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Intracerebroventricular treatment with resiniferatoxin and pain tests in mice

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TITLE:

Intracerebroventricular Treatment with Resiniferatoxin and Pain Tests in Mice

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KEYWORDS:

transient receptor potential vanilloid type 1 (TRPV1), resiniferatoxin, intracerebroventricular injection, nociception, RTX test, tail pressure test, acetaminophen

SUMMARY:

The transient receptor potential vanilloid type 1 (TRPV1) in the supraspinal region has been suggested to play some roles in the brain function. Described here is a protocol for intracerebroventricular injection of resiniferatoxin for supraspinal TRPV1 desensitization in mice. Procedures for some pain tests are also presented.

LONG ABSTRACT:

The transient receptor potential vanilloid type 1 (TRPV1), a thermosensitive cation channel, is known to trigger pain in the peripheral nerves. In addition to its peripheral function, its involvement in brain functions has also been suggested. Resiniferatoxin (RTX), an ultrapotent TRPV1 agonist, has been known to induce long-term desensitization of TRPV1, and this desensitization has been an alternative approach for investigating the physiological relevance of TRPV1-expressing cells. Here we describe a protocol for intracerebroventricular (i.c.v.) treatment with RTX in mice. Procedures are described for testing nociception to peripheral TRPV1 stimulation (RTX test) and mechanical stimulation (tail pressure test) then follow. Although the nociceptive responses of mice that had been administered RTX i.c.v. were comparable to those of the control groups, RTX-i.c.v.-administered mice were insensitive to the analgesic effect of acetaminophen, suggesting that i.c.v. RTX treatment can induce supraspinal-selective TRPV1 desensitization. This mouse model can be used as a convenient experimental system for studying the role of TRPV1 in brain/supraspinal function. These techniques can also be applied to studies of the central actions of other drugs.

INTRODUCTION:

Animals receive various physical and chemical stimuli from their environment through sensors on the peripheral nerves. The transient receptor potential vanilloid type 1 (TRPV1) is one of the thermosensitive, nonselective cation channels that act as heat sensors[1,2], and activation and/or modulation of TRPV1 is known to be a key step for nociception in both normal and inflammatory contexts[3]. Although the overall expression pattern is controversial, expression of TRPV1 has also been suggested in supraspinal regions, being involved in various brain activities (including nociception[4], thermoregulation[5], anxiety[6], attention deficit hyperactivity disorder[7], and epilepsy[8]). Moreover, it has recently been suggested that acetaminophen, a widely used painkiller, mediates the activation of central TRPV1 to elicit its analgesic action[9,10].

Administration of excess TRPV1 agonist including capsaicin and resiniferatoxin (RTX) to animals leads to the death of TRPV1-positive neurons and long-lasting desensitization to TRPV1 agonists[11,12]. Combined with the local application (intrathecal[13,14], intracisternal[15,16,17], and intraganglional[18]), this chemical ablation approach has provided an alternative way to investigate the physiological functions of TRPV1. We have recently reported that intracerebroventricular (i.c.v.) injection of RTX inhibits the analgesic effect of acetaminophen in mice, suggesting supraspinal-selective TRPV1 desensitization[19]. In this manuscript, we present the precise protocol for i.c.v. injection and subsequent pain tests.

Direct injection of drugs into the ventricles of the brain makes it possible to study their central effects while minimalizing any peripheral effects. The i.c.v. injection procedure presented here is a modification of the method reported by Haley and McCormick[20]. This method is the simple involving insertion of an injection needle into the lateral ventricles through the coronal suture and does not require any special equipment or surgical procedures for cannulation.

Peripheral local application of TRPV1 agonists evokes a burning pain sensation and neurogenic inflammation. Mice that are systemically treated with RTX, and TRPV1-KO mice, are insensitive to this stimulation[13]. We have performed intraplantar injection of RTX (RTX test) to confirm the preservation of peripheral TRPV1 in RTX-i.c.v. mice. This method is a modification of the conventional formalin test[21].

It has been reported that mice systemically treated with RTX and TRPV1-KO mice show a normal threshold to mechanical stimuli[11,13,22]. Here we present a procedure for the tail pressure test for testing changes in the analgesic effect of acetaminophen.

All of these procedures are orthodox and versatile, and can be applied to studies of other drugs.

PROTOCOL:

All of the experimental protocols used here were approved by the Animal Care and Use Committee of Musashino University. Male ddY mice (SLC, Shizuoka, Japan) were kept for at least 7 days under a 12-h light/dark cycle before experiments with water and food ad libitum. 5- or 6-week-old mice were used for the experiments.

1. Preparation of Drugs

1.1. RTX

Note: Alcoholic RTX solution can cause severe skin burns and eye damage. Make sure to use rubber gloves and glasses for protection when handling. This stock solution can be used for 6 months.

