

The mechanisms of protease-activated receptor 2 in mediating pain behaviors through nonselective cation channel signaling

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Research article

Keywords: protease-activated receptor 2 (PAR2), purinergic receptor P2X ligand-gated ion channel 3 (P2X3), transient receptor potential channel vanilloid 1 (TRPV1), transient receptor potential ankyrin 1 (TRPA1), pain

Posted Date: January 3rd, 2020

DOI: <https://doi.org/10.21203/rs.2.19972/v1>

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Abstract

Background: Activation of purinergic receptor P2X ligand-gated ion channel 3 (P2X3), transient receptor potential vanilloid type 1 (TRPV1), and transient receptor potential ankyrin 1 (TRPA1) by their specific ligands is a major mechanism contributing to magnified pain responses. The relationship between these nonselective cation channels and proteinase-activated receptor 2 (PAR2) activation mediated pain is still to be clarified.

Methods: In this study, both in vitro model of dorsal root ganglion (DRG) neurons with PAR2 agonist SL-NH2 challenge and SL-NH2-induced pain rat model were used to approach these questions. The expression of P2X3, TRPV1, and TRPA1 in DRG neurons was investigated by quantitative real-time RT-PCR, Western blot, and immunofluorescence. The involvement of the PLC β 3/PKC ϵ signaling pathway was also determined. The behavior test for mechanical allodynia and thermal hyperalgesia was performed.

Results: SL-NH2 induced upregulation of P2X3, TRPV1, and TRPA1 through phosphorylation of phospholipase C β 3 (PLC β 3) and protein kinase C ϵ (PKC ϵ) signaling pathway in DRG neurons in vitro and in vivo. SL-NH2 also elevated the proportion of P2X3-, TRPV1-, and TRPA1-expressing neurons. The upregulation of P2X3, TRPV1, and TRPA1 and phosphorylation of PLC β 3 and PKC ϵ in DRG neurons was paralleled with mechanical allodynia and thermal hyperalgesia behaviors in rats.

Conclusions: The data of the present study imply that SL-NH2 as a noxious stimulus activates PAR2 which induces TRPV1, TRPA1, and P2X3 upregulation through PLC β 3/PKC ϵ signaling pathway, thereby decreasing activation thresholds and increasing excitability, resulting in sustained nociceptive activity in DRG neurons, and then causing mechanical allodynia and thermal hyperalgesia behaviors. These data expanded our knowledge about PAR2-mediated pain sensitivity and its relationship with TRPV1, TRPA1, and P2X3 and provided new opportunities on management of pain behaviors.

Background

Protease-activated receptors (PARs) are a family of G-protein-coupled receptors (GPCRs) that possess a seven transmembrane loops that can be irreversibly activated by proteolytic cleavage of the N terminus rather than by exogenous ligand binding [1–4]. One of the PAR subtypes, PAR2 is reported to be associated with metabolism, pain, inflammation, and other diseases [5–9]. PAR2 is suspected to modulate the pathogenesis of various neurodegenerative conditions [10]. The upregulation or activation of PAR2 in dorsal root ganglion (DRG) neurons and sensory nerves is involved in the peripheral mechanisms of inflammation development and neuropathic pain [11–14].

PAR2 is a common target for multiple proteases that cleaves it at different sites to generate different PAR2-mediated intracellular signaling pathways. Different ligands are responsible for different PAR2 signaling and functions [15], therefore, signaling bias of PAR2 may lead to different functional responses. Phospholipase C (PLC) and protein kinase C (PKC) are the classical GPCR downstream molecules those

are involved in the nociceptive transmission [16]. PKC is one of the downstream pathways of PAR2 which contributes to neuropathic pain and blocking PKC can significantly diminish the hyperalgesia [17]. Moreover, PKC pathway can trigger the expression of the proinflammatory cytokines in DRG and spinal cord [18]. Since PAR2 is present in DRG neuronal populations, the role of PAR2 activation in the neuronal inflammation and the mediation of PKC activation via PAR2 remains unknown. Whether the modulation of PKC activation is mediated via PAR2 and activation of PAR2 induces the development of neuronal inflammation in DRG should be further explored. PAR2 may influence ligand-modulated calcium signaling by utilizing different important channels [19]. Therefore, there is a need to study which the sensitive gated ion channels are involved after PAR2 activation in the development of pain behaviors.

The transient receptor potential channel vanilloid 1 (TRPV1) is one of the key nociceptive receptors that is expressed in primary sensory neurons and is involved in the detection and transduction of nociceptive stimuli [20] as well as expressed in central nervous system to regulate mental disorders [21–25]. It is affected by kinase-mediated phosphorylation which leads to an increase in pain sensitivity [26]. The activation of TRPV1 induces the accumulation of cytosolic Ca^{2+} in the DRG neuron, which leads to nociceptor sensitization [27, 28]. Transient Trpv1 gene silencing or selectively inhibition of TRPV1 produces antinociceptive action on relieving pain behavior [29, 30]. Since the perception of painful thermal stimuli by sensory neurons is largely mediated by TRPV1 [31], the functional coupling of PAR2 and TRPV1 in DRG neurons may potentiate pain sensation signaling at different pathological conditions [32]. Interestingly, PAR2-triggered 'referred hyperalgesia' was inhibited by blocking TRPV1 suggesting a close relationship between PAR2 and TRPV1 in mediating pain or hyperalgesia [33].

The transient receptor potential ankyrin 1 (TRPA1) is expressed in primary sensory neurons and is activated by a variety of inflammatory mediators that can cause pain hypersensitivity during inflammation [34], nerve insult [35], and neuropathic pain [36]. Exogenous and endogenous TRPA1 activators through electrostatic binding and/or covalent attachment to this protein cause the sensation of pain or toxicology in animal models and humans [37–40]. Inhibition of TRPA1 alleviates nociception and neuropathic pain behavior [41–43]. Several pain sensing actions of PAR2 are associated with TRPA1 [44, 45], suggesting PAR2-related responses are, at least in part, through TRPA1-expressing primary sensory neurons. Thus, both PAR2-induced TRPV1 and TRPA1 downstream pathways might play a notable role in the development of mechanical and thermal hyperalgesia [46].

Purinergic P2X receptors are involved in mediating intercellular signaling via purines such as adenosine-5'-triphosphate (ATP) [47] and are responsible for the transmission of nociceptive information from the periphery to the central nervous system [48]. As an important transducer of nociceptive stimuli, purinergic receptor P2X ligand-gated ion channel 3 ($P2 \times 3$) is a trimeric cation channel that is gated by extracellular ATP and predominantly localized in small- to medium-sized neurons of the DRG [49] and acts as an attractive potential therapeutic target [50]. $P2 \times 3$ can be involved in different pain conditions [51]. Its activation contributes to acute nociceptive behavior, hyperalgesia, and allodynia [52–54]. A higher expression of $P2 \times 3$ in DRG is related to mechanical allodynia and thermal hyperalgesia during inflammation, neuropathy, and nerve injury [48, 55–61]. A high degree of specificity in pain sensation

processing actions has been attributed to the restrictive expression of P2 × 3 in small to medium diameter C- and Aδ-fiber primary afferent neurons [62]. Inhibition of P2 × 3 receptor expression in DRG neurons could relieve the neuropathic pain or inflammatory pain [63, 64]. Whether P2 × 3 could be served as a marker in the PAR2 activation induced pain should be further studied.

Despite PAR2 plays multiple roles in different diseases and healthy conditions, its activation during neuroinflammation is prominent. The signaling pathway that leads to pain including the activation of PAR2 and the downstream activation of PLC, PKC, TRPV1, TRPA1, and P2 × 3 should be further investigated. Hence, in the present study, we are utilizing pain rat model to examine the contribution of PAR2 in inflammation-related pain behavioral changes. We are also utilizing in vitro and in vivo model for exploring the expression of PAR2 related Ca²⁺ channels to investigate its mechanisms. Our findings may indicate that PAR2 plays important roles in inflammation-related changes in behavior and in cation channel-related pain disorders that should be examined further to fully elucidate its therapeutic potential.

