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

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

ABSTRACT

Background: The central amygdaloid nucleus (CeA) is involved in processing and descending regulation of pain. The amygdaloid mechanisms and cell types underlying pain processing and control as well as drugs suppressing pain-promoting actions of amygdala are still 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 the development of chronic pain and comorbid anxiety-like behavior following peripheral nerve injury.

Methods: Rats with a spared nerve injury (SNI) model of neuropathy had a chronic cannula for microinjections of drugs into the CeA or internal capsule (control site). Monofilament test was used to evaluate pain, and light-dark box (LDB) test 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. In the control injection site, perioperative TPA failed to attenuate pain development.

Conclusions: In the SNI model, perioperative treatment of the CeA with TPA attenuated the development of pain and comorbid anxiety-like behavior, while TPA treatment failed to influence maintenance of pain in the late postoperative period. Perioperative block of THIK-1 in amygdaloid microglia is among plausible mechanisms for the TPA-induced prophylactic effect, possibly together with actions of TPA on other amygdaloid receptors. TPA may provide a useful tool for the study of pain chronification and its prophylactic treatment.

Introduction

The amygdala is known to have a key role in processing of primary emotions [1]. Within the amygdala, the lateral capsular subdivision of the central nucleus (CeA) provides the principal output to the brainstem. The CeA is involved in processing 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, 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 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 NMDA [21], 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.

Materials and methods

Animals

Adult male Hannover-Wistar rats (Harlan, Horst, The Netherlands; weight: 180 to 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*.

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. 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. Additionally, a group of unoperated healthy control rats was tested in the experiments.

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

a 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], was purchased from Sigma-Aldrich (St.Louis, MO, USA). TPA was dissolved in saline and 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. Unilateral infusions of TPA, or an equivalent volume of vehicle (saline), were made 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 [30]. 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 (North Coast Medical, Inc. Morgan Hill, CA, USA) that produced forces varying from 2 g to 25 g. For the sake of clarity, the results are reported only for the force of 6 g, since the effect of TPA did not vary with the stimulus force (the interaction between stimulus force and drug effect proved not to be significant; not shown). During testing, rats were on a grid, free to move inside a transparent box. The monofilament was applied below the grid to the lateral part of the foot pad (sural nerve innervation area) in the left hind limb. At each time point, the paw was stimulated five times at a frequency of about 0.5 Hz. The withdrawal response frequency was recorded; i.e., response to all of the 5 consecutive stimulations represents a response rate of 100 %, whereas one response to 5 consecutive stimulations represents a response rate of 20 %. A response rate above the upper 95 % confidence interval (CI) of that in healthy controls was considered to represent pain hypersensitivity.

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 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 an arena that had two compartments (each 26.4 × 20.6 cm). One of the compartments was dark and the other one illuminated. 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 x 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.

Course of study

Animals were randomly divided into experimental groups and the experiments were performed in a blinded fashion. Figure 1 illustrates the course of the study for groups (n=8 in each group) in which TPA or saline was administered perioperatively

into the right CeA or the control injection site (the right internal capsule) to study influence of TPA 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 was induced, and the first intracerebral injection of TPA (30 μg) or saline was performed immediately after completing the SNI operation, after which the animal was allowed to recover from anesthesia in his home cage. Thereafter, intracerebral TPA/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 TPA/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.

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. 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.

A separate group of control animals that did not have a brain cannula nor a nerve injury was studied in the monofilament and the LDB tests following habituation to the

experimental conditions to illustrate the difference of SNI and healthy control animals under the present experimental conditions.

Histology

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

Effect of the amygdaloid administration of TPA on the development of mechanical hypersensitivity and anxiety-like behavior

Daily (right) amygdaloid microinjections of TPA (a non-selective THIK-1 channel antagonist) on D0-D3 and at a dose of 30 μg attenuated the development of mechanical pain hypersensitivity in the nerve-injured left limb during the

postoperative observation period of two weeks (main effect of TPA treatment: $F_{1,14}$ = 7.54, p = 0.016; Fig. 2 A), independent of the postoperative time point of testing (interaction between TPA treatment and postoperative time: $F_{1,14}$ = 2.29, p = 0.15). Main effect of postoperative time on the development of pain hypersensitivity was close to significance ($F_{1,14}$ = 4.07, p = 0.063). *Post hoc* testing indicated that pain hypersensitivity was significantly weaker in the TPA- than saline-treated SNI animals on D3 (Fig. 2 A). Independent of the treatment group and postoperative time point, the mean response rate of SNI animals to repetitive mechanical stimulation (i.e., the index of pain hypersensitivity) was above the 95% confidence interval (CI) of the response rate in unoperated control animals (illustrated by broken horizontal lines in Fig. 2 A).

