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CHEMOGENETIC ACTIVATION OF THE LATERAL HYPOTHALAMUS REVERSES EARLY LIFE STRESS-INDUCED DEFICITS IN MOTIVATIONAL DRIVE

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ABSTRACT

Altered motivated behaviour is a cardinal feature of several neuropsychiatric conditions including mood disorders. One well-characterized antecedent to the development of mood disorders is exposure to early life stress (ELS). A key brain substrate controlling motivated behaviour is the lateral hypothalamus (LH). Here we examined the effect of ELS on LH activation and the motivation to self-administer sucrose. We tested whether chemogenetic activation of LH circuits could modify sucrose responding in ELS rats and examined the impact on LH cell populations. Male rat pups were maternally separated for 0hrs or 3hrs on postnatal days 2-14. During adolescence, rats received bilateral injections of hM3D(Gq) into LH. In adulthood, rats were trained to self-administer sucrose and tested under a progressive ratio schedule to determine their motivation for reward following injection with either vehicle or 5mg/kg clozapine-N-oxide. Brains were processed for Fos-protein immunohistochemistry. ELS significantly suppressed lever responding for sucrose, indicating a long lasting impact of ELS on motivation circuits. hM3D(Gq) activation of LH increased responding, normalizing deficits in ELS rats, and increased Fos-

positive orexin and MCH cell numbers within LH. Our findings indicate that despite being susceptible to environmental stressors, LH circuits retain the capacity to overcome ELS-induced deficits in motivated behaviour.

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INTRODUCTION

Early life stress (ELS) is a known antecedent to the development of psychiatric conditions including disorders of mood and emotion in adulthood (Browne, 1986; Graham *et al.*, 1999). For example, cardinal symptoms of depression include behavioural despair and low motivational drive towards stimuli that are normally naturally rewarding. To study the brain pathways responsible for the expression of these symptoms in humans, preclinical research in rodents has used various animal models including separation of rodent pups from their mother in the early postnatal period. Importantly, the maternal separation model of ELS closely recapitulates the neuroendocrine and behavioural abnormalities observed in humans after early life trauma (Winslow & Insel, 1991; Plotsky & Meaney, 1993). Studies in humans and animals addressing ELS-induced changes in mood and behaviour have largely focused on the neuroendocrine hypothalamus and associated feedback sites such as the hippocampus (Plotsky & Meaney, 1993; Meaney *et al.*, 1996). However, other hypothalamic areas that play a more specific role in motivational drive are also likely to be vulnerable to ELS. For example, lesion and electrical stimulation studies have shown that the lateral hypothalamus (LH) is an important substrate for motivated behaviour. Indeed, the LH was identified as one of the first brain areas to support intra-cranial self-stimulation (Olds & Milner, 1954; Wise, 1974) and a number of recent studies have highlighted the role of distinct cell types in LH that control motivational drive towards natural rewards (Jennings *et al.*, 2013; Jennings *et al.*, 2015). To date however, there have been relatively few studies addressing the impact of ELS on LH circuits and behaviours thought to be controlled by this area of the hypothalamus.

As alluded to above, the LH contains a number of distinct cell populations including those that express GABA and glutamate some of which also co-express neuropeptide transmitters such as melanin-concentrating hormone (MCH) or orexin. Many LH neurons project to other brain regions implicated in motivated behaviour such as the ventral tegmental area (VTA) however, there are also significant numbers of local (intra-LH) inhibitory and excitatory neurons. Recently, Jennings and colleagues showed that optogenetic stimulation of a distinct population of GABAergic neurons (orexin and MCH-negative) in the LH profoundly increased food-seeking behaviours (Jennings *et al.*, 2013; Jennings *et al.*, 2015). Further, neurotensin and glutamate-expressing neurons in the LH have projections to the VTA

1 and support intra-cranial self-stimulation and reward seeking (Kempadoo *et al.*,
2 2013). Meanwhile, another set of neurons that express orexin and glutamate modulate
3 reward seeking via VTA-dependent mechanisms (Borgland *et al.*, 2006; James *et al.*,
4 2011). However, despite these studies focusing on LH manipulations that enhance
5 reward-seeking behaviour from normal (baseline) levels, it is unclear whether
6 manipulations targeting LH can overcome a state of low motivational drive produced
7 by exposure to ELS.

8 In this context, we have previously reported that ELS exposure produced a
9 lower level of activity in the open field in adult rats after a period of psychological
10 stress (James *et al.*, 2014). Notably, no changes in anxiety-like behaviour were
11 observed in ELS versus control animals suggesting that ELS produced a phenotype
12 more akin to low motivational drive. With respect to LH circuit function in these
13 animals, we found that orexin neurons were hyporesponsive to restraint stress, but the
14 impact on other LH cell populations, including MCH or GABA-interneurons, was not
15 assessed. To further validate the phenotype of animals exposed to ELS, in the present
16 study we tested maternally separated animals for sucrose self-administration (10%)
17 under progressive ratio (PR) conditions. Based on our previous data, we hypothesized
18 that ELS would reduce motivational drive for sucrose under PR conditions.
19 Consistent with this prediction, ELS animals displayed reduced lever pressing for
20 sucrose reinforcement under progressive ratio conditions compared to controls. We
21 next sought to test whether a chemogenetic strategy using designer receptors
22 exclusively activated by designer drugs (DREADD) to activate LH circuits could
23 positively modify sucrose responding in ELS animals. DREADD is a relatively new
24 technique that permits experimental control over neuronal activity. This is achieved
25 by genetic insertion of mutant human muscarinic receptors (hM3D(Gq) or hM4D(Gi))
26 into specific neuronal cell types (Armbruster *et al.*, 2007). hM3D(Gq) or hM4D(Gi)
27 receptors only bind to the inert agonist clozapine-*N*-oxide (CNO) and their actions
28 depolarise or hyperpolarise neurons. In order to characterize the impact of DREADD
29 on neuronal activity we bath applied CNO to hypothalamic slices from DREADD
30 transduced animals and made electrophysiological recordings of LH neurons. We also
31 assessed CNO-induced activity using Fos mapping in LH neuronal cell populations.