1.1.1. Add 500 µL of ethanol to 1 mg of RTX.

1.1.2. Add 500 µL of polyoxyethylene (20) sorbitan monooleate to the solution above and vortex well.

1.1.3. Add 4 mL of physiological saline to the mixture and vortex well.

1.1.4. Aliquot 40 µL of the solution into 1.5-mL screw cap tubes, and store them at -40 °C.

1.2. Acetaminophen

1.2.1. Add 20% w/v propyleneglycol solution to acetaminophen at a concentration of 30 mg/mL, and dissolve with a sonicator. Since acetaminophen may precipitate at room temperature several hours after dissolution, prepare just before use or keep the solution warm until use.

2. Subcutaneous or Intracerebroventricular Injection of RTX

2.1. Thaw the stocked solution prepared in 1.1. above and dilute it to 20 µg/mL in saline or artificial cerebrospinal fluid (ACSF) consisting of (in mM): 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 11 glucose, 1.3 $MgSO_4$, 2.5 $CaCl_2$ equilibrated with 95% O_2 and 5% CO_2 (pH 7.2).

2.2. Anesthetize mice with pentobarbital sodium salt (60 mg/kg, intraperitoneally), and check for loss of the righting reflex.

2.3. For s.c. treatment, inject RTX (20 µg/mL) into the back of the neck at a volume of 0.1 mL/10 g body weight. For the control group, inject the vehicle (10% ethanol, 10% polyoxyethylene (20) sorbitan monooleate and 80% saline) in the same way.

2.4. For i.c.v. treatment, inject 5 µL of RTX (20 µg/mL) into the right lateral ventricle. For the control group, inject the vehicle (10% ethanol, 10% polyoxyethylene (20) sorbitan monooleate and 80% ACSF) in the same way.

2.4.1. Pass a disposable 27-G needle through a metal tube (0.8 mm I.D.) to expose the 3.0-3.5 mm tip of the needle (**Figure 1A**).

2.4.2. Hold the squamosal bones of the mouse firmly with the fingers (**Figure 1B**).

Note: Pay attention to the positions of the squamosal protrusions, since these protrusions will serve as landmarks for injection.

2.4.3. Move the needle laterally on the scalp, and find the sagittal suture as the needle tip is hooked on the suture.

2.4.4. Move the tip about 1 mm to the right, then move the tip rostrally, and find the coronal suture as with 2.4.3. (**Figure 1B**).

2.4.5. Insert the needle slowly and vertically, inject the RTX solution in about 10 seconds, and hold it for about 10 seconds.

2.4.6. Withdraw the needle slowly, and return the mouse to its home cage. Bleeding is usually minimal or absent. If major bleeding occurs, use of another mouse should be considered.

2.5. Assign the pretreated mice as subjects for the RTX test or the tail pressure test (Step 3 and 4, respectively).

3. RTX Test

Note: Testing is performed between 10:00 AM and 5:00 PM. The testing room is maintained at 200 lux and 24-26 °C.

3.1. One week after pretreatments with RTX (Step 2.), transfer mice to the testing room at least 60 min prior to starting the test.

3.2. Weigh and place each mouse individually in a plexiglass cage (29.5 × 17.5 × 13.5 cm^3 height) at least 30 min prior to starting the test in order to allow it to acclimate to the environment.

Note: The order of tests should be counterbalanced across pretreatment groups.

3.3. Administer acetaminophen (300 mg/kg) to the mouse intraperitoneally 20 min before the test.

3.4. Hold the mouse loosely in a small cloth bag, and insert a 30-gauge needle into the heel of the right hind paw. Advance the needle subcutaneously to near the walking pads, and inject 20 µL of RTX solution (0.05 µg/mL).

3.5. Measure the period of licking/biting behavior in the glabrous region of the affected paw in each 5-min block.

4. Tail Pressure Test

Note: A Randall-Selitto-type pressure meter is used to assess the threshold for acute mechanical nociception. Testing is performed between 10:00 AM and 5:00 PM. The testing room is maintained at 200 lux and 24-26 °C.

4.1. One week after pretreatments with RTX (Step 2.), transfer mice to the testing room, and weigh and place each mouse individually in a plexiglass cage.

4.2. Mark the spots at 1.5 and 2.5 cm from the base of the tail.

4.3. Hold the mouse loosely in a small cloth bag, and apply pressure to the spots with a blunt probe.

Note: A cutoff pressure of 250 g is imposed to avoid tissue damage.

4.4. Determine the pressure required to elicit escape behavior (tail whisking, twisting, and squeaking), and calculate the nociceptive threshold by averaging the pressure determined at the two spots.

