Neurosteroid Modulation of GABAergic Neurotransmission in the Central Amygdala: A Role for NMDA Receptors

Chunsheng Wang[1,3], **Christine E. Marx**[1,3,5], **A. Leslie Morrow**[6,7], **Wilkie A. Wilson**[2,4,5], and **Scott D. Moore**[1,3,5]

1 Department of Psychiatry and Behavioral Sciences, Duke University Medical Center

2 Department of Pharmacology and Cancer Biology, Duke University Medical Center

3 Division of Psychiatry, Durham Veterans Affairs Medical Center Durham, North Carolina

4 Division of Neurology Research, Durham Veterans Affairs Medical Center Durham, North Carolina

5 VISN 6 Mental Illness Research, Education and Clinical Center, Durham, N.C. 27705

6 Department of Psychiatry, University of North Carolina, Chapel Hill, NC, U.S.A.

7 Department of Pharmacology, University of North Carolina, Chapel Hill, NC, U.S.A.

Abstract

The neurosteroid 3α-hydroxy-5α-pregnan-20-one (allopregnanolone or ALLO) positively modulates $GABA_A$ receptors, an action that may contribute to the anxiolytic effects of ALLO. Recent evidence suggests that ALLO's anxiolytic effects appear to be mediated by the amygdala, a key neural structure for emotional and cognitive behaviors. However, little is known regarding ALLO effects on amygdala physiology. We therefore explored ALLO effects on GABA neurotransmission in the central nucleus (Ce) of the amygdala, a major output nucleus involved in fear and anxiety. We recorded evoked $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) in Ce neurons using whole-cell patch clamp techniques. We observed that ALLO significantly reduced the amplitude of evoked $GABA_A$ receptor-mediated IPSCs. However, the effect of ALLO was occluded by the NMDA receptor antagonist D-APV. D-APV alone also reduced evoked IPSCs in Ce neurons. These results suggest that ALLO-induced reduction of GABAergic transmission in Ce appears to depend on neural network activity, possibly involving an NMDA receptor-mediated mechanism. These ALLO effects on GABAergic transmission in the central amygdala may play a role in mediating its anxiolytic actions.

Keywords

allopregnanolone; amygdala; neurosteroid; $GABA_A$; NMDA; patch clamp

Introduction

Neurosteroids are molecules formed *de novo* in the brain that modulate both inhibitory and excitatory neurotransmission (Rupprecht and Holsboer 1999). The neurosteroid

allopregnanolone (ALLO) enhances γ-aminobutyric acid type A ($GABA_A$) receptor-mediated Cl^- current (Majewska et al, 1986; Morrow et al, 1987, 1990), an action that may contribute to its anxiolytic effects (Crawley et al, 1986; Wieland et al, 1991). Allopregnanolone levels in plasma and brain increase rapidly following acute stress (Purdy et al. 1991; Barbaccia et al. 1996, Morrow et al 1998). ALLO elevations have also been observed following antidepressant (Guidotti and Costa, 1998; Uzunova et al., 1998) and antipsychotic (Marx et al. 2003; Barbaccia et al 2001) administration.

The amygdala formation is a complex structure containing multiple subnuclei and pathways (Pitkänen 2000). The central nucleus (Ce) is a major output nucleus of the amygdala that may directly mediate anxiety and the anxiolytic effects of a number of pharmacologic compounds (Roberto et al 2003, Delaney and Sah 1999, Kang-Park et al 2004). In addition, direct application of ALLO to Ce causes marked anxiolytic effects in rats (Akwa et al., 1999), suggesting that anxiolytic effects of ALLO may be mediated by the amygdala.

