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## **$\alpha_1$ -Adrenergic Receptor-Induced Heterosynaptic Long-Term Depression in the Bed Nucleus of the Stria Terminalis Is Disrupted in Mouse Models of Affective Disorders**

Zoé A McElligott<sup>1</sup> and Danny G Winder<sup>1,2,3,\*</sup>

<sup>1</sup>Vanderbilt Brain Institute, Vanderbilt University Medical Center, Nashville, TN, USA

<sup>2</sup>Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN, USA

<sup>3</sup>Kennedy Center For Human Development, Vanderbilt University Medical Center, Nashville, TN, USA

### **Abstract**

The glutamatergic synapse in specific brain regions has been shown to be a site for convergence of stress and addictive substances. The bed nucleus of the stria terminalis (BNST), a nucleus that relays between higher order processing centers and classical reward and stress pathways, receives dense noradrenergic inputs that are known to influence behavioral paradigms of both anxiety and stress-induced relapse to drug seeking.  $\alpha_1$ -Adrenergic receptors ( $\alpha_1$ -ARs) within this region have been implicated in modulation of the HPA axis and anxiety responses. We found that application of an  $\alpha_1$ -AR agonist produced a long-term depression (LTD) of excitatory transmission in an acute mouse BNST slice preparation. This effect was mimicked by a 20 min, but not a 10 min, application of 100  $\mu$ M norepinephrine (NE) in a prazosin-sensitive manner. This  $\alpha_1$ -AR LTD was independent of *N*-methyl-D-aspartate receptor (NMDAR) function unlike previously described  $\alpha_1$ -AR LTD in the hippocampus and visual cortex; however, it was dependent on the activation of L-type voltage gated calcium channels (VGCCs). In addition,  $\alpha_1$ -AR LTD was induced independently of the activation of mGluR5 which can also induce LTD in this region. Furthermore,  $\alpha_1$ -AR LTD was intact in mice receiving an intraperitoneal injection of cocaine but was disrupted in  $\alpha_{2a}$ -AR and NE transporter (NET) knockout (KO) mice. Thus a loss of this plasticity at glutamatergic synapses in BNST could contribute to affective behavioral phenotypes of these mice.

### **Keywords**

synaptic plasticity; heterosynaptic neuromodulation; catecholamine; norepinephrine; anxiety; depression

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\*Correspondence: Dr DG Winder, Department of Molecular Physiology & Biophysics, Room 724B, RRB 1, Vanderbilt University School of Medicine, Nashville, TN 37232-0615, USA, Tel: + 615 322 1144, Fax: + 615 343 0490, danny.winder@vanderbilt.edu.

### **DISCLOSURE/CONFLICT OF INTEREST**

Ms McElligott declares that except for her graduate student stipend from Vanderbilt University she has not received financial support or compensation from any individual or corporate entity for the past 4 years for research or professional service. Dr Winder has received a distribution from Columbia University for the licensing of transgenic mouse technology to Memory Pharmaceuticals and received a consultancy fee from MEDAcorp. Both authors declare that there are no personal financial holdings that could be perceived as potential conflicts of interest.

## INTRODUCTION

Relapse to drug use after extended abstinence remains a troublesome aspect of addiction. Clinical studies have implicated psychological stress as a major factor that induces relapse behavior. Although the noradrenergic system has long been implicated in withdrawal related-behaviors (Aston-Jones and Harris, 2004), recent evidence has highlighted its role in addiction (Weinshenker and Schroeder, 2007), particularly with the stress-induced relapse response (Shaham *et al*, 2000). For example, administration of an  $\alpha_2$ -AR agonist or lesioning of the ventral noradrenergic bundle (VNAB) attenuates stress-induced reinstatement to opiate seeking (Erb *et al*, 2000; Wang *et al*, 2001) and viral restoration of dopamine  $\beta$ -hydroxylase (DBH, the enzyme that produces norepinephrine (NE)) in the nucleus tractus solitarius (NTS) of mice lacking DBH restores conditioned place preference (CPP) to morphine (Olson *et al*, 2006).

The bed nucleus of the stria terminalis (BNST), a component of the extended amygdala, receives input from the VNAB, as well as glutamatergic inputs from cortical/limbic areas and sends outputs to stress and reward centers (Forsay and Gysling, 2004). The lateral BNST (lBNST), and  $\alpha_2$ - and  $\beta$ -AR signaling therein, regulates stress-induced relapse behaviors (Wang *et al*, 2001; Leri *et al*, 2002). Moreover, blockade of excitatory transmission within this same region also disrupts anxiety-related behavior (Walker and Davis, 1997). Due to these observations, our group characterized NE modulation of glutamatergic signaling in the dorsal and ventral lBNST (dlBNST, vlBNST), focusing on  $\alpha_2$ - and  $\beta$ -AR signaling (Egli *et al*, 2005). Studies, however, have also suggested a role for  $\alpha_1$ -ARs in the BNST, demonstrating that blocking  $\alpha_1$ -ARs decreases anxiety responses concurrent with reductions in hypothalamic-pituitary-adrenal (HPA) axis activation (Cecchi *et al*, 2002). Further, activation of  $\alpha_1$ -ARs also increases spontaneous inhibitory postsynaptic current (IPSC) frequency in the vlBNST of animals exposed to morphine (Dumont and Williams, 2004).

$\alpha_1$ -ARs have been reported to modulate glutamatergic transmission in other brain regions.  $\alpha_1$ -AR activation leads to depression of excitatory transmission that is long lasting in the hippocampus and visual cortex (Kirkwood *et al*, 1999; Scheiderer *et al*, 2004) and transient in the caudal NTS (Zhang and Mifflin, 2007; for review see Grueter *et al*, 2007.) In contrast, in the paraventricular nucleus of the hypothalamus (PVN),  $\alpha_1$ -AR signaling enhances excitatory transmission through both pre- and postsynaptic mechanisms (Gordon and Bains, 2003, 2005; Gordon *et al*, 2005).

Here we investigate the impact of  $\alpha_1$ -AR signaling on excitatory transmission in the lBNST. We find an extended application of NE results in robust long-term depression (LTD), which is dependent on  $\alpha_1$ -AR activation and that can be mimicked by  $\alpha_1$ -AR agonists. Intriguingly, the LTD described here differs from the previously described  $\alpha_1$ -AR LTD in the hippocampus and visual cortex in its induction characteristics. Additionally, because of the relative importance of the BNST in relapse and anxiety paradigms, and adrenergic signaling therein, we sought to determine if chronic alterations in adrenergic signaling would interfere with the expression of  $\alpha_1$ -AR LTD. We found that BNST  $\alpha_1$ -AR LTD is intact in animals acutely treated with cocaine, yet, is disrupted in both the  $\alpha_2A$ -AR knockout (KO) and the NE transporter (NET) KO lines, suggesting that chronic, but not transient, alterations in adrenergic signaling modulate the expression of  $\alpha_1$ -AR LTD.

