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Chemogenetic inhibition of amygdala excitatory neurons impairs rhEPO-enhanced contextual fear memory after TBI

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Abstract

Erythropoietin (EPO) is a hypoxia-responsive cytokine that induces neuroprotective effect in hypoxic-ischaemic, traumatic, excitotoxic and inflammatory injuries. Recently, utilizing a clinically relevant murine model of TBI and delayed hypoxemia, we have found that ongoing recombinant human EPO (rhEPO) administration influenced neurogenesis, neuroprotection, synaptic density and, behavioral outcomes early after TBI, and the impact on long-lasting outcomes 6 months after injury. We also demonstrated that the 1-month behavioral improvement was associated with mitogen-activated protein kinase (MAPK)/cAMP response element-binding protein (CREB) signaling activation and increased of excitatory synaptic density in the amygdala. However, we did not uncover which type of cells were involved in fear memory response enhancement after rhEPO treatment in the setting of TBI with delayed hypoxemia. In this report, using chemogenetic tools in our controlled cortical impact model, we were able to inactivate excitatory neurons and eliminate rhEPO-induced fear memory recall enhancement. In summary, these data demonstrate that rhEPO treatment initiated after TBI enhances contextual fear memory in the injured brain via activation of excitatory neurons in the amygdala.

Keywords

traumatic brain injury; hypoxemia; erythropoietin; designer receptors exclusively activated by designer drugs (DREADDs); fear memory

Introduction

Traumatic brain injury (TBI) affects around 1.7 million people each year in USA [18]. After the primary injury, secondary injuries, such as inflammation, hypoxia, excitotoxicity, are critical in determining the extent of injury expansion and damage to brain tissue [34]. Much of the research and studies performed in the field of TBI are focused on the secondary injury

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Author Contributions

Marta Celorrio and Stuart Friess: Conceptualization, Investigation, Writing – original draft. Stuart Friess: Supervision, Methodology, Formal analysis, Writing- Review and Editing, Project Administration, Funding acquisition.

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cascade in order to control neurogeneration and neuroinflammation; however, most of these studies have demonstrated limited translational success [22].

Erythropoietin (EPO) and its receptor (EPOr), expressed in neurons, astrocytes and oligodendrocytes [28], have been related with neuroprotective functions reducing apoptosis, neuroinflammation and excitotoxicity in the injured brain [5, 6, 13, 19]. Recombinant human EPO (rhEPO) administration in rodents has been associated with functional improvements of sensorimotor and spatial memory in TBI [1, 39, 40]. Recently, we have reported that rhEPO administration rescued of fear memory response 1 month after TBI with delayed hypoxemia by the mitogen-activated protein kinase (MAPK)/cAMP response element-binding protein (CREB) signaling activation in the amygdala [9]. Nevertheless, the particular cell type involved in the rhEPO's modulation of fear memory performance after TBI is unknown.

To further understand the mechanisms by which ongoing rhEPO treatment impacted fear memory performance, we employed Designer Receptors Exclusively Activated by Designer Drugs (DREADD)-based chemogenetic tools to inhibit excitatory neurons and to activate astrocytes, also associated with fear behaviors [2, 26]. Here we present our new findings of rhEPO efficacy on neuronal activation in the setting of TBI and delayed hypoxemia.

