EJN European Journal of Neuroscience

FENS Federation of European Neuroscience Societies

European Journal of Neuroscience, pp. 1–7, 2016 doi:10.1111/ejn.13217

Favouring inhibitory synaptic drive mediated by $GABA_A$ receptors in the basolateral nucleus of the amygdala efficiently reduces pain symptoms in neuropathic mice

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Keywords: etifoxine, neuronal network, neurosteroid

Edited by Paul Bolam

Received 27 October 2015, revised 16 February 2016, accepted 18 February 2016

Abstract

Pain is an emotion and neuropathic pain symptoms are modulated by supraspinal structures such as the amygdala. The central nucleus of the amygdala is often called the 'nociceptive amygdala', but little is known about the role of the basolateral amygdala. Here, we monitored the mechanical nociceptive thresholds in a mouse model of neuropathic pain and infused modulators of the glutamate/GABAergic transmission in the basolateral nucleus of the amygdala (BLA) via chronically-implanted cannulas. We found that an *N*-methyl-D-aspartate-type glutamate receptor antagonist (MK-801) exerted a potent antiallodynic effect, whereas a transient allodynia was induced after perfusion of bicuculline, a $GABA_A$ receptor antagonist. Potentiating $GABA_A$ receptor function using diazepam or etifoxine (a non-benzodiazepine anxiolytic) fully but transiently alleviated mechanical allodynia. Interestingly, the antiallodynic effect of etifoxine disappeared in animals that were incapable of producing 3α-steroids. Diazepam had a similar effect but of shorter duration. As indicated by patch-clamp recordings of BLA neurons, these effects were mediated by a potentiation of $GABA_A$ receptor-mediated synaptic transmission. Together with a presynaptic elevation of miniature inhibitory postsynaptic current frequency, the duration and amplitude of $GABA_A$ miniature inhibitory postsynaptic currents were also increased (postsynaptic effect). The analgesic contribution of endogenous neurosteroid seemed to be exclusively postsynaptic. This study highlights the importance of the BLA and the local inhibitory/excitatory neuronal network activity while setting the mechanical nociceptive threshold. Furthermore, it appears that promoting inhibition in this specific nucleus could fully alleviate pain symptoms. Therefore, the BLA could be a novel interesting target for the development of pharmacological or non-pharmacological therapies.

Introduction

The amygdaloid complex (or amygdala) is composed of a dozen subcortical nuclei within the temporal lobe. It can be subdivided anatomically and functionally into four distinct subregions: superficial, basolateral, central and medial (Veinante *et al.*, 2013). Functionally, it is an important structure of the limbic system providing an emotional value to sensory information and leading to adaptative behavioural responses. In line with our study, the amygdala plays a key role linking pain sensation and emotional responses as demonstrated by anatomical, electrophysiological, pharmacological and behavioural approaches (for review see Neugebauer *et al.*, 2009).

The central nucleus of the amygdala (CeA), often referred to as the 'nociceptive amygdala', serves as the output nucleus for major amygdala functions and regulates behavioural responses through projections to hypothalamic nuclei and various brainstem areas (Veinante *et al.*, 2013). This nucleus receives nociceptive information from two major pathways. The first pathway is composed of the spino-parabrachio-amygdaloid projections (Gauriau & Bernard, 2002). It provides nociceptive information of different modalities originating from a large number of tissues (e.g. skin, viscera, joints and muscles) and is mostly associated with an increase in the activity of CeA neurons. The second pathway is involved in the processing of polymodal nociceptive information originating from the thalamus and cerebral cortex. This sensory information is initially processed by neuronal circuits in the basolateral nucleus of the amygdala (BLA) before reaching the CeA, directly or indirectly via a relay in the intercalated cell clusters. Transfer of this information is ensured by a subpopulation of glutamatergic BLA neurons establishing excitatory synapses with the target neurons. In comparison to the direct BLA–CeA projections, which generate excitation, synaptic

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connections to intercalated GABAergic neurons provide an inhibitory interface capable of generating feed-forward inhibition (Royer *et al.*, 1999). Interestingly, lesion of the BLA has been recently proposed to prevent the development of chronic pain states (Li *et al.*, 2013).

The contribution of the BLA to nociceptive processing cannot be restricted to an input provider to the CeA. Its role in the control of analgesic responses has been demonstrated after local infusion of a wide variety of pharmacological substances (Veinante *et al.*, 2013). Injection of morphine into the BLA suppresses formalin-induced conditioned place aversion, at least in part, through inhibition of glutamatergic transmission mediated by *N*-methyl-D-aspartate (NMDA) receptors (Deyama *et al.*, 2007). From this study, it is also clear that the place aversion induced by intraplantar formalin is associated with excessive glutamate levels in the BLA. Of particular interest for our present study, stress-induced analgesia has been significantly reduced after bilateral injection into the BLA of the $GABA_A$ receptor agonist, muscimol, or of the positive allosteric modulator, diazepam (DZP) (Helmstetter, 1993; Rea *et al.*, 2011). These experiments and others dealing with the modulation of anxiety strongly indicated that GABAergic inhibitory transmission in the BLA plays a key role in the processing of sensory information including nociception.

