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Mu opioid receptor regulation of glutamate efflux in the central amygdala in response to predator odor

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ABSTRACT

The amygdala plays an important role in the responses to predator threat. Glutamatergic processes in amygdala regulate the behavioral responses to predator stress, and we have found that exposure to ferret odor activates glutamatergic neurons of the basolateral amygdala [BLA] which are known to project to the central amygdala [CeA]. Therefore, we tested if predator stress would increase glutamate release in the rat CeA using in vivo microdialysis, while monitoring behavioral responses during a 1 h exposure to ferret odor. Since injections of mu opioid receptor [MOR] agonists and antagonists into the CeA modulate behavioral responses to predator odor, we locally infused the MOR agonist DAMGO or the MOR antagonist CTAP into the CeA during predator stress to examine effects on glutamate efflux and behavior. We found that ferret odor exposure increased glutamate, but not GABA, efflux in the CeA, and this effect was attenuated by tetrodotoxin. Interestingly, increases in glutamate efflux elicited by ferret odor exposure were blocked by infusion of CTAP, but CTAP did not alter the behavioral responses during predator stress. DAMGO alone enhanced glutamate efflux, but did not modulate glutamate efflux during predator stress. These studies demonstrate that ferret odor exposure, like other stressors, enhances glutamate efflux in the CeA. Further, they suggest that activation of MOR in the CeA may help shape the defensive response to predator odor and other threats.

1. Introduction

Exposure to predators or predator-related cues produces hypervigilance and defensive behaviors that depend on the environmental context, and exposure to predator cues are used in animal models of psychiatric disorders such as post-traumatic stress disorder (PTSD) or phobias (Dielenberg et al., 2001; McGregor et al., 2002; Cohen and Zohar, 2004; Campeau et al., 2008; Rosen et al., 2008; Whitaker and Gilpin, 2015). Predator stress can induce defensive behaviors, behavioral inhibition, freezing, avoidance, or risk assessment, plus activation of the hypothalamic-pituitary-adrenal (HPA) axis and autonomic responses depending on the context (Dielenberg et al., 2001; McGregor et al., 2002; Hebb et al., 2004; Takahashi et al., 2005; Masini et al., 2006; Campeau et al., 2008; Rosen et al., 2008, 2015; Butler et al., 2011; Whitaker and Gilpin, 2015), as well as long-lasting increases in anxiety-related responses and sensitization of HPA activation (Adamec et al., 1999; Cohen and Zohar, 2004; Blundell et al., 2005; Masini et al., 2006; Whitaker and Gilpin, 2015).

Studies have implicated the amygdala in responses to predator threats, and predator-associated cues induce neuronal activation in various nuclei of the extended amygdala (Hebb et al., 2004; Fendt et al., 2005; Masini et al., 2005; Staples et al., 2005; Takahashi et al., 2005; Roseboom et al., 2007; Campeau et al., 2008; Butler et al., 2011, Butler et al., 2016). A variety of stressors increase glutamate release in the amygdala, and glutamatergic processes contribute to the behavioral and neuroplastic adaptations associated with acute or chronic stress (Wilson et al., 2015), however it is unknown if predator stress similarly increases glutamate release in the amygdala. We have, however, shown that exposure to predator (ferret) odor induced neuronal activation of glutamatergic neurons in the basolateral amygdala [BLA], which are known to project to the central amygdala [CeA] (Butler et al., 2011). The amygdalar administration of glutamate antagonists prior to predator stress can also block some (but not all) of the behavioral responses (Adamec et al., 1999; Blundell et al., 2005).

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Mu opioid receptor (MOR) activation or inhibition has been shown to modulate responses of CeA neurons, including postsynaptic responses as well as projections into the CeA from the BLA and intercalated nuclei and the projections from the CeA to output regions such as the periaqueductal gray (PAG), parabrachial nucleus, and bed nucleus of the stria terminalis (BNST) [see (Wilson and McDonald, 2019)]. The endogenous opioid system helps mediate many responses associated with stress or anxiety (Ribeiro et al., 2005; Wilson and Junor, 2008; Lutz and Kieffer, 2013; Poulin et al., 2013a; Berube et al., 2014; Henry et al., 2017; Wilson and McDonald, 2019) and alterations in the endogenous system are associated with stress-related psychiatric conditions (Ribeiro et al., 2005; Liberzon et al., 2007; Lutz and Kieffer, 2013; Wilson and McDonald, 2019). Opioids and opioid receptors are widely expressed in amygdala (Wilson et al., 2002; Poulin et al., 2006, 2008; Lutz and Kieffer, 2013; Wilson and McDonald, 2019), and injection of MOR agonists or antagonists into the CeA alters anxiety-related responses (Rogers and File, 1979; Narita et al., 2006; Wilson and Junor, 2008). Electrophysiological studies in the CeA suggest that MOR agonists activate distinct sets of neurons, can induce postsynaptic inhibition as well as alter presynaptic release of GABA and glutamate, and can reduce the activity of CeA projection neurons type (Zhu and Pan, 2004, 2005; Finnegan et al., 2005; Chieng et al., 2006; Chieng and Christie, 2009; Bajo et al., 2011; Blaesse et al., 2015). In the CeA, MOR co-localizes with both NMDA NR1 receptors and the GluR2 AMPA subunit, providing an anatomical substrate for MOR-glutamate interactions in this area (Glass et al., 2009; Beckerman and Glass, 2011, 2012; Scavone et al., 2011).

Since ferret odor exposure activates glutamatergic BLA neurons which project to the CeA, and MOR agonists and antagonists modulate behavioral responses when injected into the CeA (Rogers and File, 1979; Narita et al., 2006; Wilson and Junor, 2008; Butler et al., 2011; Wilson and McDonald, 2019), we hypothesized that like other stressors ferret odor exposure would increase glutamate release in the CeA, and this would be mediated via endogenous opioids acting through MOR. We used in vivo microdialysis coupled with infusion of a MOR agonist or antagonist to assess glutamate and GABA efflux in the CeA while monitoring behavioral responses during exposure to ferret odor.

2. Materials and methods

2.1. Subjects and surgical procedures

Adult male Long Evans rats (Harlan, IN; ~2 months of age) were provided *ad libitum* access to food and water, and all procedures were performed in accordance with approval by the University of South Carolina Institutional Animal Care and Use Committee (IACUC). Animals were maintained in an AAALAC accredited temperature-controlled facility, with a 12:12 light: dark cycle (lights on at 08.00 h).

To ensure ferret odor exposure was inducing a stress response, we analyzed corticosterone (CORT) responses in a separate group of male rats outfitted with jugular catheters (# R-JVC-R37 with suture anchors, Braintree Scientific, Inc., Braintree, MA) under isoflurane anesthesia. The catheters were anchored at the back and locked with 0.1 ml of a taurolidine-citrate solution (TCS) solution and a 23G port plug (#N23PP, Braintree Scientific, Inc.) Animals were habituated to brief handling while catheters were flushed daily during the recovery period using a 23G blunt end needle (Braintree Scientific, Inc. #N23). For flushing the TCS lock solution was removed and catheter was flushed with 0.15 ml of 20 Units/ml of heparin in sterile saline prior to relocking with 0.1 ml TCS solution and the port plug. Animals were allowed to recover ~4–6 days prior to transport to a separate room housing an isolation chamber for predator stress in a cylindrical chamber (20.5 cm diameter x 40.5 cm height) without bedding, similar to the stress chamber used for microdialysis. Control animals were treated similarly with exposure to a towel without any odor to assess the impact of the novel environment on the CORT response.

For microdialysis, one week before testing male rats were surgically implanted with bilateral cannulas [MD-2251, Bioanalytical Systems, Inc. (BAS), West Lafayette, IN, USA] aimed at 2 mm above the CeA using stereotaxic procedures. Rats were anesthetized with isoflurane gas (5% for induction, 1–3% for maintenance) mixed with oxygen, and given injections of local anesthetic (2% carbocaine, s.c.) at pressure points for the ears bars and the incision site. The coordinates for CeA were A/P −2.0, M/L 4.0, D/V −7.0 (from skull flat) as determined from Bregma (Paxinos and Watson, 1997) and guide cannulas were fixed to the skull with skull screws and dental cement. Stainless steel stylets were used to maintain the patency of the cannula. Nalbuphine (1 mg/kg, s.c.) was given postoperatively for pain management, and the diet was supplemented with bacon softies (Bio-serve, Frenchtown, NJ) in order to maintain postoperative weight. Animals were allowed 3–4 days recovery following surgery and dummy cannulas were removed to habituate rats to this procedure. Since animals showed a substantial CORT response to the context shift alone, to minimize the impact of this handling during microdialysis sessions, after the surgical recovery period rats were individually habituated for two days (3 h/day) in two different contexts to diminish stress responses due to transport, novelty, and the context shift. We also analyzed a separate group of rats undergoing the same protocol without ferret odor. The neutral context was the bowl-shaped chamber (42 cm diameter; 35 cm height) with bedding during the 3 h habituation and the experimental days. The stress context was a cylindrical chamber with different dimensions (31 cm × 33 cm) without bedding. This habituation included transport to a separate room that housed the microdialysis setup and tethering the animals to the microdialysis lines (without fluid) to familiarize animals with the testing procedure. Two rats were habituated and tested at a time, although microdialysis chambers were separated by partitions to isolate each animal.

