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Neuroadaptation of GABAergic Transmission in the Central Amygdala During Chronic Morphine Treatment

Michal Bajo¹, Marisa Roberto², Samuel G. Madamba¹, and George Robert Siggins^{1,*}

¹ Molecular and Integrative Neurosciences Department, The Scripps Research Institute, La Jolla, California, USA

² Committee on the Neurobiology of Addictive Disorders, The Scripps Research Institute, La Jolla, California, USA

Abstract

We investigated possible alterations of pharmacologically-isolated, evoked GABA_A inhibitory postsynaptic potentials (eIPSPs) and miniature GABA_A inhibitory postsynaptic currents (mIPSCs) in the rat central amygdala (CeA) elicited by acute application of μ -opioid receptor (MOR) agonists (DAMGO and morphine; 1 μ M) and by chronic morphine treatment with morphine pellets. The acute activation of MORs decreased the amplitudes of eIPSPs, increased paired-pulse facilitation (PPF) of eIPSPs and decreased the frequency (but not the amplitude) of mIPSCs in a majority of CeA neurons, suggesting that acute MOR-dependent modulation of this GABAergic transmission is mediated predominantly via presynaptic inhibition of GABA release. We observed no significant changes in the membrane properties, eIPSPs, PPF or mIPSCs of CeA neurons during chronic morphine treatment compared to CeA of naïve or sham rats. Superfusion of the MOR antagonist CTOP (1 μ M) increased the mean amplitude of eIPSPs in a majority of CeA neurons to the same degree in both naïve/sham and morphine-treated rats, suggesting a tonic activation of MORs in both conditions. Superfusion of DAMGO decreased eIPSP amplitudes and the frequency of mIPSCs equally in both naïve/sham and morphine-treated rats but decreased the amplitude of mIPSCs only in morphine treated rats, an apparent postsynaptic action. Our combined findings suggest the development of tolerance of the CeA GABAergic system to inhibitory effects of acute activation of MORs on presynaptic GABA release and possible alteration of MOR-dependent postsynaptic mechanisms that may represent important neuroadaptations of the GABAergic and MOR systems during chronic morphine treatment.

Keywords

addiction; drug abuse; electrophysiology; extended amygdala; opiate; tolerance

Introduction

The amygdala complex consists of several nuclei that together play a crucial role in orchestrating emotional responses, autonomic behaviors, neuroendocrine activity, and a variety of cognitive functions (Aggleton, 1993; Cahill and McGaugh, 1998; Davis and Shi, 2000; Everitt et al., 2003; Gallagher and Chiba, 1996; LeDoux, 2003, 2007; Sah et al.,

*Corresponding author: George R. Siggins, The Scripps Research Institute, Molecular and Integrative Neurosciences Department, SP30-1150, 10550 North Torrey Pines Road, La Jolla, California 92037, USA, geobob@scripps.edu.

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2003). The central nucleus of the amygdala (CeA), through its extensive efferent projections to several brain regions such as basal forebrain, hypothalamus, midbrain and brainstem nuclei, integrates and controls fear responses, reward behavior, and environmental analgesia (Davis and Shi, 2000; Everitt et al., 2003; LeDoux, 2007; Pitkanen et al., 1997; Swanson and Petrovich, 1998).

The CeA is also a part of the extended amygdala, a brain complex playing a critical role in addiction to various drugs of abuse (Koob, 1999), and is involved in the learning of stimulus-reward responses and in mediating the motivational effects of drugs of abuse (Koob et al., 1998) and stress-induced reinstatement (See et al., 2003). The opioid system in CeA is considered to be important for the CeA involvement in drug addiction (Koob and Le Moal, 2005; Benarroch, 2006) as well as for environmental analgesia (Aggleton, 1993; Fox and Sorenson, 1994; Helmstetter, 1992). The presence of diverse opioid receptors (Chieng et al., 2006; Mansour et al., 1994; Neal et al., 1999a,b) and peptides from all endogenous opioid peptide families are reported to be present in the CeA (Cassell et al., 1986; Fallon and Leslie, 1986; Gray et al., 1984; Neal et al., 1999b; Pierce and Wessendorf, 2000).

The MORs (μ -opioid receptors) are primary molecular targets of morphine in mediating morphine-dependent analgesia, reward and physical dependence (Kieffer, 2000), and are moderately expressed in the CeA (Chieng et al., 2006; Wilson et al., 2002). The acute activation of MORs can inhibit some CeA neurons through activation of potassium (GIRK) channels (Chieng et al., 2006; Zhu and Pan, 2004). Presynaptically, MORs appear to be the only functional opioid receptors localized on glutamatergic projections to the CeA, and their activation results in inhibition of glutamatergic transmission (Finnegan et al., 2005; Zhu and Pan, 2005). In addition, MOR activation has been shown to decrease GABAergic transmission in the majority of CeA neurons projecting to the periaqueductal gray area (PAG) in rats (Finnegan et al., 2005) and in the medial division of the CeA in mice (Kang-Park et al., 2009). Conversely, increased GABAergic transmission has been reported after acute inhibition of MORs and in the CeA of MOR knockout mice (Kang-Park et al., 2009). However, there is still limited information on modulation of GABAergic transmission by acute and chronic MOR activation in the general rat CeA neuronal population.

Therefore, here we have compared the effects of acute and chronic activation of MORs on GABAergic transmission in the rat CeA. Whereas acute MOR activation decreased GABAergic transmission, predominantly by presynaptic inhibition of GABA release, chronic morphine treatment elicited no significant changes compared to placebo treatment, indicating development of tolerance/neuradaptation of the GABAergic system in the CeA. However, the mechanisms of the MOR-dependent regulation of the GABAergic system are altered during chronic morphine treatment and includes an apparent alteration of postsynaptic mechanisms. In addition, our findings with a MOR antagonist suggest that MORs modulate the GABAergic system tonically in both the naïve and chronic morphine-treated rat CeA.

