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Ca<sup>2+</sup> channel  $\alpha 2\delta 1$  protein in spinal neuron sensitization

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## Electrophysiological characterization of spinal neuron sensitization by elevated calcium channel alpha-2-delta-1 subunit protein

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### Abstract

**Background**—Voltage-gated calcium channel  $\alpha_2\delta_1$  subunit is the binding site for gabapentin, an effective drug in controlling neuropathic pain states including thermal hyperalgesia. Hyperalgesia to noxious thermal stimuli in both spinal-nerve-ligated (SNL) and voltage-gated calcium channel  $\alpha_2\delta_1$  over-expressing transgenic (Tg) mice correlates with higher  $\alpha_2\delta_1$  levels in dorsal root ganglia and dorsal spinal cord. In this study, we investigated whether abnormal synaptic transmission is responsible for thermal hyperalgesia induced by elevated  $\alpha_2\delta_1$  expression in these models.

**Methods**—Behavioral sensitivities to thermal stimuli were test in L4 SNL and sham mice, as well as in  $\alpha_2\delta_1$  Tg and wild-type mice. Miniature excitatory (mEPSC) and inhibitory (mIPSC) postsynaptic currents were recorded in superficial dorsal spinal cord neurons from these models using whole-cell patch clamp slice recording techniques.

**Results**—The frequency, but not amplitude, of mEPSC in superficial dorsal horn neurons was increased in SNL and  $\alpha_2\delta_1$  Tg mice, which could be attenuated by gabapentin dose dependently. Intrathecal  $\alpha_2\delta_1$  antisense oligodeoxynucleotide treatment diminished increased mEPSC frequency and gabapentin's inhibitory effects in elevated mEPSC frequency in the SNL mice. In contrast, neither the frequency, nor the amplitude, of mIPSC was altered in superficial dorsal horn neurons from the SNL and  $\alpha_2\delta_1$  Tg mice.

**Conclusions**—Our findings support a role of peripheral nerve injury-induced  $\alpha_2\delta_1$  in enhancing presynaptic excitatory input onto superficial dorsal spinal cord neurons that contributes to nociception development.

## 1. Introduction

Neuropathic pain syndromes, including hypersensitivity to noxious stimuli (hyperalgesia), dramatically reduce the life quality of patients (Woolf & Mannion, 1999; Zimmermann, 2001; Costigan *et al.*, 2009; Baron *et al.*, 2010). Some ligands for the  $\alpha_2\delta_1$  subunit of voltage-gated calcium channels (VGCC), such as gabapentin (GBP) and pregabalin (Gee *et al.*, 1996; Lynch *et al.*, 2006), are among limited drugs that are effective in pain relief in a subpopulation of neuropathic pain patients (Field *et al.*, 2007), suggesting that  $\alpha_2\delta_1$  proteins are critical in neuropathic pain processing.

$\alpha_2\delta$  subunits are auxiliary subunits of VGCC (Tanabe *et al.*, 1987; Yaksh, 2006; Park & Luo, 2010), which include at least four distinctive genes,  $\alpha_2\delta_{1-4}$  (Klugbauer *et al.*, 1999).  $\alpha_2\delta_1$  is the most abundant subtype in the spinal cord and dorsal root ganglia (DRG) (Marais *et al.*, 2001; Cole *et al.*, 2005). Co-expression of  $\alpha_2\delta_1$  with  $\alpha_1$  and  $\beta$  VGCC subunits *in vitro* results in increased calcium current densities, accelerated activation and inactivation kinetics, and hyperpolarized activation threshold (Klugbauer *et al.*, 2003). Similar effects were also found in DRG neurons from transgenic mice (Tg) over-expressing  $\alpha_2\delta_1$  in neuronal cells (Li *et al.*, 2006).

Accumulating evidence shows that  $\alpha_2\delta_1$  proteins contribute to both induction and maintenance of nerve injury-induced neuropathic pain states through a presynaptic mechanism. For instance,  $\alpha_2\delta_1$  proteins are highly upregulated in DRG after spinal nerve ligation (SNL) injury, then translocated to primary afferent presynaptic terminals in dorsal spinal cord (Li *et al.*, 2004; Bauer *et al.*, 2009). This correlates with neuropathic pain states sensitive to intrathecal treatment with  $\alpha_2\delta_1$  antisense oligodeoxynucleotides, or GBP (Luo *et al.*, 2002; Li *et al.*, 2004; Bauer *et al.*, 2009). Since  $\alpha_2\delta_1$  Tg mice also show hyperalgesia with a similar pharmacology profile as that in the SNL model (Chaplan *et al.*, 1997; Luo *et al.*, 2002; Li *et al.*, 2006; Nguyen *et al.*, 2009), it is highly likely that increased  $\alpha_2\delta_1$  expression may mediate behavioral hypersensitivities through a similar presynaptic mechanism in both models. However, this hypothesis has not been tested thoroughly.

Peripheral nerve injuries have been shown in some studies to change excitatory (Millan, 1999; Chen *et al.*, 2009; Sandkuhler, 2009) or inhibitory (Moore *et al.*, 2002b; Scholz *et al.*, 2005) synaptic transmission in superficial dorsal horn (SDH) where projection neurons receive periphery nociceptive inputs mainly from myelinated A $\delta$  and non-myelinated C fibers as well as modulation from interneurons (Usunoff *et al.*, 2006; D'Mello & Dickenson, 2008). However, it is not known whether these changes are mediated by dysregulated  $\alpha_2\delta_1$  since nerve injuries also cause dysregulation of other factors (Wang *et al.*, 2002; Valder *et al.*, 2003; Kim *et al.*, 2009). In this study, we examined if increased  $\alpha_2\delta_1$  expression alone in the Tg mice (Li *et al.*, 2006) was sufficient to induce similar changes in miniature excitatory (mEPSC) or inhibitory (mIPSC) postsynaptic currents in superficial dorsal horn neurons as that in the SNL model (Kim & Chung, 1992).

## 2. Methods and Materials

### 2.1 Animals

Male adult 129sv mice (6-8 weeks) were purchased from Charles River laboratories, Inc. (Wilmington, MA). Male, age-matched (8-12 weeks)  $\alpha_2\delta_1$  Tg and their wild-type (WT) littermates had been backcrossed over 10 generations to the 129sv background before the experiments. All animal care and experiments were performed according to protocols approved by the Institutional Animal Care Committees of the University of California, Irvine.

