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Eleanor C. Sandhu[1,2], Anushka B. P. Fernando[1,2], Elaine E. Irvine[1,2], Kyoko Tossell[1,2], Michelle Kokkinou[1,2], Justyna Glegola[1,2], Mark A. Smith[1,2], Oliver D. Howes[1,3], Dominic J. Withers[1,2] and Mark A. Ungless[1,2]

[1]*MRC London Institute of Medical Sciences (LMS), Du Cane Road, London, W12 0NN, UK*

[2]*Institute of Clinical Sciences (ICS), Faculty of Medicine, Imperial College London, Du Cane Road, London, W12 0NN, UK*

[3]*Department of Psychosis Studies, Institute of Psychiatry, Psychology & Neuroscience, Kings College London, De Crespigny Park, London, SE5 8AF, UK*

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D.J.W. and M.A.U. are co-senior authors.

Corresponding authors: Mark A. Ungless: mark.ungless@imperial.ac.uk or Dominic J. Withers: d.withers@imperial.ac.uk

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[1] MRC London Institute of Medical Sciences (LMS), Du Cane Road, London W12 0NN, UK

[2] Institute of Clinical Sciences (ICS), Faculty of Medicine, Imperial College London, Du Cane Road, London W12 0NN, UK

[3] Department of Psychosis Studies, Institute of Psychiatry, Psychology & Neuroscience, Kings College London, De Crespigny Park, London SE5 8AF, UK.

* co-senior authors

Corresponding authors:

Mark A. Ungless: mark.ungless@imperial.ac.uk

Dominic J. Withers: d.withers@imperial.ac.uk

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Abstract

Salt intake is an essential dietary requirement, but excessive consumption is implicated in hypertension and associated conditions. Little is known about the neural circuit mechanisms that control motivation to consume salt, although the midbrain dopamine system, which plays a key role in other reward-related behaviours, has been implicated. We, therefore, examined the effects on salt consumption of either optogenetic excitation or chemogenetic inhibition of ventral tegmental area (VTA) dopamine neurons in male mice. Strikingly, optogenetic excitation of dopamine neurons decreased salt intake in a rapid and reversible manner, despite a strong salt appetite. Importantly, optogenetic excitation was not aversive, did not induce hyperactivity, and did not alter salt concentration preferences in a need-free state. In addition, we found that chemogenetic inhibition of dopamine neurons had no effect on salt intake. Lastly, optogenetic excitation of dopamine neurons reduced consumption of sucrose following an overnight fast, suggesting a more general role of VTA dopamine neuron excitation in organising motivated behaviors.

Significance Statement

Although it is well-established that midbrain dopamine neurons are involved in many types of reward-related behaviours, little is known about their role in salt intake under conditions where salt is appetitive (i.e. during salt depletion). Here, we show that optogenetic excitation of midbrain dopamine neurons can decrease salt intake. Importantly, this stimulation protocol did not affect salt concentration preferences. Furthermore, we find that this stimulation protocol can also reduce sucrose intake following an overnight fast, suggesting a broader role for dopamine neuron activity in regulating nutrient intake, which compliments findings from previous lesion- and pharmacological-based studies.

Introduction

Dietary sodium intake is essential to the regulation of fluid and electrolytes within the body. Indeed, chronic salt depletion, through diet or the use of low sodium dialysate during dialysis, has been associated with increased mortality (Alderman & Cohen, 2012). Moreover, in certain patient groups (for example the elderly and during diarrheal illness) the physiological ability to respond to salt depletion is impaired due to medication or illness. This is significant since hyponatremia has profound multi-organ consequences which may be fatal. It is therefore essential to maintain total body salt homeostasis. This is normally achieved through physiological control of loss and intake. Accordingly, a strong sodium appetite occurs in response to low levels of sodium in the body resulting in consumption of high salt foods (Richter, 1956; Denton, 1982). However, excessive salt intake, beyond metabolic need, leads to increased blood pressure and the risk of both cardiovascular disease and obesity (Ma *et al.*, 2015). There are major public health initiatives to reduce salt intake (World Action on Salt and Health; http://www.worldactiononsalt.com). Despite these, there has been little evidence of a reduction in salt consumption at a population level (McCarron *et al.*, 2013; Asayama *et al.*, 2014). There is, therefore, a pressing need to understand better the mechanisms through which salt consumption is mediated and to develop therapeutic interventions that could regulate intake.

One useful framework for understanding the neural basis of salt appetite proposes that it is regulated by three distinct neural components (Geerling & Loewy, 2008). First, salt depletion is detected by subfornical organ neurons

and nucleus of the solitary tract (NTS) neurons expressing the enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD2). These neurons are excited by salt depletion and when stimulated can drive salt appetite (Geerling & Loewy, 2008; Jarvie & Palmiter, 2016; Nation *et al.*, 2016). Second, gustatory signals transmit information about the detection of salt via non-HSD2 expressing NTS neurons (Geerling & Loewy, 2008). Third, these two signals are integrated in forebrain sites to drive motivated behaviour to consume salt (Geerling & Loewy, 2008). These forebrain sites include the mesocorticolimbic dopamine system, which plays a key role in processing information about other types of reward Wise, 2006. There is some evidence linking the reinforcing properties of salt intake to the dopamine system. For example, dopamine type 2 receptor (D2R) antagonists reduce sham drinking of sodium chloride (NaCl) where fluid empties through a gastric fistula minimizing post-ingestive inhibitory signals when compared to normal drinking of NaCl (Roitman *et al.*, 1997). Furthermore, salt depletion results in an increase in dopamine release in the nucleus accumbens upon unconditioned presentation of NaCl, which is not seen when the animal is salt replete suggesting salt appetite positively modulates dopamine signalling (Cone *et al.*, 2016). Taken together these findings suggest that during salt appetite, salt becomes appetitive and engages neural circuits (particularly the dopamine system) that are involved in mediating appetitive behaviour towards other types of reward. However, it is not well understood how changes in dopamine neuron activity affect salt intake. Interestingly, excitation of dopamine neurons has recently been shown to suppress sucrose drinking and feeding behaviour (Mikhailova *et al.*, 2016;

Boekhoudt *et al.*, 2017). We, therefore, hypothesised that under conditions of salt appetite, excitation of dopamine neurons would suppress salt intake.

Methods

Animals

Mice were housed in cages of 2-4 animals and maintained on a 12 h light/dark cycle. Prior to any changes in diet, food and water were available *ad libitum*. Male C57BL6 mice, 16-18 weeks, (Charles River, UK; IMSR Cat# CRL:27, RRID:IMSR_CRL:27) were used in non-optogenetic studies. For the optogenetic studies, male DAT-iCre heterozygous mice (DATcre+) and wild-type litter mates (DATcre-) on a C57BL/6 background were used (IMSR Cat# EM:01738, RRID:IMSR_EM:01738; Turiault *et al.*, 2007). Animal husbandry and experimental procedures were undertaken in accordance with the United Kingdom Animal (Scientific Procedures) Act of 1986.

