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Orginal article

Amygdaloid administration of tetrapentylammonium attenuates development of pain and anxiety-like behavior following peripheral nerve injury

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ABSTRACT

Background: The central amygdaloid nucleus (CeA) is involved in processing and descending regulation of pain. Amygdaloid mechanisms underlying pain processing and control are poorly known. Here we tested the hypothesis that perioperative CeA administration of tetrapentylammonium (TPA), a non-selective THIK-1 channel blocker and thereby inhibitor of microglia, attenuates development of chronic neuropathic pain and comorbid anxiety-like behavior.

Methods: Rats with a spared nerve injury (SNI) model of neuropathy or sham operation had a chronic cannula for drug microinjections into the CeA or a control injection site. Monofilament test was used to evaluate pain, and light-dark box (LDB) to assess anxiety.

Results: Perioperative CeA treatment with TPA (30 μg/day up to the third postoperative day, D3) significantly attenuated the development of pain and anxiety-like behavior. In the late phase (> D14), CeA administration of TPA (3–30 μg) failed to influence pain. Perioperative minocycline (microglia inhibitor; 25 μg), MK-801 (an N-Methyl-D-aspartate receptor antagonist; 0.1 μg), vehicle or TPA in a control injection site failed to attenuate pain development.

Conclusions: Perioperative treatment of the CeA with TPA delayed development of neuropathic pain and comorbid anxiety-like behavior, while TPA treatment failed to influence maintenance of established neuropathic pain. The failures to attenuate pain development with CeA administrations of minocycline or MK-801 do not support the hypothesis that the TPA-induced prophylactic effect was due to inhibition of amygdaloid microglia or N-methyl-D-aspartate receptors. While TPA in the CeA proved to have a prophylactic effect on SNI-induced pain behavior, the underlying mechanism still remains to be studied.

Introduction

The amygdala is known to have a key role in processing of primary emotions [1]. Within the amygdala, the central nucleus (CeA) provides the principal output to the brainstem and is involved in processing of ascending nociceptive signals and descending regulation of nociception [2]. Earlier studies indicate that peripheral nerve injuries may cause neural plasticity in the amygdala that been accompanied by chronic pain and comorbid emotional changes (*e.g.* [3–5]). Concerning non-neuronal brain cells, microglia have been shown to contribute to pain particularly at the spinal cord level [6–8], while earlier results on the role of amygdaloid microglia are less clear. It has been reported that amygdaloid microglia was not activated by common peroneal nerve ligation in mice [9] or spinal nerve ligation in rats, except when spinal nerve ligation was accompanied by olfactory bulbectomy [10]. In contrast, partial ligation or chronic constriction of the sciatic nerve in mice [11,12] induced activation of amygdaloid microglia. Moreover, spared nerve injury (SNI) induced upregulation of amygdaloid cytokines in rats as well as mice [9] that may reflect activation of microglia [6–8]. Activation of amygdaloid microglia was associated with nerve injury-induced pain hypersensitivity and comorbid anxiety in some experimental conditions [11] but not invariably [10,13]. Among explanations for

Abbreviations: ANOVA, analysis of variance; CeA, central nucleus of the amygdala; i.c., internal capsule; NMDA, N-methyl-D-aspartate; SNI, spared nerve injury; THIK-1, TWIK-related halothane-sensitive two-pore domain K^+ channel 1; TRPV1, transient receptor potentialvanilloid 1; TPA, tetrapentylammonium.

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the variability in the results on amygdaloid microglia are differences in the experimental pain models and the animal species used in previous studies. Moreover, some earlier studies in the spinal cord suggest that microglia contribute to the early rather than prolonged phase of nerve injury-induced pain [14–16]. It is not yet clear whether the role of the amygdaloid microglia varies in a similar manner with the post-injury time course.

In the brain, THIK-1 (TWIK-related halothane-sensitive two-pore domain potassium channel [17]) is highly expressed in microglia where it regulates membrane potential and surveillance activity [18]. Neurons or astrocytes do not express THIK-1 and oligodendrocytes only a little [19,20]. Tetrapentylammonium (TPA) was recently shown to be an effective tool for inhibiting THIK-1 and thereby microglial surveillance activity and release of proinflammatory cytokines [18]. TPA, however, affects also various other receptors or channels such as *e.g.* the N-methyl D-aspartate (NMDA) [21], transient receptor potential vanilloid 1 (TRPV1) [22], and inactivating Na^+ channel [23].

Here we tested the hypothesis that amygdaloid microglia contribute to the development but not maintenance of pain behavior following peripheral nerve injury. If the hypothesis is correct, perioperative treatment of the amygdala with TPA should attenuate the development of pain hypersensitivity and comorbid anxiety in the early post-injury period. Since no selective THIK-1 channel antagonist is commercially available, TPA was used to block amygdaloid THIK-1, due to which potential contribution of other amygdaloid receptors and channels affected by TPA need to be considered in the interpretation of results. Therefore, the effect of perioperative TPA treatment in the CeA was compared in a group of SNI animals with that of minocycline, another inhibitor of microglia, and MK-801, an antagonist of the NMDA receptor that has also been shown to be blocked by TPA [21].

