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DOI: https://doi.org/10.1111/dom.14937

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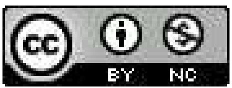

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Originally published at:
Asker, Mohammed; Krieger, Jean-Philippe; Liles, Amber; Tinsley, Ian C; Borner, Tito; Maric, Ivana; Doebley, Sarah; Furst, C Daniel; Börchers, Stina; Longo, Francesco; Bhat, Yashaswini R; De Jonghe, Bart C; Hayes, Matthew R; Doyle, Robert P; Skibicka, Karolina P (2023). Peripherally restricted oxytocin is sufficient to reduce food intake and motivation, while <scp>CNS</scp> entry is required for locomotor and taste avoidance effects. Diabetes, Obesity Metabolism, 25(3):856-877.
DOI: https://doi.org/10.1111/dom.14937

Received: 17 August 2022 | Revised: 21 November 2022 | Accepted: 2 December 2022

DOI: 10.1111/dom.14937

ORIGINAL ARTICLE

WILEY

Peripherally restricted oxytocin is sufficient to reduce food intake and motivation, while CNS entry is required for locomotor and taste avoidance effects

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Funding information

Knut och Alice Wallenbergs Stiftelse; National Institute of Diabetes and Digestive and Kidney Diseases, Grant/Award Number: DK128443-01; Swiss National Science Foundation,

Abstract

Objectives: Oxytocin (OT) has a well-established role in reproductive behaviours; however, it recently emerged as an important regulator of energy homeostasis. In addition to central nervous system (CNS), OT is found in the plasma and OT receptors (OT-R) are found in peripheral tissues relevant to energy balance regulation. Here, we aim to determine whether peripheral OT-R activation is sufficient to alter energy intake and expenditure.

Methods and Results: We first show that systemic OT potently reduced food intake and food-motivated behaviour for a high-fat reward in male and female rats. As it is plausible that peripherally, intraperitoneally (IP) injected OT crosses the blood-brain barrier (BBB) to produce some of the metabolic effects within the CNS, we screened, with a novel fluorescently labelled-OT (*f*AF546-OT, Roxy), for the presence of IP-injected Roxy in CNS tissue relevant to feeding control and compared such with BBB-impermeable fluorescent OT-B_{12} (*f*Cy5-OT-$B_{12;}$ BRoxy). While Roxy did penetrate the CNS, BRoxy did not. To evaluate the behavioural and thermoregulatory impact of exclusive activation of peripheral OT-R, we generated a novel BBB-impermeable OT (OT-B_{12}), with equipotent binding at OT-R in vitro. In vivo, IP-injected OT and OT-B_{12} were equipotent at food intake suppression in rats of both sexes, suggesting that peripheral OT acts on peripheral OT-R to reduce feeding behaviour. Importantly, OT induced a potent conditioned taste avoidance, indistinguishable from that induced by LiCl, when applied peripherally. Remarkably, and in contrast to OT, OT-B_{12} did not induce any conditioned taste avoidance. Limiting the CNS entry of OT also resulted in a dose-dependent reduction of emesis in male shrews. While both OT and OT-B_{12} proved to have similar effects on body temperature, only OT resulted in home-cage locomotor depression.

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Grant/Award Number: 183899; Vetenskapsrådet, Grant/Award Numbers: 2018-00660, 2021-01549

Conclusions: Together our data indicate that limiting systemic OT CNS penetrance preserves the anorexic effects of the peptide and reduces the clinically undesired side effects of OT: emesis, taste avoidance and locomotor depression. Thus, therapeutic targeting of peripheral OT-R may be a viable strategy to achieve appetite suppression with better patient outcomes.

KEYWORDS

food-motivated behaviour, ingestive behaviour, nausea, peripheral oxytocin, thermoregulation

1 | INTRODUCTION

Oxytocin (OT) is a small cyclic peptide hormone synthesized in the hypothalamus and secreted via the pituitary gland. OT is well-established as having a central role in parturition, milk let-down and bonding behaviours.[1] More recently, OT has also emerged as an important regulator of energy homeostasis and is considered a putative anti-obesity treatment.[2-7] Less consensus, however, characterizes the physiological role of OT in peripheral tissues; the function of which remains poorly understood, particularly in the context of food intake and motivation. Moreover, OT mRNA and OT itself has been reported in the myenteric and submucosal ganglia and nerve fibres along the entire human and rodent gastrointestinal tract.[8]

Links between feeding and circulating OT are suggested by data showing that food intake and gastric distension induce elevations in plasma OT in rodents and humans.[9,10] Further, postprandial serum levels of OT correlate with changes in brain activity, in nuclei associated with feeding behaviour control, including the hypothalamus, amygdala and hippocampus.[11] Exogenous administration of OT is sufficient to initiate a potent short-term food intake reduction in rats, mice or prairie voles after peripheral[12-15] and central administration.[12,13,16] OT also reduces food intake in humans.[17-20] Collectively, these data highlight the evolutionary conserved role of the OT system in energy balance control, warranting further exploration for potential broader therapeutic application.

The collective literature base supports the notion that plasma OT is a feeding status signal. However, the OT-R populations relevant to the anorexic effect of peripherally administered OT is poorly characterized. Here, we aim to determine whether peripheral OT alters food intake in male and female rats, and importantly whether activation of peripheral OT-R is sufficient to reduce food intake. The leading question in the current work is to understand the role of the peripheral OT and OT-R, while also examining the therapeutic potential of peripherally administered OT, with limited CNS entry. Therefore, to activate specifically peripheral OT-R only, we aimed to restrict OT from entering the brain and activating CNS OT-R by generating a new class of OT agonists that exploits vitamin B_{12} (B_{12}) conjugation ('corrination') to limit CNS entry.[21-23] Transport and trafficking of B_{12} that occurs in mammals is highly controlled and the uptake of corrinated compounds through the blood-brain barrier (BBB) and into the adult brain is extremely low.[22,24] Thus, OT-B_{12} should be an OT-R agonist that retains a peripheral site of action when systemically administered, without producing CNS-mediated effects. Here, we show its efficacy in in vitro and in vivo studies.

When food intake and motivation are reduced, it is possible that these behaviours are because of nausea or malaise. Nausea and emesis represent notable side effects of OT injections in women and can be so severe that they necessitate a follow-up anti-emetic cocktail treatment.[25] Up to one-third of women given OT during labour report nausea in a dose-dependent manner, and half also experience emetic episodes.[25] Very little is known about the mechanism of these illness behaviours beyond evidence that paraventricular nucleus (PVN) and supraoptic nucleus OT neurons are activated by emesis-inducing stimuli such as the chemotherapy agent cisplatin, and the anorexic effects of such can be blocked by CNS-specific infusion of an OT-R antagonist (L-368899), in rats.[26] Another potent emetic, LiCl, also activates PVN OT neurons and stimulates OT secretion, and the BBB-penetrant OT-R antagonist (L-368899) attenuates brain activation in response to LiCl.[27-29] Herein, we examine whether the illness-like behaviours reported in humans can be recapitulated in two animal models. In rats, we examined whether peripheral OT induces conditioned taste avoidance (CTA), a form of conditioned taste-visceral learning, where an increased drinking response to a taste stimulus can be replaced with an avoidance and rejection response, following its association with a negative visceral malaise consequence.[30] We also determine whether CTA can be mitigated when OT access to the brain is limited utilizing a novel OT-R agonist, OT-B_{12}. Furthermore, as rodents, unlike humans, are not capable of emesis here we test the emetic responses in shrews.[31] As preclinical medical research overwhelmingly studies mice or rats, using an animal model that displays emesis, the shrew, is unique. Using the shrew to evaluate the potential emetic adverse effects of pharmacological treatments, as it is one of the most phylogenetically ancestral mammalian models to humans, is capable of emesis, and shares similar emetic sensitivity to various anti-emetic medications.[21,22,32] Herein, we determine whether CNS entry of OT is required for nausea and emesis induction and hypothesize that bypassing the CNS will eliminate these OT-produced side effects.

Another behaviour that may confound or interfere with OT-mediated feeding effects is locomotor impairment. While many feeding hormones alter locomotor activity, it is necessary to parse out any anorexic effect of a substance from its effect on locomotion. At issue is the possibility that the primary effect of a substance is locomotor depression, and any observed reduction in feeding is a by-product of such. Herein, we set out to delineate any adverse effect specifically

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from peripheral OT, hypothesizing that, if OT causes locomotor depression, limiting CNS entry of OT would ameliorate the locomotor impairment.

Collectively, the current work aims to determine the array of behaviours, related to feeding and metabolism, that are controlled by systemic OT and systemic OT-R activation specifically. Importantly our main goal was to clearly differentiate those OT-mediated processes that are affected by action of systemic OT on peripheral OT-R; in contrast to those controlled by action of systemic OT on CNS OT-R. We hypothesized that limiting CNS entry by OT would preserve the hypophagic effects, while eliminating nausea and emesis driven by CNS OT-R agonism. In addition, we put particular focus on potential sex differences in the effects of systemic OT. Males and females were tested in identical, controlled conditions and in numbers sufficient to detect sex differences, which was warranted given that considerable sex differences have been detected via the CNS OT system.[33,34] Altogether, the current work aimed to elucidate the contribution of peripherally restricted OT to appetite suppression. By specifically restricting the access of peripheral OT to its peripheral OT-R with a novel agonist (OT-B_{12}), our data reveal that appetite suppression and side effects are mediated by anatomically distinct subsets of OT-R.

2 | MATERIALS AND METHODS

2.1 | Materials

All materials were used according to the manufacturer's instructions unless otherwise noted. The following were purchased from Sigma-Aldrich, St. Louis, MO, USA: acetonitrile (MeCN), methanol (MeOH), triethylamine (TEA), dimethyl sulphoxide (DMSO), diethyl ether, cyanocobalamin (B_{12}), triisopropylsilane, 1,1′-carbonyl-di-(1,2,4-trizole), n-methyl-pyrrolidone, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 1-hydroxybenzo-triazole and dihydroxybenzoic acid. The following were purchased from CEM Corporation, Matthews, NC, USA: Fmoc-Rink Amide Protide non-preloaded resin (LL), N,N′-diisopropylcarbodiimide, ethyl cyanohydroxyiminoacetate (Oxyma), Fmoc protected amino acids: Asn(Trt); Gln(OtBu); Tyr(tBu); and Trp(Boc). The following were purchased from VWR, Radnor, PA, USA: 7-trifloroacetic acid (TFA) and dimethylformamide (DMF). The following was purchased from BroadPharm, San Diego, CA, USA: sulpho-DBCO-amine. The following was purchased from Thermo Fisher, Waltham, MA, USA: AlexaFluor 564 (DBCO-AF546). The following was purchased from Lumiprobe: Sulfo-cyanine5 NHS ester. The following was purchased from Lumiprobe, Hunt Valley, MD, USA: alpha-cyano-4-hydroxycinnamic acid.