In a test of anxiety-like behavior, light-dark box (LDB), perioperative TPA-treatment (daily 30 µg on D0-D3) in the right amygdala attenuated the development of anxiety-like behavior in SNI animals as indicated by the increase in time spent in light when compared with saline-treated SNI animals (main effect of TPA treatment: $F_{1,14}$ = 10.97, p = 0.005; Fig. 2 B), independent of the postoperative time point of testing (interaction between main effect of TPA treatment and postoperative time: $F_{1,14}$ = 0.05, p = 0.83). Main effect of postoperative time on anxiety-like behavior in SNI animals was short of significance ($F_{1,14}$ = 3.46, p = 0.084). *Post hoc* testing failed to show a significant difference in anxiety-like behavior between the TPA- and saline-treated SNI animals at D3 or D14. The time spent in light was below the lower 95% CI of that in unoperated control animals (illustrated by the lower horizontal broken line in Fig. 2 B) only in the saline-treated SNI animals that were tested at D14. No obvious side-effects were observed following amygdaloid administration of TPA at the dose of 30 µg.

Attempt to attenuate mechanical hypersensitivity with 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 C). No obvious side-effects were observed after amygdaloid administration of TPA at any of the doses.

Effect of TPA in a control brain injection site on the development of pain hypersensitivity

To assess, whether TPA in a brain control injection site attenuates development of 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 the most prominent effect on the development of pain hypersensitivity (Fig. 2 A). Microinjections of TPA into the right internal capsule failed to attenuate mechanical pain hypersensitivity on D3 (t_{14} = 0.97, p = 0.35; Fig. 2 D).

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 pain indicate that perioperative CeA administration of TPA, an antagonist of THIK-1 ion channels [18], attenuates the development of pain and anxiety-like behavior as revealed by the mechanical antihypersensitivity effect and the increase of time spent in the illuminated arena, respectively. In the brain, THIK-1 is highly expressed in microglia and only little, if at all, in other types of cells [19,20]. TPA, through action on THIK-1 channels, was recently shown to inhibit microglial ramification and surveillance as well as release of proinflammatory cytokines [18]. These findings are in line with the proposal that amygdaloid microglia, through action on THIK-1, contributed to the development of pain and comorbid anxiety in SNI animals of the present study, although a contribution of other TPA-sensitive mechanisms is possible. In the late postoperative period, amygdaloid administration of TPA failed to influence pain hypersensitivity indicating that TPA-sensitive amygdaloid mechanisms are not involved in the maintenance of chronic pain behavior. Our findings in amygdala are in line with earlier results reporting that microglia in the spinal dorsal horn exerts a pronociceptive role in the early rather than prolonged phase of nerve injury-induced pain [14-16].

CeA microinjections were performed at a volume of 0.5 µl, due to which is is not possible to pinpoint the critical site of drug action at a subnucleus level. The finding that TPA failed to influence pain behavior in a brain control injection site, the internal capsule, however, supports the proposal that the TPA-induced effect was due to action on the amygdala rather than outside of it. The CeA is the origin of the main output pathways from the amygdala [1] and it contributes to the descending control of pain [2]. Therefore, it may be argued that TPA reduced hypersensitivity due to a change in the descending drive originating or relaying in the CeA. However, other amygdaloid subnuclei through their direct or indirect intra-amygdaloid projections are known to influence the CeA [1]. Therefore, we cannot exclude potential contribution of other amygdaloid subnuclei to the TPA-induced effects. For example, in addition to the CeA [3-5], neurons in the basolateral amygdala potentially contribute to nociception and are subject to plasticity in chronic pain [31].

Partial ligation or chronic constriction of the sciatic nerve have induced activation of microglia in the amygdala of mice [11,12]. This earlier result in mice is in line with the hypothesis that the present CeA administration of TPA attenuated the development of pain behavior in the rat SNI model by attenuating activation of amygdaloid microglia. Moreover, since activated microglia release proinflammatory cytokines [6-8], the upregulation of amygdaloid cytokines in rats as well as mice [13] is also in agreement with the hypothesis that the inhibitory action of TPA on amygdaloid microglia provides a plausible explanation for the TPA-induced antihypersensitivity and anxiolytic effect. The pronociceptive and anxiogenic actions of amygdaloid microglia that were attenuated by TPA need to involve e.g. a cytokine-mediated interaction of microglia with neurons [8] to be expressed as pain and anxiety-like behavior.