Materials and methods

Animals

Adult male Hannover-Wistar rats (Harlan, Horst, The Netherlands; weight: 180–280 g) were used in the experiments. The study protocol was approved by the Regional Animal Ethics Committee, and the experimental guidelines followed the European Communities Council Directive of 22 September 2010 (2010/63/EU). The animals were living in a 12-hour light/dark cycle with access to food and water *ad libitum* (room temperature 22 °C, humidity 55%). Animals were housed immediately adjacent to the experimental laboratory in single transparent cages, in which they could hear, see and smell the other rats.

Surgical procedures for producing neuropathy

The SNI model described by Decosterd and Woolf [24] was used. For the operation, anesthesia was induced with sodium pentobarbital (intraperitoneally 60 mg/kg). Anesthesia was continued by giving further doses of sodium pentobarbital (15–20 mg/kg) to keep the depth of anesthesia deep enough so that the animal did not react to noxious stimulation. The skin on the lateral surface of the left thigh was incised, after which the biceps femoris muscle was sectioned to expose the sciatic nerve trunk and its terminal branches: the sural nerve, the common peroneal nerve, and the tibial nerve. After ligating and sectioning the common peroneal and tibial nerves, their distal nerve stumps were removed, without touching the sural nerve. Sham operation was performed identically as SNI operation, except that nerves were only exposed but not ligated. As required by the animal ethics committee, animals were treated with 0.01 mg/kg of buprenorphine twice daily for 2 days to reduce postoperative pain.

Cannula insertion and drug injection procedure

A guide cannula for amygdaloid drug administrations was installed in the right hemisphere (the side opposite to the peripheral nerve injury). The reason for choosing the amygdala in the right hemisphere was that previous results indicated that the right rather than the left amygdala is involved in role in the processing of pain-related signals [25,26]. Another reason for choosing the amygdala in the right hemisphere as the treatment target was that the nerve injury was in the left hind limb and the amygdala-driven descending control of pain is stronger in the contra- than ipsilateral limb [27,28]. The guide cannula (26 gauge; PlasticsOne, Roanoke, VA, USA) for drug injections was installed after exposing the skull and drilling a hole for its placement at the same time as the SNI operation was performed. The injection target in the right amygdala was the capsule lateral of the CeA: 2.1 mm posterior from the bregma, 4.3 mm lateral from the midline, and 7.8 mm ventral from the dura mater [29]. The brain control injection site was the right internal capsule: 2.1 mm posterior from bregma, 3.6 mm lateral from the midline, and 5.0 mm ventral from the dura mater. The guide cannula tip was 2 mm above the injection target. Dental screws and dental cement were used to fix the guide cannula on the skull. When the animal was not being tested, a dummy cannula was placed in the guide cannula.

Drugs and their administration procedure

TPA, a THIK-1 channel antagonist [18], minocycline (another microglia inhibitor) and MK-801 (an NMDA receptor antagonist) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TPA was administered at the dose of 30 μg. However, when testing TPA effect in fully developed hypersensitivity, TPA doses were 3 μg, 10 μg or 30 μg. Minocycline was administered at the dose of 25 μg (which is the maximum dose that is soluble at a volume of 0.5 μl). MK-801 was administered at the dose of 0.1 μg, since at this dose MK-801 in the CeA has reversed the pronociceptive effect of amygdaloid glutamate in SNI animals [30]. Unilateral infusions of drugs, or an equivalent volume of vehicle (saline), were performed manually by using injection needles (33 Gauge) made of stainless steel (PlasticsOne). The injection needle was connected to a 10 μl Hamilton microsyringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) by polyethylene (PE-10) tubing. The injection needle protruded 2.0 mm beyond the guide cannula tip. The injection volume was a 0.5 μl. During the infusions, the animals were gently restrained for the duration of the injection (30 s). After completing the injection, the injection needle was retained within the cannula for an additional 20 s to prevent backflow of the drug. The spread of the injections was expected to be close to 1 mm [31]. Therefore, the injections were likely to cover not only the CeA but also the adjacent subnuclei of the amygdala. Consequently, the drug injection volumes of the present study may not allow pinpointing the site of the effect within the amygdala.

Assessment of mechanical pain

Before any operations or testing, the animals were habituated to the testing conditions at least in three one hour sessions during three consecutive days. Monofilament-evoked limb withdrawal response to repetitive stimulation, was used to assess pain behavior. Testing was performed using a series of calibrated monofilaments producing forces of 2 g, 4 g, 6 g, 8 g, 10 g, 15 g, and 26 g (North Coast Medical, Inc. Morgan Hill, CA, USA). During testing, rats were on a grid, free to move inside a transparent box covering the grid. The monofilament was applied below the grid to the lateral part of the left foot pad (sural nerve innervation area). At each time point, the paw was stimulated five times at a frequency

of about 0.5 Hz. The withdrawal response frequency was recorded. For example, response to all of the five consecutive stimulations represents a response rate of 100%, whereas one response to five consecutive stimulations represents a response rate of 20%.