In addition to the influence of GABAergic intercalated cell cluster neurons, the coding properties of glutamatergic BLA projection neurons can be placed under the control of local GABAergic interneurons. Although not fully characterized, they account for about 10–15% of the neuronal population and are tentatively classified based on expression of neurochemical markers such as parvalbumin, somatostatin, cholecystokinin, calbindin, calretinin and vasoactive intestinal peptide [see references in Ehrlich *et al.* (2009)]. The majority (50%) are parvalbumin-positive interneurons that establish synaptic contacts on the soma and proximal dendrites of projection neurons. The location of these inhibitory synapses is of course expected to play a critical role for the coding properties of principal BLA neurons (Veres *et al.*, 2014). This may easily explain the anxiolytic efficacy of benzodiazepine (Nagy *et al.*, 1979; Shibata *et al.*, 1982), which may preferentially target BLA sites (Niehoff & Kuhar, 1983; Thomas *et al.*, 1985; Hodges & Green, 1987). Based on these studies, anxiolytic effects have been attributed to the recruitment of α2/3-containing $GABA_A$ receptors expressed by BLA neurons. It is interesting to note here that intercalated cell cluster neurons express large amounts of extrasynaptic α4/δ-containing $GABA_A$ receptors, known to be extremely sensitive to neurosteroids, which contribute greatly to a tonic inhibition of principal BLA neurons (Marowsky & Vogt, 2014).

To date there is still little data demonstrating the influence of inhibitory/excitatory BLA microcircuits on nociceptive processes and pain responses. We therefore used a mouse model of neuropathic pain and intra-BLA injections to manipulate the balance between excitatory and inhibitory controls. Focusing mostly on GABAergic inhibitory controls, we characterized the analgesic action of benzodiazepine and non-benzodiazepine allosteric modulators of $GABA_A$ receptor function after *in vivo* intra-BLA injections. The associated changes in GABAergic synaptic transmission were also studied using patch-clamp recordings of pyramidal neurons of the BLA in brain slices.

Materials and methods

Animals and ethical issues

In the present study, behavioural testing was performed on 8–12-week-old C57Bl6J male mice (20–30 g; Janvier, Le Genest St. Isle, France), housed under standard conditions (room temperature, 22 °C; 12/12-h light/dark cycle) with *ad libitum* access to food and water. All animals were habituated to the room and to the tests at least 1 week before starting the experiments. For electrophysiological recordings, postnatal day 21–28 C57Bl6J mice of both sexes were used and housed in the same conditions. All procedures were performed in accordance with the recommendations of the directive (2010/63/EU) of the European Parliament and of the Council (September 22, 2010). The project received authorization from the French Department of Agriculture (license number 67-116 to P.P.) and from the regional ethic committee (CREMEAS AL/12/15/03/07).

Surgical procedures

All surgeries were performed under aseptic conditions and with ketamine/xylazine anaesthesia (ketamine, 17 mg/mL, i.p.; xylazine, 2.5 mg/mL, i.p., 4 mL/kg; Centravet, Taden, France).

Cannula implantation and drug infusion

The animals were anaesthetized with intraperitoneal injection of a mixture containing ketamine (75 mg/kg) and medetomidine (1 mg/kg). At the time of surgery, the animals were implanted with two 6-mm 23-gauge stainless-steel guide cannulas (outer diameter, 0.64 mm; inner diameter, 0.32 mm; PlasticsOne, USA). All animals were bilaterally implanted with cannulas targeting the basolateral amygdala (BLA relative to bregma; AP, −1.8 mm; ML, ± 3.1 mm; DV, −4.2 mm) by stereotaxic instruments according to the mouse brain atlas of Paxinos & Watson (1998). After surgery, animals received Atipamezole (10 mg/kg, i.p.) and Ketofene (5 mg/kg, i.m.), after which they had 7 days recovery before starting the experiments. Prior to drug injection, animals were lightly anaesthetized with isoflurane (2%) and 30-G cannulas (outer diameter, 0.3 mm; inner diameter, 0.15 mm; length, 6 mm; PlasticsOne) were inserted. Drugs (0.5 μL) were infused over a period of 5 min (0.1 μL/min) with a syringe pump (CMA 400; CMA Microdialysis, Sweden). The injection needles were left in place for an additional 5 min to allow diffusion. Animals were tested at 15 min after infusion after visual confirmation of full recovery from anaesthesia. After finishing the test sessions, bilateral injections of a 1% Chicago Sky Blue (Sigma) solution were targeted to the BLA (0.5 μL each side) as described in the Drug Application section below. After 30 min, the animals were anaesthetized with isoflurane (2.5%) and decapitated. Brains were removed, frozen in isopentane (Sigma) and stored at −80 °C. Sectioning (40 μm) was performed with a cryostat (Microm HM560, Thermo Scientific, USA). The sites of injections were verified according to the atlas of Paxinos & Watson (1998). Animals with injection sites located outside the BLA regions were not used in the analysis.

Neuropathic pain model

To produce a controlled constriction of the sciatic nerve, we used the cuff model, which has been well characterized previously (Benbouzid *et al.*, 2008). The common branch of the right sciatic nerve was exposed and a 2-mm-long split section of polyethylene tubing (inner diameter, 0.38 mm; outer diameter, 1.09 mm; PE-20, Harvard Apparatus, Les Ulis, France) was placed around it (Cuff group). The shaved skin layer was sutured closed. Sham-operated mice underwent the same surgical procedure as described above but without implantation of the cuff (Sham group).