2.2 | Chemistry methods

All electronic absorption spectra were obtained on either a Varian Cary 50 Bio spectrophotometer in a 2 ml quartz cuvette or a NanoDrop One^{C} between 200 nm and 800 nm. Fluorescence spectra was obtained on an Agilent (Santa Clara, CA, USA) Cary Eclipse Fluorescence Spectrophotometer in a 2-ml cuvette between 565 nm and 800 nm. Mass spectrometry data were acquired on a Shimadzu (Columbia, MD, USA) LC-MS 8040 and/or a Bruker Autoflex III Smart-beam matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) using alpha-cyano-4-hydroxycinnamic acid or dihydroxybenzoic acid as matrix. Circular dichroism was performed in a 200-μl quartz cuvette on an Applied Photophysics Chirascan VX instrument. Cell assays were prepared and ran using a Memmert iso150 Incubator and Molecular Devices FlexStation 3. Cell assays were run using a Ready-to-Assay OT receptor cell kit (Millipore Sigma HTS090RTA) for Calcium Flux FLIPR Assays. Peptides were prepared on a CEM Liberty Blue automated microwave peptide synthesizer (CEM Corporation) and cleaved from resin using a CEM Razor rapid peptide cleavage system in 95% TFA containing 2.5% triisopropylsilane and 2.5% water for 40 min at 40°C. Peptides were purified using an Agilent 1200 reverse-phase high-performance liquid chromatography (RP-HPLC). Products were dried on a 1 L Labconco FreeZone lyophilizer.

2.3 | Solid-phase peptide synthesis of oxytocin

OT was synthesized by Fmoc/tBu solid-phase peptide synthesis methodology starting with non-preloaded resin and repetitive N,N′-diisopropylcarbodiimide/Oxyma activation. Sequence synthesized was CYIQNCPXG, where X signifies an ε-azido modified lysine. OT was cleaved from resin and deprotected and the solution precipitated with cold diethyl ether and centrifuged at 4000 rpm (∼1500 *g*) for 10 min at 4°C. The supernatant was discarded, and the remaining peptide pellet was redissolved in water, flash-frozen in liquid nitrogen and freeze-dried in vacuo. The subsequent oxidation of OT to produce the C1-C6 cystine linkage was achieved by dissolving linear OT in 10% aqueous DMSO with gentle stirring over 48 h. The resulting solution was diluted with water and lyophilized to produce a white powder. The peptide was purified by semi-preparative RP-HPLC (H_2O + 0.1% TFA and MeCN from 1% MeCN/H_2O + 0.1% TFA to 70% MeCN/H_2O + 0.1% TFA over 20 min) on an Agilent ZORBAX 300SB-C8 (5 μm, 9.4 × 250 mm) column. The final cyclized OT was analysed and characterized by LC-mass spectrometry (MS). Electrospray ionization (ESI)-MS expected $m/z = 1048$, observed $m/z = [M + H^{+}]^{+}$ 1048.

2.4 | Synthesis of B_{12}-oxytocin

B_{12}-DBCO was prepared by combining B_{12} (25.0 mg, 0.018 mmol) with 1,1′-carbonyl-di-(1,2,4-trizole) (10.0 mg, 0.061 mmol) in 3 ml of n-methyl-pyrrolidone and stirring for 1 h under argon, at which time sulpho-DBCO-amine (25.0 mg, 0.059 mmol) and TEA (50 μl) were added to the solution. After an additional hour, a second equivalent of sulpho-DBCO-amine and TEA were added, and the reaction stirred overnight. B_{12}-DBCO was purified using RP-HPLC (H_2O + 0.1% TFA and MeOH from 1% MeOH/H_2O + 0.1% TFA to 70% MeOH/H_2O + 0.1% TFA in 15 min) to produce B_{12}-DBCO at 92% purity in 80% yield. MALDI-TOF-MS expected m/z = 1808, observed m/z = [M-

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$CN^{-}]^{+}$ 1782. The B_{12}-DBCO (12.0 mg, 0.011 mmol) was then reacted with OT (10.0 mg, 0.010 mmol) (Figure 3) by dissolving both compounds in 4:1 DMF/H_2O and allowing the red-coloured solution to stir gently at room temperature overnight. OT-B_{12} was purified using RP-HPLC (H_2O + 0.1% TFA and MeCN from 1% MeCN/H_2O + 0.1% TFA to 70% MeCN/H_2O + 0.1% TFA in 15 min) to produce OT-B_{12} to 95% purity in stochiometric yields. MALDI-TOF-MS expected $m/z = 2856$, observed $m/z = [M + H_2O^{+}]^{+}$ 2873.

2.5 | Synthesis of fluorescent oxytocin (*f*AF546-OT; Roxy)

AlexaFluor564 (DBCO-AF546) (2.0 mg, 0.002 mmol) was reacted with OT (2.5 mg, 0.002 mmol) by dissolving both compounds in 4:1 DMF/H_2O and allowed to stir overnight at room temperature to produce *f*AF546-OT (Roxy). Roxy was assayed by analytical RP-HPLC (H_2O + 0.1% TFA and MeOH from 1% MeOH/H_2O + 0.1% TFA to 90% MeOH/H_2O + 0.1% TFA) over 25 min, showing Roxy at 99% purity in near stochiometric yield. ESI-MS expected $m/z = 2154$, observed $m/z = [M + H_2O + 2H^{+}]^{+2}$ 1097, $[M + 2H]^{+2}$ 1078 (Figure 2). Fluorescence microscopy performed on Roxy showed a minor red-shift in excitation-maxima from 556 nm to 558 nm and emission maxima from 570 nm to 572 nm.

2.6 | Synthesis of fluorescent B_{12} oxytocin (*f*Cy5-OT-B_{12}, BRoxy)

The combination of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (13.2 mg, 0.07 mmol), 1-hydroxybenzo-triazole (22.6 mg, 0.14 mmol) and Cy5 (2.0 mg, 0.002 mmol) was dissolved in 1 ml of anhydrous DMSO and allowed to stir at room temperature under argon for 30 min before the addition of OT (2.5 mg, 0.002 mmol) then continued to stir overnight. *f*Cy5-OT was purified using RP-HPLC (H_2O + 0.1% TFA and MeCN from 1% MeCN/H_2O + 0.1% TFA to 70% MeCN/H_2O + 0.1% TFA in 20 min) to produce *f*Cy5-OT in 99% purity in near stoichiometric yields. ESI-MS expected $m/z = 1711$, observed $m/z = [M\text{-}K^{+}]^{+}$ 1672. B_{12}DBCO was then reacted with *f*Cy5-OT by dissolving both compounds in 4:1 DMF/H_2O and allowing the blue coloured solution to stir at room temperature overnight. *f*Cy5-OT-B_{12} (BRoxy) was run to 100% completion in the presence of excess B_{12}DBCO. MALDI TOF. MS expected $m/z = 3481$, observed $m/z = [M\text{-}CN + H_2O]^{+}$ 3471.

2.7 | Animals

Male and female Sprague-Dawley rats (Charles River, Italy) were housed under a 12-h light/dark cycle, in individual cages with ad libitum access to chow and water, unless otherwise stated. They were a minimum of 5 weeks of age at the start of testing (approximately 150 g). Adult male shrews (*Suncus murinus*) weighing approximately 40-60 g (n = 10 total) and adult virgin female shrews weighing approximately 35-45 g (n = 10 total), were bred and maintained at the University of Pennsylvania; they derive from a Taiwanese strain and initially were supplied by the Chinese University of Hong Kong. Shrews were single housed in plastic cages (37.3 × 23.4 × 14 cm; Innovive [San Diego, CA, USA]) under a 12-h light/dark cycle in a temperature- and humidity-controlled environment. Shrews were fed ad libitum with a mixture of feline (75%, Laboratory Feline Diet 5003; Lab Diet) and mink food (25%, High Density Ferret Diet 5LI4; Lab Diet) and had ad libitum access to tap water except where noted. All studies were carried out with ethical permissions from the Animal Welfare Committee of the University of Gothenburg, in accordance with legal requirements of the European Community (Decree 86/609/EEC) and Institutional Care and Use Committee of the at Penn State University and University of Pennsylvania. All efforts were made to minimize suffering. The following doses of peripherally (intraperitoneally [IP]) delivered OT were used in this study: 0.25 mg/kg (2.48 E-07 mg/kg), 0.5 mg/kg (4.96 E-07 mg/kg), 1.0 mg/kg (9.93 E-07 mg/kg), 2.0 mg/kg (1.99 E-06). OT-B_{12}, Roxy, and BRoxy doses were used at 4.964 E-07 mol/kg, unless otherwise noted.

2.8 | Brain cannulation

A combination of ketamine (75 mg/kg; Ketaminol Vet, Intervet International BV, AN Boxmeer, the Netherlands) and xylazine (10 mg/kg; Rompun Vet, Bayer Animal Health GmbH, Leverkusen Germany) were IP administered to achieve surgical anaesthesia. Guide cannulas (26-gauge; Plastics One, Roanoke, VA, USA) were implanted above the nucleus of the solitary tract (NTS) using coordinates adapted from Richard et al.[35] The NTS cannula was implanted at the following coordinates: ±0.75 mm from the midline/on occipital suture/–4.9 mm, with the injector aimed 6.9 mm ventral to skull.

2.9 | Testing oxytocin penetrance into the blood-brain barrier using *f*AF546-OT (Roxy)

To investigate whether OT can cross the BBB, two rats from each sex were injected with Roxy (2 μg/μl) above the NTS, while two others per sex were injected IP with Roxy (1.0 mg/kg). These doses were calculated to be equimolar to OT doses in the NTS (1 μg/μl) and IP (500 μg/kg/1 ml), doses previously reported to reduce food intake. NTS injection was done over a period of 30 s with a total injected volume of 1.0 μl (0.5 μl of BRoxy to each side of the NTS). The injection needle was left in place for a further 30 s to facilitate diffusion of the drug solution. One hour after the injection, rats were transcardially perfused and brains were dissected. Brains were then sliced in cryostat (sections of 20-μm thickness) and visualized using confocal microscope.

2.10 | Operant conditioning

The operant conditioning procedure is used to assess the motivation to obtain a reward, in this case food reward in the form of a high-fat

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(35%) pellet. Training and testing were conducted in the early to mid-light cycle in rat conditioning chambers (Med-Associates, Georgia, VT, USA), as we described previously.[36,37] Training was conducted in four stages in ad libitum fed rats. Rats are first trained on the fixed ratio 1 (FR1) schedule in 30-min sessions (single press on the active lever results in the delivery of one pellet, 45 mg, sucrose or high fat, as needed), followed by FR3 and FR5 (three and five presses per pellet, respectively), where a minimum of 30 responses per session on the active lever is required for advancement to the next schedule. Finally, the rats were trained in a progressive ratio schedule (PR[38]) until stable responding was achieved (approximately seven sessions), where the cost of a reward is progressively increased for each following reward, to determine the amount of work the rat is willing to put in to obtain a reward. Responding is considered stable when the number of pellets earned per session does not differ more than 15% between three consecutive sessions. In addition to measuring the number of rewards earned and lever presses made to earn the rewards, also food seeking is measured. Food seeking is defined as the number of head pokes into the food dispenser. All operant response testing is performed after the responses stabilized under the PR in 60-min sessions, rats were placed into the testing chambers 10 min after drug injections.

2.11 | Food intake

Chow intake was measured in the home cage. Rats had food withheld overnight to ensure a sufficient baseline food intake, against which an anorexic effect can be detected. Food was returned 30 min after injections and measured at 30 and 60 min time points. Body weight and food intake were also measured 24 h after injections.

2.12 | Open field test

Immediately after drug (OT 500 μg/kg/1 ml), equimolar dose of OT-B_{12} (1.418 mg/kg/1 ml) or vehicle (saline 1 ml/kg) injections, rats were placed in the centre of an open field arena (100 × 100 × 30 cm) that was virtually divided into centre and periphery. Behaviour in the arena was analysed for 10 min. The arena was illuminated with a light intensity of 35 lx. Behaviour was recorded using an overhead camera and tracked using EthoVision 13 XT (Noldus Information Technology, Wageningen, The Netherlands). Data were evaluated for time spent, and distance moved in the centre of the area, to evaluate anxiety-like behaviour.