## METHODS

### Animal Care

Male C57BL/6j mice 5–10 weeks old (The Jackson Laboratories, Bar Harbor, ME) and  $\alpha_2A$ -AR KOs and NET KOs which were both generated from in-house breeding on a C57BL/6 background were used in experiments. All animals were provided with food and water *ad libitum* and housed in groups within the Vanderbilt Animal Care Facilities. Experiments were performed under Vanderbilt Animal Care and Use committee approved guidelines. Animals receiving cocaine were prehandled for 5 days, receiving saline injections for 4 days.

### Brain Slice Preparations

Slicing methods were as previously described in Grueter *et al* (2006). Briefly, animals were retrieved from the colony and allowed to rest in sound attenuating boxes for a minimum of 1 h after which they were anesthetized (isoflurane) and decapitated in a separate room. 300  $\mu$ m coronal slices were made on a Leica VT1000S vibratome (Leica Microsystems, Bannockburn, IL) in a 1–4°C, oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>), high-sucrose low Na<sup>+</sup> artificial cerebral spinal fluid (ACSF in mM: 194 sucrose, 20 NaCl, 4.4 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose, 26 NaHCO<sub>3</sub>).

### Field Potential Recordings

After slicing, whole or hemisected slices were transferred immediately to interface chambers where they rested for at least 30 min in a humidified and oxygenated environment while continuously being perfused with oxygenated and heated (approximately 28–30°C) ACSF (in mM: 124 NaCl, 4.4 KCl, 2 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose, 26 NaHCO<sub>3</sub>) at a rate of 2 ml/min. Following this initial incubation, 25  $\mu$ M picrotoxin was added to the bath to block GABA<sub>A</sub> receptors and slices were allowed to rest at least another 30 min prior to recording, picrotoxin was included during the entirety of all experiments to isolate excitatory transmission. This concentration has been shown by our group to sufficiently block all inhibitory transmission via the GABA<sub>A</sub> receptor (Egli and Winder, 2003). Recording electrodes of approximately 1M $\Omega$  resistance were pulled on a Flaming/Brown microelectrode puller (Sutter Instruments, Novato, CA) and filled with ACSF. A bipolar Nichrome (A-M Systems, Carlsborg, WA) stimulating electrode was placed dorsally to the recording electrode within the dIBNST such that stimulation of the field resulted in two distinguishable negative shifts in potential: N1 (the TTX sensitive fiber volley estimate) and N2 (CNQX sensitive synaptic response) as previously reported (Weitlauf *et al*, 2004; Egli *et al*, 2005; Grueter and Winder, 2005). The amplitude (voltage) of the N2 was measured at a stimulation intensity that resulted in a voltage approximately 50% of the maximum N2 response. Slices were stimulated at a frequency of 0.05 Hz. Field potentials were recorded using Clampex 8.2 (Molecular Devices, Sunnyvale, CA). All drugs were bath applied at their final concentrations.

### Whole Cell Recordings

Following slicing, hemisected slices were allowed to rest submerged in a holding chamber filled with oxygenated and heated (28°C) ACSF for at least 30 min. After this incubation time an individual slice was moved to the recording chamber where it was submerged in oxygenated and heated (28°C) ACSF with added picrotoxin (25  $\mu$ M included for the entirety of all experiments as with the field recordings) to isolate currents evoked by glutamate receptor activation at a rate of 2 ml/min. Stimulating electrodes were the same as for field recordings in dorsal recordings and medial to the IBNST in ventral recordings. Patch electrodes (3–6M $\Omega$ ) were pulled on a Flaming/Brown microelectrode puller (Sutter

Instruments) and filled with either Cs- or K-gluconate intracellular solution (in mM: Cs- or K-gluconate 135, NaCl 5, MgCl<sub>2</sub> 2, HEPES 10, EGTA 0.6, Na<sub>2</sub>ATP 4, Na<sub>2</sub>GTP 0.4; there was no observable difference with either intracellular solution on the LTD effect). In all whole cell experiments, cells were clamped at -70 mV throughout and excitatory postsynaptic currents (EPSCs) were recorded using Clampex 9.2 (Molecular Devices). Series resistance was monitored throughout each experiment and a change greater than 20% resulted in the exclusion of the experiment from the data set. EPSCs were evoked at a frequency of 0.167 Hz and 100–400 pA EPSCs were recorded. Consistent with the field experiments, drugs were bath applied at their final concentrations.

### Analysis of Field Recordings

All recorded data were analyzed via Clampfit 9.2 (Molecular Devices). All field recordings contain a 20 min baseline recording prior to agonist application and all data points were normalized to the baseline 5 min prior to the agonist application. Plotted time courses for field experiments are represented as 1 min averages. For the majority of LTD experimental measurements, the LTD measurement was taken 55–60 min post agonist application. The exceptions are the experiments with prazosin (Figure 1c and d), in which the LTD measurement was the final 5 min of each recording.

### Analysis of Whole Cell Recordings

A 5 min baseline prior was acquired prior to agonist application and all points were normalized to minutes 3–5 within each experiment (with the exception of the low concentration methoxamine experiments where a 10 min baseline was acquired). Points are 30 s averages on plotted time course. For the majority of LTD experiments the LTD measurement is taken at 58–60 min within the experimental time course. The exceptions to this rule are the dual agonist application experiments, where the first LTD measurement is at 28–30 min within the time course and the second measurement is at min 55–57 min within the time course. The experiments with the lower concentration of methoxamine had measurements taken in the last 2 min of recording due to the shorter duration of wash.

### Statistics

All data points were reported as the mean  $\pm$  SEM and significance (determined by paired and unpaired Student's *t*-test) is reported in the text and figure legends. Significant differences were defined as having a  $p < 0.05$ .

### Reagents

(2S)-3-(((1S)-1-(3,4-Dichlorophenyl)ethyl)amino-2-hydroxypropyl) (phenylmethyl) phosphinic acid (CGP 55845, Tocris, Ellisville, MO), Cocaine (Sigma, St Louis, MO), (RS)-3,5-Dihydroxyphenylglycine (DHPG, Tocris), DL-2-amino-5-phosphonopentanoic acid (DL-APV, Sigma), methoxamine-HCl (Sigma), 2-methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP, Tocris), nimodipine (Tocris), picrotoxin (Tocris), prazosin (Tocris). Dimethylsulfoxide (DMSO) was the solvent used for stock solutions of CGP 55845, MPEP, nimodipine, picrotoxin, and prazosin where the maximum final concentration of DMSO was 0.02% by volume.