Assessment of anxiety-like behavior

When assessing anxiety-like behavior in the light-dark box (LDB) test, the rats were habituated to the laboratory but not yet exposed to the arena used for assessing anxiety until the actual testing of anxiety started. In each animal, LDB testing was performed twice: i) Once on day 3 (D3), after assessing mechanical sensitivity, but before the last drug/saline treatment of the amygdala, ii) Second time on D14, after assessing mechanical sensitivity. While repetition of the test at an interval of 10 days may *per se* influence the test result, this potential confounding factor was considered to influence identically the group treated with drug as that treated with saline. The LDB test was carried out in a commercially-available device (San Diego Instruments, San Diego, CA, USA) that had an arena with two compartments (each 26.4 × 20.6 cm). One of the compartments was dark and the other one illuminated with a xenon lamp (100 lux). Dark and light compartments were connected by a dark center chamber (15.9 × 20.6 cm) that allowed free movement between the zones. At the beginning of testing, the rat was released to the light zone. The animal was allowed to explore the experimental chambers for 10 min. The time spent in each zone during the test was recorded using an 4 × 16 array of photo beams monitored by a computer. An increase in anxiety was expected to decrease the time the animal spends in the illuminated chamber. The total distance of ambulation during the LDB test was also calculated in each experimental condition.

Course of study

Animals were randomly divided into experimental groups and the experiments were performed in a blinded fashion. Experiments were performed between 10 AM and 4 PM. Fig. 1 A illustrates the course of the study for groups (n = 8 in each group) in which TPA, MK-801, minocycline or saline was administered perioperatively into the right CeA or the control injection site (the right internal capsule) to study influence of drug treatment on the development of symptoms. Briefly, on D0 the animal was anesthetized and during anesthesia the following procedures were consecutively performed: a brain cannula was installed, SNI or sham-SNI was induced, and the first intracerebral injection of TPA (30 μg)/minocycline (25 μg)/MK-801 (0.1 μg) or saline was performed immediately after completing the SNI/sham operation, after which the animal was allowed to recover from anesthesia in his home cage. Thereafter, intracerebral drug/saline administration was performed once daily also on three following post-injury days (D1-D3). On D3, monofilament test followed by LDB test were performed just prior to the last amygdaloid administration of drug/saline. The second monofilament test followed by the second LDB test was performed on D14. However, in animals with the brain cannula in the control injection site, testing was performed only on D3, since the antihypersensitivity effect by perioperative amygdaloid administration of TPA was most prominent at D3. In sham-SNI animals, only saline was administered in the CeA.

The effect of amygdaloid TPA treatment on hypersensitivity in fully developed pain hypersensitivity was assessed in a separate group of SNI animals two to three weeks after induction of nerve injury (Fig. 1 B). In this group of animals, the brain cannula was installed into the right CeA one week before the start of the actual experiment. In the actual experiment, the monofilament test was performed just prior to and 5, 15 and 30 min following amygdaloid administration of saline or TPA at the dose of 3 μg, 10 μg or 30 μg ($n_{0\mu g\ \&\ 10\mu g} = 6$, $n_{3\mu g\ \&\ 30\mu g} = 5$). Each animal was tested at two to three treatment conditions at two day intervals, and at varying order to avoid serial effects. The maximal drug effect, if any, was used in further calculations.

Histology

After completing the experiments, rats were sacrificed by an overdose of sodium pentobarbital. Then, the brain was removed

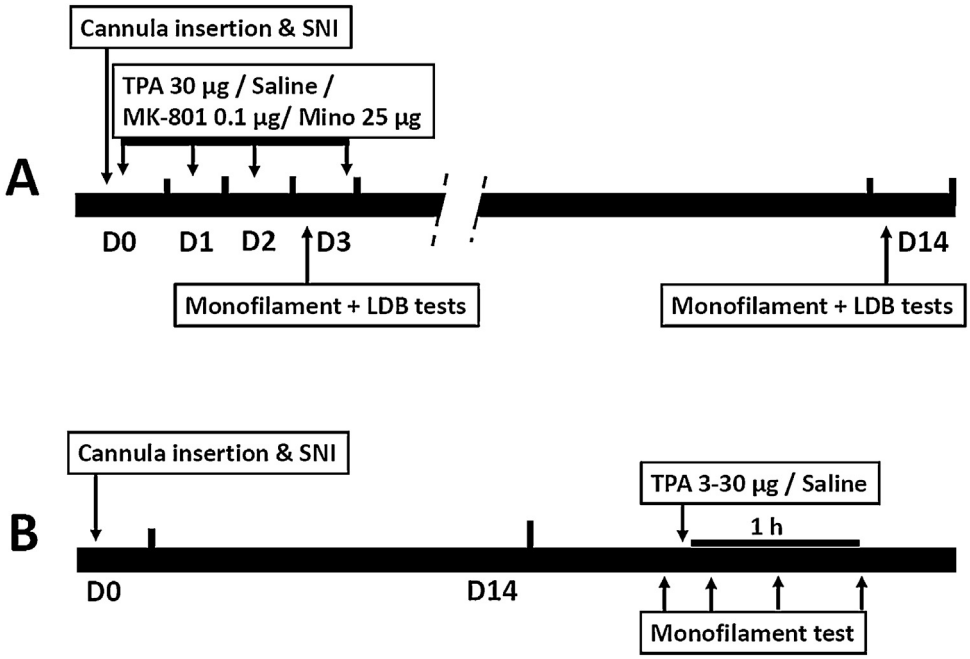

Fig. 1. Schematic drawing showing time course of the study for experimental groups in which the once daily intracerebral treatments were given during the perioperative period (**A**) or when acute treatments were given in fully developed neuropathy (**B**). D0-D14; postoperative days 0–14; SNI, spared nerve injury operation; TPA, tetrapentylammonium; monofilament, monofilament test for assessing hypersensitivity; LDB, light-dark box test for assessing anxiety-like behavior.

and immersed in 4% formaldehyde. Coronal sections of the brain were cut to verify the site of injection [29].