Measures of mechanical nociception and pain symptoms

The mechanical threshold was measured using von Frey (VF) filaments (Bioseb, Chaville, France) as previously described (Chaplan *et al.*, 1994). Mice were placed in clear Plexiglass boxes (7 × 9 × 7 cm) on an elevated mesh screen, and were allowed to habituate for 15 min before testing. Filaments were applied to the plantar surface of each hindpaw in a series of ascending forces. We approached the filament toward the plantar surface slowly until it slightly bent at contact. At that point the pressure was immediately removed. Each filament was tested five times per paw and the threshold was defined as three or more withdrawals observed among the five consecutive trials. The results were expressed in grams and both hindpaws (ipsilateral allodynic right paw and contralateral control left paw) were tested on cuff and sham animals.

Patch-clamp recordings

Slice preparation and solutions

Coronal slices containing the BLA were prepared from C57Bl6J mice (21–28 days old). Briefly, after ketamine anaesthesia (2.5 mg/100 g, i.p., Centravet), animals were decapitated, and brains were removed quickly and immersed in 4 °C Mg-artificial cerebrospinal fluid containing (in mM): 124 NaCl, 10 $MgSO_4$, 2.7 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, continuously bubbled with 95% O_2/5% CO_2 leading to pH 7.4. The brain was glued on the slicer platform, and coronal brain slices (325 μm thick) were prepared with a tissue slicer (1200vs, Leica, Germany). Slices were hemisected and incubated for 45 min at 34 °C in artificial cerebrospinal fluid containing (in mM): 124 NaCl, 2.7 KCl, 1.25 NaH_2PO_4, 1.3 $MgSO_4$, 2.0 $CaCl_2$, 26.0 $NaHCO_3$, bubbled with 95% O_2/5% CO_2. After another hour at room temperature, in artificial cerebrospinal fluid containing 0.5 μM tetrodotoxin (Abcam Biochemicals, UK) and 2 mM kynurenic acid (Sigma-Aldrich) to block sodium voltage-dependent ionic channels and ionotropic glutamatergic receptors, a single slice was transferred to the recording chamber.

Electrophysiological recordings and data acquisition

Whole-cell voltage-clamp recordings were obtained with an amplifier (MultiClamp 700B, Axon Instruments, Foster City, CA, USA). Borosilicate glass electrodes (R = 3–8 MΩ) with an inner filament (outer diameter, 1.2 mm; inner diameter, 0.69 mm; Harvard Apparatus Ltd, UK) were pulled using a horizontal laser puller (P2000; Sutter Instruments, USA). Pipettes were filled with an intracellular solution containing (in mM): 125 KCl, 2 $MgCl_2$, 10 HEPES, 2 MgATP, 0.2 Na-GTP (pH adjusted to 7.3 with KOH, osmolarity 295 mOsm adjusted with sucrose). Due to the chloride reversal potential of 0 mV and holding potential of −60 mV, inward synaptic currents corresponded to GABA miniature inhibitory postsynaptic currents (IPSCs). Series capacitances and resistances were compensated electronically throughout the experiments using the main amplifier. Recordings were filtered at 2 kHz, digitized at 10 kHz and stored with PCLAMP 8.0 software (Molecular Devices, Sunnyvale, USA) before analysis. All recordings were from neurons identified visually as having a large, pyramidal-like soma corresponding to glutamatergic BLA output neurons. Our patch-clamp configuration for miniature IPSCs did not allow us to record action potential firing; therefore, these neurons could not be identified by their accommodating firing pattern (Sah *et al.*, 2003). In a subset of experiments, however, we found that large pyramidal-like cells indeed displayed accommodated spiking when tetrodotoxin and kynurenic acid were absent from the extracellular medium (data not shown). In addition, biocytine was included in the pipette solution identifying that these pyramidal neurons had axons targeting the CeA (data not shown).

Data analysis

Synaptic currents were detected and analysed using the Strathclyde electrophysiology software packages WINEDR and WINWCP (courtesy of Dr J. Dempster, University of Strathclyde, Glasgow, UK). Detection of each single event was further confirmed by visual inspection. For each synaptic current, the peak amplitude was measured as well as the exponential decay time constant (τ) and the overall inhibitory charge (area under the curve). In some experiments KCl was replaced by K $MeSO_4$ in patch pipette solution and the excitatory : inhibitory (E : I) ratio was calculated. For each recorded cell, the excitatory charge per time unit measured at E_{Cl} was divided by the inhibitory charge per time unit measured at E_{cation}.