Virus

The DIO-ChR2-mCherry construct was kindly gifted by the Deisseroth Lab and the viral particles were produced by Vector Biolab, Philadelphia. Concentrations varied minimally with batches of virus across experiments, they ranged from 2.7-5.8*10^13 GC/ml but were diluted down to 2.0x10^12 GC/ml. DREADD (designer receptors exclusively activated by designer drugs) construct of human muscarinic acetylcholine receptor M4 fused to mCherry (hM4Di-mCherry) was constructed according to Nawaratne *et al.*, 2008 and cloned into pAAV-Eifla-DIO-WPRE vectors. DREADD construct of human muscarinic acetylcholine receptor M3 fused to mCherry (hM3Dq-mCherry) was obtained from Prof Graeme Milligan, University of Glasgow, and was inserted into pAAV-Eifla-DIO-WPRE vectors (gift from Deisseroth Lab, Stanford:http://web.stanford.edu/group/dlab/optogenetics/sequence_info.html)

. The vectors were packaged in AAV serotype 2/1 vector consisting of the AAV2 ITR genomes and the AAV1 serotype capsid gene, titer 2.1 x10^12 GC/ml.

(Vector Biolab, Philadelphia).

Surgery

Ten to twelve-week-old mice were anaesthetised with isoflurane (5% for induction; 1-2% for maintenance) and placed into a Kopf stereotaxic frame (Bilaney Consultants, UK). 0.25 % bupivacaine was injected subcutaneously beneath the scalp before an incision was made down the midline. For AAV injections, holes were drilled bilaterally to target the VTA using the coordinates AP -3.45 mm, ML ±0.4 mm, DV -5.05 mm. For optogenetic stimulation studies, to accommodate implantable fibres a 10-degree angle was used and coordinates were ML ±1.3 mm, DV -4.89 mm (injection) -4.44 mm (optical fibre). 0.5 µl of AAV was injected, bilaterally into the VTA using a 33gauge metal needle and a 5 µl Hamilton glass syringe at a rate of 0.15 µl/min. The needle was left for 5 min post injection before being slowly removed. Following this, implantable optical fibres (constructed according to Sparta *et al.*, 2011) were placed bilaterally, via the same craniotomies. Two screws were placed dorsal to lambda and anterior to bregma to anchor a dental cement cap. Mice recovered from anaesthesia in a heated chamber, were group housed, and left to recover for at least two weeks prior to handling and habituation. Microscopic inspection, under light anaesthesia, of a test cohort showed no damage to the ferrules by littermates after 2 weeks.

Handling and habituation

Mice were handled for 2 weeks, and habituated to the testing jellies and apparatus for 1 week prior to the test session.

Induction of appetite

For the salt appetite experiments, an acute sodium depletion protocol was used. Briefly, 2 weeks prior to testing, mice were switched to low sodium diet (RM<0.025%Na, Special Diets Services, Essex UK) with access to NaCl, via a glass dish, containing 0.4 % agar jelly and 0.75 M NaCl in their home cage. Two days prior to the test session, mice were injected intra-peritoneally with furosemide (20 mg/kg) (Hameln Pharmaceuticals), their cages changed and the sodium chloride jellies were replaced with 0.4 % agar jellies. This was repeated for a second day, followed by the test day. Saline controls followed the same protocol but were maintained on normal chow with NaCl jellies throughout and were injected with vehicle (NaCl; 0.9% 2ml/kg) (Animalcare Ltd) for two days prior to the test session. The experimenter was blind to group allocation and setup. A fasted state was achieved with removal of all food at 4 pm prior to the test day, although water remained *ad libitum*. This overnight-fasted state was used in experiments testing sucrose appetite and in the need-free sodium preference experiment.

Test procedure

Mice were tethered to the laser via patchcords and placed in their designated testing chamber (base of Allentown XJ cage, 19.37 x 38.13 x 13.03 cm) with a paper liner. Following 10 min of habituation, laser stimulation was started. 5

min into the stimulation protocol, three jellies of three different concentrations were placed at one end of the chamber. The order of jellies was systematically randomized and was kept constant throughout habituation and testing for each mouse. Mice were allowed to freely consume the jellies for the 30 min test session. The jellies were removed every 10 min, weighed and then returned to the chamber. For chemogenetic experiments the mice were injected with clozapine-*N*-oxide (CNO: 0.1 mg/kg; i.p.) and placed in their testing chamber. Jellies were placed in the test chamber after 30 min and the test session began.

Optical stimulation

For optical stimulation studies, implantable optic fibres were attached to a 1x2 intensity division fiber-optic rotary joint (Doric Lenses Inc.) using patch cords (Doric Lenses Inc) via a zirconia sleeve. An insulated optical fibre connected the rotary joint to a 473 nm laser source (CrystaLaser and Vortran Laser Technology Inc.). Light output was adjusted by measuring the light output from the tip of an implantable optical fibre using an optical power meter, aiming for a power of 2-3 mW from the tip of the implanted ferule. A phasic illumination pattern was used in experiments with channelrhodopsin. This consisted of 8 pulses of 5ms pulse width, spaced 37ms apart, every 5s, similar to previously published optogenetic stimulation studies (Adamantidis *et al.*, 2011; Tye *et al.*, 2013).

Immunohistochemistry

Mice were anaesthetised with Isoflurane (5%) and then 0.08 ml Euthatal 100 mg/ml i.p. and perfused transcardially with approximately 30 ml of 0.01 M phosphate buffered saline (PBS) followed by 100ml of PBS containing 4 % paraformaldehyde (4 % PFA) at 4°C. The brain was immediately removed and post fixed in 4 % PFA for 2h. Subsequently the brains were cryoprotected in 30% sucrose in PBS, embedded in optimal cutting temperature (OCT) medium and frozen in isopentane at −50°C. Brains were stored at -80°C until they were coronally sectioned at 70 µm on a cryostat (Leica CM1800, Leica Microsystems). Free floating sections were washed 4 times in PBS for 5 min, then incubated with 6% normal donkey serum in 0.2 % Triton X in PBS (PBS-Tx). Sections were then incubated simultaneously with primary antibodies, in 2 % normal donkey serum in PBS-Tx, at 4°C as follows: chicken anti-tyrosine hydroxylase (1:1000; Abcam Cat# ab76442, RRID:AB_1524535) for a minimum of 24 h; rabbit anti-cFos (1:20000; Millipore Cat# PC38, RRID:AB_2106755) for a minimum of 74 h. Sections were then washed 4 times in PBS-Tx for 5 min and then incubated with the appropriate secondary antibodies: AlexaFluor488 donkey anti-rabbit (1:1000; Thermo Fisher Scientific Cat# R37118, RRID:AB_2556546) and AlexaFluor633 goat anti-chicken (1:1000; Thermo Fisher Scientific Cat# A-21103, RRID:AB_2535756) or 488 goat anti-chicken (1:1000; Thermo Fisher Scientific Cat# A-11039, RRID:AB_2534096) alone for 2 h at room temperature or 24 h at 4°C. Sections were then rinsed for 5 min, first in PBS-Tx 3 times then PBS 2 times before being mounted in Vectorshield Mounting Medium (Vector Laboratories). Confocal laser scanning microscopy was performed using a Leica SP confocal microscope. Images were taken at a resolution of 1,024 ×

1,024 and processed using Leica Confocal Software (Leica Microsystems), Adobe Photoshop CS3 (Adobe Systems) and ImageJ. Anatomical localization of optical fibres was assessed by examining the tracts in combination with immunolabelling for tyrosine hydroxylase to identify VTA dopamine neurons.

cFos immunostaining

7 male mice underwent unilateral surgery. Following 2 weeks of recovery, mice were handled daily for 2 weeks and followed the standard habituation protocol. On the test day, mice were tethered to the laser via patchcords and placed in their designated testing chamber with a paper liner. Following a 15 min habituation period, 30 min of phasic stimulation started. The mice were left for 30 min then anaesthetised, transcardially perfused, the brain removed and sectioned at 70 µm on a cryostat. Sections were processed as above. Three areas were selected in the VTA corresponding to medial, ventral and dorsolateral regions. TH+ve cells were identified and then checked for cFos. Following cFos analysis, mCherry expression was checked in selected brain slices.