2.13 | Core-temperature and activity measurements

To monitor spontaneous home-cage locomotor activity and core body temperature rats were implanted with an intraperitoneal telemetric radio transmitter, E-mitter (G2; Minimitter, Bend, OR, USA). A small lateral incision was made, and the transmitter was implanted in abdominal cavity the transmitter was sutured to the abdominal muscle to secure the position. The skin was closed using a sterile non-absorbable silk suture. Rats were allowed to recover for 7 days post E-mitter implantation. The doses used for this study were OT 500 μg/kg, OT-B_{12} 1.418 mg/kg and vehicle (saline). All drugs were dissolved to be injected at a volume of 1 ml/kg.

2.14 | Conditioned taste avoidance

Rats were habituated to 2 h water access per day for 6 days with ad libitum access to food. Water was always presented for 2 h before the dark phase (4:30-6:30 p.m.) with the dark phase starting at 7:00 p.m. During this training period, two identical bottles of water were offered to each animal with their location periodically changed every 30 min to minimize the impact of individual side preferences. On the conditioning day, rats were offered one bottle of a 0.125% saccharin solution for 30 min before the IP injection of either saline (2 ml/kg), LiCl (0.75 M) (64 mg/kg/2 ml), OT (0.5 mg/kg/2 ml) or equimolar dose of OT-B_{12} (1.418 mg/kg/2 ml). Next, the saccharin bottle was removed, and water was offered for an additional 90 min. Conditioning was started with half of saccharin bottles placed on the right and the other half on the left with changing their locations in the middle of the 30 min. After conditioning, 1 day was allowed before the test day in which rats got two bottles of water as on training days. On the test day, one bottle of water and one bottle of the saccharin solution were offered at random locations and 30-min intakes were recorded.

2.15 | Emesis testing in shrews

Shrews were habituated to IP injections and to clear plastic observation chambers (23.5 × 15.25 × 17.8 cm) for two consecutive days before experimentation. Two hours before dark-onset, the animals were injected IP with native OT (50 μg/kg, 100 μg/kg, 500 μg/kg, 5 mg/kg or 0.05, 0.1, 0.5 and 5 μmol/kg, respectively), OT-B_{12} (143 μg/kg, 286 μg/kg, 1.4 mg/kg, 14.3 mg/kg or 0.05, 0.1, 0.5 and 5 μmol/kg, respectively) or saline, then video-recorded (Vixia HF-R62; Canon) for 120 min. After 120 min, the animals were returned to their cages. Treatments were separated by 3 days. Analysis of emetic episodes were measured by an observer blinded to treatment groups. Emetic episodes were characterized by strong rhythmic abdominal contractions associated with either oral expulsion from the gastrointestinal tract (i.e. vomiting) or without expulsion of materials (i.e. retching). Latency to the first emetic episode, total number of emetic episodes and number of emetic episodes per minute were quantified as previously described.[21,22,32]

2.16 | Acoustic startle response

Rats were habituated once to the procedure. They received mock IP injections and were subsequently placed in acoustic startle response

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(ASR) boxes for 5 min 2 days before the start of the experiment. The test was done over three experimental days where overnight fasted rats received IP injections of vehicle, OT or OT-B_{12} in a counterbalanced design. Two days were allowed between each experimental day. On each testing day, rats were left for 30 min of acclimatization in the testing room before the drug injections. Thirty minutes after injection, rats were placed in a plexiglass cylinder connected to a piezoelectric accelerometer permitting recording of the amplitude of startle responses. Cylinders were connected to and put into ventilated chambers with the light on, background noise of 50 dB, and acoustic stimuli of 90 or 120 dB (each 50 ms long). After 5 min of habituation to the chambers, the acoustic stimuli were delivered in a randomized order (20 repetitions of 90 dB and 10 repetitions of 120 dB) with inter-stimulus pauses fluctuating between 20 and 40 s using the SR Lab Software (San Diego Instruments, San Diego, CA, USA). The mean of peak amplitude response to each acoustic stimulus (in millivolts) across all repetitions was calculated. The cylinders were cleaned with 70% ethanol and water after each test.

2.17 | RNAscope

The RNAscope was done according to RNAscope Fluorescent Multiplex Kit User Manual PART 2 (catalogue number 320293). Briefly, 20-μm thick sections of a fixed frozen brain of a male rat injected with BRoxy signal in the NTS, were allowed to cool at room temperature. Slides were then immersed in cold 4% paraformaldehyde for 15 min, gradually dehydrated in ethanol 50%, 70%, 99.5% and 99.5% for 5 min each. Slides were left to dry completely before boiling them for 8 min in target retrieval where the temperature did not exceed 100°C. Slides were immediately rinsed in milli-Q water before being immersed once in 99.5% EtOH for 3 min. After complete drying at room temperature, a hydrophobic barrier was drawn around sections with the Immedge pen. Slides were then treated with protease III for 30 min at 40°C in a humidity chamber/tray within an RNAscope oven (HybEZ). Probe hybridization and amplification steps started by pipetting OTR C1 probes onto the slides and incubating for 2 h at 40°C. This step was followed by incubation with AMP I for 30 min, AMP II for 15 min, AMP III for 30 min and AMP IV C for 30 min, respectively. Slides were washed twice with wash buffer for 2 min each after each incubation and before applying the next reagent. 4′, 6-Diamidino-2-phenylindole was then applied for 45-60 s and excess 4′, 6-diamidino-2-phenylindole was flicked. Finally, Coverslip was applied with ProLong Gold Antifade mounting medium (REF P36934; Invitrogen [Waltham, MA, USA]). Sections were visualized using Confocal Laser Scanning Microscope.

2.18 | Statistical analysis

All the data are presented as mean ± SEM. Statistical significance was analysed using Student's t-test for comparisons of two groups, or for more than two groups, one- or two-way ANOVA with post-hoc Holm-Sidak tests when appropriate (GraphPad Prism 7 Software, Inc., San Diego, CA, USA). For two-way ANOVAs the interaction of sex and treatment was assessed. Emetic episodes in shrews were analysed with one-way repeated measures ANOVAs, followed by Dunnett's multiple comparison tests. Two-sided Fisher's exact tests were used to compare the percentage of shrews experiencing emesis. $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | In vivo physiological effect of peripheral native oxytocin in the rat

Peripheral application (IP) of native OT robustly reduced 30 min food intake in male and female rats (Figure 1A,B). Female rats showed a higher sensitivity to the hypophagic effect of OT (0.25 mg/kg), while the food intake suppression at the highest dose persisted longer (present at the 1 h measurement) in male rats compared with female rats, at the 2.0 mg dose. Two-way ANOVA indicated a significant effect of treatment [$F(4,86) = 25.49$; $p < .0001$] and sex [$F(1,22) = 22.94$; $p < .0001$], and there was a trend [$F(4,86) = 2.127$; $p = .08$] for interaction of sex and treatment for 30 min food intake after IP OT injections. Post-hoc tests indicate a significant effect of all OT doses in females, while in males, doses of ≥0.5 mg induce hypophagia. At 60 min after injection two-way ANOVA indicated a significant effect of treatment [$F(4,86) = 4.89$; $p = .0013$] and sex [$F(1,22) = 31.27$; $p < .0001$], and interaction of sex and treatment was not significant [$F(4,86) = 1.757$; $p = .145$]. Post-hoc tests indicate a significant effect of the highest dose in males. In male rats the highest dose also led to a reduction in 24 h food intake (Figure S1A) and a reduction in 24-h body weight gain (Figure S1B). The 24-h effect was not found in female rats (Figure S1C,D).

Intraperitoneally applied OT, at a dose of 1 mg/kg, potently reduced motivated behaviour for a food reward – high-fat (35%) 45 mg pellet—as shown by the reduced amount of effort (active lever presses) rats were willing to expend for the food reward (Figure 1D,G), resulting in the reduced number of rewards earned (Figure 1C,F). The effect was specific to motivated behaviour, as another parameter, food-seeking, was not affected (Figure 1E,H). Both male and female rats responded with reduced number of lever presses, and rewards earned, to a similar extent as, for rewards earned, there was no significant drug-sex interaction: $F(1,34) = 0.99$, $p > .05$; a strong trend to an effect of sex $F(1,34) = 5.72$, $p = .08$; and a significant effect of drug: $F(1,34) = 15.20$, $p < .0001$. The strong trend towards an effect of sex was primarily driven by a trend in the difference in performance in the vehicle $p = .09$, and not the OT condition. Statistical results were similar for active lever presses; there was no significant drug-sex interaction: $F(1,34) = 2.73$, $p > .05$; a strong trend to an effect of sex: $F(1,35) = 4.08$, $p = .05$; and a significant effect of drug: $F(1,34) = 23.57$, $p < .0001$. Again, the strong trend towards an effect of sex was primarily driven by a trend in the difference in performance in the vehicle $p = .09$, and not the OT condition.

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3.2 | Comparison of oxytocin binding in brain tissue after brain versus peripheral oxytocin application

Fluorescently labelled OT (*f*AF546-OT, Roxy) (Figure 2A) with excitation spectrum at 558 nm and emission maximum at 572 nm (Figure 2B) was injected IP or intra-CNS above the NTS, to compare brain tissue access and binding of peripherally versus centrally applied OT. Robust labelling was identified in the NTS, in the NTS-injected rat (Figure 2C). Yet, peripherally applied Roxy (IP-injected at a dose equimolar to 0.5 mg/kg of OT effective at reducing feeding behaviour in males and females) was also detected at the level of the NTS, albeit fewer cells were labelled after peripheral as compared with direct CNS OT application (Figure 2D). Collectively these data indicate that peripherally applied OT enters the brain and reaches energy balance relevant OT-R populations in brain parenchyma. Thus, it is possible that while only a small number of brain OT-R-expressing cells is accessible to peripheral OT, this may be sufficient for engaging behavioural or metabolic responses.

3.3 | Oxytocin-B_{12} synthesis and validation

Newly synthesized OT-B_{12} (Figure 3A) was purified through RP-HPLC to a 98% purity (Figure 3C).

3.3.1 | In vitro calcium flux assay at oxytocin receptor

Conjugation of B_{12} to the eighth residue of OT did not interfere with agonism at its receptor as measured in vitro in a calcium mobilization assay performed in a chem-1 cell line stably transfected with OT-R (Figure 3D).

3.3.2 | Preliminary screen of oxytocin-B_{12} in vivo

The novel compound was initially given to three female rats and compared with food intake behaviour of the same three rats on vehicle (saline) in a Latin square design. Food intake was reduced by each of the three rats when injected with OT-B_{12} (Figure 3E). Additional rats were included to compare the effect of OT-B_{12} with native OT, where both OT and OT-B_{12} reduced feeding to a similar extent (Figure 3F), with OT-B_{12} effect persisting through the 4 h of measurement (Figure 3G).

3.3.3 | Hindbrain penetrance of oxytocin-B_{12}

To confirm that peripherally applied OT-B_{12} does not gain access to CNS OT-R populations, we generated a novel fluorescent OT-B_{12} (BRoxy). When injected peripherally fluorescent OT-B_{12} was not detected in the hindbrain areas surrounding the 4th ventricle (4th V) including the NTS (Figure 3H,I and Figure S4A,B). However, importantly, when the same compound was injected directly above the NTS, robust labelling was observed in the NTS and areas surrounding the 4th V (Figure 3J-M). Peripherally injected BRoxy was, however, able to bind to peripheral OT-R in the intestinal tract (Figure S4C-G). OT-B_{12} (BRoxy, 1.728 mg/kg equimolar dose to 1.418 mg/kg of OT-B_{12}, or 0.5 mg/kg of OT) was IP-injected at a dose effective at reducing feeding behaviour in males and females.

