Neurosteroids increase tonic GABAergic inhibition in the lateral section of the central amygdala in mice

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Romo-Parra H, Blaesse P, Sosulina L, Pape HC. Neurosteroids increase tonic GABAergic inhibition in the lateral section of the central amygdala in mice. J Neurophysiol 113: 3421–3431, 2015. First published March 18, 2015; doi:10.1152/jn.00045.2015.—Neurosteroids are formed de novo in the brain and can modulate both inhibitory and excitatory neurotransmission. Recent evidence suggests that the anxiolytic effects of neurosteroids are mediated by the amygdala, a key structure for emotional and cognitive behaviors. Tonic inhibitory signaling via extrasynaptic type A γ-aminobutyric acid receptors (GABAARs) is known to be crucially involved in regulating network activity in various brain regions including subdivisions of the amygdala. Here we provide evidence for the existence of tonic GABAergic inhibition generated by the activation of δ-subunit-containing GABAARs in neurons of the lateral section of the mouse central amygdala (CeAl). Furthermore, we show that neurosteroids play an important role in the modulation of tonic GABAergic inhibition in the CeAl. Taken together, these findings provide new mechanistic insights into the effects of pharmacologically relevant neurosteroids in the amygdala and might be extrapolated to the regulation of anxiety.

NEUROSTEROIDS, also known as “neuroactive steroids” (Paul and Purdy 1992), can be synthesized de novo in both neurons and glia from progesterone (Cheney et al. 1995) or deoxycorticosterone (Khisti et al. 2005; Purdy et al. 1991) by the action of the enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase, respectively (Karavolas and Hodges 1991). A key function of neurosteroids is the modulation of neuronal excitability through interaction with certain neurotransmitter receptors (Lambert et al. 1995; Majewska et al. 1989; Paul and Purdy 1992; Rupprech 1997; Ruppurret and Holsboer 1999). Progesterone metabolites such as 3α-hydroxy-5α-pregn-20-one (allopregnanolone or 3α,5α-THPROG) and 3α,5α-tetrahydrodeoxycorticosterone (3α,5α-THDOC) are among the most potent positive allosteric modulators of type A γ-aminobutyric acid receptors (GABAARs) (Majewska et al. 1989), and their administration in pharmacological doses elicits anxiolytic, anticonvulsant, and sedative-hypnotic effects in rodents (Majewska 1992). Specifically, local infusion of allopregnanolone into the rat central nucleus of the amygdala (CeA) produced anxiolytic-like behavioral effects (Akwa et al. 1999). Thus neuroactive steroids might play a specific role in the modulation of GABAergic transmission in this region and in the regulation of anxiety.

The neurotransmitter GABA can operate in two modes: a “phasic” mode involving the activation of synaptically clustered GABAARs by quantal release of GABA and a “tonic” mode mediated by GABAARs located extrasynaptically (Farrant and Nušser 2005; Mody 2005). GABAARs are ligand-gated chloride (and bicarbonate) channels (Olsen and Sieghart 2005) consisting of five subunits that belong to various subunit classes (α1–α6, β1–β4, γ1–γ3, δ, ε, π, θ, ρ1–ρ3) (Barnard et al. 1998; Kumar et al. 2002; Whiting 1999). Synaptic and extrasynaptic receptors exhibit distinct structural and biophysical properties (Belleti et al. 2009). Most GABAARs, however, consist of two α-, two β-, and one γ- or δ-subunit (Sieghart and Sperr 2002). Synaptic GABAARs almost invariably incorporate the γ2-subunit in combination with α (α1, α2, and a3)- and β2/3-subunit isoforms, while the extrasynaptic receptors are distinct from their synaptic counterparts and are composed of the α4-, α5-, or α6-subunit in combination with β- and δ-subunits (Belleti et al. 2006).

GABAergic tonic inhibition has been described in many regions of the brain (Farrant and Nušser 2005; Glykys and Mody 2007; Semyanov et al. 2004) including the lateral amygdala (LA), the basolateral amygdala (BA; Marowsky et al. 2012), and the medial section of the CeA (CeAm; Herman et al. 2013). Besides, so far, three GABAAR subunits have been found to be expressed in mouse CeA: α1, δ, and α5, the first two of which were functionally related to the generation of tonic inhibition (Herman et al. 2013). Neurosteroids act preferentially but not exclusively on the GABAAR δ-subunit (Brown et al. 2002). Moreover, changes in the tonic GABAAR-mediated conductance are of high clinical relevance as they are related to schizophrenia, epilepsy, and Parkinson disease (for review see Brickley and Mody 2012).

The CeA is a primarily GABAergic nucleus and forms the main output station of the amygdala complex, thereby being a critical component of the neurocircuitry underlying stress, fear and anxiety, and pain (Ehrlich et al. 2009; Neugebauer et al. 2004; Pape and Pare 2010). Not only does the GABAergic system in the amygdala modulate fear and anxiety under physiological conditions, but alterations in this system may also predispose individuals to pathological anxiety traits (Parsons and Ressler 2013; Tasan et al. 2011).

It has been shown that the neurosteroid allopregnanolone affects phasic GABAergic transmission in CeA neurons by the negative modulation of evoked inhibitory postsynaptic currents (IPSCs), which might involve the activation of NMDA receptors since the effect was occluded by the NMDA receptor antagonist dl-2-amino-5-phosphonovalerate (AP-5) (Wang et
However, no neurosteroid effects on tonic GABAergic inhibition in CeA have been reported yet. Here we tested the hypotheses that 1) neurons of the lateral section of the CeA (CeAl) receive tonic GABAergic input mediated by GABA_ARs containing the δ-subunit and that 2) this tonic inhibition is modulated by the action of neurosteroids.

**MATERIALS AND METHODS**

All experiments were carried out in accordance with European Communities Council Directive 86/609/EEC. Protocols were approved by the Landesamt für Natur, Umwelt, und Verbraucherschutz Nordrhein-Westfalen (LANUV NRW; AZ 8.87-50.10.46.09.170 and 87-51-04.2010.A322).