Chronic pain, including that induced by SNI is known to induce anxiety [32]. The finding that amygdaloid administration of TPA suppressed the development of anxiety-like behavior as well as pain hypersensitivity in SNI animals suggests that an activation of a TPA-reversible amygdaloid mechanism, such as microglia, 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 potential role of microglia on behavior was studied using only pharmacological methods and with a compound that affects also various other channels (e.g. [21-23]) that may have contributed to the present findings. Therefore, further studies in which THIK-1 channels in amygdaloid microglia are selectively blocked are still needed to verify whether microglia, other TPA-sensitive amygdaloid mechanisms, or both have a key role in the present findings. Independent of the underlying mechanism, the present *in vivo* results suggest that TPA provides a powerful tool for the study and potential treatment of chronic pain and anxiety following peripheral injuries.

Conclusions

Perioperative TPA treatment of the amygdala attenuated the development of pain and anxiety-like behavior in SNI animals, while TPA failed to influence established pain hypersensitivity. TPA-induced block of THIK-1 in amygdaloid

microglia provides a plausible explanation for the present findings, although a potential contribution of other mechanisms affected by TPA (e.g. [21-23]) also needs to be taken into account.

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Legends for Figures

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.

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

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 mechanical hypersensitivity following perioperative TPA treatment. B) Development of anxiety-like behavior following perioperative TPA treatment. C) Effect of single amygdala injections of TPA at various doses on mechanical hypersensitivity in fully developed neuropathy. D) Effect of perioperative TPA treatment of a brain control injection site, the internal capsule, on mechanical hypersensitivity.

In all graphs, brain injections were performed in the right hemisphere. TPA dose was 30 μg, except in graph C the dose is shown in the X-axis. Testing of hypersensitivity was performed in the left (nerve-injured) hind paw. In A-B and D, the higher the response rate, the stronger the mechanical hypersensitivity. In C, the shorter the time spent in light, the more intense the anxiety-like behavior. Error bars

represent S.E.M. (in A-B and D, n=8, in C, n=5-6). $^{**}p < 0.01$ (t-test test with a Bonferroni correction for multiple comparisons). The broken horizontal line in graphs A-B and D represent the upper 95% confidence interval (CI) of the corresponding response in healthy control animals (n=6). In C, the broken horizontal lines represent the 95% CIs of the corresponding values obtained in healthy controls (n=6).

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; BLA, basolateral nucleus of the amygdala; LA, lateral nucleus of the amygdala; ic, internal capsule.

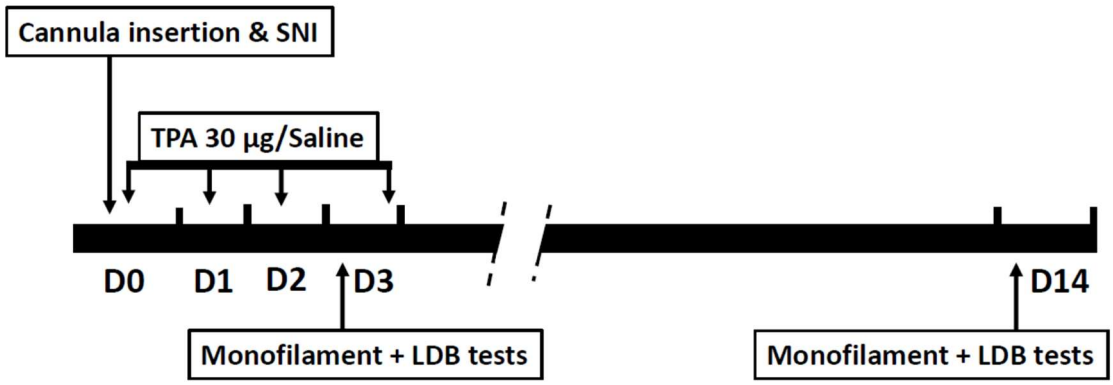

Figure 1.

