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Chemogenetic activation of oxytocin neurons: Temporal dynamics, hormonal release, and behavioral consequences

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ABSTRACT

Chemogenetics provides cell type-specific remote control of neuronal activity. Here, we describe the application of chemogenetics used to specifically activate oxytocin (OT) neurons as representatives of a unique class of neuroendocrine cells. We injected recombinant adeno-associated vectors, driving the stimulatory subunit hM3Dq of a modified human muscarinic receptor into the rat hypothalamus to achieve cell type-specific expression in OT neurons. As chemogenetic activation of OT neurons has not been reported, we provide systematic analysis of the temporal dynamics of OT neuronal responses in vivo by monitoring calcium fluctuations in OT neurons, and intracerebral as well as peripheral release of OT. We further provide evidence for the efficiency of chemogenetic manipulation at behavioral levels, demonstrating that evoked activation of OT neurons leads to social motivation and anxiolysis. Altogether, our results will be profitable for researchers working on the physiology of neuroendocrine systems, peptidergic modulation of behaviors and translational psychiatry.

1. Introduction

Being synthetized in magnocellular neurons of the paraventricular (PVN), supraoptic (SON) and accessory (AN) nuclei of the hypothalamus (Grinevich et al., 2016a; Swanson and Sawchenko, 1983), OT release has been described from axonal, somatic and dendritic neuronal compartments (Knobloch et al., 2012; Landgraf and Neumann, 2004; Ludwig and Leng, 2006; Neumann et al., 1993b). Most physiological stimuli that trigger peripheral OT secretion from terminals of magnocellular OT neurons in the posterior pituitary into systemic blood flow were also shown to stimulate OT release within distinct brain regions (Jurek and Neumann, 2018; Landgraf and Neumann, 2004) including the hypothalamic PVN and SON. For example, exposure to physical stressors such as forced swimming triggers OT release both into blood and within the PVN of rats (Torner et al., 2017). Besides, axon collaterals of magnocellular OT neurons also project to various forebrain regions, including the central amygdala, lateral septum and olfactory cortex (Knobloch et al., 2012; Menon et al., 2018; Oettl et al., 2016). To reveal the physiological and behavioral functions of OT released within these brain regions, synthetic OT or specific OT receptor antagonists have been locally applied demonstrating that OT exerts pro-social (Lukas et al., 2011; Zoicas et al., 2014), fear-attenuating (Knobloch et al., 2012; Menon et al., 2018; Viviani et al., 2011) and anxiolytic (for review see Neumann and Landgraf, 2012; Neumann and Slattery, 2016) effects in rodents. In congruency, evoked OT release in these regions, e.g. by optogenetic stimulation, modulates contextual fear (Knobloch et al., 2012), anxiety-related behavior (Jurek and Neumann, 2018) as well as olfactory-dependent social recognition (Oettl et al., 2016), among others. However technically, optogenetic stimulation of OT neurons requires the stereotaxic implantation of optic fibers, often bilaterally, which may interfere with the animal's behavioral performance. Therefore, a method allowing the remote regulation of neuronal activity without additional surgery, as provided by chemogenetics, represents a more advantageous and less invasive approach.

Chemogenetics is based on engineered G protein-coupled receptors, which have the ability to activate or inhibit neuronal firing. In detail, site-directed mutagenesis of human muscarinic acetylcholine receptor provide the basis for designer receptors exclusively activated by designer drugs (DREADD) (Armbruster et al., 2007). Depending on the DREADD-coupled G protein, DREADD-expressing neurons can be

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excited via the G_q signaling, or inhibited by G_i signaling pathway. Following vector-based expression of DREADD in the target neurons approximately three weeks after viral infection, designer receptors can be acutely activated by biologically inert drugs, such as clozapine *N*-oxide (CNO) applied either intraperitoneally (i.p.) at a dose ranging from 1 to 50 mg/kg, by intracerebral infusion into the brain region of interest at a dose of 0.1–1 mM (Lichtenberg et al., 2017; Mahler et al., 2014; McGlinchey and Aston-Jones, 2018), or chronically, i.e. via osmotic minipumps, drinking water or chow pellets (Urban and Roth, 2015). However, most laboratories apply i.p. injections to acutely activate designer receptors to avoid additional stereotaxic implantation and to allow highest precision regarding body weight-dependent CNO dosing.

Despite growing interest in OT signaling especially in the context of its effects on socio-emotional behaviors in rodents and humans (Neumann and Slattery, 2016), there is surprisingly very limited information about the application of chemogenetics for modulating the OT system. Specifically, chemogenetic activation of OT neurons has not been described so far. Here we provide comprehensive analysis of the temporal dynamics of DREADD-based acute activation on OT neuron population as reflected by intracellular calcium (Ca^{2+}) fluctuations, central and peripheral OT release and behavioral consequences specifically on social and emotional behavior.