Slice preparation. As previously described (Kamprath et al. 2011; Sosulina et al. 2010), C57BL/6J male mice (8–12 wk old; n = 30) were deeply anesthetized with isoflurane and decapitated. Their brains were rapidly removed and put into oxygenated (95% O_2–5% CO_2) ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 120 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 22 NaHCO_3, 2 MgSO_4, 2 CaCl_2, and 10 glucose; pH was 7.4. Coronal slices (250–300 μm thick) were prepared on a vibratome (Leica Microsystems, Wetzlar, Germany) at 4°C. Slices were incubated at 34°C for 20 min and then allowed to equilibrate for at least 1 h at room temperature (RT). After recovery, slices were transferred to a recording chamber continuously superfused with ACSF (~2 ml/min).

Electrophysiological recordings and data analyses. Whole-cell (current clamp and voltage clamp) recordings were made at 30°C from the CeAl (Fig. 1A, inset) with an EPC-10 single patch-clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Neurons were visualized by infrared video microscopy (camera CF8/1, Kappa, Gleichen, Germany). Cells were discarded from analysis if the resting membrane potential was more positive than ~50 mV immediately after breaking through the cell membrane. For current-clamp recordings, patch pipettes (3–4 MΩ) were filled with an intracellular solution (GTP; pH 7.2 adjusted with 1 M KOH. For each current-clamp experiment, the amplitude of the voltage deflection (V) evoked by the application of negative current injections (I) (~10 pA, 500 ms) at ~65 mV was measured and the input resistance was calculated based on Ohm’s law (R = V/I). To determine excitability action potentials were evoked by positive current injections with varying amplitudes and 500-ms duration. The amplitude of the depolarizing step was adjusted (range 50–100 pA) to evoke a minimum of two action potentials in an individual cell. Manual clamping was performed by the injection of direct positive current through the recording pipette in order to depolarize the neuron to the membrane potential prior to THDOC application. For voltage-clamp recordings the pipette solution was (in mM) 110 CsCl, 30 KCl–glucuronate, 1.1 EGTA, 10 HEPES, 0.1 CaCl_2, 1.3 MgCl_2, 3.52 pF (mean: 3.52 ± 0.33 pF; n = 30). For voltage-clamp recordings the pipette solution was (in mM) 110 CsCl, 30 KCl–glucuronate, 1.1 EGTA, 10 HEPES, 0.1 CaCl_2, 1.3 MgCl_2, 3.52 pF (mean: 3.52 ± 0.33 pF; n = 30). For voltage-clamp recordings the pipette solution was (in mM) 110 CsCl, 30 KCl–glucuronate, 1.1 EGTA, 10 HEPES, 0.1 CaCl_2, 1.3 MgCl_2, 3.52 pF (mean: 3.52 ± 0.33 pF; n = 30). For voltage-clamp recordings the pipette solution was (in mM) 110 CsCl, 30 KCl–glucuronate, 1.1 EGTA, 10 HEPES, 0.1 CaCl_2, 1.3 MgCl_2, 3.52 pF (mean: 3.52 ± 0.33 pF; n = 30).

Baseline was defined from a time frame of 2 min of stable recording under control conditions. Holding current (I_h) was determined as the current required to maintain the neuron at a holding potential of ~70 mV in the whole cell configuration. The tonic GABA current was calculated and distinguished from IPSCs with a routine written in MATLAB (The MathWorks) in which the mean I_h was obtained by a Gaussian fit to an all-points histogram from 2 min under steady-state conditions. We quantified responses as the difference in I_h between baseline and experimental conditions. Frequency, amplitude, and decay time of isolated spontaneous IPSCs (sIPSCs) were analyzed with a detection threshold of 15 pA with Mini Analysis. All events were visually confirmed, and detection threshold was readjusted in case events were not detected with standard settings. All detected events were used for event frequency analysis, but superimposed events were eliminated for amplitude and decay kinetic analysis as reported previously (Herman et al. 2013). We determined means of IPSC characteristics from baseline and experimental drug conditions containing a minimum of 60 events in a time period of 2 min under steady-state conditions. Decay kinetics was calculated with monoexponential curve fittings and is reported as half-width decay time (milliseconds).

**Immunohistochemistry.** Immunohistochemical detection of the GABA AR δ-subunit was performed with an anti-GABA AR δ-subunit antibody (AB9752, Millipore). Staining procedures were similar to those described in a previous study where the specificity of the antibody was confirmed with a GABA AR δ-subunit-knockout mouse (Maguire et al. 2009). In brief, C57BL/6J mice (8–12 wk old; n = 3 mice) were deeply anesthetized with pentobarbital (50 mg/kg) and perfused transcardially with phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde (in 0.15 M Na-phosphate buffer, pH 7.4). Brains were removed and stored in 4% paraformaldehyde overnight at 4°C. After incubation in 30% sucrose-PBS for cryopreservation, coronal sections (30 μm) were cut with a freezing microtome (Frigomobil 1205; Jung, Heidelberg, Germany). Sections were collected in 15% sucrose-PBS, followed by three washes in PBS. After 1-h incubation in 3% H_2O_2 diluted in methanol, followed by two washes in PBS, sections were blocked for 1 h in 3% bovine serum albumin (BSA), 10% goat serum, and 0.3% Triton in PBS. A guinea pig anti-MAP2 antibody against microtubule-associated protein 2 was obtained from Synaptic Systems (Göttingen, Germany). Antibodies were diluted 1:1,000 in blocking solution. Incubation with agitation was carried out at 4°C overnight. After three wash steps in PBS, sections were transferred to a solution of 0.3% Triton, 1% BSA, 1% goat serum and treated with the secondary antibodies goat anti-rabbit conjugated to Alexa Fluor 488 and goat anti-guinea pig conjugated to Alexa Fluor 594 (both diluted 1:1,000, Life Technologies) for 2 h at RT. The fluorescence nuclear stain 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich; 1:1,000) was added to label cell nuclei. After four additional washes in PBS, sections were mounted with Vectashield HardSet mounting medium (Vector laboratories, Burlingame, CA) and imaged with a laser scanning confocal microscope (Nikon eCIPlus) equipped with a CF755 LWD ×16/0.8 NA objective (Nikon). Sequential three-channel imaging with 405-, 488-, and 543-nm excitation in combination with adequate emission filters (450/30 nm; 515/30 nm, and 605/75 nm, respectively) prevented
bleed-through. Images (1,024 × 1,024 pixels) were further processed with ImageJ (http://rsb.info.nih.gov/ij/). Final images represent image mosaics generated with the ImageJ plug-in MosaicJ (Thevenaz and Unser 2007).