Conditioned place preference

Following surgery, mice were housed in pairs or trios. They were handled and scruffed for a week prior to the test day. A biased conditioned place preference (CPP) was then performed based on Tsai *et al.* (2009) with 4 days of conditioning. A three compartment CPP setup was used (Med Assoc. Inc.). The white and black compartments were scented with lemon and ethanol respectively. On day 1, mice were placed in the central grey compartment for

2 min, and gates were then opened allowing free exploration of all 3 compartments for 15 min. The time spent in each area was recorded and their preferred chamber was noted. On the first day of conditioning (day 2), the mice were placed in one compartment and the following day (day 3) the other compartment for 30 min. They were tethered in both compartments but only stimulated in the compartment they showed least preference for on day 1. Laser power output was 2 mW from the tip. Conditioning was continued for days 4 and 5. On day 6, the mice were tested for preference. As for day 1, they were placed in the central grey compartment for 2 min, gates opened and then allowed to explore for 15 min without stimulation. Time spent in each compartment on day 1 and day 6 was analysed to assess whether the mice preferred the compartment where they had received stimulation. Locomotor activity on the conditioning days was analysed to assess whether stimulation increased activity.

Open field activity

Each mouse was tested in a custom-made wooden open field arena 45 cm x 45 cm with 30 cm walls. Mice were habituated to the arena for 20 min and then injected i.p. with either CNO (0.1 mg/kg) or saline and immediately placed back in the arena for a 60 min test session. hM3Dq-expressing mice were previously injected with different CNO doses (ranging from 0.1-0.5mg/kg) or saline. Drug allocation (saline vs CNO) was randomized prior to each dose tested (data not shown). The groups were then re-randomized and injected with a dose of 0.05mg/kg CNO or saline (data shown). Their activity was recorded using a video camera suspended above the arena that

interfaced with a computerized tracking system (Ethovision XT, Noldus). Total distance travelled was recorded in 1 min bins and analysed in 5 min bins.

In vitro electrophysiology

Ten to twelve week old male mice (DATcre+ for optogenetic and chemogenetic validation experiments; C57BL6 for salt-deprivation and fasting experiments) were anaesthetised by Euthatal following isoflurane. The brain was removed by decapitation following a quick transcardial perfusion with ice-cold artificial cerebrospinal fluid (aCSF, composition in mM, NaCl 120, KCl 3.5, NaH2PO4 1.25, NaHCO3 25, glucose 10, MgCl2 1, CaCl2 2) fully equilibrated with carbogen gas (95% oxygen and 5% carbon dioxide).Two or three horizontal brain slices (190 µm thickness) encompassing the VTA were obtained using a vibratome (Leica VT1000S; Leica Microsystems, Wetzlar, Germany) and were incubated for 15min in carbogenated NMDG-HEPES recovery solution (NMDG 93, KCl 2.5, NaH2PO4 1.2, NaHCO3 30, HEPES 20, Glucose 25, sodium ascorbate 5, Thiourea 2, Soduim pyrurate 3, MgSO4 10, CaCl2 0.5, pH7.3, 300mOsm, 33°C) (Zhao et al., 2011), and transferred back to aCSF. Slices were maintained in a standard custom-made maintenance chamber gently and continuously aerated with carbogen gas for at least 60 min at room temperature (20–22 ˚C) before being used for electrophysiology.

Slices were transferred to a submersion recording chamber and were continuously perfused at a rate of 2-4 ml/min with fully oxygenated aCSF at 32°C. Neurons were visualized using infra-red differential interference

contract (IR-DIC) under an upright microscope (Olympus BXWI 51, Japan) equipped with a 40x objective (0.8 numerical aperture), an IR filter, DIC optics and a charge coupled device (CCD) video camera (Watac). For visualising recorded neurons, 0.1% neurobiotin (Vectorlab) was added to all intercellular solutions.

Whole-cell patch-clamp recordings were performed with a Multiclamp 700B amplifier (Molecular Devices, CA) and an Axopatch 200A amplifier (Axon Instruments). The signals were sampled at 20 kHz and low-pass filtered at 1 kHz. Series resistance (Rs) and input resistance (Rin) were frequently monitored throughout the experiments via a 10mV, 250ms hyperpolarizing step. Any large changes in holding current or noise characteristics were taken as early signs of cell loss and recordings were terminated. Experiments were also terminated if Rs exceeded 35 MΩ or if Rin changed more than 15% after break in the whole-cell mode. Rs (typical values of 10-30 MΩ) was compensated by 60-70% in the majority of the experiments. Membrane capacitance (Cm) was measured under voltage clamp at -50 mV using a hyperpolarizing 10 mV, 250 ms step. Cm was measured from the change in membrane charge taken from the integrated capacity transients (pClamp, Molecular Devices). All potentials cited here have not been corrected for liquid junction potentials (estimated using pClamp calculator as 9.2 mV). Slices were incubated in drug cocktails for minimum of 15 min prior to recording.

For evoked postsynaptic currents, a bipolar stimulating electrode (FHC) was placed 100-300μm rostral to the recorded neuron, and used to stimulate

afferents at 0.03 Hz. Stimulus intensity was controlled using an ISO-flex stimulus isolator (AMPI) and adjusted to evoke monosynaptic events. Therefore stimulation only elicited currents with a single peak, and fast rise and decay kinetics. $GABA_A$ receptors were blocked by picrotoxin (100 μM). The whole-cell recording electrode (4-7 MΩ) was filled with an internal solution containing (in mM): CsCH3SO3 128, HEPES 20, TEA-Cl 5, NaCl 2.8, EGTA 0.4, MgATP 2, NaGTP 0.5 (pH 7.25- 7.35, 280-285 mOsm). The putative VTA dopamine neurons were initially voltage clamped at -70mV and gradually shifted to +40mV. Once a stable clamp was achieved, a single stimulus at an interval of 20s was applied and eEPSCs were obtained. After at least 10 sweeps of stable current recording were successfully made, d-AP5 (50 μM) was applied to the slice for a minimum of 10 min and pure AMPAR-mediated eEPSCs were recorded. NMDAR-mediated currents were obtained by a digital subtraction between the mixed current and the AMPAR current using Clampfit 10.2 (Molecular Devices; Sunnyvale, CA, USA). The AMPAR/NMDAR ratio was calculated by dividing the peak amplitude of the average AMPAR-mediated eEPSC by the peak amplitude of the NMDAR-mediated eEPSC.