Results

SL-NH2 elicited P2 × 3, TRPV1, and TRPA1 expression in DRG neurons in vitro and in vivo

It is well documented that the upregulation of P2 × 3 is known to contribute to purinergic pain-sensing system [65]. In a different neuropathological perspective, PAR2-related pain sensitivity maybe attributed to the upregulation or activation of TRPV1 [66, 67] and TRPA1 [46, 68, 69] in DRG neurons. In this study, the expression of mRNA and protein level of P2 × 3, TRPV1, and TRPA1 in DRG neurons in vitro and in vivo with SL-NH2 stimulation was investigated by using quantitative real-time RT-PCR and Western blot assay. After SL-NH2 treatment, the mRNA levels of P2 × 3, TRPV1, and TRPA1 in cultured neurons are 2.13 ± 0.34 , 2.29 ± 0.30 , and 2.15 ± 0.16 -fold of the control. The protein levels of P2 × 3, TRPV1, and TRPA1 in cultured neurons are 1.76 ± 0.12 , 2.34 ± 0.30 , and 1.67 ± 0.11 -fold of the control. The mRNA levels of P2 × 3, TRPV1, and TRPA1 in DRG from homolateral side of SL-NH2 injection are 2.13 ± 0.34 , 2.30 ± 0.30 , and 2.15 ± 0.16 -fold of the control. The protein levels of P2 × 3, TRPV1, and TRPA1 in DRG from homolateral side of SL-NH2 injection are 1.86 ± 0.21 , 2.1 ± 0.20 , and 1.73 ± 0.09 -fold of the control, respectively (Fig. 1). The mRNA and protein levels of P2 × 3, TRPV1, and TRPA1 in DRG neurons have increased significantly after SL-NH2 treatment both in vitro and in vivo. These results indicated that PAR2 activation upregulates P2 × 3, TRPV1, and TRPA1 expression which may attribute to PAR2-induced pain.

The proportion of P2 × 3-, TRPV1-, TRPA1-, and PAR2-positive neurons after SL-NH2 treatment

Nonselective cation channel P2 × 3-, TRPV1-, and TRPA1-expressing DRG neurons represent a large proportion of somatic nociceptors in processing pain signaling. PAR2-expressing neurons represent a range of DRG neurons that is involved in PAR2 induced pain signaling. In this study, the percentage of P2 × 3-, TRPV1-, TRPA1-, and PAR2-positive neurons was calculated from double fluorescence labeling profiles after SL-NH2 treatment both in vitro and in vivo. The percentage of P2 × 3-, TRPV1-, TRPA1-, and

PAR2-positive neurons in culture is $50.60\% \pm 2.50\%$, $52.06\% \pm 3.81\%$, $59.14\% \pm 3.26\%$, and $52.19\% \pm 3.68\%$. After SL-NH2 exposure, the percentage of P2 × 3-, TRPV1-, TRPA1-, and PAR2-positive neurons in culture is $78.05\% \pm 0.81\%$, $78.78\% \pm 3.11\%$, $77.67\% \pm 1.38\%$, and $77.87\% \pm 2.65\%$, respectively (Fig. 2). The percentage of P2 × 3-, TRPV1-, TRPA1-, and PAR2-positive neurons in DRG from control side is $31.56\% \pm 1.65\%$, $34.33\% \pm 2.02\%$, $30.51\% \pm 2.18\%$, and $33.82\% \pm 3.26\%$. After SL-NH2 treatment, the percentage of P2 × 3-, TRPV1-, TRPA1-, and PAR2-positive neurons in DRG from treated side is $56.01\% \pm 1.05\%$, $53.22\% \pm 1.80\%$, $58.41\% \pm 2.33\%$, and $56.35\% \pm 2.34\%$, respectively (Fig. 3). The percentage of P2 × 3-, TRPV1-, TRPA1-, and PAR2-positive neurons elevated significantly ($P < 0.001$) after SL-NH2 treatment both in vitro and in vivo. These results suggest an increase in DRG neurons that express P2 × 3, TRPV1, TRPA1, and PAR2 might attribute to pain sensing signaling transmission.

The proportion of P2 × 3-, TRPV1-, and TRPA1-positive neurons in PAR2-expressing neurons after SL-NH2 treatment

To further investigate the close relationship between P2 × 3-, TRPV1-, and TRPA1-expressing neurons and PAR2-expressing neurons, the percentage of P2 × 3-, TRPV1-, TRPA1-positive neurons in PAR2-expressing neurons was also calculated from double fluorescence labeling profiles after SL-NH2 treatment both in vitro and in vivo. The percentage of P2 × 3-, TRPV1-, TRPA1-positive neurons in PAR2-expressing neurons in culture is $55.07\% \pm 4.49\%$, $56.73\% \pm 4.14\%$, and $61.17\% \pm 2.90\%$. After SL-NH2 exposure, the percentage of P2 × 3-, TRPV1-, TRPA1-positive neurons in PAR2-expressing neurons in culture is $74.15\% \pm 2.28\%$, $72.18\% \pm 2.15\%$, and $81.37\% \pm 0.82\%$, respectively (Fig. 4). The percentage of P2 × 3-, TRPV1-, TRPA1-positive neurons in PAR2-expressing neurons in DRG from control side is $31.04\% \pm 1.29\%$, $30.59\% \pm 1.99\%$, and $30.59\% \pm 0.98\%$. After SL-NH2 treatment, the percentage of P2 × 3-, TRPV1-, TRPA1-positive neurons in PAR2-expressing neurons in DRG from treated side is $57.95\% \pm 3.21\%$, $60.27\% \pm 2.31\%$, and $60\% \pm 0.98\%$, respectively (Fig. 5). The percentage of P2 × 3-, TRPV1-, and TRPA1-positive neurons in PAR2-expressing neurons is upregulated significantly ($P < 0.001$) after SL-NH2 treatment both in vitro and in vivo. The higher co-expression rate of P2 × 3, TRPV1, and TRPA1 with PAR2 in DRG neurons after PAR2 activation suggests that there is a close relationship between PAR2 with P2 × 3, TRPV1, or TRPA1 during pain sensing signaling processing.

SL-NH2 induced PLCβ3 and PKCε phosphorylation in DRG neurons

Activation of PAR2-induced phosphorylation of PLC is one of the mechanisms of receptor activation and desensitization [70]. Upregulation of PKCε in DRG neurons is involved in pain responses during inflammation [71]. Activation of PAR2-induced hyperalgesic state is dependent on PKC signaling [72]. In this study, both pPLCβ3 and pPKCε protein levels in DRG neurons in vitro and in vivo were determined by Western blot assay. After SL-NH2 treatment, the protein levels of pPLCβ3 and pPKCε in cultured neurons are 2.08 ± 0.26 and 2.13 ± 0.23 -fold of the control. The protein levels of pPLCβ3 and pPKCε in DRG from homolateral side of SL-NH2 injection are 2.04 ± 0.15 and 2.04 ± 0.14 -fold of the control, respectively (Fig. 6). These results imply that phosphorylation of PLCβ3/PKCε signaling molecule in DRG neurons

induced by PAR2 activation triggers its downstream nonselective cation channel expression which is responsible for the development and maintenance of pain.

The alterations of mechanical and thermal thresholds related to c-Fos expression after SL-NH2 treatment

The mechanical and thermal thresholds were measured from 0.5 hours to 72 hours after intraplantar injection of 10 µg SL-NH2 on right foot. The lowest mechanical and thermal thresholds were observed at 2 hours after intraplantar injection of SL-NH2 on the homolateral foot. From 2 hours to 72 hours after intraplantar injection of SL-NH2, the mechanical and thermal thresholds were persistent at very low levels compared with those on the contralateral side. After SL-NH2 treatment, the protein level of c-Fos in DRG from SL-NH2-treated rats is 2.46 ± 0.19 -fold of control (Fig. 7).

Discussion

PAR2 has been highlighted as a target for multiple proteases and it can mediate pain in different conditions [7]. PAR2 in the peripheral nerve endings are implications of the development of increased sensitivity to mechanical and thermal stimuli, especially during inflammatory states [32]. The focus on the pathophysiology and therapeutic potential of purinergic signaling has been raised more recently [73]. The close relationship between P2 × 3 and the pain-sensing system and the specific actions of P2 × 3 on pain sensation attract us to explore P2 × 3 as a novel potential therapeutic target on relieving pain. Sensitization of TRPV1 and TRPA1 via PLC and PKC kinases is correlated with the role of PAR2-mediated nociceptive signaling and pain has been recognized recently [74]. In the present study, both in vitro model of DRG with PAR2 agonist SL-NH2 challenge and SL-NH2-induced pain rat model were used to investigate the relationship between PAR2 activation and nonselective cation channels in mediating pain. The results showed that PAR2 agonist SL-NH2 induced upregulation of P2 × 3, TRPV1, and TRPA1 and phosphorylation of PLCβ3 and PKCε in DRG neurons in vitro and in vivo. SL-NH2 also elevated the proportion of P2 × 3-, TRPV1-, and TRPA1-expressing neurons. The upregulation of P2 × 3, TRPV1, and TRPA1 and phosphorylation of PLCβ3 and PKCε in DRG neurons was paralleled with mechanical allodynia and thermal hyperalgesia behaviors in rats. These findings represent a novel peripheral mechanism underlying PAR2 signaling in mechanical allodynia and thermal hyperalgesia by regulating the expression of different nonselective cation channels.

