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Hedonic drinking engages a supra-spinal inhibition of thermal nociception in adult rats

Alexander J Davies[1,5*], Doyun Kim[1,2*], Jeongrak Park[2], Jeong-Yun Lee[2], Hue Vang[1], Anthony E Pickering[3,4#], Seog Bae Oh[1,2#].

[1] Dental Research Institute and Department of Neurobiology and Physiology, School of Dentistry, Seoul National University, Republic of Korea.
[2] Department of Brain and Cognitive Sciences, College of Natural Sciences, Seoul National University, Republic of Korea.
[3] School of Physiology, Pharmacology & Neuroscience, Medical Sciences Building, University of Bristol, United Kingdom.
[4] Anaesthesia, Pain & Critical Care Sciences, Translational Health Sciences, Bristol Medical School, Bristol Royal Infirmary, University of Bristol, United Kingdom.
[5] Nuffield Department of Clinical Neuroscience, John Radcliffe Hospital, Oxford University, United Kingdom (current address).
* These authors contributed equally.

[#]Correspondance should be addressed to:

- Seog Bae Oh, D.D.S., Ph.D. Department of Brain and Cognitive Sciences, College of Natural Sciences, Seoul National University, 101 Daehak-ro, Jongno-gu, Seoul 03080, Republic of Korea. Email: odolbae@snu.ac.kr, Tel: +82 (0)2 740 8656.
- Anthony E. Pickering, B.Sc., Ph.D., M.B., Ch.B., F.R.C.A. School of Physiology, Pharmacology and Neuroscience, Medical Sciences Building, University of Bristol, Bristol, BS8 1TD, United Kingdom. E-mail: Tony.Pickering@Bristol.ac.uk, Tel: +44 (0) 117 331 2311.

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Introduction

Aversion to pain and attraction to reward are strong motivators of behavior across species [12]. The rewarding nature of pain relief [56] suggests that stimuli or contexts of opposing valence may in fact lie along a continuum [51] but the neural circuits responsible for this reciprocal processing are poorly understood [16]. Consistent with this theory reward may conversely impart analgesia. It is believed that the calming effect of giving sugar (sucrose in solution) to human infants is a direct example of the phenomenon of pain reduction by reward [59] and it is empirically applied in paediatrics [36]. A recent systematic review concluded that sucrose may offer a reduction in procedural pain in new born infants [68]; however, there is limited evidence of a direct modulation of nociceptive processing at either the spinal or cortical level [66]. Thus the role of sucrose as a bona fide analgesic in human infants remains in question until more is known about the underlying neurobiology [28].

A sucrose-analgesia phenomenon has also been demonstrated in neonatal rats [14], which at least in part, survives mid-collicular decerebration [6], can be reversed by an opioid receptor antagonist [14], and reduces Fos-like immunoreactivity in the spinal cord [61] suggesting the involvement of endogenous descending inhibitory opioidergic signalling. In adults sucrose remains a strong motivator to feed, even in potentially hazardous circumstances [30]. However, the relative contributions of descending inhibition of nociceptive input versus more cognitively driven analgesia remains unknown and could provide clues to how these circuits are disrupted in chronic pain [56].

We have developed a simple and reliable model of sucrose drinking-induced analgesia in adult Sprague-Dawley rats using the Hargreaves' test of hind paw thermal sensitivity as a nociceptive assay. We used this assay to characterise the analgesic phenomenon and the context in which it appears. Specifically, we compared previous findings in neonatal animals [5, 15, 61], by examining the potential role of endogenous analgesia mediated by opioid and catecholaminergic neurotransmitters at the spinal level via descending inhibition. We further tested the requirement of expectation and learned experience [26] by the substitution of sucrose for water in hypovolaemic and euvolaemic animals. Finally, to examine the requirement for appetitive motivation in the analgesic effect of sucrose as a reward [31] we compared the effect of voluntary sucrose drinking with passive administration via an intraoral cannula.

Our results reveal an acute, potent and robust inhibitory effect of sucrose drinking on thermal nociceptive behavior. Unlike sucrose-analgesia in neonates the phenomenon is independent of endogenous opioid signalling and does not operate via classical descending catecholaminergic inhibition of the spinal cord circuitry. Experience of sucrose drinking had a conditioning effect sufficient to elicit a short-lasting placebo-like analgesia by water drinking alone in euvolaemic animals. In chronically cannulated animals sugar analgesia only appeared after repeated conditioning by oral perfusion, and was completely prevented systemic dosing with the cannabinoid CB1 receptor antagonist rimonabant. These results suggest that rewarding drinking recruits an

endogenous analgesic circuit that is dependent on supra-spinal endocannabinoid signalling.

Methods

Animals

All experiments and procedures were approved by the Institutional Animal Care and Use Committee at Seoul National University (SNU-151113-2) and are reported here in accordance with the ARRIVE guidelines [48]. Male and female Sprague-Dawley rats were purchased from Orient Bio (Charles River, Korea) aged 5 weeks and habituated for one week prior to experiments. Animals were maintained in an air conditioned clean facility (22 ± 2°C, 50 ± 10% humidity) and provided standard lab chow and water *ad libitum,* except when food or water was removed for deprivation experiments for the duration indicated. Behavioral testing was performed between the hours of 09.00-17.00. At the end of experiments animals were killed by rising concentration of CO_2 in accordance with Schedule 1 of the UK Home Office Animals (Scientific Procedures) Act 1986.

Thermal withdrawal latency testing (Hargreaves' test)

Hind paw thermal withdrawal latencies were tested using a thermal plantar analgesia instrument (Ugo Basile, Italy). After habituation on the glass plate, when rats were resting but not sleeping, the infrared stimulator (50% intensity) was applied to the middle area of hind paw. Thermal stimuli applied during voluntary drinking were started 1-2 s after the commencement of imbibing. A positive response was determined by a rapid flinch or lifting of the paw away from the stimulus. The stimulus was repeated 4

times on each paw with an interval of at least 2 min and the time taken to withdraw was averaged per paw, per rat. A cut-off time was set at 20 s to prevent tissue damage. For examination of spinal c-fos immunoreactivity after Hargreaves' testing a manually controlled thermal stimulator was used (Plantar Analgesia Meter, IITC Life Science, USA) to deliver repetitive stimuli of 10 s duration.

Drinking task training

Training was performed daily, typically between the hours of 09.00 and 13.00. Rats were habituated in individual test cages (30 – 60 min) on an absorbent mat which was removed prior to testing. Animals were provided with 10-20 ml of either a sucrose solution (10% in water) or sucralose (0.017% in water) in a plastic tray with access via an aperture (40 mm diameter) in the cage via a removable door (Figure 1A). At the commencement of drinking, a period of 30 s access was allowed before the door was closed. After a refractory period of 5 – 10 mins rats were allowed three more access periods (four trials in total). Training was deemed complete when animals spent more than 10 s drinking during any given 30 s access period (typically within 5 sessions).

Local inflammation model

Under brief isoflurane anaesthesia, rats received an intraplantar injection of complete Freund's adjuvant (CFA, 50 µg) in one paw (left or right). CFA was prepared as a 2:1 emulsion in sterile saline. Rats received a subcutaneous injection of CFA as a 75 µl bolus using an insulin syringe with a 26 gauge needle. 24 h after injection all animals

were re-tested for thermal withdrawal latency before (resting) and during sucrose drinking.

Intra-oral cannulation

Cannula were implanted using a variation on previous methods [34, 38]. For acute cannulation [34], under isoflurane anesthesia, a curved 20G needle and 32 mm catheter (3S Cath, Dukwoo Medical) was inserted intraorally through the buccal mucosa just rostral to the lower first molar on the right side. The needle and catheter was passed subcutaneously over the masseter muscle and between the right eye and ear to emerge on the back of the head between the ears. The needle was then removed and 20 cm of PE10 tubing (16 µl dead space volume) was passed through the catheter, which was then also removed leaving the tubing in place. A 3-4mm length of PE50 tube was fashioned into a flanged end and fitted to the oral end of the tubing via cyanoacrylate glue. The flanged end was pulled into the mouth to rest flush on the inner cheek. The protruding end was secured with a silk suture to the back of the head. Rats were recovered for at least one hour after cannulation before testing.