### 2.2 Spinal nerve ligation surgery

Unilateral SNL was performed as described by Kim and Chung (1992). Briefly, under deep isoflurane anesthesia (5% for induction, and 2% for maintenance), the mouse left L4 spinal nerve, which is equivalent anatomically to L5 spinal nerve in rat (Rigaud et al., 2008), was exposed and ligated tightly with a 6.0 silk suture. We performed sham ligation by exposing the left L4 spinal nerve and loosely winding a suture around the nerve without ligation.

### 2.3 Cold test

Each mouse was acclimatized in a test compartment with a mesh floor for about 30 min. Acetone (50  $\mu$ L) was gently applied onto the plantar surface of the hindpaw using a pipette. The foot withdrawal responses were graded to a four-point scale: 0, no response; 1, brisk withdrawal or flick of the paw; 2, repeated flicking of the paw; 3, repeated flicking and licking of the paw (Choi et al., 1994). The test was repeated 3 times separated approximately by a 5-min interval on each mouse. Data were averaged for analysis.

### 2.4 Hargreaves test

Thermal paw withdrawal latencies were measured using a Hargreaves apparatus (Hargreaves et al., 1988). Briefly, each mouse was acclimatized in a test compartment on a glass surface maintained at 30 °C for at least 30 min. A radiant heat light source was positioned over the plantar surface of the hindpaw. Paw withdraw latency was recorded automatically as the duration between the time when the light was turned on and when the animals removed the paw from the thermal stimulation. The cut-off time was set to 20 sec to limit the risk of tissue damage. Three test results were recorded to calculate the average paw withdrawal latency for each mouse.

All behavioral tests were performed in a blind manner until the examiners needed to decode the data for analysis. For the SNL model, data from the injury side were compared between the SNL and sham mice. For the transgenic mouse model, data from both hindpaws were averaged, and compared between the WT and  $\alpha_2\delta_1$  Tg mice.

### 2.5 Intrathecal antisense oligodeoxynucleotides treatment

Mouse  $\alpha_2\delta_1$  (Accession number: NM\_001110846) antisense (AGCCATCTTCGCGATCGAAG) and mismatch (CGATACCTCGCTGGCTAAAG) oligodeoxynucleotides with phosphothioate modification on three nucleotides at each end were synthesized by GineLink (Hawthorne, NY), precipitated, washed in 75% ethanol

solutions and dissolved in sterile saline before use. Seven days after SNL when mice showed behavioral hypersensitivities, each solution (5  $\mu$ L/mouse) was intrathecally injected between the L4/L5 regions (Li et al., 2006; Nguyen et al., 2009) once a day for 4 days. Similar treatments in the SNL model with the same antisense oligodeoxynucleotides resulted in specific knockdown of injury-induced increase of  $\alpha_2\delta_1$  in spinal cord and behavioral hypersensitivity without causing any detectable toxicity (Li et al., 2004; Boroujerdi et al., 2008; Nguyen et al., 2009; Boroujerdi et al., 2011).

## 2.6 Patch clamp recording

Briefly, a mouse was decapitated under deep isoflurane anesthesia, and the L4 lumbar spinal cord was removed after laminectomy, sliced (300  $\mu$ m) with a vibratome (VT-1200, Leica Inc.) in ice-cold sucrose-based artificial cerebral spinal fluid (SACSF) saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub> (carbogen). The SACSF contained (mM): 250 sucrose, 2.5 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 1.2 MgCl<sub>2</sub>, 2.4 CaCl<sub>2</sub>, 26 NaHCO<sub>3</sub> and 11 glucose. Slices were first recovered at 31 °C for at least one hr in SACSF, then kept at room temperature (22-24 °C) in carbogenated ACSF containing (mM) 126 NaCl, 2.5 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 1.2 MgCl<sub>2</sub>, 2.4 CaCl<sub>2</sub>, 26 NaHCO<sub>3</sub> and 11 glucose for at least 30 min. Then a slice was placed in a recording chamber and superfused (1.5-2.0 mL/min) with carbogen-saturated ACSF continually. 1  $\mu$ M tetrodotoxin (TTX) was applied continually with perfusate when mEPSC/mIPSC was recorded. Drugs were applied with the perfusate as needed. Neurons located within or dorsal to lamina II (Substantia gelatinosa) were visualized with an upright microscope (Eclipse FN1, Nikon) with near-infrared illumination, and the lamina location of recorded sites was confirmed under low magnification after recording. Whole-cell patch clamp recording was performed at 32  $\pm$  0.5°C with MultiClamp 700B amplifiers (Axon Instruments, Molecular Devices, Union City, CA), Digidata 1440 analog-to-digital converters (Axon Instruments) and pClamp 10.2 software (Axon Instruments). Data were sampled at 10 kHz and filtered at 2 kHz. The patch electrode had a resistance of 5-7 M $\Omega$  when filled with pipette solution 1 containing (mM): 135 potassium gluconate, 5 KCl, 5 EGTA, 0.5 CaCl<sub>2</sub>, 10 HEPES, 2 Mg-ATP, and 0.1 GTP; or as indicated, with solution 2 containing (mM): 70 potassium gluconate, 65 KCl, 5 EGTA, 0.5 CaCl<sub>2</sub>, 10 HEPES, 2 Mg-ATP, 0.1 GTP. The pH of these solutions was adjusted to 7.2 with Tris-base, and the osmolarity was adjusted to 300 mOsm with sucrose. The junction potential between the patch pipette and bath solution was nulled before gigaseal formation. Series resistance was monitored without compensation throughout the experiment (MultiClamp 700B). The data were discarded if the series resistance (15-30 M $\Omega$ ) changed by more than 20% during whole-cell recording. mEPSC/mIPSC were recorded for 5 min after stabilization, counted and analyzed using clampfit 10.3 (Molecular Devices) after the traces were low-pass filtered at 2 kHz. Waveform templates were defined according to the rise and decay times and only events that match the waveform and above amplitude threshold (4 pA) were analyzed. For drug treatment, the baseline mean values of mEPSC frequency were obtained for 5 min, while the mean values during drug application were obtained for 2 min over the peak drug response. The drug effects on mEPSC frequency were compared to the baseline mean values, and expressed as percentage changes over the baseline values.