Drugs and chemicals. Drugs were applied by bath perfusion. DNQX (10 µM), AP-5 (50 µM), CGP55845A (1 µM), gabazine (GBZ, 0.5 µM), NNC-711 (5 µM), and picrotoxin (PTX, 100 µM) were from Ascent Scientific, tetrodotoxin (TTX, 1 µM), DS2 (1 µM), and THIP (10 µM) were from Sigma.

Fig. 1. Tonic GABAAergic inhibition mediated by δ- and α5-subunit-containing GABA type A receptors (GABA₆,5Rs) is present in neurons of the lateral section of the central amygdala (CeAl) and is regulated by GABA transport. A: original current trace showing spontaneous inhibitory postsynaptic currents (sIPSCs) in a CeAl neuron. Gabazine (GBZ) increased holding current (Iₜₐₜ) and blocked sIPSCs. Picrotoxin (PTX) shifted Iₜₐₜ to more positive values compared with baseline (indicated by dotted line). Inset: infrared DIC image illustrating the recording site. CeAm, medial section of central amygdala; LA, lateral amygdala; BLA, basolateral amygdala; itc, intercalated cell masses. B: original current trace from a CeAl neuron in the continuous presence of tetrodotoxin (TTX). TTX did not prevent the effects of GBZ and PTX. C: original current trace during application of the GABA transporter 1 (GAT-1) blocker NNC-711 (5 µM), followed by PTX (100 µM). Note that Iₜₐₜ was increased by NNC-711 and this effect was reversed by PTX. D: application of the δ-subunit-specific agonist THIP (10 µM) induced a fast negative shift in Iₜₐₜ followed by a small desensitization reaching a plateau. Subsequently, PTX reversed the THIP effect and caused a significant shift in Iₜₐₜ. E: current trace showing the increase in Iₜₐₜ by application of the GABA₆,5-subunit agonist DS2 (1 µM). Application of PTX shifted Iₜₐₜ to more positive values exceeding baseline. The shifts induced by DS2 and PTX were significant compared with baseline. F: representative data traces showing a positive shift in Iₜₐₜ after application of L-655,708 (500 nM, blocking α5-subunit-containing GABA₆,5Rs) and PTX. Application of PTX abolished sIPSCs and caused an additional significant shift in Iₜₐₜ. G: summary data for each pharmacological condition; data were normalized to baseline (last 2 min before drug application) of each single experiment. Significance was tested against control (control = 0). n.s., Not significant. *P < 0.05, ***P < 0.001.
finasteride (5 μM), and allopregnanolone (5 μM) were from Tocris, and THDOC (1 and 10 nM) was from Sigma-Aldrich.

Statistical analyses. Because of the variability in \( I_{\text{hold}} \) among different cells and to clearly show the full drug effect on \( I_{\text{hold}} \), we defined the baseline current as 0 for each single experiment and the drug effect was normalized to baseline. We used the one-sample \( t \)-test to compare the normalized current and the baseline value 0. A paired two-tailed \( t \)-test was used for comparisons made within the same recording with raw data (iPSC parameters), an unpaired two-tailed \( t \)-test for comparisons between two different groups, and a one-way ANOVA with Bonferroni’s post hoc analysis for comparisons of three or more groups. All statistical analyses were performed with MATLAB, Prism 5.02 (GraphPad Software, San Diego, CA), and Statistica 8 (StatSoft Europe). Data are presented as means ± SE. Statistical significance was accepted if \( P < 0.05 \).

