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RESEARCH ARTICLE

Disruption of tonic endocannabinoid signalling triggers cellular, behavioural and neuroendocrine responses consistent with a stress response

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Abstract

Background and Purpose: Endocannabinoid (eCB) signalling gates many aspects of the stress response, including the hypothalamic–pituitary–adrenal (HPA) axis. The HPA axis is controlled by corticotropin releasing hormone (CRH) producing neurons in the paraventricular nucleus of the hypothalamus (PVN). Disruption of eCB signalling increases drive to the HPA axis, but the mechanisms subserving this process are poorly understood.

Experimental Approach: Using an array of cellular, endocrine and behavioural readouts associated with activation of CRH neurons in the PVN, we evaluated the contributions of tonic eCB signalling to the generation of a stress response.

Key Results: The CB1 receptor antagonist/inverse agonist AM251, neutral antagonist NESS243 and NAPE PLD inhibitor LEI401 all uniformly increased Fos in the PVN, unmasked stress-linked behaviours, such as grooming, and increased circulating CORT, recapitulating the effects of stress. Similar effects were also seen after direct administration of AM251 into the PVN, while optogenetic inhibition of PVN CRH neurons ameliorated stress-like behavioural changes produced by disruption of eCB signalling.

Conclusions and Implications: These data indicate that under resting conditions, constitutive eCB signalling restricts activation of the HPA axis through local regulation of CRH neurons in the PVN.

KEYWORDS
Cannabinoid, CRH, Endocannabinoid, Grooming, Hypothalamus, PVN, Stress

1 | INTRODUCTION

Exposure to a threat or challenge elicits a host of physiological and psychological consequences collectively known as the stress response. Although the body's reaction to stress can be beneficial in acute conditions, unmitigated activation of stress-responsive biological systems

Abbreviations: AEA, anandamide; AP, anteroposterior; BLA, basolatera amygdala; CORT, corticosterone; DV, dorsoventral; eCB, endocannabinoid; eYFP, enhanced yellow fluorescent protein; HPA, hypothalamic pituitary adrenal; L, lateral; PBST, phosphate buffered saline and triton; PFA, paraformaldehyde; PVN, paraventricular nucleus of the hypothalamus.

can lead to negative psychological, metabolic and cellular consequences (McEwen et al., 2015). While the neural mechanisms that regulate the generation of a stress response following exposure to a challenge have been relatively well characterized, the process by which stress-responsive systems are constrained during ambient conditions is less well understood (Herman et al., 2016). As many disease states, such as mood and anxiety disorders as well as many inflammatory conditions, are associated with aberrant activation of stress-responsive systems in the absence of overt threats or challenges, understanding the neural processes involved in preventing undue activation of stress responses is an important area that has been historically neglected.

There are multiple, well-characterized arms of the stress response; amongst these, the neuroendocrine response via hypothalamic–pituitary–adrenal axis (HPA) activation is the most well understood (Pecoraro et al., 2006). Activation of the HPA axis begins with the release of **corticotropin releasing hormone** (CRH) from parvocellular neurons in the paraventricular nucleus of the hypothalamus (PVN) and ends with elevated circulating **corticosterone** (CORT) in rodents (Herman et al., 2016). Alongside HPA activation, stress exposure triggers a complex behavioural response in animals including increases in vigilance, avoidance and threat assessment. While these behavioural responses have traditionally been measured in a superficial manner, recent work has described a reliable stress-induced pattern of behavioural changes that occurs following cessation of stress and return to a familiar environment, with emphasis on an increase in stereotypic grooming (Füzesi et al., 2016; Loewen et al., 2020).

The endocannabinoid system (eCB) is intimately involved with the stress response and has been widely studied as a system which dynamically responds to stressful experiences and modulates synaptic plasticity across multiple brain circuits to influence shifts in behavioural repertoires (Morena et al., 2016). The two primary eCB ligands **anandamide** (AEA) and **2-arachidonoyl glycerol** (2-AG) are primarily synthesized postsynaptically by their primary respective synthetic enzymes **N-acylphosphatidylethanolamine phospholipase D** (**NAPE-PLD**) and **diacylglycerol lipase** (DAGL) and then act retrogradely on presynaptic cannabinoid receptors (**CB_1**) to dampen synaptic transmitter release. Both AEA and 2-AG have been implicated in the regulation of the HPA axis, particularly in the context of termination and recovery from stress exposure by modulating excitability in stress-responsive neural circuits, through acting at the CB_1 receptor (Morena et al., 2016). In addition to this dynamic role of eCB signalling in response to stress exposure, there is also some evidence that eCB signalling may also play a role in tonic suppression of the stress response in ambient conditions. For example, AEA signalling is known to rapidly decline in response to stress, and inhibition of its metabolism can blunt many aspects of stress suggesting that tonic AEA signalling may be constraining activation of the stress response in the absence of a threat (Bedse et al., 2014; 2017; Bluett et al., 2014; Gray et al., 2015; Hill et al., 2009; Sticht et al., 2019; Yasmin et al., 2020), while depletion of AEA can increase CORT secretion (Mock et al., 2020). Consistent with this, administration of a CB_1 receptor antagonist reliably increases circulating glucocorticoids, suggesting that disruption of tonic eCB signalling is sufficient to increase activation of the HPA axis (Hill et al., 2009; Newsom et al., 2012; Patel et al., 2005). While the neural circuits through which dynamic changes in eCB signalling regulate stress responses is somewhat characterized (Morena et al., 2016), there is a relative paucity of information regarding the mechanism and circuits by which tonic eCB signalling maintains quiescence of the HPA axis under ambient conditions. Using an array of anatomical, neuroendocrine and behavioural readouts, we combine multiple pharmacological approaches to disrupt tonic eCB signalling to elucidate the nature by which tonic eCB signalling constrains stress responsive processes.

What is already known

- The endocannabinoid system plays an important role in regulating multiple aspects of the stress response.
- Stress exposure disrupts tonic endocannabinoid signalling, which is thought to initiate the stress response.

What does this study add

- We identify anatomical and cellular mechanisms by which tonic endocannabinoid signalling gates stress sensitive systems.
- Using novel pharmacological tools, we identify the roles of anandamide and 2-AG in this process.

What is the clinical relevance

- Elucidating this relationship provides the foundation for manipulating the Endocannabinoid system to treat stress-related disorders.