## 2.8 Western blots

Western blots were performed as described (Boroujerdi et al., 2011). Briefly, lumbar dorsal spinal cord samples from sham or SNL mice one-week post injury were extracted in ice-cold buffer (pH 7.5) containing 50 mM Tris, 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail (Roche Applied Science). Protein concentrations were measured using the bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of proteins were separated on polyacrylamide gels, transferred to PVDF membranes. Membranes were blocked with 5% milk solution, cut in half based on the molecular weight of the  $\alpha_2\delta_1$  and  $\beta$ -actin, then probed with primary antibodies against  $\alpha_2\delta_1$  (1:1000; Sigma-Aldrich, St. Louis, MO) or  $\beta$ -actin (1:10,000; Novus, Littleton, CO), respectively, overnight at 4°C. After washing, membranes were incubated with HRP-conjugated secondary antibodies for one hr at room temperature. Protein bands were detected using chemiluminescence reagents (Pierce Biotechnology, Inc., Rockford, IL) followed by X-ray film exposure. The band densities were quantified within the linear range of the film using Image J 1.45 (NIH). Co-detection of  $\beta$ -actin on the same membrane served as a loading control. The ratio of  $\alpha_2\delta_1$  to  $\beta$ -actin band densities was taken within each sample before comparisons were made between the control and experimental samples.

## 2.9 Statistic analysis

Two-tailed Student's *t*-test was used to evaluate the statistical significance of differences in the frequency and amplitude of mEPSC/mIPSC between  $\alpha_2\delta_1$  Tg and SNL neurons and their respective controls with or without drug treatments. One-way or Two-way ANOVA analysis with Bonferroni post hoc test was used for multi-group comparisons.  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1 Elevated $\alpha_2\delta_1$ induces hyperalgesia

Behavioral sensitivities to noxious cold and heat were tested in left L4 SNL and sham mice 5-7 days after surgery. SNL, but not sham surgery, led to cold hyperalgesia (Fig. 1A) and heat hyperalgesia (Fig. 1B) with an onset time similar to that previously reported (Luo et al., 2001; Luo et al., 2002; Li et al., 2004). Data from Western blots indicated that SNL, but not sham surgery, increased  $\alpha_2\delta_1$  protein levels in DRG and dorsal spinal cord at the injury side (Fig. 1C), similar to that reported in SNL rats (Luo et al., 2001; Luo et al., 2002; Li et al., 2004).

Based on these data, we hypothesized that  $\alpha_2\delta_1$  upregulation could contribute to SNL-induced hypersensitivity. To determine whether the increase of  $\alpha_2\delta_1$  alone, without other injury factors, is sufficient to induce behavioral hypersensitivities, we tested similar behavioral sensitivities in a Tg mouse line with  $\alpha_2\delta_1$  protein overexpression in neuronal tissues (Li et al., 2006). The  $\alpha_2\delta_1$  Tg, but not WT littermate, mice showed similar cold (Fig. 2A), and heat (Fig. 2B) hyperalgesia as the SNL mice (Fig. 1A-B). These findings suggest that behavioral hypersensitivities in both models are likely mediated through elevated spinal  $\alpha_2\delta_1$  levels, forming the basis for electrophysiology studies

### 3.2 Upregulated $\alpha_2\delta_1$ enhances mEPSC frequency in superficial dorsal spinal cord

Since most nociceptive transmission is processed first in SDH, and SNL causes  $\alpha_2\delta_1$  upregulation in DRG neurons, followed by subsequent translocation to axonal terminals in dorsal spinal cord (Li et al., 2004; Bauer et al., 2009), we hypothesized that SNL induced  $\alpha_2\delta_1$  could affect presynaptic neurotransmission in SDH.

First, we examined  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/Kainate receptor mediated mEPSC from voltage-clamped ( $V_m = -60$  mV) SDH neurons of L4 spinal cord slices from 5-7 day SNL mice with behavioral hypersensitivities (Fig. 1). N-methyl-D-aspartate (NMDA) receptors were blocked with antagonist AP-5 (50  $\mu$ M) while IPSC were blocked with 10  $\mu$ M bicuculline, a GABA<sub>A</sub> receptor antagonist, and 1  $\mu$ M strychnine, a glycine receptor antagonist. Recorded mEPSC could be blocked by 20  $\mu$ M DNQX, an AMPA/Kainate receptor antagonist (data not shown), confirming that mEPSC from dorsal spinal cord neurons are mediated through AMPA/Kainate receptor activation by action potential-independent presynaptic release of glutamate.

SNL injury did not change the membrane properties, including resting membrane potential and input resistance, of SDH neurons compared with that from sham neurons (Table S1). Compared with sham control, SNL increased significantly mEPSC frequency, but not amplitude, in injured SDH neurons (Figs 3A and B, S1A and B), but not in non-injured adjacent SDH neurons (Fig. 3C and D). Thus, SNL enhances presynaptic excitatory synaptic transmission in SDH of the injury segment, which may derive from increasing glutamate release.

As blocking SNL-induced  $\alpha_2\delta_1$  with intrathecal GBP and  $\alpha_2\delta_1$  antisense oligodeoxynucleotides can reverse behavioral hypersensitivities (Luo et al., 2001; Luo et al., 2002; Li et al., 2004), we next tested whether these treatments could attenuate elevated mEPSC frequency in SDH neurons from SNL mice, which would support the involvement of elevated  $\alpha_2\delta_1$  in mediating this neuroplasticity and behavioral hypersensitivities. GBP, within the effective dose range (10-100  $\mu$ M) reported in brain and spinal cord slice preparations (Fink et al., 2000; Patel et al., 2000), dose-dependently and reversibly normalized mEPSC frequency in SDH neurons at the injury side to a level similar to that in the sham control without altering the mEPSC frequency in sham neurons (Figs. 3C, E and F).