2.2. In vivo microdialysis

Microdialysis was conducted as described previously (Reznikov et al., 2009). On the morning of microdialysis, stylets were removed and replaced with concentric microdialysis probes [BAS-MD2200] with a semipermeable membrane (cutoff 30 kDa) extending 2.0 mm beyond the guide cannula. Probes were continuously perfused (2.0 μL/min) with an artificial cerebrospinal fluid (aCSF; pH 6.5) composed of (in mM): 150 NaCl, 3.0 KCl, 1.7 $CaCl_2$, 0.9 $MgCl_2$, and 4.9 d-glucose. Dialysates were collected bilaterally in 15 min intervals beginning 3 h following probe insertion, and the first four collections were used to determine baseline efflux. Drugs were perfused through the microdialysis probes, and all drugs were obtained from Sigma -Aldrich (St. Louis, MO) and were diluted in aCSF from concentrated stocks in sterile saline on the day of testing prior to perfusion through the dialysis probes. Each rat was only analyzed during a single microdialysis testing condition.

Extracellular concentrations of glutamate and GABA in dialysates from the CeA were determined by high-performance liquid chromatography (HPLC) with electrochemical detection, after a pre-column o-phthaldialdehyde/sulfite derivatization procedure previously described [see (Reznikov et al., 2009)]. Quantification was accomplished by comparison to a standard curve (0.01, 0.1, 1.0 μM). Glutamate and GABA were separated on a 3-μm C_{18} reversed phase column and eluted with a 0.1 M sodium phosphate buffer (pH 6.4) containing 27% methanol, and were detected with a glassy carbon electrode maintained at +700 mv by an LC 4B amperometric detector (BAS) at a 100 μl/min flow rate. To ensure that assessments of GABA and glutamate were done without knowledge of the treatment condition, animals were coded by an animal ID number.

2.3. Ferret odor exposure, drug infusion, and behavioral analysis

Experiment 1: Corticosterone stress response to predator odor exposure: In order to determine the hypothalamic-pituitary-adrenal (HPA) stress response to our predator odor conditions a separate set of rats were outfitted with indwelling jugular catheters and exposed to control (no odor) condition or predator (ferret) odor for 15 min. Pre-stress baselines were taken for one or two days prior to stress, and blood samples (~250 μl) were also taken at 15, 30 and 60 min after 15 min exposure to either a control towel (no odor) or a ferret-scented towel (5 cm^2) exposure for analysis of the plasma corticosterone (CORT) [see (Butler et al., 2011, 2016)]. Fifteen minutes was selected for ferret odor exposure to coincide with the first sampling bin during microdialysis (see below). For stress exposure, rats were transferred to a novel cylindrical Plexiglas chamber (22 × 41 cm) within a HEPA-filtered isolation chamber (bioBubble, Fort Collins, CO) for 15 min, and after immediate blood sampling returned to their home cages until the 20 and 60 min time points. Cylinders contained either a 5 cm^2 piece of ferret-scented or unscented (control) towel attached ~10 cm above the chamber floor. The ferret towels were purchased from Marshall Bioresources (New Rose, NY) after several weeks in a cage with multiple ferrets and stored at −80 °C. Animals were videotaped during the 15 min stress exposure subsequent for behavioral analysis (data not shown). Rats exposed to the control no-odor condition (N = 9) were subsequently exposed to the same chamber with ferret odor, while an additional 20 rats were only exposed to ferret odor. For blood sampling, after removing TCS solution, a clean blood sample (~250 μl) was collected into a clean syringe and placed in an Eppendorf tube containing 10 μl of 30 mg/ml EDTA and 1 μl aprotinin (11,117KIU/ml) on ice prior to centrifugation at 1500 × *g* for 15 min (4 °C) and collection of serum. Blood samples were frozen (−20 °C) until analysis for CORT using an enzyme-linked immunosorbent assay [ELISA] kit with standards ranging from 20 to 20,000 pg/ml (Enzo Corticosterone EIA ADI 901-097 Enzo, Farmingdale NY) as described previously (Macht et al., 2018). Each sample was diluted 1:40 for analysis with steroid displacement reagent, processed using manufacturer's instructions, and read on a Synergy plate reader at 405 nm. CORT levels were interpolated from standards using a log-logit transformation of the data and each assay was run with an internal inter-assay standard sample. Rats without all three time points were excluded from analysis to allow use of repeated measures analysis of variance (ANOVA) and calculating area under the curve for the CORT response.

Experiment 2: Glutamate and GABA efflux during predator odor exposure: An initial experiment examined hemispheric differences in glutamate or GABA efflux in response to ferret odor exposure without drug infusion. During the microdialysis session, during washout (3 h) and baseline (1 h) collection rats were in a bowl-shaped neutral chamber (42 cm diameter; 35 cm height) with bedding. For predator odor exposure rats were transferred to a cylindrical chamber with different dimensions (31 cm × 33 cm) without bedding containing a 5 cm^2 piece of ferret-scented towel attached 10 cm above the chamber floor for 1 h (four 15 min dialysate collections). After ferret odor exposure, rats were transferred back to the neutral (bowl) context for a 1 h post-stress recovery period. Since right and left amygdala showed similar increases in glutamate efflux in response to ferret odor exposure, subsequent experiments averaged responses from the two hemispheres for each subject, unless placement was outside the CeA, in which case only one hemisphere was included.

Experiment 3. Drug infusion during ferret odor exposure: For drug infusion during predator stress, after a washout period (3 h) and four baseline collections for 1 h, animals received infusion of drugs beginning 30 min (2 collections) prior to the 1 h ferret odor exposure [drug infusion during collections #5–10 for 1.5 h]. After a 30 min pre-exposure drugs via reverse dialysis, rats were exposed to ferret odor in the stress context while dialysates were collected from the CeA for 1 h (collections #7–10, drug infusion + ferret odor). Four final post-drug washout collections were made after the rat was returned to the neutral chamber, resulting in a total of 14 collection time points.

To examine if the ferret odor exposure-induced changes in glutamate and GABA efflux in the CeA were dependent on presynaptic release, rats were infused with 2 μM tetrodotoxin (TTX) prior and during ferret odor exposure. To test if MOR function in the CeA plays a role in ferret odor–induced alterations in glutamate and GABA efflux the selective MOR agonist [D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin (300 μM DAMGO; E7384 Sigma-Aldrich) or the selective MOR antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (10 μM CTAP; Sigma-Aldrich C6352) were infused through the dialysis probe. The doses of DAMGO and CTAP were based on prior microinjection or microdialysis studies with these agents (Tang et al., 2005; Schepers et al., 2008; Wilson and Junor, 2008) showing effects on behavioral responses or neurotransmitter efflux. With each drug exposure, vehicle (aCSF) infused rats were handled separately. One set of vehicle infused rats also underwent the same protocol without ferret odor in the novel context to determine the effects of the context shift alone.

During ferret odor exposure, freezing, grooming, and rearing were digitally recorded, and hand-scored by an observer blinded to the treatment group using Observer® XT 10.0 (Noldus Information Technology, Wageningen, The Netherlands). Freezing behavior was also measured using FreezeScan (Clever Systems, Inc., Vienna VA). All behavior was expressed as duration (seconds) or number of events during each 15 min bin to correlate behavioral and microdialysis data.

Two hours after the start of ferret odor exposure, rats were anesthetized with 5% isoflurane and perfused with clearing solution followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.6). Brains were removed, post-fixed 24 h in 4% paraformaldehyde, and then placed in a sucrose solution (30% sucrose in 0.1 M sodium phosphate buffer, pH 7.6) for 48 h. Amygdala sections (45 μm) were stored in 0.1M phosphate buffer and probe placement in CeA was determined from acetylcholinesterase stained sections (Wilson and Junor, 2008). Rats without cannula placement in CeA were excluded from analysis. See SUPPLEMENTAL materials for further description and demonstration of probe placement in the CeA.