Materials and methods

Animal treatment

Male Sprague-Dawley rats (100–180 g) were housed in a temperature- and humidity-controlled room on a 12-h light/dark cycle (lights on at 6:00 am) with food and water available *ad libitum*. We carried out chronic morphine treatment according to the standard morphine pellet method, using 2 morphine (75 mg of morphine/pellet) or placebo pellets implanted subcutaneously. This method is sufficient to elicit several behavioral indices of dependence after withdrawal of the morphine (Gold et al., 1994). After surgery, the rats were housed individually for 6 days. We conducted all care and surgical procedures in

accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the Institutional Animal Care and Use Committee (IACUC) policies of The Scripps Research Institute.

Slice preparation

The rats were anesthetized with 3% halothane, decapitated and the brains quickly removed and placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF; in mM: 130 NaCl, 3.5KCl, 1.25 NaH₂PO₄·H₂O, 1.5 MgSO₄·7H₂O, 2.0 CaCl₂·2H₂O, 24 NaHCO₃, and 10 glucose) gassed with 95% O₂ and 5% CO₂. We cut coronal slices (400 μm) containing the CeA using a Leica 1000S vibrotome cutter (Campden, Lafayette, Indiana). After a 20 minute incubation in an interface configuration, we completely submerged and superfused the slices continuously (2–4 ml/min) with warm (32°C) gassed ACSF. Drugs were added to the ACSF from stock solutions in known concentrations. Because the tissue content of morphine after 1–2 hr incubation in morphine-free solutions is negligible (Chieng and Christie, 1995), and to avoid a morphine withdrawal, we made all slice preparations and recordings in ACSF containing 1 μM morphine sulphate in the slices prepared from rats chronically treated with morphine. Due to possible variability in morphine blood levels with the pellet method (Gold et al., 1994), and to match potentially higher blood levels in our animals, we used a 1 μM concentration, that is approximately 1.5 – 2 times higher than the reported morphine plasma levels in implanted rats but still significantly below a saturating concentration (10 μM).

Intracellular recording of evoked responses

In most studies, we recorded from CeA neurons with sharp electrodes filled with 3 M KCl (60–70 MΩ) using discontinuous current-clamp mode as described previously (Roberto et al., 2004). We evoked pharmacologically-isolated GABA_A-IPSPs by stimulating locally within CeA in the presence of the glutamate receptor blockers DL-2-amino-5-phosphonovaleric acid (DL-AP5; 30 μM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX; 20 μM), and the GABA_B receptor blocker CGP 55845A (1 μM). Data were acquired with an Axoclamp-2A preamplifier (Molecular Devices, Sunnyvale, CA) and stored for later analysis using the software package Clampfit 8.2 (Molecular Devices). We took all measures before MOR agonists/antagonist (control), during their superfusion (5–15 minutes), and following washout (20–30 minutes). We generated *current-voltage (I/V) curves* by applying hyperpolarizing and depolarizing steps (200 pA, 750 msec duration) in CeA neurons held near their resting membrane potentials (RMPs). To examine synaptic responses of neurons, we used an *input-output (I/O) protocol* by stimulating slices at a range of currents (50–250 μA; 0.125 Hz) in the dorsomedial part of the CeA, starting at the threshold current required to elicit an IPSP, followed by 4 more steps at increasing stimulus strength until the maximum amplitude was reached. In another protocol, we used 4 consecutive stimuli (30 second intervals) at ½ of the maximal amplitude determined from the I/O relationship. For estimation of *paired-pulse facilitation (PPF)* we used two paired stimuli of equal intensity (50% maximal amplitude) at 50 msec interpulse intervals. We calculated PPF as the ratio of the second IPSP amplitude over the first.

Whole-cell patch-clamp recording of miniature IPSCs

In a separate set of neurons, we recorded from CeA neurons using a Multiclamp 700B preamplifier (Molecular Devices) and the “blind” whole-cell patch-clamp method, in the presence of 20 μM DNQX, 30 μM DL-AP5, 1 μM CGP 55845A and 1 μM tetrodotoxin (TTX) to isolate spontaneous action potential-independent GABA_Aergic mIPSCs. All mIPSC recordings were made using pipettes (input resistance 2–3 MΩ) filled with an internal solution containing (in mM): 135 KCl, 10 HEPES, 2 MgCl₂, 0.5 EGTA, 5 ATP, and

1 GTP (the latter two added fresh on the day of recording), pH 7.3–7.4, osmolarity 275–290 mOsm.

Data analysis and statistics

To analyze data acquired from intracellular and whole cell recordings, we used Clampfit 8.2 (Molecular Devices) and Mini 5.1 software (Synaptosoft, Leonia, NJ), respectively. We used GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA) for all statistical analysis of results obtained by intracellular recording. Because not all CeA neurons are responsive to MOR agonists (Chieng et al., 2006; Zhu and Pan, 2004), we used a change of 20% of control values as a threshold for dividing the cells into MOR agonist-sensitive and –insensitive groups. We accepted statistical significance at the $p < 0.05$ level using one way ANOVA, Student's t-test or one-sample t-test/Wilcoxon signed rank test. We used a non-parametric Wilcoxon rank test to analyze the sampled data based on the opioid effect (20% margin), as these data do not represent a single population. The mIPSC results were evaluated with cumulative probability analysis, and statistical significance was determined using the Kolmogorov-Smirnov, non-parametric two-sample test (Van der Kloot, 1991) with $p < 0.05$ considered significant. All averaged values are presented as mean $\pm$ SEM.

Drugs

We purchased DAMGO, CTOP, DNQX, and DL-AP5 from Tocris Biosciences (Ellisville, MI), and TTX from Calbiochem (San Diego, CA). Morphine sulphate, and both morphine and placebo pellets, were provided by the National Institute on Drug Abuse. CGP 55845A was a gift from Novartis Pharma (East Hanover, NJ).

Results

Acute application of MOR agonists inhibits GABAergic transmission presynaptically

We recorded from total 134 CeA neurons mostly in the medial subdivision of the CeA. Using intracellular recording in current-clamp mode, we tested effects of acute application of 1 μ M morphine and DAMGO, a MOR selective agonist, on membrane properties and GABAergic transmission mediated by GABA_A receptors (GABA_ARs) in CeA neurons. Acute application of DAMGO had no significant effect on mean RMP or resistance in our sample of the CeA neurons. Similarly, acute application of morphine did not alter mean RMP or resistance of the CeA neurons (Table 1). In addition, acute superfusion of neither DAMGO nor morphine significantly altered I/V relationships ($n = 34$; data not shown).