For chronic cannulation [38] in sucrose trained animals, an 18-gauge syringe needle was inserted between the scapulae and passed subcutaneously towards the intra-oral site (buccal mucosa just rostral to the lower first molar on the right side). An intra-oral catheter made of PE90 tubing with one end heat-flared to form a 2 mm-diameter flange was inserted from the intra-oral site and externalized between the shoulders. The needle was removed, and 1 cm of silicone tubing (0.058″ ID × 0.077″ OD) was sleeved

and glued with silastic medical adhesive silicone (Dow Corning) to fix the PE90 tubing. Rats were recovered for 5 days prior to oral perfusion experiments.

Intra-oral perfusion

Cannulated animals were placed in the testing chamber for 30 mins to habituate. The open end of cannula tubing was fixed to the top of the cage and fitted to an automatic syringe pump via a 40 cm length of PE50 tubing and 25G needle. 5 ml syringes and tubing were pre-loaded with water, sucrose (10%) solution or air (sham). In all perfusion experiments thermal stimulation began 10 s after the start of oral perfusion as indicated by masticatory movement of the rat jaw in the case of water and sucrose solution. Each paw was tested twice and average latencies plotted per animal. A 20 s flush with water was performed to remove traces of the previous solution between each trial. For repetitive sucrose experience in chronically cannulated rats, all animals were given a delivery of water, sucrose (10%) solution, or air (sham) in a random order, repeated 5 times in daily sessions for 5 consecutive days

Intrathecal (lumbar spinal cord) cannulation

Intrathecal cannulation was performed in accordance with previously published methods [39]. All surgical tools, solutions and catheter materials were sterilized beforehand by autoclave or ethylene oxide (EO) gas, respectively. Animals were weighed and given a prophylactic dose of the antibiotic enrofloxacin (5mg/kg) subcutaneously prior to surgery. Under isoflurane anaesthesia the lower lumbar and mid-scapulae areas of the back were shaved and wiped with iodine solution. A small midline incision was made (1-

2cm long) and a 24 gauge catheter needle was inserted at a 45 degree angle between the 5th and 6th lumbar vertebrae. Correct placement of the needle into the vertebral canal is indicated by a slight twitch of the tail [54]. A 32 gauge intrathecal cannula with stylet (ReCathCo) was inserted into the intrathecal space by slowly advancing the cannula rostrally (6 cm). The catheter stylet was then removed and the cannula attached to length of PE10 tubing (10 cm) by cyanoacrylate glue and attached to the muscle fascia with 7-0 silk. The lumbar incision was closed with 2-3 skin clips. The PE10 tubing was passed under the back skin and exited the incision between the scapulae. Here the tubing was connected to a back-mounted pedestal system (with 6 mm side connector) (Plastics One) via a short length (1.5 cm) of thick-walled PE50 tubing, again fixed with cyanoacrylate glue. The pedestal mesh was attached to the underlying muscle fascia with 6-0 sutures and the wound around the pedestal closed with 4-0 silk sutures. The cannula system was filled with 20 µl of sterile saline to maintain patency during recovery (3-4 days). Incision sites were treated with iodine and rats were returned to warmed individual cages for recovery. Successful cannula placement was confirmed after experiments by reversible bilateral hind-limb paralysis after intrathecal administration of 2% lidocaine (20 µl), and cresyl violet dye (20 µl) labelling of lumbar spinal cord at post-mortem. Out of 12 sucrose-trained animals that underwent intrathecal catheterization, patency was maintained in seven animals which had the lumbar position of the catheter confirmed for behavioral analysis.

Intrathecal injection

Drug or vehicle-only solutions were back-loaded into a loading cannula connected to a 50 µl Hamilton syringe (with 23G needle) on an automatic syringe pump (KD Science). Drugs or vehicle solutions were delivered as a 10 µl bolus followed by 18.6 µl flush with sterile saline given at 10 µl/min. After injection the connector was removed and the back pedestal cannula was fitted with a dust-cap. Animals were then immediately returned to the testing cage for thermal sensory testing.

Drug treatments

Naloxone HCl (Sigma) was prepared as a stock (50 mg/ml) in distilled water and diluted in 0.9% saline vehicle before systemic (1 mg/kg, i.p.) [26, 53] or spinal (10 µg, i.t.) [69] administration. Yohimbine HCl (Tocris) was prepared as a stock (10 mg/ml) in DMSO and diluted in 0.9 saline (30% DMSO vehicle) for spinal (30 µg, i.t.) [39] administration. S-sulpiride HCl (Sigma) was prepared as a stock (30 mg/ml) in DMSO and diluted in saline (10% DMSO vehicle) for systemic (10 mg/kg, i.p.) [9] administration. The CB1 receptor antagonist, rimonabant (SR141716A) (Tocris) was prepared as a stock in DMSO and diluted in a vehicle containing 2% tween-80 and 20% DMSO in 0.9% saline 45] for systemic administration (5 mg/kg, i.p.) [37, 73]. Sensory testing was performed 10-15 mins after systemic and spinal injections and completed within 45 min of drug treatment. The same animals underwent alternative treatment of drug or vehicle solutions separated by at least one rest day. Spinal drug treatments were followed by a 20 µl saline flush. All testing was performed blind to the treatment.

Spinal c-fos assessment

Sucrose-trained male SD rats received thermal stimulation (IITC Life Sciences, Plantar test) to their right hind paw of a set duration (10 s) repeated 15 times at an interval of 1-2 mins, either during sucrose drinking (n=6) or while resting (control, n=6). 2 h after the final heat stimulation animals received a lethal dose of sodium pentobarbital (>60 mg/kg, i.p.) and were transcardially perfused sequentially with PBS (0.01 M) followed by paraformaldehyde (4% in PBS). c-fos immuno-labelling was performed according to previously published methods [8]. The lumbar spinal cord was removed for post-fixation overnight and cryopreserved in 30% sucrose for 3-4 weeks at 4°C. Spinal tissue blocks were embedded in OCT compound (Leica) and free-floating spinal cord sections (50 µm) were cut on a cryostat followed by immunolabelling with an anti-c-fos (Ab-5) (4-17) rabbit polyclonal antibody (1:10,000, Calbiochem) and visualised using DAB staining technique (Vector Laboratories) before mounting on gelatin-coated glass slides. Images were acquired on a light microscope and analysed for the number of c-fos-like immunoreactive nuclei per section (four sections per animal) using the “analyse particles” function on ImageJ (NIH). Image thresholding prior to particle analysis was performed by an experimenter blinded to the treatment group. Two out of 12 animals (one sucrose, one control) were removed from analysis due to poor tissue fixation.

Statistics

Comparisons before and after sucrose drinking were made with paired t test. Comparisons between three or more groups of time points were made with repeat measures one-way ANOVA followed by Bonferroni post-test for multiple comparisons.

Two-way ANOVA was used for comparison of the effect of drug treatment on thermal withdrawal latencies before and during sucrose drinking. All tests are two-sided. Behavioral data were obtained by an observer blind to the treatment group. Analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Data are presented as mean ± standard error of the mean unless otherwise stated. $p<0.05$ was considered statistically significant.

Results

In an initial study we fasted 6-8 week old rats by removing access to chow overnight (12-16 h) and placed them in a testing apparatus with access to a sucrose solution via a closable aperture (Figure 1A, B). Thermal withdrawal latencies were not different after the transient fasting compared to the pre-fasting control. Subsequent consumption of a solution of 10% sucrose in water (equivalent to the typical sugar content found in soft drinks and fruit juices [75]) increased the thermal withdrawal latency from 10.9 ± 0.8 s to 14.8 ± 0.6 s ($p<0.001$, repeat measures One-way ANOVA, n=6 rats) (Figure 1C).