Experiment 4. Effects of DAMGO on glutamate and GABA efflux without predator stress: Since in experiment 3, the MOR agonist DAMGO enhanced glutamate efflux prior to predator odor exposure, the effects of DAMGO were examined without ferret odor exposure or any context shift. First, we examined if activation of MOR receptors in the CeA induced changes in glutamate or GABA efflux by infusing the MOR agonist DAMGO through the microdialysis probe in one hemisphere and vehicle (aCSF) contralaterally into the other CeA. After the fourth baseline collection (1 h), animals received either vehicle (aCSF) or 300 μM DAMGO for six additional 15-min collections (1.5 h). Four final post-drug washout collections were made, resulting in a total of 14 collection time points (15 min each). To make sure we were not infusing a supra-physiological dose, separate rats were infused with three increasing doses of DAMGO (30, 100, 300 μM) for 2 collections of 15 min at each dose, resulting in a total of 12 collection time points (N = 5). Analysis of glutamate and GABA efflux in microdialysis probes that were out of place were also used as controls for the specificity of DAMGO's effects in the CeA (N = 3). Finally, we used different groups of animals to examine if the DAMGO-induced increases in glutamate efflux were blocked by co-infusing DAMGO with either the MOR antagonist CTAP or TTX, as well as the effects of CTAP alone. For all these experiments we implanted bilateral cannula in the CeA, and animals received DAMGO (300 μM) on one side and DAMGO plus CTAP or TTX contralaterally. Since the effects of DAMGO were seen in the first 15 min sample, TTX (alone) was infused for 30 min prior to co-infusion of DAMGO plus TTX. After the microdialysis experiment, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused via intracardiac delivery of saline (0.9%) followed by 10% formalin in 0.05 M phosphate-buffered saline. Brains were removed, sectioned and stained with acetylcholinesterase for determining cannula placement.

2.4. Statistical analysis

To ensure analyses were done without knowledge of the treatment condition, animals were coded by an ID number. For microdialysis experiments, baseline GABA or glutamate levels were averaged and data expressed a percent of baseline. Analysis of variance (ANOVA) was used to examine the effects of drug treatment and ferret odor exposure over time as a repeated measure, with post hoc Bonferroni or Fishers LSD tests. Groups were also compared by ANOVA comparing the average percent of baseline values during predator stress exposure (Experiment 3) or DAMGO infusion (Experiment 4). The number of events and duration of grooming and rearing, plus the latency to freeze and duration of freezing, were analyzed in 15 min bins during the 1 h of ferret odor exposure during microdialysis. Since most grooming and rearing behavior occurred during the initial 15 min of ferret odor exposure, behavioral results for the first 15 min bin were compared between drug treatment groups using ANOVA with post-hoc Bonferroni comparisons. Pearson's coefficients were used to determine relationships between behavioral endpoints changes in glutamate efflux in CeA for the first 15 min during ferret odor exposure. For analysis of the CORT time course, percent baseline was compared for rats exposed to control towel (no odor) or ferret-scented towel for 15 min with repeated measures ANOVA, and the area under the curve was calculated using GraphPad Prism 6.0 and compared with a Student's t-test. All data were analyzed using GraphPad Prism 6.0 (La Jolla, CA, USA) and significance was set as alpha = 0.05. Data are expressed as means ± standard error of the mean (SEM).

3. Results

3.1. Ferret odor exposure induces a prolonged corticosterone response

As seen in Fig. 1, a brief exposure to ferret odor (15 min) induced a robust and sustained elevation in plasma CORT over baseline, that was significantly greater than that seen with control (no odor) groups [$F(1,38) = 7.36$, $P < 0.01$]. There was a significant effect of time [$F(3,114) = 38.7$, $P < 0.0001$] and a significant interaction [$F(3,114) = 6.8$, $P = 0.0003$] for the CORT response, which produced a significant [$t(35) = 1.87$, $P < 0.03$] difference in the AUC between control and ferret odor groups (Fig. 1, lower panel). Both the 15 min and 60 min time points differed between the groups, but the changes at 60 min suggested that ferret odor exposure produced a prolonged elevation in CORT even after they had been returned to their home cage post-stress. Control (no odor) CORT levels were 139 ± 16 ng/ml versus 270 ± 19 ng/ml in the ferret odor exposed group at 60 min. The increase in CORT at 30 min in the control group is likely related to the novelty stress, since the animals had not been previously habituated to the testing chambers combined with transport to the isolation units. This HPA activation was similar to the increases in HPA activity seen with predator odor stresses in other studies, that seem to involve the main and accessory olfactory systems and pathways through the medial amygdala (Masini et al., 2005, 2006, 2009, 2010; Roseboom et al., 2007; Campeau et al., 2008; Whitaker and Gilpin, 2015).

3.2. Ferret odor increased glutamate efflux in the central amygdala

As seen in Fig. 2, ferret odor stress increased glutamate efflux in both hemispheres, with a main effect of time [$(F(11,110) = 2.86$, $P < 0.003)$], but no effect of hemisphere [$(F(1,10) = 0.02$, $P = 0.9)$], and no interaction [$(F(11,110) = 0.4$, $P = 0.96)$]. Glutamate efflux peaked at 15 min and lasted for the entire hour of ferret odor exposure (Fig. 2A). No changes in GABA efflux were observed during ferret odor presentation [Fig. 2B; $(F(11,110) = 1.24$, $P = 0.27)$ for time; $(F(1,10) = 0.37$, $P = 0.56$ for hemisphere]. Based on this finding, for the rest of the microdialysis experiments, right and left hemisphere changes were averaged, unless placement was outside the CeA in one hemisphere.

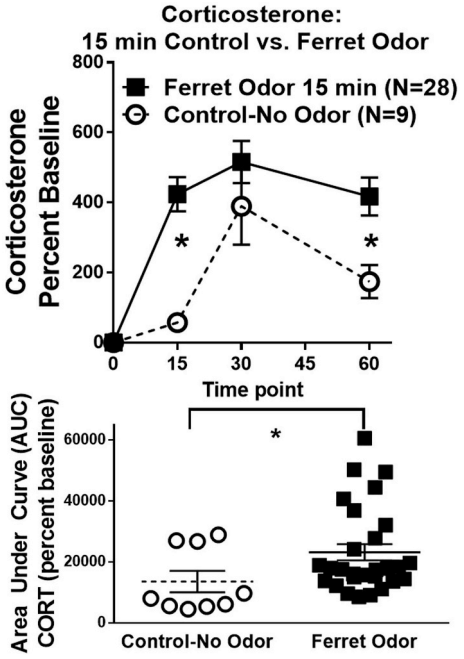

Fig. 1. A 15 min exposure to ferret odor increased plasma corticosterone over baseline. Top panel shows ferret odor exposure induced larger and more prolonged increases in CORT compared with control (no odor) groups undergoing similar context shifts into a novel environment [$F(1,38) = 7.36$, $P < 0.01$]. There was a significant effect of time [$F(3,114) = 38.7$, $P < 0.0001$] and a significant interaction [$F(3,114) = 6.8$, $P = 0.0003$] for the CORT response, which produced a significant [$t(35) = 1.87$, $P < 0.03$] difference in the AUC between control and ferret odor groups (scatter plot, bottom panel). Points (top) and lines (scatter plot) represent mean ± S.E.M with n = 9 control and N = 28 ferret exposed rats.

3.3. MOR regulation of ferret odor induced glutamate efflux in the central amygdala

In order to determine the role of MOR in glutamate and GABA efflux during predator stress, animals were infused with vehicle (aCSF), the MOR antagonist CTAP, or the MOR agonist DAMGO through the dialysis probe during ferret odor exposure. Infusion with TTX was used to determine if increases in glutamate were from sodium-dependent presynaptic release in the CeA. Drug infusion began 30 min before transferring the animal into a new context with ferret odor, but in a control group of rats the context shift (Bowl Change) was done without ferret odor during a vehicle (aCSF) infusion. As seen in Fig. 3, exposure to ferret odor during vehicle infusion resulted in an increase in glutamate efflux in the CeA that was not seen with the Bowl Change alone, and was attenuated by both CTAP and TTX. Infusion of DAMGO alone increased glutamate efflux (times #5–6), but did not affect glutamate efflux during ferret odor exposure. Two-way ANOVA with repeated measures showed a significant effect of time [$F(13,468) = 3.85$, $P < 0.0001)$] and drug infusion [$F(4,36) = 5.42$, $P = 0.0016)$], as well as a drug by time interaction [$F(52,468) = 1.56$, $P = 0.01$]. Both vehicle and DAMGO groups differed from the CTAP, TTX and no ferret odor (bowl change) groups at time points 7–10 (Fig. 3), but not differ from one another. Average changes over baseline during the 1 h ferret odor exposure (Fig. 3, bottom left), also demonstrate a significant main effect of drug infusion [$F(4,36) = 4.36$, $P < 0.0006$], with glutamate levels significantly increased in the vehicle and DAMGO groups compared with the CTAP, TTX, and Bowl Change (no ferret odor) groups. This collapsed data supports the notion that CTAP and TTX both attenuated the increases in glutamate in the CeA in response to ferret odor.