3.4 | In vivo validation of binding specificity of the fluorescent oxytocin-B_{12}

All BRoxy-positive cells co-expressed OT-R mRNA in the NTS (Figure 4C-F) and 10 N (dorsal motor nucleus of the vagus nerve) (Figure 4G-J) as indicated by the in situ RNAscope signals detected in all BRoxy-labelled cells.

3.5 | Reduced blood-brain barrier penetration of oxytocin does not affect food intake

In a new cohort of rats, we show that limiting OT brain penetration by linking it with the B_{12} preserved the anorexic effect of the compound in male and female rats (Figure 5A-D). Food intake was significantly reduced, and to a similar extent by OT and equimolar dose of OT-B_{12}, in male rats at 30 min and 60 min (Figure 5A,C). No significant differences were found between the two compounds at any time point. In female rats OT and OT-B_{12} significantly reduced food intake at 30 min (Figure 5B), and there were no significant differences between OT and OT-B_{12} at this time point or at 60 min (Figure 5B,D).

3.6 | Anxiety-like behaviour after oxytocin and oxytocin-B_{12}

To determine whether other behaviours previously linked to anorexic hormones are also altered by OT and equimolar dose of OT-B_{12}, we measured anxiety-like behaviour in male and female rats in the open field test and in the ASR test. Both OT and OT-B_{12} reduced time spent in the centre (Figure 5E), latency to enter the centre (Figure 5F), and distance the male rats travelled in the centre (Figure 5G). Overall, these data suggest that both OT and OT-B_{12}, when interpreted with the open-field test, increase anxiety-like behaviour in male rats. In female rats, OT increased anxiety-like behaviour as indicated by reduced time spent in the centre of the maze (Figure 5I) and reduced distance travelled in the centre (Figure 5K). OT-B_{12} did not significantly change any of these parameters in female rats, possibly suggesting that reduced brain penetrance interferes with the anxiogenic effect of OT in females. Although OT-B_{12} was also not significantly different from OT, possibly indicating OT-B_{12} induces an intermediate anxiety phenotype. The latency to enter the central area was similarly

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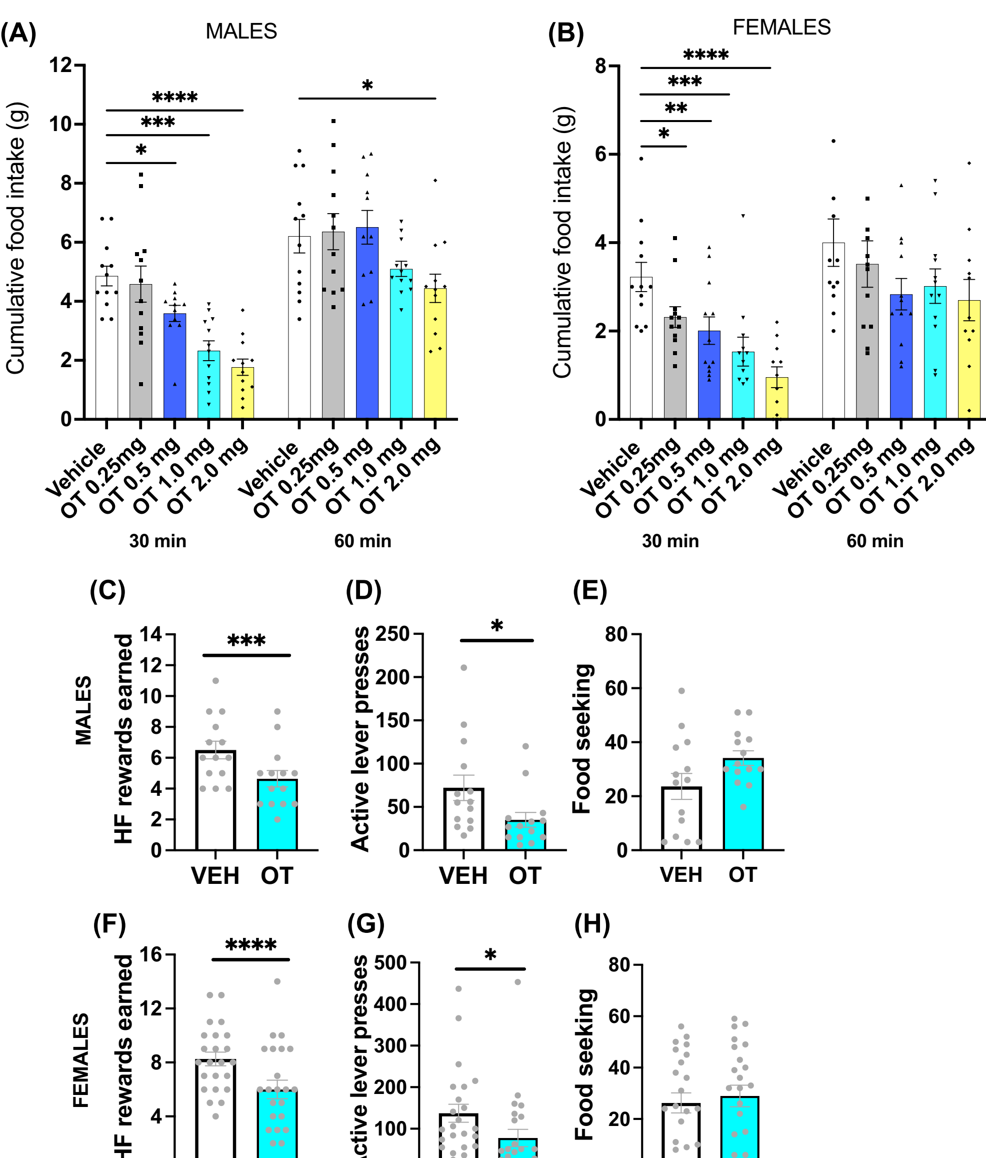

FIGURE 1 Peripherally administered oxytocin (OT) reduces food intake and food-motivated behaviour in male and female rats in a dose-dependent manner. Chow intake was significantly, and dose dependently reduced in (A) male and (B) female rats after intraperitoneal OT injections. OT (1 mg/kg) also significantly reduced food motivated behaviour as indicated by the reduced number of high-fat (HF) pellet rewards earned by (C) male or (F) female rats on a progressive ratio schedule. Similarly, the number of lever presses for the rewards was significantly reduced in (D) male and (G) female rats. However, OT injections did not affect food-seeking behaviour (number of head entries into the food receptacle) in (E) male or (H) female rats. Data are expressed as mean ± SEM. Individual data points are shown as black or grey dots. n = 12 for male rats and n = 12 for female rats, except for the progressive ratio test (males n = 14, females n = 23). $^*p < .05$, $^{**}p < .005$, $^{***}p < .0005$, $^{****}p < .00005$ compared with vehicle (saline)

reduced by both compounds compared with vehicle in females (Figure 5J). The open field test is dependent on locomotion, which can interfere with interpretation of the results in the context of anxiety. To parse out the influence of locomotion statistically on anxiety behaviour in this test, we performed an ANCOVA and determined the relationship between the total distance travelled and time or distance in the centre of the open field. In males, the covariate, total distance, was significantly related to time spent in the centre of the open field [$F(1,24) = 18.212$, $p < .001$]. There was no significant effect of the treatment on the time spent in the centre after controlling for the effect of total locomotor activity [$F(2,24) = 0.097$, $p = .908$]. While in females, neither total locomotor activity nor treatment had a significant relationship to the time spent in the centre with $F(1,24) = 0.047$, $p = .830$ and $F(2,24) = 1.969$, $p = .162$, respectively. The ANCOVA test was also done to test whether there is a relationship between the total distance and the distance travelled in the centre of the open field. In males, the covariate, total distance, was significantly related to distance travelled in the centre of the open field [$F(1,24) = 41.383$, $p < .001$]. There was no significant effect of the treatment on the distance in the centre after controlling for the effect of total locomotor activity [$F(2,24) = 0.812$, $p = .456$]. While in females, neither total locomotor activity nor treatment had a significant relation to the distance travelled in the centre with $F(1,24) = 2.547$, $p = .124$ and $F(2,24) = 0.632$, $p = .540$, respectively. Overall, the ANCOVA results indicated that the effect of treatment was not significant after controlling for total activity in both sexes. This may suggest that there is no effect of the drug on anxiety behaviour per se. However, to separate experimentally the influence of locomotion from assessment of anxiety-like behaviour we next performed the ASR test. The ASR test confirmed lack of anxiogenic effect of peripheral OT, but also, surprisingly indicates a potential anxiolytic effect of both OT and OT-B_{12}. Two-way ANOVA indicated a significant effect of treatment

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FIGURE 2 Peripherally administered oxytocin enters the central nervous system. (A) Structure of the novel fluorescently labelled oxytocin (Roxy). (B) Abosorption and fluorescence spectra of Roxy. (C,D) In vivo Roxy intraperitoneal and nucleus of the solitary tract-directed application. Roxy was detected in the brain tissue when (C) applied directly to the central nervous system and to a lesser extent when (D) applied intraperitoneally in rats. A coronal hindbrain section showing the nucleus of the solitary tract at the level of the 4th ventricle is shown in both panels

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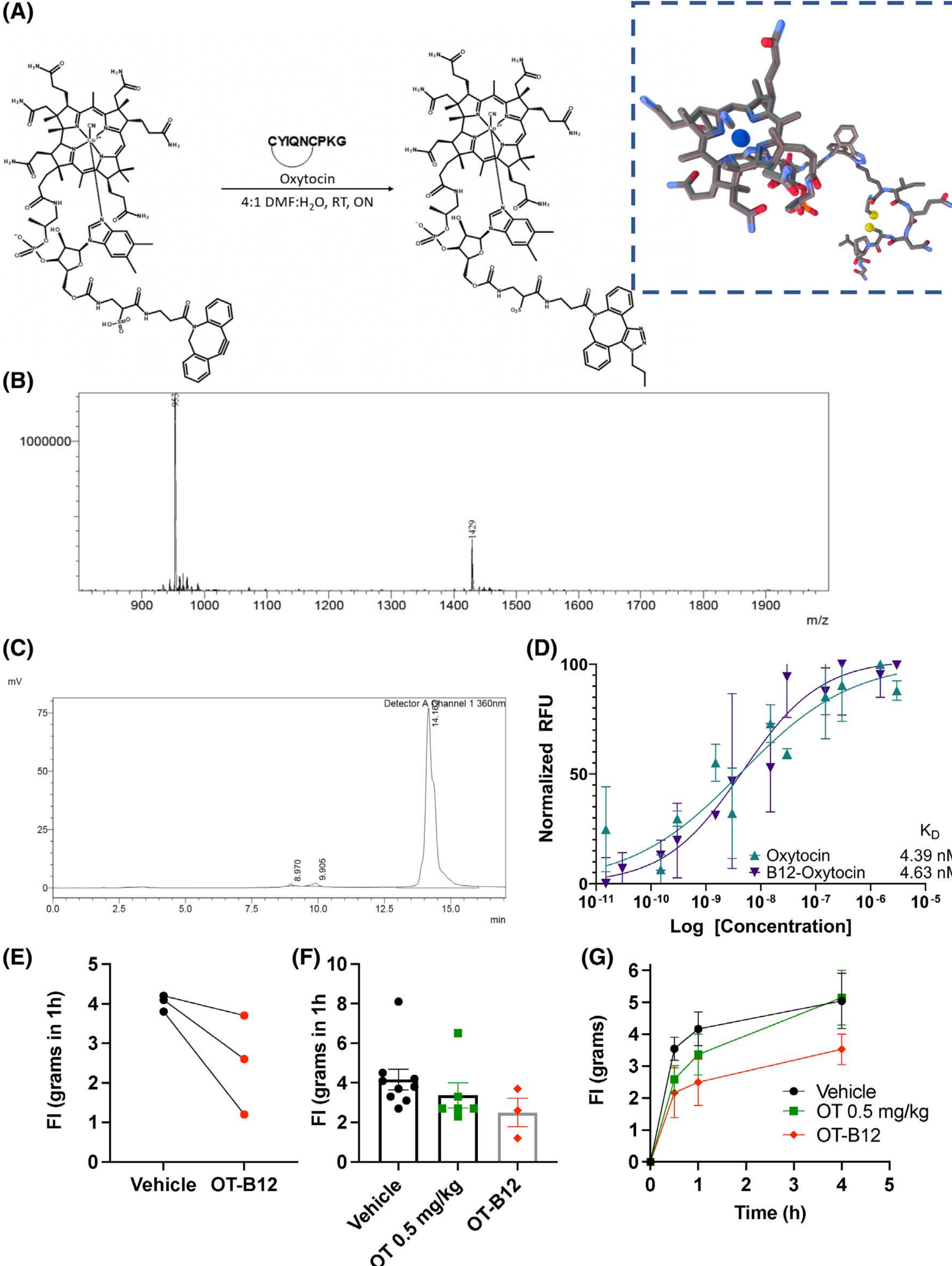