Electrophysiological characteristics of ALLO effects have been studied using patch clamp techniques with different cell preparations in a variety of CNS regions in rats (Poisbeau et al 1997; Fáncsik et al 2000; Haage et al 1999, 2002, Uchida et al 2002; Brussaard et al 1999; Sooksawate and Simmonds 1998; Puia et al 2003). These studies examined $GABA_A$ receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs) or GABA evoked currents through bath application of GABA. The consistent finding in these efforts has been that ALLO positively modulates $GABA_A$ receptors by significantly prolonging the Cl^- channel open time, allowing more Cl^- influx into neurons through $GABA_A$ receptor channels. However, little is known regarding ALLO effects on electrically evoked GABA responses involving a specific neural network. We therefore explored ALLO effects on the electrophysiology of GABAergic transmission in Ce.

Materials and Methods

All experimental procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Male rats (Sprague Dawley, 3–5 weeks) were deeply anesthetized with halothane and quickly decapitated. The brains were removed, blocked, and sliced in the coronal plane with a vibratome. Slices (300 μm thick) containing the mid-caudal amygdala formation (Bregma −2.30 to −2.80 mm) were incubated at room temperature in artificial cerebrospinal fluid (ACSF) composed of (in mM): NaCl, 124; dextrose, 10; $NaHCO_3$, 26; KCl, 2; KH_2PO_4, 1.25; $CaCl_2$, 2; and $MgSO_4$, 1; and equilibrated with 95% O_2 and 5% CO_2.

After 1-hour incubation, slices were transferred into an immersion-type recording chamber at room temperature continuously perfused with oxygenated ACSF at a rate of 3 ml/min. All experiments were conducted at room temperature (27 °C). For studies of synaptic transmission, patch pipettes were filled with intracellular solution *A* containing (in mM): Cs-gluconate, 130; CsCl, 7; HEPES, 10; Mg-ATP, 4; Tris-GTP, 0.5, QX-314, 4. For membrane property recordings, patch pipettes were filled with intracellular solution *B* containing (in mM): K-gluconate, 130; KCl, 7; HEPES, 10; Mg-ATP, 4; Tris-GTP, 0.5. The intracellular solutions were adjusted to a pH of 7.2–7.3 with KOH (or CsOH) and to an osmolarity of 280–290 mOsm. Pipette resistances were 3–6 MΩ. Intracellular recordings neurons within Ce visualized with a light microscope (Fig. 1a) (Axioskop 2, Carl Zeiss) with a water immersion lens (40X, Zeiss) (Wang et al. 2003). Once a giga-seal was obtained upon a neuron, a gentle suction was applied to the recordings pipette to cause the cell membrane to rupture, achieving the whole-cell configuration. Electrical signal were amplified through an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The junction potential was offset to zero just before approaching to the cell. All of the cells used in the study had a resting membrane potential below −55 mV

and access resistance less than 30 MΩ. Access resistance was monitored throughout recordings, and cells were rejected if it changed by more than 15% during the experiment.

IPSCs were electrophysiologically isolated with the cell membrane potential held at the reversal potential for glutamatergic currents (0 mV) in voltage-clamp mode. For evoked IPSC recordings, single stimuli were delivered every 40 seconds through a unipolar tungsten microelectrode placed in the core of the central nucleus of the amygdala. Stimulating intensity (ranged from 15 to150 μA, 200 μsec duration) was individually adjusted to be half current intensity needed to trigger the maximum eIPSC amplitude. For membrane property recordings, cell membrane potentials were held under current-clamp mode. Electrical signals were recorded and analyzed with a self-developed software using Labview (National Instruments, Austin, TX). Peak amplitude of evoked responses was measured and compared before and after drug application using paired student's t-test and one-way *ANOVA*.

3α-Hydroxy-5α-pregnan-20-one (allopregnanolone) (Steraloids Inc. Newport, RI) was first dissolved into DMSO (1 mM) and then diluted into ACSF. D-(−) 2-amino-5-phosphonovaleric acid (D-APV) (Fisher) was dissolved in water to make a stock solution (40 mM). Picrotoxin (Sigma) was directly dissolved into ACSF. 6,7-Dinitroquinoxaline-2,3 (1H, 4H)-dione (DNQX) (Sigma) was prepared as a stock solution in DMSO (0.2 M). TTX in citrate buffer (Sigma) was prepared as a stock solution (1 mM). ATP, GTP, and lidocaine N-ethyl bromide (QX-314) were directly dissolved into the intracellular pipette solution. In experiments using compounds dissolved in DMSO, the same concentration of DMSO was used in control ACSF.