Efflux of GABA in the CeA was not affected by ferret odor or drug infusion (Fig. 3, right). There was no effect of treatment group [F

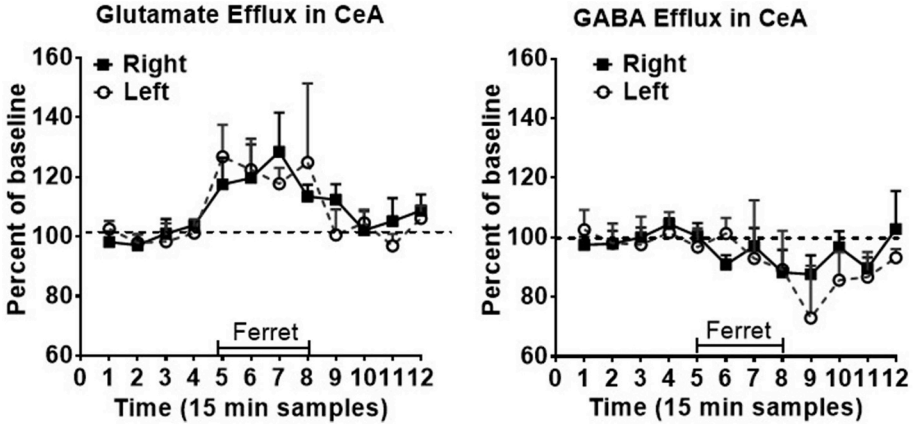

Fig. 2. Ferret odor exposure increased glutamate efflux in both right and left central amygdala (Left panel). Glutamate efflux was increased during the hour of ferret odor exposure, and there was a main effect over time [($F(11,110) = 2.86$, $P < 0.003$)], but no effect of hemisphere [($F(1,10) = 0.02$, $P = 0.9$)], and no interaction [($F(11,110) = 0.4$, $P = 0.96$)]. Right panel shows ferret odor did not affect GABA efflux in the right and left amygdala from the same rats. Time points represent 15 min dialysate samples, and data is expressed as percent of the 1 h baseline (four 15 min samples) collected just prior to placing the animal in a new context with ferret odor for 1 h (four 15 min samples). For recovery animals were returned to a neutral context without ferret odor. Points represent mean ± S.E.M with n = 6 per group.

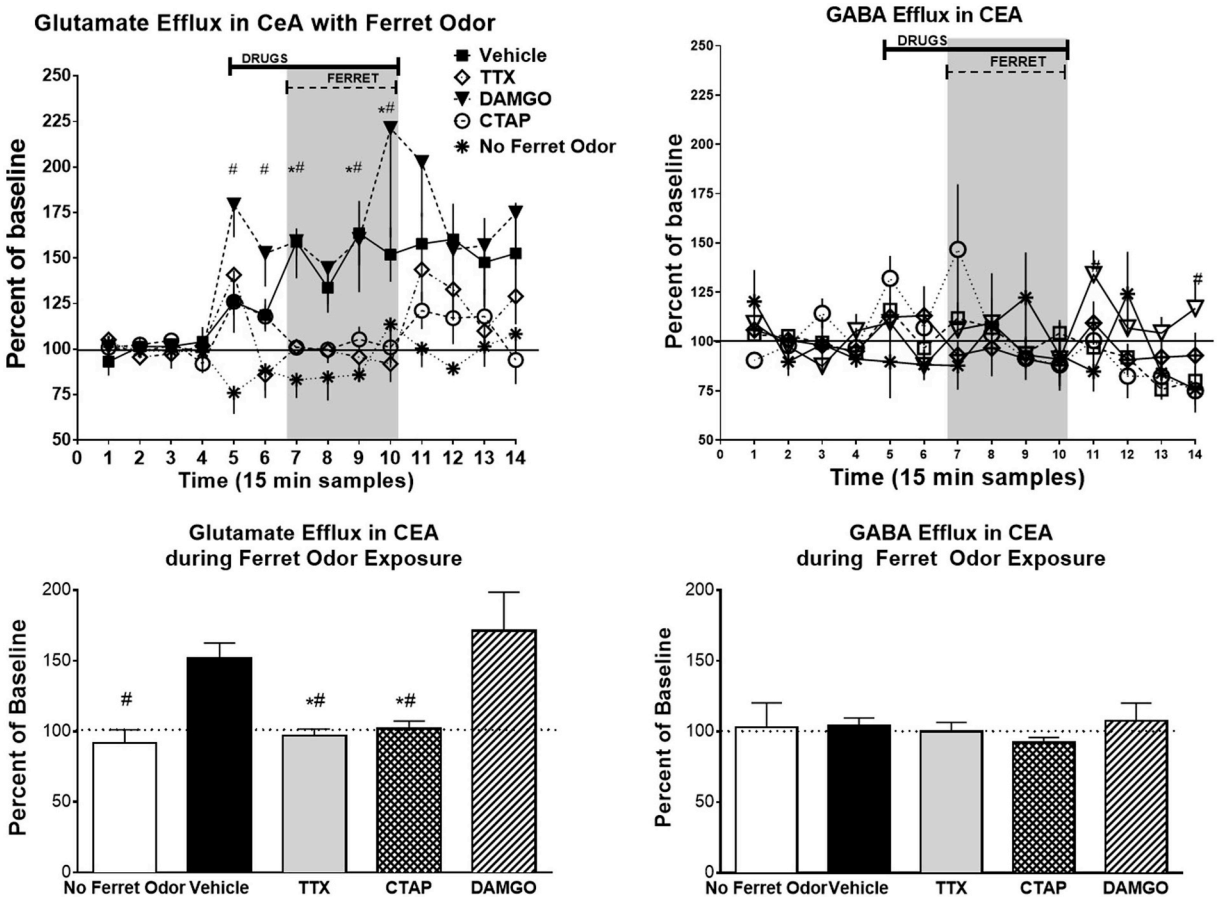

Fig. 3. Infusion of both the mu opioid receptor (MOR) antagonist CTAP and tetrodotoxin (TTX) attenuated the increased glutamate release induced by ferret odor in the CeA. Time points represent 15 min sample collections during microdialysis for glutamate (Left panels) or GABA (Right panels) during four baseline samples, followed by two collections (30 min #5,6) during infusion of Vehicle (aCSF), the MOR agonist DAMGO, the MOR antagonist CTAP, or the sodium channel blocker TTX that continued during the 1 h ferret odor exposure (shaded bar, #7–10). Four recovery samples were collected after switching the animal back to the neutral context. Ferret odor exposure significantly increased glutamate efflux in the CeA in Vehicle-treated rats, and this was blocked in the CTAP and TTX groups. The Bowl Change control group infused with aCSF and was switched to the novel context without predator odor did not show any increase in glutamate efflux. ANOVA with repeated measures showed a significant effect of time [$F(13,468) = 3.85$, $P < 0.0001$)] and drug infusion ($F(4,36) = 5.42$, $P = 0.0016$)], as well as a drug by time interaction [$F(52,468) = 1.56$, $P = 0.01$]. Post-hoc analysis indicated Vehicle (* = $P < 0.05$) and DAMGO (# = $P < 0.05$) groups differed from the CTAP, TTX or no ferret odor groups at time points 7–10, but DAMGO and Vehicle groups did not differ from one another. DAMGO alone increased glutamate efflux in the CEA, as seen during sampling points 5–6 before ferret odor exposure compared to the Vehicle, Bowl Change (no ferret odor), and TTX groups, and this elevation persisted during the recovery period. Ferret odor exposure did not alter GABA efflux in the CeA, [$F(13,468) = 2.66$, $P = 0.001$] and this was not modified by infusion of CTAP, DAMGO, or TTX [$F(4,36) = 0.39$, $P = 0.81$] (Right panels). Bottom panels showing the 1 h mean percent change over baseline during ferret odor exposure demonstrate that CTAP and TTX blocked the glutamate increase in the CeA [Left panel; $F(4,36) = 4.36$, $P < 0.0006$], and GABA efflux was not changed by predator stress or drug infusion [Right panel, $F(4,37) = 0.67$, $P = 0.62$]. (*) indicates Vehicle group differences ($P < 0.05$) and (#) indicates DAMGO group differences ($P < 0.05$) from CTAP, TTX and Bowl Change groups. Results are presented as the mean ± SEM for each time point with N's of 4–10 per group.