The relationship between nonselective cation channels P2 × 3, TRPV1, and TRPA1 and PAR2 activation in mediating mechanical allodynia and thermal hyperalgesia deepened our knowledge about PAR2-mediated pain-sensing system. P2 × 3-expressing non-peptidergic small-diameter unmyelinated C-fibers represent a large proportion of somatic nociceptors in pain-sensing processing system [65]. From the nociceptor's receptive field in peripheral tissue, excitatory P2 × 3 drives the initial nerve impulse along with the sensitizing C-fibers to facilitate pain signaling transmission through their central terminals to the spinal dorsal horn (SDH). Co-activation of PAR2 and TRPV1 or TRPA1 resulting in the release of proinflammatory neuropeptide transmitters is a new mechanisms in mediating pain responses [66, 67]. The PLC and PKC signaling in neurons is commonly considered as the pathway of pain and is usually the

target for treatment. However, the extent of nonselective cation channel P2 × 3, TRPV1, and TRPA1 as the common effector of PAR2 remains uncertain. In this way, the involvement of PLCβ3 and PKCε signaling pathway as well as P2 × 3, TRPV1, and TRPA1 in the context of PAR2 activation were investigated. In this study, we used pain behavior animal model mediated by PAR2 activation, as well as cultured DRG neurons with PAR2 activation to examine the possible signaling and cation channel mechanisms involved. The intraplantar injection of the PAR2 agonist SL-NH2 caused the pain behavior associated with c-Fos elevation in SDH. The signaling molecules in pain behavior animal model DRG and SL-NH2 treated DRG culture were upregulated. PAR2 agonist also upregulates the expression of TRPV1, TRPA1 and P2 × 3 channels suggested that the direct activation of PAR2 can induce TRPV1, TRPA1, and P2 × 3 channel expression through PLCβ3 and PKCε signaling pathway. Furthermore, pain behavior observed in animal model in the presence of PAR2 activation suggested P2 × 3, TRPV1, and TRPA1 is related to animal pain behavior.

These results provided additional support for the development of new therapeutic alternatives that can focus on pain induced by activation of PAR2. The expression of c-Fos is induced after DRG inflammation, DRG neuron hyperexcitability, and neuropathic pain hypersensitivity [75, 76], including PAR2 involved nociception [77]. c-Fos increases the expression of proinflammatory cytokines and causes neurochemical alterations in the DRG neurons [18, 78]. In the present study, the pain induced by SL-NH2 injection indicated that PAR2 is activated. The c-Fos protein was expressed in the SDH of SL-NH2-induced pain model. PAR2 contributes to the onset and maintenance of SL-NH2-induced pain hypersensitivity in a c-Fos dependent manner. PAR2 is involved in this characteristic behavior because c-Fos induces the synthesis of proinflammatory cytokines or neurotransmitters causing peripheral inflammation.

Interestingly, TRPA1-mediated heat pain sensitivity may indirectly modulate TRPV1 channels which were co-expressed with TRPA1 in nociceptors. These two receptor channels are in a synergistic and/or conditional relationship with noxious stimulation [79]. In this present study, the expression of TRPV1 and TRPA1 were significantly increased by SL-NH2-induced PAR2 activation in cultured and pain behavior animal model DRG neurons. P2 × 3 channel expression was affected similarly by PAR2 activation in this experiment.

In the peripheral terminals of sensory neurons, PLC-PKC cascade can directly activate or sensitize neighboring TRPV1 and TRPA1 channels in DRG [16, 28, 80, 81]. According to the subdivision of DRG neurons, TRPV1- and TRPA1-positive neurons are related to both peptidergic and non-peptidergic nerve fibers, whereas P2 × 3-expressing neurons represent non-peptidergic DRG neurons [82–87]. It has been shown that the co-expression of other receptors with P2 × 3 in C-fibers can alter neuronal sensitization induced P2 × 3 activation. The key role of co-expression of TRPV1 and P2 × 3 in nociceptive neuronal subpopulations in mediating severe pain complicated the concept and mechanism of nonselective cation channels on pain signaling processing [65]. The close relationship between PAR2 and TRPV1 is reflected by the co-expression of PAR2 and TRPV1 in nociceptive DRG neurons. Blocking PAR2 decreases expression of TRPV1, and the blockade of TRPV1 prevents the activation of PAR2-induced persistent thermal hyperalgesia [32, 66]. PAR2 is also co-expressed with TRPA1 in small DRG neurons [74]. The

attenuation of TRPA1 signaling by blocking PAR2 activation and inhibition of the TRPA1 inducing decreases of PAR2-related hyperactivity and pain suggests the close relationship and interaction between PAR2 with TRPA1 in mediating hyperactivity and pain [44, 67]. The difference in pain processing mechanisms between PAR2-positive neurons that co-expresses with P2 × 3-, TRPV1-, or TRPA1-expressing neurons and PAR2-positive neurons that do not co-expresses with other neurons should be further investigated in future studies. The previous studies have reported the modulation of TRPV1, TRPA1, or P2 × 3 by activation of PAR2 in a various neuropathic pain and inflammatory pain [88–92]. We draw on the previous research to get inspiration: Since TRPV1, TRPA1 or P2 × 3 are involved in the regulation of PAR2 during the process of mediating pain, is there a common signaling pathway which simultaneously mediates TRPV1, TRPA1 and P2 × 3 via PAR2 activation. We made the hypothesis and conducted the experiment and finally got a positive conclusion. This discovery provides a considerable clinical prospect. Additionally, PAR2 agonist also induces itch by activating superficial SDH neurons [93–96]. Although the effect of itching induced by SL-NH2 injection is difficult to rule out in our present study, we only focused on pain behaviors and their relationship with the modulation of TRPV1, TRPA1, and P2 × 3 by activation of PAR2. It is worth noting that SL-NH2 is not only active PAR2 but also Mrgpr GPCRs, particularly MrgprC11. But MrgprC11 activated by SL-NH2 will mediated histamine-independent itch rather than pain [97]. So this does not affect our conclusions. The transmission of pain information is closely related to the ion channel that precisely controls pain information on nociceptors. Non-selective cation channels P2 × 3, TRPV1, and TRPA1 have all been shown to play important roles in different types of initiation or transmission processes of pain. Previous studies demonstrated that inhibition of PAR2 can enhance the analgesic effect of opioids, thereby reducing the addictiveness of such drugs, while reducing the side effects such as itching after the application of opioids. This effect is based on the integration of PAR2-mediated TRPV1 and μ -opioid receptor [17, 98]. According to our results, PAR2 can mediate the progression of pain by non-selective cation channels P2 × 3, TRPV1, and TRPA1 via PLC β 3/PKC ϵ signaling pathway. Applying this discovery to the development of clinical analgesic drugs by inhibiting PAR2, which can simultaneously relieve pain related with P2 × 3, TRPV1, or TRPA1, not only enhance the efficacy and application range of analgesic drugs, but also reduce side effects. As a result, TRP channels mediated enhancement of neuronal excitation and a variety of neuronal signals is closely related to the pain sensations [99].

Conclusion

The noxious stimulus, SL-NH2, activates PAR2 inducing TRPV1, TRPA1, and P2 × 3 upregulation through PLC β 3/PKC ϵ signaling pathway and thereby decreasing activation thresholds and increasing excitability. This results in sustained nociceptive activity in DRG neurons, which causes mechanical allodynia and thermal hyperalgesia behaviors. The data from this study expand our knowledge about PAR2-mediated pain sensitivity and its relationship with TRPV1, TRPA1, and P2 × 3. These provide new opportunities for promising therapeutic alternative to manage PAR2 activated pain by targeting one or more of these nonselective cation channels or signaling pathways with pharmacological manipulation.

Materials And Methods

Animals

The animals used in this study were newborn (less than 24 hours after birth for DRG cell culture) and adult (male rat age 7 weeks with 180–220 g body weight for neuropathic experiment) Wistar rats which were from Shandong University Experimental Animal Center bred with free access to food and water and 12 h light/ 12 h dark cycle. Cultured DRG was removed from newborn rats under anesthesia with 1.5% pentobarbital sodium via peritoneal injection (2 ml/kg body weight). After finishing the final mechanical allodynia and thermal hyperalgesia behavior test, DRG and SDH were removed from adult rats under anesthesia with 1.5% pentobarbital sodium via peritoneal injection (2 ml/kg body weight).