2. Methods

2.1. Animals

Male Wistar rats (230–270 g, Charles River Laboratories, Germany) were housed in groups of 3–4 under standard laboratory conditions (food and water ad libitum, 12:12 h light/dark cycle, lights on at 0600, 21–23 °C, 55% humidity). Rats were allowed at least one week of habituation, before they were used for surgical procedures. All experiments were performed between 0800–1300 in accordance with the Guide for the Care and Use of Laboratory Animals by the NIH, and were approved by the local government.

2.2. Drug treatment

For surgical procedures, rats were injected subcutaneously with the analgesic Buprenovet (0.05 mg/kg Buprenorphine, Bayer) and the antibiotic Baytril (10 mg/kg Enrofloxacin, Baytril, Bayer, Germany) 30 min before the start of the surgery under semisterile conditions.

To activate the excitatory DREADD, water-soluble Clozapine N-oxide dihydrochloride (CNO; HB6149, HelloBio, United Kingdom) was applied i.p. at 2 mg/kg; controls received 1 ml/kg of sterile PBS (Veh).

2.3. Stereotaxic gene delivery

Details on design, cloning, production and purification of viral vectors are described elsewhere (Grinevich et al., 2016b; Grund et al., 2017; Knobloch et al., 2012). In total, 280 nl of $rAAV_{1/2}$ OTpr-hM3Dq:mCherry (4×10^{11} genomic copies per ml) or $rAAV_{1/2}$ OTpr-GCaMP6s (4×10^{10} genomic copies per ml) were slowly infused by pressure infusion at a rate of 70 nl/min in rats anesthetized with a ketamine/xylazine mixture (100 and 10 mg/kg, respectively, i.p.). All stereotaxic coordinates are summarized in Table 1.

For technical reasons, only the PVN was targeted for in vivo fiber photometry and intracerebral microdialysis, while all three nuclei were infected for monitoring chemogenetically evoked peripheral OT secretion. Infusion sites for behavioral experiments are indicated in the results section.

2.4. Optic guided implantation of optic fibers

To monitor in vivo Ca^{2+} dynamics in response to chemogenetic excitation of paraventricular OT neurons, optic fibers (M127L01 Ø 400 µm, 0.50 NA, 10 mm, Thorlabs) were implanted ~100 µm above the dorsal boarder of the PVN under 1.5% isoflurane anesthesia. Four 1 mm screws (Knupfer, Germany) and a metal implant guide (OGL, Thorlabs) were attached to skull with OptiBond FL (Kerr, Germany) and fixed by dental cement (Paladur, Heraeus Kulzer, Germany). During implantation, the optic fiber cannula was fixed in an adaptor (ADAL3, Thorlabs) attached to stereotactic holder; while the other end of the cannula was connected through an optic fiber patch cord (FP400URT, Thorlabs) to the photodetector and LED (470 nm, excitation filter 472 ± 30 nm) of fiber-optometry system (FOM, NPI Electronic, Germany). Three weeks after viral injection, the FOM optic TTL output signal was monitored and recorded via digital input/output (DIO) board (OpenEphys, USA) to the DAQ system. (OpenEphys, USA) with 0.1–20 Hz bandpass filter and 20-s time scale set in to visualize the calcium signal online, while the cannula tip was gradually lowered into the PVN at a speed of 1 mm/min. When the optic fiber tip was close to the PVN where GCaMP6s was expressed, a slight increase in the signal baseline could be visually detected. The LED power in FOM system was set with a value between 5–10 mW/mm^2 and kept constant during the whole recording. Fiber photometry raw data were sampled at 3 kHz in OpenEphys GUI.

Table 1
Stereotaxic coordinates (in mm) used for viral infusions accordant to bregma (AP, anteroposterior axis; ML, mediolateral axis; DV, dorsoventral axis; Vol, volume).

Stereotaxic coordinates	AP	ML	DV	angle	Vol
Infusion of rAAV into:					
PVN	−1.7	± 0.3	−8.3	0°	280 nl
SON	−1.4	± 1.8	−9.3	0°	280 nl
AN	−1.9	± 1.2	−8.5	0°	280 nl

2.5. Photo signal correction and estimation of OT neuron activity

Optical signal output from the FOM system was recorded as analog -1 to 0 V raw TTL signal with ambient noise. Thus a reference signal was simultaneously recorded in the empty TTL channel in the same DIO board and was used for signal correction. Both channels were sampled at 3 kHz; data were first down-sampled to 300 Hz, and then filtered (10 Hz low-pass butterworth filter) to exclude 50 Hz ADC noise and other noise above 10 Hz. Next, a nonlinear 'polyfit' function was used to correct the photo-bleaching and baseline drifting artifacts in both channels. Afterwards, the optic signal was normalized with the reference channel to exclude ambient electrical noise that appeared in both channels. 600 s of control signal were processed as described in the procedure above, and the mean of the processed data were considered as baseline fluorescence (F). The baseline was used to calculate the dF/F, which was considered as the calcium signal of OT neurons and used as a proxy for neural activity.

