Astrocyte Modulation of Fear Learning

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Spring 2015

A thesis presented to the faculty of The University of North Carolina at Chapel Hill in partial fulfillment of

the requirements for the Bachelor of Science degree with Honors in Psychology.

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Acknowledgements

Abstract

Neuro-glial signaling pathways are known to influence complex behavior, including fear learning processes that may contribute to the development of psychopathological conditions like Post-Traumatic Stress Disorder (PTSD). Recent findings from our laboratory have demonstrated a contribution of proinflammatory cytokine expression in the dorsal hippocampus to such fear learning processes. Astrocytes are thought to be the primary cellular source of this expression, but technological limitations have thus far prevented researchers from selectively isolating the precise contribution of astrocytes to this expression. We utilized Designer Receptors Exclusively Activated by Design Drugs (DREADDs) to examine the role of astrocyte signaling in the dorsal hippocampus in fear learning. We expressed a G_q-coupled DREADD virus under an astrocyte specific promoter such we could test whether activating G_q signaling in astrocytes prior to fear conditioning influenced contextual fear learning. We found that activating G_q-signaling in hippocampal astrocytes enhanced contextual fear learning. This finding suggests that gliotransmission driven by astrocytes in the dorsal hippocampus directly enhances fear learning and supports the feasibility of employing glial-expressing DREADD technology to examine astrocyte function in complex behavior.

Astrocyte Modulation of Fear Learning

Astrocytes are best known as support structures for neurons, but, in recent decades, findings have revealed them to be capable of communication with neurons through the release of gliotransmitters such as glutamate, ATP, or cytokines (Lee et al., 1993; Meeuwsen et al., 2003). These neuro-glial signaling pathways can modulate a wide range of complex behavior. For example, under basal conditions, neural cytokines are known to be involved in processes such as normal fear conditioning and anxiety behavior (Koo & Duman, 2009). Further, we recently demonstrated that neural cytokine signaling is a critical component in the development of stress-enhanced fear learning (SEFL), an animal model of Post-Traumatic Stress Disorder (PTSD) (Jones et al., 2015). Astrocytes are thought to be the primary source of cytokine expression in these processes because, though many cell types are capable of releasing neural cytokines, it has been demonstrated that, following acute stress, increased cytokine reactivity is co-localized with astroglia (Sugama et al., 2011). Such findings suggest that astrocyte signaling is crucial to these processes, but technological limitations have thus far prevented researchers from selectively isolating the activity of specific cell types in conjunction with behavioral measures in order to isolate the precise contribution of cell-type specific signaling in complex behaviors like fear learning. Recently, however, the development of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) has allowed researchers to utilize pharmacogenetic methods to manipulate isolated cell populations in vivo. The current project takes advantage of this technology to explore the role of glial cells, specifically astrocytes, in fear learning as it relates to the development of PTSD.

Proinflammatory cytokines are important gliotransmitters that are secreted from astrocytes and can directly alter central signaling (Lee et al., 1993; Meeuwsen et al., 2003).

Under basal conditions, the regular secretion of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) is important for the facilitation of learning, memory, and neural plasticity (for a review, see Yirmiya et. al., 2010). For example, during LTP induction in the hippocampus, 1L-1β gene expression increases rapidly to low levels (Schneider et al., 1998). This low-level cytokine expression is necessary for the maintenance of LTP, as, when IL-1 signaling is blocked through transgenic manipulations or administration of IL-1 receptor antagonist (IL-1ra), LTP induction is prevented (Schneider et al., 1998).

Although low levels of proinflammatory cytokines are beneficial and necessary for learning and hippocampus-dependent memory, it is apparent that deviation from these basal levels results in the impairment of these functions. For example, TNF-α appears to facilitate proliferation of neurons at low levels, while at high levels it can cause neuron death (Bernardino et al., 2008), and IL-1β has been shown to impair hippocampal-dependent memory when administered immediately after learning (Gonzalez et al., 2009). These observations have lent credence to the suggestion that proinflammatory cytokines such as IL-1β and TNF-α follow an inverted U-shaped pattern of activation for contributions to hippocampal memory, such that extremely low or high levels of expression result in impairment of hippocampal-dependent memory while moderate levels of expression facilitate this type of memory (Goshen et al., 2007).