33 METHODS AND MATERIALS

34 *Ethics statement*

All experiments performed were approved by the University of Newcastle Animal Care and Ethics Committee, and carried out in accordance with the National Health and Medical Research Council Australian Code of Practice for the care and use of animals for scientific practice.

Animals

12 experimentally naïve Wistar dams were obtained from the Animal Resources Centre, Canning Vale, Western Australia, and bred with six experimentally naïve males at the University of Newcastle. A total of 52 male rats were included in the study. On PND1, the day of birth, each litter was randomly allocated to the ELS group (3hr maternal separation) or the No ELS group. On PND21, rats were weaned to two rats per cage (Able Scientific, Sydney). Food (Rat and Mouse Pellets, Glen Forest, Western Australia) and water were available ad libitum and rats were maintained on reverse 12hr light/dark cycle (lights off at 0700hrs).

Early life stress (ELS)

The maternal separation procedure was performed as described by James et al. (James et al., 2014). Briefly, from PND2-14, litters in the ELS condition were removed from their home cage and individually separated in an alternate temperature controlled room (30-34°C) for 3hrs/day, from 9am–12pm. Pups in the No ELS condition remained undisturbed during this period except for weekly weighing.

Surgery

All surgeries were performed on rats between PND42-44 (~200grams). Rats were anesthetized with isoflurane prior to placing them in the stereotaxic frame (Stoelting instruments). Rats were injected intramuscularly with 0.3mL of a broad-spectrum antibiotic (150mg/mL procaine penicillin, 112.5mg/mL benzathine penicillin; Norbrook Laboratories, UK) and subcutaneously with 0.2mL of a 50mg/mL solution of carprofen (Norbrook Laboratories, UK).

Viral injections. rAAV5/hSyn-HA-hM3D(Gq)-IRES-mCitrine (UNC vector core, Lot# AV4619F) was bilaterally injected into the LH (AP: -2.4, ML: $\pm$ 1.9, DV: -8.4). 1 μ L was injected over 4mins and the needle was left in place for 2mins. Injections were made using a 2 μ L Hamilton Neuros syringe (30G) attached to a Stoelting Quintessential Stereotaxic Injector pump (Cat# 53311).

Behavioural procedures

Sucrose self-administration. On PND70-75, all animals were trained to self-administer sucrose (10% w/v) in daily 30min sound-attenuated operant chamber

1 sessions under a FR1 administration schedule (Med Associates, VT, USA). During
 2 FR1 training, responding on the active (right) lever resulted in the delivery of 0.1ml
 3 of sucrose into a receptacle followed by a 4sec light stimulus located above the active
 4 lever, indicating the availability of sucrose. The inactive (left) lever was not extended
 5 during FR1 training. FR1 sessions continued for eight days where on the first four
 6 days of FR1 training, all rats had restricted access to water, 2hrs/day (from 3-5pm), to
 7 facilitate acquisition of operant conditioning. Following FR1 training, rats progressed
 8 to a FR3 schedule for four days where both the active and inactive (left) levers were
 9 presented. Responding on the inactive lever was recorded but had no scheduled
 10 consequence. During the final stage of sucrose self-administration, PR responding
 11 was assessed during five 90min sessions using a reinforcement schedule where the
 12 number of lever presses required for sucrose reward delivery increased by 1 following
 13 each reward, ie. 1, 2, 3, 4, 5, etc. (Besheer *et al.*, 2008; Jupp *et al.*, 2011). Breakpoint
 14 was defined as the highest response ratio completed (ie. total number of rewards
 15 received) throughout the PR session. All behavioural testing took place between 9am
 16 and 3pm daily.

17 *Forced swim test.* A subgroup of animals was exposed to a forced swim test
 18 immediately prior to the last PR session. Briefly, rats were placed in 40cm of tepid
 19 water (25-30°C) and swimming, immobility and climbing behaviour were recorded
 20 for 10mins.

21 *Sacrifice and tissue harvesting.*

22 Two hours following the initiation of the final PR test, rodents were anaesthetized
 23 with sodium pentobarbitone (1ml intraperitoneal, i.p, Virbac, Australia) and perfused
 24 with 200mL of 0.1M Phosphate Buffered Saline (PBS) followed by 500mL of 4%
 25 paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde at
 26 4°C for 2hrs, then transferred to 30% sucrose in 0.1M PBS for 48hrs at 4°C. Brains
 27 were frozen on dry ice and stored at -80°C until sectioning.

28 *Dual Fos-protein and mCitrine immunohistochemistry.* Serial 40µm thick
 29 coronal sections of the forebrain were cut using a cryostat (Leica Biosystems
 30 CM1900, North Ryde, NSW, Australia) and a 1-in-4 series of hypothalamic sections
 31 (Bregma -2.60mm to -2.92mm) were processed for immunohistochemical detection of
 32 Fos-protein and mCitrine (GFP). The mCitrine reporter protein has efficient labeling
 33 for somata and proximal processes of virally transduced neurons (Ye *et al.*, 2017).
 34 Tissue was incubated for 48hrs in a 1:8000 primary Fos antibody concentration

(rabbit monoclonal, 5348S, Cell Signaling Technology, MA, USA) followed by a two hour incubation in a 1:1000 concentration donkey anti-rabbit secondary (711-065-152, Jackson IR, PA, USA). After visualisation of Fos-protein with glucose oxidase, sections were incubated for 72hrs in a 1:2000 primary anti-GFP antibody concentration (chicken polyclonal, ab13970, Abcam, MA, USA) followed by a two hour incubation in a 1:400 concentration donkey anti-chicken secondary (703-005-155, Jackson IR, PA, USA).