RESULTS

Tonic inhibitory transmission is present in CeA neurons. GABAergic currents were recorded from visually identified neurons located in the CeA (Fig. 1A, inset). The use of CsCl inside the pipette, however, prevented the characterization of the recorded neurons based on their biophysical properties. The CsCl intracellular solutions has a calculated chloride reversal potential of 0 mV; therefore, GABA\(_{\text{A}}\)R-mediated sIPSCs appeared as fast inward currents at a holding potential of 70 mV in the presence of NMDA receptor, non-NMDA glutamate receptor, and GABA\(_{\text{A}}\)R antagonists (see MATERIALS AND METHODS; Fig. 1A). The frequency of sIPSCs under control condition was 6.9 ± 0.84 Hz. The GABA\(_{\text{A}}\)R-mediated tonic conductance was estimated by the shift in the baseline \( I_{\text{hold}} \) obtained by blocking GABA\(_{\text{A}}\)Rs with selective antagonists. To isolate the contribution of action potential-dependent vesicular release of GABA from neighboring synapses, we used low concentrations of GBZ, a competitive GABA\(_{\text{A}}\)R antagonist displacing GABA from its binding site (Hamann et al. 1988). A concentration of 0.5 μM has been reported to selectively abolish spontaneous GABA\(_{\text{A}}\)R-mediated synaptic currents but not tonic currents (Bai et al. 2001; Semyanov et al. 2003). In CeA neurons, GBZ (0.5 μM) abolished spontaneous synaptic events (Fig. 1A) and produced a small inward shift of the baseline \( I_{\text{hold}} \) (\( \Delta I_{\text{hold}} = -2.63 ± 2.3 \) pA). Normalization to cell size showed no significant change in current density (\( \Delta C D = -0.035 ± 0.03 \) pA/pF; 1-sample \( t \)-test; \( P = 0.2628 \) vs. baseline; \( n = 11 \); Fig. 1, A and G). To subsequently and completely block GABA\(_{\text{A}}\)R-mediated conductance in the same neurons, we used the GABA\(_{\text{A}}\)R channel blocker PTX. PTX is an open channel blocker and acts as a noncompetitive GABA\(_{\text{A}}\)R antagonist that has equivalent efficacy in blocking low-affinity synaptic and high-affinity extrasynaptic GABA\(_{\text{A}}\)Rs (Stell and Mody 2002). Thus application of PTX (100 μM) on top of GBZ produced an outward shift in \( I_{\text{hold}} \) (\( \Delta I_{\text{hold}} = 57.19 ± 17.38 \) pA, \( \Delta C D = 0.9155 ± 0.32 \) pA/pF; 1-sample \( t \)-test, \( P = 0.0181 \) vs. baseline; Fig. 1, A and G), reflecting the blockade of a tonic GABA\(_{\text{A}}\)R-mediated current. To determine whether or not the induced shift in \( I_{\text{hold}} \) was due to the generation of action potential-dependent GABA release under basal conditions, we performed the same type of experiment in the presence of TTX (1 μM). Under these conditions, action potential-independent miniature IPSCs (mIPSCs) occurred at a frequency of 3.9 ± 0.4 Hz. Application of GBZ blocked mIPSCs and induced an inward shift of \( I_{\text{hold}} \) (\( \Delta I_{\text{hold}} = -4.2 ± 0.63 \) pA; \( \Delta C D = -0.07108 ± 0.15 \) pA/pF; 1-sample \( t \)-test, \( P = 0.0031 \) vs. baseline, \( n = 7 \); Fig. 1, B and G). It should be noted that GBZ also increases the chloride conductance (Bai et al. 2001; Scimemi et al. 2005; Semyanov et al. 2004). This inverse antagonist mechanism at low concentrations (0.2–0.5 μM; Włodorczyk et al. 2013) might contribute to the small inward shift in \( I_{\text{hold}} \) seen in parallel with the expected block of spontaneous synaptic events (Fig. 1A). In the same seven neurons, the successive application of PTX shifted \( I_{\text{hold}} \) to more positive values (\( \Delta I_{\text{hold}} = 48.6 ± 16.8 \) pA, \( \Delta C D = 0.7877 ± 0.24 \) pA/pF; 1-sample \( t \)-test, \( P = 0.0173 \) vs. baseline; Fig. 1, B and G). The fact that the PTX-induced shift in \( I_{\text{hold}} \) persisted in the presence of TTX indicates that presynaptic GABA release during spontaneous action potential-dependent network activity is not a major source of ambient GABA mediating the tonic current.

Next, we tested for the effect of an increased ambient GABA concentration. To do so, we applied NNC-711 (5 μM), a specific inhibitor of GABA transporter 1 (GAT-1), to block GABA reuptake (Suzdak et al. 1992). Consistent with previous reports in hippocampus (Holter et al. 2010; Stell and Mody 2002), NNC-711 shifted \( I_{\text{hold}} \) to more negative values in six of six cells (\( \Delta I_{\text{hold}} = -9.05 ± 5.96 \) pA, \( \Delta C D = -0.24 ± 0.056 \) pA/pF; 1-sample \( t \)-test, \( P = 0.0080 \) vs. baseline; Fig. 1, C and G). The addition of PTX in the continuous presence of NNC-711 shifted \( I_{\text{hold}} \) back to baseline (\( \Delta I_{\text{hold}} = 0.1 ± 4.4 \) pA, \( \Delta C D = -0.01 ± 0.05 \) pA/pF; 1-sample \( t \)-test, \( P = 0.8975 \) vs. baseline; Fig. 1C). Hence, while the effect of NNC-711 was small, GAT-1-mediated GABA transport reduced tonic GABAergic inhibition.

GABA\(_{\text{A}}\)R δ-subunit is functionally expressed in CeA neurons. Tonic GABAergic currents are usually mediated by extrasynaptic GABA\(_{\text{A}}\)Rs containing α4-, α5-, or α6-subunits in combination with β- and δ-subunits (Belelli et al. 2006). Recent immunohistochemical and electrophysiological data demonstrated the expression of α1- and δ-subunits in the CeAm in mice (Herman et al. 2013). Because there is evidence that the expression of the α1-subunit is much lower in CeA compared with CeAm (Hortnagl et al. 2013) and because the GABA\(_{\text{A}}\)R δ-subunit is the main target of neurosteroid action, we focused on this subunit.

Application of the δ-subunit-specific agonist THIP hydrochloride (10 μM; Krosggaard-Larsen et al. 2004) resulted in an inward shift of \( I_{\text{hold}} \) in CeA neurons (\( \Delta I_{\text{hold}} = -125.6 ± 23.2 \) pA, \( \Delta C D = -2.281 ± 0.58 \) pA/pF; 1-sample \( t \)-test, \( P = 0.0109 \) vs. baseline, \( n = 6 \); Fig. 1, D and G), providing further evidence that GABA\(_{\text{A}}\)Rs containing δ-subunits are involved in the tonic current component. When PTX was added, \( I_{\text{hold}} \) shifted back to baseline without exceeding it (\( \Delta I_{\text{hold}} = 9.72 ± 20.55 \) pA, \( \Delta C D = -0.046 ± 0.35 \) pA/pF; 1-sample \( t \)-test, \( P = 0.9 \) vs. baseline; Fig. 1D).