4.5. Repeat steps 4.3. to 4.4. every 15 min.

4.6. After obtaining the baseline, administer acetaminophen (300 mg/kg) to the mouse intraperitoneally. After administration, repeat steps 4.3. and 4.4 every 15 min.

REPRESENTATIVE RESULTS:

The i.c.v.-treated mice show no apparent abnormalities in their appearance, spontaneous activities, body weight[19] and core body temperature (Vehicle-treated group, 38.4 ± 1.3 °C, n = 6; RTX-treated group, 38.7 ± 0.2 °C, n = 6).

Figures 2A-B show the responsiveness of s.c.- or i.c.v.-treated mice to the intraplantar injection of RTX. The licking/biting behavior of vehicle-treated mice was remarkable in the first 10 min[19]. Although the s.c.-pretreated mice did not show licking/biting behavior at all, the i.c.v.-pretreated mice normally responded to the plantar injection of RTX. Moreover, as shown in **Figure 2B**, intraperitoneal administration of acetaminophen (300 mg/kg) reduced the licking/biting behavior of vehicle-i.c.v.-treated mice but not that of RTX-i.c.v.-treated mice.

Figure 2C shows the analgesic effects of acetaminophen (300 mg/kg) in the tail pressure test. Acetaminophen reduced the nociceptive response of vehicle-pretreated mice in both tests, but the analgesic effects of acetaminophen were inhibited in mice that were pretreated i.c.v. with RTX.

FIGURE AND TABLE LEGENDS:

Figure 1: Photographic and schematic views of i.c.v. injection. (A) Needle used for i.c.v.

injection. **(B)** Schema of the mouse skull and the movement of the needle tip. Squamosal bones are shown in blue.

Figure 2: Nociceptive responses of mice that were pretreated s.c. or i.c.v. with RTX. (A) Time course (left panel) and total time of licking/biting behavior (right panel) of s.c.-pretreated mice. RTX was injected into the plantar area at time zero (indicated by arrow head). **(B)** Time course (left panel) and total time of licking/biting behavior (right panel) of i.c.v.-pretreated mice. Either acetaminophen (300 mg/kg) or its vehicle (20% propyleneglycol) was administered intraperitoneally 20 min before intraplantar injection of RTX (indicated by arrow head). **(C)** Mechanical pain threshold in the tail of i.c.v.-pretreated mice and the analgesic effect of acetaminophen. All data were expressed as mean ± SEM. The number of mice in each group is shown in parentheses. The two-tailed Mann-Whitney U-test was used to compare the data for two groups. Differences at $P<0.05$ were considered to be significant. AcAP, acetaminophen; PG, propyleneglycol; n.s., not significant; i.pl., intraplantar injection. These figures have been modified from Fukushima et al[19].

DISCUSSION:

The most critical step in these experiments is the success of the i.c.v. injection. The i.c.v. injection technique used here is quite simple but requires some practice. Prior to experiments, practice with dyes (e.g. 0.5% trypan blue in saline) is recommended. If the injection is performed correctly, a needle mark should be evident on the coronal suture and the injected dye should be present in the contralateral ventricle and the third ventricle. Moreover, forcible insertion should be avoided during injection. If the needle tip is correctly placed on the coronal suture, the needle should penetrate the skull smoothly.

This i.c.v. technique can also be applied to awake, non-anesthetized mice, and we have reported the acute central effects of drugs examined using this technique[23,24]. Although the present procedure is advantageous in that no special equipment for cannulation is necessary, the i.c.v. injection can be performed only once. If repeated administration of drugs is required, cannulation is necessary.

The RTX test presented here is an easy-to-use approach for assessing the function of peripheral TRPV1[3,19]. Nociceptive behavior can be observed most prominently at a dose of 1-10 ng RTX and inhibited by co-injection of capsazepine, a TRPV1 antagonist[19,25]. In the formalin test some groups video-tape the experiments, but the post-hoc observation is often difficult because mice tend to cover the affected paw with the head and body. Therefore, experimenters in our laboratory observe and measure the licking/biting behavior directly. In this scenario, care should be taken not to disturb the mice. In addition, in pain tests, it is very important to sufficiently calm the mouse. Excessively strong gripping and a noisy environment could produce stress-induced analgesia and delay the nociceptive response.

Mice that are i.c.v.-pretreated with RTX show a normal nociceptive response in the RTX test and the tail pressure test. However, these mice are insensitive to the analgesic effects of acetaminophen, which has been suggested to mediate central TRPV1[9,10]. These results suggest

that supraspinal-selective TRPV1 desensitization can be induced in RTX-i.c.v. mice. Although TRPV1 desensitization has been performed with local application of agonists[13-18], supraspinal-selective desensitization has not yet been achieved. The RTX-i.c.v. injection protocols presented here will provide a convenient experimental model for studying the role of TRPV1 in supraspinal function.