2.6. Implantation of jugular vein catheter and blood sampling

To monitor OT plasma concentration in response to chemogenetic excitation of hypothalamic OT neurons a jugular vein catheter was implanted under urethane (25%, 1.2 ml/kg) anesthesia 19 d following viral infusion as previously described (Neumann et al., 1998). After surgery, the anesthetized rat was placed onto a heating plate to maintain homeostasis of body temperature and remained there for the blood-sampling period starting 1 h after surgery. The jugular vein catheter was connected to a 1-ml plastic syringe filled with sterile heparinized saline (30 IU/ml). Forty-five min before and after i.p. injection of CNO or Veh, 400 µl of blood was taken. The same volume of blood was replaced with sterile saline before the catheter was filled with heparinized saline again. All blood samples were collected on ice in EDTA-coated tubes (Bayer AG, Germany) and centrifuged (13,000 rpm, 10 min, 4 °C).

200 µl of plasma was separated and stored at −80 °C until subsequent extraction and quantification of plasma OT by radioimmunoassay (de Jong et al., 2015).

2.7. Implantation of microdialysis probe and intracerebral microdialysis

To monitor OT release within the PVN, a microdialysis probe (Horn and Engelmann, 2001; Neumann et al., 1993a) (molecular cut-off 18 kDa) was stereotaxically implanted with its U-shaped tip resting in the right PVN (AP -1.4 mm, ML + 1.8 mm, DV -8.3 mm, angle 10°), and anchored to two stainless steel screws using dental cement 18 days after viral infusion. Following surgery under ketamine/xylazine anesthesia (100 / 10 mg/kg, i.p.) animals were housed singly and handled daily to habituate to the microdialysis procedure. Three days after surgery, the microdialysis probe was connected to a syringe mounted onto a microinfusion pump via polyethylene tubing and perfused with sterile Ringer's solution (3.3 µl/min) starting at 0800 for 2 h to establish an equilibrium between inside and outside of the microdialysis membrane before sampling of microdialysates. The outflow of the microdialysis probe was equipped with a tube holder that allowed direct sample collection into a 1.5-ml Eppendorf tube containing 10 µl of 0.1 M HCl. Five consecutive 30-min dialysates were collected on both experimental day 1 and day 2. On day 1, samples 1 and 2 were taken before, and sample 3, 4 and 5 after i.p. administration of vehicle or CNO without further manipulation of the animals. On day 2, sample 1 was taken before, and samples 2 and 3 after i.p. injection of vehicle or CNO. Thereafter, rats were exposed to 5 min of forced swimming (water temperature 22–24 °C), before samples 4 and 5 were collected. Microdialysate samples were immediately frozen on dry ice and subsequently stored at −80 °C until quantification of OT by radioimmunoassay without extraction.

2.8. Quantification of OT

OT content was analyzed in extracted plasma samples and in evaporated microdialysates by a highly sensitive radioimmunoassay (RIAgnosis, Germany). The detection limit of the assay is 0.1 pg per sample. Cross-reactivity with arginine-vasopressin, ring moieties and other related peptides are < 0.7% throughout. To eliminate inter-assay variability (< 10%), all dialysates to be compared were measured in the same assay (de Jong et al., 2015; Landgraf et al., 1995).

2.9. Analysis of social behavior

To analyze the effect of chemogenetically activated hypothalamic OT neurons on social behavior 21 days following viral injection, we performed the social defeat paradigm as previously described (Lukas et al., 2011). Briefly, aggressive male rats, i.e. rats selectively bred for low anxiety-related behavior (Veenema et al., 2007), were used as residents (defeater). Starting at 930, defeated rats were tested in the social preference paradigm 2 h after the onset of the 30-min social defeat using the former defeater rat as social stimulus.

2.10. Analysis of anxiety-related behavior

The effect of chemogenetic activation of paraventricular OT neurons on anxiety-like behavior was tested in the light-dark box 21 days following viral infusion between 0900 and 1200. The light-dark box consisted of a lit (40 x 50 cm, 100 lx; light box) and a dark (40 x 30 cm, 0 lx) compartment connected via a small opening (7.5 x 7.5 cm) enabling transition between the two compartments. One hour after i.p. CNO administration, the rat was placed in the lit compartment for 5 min (Crawley and Goodwin, 1980), and the rat's behavior was analyzed using an automated video tracking system (Noldus, EthoVision X7).

Moreover, self-grooming was scored every fifth minute during ongoing microdialysis in their home cage on day 1 by an experienced observatory blind to the treatment.

2.11. Quantitative analysis of vector expression

The transgene expression was verified in perfused, 40-µm thick coronal slices containing the PVN, SON and AN by immunofluorescent staining of OT-associated neurophysin (1:500, PS38 mouse monoclonal (Ben-Barak et al., 1985; Whitnall et al., 1985)) and mCherry (1:1,000, abcam, ab167453), and visualized using sequential imaging on a Leica TCS SP8. Digitized images were analyzed using FIJI (NIMH, Bethesda, MD, USA).

