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Ventral hippocampal diacylglycerol lipase-alpha deletion decreases avoidance behaviors and alters excitation-inhibition balance

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ABSTRACT

The endogenous cannabinoid, 2-arachidonoylglycerol (2-AG), plays a key role in the regulation of anxiety- and stress-related behavioral phenotypes and may represent a novel target for the treatment of anxiety disorders. However, recent studies have suggested a more complex role for 2-AG signaling in the regulation of stress responsivity, including increases in acute fear responses after 2-AG augmentation under some conditions. Thus, 2-AG signaling within distinct brain regions and circuits could regulate anxiety-like behavior and stress responsivity in opposing manners. The ventral hippocampus (vHPC) is a critical region for emotional processing, anxiety-like behaviors, and stress responding. Here, we use a conditional knock-out of the 2-AG synthesis enzyme, diacylglycerol lipase α (DAGLα), to study the role of vHPC 2-AG signaling in the regulation of affective behavior. We show that vHPC DAGLα deletion decreases avoidance behaviors both basally and following an acute stress exposure. Genetic deletion of vHPC DAGLα also promotes stress resiliency, with no effect on fear acquisition, expression, or contextual fear generalization. Using slice electrophysiology, we demonstrate that vHPC DAGLα deletion shifts vHPC activity towards enhanced inhibition. Together, these data indicate endogenous 2-AG signaling in the vHPC promotes avoidance and increases stress reactivity, confirming the notion that 2-AG signaling within distinct brain regions may exert divergent effects on anxiety states and stress adaptability.

1. Introduction

Anxiety disorders are the most prevalent psychiatric illness; it is estimated that up to one in every three adults will suffer from an anxiety disorder at one point in their lives, yet current pharmacotherapies establish full remission in only a minority of patients (Berger et al., 2009; Kessler et al., 2005; Lee et al., 2016; Stein et al., 2006). This suboptimal performance has pushed research towards other avenues for treatment, and one promising target is the endocannabinoid (eCB) system, which has been demonstrated to be a potent regulator of brain regions and neural circuits implicated in emotional processing, negative affect, and stress responding (Bedse et al., 2020; Hill et al., 2018; Patel et al., 2017).

One brain region that is both highly stress-responsive and regulated by eCBs is the hippocampus (HPC). The cannabinoid receptor type 1 (CB1R) is highly expressed in the HPC (Hajos and Freund, 2002a; b; Hajos et al., 2001; Katona et al., 2000; Katona et al., 1999; Mackie, 2005), along with key synthesis and degradation enzymes of its primary endogenous ligands, 2-arachidonolyglycerol (2-AG) and N-arachidonoylethanolamine [anandamide (AEA)] (Rivera et al., 2014; Yoshida et al., 2006). The HPC has been divided along its dorsal-ventral axis into two separate brain regions with distinct functions and brain connectivity: the dorsal HPC (dHPC) which plays a role in learning and spatial navigation, and the ventral HPC (vHPC) which is closely related to emotional processing and affect (Fanselow and Dong, 2010). Most literature to date has focused on eCB signaling within the dorsal portion, clearly establishing that eCBs potently regulate synaptic transmission, short-term and long-term plasticity, and subsequent learning and memory mechanisms (Carlson et al., 2002; Chevaleyre and Castillo, 2004; De Oliveira Alvares et al., 2008a; de Oliveira Alvares et al., 2008b;

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Edwards et al., 2006; Micale et al., 2017; Wilson et al., 2001). Studies have also demonstrated that eCB signaling in the dHPC modulates anxiety-like behaviors: activation of the CB1R in the dHPC produces bidirectional effects, with low doses promoting an anxiolytic response and high doses producing an anxiogenic phenotype (Hakimizadeh et al., 2012; Hill and Gorzalka, 2004). Yet, eCB signaling in the vHPC remains understudied despite accumulating evidence that vHPC activity is critical for the development or expression of anxiety-like behaviors (Felix-Ortiz et al., 2013; Goosens, 2011; Padilla-Coreano et al., 2016; Parfitt et al., 2017; Peng et al., 2019).

Limited data suggests that eCB signaling within the vHPC plays a role in the expression of aversive behavior (Rubino et al., 2008). Pharmacological elevation of AEA specifically within the vHPC increases innate anxiety-like behaviors in rats (Campos et al., 2010; Roohbakhsh et al., 2009). Similarly, increasing vHPC AEA levels also enhances the persistence of conditioned fear (Balogh et al., 2019). These data suggest that the vHPC eCB system may serve to promote aversive processing. However, even less is known about how the other main eCB, 2-AG, regulates vHPC-dependent behavior; one study reported that pharmacological elevation of vHPC 2-AG has no effect on fear learning, while another demonstrated that bilateral infusion of 2-AG directly into the vHPC decreases freezing to a shock-paired context (Balogh et al., 2019; Rea et al., 2014).

Here, we examined the role of 2-AG signaling in the vHPC using a genetic conditional knock-out of the primary 2-AG synthesis enzyme, diacylglycerol lipase α (DAGLα). Given that enhancing AEA signaling promotes anxiety-like responses, we hypothesized that site-directed deletion of DAGLα in the vHPC, which decreases 2-AG levels, would *decrease* anxiety-like behaviors (Bluett et al., 2017; Marcus et al., 2020). Indeed, we found that vHPC DAGLα KO decreases basal and stress-induced avoidance behaviors, as well as promotes stress resiliency. Mechanistically, we reveal that genetic deletion of vHPC DAGLα shifts vHPC synaptic activity towards enhanced inhibition. Importantly, this effect was replicated with acute pharmacological DAGLα inhibition. These data ultimately demonstrate that 2-AG signaling in the vHPC functions to promote avoidance behavior and supports the notion that 2-AG can produce opposing effects on anxiety-like behavior and stress reactivity via actions within distinct brain regions.

2. Methods and materials

Subjects. All studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Vanderbilt University Institutional Animal Care and Use Committee. DAGLα$^{fl/fl}$ and wild-type mice were housed in a temperature and humidity-controlled housing facility under a 12 h light/dark cycle with *ad libitum* access to food. For generation of DAGLα$^{f/f}$ mice, see (Bluett et al., 2017). Mice underwent viral injections at 5–10 weeks. At least 5 weeks later, mice underwent behavioral testing as described below. Mice used for EPM, EZM, fear conditioning, and electrophysiology experiments were group-housed; for NIH testing, mice were singly housed. Male and female mice are designated by circles (males) and triangles (female).

Surgeries. Mice were anesthetized with isoflurane and then transferred to the stereotax (Kopf Instruments, Tujunga, CA) and kept under 3% isoflurane anesthesia. Mice were injected with 10 mg/kg ketoprofen (AlliVet, St. Hialeah, FL) as an analgesic. The hair over the incision site was shaved and the skin was prepped with alcohol and iodine. A midline sagittal incision was made to expose the skull; a hole was drilled above the vHPC (AP, −3.6; ML, ±3.0; DV, −4.0) on each side. A virus expressing Cre-recombinase [AAV8-Ef1a-mCherry-IRES-Cre (AAV-Cre; Addgene, Watertown, MA, #55632-AAV8) or AAV5-CMV-HI-eGFP-Cre-WPRE.SV40 (AAV-Cre; Penn Vector Core, Philadelphia, PA)] or a virus expressing GFP [AAV5-CMV-PI-eGFP-WPRE-bGH (AAV-GFP, Penn Vector Core, Philadelphia, PA)] was delivered bilaterally into the vHPC at a volume of 500–650 nL using a motorized digital software (NeuroStar, Stoelting CO., Wood Dale, IL), a 10 μL microinjection syringe (Hamilton CO., Reno, NV), and a Micropump Controller (World Precision Instruments, Sarasota, FL) at 0.1 μL per minute. A local, topical anesthetic, benzocaine (Medline Industries, Brentwood, TN) was applied to the incision area. After surgery, post-operative treatment with ketoprofen was administered for at least 48 h, up to 72 h.

2.1. Behavior

Elevated Plus Maze: The elevated plus maze (EPM) consists of two pairs of open and closed arms that intersect in an open center platform. The total length of each set of arms is 68 cm, and the closed arms have a wall that is 15 cm in height. The maze is elevated 40 cm off the floor. At the start of the test, mice were placed in the closed arm, facing the center. Light levels in the open arm were approximately 250 lux, while the closed arms were <100 lux. Behavior was tracked for 6 min as mice freely explored the apparatus. The apparatus was cleaned in-between mice with a 70% ethanol solution. All behavior was recorded and quantified with ANY-maze software (Stoelting, Wood Dale, IL). A total of four separate cohorts of male (AAV-GFP: 21; AAV-Cre: 24) and female (AAV-GFP: 4; AAV-Cre: 4) were run through EPM.