Material and methods

Virus delivery of DREADDs and confirmation of virus expression location

All procedures were approved by the Washington University Animal Studies Committee (Protocol 19-0864) and are consistent with the National Institutes of Health guidelines for the care and use of animals. Animals were housed 5/cage and had free access to water and food with a 12-hour light/dark cycle. C57BL/6J 8 week-old male mice (Jackson Laboratory, Bar Harbor, ME) weighing 20-25 grams (g) were used. To evaluate how the ongoing rhEPO treatment impacts fear memory performance, we used DREADDs expression mediated by adeno-associated viral vectors (AAVs)-induced neuronal and astrocytes transfection [7, 30] in the basolateral amygdala (BLA). BLA glutamatergic neurons and astrocytes were transduced with an AAV8 containing a *CaMKIIα* [25, 36] and *Gfap* [2, 3, 12] promoter-driven gene that encodes the evolved human M4-muscarinic receptor fused to mCherry (hM4D(Gi)-mCherry). The hM4D(Gi) couples the Gi protein-mediated signaling to inhibit neurons and glia upon biding of clozapine-N-oxide (CNO) [7]. AAV8-CAMKII-hM4D(Gi)-mCherry and AAV8-GFAP-hM4D(Gi)-mCherry (adenovirus serotype 8, 2×10^{12} virus molecules per ml; Addgen, Watertown, MA) and their control viruses (AAV8-CAMKII-mCherry and AAV8-GFAP-mCherry) were used. Stereotaxic left side unilateral injections (300 nl at 100 nl min^{-1}) were made into the BLA (anterior-posterior, −1 mm; medial-lateral, ±2.85 mm; dorsal-ventral, 5.15 mm; from bregma) 3 weeks before controlled cortical impact (CCI). The injection volume and flow rate were controlled by a syringe pump (Harvard apparatus, Holliston, MA). The needle was left in place for an additional 5 min after each injection to minimize upward flow of viral solution. The location of the virus was confirmed with immunohistochemical staining of mCherry expression after sacrifice. Animals in which mCherry was not to be primarily located in the BLA region were excluded from all analysis.

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Traumatic brain injury and delayed hypoxemia

We performed CCI as previously described [8, 10, 11] three weeks after the DREADDs injection. Briefly, mice were anesthetized with 5% isoflurane at induction, followed by maintenance at 2% isoflurane for the procedure's duration. Buprenorphine sustained release (0.5 mg/kg subcutaneously) was administered before scalp incision. The head was shaved and ear bars were used to stabilize the head within the stereotaxic frame (MyNeurolab, St. Louis, MO). Then, the craniotomy was performed over the left parietotemporal cortex using a 5-mm trephine attached to an electric drill. Then, the impactor tip was positioned over the left fronto-parietal cortex by moving it 1.5 mm anteriorly and 1.2 mm to the left from lambda. The impact was delivered at 2 mm depth (velocity 5 m/s, dwell time 100 ms). The ears bars were released immediately after the injury. All animals then received a loose fitting plastic cap secured over the craniectomy with Vetbond (3M, St. Paul, MN). The skin was closed with interrupted sutures and was treated with antibiotic ointment before the mouse was recovered from anesthesia on a warming pad. One day after surgery, animals who had undergone CCI experienced hypoxemia (8% O_2, 4% CO_2) for 60 min in a hypoxia chamber (Coy Laboratory, Grass Lake, MI). A mixture of N_2, O_2, and CO_2 was utilized to maintain normocarbic hypoxemia.

Recombinant human erythropoietin treatment

Male mice were injected intraperitoneally (i.p.) with 5000 U/kg rhEPO (Amgen, Thousand Oaks, CA), the most common dose utilized in murine models [1, 9]. Mice received a total of 6 of rhEPO, with the first dose of rhEPO administrated 8 h before hypoxemia. Daily doses were given for the next 2 consecutive days, followed by weekly rhEPO injections up to 1 month. Injured animals were randomized to receive rhEPO or saline vehicle administration using computer-generated number randomization.

Clozapine-N-oxide injection

Clozapine-N-oxide (CNO) was dissolved in 0.5% of DMSO and then diluted in 0.9% saline. 3 mg/kg was injected intraperitoneal 15 min prior to contextual and cued fear test [26].

Contextual and cued fear conditioning

To study fear memory response in mice we used a fear conditioning protocol as previously describe [8, 10, 11]. Briefly, we used a 3-day fear conditioning experimental paradigm (Fig. 1). On day 1 (conditioning), mice were placed in context A for 10.5 min and received 5 tone-shock pairings (30 s tone with 0.5 mA and shock during the last 2 s). Freezing time in 30 s epochs was measured by a blinded observer. On day 2 (contextual test), mice were placed in context A for 10.5 min and freezing time was measured to assess contextual fear memory. On day 3 (cued test), mice were placed in a novel context B (checkered walls and white hard cover on the floor) to eliminate any confounding interactions of contextual fear for 10.5 min and subsequently given five 30 s tones without any shocks. On day 1, after the final tone-shock pairing, mice remained in the conditioning chamber for 30 s before being returned to their home cages. Freezing was defined as the absence of visible movement except that required for respiration.