To examine possible modulation of GABAergic synaptic responses by acute DAMGO and morphine we used two protocols: 1) input-output (I/O) relationships (Figure 1A and B), and 2) averages of 4 consecutive responses at 50% of the maximal amplitude determined from the I/O relationship (Figure 1C and D). Superfusion of both morphine and DAMGO significantly reduced the mean amplitude of evoked GABA_A-IPSPs, by $33.3 \pm 6.0\%$ in 7 of 15 cells (46%) and by $35.1 \pm 6.6\%$ in 10 of 17 CeA neurons (59%), respectively. We observed no effect (80–120% of control) in 33 and 29% of the CeA neurons (morphine: 5 of 15, DAMGO: 5 of 17 cells), respectively. Interestingly, morphine and DAMGO increased the mean IPSP amplitude by $58.6 \pm 23.1\%$ in 3 of 15 (20%) cells and by $62.4 \pm 40.5\%$ in 2 of 17 cells (11%), respectively, without recovery on washout.

We asked whether the effects of morphine and DAMGO on CeA GABA_Aergic transmission might be mediated by pre- or postsynaptic mechanisms by using the PPF protocol (Figure 1E and F). Acute morphine significantly increased PPF by $45.7 \pm 13.7\%$ in 9 of 14 cells (64%) neurons, suggesting decreased presynaptic GABA release in a majority of cells (Figure 1F). PPF was not altered in 3 of 14 (23%) cells and decreased by $23.6 \pm 7.5\%$ in 2 of

14 cells (13%). Similarly, DAMGO increased PPF by $56.1 \pm 8.8\%$ in 9 of 17 (53%) cells (Figure 1E), had no effect in 18% (3 of 17) and decreased PPF by $36.4 \pm 4.90\%$ in 5 of 17 (29%) neurons in the CeA. Thus, although there were a few cells ($n = 4$ for DAMGO and 3 for morphine) in which changes in the IPSP amplitudes did not correlate with the changes in PPF, the great majority of cells with reduced amplitude of the IPSPs showed increases in PPF. These data suggest that morphine and DAMGO effects on GABA_Aergic transmission are mediated predominantly by presynaptic mechanisms. Pretreatment with 1 μ M CTOP, a MOR specific antagonist, prevented the DAMGO-dependent decrease in the mean amplitude of the IPSPs (CTOP: $107.6 \pm 5.8\%$ alone and with DAMGO: $107.5 \pm 8.3\%$ of control, $n = 6$) and the change in PPF (CTOP: $102.8 \pm 10.2\%$ and DAMGO: $99.8 \pm 11.8\%$ of control, $n = 6$) in the CeA (data not shown).

A presynaptic inhibition of vesicular GABA release, as a predominant effect of acute MOR activation, is further supported by our results from whole-cell recording of GABA_A mIPSCs following acute application of 1 μ M morphine (Figure 2). Here, the mean frequency of the mIPSCs was significantly decreased by $34.3 \pm 4.3\%$ in 60% (6 of 10) of CeA neurons (Figure 2B and D), suggesting a presynaptic effect. By contrast, the mean amplitude of mIPSCs, corresponding to postsynaptic effects, was not significantly changed in a majority (6 of 10) of the CeA neurons (Figure 2C and E). Only 2 cells showed decreased frequency as well as amplitude of mIPSCs. In addition, 2 cells showed increased frequency or amplitude of mIPSCs induced by acute morphine (not shown), in accord with our intracellular recording data. The mean decay ($101.6 \pm 3.6\%$ of control in 8 of 10 cells) and rise-time ($101.6 \pm 6.5\%$ of control in 5 of 10 cells) of mIPSCs were not significantly altered in the majority of the cells, suggesting a lack of postsynaptic effects of acute morphine application in these cells.

Basal CeA GABAergic transmission is not altered during chronic morphine treatment

Because our study of acute morphine was performed on CeA from naïve rats without pellet implantation, we asked if subcutaneous pellet implantation may affect basal GABAergic transmission in CeA. The mean amplitude of the eIPSPs was significantly decreased in the naïve/sham (placebo pellet-treated) rats (7.5 ± 0.6 mV; $n = 27$) compared to naïve (no pellets) rats (9.8 ± 1.0 mV; $n = 15$), whereas PPF was not altered (naïve/sham: 1.1 ± 0.1 , $n = 25$; naïve rats: 1.0 ± 0.1 , $n = 14$). Using whole-cell recording of mIPSCs, we found a significant decrease in basal rise-times of the mIPSCs in CeA of naïve/sham rats (2.3 ± 0.2 ; $n = 12$) compared to naïve rats (3.6 ± 0.4 ; $n = 10$), whereas there were no significant differences between CeA neurons from naïve and placebo pellet-treated (naïve/sham) rats in basal amplitude, frequency or decay time (data not shown). These findings suggest that the morphine/placebo pellet treatment had no effect on vesicular GABA release or the amplitude of mIPSCs, but may have affected the amplitude of eIPSPs and some kinetic properties of the GABA_A receptors.

Of the CTOP- and DAMGO-sensitive cells (see below), we did not observe significant changes in basal I/V relationships (not shown), RMPs, or input resistance between naïve/sham and chronic morphine rats (Table 2). Although the data from I/O relationships showed a tendency toward decreased amplitude of the eIPSPs in CeA neurons from chronic morphine rats compared to naïve/sham rats, this did not reach statistical significance (Figure 3A). We also found no significant alteration of the mean amplitude of eIPSPs, obtained by averaging 4 responses at half maximal stimulus intensity, between CeA of naïve/sham and chronic morphine rats (Figure 3B).

Because acute morphine appears to elicit its effects predominantly by presynaptic modulation of GABA release, we also examined PPF to assess modulation of basal GABA release in the CeA during chronic morphine treatment. PPF in the CeA of chronic morphine

rats did not differ significantly from PPF measured in the CeA of naïve/sham rats (Figure 3C). These results are supported further by the data from whole-cell recordings showing no significant alterations of the mean amplitude, frequency, rise or decay time of mIPSCs in CeA neurons from the chronic morphine-treated rats compared to the naïve/sham rats (Table 3). The lack of significant changes in basal GABA_A-transmission and GABA release in the CeA during chronic morphine treatment may indicate the development of tolerance or neuroadaptations of the GABAergic system in the CeA to continuous morphine exposure.