## RESULTS

### $\alpha_1$ -AR Activation Produces LTD of Excitatory Responses in the BNST

Both dIBNST and vIBNST are activated in response to various stressors (footshock, yohimbine, restraint, etc; Funk *et al.*, 2006). Additionally electrical stimulation of dIBNST and vIBNST produces behavior similar to that observed after experiencing a stressor

(Casada and Dafny, 1991). We began our investigation into the modulation of excitatory synapses via  $\alpha_1$ -AR in the BNST by recording extracellular excitatory potentials in the dBNST (Figure 1a). We first applied the  $\alpha_1$ -AR selective agonist methoxamine (100  $\mu$ M) for 20 min and observed a long lasting depression in excitatory transmission ( $79.5 \pm 4.7\%$ ,  $p < 0.01$ ,  $N = 6$ ; Figure 1b) that was absent in the presence of the  $\alpha_1$ -AR antagonist 10  $\mu$ M prazosin ( $101.9 \pm 5.6\%$ ,  $N = 6$ ; Figure 1c). To verify that this phenomenon was LTD and not the result of a constitutively activated  $\alpha_1$ -AR, we applied prazosin (10  $\mu$ M) to slices 60 min after a 20 min application of methoxamine (100  $\mu$ M) was terminated. This treatment failed to reverse the depression observed after agonist application ( $67.3 \pm 3.0\%$ ,  $p < 0.001$ ,  $N = 5$ ; Figure 1d) suggesting that the 20 min activation of  $\alpha_1$ -ARs results in an LTD of excitatory inputs. The BNST, however, is composed of a heterogeneous population of dendrites and cell bodies and therefore extracellular recordings may potentially reflect effects on excitability of the postsynaptic dendrites/cell. We therefore utilized whole-cell voltage clamp recordings to measure isolated EPSCs in dBNST and vBNST neurons. Application of methoxamine (10 or 100  $\mu$ M) for 15 min produced a robust depression of the evoked EPSC that persisted for at least 40 min after washout of agonist (dBNST: 10  $\mu$ M,  $65.6 \pm 6.3\%$ ,  $N = 5$ ,  $p < 0.001$ , Figure 2a; 100  $\mu$ M,  $46.1 \pm 9.8\%$ ,  $N = 6$ ,  $p < 0.001$ , Figure 2b; 10 vs 100  $\mu$ M was not statistically different  $p > 0.15$ ; vBNST 100  $\mu$ M:  $63.9 \pm 8.3\%$ ,  $N = 9$ ,  $p < 0.001$ , Figure 2c; representative experiment from the dBNST 100  $\mu$ M methoxamine recording Figure 2d). We did not see changes in the input resistance (IR) following application of methoxamine in either the dBNST (10 and 100  $\mu$ M) nor the vBNST (100  $\mu$ M) ( $97.3 \pm 5.5\%$ ,  $p > 0.7$ ,  $N = 19$ , Figure 2d representative trace). Additionally, we did not observe a significant change to a second 15 min application of methoxamine in the dBNST (second methoxamine application vs first methoxamine application  $p > 0.05$ ,  $N = 6$ ; Figure 2e). This, along with the extent of the depression, suggests that the initial induction of  $\alpha_1$ -ARLTD was saturated under our conditions although we cannot rule out that receptors may have become desensitized to the first agonist application.

### Prolonged Exposure to NE Results in $\alpha_1$ -AR-Dependent LTD

NE application can elicit LTD of excitatory inputs in the visual cortex and the hippocampus (Kirkwood *et al*, 1999; Scheiderer *et al*, 2004). Thus, we decided to test whether NE induces similar LTD in the BNST, a nucleus heavily innervated by adrenergic fibers. Our group has previously reported that a 10 min application of 100  $\mu$ M NE results in a transient bimodal response (an increase or decrease) in the extracellular field potential that is mediated by  $\alpha_2$ - and  $\beta$ -ARs (Egli *et al*, 2005). This application, however, was insufficient to produce a sustained depression in excitatory transmission ( $98.4 \pm 3.4\%$ ; Figure 3a,  $N = 6$ , Egli *et al*, 2005) in field recordings. In the BNST and other regions, chronic stressors are thought to promote lasting increases in extracellular NE levels by shifting the firing patterns of noradrenergic cells from phasic firing to burst firing (Forsay and Gysling, 2004). We found that increasing the duration of application of NE to 20 min at the same concentration produced a robust LTD of excitatory responses ( $67.1 \pm 7.3\%$ ,  $p < 0.01$ ,  $N = 6$ ) that persisted for over 60 min after agonist application (Figure 3a). Moreover, the experiments where we applied 100  $\mu$ M NE for 20 min were significantly different from the experiments where we applied 100  $\mu$ M NE for 10 min ( $p < 0.05$ ) during the LTD phase of the recording. With the extended application time we still observed the same bimodal effect to NE in our transient responses. (Two of the six experiments resulted in an initial increase in synaptic efficacy and four of six in an initial decrease in synaptic efficacy.) The sustained depression, however, was observed irrespective of the polarity of the initial response to NE (Figure 3b). To test whether the persistent depression induced by 100  $\mu$ M NE was due to activation of  $\alpha_1$ -ARs we applied the  $\alpha_1$ -AR specific antagonist prazosin (10  $\mu$ M) to the slice prior to NE application and throughout the experiment. The application of prazosin completely ablated the ability of NE (20 min at 100  $\mu$ M) to induce a long-lasting depression in excitatory

responses ( $94.6 \pm 4.5\%$ ,  $N = 5$ ; Figure 3c); however, we still observed the initial bimodal response (3 of 5 increases in synaptic efficacy, 2 of 5 decreases in synaptic efficacy).

### **$\alpha_1$ -AR LTD in the BNST Is Not Dependent on NMDAR Activation or Concurrent Stimulation of Presynaptic Fibers but Is Dependent on L-Type VGCCs**

$\alpha_1$ -AR LTD has been previously described in the visual cortex and most recently in the hippocampus where it has been shown to require concurrent activation of presynaptic inputs and *N*-methyl-D-aspartates (NMDARs) (Kirkwood *et al*, 1999; Scheiderer *et al*, 2004). We found, however, that applying methoxamine in the absence of stimulation resulted in significant LTD ( $82.3 \pm 2.4\%$ ,  $p < 0.01$ ,  $N = 6$ ; Figure 4a) that was indistinguishable from that observed with concurrent stimulation. The absence of concurrent stimulation itself, however, had no effect on the amplitude of subsequent field potentials ( $97.4 \pm 9.5\%$ ,  $N = 5$ ; inset, Figure 4a). To examine the influence of NMDAR activation on  $\alpha_1$ -AR LTD in the BNST, we recorded EPSCs at  $-70$  mV in the presence of  $100 \mu\text{M}$  DL-APV. Again under these conditions, a 15 min application of methoxamine still produced robust LTD ( $66.7 \pm 13.3\%$ ,  $N = 5$ ,  $p < 0.05$ , not significantly different from single methoxamine application  $p > 0.05$ ; Figure 4b). LTD induced by group 1 mGluR receptors (that are coupled to Gq) in other brain regions has been shown to involve L-type voltage gated calcium channels (VGCCs; for review see Grueter *et al*, 2007). Thus, we hypothesized that  $\alpha_1$ -AR LTD might also require L-type calcium signals. We applied methoxamine ( $100 \mu\text{M}$ ) in the presence of the L-type VGCC blocker nimodipine ( $10 \mu\text{M}$ ) and found that, although there was a small early depression (minutes 47–49,  $86.6 \pm 3.4\%$ ,  $p < 0.01$ ), LTD was blocked ( $99.5 \pm 4.8\%$ ,  $N = 7$ ; Figure 4c). Our group has previously described LTD in the dBNST that can be induced by activating the group 1 mGluR, mGluR5 (Grueter *et al*, 2006). To ensure that  $\alpha_1$ -AR LTD was not the result of increased glutamate inducing mGluR5-LTD, we applied methoxamine in the presence of the mGluR5 antagonist MPEP ( $10 \mu\text{M}$ ) at a concentration that prevents the induction of mGluR-LTD (Grueter *et al*, 2006). We found that MPEP had no effect on  $\alpha_1$ -AR LTD ( $54.3 \pm 6.7\%$ ,  $p < 0.005$ ,  $N = 5$ ; Figure 4d). These data thus suggest that  $\alpha_1$ -ARs heterosynaptically induce LTD via a non-Hebbian mechanism in the dBNST in an L-type VGCC dependent manner.