Drug application

We used bicuculline methiodide (10 μM; Sigma-Aldrich) or MK-801 (1 μM; Sigma-Aldrich) as antagonists of $GABA_A$ receptor and NMDA glutamate receptor currents, respectively. They were prepared as 1000 times concentrated solutions in water. DZP (Roche, France) was dissolved in absolute ethanol at a final concentration of 10 μM (final ethanol concentration < 0.1%). Etifoxine (EFX) (2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride) (Stresam™, Biocodex, Gentilly, France) was prepared in dimethylsulphoxide (Sigma-Aldrich) and injected at a final concentration of 60 μM (final concentration of dimethylsulphoxide < 0.1%). Drugs were either infused (0.5 μL) in the basolateral nuclei *in vivo* or bath-applied at the same concentration on amygdala slices *in vitro*. Finasteride (FIN) [1,(5α)-androsten-4-aza-3-one-17β-(N-tert-butyl-carboxamide)] (Steraloids, Newport, RI, USA) solubilized in ethanol and diluted in olive oil was used to inhibit 5α-reductase activity. It was subcutaneously injected (100 μL, 25 mg/kg) three times every 2 days.

Statistics

Data are presented as mean ± SEM. Statistical analyses were performed with Prism software (GRAPHPAD) using multifactor ANOVA or *t*-test. The Tukey *posthoc* test and Bonferroni comparisons were used when appropriate for multiple comparisons in the behavioural as well as the electrophysiological experiments. Differences were considered to be statistically significant when $P < 0.05$.

Results

The influence of inhibitory and excitatory controls in the BLA was characterized in a mouse model of mono-neuropathy resulting from a chronic constriction of the sciatic nerve. As shown in Fig. 1A, the surgery aimed at installing a polyethylene cuff around the main branch of the right sciatic nerve triggered a significant ipsilateral (right paw) reduction in the VF threshold as a measure of mechanical nociceptive thresholds (repeated-measures two-way ANOVA, time × paw side, $F_{51,561} = 4.406$, $P < 0.0001$). Compared with the value before surgery (4.75 ± 0.55 g, n = 12), the threshold was strongly reduced to 0.62 ± 0.04 g (n = 12, Tukey, $P < 0.0001$) after 7 days and remained significantly lower for > 70 days (Fig. 1A). During this period, the VF values of the ipsilateral paw

were significantly different for every time-point from the respective mean values of the contralateral paw and of both paws of sham-operated mice. This result is fully in agreement with the presence of a mechanical allodynia resulting from the 'cuff' compression of the right sciatic nerve.

Excitation/inhibition balance control of nociception and pain in the basolateral nucleus of the amygdala in mice

Using mice preimplanted with BLA cannulas and bilateral injections, the effects of drugs modulating the balance between fast excitation and inhibition were analysed at 2 weeks after the cuff surgery. As shown above, VF thresholds measured at 2 weeks after surgery were significantly different (repeated-measures two-way ANOVA, time, $F_{5,90} = 21.9$, $P < 0.0001$; Bonferroni, $P < 0.0001$) between intact left and cuffed right paws. As early as 15 min after BLA injection of the NMDA receptor antagonist MK-801 (1 μM, Fig. 1B), the VF threshold increased significantly from 1.36 ± 0.04 to 4.62 ± 0.31 g ($n = 10$; Tukey, $P < 0.001$) and also remained not significantly different from the intact contralateral paw after 30 min (Bonferroni, $P = 0.073$). This strong analgesic effect was transient and thresholds returned to basal (neuropathic) levels at 60 min after the BLA infusion (1.16 ± 0.07 g, $n = 10$; compared with before injection: Tukey, $P = 0.9$). We next used bicuculline to inhibit $GABA_A$ receptor-mediated inhibition in the BLA (Fig. 1C). If bicuculline (10 μM) had no significant effect on the allodynic right paw (from 1.47 ± 0.12 to 1.20 ± 0.06 g after 15 min, $n = 12$; Bonferroni, $P = 0.99$), it induced a rapid decrease in the mean VF threshold of the left (intact) hindpaw (from 4.33 ± 0.22 to 1.07 ± 0.04 g after 15 min, $n = 12$; repeated-measures two-way ANOVA, time × paw side, $F_{5,45} = 25.21$, $P < 0.001$; Tukey, $P < 0.0001$). These low VF thresholds were not different from the cuffed paw at 15 and 30 min after bicuculline injection (Bonferroni, $P = 0.95$). Note that the effects of MK-801 and bicuculline were short-lasting (< 1 h) but particularly efficient at producing analgesia or allodynia, respectively. This effect seemed specific as vehicle injections were ineffective at modulating VF thresholds in all cases ($n = 10$ for MK-801 and $n = 12$ for bicuculline).