Some effects of acute MOR agonism on GABAergic transmission are altered by chronic morphine treatment, but effects of MOR antagonism are not

To test a functional adaptation of the GABAergic system to chronic morphine, we superfused both the MOR antagonist (CTOP) and agonist (DAMGO), respectively, and compared membrane properties and synaptic responses in CeA neurons from chronic morphine-treated and naïve/sham rats. Acute application of a relatively high concentration (1 μ M) of CTOP significantly depolarized the naïve/sham CeA neurons sensitive to CTOP (see below; mean RMP control: -71.4 ± 2.1 mV vs. CTOP: -68.8 ± 3.1 mV, $n = 13$) but not those from morphine-treated rats (control: -69.0 ± 1.7 mV vs. CTOP: -67.8 ± 1.9 mV, $n = 18$). However, CTOP significantly decreased input resistance of the CeA neurons from morphine-treated (control: 95.1 ± 6.4 M Ω vs. 86.8 ± 5.4 M Ω , $n = 18$) but not from naïve/sham rats (control: 108.5 ± 9.5 M Ω vs. CTOP: 99.9 ± 7.5 M Ω , $n = 13$). The CTOP effects on resistance were reversed by washout of CTOP, whereas the effects on RMP were unchanged by washout. DAMGO had no significant effects on membrane potential in the CTOP sensitive neurons from naïve/sham (control: -75.6 ± 2.2 mV vs DAMGO: -74.0 ± 2.4 mV, $n=7$) nor morphine-treated rats (control: -75.9 ± 1.1 mV vs DAMGO: -76.2 ± 1.0 mV, $n=7$). Similarly, we did not observe any significant changes in the input resistance of the CeA neurons from naïve/sham (control: 84.8 ± 16.9 M Ω vs DAMGO: 83.3 ± 16.5 M Ω , $n=7$) and morphine-treated rats (control: 103.8 ± 8.1 M Ω vs DAMGO: 113.0 ± 12.3 M Ω , $n=7$). Both CTOP and DAMGO showed no significant changes in I/V relationships in either naïve/sham or chronic morphine-treated rats (data not shown).

CTOP antagonism of MORs resulted in a significant increase in eIPSP amplitudes in CeA of both naïve/sham and chronic morphine rats. In naïve rats, CTOP superfusion increased the mean amplitude of eIPSPs by $56.7 \pm 9.1\%$ compared to control in 11 of 17 (65%) neurons, as determined from the mean of 4 responses (Figure 4), with partial recovery on washout. In the remaining CeA neurons, CTOP had no effect (5 of 17 cells: 29%) or decreased the mean eIPSP amplitude by 36.0% in (1 of 17 cells: 6%; data not shown). In CeA of chronic morphine rats, a mean CTOP-induced increase by $64.5 \pm 8.7\%$ in IPSP amplitude was observed in 17 of 22 cells (77%; Figure 4). Of the remaining cells, we found no change in 3 and a decrease in mean eIPSP amplitude by $34.2 \pm 0.8\%$ in 2 neurons. These results suggest a MOR-dependent tonic inhibition of the GABAergic transmission in the CeA of both naïve/sham and chronic morphine rats. Statistical comparison of the CTOP-induced increase in the eIPSP amplitudes between naïve/sham and chronic morphine rats showed no significant difference.

In accord with our results from the naïve (non-treated) rats, acute superfusion of DAMGO led to a decrease in the eIPSP amplitudes in the CeA neurons from both naïve/sham and chronic morphine treated rats. In CeA of naïve/sham rats, DAMGO decreased the mean amplitude of IPSPs by $51.9 \pm 8.4\%$ compared to control in 6 of 8 (75%) neurons (Figure 5A). In the remaining cells, DAMGO had no effect (1 of 8 cells: 12.5%) or increased the eIPSP amplitude by 52.7% with partial recovery during washout (1 of 8 cells: 12.5%), respectively. In the morphine-treated rats, DAMGO superfusion decreased the mean amplitude of eIPSPs by $34.4 \pm 3.8\%$ in 8 of 9 (88%) neurons, and had no effect in one (12%) of the recorded neurons. Although the reduction of the mean eIPSP amplitude was

bigger in the naïve/sham rats than in morphine-treated ones, this difference did not reach significance.

Although CTOP increased IPSP amplitudes in most CeA neurons from both naïve/sham and chronic morphine rats, it had a dual effect on PPF (Figure 4C). In naïve/sham rats, CTOP decreased PPF (suggesting increased GABA release) in 8 of 18 neurons (44%), but increased PPF (decreased GABA release) in another 8 of 18 neurons (44%). In the remaining cells (2 of 18; 11%) CTOP had no significant effect on PPF. Similarly, in CeA from chronic morphine rats, CTOP significantly decreased PPF in 7 of 22 cells (32%) but increased PPF in another 9 of 22 CeA cells (41%). CTOP had no effect on PPF in the remaining 6 of 22 (27%) cells from the chronic morphine rats. None of the CTOP effects on PPF differed significantly between naïve/sham and chronic morphine-treated rats.

In the both treated groups, the acute superfusion of DAMGO significantly altered PPF in most of the CeA neurons (Figure 5B). In CeA of naïve/sham rats we found a DAMGO-dependent increase in PPF by $43.2 \pm 8.4\%$ in 5 of 6 (83%) neurons and no effect in one of the CeA neurons (17%). In the morphine-treated rats, DAMGO increased PPF by $57.2 \pm 13.9\%$ in 4 of 8 (50%) CeA neurons and decreased PPF in one neuron (12.5%). In the remaining three cells (37.5%), DAMGO had no effect on PPF ratio. Like the CTOP effects, the DAMGO-elicited effects on PPF were not significantly different between naïve/sham and morphine-treated rats. Although the lack of significant differences in the synaptic responses to CTOP and DAMGO between naïve/sham and chronic morphine rats further supports a tolerance or possible neuroadaptation of the GABAergic system in the CeA following chronic morphine treatment, the reduction in the DAMGO-induced inhibition of the eIPSPs in morphine-treated rats might suggest a functional alteration of MOR-dependent regulation of GABAergic transmission in the CeA during chronic morphine treatment.