Intrathecal treatments with  $\alpha_2\delta_1$  antisense, but not mismatch, oligodeoxynucleotides (5  $\mu$ g/mouse, once per day) for 4 days in 1-week SNL mice also caused a significant reduction in averaged frequency (Figs. 3G, S1C), but no significant change in amplitude (Figs. 3H, S1D) of mEPSC in L4 injury side SDH neurons. The antisense oligodeoxynucleotide effect was specific since it also blocked the inhibitory effects of GBP (100  $\mu$ M) on SNL-induced mEPSC frequency (Fig. 3I). Similar antisense oligodeoxynucleotide treatments in SNL rats have been shown to block SNL-induced behavioral hypersensitivity and  $\alpha_2\delta_1$  upregulation in dorsal spinal cord (Li et al., 2004; Boroujerdi et al., 2008; Boroujerdi et al., 2011). Together, these data further support that upregulated  $\alpha_2\delta_1$  likely contributes to SNL-induced increase of mEPSC frequency, which contributes to behavioral hypersensitivity.

To test whether  $\alpha_2\delta_1$  overexpression alone was sufficient to enhance mEPSC frequency in SDH neurons, we performed similar recordings in L4 spinal cord slices from the  $\alpha_2\delta_1$  Tg and WT mice. Similar to that seen for the SNL mice, Tg SDH neurons have similar resting membrane potential and input resistance as WT neurons (Table S1). The averaged frequency (Figs. 4A and B, S2A), but not amplitude (Figs. 4A and C, S2B), of mEPSC in Tg neurons appeared significantly higher than that from WT neurons (Fig. 4B). Thus,  $\alpha_2\delta_1$  upregulation alone is sufficient to increase mEPSC frequency in SDH neurons. GBP dose-dependently and reversibly normalized mEPSC frequency in Tg SDH neurons to a level similar to that in WT control without changing the mEPSC frequency in WT SDH neurons (Fig. 4B, D and E). These results suggest that GBP modulates mEPSC only when  $\alpha_2\delta_1$  is upregulated, consistent with findings from a previous study (Moore et al., 2002a).

### 3.3 Inhibitory synaptic transmission in superficial dorsal horn is not altered in SNL and $\alpha_2\delta_1$ Tg mice

Inhibitory interneurons in spinal dorsal horn release glycine and GABA, which activate glycine and GABA<sub>A</sub> receptors, and cause hyperpolarization of postsynaptic neurons. To test whether  $\alpha_2\delta_1$  upregulation affected inhibitory synaptic transmission in SDH neurons from SNL (5-7 days post injury) and  $\alpha_2\delta_1$  Tg mice, we examined mIPSC from L4 spinal slices of these models using high Cl<sup>-</sup> (65 mM) intrapipette solution (solution 2 in Methods) to set Cl<sup>-</sup> reversal potential at -18 mV so that at -60 mV, both mEPSC and mIPSC are inward currents. We blocked mEPSC with 20  $\mu$ M DNQX and 50  $\mu$ M APV, and the remaining mIPSC could be eliminated by 10  $\mu$ M bicuculline and 1  $\mu$ M strychnine (data not shown), confirming that the recording condition was suitable for recording mIPSC (Fig. 5A).

Interestingly, both SNL (Figs. 5A, B and C, S3A and B) and Tg (Figs. 5D and E, S3C and D) mice showed similar mIPSC frequencies and amplitudes in SDH neurons as corresponding controls (SNL vs Sham; Tg vs WT). These data suggest that increased  $\alpha_2\delta_1$  does not alter SDH inhibitory synaptic transmission in these models.

## 4. Discussion

SNL increases  $\alpha_2\delta_1$  proteins in DRG neurons, and subsequent axonal transport of  $\alpha_2\delta_1$  proteins to presynaptic central terminals in dorsal spinal cord that contributes to neuropathic pain states (Li et al., 2004; Bauer et al., 2009). SNL also causes dysregulation of other genes in DRG and spinal cord (Wang et al., 2002; Valder et al., 2003; Kim et al., 2009). To study the mechanism of  $\alpha_2\delta_1$  mediated nociception without the influence from other injury factors, we included the non-injury  $\alpha_2\delta_1$  Tg mice as a complementary model in which  $\alpha_2\delta_1$  expression is elevated in spinal cord and DRG neurons in addition to other neuronal cells (Li et al., 2006). Our data show that both the SNL and  $\alpha_2\delta_1$  Tg models have similar cold and heat hyperalgesia, validating the use of these models for our studies.

Taking the advantage of spinal cord slice patch-clamp recording from both models, we could focus on changes in spinal cord synaptic neurotransmission without worrying about influence from the peripheral and descending contributions. Since elevated  $\alpha_2\delta_1$  proteins and behavioral hypersensitivities are common correlates in the SNL and  $\alpha_2\delta_1$  Tg mouse models, we tested if elevated  $\alpha_2\delta_1$  proteins contribute to SDH neuron sensitization through

a similar synaptic mechanism in these models. Our data showed that SDH neurons in both models received higher frequency of glutamate input than their respective controls (Figs. 3, 4, S1, 2). The mEPSC frequency increase occurred only in injured L4, but not adjacent non-injured L3 SDH neurons from the SNL model (Fig. 3C), supporting that this change is injury segment specific. Intrathecal GBP, which blocks behavioral hypersensitivities in both models secondary to its binding to  $\alpha_2\delta_1$  proteins (Luo et al., 2001; Luo et al., 2002; Field et al., 2006; Li et al., 2006; Lynch et al., 2006), also diminishes elevated mEPSC frequency dose-dependently in SDH neurons from both models (Figs. 3, 4). The specificity of elevated  $\alpha_2\delta_1$  in this injury-induced neuroplasticity is further confirmed by the following findings. Intrathecal  $\alpha_2\delta_1$  antisense oligodeoxynucleotide treatment, which can block injury-induced behavioral hypersensitivities and  $\alpha_2\delta_1$  upregulation in dorsal spinal cord (Li et al., 2004; Boroujerdi et al., 2008; Boroujerdi et al., 2011), also diminishes (1) increased mEPSC frequency (Fig. 3G), and (2) the GBP's inhibitory effects on elevated mEPSC (Fig. 3I) in SDH neurons from SNL mice.