To mitigate the confounds of fasting in rats, such as metabolic stress and polyuria causing dehydration [55], we adopted a training schedule of sucrose drinking (see Methods). Prior to training, non-fasted animals displayed a long and variable latency to drink, taking an average of 72 ± 23 s (n=12) once given access to the sucrose solution. After several days of training, however, rats readily drank the sucrose solution upon access, with the average time taken to drink significantly reduced to less than 2 s by the sixth training session (Figure 2A). Trained rats consistently displayed an increase in withdrawal latency while drinking sucrose (10.7 ± 0.5 s) compared to resting baseline (8.6 ± 0.3 s, $p=0.0002$, paired t test, n=12 rats) (Figure 2B). Rats were often observed to continue consuming sucrose after withdrawal of the hind paw from the thermal stimulus, at which point the access window was closed. The sucrose effect was transient and the withdrawal latency returned to baseline when tested 1-2 min later. We also found the same phenomenon in female rats, showing that they were also capable of being trained to drink sucrose, with the average time to drink decreasing from 158 ± 23 s to 34 ± 7 s

by the fifth training session (n=12 rats, Repeat measures One-way ANOVA: $F(4, 44)=5.175$, $p=0.0017$. Dunnett's post -test: Session 1 vs 5, $t=3.806$, $^{**}p<0.01$), and paw withdrawals increasing from 10.3 ± 0.3 s at rest to 12.6 ± 0.3 s during sucrose drinking (n=12 rats, paired t test: $t=8.919$, $p<0.0001$)

In a subsequent session, male sucrose-trained rats were given access to the drinking area but not provided with any solution. In this experiment animals showed the same movement to the window, inserting their head through the aperture, but displayed no consistent change to their withdrawal latency ($p=0.1247$, paired t-test, $t=1.662$; n=12 rats) compared to rest (Figure 2C), suggesting that contextual expectancy alone is not sufficient for the analgesic response to a sweet reward. In a separate experiment rats were trained to consume the non-nutritive sweetener sucralose (0.017% w/v in water) at a concentration equivalent to the sweetness of 10% sucrose (1:600) [76]. Withdrawal latencies recorded during sucralose drinking were also elevated (11.1 ± 0.7 s; $p=0.0162$, n=12 rats, paired t-test) compared to prior resting values (9.3 ± 0.5 s) (Figure 2D). There was no difference in the percentage change in withdrawal latency during sucrose (125 ± 4% of resting) or sucralose drinking (120 ± 7% of resting) ($p=0.5676$, unpaired t-test, $t=2.074$; n=12 rats per group). These results indicate that sweet substances do not need calorific value to produce analgesia.

Next we asked whether sucrose-drinking analgesia persisted in rats in an already sensitised state. At baseline, prior to sensitisation with the inflammatory reagent complete Freund's adjuvant (CFA), rats displayed analgesia in both paws tested during

sucrose drinking (Figures 3A and 3B). A single bolus of CFA (50 µg) given subcutaneously to the plantar surface of the hind paw resulted, 24 h later, in a significant reduction in withdrawal latency in the injected (ipsilateral) paw, indicative of inflammatory hyperalgesia (Figure 3A). After CFA treatment, subsequent drinking of sucrose solution still produced an increase in withdrawal latency by an equivalent degree to that seen before sensitisation (Figure 3A), suggesting a potent analgesic mechanism capable of partially reversing inflammatory hypersensitivity. The effect of sucrose on the withdrawal latency in the untreated contralateral paw was also preserved, albeit at a slightly reduced magnitude (though not significantly) at Day 2 compared to Day 0 (Figure 3B), likely due to the increased weight-bearing on the uninflamed paw. These results complement previous findings in neonates [61] and suggest that the sucrose analgesia phenomenon in adults is sufficient to attenuate responses from hypersensitive thermal afferents.

Sucrose-analgesia in neonates has been shown to be an opioid receptor-dependent phenomenon [5, 14]. However, pre-treatment with the opioid receptor antagonist naloxone at a dose shown to block both endogenous and exogenous opioid analgesia in rats (1 mg/kg, i.p.) [26, 53] (Figure 4A) or given directly to the spinal cord (10 µg, i.t.) [69] (Figure 4B), did not block the analgesia induced by sucrose drinking.

Dopaminergic and noradrenergic neurotransmitter systems are involved in directing behavior towards sweet reward [17, 62] and are both capable of mediating analgesia via descending inhibitory signalling [42]. For example, consumption of sugar leads to the

release of dopamine in the striatum, a critical component of the brain's reward system and a driver of hedonic behavior even in the face of unpleasant stimuli [71]. In the spinal cord dopamine produces thermal analgesia via D2-like receptors [43, 70], therefore we treated rats systemically with the D2-like receptor antagonist sulpiride at 10 mg/kg, a dose sufficient to prevent analgesia induced by spinal administration of the dopaminergic agonist apomorphine [9]. The dopamine receptor antagonist did not alter sucrose analgesia (Figure 4C).

The actions of noradrenaline on α2-adrenoceptors to inhibit spinal nociception are well characterised [57, 67]. Sweet reward also increases the activity of noradrenergic locus coeruleus (LC) neurons, helping to direct attention to reward by releasing noradrenaline in the prefrontal cortex [17]. Conversely chemical ablation of LC projections dramatically reduces voluntary sucrose intake [4]. LC neurons are also among the brainstem nuclei to have been implicated in the phenomenon of sucrose analgesia in neonates [6, 49]. As systemic administration of α2 antagonists can have confounding autonomic and sedating side effects, we targeted the spinal cord directly with intrathecal injection of 30 µg yohimbine, a dose sufficient to block endogenous noradrenergic spinal analgesia [39]. However, intrathecal yohimbine also had no effect upon sucrose analgesia (Figure 4D). Together these results suggest that these descending catecholaminergic pathways are unlikely to be involved in the analgesic response to sucrose.

To examine whether sucrose drinking is inhibiting nociception at the spinal level (by another mechanism) we performed immuno-labelling for c-fos in the spinal cord as a

measure of activity in nociceptive circuits [40]. Animals received repetitive thermal stimulation to one hind paw either at rest or during concurrent sessions of sucrose drinking. Spinal cords were fixed 2 h after the final stimulation and processed for c-fos-like immuno-reactivity. The pattern of c-fos-like immunolabelling in the medial region of the superficial dorsal horn of the lumbar spinal cord with the repetitive stimulation protocol (10 s thermal stimulation repeated once per minute for 15 minutes) was consistent with previous observations of hind paw thermal stimulation [18, 77] and confirmed activation of the spinal nociceptive circuity (Figures 5A and B, Control). We also observed a small number of positive nuclei in the neck region of the deep dorsal horn, corresponding to laminae V-VI of the spinal cord. No c-fos-like reactivity could be discerned in the contralateral dorsal horn or ipsilateral ventral horn. Animals which drank sucrose during each thermal stimulation also showed similar pattern of c-fos expression in the spinal cord (Figure 5A), and quantification of c-fos-like immuno-reactivity showed no difference between groups in either superficial or deeper laminae of the spinal dorsal horn (Figure 5B). This result suggests that sucrose drinking does not significantly alter the activity in nociceptive circuits at a spinal level.

Next, we asked whether sweet taste was required for the analgesic effect by providing motivation to drink water instead of a sucrose solution. Rats deprived of water for 24 h readily drank water upon its presentation and this also produced an increase in withdrawal latency (Figure 6A), while water fasting alone had no effect on baseline latency (Figure 6A). By presenting water instead of sucrose to sucrose-trained rats we were able to examine the effect of water drinking in euvolemic animals. In this context

water drinking alone was sufficient to increase withdrawal latency (Figure 6B). Animals trained to drink non-nutritive sucralose also displayed a significant increase in mean withdrawal latency from 8.5 ± 0.5 s to 12.3 ± 0.9 s ($p=0.0026$, paired t-test, $t=4.112$; n=10 rats) when presented with water instead of sucralose. A trial-to-trial comparison of drinking performance during the four repeat thermal stimulations per session revealed that the total time spent drinking water decreased progressively compared to sucrose over the same number of trials (Figure 6C), though was still sufficient to elicit analgesia equivalent to sucrose over the four trials tested (Two-Way repeat measures ANOVA: sucrose v water: $F(1,66)=0.05$, $p=0.8317$, n=12 rats). This suggested that the conditioning effect of sucrose driving water drinking in euvolaemic animals rapidly extinguishes after repeated water exposure. Systemic treatment with naloxone had no effect on the acute analgesia induced by sucrose-conditioned water drinking, suggesting it was not mediated by endogenous opioidergic mechanisms (Figure 6D).