Dual Fos-protein and orexin immunohistochemistry. A second series of tissue was processed for immunohistochemical detection of Fos-protein and orexin. Briefly, free floating brain sections were incubated for 48hrs at room temperature in a solution of PBS azide containing 0.5% Triton X-100, 2% normal horse serum and a cocktail of primary antibodies (1:2000 c-Fos antibody, rabbit monoclonal, 5348S, Cell Signaling Technology, MA, USA and 1:1000 orexin A antibody, goat polyclonal, sc-8070, Santa Cruz Biotechnology, CA, USA). The selectivity of this antibody has been illustrated by Blanco-Centurion et al. (2013) and Campbell et al. (2015). Sections were then incubated for 2hrs at room temperature in a cocktail of secondary antibodies (1:1000, Alexa Fluor 594 donkey anti rabbit, Invitrogen, and 1:500, AMCA anti-goat, Jackson Immunoresearch). Sections were finally mounted onto gelatin-coated slides and coverslipped with gelvatol.

Dual Fos-protein and MCH immunohistochemistry. An adjacent series of tissue was processed for immunohistochemical detection of Fos-protein and MCH. Free floating sections were incubated for 72hrs at 4°C in a solution of PBS containing 0.5% Triton X-100, 2% normal horse serum and a cocktail of primary antibodies (1:2000 c-Fos antibody, rabbit monoclonal, 5348S, Cell Signaling Technology, MA, USA and 1:1000 MCH antibody, goat polyclonal, sc-14507, Santa Cruz Biotechnology, CA, USA). The concentration of MCH primary antibody used was the same as Petrovich et al. and Sharpe et al. (Petrovich *et al.*, 2012; Sharpe et al., 2017). Sections were then incubated for 3hrs at room temperature in a cocktail of secondary antibodies (1:200, Alexa Fluor 488 donkey anti rabbit, Invitrogen, and 1:200, Alexa Fluor 594 anti-goat, Jackson Immunoresearch).

HA and orexin or MCH immunohistochemistry. A forth series of tissue was processed for hemagglutinin (HA) immunoreactivity, the epitope tag fused to the hM3D(Gq) DREADD providing more precise localization of the DREADD receptors (Ye *et al.*, 2017), with MCH or orexin. All immunohistochemical methods were as

described above with HA in place of Fos. A 1:1000 primary HA antibody (mouse monoclonal, 901513, BioLegend, CA, USA) concentration was used with 1:400 Alexa Fluor 488 anti-mouse (Jackson ImmunoResearch) as the secondary.

Neuronal quantification

Tissue was imaged using Olympus cellSens imaging software (version 1.3) under a 10x microscopic objective (Olympus BX51). A 40x microscopic objective was used for HA immunohistochemistry. Images were quantified using iVision (Biovision) software where bilateral counts of Fos, MCH, orexin, HA and mCitrine were analyzed across Bregma levels (-2.60mm, -2.76mm and -2.92mm) by one observer, blind to treatment. Dual labeled cells were quantified by merging the two cell images in iVision. Given the spread of the virus, immunohistochemical quantification occurred in two regions of the hypothalamus, the perifornical/lateral hypothalamic area (LH) and the dorsomedial hypothalamus (DMH). The LH was defined as the area from the medial side of the fornix to the optic tract and the DMH was defined as the region from the medial side of the fornix to the third ventricle (James *et al.*, 2014; Laorden *et al.*, 2012; Paxinos, 2007). The total number of Fos-positive cells that were orexin and MCH negative was calculated by subtracting the number of Fos-positive orexin cells and Fos-positive MCH cells from total Fos counts.

Electrophysiology procedures

Drugs. CNO was obtained from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program, RTI International (USA) and dissolved in 5% DMSO to a concentration of 10mM. CNO was further diluted to a final concentration of 5 μ M for patch-clamp electrophysiology

Slice preparation for electrophysiology experiments. Rats were deeply anaesthetized with ketamine (1ml kg⁻¹ i.p) and decapitated. Brains were rapidly removed and immersed in ice-cold oxygenated (95% O₂, 5% CO₂) sucrose substituted artificial cerebrospinal fluid (S-ACSF, containing in mM: 236.2 sucrose, 25 NaHCO₃, 13.6 glucose, 2.5 KCl, 1 CaCl₂, 1 NaH₂PO₄ and 6 MgCl₂). Coronal slices (250 μ m) containing hypothalamus were obtained using a vibrating microtome (Campden 7000smz, Leicester, England), then transferred to an oxygenated storage chamber containing artificial cerebrospinal fluid (ACSF - 119.4 mM NaCl substituted for sucrose) and incubated for a minimum of one hour at room temperature prior to recording.

Electrophysiology. Slices were transferred to a recording chamber and continually superfused with oxygenated ACSF. Cells were visualized using infrared differential interference contrast microscopy attached onto an electrophysiology rig (East Sussex, United Kingdom). Whole-cell recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), digitized at 10kHz, via NI USB-6361 (National Instruments, Texas, USA) and recorded onto a PC running Axograph X software (Axograph). All recordings were restricted to the hypothalamic region in brain slices spanning between Bregma -1.72mm and -3.00mm³¹. After obtaining the whole-cell recording configuration, series resistance and input resistance were monitored and only stable recordings (<20% changes) were included. Recording pipettes (6-9 MΩ) were filled with an internal solution containing (in mM): 135 K-Gluconate, 6 NaCl, 2 MgCl₂, 10 HEPES, 0.1 EGTA, 2 MgATP, 0.3 NaGTP (pH 7.3 with KOH). Action potential (AP) discharge was studied in current-clamp mode. The membrane potential recorded 30sec after switching from voltage-clamp to current clamp was designated as the resting membrane potentials (RMP). A small bias current (<10pA) was injected into all cells. This hyperpolarised membrane potential and reduced the likelihood of spontaneous action potential firing at baseline. Following this, we ran a protocol that depolarised membrane potential from -80mV to -40mV, as demonstrated by Schöne et al. (2011).