DRG neuronal culture with SL-NH2 stimulation

DRG neurons from newborn rats were cultured as our previous protocols [100]. Briefly, DRG explants were digested with 1.25 mg/ml collagenase, triturated with glass pipette, filtered with 130 μ m filter, centrifuged at 1500 rpm for 5 minutes, resuspended in culture medium, and plated in 6-well or 24-well clusters. After incubation at 37 °C with 5% CO₂ for the first 24 hours, DRG cells in culture were treated with 5 μ g/ml cytosine arabioside for the second 24 hours. After that, DRG cultures were incubated with SL-NH2 (100 μ mol/L) for additional 24 hours. DRG cells were incubated only with DMEM/F-12 culture medium as control group.

Mechanical allodynia and thermal hyperalgesia behavior test after SL-NH2 injection

Fifteen male rats with the body weight of 180–220 g were used in this behavior test. For induction of mechanical allodynia and thermal hyperalgesia behaviors, intraplantar injection of 10 μ g in 100 μ l SL-NH2 on the right hind paw was applied for each rat 3 times in the successive 3 days. Intraplantar injection of 100 μ l normal saline solution on the contralateral (left) side was used for control. Mechanical allodynia and thermal hyperalgesia behavior test timepoint was at 0 (baseline), 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72 hours, respectively. The mechanical allodynia and thermal hyperalgesia behavior test protocol was as our previous study [101]. The rats were acclimatized in a plastic box on a metal mesh for 30 minutes before behavior test. For the measurement of mechanical thresholds, the hind paw threshold score with different intensive von Frey filaments was recorded and the average score was calculated from 3 thresholds measured in 3 successive tests each with 3 minutes interval. For the measurement of thermal thresholds, the average withdrawal latency was obtained from 3 thermal thresholds measured in 3 successive tests each with 5 minutes interval. The mechanical allodynia and thermal hyperalgesia behavior was measured with blind test. After finishing the final mechanical allodynia and thermal hyperalgesia behavior test at 72 hours, DRG from SL-NH2-treated rats was used for detecting mRNA and protein levels.

Measurement of mRNA levels with quantitative real-time RT-PCR

The mRNA levels of P2 × 3, TRPV1, and TRPA1 in cultured DRG neurons with SL-NH2 stimulation and in DRGs from SL-NH2-treated rats were measured with quantitative real-time RT-PCR. L4-L6 DRGs were removed from SL-NH2 injected side (right side) and normal saline injected side (left side), respectively, and examined separately. Extraction of total RNA was by using RNA Fast200 kit (Fastagen). Synthesis of cDNA was by using cDNA synthesis kit (Termo Scientific). Quantitative PCR was performed with SYBR Green master mixes (Takara) and amplification was performed with the synthetic oligonucleotide primers. The PCR reaction was performed at 55 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles at 94 °C for 5 seconds, 58 °C for 30 seconds, and 72 °C for 45 seconds and the final results carried out by using $2^{-\Delta\Delta Ct}$ method [102]. The primer sequences used in this PCR reaction system were shown in Table 1.

Table 1
|The sequences of oligonucleotide primers.

Genes	Primer sequences
P2 × 3	5'-AGT CGG TGG TTG TGA AGA GC-3' (coding sense)
	5'-GTG TCC CTC ACT TGG TAG GC-3' (coding antisense)
TRPV1	5'-AGA GAG CCA TCA CCA TCC TG-3' (coding sense)
	5'-TAC CTC GTC CAC CCT GAA AC-3' (coding antisense)
TRPA1	5'-CCG TTG CTT TCC TTA ATC CA-3' (coding sense)
	5'-AAT AAG CTG CCC AAA GGT GA-3' (coding antisense)
β-actin	5'- CGT TGA CAT CCG TAA AGA CC-3' (coding sense)
	5'-AAC AGT CCG CCT AGA AGC AC-3' (coding antisense)

Measurement of protein levels with Western blot assay

The protein levels of P2 × 3, TRPV1, TRPA1, pPLCβ3, and pPKCε in cultured DRG neurons with SL-NH2 stimulation and in L4-L6 DRGs from SL-NH2-treated rats and the protein levels of c-Fos in SDH were determined with Western blot assay. Cultured DRG neurons and DRGs from SL-NH2-treated rats were lysed in RIPA buffer (Beyotime Biotechnology) containing protease and phosphatase inhibitors (Roche) on ice for 30 minutes and removed to a refrigerated centrifuge at 13000 rpm for 30 minutes to collect supernatant. The protein samples (50 μg) were loaded for each lane and total protein was separated in SDS-PAGE gel and transferred to nitrocellulose membranes. It is then incubated with each corresponding primary antibody overnight at 4 °C and the corresponding secondary antibody at room temperature for 2

hours. The information of the primary antibodies and secondary antibodies used in immunoblotting was shown in Table 2. Immunoreactive images were then analyzed by using ImageJ software.

Table 2
The primary and secondary antibodies for immunoblotting.

category	antibodies	concentration	source
primary	rabbit polyclonal anti-P2 × 3	1:1000	Abcam, Cambridge, MA
primary	mouse monoclonal anti-TRPV1	1:1000	Abcam, Cambridge, MA
primary	rabbit polyclonal anti-TRPA1	1:1000	Abcam, Cambridge, MA
primary	rabbit polyclonal anti-c-Fos	1:1000	Abcam, Cambridge, MA
primary	rabbit polyclonal anti-pPLCβ3	1:1000	Sigma, St.Louis, MO
primary	rabbit monoclonal anti-pPKCε	1:1000	Abcam, Cambridge, MA
primary	mouse monoclonal anti-β-actin	1:1000	Abcam, Cambridge, MA
secondary	goat anti-mouse IgG (HRP)	1:4000	Abcam, Cambridge, UK
secondary	goat anti-rabbit IgG (HRP)	1:4000	Abcam, Cambridge, UK

Double fluorescence labeling of DRG neurons

Cultured DRG neurons with SL-NH2 stimulation and L4-L6 DRGs sections from SL-NH2-treated rats were processed for immunofluorescence staining. The P2 × 3-, TRPV1-, TRPA1-, and PAR2-positive neurons in total DRG neurons were determined by double fluorescence labeling of MAP2 with P2 × 3, TRPV1, TRPA1, and PAR2, respectively. The P2 × 3-, TRPV1-, and TRPA1- -positive neurons in PAR2-positive neurons were determined by double fluorescence labeling of PAR2 with P2 × 3, TRPV1, and TRPA1, respectively. The double fluorescence labeling was processed as our previous study [14]. The information of the primary antibodies and secondary antibodies used in fluorescence staining was shown in Table 3.

Table 3

|The primary and secondary antibodies for fluorescence staining.

category	antibodies	concentration	source
primary	chicken polyclonal anti-MAP2	1:500	Abcam, Cambridge, MA
primary	mouse monoclonal anti-MAP2	1:500	Abcam, Cambridge, MA
primary	rabbit polyclonal anti-MAP2	1:500	Abcam, Cambridge, MA
primary	guinea pig polyclonal anti-P2 × 3	1:500	Abcam, Cambridge, MA
primary	rabbit polyclonal anti-P2 × 3	1:500	Abcam, Cambridge, MA
primary	mouse monoclonal anti-PAR2	1:500	Santa Cruz, Biotechnology, Santa Cruz, CA
primary	rabbit polyclonal anti-PAR2	1:200	Abcam, Cambridge, MA
primary	mouse monoclonal anti-TRPV1	1:500	Abcam, Cambridge, MA
primary	rabbit polyclonal anti-TRPA1	1:500	Abcam, Cambridge, MA
secondary	goat anti-chicken IgG (FITC)	1:200	Abcam, Cambridge, MA
secondary	goat anti-guinea pig IgG (TRITC)	1:200	Abcam, Cambridge, MA
secondary	goat anti-mouse IgG (FITC)	1:200	Abcam, Cambridge, MA
secondary	goat anti-mouse IgG (TRITC)	1:200	Abcam, Cambridge, MA
secondary	goat anti-rabbit IgG (FITC)	1:200	Abcam, Cambridge, MA
secondary	goat anti-rabbit IgG (TRITC)	1:200	Abcam, Cambridge, MA

Statistical analysis

SPSS (version 22.0) software was used for statistical analysis of the data in this study. All quantitative data were reported as the mean \pm SEM. All the data were processed for normal distribution test and all the data were meet the criteria of normal distribution. And the variances of two populations are equal. Hence, two independent sample t-test was carried out for comparing the difference between two groups of the data in this study. Statistical significant was defined by the value of $P < 0.05$.