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None.

DISCLOSURES:
The authors have no conflicts of interest to declare

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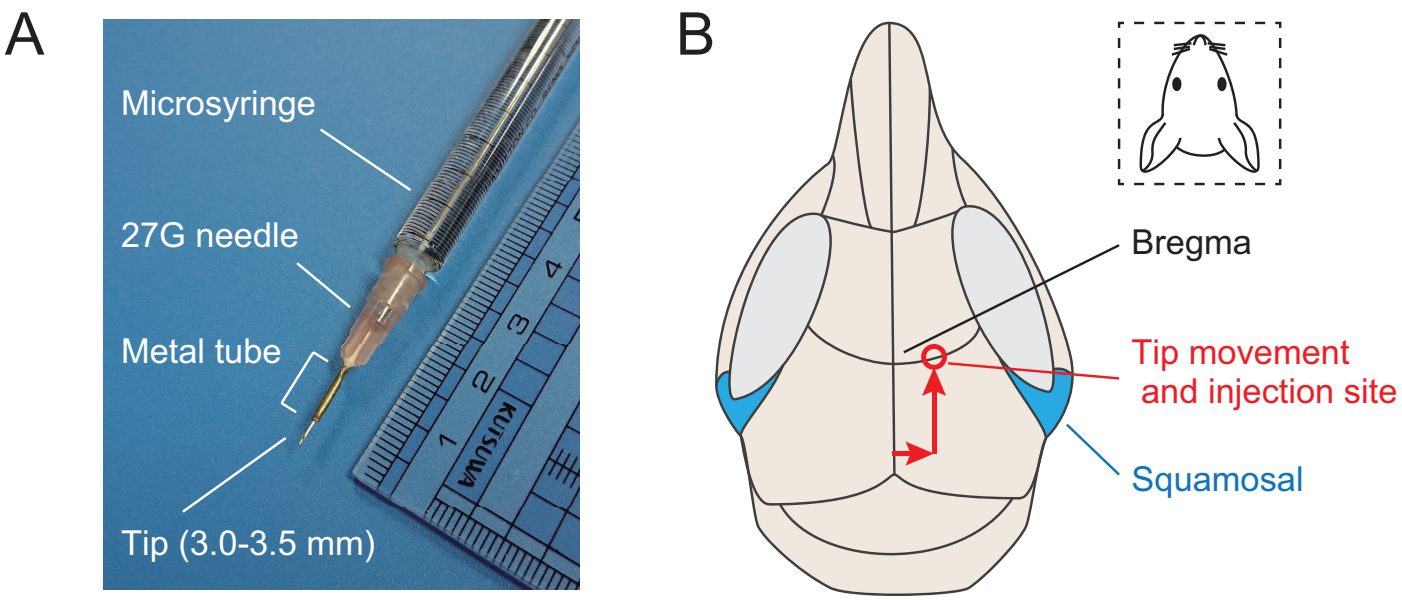