(4,36) = 0.39, P = 0.81] on GABA efflux over time, and the average GABA efflux during 1 h ferret odor exposure showed no differences between groups [F(4,37) = 0.67, P = 0.62]. Although there was an effect of time [F(13,468) = 2.66, P = 0.001], this may have been related to the oscillations in GABA levels in some groups (CTAP) which was seen previously in the amygdala during stress (Reznikov et al., 2009).

3.4. MOR regulation of fear behaviors elicited by predator stress

Fig. 4 shows the freezing, rearing and grooming behaviors observed during the first 15 min of ferret odor exposure in the new context. Vehicle-treated rats showed high levels of rearing and grooming during the first 15 min, and enhanced freezing at later time points (data not shown). While CTAP did not affect behaviors, DAMGO and TTX enhanced freezing, and reduced rearing during the first 15 min of ferret odor exposure. Of note, rats infused with TTX showed general lethargy, and the increase in freezing appeared more like a general decrement in overall movement, which was supported by a decrease in both rearing and grooming. Pharmacological manipulations in the CeA significantly changed both the latency to freeze [F(3,30) = 49.8; P < 0.0001] and the duration of freezing [F(3,32) = 5.7; P < 0.003], with the DAMGO and the TTX groups showing shorter latencies to freeze and more freezing (seconds) than both the vehicle and CTAP groups (P < 0.05). DAMGO and TTX reduced the rearing events [F(3,31) = 6.7; P < 0.002] and duration [F(3,31) = 10.0; P < 0.0001] compared to vehicle and CTAP groups. The number of grooming events [F(3,31) = 2.9; P = 0.05], but not duration [F(3,31) = 1.13; P = 0.35], was affected by drug exposure (Fig. 4, bottom panels), although the differences were between the DAMGO and TTX groups; DAMGO infusion enhanced grooming behaviors, particularly during the later time points (not shown), while TTX infusion attenuated active behaviors in general.

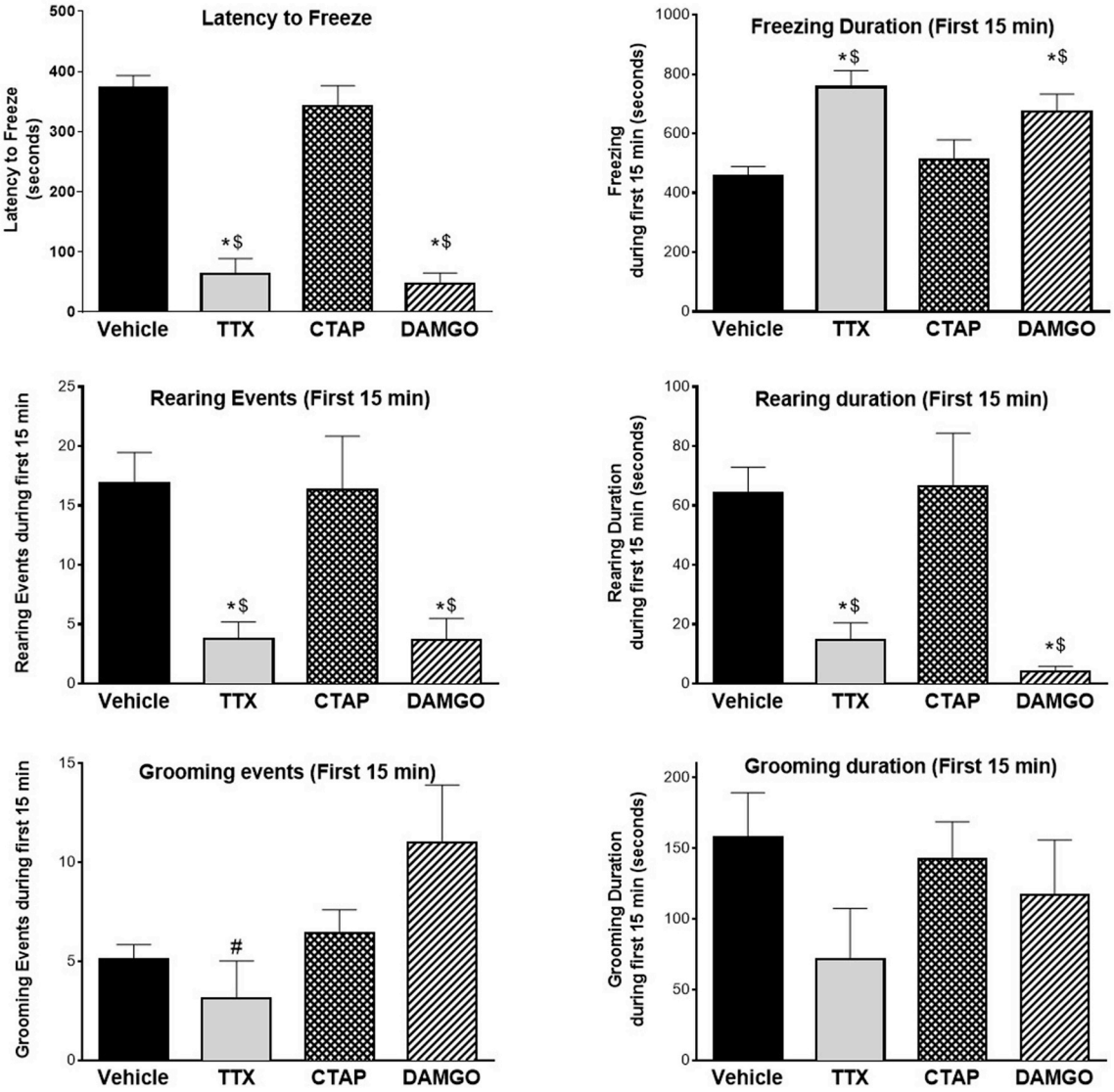

Fig. 4. Effects of infusion of the MOR agonist DAMGO, the MOR antagonist CTAP and TTX into the CeA on defensive behaviors during the first 15 min of ferret odor exposure. Vehicle-treated rats showed with low levels of freezing behavior (top panel), and high levels of rearing and grooming (middle and bottom panels), and this pattern was not altered by infusion of CTAP. In contrast, both DAMGO and TTX decreased the latency to freeze (top left) and increased freezing duration (top right), while decreasing rearing events and duration (middle panels). Treatment group significantly changed both the latency to freeze [F(3,30) = 49.8; P < 0.0001] and the duration of freezing [F(3,32) = 5.7; P < 0.003], with the DAMGO and the TTX groups showing shorter latencies to freeze and more freezing (seconds) during the first 15 min of ferret odor exposure than both the Vehicle (* = P < 0.05) and CTAP ($ = P < 0.05) groups. Infusion of DAMGO and TTX both reduced the rearing events [F(3,31) = 6.7; P < 0.002] and duration [F(3,31) = 10.0; P < 0.0001], with Vehicle (* = P < 0.05) and CTAP ($ = P < 0.05) groups differing from both the DAMGO and TTX groups. The number of grooming events [F(3,31) = 2.9; P = 0.05], but not duration [F(3,31) = 1.13; P = 0.35], were affected by drug infusion, with significant differences between the DAMGO and TTX group (# = P < 0.05). Bars represent mean ± SEM of N = 7–10 rats per group.

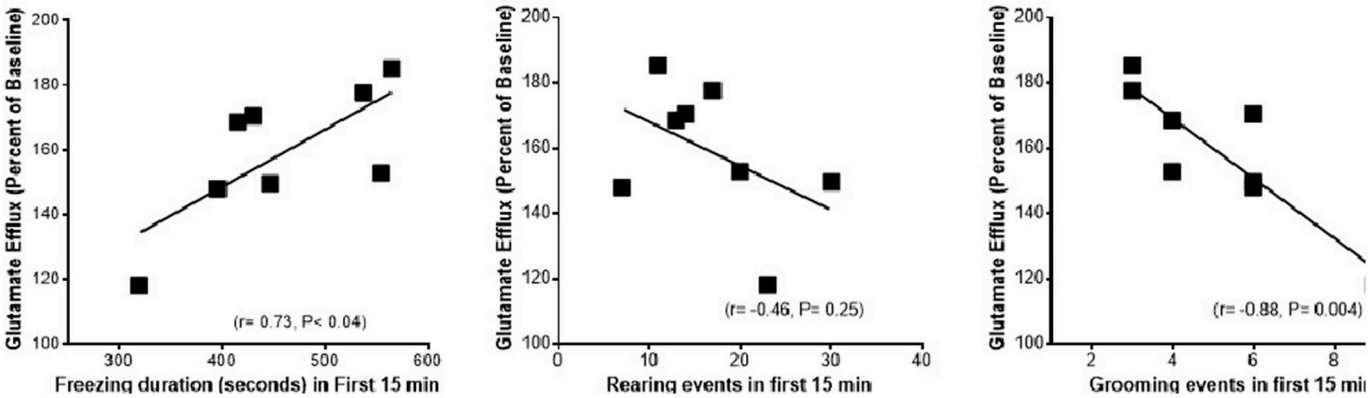

Fig. 5. In Vehicle-infused rats, correlations were seen between ferret odor induced glutamate efflux in the CeA and freezing or grooming behaviors. Scatter plots show glutamate efflux during the first 15 min of ferret odor exposure was positively correlated with freezing duration (Left panel) and negatively correlated with grooming events (Right panel). No correlations were seen with rearing events (Middle panel).