Statistical analyses

Data were analyzed using one-way or two-way mixed-design ANOVA and *t*-test (with Bonferroni correction for multiple comparisons when appropriate). $p < 0.05$ was considered to represent a significant difference.

Results

Development of mechanical pain hypersensitivity

Daily (right) amygdaloid microinjections of TPA (a non-selective THIK-1 channel antagonist) on D0-D3 and at a dose of 30 μg attenuated development of mechanical pain hypersensitivity in the nerve-injured left limb in the early postoperative period. On postoperative D3, mechanical sensitivity was significantly different among the TPA- and vehicle- treated SNI animals, and the vehicle-treated sham group (main effect of experimental group: $F_{2,20} = 10.0$, $p = 0.001$; Fig. 2 A). *Post hoc* testing indicated that mechanical sensitivity was significantly attenuated by TPA in SNI animals and that mechanical sensitivity in the vehicle-treated sham group was lower than that of the vehicle- or TPA-treated SNI group (Fig. 2 A). On postoperative D14, mechanical pain sensitivity was significantly different among the experimental groups ($F_{6,120} = 86.7$, $p < 0.0001$; Fig. 2 B). However, on D14, unlike on D3, *post hoc* testing indicated that there was no significant difference in mechanical sensitivity between the TPA- and vehicle-treated SNI groups, while mechanical sensitivity of the vehicle-treated sham group differed significantly from that of the TPA- and vehicle-treated SNI groups (Fig. 2 B).

To assess, whether TPA in a brain control injection site attenuates development of mechanical pain hypersensitivity, TPA was microinjected daily at a dose of 30 μg into the right internal capsule in SNI animals. Pain hypersensitivity was tested at D3, at which postoperative time point amygdaloid administration of TPA had a significant effect on development of mechanical pain hypersensitivity (Fig. 2 A). Perioperative treatment with TPA produced a significantly weaker attenuation of early (D3) mechanical pain hypersensitivity when administered in the brain control injection site (internal capsule) than in the CeA ($F_{1,12} = 5.2$, $p = 0.042$; Fig. 2 C).

Perioperative treatments of the CeA with minocycline (another microglia inhibitor; 25 μg) or MK-801 (an NMDA receptor antagonist; 0.1 μg) failed to produce significant attenuations in development of mechanical pain hypersensitivity when compared with the vehicle-treated SNI group (main effect of experimental group on D3: $F_{2,17} = 0.8$; Fig. 2 D).

Development of anxiety-like behavior

In a test of anxiety-like behavior, light-dark box (LDB), there was a significant difference in time spent in light among the TPA-treated SNI group, vehicle-treated SNI group, and the vehicle-treated sham group (main effect of the experimental group: $F_{2,20} = 6.0$, $p = 0.009$; Fig. 2 E), independent of the postoperative test day ($F_{2,20} = 0.1$). While the average time spent in light by the TPA-treated SNI group tended to be longer than that in other groups, *post hoc* testing failed to reveal significant differences among the groups. LDB test on D3 after SNI indicated that the time spent in light by animals receiving TPA in the brain control injection site tended to be shorter than that by animals receiving TPA in the CeA ($29 \pm 4.5\%$ *vs* $42.9 \pm 4.7\%$; $t_{14} = 2.0$, $p = 0.05$; not shown).

Anxiety-like behavior of SNI animals was not significantly influenced by perioperative minocycline or MK-801 treatments of