In support of this inverted U-shaped pattern of activation for proinflammatory cytokines, preclinical rodent models have consistently demonstrated that proinflammatory cytokine gene expression increases dramatically in the hippocampus following contextual fear learning (Yirmiya et. al, 2010). Critically, this expression does not increase immediately, but rather exhibits a temporal delay of about 24 hours (Goshen et al., 2007), which suggests that increased IL-1β gene expression is a crucial component of contextual fear learning that occurs as a

consequence of exposure to a stressor. In support of this idea, Goshen et al. (2007) showed that IL-1ra, which prevents IL-1β from binding to IL-1 receptors, prevents contextual fear learning. It is important to note that numerous studies have isolated the impact of proinflammatory cytokines on memory to hippocampus-dependent memory processes (for a review, see Yirmiya et. al, 2010). These studies demonstrate that an elevation in hippocampal levels of proinflammatory cytokines results in a reduction in contextual memory, a form of memory dependent upon the hippocampus, but does not have an effect on non-contextual memory, which is hippocampal-independent (Gonzalez et al., 2009).

The detrimental effect of elevated proinflammatory cytokine levels has particular relevance to the study of anxiety disorders such as Post-Traumatic Stress Disorder (PTSD). PTSD is a clinical condition that may develop after exposure to a traumatic event. The hallmark symptoms of PTSD include intrusions, or flashbacks in the form of dreams or vivid memories, avoidance, meaning both avoidance of reminders of the experienced trauma as well as withdrawal from activities and loved ones, and hyperarousal, or a heightened sensitivity to potentially stressful experiences (American Psychiatric Association, 2013). This third symptom, also referred to as hyperreactivity, describes situations in which fear is inappropriately regulated such that, after exposure to a severe stressor, subsequent exposure to a mild stressor evokes a severe response akin to a re-exposure to the severe stressor. This response can be described as a generalization of the context of the original trauma to a distinct context in which the severity of the patient's response is inappropriate. As such, enhanced fear learning that is resistant to extinction can be thought of as an important component of PTSD.

Because PTSD cannot be studied experimentally, animal models of the disorder are invaluable for understanding the neuroplastic changes that occur following exposure to a severe

stressor that may lead to the development of PTSD. Stress-enhanced fear learning (SEFL) is an animal model of PTSD that excellently captures the hyperreactivity to future fear learning events that occurs in PTSD (Rau, DeCola, & Fanselow, 2005). Work in our laboratory with this model has demonstrated the involvement of proinflammatory cytokines in this process. For example, in the Lysle laboratory, we recently observed a time-dependent increase in IL-1β expression in the dorsal hippocampus following exposure to a severe stressor, and showed that central administration of IL-1ra after exposure to this stressor prevents enhanced fear learning (Jones et al., 2015). These findings are consistent with clinical evidence that demonstrates that elevated serum levels of proinflammatory cytokines are often linked with PTSD (for a review, see Gill et al., 2009). As such, these findings provide further evidence that neuroimmune signaling in the dorsal hippocampus contributes to altered fear learning in PTSD, though the exact mechanism of this action and the cellular source of this proinflammatory cytokine expression remain unknown.