3.5. Correlations between CeA glutamate efflux and defensive behaviors during ferret odor exposure

In the vehicle-treated rats, significant correlations were seen between increases in glutamate efflux during the first 15 min of ferret odor exposure and freezing duration [Fig. 5 Left Panel; $r = 0.73$, $P = 0.04$), grooming events [Fig. 5, Right Panel; $r = -0.88$, $P = 0.004$], and grooming duration [$r = -0.78$, $P = 0.02$; not shown]; these correlations were abolished with infusion of TTX, CTAP or DAMGO in the CeA (not shown). No significant correlations were seen between rearing events [Fig. 5 Middle Panel; $r = -0.46$, $P = 0.25$] or duration [$r = -0.54$, $P = 0.29$].

3.6. DAMGO effects on CeA glutamate efflux in unstressed animals

Since the MOR agonist DAMGO enhanced glutamate efflux prior to predator odor exposure, but did not alter influences of ferret odor on glutamate efflux, additional experiments tested the effects of DAMGO without any stress exposure. As seen in Fig. 6, infusion of DAMGO (300 μM) in one hemisphere significantly increased glutamate efflux compared to aCSF (Vehicle) infusion in the contralateral hemisphere [$F(1,15) = 6.2$, $P < 0.03$ for difference between DAMGO and Vehicle; $F(13,195) = 2.2$, $P < 0.02$ for interaction of treatment over time]. Fig. 6 inset shows the increases in glutamate efflux with increasing cumulative doses (0, 30, 100 and 300 μM) of DAMGO, and the site specificity of the effects which was not seen in probes outside the CeA, although this effect was marginally significant due to the low number of subjects [$F(1,6) = 5.4$, $P = 0.059$; N = 5 DAMGO and N = 3 out of place]. Infusion of DAMGO had no effect on GABA efflux [$F(1,10) = 0.4$, $P = 0.5$ for treatment; $F(13,130) = 1.4$, $P = 0.2$ for interaction]. The mean increases over the six collections (1.5 h) are shown in the lower left panel (Fig. 6), showing the increases in glutamate efflux with DAMGO compared to vehicle infusion, and the attenuation of DAMGO's effects with the co-infusion of the MOR antagonist CTAP, but not TTX [$F(4,49) = 3.9$, $P < 0.008$]. Although CTAP and DAMGO + CTAP groups appeared to have slightly elevated levels of glutamate efflux, this was not statistically different from the vehicle condition. None of these pharmacological infusions in the CeA influenced GABA efflux [$F(4,49) = 0.8$, $P = 0.5$].

4. Discussion

Glutamatergic changes in the amygdala contribute to the behavioral adaptations and neuroplasticity associated with several types of stress (Wilson et al., 2015). Similar to other stressors, we demonstrate enhanced glutamate efflux in the CeA in response to ferret odor exposure. Further, these increases in glutamate efflux in the CeA were correlated with both freezing duration (positively) and grooming behavior (negatively), although these correlations were abolished by infusion of DAMGO, CTAP or TTX into the CeA during ferret odor exposure. These changes appeared to be specific to the CeA, since they were not seen in animals with microdialysis probes outside the CeA (data not shown). Furthermore, rats that underwent the same microdialysis protocol but were not exposed to a ferret odor (change in context alone) failed to show any alteration in glutamate or GABA efflux in the CeA. Although the sustained glutamate extracellular concentrations in response to ferret odor could reflect both neuronal and glial (especially astrocytic) sources, as well as both enhanced release and reduced reuptake, the attenuated response with the voltage-gated sodium channel blocker TTX indicates that axon depolarization-dependent release is at least in part responsible for the enhanced extracellular glutamate efflux from predator stress. Further, the MOR antagonist CTAP blocked glutamate release in the CeA in response to ferret odor suggesting endogenous opioids mediate this response, although CTAP infusion failed to modify ferret odor-induced behaviors. This suggests that while behavioral responses and glutamate efflux in the CeA are correlated, glutamate efflux in this region is unlikely to be involved in inducing the immediate behavioral responses during predator stress.

The ability of the MOR antagonist CTAP to block glutamate release in the CeA in response to ferret odor suggests induction of this response by endogenous opioids. Since enkephalin-containing neurons in the BLA are glutamatergic pyramidal cells (Poulin et al., 2008), this might suggest the co-release of glutamate and ENK during predator stress. This is also supported by the co-localization of MOR with both NMDA NR1 receptors and the GluR2 AMPA subunits in the CeA, providing an anatomical substrate for the opioid-glutamate interactions (Glass et al., 2009; Beckerman and Glass, 2011, 2012; Scavone et al., 2011). Surprisingly however, despite the correlations between glutamate efflux and freezing or grooming behaviors in vehicle-treated rats, the attenuation of glutamate efflux in the CeA with CTAP did not alter the behavioral responses to ferret odor compared with vehicle-treated rats. The lack of behavioral effects of CTAP in the CeA is similar to what we found previously with CTAP microinjection into the CeA in a defensive burying task (Wilson and Junor, 2008). Other studies have suggested that while activation of all amygdala nuclei plays a significant role during predator exposure, only the medial amygdala and the BLA are critical for unconditioned and conditioned behavioral responses to a predator cues (Vazdarjanova et al., 2001; Takahashi et al., 2005; Campeau et al., 2008; Martinez et al., 2011; Rosen et al., 2015). Studies have also failed to show neuronal activation in the CeA with predator exposure, including ferret odor (Staples et al., 2005; Campeau et al., 2008), but see (Sun et al., 2019). In contrast, TTX treatment also attenuated glutamate efflux, but decreased rearing, grooming and freezing behaviors, suggesting inactivity or lethargy. This may reflect