2 | METHODS

2.1 | Animals

All experiments were approved by the University of Calgary Animal Care and Use Committee in accordance with Canadian Council on Animal Care Guidelines. A total of 139 male mice were used in this study, 8–11 weeks old and weighing an average of 29 g (±5 g) immediately prior to treatment. Transgenic Crh-IRES-Cre (B6(Cg)-Crhtm1(cre)Zjh/J; stock number 012704)/Ail4 (B6.Cg-Gt (ROSA)26Sortm14(CAG-TdTomato0Hze/J; stock number 007914) mice, which have been previously described in Wamsteeker et al. (2010), were bred in house to express td-tomato in CRH expressing neurons. Wild-type (C57BL/6J; stock number 000664) mice were purchased from Jackson Laboratories (Bar Harbor, US). For viral and CRH neuron activity experiments,

offspring derived from crosses of homozygous Crh-IRES-Cre and Ai14 genotypes were used. Mice were single housed on a 12:12 h light dark schedule where lights were turned on at 8:00 AM. Before testing at 10–12 weeks old, animals were housed in Greenline ventilated cages containing small wood chip bedding, shredded paper and a paper or plastic house for environmental enrichment. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

2.2 | Drugs

All systemically administered drugs were dissolved in 5% polyethylene glycol, 5% Tween-80 and 90% saline on the day of each experiment. The CB_1 receptor antagonist/inverse agonist AM251 (3 mg·kg^{-1}; Cayman Chemical, Ann Arbor, USA) and the neutral CB1 receptor antagonist NESS0327 (0.3 mg·kg^{-1}; Cayman Chemical, Ann Arbor, MI, USA) were administered 15 min prior to behavioural recording. The NAPE-PLD inhibitor LEI401 (30 mg·kg^{-1}; was synthesized as previously described in Mock et al., 2020) and the DAGL inhibitor DO34 (30 mg·kg^{-1}; as previously described in Ogasawara et al., 2016) were administered i.p. 120 min before behavioural recording. A 30 mg·kg^{-1} dose for each of these drugs produces a robust decrease in AEA and 2-AG content in the brain following peripheral drug administration at the times in which the animals were tested herein (Mock et al., 2020; Ogasawara et al., 2016). For central administrations, AM251 (Cayman Chemical) was mixed in 80% DMSO and 20% saline to make a 1 µg/200 nl solution, and 200 nl was administered unilaterally into the PVN, or bilaterally into the striatum, 15 min before behavioural recording. Drugs were infused through 30-gauge injection needle connected to a 10-µl Hamilton micro syringe by polyethylene tubing and driven by a minipump (Harvard Apparatus, Holliston, USA). A 3-min infusion protocol consisted of 1-min active infusion and a 2-min diffusion period to allow for proper spread and prevent back flow into the cannula.

2.3 | Behavioural protocol

All animals were handled for 4 days where they were familiarized with the experimenter and drug administration procedures. After handling, animals were habituated for one hour a day for 3 days to their testing room and "homecage" environment, which consisted of wood chip bedding, while their shredded paper, house, food hopper and water bottle were removed. Animals that did or did not undergo surgery were single housed for 17–21 days or 14–16 days, respectively, with seven of those days containing handling as described above. On test day, animals had their enrichment and food/water removed an hour before behavioural measurements. Mice then received drug, or vehicle, administrations before behavioural assessment. Drug assignment was balanced according to the time of day the animal was tested (between 1 and 5 h subsequent to lights turning on). Following drug administration, behaviours were recorded in their homecage for a 15-min epoch.

Recorded behaviour videos were scored using a custom python behavioural scoring program by an individual blinded to each subject's treatment group. Seven behaviours were scored (as described by Füzesi et al., 2016) as time spent: walking (movement of front and back paws to change location or direction); surveying (sniffing of environment while back legs remained stationary); rearing (achieving an upright position by lifting of front legs and simultaneous extension of back legs); digging (displacing bedding); chewing (chewing of any inanimate object in the cage); grooming (body licking, rubbing or scratching); resting (no observable movement).

2.4 | Immunohistochemistry

On each test day, 90 min after behavioural monitoring began, animals were deeply anaesthetized with a lethal dose (800 mg·kg^{-1}) of pentobarbital and perfused intracardially with 4% paraformaldehyde (PFA) and phosphate buffered saline (PBS). Mice were tested for reflex prior to perfusion. Following perfusion, the brains were extracted and post-fixed in 4% PFA at 4°C overnight and then submerged in 30% sucrose in PBS at 4°C for 48 h. Brains were flash frozen, mounted and sectioned (40 µm) on a Leica SM 2010R sliding microtome. Slices containing the PVN were collected according to Paxinos and Franklin's mouse atlas and placed in an anti-freeze solution until antibody treatment.

Sections were washed three times (all washes are 10 min in duration) in a PBS solution and then three more times in a PBS and 0.1% Triton X-100 solution (PBST) before incubation in PBS blocking buffer containing 5% normal donkey serum at room temperature for 1 h. Sections were then incubated in primary antibody (rabbit anti-Fos; 1:300 Cell Signaling Technology, Danvers, USA) at 4°C overnight. Tissue was then washed in PBST solution three times and then incubated in secondary antibody at room temperature for 2 h (donkey anti-rabbit Alexa Fluor 488 [1:100 Jackson Laboratory, Farmington, USA]). Sections were then mounted in PBS onto glass slides using Fluoroshield with DAPI (Sigma-Aldrich) and examined using a Leica TCS SPE-II confocal microscope (confocal is Leica TCS SPE-II and a fluorescent is Leica DM4000 B/M LED). Two or three slices containing the PVN were collected from each specimen between 0.7 mm and 1.1 mm posterior to bregma. Grey-scale images were taken of both hemispheres separately for each slice using I3 and N21 filter cubes. All images from the same experiment were obtained under the same exposure and magnification (20×; NA 0.55). Images were processed in ImageJ, where they were subjected to an identical brightness threshold before Fos labelled cells were counted based on size criteria set for each experiment. A boundary of the entire PVN was drawn according to the Paxinos and Franklin's mouse atlas, and the total number of FOS+ cells in this area were counted. This was then normalized to the size of the PVN for each slice by dividing number of FOS+ cells by area, to give a cells mm^{-2} value that could be

compared across drug groups. For colocalization studies, slices were imaged using a Leica TCS SPE-II confocal microscope. Cells expressing both td-tomato (CRH expressing neurons) and Fos were identified in the manner described above, and z stacks (nine stacks at 4.4 µm per layer) were manually assessed on the microscope to confirm colocalization. Colocalization was determined by manually scrolling through z-stacks to rule out overlapping cells. Number of Fos +/td-tomato+ co-expressing cells was divided by the total number of td-expressing cells within the same region to obtain a percent value for each animal. The proportion of Fos expressing cells that were non-CRH expressing cells were calculated by dividing the total Fos labelled cells by the amount of Fos labelled cells that did not colocalize with td-tomato. The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

For histology, perfusions and slicing were performed in the same way as described for immunohistochemistry. Brain slices were mounted and stained for DAPI to identify cell bodies. Slices were then imaged using a fluorescent microscope using the DAF filter cube to visualize DAPI. Only animals that had correct placements were included.

2.5 | Corticosterone analysis

Tail blood (200 µl) was taken from an awake mouse 30 min following the start of behavioural recordings and 60 min before being euthanized using microvette collection tubes and stored in wet ice until spinning. Blood samples were centrifuged at 10,000 × *g* for 5 min at 20°C. Serum was stored at −20°C until analysis in triplicates with a Corticosterone ELISA kit (Arbor Assays, Ann Arbor, USA) by following the manufacturer's instructions.