The overall goal of the present study was to elucidate the precise contribution of hippocampal astrocytes to the memory impairment caused by the brain's immune response to stress by isolating and manipulating the activation of G_q signaling in hippocampal astrocytes. This was achieved through the transduction of an adeno-associated virus (AAV) in the dorsal hippocampus to express G_q-coupled Designer Receptors Exclusively Activated by Designer Drugs (G_q-DREADDs) under the glial fibrillary acidic protein (GFAP) promoter. DREADDs are pharmacogenetically modified G protein-coupled receptors (GPCRs) that respond specifically to clozapine-N-oxide (CNO) (Armbruster et al., 2007). As such, DREADD-expressing astrocytes are unaffected by endogenous activity, meaning that G_q signaling can be selectively activated in astrocytes within the dorsal hippocampus through intraperitoneal (i.p.) administration of CNO. Importantly, the effects of CNO persist only temporarily, meaning that targeted cells are

activated for a short span of time, allowing for much flexibility in experimental procedure (Armbruster et al., 2007). Thus, we used glial-expressing DREADD technology to test whether activating G_q signaling in astrocytes for six hours prior to a traditional foot shock fear conditioning paradigm would alter fear learning. Given that our previous studies showed that stress-induced IL-1β signaling was a critical component to the development of enhanced fear learning, we predicted that increased neuroinflammatory activity in the dorsal hippocampus would render animals hypersensitive to fear learning events. Thus, we predicted that activating a G_q-signaling pathway in astrocytes prior to foot shock conditioning would enhance fear learning.

Method

Animals

Male Sprague–Dawley rats (250–400 g in weight, Charles-River Laboratories, Raleigh, NC) were housed individually in a colony room under a reversed day–night (12-hour) cycle. The rats were allowed *ad libitum* access to food and water throughout the experiment. During a four-week acclimation period prior to experimentation, the rats were handled regularly. All manipulations were initiated 1 hour into the dark phase of the day–night cycle. All procedures were conducted in accordance with and approval by the UNC Institutional Animal Care and Use Committee (IACUC).

Surgical Procedures

Stereotaxic virus infusion. Animals were surgically infused with the viral vector, rAAV5/GFAP-HA-hm3D-IRES-mCitrine, purchased from the UNC Gene Therapy Center, Chapel Hill, NC. Animals were anesthetized with a 1.0 mg/kg intraperitoneal injection of 9:1 (vol:vol) ketamine hydrochloride (100 mg/ml) mixed with xylazine (100 mg/ml) and placed in a stereotaxic apparatus. Two small holes were opened in the skull and an injection micropipette

was used to micro-inject 0.7 µl of the viral vector. Injectors were directed bilaterally at the dorsal hippocampus (AP -3.4 mm, ML ±3.1, DV -2.15 mm, 15° relative to bregma; infusion speed 23 nl/min; Nanoliter 2000 Injector, WPI). Purified virus was obtained pre-dialyzed (350mM NaCl, 5% D-sorbitol in phosphate buffered saline) and microinjected at 4 x 10^{12} particles/ml, 0.7 µl per hemisphere over 7 minutes. Injectors were left in place for 15 minutes to allow for diffusion and to avoid backflow of the virus to the surface. Animals were given three weeks for post-operative recovery, after which they were exposed to additional procedures before beginning behavioral testing. Upon completion of the experiment, correct virus expression was verified and animals with incorrect virus expression were dropped from the analysis.

Behavioral Procedures

Fear conditioning was conducted in a standard rodent chamber (BRS/LVE, Laurel, MD; 26.7 × 24.8 × 30.7) housed in a metal sound-attenuating cubicle (BRS/LVE, Laurel, MD) in a room separate from the animal colony. The chamber had a 16 bar grid floor design (.48 cm in diameter spaced 1.8 cm apart), smooth metal side walls, and clear plastic front and rear walls. Cedar bedding and vanilla extract were used in the chambers to create a distinct environment from the home cages. Grid floors were wired to a BRS/LVE shock generator (SG903) and scrambler (SC922). The interior lights were kept on. The grid floor was wired to a Med Associates shock generator and scrambler (ENV-414). Behavior was recorded using a video recording system (Sony Video Camera Model HDR-CX150) directed at the clear front wall of the chamber.