For optogenetic and pharmacogenetic stimulation, VTA dopamine neurons were identified by the expression of mCherry or YFP. The whole-cell recording electrode (5-7 MΩ) was filled with an internal solution containing (in mM): K-Gluconate 140, KCl 5, HEPES 10, EGTA 0.1, MgCl2 2, MgATP 2, NaGTP 0.2 (pH 7.3-7.4, 280-285 mOsm). A blue light (470nm) was delivered by TTL-control from a microscope-mounted LED to the entire field through the

365 objective. After the achievement of stable current clamp, a rapid flash of light
366 for 5ms (25Hz) with an inter-stimulus interval of 5s was given for 4 times at
367 the 60s inter-sweep interval. The light intensity was adjusted according to the
368 magnitude of its response. The yellow light (585 nm) was delivered by TTL-
369 control from a microscope-mounted LED to the entire field through the
370 objective. After the stable current clamp was achieved, +75pA current step
371 (12s) was given and 2 sets of 8s continuous light stimulation was applied at
372 2s intervals. The light intensity was adjusted according to the magnitude of its
373 response. For the hM4Di experiments, 100μM CNO (C0832, Sigma) was
374 pipetted directly into the bath chamber after obtaining a stable spontaneous
375 firing for 10min. After the membrane potential was hyperpolarised, CNO was
376 washed off with aCSF. For hM3Dq, 1uM CNO was perfused onto the brain
377 slice after obtaining a stable spontaneous firing for 10 mins and change of
378 membrane potential and firing frequency was monitored for 20 min.
379
380 In vivo *electrophysiology*
381 C57BL6 mice were anaesthetised with isofluorane and maintained with
382 urethane during recording. Body temperature was maintained at 35^0C ±0.5
383 with a homeothermic heating blanket connected to a rectal thermometer
384 (Harvard Apparatus, Edenbridge, Kent UK). Hydration was maintained with
385 injections of 0.45% saline or 0.9% saline every 3h for mice in the salt depleted
386 experiment and mice in the overnight fasted experiment. A craniotomy was
387 performed above the VTA, removing a rectangular section of skull 3-3.6 mm
388 from bregma, and 0.5 mm either side of the midline in length, avoiding
389 damage to the underlying dura and mid-sagittal sinus. A glass electrode was

then positioned ±0.4 mm mediolateral to bregma and within the range of -3.2 to -3.5 mm anterior-posterior to bregma. The glass electrode was then lowered at a speed of 10μm/s to a depth of -3.5 mm from the dura. The electrode was then slowly lowered at 1 μm/s, stopping at spike detection. Extracellular recordings were made with glass microelectrodes (tip diameter, 1-1.5 um; 15-25 MΩ) lowered into the VTA with a micromanipulator (single-axis IVM) controlled via LINLAB software and a PatchPad (all from Scientifica, Uckfield, UK). Signals were AC-coupled, amplified (x 1000) and bandpass filtered (0.3-5 kHz) with a Neurolog system (NL102G head-stage and DC preamplifier; Digitimer), and acquired on-line with a Micro1401 interface and SPIKE2 software (v6; Cambridge Electronic Design, Cambridge, UK). Mains noise (50 Hz) was eliminated with 'Humbug' filters (Quest Scientific, North Vancouver, BC, Canada). Electrophysiological recordings were collected from putative dopamine neurons (identified using electrophysiological criteria; Ungless & Grace, 2012) sampled using multiple penetrations within the VTA in a random order across an AP gradient of -3.2 to -3.5 mm in the left hemisphere and right hemisphere. Neurons were recorded once their baseline firing had stabilized, data were collected for a 3 min spike train. Five spike firing parameters were extracted and analysed from in vivo recordings: firing rate, coefficient of variation of the interspike interval (CV ISI), spike waveform shape and duration from onset (defined as a change of >0.02mV from baseline) to the negative trough (Ungless *et al.*, 2004) and percentage of spikes within a burst (Grace & Bunney, 1984). Single unit recordings were performed by an experimenter blind to condition. All parameters were analysed with scripts and algorithms within Spike2 (CED, Cambridge, UK).

Experimental Design and Statistical Analyses

Behavioural data were analysed using a mixed ANOVA with Time and Concentration, where appropriate to analysis, as within subjects factors; and Genotype ($DATcre^+$ vs $DATcre^-$) as a between subjects factor. An ANOVA was performed to test the consumed weight of the jellies, total beam breaks or preference score (this is the intake of one concentration jelly over the course of the session divided by total intake of the jellies in that session). Where significant interactions were observed, follow-up pairwise comparisons were conducted. Violations of sphericity were adjusted for using the Huynh-Feld adjustment. Violations of Normality were assessed by plotting the residuals of the data. All electrophysiological data were analysed using non-parametric Mann-Whitney U tests. The significance level for all statistics was $p < 0.05$ (two-tailed).

Results

Optogenetic excitation of VTA dopamine neurons selectively decreases intake of high concentration salt jellies during salt appetite

To optogenetically excite VTA dopamine neurons, DATcre+ and DATcre- mice (Turiault *et al.*, 2007) were stereotaxically injected with a cre-dependent adeno-associated virus (AAV) containing an EF1α promoter-driven channelrhodopsin (ChR2) fused to mCherry (AAV-ChR2-mCherry; Figure 1A). We observed strong mCherry expression, and colocalisation with TH+ neurons, in VTA sections of DATcre+ mice (Figure 1B). We then confirmed with *ex vivo* recordings that our stimulation protocol (Figure 1C) excited identified VTA dopamine neurons (Figure 1D). Furthermore, following photostimulation in awake behaving mice, we observed increased cFos expression selectively in dopamine neurons, providing evidence of *in vivo* activation (Figure 1E).

We next investigated the effect of optogenetically exciting dopamine neurons on salt intake. Salt appetite was induced in mice by placing them on a low sodium diet and administering the sodium-wasting loop diuretic furosemide (Rowland *et al.*, 2004; Figure 1F). Salt intake was assessed using a three-choice salt jelly assay with three different concentrations (0.3 M, 0.15 M, & 0.075 M NaCl; Figure 1F; no effect of genotype was observed on this assay in the absence of stimulation; data not shown). Optogenetic excitation of VTA

dopamine neurons, initiated 5 min before exposure to salt jelly, selectively decreased consumption of the 0.3 M salt jelly in DATcre+ mice compared to DATcre- mice during the first 10 min of the test session, when salt intake was greatest (Figure 1G-I). When mice were tested again one week later, in the absence of photo-stimulation, no group difference was observed, indicating that the effects of optogenetic stimulation were reversible (Figure 1J-L).

One possible explanation of our results is that stimulation of VTA dopamine neurons induces an aversive state. Indeed, stimulation of mesocortical dopamine neurons (or dorsal raphe dopamine neurons) can induce a conditioned place aversion (Lammel *et al.*, 2012; Gunaydin *et al.*, 2014; Matthews *et al.*, 2016). However, given the relatively lateral position of our laser fibre we considered it unlikely that we were stimulating mesocortical dopamine neurons. Nonetheless, we used a biased conditioned place preference (CPP) test to assess the aversive properties of our optogenetic stimulation protocol. DATcre+ and DATcre- mice injected with AAV-ChR2 were first habituated to the three-compartment apparatus and their preferred compartment noted. The same optical stimulation procedure as used during the behavioural salt-jelly tests was then conducted in the non-preferred compartment for two days with alternating days in the preferred compartment without stimulation (Figure 1M). Following this, mice were tested for their compartment preference in the absence of optical stimulation. We replicated the effect of Tsai *et al.*, 2009) demonstrating that DATcre+ mice spent an increased amount of time in the compartment where they received stimulation versus time spent in this compartment prior to stimulation, whereas DATcre-

mice spent a similar amount of time in the stimulation compartment before and after conditioning (Figure1NO). This suggests that VTA dopamine neuron stimulation does not result in an aversive state.