Specific experiments

Experiment one: Effect of ELS on depressive-like behaviour in adulthood ($n=29$). Rats were exposed to either ELS or No ELS and remained undisturbed until adulthood. On PND70-75, all rats were trained to self-administer 10% sucrose on FR1, FR3 and PR schedules of reinforcement. Between PND86-91, rats were given one final PR session (PR day five) with a subset of these rats ($n=8$) being exposed to the forced swim test immediately prior to PR day five. These animals were not included in statistical analysis for PR day five. $n=6$ rats were exposed to the forced swim test on PR day six and were included in repeated measures statistics for PR day five. For experimental timeline, please refer to Fig 1.

Experiment two: Effect of chemogenetic activation of the lateral hypothalamus on ELS-induced deficits in motivational drive ($n=23$). Rats were exposed to either ELS or No ELS. On PND 42-44, all rats received intracranial hM3D(Gq) viral injections into the lateral hypothalamus. On PND70-75, all rats were trained to self-

administer 10% sucrose on FR1, FR3 and PR schedules of reinforcement. Rats received three lots of 5% DMSO in 0.9% saline solution (1mL/kg) habituation injections (i.p) throughout operant training. On PR day five, rats were given either vehicle (5% DMSO; 1mL/kg; i.p) or clozapine-*N*-oxide (CNO dissolved in 5% DMSO; 5mg/kg; i.p; National Institute of Mental Health Chemical Synthesis and Drug Supply Program, RTI International) injections 30mins prior to the PR self-administration session. To ensure the CNO had completely metabolised, rats were given an additional self-administration session the following day, PR day six. On PR day seven, the administration of vehicle or CNO 30mins prior to self-administration was reversed (counterbalanced order) and rats were deeply anaesthetized with sodium pentobarbitone two hours following the initiation of the session and perfused. Brains were then processed for immunohistochemical detection of Fos-protein, orexin, MCH and HA. Food and water consumption was monitored every second day throughout sucrose self-administration training to examine any potential non-specific effects of hM3D(Gq) viral transduction and whether any differences in sucrose self-administration observed were compensated for with normal rat chow or water intake. Both food and water consumption were estimated across all groups by weighing food and water, dividing this number by two to assess consumption per day and dividing this number by the number of animals per cage. For experimental timeline, please refer to Fig 3.

Data Analysis

Statistical analyses were conducted using IBM SPSS V22. Sucrose preference was analysed using an analysis of variance (ANOVA) with the between-subjects factor of Neonatal Treatment (No ELS, ELS) and the within-subjects factor of Session for FR1, FR3 and PR. Forced swim data, body weight and food and water consumption were analysed using between-subjects ANOVAs. The LH DREADD experiment was analysed using the between-subjects factor of Neonatal Treatment and the within-subjects factor of Drug (Vehicle, CNO) with litter size as a covariate. For immunohistochemical analyses, all cell counts were averaged across each animal for each rostrocaudal level of both the LH and DMH. Immunohistochemical data was analysed using a two-way between-subjects ANOVA comparing Neonatal Treatment and Drug. The reactivity of orexin, MCH and mCitrine neurons was assessed using the percentage of orexin, MCH and mCitrine cells expressing Fos-protein. Data from electrophysiology experiments was analyzed using paired *t*-tests to determine the

1 extent of depolarization caused by the application of CNO. All figures are presented
2 as mean + SEM. An alpha value less than or equal to 0.05 was adopted for all
3 statistical analyses.

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RESULTS

Experiment one: Effect of chronic ELS on sucrose self-administration and PR responding in adulthood (n=29)

Sucrose self-administration. For experimental timeline, please refer to Fig 1. Sucrose self-administration was used to examine motivational drive. Self-administration training involved eight fixed ratio 1 (FR1) sessions where the delivery of a sucrose reward was contingent upon one active lever press. This was followed by four FR3 sessions where sucrose delivery required three active lever presses. Finally, all rats were trained on a progressive ratio (PR) schedule where the number of lever presses required for sucrose reward delivery increased by 1 following each reward, ie. 1, 2, 3, 4, 5, etc. (Besheer *et al.*, 2008; Jupp *et al.*, 2011). Analysis of active lever presses during FR1 training revealed no significant effect of Neonatal Treatment (No ELS, ELS) ($F_{(1,27)}=0.3$, $p=0.857$; Fig 2A). There was also no significant effect of Neonatal Treatment on the number of active lever presses ($F_{(1,27)}=3.1$, $p=0.090$; Fig 2B) or inactive lever presses during FR3 training ($F_{(1,27)}=0.5$, $p=0.491$; Fig S1A). In contrast, analysis of active lever presses under a PR schedule of reinforcement revealed a significant interaction between PR Day (1-5) and Neonatal Treatment ($F_{(4,76)}=2.8$, $p=0.032$). There was also a significant effect of Neonatal Treatment on the number of active lever presses during PR responding ($F_{(1,19)}=9.8$, $p=0.006$) with ELS rats displaying a significantly lower number of active lever presses (Fig 2C). Neonatal Treatment had no significant effect on the number of inactive lever responses ($F_{(1,19)}=2.8$, $p=0.108$; Fig S1B). An analysis of PR breakpoint data revealed a significant interaction between Neonatal Treatment and PR Day ($F_{(4,76)}=3.9$, $p=0.006$). There was also an effect of Neonatal Treatment on PR breakpoint ($F_{(1,19)}=10.9$, $p=0.004$) with ELS rats exhibiting a significantly lower breakpoint than No ELS rats (Fig 2D). In summary, ELS reduced responding for sucrose under a PR schedule of reinforcement.

Forced swim test. The forced swim test was used as a measure of behavioural despair and involved placing animals in a water filled chamber and characterizing behavioural responses. There was no significant effect of Neonatal Treatment on the time spent climbing, swimming or time in immobility in the forced swim test ($F_{(1,12)}=0.5$, $p=0.483$; $F_{(1,12)}=3.6$, $p=0.08$; $F_{(1,12)}=0.8$, $p=0.391$; Fig 2E, F, G).