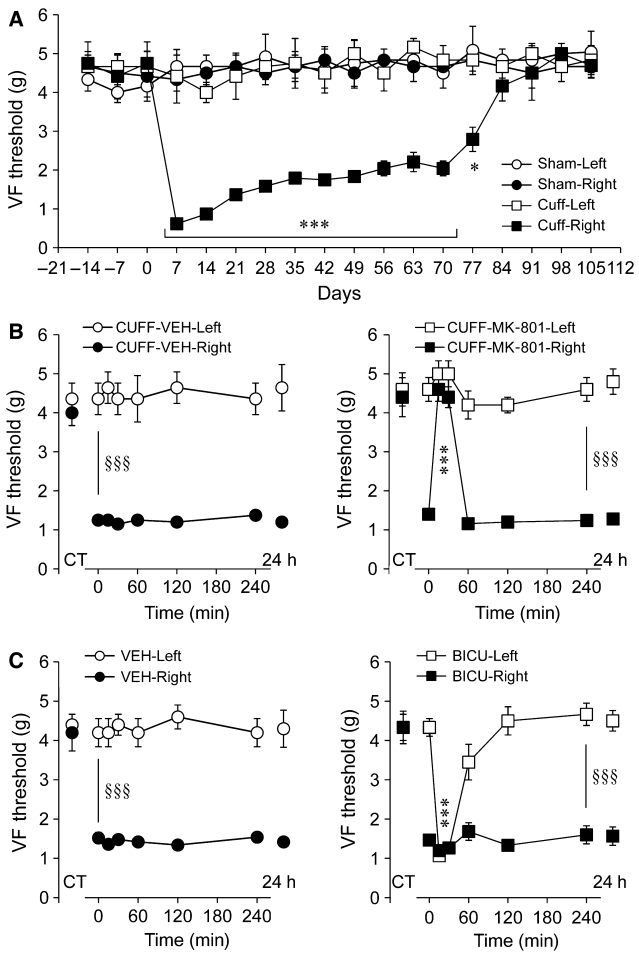

FIG. 1. Changes in mechanical nociceptive thresholds measured with VF filaments in mice that were sham-operated (Sham) (A) or with a chronic compression of the right sciatic nerve (Cuff) (A–C). Values are shown for left and right hindpaws. (A) Time-course of VF threshold before and after surgery (day 0) for both hindpaws in sham and cuffed mice. Note that mechanical allodynia developed rapidly for the right hindpaw of cuffed mice and persisted for about 3 months. (B and C) Changes in VF thresholds in neuropathic mice after a bilateral BLA injection at time 0 of the vehicle (VEH) solutions (left graphs): 1 μM MK-801 (B, right graph) or 10 μM bicuculline (BICU) (C, right graph). VF thresholds are also given in control (CT) (i.e. before cuff surgery). Tukey (*compared with paw value at time 0) and Bonferroni (§ for a time-point between paws) multiple comparisons are indicated: ***,§§§$P < 0.001$, *$P < 0.05$.

Analgesia mediated by the potentiation of inhibition in basolateral nucleus of the amygdala microcircuits

As the function of BLA microcircuits also relies on the activity of GABAergic interneurons, we next evaluated the consequences of injecting $GABA_A$ receptor-positive modulators on mechanical nociception and allodynia. We first used the classical benzodiazepine DZP (Fig. 2A, left graph). The injection of DZP at 1 μM (final volume 0.5 μL) was associated with a rapid and significant increase in the mean VF threshold for the left and right hindpaws (repeated-measures two-way ANOVA, time × paw side, $F_{5,55} = 17.97$, $P < 0.0001$). This increase was modest but statistically significant in the case of the left hindpaw as the maximal antinociceptive effect was reached at 30 min after injection of DZP when the VF mean threshold reached 6.33 ± 0.22 g (compared with before injection: 4.67 ± 0.28 g, $n = 12$; Tukey, $P < 0.0001$). The DZP analgesic effect was more pronounced for the ipsilateral (allodynic) paw (from 1.33 ± 0.04 to 5.33 ± 0.38 g, 30 min after DZP, $n = 12$; Tukey, $P < 0.001$). VF threshold changes followed a similar time-course in both paws. The DZP-induced analgesic effect was still present at 1 h after the injection and thresholds were back to their respective basal values after 2 h. We also used the recently described non-benzodiazepine compound EFX (Fig. 2B, left graph), which potentiates $GABA_A$ receptor function after binding to a site distinct from the benzodiazepines. Injected at a concentration of 60 μM, we found that it significantly increased the VF threshold of the allodynic paw (repeated-measures two-way ANOVA, time × paw side, $F_{5,45} = 10.08$, $P < 0.0001$) but was without apparent effect on the left paw (from 4.80 ± 0.33 to 5.20 ± 0.33 g, 2 h after EFX, $n = 10$; Tukey, $P = 0.99$). The mean VF thresholds of the right hindpaw were increased from 1.28 ± 0.06 g to a maximal value of 4.40 ± 0.40 g ($n = 10$; Tukey, $P < 0.0001$) at 2 h after EFX bilateral injection. No statistical differences could be found between the two paws of the cuffed EFX-treated mice at 60 and 120 min. It should be noted here that the antiallodynic effect was much slower to establish, compared with DZP, but was also transient as thresholds were back to their original level at 4 h after the injection.

Apart from a direct allosteric modulation of $GABA_A$ receptor function, DZP and EFX are also known to bind to mitochondrial