Figure 1

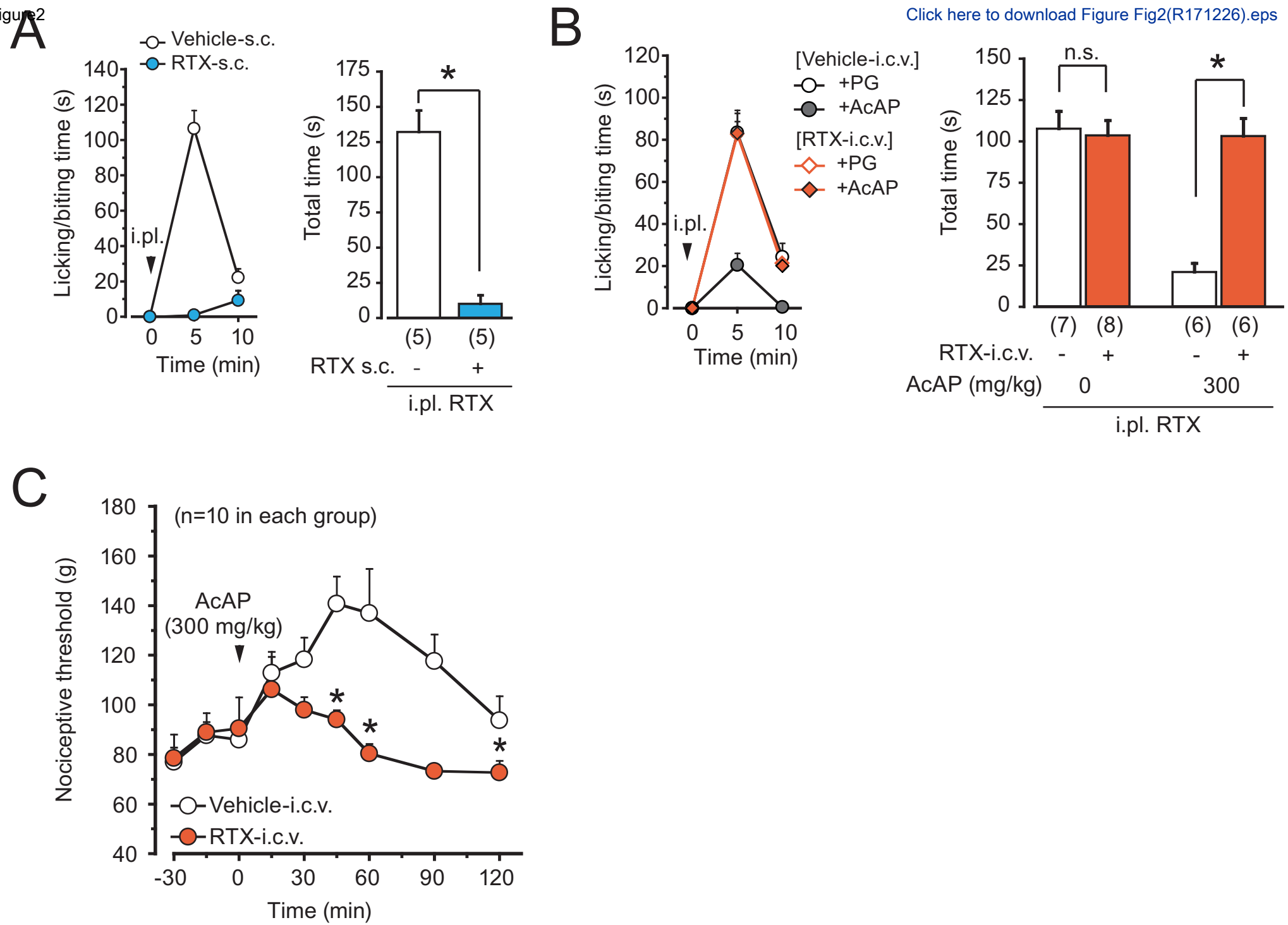

Figure 2

Materials Table

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Name of Material/ Equipment	Company	Catalog Number
Resiniferatoxin	LKT Laboratories	R1774
Acetaminophen	IWAKI SEIYAKU	
Pentobarbital sodium salt	Tokyo Chemical Industry	P0776
Ethanol (99.5)	Wako Pure Chemical Industries	057-00456
Polyoxyethylene(20) Sorbitan Monooleate	Wako Pure Chemical Industries	161-21621
25 mL microsyringe	Hamilton	1702LT
100 mL microsyringe	Hamilton	1710LT
26-gauge disposable needle	TERUMO	NN-2613S
30-gauge disposable needle	NIPRO	01134
Pressure meter	Ugo Basile	Analgesy-Meter Type 7200

Comments/Description

used for s.c./i.c.v. pretreatments and the RTX test

gifted from IWAKI SEIYAKU
used for anesthesia

used for dissolving RTX

used for dissolving RTX

used for i.c.v. injection
used for intraplantar injection
used for i.c.v. injection
used for intraplantar injection

used for tail pressure test

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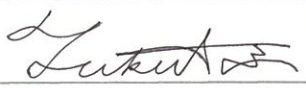
Signature:	 Date: December 28th, 2017

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Responses to editor and reviewers

We take this opportunity to express our gratitude to the editor and the reviewers for their constructive and useful remarks.

Replies to editorial comments:

We thank you very much for taking your time in evaluating our manuscript. Changes we have made and answers to the editorial comments are marked in green in the revised manuscript.

Comment #1

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Reply #1

We have thoroughly proofread the manuscript.

Comment #2

Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi: DOI (YEAR).] For more than 6 authors, list only the first author then et al.

Reply #2

Page numbers of nine references have now been corrected (Refs 3, 5, 11, 14, 15, 18, 20, 23 and 25).

Comment #3

Please define all abbreviations before use.

Reply #3

Lines 31 and 34 in the revised manuscript: Definitions of “RTX” and “i.c.v.” have been added.
Line 51 in the revised manuscript: The word “ADHD” has been changed to “attention deficit hyperactivity disorder”.

Comment #4

Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

Reply #4

Comments/Descriptions have been added in the revised table.

Comment #5

Figure 2: Please change second (sec) to s. Panel 1 A second graph, y-axis, is it sec or min?

Reply #5

> Please change second (sec) to s.