The reduction in salt intake with optogenetic excitation of VTA dopamine neurons might be attributed to a general increase in locomotor activity competing with consumption (Carlton, 1963). However, analysis of locomotor activity during the four conditioning days revealed no differences in total activity between DATcre+ and DATcre- mice, with or without stimulation (Figure 1P).

Chemogenetic inhibition of VTA dopamine neurons does not affect intake of high concentration salt jellies during salt appetite

Next, we wanted to examine the effects of inhibition of VTA dopamine neurons on salt intake. To ensure robust inhibition of dopamine neurons we used a chemogenetic approach which tonically inhibits the spontaneous activity of neurons (Stachniak *et al.*, 2014). A cre-dependent adeno-associated virus (AAV) containing the Gi-coupled human M4 muscarinic DREADD coding sequence (hM4Di; a G-protein coupled receptor that decreases cell excitability) conjugated to the fluorescent protein mCherry (AAV-hM4Di-mCherry)**,** was injected into the VTA of DATcre+ and DATcre- mice (Figure 2A). We observed strong mCherry expression, and colocalisation with TH+ neurons, in VTA sections of DATcre+ mice (Figure 2B). We then confirmed with *ex vivo* recordings that application of CNO inhibited action potential firing of identified VTA dopamine neurons (Figure

2C). CNO was injected 30 min prior to testing, and had no effect on intake of jellies (Figure 2D and 2E). As no obvious change in behaviour was observed during the salt assay, we wanted to confirm we had an effective CNO dose to activate hM4Di *in vivo*. The same mice were placed in an open field chamber for 20 min, then injected with the same dose of CNO as used previously and returned to the chamber for 60 min. DATcre+ mice demonstrated significant decreases in their locomotor activity compared to DATcre- mice, suggesting that our chemogenetic approach was capable of inhibiting dopamine neurons (Figure 2F). We conclude, therefore, that inhibiting dopamine neurons does not affect salt intake.

We also carried out complimentary chemogenetic experiments to excite dopamine neurons, by expressing hM3Dq in the VTA (Figure 3A-C). However, this manipulation induced very high levels of locomotor activity (consistent with previous reports; Wang *et al.*, 2013) confounding interpretation of the apparent reduction in intake seen across all salt jelly concentrations (Figure 3D-F). This effect of chemogenetic excitation on locomotion, not seen with optogenetic excitation, may be due to the different temporal dynamics of the chemogenetic approach which is likely to have a slower onset and offset than the optogenetic stimulation, and/or the possibility that the chemogenetic approach excited a larger population of dopamine neurons compared to the more localised optogenetic approach.

Optogenetic excitation of VTA dopamine neurons selectively decreases intake of high concentration sucrose jellies following an overnight fast

We next sought to test whether the effects of optogenetic excitation of VTA dopamine neurons generalised to other types of appetite, in particular appetite for sucrose following an overnight fast. DATcre+ and DATcre- mice were again injected with AAV-ChR2 in the VTA, implanted with optical fibres, and then allowed to recover for one week. Mice were then handled and habituated to the testing apparatus and sucrose jellies as in the salt experiment. At ~ 4pm the day prior to testing, home-cage chow was removed and mice were fasted overnight. The following day, optogenetic excitation of VTA dopamine neurons, which began 5 min prior to testing, selectively decreased consumption of the high concentration sucrose jelly (assayed using three jellies of different sucrose concentrations: 10%, 20%, and 30%) in DATcre+ mice compared to DATcre- mice (Figure 4A-B). This selective decrease in consumption of the high concentration sucrose jelly in DATcre+ mice was observed during the first 10 min of the session when appetite was strongest (Figure 4C). These results parallel those seen during salt appetite, suggesting that excitation of VTA dopamine neurons may have a general effect on appetites.

Salt appetite and sucrose appetite do not affect firing activity or excitatory synaptic strength in dopamine neurons

Given the effect of increasing firing activity on salt and sucrose intake, we wanted to know what effect these appetites had on baseline dopamine neuron firing activity. Interestingly, despite the intensive study of the role of dopamine neurons in reward, little is known about how appetite affects their firing activity. One recent study reported an increase in burst firing, but not firing

553 rate, of substantia nigra dopamine neurons in response to prolonged food
554 restriction, but not an overnight fast (Branch *et al.*, 2013). In addition, they
555 observed an increase in excitatory synaptic strength following food restriction.
556 We, therefore, examined *in vivo* firing activity and *ex vivo* excitatory synaptic
557 strength in putative dopamine neurons in the VTA either after an overnight
558 fast or during salt appetite. First, we conducted single-unit extracellular
559 recordings of action potential activity from putative dopamine neurons in the
560 VTA of anaesthetised mice. We observed no effect of either an overnight fast
561 or salt appetite on firing frequency, burst activity, or firing regularity (Figure
562 5A-F). Second, we conducted whole-cell recordings of synaptic currents in
563 putative dopamine neurons in *ex vivo* acute brain slices. In particular, we
564 assayed AMPAR/NMDAR ratios (a commonly used measure of synaptic
565 strength in dopamine neurons; Ungless *et al.*, 2001; Branch *et al.*, 2013). We
566 observed no effect of either an overnight fast or salt appetite on
567 AMPAR/NMDAR ratios (Figure 5G-J). Taken together, these results suggest
568 that the acute induction of either a salt or sucrose appetite does not affect
569 baseline firing activity, or excitatory synaptic strength, in VTA dopamine
570 neurons.
571

572 **Optogenetic excitation of VTA dopamine neurons does not disrupt**
573 **salt concentration preference following an overnight fast**

574 Our results show that optogenetic excitation of VTA dopamine neurons
575 selectively can reduce intake of both high-concentration salt and high-
576 concentration sucrose. One possible interpretation of this effect is that the
577 optogenetic excitation somehow leads to the inability to perceive differences

in concentration or to exhibit preference behaviour. To test this, following an overnight fast, we presented mice with three jellies of differing salt concentration, but the same concentration of sucrose (Jelly 1 - 0.075 M NaCl + 10 % sucrose; Jelly 2 - 0.15 M NaCl +10 % sucrose; Jelly 3 - 0.3 M NaCl + 10 % sucrose) to assess whether mice could discriminate the differing concentrations of NaCl. DATcre+ mice and DATcre- mice exhibited a clear preference for the low concentration salt jelly (0.075 M NaCl + 10 % sucrose), as would be expected in this salt replete state (Figure 6A-C). Importantly, as expected, optogenetic excitation of dopamine neurons reduced overall jelly intake in the DATcre+ mice, but there was no interaction with salt concentration. Furthermore, when intake was expressed as preference scores it was clear that there was no effect of optogenetic stimulation on preference (Figure 6D).

Discussion

Here we showed that optogenetic excitation of VTA dopamine neurons specifically reduced intake of high concentration salt during salt appetite. This effect was relatively rapid (i.e., occurring within minutes of stimulation) and reversible (i.e., it was not present a week later in the absence of excitation). We did not detect any aversive properties of the optogenetic excitation of VTA dopamine neurons, nor did it lead to an increase in locomotor activity. We also found that chemogenetic inhibition of dopamine neurons had no effect on salt-intake. Furthermore, we found that optogenetic excitation of VTA dopamine neurons also reduced intake of a high concentration sucrose jelly following an overnight fast, complimenting recent reports of optogenetic excitation of VTA dopamine neurons inhibiting sucrose drinking (Mikhailova *et al.*, 2016) and chemogenetic excitation of dopamine neurons inhibiting food intake (Boekhoudt *et al.*, 2017). Taken together, these results suggest a general role of VTA dopamine neuron excitation in modulating intake during appetite. Importantly, we found that the specific reduction in intake of the high salt concentration jelly during salt appetite was not due to a disruption in the ability of the mice to demonstrate a preference. Although it is not possible for us to know which projection-specific populations of dopamine neurons we excited, it should be noted that because of the position of our optic fibres it is likely that we preferentially stimulated dopamine neurons in more lateral parts of the VTA, which are more likely to project to the striatum, and avoided more medially located mesocortical dopamine neurons which can drive aversive behaviour (Lammel *et al.*, 2012). Consistent with this, we found that our

stimulation protocol could generate a conditioned place preference, similar to that seen in previous studies (e.g., Tsai *et al.*, 2009).