2.12. Statistical analyses

Statistical analyses were performed using SigmaPlot 13 (Systat). Fiber photometry data were analyzed using custom MATLAB (MathWorks) scripts. For repeated or multiple comparisons, the appropriate ANOVA test was applied as indicated in results section. Any overall statistical differences, which were set at $p < 0.05$, were further analyzed using Tukey's post hoc test. When two groups were compared, unpaired *t*-test or nonparametric Mann-Whitney *U* test was used.

3. Results

3.1. Specificity of virally introduced gene expression

We induced the expression of an excitatory DREADD (hM3Dq) and GCaMP6s selectively under the control of an OT promoter fragment using a rAAV injected into the PVN, or PVN, SON and AN depending on the experimental design. The local injection of the rAAVs resulted in selective expression of the respective transgenes in OT neurons (Fig. 1). Cell counting (n = 3 rats, with 4 PVN sections per rat) revealed that 860 out of 944 cells (91.0 ± 2.7%) were immunoreactive for OT, GCaMP6s and hM3Dq:mCherry revealing an efficient and highly specific virus expression. Further, 96.4 ± 3.8% of OT neurons were at least positive for one of the fluorescent markers. The misexpression of hM3Dq:mCherry in non-OT cells was 1.3 ± 0.8% and 0.9 ± 1.2% for GCaMP6s.

3.2. Chemogenetic excitation of OT neurons evokes increased intracellular Ca^{2+} levels

Based on co-infection with GCaMP6s, we monitored Ca^{2+} dynamics in chemogenetically excited PVN-OT neurons using in vivo fiber photometry (Fig. 1). Here, we quantified Ca^{2+} signal dF/F prior and after i.p. CNO injection. One-way repeated measures ANOVA followed by post-hoc corrections revealed an increase in Ca^{2+} signal dF/F starting 20 min after CNO administration ($F_{12,35} = 7.56$, $p = 0.018$) and lasting up to 1 h (repeated measure multiple comparison; (+20 min): $p = 0.013$, (+30 min): $p = 0.012$, (+40 min): $p = 0.018$, (+50 min): $p = 0.03$, (+60 min): $p = 0.026$).

3.3. Chemogenetic activation of hypothalamic OT neurons evokes peripheral OT secretion

In anesthetized rats, we monitored peripheral OT secretion in plasma sampled before and after stimulation (Fig. 2A). Here, chemogenetic excitation of hypothalamic OT neurons induced a rise in plasma OT concentration indicative for peripheral release from neurohypophysial terminals ($F_{1,29} = 11.60$, $p = 0.005$, two-way repeated measures ANOVA). While basal plasma OT did not differ between the treatment groups ($p = 0.61$), post-hoc analysis revealed reliably increased plasma OT ($p < 0.001$) when OT neurons were chemogenetically excited. Within-group comparison demonstrated that chemogenetic activation of OT neurons evokes peripheral OT release as OT plasma content was increased ($p < 0.001$). In contrast, plasma OT did