SDH projection neurons receive inputs mainly from small myelinated A $\delta$  and nonmyelinated C-fibers carrying nociceptive information such as cold and heat hyperalgesia (Light & Perl, 1979; Brown, 1982; Millan, 1999; Graham et al., 2007), and form synaptic connections with heterogeneous neurons (Millan, 1999; Prescott & De Koninck, 2002; Lu & Perl, 2005). In combination with pharmacological findings that intrathecal injection of glutamate receptor antagonists (Chaplan et al., 1997; Nguyen et al., 2009), or similar GBP and  $\alpha_2\delta_1$  antisense oligodeoxynucleotides (Luo et al., 2001; Luo et al., 2002; Li et al., 2004) relieves behavioral hypersensitivity in these models, our data support that increased presynaptic  $\alpha_2\delta_1$  at the injury segment is likely contributing to the central sensitization and nociception development in the SNL model.

It has been reported recently that transient overexpression of  $\alpha_2\delta_1$  with P/Q-type VGCC subunits in vitro renders the accumulation of P/Q-type VGCC at presynaptic boutons and subsequent higher probability of vesicle release (Hoppa et al., 2012). We provide complementary in vivo evidences here indicating that this  $\alpha_2\delta_1$ -associated phenomenon also occurs in mouse spinal cord after peripheral nerve injury that mediates nociceptions. We have shown that GBP attenuates increased mEPSC frequency in SNL and  $\alpha_2\delta_1$  mice, suggesting GBP's presynaptic modulatory role. However, GBP fails to affect mEPSC in sham and WT mice, which is similar to that observed previously by Moore, et al. (Moore et al., 2002a), and in line with animal behavioral results showing that intrathecal GBP has no effect on behavioral sensitivities under control conditions (Stanfa et al., 1997; Luo et al., 2001; Li et al., 2006). While in vitro data support that  $\alpha_2\delta_1$  is critical in regulating VGCC trafficking to the cell membrane and nerve terminals and GBP disrupts this process (Hendrich et al., 2008; Bauer et al., 2009), the onset time of gabapentin's inhibitory action in our experiments (mins) is faster than the expected time for regulating VGCC trafficking, which takes at least hrs. Thus, our data support a fast action of GBP in blocking synaptic hyperexcitability and behavioral hypersensitivity in neuropathic pain models (Hunter et al., 1997; Hwang & Yaksh, 1997; Abdi et al., 1998; Chapman et al., 1998; Luo et al., 2002; Li et al., 2004; Mixcoatl-Zecuatl et al., 2004; Suzuki et al., 2005; Li et al., 2006; Suzuki & Dickenson, 2006) through a yet clearly defined mechanism secondary to its binding to  $\alpha_2\delta_1$  proteins (Lynch et al., 2006). This could be different from its effects in inhibiting VGCC

trafficking. Recent findings supporting this notion include that SNL injury-induced and  $\alpha_2\delta_1$ -mediated behavioral hypersensitivities are modulated by descending serotonergic facilitation at the spinal level (Suzuki et al., 2005; Chang et al., 2013), which is indeed sensitive to blockade by gabapentinoids (Suzuki et al., 2005; Bee & Dickenson, 2008). Interestingly, the descending serotonergic facilitatory pathway is thought to play a critical role in recruitment and activation of silent glutamatergic synapses in the spinal cord (Li & Zhuo, 1998), which could also facilitate the priming of dorsal spinal cord neurons for sensitization (Nguyen et al., 2009). It is possible that priming of dorsal horn neurons by increased presynaptic  $\alpha_2\delta_1$  proteins (Nguyen et al., 2009) triggers recruitment of silent synapses through a yet identified process that promotes the formation of descending serotonergic facilitatory circuit (Li & Zhuo, 1998) in mediating behavioral hypersensitivity. Further investigations are warranted for testing this hypothesis.

GABA<sub>A</sub> and glycine receptors mediate IPSC in the spinal cord (Todd & Sullivan, 1990; Yoshimura & Nishi, 1993). However, experimental data on whether inhibitory synaptic transmission is altered in the spinal cord post peripheral nerve injuries are not consistent. Some studies show neither GABAergic nor glycinergic tone changes in neuropathic rodent models (Somers & Clemente, 2002; Polgar et al., 2003; Polgar et al., 2005; Wang et al., 2007), while some show loss of GABAergic inhibition in peripheral nerve injury models (Ibuki et al., 1997; Moore et al., 2002b; Yowtak et al., 2011). We found no change in mIPSC in SDH neurons from SNL and  $\alpha_2\delta_1$  Tg mice. In addition to the differences in recording conditions and animal strains used, this discrepancy may be due to the fact that we did not separate GABA<sub>A</sub> and glycine receptor mediated IPSC. Thus, any change in GABA<sub>A</sub> receptor mediated IPSC could have been diluted to a level below the sensitivity of detection in our studies. However, our findings is consistent with that from studies indicating, for instance, neither GABAergic nor glycinergic IPSC changes in diabetic neuropathic or SNL rats (Polgar et al., 2003; Polgar et al., 2005; Wang et al., 2007). Our data are also supported by a previous electron microscopy study showing that SNL causes elevation of  $\alpha_2\delta_1$  in excitatory, but not inhibitory, synapses (Bauer et al., 2009).

In this study, we systematically compared AMPA receptor mediated excitatory and Glycine/GABA<sub>A</sub> receptor mediated inhibitory synaptic transmission in SDH of the SNL model. Our conclusion is consistent with findings from several other studies indicating that neuropathic pain syndromes in various peripheral nerve injury animal models may result from enhanced excitatory synaptic transmission in the spinal cord, even though the reported endpoint measurements in different peripheral nerve injury models may differ (Kohno et al., 2003; Wang et al., 2007; Zhang et al., 2009; Zhou et al., 2011). However, our data would not allow us to exclude the contribution of other postsynaptic mechanisms, such as NMDA receptor-mediated dorsal spinal cord neuron hyperexcitability, to behavioral hypersensitivity in the nerve injury model (Tao et al., 2003a; Tao et al., 2003b) since NMDA-receptor was blocked in our recording conditions.