The experimental design is summarized in Table 1. Animals (N = 21) were randomly assigned to a drug treatment (*CNO* or *saline vehicle*) and to a shock treatment (*foot shock* or *no foot shock*). On the treatment day, animals were administered 3.0 mg/kg of CNO or saline

vehicle (i.p.) at three time points prior to conditioning: 4.5 hours, 2.5 hours, and 30 minutes. After the final CNO administration, animals were placed in the standard rodent chamber. Each animal was placed in the conditioning chamber for 3 min 44 s and received either 5 scrambled foot shocks (2 mA, 1 s, 30 s variable interval schedule) or no shocks. Animals were then removed from the conditioning chamber and returned to their home cages. Twenty-four hours later, behavioral testing began. Animals were tested in the conditioning chamber for the assessment of freezing behavior, a behavioral quantification of learned fear, during an 8 min 32 s session (no shocks administered). Freezing behavior was measured as the time spent immobile, except for movement necessary for respiration (Rau, DeCola & Fanselow, 2005). The complete session recording was analyzed by raters blind to the treatment condition. Following the completion of the above behavioral procedures, animals were sacrificed via transcardial perfusion and brains were extracted for immunohistochemical analysis of virus expression.

Immunohistochemical Analysis

Animals were deeply anesthetized with 9:1 (vol:vol) ketamine hydrochloride (100 mg/ml) mixed with xylazine (100 mg/ml) and transcardially perfused with cold phosphate buffer (PB; pH = 7.4) followed by 4% paraformaldehyde in 0.1 M PB. Brains were placed in 30% sucrose for cryoprotection and sliced into 40 μm sections. To ensure that the virus was expressed in the dorsal hippocampus and only in astrocytes, we conducted fluorescence immunohistochemical staining for virus expression (using an antibody against the HA-tag in the viral construct we utilized) and GFAP, an astrocyte marker. Tissue sections were washed for 15 minutes with 0.1 M PB, and pre-incubated for 60 minutes in 0.4% TritonX-100 in 0.1M PB. Sections were then incubated for 60 minutes with 3% normal goat serum and 0.4% Triton X-100 in 0.1 M PB. Sections were incubated overnight with rabbit anti-HA (1:500; Cell Signaling,

Danvers, Ma) in 0.1 M PB with 3% normal goat serum and 0.4% TritonX-100. The following

day, sections were washed three times in 0.1 M PB and incubated overnight with mouse anti-

GFAP (1:1000, Thermo-Fisher Scientific, Waltham, Ma). Alexafluor488-conjugated and

Alexfluor-594-conjugated secondary antibodies (1:1000, Life Technologies, Grand Island, NY)

were used for visualization. Tissue sections labeled with only secondary antibodies were used as

secondary controls to ensure specificity of our primary antibodies. Sections were mounted onto

SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA) using Vectashield with DAPI hardset

mounting medium (Vector Laboratories, Burlingame, CA).

Color images were captured through a digital camera (Roper Scientific), mounted on an

optical microscope (BX-51, Olympus). Images captured were between -2.76 mm and -4.2 mm

relative to bregma (Paxinos and Watson, 1998).

Statistical Analyses

The effect of drug treatment (CNO or saline vehicle) and shock treatment (foot shock or

no foot shock) on freezing behavior during the test day was analyzed for statistical significance

using a standard 2×2 analysis of variance (ANOVA). Percent freezing during the test session

was analyzed with drug treatment (CNO or saline vehicle) and shock treatment (foot shock or

no foot shock) as between-subject variables. For all experiments, the significance level was set at

.05. Significant interactions were examined using LSD post-hoc comparisons.

<center>**Results**</center>

G_q- DREADD was expressed in astrocytes in the dorsal hippocampus

For the final data analysis, only data from animals that expressed the G_q-DREADD virus

in hippocampal astrocytes was included. We observed dense virus expression that was co-

localized with GFAP in the dorsal hippocampus of rats infused with the virus in 16 rats. See

Figure 1. These data provide evidence that the DREADD virus was expressed in astrocytes in the dorsal hippocampus. Unfortunately, we observed neuronal expression of the virus in a significant portion of rats infused with the virus. Thus, these animals were dropped from our analysis to avoid confounding effects that may have resulted from activation of G_q signaling in neurons during our behavioral paradigm.