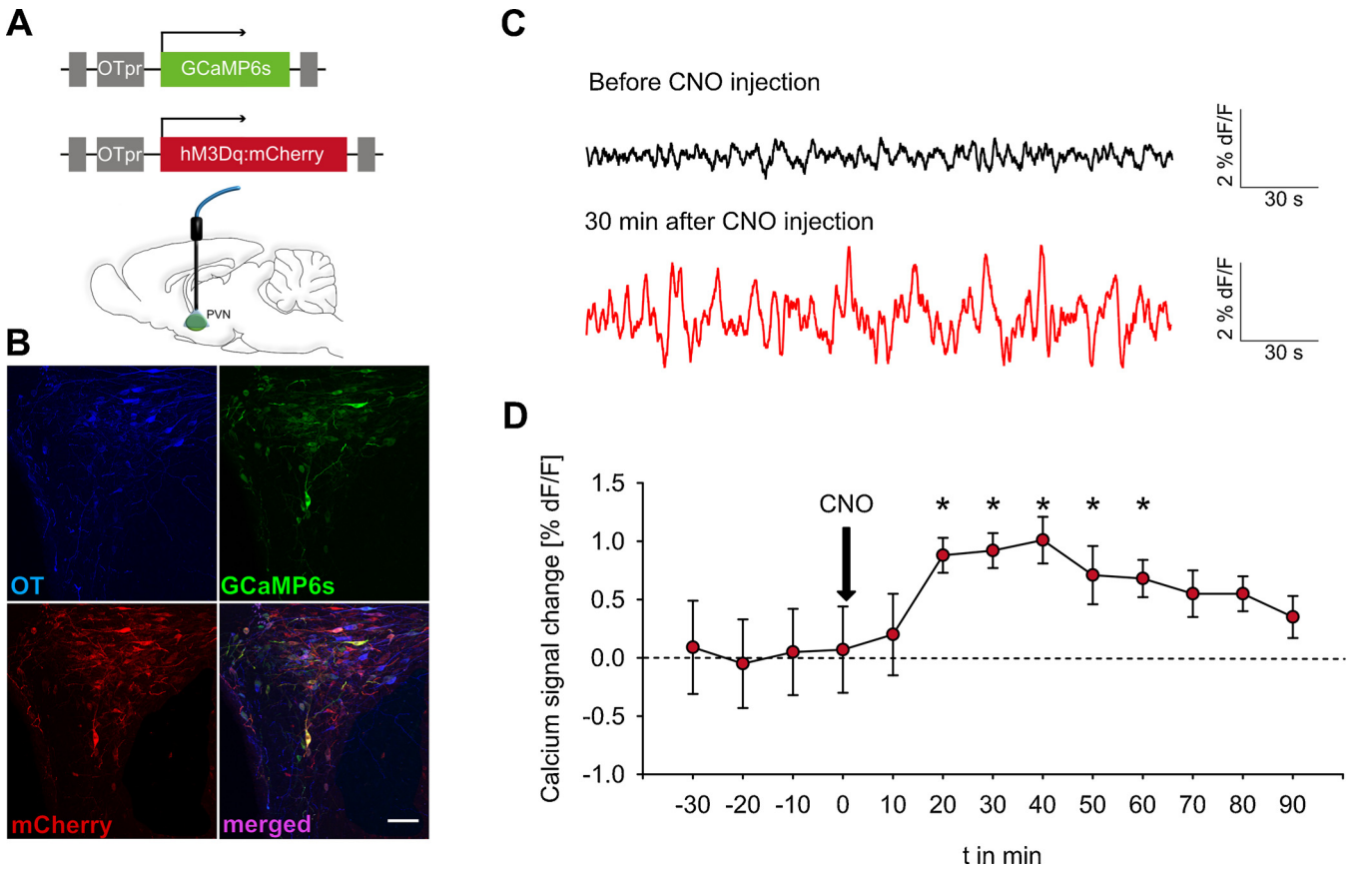

Fig. 1. Calcium dynamics in OT neurons in response to chemogenetic excitation.
A) Schema of viral vectors $rAAV_{1/2}$ OTpr-GCaMP6s and OTpr-hM3Dq:mCherry injected into the PVN.
B) Panels of immunostained section of the PVN showing co-localization of OT (blue), GCaMP6s (green), and Gq-mCherry (red) signals. Scale bar: 50 µm.
C) Example of raw traces of recorded Ca^{2+} signal in OT neurons before and 30 min after i.p. CNO injection in lightly anaesthetized adult Wistar rats (n = 3).
D) Quantification of Ca^{2+} signal dF/F prior and after i.p. CNO injection, showing significant increase of OT neuron Ca^{2+} activity following CNO administration. Average dF/F values were calculated over 10-min time bins. Data are presented as difference respect to the mean baseline (dotted line).
Line chart represents mean ± SEM, *$p < 0.05$ vs control basal, n = 3.

not change in rats treated with sterile PBS ($p = 0.84$). These findings are also reflected by higher delta values in absolute OT content ($sample^{+45\ min}$ – $sample^{-45\ min}$, $U = 81.0$, $p = 0.002$).

3.4. Chemogenetic excitation of PVN-OT neurons evokes local OT release

To test whether chemogenetic excitation of PVN-OT neurons evokes local OT release in conscious rats, intracerebral microdialysis was performed on two consecutive days either under basal, unstressed conditions (day 1), or before and after exposure to forced swim (day 2, Fig. 2B). Importantly, comparison of three groups (vector/i.p.: control/Veh, control/CNO, hM3Dq/Veh) revealed that the relative OT content in microdialysates did not change over time (two-way ANOVA for repeated measures; day 1: $F_{8,57} = 1.02$, $p = 0.44$; day 2: $F_{8,55} = 0.29$, $p = 0.97$). Therefore these three groups were combined and below defined as combined control group.

For day 1, two-way repeated measures ANOVA including combined control group and hM3Dq/CNO showed an interaction of treatment x time ($F_{4,90} = 3.49$, $p = 0.012$). Specifically, post-hoc analysis revealed an increased OT release in the hM3Dq/CNO group at both 60 ($p = 0.002$) and 90 min ($p = 0.005$) vs. respective combined control group indicative for chemogenetically evoked OT release.

On day 2, two-way repeated measures ANOVA revealed an interaction of treatment x time ($F_{4,89} = 2.70$, $p = 0.038$). In line, relative OT release was increased 60 min after CNO injection in comparison to both the respective combined control ($p = 0.002$, between subjects) and hM3Dq/CNO basal level ($p = 0.030$, within subjects). In response to forced swimming, separate one-way repeated measures ANOVA revealed an increase in the relative OT content within the comined control group ($F_{4,55} = 6.12$, $p < 0.001$). Importantly, physical stress failed to further enhance chemogenetically evoked OT release (within group analysis; sample 3 (+60 min) vs. sample 4 (+90 min): $p = 0.85$; sample 3 vs. sample 5 (+120 min): $p = 0.94$). In detail, hM3Dq/CNO rats still responded with a more pronounced OT release compared to the respective combined control group (sample 4 (+90 min): trend with $p = 0.068$, sample 5 (+120 min): $p = 0.006$) following forced swim.