FIGURE 3 Novel oxytocin (OT)-vitamin B_{12} (B_{12}) compound biochemistry and initial in vivo data. (A) To hinder the blood-brain barrier crossing, OT is linked to B_{12} at the K8 residue with disulphide located at C1-C6 and molecular weight of 2856.7 g/mol. (B) matrix-assisted laser desorption/ionization time of flight mass spectrometry of OT-B_{12}, expected $m/z = 2856$, observed $m/z = [M + H_2O]^+$ 2873. (C) Sample purification through reverse-phase high-performance liquid chromatography with a purity of approximately 98%, tracked at 360 nm. (D) In vitro agonism at the OT receptor, which confirms equipotency for OT-B_{12} versus OT. (E) Preliminary in vivo rat data ($n = 3$) showing an anorexic effect of the new compound, where three of three female rats consumed less chow when injected with OT-B_{12} 1.418 mg/kg equimolar to 0.5 mg/kg OT. (F) Effect size seems comparable with that achieved by 0.5 mg/kg of OT. (G) 4-h time course of the anorexic effect of OT and OT-B_{12} compared with the vehicle (saline). (H,I) BRoxy, injected intraperitoneally was not detected in the hindbrain areas surrounding the 4th ventricle (4th V) including the nucleus of the solitary tract (NTS; also see Figure S4A,B). (J-M) When the same compound was injected directly above the NTS robust labelling was observed in the NTS and surrounding 4th V areas. Peripherally injected BRoxy was, however, able to bind to peripheral OT receptor in the intestinal tract (Figure S4C-G)

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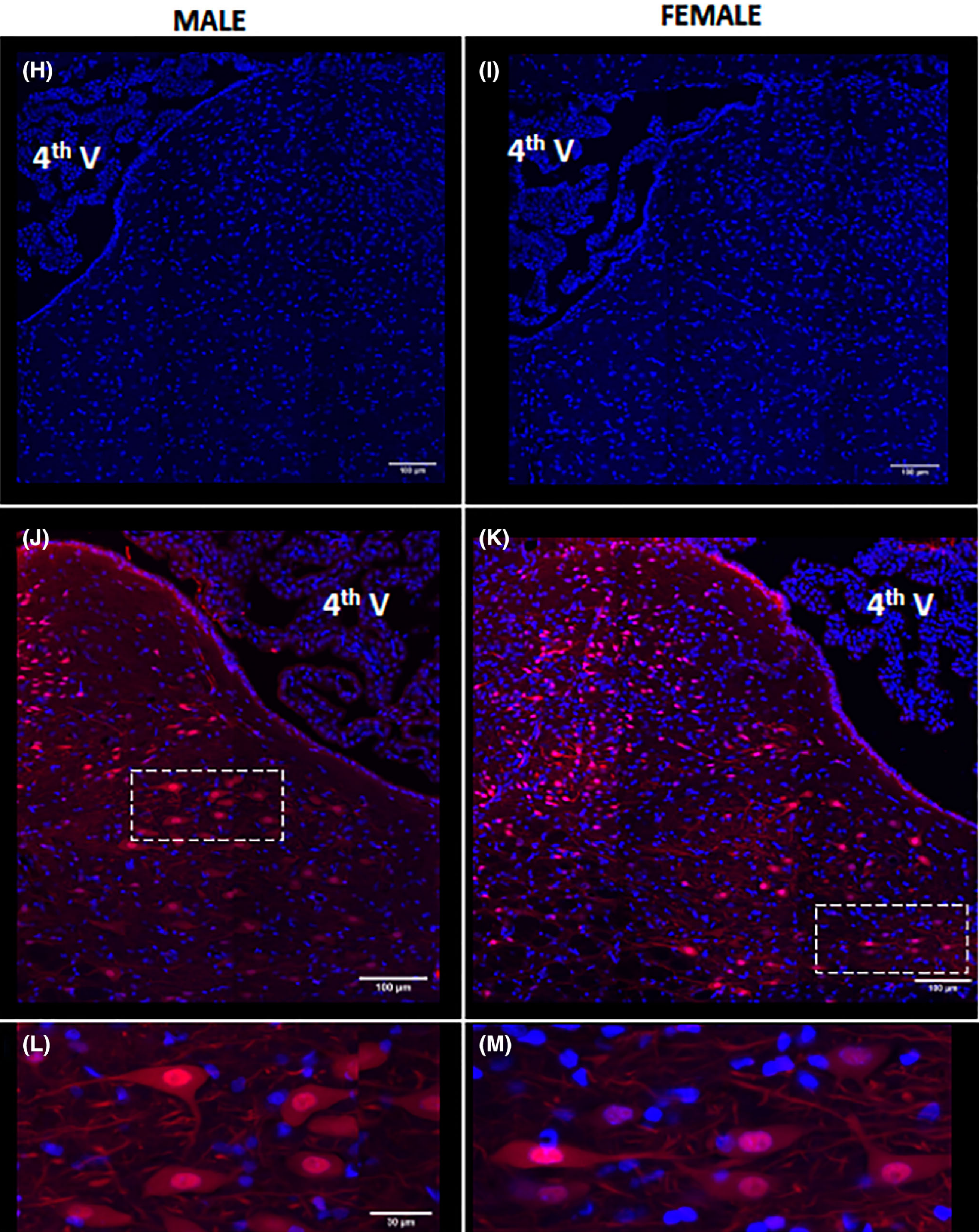

FIGURE 3 (Continued)

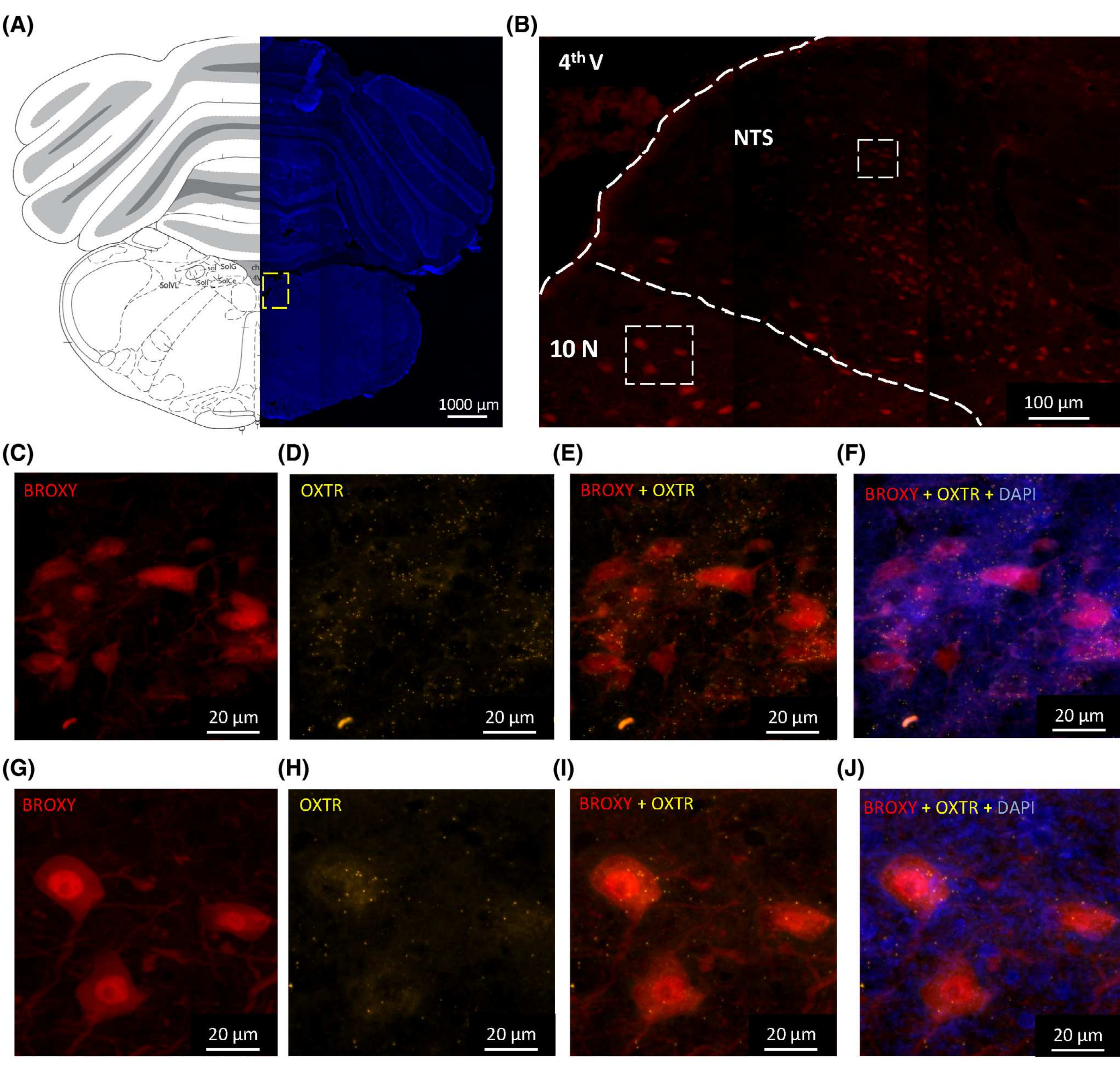

FIGURE 4 Labelled oxytocin (OT)-vitamin B_{12} (B_{12}) binds exclusively to OT receptor(-R)-expressing cells in the rat brain. Images show in situ fluorescent hybridization (RNAscope) for OT-R in the nucleus of the solitary tract (NTS) and 10 N (dorsal motor nucleus of the vagus nerve) in a male rat injected with BRoxy (fCy5-OT-B12) above the NTS. (A) The whole brain coronal section at the level of the 4th ventricle (4th V), imaged with a ×5 objective and its corresponding rat brain atlas figure. (B) BRoxy was detected in cells throughout the NTS and 10 N (×20 objective). (C-J) Representative images of BRoxy (red), OT-R detected by RNAscope (orange dots), and nuclear stain, DAPI (blue). All BRoxy positive cells co-expressed OT-R mRNA in the (C-F) NTS and (G-J) 10 N (63× objective)

[$F(2,36) = 16.00$; $p < .0001$], no significant effect of sex [$F(1,21) = 0.0022$, $p > .96$], and no significant interaction between these two factors [$F(2,36) = 1.252$, $p = .3$] for the 90-dB stimulus. Post-hoc tests indicated a significant effect of OT and a strong trend for OT-B_{12} in males (Figure 5M), and a robust effect of OT and OT-B_{12} in females (Figure 5N). For the 120 dB stimulus there was also a significant effect of treatment [$F(2,36) = 3.295$; $p = .048$] and sex [$F(1,21) = 6.812$; $p = .02$], and no significant interaction of sex and treatment [$F(2,36) = 1.171$; $p = .32$]. Post-hoc tests indicated a significant effect of both OT and OT-B_{12} in males (Figure 5O), but not females (Figure 5P).