LTD can be maintained at synapses via either a pre- or postsynaptic mechanism. To begin to address questions of the synaptic locus of  $\alpha_1$ -AR LTD we conducted paired pulse ratio (PPR) analysis. Increases observed in the PPR associated with a decrease in EPSC amplitude are suggestive of a presynaptic mechanism. Evoked EPSCs to two paired stimuli with a 50ms inter-stimulus interval were acquired during whole cell recordings and we analyzed the ratio of the second response to the first response. In the dBNST we did not observe a change in the PPR upon application of methoxamine ( $10$  and  $100 \mu\text{M}$ ) nor at the LTD time point ( $N = 11$ ,  $p > 0.15$ ; Figure 4e).

### **$\alpha_1$ -AR LTD Is Disrupted in Mice with Aberrant Noradrenergic Signaling**

Alterations in noradrenergic signaling may underlie several affective disease states (Raskind *et al*, 2000; Stone *et al*, 2007). A single administration of cocaine elicits a transient increase in extracellular NE, while depression, anxiety and alcoholism are thought to involve more chronic alterations in adrenergic tone. Noradrenergic signaling in the BNST has been implicated in anxiety, depression, and drug abuse (Shaham *et al*, 2000; Forray and Gysling, 2004; Morilak *et al*, 2005) and synaptic plasticity is altered by multiple substances of abuse and stress in reward nuclei (Saal *et al*, 2003). We therefore chose to examine  $\alpha_1$ -AR LTD in the BNST in animals treated with cocaine ( $20 \text{ mg/kg}$ ) 30 min prior to slicing, and two animal models of affective disorders,  $\alpha_2\text{-AR}$  and NET KOs. Both of these KO lines of mice have altered adrenergic systems and behavior (Bohn *et al*, 2000; Xu *et al*, 2000; Schramm *et al*, 2001; Lahdesmaki *et al*, 2002; Dziedzicka-Wasylewska *et al*, 2006; Keller *et*

*al*, 2006). Animals receiving cocaine 30 min prior to slicing still showed robust  $\alpha_1$ -AR LTD ( $54.1 \pm 9.4\%$ ,  $p < 0.005$ ,  $N = 5$ ; Figure 5a). In both KO animal models,  $\alpha_1$ -AR LTD was not observed upon application of methoxamine ( $\alpha_{2A}$ -AR KO:  $96.0 \pm 2.8\%$ ,  $p > 0.05$ ,  $N = 5$ ; NET KO:  $104.2 \pm 6.0\%$ ,  $p > 0.05$ ,  $N = 7$ ; Figure 5b and d); however, application of the mGluR5 agonist DHPG still resulted in robust depression in the  $\alpha_{2A}$ -AR KO mouse indicating that LTD via  $G_{\alpha q}$  coupled mechanisms is still intact ( $57.6 \pm 3.7\%$ ,  $p < 0.001$ ,  $N = 5$ ; Figure 5c). This is additionally intriguing because induction of  $\alpha_1$ -AR LTD occludes further depression of EPSCs in response to subsequent application of DHPG (100  $\mu$ M) ( $p > 0.05$ ,  $N = 5$ ; Figure 5c inset).

## DISCUSSION

Previously, our group reported on noradrenergic modulation of excitatory synapses within the dIBNST, finding that both  $\alpha_2$ - and  $\beta$ -ARs contributed to effects observed with a 10 min application of 100  $\mu$ M NE and that  $\alpha_2$ -ARs contributed to effects in the vIBNST (Egli *et al*, 2005). Egli *et al* found that in the dIBNST, 100  $\mu$ M NE resulted in either a transient increase or decrease in excitatory transmission, while in the vIBNST it resulted in a transient decrease in excitatory transmission. Here we investigated the possibility that  $\alpha_1$ -ARs modulate glutamatergic synapses in this region as well. We found that a 20 min, but not a 10 min, 100  $\mu$ M NE application resulted in an LTD of excitatory transmission that was mediated by the  $\alpha_1$ -AR and L-type VGCCs, however, this LTD was independent of NMDAR, and mGluR5 activation, or concurrent stimulation. Finally, we found that  $\alpha_1$ -AR LTD in the dIBNST was disrupted in two KO mice that have genetically manipulated adrenergic systems, and exhibit altered anxiety, depression and reward phenotypes; but, not in animals that received a transient alteration of their adrenergic system—a single intraperitoneal (i.p.) injection of cocaine 30 min prior to slicing.

### NE Induces LTD in a Time-Dependent Manner

Our group has shown that while a 10 min application of NE in the dIBNST results in a bimodal transient regulation of the glutamatergic field potential (Egli *et al*, 2005), it fails to induce LTD via the  $\alpha_1$ -AR. Doubling the duration of NE application, however, results in  $\alpha_1$ -AR LTD. This duration-dependent result was an intriguing finding. It is clear that in the 10 min NE application experiments agonist wash-in has occurred and GPCRs are being activated based on data from our group (Egli *et al*, 2005) and the data shown here. Additionally, Dumont and Williams (2004) demonstrated that a brief (<2 min) application of 100  $\mu$ M NE (in a submerged recording chamber) activated  $\alpha_1$ -ARs in vIBNST to transiently increase spontaneous IPSCs, thus, the  $\alpha_1$ -AR is also presumably activated in our recordings, but at a level below threshold for LTD. The duration-dependence may play a role physiologically, as it may be disadvantageous to induce this plasticity with transient NE increases in the BNST. Therefore this LTD may be activated when the animal experiences a lasting stressor. Microdialysis studies have demonstrated that NE levels remain elevated above the baseline in the BNST over an hour after restraint stress and blocking  $\alpha_1$ -ARs attenuates stress-induced rises in adrenocorticotrophic hormone (ACTH) (Pacak *et al*, 1995; Cecchi *et al*, 2002). Moreover, Banihashemi and Rinaman (2006) showed that ablating BNST NE inputs prevents increases in corticosterone to i.p. yohimbine 90 min post injection. Therefore, under stress-producing conditions,  $\alpha_1$ -AR LTD may be recruited to alter the engagement of the HPA stress axis by the BNST over an hour after the initial stress insult. Thus, this  $\alpha_1$ -AR LTD may specifically participate in stress responses generated by long-term increases in NE in normal subjects and may be dysregulated in addictive states such as alcoholism and anxiety disorders like post traumatic stress disorder (PTSD).