2.6 | Injection and implantation

An automatic (Neurostar) stereotaxic apparatus (Kopf Instruments) fitted with an isoflurane delivery setup was used for all surgical procedures. Mice were placed under deep anaesthesia via isoflurane prior to being securely placed into ear bars. All animals received subcutaneous injections of saline (1 ml) and Metacam (2 $mg{\cdot}kg^{-1}$). The scalp was shaved, and 30% ethanol was applied prior to incision. The skull was exposed, and holes were drilled to allow for implantation of cannulae or injection. For drug infusion experiments, a unilateral cannula was lowered into the PVN (anteroposterior [AP], −0.25 mm; lateral [L], 0.3 mm from bregma; dorsoventral [DV], −4.5 mm from dura) and secured with Metabond and dental cement. For optogenetic studies, a pulled glass capillary was lowered into the PVN (AP, −0.3 mm; L, 0.2 mm from bregma; DV, −4.8 mm from dura), and 210 nl of Arch 3.0-eYFP (rAAV2-EF1a-double floxed-eArch3.0-eYFP; 5×10^{11} GC ml^{-1}; UNC Vector Core) or eYFP (Addgene plasmid 20296, pAAV-EF1a-double floxed-eYFP-WPRE-HGHpA; 5×10^{11} GC ml^{-1}; Penn Vector Core, Philadelphia, USA) was infused using a Drummond Nanoject III in three 70-nl boluses. Mice were sutured and allowed to recover. Two weeks later, mice had 400-µm diameter optical ferrules (400/460/0.48; Doric, Franquet, CA) lowered over the PVN (AP, −0.3 mm; L, 0 mm from bregma; DV, −4.5 mm from dura) and secured similarly to cannula implants. All animals were given at least 1 week to recover following surgical procedures before the initiation of behavioural experiments.

2.7 | Optogenetic procedures

A light source (593 nm, IKE-593-100-OP, IkeCool Corporation) was connected to the implanted ferrule with a 2-m fibre optic cable (400/460/1100-0.48; Doric, Franquet, CA). Animals underwent four handling and three habituation days prior to behavioural testing. During habituation, mice were attached to the fibre and allowed to wander their homecage for an hour. AM251 (3 $mg{\cdot}kg^{-1}$) or VEH (10 $\mu l{\cdot}g^{-1}$) injections were administered i.p. 15 min prior to a 15-min behavioural recording during which all animals underwent exposure to continuous yellow light (532 nm; 15 mW). Following behavioural testing, mice were perfused and PVN CRH neurons were assessed for viral expression using a Leica DM4000 B/M LED fluorescent microscope at 10× magnification.

2.8 | Data and analysis

Studies were designed to generate groups of equal size, balanced for time of experimentation and blinded analysis. All comparisons between vehicle and drug groups were analysed using independent samples *t* tests. In the experiment utilizing optogenetics, the three groups were compared using a one-way ANOVA. Post-hoc tests were run only if F achieved P<0.05 and there was no significant variance inhomogeneity. All variability data are represented as standard error of the mean (SEM) in each graph. Different group sizes in experiments where surgery was performed were a result of exclusions due to inaccurate viral expression or cannula/ferrule placements. Statistical analysis was undertaken only for studies where each group size was at least n = 5. The declared group size of each experiment represents the number of independent values, and no replicate values were used in any statistical analysis. Sample sizes for behaviour, CORT and Fos measurements were designed to be n = 6–8. In attempts to improve animal welfare, this is the lowest numbers of animals that can be used while still providing statistically relevant effects using these techniques. Groups containing n = 5 were a result of exclusions as a result of incorrect viral, ferrule or cannula placements. For all analysis performed, the threshold for statistical significance is $P < 0.05$. All outliers were included in data analysis and presentation. Descriptive statistics are reported in (Table S1). The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022).

2.9 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/2022 (Alexander, Christopoulos, et al., 2021; Alexander, Fabbro, et al., 2021; Alexander, Kelly, 2021).

3 | RESULTS

3.1 | Disruption of eCB signalling activates a stress-like response

To investigate the effects of CB_1 receptor blockade on the stress response, we administered the CB_1 receptor antagonist/inverse agonist AM251 (3 mg·kg^{-1}, i.p.) before probing three well established arms of the stress response: (1) Fos induction of CRH expressing neurons in the PVN; (2) elevations in circulating CORT; (3) increases in repetitive behaviours (e.g., grooming) in a homecage environment (Figure 1a). When compared to an intraperitoneal (i.p.) vehicle administration, AM251 (i.p.) significantly altered the homecage behavioural profile of mice, as shown by the raster plots depicting the behaviours of each mouse over the 15-min epoch (Figure 1b). Specifically, AM251 administered animals spent significantly more time walking, rearing, and grooming, than vehicle animals, paralleling behavioural changes in mice exposed to acute stress (Füzesi et al., 2016; Figure 1c). AM251 also decreased surveying, $t(14) = 3.415$, $P = 0.0042$, while having no effect on chewing, $t(14) = 2.142$, $P = 0.0503$, digging, $t(14) = 0.06254$, $P = 0.5418$, or resting $t(14) = 1.534$, $P = 0.1474$. To further support eCB signalling disruption producing a stress-like response, CORT levels were measured in the blood 30 min following behaviour recording onset. AM251 significantly increased circulating CORT levels (Figure 1d). AM251 also increased Fos expression in the PVN. Furthermore, focusing in on the stress-integrative PVN CRH neurons, AM251 administration dramatically increased Fos expression in these neurons (Figure 1e,f). Moreover, the proportion of Fos expression in non-CRH expressing neurons did not differ between treatments (Figure S2). Together, these results highlight the importance of eCB signalling in constraining activation of a stress-like response in a low stress, familiar environment.

3.2 | Pharmacological characterization of tonic eCB regulation of the stress-like response

The CB_1 receptor is a G protein-coupled receptor with a high degree of constitutive signalling: tonic eCB signalling can be both agonist-dependent (mediated by the release of AEA or 2-AG) and agonist-