In Figs.2A-D, the word “*sec*” has now been changed to “*s*”.

> Panel 1 A second graph, y-axis, is it sec or min?

As mentioned in figure legends in the former manuscript (lines 214 and 216), the y-axes of second panels of Fig 2A,B indicate total time spent licking/biting during the first 10 minutes of the RTX test. In the revised manuscript, these sentences have been revised (please see also Reply #7 below). We have revised the axis labels.

Comment #6

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Reply #6

Please refer to the attached document concerning copyright permission. We have revised the sentence on the last line of the legend for Figure 2 (line 234-235 in the revised manuscript).

Comment #7

Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 29, 37, 44, 115, 117, 162, 164, 184-186, 200, 213-214, 216-217, 246-248.

Reply #7

As listed below, we have revised the sentences that you pointed out.

Line 29 in the former manuscript:

"*The transient receptor potential vanilloid type 1 (TRPV1) is a thermosensitive cation channel Although one of the major functions of TRPV1 is to trigger pain in peripheral nerves, its involvement in the brain has also been suggested.*"

> Lines 29-31 in the revised manuscript:

"*The transient receptor potential vanilloid type 1 (TRPV1), a thermosensitive cation channel, is known to trigger pain in peripheral nerves. In addition to its peripheral function, its involvement in brain functions has also been suggested.*"

Line 37 in the former manuscript:

"*Mice that had been i.c.v.-administered RTX showed normal nociceptive responses but were insensitive to the analgesic effect of acetaminophen,*"

> Lines 36-38 in the revised manuscript:

"*Although the nociceptive responses of mice that had been administered RTX i.c.v. were comparable to those of the control groups, RTX-i.c.v.-administered mice were insensitive to the analgesic effect of acetaminophen,*"

Line 44 in the former manuscript:

"*The transient receptor potential vanilloid type 1 (TRPV1) is a nonselective cation channel predominantly in primary afferents (especially C-fibers)1. This channel responds to several types of pain stimuli including heat (>43 °C)2, and activation and/or modulation of TRPV1 is known to be a key step for nociception in both normal and inflammatory contexts.*"

> Lines 45-49 in the revised manuscript:

"*Animals receive various physical and chemical stimuli from their environment through sensors on peripheral nerves. The transient receptor potential vanilloid type 1 (TRPV1) is one of the thermosensitive,*

nonselective cation channels that act as heat sensors, and activation and/or modulation of TRPV1 is known to be a key step for nociception in both normal and inflammatory contexts"

Line 115 in the former manuscript:
"*Pretreatment with resiniferatoxin*"
> Line 113 in the revised manuscript:
"*Subcutaneous or intracerebroventricular injection of RTX*"

Line 117 in the former manuscript:
"*One week before pain tests, mice were pretreated with RTX subcutaneously (s.c.) or intracerebroventricularly (i.c.v.).*"
> This sentence has been deleted in the revised manuscript (please see also Reply #9 below).

Line 162 in the former manuscript:
"*inject 20 µL of RTX solution (0.05 µg/mL) subcutaneously into the plantar surface of the right hindpaw using a 30-gauge needle*."
> Lines 170-172 in the revised manuscript:
"*and insert a 30-gauge needle into the heel of the right hindpaw. Advance the needle subcutaneously to near the walking pads, and inject 20 µL of RTX solution (0.05 µg/mL).*"

Line 164 in the former manuscript:
"*Measure the period of nociceptive behavior characterized by licking/biting of the affected paw in each 5-min block.*"
> Lines 174-175 in the revised manuscript:
"*Measure the period of licking/biting behavior in the glabrous region of the affected paw in each 5-min block.*"

Lines 184-186 in the former manuscript:
"*Determine the pressure required to elicit a response (tail whisking, twisting and squeaking). This pressure is defined as the nociceptive threshold. The mean of the two values is used for calculations.*"
> Lines 193-195 in the revised manuscript:
"*Determine the pressure required to elicit escape behavior (tail whisking, twisting and squeaking), and calculate the nociceptive threshold by averaging the pressure determined at the two spots.*"

Line 200 in the former manuscript:
"*the i.c.v.-pretreated mice showed licking/biting behavior comparable to that of vehicle-pretreated mice.*"
> Lines 209-210 in the revised manuscript:
"*the i.c.v.-pretreated mice normally responded to the plantar injection of RTX.*"

Lines 213-214 in the former manuscript:
"*Time course of licking/biting behavior of s.c.-pretreated mice evoked by i.pl. injection of RTX (left) and total time spent licking/biting during the first 10 minutes of the test (right).*"
> Lines 224-226 in the revised manuscript:
"*Time course (left panel) and total time of licking/biting behavior (right panel) of s.c.-pretreated mice.*

RTX was injected into the plantar area at time zero (indicated by arrow head)."