CNO enhanced fear learning

A 2 x 2 ANOVA revealed a significant main effect of drug treatment, $F(1, 17) = 5.37$, $p < .05$ and a significant main effect of foot shock treatment, $F(1,17) = 6.047$, $p < 0.05$. LSD post hoc comparisons showed that animals that received CNO and were exposed to moderate foot shock exhibited significantly more freezing behavior ($M = 76.33\%$, $SD = 23.24$) than animals that received saline vehicle and were exposed to moderate foot shock ($M = 39.11\%$, $SD = 25.77$), $p < .05$. See Figure 2. Thus, these results show that activation of G_q signaling in astrocytes in the dorsal hippocampus prior to exposure to a moderate foot shock enhanced contextual fear learning.

Discussion

The goal of the current study was to apply new pharmacogenetic technology to a traditional fear-conditioning paradigm in order to explore the role of astrocytes in fear learning. Recent pharmacogentic developments allowed us to selectively activate G_q signaling in dorsal hippocampal astrocytes in vivo. We predicted that activating G_q signaling in astrocytes for four hours prior to fear conditioning would enhance fear learning. Thus, we expected to find that G_q-DREADD-expressing animals that received CNO would exhibit an exacerbated behavioral fear response following exposure to a moderate stressor. This hypothesis was supported, and the

current findings suggest that activating G_q signaling in astrocytes in the dorsal hippocampus renders animals more susceptible to enhanced fear learning.

The current findings are of particular relevance to the study of PTSD, a disorder in which enhanced fear learning becomes maladaptive. Clinical evidence demonstrates that elevated serum levels of proinflammatory cytokines are often linked with PTSD (Spivak et al., 1997; Gill et al., 2009). As such, it may be that neuroinflammation caused by astrocyte immune signaling contributes to the detrimental effect that severe stress and psychological trauma can have on the brain, leading to the development of PTSD. This hypothesis is also supported by previously published preclinical data from our laboratory that suggests that neuroinflammation in the dorsal hippocampus that is induced following an initial severe stressor is a critical component of stress-enhanced fear learning (Jones et al., 2015). The current data are consistent with this hypothesis, suggesting that excess inflammatory activity induced by astrocyte signaling in the dorsal hippocampus prior to fear conditioning hypersensitizes the animal to future fear learning experiences.

The results of the current study provide early support for this hypothesis and demonstrate the potential value of DREADD technology to future studies of fear learning. However, it is important to note that one limitation of the current study was our sample size. Virus expression in non-astrocyte cells in some animals forced us to exclude data from a large portion of our sample. Future studies should optimize glial-expressing DREADD technology to eliminate virus expression in non-astrocyte cells and replicate the current findings. Still, the current study is the first to employ glial-expressing DREADDs in a fear learning paradigm and our findings suggest that DREADD technology's ability to manipulate isolated cell populations at high levels of spatial and temporal specificity could make it a powerful tool in future studies of fear learning.

Future studies may apply this technology in the effort to better understand and contextualize the implications of the present findings regarding the G_q signaling of dorsal hippocampal astrocytes as they relate to PTSD. Specifically, though the current study suggests that the selective activation of G_q-DREADD signaling in astrocytes in the dorsal hippocampus successfully enhances fear learning, we did not examine physiological components of learning in the hippocampus. As such, it may be prudent to utilize DREADD technology in studies of other memory processes that may also involve astrocyte signaling. For example, proinflammatory cytokines like IL-1β have been shown to facilitate Long-Term Potentiation (LTP) (Lynch, 2004). To this end, an application of DREADD technology to established animal models of memory could prove invaluable to identifying the specific role of astrocytes in this context. Further, because the dorsal hippocampus does not function in isolation, using G_q-DREADD technology to selectively activate G_q signaling in astrocytes in other areas of the brain involved in fear learning, such as the basolateral amygdala or perirhinal cortex (Kim et al., 2011) would allow for a better understanding of how this network functions in response to severe stressors.