The opioid-independent nature of the analgesic response evoked by sucrose-conditioned water drinking, and it's rapid extinction are characteristic of non-opioid placebo analgesia [3, 22], which is thought to be mediated by cannabinoid CB1 receptors [10]. We therefore investigated the potential role of endocannabinoid signalling using the well-characterised CB1 antagonist rimonabant (SR141716A). Consistent with the role of CB1 receptors in controlling appetite [7, 24] systemic treatment with rimonabant at (5 mg/kg i.p.) in sucrose-trained rats led to a dramatic increase in the time taken to start drinking sucrose compared to vehicle (Figure 7A) animals receiving rimonabant showed little interest in the drinking access window and

latency times were not significantly different from pre-training values (p=0.5550, unpaired t test; t=0.6088, n=7 rats). Therefore to bypass the suppressive effect of CB1 antagonism on voluntary sucrose drinking we applied solutions directly to the oral cavity via an intraoral catheter [34, 38]. Administration of 20 µl sucrose solution intraorally elicited tongue and mouth movements in the animals without spillage indicative of tasting and active ingestion [34]. However, passive administration of sucrose solution in naïve animals was not sufficient to elicit thermal analgesia in rats compared with water perfusion or 'sham' dry running of the peristaltic pump as controls (Figure 7B), consistent with the reported loss of passive sucrose analgesia in development (>3 weeks old rats) [5].

This raised the question of whether the analgesic effect of oral sucrose requires prior experience of sucrose drinking. To address this question rats had chronic intraoral cannulation after sucrose drinking training. In the first session after recovery from cannulation we again saw no effect of oral sucrose perfusion compared to water or sham (air only) (Figure 7C). Interestingly, with repeated exposure via the oral cannula rats continued to consume sucrose (Movie 1) but in contrast allowed water to drip from their mouth (Movie 2), suggesting that a preference for sucrose had developed and that as a consequence water consumption was being avoided in these animals. By the fifth repeat session of oral perfusion we observed a significant increase in withdrawal latency during sucrose perfusion (Figure 7D). In these animals, withdrawal latencies were consistently elevated during sucrose perfusion compared to water (Figure 7E). Under these conditions, pre-treatment with the CB1 antagonist rimonabant completely

blocked the analgesic effect of sucrose (Figure 7F). Together, these results suggested that taste experience alone is not sufficient to reveal the analgesic effect of sucrose drinking. Instead, the development of a preference for sucrose after repeated exposure appears to engage an analgesic mechanism via endogenous activation of cannabinoid CB1 receptors.

Discussion

We found that sucrose drinking in trained adult rats produces a robust and reproducible thermal analgesia which was sufficiently potent to oppose inflammatory thermal hyperalgesia. The magnitude of the analgesic effect was similar to that previously seen in neonates [61] and was seen in female as well as male animals. The analgesic effect was reproducible on repeated testing over hours and days and was temporally aligned to the periods of drinking behavior, consistent with previous reports of ingestive analgesia [29]. The effect extended to sucralose and to water (if fluid deprived or when consumption was conditioned by previous sucrose experience) indicating the analgesia was seen with hedonic drinking rather than simply nutritive or hydrative consumption [31].

The main central projection of the gustatory nerves is to the nucleus of the solitary tract (NTS) [35], which in turn sends projections to the parabrachial (PB) nucleus [21]. Indeed, Fos-like immuno-reactivity is induced in the NTS and PB by analgesic sucrose consumption [6]. Direct electrical stimulation of the NTS produces analgesia in rats [52] and this has been linked to the release of beta-endorphin within the brainstem to produce an opioidergic analgesia [19]. The NTS and PB also make extensive connections to the key trimverate of this descending system namely the periaqueductal gray (PAG), locus coeruleus (LC) and the rostro-ventral medulla (RMV) [58]. We therefore reasoned that targeting the opioidergic neurotransmitter system may modulate the analgesic effect of sucrose. However, treatment with naloxone (either spinally or systemically) failed to prevent the change in paw withdrawal latency observed during

sucrose drinking. Additionally, pre-treatment with D2-like receptor and α2-adrenergic receptors antagonist at doses sufficient to block spinal analgesia by dopaminergic [9] and noradrenergic agonists [39] also had no effect on the analgesic response of sucrose drinking. Furthermore, the number of Fos-like nuclei in either the superficial or deep lamina of the spinal cord were not different between sucrose-fed and control groups, suggesting that sucrose analgesia is achieved without affecting either the input of noxious stimuli to the spinal cord [1] or interneuron circuits that drive the spinal reflexes [44]. Together these data suggest that the neonatal phenomenon [5, 15, 61] and the adult behavioral paradigm reported here are engaging distinct neural circuitry.

A form of naloxone-sensitive sucrose analgesia has been reported in adult rats using the tail flick assay [23]. In our study we used the Hargreaves' model of thermal nociception. Unlike the tail flick response, which is preserved in spinalized rats [41] the behavioral response to thermal stimulation of the hind paw (hot plate; Hargreaves) is thought to engage supra-spinal processing [20]. The latency of both the tail flick (a spinal reflex) and hot plate response are increased by stimulation of the PAG and RVM [42]. However, while the analgesic effect of RVM stimulation on the tail flick response can be inhibited by spinal administration of either opioidergic or monoaminergic antagonists, none of these treatments applied spinally antagonised the effect of RVM stimulation on the hot plate response [42] suggesting that such brainstem nuclei are indeed capable of mediating a form of analgesia without engagement of the descending spinal projections.

Water is a strong motivator in water-deprived rats [63]. In our study water consumption by hypovolaemic rats also increased paw withdrawal latencies. Like sucrose in satiated rats, this observation is consistent with the potential role of reward pathways in the analgesic effect. Euvolaemic rats, as expected, were not motivated to drink water. However, by presenting water to rats after voluntary sucrose or sucralose training, we were not only able to motivate euvolaemic animals to drink water, but also to mimic the analgesic effect of both substances, suggesting that caloric consumption is not required and that brain pathways related to the expectation of gustatory reward [71] are sufficient for the analgesic effect.

The effect of water consumption when presented to an animal expecting sugar reward is consistent with the definition of placebo analgesia [74]. In our study, the analgesic response evoked by sucrose-conditioned water drinking was not blocked by naloxone which suggested that this paradigm may be an example of non-opioid placebo analgesia [3]. Interestingly, over the course of the four trials we recorded progressively shorter drinking times for water than for sucrose in sucrose-trained animals, such that by the fifth trial rats did not drink water and therefore could not be tested further. This apparent extinction of the effect is again consistent with a placebo-like response [22].

The neuropharmacology of placebo responses are thought to differ depending on the context of the conditioning paradigm, with expectation-evoked placebo driven by endogenous opioids and conditioning-evoked placebo driven by other specific

transmitter systems [3]. In one of the original descriptions of sweet-substance analgesia by Dum & Herz it was shown that naloxone-sensitive analgesia could be elicited by expectation of sweet reward alone. [26]. In our paradigm we observed that simply expectation of reward in the absence of consumption was not sufficient to increase withdrawal latencies. This result could be due to the lack of a distinct conditioning cue, which in the case of a learned reward response is necessary to engage attentional circuits in the brain stem [17]. The opioid-dependency of sucrose analgesia may therefore depend on differences in the past learning experience of sucrose consumption [3].

The similarity with the characteristics of non-opioid placebo analgesia [3, 22] led us to investigate the possible contribution of cannabinoid CB1 receptors [10] to the sucrose analgesia phenomenon. Treatment with the CB1 receptor antagonist rimonabant significantly reduced the motivation of the animals to drink sucrose, consistent with the known suppressive effect of the drug on the consumption of palatable foods [7, 65]. Therefore, in order to test the effect of the drug on withdrawal latencies during sucrose drinking we switched to a system of direct perfusion via an intraoral cannula. In contrast to previous studies in neonatal animals [5, 15, 61], passive intraoral administration of sucrose solution alone did not affect withdrawal latencies in naïve animals, nor when animals had previously been trained to voluntarily drink sucrose. Instead, the sucrose analgesia phenomenon was revealed by repeated exposure via the intraoral cannula which resulted in a clear preference for sucrose compared to water perfusion. This suggests that rather than simply taste experience, there is a requirement for the

development of a conscious preference for sucrose drinking in a specific context in order to endow the passively administered sucrose with sufficient hedonic value to elicit analgesia [31, 60].

