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Reduced acute nociception and chronic pain in *Shank2*$^{-/-}$ mice

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Abstract

Autism spectrum disorder is a debilitating mental illness and social issue. Autism spectrum disorder patients suffer from social isolation, cognitive deficits, compulsive behavior, and sensory deficits, including hyposensitivity to pain. However, recent studies argued that autism spectrum disorder patients show physiological pain response and, in some cases, even extremely intense pain response to harmless stimulation. Recently, *Shank* gene family was reported as one of the genetic risk factors of autism spectrum disorder. Thus, in this study, we used *Shank2*$^{-/-}$ (Shank2 knock-out, KO) mice to investigate the controversial pain sensitivity issue and found that Shank2 KO mice showed reduced tactile perception and analgesia to chronic pain.

Keywords

Autism, pain, shank2, nociception

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Introduction

Autism spectrum disorder (ASD) is a mental disorder but also social issue. ASD patients show impairment in social activities, cognitive deficits, repetitive/compulsive behavior, and sensory deficits. Related to sensory deficits, although there are some debates on this issue, ASD patients have been reported to have a high sensitivity threshold to several noxious stimuli. Many case studies described children with ASD who did not express pain when physically hurt or injured.[1,2] In addition to patients' self-reports, parents reports and clinical observations also described ASD patients showing a hyposensitivity to painful stimulation.[1,2] However, recent studies challenged the notion of low pain sensitivity in ASD patients.[3,4] For example, some case studies reported ASD children strongly complaining of abdominal pain (sometimes even extreme pain) caused by a mild tactile stimulation such as having their haircut. Clinical examinations also reported that ASD children undergoing venipuncture experienced the same level of pain as non-ASD children did.[5,6] It is plausible that ASD children actually do feel pain, but they just do not express it. Thus, it is important to clarify whether ASD patients have sensory deficits, especially low pain sensitivity.

Mutations in the *Shank* genes have been reported as one of the genetic factors, causing autism-like phenotypes in human and mice. Shank is a scaffolding protein mainly located in the postsynaptic density.[7] It is well known that Shank regulates structural change of spine and is involved in synaptic plasticity by controlling the function of N-methyl-D-aspartate receptor.[8,9] Recently, it was reported that in mice, the deletion of any major Shank isoform (Shank1, Shank2, or Shank3) induced autistic-like behaviors like reduced social interactions,

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repetitive behavior, and hyperactivity.[7,8,10,11] Thus, we used *Shank2*$^{-/-}$ (Shank2 knock-out, KO) mice to investigate whether low pain sensitivity is a real symptom in ASD patients.

Materials and methods

Animals

Male Shank2 KO ($n = 30$) and wild-type (WT) ($n = 29$) mice older than two months were used for all experiments. Although some of ASD phenotypes begin to appear in younger age, ASD phenotypes seem to be more obvious in adult Shank2 KO mice as we reported in previous work[8] and other similar works.[10,12] The animals were group housed in standard laboratory cages on a 12-h light–dark cycle with food and water available ad libitum. All the experiments were approved by the Institute of Laboratory Animal Resources of Seoul National University.

Behavioral tests

All behavioral tests were performed in the daytime. The investigator doing the tests was blind to the mice genotype.

Mechanical withdrawal threshold measurement. The mechanical withdrawal threshold was measured using an electronic von Frey apparatus (Dynamic Plantar Aesthesiometer, Ugo Basile). The mice were placed on the iron mesh of the apparatus at least 2 h before the experiment to allow them to become accustomed to the equipment. Briefly, an electronic von Frey tip was placed on the mouse left hind paw sole and increasing force was applied at a 0.5 g/s rate. The mechanical withdrawal threshold was measured as the force applied when the mouse withdrew his paw (g). The cut-off time was 20 s (10 g). The experiment was repeated five times with 10 min intervals between measurements. The maximum and minimum values are excluded, and the average of remained three values was used for mechanical threshold.

Hot plate test. The mice were placed in the behavior room at least 2 h before the test to allow them to become accustomed to the experimental apparatus. Briefly, the mice were placed on the hot (55°C) plate and the latency to their first reaction (licking, shaking, jumping, or lifting of the hind paw) was recorded manually. If the mouse did not show any response within 20 s, the test was terminated to avoid tissue damage and the latency to the response was recorded as 20 s. Three values were used for average of latency to response.