In summary, combinational analysis with data from the SNL and  $\alpha_2\delta_1$  Tg models supports our hypothesis that  $\alpha_2\delta_1$  upregulation at the presynaptic central terminals of primary afferents is sufficient to enhance excitatory glutamatergic neurotransmission in superficial dorsal spinal cord that contributes to nociception. Therefore, blocking injury-induced  $\alpha_2\delta_1$

upregulation and glutamatergic pathways in a sensory neuron specific manner may improve therapeutic efficacy and reduce drug side effects in neuropathic pain management.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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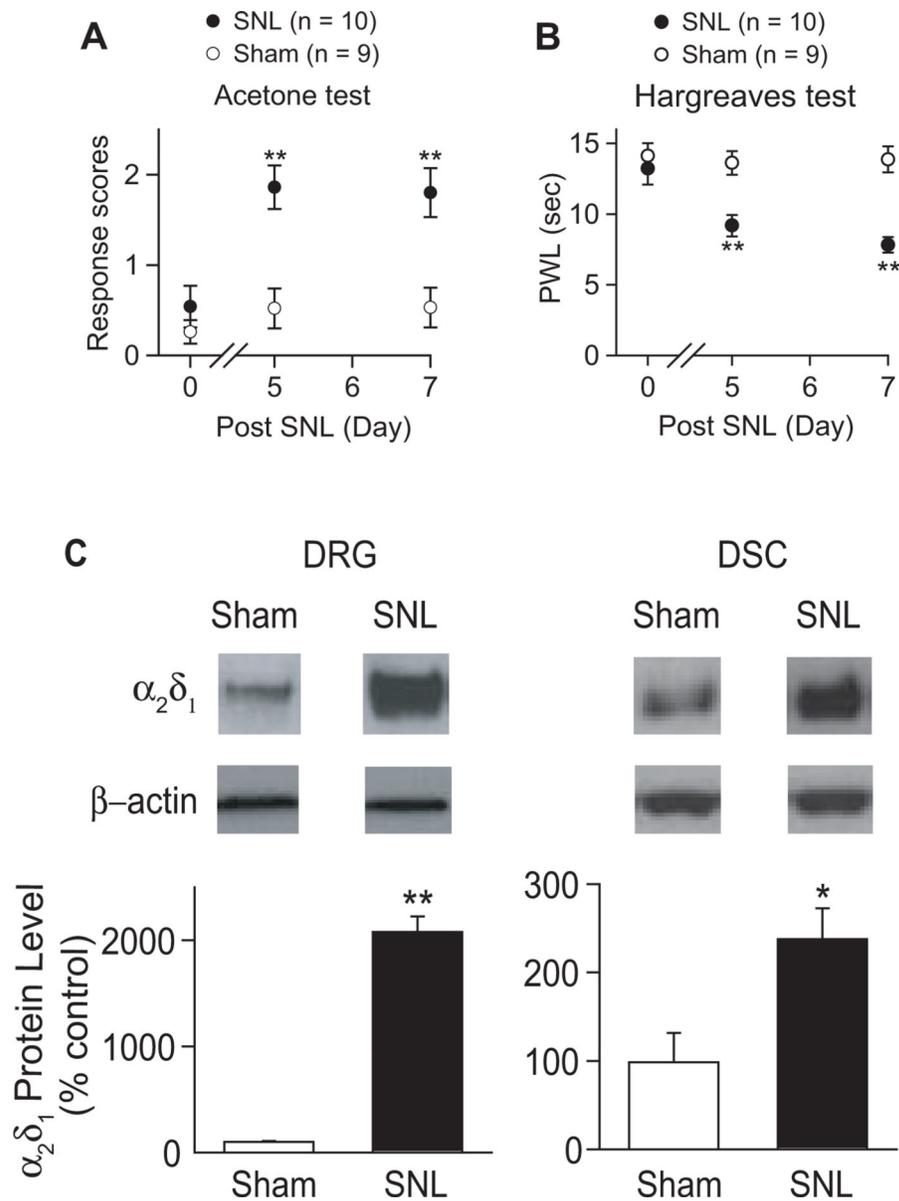
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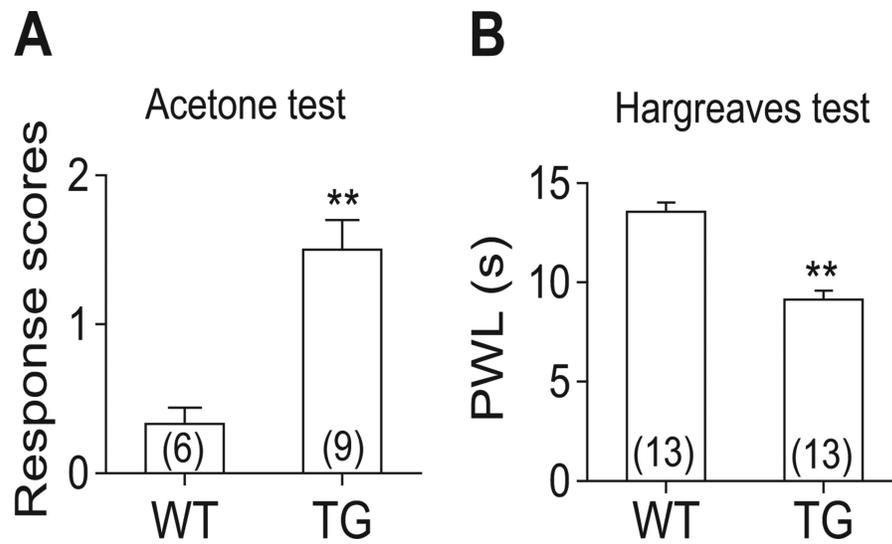
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- It is known that elevated  $\alpha_2\delta_1$  proteins in spinal cord contribute to nerve injury induced neuropathic pain states.
- Findings from this study added electrophysiological evidences to support that elevated  $\alpha_2\delta_1$  proteins contribute to central sensitization by increasing excitatory presynaptic inputs onto superficial dorsal spinal cord neurons, leading to the development of behavioral hypersensitivities.