Lines 216-217 in the Former manuscript:
"*Analgesic effect of acetaminophen in the tail pressure test.*"
> Lines 230-231 in the revised manuscript:
"*Mechanical pain threshold in the tail of i.c.v.-pretreated mice and the analgesic effect of acetaminophen.*"

Lines 246-248 in the former manuscript:
"*Although it has been suggested that TRPV1 plays some roles in brain function25, brain-specific TRPV1-deficient mutants have not yet been generated. The present RTX-i.c.v. mouse model may have potential for investigating the physiological function of supraspinal TRPV1.*"
> Lines 265-269 in the revised manuscript:
"*Although TRPV1 desensitization has been performed with local application of agonists, supraspinal-selective desensitization has not yet been achieved. The RTX-i.c.v. injection protocols presented here will provide a convenient experimental model for studying the role of TRPV1 in supraspinal function.*"

Comment #8
Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
Reply #8
A sentence in Step 1.1.4. in the former manuscript has been move to NOTE (lines 94-95 in the revised manuscript).
A sentence in Step 3.1.2. in the former manuscript has been move to NOTE (line 165 in the revised manuscript).
A sentence in Step 3.2.3. in the former manuscript has been move to NOTE (line 191 in the revised manuscript).

Comment #9
The Protocol should be made up almost entirely of discrete steps without paragraphs of text between sections.
Reply #9
We have deleted a sentence on lines 117-118 in the former manuscript, and we have alternatively added the words "*One week after pretreatment with RTX (Step 2.)*" to Steps 3.1. and 4.1. (lines 158 and 183 in the revised manuscript).
The words in Step 3.2.1. in the former manuscript "*As for the RTX test (3.1.1. and 3.1.2. above)*" have been deleted.
Paragraphs at the heads of Step 3. and 4. in the revised manuscript have now been described as NOTE (lines 155-156 and 180-181 in the revised manuscript).

Comment #10

Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Reply #10

As mentioned in Reply #2 to Reviewer #1 below, we have added details of the procedures used for finding the sagittal and coronal sutures (Steps 2.4.3. and 2.4.4. in the revised manuscript).

Comment #11

1.1.2 500 ul of polyoxyethylene is added to the mixture above?

Reply #11

Indeed. We have now added the words "to the solution above" to the sentence (line 99 in the revised manuscript).

Comment #12

1.1.5: What is the desired concentration?

Reply #12

We have changed the term "desired concentration" to "20 μg/mL" and moved this step to Step 2.1. in the revised manuscript. Due to this change, the numbering of the following steps has been changed (2.1.-2.3.5. in the former manuscript; 2.2.-2.4.6. in the revised manuscript).

Comment #13

2.1: Is the anesthesia procedure approved by the IACUC? How do you check the depth of anesthesia before proceeding? Do you apply eye ointment? Do you perform shaving of the hair?

Reply #13

> Is the anesthesia procedure approved by the IACUC?

As described in the header of the Protocol section, all of the protocols were approved by the ethics committee of Musashino University (lines 82-83 in the former manuscript and lines 84-85 in the revised manuscript).

> How do you check the depth of anesthesia before proceeding?

After checking for loss of the righting reflex, the following steps were performed. We have added this point to Step 2.2. in the revised manuscript

> Do you apply eye ointment? Do you perform shaving of the hair?

We did not perform any other additional treatments including eye ointment application or hair shaving.

Comment #14

3: How do you perform the pain test? Why do you perform in the given time period? How many times a day?

Reply #14

> How do you perform the pain test?

In the former manuscript, the protocols for pain tests (the RTX test and the tail pressure test) had been described in the sub-steps, Steps 3.1 and 3.2. In the revised manuscript, we have added a further step (Step 2.5.) and re-organized the steps as follows: Step 3.1. in the former manuscript has been re-numbered as Step 3, and Step 3.2. in the former manuscript has been re-numbered as Step 4.

> Why do you perform in the given time period?

In our previous report (Ref. 19), we had stated that licking/biting behavior was remarkable in the first 10 min, and then became gradually reduced. Based on this finding, we assessed the total licking/biting time during the first 10 min in subsequent experiments. We have added a sentence to this effect on line 208 in the revised manuscript.

> How many times a day?

We never performed the pain tests repeatedly using the same mouse because repetitive application of RTX or acetaminophen might have affected the results.

Comment #15

3.1.5: Does acetaminophen have any biasness in the study?

Reply #15

We are not completely sure what you mean by "biasness" here, but we think that use of acetaminophen would not have introduced bias because acetaminophen was administered to both groups (vehicle-i.c.v. and RTX-i.c.v.) and acetaminophen solution is colorless.