Using whole-cell recording, we found a significant alteration of the frequency but not the amplitude of mIPSCs by acute application of CTOP in a majority of CeA neurons in both animal groups (Figure 6). In naïve/sham rats CTOP significantly increased the mean frequency of mIPSCs by $77.8 \pm 36.3\%$ in 4 of 7 CeA neurons (57% of cells) but decreased it by $26.7 \pm 7.5\%$ in 2 cells (Figure 6A and B). Only one cell showed no effect of CTOP on mIPSC frequency. The mean amplitude of mIPSCs was not altered in 4 of 7 of neurons (57% of cells; Figure 6C). In the remaining cells, the amplitude of mIPSCs was increased by $47.7 \pm 3.5\%$ (2 of 7 cells) and decreased by 33.5% in one cell. Similarly, in chronic morphine-treated rats CTOP changed the mean frequency of mIPSCs in a majority of cells (66%): an increase by $52.9 \pm 15.7\%$ in 4 of 9 cells and a decrease by $29.0 \pm 8.0\%$ in 2 neurons. The amplitude of mIPSCs was not significantly altered by CTOP in a majority of morphine-treated cells (56%), but was increased by $35.5 \pm 16.5\%$ and decreased by $21.7 \pm 0.2\%$ in 2 of 9 cells (22%), respectively. Comparison of the CTOP effects on the frequency and amplitude of the mIPSCs between naïve/sham and chronic morphine rats did not show significant differences. The CTOP effects on mean decay times of mIPSCs between naïve/sham and chronic morphine rats were also nearly equivalent. CTOP did not change significantly the mean decay time of mIPSCs in most cells from naïve/sham (6 of 7) and chronic morphine rats (7 of 9 cells). The decay time was changed in the remaining cell (decreased by 33.2%) and 2 cells (increased by $29.3 \pm 5.2\%$) from naïve/sham and chronic morphine rats, respectively. However, we did observe differences in the CTOP effects on a rise-time of mIPSCs between naïve/sham and chronic morphine rats. Thus, CTOP altered the mean rise-time of mIPSCs in a majority of the cells (57%) from naïve/sham rats, with an increase by $43.6 \pm 16.7\%$ in 3 of 7 cells and a decrease by 27.2% in one neuron, whereas it had no effect on the rise time in any tested neurons from chronic morphine rats. These data may suggest an alteration of the kinetics of the mIPSCs by chronic morphine treatment, perhaps related to single channel gating.

The DAMGO effects on the mIPSCs in whole-cell recordings differed somewhat between naïve/sham and morphine-treated rats (Figure 7). In naïve/sham rats, acute application of DAMGO decreased the mean frequency of mIPSCs by $35.0 \pm 9.4\%$ and had no effect on the mean amplitude in 4 of 5 (80%) neurons (Figure 7A–E). In the remaining neuron (20%), DAMGO had no effect on the mean frequency but decreased the mean amplitude of mIPSC by 22.8%. In morphine-treated rats, both the frequency and amplitude of mIPSCs were significantly altered by DAMGO in the majority of the CeA neurons (Figure 7F–J). The mean frequency of mIPSCs was decreased by $51.0 \pm 5.4\%$ in all 7 of the tested neurons. Contrary to the naïve/sham rats, DAMGO significantly decreased also the mean amplitude of mIPSCs by $32.0 \pm 7.0\%$ in almost all (6 of 7: 86%) CeA neurons tested, with no effect in the remaining cell. The mean rise-time was not significantly changed by acute application of DAMGO in 4 of 5 neurons but was increased by 32.2% in the remaining cell from the naïve/sham rats. By contrast, the mean decay time was significantly increased by $21.4 \pm 5\%$ in 4 of 5 neurons, with no change in one cell. In a majority (6 of 7: 86%) of CeA neurons from the chronic morphine-treated rats, DAMGO had no significant effects on the mean rise- or decay-times. In the remaining neuron, DAMGO increased rise-time by 26.6% and decay-time by 31.0%. Despite the significant increase in the decay-time by DAMGO in the naïve/sham rats, there were no significant differences between the DAMGO-effects on the decay- and rise-times between naïve/sham and morphine-treated rats. Comparison of DAMGO evoked effects on the mIPSCs between the animal groups showed significant differences in the MOR-dependent modulation of the amplitude and partially also decay-time, but not the frequency or rise-time, of the mIPSCs by chronic morphine treatment. These data may suggest an alteration of presynaptic vesicular filling or of postsynaptic mechanisms of MOR-elicited regulation of GABA receptors during chronic morphine treatment that indicate a neuroadaptation of the CeA GABAergic system.

Discussion

As far as we are aware, this study represents the first assessment of the effects of both acute and chronic administration of MOR agonists on evoked and spontaneous GABAergic transmission in the CeA. Acute superfusion of both DAMGO and morphine decreased GABAergic transmission in a majority of the CeA neurons, predominantly by a presynaptic inhibition of vesicular GABA release. During chronic morphine treatment, we found no significant alterations of basal GABAergic transmission in the CeA compared to naïve/sham rats. The CTOP and DAMGO effects in both animal groups were characterized by an increase and decrease in GABAergic transmission, respectively, involving modulation of GABA release, in a majority of CeA neurons. However, we found significant differences in the effects of the MOR agonist DAMGO, but not the antagonist CTOP, on GABAergic transmission in the CeA between naïve/sham and chronic morphine-treated rats. Thus, our combined data suggest a neuroadaptation of the GABAergic system in the CeA and possible functional alteration of the MOR-dependent regulation of GABAergic transmission during chronic morphine treatment. In addition, the CTOP effects observed in the naïve/sham rats indicate a tonic modulation of the GABAergic transmission mediated by basal MOR activation in the rat CeA.