Experiment two: Effect of chemogenetic activation of the LH on chronic ELS-induced deficits in motivational drive (n=23).

Sucrose self-administration. This experiment repeated the protocol of experiment one with the addition of DREADD activation. For experimental timeline, please refer to Fig 3. Similar to experiment one, we found no significant effect of Neonatal Treatment on FR1 ($F_{(1,21)}=0.6$, $p=0.448$) or FR3 ($F_{(1,21)}=1.9$, $p=0.178$) active lever responding. There was a significant reduction in PR active lever responding in ELS rats compared to No ELS controls ($F_{(1,21)}=6.2$, $p=0.044$). Experiment two aimed to determine whether chemogenetic activation of the LH prior to a final PR test session could reverse effects produced by ELS. An analysis of active lever presses during PR revealed a significant effect of Drug (Vehicle, CNO) ($F_{(1,20)}=16.3$, $p=0.001$) with CNO increasing lever responding for sucrose (Fig 4A) without affecting inactive lever responding ($F_{(1,20)}=1.9$, $p=0.185$; Fig S1C). There was an effect of Neonatal Treatment on the number of active lever presses during PR responding ($F_{(1,20)}=6.0$, $p=0.049$) with ELS rats exhibiting significantly lower lever presses compared to No ELS ($F_{(1,21)}=4.3$, $p=0.050$; Fig 4A) but only under vehicle-treated conditions. Interestingly, under vehicle conditions, ELS rats only responded significantly lower than No ELS rats in the first 15 minutes of the PR task (Fig 4B). An analysis of PR breakpoint revealed a significant effect of Drug on PR breakpoint ($F_{(1,20)}=14.5$, $p=0.001$) with CNO increasing breakpoints for sucrose (Fig 4C). Notably, subsequent one-way ANOVAs revealed no difference between ELS or No ELS rats under CNO ($F_{(1,21)}=0.1$, $p=0.746$). There was no significant main effect of Neonatal Treatment on PR breakpoint ($F_{(1,20)}=5.1$, $p=0.063$; Fig 4C). In summary, CNO increased lever responding in ELS rats to levels seen in No ELS controls.

Body weight, food and water consumption. To ensure ELS did not influence baseline nutrition, body weight, along with food and water consumption were monitored. There was no effect of Neonatal Treatment on postnatal day (PND) 86 body weight ($F_{(1,21)}=0.6$, $p=0.817$; Fig 4D). There was also no significant difference between ELS and No ELS rats in food or water consumption throughout the sucrose self-administration period ($F_{(1,21)}=0.7$, $p=0.398$; $F_{(1,21)}=1.5$, $p=0.228$; Fig 4E, F).

Counts of Fos-positive mCitrine cells. To examine the effect of CNO on the activity of hM3D(Gq) transduced neurons, we quantified Fos-protein in mCitrine cells (the reporter protein of our DREADD). Figure 5A illustrates representative hM3D(Gq) viral expression observed in the LH. Supplementary figures S2 and S3 illustrate the extent of hM3D(Gq) viral expression in the LH. There was no effect of Neonatal Treatment and Drug on the number of mCitrine-positive cells in the LH

($F_{(1,20)}=3.5$, $p=0.074$) (Table 1). There was a significant interaction between Neonatal Treatment and Drug on the number of mCitrine-positive cells in the DMH ($F_{(1,20)}=4.2$, $p=0.053$) however, the total number of these cells was small (<20) (Table 1). In the LH, there was a significant effect of Neonatal Treatment with ELS rats having overall increases in the percentage of Fos-positive mCitrine cells ($F_{(1,20)}=4.3$, $p=0.052$). Treatment with CNO also significantly increased the percentage of Fos-positive mCitrine cells in LH ($F_{(1,20)}=91.9$, $p<0.0001$; Fig 5B). In contrast, there was no effect of Neonatal Treatment on the percentage of Fos-positive mCitrine cells in DMH ($F_{(1,20)}=0.4$, $p=0.555$). However, despite the small number of mCitrine cells present in the DMH, CNO-treatment increased the number that were Fos-positive ($F_{(1,20)}=10.2$, $p=0.012$; Fig 5B). In summary, the number of hM3D(Gq) transduced neurons that were Fos-positive was increased after CNO treatment.

Counts of Fos-positive orexin cells There was no effect of Neonatal Treatment and Drug on the number of orexin cells in the LH ($F_{(1,20)}=1.2$, $p=0.294$) or DMH ($F_{(1,20)}=0.2$, $p=0.629$) (Table 1). In the LH, there was no significant effect of Neonatal Treatment ($F_{(1,20)}=0.0$, $p=0.993$) on the percentage of Fos-positive orexin neurons (Fig 5C). However, there was a significant effect of Drug ($F_{(1,20)}=83.7$, $p<0.0001$) with CNO-treated rats displaying a greater percentage of Fos-positive orexin cells compared to Vehicle-treated rats (Fig 5C). In the DMH, there was no effect of Drug and Neonatal Treatment on the percentage of Fos-positive orexin cells ($F_{(1,20)}=0.1$, $p=0.747$) (Fig 5C).

Counts of Fos-positive MCH cells. There was also no effect of Neonatal Treatment or Drug on the number of MCH neurons in the LH ($F_{(1,20)}=0.1$, $p=0.770$) or DMH ($F_{(1,20)}=0.8$, $p=0.392$) (Table 1). In the LH, there was no significant difference across Neonatal Treatment groups on the percentage of Fos-positive MCH cells ($F_{(1,20)}=3.8$, $p=0.067$; Fig 5D). Interestingly, CNO-treated rats had overall increases in the percentage of Fos-positive MCH cells compared to Vehicle-treated rats ($F_{(1,20)}=85.9$, $p<0.0001$; Fig 5D). In the DMH, there was no significant interaction between Neonatal Treatment and Drug on the percentage of Fos-positive MCH neurons ($F_{(1,20)}=0.0$, $p=0.910$; Fig 5D).