Restraint Stress + Elevated Zero Maze: Mice were restrained in custom made (Vanderbilt Machine Shop, Nashville, TN) restraint tubes for 1 h. Following this restraint session, mice were placed back in their home cage for 30 min, before being tested in the elevated zero maze (EZM) (San Diego Instruments, San Diego, CA). The EZM is a white platform consisting of two open arms and two closed arms. The outer and inner diameters of the EZM were 60.9 cm and 50.8 cm respectively, and the apparatus is elevated 60.9 cm from the floor. Light levels were measured at the beginning of each test, with the open arms ~250 lux, and the closed arms <100 lux. At the start of the test, mice were placed in a closed arm of the maze. Behavior was tracked for 5 min as mice freely explored the apparatus. The apparatus was cleaned in-between mice with a 70% ethanol solution. All behavior was recorded and quantified with ANY-maze software (Stoelting, Wood Dale, IL). 3 cohorts of male (AAV-GFP: 6; AAV-Cre: 10) and female (AAV-GFP: 4; AAV-Cre: 4) mice were run through restraint stress and EZM.

Repeated Novelty-Induced Hypophagia (rNIH): rNIH was performed as previously described (Bluett et al., 2017). All mice were acclimated to the testing rooms under red light (<50 lux) for at least 30 min before home-cage training and novel-cage testing. Briefly, mice were habituated to liquid vanilla Ensure (Abobott Laboratries, Abbott Park, IL) in their home cages for 30 min per day for 4 days ("Training"). On test day, mice were transferred to a novel, empty cage in a brightly lit room (~300 lux) and given access to liquid vanilla Ensure for 30 min ("NIH Test"). The next week, mice underwent home-cage "Training" for 2 consecutive days. After the 2nd home-cage training, mice were exposed to foot shock stress as previously described (Bluett et al., 2017). This foot shock stress is a 7.5-min session which consists of six 0.7 mA foot shocks delivered 1 min apart using a MED Associates fear-conditioning chamber (St. Albans, VT). Each shock coincided with the last 2 s of a 30 s auditory tone. After stress exposure, mice were returned to their home cages. Approximately 24 h after foot shock, mice underwent novel-cage NIH testing ("1 FS NIH"). Mice were exposed to five days of consecutive foot shock stress and returned back to their home-cages after each foot shock session. 24 h after the 5th foot shock, mice underwent NIH testing ("5 FS NIH"). One cohort of mice were run through rNIH.

Fear Conditioning: Following rNIH testing, this same cohort of mice underwent fear conditioning training as described previously (Hartley et al., 2016). On day 1, mice were placed in context A for fear conditioning, which included a chamber with 30.5 × 24.1 × 21.0 cm^3 dimensions and an automated freezing analysis software (Med Associates, St. Albans, VT). Mice were presented with six conditioned stimulus–unconditioned stimulus pairings (tone-foot shock) separated by a 30 s intertrial interval. Each tone (80 dB, 3,000 Hz) lasted 30 s. Mice were presented with an electric foot shock (US) at 0.7 mA during the last

2 s of each 30 s tone (CS+). On day 2, mice were placed in context B, which consisted of the same chamber with a white floor, a curved white wall contextual insert and a distinct vanilla extract olfactory cue (McCormick). A short 30 s baseline was used to test initial freezing as a measure of fear generalization to the new context. Mice were then presented with 6 tone (CS+) presentations (30 s) with a 30 s intertrial interval to test conditioned freezing to the CS+. On day 3, mice were placed in Context A to assess contextually conditioned freezing, and exposed to 6 tone (CS+) presentations (30s) in the absence of shock. Mice that were statistically assessed to be outliers during baseline freezing were excluded from analysis for that day. The chamber was cleaned in between mice with a 70% ethanol solution.

Ex vivo electrophysiology. Male and female DAGLα (Fig. 4) or wild-type (Fig. 5) mice were briefly anesthetized with isoflurane and transcardially perfused with ice-cold oxygenated (95% v/v O_2, 5% v/v CO_2) N-methyl-D-glucamine (NMDG) based ACSF (Ting et al., 2014) comprised (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Na-ascorbate, 3 Na-pyruvate, 5 N-acetylcyctine, 0.5 $CaCl_2{\cdot}4H_2O$ and 10 $MgSO_4{\cdot}7H_2O$. The brain was quickly removed and 250 μm coronal slices containing vHPC were cut using a vibratome