**Figure 1. Unilateral L4 SNL injury caused nociceptions that correlated with  $\alpha_2\delta_1$  upregulation in dorsal spinal cord and DRG**

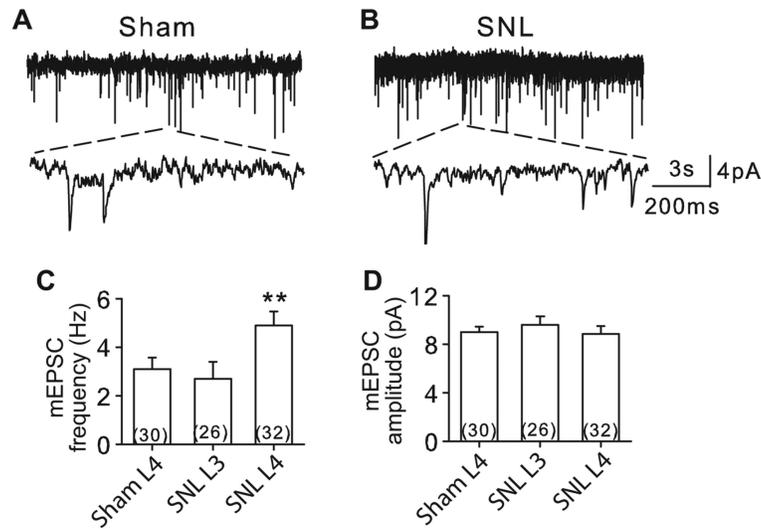
Responses to noxious cold (A), and paw withdrawal latency (PWL) to heat (B) stimuli in the hindpaws of injury-side from sham and SNL mice were tested blindly at designated time points post surgery. Data presented are the means  $\pm$  SEM from the number of animals indicated. \*\* $p < 0.01$  compared with control values by one-way ANOVA test. The  $\alpha_2\delta_1$  levels in injury-side of dorsal spinal cord and DRG were examined one-week post sham or SNL surgery with Western blots (C).  $\beta$ -actin was used as loading control as described in the Method. Representative Western blots were shown on top of each corresponding bar graph presented as the means  $\pm$  SEM from 8 each of sham and SNL mice in 3 independent Western blots. \* $p < 0.05$ , \*\* $p < 0.01$ , compared with control values by Student's *t* test.



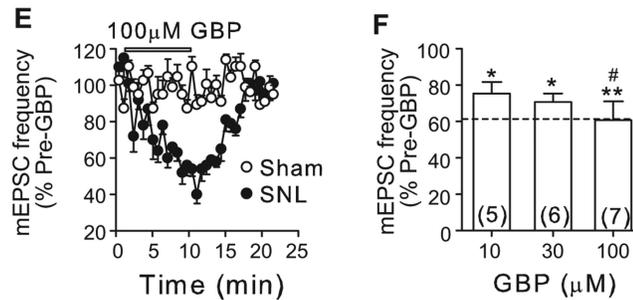
**Figure 2. Over-expression of  $\alpha_2\delta_1$  in the Tg mice caused similar behavioral hypersensitivities as in the SNL mice**

Responses to noxious cold (A), and paw withdrawal latency (PWL) to heat (B) stimuli in the hindpaws of adult injury-free WT and  $\alpha_2\delta_1$  Tg mice were tested blindly. Data presented are the means  $\pm$  SEM from the number of animals indicated in the parentheses. \*\* $p < 0.01$  compared with WT mice by Student's  $t$ -test.

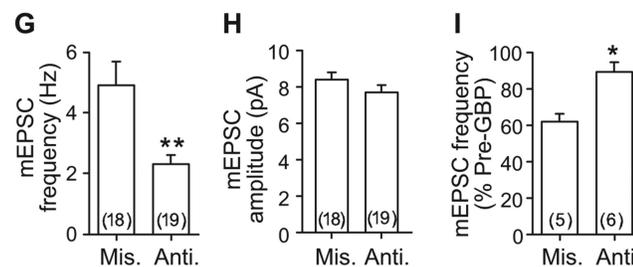
## Increased mEPSC frequency in SDH neurons post SNL



## GBP blocked mEPSC frequency increase in SNL SDH neurons



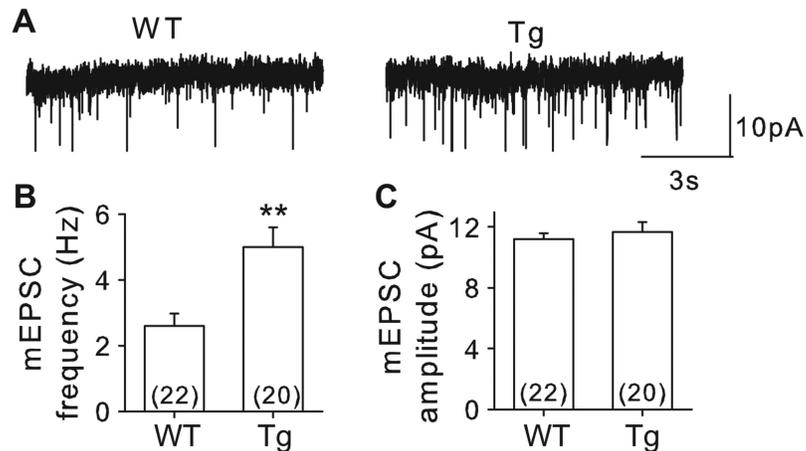
## Antisense oligodeoxynucleotide treatment blocked mEPSC frequency and inhibitory effects of GBP in SNL SDH neurons

**Figure 3. SNL injury led to elevated mEPSC frequency in SDH neurons that was sensitive to  $\alpha_2\delta_1$  blockade**

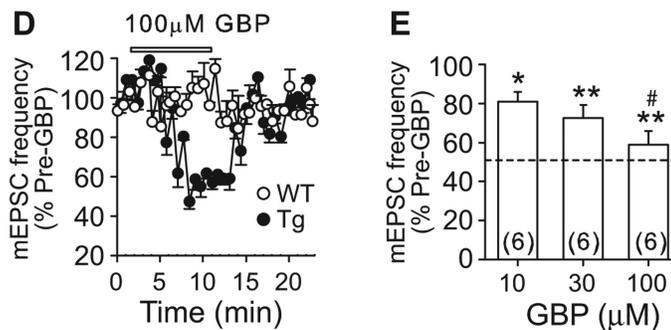
**A** and **B**. Representative mEPSC traces from injury-side of L4 spinal cord slices of sham (**A**) and SNL (**B**) mice 5-7 days after surgery. The upper panels were 10 s traces and the lower panels were 600 ms traces derived from each top trace as indicated by the dot lines. **C** and **D**. Summary of averaged mEPSC frequency (**C**) and amplitude (**D**) in L3 and L4 SDH neurons from injury side of sham and SNL mice. \*\* $p < 0.01$  compared with L4 sham neurons by Student's *t*-test. **E**. Time courses for the effects of 100 μM GBP on mEPSC