Additionally, though the current study approached fear learning by targeting G_q signaling in astrocytes, an examination of alternate astrocyte signaling pathways may provide further insights into how astrocyte signaling contributes to the development of PTSD. PTSD imposes a severe fiscal and emotional cost on society and a prophylactic pharmaceutical treatment remains a highly sought after clinical tool. Recent evidence from our laboratory has suggested that anti-inflammatory manipulations in the dorsal hippocampus following trauma can prevent the development of SEFL. The activation of G_i signaling in astrocytes is known to counteract glutamate-evoked Ca^{2+} elevations in astrocytes, which we hypothesize may directly alter cytokine release (Block et al., 2013). If activating G_i signaling in hippocampal astrocytes

following trauma can prevent SEFL in animals, these findings would provide a new target for the development of such preventative treatments. Viral constructs for G_i-coupled DREADD receptors, which selectively activate G_i signaling in astrocytes, could be utilized in both traditional fear learning paradigms and animal models of PTSD, such as SEFL, in order to assess the involvement of G_i astrocyte signaling in fear learning. G_i-coupled GPCR signaling in astrocytes counteracts intracellular Ca^{2+} release evoked by glutamate, which we hypothesize could directly attenuate the release of proinflammatory cytokines known to be critical to stress-enhanced fear learning. Empirical confirmation of this hypothesis would provide further evidence for a critical role of astrocyte signaling in fear learning processes.

In sum, the current study provides the first evidence that astrocyte function is important in fear learning. Our data demonstrate that activating G_q signaling in hippocampal astrocytes prior to fear conditioning enhances contextual fear learning. Further, these data support the feasibility of applying glial-DREADD technology in vivo to study the importance of astrocyte function in complex behavior.