3.5. Chemogenetic activation of hypothalamic OT neurons reverses social defeat-induced lack of social preference

To test whether stimulation of endogenous OT can reverse social defeat-induced lack of social preference, hypothalamic OT neurons (PVN, SON, AN) were chemogenetically activated. Two-way repeated measures ANOVA revealed an interaction of treatment x interval ($F_{2,53} = 5.52$, $p = 0.011$). Here, exposure of the defeated control group to their individual defeater rat resulted in a lack of social preference ($p = 0.90$, Fig. 3B) also seen in DREADD-free animals treated with CNO ($p = 0.92$). Importantly, chemogenetic excitation of hypothalamic OT neurons resulted in a reinstatement of social preference toward the former defeater ($p < 0.001$).

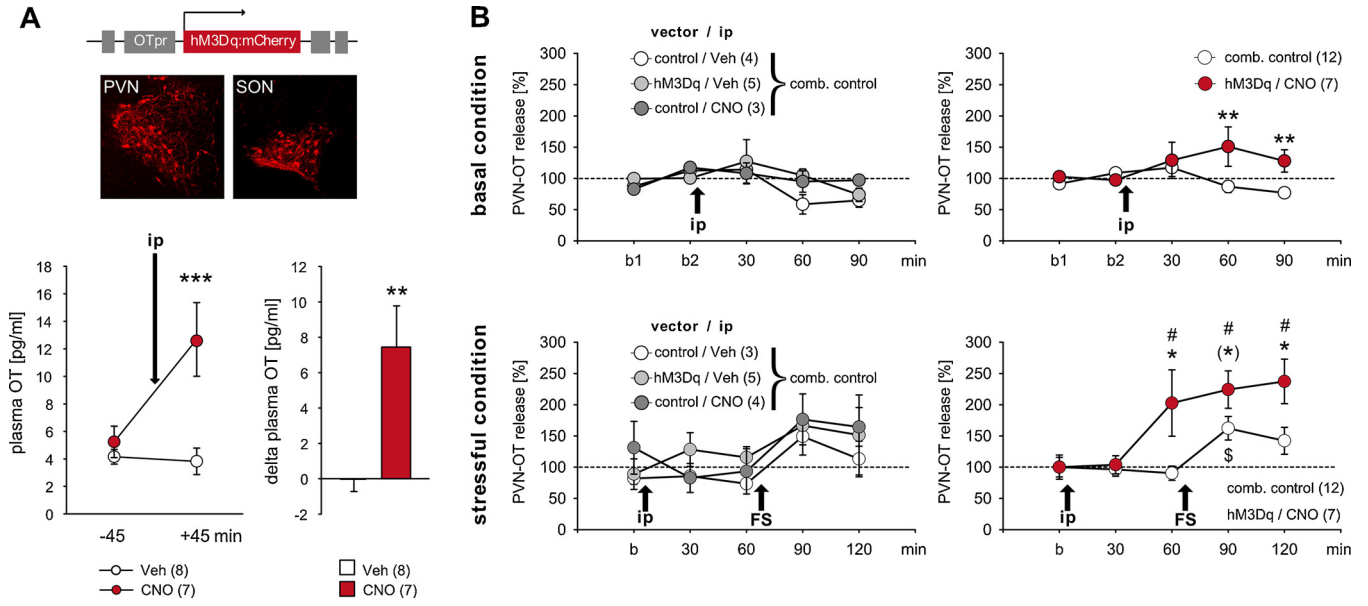

Fig. 2. Chemogenetic activation of hypothalamic OT neurons evokes peripheral and central OT release in male Wistar rats.
A) Bilateral injection of rAAV$_{1/2}$ OTpr-hM3Dq:mCherry into PVN, SON and AN induced the expression of an excitatory DREADD specifically in OT neurons. Three weeks later, blood was sampled via jugular vein catheter to monitor absolute plasma OT concentration in response to i.p. injection of control (Veh; PBS, pH 7.4) or CNO (2 mg/kg). Chemogenetic excitation of OT neurons evoked increased plasma OT concentration indicative for peripheral OT release from magnocellular neurons. B) Intracerebral microdialysis within the PVN was performed to monitor somato-dendritic OT release in response to chemogenetic excitation of paraventricular OT neurons under basal (day 1) and stressed (day 2) conditions on two consecutive days. On day 1 (upper panel), 30-min dialysates were sampled before (b1 and b2) and after i.p. injection of Veh or CNO (2 mg/kg). On day 2 (lower panel), one 30-min dialysate was sampled before (basal (b)) and four after i.p. injection of Veh or CNO. Samples 4 (90 min) and 5 (120 min) were collected following a 5-min forced swim stress (FS). Only CNO injection in animals expressing an excitatory DREADD specifically in OT neurons induced a rise in somatodendritic OT secretion. All data are expressed as percentage of baseline (= 100%; dotted line).
Bar chart represents mean + SEM; line charts ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, (*)p = 0.068 vs. respective control, $^{\#}$p < 0.05 vs. CNO basal (sample 1, b), $^{\$}$p < 0.05 vs. control basal (sample 1, b); group size (n) is depicted in brackets.