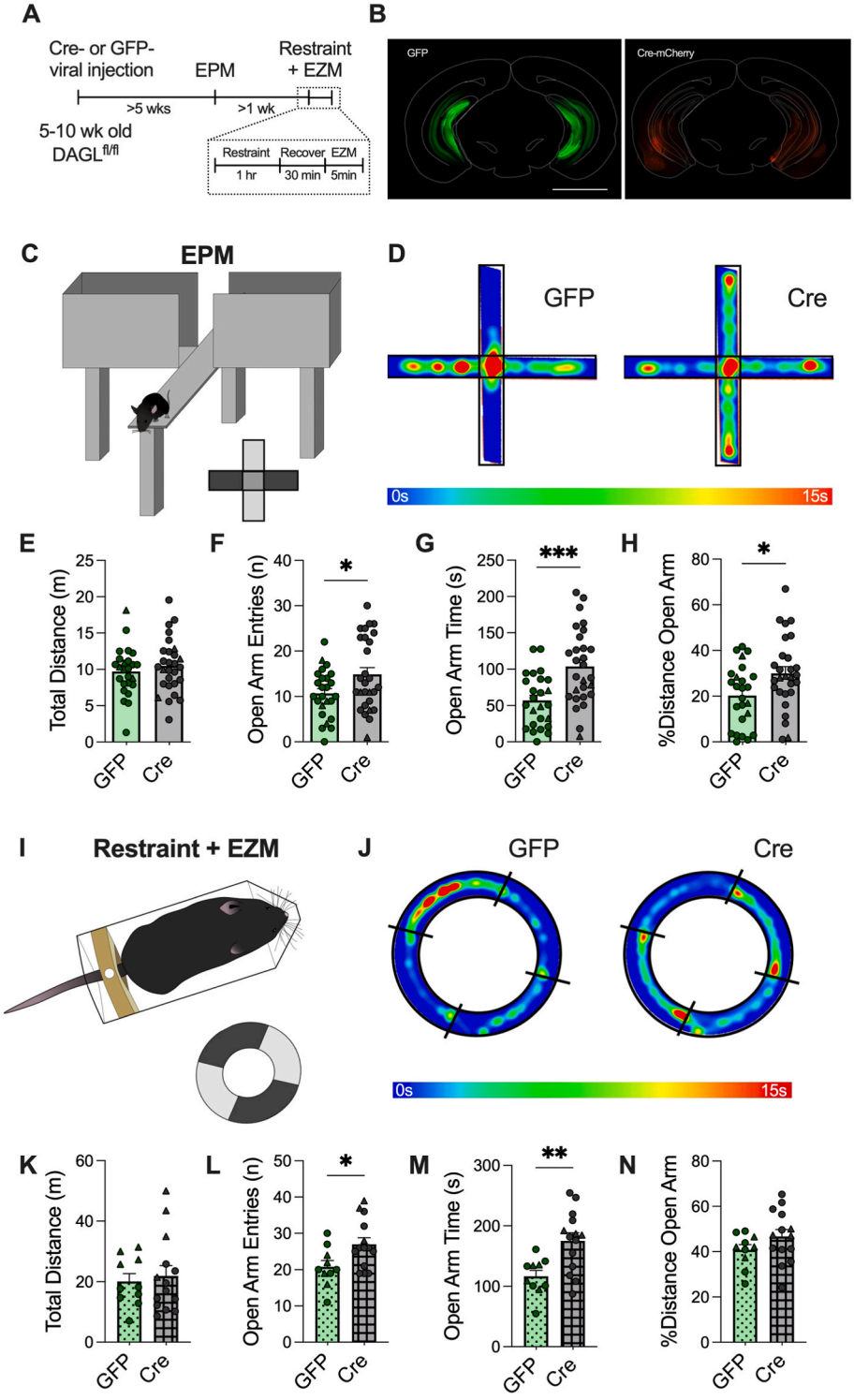

Fig. 1. vHPC DAGLα deletion decreases avoidance behaviors basally and following acute restraint stress A) Schematic of experimental design and timeline. DAGLα$^{fl/fl}$ male and female mice were bilaterally injected with virus expressing AAV-GFP (25) or AAV-Cre (28) into vHPC. Unpaired, two-tailed *t*-test used for all analysis. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ B) Representative image of AAV-GFP injection (left) and AAV-Cre injection (right); scale bar represents 2000μm C) Schematic of elevated plus maze (EPM). Darker shaded regions represent the closed arm; lighter shader regions correspond with the open arm D) Representative heat maps of AAV-GFP (left) or AAV-Cre (right) injected mice during the EPM test E) Quantification of total distance travelled during EPM in mice injected with AAV-GFP (n = 25) or AAV-Cre (n = 28) F) Quantification of the number of open arm entries during EPM test (GFP: n = 25; Cre: n = 28) G) Assessment of total time spent in the open arm during EPM test (GFP: n = 25; Cre: n = 28) H) Quantification of the %distance travelled in the open arm (GFP: n = 25; Cre: n = 28) I) Schematic of restraint stress and subsequent elevated zero maze (EZM) testing of mice injected with AAV-GFP (10) and AAV-Cre (14); darker shaded regions represent the closed arm; lighter shader regions correspond with the open arm J) Representative heat maps during EZM of AAV-GFP (left) or AAV-Cre (right) injected mice K) Quantification of total distance travelled during EZM in mice injected with AAV-GFP (n = 10) or AAV-Cre (n = 14) in the vHPC L) Assessment of the total number of entries into open arm during the EZM test (GFP: n = 10; Cre: n = 14) M) Quantification of the total time spent in open arm during EZM test (GFP: n = 10; Cre: n = 14) N) Assessment of the % distance travelled in open arm during EZM (GFP: n = 10; Cre: n = 14).

(Leica Biosystems, model # VT1000S) in the NMDG solution. Slices were incubated for 13–20 min at 32 °C in oxygenated NMDG-ACSF then stored at 24 °C until recordings were performed in HEPES-based ACSF containing (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 ascorbate, 3 Na-pyruvate, 5 N-acetylcyctine, 2 $CaCl_2{\cdot}4H_2O$ and 2 $MgSO_4{\cdot}7H_2O$.

Recordings were performed in a submerged recording chamber during continuous perfusion of oxygenated ACSF containing (in mM): 113 NaCl, 2.5 KCl, 1.2 $MgSO_4{\bullet}7H2O$, 2.5 $CaCl_2{\bullet}2H2O$, 1 NaH_2PO_4, 26 $NaHCO_3$, 1 ascorbate, 3 Na-pyruvate and 20 glucose; at a flow rate of 2.5–3 ml/min. Slices were visualized using a Nikon microscope (Eclipse FN1, Nikon Instruments Inc., Melville, NY) equipped with differential interference contrast microscopy. Whole-cell current clamp recordings were obtained under visual control using a 40x objective. 2–6 MΩ borosilicate glass pipettes were filled with a Cs^+ based internal solution (in mM): 120 CsOH, 120 D-gluconic acid, 2.8 NaCl, 20 HEPES, 5 TEA-Cl, 2.5 Mg-ATP, 0.25 Na-GTP.

Spontaneous excitatory post-synaptic currents (sEPSCs) and spontaneous inhibitory post-synaptic currents (sIPSCs) were measured at −70mV and 0 mV, respectively, for 1 min. To confirm that these voltages isolate sEPSCs or sIPSCs, CNQX (20 μM)/D-AP5 (50 μM) or picrotoxin (50 μM) was washed on for 10min. Data collection was coordinated using pClamp 10 (Molecular Devices, San Jose, CA) and cell electrical properties were monitored using a Molecular Devices 700B MultiClamp amplifier and Digidata 1440A low-noise data acquisition digitizer. Cells with an access resistance of >25MΩ or that exhibited greater than a 20% change of access resistance between sEPSC and sIPSC recordings were not included in our datasets. For Fig. 5, slices were incubated in DO34 (2.5 μM). Cells from male verse female mice are represented as circles and triangles, respectively.

Virus validation. Mice were anesthetized using isoflurane and transcardially perfused with ice-cold phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA) solution. Brains were dissected and stored overnight in 4% PFA, and then transferred to a 30% sucrose solution for at least two days. 100 μm brain sections were taken using a Leica CM3050 S cryostat (Leica Microsystem, Weitzlar, Germany). Brain sections were washed in Tris-Buffered Saline (TBS) then mounted on glass slides with VectaShield H-1200 DAPI mounting medium (Vector Laboratories, Burlingame, CA) before coverslipping. Images were taken using an Axio Imager M2 epifluorescent microscope. Whole slice images were acquired using a 5x objective. Mice were excluded from behavioral experiments if AAV-mediated fluorescence was not in the vHPC or if there was no fluorescence. For representative images in Fig. 1, scale bar was added post-hoc using brain atlas as reference.

Experimental design and statistical analysis. Experimental design for experiments can be found in results section and figure legends. For direct comparison of two groups, an unpaired, two-tailed *t*-test was performed; statistics presented as $t_{(df)} = t$; p-value. For analysis of two groups across two or more treatments or time points, a two-way ANOVA with Holm-Sidak post-hoc correction was used; statistics presented as $F_{\text{Source of Variation}}(DFn, DFd) = F$; p-value. To compare proportion of stress resilient versus susceptible phenotypes, a Chi-square test was performed; statistics presented as $z_{(df,\ \text{Chi-square})} = z$; p-value. For all data sets, significance was defined by a p value of <0.05. A ROUT outlier test was performed on all data sets and outliers were excluded. Data are presented as mean ± SEM.

3. Results

3.1. Genetic deletion of vHPC DAGLα reduces basal avoidance behavior

To elucidate the contribution of 2-AG signaling in the vHPC to anxiety-like behavior, we used a Cre-Lox strategy to selectively delete the 2-AG synthesis enzyme, DAGLα. A virus expressing Cre-Recombinase driven under a general eukaryotic promoter (AAV-Cre) or a virus expressing GFP, also under a general eukaryotic promoter (AAV-GFP), was bilaterally delivered into the vHPC of $DAGL\alpha^{fl/fl}$ mice. This approach has been previously shown to reduce DAGLα protein and decrease 2-AG content (Bluett et al., 2017; Marcus et al., 2020).

Given overwhelming evidence that the vHPC regulates anxiety states, as well as previous literature revealing that eCB manipulation in the vHPC can regulate anxiety-like states, we assessed how vHPC DAGLα KO alters innate avoidance behaviors using the elevated plus maze (EPM). vHPC $DAGL\alpha^{fl/fl}$ mice were virally injected with either AAV-Cre or AAV-GFP, and at least 4 weeks later, were tested in the EPM (Fig. 1A–D). Viral injection (Cre versus GFP) had no effect on total distance travelled ($t_{(51)} = 0.7916$; $p = 0.4323$, unpaired *t*-test), demonstrating no locomotor impairment (Fig. 1E). However, AAV-Cre injection reduced avoidance behavior evident by an increase in the number of entries ($t_{(51)} = 2.246$; $p = 0.0291$, unpaired *t*-test) and time ($t_{(51)} = 3.760$; $p = 0.0004$, unpaired *t*-test) spent in the open arm compared to mice that were injected with AAV-GFP (Fig. 1F–G). In addition, vHPC DAGLα deletion also increased the % distance travelled in the open arm ($t_{(51)} = 2.447$; $p = 0.0179$, unpaired *t*-test) (Fig. 1H). Further analysis into sex as a variable revealed no significant effect of sex on the number of entries ($F(1,38) = 1.861$; $p = 0.1805$), time spent ($F(1,44) = 1.013$; $p = 0.3198$), or % distance travelled ($F(1,48) = 0.01571$; $p = 0.9008$) in the open arm. These data demonstrate that vHPC DAGLα KO decreases innate avoidance behaviors in male and female mice.

3.2. *vHPC DAGLα deletion reduces avoidance after acute stress exposure*

It has been previously demonstrated that eCB signaling in the vHPC can switch from being anxiogenic to anxiolytic, depending on previous stress exposure (Campos et al., 2010). Facilitation of AEA signaling in the vHPC has been shown to promote anxiety-like behaviors in naïve rats, but reduce anxiety after an acute session of restraint stress. To assess whether vHPC 2-AG also produces bidirectional effects on avoidance behaviors depending on previous stress exposure, the same cohort of mice was acutely stressed before being tested for avoidance behaviors in the comparable paradigm, the elevated zero maze (EZM).