Furthermore, we tested the effect of the δ-subunit positive allosteric modulator DS2. At a low concentration (1 μM), DS2 shifted \( I_{\text{hold}} \) to more negative values (\( \Delta I_{\text{hold}} = -13.16 ± 4.7 \) pA, \( \Delta C D = -0.30 ± 0.096 \) pA/pF; 1-sample \( t \)-test, \( P = 0.0366 \) vs. baseline, \( n = 5 \); Fig. 1, E and G). The addition of PTX shifted \( I_{\text{hold}} \) to more positive values not different from baseline (\( \Delta I_{\text{hold}} = 6.4 ± 5.4 \) pA, \( \Delta C D = 0.2104 ± 0.1391 \) pA/pF; 1-sample \( t \)-test, \( P = 0.2048 \) vs. baseline, \( n = 4 \); Fig. 1E).
While the GAB\(A_\delta\)R \(\delta\)-subunit is the most relevant subunit in terms of neurosteroid action, we also checked for the contribution of the \(\alpha_5\)-subunit to tonic inhibition in CeAl neurons, using the \(\alpha_5\)-subunit partial inverse agonist L-655,708 (500 nM) (Prenosil et al. 2006; Quirk et al. 1996). While L-655,708 had no effect in the CeAm (Herman et al. 2013), bath application of this drug induced a small but significant effect on \(I_{\text{hold}}\) in CeAl neurons (\(\Delta I_{\text{hold}} = 15.2 \pm 1.6\) pA, \(\Delta CD = 0.3207 \pm 0.82\) pA/pF; 1-sample t-test, \(P = 0.030\) vs. baseline, \(n = 4\); Fig. 1, \(F\) and \(G\)). An only minor contribution of the \(\alpha_5\)-subunit is in line with a rather low expression level of this subunit in the CeA (Marowsky et al. 2012; Pirker et al. 2000; Prut et al. 2010; but see Hortnagl et al. 2013). A summary of the pharmacological effects is depicted in Fig. 1G.

In agreement with the functional data (Fig. 1) and previous studies, immunostaining confirmed the expression of the \(\delta\)-subunit in the CeA, with a stronger signal in CeAl compared with CeAm (Fig. 2, \(A–D\)). Indicating specificity of the staining, strong GAB\(A_\delta\)R \(\delta\)-subunit labeling was seen in the dentate gyrus (Fig. 2E; Maguire et al. 2009).

**Neurosteroids increase \(I_{\text{hold}}\) in CeAl neurons.** The progestrone metabolites THDOC and allopregnanolone induce opening of the GAB\(A_\delta\) receptor channel at nanomolar concentrations in vitro (Lambert et al. 1995; Majewska 1992), acting preferentially, but not exclusively, on \(\delta\)-containing subunit assemblies of tonic GAB\(A_\delta\)Rs (Brown et al. 2002; Stell et al. 2003). To determine the effect of neurosteroids on GABAergic tonic inhibition, we tested THDOC and allopregnanolone in the presence of ionotrophic glutamate receptor antagonists and GAB\(A_\delta\)R inhibition.

Superfusion of THDOC (10 nM) produced a significant negative shift in baseline \(I_{\text{hold}}\) (\(\Delta I_{\text{hold}} = -68.76 \pm 19.6\) pA, \(\Delta CD = -1.4 \pm 0.52\) pA/pF; 1-sample t-test, \(P = 0.0295\) vs. baseline, \(n = 8\); Fig. 3, \(A\) and \(O\)). This effect was reversed by the subsequent application of PTX in eight of eight cells recorded (\(-26.78 \pm 20.38\) pA, \(\Delta CD = 0.75 \pm 0.56\) pA/pF; 1-sample t-test, \(P = 0.2271\) vs. baseline; Fig. 3A). Interestingly, the THDOC-induced \(\Delta CD\) was almost as big as the \(\Delta CD\) induced by THIP (\(-2.28 \pm 0.58\) pA/pF; see Fig. 1G; unpaired t-test, \(P = 0.1066\)). At this concentration, THDOC affected sIPSCs by increasing the half-width decay time (control: 14.6 \(\pm\) 2.0 ms, THDOC: 27.7 \(\pm\) 5.8 ms; paired t-test, \(P = 0.0396\); Fig. 3, \(B\) and \(C\)), without changing sIPSC instantaneous frequency (control: 3.1 \(\pm\) 0.6 Hz, THDOC: 2.4 \(\pm\) 0.7 Hz; paired t-test, \(P = 0.1172\); Fig. 3D) and sIPSC amplitude (control: 42.7 \(\pm\) 11.4 pA, THDOC: 38.3 \(\pm\) 6.3 pA; \(P = 0.5541\); Fig. 3E). Similar effects have been previously observed in hippocampal neurons (Belelli et al. 2006; Belelli and Lambert 2005; Jo et al. 2011). The THDOC-induced changes in \(I_{\text{hold}}\) were not prevented by the preceding application of TTX (\(\Delta I_{\text{hold}} = -59.5 \pm 18.6\) pA, \(\Delta CD = -0.9 \pm 0.33\) pA/pF; 1-sample t-test, \(P = 0.0412\) vs. baseline, \(n = 6\); Fig. 3, \(F\) and \(O\)), suggesting that the effects of THDOC are not mediated by the generation of action potential-dependent GABA release under basal conditions. This increase was reversed by the successive application of PTX (\(\Delta I_{\text{hold}} = 22.1 \pm 4.9\) pA, \(\Delta CD = 0.5036 \pm 0.2127\) pA/pF; 1-sample t-test, \(P = 0.0987\) vs. baseline; Fig. 3G). Furthermore, a low dose of THDOC (1 nM), which is considered to be below physiological plasma concentrations (\(-8\) nM) (Stell et al. 2003), was sufficient to increase \(I_{\text{hold}}\) (\(\Delta I_{\text{hold}} = -26.5 \pm 4.9\) pA, \(\Delta CD = -0.5213 \pm 0.94\) pA/pF; 1-sample t-test, \(P = 0.0117\), \(n = 5\); Fig. 3, \(G\) and \(O\)), but to a lesser extent than 10 nM (10 nM THDOC vs. 1 nM THDOC, unpaired t-test, \(P = 0.0065\)).