After pharmacological isolation of GABA_A-IPSPs/IPSCs, we found no significant MOR agonist effects on postsynaptic membrane properties of the CeA neurons following acute application of the agonists. In general, one of the commonly reported MOR agonist effects on neuronal membrane properties is hyperpolarization mediated by GIRK potassium channels. For example, acute application of MOR agonists has been reported to hyperpolarize some CeA neurons (Chieng et al., 2006; Zhu and Pan, 2004). Therefore, we were surprised to find no effects of the MOR agonists on membrane properties of the CeA neurons in our study. One explanation may be our use of pharmacological isolation of

synaptic potentials and currents. In our studies, as well as others showing no effects of the MOR agonists on CeA membrane properties (Finnegan et al., 2005; Kang-Park et al., 2009), pharmacological isolations of GABA or glutamate PSP/Cs were performed, whereas no such isolation was used in any of the studies showing the hyperpolarization of CeA neurons by acute application of MOR agonists (Chieng et al., 2006; Zhu and Pan, 2004). Thus, it is possible that the hyperpolarizations were elicited by MOR agonist decreases of tonic glutamate release.

Our data from intracellular and whole-cell recordings indicate that acute activation of MORs reduces GABA_Aergic transmission in a majority of the CeA neurons, predominantly by a presynaptic inhibition of vesicular GABA release. This is in accord with studies showing decreased GABAergic transmission via a presynaptic inhibition of quantal GABA release in a majority of CeA neurons in mice (Kang-Park et al., 2009) and a subpopulation of CeA neurons projecting to periaqueductal gray (PAG) in rats (Finnegan et al., 2005). Interestingly, we detected a subpopulation of the CeA neurons showing a positive modulation of GABAergic transmission by the acute activation of MORs. Dual modulation of synaptic transmission by morphine has been reported in habenular nucleus, where morphine inhibited and facilitated excitatory transmission in a cell-dependent manner (Hashimoto et al., 2009). One possible explanation for the positive modulation of GABAergic transmission by MOR agonists might be an inhibition of inhibitory afferent projections and/or interneurons projecting to the recorded neurons.

We used the morphine pellet method of chronic morphine treatment that has been shown to induce development of tolerance to and dependence on morphine (Gold et al., 1994; Williams et al., 2001). This model allows the study of effects of continuous morphine exposure (in our study, 6 days) on GABA_Aergic transmission in the CeA. Our findings of a lack of significant change in basal GABA_Aergic transmission in the CeA during chronic morphine treatment compared to naïve/sham controls indicate the development of neuroadaptation and tolerance of the GABA_Aergic system in the CeA to the acute inhibitory effects of MOR agonists. This is in accordance with the concept of between-systems neuroadaptation, which is characterized by adaptation and tolerance of a different molecular system (GABA_Aergic system in our study) triggered by changes in neuronal responses to a primary drug (opioids) (Koob and Bloom, 1988). Because the predominant effect of acute MOR agonists is presynaptic inhibition of GABA release, the chronic morphine-induced neuroadaptation of the CeA GABA_Aergic system most likely occurs presynaptically in GABAergic terminals of the CeA interneurons and/or afferent projections from the other amygdala nuclei and brain areas (e.g. cortex, brain stem, thalamus, etc. (Sah et al., 2003)). Neuroadaptation of CeA neurons to chronic morphine exposure also is supported by studies showing induction of c-Fos protein expression in the CeA following acute morphine injection (Singh et al., 2004), but lack of induction of c-Fos expression after chronic morphine pellet treatment (Georges et al., 2000; Stornetta et al., 1993).

We also tested effects of acute block and activation of MORs on the GABA_Aergic system in the CeA during chronic morphine exposure. In the majority of CeA neurons, the MOR antagonist CTOP increased the amplitude of GABA_A-IPSPs and modulated GABA release in both naïve/sham and chronic morphine rats. The acute activation of MORs by DAMGO decreased eIPSP amplitudes and modulated GABA release, predominantly negatively, in majority of the CeA neurons. Our finding of the lack of significant differences in CTOP effects between the animal groups further supports the development of neuroadaptation/tolerance of GABAergic transmission in the CeA to chronic morphine treatment. The lack of CTOP effects on GABAergic transmission, as a sign of morphine withdrawal, does not rule out other mechanisms that might be involved, e.g. modulation of glutamatergic transmission by chronic morphine and withdrawal. Lack of significant difference in DAMGO effects on

GABA release, as determined by PPF and the frequency of mIPSCs, between naïve/sham and morphine treated rats, suggests development of neuroadaptation/tolerance of presynaptic mechanisms of the MOR-dependent modulation of the GABAergic transmission in the CeA. However, acute application of DAMGO revealed a possible alteration of the MOR-dependent regulation of GABAergic transmission in the CeA during chronic morphine treatment. First, there was a suggestive reduction, although not significant, in the DAMGO inhibitory effects on eIPSP amplitudes in CeA neurons from the morphine-treated rats compared to naïve/sham rats. Second, acute application of DAMGO significantly decreased the mean amplitude of mIPSCs, suggesting either a change in presynaptic vesicular filling or a possible postsynaptic action of DAMGO, in a majority of the CeA neurons from the morphine-treated rats, but not from the naïve/sham rats. We speculate that chronic morphine treatment may activate postsynaptic mechanisms of MOR-dependent modulation of the GABAergic system in the CeA. During chronic morphine treatment, we did not observe any significant DAMGO effects on the membrane properties of the CeA neurons, and no changes in MOR binding or DAMGO-stimulated G-protein coupling have been found in amygdala (Sim et al., 1996; Maher et al., 2001; Kirschke et al., 2002). Thus, to date we do not know what homologous and/or heterologous postsynaptic mechanisms (Williams et al., 2001; Christie, 2008) might be altered during chronic morphine treatment.