Mice were exposed to restraint stressed for 1 h, and then subject to EZM (Fig. 1I–J). Following an acute restraint stress exposure, mice injected with AAV-Cre continued to exhibit decreased avoidance. AAV-Cre-injected mice displayed an increase in the total number of entries ($t_{(22)} = 2.407$; $p = 0.0249$, unpaired *t*-test) and time spent ($t_{(22)} = 3.275$; $p = 0.0035$, unpaired *t*-test) in the open arm of the apparatus, with no differences in total locomotor distance ($t_{(22)} = 0.3875$; $p = 0.7021$, unpaired *t*-test) or % distance travelled in the open arm ($t_{(22)} = 1.424$; $p = 0.1685$, unpaired *t*-test) (Fig. 1K-N) compared to GFP controls. Taken together, these data indicate that vHPC DAGLα deletion reduces avoidance behavior both basally and following an acute stress exposure.

We next used the novelty induced hypophagia (NIH) test to confirm that vHPC DAGLα deletion decreases stress-induced avoidance behaviors. A separate cohort of $DAGL\alpha^{fl/fl}$ were bilaterally injected with AAV-Cre or AAV-GFP into the vHPC, as before; at least 4 weeks later, mice were trained to drink the palatable substance, Ensure (Fig. 2A). The latency to drink the Ensure and the total Ensure consumed were assessed during training and on NIH test days.

AAV-Cre significantly reduced the latency to consume the palatable food in the NIH test ($t_{(25)} = 2.480$; $p = 0.0203$, unpaired *t*-test), confirming that vHPC DAGLα deletion decreases stress-induced avoidance behavior (Fig. 2D). Mice injected with AAV-Cre also exhibited an increase in total consumption ($t_{(25)} = 6.617$; $p < 0.0001$, unpaired *t*-test) compared to mice injected with AAV-GFP (Fig. 2G), possibly suggesting decreased stress-induced anhedonia. Importantly, there were no significant differences in either latency to consume or total consumption across home cage training, providing compelling evidence that vHPC DAGLα deletion decreases avoidance behavior without affecting appetitive or consummatory drive at baseline (Fig. 2C, F).

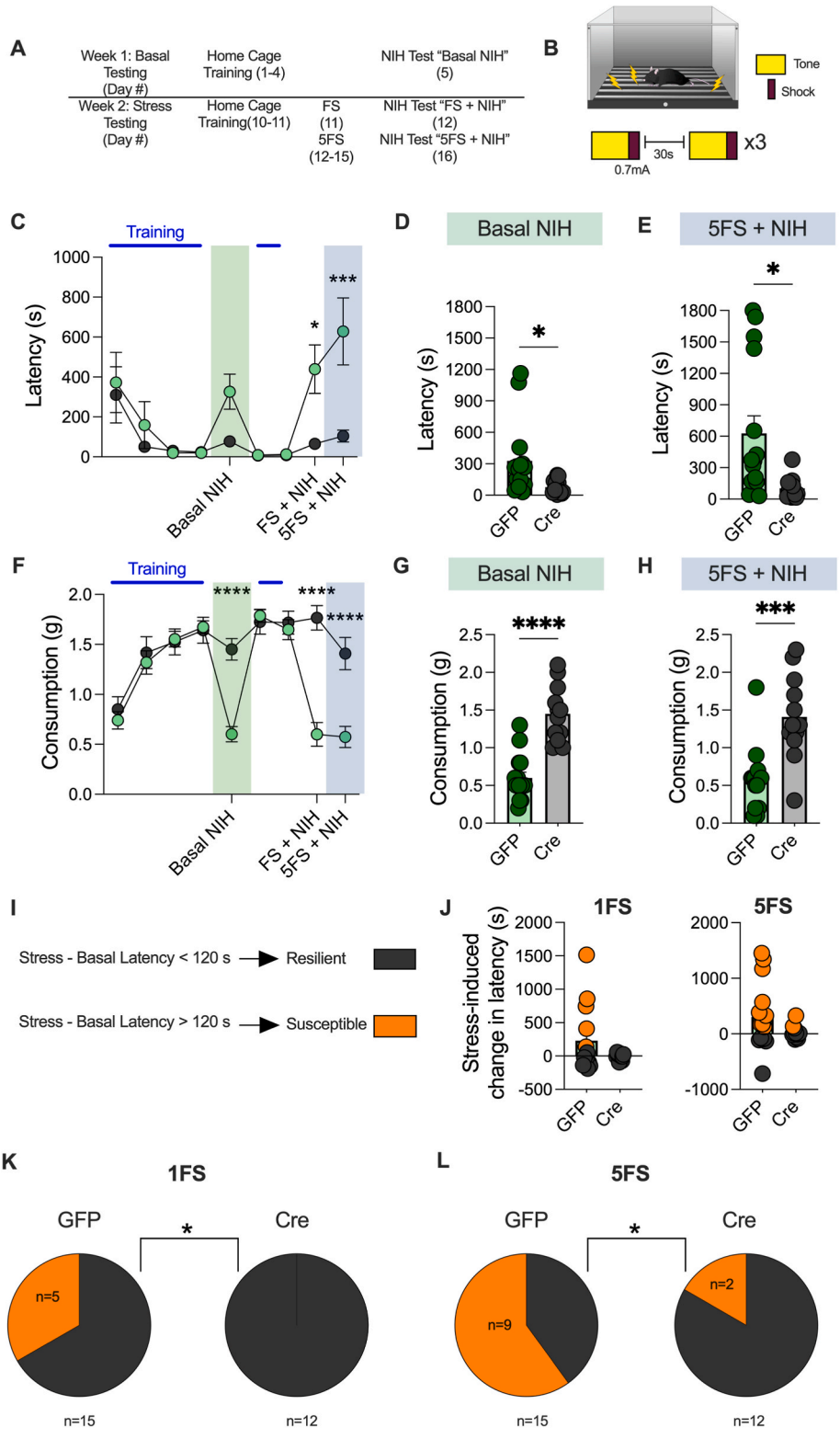

Fig. 2. vHPC DAGLα deletion decreases stress-induced avoidance in the NIH assay and promotes stress resiliency A) Timeline of repeated novelty-induced hypophagia (rNIH) experiment in a separate cohort of mice. DAGLα$^{fl/fl}$ male mice were bilaterally injected with virus expressing AAV-GFP (15) or AAV-Cre (12) into the vHPC. 2-Way ANOVA (C, F), unpaired, two-tailed, *t*-test (D-E, G-H, J), or Chi square (K–L) used for analysis. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$ B) Schematic of foot shock stress. Mice were exposed to 5 days of foot shock stress, which consisted of 6 tone (30s)-shock (2s, 0.7 mA) pairings C) Quantification of the latency to consume Ensure over training days and NIH test days D) Quantification of feeding latency during basal NIH test, without shock (GFP: n = 15; Cre: n = 12) E) Assessment of feeding latency after 5 consecutive days of foot shock (GFP: n = 15; Cre: n = 12) F) Quantification of total Ensure consumption across training and NIH test days G) Assessment of consumption of Ensure on basal NIH test (GFP: n = 15; Cre: n = 12) H) Quantification of total consumption on NIH test, after 5 days of foot shock stress (GFP: n = 15; Cre: n = 12) I) Criteria used to separate mice into susceptible or resilient following NIH. Mice with a stress-induced latency of <120s were classified as resilient J) Quantification of the average stress-induced change in latency after one day (left) or 5 days (right) of foot shock stress (GFP: n = 15; Cre: n = 12) K) Assessment of the proportion of stress resilient mice that were injected with AAV-GFP compared to AAV-Cre, after one day of foot shock L) Assessment of the proportion of stress resilient mice that were injected with AAV-GFP compared to AAV-Cre, after 5 days of foot shock.

2-AG signaling has been shown to play a prominent role in modulating stress effects after repeated homotypic stress exposures (Bluett et al., 2017; Patel and Hillard, 2008; Rademacher et al., 2008). Thus, we also exposed mice to five consecutive days of foot shock stress, as previously described (Fig. 2A–B) (Bluett et al., 2017). Importantly, stress significantly increased the latency to drink Ensure in control mice injected with AAV-GFP ($F_{DayxVirus}(8,200) = 2.961$; $p = 0.0037$, 2-Way ANOVA) (Fig. 2C). However, after five days of foot shock stress, mice with AAV-Cre viral injection demonstrated significantly decreased feeding latency, further confirming that vHPC DAGLα deletion decreases stress-induced avoidance behaviors ($t_{(25)} = 2.752$; $p = 0.0109$, unpaired *t*-test) (Fig. 2E). vHPC DAGLα deletion also promoted an increase in the total consumption on all NIH tests ($F_{DayxVirus}(8,200) = 20.26$; $p < 0.0001$, 2-Way ANOVA), including after five days of consecutive foot shock stress ($t_{(25)} = 4.469$; $p = 0.0001$, unpaired *t*-test) (Fig. 2F, H). These data ultimately confirm, as we show in Fig. 1, that vHPC DAGL

deletion decreases stress-induced anxiety-like behaviors.