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Tissue processing

Mice were anesthetized with isoflurane followed by transcardial perfusion with ice-cold 0.1 M heparinized phosphate-buffered saline (PBS, pH 7.4) and 4% paraformaldehyde (PFA, Sigma-Aldrich, St Louis, MO). Perfused brains were kept in 4% PFA at 4°C overnight, followed by equilibration in 30% sucrose for at least 48 h before sectioning.

Immunohistochemistry

Histological sections were examined to assess the injection site and the neuronal activation in amygdala. Brains were taken 90 min after the completion of the fear conditioning test. Fifty-μm thick serial sections were cut on a freezing microtome starting with a visibly complete corpus callosum and continuing caudally to bregma −3.08 mm. Sets of 12 cryosections spaced every 300 μm were used for immunohistochemical studies. Immunofluorescence staining was performed on free-floating sections (50-μm thick). Staining was performed on free-floating sections washed in PBS between applications of primary and secondary antibodies. Normal donkey serum (NDS, 20%) in PBS was used to block nonspecific staining for all antibodies. Slices were then incubated at 4°C overnight with the primary antibodies (Table 1). The following day, antibody binding was detected by incubating sections with Alexa fluorescence secondary antibody (Table 1) for 2 hours. Sections were mounted on glass slides in PBS, dried, and coverslipped with mounting medium for fluorescence with DAPI.

Quantification of immunohistochemistry

Fluorescence images were taken on a Zeiss Axio Imager Z2 Fluorescence Microscope (Zeiss) with ApoTome 2 optical sectioning grid imager with 20X objective. 20 μm z stacks with 1 μm interval were obtained of the ipsilateral amygdala. The BLA of three serial dorsal amygdala slices 300 μm apart from each mouse were counted for co-localization of pCREB and AAV8-CAMKII-hM4D(Gi)-mCherry virus (Table 1). The cellular specificity of DREADDs expression was tested by immunohistochemical analysis of randomly selected areas of BLA.

Statistical analysis

All data for each animal was entered and tracked utilizing a REDCap database to maintain data integrity [20]. The histological data and behavioral data have normal distribution and repeated measures two-way ANOVA was performed. All analysis was performed with Statistica v13.3 (TIBCO software. Palo Alto, CA).

Results

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)-based chemogenetic tools to inhibit excitatory neurons and to activate astrocytes.

Previously we have been reported that rhEPO administration has been associated with improvement of fear memory in our combined injury model of TBI and delayed hypoxemia [9]. rhEPO effect was associated with MAPK signaling pathway exclusively in the amygdala [9]. However, it is unclear if MAPK signaling activation by rhEPO was specific to

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astrocytes, neurons, or both cell types. A growing body of literature suggests that astrocytes are involved in the modulation of neuronal activity and synaptic physiology in hippocampus-amygdala circuitry in fear memory responses [2, 26]. We next asked whether the astrocyte activation and/or glutamatergic-neuron inhibition in the amygdala could modulate the fear memory enhancement observed after rhEPO treatment. For this purpose, we delivered AAV8-CAMKII-hM4D(Gi)-mCherry in excitatory neurons and AAV8-GFAP-hM4D(Gi)-mCherry in astrocytes in the BLA region three weeks before CCI (Fig. 1). After 6 doses of rhEPO, we performed conditioning fear memory injecting CNO (DREADDs agonist) 15 min prior conditioning, contextual, and the cued text (Fig. 1). In the BLA, we observed that the hM4Di receptor was transfected in neurons and in astrocytes (Fig. 2A) with high specificity and penetrance in neurons (Fig. 2B and C). To verify that hM4Di inhibited neurons *in vivo* upon i.p. CNO injection (3 mg/kg), we quantified pCREB staining to evaluate neuronal activation, as previously described [37]. CNO injections dramatically decreased pCREB levels in the neurons in both CAMKII-hM4D(Gi) CCI-Veh and CCI-EPO (Fig. 2D and E). We also confirmed the transfection of the hM4Di receptor in the astrocytes of the BLA (Fig 2F) with high specificity and penetrance in astrocytes (Fig. 2G and H). The activation of the transfected astrocytes after CNO injections did not affect the neuronal pCREB activation (Fig. 2I).