Results

We first examined ALLO effects on membrane properties of Ce neurons. Ce neurons were recorded in current clamp mode with intracellular solution *B* (see methods). The average resting membrane potential in Ce neurons was -67.6 ± 1.2 mV in control conditions and -67.7 ± 1.3 mV after bath application of ALLO (100 nM), with no significant change (n = 8). The input resistance of Ce neurons was 262 ± 33 MΩ before and 275 ± 38 MΩ after ALLO (n = 8). ALLO did not change the current-voltage (I-V) relationship in Ce neurons. Neurons showed a wide range in the number of spikes in response to a depolarizing current injection (150 pA, 600ms) ranging from 2 to 18. All neurons recorded in current clamp mode in Ce (n = 8) showed spike accommodation and the pattern of spike accommodation was very similar before and after ALLO application. These results indicate that membrane properties of Ce neurons are not altered by ALLO.

To examine GABAergic transmission in the central amygdala, we recorded evoked IPSCs (eIPSCs) in Ce neurons in response to stimulation at the lateral margin of Ce. We first confirmed that the reversal potential of the eIPSC was between -65 and -70 mV in Ce neurons (Fig. 1a). However, to evaluate the possibility that ALLO was having effects on intact neural networks, we subsequently isolated IPSCs using a holding potential of 0 mV (near the reversal potential for the glutamate receptor-mediated responses). As shown in Fig. 1b, picrotoxin (PTX, 100 μM, a GABA$_A$ receptor channel blocker) completely blocked the eIPSCs, with little residual current remaining from potential excitatory postsynaptic currents (including NMDA or AMPA currents). These data demonstrate that the recorded eIPSCs reflect a relatively pure GABAergic current.

After bath application of ALLO at a physiological concentration (100 nM), the amplitude of eIPSCs in Ce neurons was significantly reduced, with an average reduction of $31.0 \pm 4.0\%$ (n = 8, p< 0.01, paired t-test, Fig. 1c, d). In general, we saw no reversal of the ALLO effect upon re-superfusion with control medium up to 30 minutes (n=4). No rundown of the eIPSCs was observed in the recorded neurons in control conditions. Since ALLO is known to directly

enhance GABA$_A$ receptor function in other systems involving dissociated neurons (Poisbeau et al 1997; Haage et al 1999; Sooksawate and Simmonds 1998), we determined if ALLO-induced decreases in GABAergic transmission in Ce depend on neural network activities.

To further investigate possible involvement of glutamate in the mechanism of ALLO-induced reduction of GABAergic transmission, we examined the role of NMDA receptors in ALLO actions on GABAergic transmission in Ce. To test this possibility, the NMDA receptor antagonist D-APV (50 μM) was applied to the amygdala slice preparation. As shown in Fig. 2, D-APV occluded the reduction of eIPSCs in Ce neurons by ALLO (n = 7, p > 0.05, paired *t*-test). This result suggests that NMDA receptor function is relevant to ALLO reduction of GABAergic transmission in Ce.

If ALLO-induced reduction of GABAergic transmission is mediated by the negative modulation of NMDA receptors, we would expect a reduction of GABAergic transmission by NMDA blockers such as D-APV. We tested this possibility by applying the NMDA receptor antagonist D-APV (50 μM) to the amygdala slice preparation. As shown in Fig. 3, D-APV significantly reduced the eIPSC in Ce neurons by $46 \pm 8\%$ (n = 7, p < 0.01, paired *t*-test).