Next we tested the effect of allopregnanolone on \(I_{\text{hold}}\) in CeAl neurons. Bath application of allopregnanolone (5 nM) induced a negative shift in \(I_{\text{hold}}\) (\(\Delta I_{\text{hold}} = -22.53 \pm 9.1\) pA, \(\Delta CD = -0.2942 \pm 0.088\) pA/pF; 1-sample t-test, \(P = 0.0202\), \(n = 6\); Fig. 3H), without affecting sIPSC half-width decay time (control: 8.8 \(\pm\) 0.7 ms, allopregnanolone: 13.3 \(\pm\) 4.7 ms; paired t-test, \(P = 0.6905\); Fig. 3, \(I\) and \(J\)), sIPSC instantaneous frequency (control: 5.0 \(\pm\) 1.1 Hz, allopregnanolone: 5.1 \(\pm\) 0.5 Hz; paired t-test, \(P = 0.6250\); Fig. 3K), or sIPSC amplitude (control: 46.1 \(\pm\) 12.0 pA, allopregnanolone: 40.2 \(\pm\) 10.2 pA; paired t-test, \(P = 0.0625\); Fig. 3L). The effect on \(I_{\text{hold}}\) was reversed by the application of PTX, which shifted \(I_{\text{hold}}\) back to baseline in all six neurons tested (\(\Delta I_{\text{hold}} = 1.633 \pm 4.65\) pA, \(\Delta CD = 0.4236 \pm 0.057\) pA/pF; 1-sample t-test, \(P = 0.4940\); Fig. 3H). The blockade of action potentials by TTX did not prevent the allopregnanolone-induced effects (\(\Delta I_{\text{hold}} = -42.7 \pm 13.3\) pA, \(\Delta CD = -0.4567 \pm 0.1496\) pA/pF; 1-sample t-test, \(P = 0.0117\), \(n = 5\); Fig. 3M). In the same neurons, the successive application of PTX reduced \(I_{\text{hold}}\) and reversed the effect produced by allopregnanolone (\(\Delta I_{\text{hold}} = 58.78 \pm 21.6\) pA, \(\Delta CD = 0.67 \pm 0.28\) pA/pF; 1-sample t-test, \(P = 0.0960\), \(n = 5\); Fig. 3M), suggesting that the allopregnanolone effect is

Fig. 2. GAB\(A_\delta\)R \(\delta\)-subunit is expressed in CeAl. \(A\): within the amygdala complex, GAB\(A_\delta\)R \(\delta\)-subunit immunoreactivity is highest in CeAl. \(B\) and \(C\): cytoskeleton protein MAP2 (\(B\)) and nuclear stain DAPI (\(C\)) were used to identify anatomical structures. \(D\): merged channels. \(E\): hippocampal formation served as a reference structure to test for GAB\(A_\delta\)R \(\delta\)-subunit antibody specificity. Dentate gyrus shows strong GAB\(A_\delta\)R \(\delta\)-subunit labeling. BA, basolateral amygdala; d, dorsal; m, medial. Scale bars in \(D\) and \(E\), 200 \(\mu m\).
due to the activation of nonsynaptic GABA<sub>A</sub>Rs. The amplitude of the allopregnanolone effect might be underestimated because of the rather low concentration used (5 nM). However, it has been shown that high doses of allopregnanolone (300 nM) have a direct effect on chloride channels (Twyman and Macdonald 1992), and such an effect would lead to changes in \( I_{\text{hold}} \).

It is known that the biosynthesis of THDOC and allopregnanolone is limited by the 5α-reductase pathway (Sanna et al. 2004).
To test whether or not the inhibition of biosynthesis of neurosteroids would affect tonic GABAergic inhibition, we bath applied finasteride, a specific inhibitor of the 5α-reductase (Finn et al. 2006; Scarduzio et al. 2013). Finasteride application induced a trend toward an increased IPSC decay half-width (control: 14.48 ± 2.33 ms, finasteride: 21 ± 1.84 ms; *P = 0.0522; n = 5), which, however, was not significant. In addition, neither the frequency (P = 0.9932) nor the amplitude (P = 0.8133) of the sIPSCs was affected by finasteride. Furthermore, as expected, finasteride (5 μM) was able to partially reduce I_{hold} (ΔI_{hold} = 9.6 ± 1.3 pA, ΔCD = 0.1274 ± 0.03 pA/pF; 1-sample t-test, *P = 0.0050; time of onset of finasteride effect: 7.57 ± 0.74 min; n = 7; Fig. 3N). The application of PTX on top of finasteride further decreased I_{hold} (ΔI_{hold} = 21.47 ± 3.76 pA, ΔCD = 0.29 ± 0.09 pA/pF; 1-sample t-test, *P = 0.0159 vs. baseline). These data suggest that neurosteroids are locally synthesized. Figure 3O shows a quantitative summary of the neurosteroid and finasteride effects.

**THDOC hyperpolarizes CeAl neurons.** To test the effect of THDOC on the membrane potential and action potential activity, we recorded CeAl neurons under current-clamp conditions. Neurons were held at approximately −65 mV through direct current injection to minimize variability in membrane resting potential and resulting driving forces. Action potentials were evoked by a depolarizing square pulse every 20 s (see MATERIALS AND METHODS). THDOC (10 nM) induced a hyperpolarization of the membrane potential resulting in a block of action potentials and reaching steady state after −20 min (hyperpolarization from −63.21 ± 0.9 mV to −69.34 ± 1.9 mV; paired t-test, *P = 0.0286, n = 4; Fig. 4. A–C). Bringing back the membrane potential to preapplication level through injection of positive current [manual clamping (MC) to −63.08 ± 1.0 mV] during near-maximal effects of THDOC confirmed that the injection of positive current was not able to induce action potentials (Fig. 4. A–C).