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Table 1
Experimental design

|	N	Drug treatment	Shock treatment	Test day
Group 1	5	CNO (3 mg/kg) 4.5 hr, 2.5 hr, 30 min before conditioning	4 min with 5 scrambled shocks (2 mA, 1 s)	Conditioning chamber 8 min 32 s
Group 2	5	Saline vehicle (3 mg/kg) before conditioning	4 min with 5 scrambled shocks (2 mA, 1 s)	Conditioning chamber 8 min 32 s
Group 3	4	CNO (3 mg/kg) 4.5 hr, 2.5 hr, 30 min before conditioning	3 min 44 s with no shock	Conditioning chamber 8 min 32 s
Group 4	7	Saline vehicle (3 mg/kg) before conditioning	3 min 44 s with no shock	Conditioning chamber 8 min 32 s
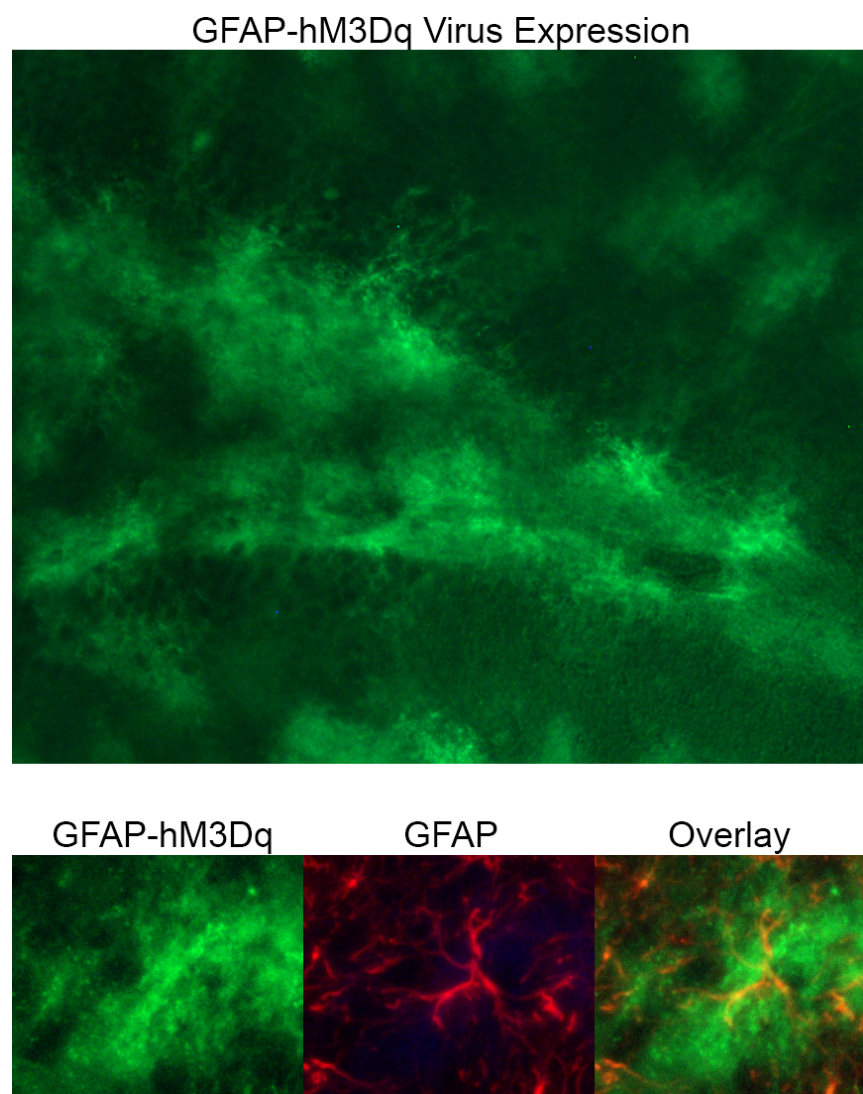
Figure 1. G_q-DREADD virus expression. Top panel shows G_q-DREADD expression in the dorsal hippocampus. Anti-Ha antibody (Cell signaling, Danvers, MA) is shown in green, 10x. Bottom panel shows co-localization of the G_q-DREADD virus (green) with GFAP (red), showing that virus is expressed in astrocytes.
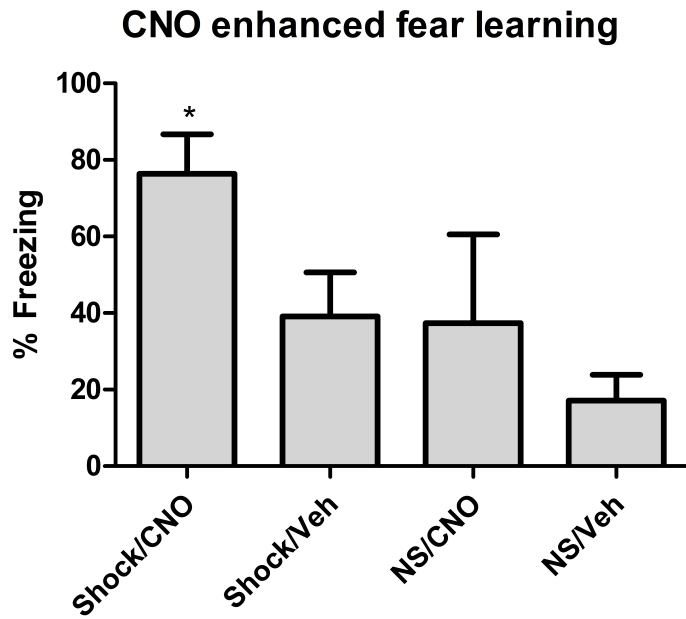
CNO enhanced fear learning

Figure 2. Repeated CNO administration (3 mg/kg, i.p. 4.5, 2.5, 0.5 hours prior to fear conditioning) enhanced fear learning. Animals that received CNO prior to foot shock exhibited exacerbated freezing behavior, * greater than Shock/Veh (vehicle), $p < .05$.