In cingulate cortex inhibition of NMDA receptor-mediated functions causes a reduction of GABAergic transmission (Li et al 2002). We reasoned that ALLO may be suppressing NMDA-mediated functions in Ce neurons, and therefore examined ALLO effects on evoked NMDA-mediated currents. NMDA currents were observed at a holding potential of +40 mV in conjunction with application of PTX (100 μM) and DNQX (20 μM, an AMPA receptor antagonist) in the bath (Fig. 4). Our results showed a small but significant reduction ($12 \pm 2\%$; n = 11, p< 0.05, one-way *ANOVA*) of NMDA current (D-APV sensitive) in Ce neurons in the presence of ALLO (100 nM) (Fig. 4). When ALLO concentration was increased to 4 μM, the average reduction of NMDA current was $14 \pm 4\%$ of ALLO (n = 5, p< 0.05, one-way *ANOVA*). There was no significant difference between the two concentrations.

Discussion

In this study, we explored the electrophysiological effects of ALLO on GABAergic neurotransmission in the central amygdala. Specifically, ALLO negatively modulates eIPSCs, in contrast to previous studies of ALLO effects on mIPSCs in different brain regions (Poisbeau et al 1997; Haage and Johansson 1999; Patenaude et al 2001). Unlike mIPSCs, eIPSCs involve membrane excitability and network functioning. However, our results revealed that ALLO did not significantly affect membrane properties of Ce neurons. This left the possibility that other neural network effects of ALLO may underlie the mechanism of ALLO-induced reduction of evoked GABAergic transmission in Ce. As shown previously, glutamatergic NMDA receptors have significant effects on GABAergic transmission in CNS neurons (Aguayo et al. 1998; Li et al. 2002). Specifically, these prior studies demonstrated that activation of NMDA receptors enhances GABAergic transmission, whereas blocking NMDA receptors reduces GABAergic transmission. We therefore examined the effects of the NMDA receptor antagonist D-APV on the ability of ALLO to suppress eIPSCs. Both ALLO and D-APV reduced evoked IPSCs in Ce, and D-APV prevented further reduction of eIPSCs by ALLO. These results suggest that the effects of ALLO on eIPSCs possibly involve an NMDA receptor-mediated mechanism. Although the direct inhibitory effect on ALLO on isolated NMDA receptor-mediated currents was relatively modest compared to ALLO effects on eIPSCs, we speculate that this could be due to amplication of ALLO effects in an intact, complex network.

Since inhibition of NMDA receptor function occluded ALLO-induced reduction of eIPSCs, we suggest that an NMDA receptor-dependent mechanism may mediate ALLO effects on GABA neurotransmission in Ce. However, the concentration of GABA has also been shown

to play a role in ALLO effects on GABA$_A$ receptors in rat hypothalamic cultured neurons (Poisbeau et al 1997) and dissociated medial preoptic neurons (Haage and Johansson 1999). Specifically, at low GABA concentrations, ALLO potentiates GABA$_A$ receptor responses, but has no effect or significantly decreases GABA$_A$ receptor responses at high GABA concentrations. The exact mechanism for this phenomenon is not clear, but it has been suggested that changes in kinetic properties of the GABA$_A$ receptor channel and desensitization of GABA$_A$ receptors by ALLO may be involved (Haage and Hohansson 1999). In our experiments, electrical stimulation of GABAergic neurons in Ce would likely cause a large amount of GABA release and significantly elevate the concentration of GABA, potentially leading to a change in the way of effects of ALLO on GABA$_A$ receptor-mediated responses. In addition, GABA receptor desensitization in high concentrations of ALLO has been demonstrated previously (Haage and Hohansson 1999). However, this may not fully explain our results. Moreover, this explanation is inconsistent with the suppression of eIPSCs by the NMDA antagonist D-APV and the subsequent occlusion of the ALLO effect.

The possibility that NMDA receptors contribute to the effects of ALLO is supported by the finding that ALLO decreases NMDA receptor-mediated current in Ce neurons. Although direct ALLO effects on NMDA receptors have not been reported previously, prior studies have shown that the sulfated ALLO stereoisomer pregnanolone sulfate (3α-hydroxy-5β-pregnan-20-one sulfate or PS) negatively modulates NMDA current in different cell types (Irwin et al 1994; Park-Chung et al 1994, Weaver et al 2000), as does its synthetic homologue pregnanolone hemisuccinate (Weaver et al 1997). The exact mechanism(s) of possible ALLO regulation of NMDA receptor-dependent processes remains unclear.