3.3. *vHPC DAGLα deletion promotes stress resiliency*

Our data thus far demonstrate that vHPC DAGLα deletion reduces both basal and stress-induced avoidance behavior; however, it is still unknown whether DAGLα activity modulates stress susceptibility. To directly answer this question, we used the repeated NIH testing paradigm (rNIH) that is highly sensitive to eCB manipulation (Bluett et al., 2017). This rNIH paradigm has been previously described as a method to examine the susceptibility to develop a generalized avoidance response to stress (Bluett et al., 2017). The stress-induced change in latency of greater than 120s is used to categorize mice as stress susceptive or resilient (Fig. 2I); this model has been previously shown to induce a stress susceptible phenotype in approximately one-third of mice. The stress-induced change in feeding latency was not found to be statistically significant between AAV-GFP and AAV-Cre injected mice after one or five days of foot shock stress ($t_{(25)} = 0.7432$; $p = 0.4643$, unpaired *t*-test; $t_{(25)} = 1.538$; $p = 0.1366$, unpaired *t*-test) (Fig. 2J).

As expected, following one day of foot shock stress, one-third of the control mice [5 out of 15 AAV-GFP injected mice] developed a stress susceptible phenotype. AAV-Cre produced a significant decrease in the proportion of stress susceptible mice following one day of foot shock stress ($z_{(1,4.909)} = 2.216$; $p = 0.0267$, Chi-square) (Fig. 2K). After five days of foot shock, viral injection of AAV-Cre into the vHPC continued to produce a significant decrease in the proportion of stress susceptible mice ($z_{(1,\ 5.185)} = 2.277$; $p = 0.0228$, Chi-square) (Fig. 2L). These data ultimately reveal that vHPC DAGLα deletion promotes stress resiliency after acute and repeated homotypic stress.

3.4. *vHPC DAGLα deletion has no effect on conditioned freezing*

Several studies have demonstrated a role for vHPC in the regulation of fear acquisition and expression (Bast et al., 2001; Kjelstrup et al., 2002; Sanders et al., 2003; Sierra-Mercado et al., 2011). However, studies assessing the role of vHPC 2-AG signaling in the acquisition and expression of conditioned fear are contradictory. One study reported no effect of pharmacological elevation of vHPC 2-AG during fear conditioning, while another reported that bilateral infusion of 2-AG directly into the vHPC can reduce contextually-induced freezing to a previously

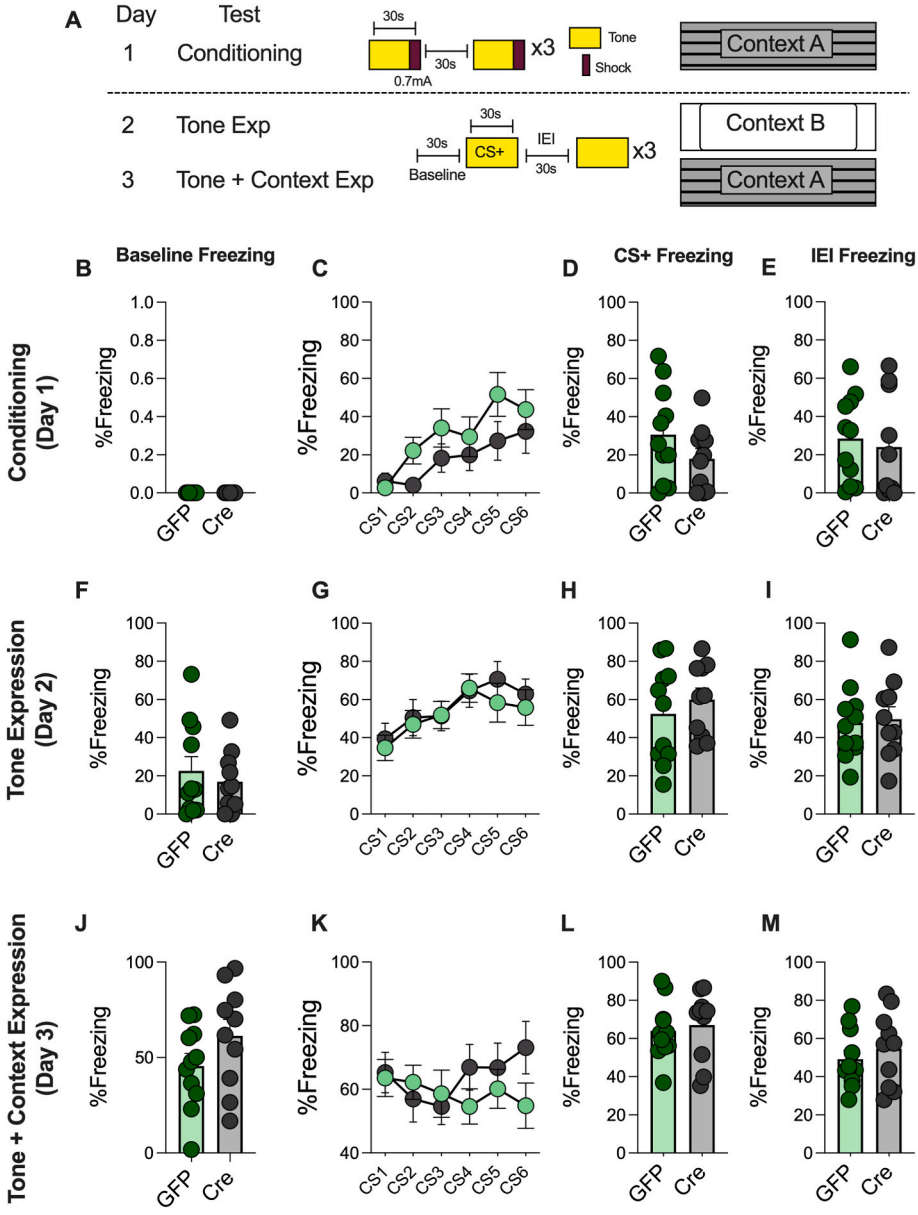

Fig. 3. vHPC DAGLα deletion has no effect on conditioned freezing or generalization A) Schematic of fear conditioning paradigm. Following rNIH testing, the same cohort of mice were fear conditioned in Context A, during which mice were exposed to 6 tone (CS+, 30s)-shock (US, 2s, 0.7 mA) pairings. 24 h after conditioning, mice were tested for tone (CS+)-induced fear expression in Context B, where mice were exposed to 6 tones in the absence of shock. On day 3, mice were placed back in Context A to assess contextually conditioned fear behavior, and exposed to 6 tones in the absence of shock. Unpaired, two-tailed, *t*-test (B, D-F, H-J, L-M), or 2-Way ANOVA (C, G, K) used for analysis. B) Quantification of baseline %freezing time during fear conditioning (GFP: n = 11; Cre: n = 10) C) Assessment of the % time spent freezing over 6 tone (CS+) presentations during conditioning day D) Quantification of the average %time spent freezing during CS+ (GFP: n = 11; Cre: n = 10) E) Quantification of the average % freezing time in-between tone/shock presentations (IEI) (GFP: n = 11; Cre: n = 10) F) Assessment of generalized freezing in Context B (GFP: n = 11; Cre: n = 10) G) Quantification of conditioned freezing across tones (CS+) in Context B. H) Quantification of the average conditioned freezing to the CS+ (GFP: n = 11; Cre: n = 10) I) Assessment of %time spent freezing during IEI in Context B (GFP: n = 11; Cre: n = 10) J) Quantification of contextually conditioned freezing during re-exposure to Context A (GFP: n = 11; Cre: n = 10) K) Quantification of %time spent freezing across tones (CS+) in Context A L) Assessment of the average %freezing time during CS+ (GFP: n = 11; Cre: n = 10) M) Quantification of the %time spent freezing in-between tones in Context A (GFP: n = 11; Cre: n = 10).

foot shock-paired context, suggesting that 2-AG signaling may disrupt fear expression (Balogh et al., 2019; Rea et al., 2014). To examine how vHPC DAGLα deletion affects fear learning, mice underwent classical auditory fear conditioning following rNIH testing (Fig. 3A). Mice were fear conditioned in Context A. Importantly, mice injected with AAV-Cre demonstrated the same amount of baseline freezing compared to AAV-GFP controls (Fig. 3B). Over the six tone (CS)-shock (US) presentations, there was no significant difference in the % time spent freezing during tone presentation between AAV-Cre and AAV-GFP injected mice ($F_{CSxVirus}(5,95) = 1.195$; $p = 0.3177$, 2-Way ANOVA) (Fig. 3C). Virus injection did not produce any significant change in the average % time spent freezing during the tone ($t_{(19)} = 1.356$; $p = 0.1909$) or between tones ($t_{(19)} = 0.4135$; $p = 0.6838$, unpaired *t*-test) (Fig. 3D–E). These data together demonstrate that loss of vHPC DAGLα activity does not affect the acquisition of conditioned freezing, consistent with Balogh et al., 2019.