Abbreviations

DRG, dorsal root ganglion; P2X3, purinergic receptor P2X ligand-gated ion channel 3; PAR2, proteinase-activated receptor 2; PKC, protein kinase C; PLC, phospholipase C; SDH, spinal dorsal horn; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid type 1.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Z.L. and Z.L. conceived and designed the study, obtained funding for the study, wrote and revised the manuscript. Y.Y. assessed the cultured DRG neurons and DRG tissue from pain rat model, performed the mechanical and thermal pain behavior test, analyzed the data, and assisted in writing and revising the manuscript. N.W. and Y.L. cultured and harvested DRG neurons. Y.Y. and Y.F. established SL-NH2-induced pain rat model. All authors read and approved the final version of the manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study were included in this published article and are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All experimental protocols for animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Ethical Committee for Animal Experimentation of the School of Basic Medical Sciences at Shandong University (Document No. ECSBMSSDU-2018-2-007).

Funding

This research was supported by the Shandong Provincial Key Research and Development Project in China (No. 2019GSF107062).

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Figures

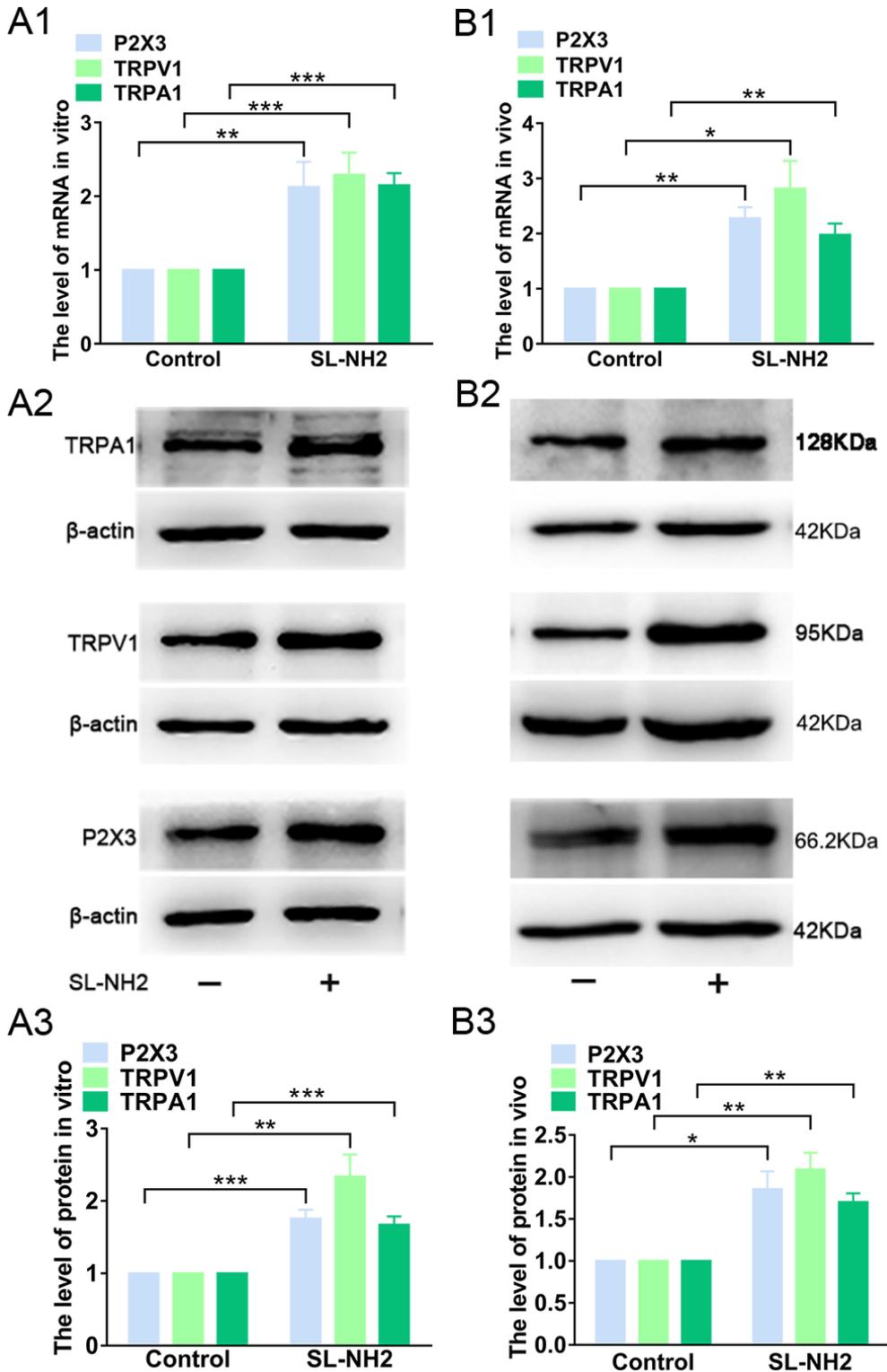


Figure 1

P2X3, TRPV1, and TRPA1 mRNA and protein levels in DRG neurons in vitro and in vivo after SL-NH2 treatment. (A1), P2X3, TRPV1, and TRPA1 mRNA levels in vitro; (A2), P2X3, TRPV1, and TRPA1 immunoreactive bands in vitro; (A3), P2X3, TRPV1, and TRPA1 protein levels in vitro; (B1), P2X3, TRPV1,

and TRPA1 mRNA levels in vivo; (B2), P2X3, TRPV1, and TRPA1 immunoreactive bands in vivo; (B3), P2X3, TRPV1, and TRPA1 protein levels in vivo. n=5, mean \pm SEM, *P<0.05, **P<0.01, ***P<0.001.

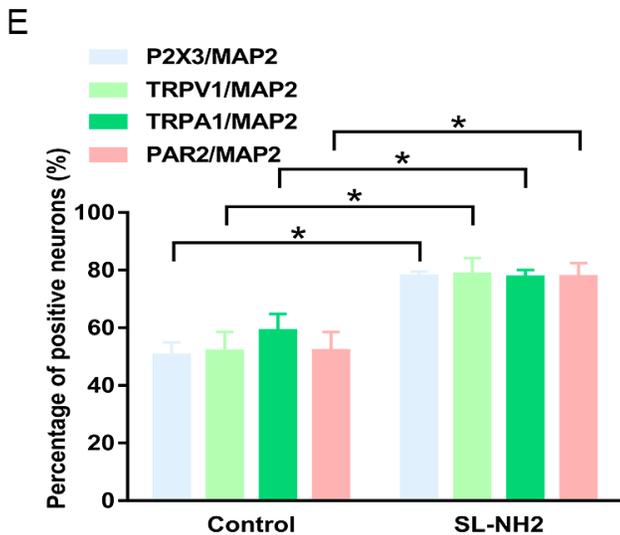
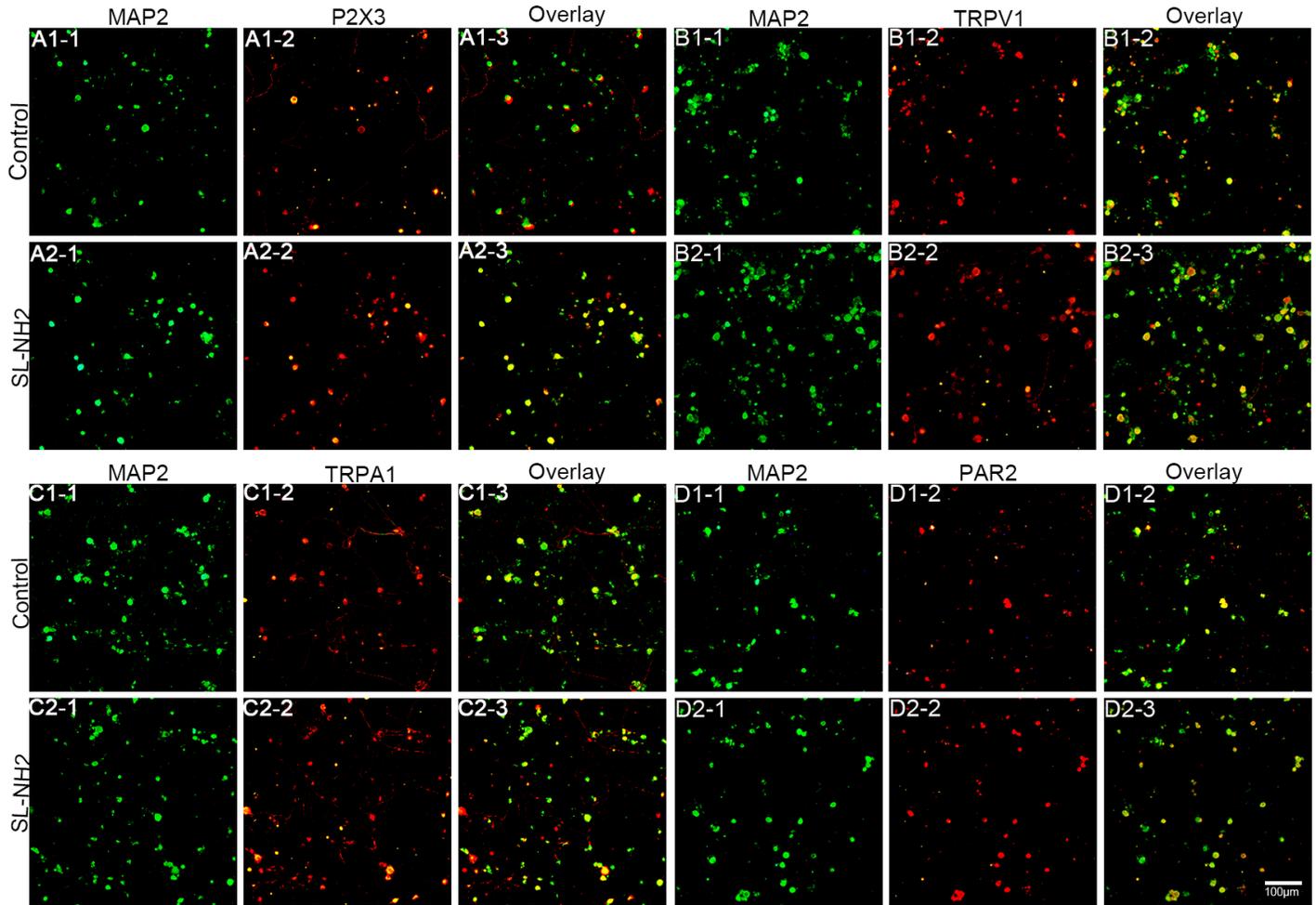