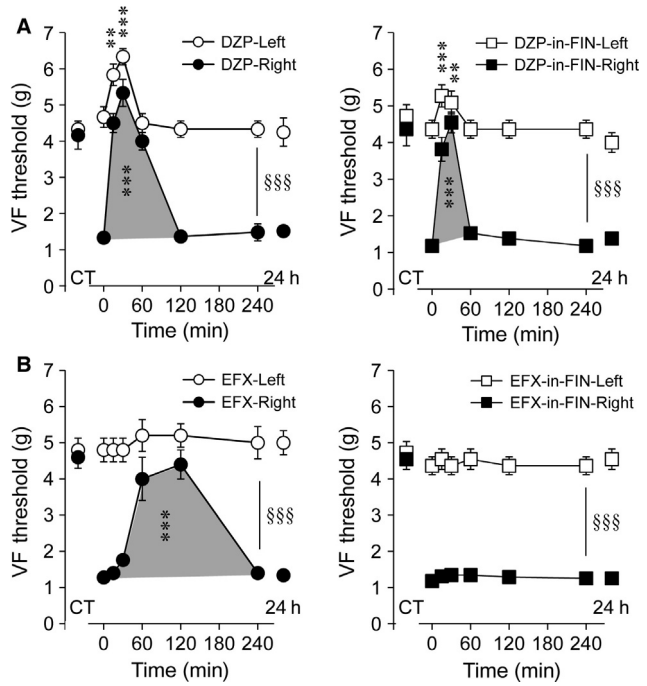

FIG. 2. Effects of BLA bilateral injections of DZP (1 μM) (A) and EFX (60 μM) (B) at time 0 on VF thresholds. The graphs on the right were obtained in FIN-treated animals. In all cases, VF thresholds are also given in control (CT) (i.e. before cuff surgery). Tukey (*compared with paw value at time 0) and Bonferroni (§ for a time-point between paws) multiple comparisons are indicated: ***,§§§$P < 0.001$, ** $P < 0.01$.

translocator protein (TSPO). In several brain structures, this action has been shown to promote the intracellular synthesis of allopregnanolone-like compounds (neurosteroids), which are the most potent endogenous modulators of $GABA_A$ receptor function (Poisbeau *et al.*, 2014). To verify this hypothesis, the synthesis of allopregnanolone-like compounds was inhibited by treating the mice with three subcutaneous injections of FIN every 2 days before BLA infusions (see Materials and methods). As illustrated in Fig. 2 (right graphs), DZP analgesia persisted in FIN-treated mice (repeated-measures two-way ANOVA, time × paw side, $F_{5,50} = 18.51$, $P < 0.0001$), whereas EFX action was fully prevented (repeated-measures two-way ANOVA, $F_{5,50} = 0.20$, $P = 0.99$). As the DZP analgesic effect was shorter (< 1 h) in FIN-treated mice, this result suggests that part of the transient analgesic effect was also controlled by endogenous neurosteroid. In the case of EFX and at this concentration, the endogenous production of allopregnanolone-like compounds was necessary to observe the antiallodynic effect.

Modulation of inhibitory synaptic controls by etifoxine in basolateral nucleus of the amygdala neurons in mice

To go one step further, the effect of these modulators was more directly characterized by using patch recording of BLA pyramidal neurons and analysis of $GABA_A$ receptor-mediated synaptic currents ($GABA_A$ receptor IPSCs) (Fig. 3). As expected, DZP applied at 1 μM on BLA neurons potentiated the apparent affinity of $GABA_A$ receptors. This was clearly seen by a prolongation of the decaying time constant (from 16.84 ± 2.17 to 30.62 ± 3.82 ms, $n = 6$; Student's *t*-test, $t = 6.21$, df = 5, $P = 0.0016$). If no significant change could be seen for the mean amplitude (from 15.54 ± 3.27 to 22.39 ± 4.31 pA, $n = 6$; Student's *t*-test, $t = 1.77$, df = 5, $P = 0.13$), a clear increase was observed for the frequency of occurrence (from 2.78 ± 0.32 to 5.21 ± 0.88 ms, $n = 6$; Student's *t*-test, $t = 3.65$, df = 5, $P = 0.015$). In the case of EFX, potentiation was associated with an increase in the mean frequency of occurrence of spontaneously-occurring $GABA_A$ IPSCs and of their amplitude and duration (see Fig. 3 and Table 1). This corresponded to an overall increase in the inhibitory charge (e.g. increase of both amplitude and duration) carried by individual $GABA_A$ IPSCs from 222.6 ± 20.6 to 334.9 ± 26.7 pA.ms ($n = 17$; one-way ANOVA, $F_{3,32} = 6.344$, Tukey, $P = 0.037$). When applied to BLA slices and within the time-course of a patch-clamp recording (about 1 h), we failed to see a proper washout of EFX. However, similar experiments performed on slices collected from FIN-treated mice revealed that the EFX increase of IPSC inhibitory charge was absent (Table 1). This was not the case for the transient increase in IPSC frequency, which persisted in the FIN condition.

To investigate, *in vitro*, the consequences of EFX on BLA network activity, we calculated the E : I ratio in control condition ($n = 13$) or in the presence of BIC ($n = 4$), EFX ($n = 6$) or MK-801 ($n = 6$) in 23 cells. In control conditions, excitation was more important than inhibition, and the mean E : I ratio was 2.62 (ranging from 0.99 to 6.71). Bicuculline fully suppressed inhibition. EFX, as well as MK-801, reversed the ratio, but in different ways (mean EFX E : I ratio, 0.26 ranging from 0.025 to 0.48; mean MK-801 E : I ratio, 0.31 ranging from 0.054 to 0.64). EFX increased the inhibitory charge by 66.51%, whereas the excitatory charge decreased (−605.72%) due to the large overall network inhibition. For its part, MK-801, as expected, decreased the excitatory charge (−89.71%) but also reduced the inhibitory charge (−15.93%) to lead to a net imbalance in favour of inhibition.