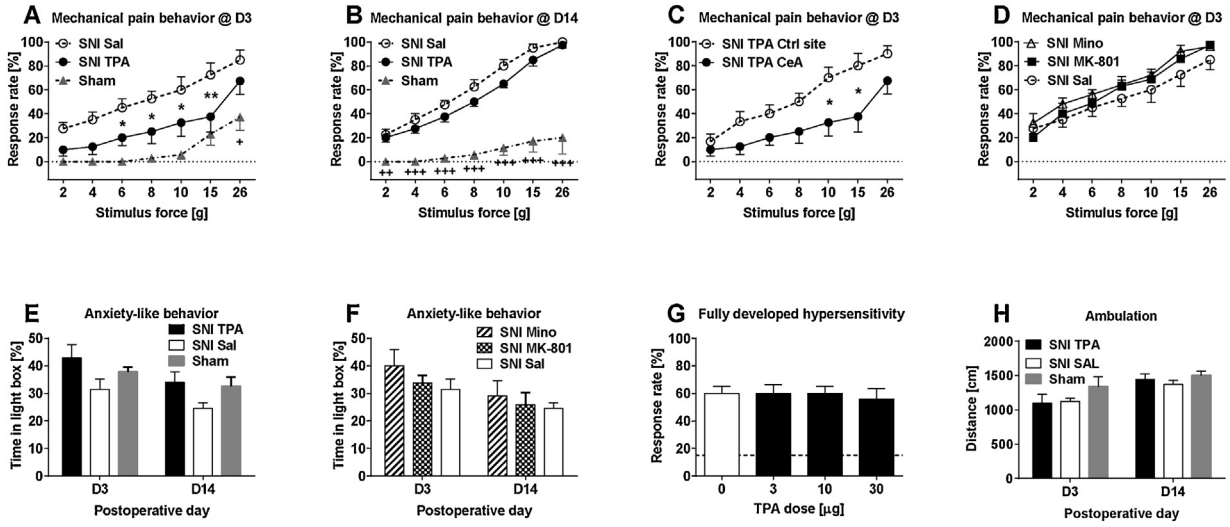

Fig. 2. Effect of amygdaloid tetrapentylammonium (TPA) treatment on mechanical hypersensitivity, assessed in the monofilament test, and anxiety-like behavior, assessed in the light-dark box (LDB) test, in rats with a spared nerve injury (SNI) model of neuropathy. A) Development of early mechanical hypersensitivity following perioperative TPA treatment. B) Development of late mechanical hypersensitivity following perioperative TPA treatment. C) Development of early mechanical hypersensitivity following perioperative TPA treatment of a brain control injection site, the internal capsule. D) Development of early mechanical hypersensitivity following perioperative minocycline (Mino) or MK-801 treatment. E) Development of anxiety-like behavior following perioperative TPA treatment. F) Development of anxiety-like behavior following perioperative minocycline or MK-801 treatment. G) Attempt to attenuate maintenance of mechanical hypersensitivity by acute amygdala injections of TPA in fully developed neuropathy. H) Ambulation distances during the LDB test. Sham, saline-treated sham-operated control group. In all graphs, brain injections were performed in the right hemisphere. TPA dose was 30 μg, except in graph G the dose is shown in the X-axis. Minocycline dose was 25 μg, and MK-801 dose was 0.1 μg. Testing of hypersensitivity was performed in the left (nerve-injured) hind paw. In A–D and G, the higher the response rate, the stronger the mechanical hypersensitivity. In E and F, the shorter the time spent in light, the more intense the anxiety-like behavior. Error bars represent S.E.M. (in A–F and H, n = 7–8, in G, n = 5–6). */+$p < 0.05$, **/++$p < 0.01$, +++$p < 0.005$ (t-test test with a Bonferroni correction for multiple comparisons; asterisks indicate difference between SNI-Sal and SNI-TPA groups, crosses indicate difference between Sham and SNI-TPA groups). In G, the broken horizontal line represents the upper 95% CIs of the corresponding value obtained in unoperated controls (n = 6).

the CeA when compared with vehicle treatment (main effect of the experimental group: $F_{2,18}$ = 1.2; Fig. 2 F), independent of the postoperative test day ($F_{2,18}$ = 0.1).

No obvious side-effects were observed following perioperative treatment of the CeA with TPA, minocycline or MK-801.

Effect of amygdaloid TPA treatment in fully established SNI

In a separate group of SNI animals, TPA was administered in the right CeA two to three weeks after the nerve-injury to assess whether amygdaloid TPA treatment influences maintenance of fully established pain hypersensitivity. Amygdaloid treatment with TPA at various doses (3–30 μg) failed to influence maintenance of mechanical pain hypersensitivity of SNI animal in the late postoperative period ($F_{3,18}$ = 0.11, p = 0.96; Fig. 2 G).

Locomotor activity

Ambulation distances in the LDB test were determined to assess whether changes in motor activity could explain the antihypersensitivity or anxiolytic-like effects of TPA in SNI animals. Ambulation distances were not significantly different among the TPA-treated SNI group, vehicle-treated SNI group and the vehicle-treated sham group (main effect of experimental group: $F_{2,20}$ = 1.6; Fig. 2 H), independent of the postoperative test day ($F_{2,20}$ = 0.5). Ambulation distances were significantly longer at the late (D14) than the early (D3) postoperative test day ($F_{1,20}$ = 13.1, p = 0.0017; Fig. 2 H).

Centers of brain injection sites

The centers of the amygdaloid injection sites were in or immediately adjacent to the CeA in the right hemisphere (Fig. 3). The centers of control injection sites were in the right internal capsule (Fig. 3).