Modulation of the GABAergic system by chronic exposure to morphine has been studied predominantly by examining alterations after morphine withdrawal, rather than during chronic morphine treatment. Thus, data on alterations of the GABA system *during* chronic morphine treatment are limited to only a few brain regions compared to data obtained with morphine withdrawal. In rat PAG slices, MOR antagonists had no effect on either amplitude or frequency of evoked and miniature IPSCs in vehicle treated rats, whereas they increased the frequency of IPSCs during chronic morphine treatment, suggesting sensitization of the MORs in this brain region (Ingram et al., 1998). By contrast, in mouse PAG there was no alteration of basal mIPSCs during chronic morphine treatment compared to vehicle treatment (Hack et al., 2003) and no difference in the effects of acute naloxone application, characterized by an increase in the frequency of mIPSCs, between the treatment groups (Hack et al., 2003). In the dorsal raphe nucleus (DRN), the GABAergic system plays a critical role in regulation of serotonin levels via inhibition of DRN neurons (Jolas et al., 2000; Tao and Auerbach, 2000; Tao et al., 2000). Acutely administered morphine increased serotonin levels through an inhibition of GABAergic transmission, whereas during chronic morphine treatment serotonin returned to control levels (Tao and Auerbach, 1994), suggesting neuroadaptation or tolerance of the GABAergic system in DRN. However, in the rat spinal cord, a significant decrease in GABA levels during chronic morphine treatment has been reported (Dunbar et al., 2006). Overall, these data, together with our results, suggest a neuroadaptation of the GABAergic system to chronic morphine exposure that varies from sensitization (Ingram et al., 1998), to tolerance (Hack et al., 2003; Tao and Auerbach, 1994) to inhibition (Dunbar et al., 2006) that is both brain region- and species-dependent.

The CTOP-dependent modulation of GABAergic transmission in CeA from naïve/sham rats indicates a tonic regulation of GABAergic transmission mediated by MORs, in accord with a previous study showing MOR-dependent tonic inhibition of GABAergic transmission in the mouse CeA (Kang-Park et al., 2009). Moreover, tonic inhibition of CeA neurons by endogenous opioids has also been suggested by studies showing increased numbers of c-Fos-positive cells in the CeA following administration of MOR antagonists. Prominent distribution of c-Fos positive neurons has been found in capsular and lateral divisions of the CeA, with few c-Fos positive neurons in the medial division (Carr et al., 1999; Gestreau et al., 2000; Veinante et al., 2003). In neurons that receive mostly inhibitory inputs, MOR antagonists may actually induce inhibition by lifting the tonic suppression of GABAergic

input. Thus, one explanation of the lack of significant alteration of the number of c-Fos-positive neurons in the CeA medial subdivision by acute MOR antagonists may include increased inhibition of these medial neurons by intrinsic inhibitory inputs from the lateral and capsular subdivisions of CeA (Cassell et al., 1999), accompanied by unaltered excitatory glutamatergic transmission in the medial subdivision (Kang-Park et al., 2009). Of the endogenous opioids, endorphins and enkephalins are expressed in the highest levels in the CeA (Lam et al., 2008) and are considered to mediate the tonic inhibition of CeA neurons.

The selective MOR-dependent tonic inhibition of GABAergic transmission (Kang-Park et al., 2009) and inhibition of both GABAergic and glutamatergic transmission by acute application of MOR agonists (Finnegan et al., 2005; Zhu and Pan, 2004, 2005) suggest the presence of two pools of presynaptic MORs in the CeA. The first might be represented by the MORs localized exclusively on GABAergic terminals and activated tonically by endogenous opioids, whereas the second pool of MORs localized at both excitatory and inhibitory terminals in the CeA appears to be activated by any stimuli that increase the levels of the endogenous opioids and/or exogenous MOR agonists.

In summary, our studies indicate that under basal conditions GABAergic transmission in the rat CeA is tonically inhibited by MORs. Acute application of MOR agonists results in suppression of GABAergic transmission, predominantly via presynaptic inhibition of vesicular GABA release. During chronic morphine treatment, the GABAergic system in the rat CeA shows a neuroadaptation or tolerance to the inhibitory effects of acute activation of MORs but possible presynaptic vesicular or postsynaptic mechanisms of MOR-dependent modulation of the GABAergic system may be activated. These findings have considerable relevance for the pharmaceutical treatment of pain and for opiate dependence, as well as for the role of endogenous opioids in a brain region known to be involved in fear-, stress- and anxiety-like responses.

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Figure 1. Acute application of both DAMGO and morphine decrease GABA_A-IPSPs in most CeA neurons

We applied 1 μ M DAMGO and 1 μ M morphine acutely by rapid superfusion and carried out intracellular recordings using sharp electrodes in current-clamp mode from CeA neurons held close to their RMPs. **A) and B):** normalized input-output curves showing a decrease in the amplitude of eIPSPs elicited by DAMGO and morphine, at all and most of the stimulation intensities, respectively. **C) DAMGO and D) morphine** also decreased the mean amplitudes of eIPSPs evoked by the 1/2 maximal IPSP protocol, consisting of 4 repetitive stimuli at a half-maximal intensity. The upper half of the panels shows representative IPSP traces. **E) and F):** Paired-pulse ratios (mean amplitudes of IPSP2/IPSP1) used to estimate PPF suggest that DAMGO and morphine effects on eIPSPs are predominantly mediated by inhibition of presynaptic GABA release. The upper half of the panels shows representative traces from the PPF protocol using 50 ms inter-stimulus intervals. All results expressed as mean $\pm$ s.e.m., and statistical significance (* $P < 0.05$) calculated by one-sample *t*-test/ Wilcoxon signed rank test.

Figure 2. Acutely applied morphine decreases frequency but not amplitude of miniature GABA_A-IPSCs

A) Representative traces of whole-cell recordings of spontaneous mIPSCs from CeA neurons, with 1 μ M TTX in the bath to block action potential-dependent events. **B)** Morphine (1 μ M) applied acutely shifted the cumulative frequency of mIPSCs to the right (suggesting a decrease), but had no effect on the amplitude of the mIPSCs as shown in a representative cell (**C**). The results represent cumulative probability determined by the Kolmogorov-Smirnov test in a representative CeA neuron. **D)** The averaged mIPSC frequencies recorded from multiple CeA neurons were decreased by acute application of morphine, whereas the averaged amplitudes of mIPSCs were unchanged (**E**). The results are expressed as mean $\pm$ s.e.m., and statistical significance (*) calculated by one-sample *t*-test/ Wilcoxon signed rank test was set at $P < 0.05$.