rhEPO administration enhanced fear memory through neuronal activation in the amygdala

Four weeks after CCI, we performed contextual and cued-fear conditioning. No differences in behavior were observed between the GFAP-mCherry and CAMKII-mCherry control groups within rhEPO or vehicle-treated mice and these groups were combined for further analysis. In mice expressing control virus, we observed that rhEPO administration increased contextual freezing response compared with CCI-Veh (Fig. 3A). In rhEPO-treated mice, amygdala neuronal inactivation with CNO (CCI-EPO CAMKII) decreased freezing time compared with CNO-induced astrocyte activation (CCI-EPO GFAP) or control virus (CCI-EPO CONTROL, Fig. 3B). However, no differences in freezing response were observed in Veh-treated mice across the three DREADDs groups (CCI-Veh CAMKII, CCI-Veh GFAP and, CCI-Veh CONTROL, Fig. 3C). In contrast, we found no differences in cued-fear memory between the DREADDS groups in both rhEPO-treated (Fig. 3E) and untreated mice (Fig. 3F). In conclusion, hM4Di activation in excitatory amygdala neurons impaired rhEPO contextual fear memory recovery upon CNO administration.

Discussion

Using a chemogenetic approach, we demonstrated that silencing excitatory neurons in amygdala during ongoing rhEPO treatment impaired contextual fear memory 1 month after TBI with delayed hypoxemia. These data support that chronic rhEPO administration in the setting of TBI improved fear memory performance through neuronal MAPK pathway activation in an amygdala-dependent matter.

The hippocampus-amygdala pathway have been implicated in the retrieval of contextual fear memory [32, 33]. Basal amygdala receives excitatory hippocampal inputs from contextual-responding ventral CA1 neurons that contributes to encoding fear memory for the context

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[23]. In our model of delayed hypoxemia after TBI, we have previously found that rhEPO treatment for 1 month influenced fear memory response through neuronal MAPK/CREB signaling activation in the amygdala [7]. Nevertheless, the mechanism what rhEPO activates hippocampal-amygdala circuit remained unclear. EPO/EPOr system is upregulated and expressed in neurons, astrocytes and oligodendrocytes upon brain injury [21, 35]. Using only CCI animals, we wanted to explore the neuronal vs astrocytes activation in the EPO effect specifically after injury by using DREADDs-based chemogenetic tools and to understand the possible mechanism which rhEPO induces fear memory enhancement. DREADDs-based chemogenetic approach can modulate excitatory neurons activity (CAMK-positive neurons) inducing alteration in behavior performance by CNO administration [41–43]. The amygdala plays an important role in the fear memory response [24]. The conditioned stimulus (e.g. tone, context) and the unconditioned stimulus (e.g. foot shock) inputs converge and associate in the BLA [24]. Then, the BLA glutamatergic neurons project directly or indirectly to the central amygdala, which is the major source of output pathways for fear responses such as freezing [17, 38]. Previously reported, we demonstrated that after rhEPO treatment induced MAPK/CREB signaling activation specifically in the amygdala associated with an increase of fear conditioning [9]. Then, we used CaMKII-driven Gi-DREADDs to inactivate excitatory neurons in the BLA in order to confirm whether rhEPO-induced fear memory enhancement is dependent on amygdala neuronal activation. We observed contextual-fear memory impairment in CCI-Veh CONTROL compare with CCI-EPO CONTROL. In the present study, silencing excitatory neurons in the amygdala decreased contextual-fear memory in EPO-DREADDs injured mice compared with EPO-CONTROL injured mice. However, we did not observe cued-fear memory impairments when we inhibited neuronal response. While contextual learning involves inputs from the hippocampus, others have reported that this specific input is not necessary for cued-fear learning [14]. After CCI, the hippocampus is highly affected by the injury with increased neuronal loss and inflammation [15, 29] that can produce deficits explicitly in contextual fear memory [9]. This finding was not reproduced in Veh-DREADDS injured mice providing evidence for rhEPO-induced neuronal activation in the amygdala as a mechanism for fear memory enhancement.