Counts of Fos-positive orexin- and MCH-negative cells. An analysis of the number of Fos-positive cells that were orexin and MCH negative in the LH revealed an interaction between Drug and Neonatal Treatment ($F_{(1,20)}=6.1$, $p=0.023$). There was a significant main effect of Neonatal Treatment ($F_{(1,20)}=15.8$, $p=0.01$) with the

ELS-CNO group having an increased number of Fos-positive cells compared to the No ELS-CNO group ($p=0.006$; Fig 5E). There was also a significant effect of Drug on the number of Fos-positive cells in the LH ($F_{(1,20)}=485.2$, $p<0.0001$) with CNO resulting in increased Fos counts compared to Vehicle controls (Fig 5E). In the DMH, there was no significant interaction between Neonatal Treatment and Drug on the number of Fos-positive cells in the DMH ($F_{(1,20)}=1.1$, $p=0.310$; Fig 5E).

HA-MCH and HA-orexin immunohistochemistry. Hemagglutinin (HA) immunoreactivity, the epitope tag fused to the hM3D(Gq) DREADD, was used in combination with orexin or MCH immunohistochemistry to determine which LH populations expressed virally transduced DREADD. These data showed an absence of colocalization between hM3D(Gq) virally transduced cells and orexin ($n=102\pm4$ orexin cells, $n=3\pm1$ HA+orexin cells) or MCH ($n=134\pm22$ MCH cells, $n=7.5\pm1$ HA+MCH cells) in the LH (Fig 6A, B).

Effect of CNO on LH cell activity in hypothalamic slices. To verify that hM3D(Gq) cell transduction was sufficient to promote increased neuronal activity we made blind patch clamp recordings of cells in the LH in the presence or absence of bath-applied CNO ($5\mu\text{M}$). Of 11 LH cells recorded, 7 showed a significant membrane depolarisation after CNO that was sufficient to provoke action potential discharge (spiking) ($n=11$, $p<0.05$, paired t -test, Fig 6C, D).

DISCUSSION

In the present study we show that exposure to ELS promotes deficits in motivated responding for sucrose on a PR schedule of responding. These findings support a hypothesis that the brain circuits controlling motivated behaviour are vulnerable to long lasting remodelling by ELS. Importantly, activation of LH circuits using hM3D(Gq) DREADD increased responding for sucrose in ELS rats to No ELS control levels. We confirmed in slice-based electrophysiology that increased membrane depolarisation and produced action potential spiking in LH neurons. CNO-treatment was associated with an increase in spike firing and number of Fos-positive cells in the LH including MCH, orexin and putative GABA and glutamatergic neurons. Together these data suggest that deficits in responding for sucrose produced by ELS can be overcome by selective pharmacological targeting of LH circuits.

Effect of ELS on motivated responding for sucrose

1 We have previously shown that prior ELS exposure promoted hypoactivity in the
2 open field and reduced stress-induced activation of LH orexin neurons in adult rats
3 (James *et al.*, 2014). Here we aimed to determine the impact of ELS on responding
4 for sucrose in adulthood using a more sensitive measure of altered motivational state.
5 We found that responding for sucrose under PR was suppressed in ELS rats compared
6 to No ELS controls. In contrast, ELS animals displayed normal behaviour during
7 forced swim testing. Thus, in our hands, maternal separation produced a phenotype in
8 male rats that more closely resembles deficits in motivational drive, rather than
9 behavioural despair, the phenotype most often used to describe deficits in forced
10 swim behaviour, findings consistent with Rüedi-Bettschen and colleagues (2005).

11 Notably, animals in both conditions learnt to acquire lever pressing for sucrose
12 at similar rates suggesting that ELS did not impair motor performance or affect
13 acquisition or learning of the instrumental task. Although ELS rats displayed a trend
14 towards reduced responding under FR3, significant differences in motivated
15 responding for sucrose only emerged after repeated PR testing. It is important to
16 highlight that in our second cohort of rats, the breakpoint in ELS animals was not
17 quite statistically different from controls, despite an identical trend to the first cohort.
18 However, ELS rats displayed significantly reduced active lever responding during the
19 PR session, consistent with cohort one. We don't think this reflects a major difference
20 in vulnerability to ELS between experimental cohorts, although it is important to note
21 that only the second cohort of rats received surgery for DREADD injections.
22 Moreover, while the breakpoint is a commonly used index of performance under PR
23 testing, the number of active lever presses during this schedule is considered an
24 equally sensitive measure of effort or willingness to obtain a reward, particularly
25 when using small ratio step sizes as we did here (Rickard et al., 2009; Stafford &
26 Branch, 1998).

27 *Effect of DREADD activation of LH circuits on motivated responding after ELS*

28 hM3D(Gq) DREADD dramatically increased lever responding for sucrose under PR
29 conditions in both ELS and No ELS groups. These data demonstrate that motivation
30 to lever press for sucrose can be enhanced by LH activation and importantly,
31 plasticity remains within the LH circuitry to overcome a state of low motivational
32 drive produced by ELS. Interestingly, when we assessed active lever pressing during
33 the first 30 min of responding under PR, we found that the differences between ELS
34 and No ELS animals under vehicle conditions were mainly confined to the first 15

minutes of responding. Noticeably, CNO-treated animals in both ELS and No ELS groups displayed increased responding across these periods but converged at the same time point. In contrast, inactive lever responding was not altered by CNO in either treatment condition suggesting that the increased responding was not a function of non-specific locomotor activity or arousal.

Effect of ELS on Fos-positive cell counts in the LH

To understand the neural circuits impacted by ELS we made counts of Fos-positive cells in the LH including cells immunoreactive for orexin and MCH. We have previously found suppressed orexin cell reactivity in ELS animals after restraint stress in adulthood corresponding to hypoactivity in the open field (James *et al.*, 2014).

