitivity, and the mechanical PWT was evaluated 24 h after DAPT administration. As shown in Fig. 3B, administration of DAPT following the appearance of pain sensitivity dose-dependently increased the mechanical PWT of the rats (P<0.05). These results indicated that inhibition of the notch signaling pathway prevented and reversed the mechanical allodynia of neuropathic pain in a dose-dependent manner.

Administration of JAG-1 peptide, a ligand of the notch signaling pathway, dose-dependently induces neuropathic pain-like behavior in normal rats. In order to further investigate the roles of the notch signaling pathway in neuropathic pain, normal rats were administered with different concentrations of the JAG-1 peptide (1, 10 and 100 μM), and the mechanical PWT was measured 4, 12 and 24 h after treatment. SC-JAG-1 peptide was administered as a negative control. As shown in Fig. 4, a single administration of the JAG-1 peptide dose-dependently decreased the mechanical PWT of the normal animals compared with the SC-JAG-1 peptide group (P<0.05). In addition, the administration of DAPT had no effect on the mechanical PWT of normal rats (data not shown). These results further suggested that activation of the notch signaling pathway contributed to the induction and maintenance of mechanical allodynia in neuropathic pain.

Discussion

In the present study, it was found that early inhibition of the notch signaling pathway prior to the appearance of pain sensitivity prevented the induction of mechanical allodynia in a rat model of SNI-induced neuropathic pain. In addition, late inhibition of the notch signaling pathway following appearance of pain sensitivity reversed the mechanical allodynia of neuropathic pain. Early and late inhibition of the notch signaling pathway produced antinociceptive effects in a dose-dependent manner. Furthermore, activation of the notch signaling pathway dose-dependently induced mechanical allodynia in normal animals. Therefore, the activation of notch signaling contributed to the induction and maintenance of neuropathic pain.

Chronic neuropathic pain can result from tissue damage, inflammation or injury of nervous system, and symptoms include hyperalgesia, allodynia and spontaneous pain (2,13). It is well established that mechanical allodynia is characteristic of neuropathic pain (13). The SNI model has proven to be robust, with substantial and prolonged changes in mechanical sensitivity and thermal responsiveness that resemble those of clinical neuropathic pain (33,34). Neuropathic pain affects areas innervated by the sural nerve and, to a lesser extent, the saphenous nerve; however, the contralateral hindpaw is unaffected (33,34). The SNI model also exhibits marked hypersensitivity to

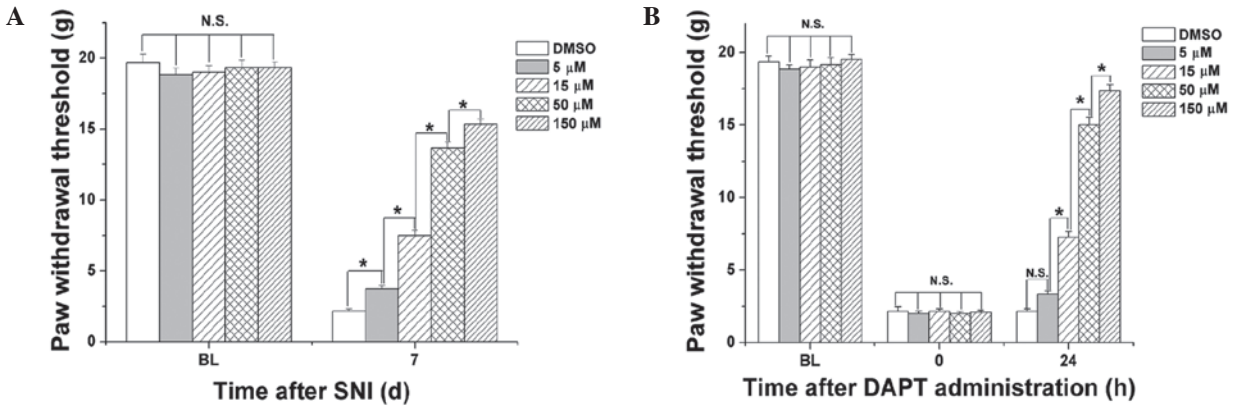

Figure 3. Administration of DAPT dose-dependently attenuates mechanical allodynia in SNI-induced neuropathic pain. (A) Various doses of DAPT (5, 15, 50 and 150 μM) or DMSO were administrated 0.5 h prior to SNI surgery. Mechanical PWT was measured prior to (BL) and 7 days after SNI surgery. (B) Different doses of DAPT (5, 15, 50 and 150 μM) or DMSO were administred following the appearance of mechanical allodynia. Mechanical PWT was measured prior to SNI surgery (BL), prior to administration (0 h) and 24 h following DAPT or DMSO administration. Data are presented as the mean ± standard error of the mean (n=6 per group). *P<0.05; N.S, no significant differences. SNI, spared nerve injury; PWT, paw withdrawal threshold; DMSO, dimethyl sulfoxide; BL, baseline; d, days; N.S. not significant.