### $\alpha_1$ -AR LTD in the BNST Is a Heterosynaptic Form of Plasticity

LTD mediated via group I mGluRs remains the best characterized  $G_{\alpha q}$ -coupled receptor LTD and has been described in the cerebellum, hippocampus, cortex, dorsal and ventral striatum, ventral tegmental area, and BNST (Ito, 2001; Robbe *et al*, 2002; Malenka and Bear, 2004; Bellone and Luscher, 2005; Grueter *et al*, 2006, for review see Grueter *et al*, 2007). An interesting aspect exhibited by group I mGluR-LTD is a degree of promiscuity of mechanism depending on the synapse where the LTD is expressed. This notion can now be extended to the less characterized  $\alpha_1$ -AR LTD. Unlike in the hippocampus and visual cortex (Kirkwood *et al*, 1999; Scheiderer *et al*, 2004),  $\alpha_1$ -AR LTD within BNST is independent of the activation of NMDARs (in both the induction phase and the maintenance phase of the LTD) and concurrent presynaptic stimulation. Intriguingly, we found that  $\alpha_1$ -AR LTD expression in the BNST is dependent on L-type VGCC activity. L-type VGCC activity (particularly  $Ca_{V1.3}$ ) is required in the dorsal striatum to induce corticostriatal group I mGluR-LTD (Wang *et al*, 2006). Furthermore, it has recently been demonstrated in the hippocampus that signaling via phospholipase C can increase the conductance of  $Ca_{V1.3}$  at negative potentials (Gao *et al*, 2006). Consistent with the role of the L-type VGCC in the induction of group I mGluR-LTD in the dorsal striatum, postsynaptic cells must be depolarized to  $-50$  mV (Adermark and Lovinger, 2007). In contrast, however, we were able to induce  $\alpha_1$ -AR LTD holding the cell at  $-70$  mV and in the absence of concurrent stimulation; although, subsequent stimulation, following the activation of  $\alpha_1$ -AR, may activate L-type channels in the maintenance phase of the LTD. Norepinephrine can modulate cell excitability within BNST, causing depolarization especially in nonprojection cells (Dumont and Williams, 2004) and, therefore, it is possible that the application of methoxamine directly depolarized the cell sufficiently to activate L-type VGCCs. Additionally, the concentrations of our extracellular and intracellular recording solutions may have produced sufficiently depolarizing conditions. These seem unlikely given previous sharp microelectrode studies under identical conditions that indicated resting membrane potentials of  $-64$  to  $-66$  mV (Egli and Winder, 2003) coupled with a lack of change in the input resistance. Another possibility was that  $\alpha_1$ -AR LTD may be dependent on signaling via mGluR5 downstream of  $\alpha_1$ -AR activation. We found, however, that blockade of mGluR5 during the induction of  $\alpha_1$ -AR LTD does not impact the expression of this LTD. This suggests that in this region adrenergic afferents may influence plasticity independently of descending glutamatergic inputs to the BNST from areas like the limbic cortex, hippocampus, and amygdala.

The postulated heterosynaptic mechanism of this  $\alpha_1$ -AR LTD could be of behavioral significance. A hallmark of some affective disorders is the inherent inability to overcome the disorder by reasoning (ie feelings/urges are beyond the cognitive control of the patient) (DSM-IV).  $\alpha_1$ -ARs in the BNST modulate ACTH levels in animals who have been exposed to a stressor (Cecchi *et al*, 2002). Moreover it has recently been shown that the same axon collaterals from the nucleus tractus solitarius that project to the BNST also may project to the PVN (Banihashemi and Rinaman, 2006). NE may therefore modulate this circuitry regardless of input from cortical structures. It is intriguing to think that this dissociation of induction of  $\alpha_1$ -AR LTD from the glutamatergic input of cognitive centers innervating the BNST potentially contributes to alterations in behavior in a disease state such as generalized anxiety or addiction. Clearly, however, additional work would be needed to provide support for this notion.

### $\alpha_1$ -AR LTD Is not Observed in Mice with Chronically Altered Adrenergic Signaling

Previously our group reported that a 10 min application of  $100 \mu\text{M}$  NE failed to alter glutamatergic transmission in  $\alpha_{2A}$ -AR KOs, mice with altered anxiety phenotypes. It was concluded that the  $\alpha_{2A}$ -AR may gate responses to NE within BNST via its interactions with



other receptors (Egli *et al*, 2005). Due to the shorter duration of agonist application, however, contributions by the  $\alpha_1$ -AR in the  $\alpha_{2A}$ -AR KO mice may not have been observed. Therefore, we decided to examine  $\alpha_1$ -AR LTD in the  $\alpha_{2A}$ -AR KO mice. Surprisingly,  $\alpha_1$ -AR LTD could not be induced via a 20 min application of methoxamine. This implied that perhaps the lack of LTD was due to functional desensitization of  $\alpha_1$ -AR or *in vivo* induction of  $\alpha_1$ -AR LTD as a result of increased extracellular concentration of NE. These ideas are supported by increased metabolite/transmitter ratios within several brain regions in the  $\alpha_{2A}$ -AR KO mice (Lahdesmaki *et al*, 2002), although these ratios can also be interpreted as increases in catabolism and therefore have caveats (Commissiong, 1985). To support our data with the  $\alpha_{2A}$ -AR KO mice we next used the NET KO mice, another mouse model with altered behavioral phenotypes and adrenergic transmission. Fast cyclic voltammetry experiments in the BNST in the NET KO mice have demonstrated that NE clearance rates are over six times slower in the KO mice as compared to wild-type controls (Xu *et al*, 2000). As was observed with the  $\alpha_{2A}$ -AR KO mice, the NET KO mice also failed to express LTD after a 20 min exposure to methoxamine. Interestingly, the  $\alpha_{2A}$ -AR KO mice express mGluR5-LTD after an application of DHPG, demonstrating that signaling via GPCRs, and more specifically those coupled to  $G_{\alpha q}$ , are still intact. Autoradiography data in the NET KO mice shows a reduction in the cell surface expression of  $\alpha_1$ -ARs in several brain regions (Bohn *et al*, 2000; Xu *et al*, 2000; Dziedzicka-Wasylewska *et al*, 2006) but an upregulation of  $\alpha_{2A/C}$ -AR within the BNST (Gilsbach *et al*, 2006). One possibility of our results, taken together with this data, suggest that within these mouse models  $\alpha_1$ -ARs and/or their signaling pathways are desensitized, preventing induction of  $\alpha_1$ -AR LTD. An intriguing observation was that  $\alpha_1$ -AR LTD can occlude mGluR5-LTD in the BNST. This suggests that the two LTDs share a common mechanism as they do in the visual cortex (Choi *et al*, 2005). There is evidence, however, in dopaminergic cells in the VTA that  $\alpha_1$ -ARs desensitize group I mGluRs (Paladini *et al*, 2001). The L-type VGCC experiments provide indirect support for the desensitized receptor hypothesis as well. In these experiments there is a significant transient depression in response to the application of methoxamine that is not observed in either of the mouse models. One possibility for this transient depression would be GABA<sub>B</sub> receptor activation due to the activation of  $\alpha_1$ -ARs on interneurons (Dumont and Williams, 2004). This transient depression is not observed in either KO animal suggesting that there may be a tonic reduction in  $\alpha_1$ -ARs. Additional experiments will need to be conducted to confirm these hypotheses.