Somewhat surprisingly therefore, in the present study there were no significant differences between ELS and control rats after sucrose PR – even though PR responding was lower in ELS rats. In addition, very few Fos-positive MCH cells were observed in the LH of ELS and No ELS rats suggesting that this population of cells is not strongly impacted by maternal separation. A number of differences in design between our two studies (James *et al.*, 2014) may account for these discrepancies. First, hypoactivity of orexin cells after ELS in our previous work was only unmasked after exposure to restraint stress in adulthood. Second, in the present study rats were trained for sucrose self-administration and although sucrose consumption was low under PR conditions, this may have been sufficient to mask any differences in LH circuit activity caused by ELS. Last, Fos-mapping has well known limitations, including limited temporal resolution, making future studies using electrophysiological recordings a logical next step to a detailed understanding of ELS remodelling of LH circuits.

Effect of DREADD activation on Fos-positive cell counts in the LH

CNO-induced activation of hM3D(Gq) significantly increased the numbers of Fos-positive cells in the LH including those immuno-positive for mCitrine, the reporter protein of the hM3D(Gq) DREADD, MCH, and orexin. Notably, while CNO increased the recruitment of these peptide-expressing neurons, orexin and MCH neurons represented only 20-30% of the total population of cells exhibiting Fos-protein after CNO (Fig 5E). Indeed, it is unlikely that the low level of MCH neurons would have any significant effect on behaviour. Thus, based on the large number of non-orexin and non-MCH neurons exhibiting Fos after CNO in DREADD animals, it is highly likely that a majority of responsive neurons are GABAergic or glutamatergic

projection neurons. This is consistent with the fact that our immunolabeling showed very little colocalization of reporter proteins HA or mCitrine with orexin or MCH (Fig 6A, B). Thus, while we can only speculate as to the specific pathways transduced by DREADD, activation of GABAergic neurons in the LH that project to the VTA has recently been shown to drive both consummatory and reward behaviours (Navarro et al., 2016). Indeed, using optogenetics, Barbano et al. (2016) showed that the expression of consummatory versus reward behaviours depends on the frequency of stimulation of GABA LH → VTA neurons. A role for LH glutamatergic neurons in reward-based behaviours also exists. In a study by Kempadoo and colleagues (2013), LH neurotensin neurons were transduced with channel-rhodopsin and fibre optics were placed over the VTA. Lever pressing for delivery of 20 Hz blue light stimulation was found to support intra-cranial self-stimulation through glutamate-mediated transmission. Further, a role for local intra-LH glutamatergic neurons in enhancing or synchronising LH output cannot be ruled out (Allen mouse brain atlas., 2004; Erlander *et al.*, 1991; Feldblum *et al.*, 1993; Lein *et al.*, 2007; Jennings *et al.*, 2015).

Conclusions

In conclusion, we show here that chemogenetic activation of LH circuits can enhance motivated responding for sucrose under PR conditions and overcome a state of low motivational drive produced by ELS. Interestingly, AAV5-hSyn DREADD appeared to promote this behaviour by transduction of intra-hypothalamic neurons that modulate LH output via projections to targets such as the VTA. However, our approach did not directly test this possibility. Overall these data have important implications clinically because they show that while LH circuitry can be maladapted by ELS, selective pharmacological activation of appropriate cell types in LH can overcome low motivated arousal; a debilitating symptom of neuropsychiatric diseases such as depression. While further work is required to dissect the specific LH circuits that mediate these effects, this knowledge may aid in developing more effective treatments for depressive illnesses.

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4

5 **AUTHOR CONTRIBUTIONS**

6 EJC, CVD, BAG and DMH designed research; EJC, CSM, CDA and JWY performed
7 research; EJC, CVD, CSM and JWY analyzed data; all authors wrote and reviewed
8 the manuscript.

9

10 **DATA ACCESSIBILITY**

11 All data generated or analyzed during this study are included in this published article
12 (and its Supplementary Material). For additional information, please contact the
13 corresponding author.

14

ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
ANOVA	Analysis of variance
AP	Action potential
CNO	Clozapine- <i>N</i> -oxide
DMH	Dorsomedial hypothalamus
DMSO	Dimethyl sulfoxide
DREADD	Designer receptors exclusively activated by designer drugs
ELS	Early life stress
FR	Fixed ratio
HA	Hemagglutinin
LH	Lateral hypothalamus
MCH	Melanin-concentrating hormone
PBS	Phosphate buffered saline
PND	Postnatal day
PR	Progressive ratio
RMP	Resting membrane potential
VTA	Ventral tegmental area

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FIGURE LEGENDS AND TABLE

Table 1. Orexin, MCH and hM3D(Gq) cell expression in each subregion of the

Treatment	Region	Orexin number	MCH number	hM3D(Gq) number
No ELS - Vehicle (n = 5)	LH	127.6 ± 4.9	197.2 ± 7.9	46.3 ± 6.1
	DMH	28.2 ± 1.9	45.9 ± 6.7	9.8 ± 1.6
ELS – Vehicle (n = 7)	LH	139.9 ± 6.0	182.9 ± 14.2	66.6 ± 8.0
	DMH	29.3 ± 1.9	24.0 ± 4.4	13.3 ± 2.5
No ELS – CNO (n = 5)	LH	125.2 ± 4.9	202.0 ± 6.7	82.9 ± 12.4
	DMH	29.4 ± 2.5	42.4 ± 8.1	16.7 ± 1.6
ELS – CNO (n = 6)	LH	148.3 ± 4.9	193.4 ± 7.6	70.2 ± 7.3
	DMH	32.6 ± 1.9	31.9 ± 8.2	10.3 ± 3.4

hypothalamus. Data presented as average number of orexin, MCH or mCitrine cells per hemisphere across Bregma levels -2.60mm, -2.76mm and -2.92mm ± standard error mean. Early life stress (ELS); clozapine-N-oxide (CNO); lateral hypothalamus (LH); dorsomedial hypothalamus (DMH).

Figure 1. Schematic illustration of the experimental design for experiment 1.