Previous studies have revealed that astrocytes are actively involved in neuronal activation and that they are necessary for plasticity and memory [2]. Unlike in neurons where Gq-DREADDs activations leads to cellular activation and Gi-DREADDs activation leads to cellular inhibition, astrocyte cellular activation can occur via either Gq- or Gi-DREADDs [16]. Gi-DREADDs activation of astrocytes in hippocampus has been shown to enhance memory associated with conditioned place preference test [27]. Surprisingly, Gq-DREADDs activation of astrocytes in amygdala induced fear memory impairment [26]. Based on these findings, we employed Gi-DREADDs-driven astrocytes activation in amygdala to induce fear memory impairment to neutralize rhEPO-induced fear memory enhancement. rhEPO fear-memory improvements were not altered by CNO Gi-DREADDs-driven astrocyte activation. Future investigation will have to address whether hippocampal neuronal inactivation may influence amygdala-dependent fear memory. The role of rhEPO administration in inhibitory neurons and/or synapsis and its impacts on fear memory are unknown. Future studies will be required to characterize the influence of rhEPO on the

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balance between excitation and inhibition that may regulate the plasticity of this neural network [4].

Epidemiologic studies have provided evidence of sex-dependent differences following TBI. A growing body of clinical evidence show that sex may be an important factor conferring risk from TBI, defining the underlying mechanisms using animal models remains an important goal [31]. We did not use female mice in this report to test sex-dependent differences after TBI in fear memory. However, these studies suggest that future investigations of rhEPO administration should include the interaction of age and sex on the candidate therapeutic evaluation.

Conclusions

In conclusion, our data demonstrate for first time that chemogenetic silencing of neurons in the amygdala after TBI with delayed hypoxemia impairs rhEPO-induced contextual-fear memory enhancement. This data provides an insight that injured ventral hippocampus might decrease the network excitability towards the amygdala after TBI that is restored upon rhEPO treatment. Studies on characterizing the ongoing rhEPO impact on inhibition and/or excitation balance in the brain will be essential to understanding the mechanism underlying how rhEPO modulates neuronal activation within hippocampus-amygdala circuitry associated with behavioral changes after injury. Targeting EPO/EPOr in neurons in the hippocampus-amygdala circuitry may yield promising treatment approaches in TBI.

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Abbreviations:

TBI	traumatic brain injury
EPO	erythropoietin
rEPO	erythropoietin receptor
CCI	controlled cortical impact
MAPK/CREB	mitogen-activated protein kinase/cAMP response element-binding protein
BLA	basolateral amygdala
DREADDs	designer receptors exclusively activated by designer drugs
CNO	Clozapine-N-oxide