Our data provide an initial electrophysiological exploration of ALLO effects on GABA and NMDA receptor-mediated neurotransmission in the Ce, and raise the possibility that NMDA receptors play an important role in ALLO actions. These results also suggest that ALLO effects on GABAergic transmission in a neural network may be different from effects on isolated neurons. These findings may provide insights into potential mechanisms mediating ALLO's pronounced anxiolytic effects, and to the pathophysiology of psychiatric disorders with anxiety components.

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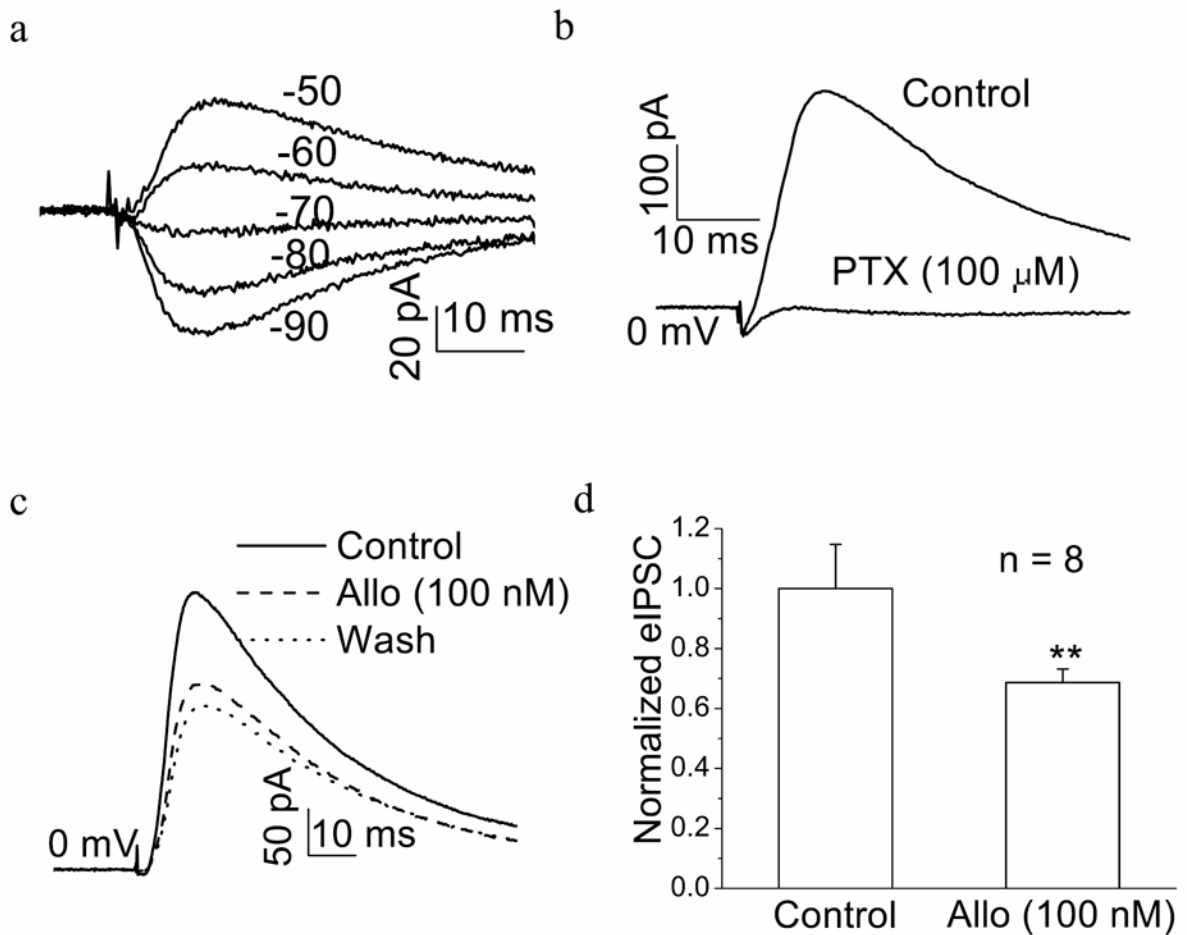
Figure 1. Allopregnanolone significantly reduces GABAergic transmission in Ce