3.7 | Oxytocin, but not oxytocin-B_{12}, induces a potent conditioned taste avoidance

Rats were conditioned with OT, equimolar dose of OT-B_{12}, or LiCl followed by 30-min saccharine exposure. Two-way ANOVA (drug × liquid)

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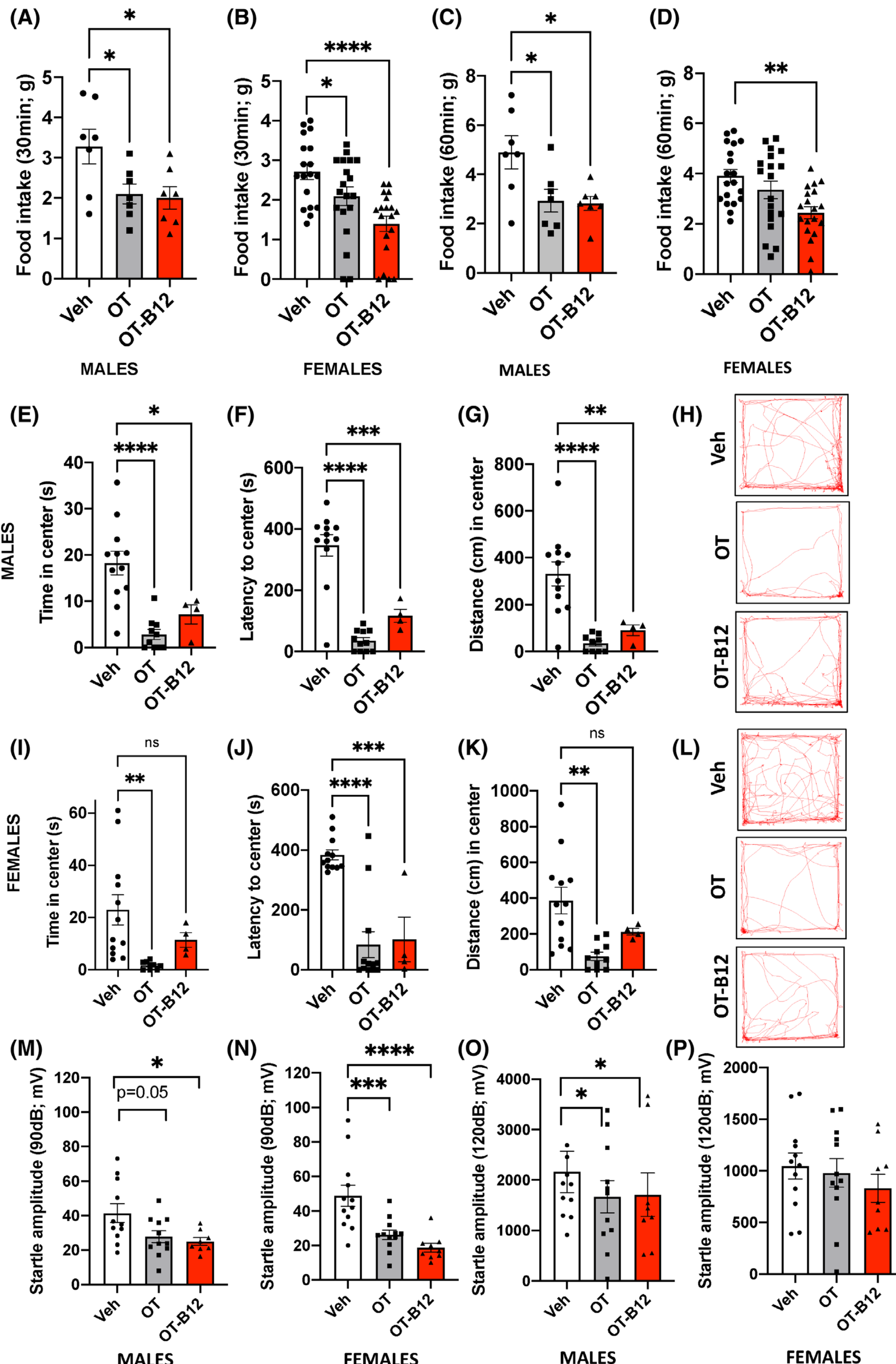

FIGURE 5 Legend on next page.

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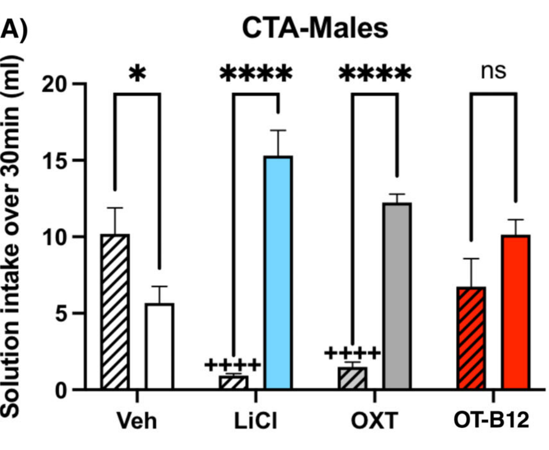
indicated a significant interaction [$F(3,40) = 26.38$; $p < .0001$]. As expected, previous injection of LiCl was associated with potently reduced saccharine consumption during the test day compared with saline injected control rats ($p < .0001$, Figure 6). Interestingly OT also induced a robust suppression of saccharine intake during the test day ($p < .0001$, Figure 6). Post-hoc tests indicate no significant difference between the saccharine level consumed by LiCl and OT injected rats ($p = .93$), remarkably indicating that OT is as potent as LiCl at inducing CTA. Importantly however, rats that received OT-B_{12} on the conditioning day did not develop the avoidance and consumed the same amounts of saccharine as saline injected control rats (Figure 6). No sex differences were identified in the effect of LiCl, OT or OT-B_{12}.

3.8 | Emesis in shrews

In males, OT dose-dependently induced emesis with most of the shrews experiencing emesis after 0.1, 0.5 and 5 mg/kg OT dosing (Figure 7A). All doses of native OT triggered emesis within minutes after administration (Figure 7B and Figure S8). Indeed, 30% of the animals exhibited emesis upon administration of the lowest dose of native OT, 80% with the intermediate dose and 100% with the two higher doses tested (Figure 7C). OT-B_{12} also induced emesis in a dose-dependent fashion. However, we observed a trend toward a general reduction in the prevalence of emesis following equimolar OT-B_{12} treatments. These differences were particularly striking at the 0.1 mg/kg OT versus 0.286 mg/kg OT-B_{12} dose, which shows a significant reduction of the shrews experiencing emesis following OT-B_{12} compared with its native form ($p < .05$) as represented by the heatmaps of OT and OT-B_{12} (Figure 7B). Thus, the conjugation of OT to B_{12} alters its pharmacodynamic profile reducing the centrally mediated emetic response in male shrews, while retaining the peripheral site-of-actions upon systemic administration. In females, only the highest dose tested of OT induced a significant increase in the number of emetic episodes (Figure 7D,E). All doses of OT-B_{12} were ineffective at causing significant emesis. The percentage of female shrews experiencing emesis following matched equimolar doses of native and OT-B_{12} did not differ (Figure 7F). When the emetic sensitivity of female and male shrews was directly compared, we observed a strong



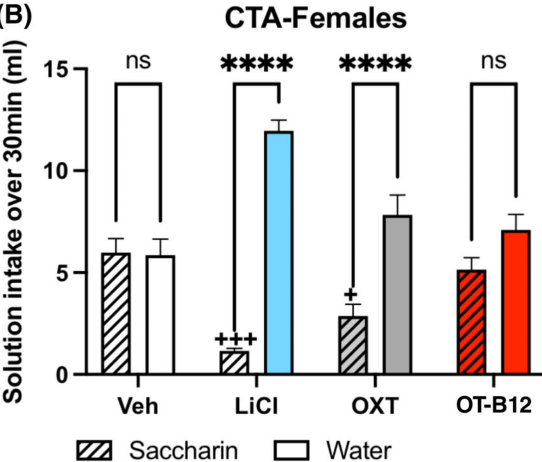


FIGURE 6 Oxytocin (OT)-induces conditioned taste avoidance (CTA) similar to LiCl and requires unhindered central nervous system entry of the peptide to achieve the CTA. (A) Male or (B) female rats conditioned with OT and LiCl but not those receiving OT-vitamin B_{12} (B_{12}) reduced their saccharine intake during the test day of the CTA test. Sample size was as follows OT; n = 7, OT-B12; n = 5, LiCl; n = 5, vehicle; n = 7 for males as well as females. Data are expressed as mean ± SEM. $^{*}p < .05$, $^{****}p < .0001$, saccharine compared with water intake for each drug condition; $^{++++}p < .0001$ saccharine intake for each drug compared with saccharine intake with vehicle (Veh; saline)

FIGURE 5 Equipotency of the behavioural impact of the blood-brain barrier-impenetrable oxytocin (OT)-vitamin B_{12} (B_{12}) and native OT. Equimolar doses of OT and OT-B_{12} are equally potent at reducing food intake at 30 min post-injection in (A) male and (B) female rats. (C) Both OT-B_{12} and OT remain equally effective at food intake suppression in male rats at 1 h. (A-D) In female rats, only OT-B_{12} reduced feeding behaviour at 1 h n = 7 for male rats and n = 18-19 for female rats. In an open field test, (E) male rats reduce the time spent in the centre, and (F) latency to enter central part of the field after both OT and OT-B_{12}. (G) Male rats also reduce the distance travelled in the centre in response to either OT or OT-B_{12}. (I) In females time spent in the centre is reduced by OT but not OT-B_{12}. (J) Both compounds reduce the latency to enter the central area. (K) However, only OT reduces the distance travelled in the centre. Track visualizations display a representative open field locomotion in (H) males and (L) females during each drug condition. Data are expressed as mean ± SEM. (E-K) Individual data points are shown as black dots, squares, or triangles. n = 12 for OT and vehicle (Veh) and n = 4 for OT-B_{12} in male rats and female rats. The acoustic startle response test indicated an anxiolytic response to both OT and OT-B_{12} in (M) males and (N) females when challenged with a 90 dB stimulus. An anxiolytic response was also noted at the 120 dB intensity, with both OT and OT-B_{12} showing an anxiolytic response in (O) males but not (P) females. n = 11-12 for OT and vehicle, and 8-9 for OT-B_{12}. $^{*}p < .05$, $^{**}p < .005$, $^{***}p < .0005$, $^{****}p < .00005$, compared with vehicle (saline)