Tail flick test. The mice were placed in the behavior room at least 2 h before the test to allow them to become accustomed to the experimental apparatus. Briefly, the mouse tail was placed below a heat source. Heat was applied on the area around 1 cm from the tail tip, and the latency to the tail withdrawal reflex was recorded manually. If the mouse did not show any response within 20 s, the test was terminated to avoid tissue damage and the latency to the response was recorded as 20 s. The test was repeated three times with 10 min intervals between measurements. Three values were used for average of latency to response.

Allodynia response measurement. Mechanical allodynia response was measured on Day 0 (before chronic pain induction), Day 3, and Day 7 after chronic pain induction (complete Freund's adjuvant (CFA) dorsum injection or common peroneal nerve (CPN) ligation). The mice were allowed to become accustomed to the experimental equipment (a round bucket) for around 1 h before the test. Mechanical allodynia was assessed based on response to the application of von Frey filaments (Stoelting, Wood Dale, IL, USA). The filament was applied on the each hind paw dorsum while the animal was resting. Based on previous experiments, we used 1.65 filaments.[13] Positive responses included licking, biting, or sudden withdrawal of the hind paw. The test was repeated every 5 min for nine times, and the results were expressed as the percentage of positive responses.

Surgery and CFA injection. The surgical procedure used to induce neuropathic pain was substantially based on the previous report.[14] Briefly, the mice were anesthetized with a ketamine/xylazine mixture in saline. Their eyes were protected by a coating of artificial tear jelly. The left leg was shaven using scissors and sterilized with 70% alcohol and povidone iodine solution. About 1 cm of the left thigh skin was cut to expose the muscles. Then, an incision was made in the muscle using scissors, and sterile saline was applied to the exposed region. CPN was ligated with a wax-coated braided suture 4–0 without disturbing the blood vessels. Next, the ligature was slowly tightened until twitching of the dorsiflexors of the foot became visible at the toes. The ligature was then tied with a knot and the skin was sutured using a 5–0 silk suture and cleaned with povidone iodine solution. To induce inflammatory pain, 50% CFA in saline (10 µl, Sigma, St. Louis, MO, USA) was injected subcutaneously in the dorsum (for the allodynia response test) or sole (for the mechanical withdrawal threshold measurement) of the left hind paw using a Hamilton syringe, carefully avoiding leakage of the injected solution.

Statistical analysis

The basal mechanical withdrawal threshold and withdrawal latency in the tail flick and hot plate test were analyzed by unpaired *t* test. Allodynia response was analyzed using repeated measure two-way ANOVA with Bonferroni post hoc test. One-way ANOVA was used for analysis of allodynia response in Shank2 KO mice which were injected with CFA in the dorsum. Data are presented as mean ± standard error of the mean, unless otherwise stated.

Results

To study Shank2 KO mice pain sensitivity, we measured the basal mechanical threshold and the responses to acute and chronic pain (Figure 1(a)). First, we examined whether Shank2 KO mice have a physiologic tactile perception by measuring their basal mechanical withdrawal threshold. Shank2 KO mice showed significantly higher withdrawal threshold to the application of increasing force on their paw than WT control mice (Figure 1(b)). Moreover, in Shank2 KO mice, acute pain perception was impaired. In the hot plate test, latency to the response was delayed in Shank2 KO mice compared to WT mice (Figure 1(c)). In addition, similar results were observed in the tail flick test (Figure 1(d)). These findings revealed that Shank2 KO mice have impaired basal tactile perception and acute pain response.

Next, we examined Shank2 KO mice chronic pain response using neuropathic or inflammatory pain models. In the neuropathic pain model, we ligated the CPN; in the inflammatory pain model, we injected the CFA in the left hind paw sole or dorsum. Three days after measuring the basal mechanical threshold and acute pain response, the mice were divided into three groups. In one group, the basal mechanical withdrawal threshold was measured again before chronic inflammatory pain was induced (Day 0) via CFA sole injection. Allodynia response was measured in the remaining mice before chronic pain was induced (Day 0) via CFA dorsum injection (inflammatory pain) or CPN ligation (neuropathic pain) (Figure 1(a)). Pain response was retested at Day 3 and Day 7 after chronic pain induction. Shank2 KO mice did not show any difference in the mechanical withdrawal threshold compared to WT mice on Day 3 and Day 7. CFA sole injection reduced the mechanical withdrawal threshold in both Shank2 KO and WT mice comparably (Figure 2(a)).