It is possible that the revelation of the analgesic phenomenon by repeated sucrose exposure also represents the diminishment of an aversive component over time, which may otherwise mask an innate reward-analgesia by sucrose [16]. Conversely, the extinction of conditioned voluntary water consumption, and the failure to ingest orally perfused water could represent the *development* of an aversion to the previously neutral water in parallel to the learned preference for sucrose. Given the positive association between the preference for sweet drinks and the frequency of their consumption [33] our findings have implications for strategies to reduce the consumption of sugar-sweetened beverages, particularly in young children and adolescents [27].

We found that CB1 receptors play a critical role in the context of sucrose-conditioned analgesia. The neural pathway underlying this effect remains unknown but we may find clues in the role of endocannabinoids in food intake [24]. Feeding during satiety (a mark of hedonic consumption) has been linked to CB1 receptor signalling in both the hypothalamus [50] and PBN [25]. As a site of convergence of gustatory [21] and hypothalamic [2] pathways, and an important relay for spinal projection neurons [11], the PBN is well placed to modulate ascending nociceptive signalling by hedonic consumption. Interestingly, hypothalamic projections to the PBN have recently been shown to be sufficient to elicit analgesia during hunger [2]. We may speculate that

endocannabinoid signalling in the hypothalamic-PBN pathway could be sufficient for sucrose analgesia; however, important questions remain as to how this pathway could be specifically activated after learned sucrose–conditioning.

In humans, evidence of an analgesic-like response to oral sucrose is consistently observed in newborns [68, 72], while reports in adults are mixed [13, 46, 60]. The distinction between the supra-spinal analgesia we have observed here in the adult rat, and the descending spinal analgesia reported in neonatal animals [5, 15, 26], could help to explain some of the discrepancy. Intriguingly, the analgesic effect of oral glucose administration in newborns undergoing a minor procedure was not blocked by intravenous naloxone [32]. This raises the question of whether in fact the opioid-independent, supra-spinal analgesia we have observed in adult rats has more in common with human newborns, which also lack observable spinal nociceptive modulation during sucrose analgesia [66]. The prospect of sucrose mediating a placebo-like phenomenon also raises important ethical questions for the use of sucrose as an analgesic in newborn infants [28]. Further knowledge of the neural circuits involved will help not only inform the use of sucrose as an analgesic on the pediatric wards [66, 68], but also the neurological drivers of hedonic overconsumption leading to addiction and obesity [47] and their relationship to chronic pain [64].

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Conflict of interest statement

All authors declare no conflict of interest.

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Figure Captions

Figure 1.

Active consumption of sucrose (10%) solution in fasted rats increases hind paw withdrawal latency to heat. (**A**) Reward drinking and hind paw thermal sensory testing apparatus. (**B**) Naïve rats were food deprived overnight (16h) before being offered a sucrose (10%) solution through a window in the testing chamber. (**C**) Thermal sensitivity was tested at rest before and after fasting, and re-tested on commencement of drinking with sucrose solution. Heat stimulus was applied to the right hind paw four times (during separate trials) and the average latency plotted. Bars represent mean ± SEM. Repeat measures one-way ANOVA $F(2,15)=26.00$, n=6, $p=0.0001$; Bonferroni post-test, ***$p<0.001$. *ns*, not significant.

Figure 2.

Training reveals analgesic effect during active sucrose drinking in non-fasted rats. (**A**) Time to drink reward from window opening. Rats were trained in daily sessions to drink 10% sucrose solution provided through window (30s access periods, 4 repeat trials per session, 6-8 session repeats). Session 3, not determined. Bars represent mean ± SEM. One way repeat measures ANOVA, $F(4,44)=7.571$ with Dunnett's multiple comparisons to session 1, **$p<0.01$, ***$p<0.001$. (**B**) Non-fasted, sucrose trained rats. Withdrawal latency at rest and during sucrose drinking. n=12 rats, Paired Student's t-test. (**C**) Thermal withdrawal latency at rest and during access to the solution reservoir in the absence of sucrose solution. n=12 rats, paired Student's t-test. (**D**) Rats trained to drink

sucralose (0.017% w/v). Withdrawal latency at rest and during sucralose drinking. n=12 rats, paired Student's t-test. The large, filled data points in B-D represent mean values ± SEM.

Figure 3.

Sucrose drinking attenuates thermal hypersensitivity after local inflammation. The analgesic effect of sucrose drinking on heat withdrawal latencies on both paws was checked on all animals in a pre-test session (Day 0) and again on Day 2. On Day 1 all rats received an intraplantar injection of complete Freund's adjuvant (CFA, 50 µg, 2:1 emulsion in sterile saline, arrow) in one paw (ipsilateral). (**A**) Thermal sensitivity in CFA inflamed (ipsiateral) hind paw. Absolute withdrawal latencies over time (left). Repeat measures One-way ANOVA: $F(3,27)=111.3$, $p<0.0001$ with Bonferroni post-test, *p<0.05, ***p<0.001 (n=10 rats). Percentage change in latency (right). Paired t-test, p=0.6846. (**B**) Thermal sensitivity in untreated (contralateral) hind paw. Absolute withdrawal latencies over time (left). Repeat measures One-way ANOVA: $F(3,27)=44.46$, $p<0.0001$ with Bonferroni post-test, ***p<0.001 (n=10 rats). *ns*, not significant. Percentage change in latency (right). Paired t-test, p=0.0554.

Figure 4.

Sucrose drinking analgesia is not prevented by systemic or spinal naloxone, systemic dopamine D2-like receptor or spinal alpha2-adrenergic receptor blockade. (**A**) 8-10 week male SD rats treated with naloxone (1 mg/kg) or vehicle (saline) i.p. 10 min before Hargreaves' testing. Two way ANOVA: Effect of naloxone $F(1,10)=1.62$ p=0.2321.

Effect of sucrose F(1,10)=39.63, p<0.0001; Bonferonni post-test, *p<0.05, ***p<0.001. (**B**) Spinal treatment with naloxone (10 µg) or vehicle (saline) i.t. 10 min before thermal latency testing. Two way ANOVA: Effect of naloxone F(1,9)=0.54 p=0.4799. Effect of sucrose F(1,10)=98.74, p<0.0001; Bonferonni post-test, ***p<0.001. (**C**) 8-10 week male SD rats treated with sulpiride (10 mg/kg) or vehicle (10% DMSO in saline) i.p. 10 min before thermal latency testing. Two way ANOVA: Effect of sulpiride F(1,10)=0.05 p=0.8215. Effect of sucrose F(1,10)=22.15, p=0.0008; Bonferonni post-test, *p<0.05, **p<0.001. (**D**) Spinal treatment with yohimbine (30 µg) or vehicle (30% DMSO in saline) i.t. 10 min before thermal latency testing. Two way ANOVA: Effect of yohimbine F(1,11)=1.91 p=0.1948. Effect of sucrose F(1,11)=20.19, p=0.0009; Bonferonni post-test, *p<0.05, **p<0.01.

Figure 5.

Sucrose consumption does not affect expression of spinal c-fos during thermal stimulation of the hind paw. (**A**) c-fos-like immunoreactivity in the ipsilateral dorsal horn of the lumbar L4-6 spinal cord in control (*above*) and sucrose fed (*below*) male rats after repeated hind paw thermal stimulation. Note dark stained nuclei in the dorso-medial region of the superficial lamina (outlined by white dashed line) and neck region of the deep lamina. (**B**) Quantification of c-fos-like nuclei in control and sucrose-fed groups. Two-way ANOVA. Effect of sucrose: F(1,16)=0.01, p=0.9084; n=4 sections per rat, 5 rats per group. *ns*, not significant.