Interestingly, both of these KO mice have altered anxiety/depression phenotypes (Schramm *et al*, 2001; Lahdesmaki *et al*, 2002; Dziedzicka-Wasylewska *et al*, 2006; Keller *et al*, 2006) including increased anxiety-like behavior in the elevated plus maze ( $\alpha_{2A}$ -AR KO mice), increased response to injection stress ( $\alpha_{2A}$ -AR KO mice), decreased struggling/mobility in the forced swim and tail suspension tests (NET KO mice) and bradycardia to stressful stimuli (NET KO mice). In addition the NET KO mice have heightened sensitivity to psychostimulants, enhanced conditioned place preference to cocaine, and increased analgesia to opiates (Bohn *et al*, 2000; Xu *et al*, 2000; Hall *et al*, 2002). Furthermore it has been shown that the BNST sends monosynaptic projections to dopaminergic VTA neurons to modulate reward (Georges and Aston-Jones, 2002). During withdrawal from morphine there is an inhibition of the firing of these dopaminergic cells that can not only be reversed with the  $\alpha_2$ -AR agonist clonidine, but potentiated by its administration (Georges and Aston-Jones, 2003). It is an interesting notion that the NET KO mice lack a mechanism ( $\alpha_1$ -AR LTD) that may contribute to the inhibition of the dopaminergic cells in the withdrawal state while simultaneously demonstrating increased behavioral sensitization and reward mediated behaviors to drugs of abuse. A lack of functioning  $\alpha_1$ -ARs and/or their signaling pathways may impact such behavior. Although  $\alpha_1$ -AR activation can affect the excitability of cells in various ways, the lack of a long term change in cell function, like synaptic plasticity induced by the  $\alpha_1$ -ARs, in these animals could have implications for their exhibited behavior.

Clinical data have highlighted the  $\alpha_1$ -AR as a therapeutic target for anxiety disorders. Raskind and colleagues reported that  $\alpha_1$ -AR antagonists are efficacious for patients with combat/noncombat induced post-traumatic stress disorder (Raskind *et al*, 2000, 2002; Taylor and Raskind, 2002; Peskind *et al*, 2003; Taylor *et al*, 2006). Additionally,  $\alpha_1$ -AR antagonists are used to treat ailments like benign prostatic hypertrophy and hypertension and thus specific pharmacological agents are available for further investigation into their benefits in the treatment of affective disorders. Furthermore, the notion that alterations in adrenergic mediated synaptic plasticity within nuclei like the BNST, and others implicated in stress and anxiety, may contribute to the pathological learning (learned fear) that may mediate PTSD and similar disorders remains an interesting possibility.

Previous work has highlighted the role of adrenergic modulation in BNST in behavioral paradigms of stress-induced relapse to drug seeking and anxiety. Our findings showed that NE modulates excitatory synapses in the dBNST by inducing an LTD that is dependent on the  $\alpha_1$ -AR and L-type VGCC activation and independent of the NMDAR and stimulation from presynaptic inputs. A crucial element to  $\alpha_1$ -AR LTD is that the induction depends on the length of exposure to NE, preventing transient increases in NE to elicit plasticity. Furthermore, a lack of this plasticity in animal models of affective disorders may impact their behavioral phenotypes. Together, our results demonstrate a mechanism by which NE may modulate BNST functional output under conditions of psychological stress.

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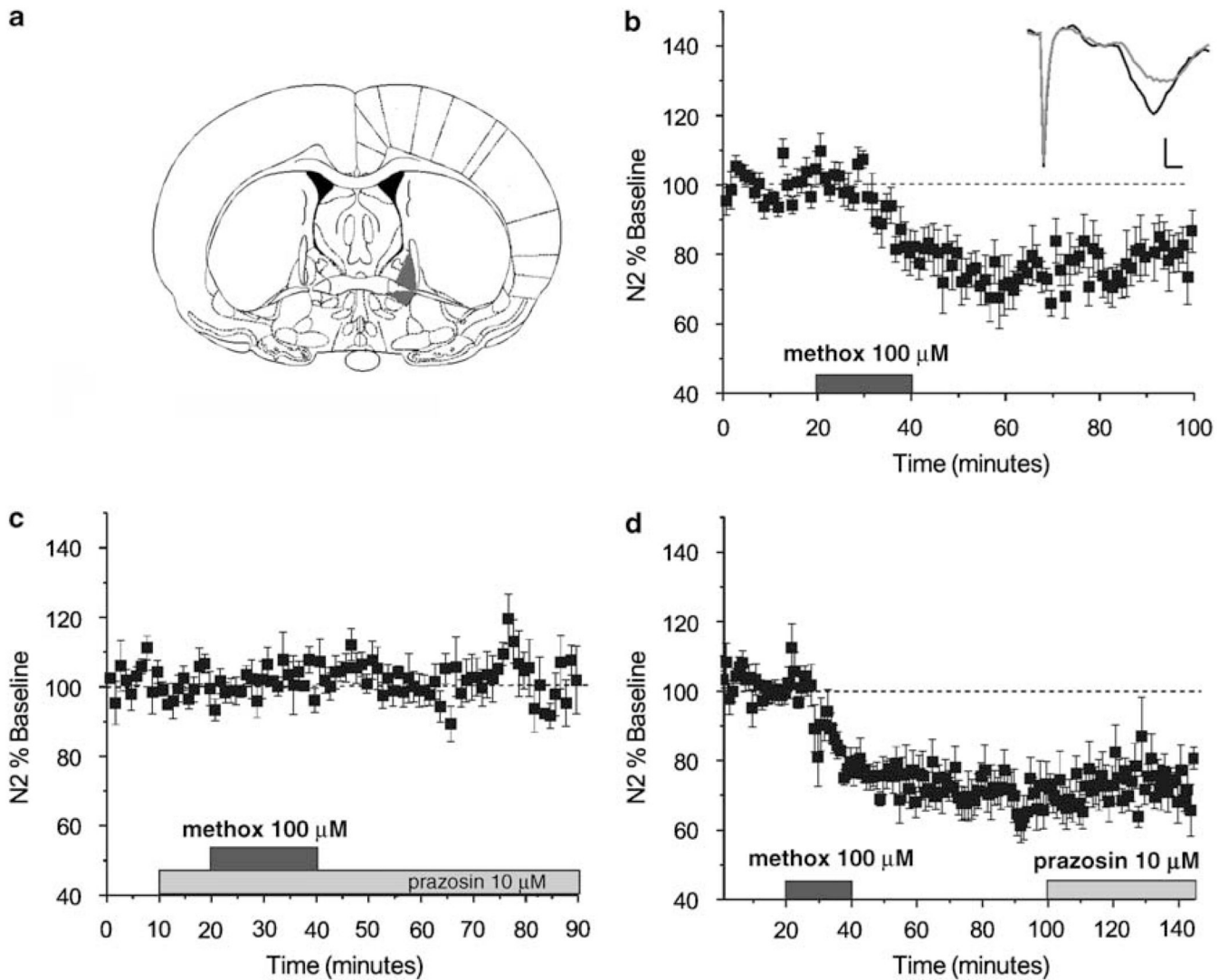
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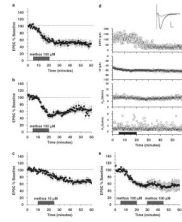
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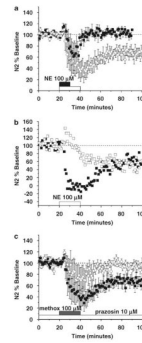
**Figure 1.**