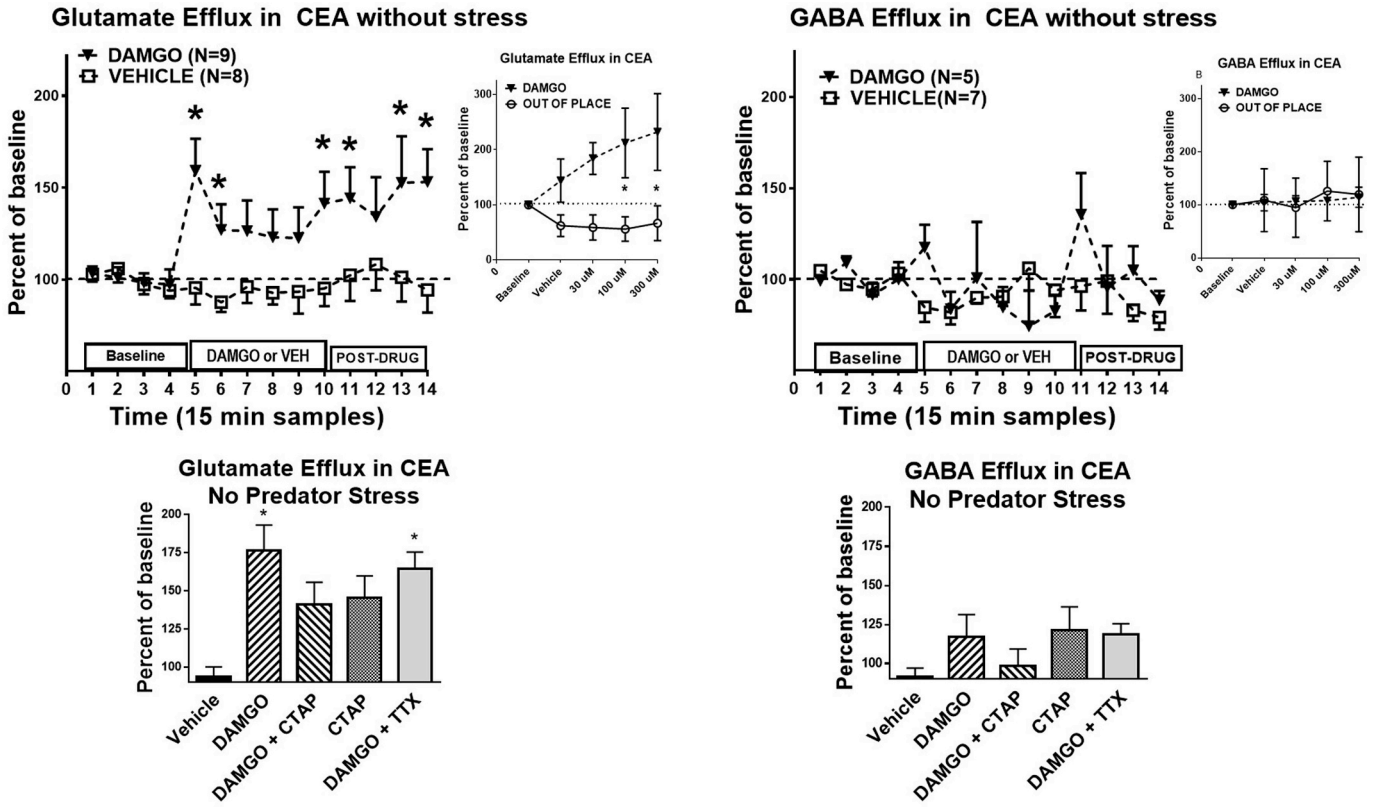

Fig. 6. DAMGO increases CeA glutamate efflux in the absence of any stress exposure. Each animal was infused with DAMGO in one hemisphere and either Vehicle or DAMGO plus CTAP or TTX in the other hemisphere. Top panels show infusion of DAMGO (300 μM) CeA significantly increased glutamate efflux compared with aCSF (Vehicle) infusion [$F(1,15) = 6.2$, $P < 0.03$] and there was a significant [$F(13,195) = 2.2$, $P < 0.02$] interaction of treatment over time. The left inset shows a strong trend for dose-dependent increases with DAMGO ($N = 5$) compared to effects seen with probes with placement outside the CeA ($N = 3$), [$F(1,6) = 5.4$, $P = 0.059$]. Infusion of DAMGO had no effect on GABA efflux [$F(1,10) = 0.4$, $P = 0.5$ for DAMGO vs. Vehicle treatment; $F(13,130) = 1.4$, $P = 0.2$ for interaction]. The mean increases over the six collections (1.5 h) are shown in the lower left panel, showing the increases in glutamate efflux with DAMGO compared to vehicle infusion, which were attenuated with the co-infusion of the MOR antagonist CTAP, but not TTX [$F(4,49) = 3.9$, $P < 0.008$]. Although CTAP and DAMGO + CTAP groups appeared to have slightly elevated levels of glutamate efflux, this was not statistically different from the vehicle condition. None of these pharmacological infusions in the CeA influenced GABA efflux [$F(4,49) = 0.8$, $P = 0.5$]. * = $P < 0.05$ vs Vehicle group. Bars represent mean ± SEM of $N = 18$ rats in the DAMGO alone group and $N = 5$–12 rats in the other drug treatment groups.

TTX inhibition of all presynaptic neurotransmitter release in the CeA, while CTAP prevented glutamate efflux in the CeA via selectively blocking activation of MOR receptors.

Nevertheless, the results suggest that the opioid-regulated increase in extracellular glutamate in the CeA does not appear to regulate the overt initial behavioral responses during the predator odor exposure. Combined with the correlation between behavioral responses and glutamate efflux in the vehicle-treated animals, this might suggest that some other aspect of predator odor exposure is inducing both responses. One possibility is individual differences in the animal's perception of the level of threat induces a primary effect, such as HPA activation during predator stress, that subsequently influences both opioid-regulated glutamate efflux in CeA and behavioral responses. The dissection of this possibility will require further studies, however, since the need for analyzing recovery for 1 h after predator stress during microdialysis prohibited the analysis of CORT responses that could assess any individual differences in acute HPA responses to predator odor and/or the effects of opioid manipulations in the CeA on HPA activation. Several studies have suggested that individual variations in behavioral responses, including showing active or passive coping styles with stress, are related to differences in either HPA or autonomic stress reactivity (Koolhaas et al., 2010; Szklarczyk et al., 2016). Mice strains that show greater restraint stress-induced passive coping (e.g., immobility or freezing) also show increased HPA activation and altered expression of glucocorticoid-regulated genes in the amygdala (Szklarczyk et al., 2016). Interestingly, both stress-related CORT responses and gene transcription in amygdala were enhanced by systemic administration of the MOR antagonist naltrexone (Szklarczyk et al., 2016), which suggests that HPA activation is likely downstream from opioid regulation induced by stress. Alternatively, it is possible that predator stress might activate the opioid system in multiple brain sites to induce glutamate efflux in CeA, as well as adaptive behavioral responses and HPA activation perhaps through effects in other brain regions such as BNST. Interestingly, intracerebroventricular administration of a met-enkephalin analog modulated locomotion and rearing behaviors, as well as activating the HPA axis (Csabafi et al., 2011), while proenkephalin knockout animals showed shifts in anxiety-related behaviors and decreased peak HPA responses (Bilkei-Gorzo et al., 2008). In the latter study behavior and CORT responses were correlated in wildtype mice, but not the proenkephalin knockouts. Since these enkephalin effects were related to global brain alterations in opioid signaling, and predator stress influences numerous brain areas, it is likely that the behavioral responses in our studies were mediated via activation of complex brain circuits involving multiple transmitter systems as opposed to just glutamate efflux in the CeA. Perhaps antagonizing opioid-induced glutamate release in the CeA regulates a selective aspect of the behavioral response to predator odor (for example freezing or HPA activation), but this was not sufficient to modify the full behavioral output without concurrent shifts in other brain areas regulating mutually exclusive behaviors (such as grooming and rearing).

This does not necessarily mean the predator odor-induced elevation in CeA glutamate and its modulation by opioids is not without some

consequence, since such changes could play a significant role in other responses to predator stress and/or the long lasting effects of predator stress on anxiety-like behaviors and the sensitization of fear responses (Adamec et al., 1999; Cohen and Zohar, 2004; Blundell et al., 2005; Masini et al., 2006; Whitaker and Gilpin, 2015). Colocalization of MOR with GluR2 expressing AMPA receptors and NMDA NR1 subunits in the CeA and BNST position the opioid system to influence glutamate-induced synaptic plasticity in this circuit, as well as long-term behavioral adaptations (Glass et al., 2009; Beckerman and Glass, 2011, 2012; Scavone et al., 2011). Glutamatergic process in the BLA and CeA also mediate some of the sensitized fear responses in rats exposed to a cat (Adamec et al., 1999; Blundell et al., 2005). This notion is further supported by electrophysiological studies demonstrating MOR regulation of plasticity in the CeA induced by theta burst stimulation of the BLA (Blaesse et al., 2015). The ability of MOR antagonists to block glutamate efflux and modulate neural adaptations and plasticity induced by predator stress will require additional studies, although this would fit with the suggestion that opioids modulate adaptations and resilience to chronic stress and perhaps long term consequences of traumatic stressors (Liberzon et al., 2007; Henry et al., 2017). Finally, the opioid regulated glutamate efflux with predator stress could be modulating other adaptive responses, such as HPA activation, autonomic responses, or changes in nociception associated with predator odor exposure (Dielenberg et al., 2001; McGregor et al., 2002; Hebb et al., 2004; Takahashi et al., 2005; Masini et al., 2006; Campeau et al., 2008; Rosen et al., 2008, 2015; Butler et al., 2011; Whitaker and Gilpin, 2015; Itoga et al., 2016). Interestingly, the time to the maximal CORT response with ferret odor at 30 min suggests that the increases in glutamate efflux seen at the first microdialysis sampling time point (15 min) were likely not directly dependent on prior CORT responses, but present a time frame that would allow the CeA glutamate response to help modulate the endocrine stress responses.