Figure 3. No difference in basal CeA IPSPs between naïve/sham and chronic morphine-treated rats

We implanted 2 placebo or morphine pellets s.c. and performed intracellular recording on day 6 after the pellet implantation. In CeA slices from chronic morphine-treated rats, we added 1 μ M morphine to the bath to avoid morphine withdrawal. **A)** Neither input-output curves nor **B)** the mean $\frac{1}{2}$ max IPSP protocol showed significant alteration of the amplitude of eIPSPs during chronic morphine treatment. **C)** Basal eIPSP PPF in CeA neurons from chronic morphine rats also was not different from that of naïve/sham rats. Results expressed as mean $\pm$ s.e.m and statistical significance $P < 0.05$ determined by Student's *t*-test.

Figure 4. Antagonism of MORs increases IPSP amplitudes in CeA neurons from both naïve/sham and chronic morphine-treated rats
 MOR antagonist CTOP (1 μ M) superfused acutely during intracellular recordings with sharp electrodes. **A)** There was significant alteration of input-output curves by CTOP represented by an increase in the mean eIPSP amplitudes at the 2 and 1 intensity (normalized) stimuli in naïve/sham and chronic morphine rats, respectively. **B)** CTOP (1 μ M) significantly increased the mean amplitude of eIPSPs determined by the $\frac{1}{2}$ maximal IPSP protocol in CeA of both animal groups, with little recovery after washout of CTOP. **C)** CTOP increased eIPSP PPF in approximately half the CeA neurons (right bars) and decreased PPF in the other half. All the results expressed as mean $\pm$ s.e.m. and statistical significance * $P < 0.05$ calculated by one-sample t -test/Wilcoxon signed rank test.

Figure 5. MOR-dependent decrease in IPSP amplitudes in CeA neurons persists in chronic morphine-treated rats compared to naïve/sham rats

MOR agonist DAMGO (1 μ M) superfused acutely during intracellular recordings with sharp electrodes. In the morphine treated rats, morphine sulphate (1 μ M) was present in the bath during recording to prevent morphine withdrawal **A)** DAMGO (1 μ M) significantly decreased (**) the mean amplitude of eIPSPs determined by the $\frac{1}{2}$ maximal IPSP protocol in CeA of both animal groups. **B)** DAMGO increased PPF in most of the CeA neurons from both animal groups. All the results are expressed as mean $\pm$ s.e.m. and statistical significance *, # $P < 0.05$ and ** $P < 0.01$ were calculated by one-sample *t*-test/Wilcoxon signed rank test.

Figure 6. Antagonism of MORs increases the frequency of miniature IPSCs: whole-cell recordings with 1 μ M TTX in the bath and 1 μ M CTOP superfused acutely
A) and F): representative recordings of the mIPSPs. In a representative CeA neuron of naïve/sham rats, CTOP significantly shifted frequency to the left (frequency increase) **(B)**, with no significant change in cumulative amplitude **(C)**. The graphs represent cumulative probability determined by the Kolmogorov-Smirnov test. The averaged data from multiple cells also showed increased frequencies of mIPSCs **(D)** elicited by acute application of CTOP and unchanged mIPSC amplitudes **(E)**. Results are expressed as mean $\pm$ s.e.m. and statistical significance (*) $P < 0.05$ was calculated by one-sample t -test/Wilcoxon signed rank test. **G)** During chronic morphine treatment, CTOP shifted the cumulative probability of frequency to the left with little change in the amplitude **(H)**, as recorded in a representative CeA neuron. Acute application of CTOP increased the averaged mIPSCs

frequency (**I**) determined in multiple CeA neurons, but had no significant effects on the averaged amplitudes of mIPSCs (**J**).

Figure 7. Activation of MORs decreases only the frequency of CeA mIPSCs in naive/sham rats but decreases both the frequency and amplitude of mIPSCs in morphine-treated rats: whole-cell recordings with 1 μ M TTX in the bath and 1 μ M DAMGO superfused acutely
A) and **F)**: representative recordings of the mIPSPs. **B)** and **C)** represent cumulative probability determined by the Kolmogorov-Smirnov test in a representative CeA neuron from a naïve/sham rat. DAMGO significantly shifted inter-event intervals to the right (frequency decrease), with no significant change in cumulative amplitude. **D)** The averaged data from multiple cells also showed decreased frequencies of mIPSCs elicited by acute application of DAMGO, but unchanged mIPSC amplitudes (**E**). **G)** During chronic morphine treatment, DAMGO shifted the cumulative probability of mIPSC intervals to the right (frequency increase) and of amplitude to the left (amplitude decrease) (**H**), as recorded in a representative CeA neuron. Acute application of DAMGO decreased both the mean mIPSC frequency (**I**) as well mean amplitude of mIPSCs (**J**), as determined in multiple CeA neurons. Results are expressed as mean $\pm$ s.e.m. and statistical significance (*) $P < 0.05$ and

(**) $P < 0.01$ were calculated by one-sample t -test/Wilcoxon signed rank test. $n = 5$ CeA neurons from naive/sham rats; $n = 7$ from chronic morphine rats.

Table 1

The effects of acute application of MOR agonists on passive membrane properties of the CeA neurons.

	Morphine (n = 10)		DAMGO (n = 12)	
Drug	Control	Morphine	Control	DAMGO
Resting membrane potential (mV)	-72.1 ± 1.6	-71.9 ± 1.9	-73.3 ± 1.1	-73.4 ± 1.2
Input resistance (MΩ)	98.3 ± 6.5	98.9 ± 5.6	109.0 ± 7.0	104.4 ± 7.2

Table 2

Basal membrane properties of the CeA neurons from naïve/sham and chronically morphine treated rats.

	Naïve/Sham (n = 21)	Chronic Morphine (n = 27)
Resting membrane potential (mV)	-72.9 ± 1.5	-70.9 ± 1.4
Input resistance (M Ω)	98.9 ± 7.9	97.7 ± 4.9

Table 3

Basal whole-cell recording parameters of the CeA neurons from naïve/sham and chronically morphine treated rats.

mIPSC	Naïve/Sham (n = 10)	Chronic Morphine (n = 14)
Amplitude (pA)	56.1 ± 6.7	52.2 ± 6.2
Frequency (Hz)	0.9 ± 0.2	0.9 ± 0.2
Rise Time (ms)	2.1 ± 0.2	2.2 ± 0.2
Decay Time (ms)	15.3 ± 0.9	15.6 ± 1.1