Figure 2

P2X3-, TRPV1-, TRPA1-, and PAR2-positive neurons in culture after SL-NH2 exposure. (A1), P2X3-positive neurons in control culture; (A2), P2X3-positive neurons after SL-NH2 exposure; (B1), TRPV1-positive neurons in control culture; (B2), TRPV1-positive neurons after SL-NH2 exposure; (C1), TRPA1-positive

neurons in control culture; (C2), TRPA1-positive neurons after SL-NH2 exposure; (D1), PAR2-positive neurons in control culture; (D2), PAR2-positive neurons after SL-NH2 exposure. Scale bar = 100 μ m. E, Quantification of the percentage of P2X3-, TRPV1-, TRPA1-, and PAR2-positive DRG neurons in vitro. n=5, mean \pm SEM, *P<0.001.

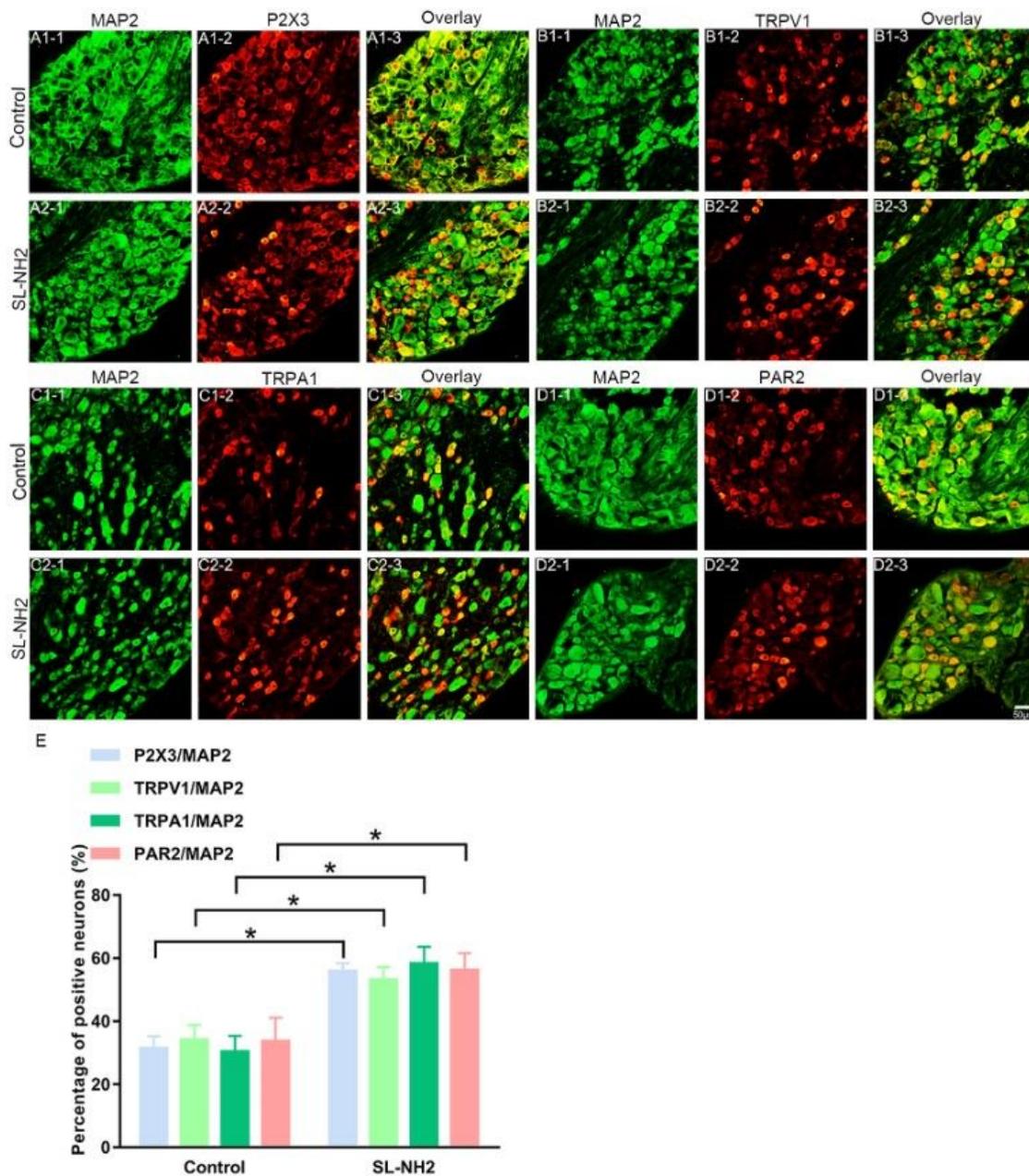


Figure 3

P2X3-, TRPV1-, TRPA1-, and PAR2-positive neurons in DRG from SL-NH2-treated rats. (A1), P2X3-positive neurons in control group; (A2), P2X3-positive neurons after SL-NH2 treatment; (B1), TRPV1-positive neurons in control group; (B2), TRPV1-positive neurons after SL-NH2 treatment; (C1), TRPA1-positive neurons in control group; (C2), TRPA1-positive neurons after SL-NH2 treatment; (D1), PAR2-positive neurons in control group; D2, PAR2-positive neurons after SL-NH2 treatment. Scale bar = 50 μ m. E,

Quantification of the percentage of P2X3-, TRPV1-, TRPA1-, and PAR2-positive DRG neurons in vivo. n=5, mean \pm SEM, *P<0.001.