Figure 6.

Water drinking by water-deprived or sucrose-expectant rats increases paw withdrawal latency to heat. (**A**) Naïve rats were Hargreaves' tested before and after water deprivation (24h) at rest, and again at the commencement of drinking sucrose (10%) solution. Repeat measures one way ANOVA: $F(2, 15)=49.48$, $p<0.0001$. Bonferroni post-test: ***$p<0.001$. (**B**) Unlike naïve animals, previously sucrose-trained rats drank water, increasing withdrawal latency. Paired Student's t -test. (**C**) Total licking time declines during water drinking in sucrose-conditioned rats compared to sucrose drinking. Two-way ANOVA: Sucrose versus water drinking, $F(1,30)=15.38$, $p=0.0029$; Bonferonni post-test, *$p<0.05$, **$p<0.01$. (**D**) Systemic naloxone (1 mg/kg) did not prevent the analgesic effect of conditioned water drinking. Two-way ANOVA: Effect of naloxone, $F(1,10)=2.79$, $p=0.1257$; Effect of water, $F(1,10)=39.86$, $p=0.0002$; Bonferonni post-test, *$p<0.05$, **$p<0.01$.

Figure 7.

Sucrose analgesia in trained rats is depedent on endogenous activation of cannabinoid CB1 receptors. (**A**) CB1 receptor antagonist SR141716A (5 mg/kg, i.p.) increased the latency to start drinking sucrose. Sucrose trained animals were divided randomly into two groups. Each group received either the antagonist or vehicle and received the alternate injection two days later ($p=0.0004$, paired t test, $t=6.951$; $n=7$ rats). (**B**) Passive consumption of sucrose in naïve rats has no effect on thermal nociception. Naïve rats had an intra-oral catheter implanted under anaesthesia and then recovered for 1 h before Hargreaves' testing. Thermal withdrawal latencies were tested during oral

perfusion of water, sucrose (10%) or air (sham) at 2 µl/s. Bars represent mean ± SEM. Repeat measures one-way ANOVA, F(2, 17)=0.7544, p=0.4953; n=6 rats, with Bonferroni post-test for multiple comparisons. *ns*, not significant. (**C**) Sucrose-trained, chronically cannulated rats provided sucrose or water via intraoral cannula for the first time. Thermal withdrawal latencies were tested during oral perfusion of water, sucrose (10%) or air (sham) at 15 µl/s. p=0.1494, repeat measures one-way ANOVA F(2 23)=2.184, n=8 rats, with Bonferroni post-test for multiple comparisons, *ns*, not significant. (**D**) Sucrose-trained, chronically cannulated rats provided sucrose or water via intraoral cannula after five repeat sessions. Thermal paw withdrawal latencies were significantly increased during oral perfusion of sucrose (10%) compared to water or air (sham) at 15 µl/s. p=0.001, repeat measures one-way ANOVA F(2, 23)=18.04, n=8 rats, with Bonferroni post-test for multiple comparisons, **p<0.01 (t=4.304), ***p<0.001 (t=5.781). (**E, F**) Effect of CB1 receptor antagonist rimonabant or vehicle treatment on thermal paw withdrawal latencies during perfusion of water or sucrose in cannula experienced rats. (**E**) Withdrawal latencies were significantly increased during sucrose perfusion compared to water in vehicle-treated animals (p=0.0066, paired t-test, t=3.813; n=8 rats). (**F**) Rimonabant (5 mg/kg, i.p.) given 15 min prior to testing prevented the increase in latency during sucrose perfusion compared to water (p=0.9397, paired t-test, t=0.0786; n=8 rats).

Movie 1.

Intra-oral perfusion of sucrose (10% in water; 15 µl/s) in chronically cannulated rat after 5 repeat perfusion sessions. Note the movements of the tongue and mouth in the without spillage.

Movie 2.

Intra-oral perfusion of water only (15 µl/s) in chronically cannulated rat after 5 repeat perfusion sessions. Note the spillage of the water from the mouth in the absence of significant movements of the tongue or the jaw.

Summary (25 words max)

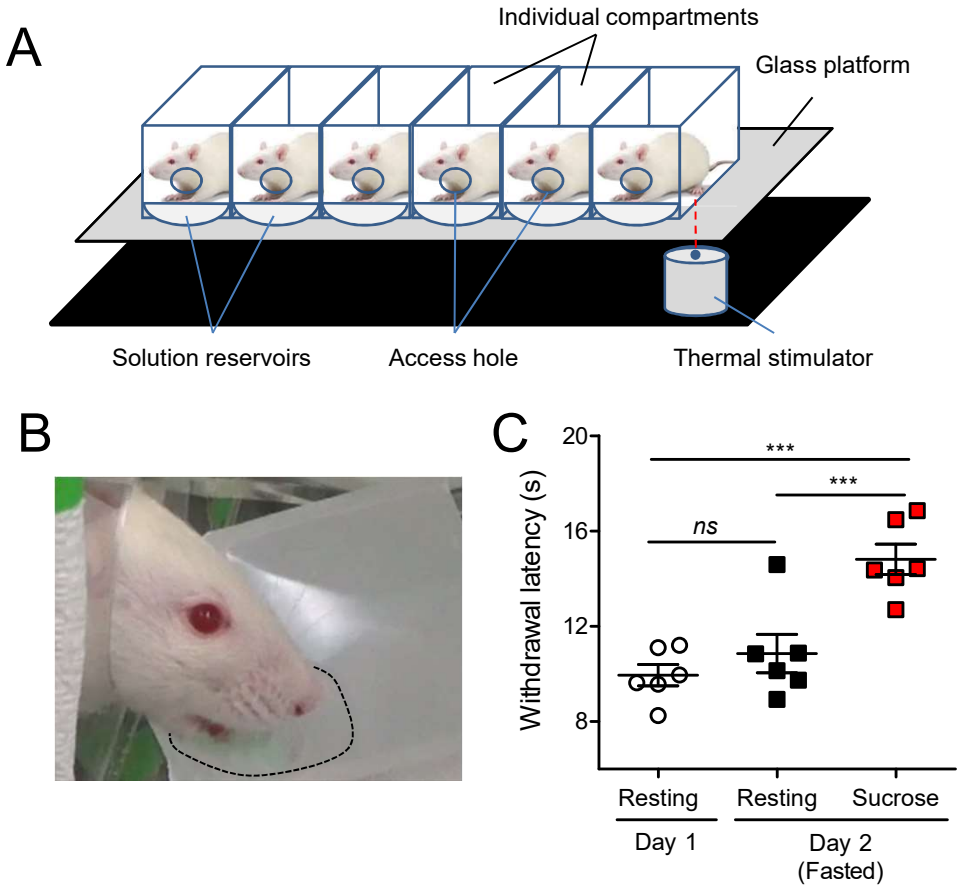
Scientific:

Hedonic drinking induces a potent thermal analgesia mediated supra-spinally by endocannabinoid signalling at CB1 receptors.

Lay:

Drinking sweet beverages suppresses the behavioural response to heat pain due to the release of cannabis-like chemicals in the brain.

Figure 1.