a). Traces of the evoked inhibitory postsynaptic current (eIPSC) recorded from a Ce neuron under voltage-clamp mode and in the presence of DNQX (20 μM) and D-APV (50 μM). eIPSCs at different holding potentials show a reversal potential between −60 to −70 mV. b). The eIPSC is PTX sensitive, and when held at 0 mV without glutamate receptors blockers, has virtually no contamination from other sources (such as the EPSC). c). Traces from another Ce neuron show a reduction of the eIPSC after allopregnanolone (100 nM). This effect could not be washed off after 30 min of washing. Each trace was an average of 10 consecutive responses under the same condition. d). Bar graph of the averaged results shows a significant inhibition of $31.0 \pm 4.0\%$ by ALLO. ** $p < 0.01$ (*paired t-test*).
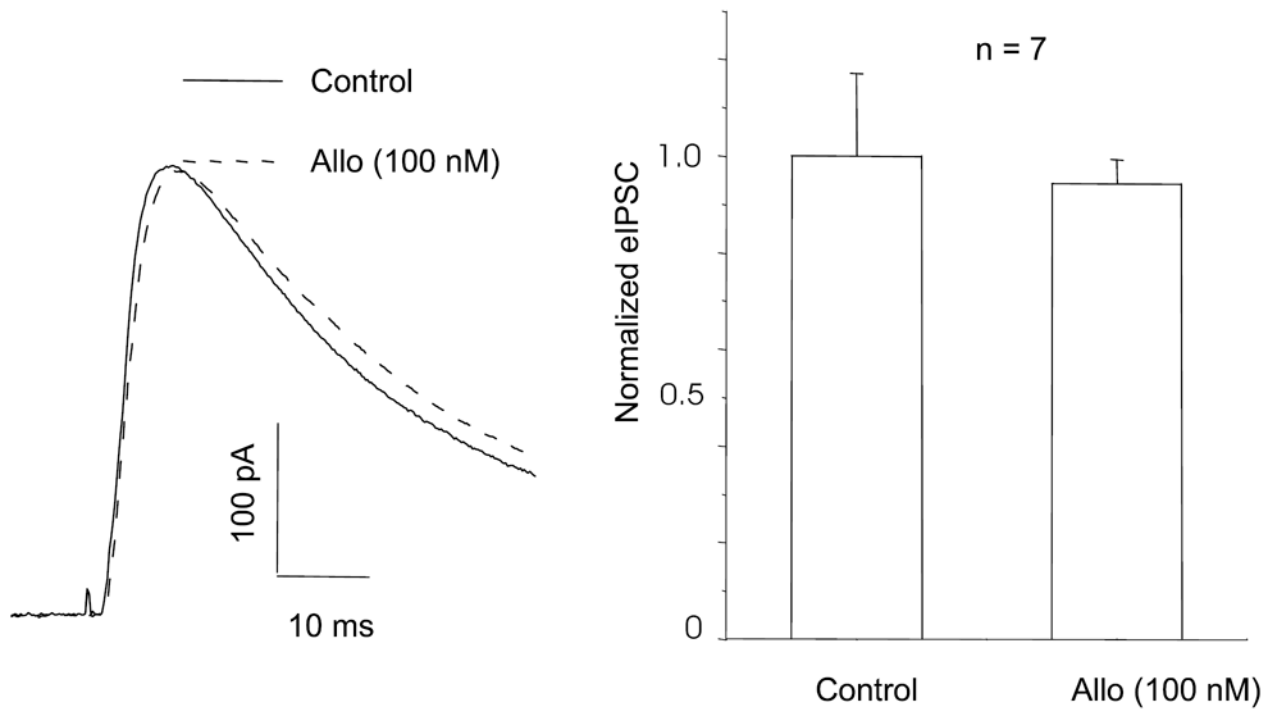
Figure 2. D-APV prevents reduction of GABAergic transmission by ALLO in Ce