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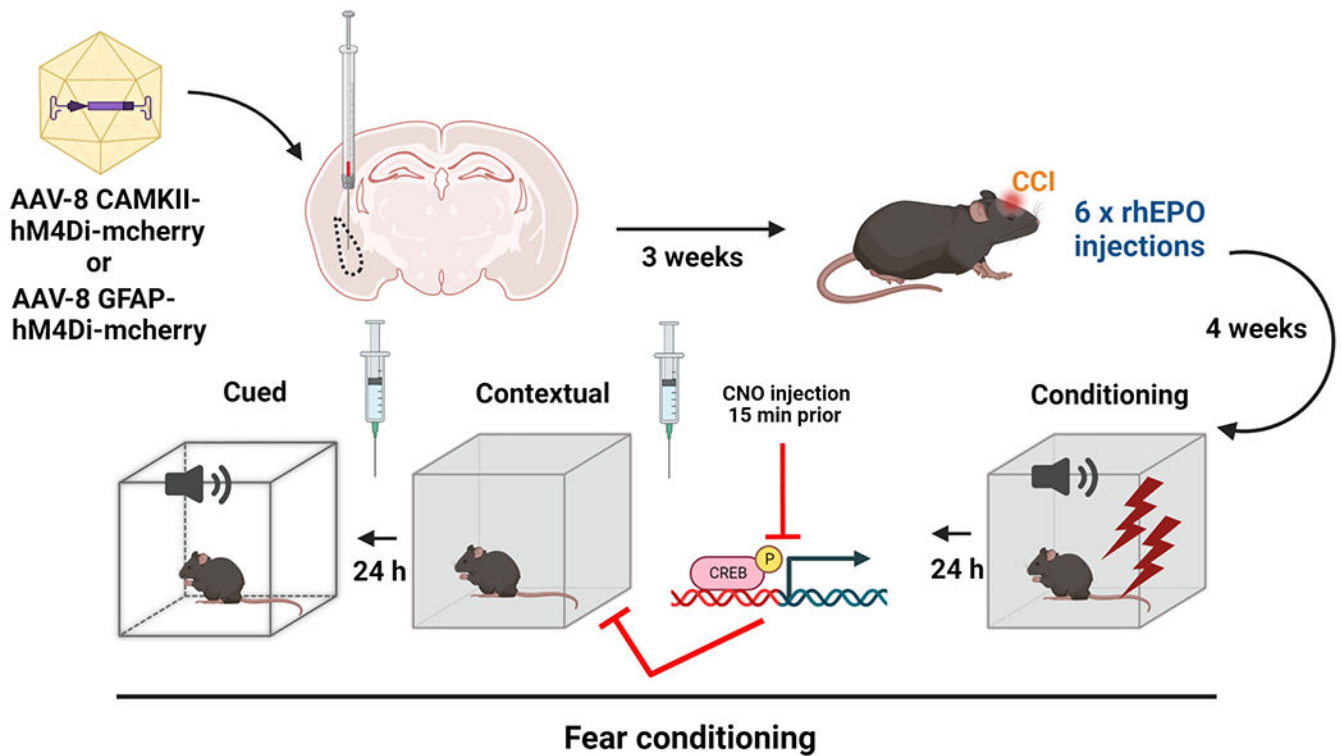

Figure 1. Experimental design.

AAV8-CAMKII-hM4D(Gi)-mCherry in excitatory neurons or AAV8-GFAP-hM4D(Gi)-mCherry in astrocytes were locally injected in the BLA region three weeks before CCI. After 6 doses of rhEPO and 4 weeks post-injury, we evaluated fear memory injecting CNO (DREADDs agonist) 15 min prior to contextual and cued testing.

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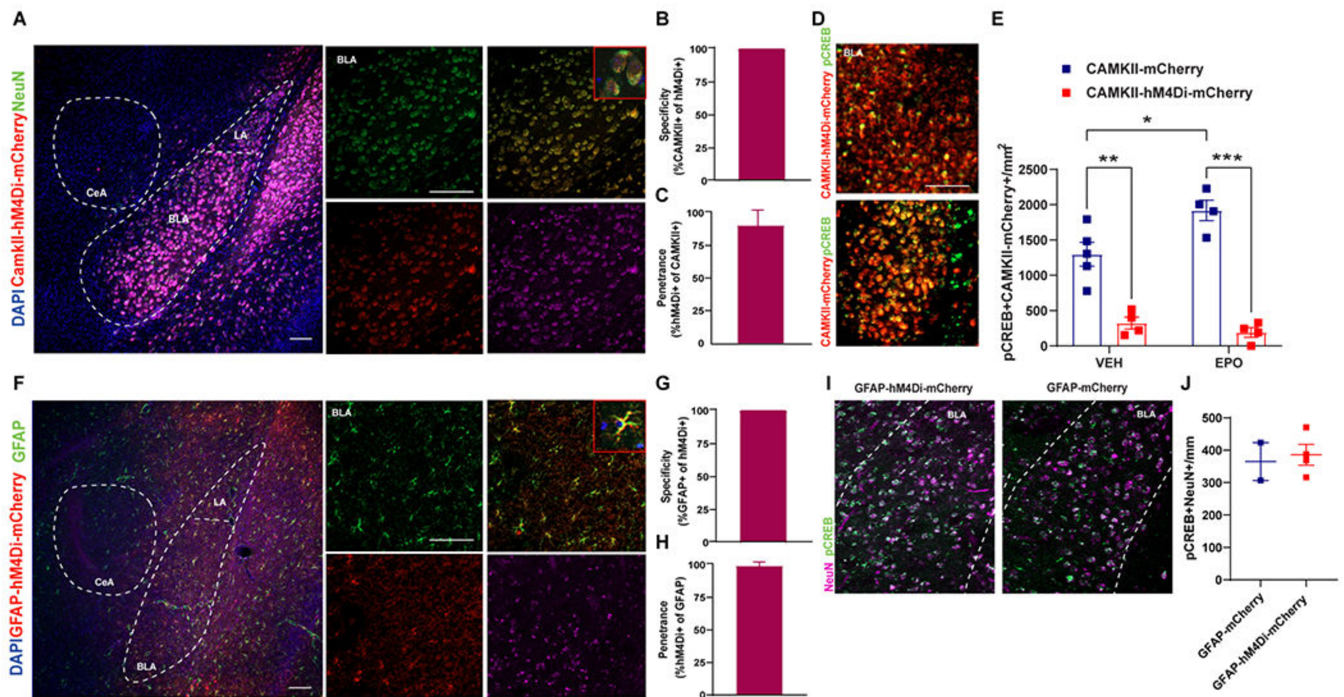