$\alpha_1$ -AR activation induces long-term depression (LTD) in bed nucleus of the stria terminalis (BNST). (a) BNST schematic adapted from Paxinos and Franklin (2001). Gray shading represents the lateral BNST, above anterior commissure dorsal lateral BNST, below anterior commissure ventral lateral BNST. (b) Application of the  $\alpha_1$ -AR selective agonist methoxamine (100  $\mu$ M) induces a depression of extracellularly recorded excitatory responses that persists for over 60 min post wash ( $N = 6$ ). (Inset) Representative traces of N1 and N2, 5 min average of baseline (black) and LTD (gray) (scale bars 0.2 mV by 0.5 ms). (c) The  $\alpha_1$ -AR antagonist prazosin (10  $\mu$ M) blocks induction of the depression by methoxamine (100  $\mu$ M; ( $N = 6$ )). (d) Prazosin (10  $\mu$ M) cannot reverse the depression induced by methoxamine (100  $\mu$ M) when applied in wash ( $N = 5$ ).



**Figure 2.**

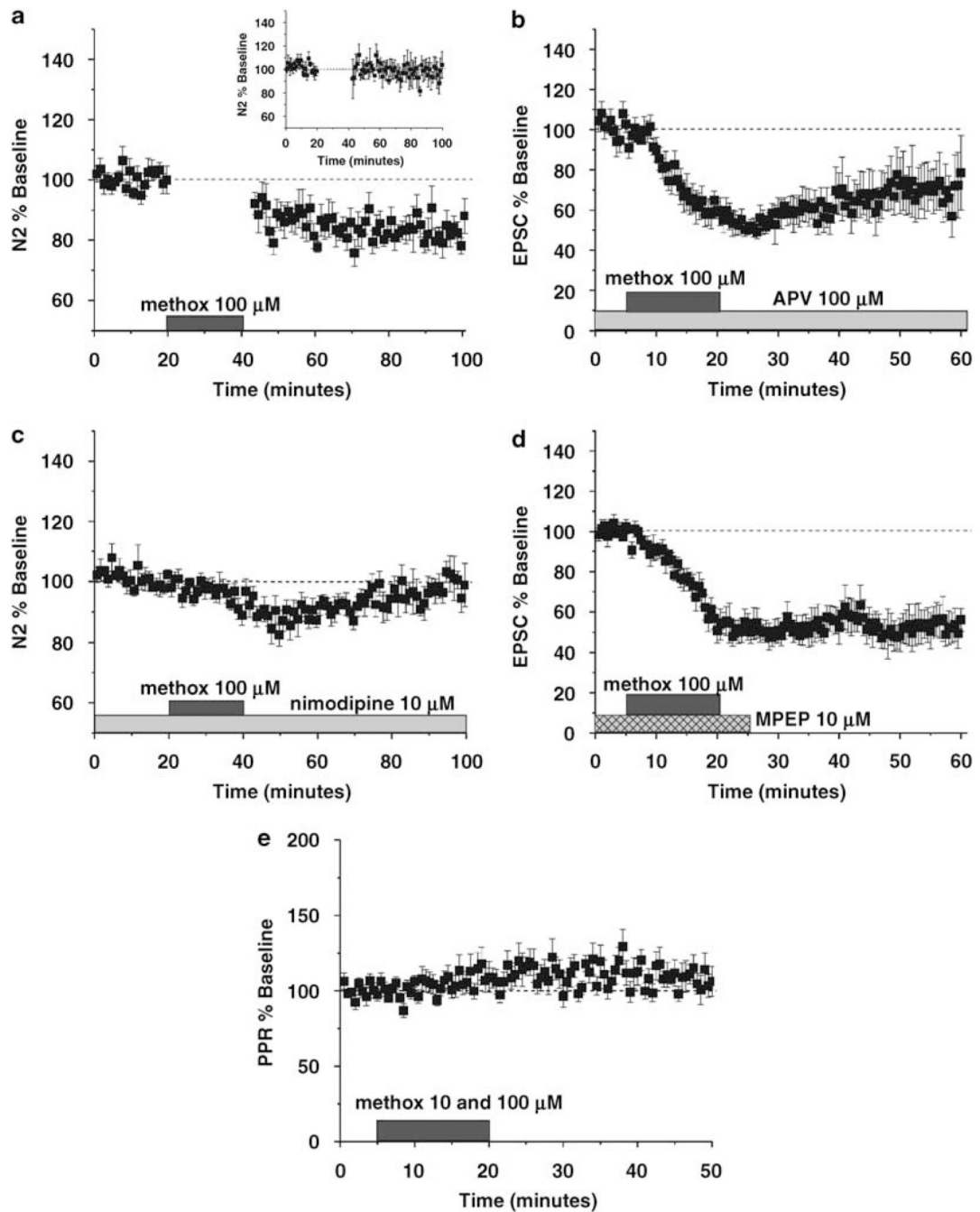
Excitatory postsynaptic currents (EPSCs) are depressed by  $\alpha 1$ -AR agonist application. (a) Whole cell voltage clamp ( $-70$  mV holding potential) experiments were performed to assess long-term depression (LTD) in the dorsal lateral BNST (dlBNST). Methoxamine ( $100 \mu\text{M}$ ) was applied for 15 min and washed out for 40 min resulting in LTD ( $N = 6$ ). (b) Under the same conditions  $100 \mu\text{M}$  methoxamine also induced LTD in the ventral BNST (vBNST). ( $N = 8$ ) (c) A lower concentration of methoxamine ( $10 \mu\text{M}$ ) applied for the same duration also can induce significant LTD in the dlBNST. (d) A representative experiment in the dlBNST: EPSC (pA) = EPSC amplitude, HI (pA) = holding current,  $R_A$  ( $\text{M}\Omega$ ) = Access Resistance, and  $R_I$  ( $\text{G}\Omega$ ) = Input resistance. (d inset) Representative traces of EPSCs, each a 2 min average of baseline (black line) and LTD (gray line; scale bar  $40$  pA by  $5$  ms). (e) Applying  $100 \mu\text{M}$  methoxamine after previous induction of LTD fails to further depress EPSCs ( $N = 6$ ).