Left: superimposed traces of eIPSCs from a Ce neuron before and after ALLO (100 nM) application in the presence of D-APV (50 μM). Each tracing represents an average of 10 responses in the same condition. Right: Averaged data of 7 neurons shows no significant inhibition of eIPSC by ALLO in the presence of D-APV
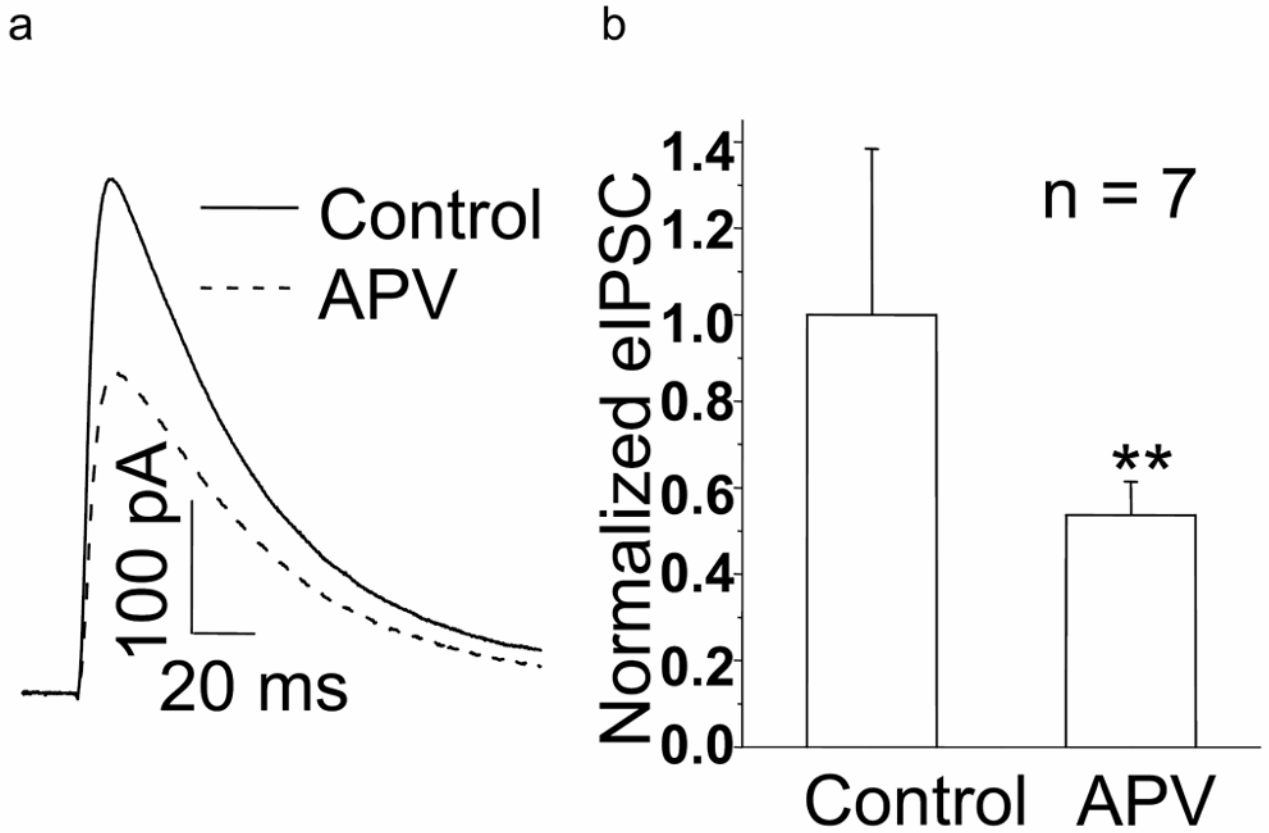
Figure 3. D-APV significantly reduces GABAergic transmission (eIPSCs) in Ce
a). Superimposed GABAergic responses in a Ce neuron before and after D-APV (50 μM). b).
Averaged results show a significant decrease of peak amplitude of eIPSC after D-APV, ** p
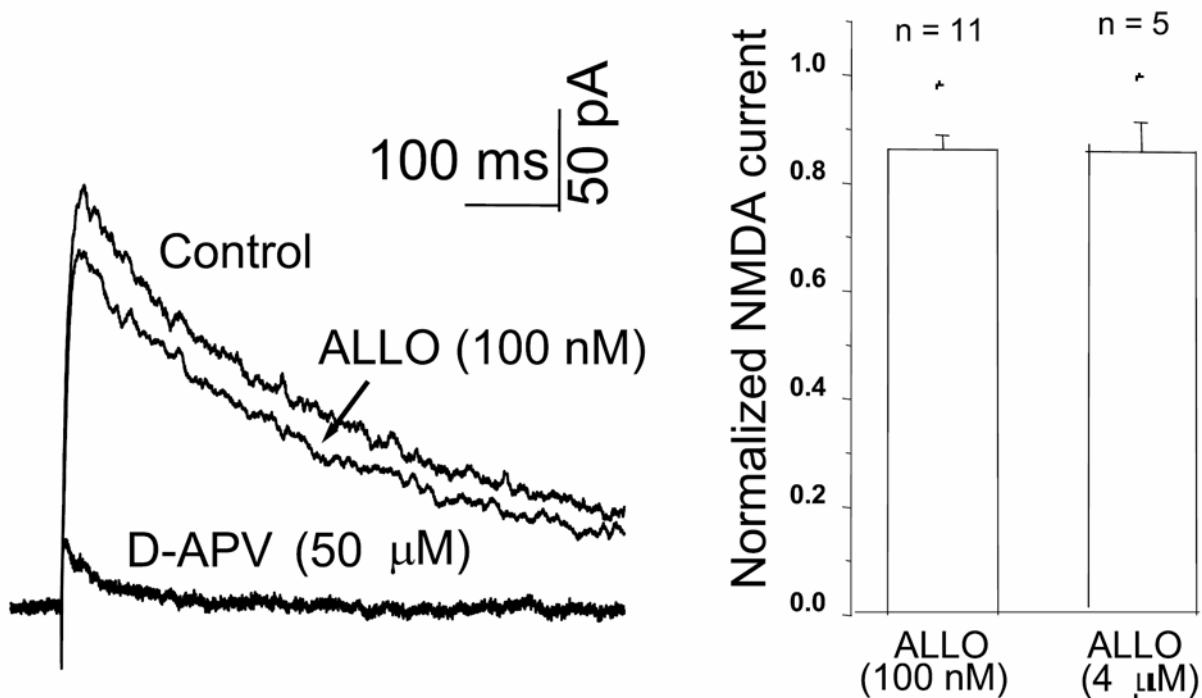< 0.01, paired *t*-test.

Figure 4. Allopregnanolone significantly reduces NMDA (N-methyl-D-aspartate) current in Ce neurons
Left: superimposed traces of NMDA currents in control and ALLO (100 nM) conditions in a Ce neuron. NMDA currents were isolated by application of PTX (100 μM) and DNQX (20 μM) with a holding potential at +40 mV during the recording. Because DNQX blocks excitatory transmission in neural network, NMDA currents were generated by local stimulation within Ce. This current was blocked by the NMDA receptor blocker D-APV (50 μM). Each trace represents an average of 10 responses under the same conditions. Right: Averaged data demonstrates that ALLO (100 nM and 4μM) significantly reduces of the peak amplitude of the evoked NMDA current by $13 \pm 2\%$ (n = 11, $p < 0.05$, one-way *ANOVA*) and $14 \pm 4\%$ (n = 5, $p < 0.05$, one-way *ANOVA*), respectively. There is no significant difference in reduction of the peak amplitude of NMDA current between the two ALLO concentrations.