Despite the intensive study of the role of VTA dopamine neurons in reward processing, relatively little is known about how appetite affects their firing activity or synaptic properties. Our *in vivo* recordings in both salt-depleted and fasted mice revealed no effect of these appetite manipulations on spontaneous firing activity of putative VTA dopamine neurons. Consistent with this, an overnight fast does not change the spontaneous firing of substantia nigra dopamine neurons (Branch *et al.*, 2013). However, more prolonged fasting or long-term food restriction has been shown to increase burst firing of dopamine neurons (Marinelli *et al.*, 2006; Branch *et al.*, 2013). Taken together these findings suggest that an acute appetite does not change dopamine neuron firing activity, but that more chronic manipulations of appetite may increase dopamine neuron firing activity possibly by engaging stress-related mechanisms. The excitatory inputs of midbrain dopamine neurons are highly sensitive to motivationally-significant events (e.g., a single exposure to addictive drugs such as cocaine, reward learning, stress, and long-term food restriction; Ungless *et al.*, 2001; Stuber *et al.*, 2008; Saal *et al.*, 2003; Branch *et al.*, 2013). We, therefore, tested whether manipulations of appetite used in this study changed synaptic strength in VTA dopamine neurons. No change was seen in AMPAR/NMDAR ratios following overnight fast or salt depletion, indicating that an acute appetite *per se* does not affect glutamatergic synaptic strength in dopamine neurons. We sampled from a broad population of putative dopamine neurons in the lateral parts of the VTA with unknown

640 projection targets, and it is therefore possible that our sample contained some
641 mesocortical dopamine neurons which do not exhibit synaptic plasticity to
642 appetitive events (Lammel *et al.*, 2011).
643
644 Our finding that optogenetic excitation of VTA dopamine neurons resulted in a
645 reduction in intake during appetite is consistent with reports of suppressed
646 intake with the systemic administration of either d-amphetamine (Kraeuchi *et*
647 *al.*, 1985 et al), cocaine (Wellman *et al.*, 2002 et al) or dopamine receptor
648 agonists (Chen *et al.*, 2008 ; Cincotta *et al.*, 1997, Kuo, 2002). A number of
649 studies have attributed the anorexic effects of amphetamine to its modulation
650 of the dopaminergic system. Supporting this, dopamine antagonists
651 (Leibowitz, 1975; Garattini *et al.*, 1976); electrolytic lesions and 6-OHDA
652 lesions of the nigrostriatal pathway (Carey & Goodall, 1975; Fibiger *et al.*,
653 1973) and 6-OHDA lesions of the neostriatum (Joyce & Iversen, 1984) all
654 alleviate the hypophagic effects of amphetamine. Direct evidence of the role
655 of dopamine in amphetamine-induced hypophagia is found in dopamine-
656 deficient (DD) mice that are insensitive to the hypophagic effects of
657 amphetamine (Cannon *et al.*, 2004). Moreover, viral restoration of dopamine
658 to the caudate putamen of these mice reinstated amphetamine-induced
659 hypophagia implicating dopamine signalling within the dorsal striatum in this
660 phenomenon (Cannon *et al.*, 2004). The specificity of these effects to the
661 dopamine system within this region is supported by the failure to ameliorate
662 amphetamine-induced hypophagia using a variety of manipulations targeting
663 alternative neurochemical systems thought to be altered by amphetamine
664 administration (Cannon *et al.*, 2004; Sotak *et al.*, 2005). Furthermore, no

665 effect was found with viral restoration of dopamine signalling in the nucleus
666 accumbens, which is consistent with a large body of literature on the failure to
667 disrupt the primary motivational properties of food with lesions to the nucleus
668 accumbens or dopamine antagonism within this region (Caine & Koob, 1994;
669 Roberts *et al.*, 1977; Salamone *et al.*, 2005).
670
671 A reduction in intake with increased dopamine activity may appear
672 counterintuitive considering dopamine's role in behavioural activation
673 (Salamone *et al.*, 2009; Robbins & Everitt, 2007; Syed *et al.*, 2016) and food-
674 seeking behaviour (Wise, 2006). However, it has been proposed that optimal
675 levels of dopaminergic activity are essential for the activation of
676 motivationally-relevant behaviour (Heffner *et al.*, 1977, Robbins, 2010;
677 Palmiter, 2007). The activation of motivated behaviour is dependent on an
678 inverted u-shaped function of dopamine activity. In the case of appetite,
679 optimal levels of dopamine activity result in food-seeking behaviour (Roitman
680 *et al.*, 2004). However, too little dopamine, exemplified in dopamine-deficient
681 mice, inhibits feeding as these mice die of starvation unless maintained with
682 daily injections of L-DOPA (Szczypka *et al.*, 1999; Zhou & Palmiter, 1995).
683 Too much dopamine, as may be the case with the present optogenetic study
684 and previous studies (van der Hoek & Cooper, 1994; Alnaser & Cooper, 1994;
685 Scislowski *et al.*, 1999; Mikhailova *et al.*, 2016), also results in inhibition of
686 intake during appetite. Our optogenetic excitation protocol may, therefore,
687 have resulted in dopamine activity beyond the optimal levels for engaging in
688 food consumption during a state of appetite. Importantly, the use of
689 pharmacological manipulations often produces confounding results to those

690 observed with more rapid optogenetic manipulations (Otchy *et al.*, 2015). Our
691 findings, therefore, usefully build on these previous pharmacological
692 manipulations by showing that direct excitation of dopamine neurons can
693 relatively rapidly suppress intake. Lastly, our observation that chemogenetic
694 inhibition had no effect on intake is reminiscent of the failure to affect food
695 consumption with 6-OHDA lesion and dopamine antagonism of the striatum
696 (Baldo *et al.*, 2002; Aberman & Salamone, 1999).
697
698 We propose that optogenetic excitation of VTA dopamine neurons reduces
699 salt or sucrose appetite such that mice are no longer driven to consume a
700 high concentration of a relevant reinforcer. Alternative theoretical accounts of
701 these results could be that increased tonic dopamine levels switch behaviour
702 from exploitation of a current food resource to exploration of the environment
703 for alternative food resources (Cohen *et al.*, 2007; Beeler *et al.*, 2010; Beeler
704 *et al.*, 2012; Humphries *et al.*, 2012) perhaps by increasing behavioural vigor
705 (Niv *et al.*, 2007). Although it is difficult to separate the different contributions
706 of phasic versus tonic dopamine in our task, and the effects of our stimulation
707 protocol on them, this account of our results seems unlikely for several
708 reasons. First, optogenetic excitation of VTA dopamine neurons during the
709 stimulation days of the CPP test showed no difference in locomotor activity
710 compared to non-stimulated sessions. Second, intake was decreased
711 throughout the 30 min intake test. If stimulation had led to more exploration,
712 but no suppression of appetite, then we might have expected mice to
713 eventually return to consume the jellies. Another possibility we addressed is
714 that the stimulation procedure disrupted the ability of the mice to demonstrate