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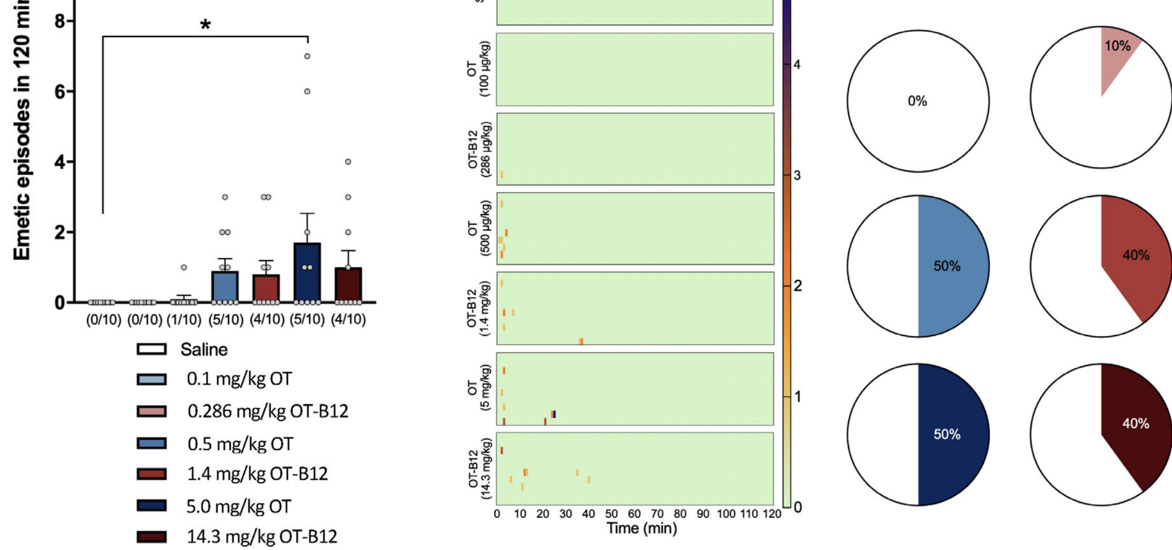

FIGURE 7 Oxytocin (OT)-induced acute emesis in male shrews is ameliorated by restricting OT central nervous system entry in a dose-dependent manner. (A) Emetic episodes measured throughout 2 h post-drug injection in male shrews. The number of animals exhibiting emesis, expressed as a fraction of the total number of animals tested is indicated underneath each treatment group (n = 10 per group). (B) Heat map of emetic episodes in male shrews during the 2-h period post-drug injection. (C) Percentage of male shrews experiencing emesis in each drug condition. (D) Emetic episodes measured throughout the 2-h post-drug injection in female shrews. Number of animals exhibiting emesis, expressed as a fraction of the total number of animals tested is indicated underneath each treatment group (n = 10 per group). (E) Heat map of emetic episodes in female shrews during the 2-h period post-drug injection. (F) Percentage of female shrews experiencing emesis during each drug condition. Data in (A) and (D) are expressed as mean ± SEM and analysed with one-way repeated measures ANOVAs, followed by Dunnett's multiple comparison tests. Data in (C) and (F) analysed with Fisher's exact tests. *$p < .05$, **$p < .01$, ***$p < .001$

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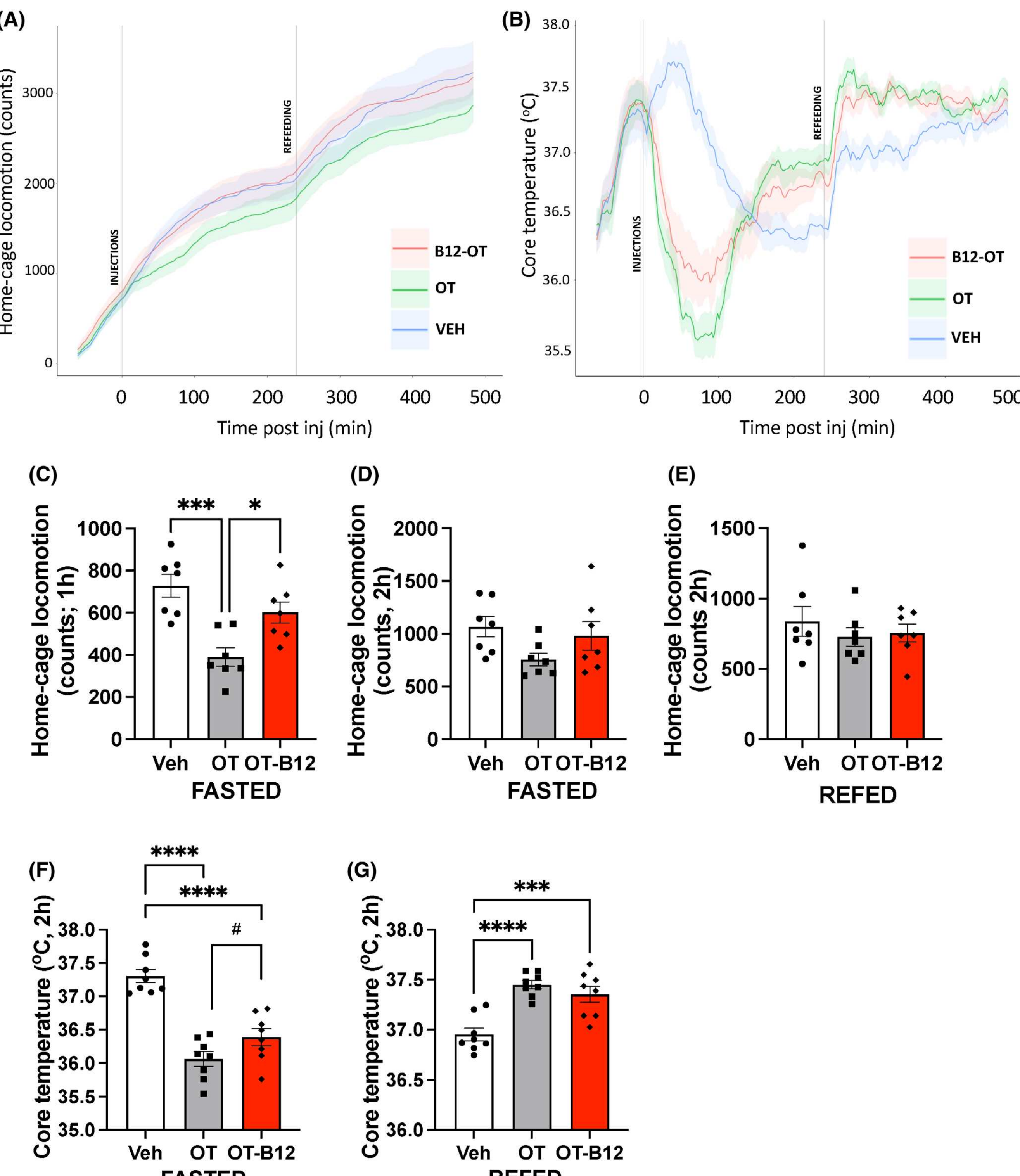

FIGURE 8 OT-induced locomotor depression, but not thermoregulatory impact, requires BBB penetration. (A) Line graphs displaying telemetrically recorded home-cage spontaneous locomotor activity after OT, OT-B_{12} or vehicle injections. (B) Line graphs displaying telemetrically recorded home-cage core body temperature after OT, OT-B_{12} or vehicle injections. (C) OT but not OT-B_{12} reduces home-cage locomotor activity for 1 h post-injection in rats without access to food. (D) 2 h average activity post-injection in fasted rats. (E) Upon refeeding neither OT nor OT-B_{12} altered home-cage locomotion. (F) Surprisingly both OT and OT-B_{12} robustly reduce body temperature post-injection, an effect lasting 2 h. (G) In contrast, past the 2-h period and upon refeeding both compounds consistently produce a hyperthermic effect; bar graph represents 2-h average of core temperature starting immediately after food presentation. Data are expressed as mean ± SEM. Individual data points are shown as black dots, squares or triangles. n = 8 for male rats. *$p < .05$, ***$p < .0005$, ****$p < .0005$ compared with vehicle (saline)

effect of sex for all doses of native OT, as well as, for the highest dose of OT-B_{12} tested (all $p < .05$, Table S1).

3.9 | Energy expenditure parameters are differentially altered by oxytocin and oxytocin-B_{12}

Home-cage locomotor activity is robustly reduced by OT but not OT-B_{12} for 60 min post-drug injection (Figure 8C), indicating that BBB penetrance of OT is required for the locomotor depression induced by OT. No significant activity changes with any of the compounds were detected at 2 h in fasted rats (Figure 8D) or for the 1-h duration of refeeding (Figure 8E). Both OT and OT-B_{12} had a biphasic effect on body temperature; they induced a robust hypothermia lasting 2 h post-injection (Figure 8F), and after 2 h the effect turned to hyperthermia while the rats were still without food. The hyperthermia persisted throughout the 2-h period of refeeding, while body temperature increased at the point of refeeding in all rats irrespective of treatment (Figure 8B). OT and OT-B_{12} rats elevated their temperature to a significantly higher extent than vehicle controls (Figure 8G).

4 | DISCUSSION

We show that peripheral application of native OT reduces food intake and motivated behaviour for palatable food, induces emesis and CTA, reduces anxiety-like behaviour and locomotor activity, and has a biphasic effect on body temperature. As peripherally applied OT enters the brain, to facilitate the delineation of the peripheral versus central effects of peripherally applied OT, we generated a novel compound: OT linked to vitamin B_{12}, which enabled us to separate OT effects requiring unhindered brain access versus those where brain access is not required. By utilizing OT-B_{12} we found that unrestricted brain penetration is not required for the anorexic and anxiolytic effects of OT. In contrast, restricting brain access of OT attenuated or abolished nausea, CTA, emesis and locomotor depression induced by OT. These results are consistent with our hypothesis and indicate that peripheral OT-R activation is sufficient for the anorexic effect of OT, and that several potentially clinically concerning effects of OT can be eliminated by restricting brain access for OT.