A
Individual compartments
Glass platform
Solution reservoirs
Access hole
Thermal stimulator
B
C
Withdrawal latency (s)
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16
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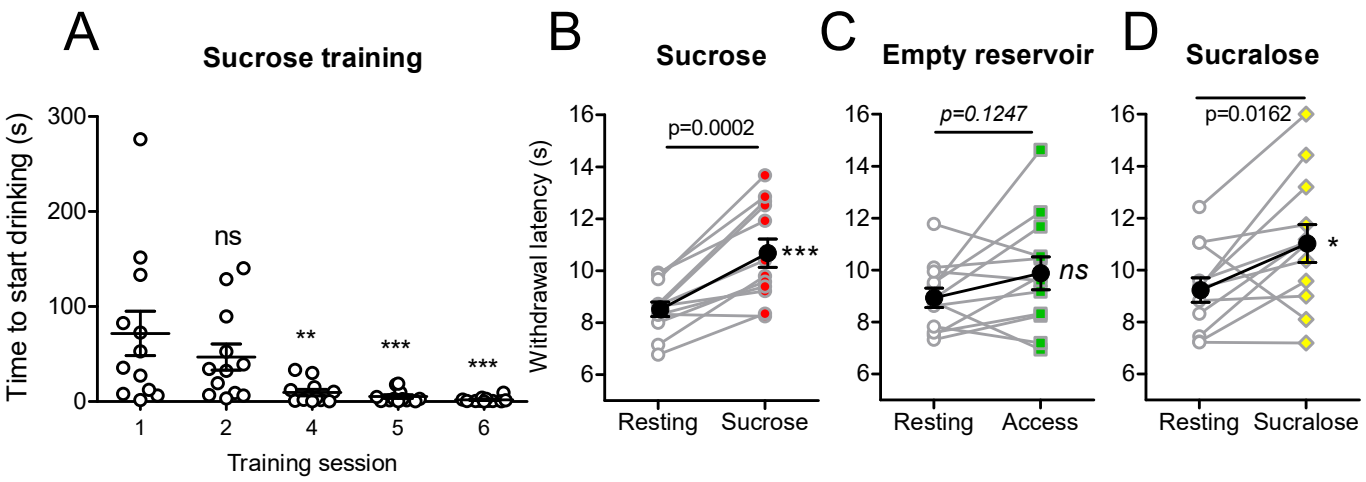
Resting
Resting
Sucrose
Day 1
Day 2
(Fasted)

Figure 2.


A
Sucrose training
Time to start drinking (s)
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0
ns
**
***
***
1
2
4
5
6
Training session
B
Sucrose
Withdrawal latency (s)
16
14
12
10
8
6
p=0.0002
***
Resting
Sucrose
C
Empty reservoir
p=0.1247
ns
Resting
Access
D
Sucralose
p=0.0162
*
Resting
Sucralose

Figure 3.

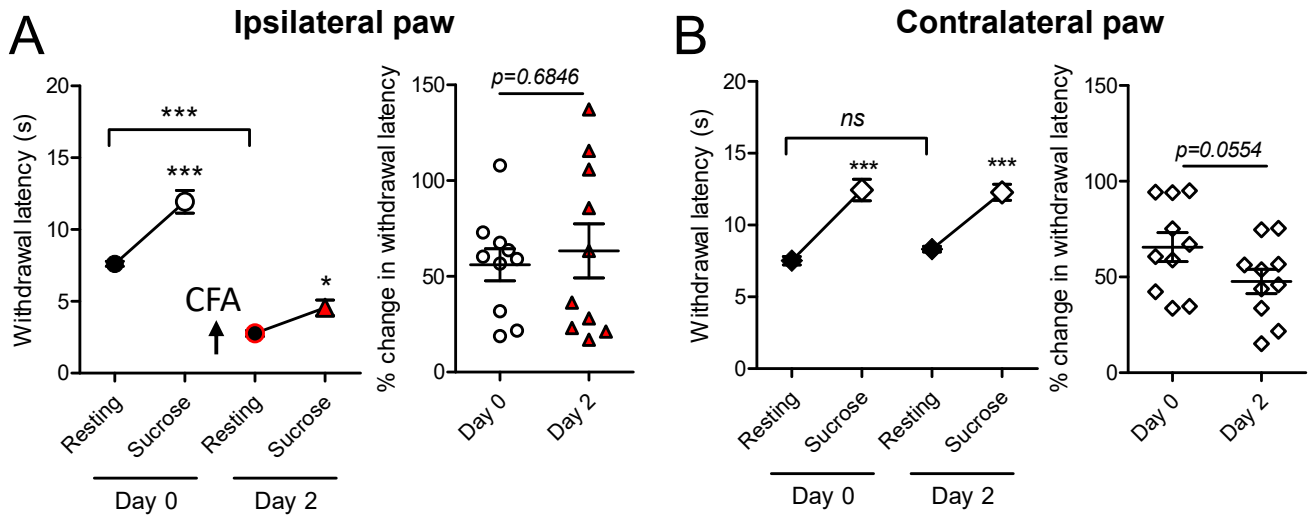

A
Ipsilateral paw
Withdrawal latency (s)
20
15
10
5
0
***
***
*
CFA
Resting
Sucrose
Resting
Sucrose
Day 0
Day 2
% change in withdrawal latency
150
100
50
0
p=0.6846
Day 0
Day 2
B
Contralateral paw
Withdrawal latency (s)
20
15
10
5
0
ns
***
***
Resting
Sucrose
Resting
Sucrose
Day 0
Day 2
% change in withdrawal latency
150
100
50
0
p=0.0554
Day 0
Day 2

Figure 4.

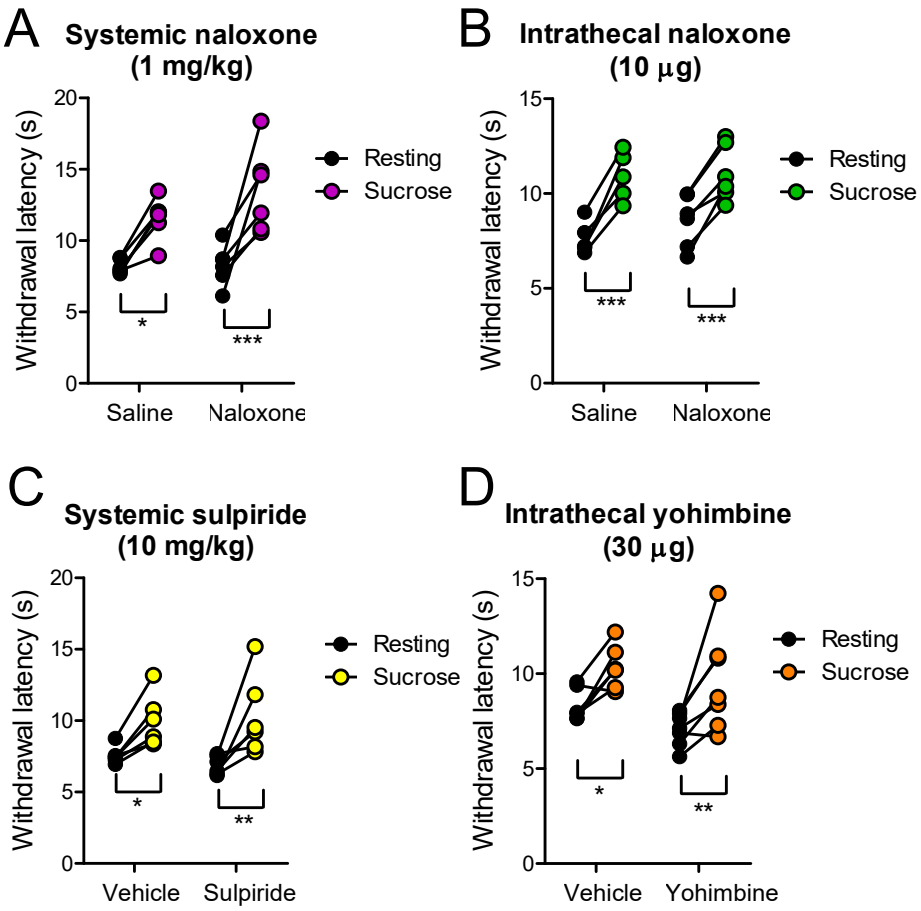

A
Systemic naloxone
(1 mg/kg)
Withdrawal latency (s)
20
15
10
5
0
*
***
Saline
Naloxone
Resting
Sucrose
B
Intrathecal naloxone
(10 μg)
Withdrawal latency (s)
15
10
5
0
***
***
Saline
Naloxone
Resting
Sucrose
C
Systemic sulpiride
(10 mg/kg)
Withdrawal latency (s)
20
15
10
5
0
*
**
Vehicle
Sulpiride
Resting
Sucrose
D
Intrathecal yohimbine
(30 μg)
Withdrawal latency (s)
15
10
5
0
*
**
Vehicle
Yohimbine
Resting
Sucrose

Figure 5.