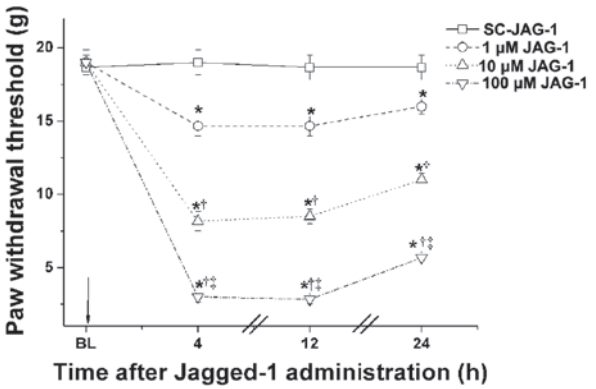

Figure 4. Administration of JAG-1 peptide dose-dependently induces neuropathic pain-like behavior in normal rats. Different doses of JAG-1 peptide (1, 10 and 100 μM) or SC-JAG-1 were administered into normal rats. The mechanical paw withdrawal threshold was measured prior to (BL) and at 4, 12 and 24 h following JAG-1 or SC-JAG-1 administration. Data are presented as the mean ± standard error of the mean (n=6 per group). *P<0.05, vs. SC-JAG-1 group; †P<0.05, vs. 1 μM JAG-1 group; ‡P<0.05, vs. 10 μM JAG-1 group. JAG-1, Jagged-1; SC-JAG-1, scrambled control Jagged-1 peptide; BL: baseline.

normally innocuous mechanical stimuli (33), and may assist in elucidating the mechanisms underlying the development of neuropathic pain and be used to screen for the efficacy of novel therapeutic agents. In the present study, all the animals developed significant mechanical allodynia following the induction of SNI neuropathic pain, which was consistent with previous studies (33,34).

Numerous mechanisms have been investigated, which may be involved in the pathogenesis of neuropathic pain. Several mechanisms at various sites may operate independently, in combination or at different time-points, which result in the onset of the characteristic symptoms of neuropathic pain (13). These mechanisms may include changes in terminal and peripheral sensitization (1); phenotypic switches and excitability of injured axons; collateral sprouting in the periphery or central terminal sprouting (2); hyperexcitability in the affected DRG neurons (1); splitting, detachment and loss of myelin (3,4); synaptic plasticity in the spinal cord (5,6); loss of inhibitory interneurons (7); and modifications of brain stem input to the spinal cord (7). In addition, excitatory and inhibitory interneurons integrate and transduce sensory information from the periphery in the spinal dorsal horn, therefore, alterations in the number or transmission properties of these interneurons are considered to be major contributors to the development of chronic sensory neuropathies, including hyperalgesia and allodynia (35,36). These changes result predominantly from *de novo* gene transcription (8), post-translational modifications (8), alterations in ion channel conductivity and receptor function (9,10), neuroimmune interactions (11) and neuronal apoptosis (12). However, the primary mechanisms involved in the induction and maintenance of neuropathic pain remain to be elucidated.

Notch signaling is an evolutionarily conserved pathway, which is essential for numerous biological processes, including development (16), immunology (17), inflammation (18), vasculogenesis (19), tumor formation (20), and learning and memory (21). Notch is a cell-surface receptor, which is involved in the regulation of cell-fate decisions during nervous system development (14,15), and is essential for synaptic plasticity (16) in the adult CNS. Proteolytic cleavage of the notch extracellular and transmembrane domains is mediated by the binding of ligands, including Delta and Jagged (17,18). The latter cleavage is induced by the γ-secretase enzyme complex, resulting in the release of a notch intracellular domain, which translocates into the nucleus and regulates transcription (17,18). All the components of the notch signaling pathway, including ligands, receptors and enzymes involved in notch receptor cleavage, are expressed in the adult CNS, and are significantly increased following nerve injury, suggesting that they are involved in its repair (16,30). Previous findings have suggested that the activation of notch signaling may contribute to neuronal death (22,23), microglial cell (24) and astrocyte generation and activation (25), neurite growth inhibition (26), increased dendritic branching (26), oligodendrocyte progenitor cell differentiation (27) and demyelination (28) in the PNS and CNS (14). In addition, the notch signaling pathway regulates the excitatory or inhibitory cell-fate decision in the developing spinal cord; therefore, activation of the notch signaling pathway was reported to promote the generation

of excitatory neurons from the sensory interneuron progenitors (29). Therefore, notch signaling activation may contribute to the generation and maintenance of neuropathic pain.

In order to investigate the roles of the notch signaling pathway in neuropathic pain, a γ-secretase enzyme (a key enzyme of notch signaling pathway) inhibitor, DAPT, was administered prior to or following the appearance of pain sensitivity in SNI-induced neuropathic pain model rats. The results revealed that early or late inhibition of notch signaling prevented or reversed mechanical allodynia in rats with neuropathic pain, in a dose-dependent manner. In addition, administration of JAG-1 peptide, a ligand of notch signaling pathway, induced mechanical allodynia in normal rats, in a dose-dependent manner. These results indicated that activation of notch signaling may contribute to the induction and maintenance of neuropathic pain.

However, the roles of the notch signaling pathway in thermal or cold allodynia of neuropathic pain, and the involvement of notch signaling in inflammatory pain remain to be elucidated. Furthermore, the mechanisms underlying notch signaling in neuropathic pain require further investigation.

In conclusion, the results of the present study demonstrated that early and late inhibition of the notch signaling pathway led to the prevention and reversal of mechanical allodynia in neuropathic pain, respectively; activation of notch signaling induced mechanical allodynia in normal animals; and inhibition of notch signaling produced an antinociceptive effect, in a dose-dependent manner. These results suggest a novel therapeutic target for the treatment of patients with neuropathic pain.

Acknowledgements

This study was supported by research grants from the National Natural Science Foundation of China (no. 30872434 to Dr Yanyan Sun; and no. 30972847 to Dr Guolin Wang) and the Natural Science Foundation of Tianjin (no. 08JCYBJC08000 to Dr Guolin Wang).

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