Discussion

The BLA is the initial site of sensory convergence and integration in the amygdala. Indeed, infusion in the BLA of mu opioid receptor agonist decreases thermal nociceptive sensitivity (Helmstetter *et al.*, 1995; McGaraughty & Heinricher, 2002). Moreover, the BLA controls stress-induced and fear-induced analgesia, which are both reduced by intra-BLA infusions of the $GABA_A$ receptor agonist muscimol (Rea *et al.*, 2011) or by the allosteric modulator DZP

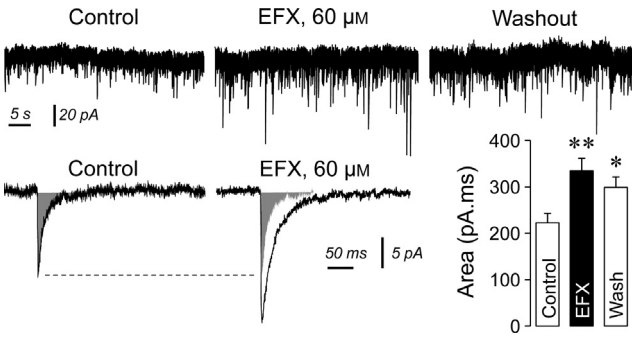

FIG. 3. Properties of $GABA_A$ receptor-mediated IPSCs recorded in BLA neurons in amygdala slices from 21–28-day-old mice using the whole-cell patch-clamp technique. As illustrated with the raw traces, bath application of EFX increased the frequency of occurrence of IPSCs and their amplitudes (see also Table 1). Histogram gives the mean inhibitory charge carried by the $GABA_A$ receptor IPSCs (area) in control, EFX and after washout of the drug. Note that, during the course of the recording (maximum recording time of 40 min), increase in inhibitory charge was hardly reversible after EFX bath application. Statistical significance with Tukey *posthoc* test: **$P < 0.01$, *$P < 0.05$.

TABLE 1. Changes in miniature $GABA_AR$-mediated IPSCs after application of EFX (60 μM) in the absence (top three lines) and presence (bottom three lines) of the 5α–reductase inhibitor finasteride (FIN, 10 μM)

Conditions	Freq. (Hz)	Area (pA.ms)	Amp. (−pA)	Decay (ms)	N
Control	3.2 ± 0.5	222.6 ± 20.6	13.6 ± 1.2	17.3 ± 1.3	17
EFX (60 μM)	**5.4 ± 0.8*** **	**334.9 ± 26.7****	**18.3 ± 1.3****	**21.0 ± 1.2***	17
Washout	3.9 ± 0.5	**299.0 ± 22.6***	15.1 ± 1.3	**21.3 ± 2.0***	13
Control in FIN	2.8 ± 0.8	192.3 ± 27.8	15.3 ± 2.9	17.8 ± 1.2	8
EFX (60 μM) in FIN	**5.6 ± 1.0***	209.9 ± 33.5	14.5 ± 2.7	17.6 ± 1.2	8
Washout in FIN	2.5 ± 0.6	206.8 ± 31.8	16.6 ± 2.3	17.7 ± 1.6	7

Mean frequency of occurrence (Freq.), inhibitory charge carried by IPSCs (Area), absolute current amplitude (Amp.) and half decay time are given for the IPSCs in the two different conditions. Data in bold were found to be statistically different at $***P < 0.001$, $**P < 0.01$, and $*P < 0.05$ with the Tukey *posthoc* test after one–way ANOVA. Statistical analysis was performed on BLA neurons submitted to at least 10 min of EFX washout (i.e. 13 out of 17 in control; 7 out of 8 in finasteride). In finasteride condition (FIN), note that only changes in frequency persisted during the application of EFX.

(Helmstetter, 1993). Based on this experimental evidence, it can be concluded that GABAergic inhibition in the BLA controls nociceptive responses as well as analgesia induced by fear or stress. Among many possibilities, BLA inhibition is likely to affect CeA neuronal activity, which has been shown to modulate pain behaviour through projections to descending pain control centres in the brainstem (Neugebauer *et al.*, 2009) and to control pain modulation systems (Helmstetter *et al.*, 1998). To fully understand the involvement of BLA inhibition in pain modulation, we also have to take into account the excitatory/inhibitory balance regulating BLA network excitability and therefore its impact on the activity of downstream structures. To date, no studies have shown any modulation of excitation in the BLA in pain conditions or in stress-induced or fear-induced analgesia. However, it has been shown that infusion of NMDA and non-NMDA receptor antagonists in the BLA reduced anxiety (for review, see Bergink *et al.*, 2004), demonstrating that modulation of excitation in the BLA can influence behaviour. Accordingly our results show that altering the BLA excitatory/inhibitory balance either alleviates neuropathic pain symptoms or induces pain. Indeed, a direct blockade of NMDA receptor suppressed mechanical allodynia, whereas a blockade of GABAergic inhibition produced allodynia in the healthy paw.