Conversely, perfusion of the MOR agonist DAMGO alone in the CeA increased glutamate efflux, which was not altered by subsequent ferret odor exposure. Further, DAMGO-infused rats displayed an increase in freezing behavior, and a significant decrease in rearing behavior to ferret odor. This change in freezing in the DAMGO treated rats could be seen as a shift in coping strategy to the threat (Kalueff and Tuohimaa, 2005), and was similar to shifts in defensive burying and other behavioral responses (rearing/escape) to predator odor seen with DAMGO microinjection into the CeA in our earlier studies (Wilson and Junor, 2008). Many behavioral response to predator stress are dependent upon the testing conditions and the behavioral options available to the animal (Dielenberg et al., 2001; McGregor et al., 2002; Hebb et al., 2004; Masini et al., 2006; Campeau et al., 2008; Rosen et al., 2008). The influences of MOR activation with DAMGO might suggest the endogenous opioids in specific circuits might help guide the selection of behavioral adaptations appropriate for the specific situation, which is over-ridden with direct MOR activation on multiple cell types seen with microinjection of microdialysis approaches. Several studies by Drolet and colleagues support this concept, since differential expression of ENK mRNA is associated with individual differences in anxiety related responses to predator odor as well as vulnerability to social defeat (Hebb et al., 2004; Berube et al., 2014). Similarly, our microinjection studies demonstrated that DAMGO injections into the CeA in a larger arena with bedding reduced in burying behavior, increased the latency to bury, reduced the number of animals showing burying behavior, and increased rearing (Wilson and Junor, 2008). Although DAMGO reduced burying behavior in the defensive burying task in a manner similar to the effects of anxiolytic compounds, this interpretation fails to encompass a potential shift in the full behavioral profile elicited during this test, including the increases in rearing and escape-type behaviors observed with DAMGO injections in the CeA. One potential explanation of these results in the defensive burying test and the current study is direct MOR activation in the CeA, perhaps via interactions with a distinct set of output neurons in the CeA, induced distinct shifts in behavior depending on the contextual framework of the predator odor exposure. Thus, DAMGO in the presence of predator odor induced a shift toward more escape-like or avoidance behaviors rather than burying in a defensive burying paradigm, and shifts in rearing and freezing in a more confined context without bedding used for microdialysis. This might also explain the decrease in open arm time in the plus maze with DAMGO injections in the CeA (Wilson and Junor, 2008), since a shift in behavioral response toward avoidance might not only activate escape type behavior to a predator (discrete) threat, but also avoidance of an unpredictable threatening environment (open arms of the maze). Interestingly, injections of opiate antagonists into the CeA of morphine-dependent rats also induced a withdrawal profile that includes escapes and jumps (Lagowska et al., 1978; Calvino et al., 1979).

The CeA is comprised of several neurochemically-distinct cell populations, which are differentially responsive to both stress and MOR activation (Day et al., 1999; Zhu and Pan, 2005; Haubensak et al., 2010; Poulin et al., 2013a). Electrophysiological studies in amygdala slices have suggested that the effects of MOR agonists in CeA are dependent upon characterization of the cell type in this area (Zhu and Pan, 2004, 2005; Finnegan et al., 2005; Chieng et al., 2006; Chieng and Christie, 2009; Blaesse et al., 2015). Recordings from CeA neurons that selectively project to the vlPAG demonstrated that DAMGO decreased the frequency of IPSCs in approximately half of these neurons (Finnegan et al., 2005; Chieng et al., 2006), and the PAG is known to be important for shaping the behavioral defensive responses to predator threat [see (Assareh et al., 2016)]. Alternatively, MOR-induced inhibition of one population of CeA neurons with DAMGO could offset the balance of enkephalin-mediated versus corticotropin releasing hormone (CRF)-mediated influences, since these two peptides have opposing effects on many behavioral outputs and are localized in distinct neurons in CeA (Veinante et al., 1997; Day et al., 1999). MOR and CRF R1 receptors are colocalized in many CeA neurons (Jaferi and Pickel, 2009). Another possibility is that the shift in behavioral responses in the DAMGO-treated rats may involve the MOR-containing neurons in the CeA that project to the BNST (Glass et al., 2009; Beckerman and Glass, 2012; Poulin et al., 2013b), since DAMGO infusion in the CeA also induced Fos in several BNST subnuclei compared with other groups (data not shown; see SUPPLEMENTAL data). The BNST has been shown to mediate the behavioral responses to predator threat (Fendt et al., 2005; Staples et al., 2005; Takahashi et al., 2005; Campeau et al., 2008; Xu et al., 2012; Rosen et al., 2015; Butler et al., 2016) and predator-related cues induce neuronal activation in the BNST (Staples et al., 2005; Campeau et al., 2008; Butler et al., 2016). Fos activation in the BNST was also induced by morphine, and this was attenuated by knockdown of NMDA NR1 subunits in the CeA (Beckerman and Glass, 2012). Thus, our results support the idea of intersecting opioid and glutamatergic modulation of fear-related response to predator cues in the CeA in response to MOR activation that may involve projections to the BNST (Scavone et al., 2011; Beckerman and Glass, 2012) or PAG (Finnegan et al., 2005; Chieng et al., 2006), although the direct role of endogenous opioid-induced glutamate release within the CeA on acute responses to predator stress will require further investigation given the relative lack of effects of MOR antagonists on behavioral outcomes as discussed above.

DAMGO infused into the CeA also enhanced glutamate efflux in the absence of any stressor. These studies demonstrated a reliable and dose-dependent increase in glutamate efflux by DAMGO that was attenuated by the co-infusion of the MOR antagonist CTAP, but not TTX. The ability of CTAP to block this effect and the dose-dependent responses suggest this is not related to a supra-pharmacological dose effect at another receptor. This increase in glutamate efflux with DAMGO alone is somewhat surprising, since electrophysiological studies rather consistently suggest that MOR agonists alter presynaptic release of glutamate and these studies would have predicted that DAMGO would decrease (rather than increase) glutamate release [see (Wilson and

McDonald, 2019) for review]. This discrepancy is likely due to electrophysiological analysis assessing synaptic release, while microdialysis samples the extracellular pool that could reflect glutamate efflux from many terminals not only CeA, but potentially also the nearby medial amygdala and intercalated nuclei. In addition, these techniques sample on a different time scale. Reverse perfusion via the microdialysis probe would influence intercalated neurons, and electrophysiological analysis of CeA neurons combined with uncaging of glutamate to selectively activate intercalated neurons demonstrated that DAMGO decreased the probability of GABA release in the CeA from this area, while not affecting excitatory responses (Blaesse et al., 2015). Thus, glutamate might be increased via an indirect effect on other CeA inputs. Conversely, several aspects of the DAMGO-induced glutamate efflux suggest this may involve a non-synaptic mechanism, perhaps involving MOR on glia in the CeA. The lack of attenuation by TTX suggests that DAMGO activation of MOR increases glutamate efflux independent of sodium dependent presynaptic neuronal release. One possibility is MOR-related effects on glial activation via interactions with other receptors, such as the toll-like receptor 4, might indirectly modulate glutamate efflux (Watkins et al., 2009). Further, MOR are also found in astrocytes in several limbic regions (Ruzicka et al., 1996; Nam et al., 2018) and recent studies indicate that activation of astrocytic MOR induce rapid glutamate release in the hippocampus (Woo et al., 2018); a similar effect might be occurring in CeA. These MOR-related effects involving glial regulation of glutamate might link predator stress effects with neuroinflammatory responses, representing a novel future direction for this work.

Interestingly, in the absence of stress, CTAP alone or when co-infused with DAMGO appeared to non-significantly elevate glutamate efflux, perhaps supporting other studies suggesting endogenous opioid tone in the CeA using antagonist administration (Carr et al., 1999; Gestreau et al., 2000; Kang-Park et al., 2009; Bajo et al., 2011, Bajo et al., 2014). This MOR antagonist effect would also be more consistent with the electrophysiological evidence showing MOR activation decreases presynaptic glutamate release [see (Wilson and McDonald, 2019) for review]. It is possible this CTAP effect is also related to the within subjects design used in these studies where animals were receiving DAMGO infusion in the contralateral amygdala during CTAP infusion, and there was some crosstalk between the two hemispheres. Alternatively, the agonist and antagonist effects might be related to different mechanisms, where the CTAP effect is related to endogenous opioid tone that is inhibiting MOR induced synaptic (TTX sensitive) glutamate release, while MOR receptor activation with DAMGO is acting via non-synaptic (perhaps glial) glutamate release in a TTX insensitive manner.

4.1. Conclusion

This study showed that predator odor exposure, which robustly enhances corticosterone, increased glutamate efflux in the CeA in a manner that correlated with defensive behavioral responses. This increase in glutamate efflux in the CeA was blocked with a MOR antagonist, although this attenuation in glutamate efflux failed to alter the initial behavioral responses to predator odor. This may suggest that opioids modulate the long-term consequences of predator stress by shifting glutamate signaling, and/or influence other physiological responses such as HPA activation, nociception or autonomic responses, although additional experiments will be required to define the circuits and contexts underlying these relationships, as well as if similar effects are seen in females. Direct activation of MOR receptors with the agonist DAMGO also enhanced glutamate efflux, even in the absence of predator stress.

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