715 preferential consumption of one jelly. However, when fasted mice were
716 presented with three jellies with differing concentrations of salt, but the same
717 concentration of sucrose, optogenetic excitation of VTA dopamine neurons
718 did not affect preferential consumption of the low concentration salt jelly. This
719 suggests the ability to exhibit preference behaviour is unaffected by
720 optogenetic stimulation of dopamine neurons and is consistent with the
721 observation that hyperdopaminergic mutant mice exhibit normal hedonic
722 'liking' responses to sweet tastes (Pecina *et al.*, 2003).
723
724 The reduction in intake of both salt and sucrose with optogenetic excitation of
725 VTA dopamine neurons suggests a common mechanism may have been
726 disrupted for both nutrient rewards. Dopamine within the dorsal striatum has
727 been shown to play a specific role in feeding behaviour as viral restoration of
728 dopamine signalling in dopamine deficient mice rescues feeding behaviour
729 (Cannon *et al.*, 2004). Our optical fibre was positioned preferentially above the
730 dorsolateral ventral tegmental area. Considering the topography of projections
731 of the ventral tegmental area it is possible that we excited neurons projecting
732 to more dorsal regions of the striatum. Activation of dopamine projections
733 from the dorsolateral VTA to more dorsal regions of the striatum may provide
734 a nutritional signal despite a state of hunger such that mice are no longer
735 motivated to consume the highest concentration jelly.

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Figure Legends

Figure 1: Optogenetic excitation of VTA dopamine neurons selectively decreases intake of high concentration salt jellies during salt appetite. A) DATcre- and DATcre+ mice were injected with a cre-dependent adeno-associated virus carrying channelrhodopsin, conjugated to the fluorescent protein mCherry (AAV-ChR2-mCherry). B) Co-localisation of mCherry and TH confirmed expression of channelrhodopsin in VTA dopamine neurons in DATcre+ mice. C) Optical stimulation consisted of 8 pulses: 5ms on of a blue light laser, 37ms off. D) *Ex vivo* whole cell patch recordings confirmed the optical blue light stimulation protocol was sufficient to depolarise the VTA TH+ positive cells leading to phasic bursts of activity. E) DATcre+ (n=4) and wildtype litter mates (n=3) injected with AAV-ChR2-mCherry in the VTA were optogenetically stimulated using the same protocol. A significant increase in the number of dopamine neurons exhibiting cFos expression was observed in DATcre+ mice compared to DATcre- mice (23.1±3.6 vs. 12.9±1.5; t=2.326, p=0.028; N = 16 & 12 (sections); immunostaining for tyrosine hydroxylase (TH) and cFos), confirming VTA dopamine neurons were activated *in vivo* by optical blue light stimulation. F) An acute salt appetite was induced and assayed by injecting mice with the diuretic furosemide for two days before a preference test between three jellies of three different NaCl concentrations. DATcre- and DATcre+ mice were optogenetically stimulated with blue light during the preference test. G) DATcre- (n=8) and H) DATcre+ mice (n=6) differed in their intake of the different concentration salt jellies, Time x Genotype x Concentration $F(4, 48)=3.6$ $p<.05$. Main effects of Time

F(2,24)=10.3 p<0.005, Concentration F(1.5,18.2)=9.4 p<.005 and Time x Concentration F(2.5,30.4)=18.4 p<0.001) were also revealed with statistical analysis. There was no significant interaction of Time x Genotype (F(2,24)=1.7 p>0.1 N.S. I) A selective reduction in intake of the high concentration salt jelly occurred during the first 10 min of the test session in the DATcre+ mice, Concentration x Genotype F(2,24)=7.1 p<.005, Concentration F(1.5, 17.6)=34.11 p<.001), pairwise comparisons 0.3M salt DATcre- vs DATcre+ p<.05. J) and K) When tested a week later in the absence of stimulation, mice preferentially consumed the highest concentration salt jelly (Concentration F(1.3, 16.1)=40.6 p<.001). Overall mice decreased their consumption across time (F(1.7,20.0)=56.4 p<.001) presumably due to satiation which resulted in an overall change in preference for the high concentration salt jelly (Time x Concentration F(2.5,16.4) p<.001). No differences in intake were observed between salt-depleted groups across the session (Time x Genotype x Concentration F(4,48)= 1.7 p>0.1 N.S., Concentration x Genotype, Time x Genotype and Genotype all F's<1 p>0.5 N.S), L) nor during the first 10 min of the session (Genotype, Concentration x Genotype all F's <1, p's>0.3). M) DATcre- (n=9) and DATcre+ (n=6) mice injected with the cre dependent AAV-ChR2-mcherry virus in the VTA and were tested using an biased CPP test. Preference was assessed for one of two chambers (pre-test) followed by optogenetic stimulation for two days in the non-preferred chamber (days 2 and 4) and two days of no stimulation in the preferred chamber (days 3 and 5). The last day (post-test) mice were tested for their preference in the absence of stimulation. N) DATcre- mice (n=6) showed no preference for the chamber where they had previously

received stimulation (Stimulation F(1,5)= 2.9 p>0.1 N.S.; Session F(1,5)= 2.6 p>0.1 N.S.; Session x Stimulation F<1 p>0.7 N.S). O) DATcre+ mice (n=9) showed a significant preference for the chamber in which they had previously received stimulation (Session x Stimulation F(1,8)=7.0 p<.05), pairwise comparisons revealed this was specific to the stimulated chamber (pre vs post session p<.01). P) Optogenetic stimulation did not change locomotor activity as measured during the stimulation days of the unbiased CPP test (Stimulation; Stimulation x Genotype; Genotype all F's<1 p's>0.4 N.S.).

Figure 2: Chemogenetic inhibition of VTA dopamine neurons does not affect intake of high concentration salt jellies during salt appetite. A) DATcre+ mice (n=9) and DATcre- mice (n=11) were injected in the VTA with cre-dependent adeno-associated virus carrying the Gi-coupled human M4 muscarinic DREADD coding sequence conjugated to the fluorescent protein mCherry (AAV-hM4Di-mCherry). B) Co-localisation of mCherry and TH staining confirmed expression of AAV-hM4Di-mCherry in VTA dopamine neurons of DATcre+ mice. C) *Ex vivo* recordings confirmed CNO application to coronal VTA slices resulted in hyperpolarisation of VTA TH+ve neurons in DATcre+ mice that had been previously injected with AAV-hM4Di-mCherry. D) and E) Systemic CNO activation of AAV-hM4Di-mCherry prior to the salt appetite assay resulted in no significant effects on intake between genotypes (Time x Concentration x Genotype, Concentration x Genotype, Time x Genotype all F's <1 all p's>0.5). Intake decreased over time (F(1.3,23.9)=100.3 p<.001) with preference in concentration also changing over time (F(1.9,33.8)=6.0 p<.001). F) Systemic CNO activation of AAV-

hM4Di-mCherry in the VTA of DATcre+ (n=10) and DATcre- (n=12) mice resulted in a significant reduction in locomotor activity in DATcre+ mice, Treatment x Genotype $F(1,18)=8.5$ $p<.01$ pairwise comparisons revealed significant effects only following CNO treatment, DATcre+ vs DATcre- $p<.005$. There was a significant interaction between treatment and time, with locomotor activity continuing to reduce with time in the CNO DATcre+ group (Time x Treatment $F(11,198)=2.8$ $P<.005$). However, this did not differ between genotypes (Time x Treatment x Genotype $F(11,198)=1.1$ $p>0.3$; Time x Genotype $F<1$ $p>0.7$). Data represented as means ± SEM.