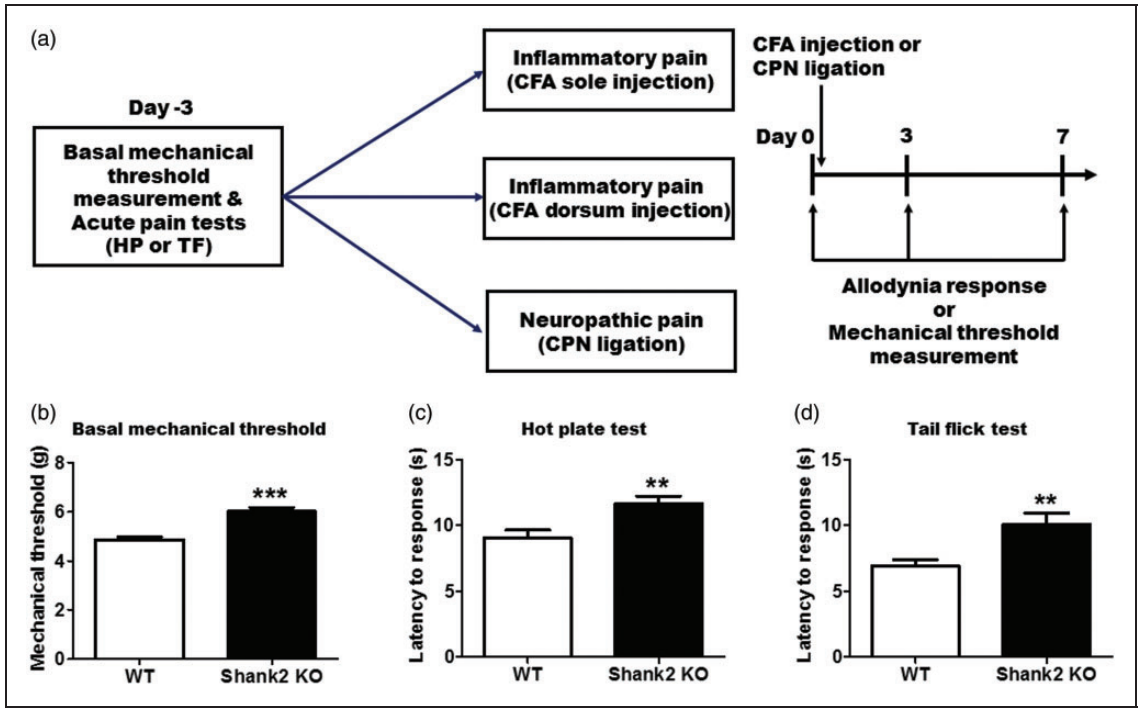

Figure 1. Shank2 KO mice show impaired basal tactile perception and acute pain response. (a) Experimental schedule to evaluate pain response in Shank2 KO mice. HP, hot plate; TF, tail flick. (b) Measurement of basal mechanical withdrawal threshold in Shank2 KO mice. Increasing force was applied on the left hind paw using electronic von Frey filaments and the latency to the withdrawal response was measured automatically ($n = 29$ for WT, $n = 30$ for Shank2 KO, unpaired t-test, $t_{57} = 5.635$, *** $p < 0.001$). (c, d) Acute pain perception in Shank2 KO mice was evaluated by the hot plate (c) and tail flick (d) tests (hot plate test, $n = 10$ for each group, unpaired t-test, $t_{18} = 3.134$, ** $p < 0.01$; tail flick test, $n = 19$ for WT, $n = 21$ for Shank2 KO, unpaired t-test, $t_{38} = 3.092$, ** $p < 0.01$).