Postnatal day (PND); fixed ratio 1 or 3 (FR1, FR3); progressive ratio (PR).

Figure 2. Effect of early life stress (ELS) on sucrose operant responding and forced swim behaviour.

(A, B) There was no effect of ELS on the number of lever presses for sucrose under fixed ratio (FR) 1 and 3 schedules of reinforcement. No ELS: n=14; ELS: n=15. (C, D) ELS resulted in a significant reduction in the number of active lever presses and the breakpoint for sucrose under a PR schedule of reinforcement. Bar graphs represent average lever responding and breakpoint over the 5 day PR task. No ELS: n=10; ELS: n=11. (E, F, G) ELS resulted in no significant differences in forced swim behaviour. No ELS: n=7; ELS: n=7. *p<0.05; **p<0.01.

Figure 3. Schematic illustration of the experimental design for experiment 2.

Following early life maternal separation, rats received bilateral injections of hM3D(Gq) virus into the LH during adolescence (postnatal days (PND) 42-44). This designer receptor exclusively activated by designer drugs (DREADD) allowed for the reversible activation of neurons within the LH but only following a systemic injection of the synthetic ligand clozapine-*N*-oxide (CNO). Rats were then allowed to self-administer sucrose from PND70-85 followed by progressive ratio testing after vehicle or CNO injections. Fixed ratio 1 or 3 (FR1, FR3); progressive ratio (PR); intraperitoneal, i.p.

Figure 4. Effect of early life stress (ELS) and lateral hypothalamic (LH)

DREADD on sucrose operant responding, body weight, food and water

consumption. (A) ELS resulted in reduced lever presses for sucrose under vehicle conditions during the progressive ratio (PR) test. Clozapine-*N*-oxide (CNO) increased lever responding for sucrose in ELS rats equivalent to No ELS levels. (B) Time bin data of active lever presses shows that ELS reduced lever presses for sucrose in the first 15 minutes of PR in the vehicle condition only. (C) A similar trend was seen for breakpoint with ELS reducing breakpoint under vehicle conditions but not in response to CNO. No ELS - Vehicle: n=10; ELS - Vehicle: n=13; No ELS - CNO: n=10; ELS - CNO: n=13. *p<0.05 No ELS – Vehicle versus ELS - Vehicle. (D, E, F) ELS and LH

viral transduction had no effect on final body weight (postnatal day (PND) 86) and food or water consumption over the sucrose self-administration period.

Figure 5. Effect of early life stress (ELS) and DREADD on lateral hypothalamic (LH) Fos-protein expression in orexin, melanin-concentrating hormone (MCH) and LH neurons. (A) Representative image of hM3D(Gq) viral expression (HA-tag immunostain) in the LH. Scale bars, 200 μ m and 20 μ m respectively. Third ventricle (3V); optic tract (OT); mammillary tract (MT); fornix (F). (B) Photomicrograph of Fos (black) and mCitrine (brown) cells. Clozapine-N-oxide (CNO) resulted in a significant increase in the percentage of Fos-positive mCitrine cells in the LH and DMH. No ELS - Vehicle: n=2; ELS - Vehicle: n=2; No ELS - CNO: n=2; ELS - CNO: n=2. **p<0.01, ***p<0.0001 Vehicle-treated rats versus CNO-treated rats. Scale bar, 20 μ m. (C) Photomicrograph of Fos-positive orexin cells. CNO resulted in significantly increased numbers of Fos-positive orexin neurons in the LH but not the DMH. No ELS - Vehicle: n=5; ELS - Vehicle: n=7; No ELS - CNO: n=5; ELS - CNO: n=6. ***p<0.001 Vehicle-treated rats versus CNO-treated rats. Scale bar, 50 μ m. (D) Photomicrograph of dual Fos-MCH immunohistochemistry. CNO resulted in significantly greater numbers of Fos-positive MCH neurons in both the LH but not the DMH. No ELS - Vehicle: n=5; ELS - Vehicle: n=7; No ELS - CNO: n=5; ELS - CNO: n=6. ***p<0.001 Vehicle-treated rats versus CNO-treated rats. Scale bar, 50 μ m. (E) ELS resulted in a significant increase in the number of Fos-positive neurons in the LH (that were orexin and MCH negative) under CNO-treatment conditions. Additionally, CNO significantly increased the number of Fos-positive cells in the LH. There were no effects of Neonatal Treatment or Drug on the number of Fos-positive cells in the dorsomedial hypothalamus (DMH). No ELS - Vehicle: n=5; ELS - Vehicle: n=7; No ELS - CNO: n=5; ELS - CNO: n=6. **p<0.01 No ELS - CNO versus ELS - CNO. ***p<0.001 Vehicle-treated rats versus CNO-treated rats. Scale bar, 200 μ m.

Figure 6. hM3D(Gq) modulation of lateral hypothalamus (LH) neurons. (A) Representative photomicrograph of the LH demonstrating limited colocalization between HA (green; tag for hM3D(Gq) DREADD) and MCH (red) immunolabeling (n=134 $\pm$ 22 MCH cells, n=7 $\pm$ 1 HA+MCH cells). (B) Representative photomicrograph of the LH demonstrating limited colocalization between HA (green) and orexin (blue)

immunolabeling ($n=102\pm4$ orexin cells, $n=3\pm1$ HA+orexin cells). $n=2$ clozapine-*N*-oxide (CNO)-treated rats. Scale bar, 20 μ m. **(C)** Effects of CNO on membrane potential in current clamp configuration of LH neurons. Horizontal dashed line indicates baseline membrane potential. $n=11$. **(D)** Population data for the effects of CNO on membrane potential in LH neurons (* $p<0.05$).

Experiment 1: Behavioural timeline

ejn_13674_f1.eps

Experiment 2: Behavioural timeline

ejn_13674_f3.eps

ejn_13674_f4.eps

B

Lateral hypothalamus

Dorsomedial hypothalamus

□ No ELS
■ ELS

C

D

E

A

B

C

D

ejn_13674_f6.eps

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