Discussion

The present results in the rat SNI model of chronic neuropathic pain indicate that perioperative CeA administration of TPA significantly delays development of pain hypersensitivity as indicated by attenuation of mechanically evoked withdrawal responses at an early (D3) but not at a late (D14) postoperative test day. Moreover, anxiety-like behavior in SNI animals was attenuated by perioperative treatment of the CeA with TPA, although the effect on anxiety-like behavior was weak. In fully

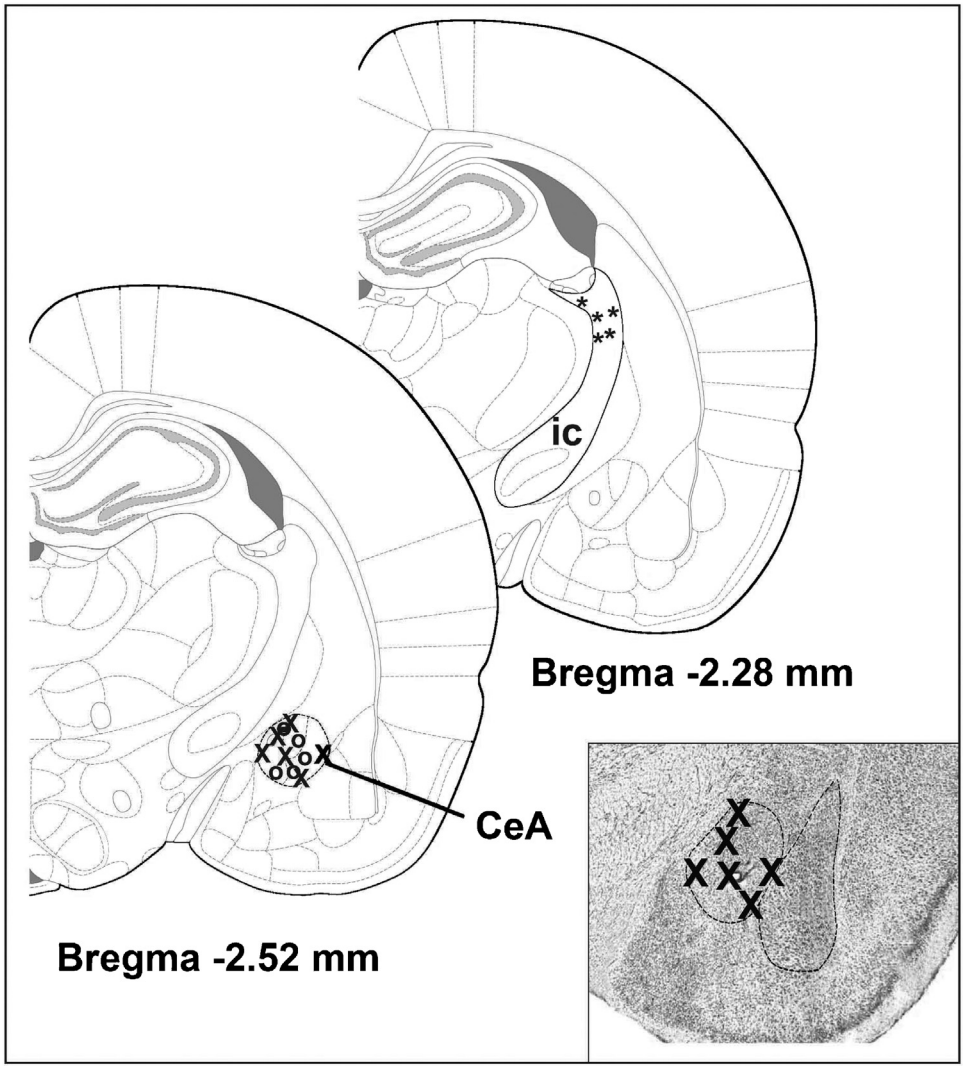

Fig. 3. Centers of the injection sites in the right hemisphere. Each X represents an amygdaloid injection site in a nerve-injured animal; each asterisk represents a control injection site in the internal capsule in a nerve-injured animal; each open circle represents an amygdaloid injection site in a healthy control animal. Overlapping injections sites are shown with one symbol. CeA, central nucleus of the amygdala; ic, internal capsule.

developed neuropathy, TPA in the CeA failed to influence maintenance of pain behavior. Mechanically evoked limb withdrawal responses were stronger both in the TPA- and vehicle-treated SNI groups than in the vehicle-treated sham group indicating that the present TPA-induced attenuation of pain behavior in SNI animals reflects reduction of neuropathic rather than musculoskeletal postoperative pain.

The attenuation of early pain hypersensitivity by perioperative treatment of the CeA with TPA, a non-selective inhibitor of microglia [18], is in line with our working hypothesis according to which amygdaloid microglia contributes to development of neuropathic pain behavior. However, perioperative treatment of the CeA with minocycline, another microglia inhibitor, failed to attenuate development of neuropathic pain hypersensitivity. This finding does not support the explanation that the TPA-induced preemptive effect in the present study was due to inhibition of amygdaloid microglia. Earlier studies have shown that TPA can block the NMDA receptor [21]. In the present study, however, perioperative treatment of the CeA with MK-801, an NMDA receptor antagonist, failed to attenuate development of pain hypersensitivity in SNI animals. This finding is not in line with the proposal that a TPA-induced block of amygdaloid NMDA receptors explains the TPA-induced attenuation of early pain hypersensitivity. As always with negative findings of drug effects, one needs to consider whether the currently used drug doses were appropriate. However, it should be pointed out that perioperative minocycline treatment of the anterior cingulate cortex at a dose lower than the currently used dose (5 μg) has significantly attenuated peripheral nerve injury-induced activation of cortical microglia and mechanical pain hypersensitivity in mice [32]. Moreover, the currently used low dose of MK-801 (0.1 μg) has proved effective in preventing the pronociceptive effect induced by glutamate administration into the CeA of SNI animals [30].