Figure 2. DREADDs-based chemogenetic tools to inhibit excitatory neurons and to activate astrocytes.

A Unilateral injection of AAV8-CAMKII-hM4D(Gi)-mCherry (red) in the BLA (3 weeks before injury) resulted in hMD4i expression in neurons (NeuN, green) with a zoomed in insert. Scale bar: 50 µm. CAMKII-hM4D(Gi)-mCherry was expressed in the (**B**) 100% of the neurons (662/662 neurons from 2 mice) with (**C**) 90% specificity (662/595 neurons from 2 mice). **D** Representative fluoroscopy of the BLA labeled with pCREB (green) and AAV8-CAMKII-hM4D(Gi)-mCherry (red) with a zoomed in insert. **E** Quantification of pCREB density in the BLA. Two-way ANOVA (DREADDs and Treatment) followed by post hoc Tukey. DREADDs $F_{(1,13)} = 103.0$ $p < 0.0001$. Treatment $F_{(1,13)} = 3.37$ $p = 0.09$. DREADDs*Treatment $F_{(1,13)} = 8.01$ $p = 0.014$. *$p<0.05$, n=4-5 mice per group. **F** Unilateral injection of AAV8-GFAP-hM4D(Gi)-mCherry (red) in the BLA (3 weeks before injury) resulted in hMD4i expression in astrocytes (GFAP, green) with a zoomed in insert. Scale bar: 50 µm. GFAP-hM4D(Gi)-mCherry was expressed in the (**G**) 100% of the neurons (332/332 neurons from 2 mice) with (**H**) 98.5% specificity (332/325 neurons from 2 mice). **I** Representative fluoroscopy of the BLA labeled with pCREB (green) and NeuN (purple). **J** Quantification of pCREB positive neurons in the BLA.