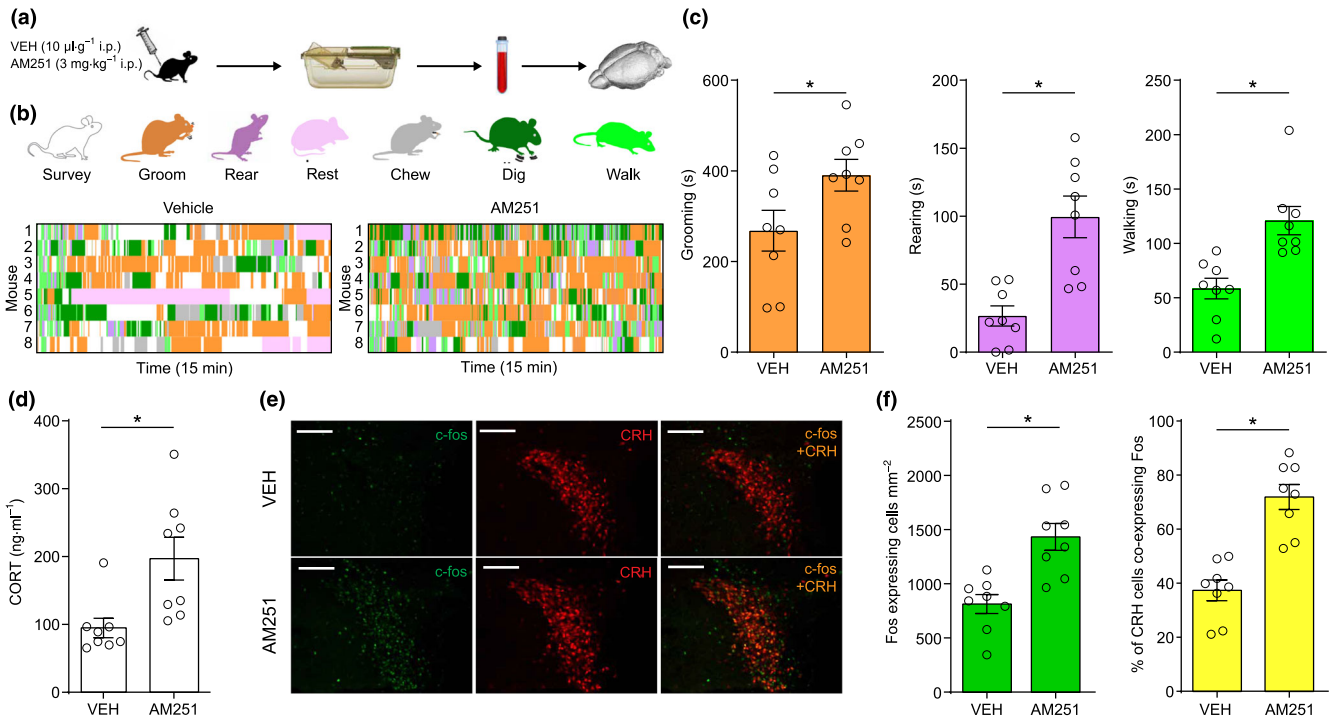

FIGURE 1 CB_1 receptor blockade precipitates a stress-like phenotype. (a) Procedural outline showing drug administration 15 min before homecage behavioural recording, 45 min before blood collection and 105 min before brain extraction. (b) Raster plot shows the detailed behavioural output throughout the 15-min epoch, where each behaviour is colour coded based on the provided index. (c) Quantification of grooming, $t(14) = 2.153$, $P = 0.0492$, rearing, $t(14) = 4.291$, $P = 0.0007$, and walking, $t(14) = 3.892$, $P = 0.0016$, behaviours following vehicle (VEH) or AM251 (VEH: n = 8/AM251: n = 8). (d) Corticosterone (CORT) levels following vehicle (VEH) or AM251 administration, $t(14) = 2.960$, $P = 0.0103$, and (e) Fos immunohistochemical activity (green) in CRH expressing neurons (red) in the PVN following VEH (left) or AM251 (right). (f) Quantification of Fos expressing cells (left), $t(14) = 4.086$, $P = 0.0011$, and % of CRH expressing cells that co-expressed Fos (right), $t(14) = 5.769$, $P < 0.001$. Scale bars: 150 µm, *$P < 0.05$. Total sample size: n = 16.

independent (constitutive G protein signalling). To establish if tonic eCB regulation of the stress-like response is driven by eCB signalling itself or via constitutive receptor activity, we employed the use of the neutral CB_1 receptor antagonist NESS0327 (i.p.; Figure 1a), which, unlike AM251, blocks ligand-dependent activation of CB_1 receptors but does not prevent agonist-independent constitutive signalling (which is blocked by AM251). In comparison to VEH, mice administered NESS showed significantly more homecage grooming (Figure 2b) and digging behaviours, $t(13) = 2.243$, $P = 0.0429$, elevated circulating CORT levels (Figure 2c) and elevated Fos expression in PVN neurons (Figure 2d,e). Beyond stereotypic behaviours, NESS administration had no influence on surveying, $t(13) = 1.875$, $P = 0.0834$, walking, $t(13) = 1.277$, $P = 0.2238$, rearing, $t(13) = 1.346$, $P = 0.2012$, or sleeping, $t(13) = 1.075$, $P = 0.3019$, but decreased time spent chewing, $t(13) = 3.111$, $P = 0.0083$. One mouse was excluded due to incorrect injection volume. These results suggest that active, tonic eCB signalling of AEA and/or 2-AG at CB_1 receptors governs inhibition of stress responsive networks at rest.

To determine which eCB ligand mediates tonic suppression of stress-like responses, we utilized pharmacological inhibition of both AEA and 2-AG biosynthesis. AEA is primarily synthesized by NAPE-PLD, and an inhibitor of this enzyme, LEI401, has recently been developed and produces robust reductions in central AEA content (Mock et al., 2020). Using this tool, we sought to determine if depletion of AEA produced alterations in homecage grooming behaviour and HPA axis activity, similar to AM251, NESS0327 and stress exposure (Figure 3a). The NAPE-PLD inhibitor, LEI401, was administered systemically 2 h before recording behaviours. Interestingly, LEI401 treated mice exhibited significantly more stereotypic grooming behaviours when compared to animals who received VEH injections (Figure 3c). Consistent with this, LEI401 administration also activated the HPA axis with marked increases in circulating CORT levels