Among alternative mechanisms that might explain the TPA-induced attenuation in development of neuropathic pain behavior is its inhibitory action on pronociceptive TRPV1 channels [22]. TRPV1 channels have been shown to contribute to synaptic plasticity in the basolateral amygdala [33], an amygdaloid subnucleus that contributes to control of pain behavior [34] and to which TPA at the currently used injection volume of 0.5 μl was likely to spread from the injection site in the CeA [31]. Other mechanisms potentially contributing to the TPA-induced preemptive effect are TPA-induced inhibitions of the inactivating Na^+ current [23], high voltage-gated Ca^{2+} current, and A-type K^+ current [35].

Chronic pain, including that induced by SNI is known to induce anxiety [36]. The finding that amygdaloid administration of TPA slightly but significantly attenuated the development of anxiety-like behavior in SNI animals suggests that an activation of a TPA-reversible amygdaloid mechanism contributes to the comorbidity of pain and affect in SNI. This proposal still leaves open whether the TPA-reversible amygdaloid mechanism might have had a direct anxiogenic effect on neuronal circuitry involved in anxiety. Alternatively, the TPA treatment-sensitive anxiety could be indirectly caused by a TPA-reversible pronociceptive mechanism enhancing the magnitude of pain that was driving neuronal circuitry underlying anxiety.

Among limitations of the present study is that the main working hypothesis on the potential role of amygdaloid microglia in development of neuropathic pain behavior was studied using only pharmacological methods. Moreover, present pharmacological findings do not allow excluding involvement of TRPV1 and various other cation channels that according to *in vitro* studies are known to be sensitive to TPA [22,23,35] and that thereby might have a role in TPA-induced attenuation in development of neuropathic pain behavior.

Conclusions

Perioperative treatment of the amygdala with TPA, a non-selective microglia inhibitor, delayed development of neuropathic pain behavior, while TPA failed to influence established pain hypersensitivity. Since perioperative treatment of the amygdala with minocycline, another microglia inhibitor, or an NMDA receptor antagonist did not delay development of pain behavior, amygdaloid microglia or NMDA receptors may not have a critical role in the TPA-induced preemptive effect in the SNI model-induced pain. Among alternative explanations are a TPA-induced suppression of TRPV1 or other cation channels in the amygdala.

Conflict of interest

One of the authors (AK) is an employee of a pharmaceutical company (Orion Corporation, OrionPharma, Turku, Finland). Other authors declare no conflict of interest.

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References

[1] LeDoux J.. The amygdala. Curr Biol 2007;7:R868–74.
[2] Neugebauer V, Galhardo V, Maione S, Mackey S. Forebrain pain mechanisms. Brain Res Rev 2009;60:226–42.
[3] Ikeda R, Takahashi Y, Inoue K, Kato F. NMDA receptor-independent synaptic plasticity in the central amygdala in the rat model of neuropathic pain. Pain 2007;127:161–72.
[4] Gonçalves L, Silva R, Pinto-Ribeiro F, Pêgo JM, Bessa JM, Pertovaara A, et al. Neuropathic pain is associated with depressive behaviour and induces neuroplasticity in the amygdala of the rat. Exp Neurol 2008;213:48–56.
[5] Gonçalves L, Dickenson AH. Asymmetric time-dependent activation of right central amygdala neurones in rats with peripheral neuropathy and pregabalin modulation. Eur J Neurosci 2012;36:3204–13.
[6] Watkins LR, Maier SF. Beyond neurons: evidence that immune and glial cells contribute to pathological pain states. Physiol Rev 2002;82:981–1011.
[7] Mika J. Modulation of microglia can attenuate neuropathic pain symptoms and enhance morphine effectiveness. Pharmacol Rep 2008;60:297–307.
[8] Ji RR, Chamessian A, Zhang YQ. Pain regulation by non-neuronal cells and inflammation. Science 2016;354:572–7.
[9] Zhang F, Vadakkan KI, Kim SS, Wu LJ, Shang Y, Zhuo M. Selective activation of microglia in spinal cord but not higher cortical regions following nerve injury in adult mouse. Mol Pain 2008;4:15.
[10] Burke NN, Geoghegan E, Kerr DM, Moriarty O, Finn DP, Roche M. Altered neuropathic pain behaviour in a rat model of depression is associated with changes in inflammatory gene expression in the amygdala. Genes Brain Behav 2013;12:705–13.
[11] Sawada A, Niiyama Y, Ataka K, Nagaishi K, Yamakage M, Fujimiya M. Suppression of bone marrow-derived microglia in the amygdala improves anxiety-like behavior induced by chronic partial sciatic nerve ligation in mice. Pain 2014;155:1762–72.
[12] Taylor AM, Mehrabani S, Liu S, Taylor AJ, Cahill CM. Topography of microglial activation in sensory- and affect-related brain regions in chronic pain. J Neurosci Res 2017;95:1330–5.
[13] Gui WS, Wei X, Mai CL, Murugan M, Wu LJ, Xin WJ, et al. Interleukin-1β overproduction is a common cause for neuropathic pain, memory deficit, and depression following peripheral nerve injury in rodents. Mol Pain 2016;12:, doi:http://dx.doi.org/10.1177/1744806916646784.
[14] Raghavendra V, Tanga F, DeLeo JA. Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. J Pharmacol Exp Ther 2003;306:624–30.
[15] Li Z, Wei H, Piirainen S, Chen Z, Kalso E, Pertovaara A, et al. Spinal versus brain microglial and macrophage activation traits determine the differential neuroinflammatory responses and analgesic effect of minocycline in chronic neuropathic pain. Brain Behav Immun 2016;58:107–17.
[16] Peng J, Gu N, Zhou L, Eyo UB, Murugan M, Gan WB, et al. Microglia and monocytes synergistically promote the transition from acute to chronic pain after nerve injury. Nat Commun 2016;7:12029.
[17] Rajan S, Wischmeyer E, Karschin C, Preisig-Müller R, Grzeschik KH, Daut J, et al. THIK-1 and THIK-2, a novel subfamily of tandem pore domain K^+ channels. J Biol Chem 2001;276:7302–11.
[18] Madry C, Kyrargyri V, Arancibia-Cárcamo IL, Jolivet R, Kohsaka S, Bryan RM, et al. Microglial ramification, surveillance, and interleukin-1β release are