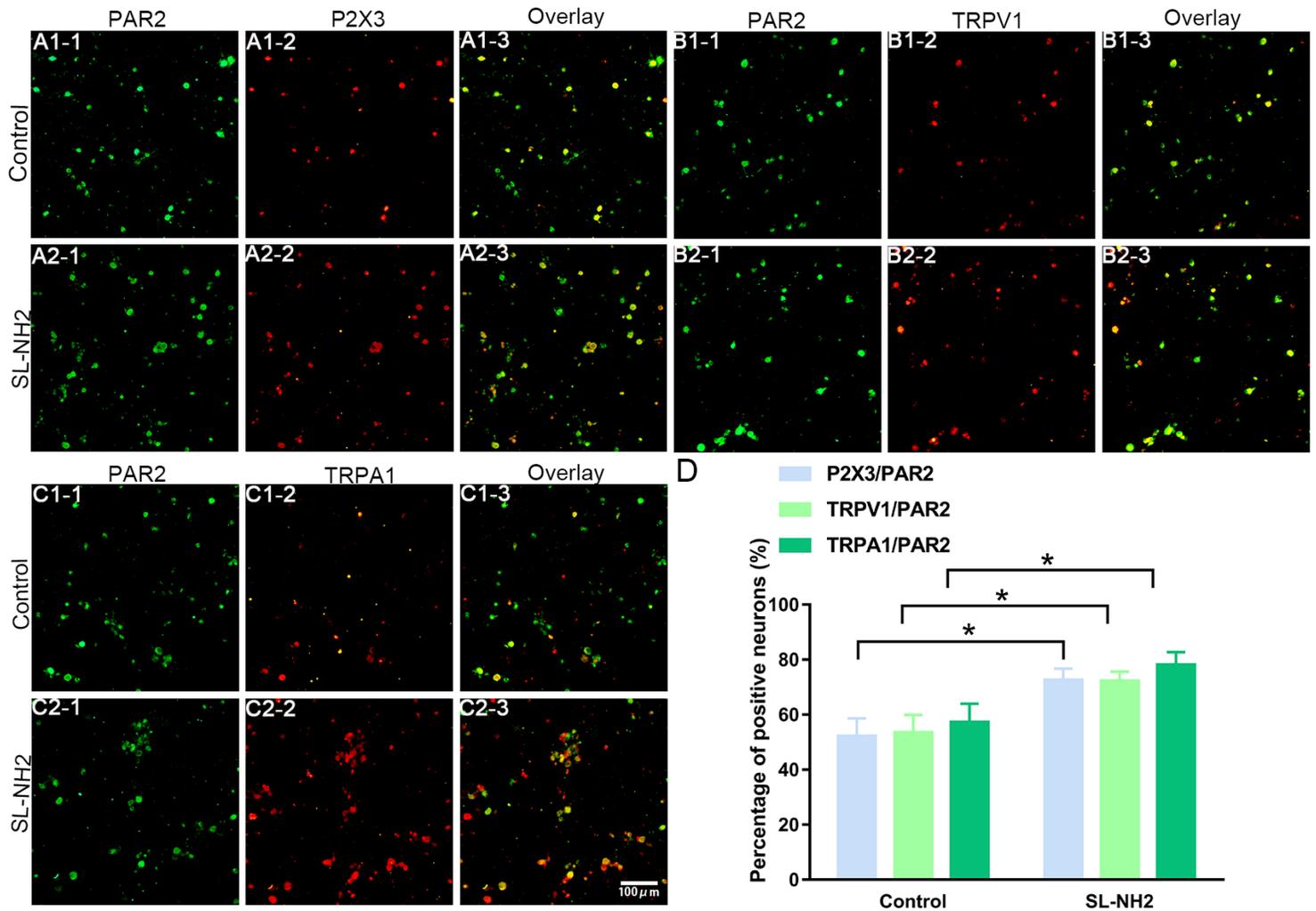


Figure 4

P2X3-, TRPV1-, and TRPA1-positive neurons in PAR2-expressing neurons in culture after SL-NH2 exposure. (A1), P2X3-positive neurons in control culture; (A2), P2X3-positive neurons after SL-NH2 exposure; (B1), TRPV1-positive neurons in control culture; (B2), TRPV1-positive neurons after SL-NH2 exposure; (C1), TRPA1-positive neurons in control culture; (C2) TRPA1-positive neurons after SL-NH2 exposure. Scale bar = 100 μ m. (D), Quantification of the percentage of P2X3-, TRPV1-, and TRPA1-positive neurons in PAR2-expressing neurons in vitro. n=5, mean \pm SEM, *P<0.001.

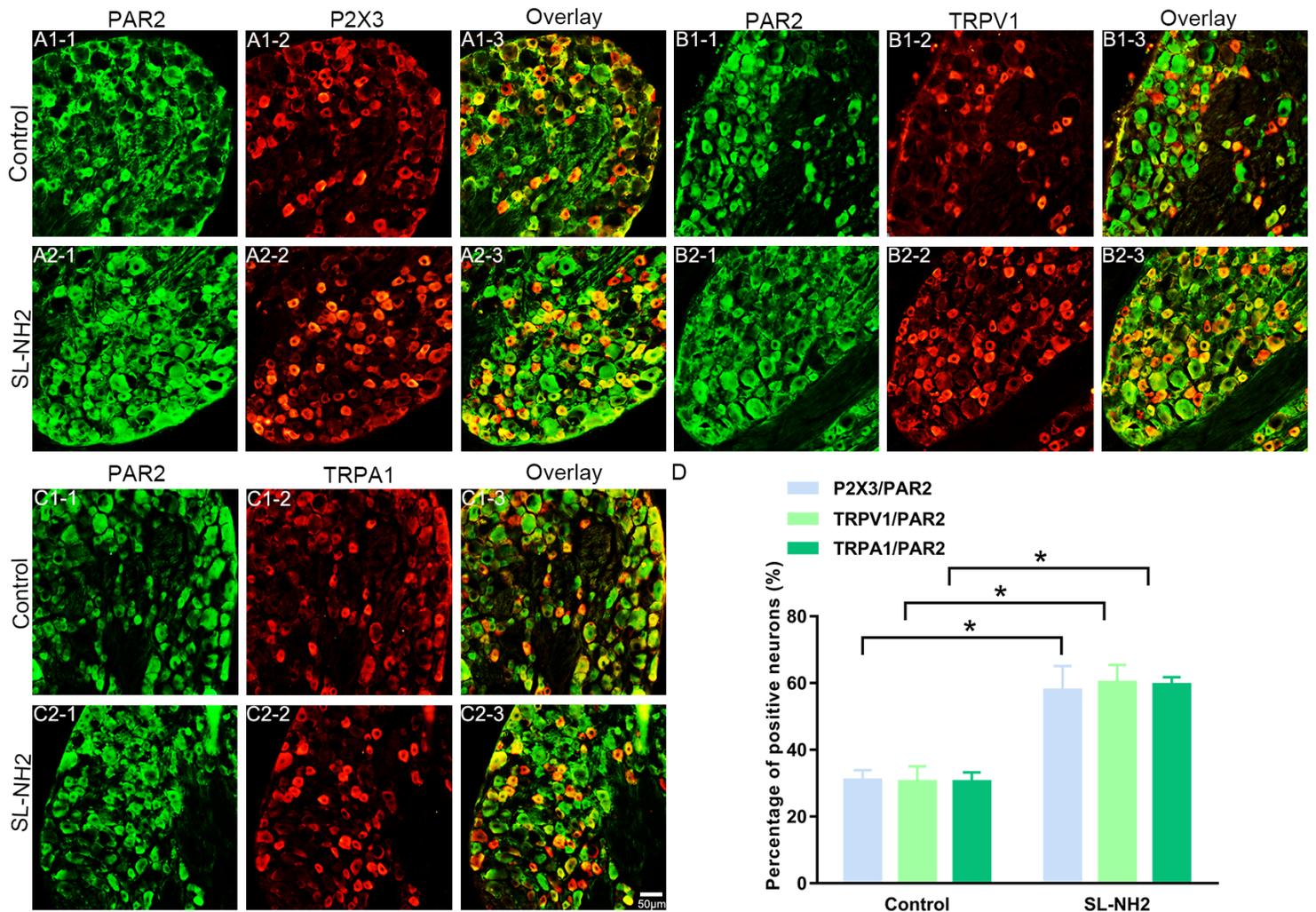


Figure 5

P2X3-, TRPV1-, and TRPA1-positive neurons in PAR2-expressing neurons in DRG from SL-NH2-treated rats. (A1), P2X3-positive neurons in control group; (A2), P2X3-positive neurons after SL-NH2 treatment; (B1), TRPV1-positive neurons in control group; (B2), TRPV1-positive neurons after SL-NH2 treatment; (C1), TRPA1-positive neurons in control group; (C2), TRPA1-positive neurons after SL-NH2 treatment. Scale bar = 50 μ m. (D), Quantification of the percentage of P2X3-, TRPV1-, and TRPA1-positive neurons in PAR2-expressing neurons in vivo. n=5, mean \pm SEM, *P<0.001.