We next assessed whether vHPC DAGLα deletion effects conditioned (tone or context) fear expression. Following conditioning in Context A, mice were probed for auditory fear expression in a novel Context B (Fig. 3A). As expected, 24 h after fear conditioning, both control and vHPC DAGLα deletion groups exhibited higher baseline freezing in Context B than in Context A before conditioning (Fig. 3B; F). There were, however, no differences in baseline freezing between the two groups ($t_{(19)} = 0.6182$; $p = 0.5438$, unpaired *t*-test), indicating no effect of vHPC DAGLα deletion on contextual fear generalization (Fig. 3F). Mice were then exposed to 6 tone (CS+) presentations in the absence of shock. AAV-Cre had no significant effect on % freezing time within session ($F_{CSxVirus}(5,95) = 0.7020$; $p = 0.6233$, 2-Way ANOVA), or the average % time spent freezing during the tone ($t_{(19)} = 0.7532$; $p = 0.4606$, unpaired *t*-test) or between tones ($t_{(19)} = 0.2218$; $p = 0.8269$, unpaired *t*-test), ultimately indicating that vHPC DAGLα has no effect on auditory conditioned freezing behavior (Fig. 3G–I). On day 3, mice were placed back in Context A to assess contextually conditioned freezing; there were no significant differences in baseline freezing when mice were placed back in the shock-paired context, Context A ($t_{(19)} = 1.483$; $p = 0.1544$; unpaired *t*-test) (Fig. 3J). Furthermore, AAV-Cre injection had no effect on tone-induced freezing within-session ($F_{CSxVirus}(5,95) = 0.7658$; $p = 0.5767$, 2-Way ANOVA), or average % time spent freezing during the tone ($t_{(19)} = 0.4156$; $p = 0.6823$, unpaired *t*-test) or between tones ($t_{(19)} = 0.7144$; $p = 0.4836$, unpaired *t*-test) (Fig. 3K-M). Together these data demonstrate that vHPC DAGLα deletion does not affect acquisition or expression of conditioned freezing, or contextual fear generalization.

3.5. vHPC DAGLα deletion shifts excitation/inhibition balance towards inhibition

To determine consequences of DAGLα deletion on vHPC activity, we used whole-cell patch-clamp electrophysiology (Fig. 4A). DAGLα$^{fl/fl}$ mice were injected with AAV-GFP or AAV-Cre into the vHPC and brain slices were obtained for electrophysiology experiments. It has been proposed that the CB1R is localized primarily on presynaptic GABA terminals, suggesting that hippocampal eCBs regulate GABA release (Hajos et al., 2001; Hoffman and Lupica, 2013; Katona et al., 1999, 2000; Tsou et al., 1999). In support of this hypothesis, CB1R activation causes depression of GABAergic inhibitory postsynaptic currents (IPSCs) in hippocampal slices (Hajos et al., 2001; Hoffman and Lupica, 2000). However, studies have also reported the involvement of hippocampal eCB signaling in the regulation of glutamatergic transmission (Hoffman

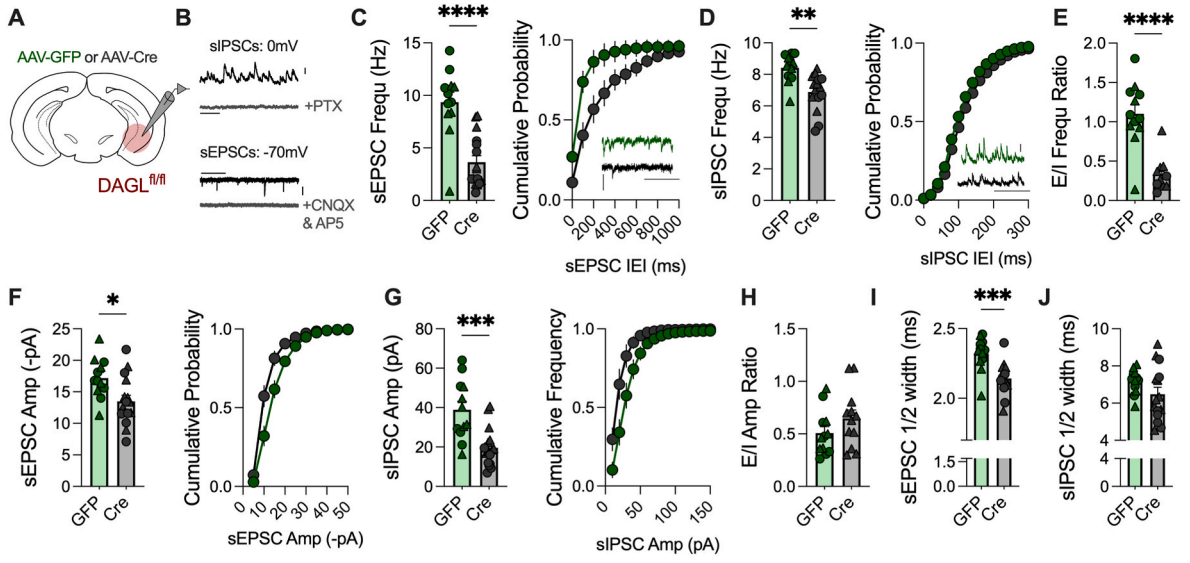

Fig. 4. vHPC DAGLα deletion shifts vHPC activity towards greater synaptic inhibition. A) Schematic of experimental design. DAGLα$^{fl/fl}$ mice were injected with AAV-GFP or AAV-Cre. At least 5 weeks later, brain slices containing the vHPC from male (circle) and female (triangle) mice were obtained for whole-cell slice electrophysiology. Glutamatergic and GABAergic synaptic transmission was assessed from the same cell. *N,n* reflect N = number of cells, from *n* = number of mice. Unpaired, two-tailed *t*-test used for analysis *, $p < 0.05$; **, $p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. B) Representative trace of spontaneous inhibitory postsynaptic currents (sIPSCs) (top, black) that can be blocked with $GABA_A$ receptor antagonist, picrotoxin (50 μM) (top, gray) and representative trace of spontaneous excitatory postsynaptic currents (sEPSCs) (bottom, black) that can be blocked with AMPA/NMDA receptor antagonists, CNQX (20 μM)/D-AP5 (50 μM) (bottom, gray). C) Quantification of sEPSC frequency from slices obtained from AAV-GFP (n = 12,3) or AAV-Cre (n = 16,4) injected mice (left). Cumulative probability plot of sEPSC interevent interval (IEI) with inset of representative traces from AAV-GFP (green) and AAV-Cre (black) injected mice (right). D) Quantification of sIPSC frequency (AAV-GFP: n = 12,3; AAV-Cre: n = 15,4) (left). Cumulative probability plot of sIPSC IEI with inset of representative traces from AAV-GFP (green) and AAV-Cre (black) injected mice (right). E) Assessment of the excitatory (sEPSC)/inhibitory (sIPSC) (E/I) frequency ratio (AAV-GFP: n = 12,3; AAV-Cre: n = 10,4). F) Quantification of the amplitude of sEPSCs in the vHPC from AAV-GFP (n = 12,3) or AAV-Cre (n = 16,4) injected mice. G) Quantification of sIPSC amplitude in slices obtained from DAGLα$^{fl/fl}$ mice injected with AAV-GFP (n = 12,3) or AAV-Cre (n = 16,4). H) Assessment of the E/I amplitude ratio comparing AAV-GFP (n = 12,3) injected mice to AAV-Cre (n = 12,4) injected mice. I) Quantification of sEPSC ½ width (AAV-GFP: n = 12, 3; AAV-Cre: n = 14, 4). J) Quantification of sIPSC ½ width (AAV-GFP: n = 12, 3; AAV-Cre: n = 15, 4). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

et al., 2010; Hoffman and Lupica, 2013; Misner and Sullivan, 1999; Stella et al., 1997; Straiker and Mackie, 2005). Therefore, we examined how DAGLα deletion alters both glutamatergic and GABAergic neurotransmission in the vHPC by recording spontaneous excitatory post-synaptic currents (sEPSCs) and spontaneous inhibitory post-synaptic currents (sIPSCs) from the same cell (Fig. 4B).