Based on these results and focusing on GABAergic transmission, we have explored the consequences of an increase of this neurotransmission on neuropathic-induced allodynia. Previously, it has been shown that BLA infusion of DZP attenuated stress-induced analgesia (Helmstetter, 1993) or produced antinociceptive effects (Jimenez-Velazquez *et al.*, 2010). Our results confirmed that DZP alleviates mechanical allodynia and produces analgesia in both neuropathic and healthy paws. In addition, we used another allosteric modulator of $GABA_A$ receptor (i.e. EFX, a non-benzodiazepine anxiolytic), which has a different pharmacological profile and binding sites that are distinct from those of benzodiazepines (Hamon *et al.*, 2003). A single infusion of EFX produced a longer-lasting alleviation of neuropathic pain symptoms without effect on the healthy paw. As shown previously in a model of neuropathic pain, this effect is likely to be contributed by the action of EFX on neurosteroidogenesis through its action on the mitochondrial translocator protein TSPO (Poisbeau *et al.*, 2014). Indeed, blockade of a key enzyme in the production of neurosteroid by FIN fully suppresses the antiallodynic action of EFX. A similar mechanism has already been proposed by our group (Zell *et al.*, 2014, 2015) to explain analgesia produced by high plasma corticosterone levels through the modulation of spinal GABAergic inhibition. We may then conclude that EFX, by increasing mitochondrial cholesterol entry, increases the synthesis of neurosteroids that are, in turn, potent positive modulators of GABAergic synaptic transmission (Poisbeau *et al.*, 1997) resulting in an increased inhibition within the BLA neuronal network.

To dissect the action of EFX further, we recorded GABAergic synaptic transmission on BLA output neurons and compared the effect of the well-characterized benzodiazepine DZP with that of EFX. DZP prolonged the decay time constant and increased the amplitude and frequency of IPSCs. The increase in IPSC duration and amplitude are mostly contributed by postsynaptic mechanisms (Vicini *et al.*, 1986), whereas the frequency increase generally results from a presynaptic mechanism as demonstrated for glycine receptors in rat spinal sacral dorsal commissural nucleus neurons (Jeong *et al.*, 2003). Presynaptic $GABA_A$ receptors have previously been identified in the BLA where they produced excitation (Woodruff *et al.*, 2006). Indeed, depolarization of the presynaptic terminal is expected to increase synaptic release and neuronal excitability, especially if the neurotransmitter is excitatory (Pugh & Jahr, 2011; Dellal *et al.*, 2012). In comparison with DZP, the effects of EFX are likely to be similar, as seen by the increase in frequency, amplitude and duration of synaptic currents. Our results show that the latter two depend greatly on EFX-induced neurosteroidogenesis, whereas the presynaptic increase of synaptic current frequency is not affected by FIN. Accordingly, at the network level, the cellular effect of EFX on synaptic transmission impacts network excitability. Indeed, EFX increases network inhibition, which in turn drastically reduces network excitation leading to a change of network excitatory/inhibitory balance and a decrease in network excitability.

Altogether, this overall increase of GABAergic inhibition impacts CeA neuronal activity. However, the nature of the impact on CeA neuronal activity is difficult to predict. There are many pathways between the BLA and CeA, either direct from glutamatergic output BLA neurons or through a relay in the intercalated mass (Veinante *et al.*, 2013). In the former case, the working hypothesis will predict that a decrease in BLA excitatory drive will cause a smaller activation of the GABAergic CeA neuronal network. In the latter case, a decrease of the excitatory drive on inhibitory intercalated cells will reduce their feed-forward inhibition on CeA neurons. To add layers of complexity, it has also been shown that feed-forward inhibition is itself controlled by inhibition coming from neighbouring intercalated cells (Royer *et al.*, 1999, 2000) and that some specific intercalated cells may respond to noxious stimuli and project back on BLA interneurons (Bienvenu *et al.*, 2015). Therefore, a large amount of work remains to be done in order to fully dissect the BLA–CeA microcircuits. However, our *in vivo* experiments clearly demonstrate that stimulating endogenous GABAergic inhibition in the BLA with DZP or EFX alleviates allodynia in neuropathic mice. This analgesic

action is mediated by a direct and indirect (i.e. neurosteroid-based) potentiation of $GABA_A$ receptor function at presynaptic and postsynaptic sites.

Conflict of interest

P.P. received financial support from Biocodex to establish its molecular mechanisms of action. The other authors declare no conflict of interest.

Acknowledgements

The Centre National de la Recherche Scientifique and Université de Strasbourg, Région Alsace supported the project (A.Z. and N.K.) FP7 Career Integration grant 334455, Initiative of Excellence (IDEX) Attractiveness grant 2013-15 and University of Strasbourg Institute for Advanced Study (USIAS) fellowship 2014-15 (A.C.). We thank the Biocodex laboratories (Gentilly, France) and, especially, Dr Marc Verleye for kindly providing us with EFX. Dominique Ciocca (Chronobiotron, UMS 3415) for helping us to set up cannula implantation and Tando Maduna for English proofreading and editing.

Abbreviations

BLA, basolateral nucleus of the amygdala; CeA, central nucleus of the amygdala; DZP, diazepam; E : I, excitatory : inhibitory; EFX, etifoxine; FIN, finasteride; ISPC, inhibitory postsynaptic current; NMDA, *N*-methyl-D-aspartate; VF, von Frey.

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