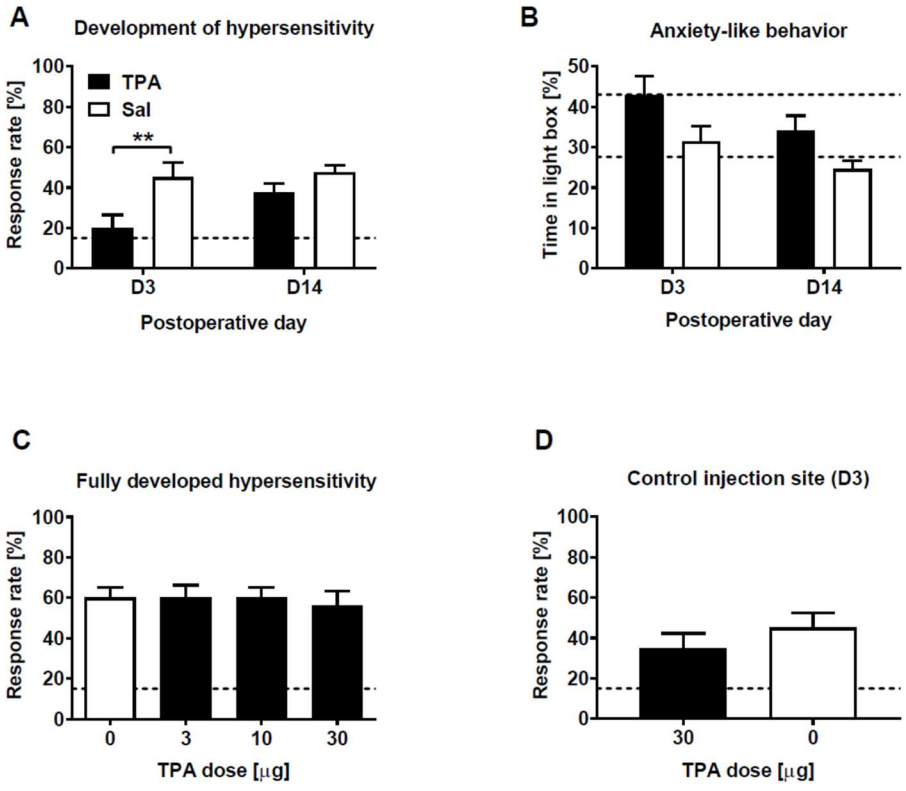

Figure 2.

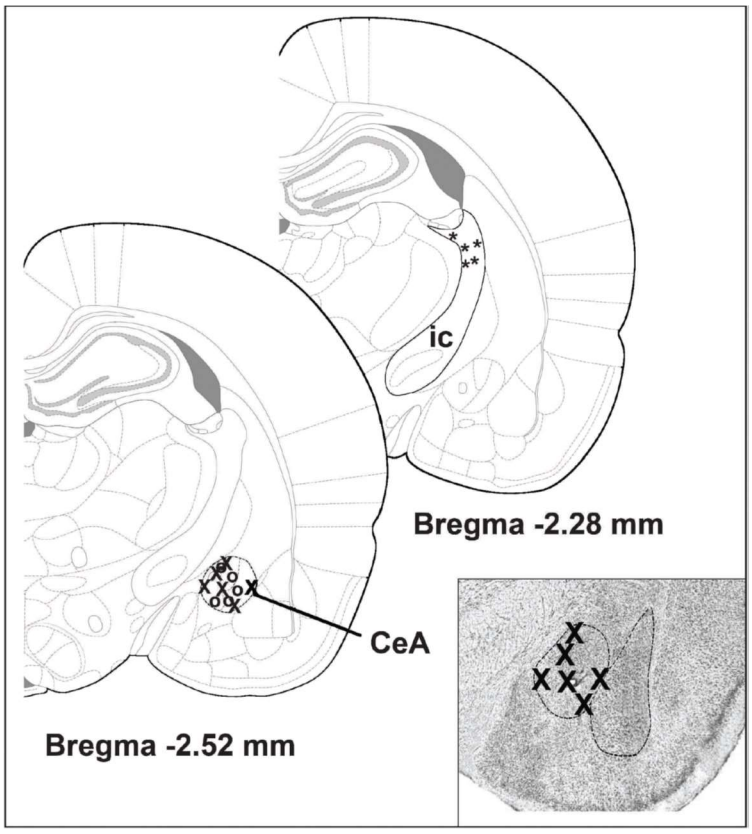

Figure 3.