3.6. Chemogenetic activation of hypothalamic OT neurons reduces anxiety-related behavior and promotes self-grooming

Here, we analyzed the effects of increased brain OT availability induced by chemogenetic activation on non-social behavior, which resulted in reduced anxiety-like behavior (one-way ANOVA, $F_{2,18} = 5.46$, $p = 0.028$) and increased self-grooming (two-way repeated measures ANOVA, $F_{4,74} = 2.56$, $p = 0.049$).

In detail, chemogenetic activation of PVN-OT neurons evoked anxiolysis (Fig. 3D-F), since CNO-treated animals expressing the excitatory DREADD spent more time in the lit compartment of the light/dark box ($p = 0.028$) compared to controls (Veh). None of the treatments altered the number of light box entries ($F_{2,18} = 1.54$, $p = 0.27$) or locomotor activity ($F_{2,18} = 2.67$, $p = 0.12$). Importantly, CNO alone in DREADD-free animals did not affect anxiety-like behavior compared to control.

Moreover, behavioral analysis during ongoing intra-PVN microdialysis on day 1 revealed increased grooming behavior, when OT neurons were chemogenetically excited. The frequency of self-grooming peaked 60 min after i.p. injection of CNO compared to control ($CNO^{+60\ min}$ vs. $Veh^{+60\ min}$: $p = 0.002$, Fig. 3G). Within-group analysis revealed that control rats spent more time on grooming behavior during the first 30-min period after i.p. injection of Veh compared to baseline 1 ($Veh^{+30\ min}$ vs. Veh^{b1}: $p = 0.016$). Other behaviors such as sleep ($F_{4,74} = 0.74$, $p = 0.57$), exploration ($F_{4,74} = 0.31$, $p = 0.87$) or food/water-intake ($F_{4,74} = 0.06$, $p = 0.99$) did not differ between groups (data not shown).

4. Discussion

The current study demonstrates functional evidence for the chemogenetic activation of hypothalamic OT neurons. In detail, we provide evidence for the efficiency and the associated temporal dynamics of activation of excited OT neurons (1) at cellular level by in vivo monitoring of Ca^{2+} fluctuations, (2) at neuroendocrine level as demonstrated by peripheral and central OT release, and (3) at behavioral level as demonstrated by social motivation and anxiolysis.

Chemogenetics represents a highly innovative neurobiological tool that allows cell type-specific remote control of a certain neuronal population. Although OT has attracted the attention of the scientific community due to its beneficial effects on socio-emotional behavior in rodents and humans (Neumann and Slattery, 2016), there is surprisingly very limited information about the application of chemogenetics for modulating the OT system. In fact, there are only three publications from our labs, which employed an inhibitory DREADD to silence OT neurons (Eliava et al., 2016; Grund et al., 2017; Menon et al., 2018). In these studies, we applied cell type-specific OT promoter driving the expression of hM4Di and showed an exquisite specificity of the used promoter for the specific expression of the transgene in 90–100 % of OT neurons after approximately three weeks. Importantly, chemogenetic silencing of OT neurons was efficient at cellular, network and behavioral levels. In the present study, we confirmed the cell type-specific expression of the transgenes with superb efficiency, which provides the basis to characterize the consequences and temporal dynamics of chemogenetically activated OT neurons.

Previous in vitro studies revealed Ca^{2+} mobilization following chemogenetic excitation of transfected cells (Armbruster et al., 2007). To quantify Ca^{2+} fluctuations in chemogenetically activated PVN-OT neurons, we applied in vivo fiber photometry by co-infection with GCaMP6s – a genetically encoded Ca^{2+} biosensor. Here, we identified a maximal Ca^{2+} response 20–60 min after i.p. CNO injection, hence providing a meaningful time window important for the optimal design of experimental protocols.