regulated by the two-pore domain K^+ channel THIK-1. Neuron 2018;97:299–312.
[19] Butovsky O, Jedrychowski MP, Moore CS, Cialic R, Lanser AJ, Gabriely G, et al. Identification of a unique TGF-β-dependent molecular and functional signature in microglia. Nat Neurosci 2014;17:131–43.
[20] Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J Neurosci 2014;34:11929–47.
[21] Sobolevsky AI. Quantitative analysis of tetrapentylammonium-induced blockade of open N-methyl-D-aspartate channels. Biophys J 2000;79:1324–35.
[22] Jara-Oseguera A, Llorente I, Rosenbaum T, Islas LD. Properties of the inner pore region of TRPV1 channels revealed by block with quaternary ammoniums. J Gen Physiol 2008;132:547–62.
[23] Ghatpande AS, Rao S, Sikdar SK. Tetrapentylammonium block of chloramine-T and veratridine modified rat brain type IIA sodium channels. Br J Pharmacol 2001;132:1755–60.
[24] Decosterd I, Woolf CJ. Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 2000;87:149–58.
[25] Carrasquillo Y, Gereau RW. Hemispheric lateralization of a molecular signal for pain modulation in the amygdala. Mol Pain 2008;4:24.
[26] Ji G, Neugebauer V. Hemispheric lateralization of pain processing by amygdala neurons. J Neurophysiol 2009;102:2253–64.
[27] Kolber BJ, Montana MC, Carrasquillo Y, Xu J, Heinemann SF, Muglia LJ, et al. Activation of metabotropic glutamate receptor 5 in the amygdala modulates pain-like behavior. J Neurosci 2010;30:8203–13.
[28] Bourbia N, Sagalajev B, Pertovaara A. Descending effect on spinal nociception by amygdaloid glutamate varies with the submodality of noxious test stimulation. Neurosci Lett 2014;570:26–31.
[29] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. New York: Academic Press; 1982.
[30] Sagalajev B, Bourbia N, Beloushko E, Wei H, Pertovaara A. Bidirectional amygdaloid control of neuropathic hypersensitivity mediated by descending serotonergic pathways acting on spinal $5\text{-}HT_3$ and $5\text{-}HT_{1A}$ receptors. Behav Brain Res 2015;282:14–24.
[31] Myers RD. Injection of solutions into cerebral tissue: relation between volume and diffusion. Physiol Behav 1966;1:171–4.
[32] Miyamoto K, Kume K, Ohsawa M. Role of microglia in mechanical allodynia in the anterior cingulate cortex. J Pharmacol Sci 2017;134:158–65.
[33] Zschenderlein C, Gebhardt C, von Bohlen Und Halbach O, Kulisch C, Albrecht D. Capsaicin-induced changes in LTP in the lateral amygdala are mediated by TRPV1. PLoS ONE 2011;6:e16116.
[34] Zeitler A, Kamoun N, Goyon S, Wahis J, Charlet A, Poisbeau P, et al. Favouring inhibitory synaptic drive mediated by $GABA_A$ receptors in the basolateral nucleus of the amygdala efficiently reduces pain symptoms in neuropathic mice. Eur J Neurosci 2016;43:1082–8.
[35] Wang X, Xiao AY, Ichinose T, Yu SP. Effects of tetraethylammonium analogs on apoptosis and membrane currents in cultured cortical neurons. J Pharmacol Exp Ther 2000;295:524–30.
[36] Leite-Almeida H, Cerqueira JJ, Wei H, Ribeiro-Costa N, Anjos-Martins H, Sousa N, et al. Differential effects of left/right neuropathy on rats' anxiety and cognitive behavior. Pain 2012;153:2218–25.