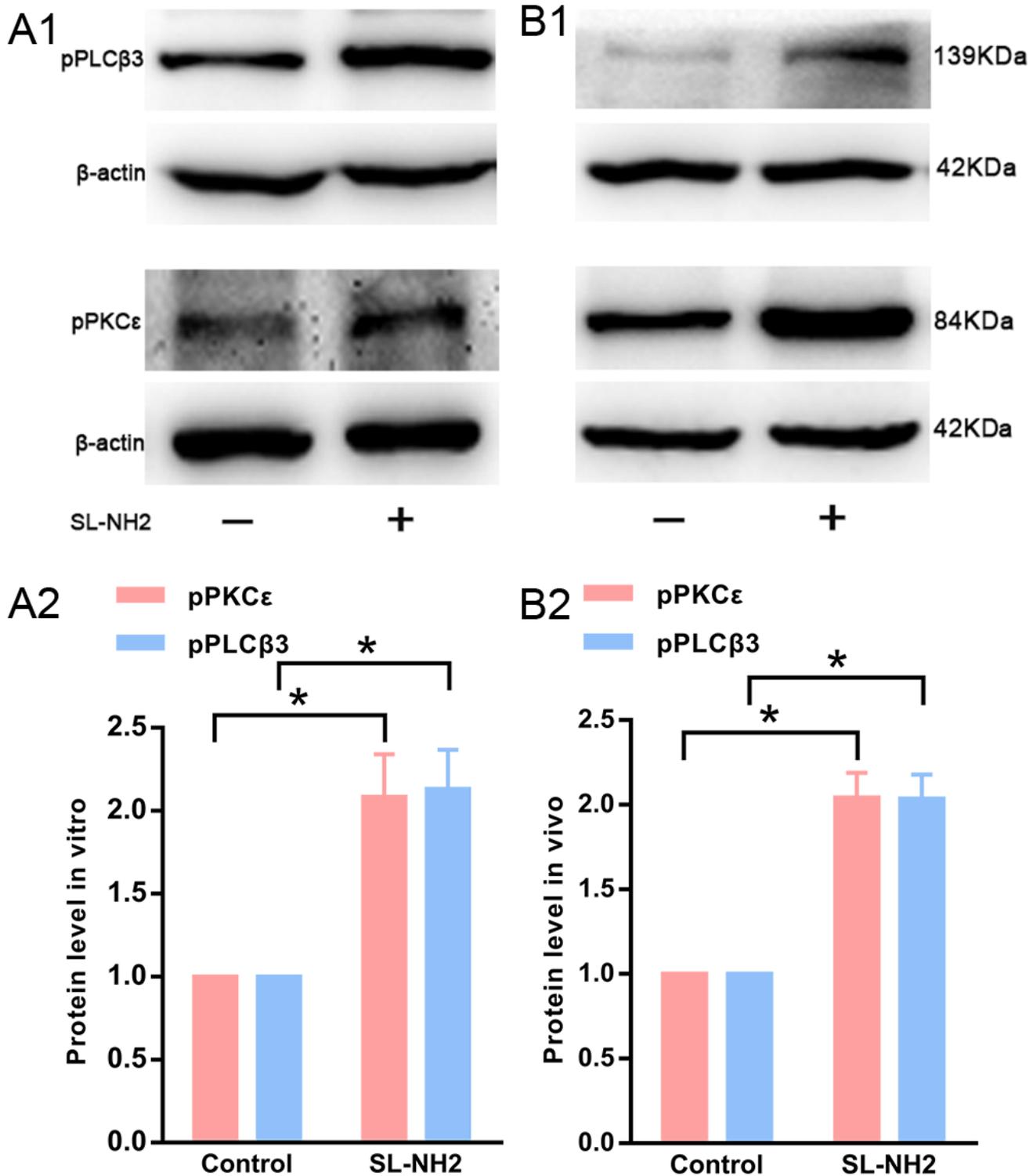


Figure 6

The protein levels of pPLCβ3 and pPKCε in DRG neurons after SL-NH2 treatment in vitro and in vivo. (A1), pPLCβ3 and pPKCε immunoreactive bands in vitro; (A2), Quantification of pPLCβ3 and pPKCε protein levels in vitro; (B1), pPLCβ3 and pPKCε immunoreactive bands in vivo; (B2), Quantification of pPLCβ3 and pPKCε protein levels in vivo. n=5, mean ± SEM, *P<0.001.

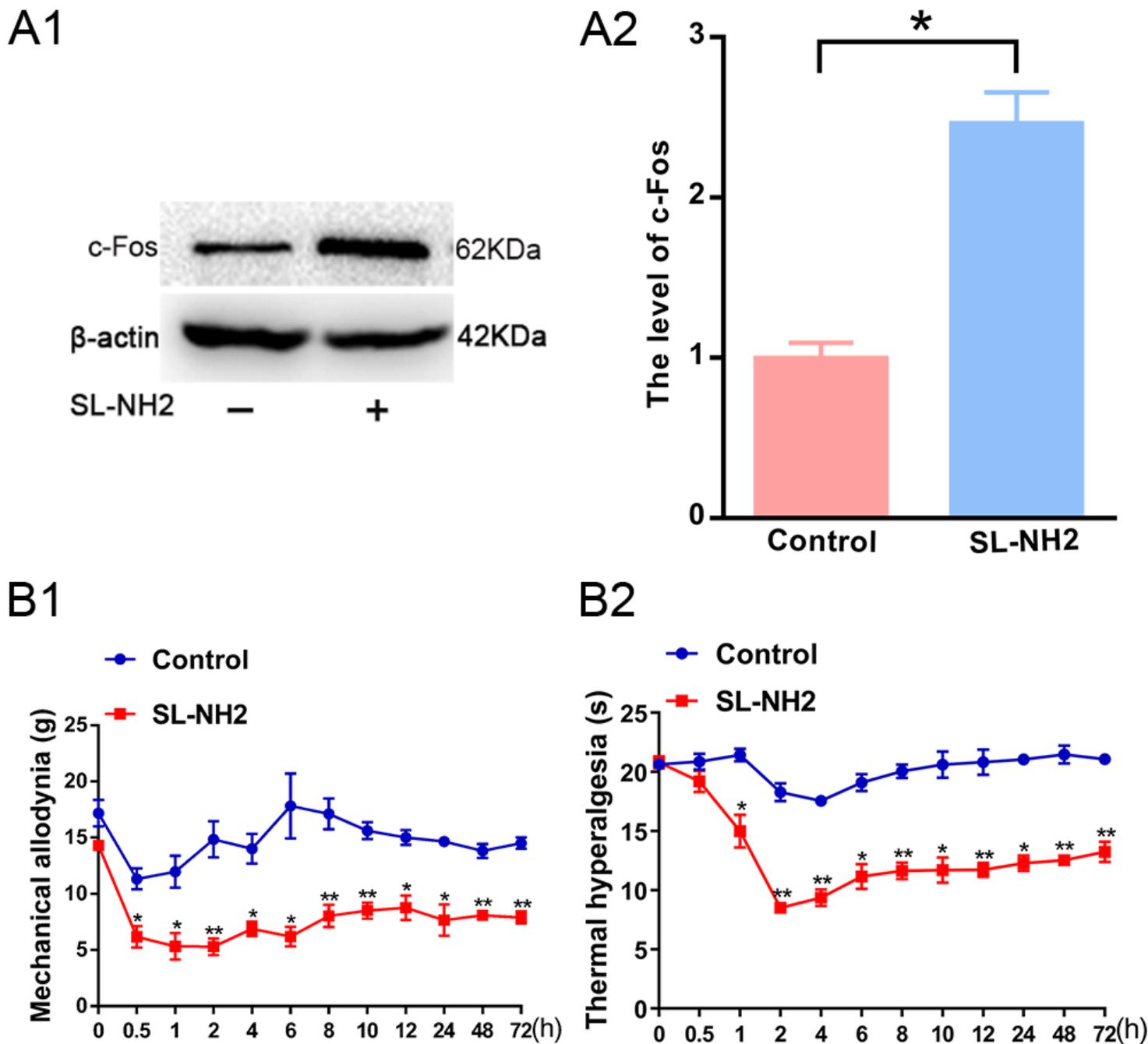


Figure 7

The alterations of mechanical and thermal thresholds related to c-Fos expression after SL-NH2 treatment in rats. (A1), c-Fos immunoreactive bands in vitro; (A2), Quantification of c-Fos protein levels in SDH; (B1), The mechanical thresholds after SL-NH2 treatment; (B2), The thermal thresholds after SL-NH2 treatment. n=15, mean \pm SEM, *P<0.001, **P<0.0001.

Supplementary Files

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