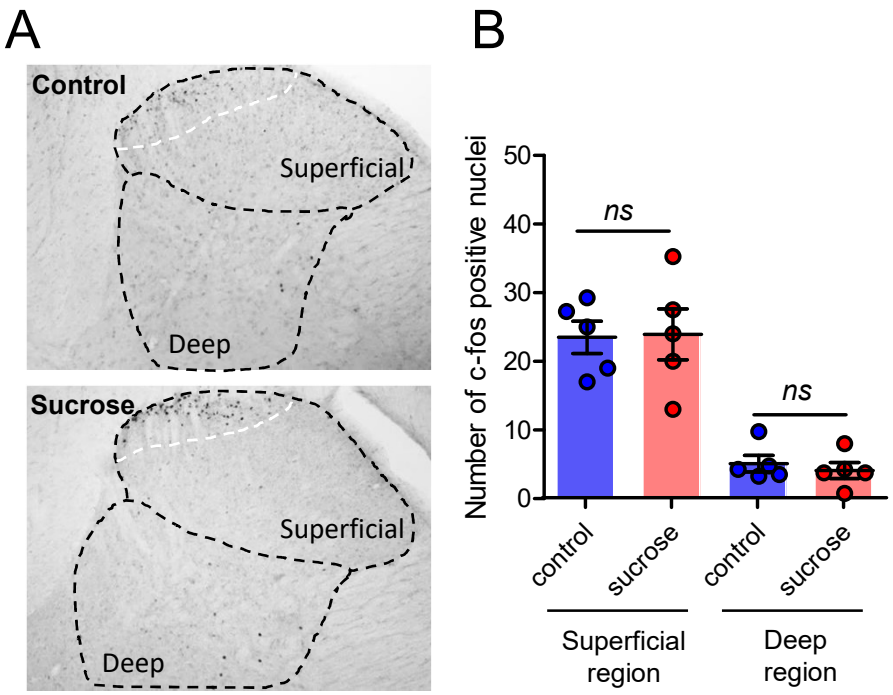

A
Control
Superficial
Deep
Sucrose
Superficial
Deep
B
Number of c-fos positive nuclei
50
40
30
20
10
0
ns
ns
control
sucrose
control
sucrose
Superficial region
Deep region

Figure 6.

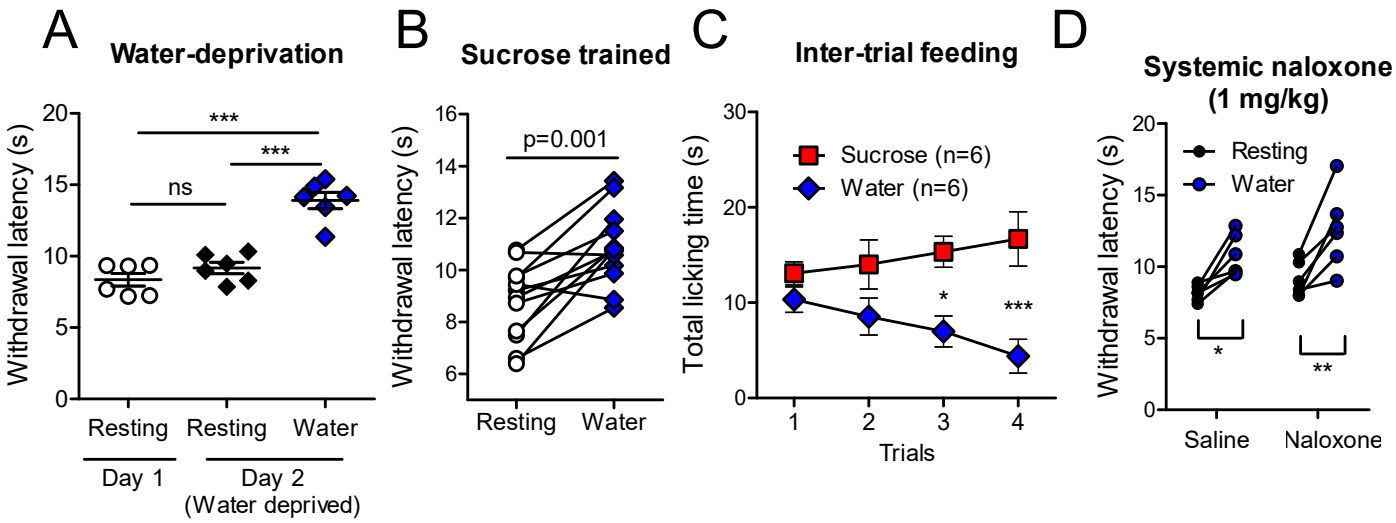
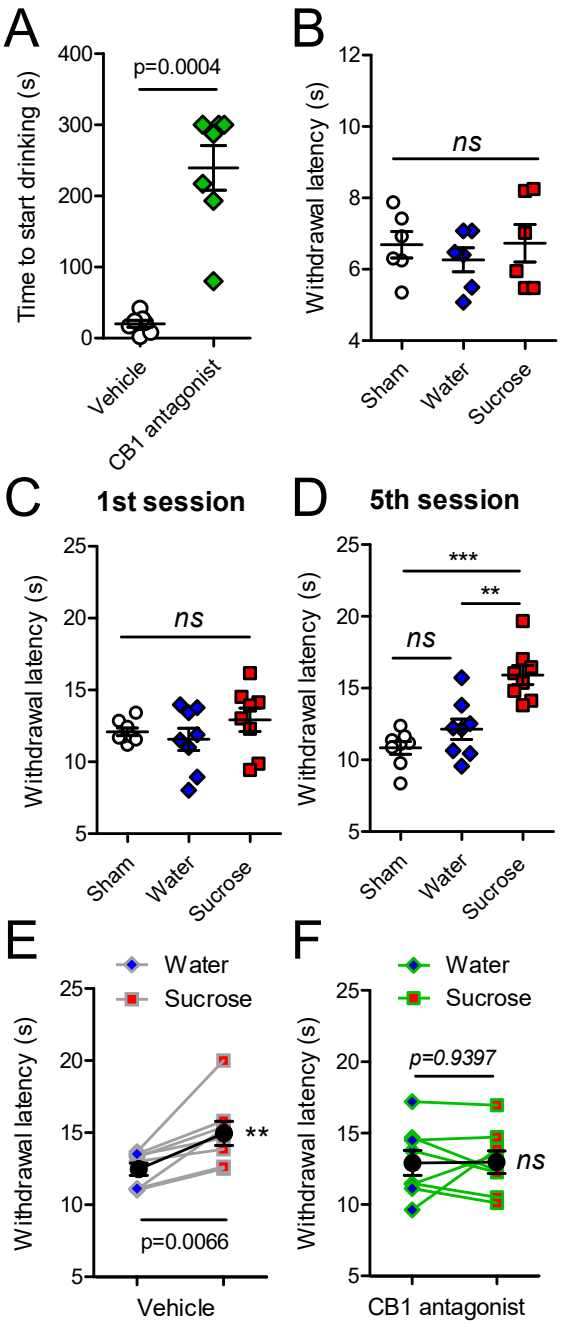

A
Water-deprivation
Withdrawal latency (s)
***
***
ns
Resting
Resting
Water
Day 1
Day 2
(Water deprived)
B
Sucrose trained
Withdrawal latency (s)
p=0.001
Resting
Water
C
Inter-trial feeding
Total licking time (s)
Sucrose (n=6)
Water (n=6)
*
***
Trials
D
Systemic naloxone
(1 mg/kg)
Withdrawal latency (s)
Resting
Water
*
**
Saline
Naloxone

Figure 7.


A
Time to start drinking (s)
400
300
200
100
0
p=0.0004
Vehicle
CB1 antagonist
B
Withdrawal latency (s)
12
10
8
6
4
ns
Sham
Water
Sucrose
C
1st session
Withdrawal latency (s)
25
20
15
10
5
ns
Sham
Water
Sucrose
D
5th session
Withdrawal latency (s)
25
20
15
10
5
***
**
ns
Sham
Water
Sucrose
E
Water
Sucrose
Withdrawal latency (s)
25
20
15
10
5
**
p=0.0066
Vehicle
F
Water
Sucrose
Withdrawal latency (s)
25
20
15
10
5
p=0.9397
ns
CB1 antagonist