Peripheral application of OT dose dependently reduced food intake, with a short latency of the anorexic effect, that peaked at 30 min post-injection, in line with the proposed role of peripheral OT as an anorexic hormone. At the two highest doses tested the intake reduction was about 50% for both sexes. The hypophagic response was largely similar between sexes, although females showed a greater sensitivity to OT at the lowest dose tested, while response to the highest dose lasted longer in males. The reduction in food intake was not impaired, in either sex, by restriction of OT brain access via the OT-B_{12} link. Importantly, our data also show that food intake reduction is independent of the taste avoidance and locomotor depression induced by OT, as OT-B_{12} was effective at reducing food intake, but not locomotor depression or CTA. This separation of the distinct behavioural responses produced by OT is important not only for the mechanistic understanding of OT-induced hypophagia, but also a promising discovery for the potential therapeutic considerations of OT. The idea that direct peripheral activation of OT-R is able to carry an anorexic response is supported by the peripheral location of OT-R in organs key to feeding and metabolism regulation, for example the gastrointestinal vagal afferents, liver or the adipose tissue.[39-41] Moreover, peripherally administered oxytocin is reported to suppress eating in a vagal-dependent manner.[42,43]

Systemic application of OT in humans suggests that OT effects may extend beyond control of ingestive behaviour and into motivated behaviour, at least at the level of the brain activation: in overweight and obese men, OT injection attenuates the functional connectivity between the ventral tegmental area, where dopaminergic cell bodies reside, and linked food motivation brain regions in response to high-calorie visual food images.[44] Given this potential link of peripheral OT and food reward, here we determined whether peripherally applied OT reduces motivated behaviour for a high-calorie food reward, in both male and female rats. Our data show that peripheral application of OT is sufficient to reduce motivated behaviour for a high-fat reward. This effect was specific to motivated behaviour, as another behaviour measured during the operant task, food seeking, was not altered by the treatment. This dissociation also supports the idea that the effect is specific and not the result of general behavioural suppression, where one would expect all parameters requiring physical and locomotor activity to be reduced. These data contrast with two previous studies, in mice or rats, suggesting that systemic OT does not alter motivated behaviour for sucrose, measured by progressive ratio operant responding for sucrose solution or pellets.[45,46] Another report indicates a significant effect of peripherally injected OT in rats on progressive ratio-measured sucrose-motivated behaviour, with females displaying a much higher sensitivity to OT compared with the marginal response observed in males that required a dose 10-fold higher than females.[47] Here, we found a potent and similar suppression of food-motivated behaviour in male and female rats. The robust effect observed in males may be explained by the fact that, in contrast to previous studies, we used a high-fat rather than sucrose reward; previous literature indicates that males tend to prefer high-fat to high-sugar foods. Moreover, these results are in line with a growing body of literature showing that peripherally applied OT reduces reward derived from drugs of abuse and alcohol.[45,46,48-54] Although much of that literature operates under the implicit assumption that the anti-reward effects are exerted directly on OT-R expressed at the level of the CNS. Both direct the effect on CNS but also activation of peripheral OT-R remains a possible mechanism of the reward reducing function of OT. For example, systemically applied OT reduces methamphetamine motivation, seeking, and ameliorates withdrawal, and does so in a vagus-dependent manner.[55]

Native OT produced a powerful CTA in male and female rats, it also dose-dependently produced emesis in shrews. The avoidance response was comparable with that induced by the gold standard of the CTA test, namely LiCl. This is perhaps not surprising as LiCl was previously shown to increase central OT neuron activation, and,

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importantly to rely on OT for its avoidance generation.[27-29] Our CTA results are not in line with some existing literature.[42,56-58] There are, however, important differences between our experimental setup and these published results; for example, dose used, route of administration, animal model and acute versus chronic administration.[42,56-58] Previous studies indicated that the ventromedial hypothalamus (VMH)[58] or the nucleus accumbens core[57] OT application does not induce CTA. Our drug application was via IP injections allowing drug access to many other OT-R populations in the periphery and the brain than just VMH or nucleus accumbens. Thus, integrating our results with those previous studies suggests that OT induces CTA, but probably not via OT-Rs in VMH or the nucleus accumbens core. Iwasaki et al.[42] in a study using mice, found that while both 200 and 400 μg/kg OT doses significantly reduced food intake, only the higher dose decreased food intake despite vagotomy or capsaicin pretreatment, suggesting that the lower dose does not enter the brain in concentrations sufficient to affect feeding behaviour. Only this lower dose was tested for CTA, and it did not induce any CTA. As we hypothesized that OT induced CTA by entering the brain, the results of the Iwasaki et al. article are, in fact, consistent with the current findings, as we would not expect a dose ineffective centrally to induce CTA. In the current work we selected the OT dose for the CTA study to be the first effective dose at evoking anorexia in both sexes: OT 500 μg/kg. We also determined that OT at this dose crosses the BBB and reaches its receptors in the brain at least in the NTS and surrounding nuclei. However, many previous studies do link OT with CTA or nausea in animal models.[27-29] Importantly, OT-R are required for CTA development as the OT-R blockade reduces acquisition of taste aversion and blunts responsiveness of amygdala neurons to an aversive stimulus in mice.[29] The current data are also in line with the clinical reports indicating nausea and emesis after OT infusions in women.[25,59-67] For women receiving iv injections of oxytocin, pitocin, or carbetocin during labour, nausea and vomiting are common side effects, as noted by many studies.[59-67]

The fact that OT-induced emesis can be captured in an animal model is crucial from a clinical perspective. This is because despite some human evidence, there is a paucity of data on the mechanisms of OT-induced nausea and emesis, and the human data are primarily gathered from OT infusions during labour, which is a complex context that could be predisposing the subject to nausea and emetic responses. Other anorexic peptides, for example glucagon-like peptide (GLP)-1, are well known to increase nausea, and it remains a problematic side effect in a significant fraction of patients receiving GLP-1-based therapeutics. Interestingly, this effect of GLP-1 is exclusively driven by its CNS receptors,[68] and limiting the CNS entry of GLP-1 preserves its glucoregulatory effect but eliminates nausea, aversion and emesis associated with GLP-1.[21-23] In the current study, limiting OT entry by conjugation with B_{12} retained the anorexic effects of OT but reduced emesis and abolished CTA-induced by native OT, supporting the idea that the malaise-inducing impact of OT is mediated by centrally located OT-R populations. Indeed, the neuroanatomical location of central OT-R further boosts this hypothesis, as OT-R is found in the area postrema, NTS and amygdala, all tightly associated with the generation of conditioned taste aversion, emesis and nausea. The fact that brain OT-producing neurons are a key element in taste avoidance responses further cements the idea of the central OT system having a key role in these processes, a role separable from that of the peripheral OT.

Many feeding hormones alter locomotor activity, and it is particularly valuable to parse out the anorexic effect of a substance from its effect on locomotion, as otherwise it will remain plausible that the primary effect of a substance is to cause locomotor depression and reduced feeding is just a by-product of this depression. Both native OT and OT-B_{12} were effective at reducing food intake to a similar extent, yet OT-B_{12} did not alter home-cage locomotion, indicating that locomotor depression is not responsible for driving anorexia and that locomotor depression induced by OT requires OT to enter the CNS in an unhindered manner. Thus, here we show that OT causes locomotor depression, limiting CNS entry of OT ameliorates the locomotor impairment, and anorexic effect of OT is not simply a by-product of locomotor depression.

Similarly, it is also common for feeding hormones and neuropeptides to affect thermoregulation. A growing body of work[2-6] indicates that CNS OT and OT-Rs are an essential element of the thermoregulatory control. Hindbrain OT administration increases brown adipose tissue (BAT) thermogenesis and white adipose tissue browning in mice and rats, mechanisms probably contributing to the weight loss induced by chronic CNS OT administration.[2,69,70] Whether peripheral OT-R follow suit to their CNS counterparts and increase body temperature remains unknown, and thus was investigated here. Surprisingly, the immediate effect of peripheral OT injections was to reduce the body temperature. The relatively rapid reduction in temperature noted here after OT or OT-B_{12} injections suggest an active heat release, through for example, vasodilation particularly in the tail of the rat or coordinated salivation and grooming. Bearing in mind that current data indicate that a hypothermic effect is present irrespective of the ability of OT to cross the BBB, direct action of OT on the nodose receptors and/or dendritic terminals of vagal afferents remains a strong possible mechanism for the observed hypothermia. The vagus nerve is argued to have diverse contributions to body temperature control, and both hypo- and hyperthermic responses have been observed after vagal stimulation[71]; and for review see Chang.[72] Vagal afferents mediate inhibition of BAT activity in response to other gastrointestinal hormones, for example, ghrelin or GLP-1.[73-75] Although, considering that the hypothermic response tended to be more potent with native OT, as compared with OT-B_{12}, it is probable that OT-R populations within the CNS also contribute to the hypothermic response. Several nuclei expressing OT-Rs within the hindbrain or PVN contain sympathetic premotor neurons,[76,77] inhibition of which can result in the observed hypothermia. Experiments evaluating OT-B_{12} and OT effects on the sympathetic neuron and BAT activity are needed to evaluate this possibility. Hypothermia can also be driven by sympathetically mediated vasodilatation. Neurotransmitters, such as serotonin and dopamine, are associated with hypothermic responses[78,79] and have been previously linked with OT.[80-84] The 2-h period of lower body temperature was immediately followed by a

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period of increased body temperature, which persisted even when all rats received access to food. The expected thermic effect of feeding, probably a mix of locomotion-induced increase in temperature along with the thermic effect of food, was found in all treatment groups. As hyperthermia followed a period of discrete hypothermia, it is difficult to parse out whether the hyperthermic response was an overcompensation for the hypothermic phase, or whether it was a distinct thermoregulatory response. However, given that previous studies clearly indicate that at least central OT induces BAT activation,[2,69,70] it is probable that the hyperthermic phase is a response to OT in its own right, with multiple sites of induction, that is, by both peripheral and central OT-R populations.

OT has long been considered a key player in the regulation of anxiety based on evidence from preclinical, as well as clinical studies.[85] In the current study, the open field test possibly suggested that male and female rats exhibited increased anxiety-like behaviour after peripherally applied native OT, but also, contrary to our hypothesis, after OT-B_{12}. These results could suggest that it is sufficient to activate directly the peripheral OT-R to engage the anxiogenic response. It is possible that increased anxiety in animals naturally follows a meal and is engaged by the same hormones that signal satiety, as with reduced need for finding food, increased anxiety reduces the motivation to take the risks that come with foraging. This is in line with data indicating that many satiety peptides (e.g. GLP-1) increase anxiety, while hunger hormones (e.g. ghrelin) reduce anxiety.[86-89] However, further analysis of the open field data indicated that the reduced time spent in the centre of the field could be explained by the overall reduced locomotion, potentially questioning any effect of OT or OT-B_{12} on anxiety-like behaviour per se. Importantly, an anxiolytic effect of OT but also OT-B_{12} was revealed by a test of anxiety-like behaviour, which does not depend on locomotion, the ASR test. These data are in line with reports of central applications of OT resulting in reduced anxiety-like behaviour.[90-93] Moreover, other reports also indicate an anxiolytic effect of subcutaneously applied OT in mice.[94,95]

As the metabolic effects of central OT have been suggested to depend on the body weight and adiposity status, such that animals with higher adiposity and body weight show a more pronounced weight loss and anorexic response, it is plausible that this observation would extend to the peripheral OT. While here, we focused on a comprehensive evaluation of the role of peripheral OT in healthy rats, future studies using rodent obesity models would be important to evaluate whether also peripherally restricted OT shows greater efficacy at appetite reduction and weight loss.

OT administration in obese humans represents a novel weight loss therapeutic strategy. Yet, the exact mechanisms (i.e. the contribution of distinct OT-R populations) are unclear and nausea and emesis represent notable side effects of native OT. Current data represent a proof-of-concept study showing that the anorexic effects of OT are separable from the nausea, taste avoidance and locomotor depression. In our work, we specifically restricted the access of peripheral OT to its peripheral OT-R with a novel OT-B_{12} agonist revealing the intriguing opportunity to separate peripherally mediated appetite suppression from centrally mediated side effects. Thus, we suggest that limiting CNS OT entry is a potential step forward in the therapeutic use of OT in humans, and the novel OT-B_{12} OT-R agonist represents a new agonist effective at appetite suppression devoid of nausea and emesis.

ACKNOWLEDGMENTS

This research was funded by the Wallenberg Foundation (WCMTM, to KPS), Swedish Research Council (2018-00660, to KPS, 2021-01549 to J-PK), Ragner Söderberg Foundation (KPS), the Swiss National Science Foundation (183899 to J-PK) and the US National Institutes of Health R01 DK128443-01 (to RPD, MRH, BCDJ). We would also like to thank Dr Linda Engström Ruud for RNAscope assistance.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/dom.14937.

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How to cite this article: Asker M, Krieger J-P, Liles A, et al. Peripherally restricted oxytocin is sufficient to reduce food intake and motivation, while CNS entry is required for locomotor and taste avoidance effects. *Diabetes Obes Metab*. 2023;25(3):856-877. doi:10.1111/dom.14937

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