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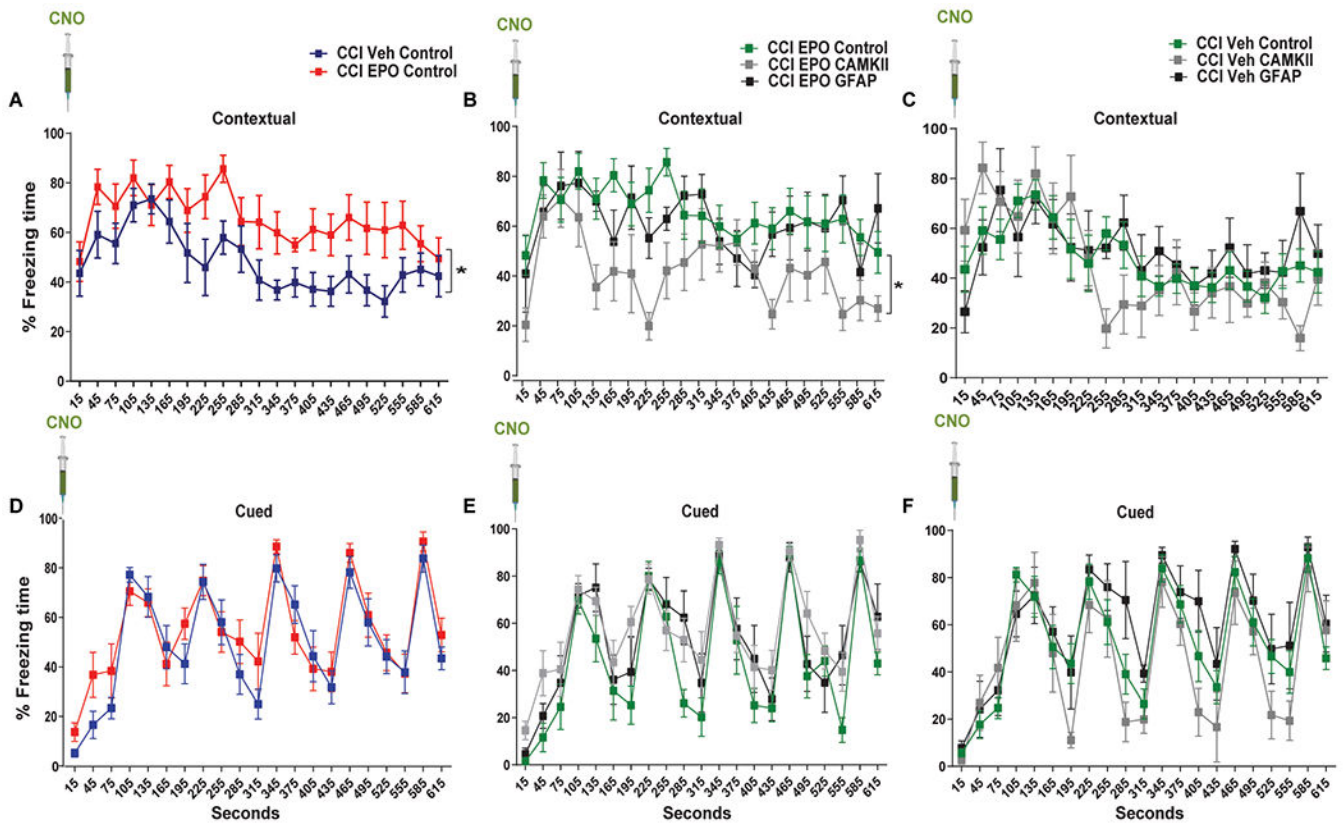

Figure 3. rhEPO treatment enhanced contextual fear memory through excitatory neuronal activation in the amygdala.

Fear conditioning quantification of percentage freezing time of (**A**) contextual control groups. Two-way (Treatment and Time) repeated measures ANOVA. Treatment $F_{(1,18)}$ = 5.09 p = 0.037. Time $F_{(20,360)}$ = 5.46 p < 0.0001, n = 4-5 mice per group **(B)** contextual CCI-EPO groups. Treatment $F_{(2,16)}$ = 3.87 p = 0.042. Time $F_{(20,320)}$ = 4.18 p < 0.0001, n = 5-9 mice per group. **C** contextual CCI-Veh groups. Treatment $F_{(2,18)}$ = 0.15 p = 0.86. Time $F_{(20,320)}$ = 6.81 p < 0.0001, n = 5-9 mice per group. **D-F** cued memory.

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Table 1.

Overview of the primary antibodies used in the present study

Antibody	Fluorophore	Species	Source	Product number
pCREB		Rabbit polyclonal	Cell Signaling	9198
GFAP		Rabbit polyclonal	DAKO North America	Z033429-2
mCherry		Goat polyclonal	Novus Biologicals	NBP3-05558
Secondary antibody	AF598	Donkey anti-goat	Thermo Fisher	A-11055
Secondary antibody	AF488	Donkey anti-rabbit	Thermo Fisher	A-21206