Figure 3: Chemogenetic excitation of VTA dopamine neurons induces behavioural hyperactivity and non-selective reduction in salt intake. A) DATcre+ mice and DATcre- mice were injected with cre-dependent adeno-associated virus carrying the Gq-coupled human M3 muscarinic DREADD coding sequence conjugated to the fluorescent protein mCherry (AAV-hM3Dq-mCherry). B) Co-localisation of mCherry and TH staining confirmed expression of AAV-hM4Di-mCherry in VTA dopamine neurons of DATcre+ mice. C) *Ex vivo* recordings confirmed CNO application to coronal VTA slices resulted in depolarisation of VTA TH+ve neurons in DATcre+ mice that had been previously injected with AAV-hM3Dq-mCherry. D) and E) Systemic CNO activation of AAV-hM4Di-mCherry prior to the salt appetite assay resulted in a significant reduction in intake across the session of DATcre+ mice (n=11) compared to DATcre- mice (n=12), Genotype x Time $F(2,42)= 5.7$ $p<.01$. Overall, intake decreased with time ($F(2,42)=140.8$ $p<.001$) and DATcre+ differ in intake to DATcre- ($F(1,21)=16.5$ $p<.005$). Unlike optogenetic

excitation of VTA dopamine neurons, this difference in intake between DATcre+ and DATcre- mice was not due to changes in preference of concentration (Time x Concentration x Genotype $F<1$ $p>0.5$ N.S., Concentration x Genotype $F(2,42)=2.1$ $p>0.1$ N.S, Concentration $F(1.6,34.5)=27.5$ $p<.001$). Changes in preference of salt jelly changed overall over the course of the session regardless of the genotype of the mouse (Time x Concentration $F(3.1,64.4)=10.4$ $p<.001$). Significant differences in intake occurred primarily in the first and last thirds of the session, pairwise comparisons; p's<.005 for intake during both 0-10 min and 20-30 min of the session for DATcre+ vs DATcre-. F) DATcre+ (n=15) and DATcre- mice (n=12) infused with the same hM3Dq virus in the VTA and injected systemically with the same dose of CNO, significantly increased in locomotor behaviour confirming the activation of the virus and effectiveness of the CNO dose Genotype x Drug $F(1,23)=46.2$ $p<.001$, Drug $F(1,23)=23.5$ $p<.001$; Genotype $F(1,23)=71.1$ $p<.001$. Data represented as means ± SEM.

Figure 4: Optogenetic excitation of VTA dopamine neurons selectively decreases intake of high concentration sucrose jellies following an overnight fast. A+B) Optogenetic excitation of VTA dopamine neurons resulted in a selective decrease in consumption of the high concentration sucrose jelly in B) DATcre+ mice (n=8) with respect to A) DATcre- mice (n=15), Time x Concentration x Genotype $F(4,84)=5.2$ $p<.005$, Concentration x Genotype $F(2,42)=3.6$ $p<.05$. There was no overall preference for one concentration ($F(2,42)=1.6$ $p>0.2$), nor did a preference occur over time (Time x Concentration $F<1$ $p>0.5$), or overall intake differ between mice (Time x

1170 Genotype, F<1, p>0.5; Genotype F<1, p>0.4). The selective reduction in
1171 intake of the highest concentration sucrose jelly in DATcre+ (n=8) mice with
1172 respect to DATcre- (n=16) mice was specific to the first 10 min of the session,
1173 Concentration x Genotype F(2,44)=5.3 p<.01, pairwise comparisons 30%
1174 sucrose DATcre- vs DATcre+ p<.01.
1175
1176 **Figure 5: Salt appetite and sucrose appetite do not affect firing activity**
1177 **or excitatory synaptic strength in putative VTA dopamine neurons.** A)
1178 Traces of firing activity from putative dopamine neurons from C57BL6 mice
1179 either salt-depleted or non-depleted saline controls. B) No differences in firing
1180 frequency of putative dopamine neurons were seen following salt depletion
1181 (n=22(13)) with respect to control saline injected controls (n=18(9)) (U=135
1182 p>0.5 N.S.). No differences in % spikes in bursts between groups (U=148
1183 p>0.1 N.S.). The coefficient of variation of the interspike interval did not differ
1184 between groups (U=186 p>0.7 N.S.). C) Individual frequency of firing of each
1185 putative dopamine neuron against its % spikes in a burst. D) Traces of firing
1186 activity from putative VTA dopamine neurons from C57BLk6 mice either
1187 fasted or non-fasted controls. E) No differences in firing frequency of putative
1188 dopamine neurons were seen following fasting (n=27(10)) with respect to
1189 controls (n=22(10)) (U=246 p>0.3 N.S.). No differences in % spikes in bursts
1190 between groups (U=209 p>0.8 N.S.). The coefficient of variation of the
1191 interspike interval did not differ between groups (U=265, p>0.5 N.S.). F)
1192 Individual frequency of firing of each putative dopamine neuron against its %
1193 spikes in a burst. G) Example traces of excitatory postsynaptic potentials in
1194 putative dopamine neurons from salt-depleted and non-depleted mice. H)

AMPA/NMDA ratio was unaffected in dopamine neurons of salt-depleted (n=10(5)) vs non-depleted mice (n=9(4)) (U=34 p>0.1 N.S.). I) Example traces of excitatory postsynaptic potentials in putative dopamine neurons from fasted and non-fasted mice. J) AMPAR/NMDAR ratio was unaffected in putative dopamine neurons of fasted (n=10(5)) vs non-fasted mice (n=11(6)) (U=50 p>0.1 N.S.) Ns are cells (animals).

Figure 6: Optogenetic excitation of VTA dopamine neurons does not disrupt salt concentration preference following an overnight fast. A+B) Although optogenetic excitation of VTA dopamine neurons reduced overall intake in the DATcre+ (n=16) compared to DATcre- mice (n=14) following an overnight fast throughout the session, it did not affect salt concentration preference. Time x Genotype x Concentration $F(4,112) = 1.1$ $p>0.3$ N.S. Overall intake was significantly reduced in DATcre+ mice (Genotype $F(1,28)=25.9$ $p<.001$) which differed across time, Time x Genotype $F(2, 56) = 3.6$ $p<.05$. C) DATcre+ and DATcre- mice preferentially consumed the low concentration salt +10% sucrose jelly, Concentration $F(1.7, 48.8)=21.3$ $p<.005$ (pw comparisons-, intake was significantly different between all concentrations p's<.05), which did not differ between genotypes, Concentration x Genotype $F<1$ $p>0.5$. Preference changed with time (Time x Concentration $F(2.4, 68.2)=10.3$ $p<.001$). D) Concentration preference did not differ between DATcre+ and DATcre- mice (Concentration x Genotype $F<1$ $P>0.5$) with all mice preferring the low salt concentration jelly (Concentration $F(1.9,45.6)=21.0$ $p<0.001$). Data represented as means ± SEM.