frequency from sham and SNL neurons. **F.** Dose-dependent normalization of mEPSC frequency on SNL neurons by GBP.  $*p < 0.05$ ,  $**p < 0.01$  compared with pre-GBP treatment,  $^{\#}p < 0.05$  compared with 10  $\mu\text{M}$  GBP by one-way ANOVA test. Dot-line represents baseline mEPSC frequency level in L4 sham neurons shown in **C**, **G** and **H**. Summary of averaged mEPSC frequency (**G**) and amplitude (**H**) data in SDH neurons from SNL mice that were treated with intrathecal  $\alpha_2\delta_1$  antisense (Anti.) or mismatch (Mis) oligodeoxynucleotides (5  $\mu\text{g}/\text{day}$ ) for 4 days, starting 1-week post injury. **I.** Effects of GBP (100  $\mu\text{M}$ ) on elevated mEPSC frequency in L4 SDH neurons from injury-side of SNL mice post  $\alpha_2\delta_1$  antisense or mismatch oligodeoxynucleotide treatments. Summarized data are shown as the means  $\pm$  SEM from the number of neurons indicated in the parentheses.  $*p < 0.05$ ,  $**p < 0.01$  compared with  $\alpha_2\delta_1$  mismatch treated groups by Student's *t*-test.

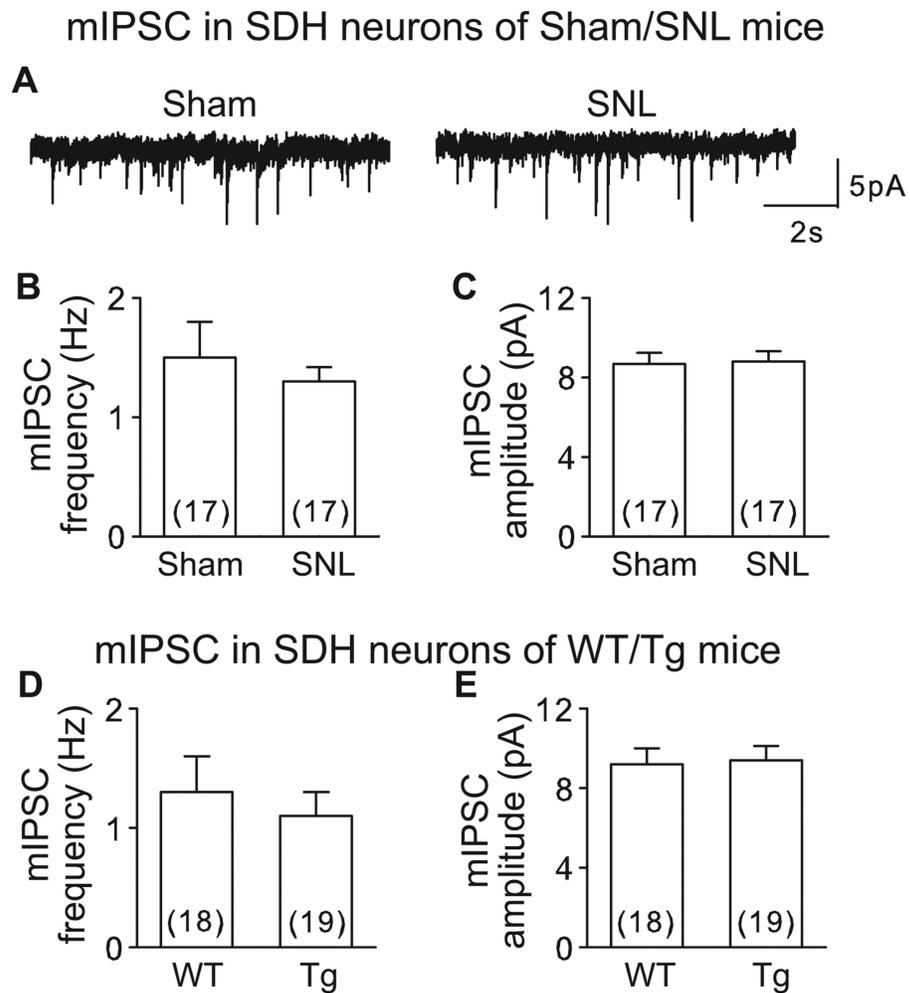
## Increased mEPSC frequency in Tg SDH neurons



## GBP blocked mEPSC frequency increase in Tg SDH neurons

**Figure 4. Enhanced mEPSC in SDH neurons from  $\alpha_2\delta_1$  Tg mice**

**A.** Representative mEPSC traces from WT (left) and  $\alpha_2\delta_1$  Tg (right) SDH neurons. **B** and **C.** Summary of averaged mEPSC frequency (**B**) and amplitude (**C**) data. \*\* $p < 0.01$  compared with WT group by Student's  $t$ -test. **D.** Time courses for the effects of 100  $\mu$ M GBP on mEPSC frequency from WT and Tg L4 SDH neurons. **E.** Dose-dependent normalization of mEPSC frequency in Tg SDH neurons by GBP. Dot-line represents baseline mEPSC frequency level in WT SDH neurons shown in **B**. Summarized data are shown as the means  $\pm$  SEM from the number of neurons indicated in the parentheses. \* $p < 0.05$ , \*\* $p < 0.01$  compared with pre-GBP treatment, # $p < 0.05$  compared with 10  $\mu$ M GBP, by one-way ANOVA test.



**Figure 5. mIPSC was not altered in SDH neurons from SNL and  $\alpha_2\delta_1$  Tg mice**  
**A.** Representative mIPSC traces from a sham (left) or SNL (right) SDH neuron. **B-E.** Summary of averaged mIPSC frequency (**B, D**) and amplitude (**C, E**) from sham/SNL (**B, C**) or WT/Tg (**D, E**) mice. Summarized data are shown as the means  $\pm$  SEM from the number of neurons indicated in the parentheses.  $p > 0.05$  by Student's *t*-test for all the comparisons between sham and SNL as well as WT and Tg mice.