**Figure 3.**

Norepinephrine (NE) induces  $\alpha$ 1-AR LTD via a time-dependent mechanism. (a) Expressed as percent of baseline, a 20 min application of NE (100  $\mu$ M) results in a sustained depression of extracellularly recorded excitatory response that lasts for over 60 min post drug application (open symbols;  $N = 6$ ). However, responses following a 10 min application of NE (100  $\mu$ M) fail to induce a sustained depression of the excitatory response (closed symbols;  $N = 6$ ). (b) Two representative experiments with 20 min applications of NE (100  $\mu$ M) demonstrate the transient bimodal effect, open symbols: increase in EPSP response followed by LTD, closed symbols: decrease in EPSP response followed by LTD. (c) 10  $\mu$ M of the  $\alpha$ 1-AR specific antagonist prazosin blocks the sustained depression of the excitatory field potential (open symbols;  $N = 5$ ).

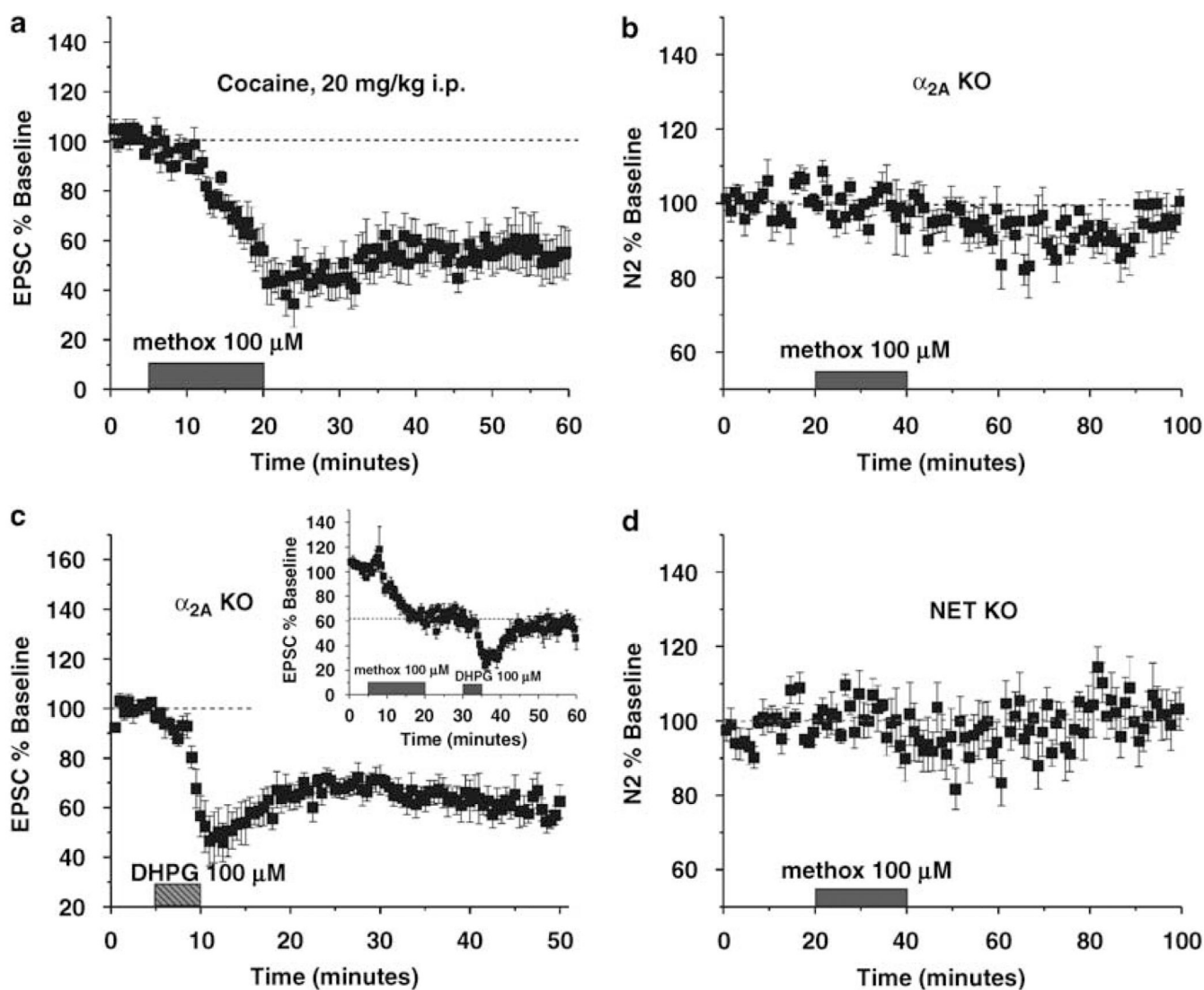




**Figure 4.**

$\alpha$ 1-AR LTD (as measured in the dIBNST) is induced independently of evoked glutamatergic synaptic activity but dependent on L-type voltage gated calcium channels (VGCCs). (a) To address the involvement of presynaptic stimulation the stimulus was turned off prior to 100  $\mu$ M methoxamine application and turned back on 2 min post  $\alpha$ 1-AR agonist removal. This did not disrupt LTD expression. ( $N = 7$ ). (a inset) To control for the lack of stimulation interleaved experiments were run without the presence of agonist ( $N = 5$ ). (b) To assess the role of *N*-methyl-D-aspartate receptor (NMDAR) activation  $\alpha$ 1-AR LTD experiments were performed in whole cell voltage clamp ( $-70$  mV holding potential) and DL-APV (100  $\mu$ M) was included throughout the duration of the experiment ( $N = 5$ ). (c) The L-type calcium

channel blocker nimodipine (10  $\mu\text{M}$ ) prevented the induction of  $\alpha\text{1-AR LTD}$  by 100  $\mu\text{M}$  methoxamine, however it did not prevent a significant transient depression. ( $N = 7$ ) (d) To verify that  $\alpha\text{1-AR LTD}$  does not require mGluR5 signaling we applied 100  $\mu\text{M}$  methoxamine in the mGluR5 antagonist MPEP (10  $\mu\text{M}$ ;  $N = 5$ ). (e) Paired pulse ratios do not change in response to methoxamine (10 or 100  $\mu\text{M}$ ) in dIBNST ( $N = 11$ ).



**Figure 5.**  
 $\alpha$ 1-AR is disrupted in animal models of affective disorders. (a) Cocaine (20 mg/kg) injected 30 min prior to slicing does not prevent the induction/expression of  $\alpha$ 1-AR LTD ( $N = 5$ ). (b) A 20 min application of methoxamine (100  $\mu$ M) fails to induce  $\alpha$ 1-AR LTD in  $\alpha$ 2A-AR KO mice ( $N = 5$ ). (c) DHPG (100  $\mu$ M) induces mGluR5-LTD in  $\alpha$ 2A-AR KO mice ( $N = 5$ ). (d) Methoxamine (100  $\mu$ M) fails to induce  $\alpha$ 1-AR LTD in NET KO mice ( $N = 7$ ).