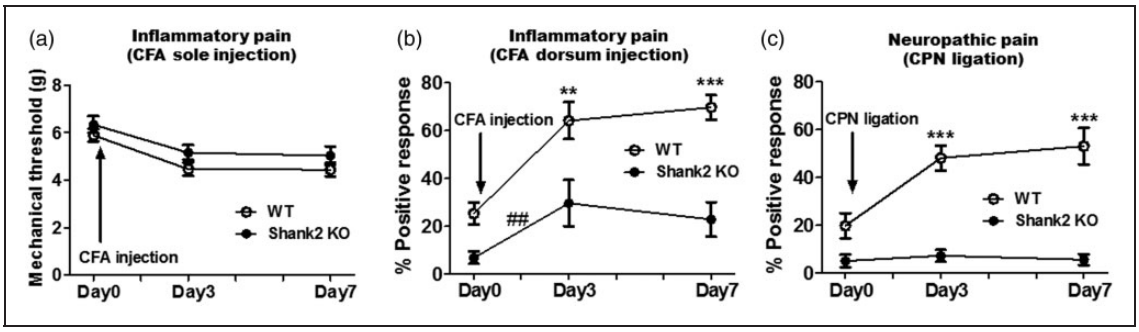

Figure 2. Shank2 KO mice show impaired induction of chronic pain. (a) CFA was injected in the left hind paw sole after measuring basal mechanical withdrawal threshold (Day 0). Thresholds were reduced at Day 3 and Day 7 after CFA injection in both WT and Shank2 KO groups; however, there was no significant difference ($n = 10$ for WT, $n = 9$ for Shank2 KO; repeated measure two-way ANOVA: effect of time, $F_{(2,36)} = 15.95$, $p < 0.001$; effect of genotype, $F_{(1,36)} = 3.456$, $p > 0.05$; effect of interaction, $F_{(2,36)} = 0.1038$, $p > 0.05$). (b) After measuring basal allodynia response (Day 0), CFA was injected in the left hind paw dorsum and again allodynia response was measured at Day 3 and Day 7 after CFA injection ($n = 9$ for each group, repeated measure two-way ANOVA followed by Bonferroni posttest: effect of time, $F_{(2,16)} = 31.73$, $p < 0.001$; effect of genotype, $F_{(1,16)} = 18.34$, $p < 0.001$; effect of interaction, $F_{(2,16)} = 5.165$, $p < 0.05$, posttest ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA followed by Tukey's multiple comparison test for Shank2 KO, $F_{(2,16)} = 6.306$, $p < 0.01$, posttest ## $p < 0.01$). (c) After measuring basal allodynia response (Day 0), the left CPN was ligated to induce neuropathic pain and allodynia response was measured again at Day 3 and Day 7 after CPN ligation ($n = 9$ for WT, $n = 10$ for Shank2 KO; repeated measure two-way ANOVA followed by Bonferroni posttest: effect of time, $F_{(2,34)} = 11.27$, $p < 0.001$; effect of genotype, $F_{(1,34)} = 58.74$, $p < 0.001$; effect of interaction, $F_{(2,34)} = 9.696$, $p < 0.001$, posttest *** $p < 0.001$). Interestingly, Shank2 KO mice showed decreased allodynia response after CPN ligation.

However, allodynia response after CFA dorsum injection was increased only in WT mice. Although Shank2 KO mice showed increased allodynia response after CFA dorsum injection compared to baseline, they showed reduced allodynia response compared to WT mice (Figure 2(b)). Finally, we examined Shank2 KO mice response to chronic neuropathic pain induction. Again, Shank2 KO mice showed reduced allodynia response at Day 3 and Day 7 after CPN ligation, whereas in WT mice, allodynia response significantly increased compared to baseline (Day 0) (Figure 2(c)). Taken together, our results indicate that Shank2 KO mice have decreased sensitivity to chronic pain.

Discussion

In this study, we demonstrated that Shank2 KO mice have impaired basal tactile sensitivity and acute pain response. Furthermore, Shank2 KO mice have reduced sensitivity to chronic neuropathic and inflammatory pain. In Shank2 KO mice, Shank2 exons 6 and 7 are deleted in the whole body. Thus, the altered pain response in Shank2 KO mice could be caused by defects in the central or peripheral nervous system. For example, the impaired basal tactile perception and delayed acute pain response in Shank2 KO mice may result from synaptic dysfunctions in the spinal cord, rather than in the brain. However, defects in the brain such as anterior cingulate cortex and somatosensory cortex can cause these impaired pain responses in Shank2 KO mice.[15,16] Shank2 is expressed in the nervous system, including spinal cord and brain (source: GeneCards, GCID: GC11M070467). Dysfunctions in the N-methyl-D-aspartate receptor-mediated synaptic transmission in the spinal cord can cause deficits in tactile perception and acute pain response.[17,18] On the other hand, it is well known that in many cases ASD children also have attention deficit problems such as ADHD;[19] therefore, it is possible that the altered tactile perception and pain response may stem from this issue. Further studies will be required to reveal how Shank2 regulates pain response at the spinal cord and brain levels and whether other ASD genetic risk factors also cause deficits in pain sensitivity.

Authors' contributions

H-GK carried out all the experiments and outlined the manuscript, designed the studies, and wrote the manuscript. S-BO, B-KK, and MZ supervised the experiments, participated in the interpretation of data, and wrote the manuscript. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

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