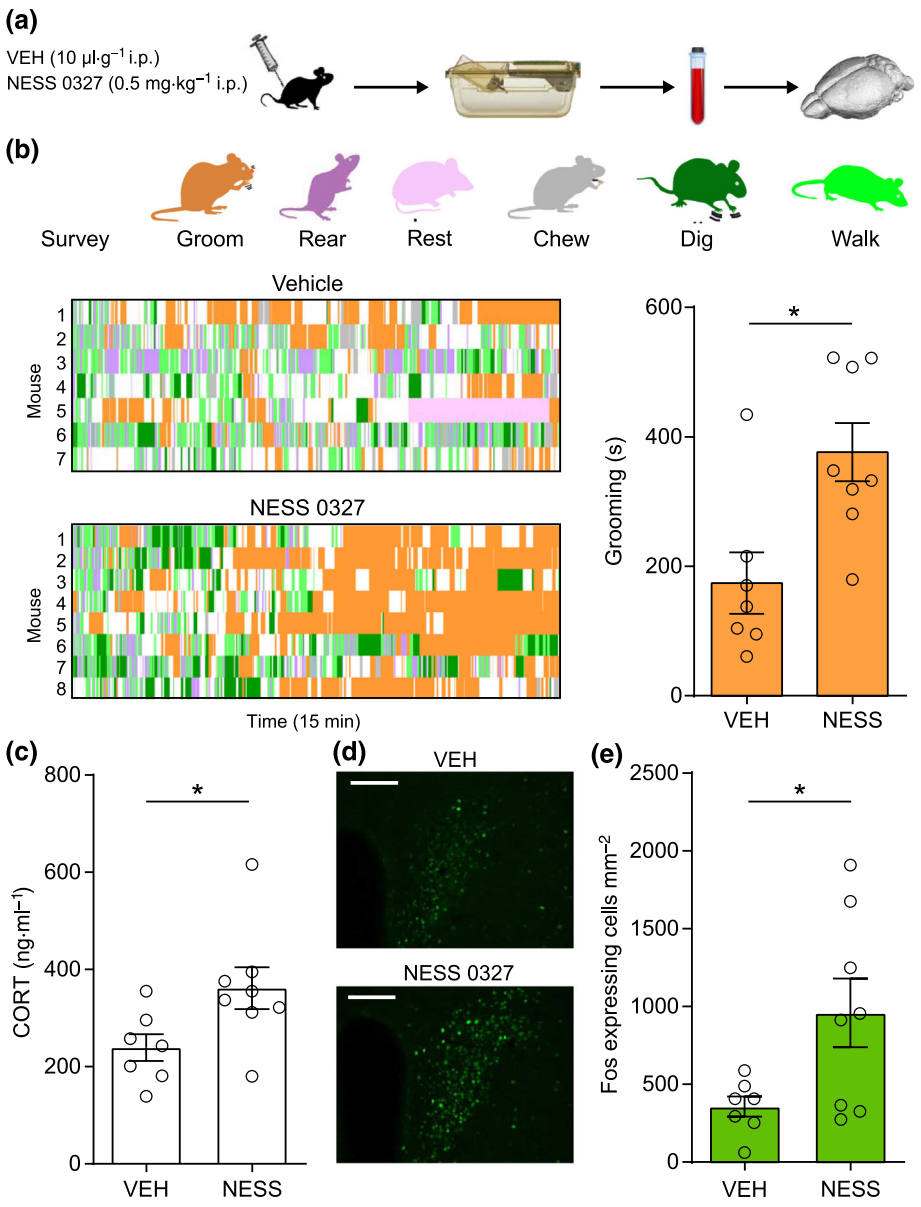

FIGURE 2 A neutral CB_1 receptor antagonist initiates the stress response. (a) Mice were administered vehicle (VEH) or NESS0327 15 min before homecage behaviour recording, 45 min before blood collection and 105 min before brain extraction (VEH: n = 8/NESS0327: n = 8). (b) Quantification of grooming in seconds, $t(13) = 3.089$, $P = 0.0086$, and a detailed homecage behavioural sequelae following VEH (top) or NESS (bottom) administration. (c) Circulating corticosterone (CORT) levels following VEH or NESS administration, $t(13) = 2.318$, $P = 0.0374$. (d) Fos immunohistochemistry expression following VEH (left) or NESS (right). Scale bars: 150 µm, $^*P < 0.05$. (e) Quantification of Fos density in the PVN following VEH or NESS, $t(13) = 2.459$, $P = 0.0287$. Total sample size: n = 16. Animals excluded from analysis and data visualization are due to experimenter error.

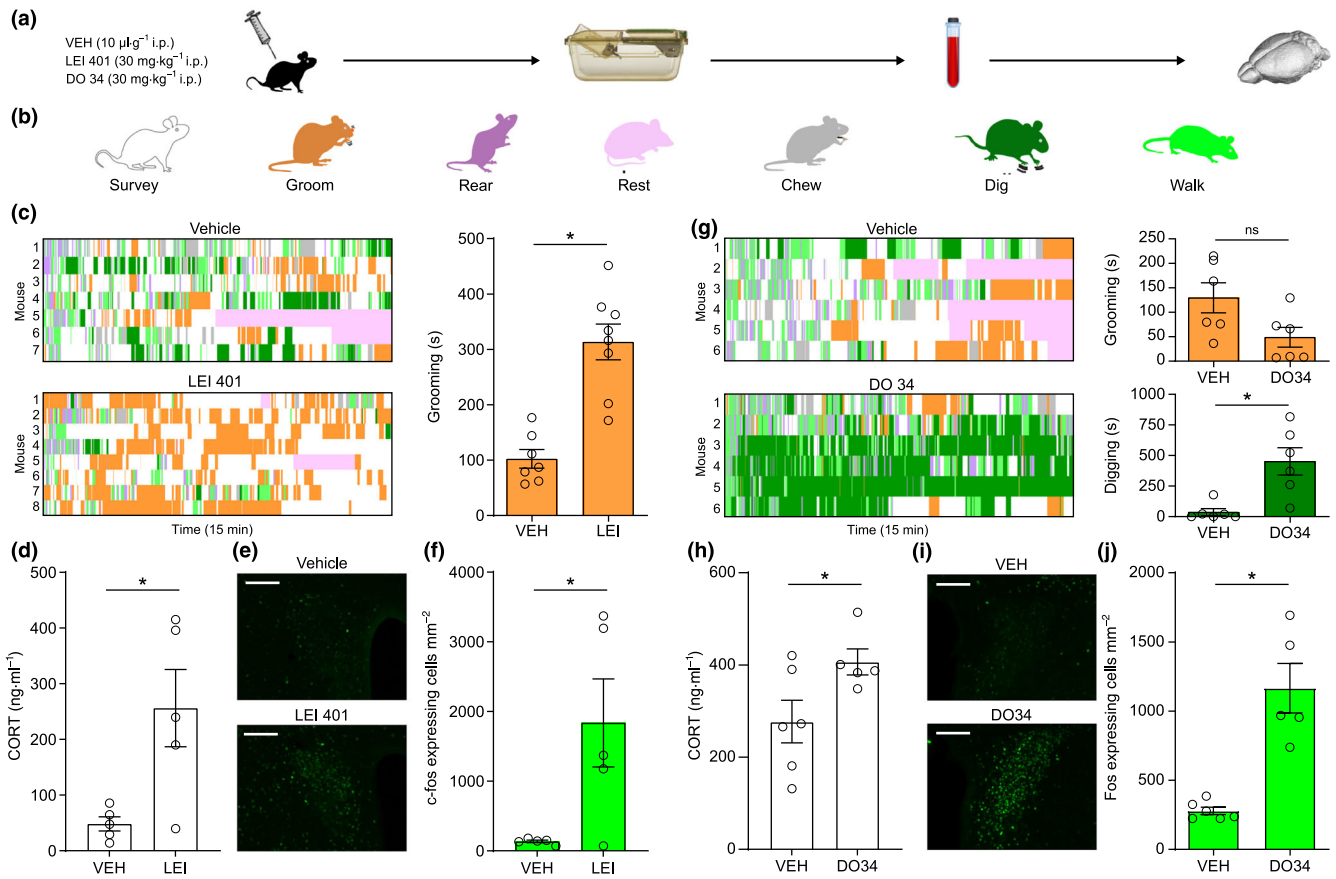

FIGURE 3 Inhibition of endocannabinoid biosynthesis activates a stress response. (a) LEI401 or DO34 were administered 2 h before homecage behaviour recording, 2.5 h before blood collection and 3.5 h before brain extraction. (b) Colour index of quantified behaviours throughout the behavioural epoch. (c) Detailed homecage behavioural sequelae following vehicle (VEH) (top) or LEI401 (bottom) administration and quantification of grooming in seconds (s), $t(13) = 5.535$, $P < 0.0001$. (d) Quantification of circulating corticosterone (CORT), $t(8) = 2.947$, $P = 0.0185$. (e) Representative images of Fos immunohistochemistry expression following VEH (left) or LEI401 (right); scale bars: 150 µm. (f) Quantification of Fos in the PVN following VEH or LEI401, $t(8) = 2.694$, $P = 0.0273$. (g) Detailed homecage behavioural sequelae following VEH (top) or DO34 (bottom) administration and quantification of digging, $t(10) = 3.602$, $P = 0.0048$, and grooming, $t(10) = 2.192$, $P = 0.0532$, in seconds (s). (h) Circulating corticosterone (CORT) levels following VEH or DO34 administration, $t(17) = 2.275$, $P = 0.049$. (i) Representative images of Fos immunohistochemistry expression in the PVN; scale bars: 150 µm. (j) Quantification of Fos density in the PVN, $t(9) = 5.402$, $P = 0.0004$, following VEH (left) or DO34 (right), $^*P < 0.05$. Group sizes for LEI401 experiment: Total (n = 16); VEH (n = 8); LEI401 (n = 8). Behaviour and HPA measures were taken from different cohorts. Group sizes for DO34 experiment: Total (n = 12); VEH (n = 6); DO34 (n = 6). Animals excluded from analysis and data visualization were a result of experimenter error.

(Figure 3d) and Fos expression in the PVN (Figure 3e,f). It is worth noting that LEI401 also decreased walking, $t(13) = 3.345$, $P = 0.0053$, rearing, $t(13) = 2.178$, $P = 0.0484$, and digging, $t(13) = 2.912$, $P = 0.0121$, behaviours while having no effect on chewing, $t(13) = 2.096$, $P = 0.0562$, resting, $t(13) = 1.00$, $P = 0.3356$, or surveying, $t(13) = 1.236$, $P = 0.2384$.