The apparent input resistance was significantly decreased by THDOC [control: 477.3 ± 35.11 MΩ, THDOC: 237.2 ± 19.84 MΩ, MC: 247.2 ± 19.8 MΩ, post-MC: 267.1 ± 25.6 MΩ; 1-way ANOVA F(3,16) = 21.93, *P < 0.0001, n = 4; Fig. 4D]. A Bonferroni’s multiple-comparison test revealed differences between control and THDOC (t = 6.969, *P < 0.05), MC (t = 6.979, *P < 0.05), and post-MC (t = 6.102, *P < 0.05), indicating that the membrane conductance was increased by THDOC application leading to a reduction in excitability.

**DISCUSSION**

In the present study, we show that a tonic GABAergic current mediated by GABA_ARs containing the δ-subunit is present in neurons of the CeAl and that this current is under the control of the neurosteroids THDOC and allopregnanolone. According to the converging immunohistochemical and pharmacological evidence provided here, the GABA_Aδ subunit contributes substantially to the tonic inhibition in the CeAl. In detail, the δ-subunit was detected at the protein level in the neurons of the CeAl. Moreover, GABA_Aδ current was enhanced by THDOC application, leading to a reduction in excitability and increase in membrane input resistance. Additionally, the results of this study suggest a functional role for THDOC in the inhibition of neuronal activity in the CeAl, which is consistent with previous findings in other brain regions (Farber et al. 2000; Morishita et al. 2000; Seo et al. 2002).

**Fig. 3.** Neurosteroids modulate tonic GABAergic inhibition in CeAl neurons. A: current trace showing the increase of the tonic current induced by the neurosteroid tetrahydrodeoxyxestosterone (THDOC, 10 nM). The effect was reversed by PTX. B: sIPSCs (mean of 10 traces) recorded at −70 mV in control and after application of THDOC (10 nM) displaying the increase in decay time (C), instant frequency (D), and amplitude (E) of sIPSCs before (black) and after (white) THDOC application. While decay time was increased by THDOC, instant frequency and amplitude remained unaffected. F: same as in A but in the continuous presence of PTX. Note that the effect of THDOC on the neuron recorded was a postsynaptic effect since TTX did not prevent the increase in I_{hold}. G: even at a low concentration of 1 nM, THDOC was effective in enhancing the tonic current. H: current traces showing the increase in the tonic current induced by the neurosteroid allopregnanolone (ALLO, 5 nM). This effect was reversed by the successive application of PTX. I: sIPSCs (mean of 10 traces) recorded at −70 mV in control and after application of allopregnanolone. J–L: decay time half-width (J), instant frequency (K), and amplitude (L) of sIPSCs. Note that although allopregnanolone increased the decay time this was not significantly different from control (paired t-test, *P = 0.6905). M: same as in H but under the blockade of action potentials by TTX. N: representative current traces showing a positive shift in I_{hold} after application of the 5α-reductase inhibitor finasteride (FIN) and PTX. Application of PTX caused an additional significant shift in I_{hold}. O: summary of the different pharmacological conditions: effects of THDOC, allopregnanolone, and finasteride on tonic inhibitory currents. Data were normalized to baseline (last 2 min before drug application) of each single experiment, and significance was tested against control (control = 0). *P < 0.05, **P < 0.01.

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CeA when using a knockout-tested δ-subunit-specific antibody (Maguire et al. 2009). Furthermore, in vitro application of the specific GABA$_{A,R}$ δ-subunit agonist (THIP) and the nonselective GABA$_{A,R}$ δ-subunit modulator (DS2) significantly evoked a tonic GABAergic current in CeAl neurons.

It is known that two subunit-specific forms of tonic inhibition exist in CeAm neurons in mouse and rat (Herman et al. 2013; Herman and Roberto 2014). A sustained tonic conductance mediated by the GABA$_{A,R}$ α1-subunit is present in mouse neurons expressing corticotropin-releasing factor (CRF) receptor-1 (CRFIR). CRFIR-negative neurons, however, possess no intrinsic tonic inhibition but demonstrate a δ-subunit-mediated tonic conductance in the presence of an increased local GABA concentration (Herman et al. 2013). In contrast, we did not find any cell type specificity in the CeAl since all the neurons recorded displayed an ongoing and δ-subunit-mediated tonic GABAergic current. GABAergic tonic inhibition has also been shown in LA and BA, where it is mediated by GABA$_{A,R}$s containing the α3-subunit and where the expression of the δ-subunit is rather low (Marowsky et al. 2012). The evidence for δ-subunit-mediated tonic GABAergic inhibition in CeAl neurons is in agreement with the observed strong effect of the neurosteroids THDOC and allopregnanolone. Interestingly, in paracapsular cells of the amygdala, which are also known to express GABA$_{A,R}$s containing the δ-subunit, THDOC (at a concentration of 300 nM) affected the tonic current only when applied in combination with 5 μM GABA (Marowsky and Vogt 2014), suggesting that CeAl neurons are a dominant target of neurosteroid action within the amygdala. PTX clearly unmasked an intrinsic tonic current in (e.g., Fig. 1, A and B) as indicated by the induced outward current. However, when PTX was applied successively to drugs that induced a strong increase in the tonic current (e.g., THIP and THDOC; Fig. 1D and Fig. 3A, respectively), PTX still induced a shift in $I_{\text{hold}}$ back to baseline, but without exceeding it. While similar effects have been seen elsewhere (Herman et al. 2013; Jo et al. 2011; Kolbaev et al. 2012), a clear explanation for this phenomenon is missing. One possible explanation is that the activation of δ-subunit-containing GABA$_{A,R}$s by drugs like THIP and THDOC directly or indirectly also leads to an activation of a PTX-insensitive non-GABAergic conductance.