Canonical 2-AG signaling involves retrograde presynaptic suppression of afferent neurotransmitter release (Katona and Freund, 2012). We hypothesized that reducing 2-AG signaling via vHPC DAGLα deletion would disinhibit GABAergic transmission, thus enhancing inhibitory input. Unexpectedly, AAV-Cre injection promoted a significant *decrease* in the frequency of both sEPSCs ($t_{(26)} = 5.241$; $p < 0.0001$, unpaired *t*-test) and sIPSCs ($t_{(25)} = 3.723$; $p = 0.0010$, unpaired *t*-test) (Fig. 4C–D). We wanted to determine the net effect of vHPC DAGLα KO, and so the excitatory/inhibitory (E/I) frequency ratio was assessed. Interestingly, mice injected with AAV-Cre exhibited an overall decrease in the E/I frequency ratio ($t_{(20)} = 5.252$; $p < 0.0001$, unpaired *t*-test) (Fig. 4E). These data suggest that vHPC DAGLα deletion shifts net synaptic activity towards enhanced inhibition.

Furthermore, we also found a significant decrease in the amplitude of both sEPSCs ($t_{(26)} = 2.719$; $p = 0.015$, unpaired *t*-test) and sIPSCs ($t_{(26)} = 4.060$; $p = 0.0004$, unpaired *t*-test) of AAV-Cre-injected mice, which could suggest a decrease in the number of synapses or concomitant changes in postsynaptic efficacy (Fig. 4F–G). As before, the net effect of this decrease in amplitude between sEPSCs/sIPSCs was assessed. However, there was no net shift in E/I balance of the size of these spontaneous events ($t_{(22)} = 1.400$; $p = 0.1755$, unpaired *t*-test) (Fig. 5H). To further probe changes mediated by vHPC DAGL KO, we analyzed sEPSC and sIPSC kinetics. Interestingly, we found a significant decrease in sEPSC ½ width ($t_{(24)} = 3.779$; $p = 0.0009$, unpaired *t*-test), with no change in sIPSC ½ width ($t_{(25)} = 1.535$; $p = 0.1374$, unpaired *t*-test) (Fig I-J). Together, these data demonstrate that vHPC DAGLα deletion causes an overall decrease in the frequency and amplitude of spontaneous glutamatergic and GABAergic transmission, ultimately biasing population activity towards greater synaptic inhibition.

3.6. Pharmacological vHPC DAGLα inhibition also shifts biases vHPC activity towards inhibition

To confirm that DAGLα regulates vHPC E/I balance, we assessed the effects of acute pharmacological DAGLα inhibition in slices obtained from wild-type mice (Fig. 5A). Slices were incubated in the DAGLα inhibitor, DO34 (2.5 μM) and sEPSCs and sIPSCs were obtained as in Fig. 4. Following DO34 incubation, we observed a significant decrease in the frequency ($t_{(28)} = 2.425$; $p = 0.0220$, unpaired *t*-test) and amplitude ($t_{(27)} = 2.245$; $p = 0.0331$, unpaired *t*-test) of sEPSCs, thus paralleling our genetic DAGLα deletion (Fig. 5B, E). However, we did not observe any differences in sIPSC frequency ($t_{(29)} = 0.8641$; $p = 0.3946$, unpaired *t*-test) or amplitude ($t_{(29)} = 1.171$; $p = 0.2511$, unpaired *t*-test), or the kinetics of either sEPSCs ($t_{(29)} = 1.275$; $p = 0.2123$, unpaired *t*-test) or sIPSCs ($t_{(28)} = 1.751$; $p = 0.0908$, unpaired *t*-test) (Fig. 5C, F, H-I). Importantly, our data revealed a significant bias towards inhibition following acute vHPC DAGL inhibition, evident as a decrease in E/I frequency ratio ($t_{(26)} = 3.516$; $p = 0.0016$, unpaired *t*-test) and E/I amplitude ratio ($t_{(27)} = 2.235$; $p = 0.0339$, unpaired *t*-test) (Fig. 5D, G). Together, these results provide a potential mechanism by which vHPC DAGLα activity modulates anxiety-like behaviors, through an increase in local, synaptic inhibition. These data also suggest genetic and acute pharmacological inhibition produce similar, albeit not identical, changes in synaptic signaling in vHPC neurons.

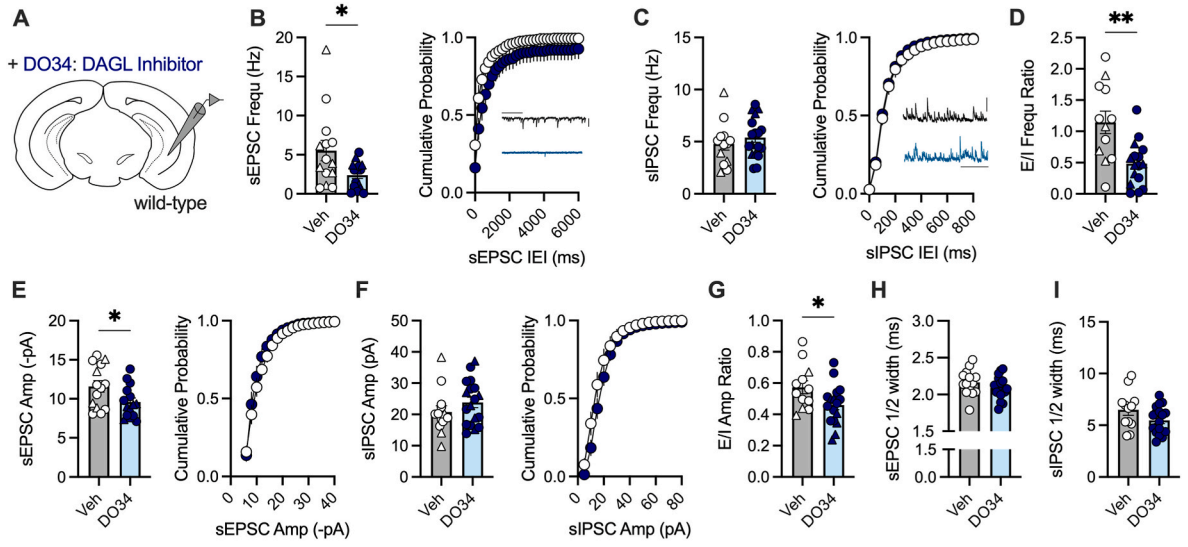

Fig. 5. Acute pharmacological DAGL inhibition biases vHPC output towards greater inhibition. A) Schematic of experimental design. Slices from male (circle) and female (triangle) wild-type mice were incubated in Veh (Vehicle; ACSF) or DO34 (ACSF + DO34; 2.5 μM). sEPSCs and sIPSCs were recorded from the same cell. *N,n* reflect N = number of cells, from *n* = number of mice. Unpaired, two-tailed *t*-test used for analysis *, $p < 0.05$; **, $p < 0.01$. B) Quantification of sEPSC frequency in slices incubated in Veh (n = 14,5) or DO34 (n = 16,5) (left). Cumulative probability plot of sEPSC interevent interval (IEI) (right) and representative traces of sEPSCs from Veh (black) and DO34 (blue) incubated slices (inset). C) Quantification of sIPSC frequency (Veh: n = 13,5; DO34: n = 18,5) (left). Cumulative probability plot of sIPSC IEI with inset of representative traces of sIPSCs from Veh (black) and DO34 (blue) incubated slices (right). D) Analysis of E/I ratio frequency (Veh: n = 12,5; DO34: n = 16,5). E) Quantification of sEPSC amplitude in slices incubated in Veh (n = 14,5) or DO34 (n = 15,5). F) Quantification of sIPSC amplitude (Veh: n = 13,5; DO34: n = 18,5)
G) Analysis of E/I amplitude ratio following DO34 incubation (n = 17,5) compared to Veh (n = 12,5). H) Assessment of sEPSC ½ width from slices incubated in Veh (n = 14,5) or DO34 (n = 17,5). I) Quantification of the ½ width of sIPSCs (Veh: n = 12,5) (DO34: n = 18,5). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

One brain region critical for the development and expression of anxiety states is the vHPC. Activity within the vHPC of rodents, and its human analogue, the anterior hippocampus, has been consistently found to correlate with anxiety phenotypes (Caliskan and Stork, 2019; Gulyaeva, 2015; Satpute et al., 2012). Despite substantial evidence indicating strong eCB regulation of hippocampal activity, the role of 2-AG signaling in modulating vHPC activity and subsequent anxiety-like behaviors is not well established (Katona and Freund, 2012). Here, we demonstrate that vHPC-specific genetic deletion of the 2-AG synthesis enzyme, DAGLα, decreases anxiety-like behavior in several well-validated behavioral paradigms (EPM, EZM, NIH). We also show that this phenotype is associated with changes in excitatory/inhibitory balance, shifting vHPC activity towards greater synaptic inhibition.