While many studies have suggested that AEA may be important for tonic eCB signalling (Gray et al., 2015; Hill et al., 2009; Natividad et al., 2017; Yasmin et al., 2020), there is also evidence that 2-AG may have tonic signalling capabilities (Lee et al., 2015; Marcus et al., 2020). To examine the impact of pharmacological depletion of 2-AG on stress-related measures, we inhibited 2-AG biosynthesis via administration of the DAGL inhibitor DO34 (Ogasawara et al., 2016). Two hours following DO34 administration, behavioural recordings revealed no significant differences in grooming behaviours when compared to VEH administration (Figure 3g). We further investigated the behavioural profile, which revealed that DO34 produced dramatic significant increases of repetitive digging behaviours (Figure 3g), suggesting the possibility that the excess engagement in this behaviour masked any notable differences in grooming behaviour. However, animals receiving the DAGL inhibitor exhibited robust elevations in circulating CORT (Figure 3h), and Fos expression in the PVN (Figure 3i,j), indicating an activation of the HPA axis and stress-like response. CORT and Fos measurements were excluded for one animal due to a stressful disruption following their behavioural measurements. Meanwhile, DO34 administration did not alter walking, $t(10) = 0.6030$, $P = 0.5599$, rearing, $t(10) = 0.7796$, $P = 0.4537$, chewing, $t(10) = 1.257$, $P = 0.2373$, or resting, $t(10) = 0.6758$, $P = 0.5145$, and led to a decrease surveying behaviour, $t(10) = 3.377$, $P = 0.0070$. Overall, it appears that both AEA and 2-AG tonically regulate the HPA axis in

a low stress environment, but the behavioural profiles evoked by these two treatments do differ, with depletion of AEA directly mirroring the effects of CB_1 receptor antagonism or stress exposure (Füzesi et al., 2016), while depletion of 2-AG produces large-scale changes in different repetitive behaviours but produces activation of neurons in the PVN and elevations in circulating CORT.

3.3 | The PVN is a critical hub for eCB regulation of the HPA axis

CRH neurons in the PVN orchestrate many aspects of the stress response, and previous work has suggested that the ability of stress to increase grooming behaviour also requires activation of these neurons (Füzesi et al., 2016). To determine if disruption of CB_1 receptor signalling activated CRH neurons in the PVN to drive stress-induced behavioural changes, we utilized optogenetics. Using the same technique employed in prior acute stress studies (Füzesi et al., 2016), the PVN of CRH-Cre mice was infected with a cre-dependent inhibitory opsin, Arch3.0-eYFP or a control eYFP virus (Figure 4a). Following expression of the virus and implantation of ferrules over the PVN, mice were given either AM251 or VEH prior to behavioural recording.

During homecage recording, continuous yellow light (532 nm) was delivered over the PVN (Füzesi et al., 2016) (Figure 4b). One-way ANOVA reported an effect of treatment, and post hoc analysis revealed that within the group expressing eYFP, consistent with our initial study, AM251 significantly increased grooming behaviours when compared to VEH administered mice ($P = 0.0012$). In contrast, animals expressing Arch3.0 exhibited less AM251 induced grooming than animals expressing eYFP ($P = 0.0152$) and did not differ from eYFP animals administered VEH ($P = 0.1971$) (Figure 4c,d). Thus, inhibiting CRH neurons in the PVN prevents AM251 from elevating stereotypic grooming similar to what has been established with stress. Otherwise, there were no differences in surveying, $F(2, 15) = 0.8767$, $P = 0.4364$, walking, $F(2, 15) = 0.3426$, $P = 0.7154$, rearing, $F(2, 15) = 1.436$, $P = 0.2687$, digging, $F(2, 15) = 0.6578$, $P = 0.5323$, chewing, $F(2, 15) = 0.2272$, $P = 0.7995$, or sleeping, $F(2, 15) = 2.643$, $P = 0.1040$.

Based on the optogenetic data provided above, local CRH neurons in the PVN appear to be orchestrating the stress-like stereotypic grooming following AM251 administration, similar to what is seen following acute stress. CB_1 receptors are expressed within the PVN proper, and activation of these receptors is known to locally influence excitatory and inhibitory synaptic transmission onto CRH neurons and regulate the stress response (Colmers & Bains, 2018; Di et al., 2003; Evanson et al., 2010; Nahar et al., 2015; Wamsteeker et al., 2010). As such, we next investigated whether local tonic eCB signalling in this region acts to gate the stress-like responses produced by AM251. Following intracranial cannula implantation, AM251 was administered directly into the PVN 15 min before behavioural recording (Figure 5a). Mice that received direct intra-PVN infusion of AM251 exhibited significantly increased stereotypic grooming behaviours when compared to VEH infused animals (Figure 5d,e). Furthermore, intra-PVN AM251 infusions also resulted in elevated circulating CORT levels (Figure 5f). These results suggest that tonic eCB signalling within the PVN proper is restricting activation of a stress response in ambient conditions and that disruption of this tone can produce the generation of a stress-like response. Central AM251 administration had no effects on walking, $t(12) = 0.1237$, $P = 0.9036$, rearing, $t(12) = 0.4205$, $P = 0.6816$, chewing, $t(12) = 1.417$, $P = 0.1820$, sleeping, $t(12) = 1.265$, $P = 0.2298$, digging, $t(12) = 1.357$, $P = 0.1997$, or surveying $t(12) = 0.4842$, $P = 0.6370$. Based on the abundance of research pointing to the striatum as a regulator of grooming behaviours, we also investigated whether tonic eCB signalling was regulating self-directed behaviours here as well. Interestingly, administration of AM251 directly to the striatum had no effect on grooming behaviours when compared to VEH administrations (Figure 5h,i), indicating that this

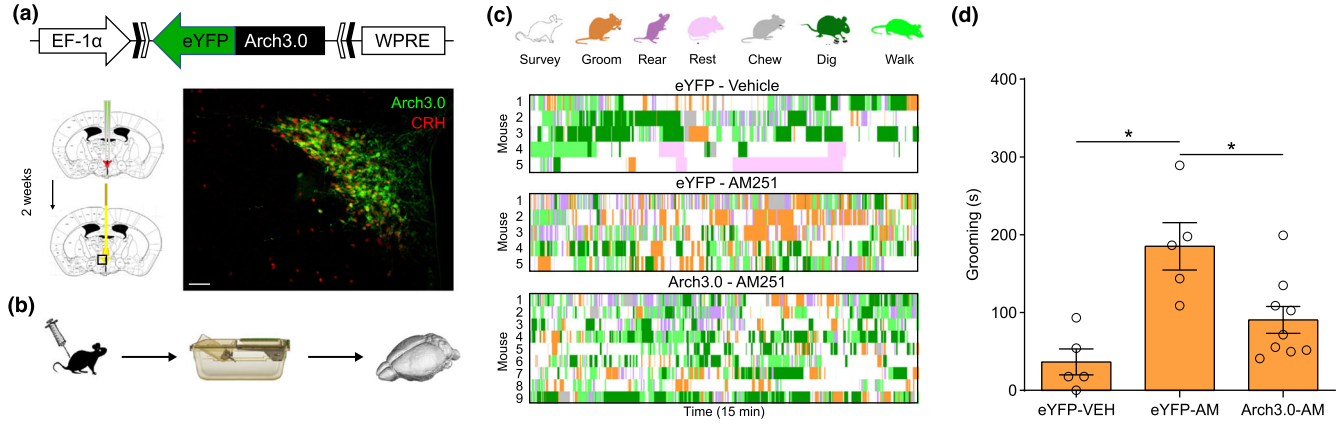

FIGURE 4 Inhibition of CRH neurons in the PVN blocks AM251 induced self-directed behaviours. (a) Cre-dependent AAV DIO Arch3.0-eYFP virus injected bilaterally into the PVN. (b) Animals were injected with vehicle (VEH) or AM251 15 min prior to constant 593 nm light exposure inside the homecage. (c) Detailed behavioural output over the entire 15-min behavioural epoch for each mouse. (d) Histogram analysing grooming behaviour in seconds (s) between groups, $F(2, 15) = 9.425$, $P = 0.0022$. *$P < 0.05$. Group sizes: Total (n = 31); eYFP-VEH (n = 10); eYFP-AM (n = 10); Arc3.0-AM (n = 11). Animals excluded from analysis and data visualization were a result of incorrect virus or ferrule placements.