Comment #16

3.1- 3.2: Are both the tests performed on the same mouse?

Reply #16

As mentioned in Reply #14 above, each mouse was subjected to the RTX test or the tail pressure test.

Comment #17

What are the control used? What is the sex and age of the mice?

Reply #17

> What are the control used?

In Fig. 2, the control groups were pretreated with the vehicle for RTX (10% ethanol, 10% polyoxyethylene and 80% saline/ACSF). We have added sentences describing the control group in Steps 2.3. and 2.4. in the revised manuscript.

> What is the sex and age of the mice?

The sex and age of the mice have been described in the header of the Protocol section (lines 83 and 85 in the former manuscript and lines 85-87 in the revised manuscript).

Comment #18

As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Reply #18

To revise the Discussion, two NOTE paragraphs in the Protocol have been moved to Discussion (lines 139-140 and 180-182 in the former manuscript).

> a) Critical steps within the protocol

This point has been discussed in the first paragraph of the Discussion (lines 238-244).

> b) Any modifications and troubleshooting of the technique

These points have been described in lines 246-247.

> c) Any limitations of the technique

These points have been described in lines 247-250.

> d) The significance with respect to existing methods

This point has been discussed in lines 265-267.

> e) Any future applications of the technique

This point has been described in lines 267-269.

Replies to Reviewer #1

We greatly appreciate your helpful comments on our manuscript. As indicated in the following responses, we have taken all of these comments and suggestions into account in the revised version of our manuscript. The modified sentences/words are marked in blue in the revised manuscript.

Comment #1

The use of "resiniferatoxin" and its abbreviation "RTX" seems to be random throughout the manuscript. I would suggest spelling it out as "resiniferatotoxin (RTX)" when it first appears in the Abstract, and also in the Introduction section. For the rest of the manuscript, it can be just written as "RTX".

Reply #1

We apologize for any confusion. In the revised manuscript, we have changed the word “resiniferatoxin” to “RTX” (lines 35, 72, 91, 93, 97, 113 and 153 in the revised manuscript).

Comment #2

Protocol 2.3.3: This section could be described in a bit more detail. How do you "find the sagittal suture with the needle tip over the skin"? Do you need to poke through the scalp?

Reply #2

> How do you "find the sagittal suture with the needle tip over the skin"?

Thank you for your helpful advice. We have divided Step 2.3.3. in the former manuscript into Steps 2.4.3. and 2.4.4. in the new one We have also modified the descriptions of these steps.

>Do you need to poke through the scalp?

Yes, we performed these steps (finding the sutures and injection sites) without making any incisions in the scalp.

Comment #3

Protocol 3.1.2 and 3.2.1: plexiglass is misspelled.

Reply #3

Thank you for pointing this out. We have corrected the words in the revised manuscript (lines 161 and 184).

Comment #4

Protocol 3.2.4: It is not clear what exactly "two values" mean. Does this mean pressure value for tail whisking and another pressure value for squeaking?

Reply #4

We apologize for the lack of explanation. The term “two values” means the pressure applied to the spots

at 1.5 and 2.5 cm from the base of the tail (Step 4.2.). In the revised manuscript, we have modified the sentences in Step 4.4.

Comment #5

Protocol 3.2.6: It might be nicer to say "After obtaining the baseline, administer acetaminophen (300 mg/kg) to the mouse intraperitoneally. Following the administration, steps 3.2.3. and 3.2.4 are repeated every 15 min again."

Reply #5

Thank you for your helpful advice. We have changed the sentence (Step 4.6. in the revised manuscript).

Comment #6

Representative Results section, line 202: Figure 2C shows only the tail pressure test, and not the RTX test.

Reply #6

We are sorry for this error. In the revised manuscript, the words "the RTX test and (line 202 in the former manuscript)" have been deleted. A sentence about the analgesic effect of acetaminophen in the RTX test has been added (lines 210-212 in the revised manuscript).

Reply to Reviewer #2:

We greatly appreciate your important comments regarding the manuscript.

Comment #1

Alcoholic RTX solution is irritating, It is worth mentioning.

Reply #1

Thank you for pointing this out. To draw attention to this point, sentences on lines 95-96 in the former manuscript have been moved to the head of Step 1.1. (lines 93-94 in the revised manuscript). This change is marked in yellow in the manuscript.

Reply to Reviewer #3:

Thank you very much for your positive comments. We greatly appreciate the fact that you have recognized the importance of the present manuscript.

Other modifications

1. A sentence has been added in the revised manuscript (lines 227-229). This sentence is marked in gray.
2. The words on line 237 in the former manuscript "*post-hoc analysis*" have been changed to "*post-hoc observation*" in the revised manuscript (line 255). This modification is marked in gray.

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