First, we reveal that 2-AG signaling in the vHPC promotes basal anxiety-like behavior. Over the last decade, 2-AG signaling has been identified as an important regulator of anxiety states and stress responsivity (Bedse et al., 2018, 2020; Bluett et al., 2017; Hill et al., 2018; Kondev et al., 2021; Patel et al., 2017). Systemic elevations of 2-AG levels have been shown to counteract the negative effects of stress, promote stress resiliency, and decrease innate avoidance in mice (Bluett et al., 2017; Cavener et al., 2018; Jenniches et al., 2016; Shonesy et al., 2014). However, some studies have reported that 2-AG augmentation can promote avoidance and stress reactivity under some conditions (Balogh et al., 2019; Hartley et al., 2016; Heinz et al., 2017; Imperatore et al., 2015; Kondev et al., 2022; Llorente-Berzal et al., 2015). This seemingly contradictory data could be explained by divergent effects of 2-AG signaling within distinct brain regions or neuronal circuits that regulate affective behaviors in opposing ways. It has been previously shown that pharmacological elevation of AEA levels in the vHPC increases innate anxiety-like behaviors in the EPM (Campos et al., 2010; Roohbakhsh et al., 2009), suggesting that eCB signaling via CB1R activation in the vHPC promotes avoidance. Our data are consistent with these findings and suggest that 2-AG, similar to AEA, signaling in the vHPC may enhance anxiety-like behaviors. However, one study reported that THC infused into the vHPC reduced avoidance (Rubino et al., 2008); these mixed effects may be due to differences in the level of CB1R activation since CB1R agonists can dose-dependently produce bimodal anxiety-states, with low doses alleviating anxiety and high doses promoting anxiety (Hakimizadeh et al., 2012; Hill and Gorzalka, 2004; Roohbakhsh et al., 2009).

We further demonstrate that vHPC DAGLα deletion decreases anxiety-like behaviors following acute and repeated stress exposure. Stress has been consistently shown to dysregulate eCB signaling in the HPC; for example, exposure to restraint stress, social defeat stress, and early life stress have each been demonstrated to increase 2-AG levels in the HPC (Dubreucq et al., 2012; Llorente et al., 2008; Wang et al., 2012). Our data suggest that these stress-induced increases in 2-AG levels in the vHPC may promote stress-induced avoidance, given that decreasing vHPC 2-AG synthesis prevents the increase in anxiety-like behavior observed after both acute (restraint and foot shock) and repeated (5 days of foot shock) stress. This effect appears to be specific to 2-AG, as one study found that pharmacological AEA enhancement promotes anxiolysis in rats exposed to an acute restraint stress (Campos et al., 2010). These differences in the behavioral effects of eCB manipulation may be due to differential effects of stress on eCB levels: unlike 2-AG, acute and chronic stress have been shown to decrease AEA levels in the HPC (Dubreucq et al., 2012; Gunduz-Cinar et al., 2013; Llorente et al., 2008; Wang et al., 2012).

Behaviorally, we also reveal that genetic deletion of vHPC DAGLα has no effect on acquisition or expression of conditioned freezing. The vHPC has been heavily implicated in auditory and contextual fear conditioning (Bast et al., 2001; Maren and Holt, 2004; Sierra-Mercado et al., 2011; Yoon and Otto, 2007; Zhang et al., 2014). Previous studies assessing the role of vHPC 2-AG signaling in fear conditioning have enhanced 2-AG signaling, either pharmacologically or through direct infusion of 2-AG directly into the vHPC (Balogh et al., 2019; Rea et al., 2014). Our data here assessing how reduced 2-AG levels effects fear conditioning, suggests that vHPC 2-AG signaling is not *necessary* for the acquisition or expression of conditioned fear behavior in male mice. Furthermore, while the HPC has been repeatedly shown to play a role in fear extinction, we did not directly assess the contribution of vHPC DAGLα activity in modulating extinction here (Sierra-Mercado et al., 2011). This remains an important question for future studies.

We further demonstrate that genetic deletion and pharmacological inhibition of DAGLα promote a decrease in the E/I ratio, shifting vHPC activity towards synaptic inhibition. It has been previously reported that silencing of the vHPC, either through lesions or chemogenetic inhibition, decreases avoidance behaviors in rodent behavioral models (Bannerman et al., 2004; Bertoglio et al., 2006; Goosens, 2011; Kjelstrup et al., 2002; Padilla-Coreano et al., 2016; Parfitt et al., 2017). However, future studies will need to examine the role of DAGLα in mediating vHPC activity *in vivo* using optical imaging tools.

Surprisingly, we show that both genetic deletion and pharmacological inhibition of vHPC DAGLα *reduces* the frequency of spontaneous glutamatergic neurotransmission. These data suggest that 2-AG signaling in the vHPC serves to *promote* glutamate release. One potential mechanism whereby 2-AG facilitates synaptic neurotransmitter release is through activation of CB1R on astrocytes, which has been implicated in LTP modulation and synaptic potentiation (Araque et al., 2017; Covelo and Araque, 2018; Martin-Fernandez et al., 2017; Navarrete and Araque, 2008; Perea and Araque, 2007; Robin et al., 2018). Navarrete and Araque (2008) have demonstrated that activation of astrocytic CB1R can stimulate glutamate release in the dHPC, making it plausible that a similar mechanism may exist in the ventral portion (Navarrete and Araque, 2008; Araque and Navarrete, 2010). Based on this hypothesis, decreasing 2-AG synthesis and subsequent 2-AG signaling may prevent this astrocyte-mediated potentiation, thus resulting in a decrease in glutamatergic transmission as we observe here. However, the downstream signaling cascades mediating how astrocytic CB1R activation promotes synaptic glutamate neurotransmission is also not well understood, but may reflect astrocyte-driven changes in glutamate-mediated slow-inward currents (Navarrete and Araque, 2008). Ultimately, use of astrocyte-specific manipulation of eCB system components will need to be used to directly address the contribution of astrocytes in these effects.

Finally, two important limitations require consideration; first, in our behavioral manipulation we did not explicitly test the role of sex in mediating vHPC DAGLα deletion effects. It has been previously described that sex can affect hippocampal function and stress responsivity and adaptability (Koss and Frick, 2017; Shors et al., 2001). Here, we did not observe an effect of sex on basal avoidance in the EPM, but this may be due to differences in the number of male (25) verse female mice (8). Additionally, our rNIH and fear conditioning experiments were performed only in male mice. Future studies will need to explicitly test the role of sex in mediating vHPC DAGLα effects.

Second, the possibility remains that our genetic knock-down strategy may result in compensatory changes in components of the eCB system. Indeed, comparison of the electrophysiological effects of genetic DAGLα deletion (Fig. 4) and pharmacological DAGLα inhibition (Fig. 5) reveal that only spontaneous glutamatergic transmission is affected in both conditions. Therefore, it remains possible that our behavioral effects here could be a consequence of prolonged (weeks) adaptations to DAGLα deletion and subsequent 2-AG depletion. Future studies will need to test how an acute pharmacological enhancement or depletion of vHPC 2-AG modulates avoidance behaviors and stress reactivity. Finally, while we demonstrate functional validation of viral-mediated DAGLα deletion given that pharmacological DAGLα inhibition with DO34 produces similar effects, and we have reported and validated this approach previously, we did not directly assess 2-AG levels after Cre-mediated DAGLα deletion here (Bluett et al., 2017; Marcus et al., 2020).

Together, these data suggest 2-AG signaling in the vHPC promotes avoidance and stress reactivity. We suggest here that vHPC 2-AG signaling, unlike in other brain regions, may promote anxiety-like states and worsen the adverse consequences of stress exposure. Understanding how and where 2-AG regulates affective behavior is critical for our understanding of how eCBs, and eCB-related drugs, ultimately modulate anxiety states. These data may be important in guiding the development of future eCB-based pharmacotherapies for stress-related psychiatric disorders.

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Conflict of interest

Sachin Patel is a scientific consultant for Psy Therapeutics, Janssen, and Jazz Pharmaceuticals. All other authors declare no financial conflicts of interest.

CRediT authorship contribution statement

Veronika Kondev: Writing – original draft, Writing – review & editing, Investigation, Validation, Formal analysis, Visualization. **Rebecca Bluett:** Writing – review & editing, Investigation, Formal analysis. **Mustafa Najeed:** Investigation, Formal analysis. **Luis E. Rosas-Vidal:** Investigation, Funding acquisition, Writing – review & editing. **Brad A. Grueter:** Resources, Writing – review & editing. **Sachin Patel:** Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

Sachin Patel is a scientific consultant for Psy Therapeutics, Janssen, and Jazz Pharmaceuticals. All other authors declare no financial conflicts of interest.

Data availability

Data will be made available on request.

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