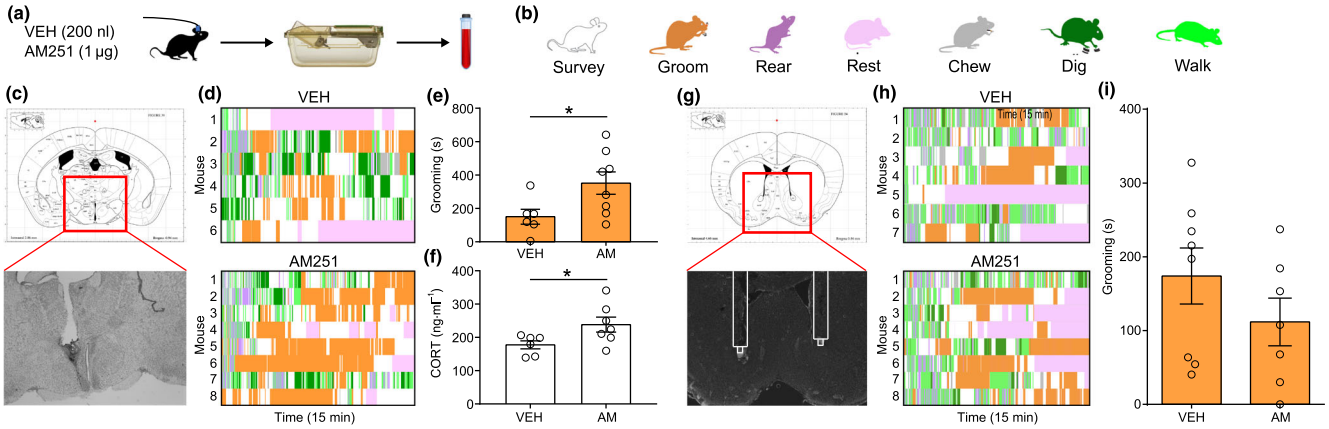

FIGURE 5 Local AM251 administration into the PVN initiates a stress-like phenotype. (a) Unilateral administration of vehicle (VEH) or AM251 15 min prior to homecage behavioural recording, 45 min before blood collection and 105 min before brain extraction. (b) Colour index of quantified behaviours throughout the behavioural epoch. (c) Histology of cannula placements into the PVN. (d) Detailed analysis of homecage behaviours following intra-PVN vehicle or AM251 over a 15-min epoch and (e) histogram quantification of grooming behaviour in seconds (s) following VEH or AM251 infusions, $t(12) = 2.317$, $P = 0.0390$. (f) Circulating corticosterone (CORT) levels following VEH or AM administration, $t(11) = 2.255$, $P = 0.0455$. (g) Bilateral administration of VEH or AM251 into the striatum 15 min prior to (h) homecage behavioural recording where (i) grooming was quantified, $t(13) = 1.231$, $P = 0.24$, alongside all other behaviours. $^*P < 0.05$. Group sizes for the PVN experiment: Total (n = 24); VEH (n = 12); AM251 (n = 12). Group sizes for the striatum experiment: Total (n = 24); VEH (n = 12); AM251 (n = 12). Animals excluded from analysis and data visualization are a result of incorrect cannula placements.

effect was at least partially specific to the PVN. Consistent with this, striatal administrations of AM251 also had no effect on walking, $t(13) = 0.5981$, $P = 0.5601$, rearing, $t(13) = 0.6569$, $P = 0.5227$, chewing, $t(13) = 0.7443$, $P = 0.4699$, resting, $t(13) = 0.2804$, $P = 0.7836$, digging, $t(13) = 0.3881$, $P = 0.7042$, or surveying, $t(13) = 1.072$, $P = 0.3033$.

4 | DISCUSSION

The current study has expanded previous work regarding eCB regulation of the HPA axis and stress response by demonstrating that tonic signalling of both AEA and 2-AG, acting at the CB_1 receptor within the PVN, restricts activation of the HPA axis at rest. Disruption of tonic eCB signalling produces rapid activation of the HPA axis and the manifestation of a behavioural stress-induced response. Importantly, the manifestation of the stress-like response was driven by local actions of eCB signalling within the PVN proper and that optogenetic silencing of CRH neurons in the PVN inhibited this response. Together, these data indicate that tonic eCB signalling in the PVN restricts activation of CRH neurons in the absence of a threat or challenge, thereby providing an active mechanism of restraint on the HPA axis in ambient conditions.

AM251, as well as many other pharmacological tools used to block CB_1 receptor signalling, are both antagonist and inverse agonist, making the mechanism by which they disrupt tonic eCB signalling complex. Disruption of eCB signalling could be due to blockade of eCB molecules binding to the CB_1 receptor or it could be due to suppression of agonist-independent constitutive signalling of the CB_1 receptor. Differential physiological and behavioural processes have been ascribed to both forms of tonic eCB signalling suggesting that both pathways are potential mechanisms (Lee et al., 2015; Meye et al., 2013; Sink et al., 2010). To address this question, we utilized a neutral antagonist, NESS0327, which only blocks agonist driven CB_1 receptor activation but does not influence constitutive receptor signalling (Ruiu et al., 2003). Similar to what was found following administration of AM251, NESS0327 increased Fos expression in the PVN, elevated CORT and increased grooming behaviour. This indicates that tonic eCB regulation of CRH neurons in the PVN, and activation of the HPA axis, is driven by eCB molecules signalling directly on the CB_1 receptor, and not by constitutive CB_1 receptor signaling.