Changes in neurosteroid levels (e.g., during the ovarian cycle) and the consequent changes in tonic GABAergic signaling via δ-subunit-containing GABA$_{A,R}$s have been linked to an altered seizure susceptibility and to anxiety (Maguire et al. 2005). Similarly, a reduction of endogenous THDOC and allopregnanolone levels has been found to lower the potency of GABA in eliciting GABAergic IPSCs (Barbaccia 2004; Dong et al. 2001). In the present study, THDOC hyperpolarized CeAl neurons, thereby decreasing their excitability. The CeAl neurons are mutually interconnected via GABAergic synapses, and they connect to the CeAm. An enhancement of tonic GABAergic inhibition in the CeAl might thus result in either an inhibitory or a disinhibitory overall influence on CeAm output neurons. In addition, modulatory effects of THDOC on tonic GABAergic currents are context dependent. For the hypothalamic-pituitary-adrenal (HPA) axis it has been shown that THDOC potentiates the inhibitory action of GABA on CRF neurons under control conditions, whereas THDOC activates the HPA axis after stress (Sarkar et al. 2011). This change in the direction of the THDOC effect on network activity is due to a stress-induced collapse of the chloride gradient in CRF neurons that results in an excitatory action of GABA (Sarkar et al. 2011).

Behaviorally, a reduction of endogenous THDOC and allopregnanolone levels is related to anxiety-like responses, increased aggression, and a reduced sensitivity to the loss of righting reflex induced by GABA$_{A,R}$ agonists or positive modulators, but also to an enhanced expression of fear conditioning responses in rodents. Conversely, enhanced neurosteroid action results in anxiolysis, sedation/hypnosis, and anxioreducing action (Barbaccia 2004). Furthermore, increases in context-dependent fear can be abolished by treatment with drugs that normalize the corticolimbic levels of allopregnanolone (Agis-Balboa et al. 2009). Therefore the enhancement of tonic inhibition through neurosteroid action in the CeAl is likely to reduce activity in CeAm neurons mediating fear responses.

Inhibition of the synthesis of neurosteroids with finasteride, a specific inhibitor of the 5α-reductase (Finn et al. 2006; Scarduzio et al. 2013), led to a decrease in the tonic GABAergic current in CeAl neurons. Although the bath application of finasteride does not reveal the cellular source of the neurosteroids, the observed effect indicates that there is an ongoing local neurosteroid synthesis. While we cannot fully exclude nonspecific effects of finasteride on other ligands or conductances, additional evidence for the local synthesis is provided by the demonstration of immunoreactivity of neurosteroids (Saalmann et al. 2007; Tokuda et al. 2010) and 5α-reductase type II expression (Castelli et al. 2013) in neurons of the amygdala. Interestingly, mice with enhanced contextual fear responses and impaired fear extinction show decreased 5α-reductase type 1 mRNA expression and allopregnanolone levels in the amygdala and other brain regions (Pibiri et al. 2008).

The combination of rapid local neurosteroid synthesis and GABA potentiation makes the neurosteroid system a target for local modulation of GABAergic inhibition, thereby contributing to synaptic plasticity. In line with this, finasteride has an effect on fear learning (Disney and Calford 2001). In addition, midazolam (a benzodiazepine acting on GABA$_{A,R}$s) enhances a specific form of network inhibition and inhibits long-term potentiation and contextual fear learning, an effect that is blocked when finasteride is administrated 1 day before midazolam (Tokuda et al. 2010). The present study provides for the first time evidence that finasteride is able to decrease tonic inhibition in CeA neurons. GABA$_{A,R}$s containing the α5-subunit contribute to tonic inhibition only when the ambient GABA concentration increases but are not activated by endogenous ambient GABA (Scimemi et al. 2005). The majority of CeAl neurons are GABAergic cells (Ehrlich et al. 2009; Haubensak et al. 2010), which could contribute to an enhanced ambient GABA concentration. A higher ambient GABA concentration in CeAl versus CeAm would explain the small α5-subunit-mediated tonic current in CeAl, which is completely absent in CeAm (Herman et al. 2013). Furthermore, Li et al. (2013) suggested that excitatory synapses onto somatostatin-positive neurons in CeAl are strengthened while the somatostatin-negative neurons are weakened after fear conditioning. Because the somatostatin-positive neurons are forming intra-CeAl GABAergic synapses, an increased activity of these neurons might result in higher extracellular GABA levels. Thus, while activation of δ-subunit-containing GABA$_{A,R}$s predominates at low ambi-
ent GABA concentrations, α5 subunit-containing GABA$\alpha$Rs would be activated after fear conditioning.

It has been suggested that synaptic spillover after vesicular release (Attwell et al. 1993), reversal of GABA transporters (Gaspary et al. 1998), and a GABA “leak” through bestrophin channels (Lee et al. 2010) are sources of ambient GABA. As shown recently, reactive astrocytes seem to produce and release GABA through bestrophin channels under pathological conditions (Jo et al. 2014). Active uptake maintains GABA at sufficiently low concentrations to prevent tonic GABA$\beta$R activation (Isaacson et al. 1993). The enhanced tonic GABAergic current seen in CeAl neurons after the application of NNC-711, a selective inhibitor of GABA uptake by GAT-1, confirms the presence of active GABA uptake but excludes reverse GABA transport as a source of extracellular GABA under these conditions. It should be noted that the effect of NNC-711 was relatively small, which might be due to a relatively high ambient GABA level in the CeAl (see above).

In conclusion, here we provide evidence that CeAl neurons contain GABA$\alpha$Rs that mediate inhibitory tonic currents that are likely attributable to the expression of the GABA$\alpha$R δ-subunit. Furthermore, we show that these tonic currents are modulated by neurosteroids and play an important role in regulating the excitability of CeAl neurons. As a consequence, the tonic GABAergic current and the neurosteroid-mediated regulation may be potential targets for a pharmacological manipulation of the imbalance in the amygdala output underlying anxiety disorders.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: H.R.-P. and H.-C.P. conception and design of research; H.R.-P., P.B., and L.S. performed experiments; H.R.-P., P.B., and L.S. analyzed data; H.R.-P., P.B., L.S., and H.-C.P. interpreted results of experiments; H.R.-P. and P.B. prepared figures; H.R.-P., P.B., and H.-C.P. drafted manuscript; H.R.-P., P.B., and L.S. edited and revised manuscript; H.R.-P., P.B., L.S., and H.-C.P. approved final version of manuscript.

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NEUROSTEROID EFFECTS IN CeAl


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