Previous work has suggested that AEA is likely to be the eCB molecule mediating tonic inhibition of the HPA axis. AEA levels are known to decline in response to stress, and inhibition of AEA metabolism by fatty acid amide AEA hydrolase (**FAAH**) attenuates many endocrine and neurobehavioral responses to stress (Morena et al., 2016). The amygdala, particularly the basolateral nucleus (BLA), is a hub for some of the stress constraining effects of AEA (Bedse et al., 2014; Ganon-Elazar & Akirav, 2009; Gray et al., 2015; Hill et al., 2009). AEA provides tonic inhibition onto glutamatergic afferents into the BLA, and a loss of AEA signalling following stress exposure removes this tonic inhibition, resulting in increased excitatory drive onto BLA pyramidal neurons (Bedse et al., 2017; Yasmin et al., 2020). It is possible that a similar mechanism is at play within the PVN as CB_1 receptors have been found to regulate excitatory afferents impinging upon CRH neurons in the PVN (Di et al., 2003; Wamsteeker et al., 2010). However, synaptic studies do not indicate that this input is subject to tonic inhibition by eCB signalling (Di et al., 2003; Wamsteeker et al., 2010), like it is in the BLA (Bedse et al., 2017; Yasmin et al., 2020). Alternatively, local CRH neuron

microcircuits, which regulate GABAergic inputs onto parvocellular neurons in the PVN (Jiang et al., 2018), could represent a different circuit through which alterations in eCB signalling could be acting to modulate the stress response. This effect could also involve local CB_1 signalling on astrocytes, as eCB signalling can regulate the release of gliotransmitters (Covelo et al., 2021; Navarrete et al., 2014), and CB_1 signalling on astrocytes is known to be important for other stress-related processes such as fear learning (Martin-Fernandez et al., 2017). Perhaps disruption of eCB signalling onto, or from, neighbouring astrocytes could influence the excitability of CRH neurons, thus regulating HPA axis activity. Further work is required to understand the mechanism by which local eCB signalling gates activation of CRH PVN neurons.

As disruption of 2-AG produced similar neuroendocrine effects to that of antagonism of the CB_1 receptor or depletion of AEA, it seems likely that there is tonic 2-AG signalling that is constraining HPA axis activity. The lack of effect of 2-AG depletion on grooming behaviour could be due to this pharmacological approach triggering a dramatic elevation of a different stereotyped behaviour which masked changes in grooming behaviour. Animals treated with DO34 spent the overwhelming majority of their time burying their noses in the bedding then ploughing them back and forth throughout the cage. Others have referred to this behaviour as "inchworming," and it has been identified as a stereotyped behaviour sometimes seen in rodent models of autism (Smith et al., 2014). It is unclear what is driving this behaviour to occur, but given that 2-AG signalling in the striatum is known to regulate motor behaviour (Lerner et al., 2010; Shonesy et al., 2013), it seems possible that depletion of 2-AG results in the engagement of a striatal circuit that triggers this behavioural stereotypy, which, in turn, overrides the expression of grooming behaviour. Further work is required to understand the nature and significance of this behaviour, but regardless, the neuroendocrine evidence seen herein indicates that 2-AG signalling does indeed provide tonic inhibitory control over the HPA axis.

While the canonical view is that CRH neurons are primarily neuroendocrine regulators, accumulating evidence over the past few years has demonstrated that this discrete cluster of neurons also has important and complex roles in coordinating behavioural responses to varying degrees of threat and reward (Daviu et al., 2020; Li et al., 2020; Sterley et al., 2018; Yuan et al., 2019). Interestingly, many of these processes are similarly influenced by changes in eCB signalling. As the ability of AM251 to trigger grooming responses was inhibited by optogenetic silencing of PVN CRH neurons, it would be important to understand whether eCB modulation of other aspects of threat and defensive behaviour will similarly involve modulation of PVN CRH neurons. Despite grooming being a relatively non-specific behaviour that can be triggered by a host of stimuli and be mediated through different circuits, the current data do indicate that its expression is dependent on activation of PVN CRH neurons, similar to what is seen following stress (Füzesi et al., 2016). This is consistent with the fact that direct intra-cranial administration of CRH reliably triggers grooming behaviour as well (Sherman & Kalin, 1987). Despite the well-known importance of striatal circuits in driving grooming behaviours (Ahmari et al., 2013; Ramírez-Armenta et al., 2022; Zhang et al., 2021), consistent with our other data, local administration of AM251 into the PVN produced an increase in grooming, while a comparable manipulation into the striatum had no effect. That being said, a direct connection from CRH PVN neurons to the globus pallidus has recently been identified (Hunt et al., 2018), providing an anatomical route through which changes in CRH PVN neuron activity could rapidly modulate motor circuits to trigger grooming.

In terms of limitations of the current study, while we saw low non-specific labelling (Mean = 4.1%; Figure S1), we cannot completely rule out the possibility that the opsin used here did not spread to non-CRH cells in the PVN. Additionally, these studies were performed using only male mice. Investigating sex differences is an important part of understanding neural physiology, especially in the context of stress. However, previous work has provided evidence for both sexes exhibiting similar eCB responses to stress (Vecchiarelli et al., 2022), and the ability of AM251 to increase CORT levels during the daily trough of the HPA axis (which was when we tested mice in the current study) is seen in both males and females (Atkinson et al., 2010).

Taken together, these data clearly establish that tonic signalling of AEA and/or 2-AG, within the PVN, regulates the activation of CRH neurons in the PVN. Disruption of this tonic eCB signalling triggers the assembly of a neurobehavioral and endocrine phenotype that parallels what occurs in response to stress exposure. These data confirm that disruption of eCB signalling is sufficient to generate a stress-like response using multiple readouts and various pharmacological approaches. Both rodent and human studies have found that stress can produce declines in eCB signalling (Hill et al., 2009; Mayo, Asratian, Lindé, Holm, et al., 2020; McLaughlin et al., 2012; Patel et al., 2005; Rademacher et al., 2008; Spohrs et al., 2022; Yasmin et al., 2020), suggesting that a loss of eCB signalling may not only be sufficient, but also necessary, for the initiation of a stress response. A high proportion of humans who were taking the anti-obesity drug Rimonabant, which is a CB_1 receptor antagonist, discontinued their use due to the onset of stress-related psychiatric symptoms, particularly anxiety and depression (Christensen et al., 2007; Hill & Gorzalka, 2009). These outcomes are consistent with the consequence of a persistently enhanced drive on PVN CRH neurons due to chronic disruption of eCB signalling. These data may also help to explain the low stress/anxiety phenotype seen in humans carrying a mutation in the FAAH gene, which results in reduced AEA metabolism and tonic elevations in eCB signalling (Dincheva et al., 2015; Gunduz-Cinar et al., 2013; Hariri et al., 2009; Mayo, Asratian, Lindé, Holm, et al., 2020; Spagnolo et al., 2016). Perhaps the basal elevations in eCB tone produced by this gene variant provide enhanced restriction of stress responsive neural circuits, centrally involving CRH neurons in the PVN. As FAAH inhibitors are currently under investigation for the management of anxiety and stress-related conditions (Mayo, Asratian, Lindé, Morena, et al., 2020; Paulus et al., 2021; Schmidt et al., 2021), with some encouraging data published to date from human clinical trials, this ability of tonic eCB signalling to constrain PVN CRH neuron activity may prove to be an important mechanism

in this process. Collectively, these data highlight the importance of eCB signalling in the regulation of stress and the HPA axis and indicate that CRH neurons in the PVN are an important nexus for this interaction.

AUTHOR CONTRIBUTIONS

Gavin N. Petrie and Matthew N. Hill designed the experiments; Gavin N. Petrie performed the majority of experiments and wrote the initial draft of the manuscript and made the figures and worked with Matthew N. Hill on revising and finalizing the manuscript. Georgia Balsevich, Tamás Füzesi and Robert J. Aukema all made substantial contributions to the acquisition of data, revising the manuscript and assisting with figure development. Wouter P. F. Driever and Mario van der Stelt contributed to the acquisition of data by providing critical pharmacological agents and assisting with revising the manuscript. Jaideep S. Bains contributed to the conception and design of the studies and provided substantial input on the revisions of the manuscript.

CONFLICT OF INTEREST STATEMENT

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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