A rise in intracellular Ca^{2+} levels specifically in OT neurons is a

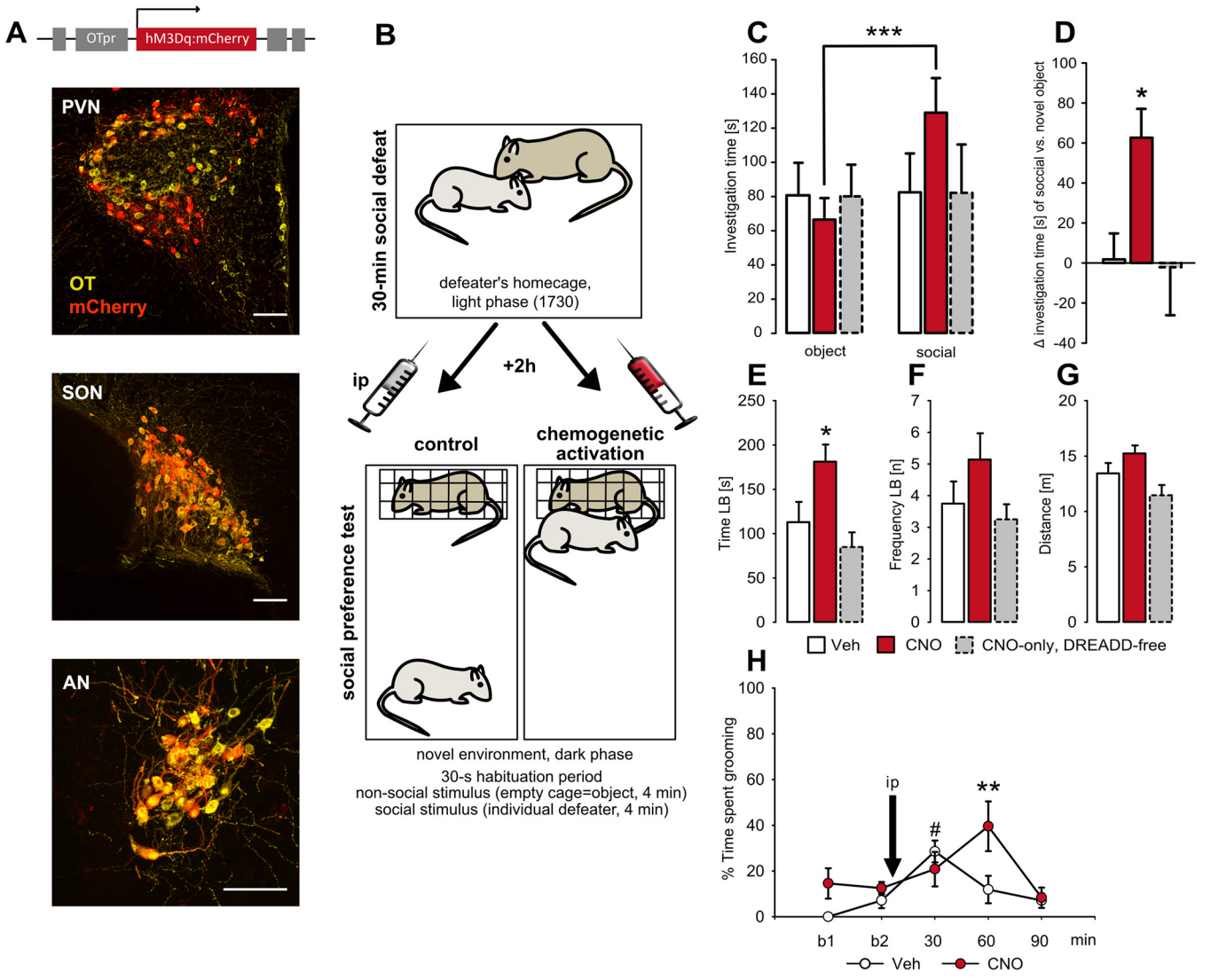

Fig. 3. Chemogenetic activation of hypothalamic OT neurons modulates social and emotional behavior, such as social preference, self-grooming and anxiety-related behavior in male Wistar rats.

A) Bilateral infusion of rAAV$_{1/2}$ OTpr-hM3Dq:mCherry into PVN, SON and AN resulted in the expression of an excitatory DREADD specifically in OT neurons. Scale bar 100 µm.

B) Scheme of social defeat-induced social avoidance paradigm.

C) Socially defeated rats were exposed to their individual defeater rat as social stimulus during the social preference paradigm 2 h later. Control (Veh; PBS, pH 7.4) or CNO (2 mg/kg) were injected i.p. 45 min prior to social preference test. Here, social defeat induced a lack of social preference in controls (n = 12), which was reinstated by chemogenetic activation of hypothalamic OT neurons (n = 10). CNO in DREADD-free rats did not affect investigative behavior per se (n = 5).

D) Delta values reflect the preference of social vs. object investigation.

E) Time spent in light compartment of the light/dark box, n(Veh) = 8, n(CNO) = 7, n(CNO only, DREADD free) = 4.

F) Entries into the light compartment.

G) Traveled distance in the light/dark box.

H) During ongoing microdialysis on experimental day 1, behavioral assessment revealed increased self-grooming, when OT neurons were activated chemogenetically. Other behavior, such as food/water-intake, sleep and exploration were not affected, n = 7–8.

Bar charts represent mean + SEM; line chart ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001 vs. Veh or as indicated, $^{\#}$p < 0.05 vs. Veh basal (basal 1 and 2).

prerequisite for OT release (Jurek and Neumann, 2018). As magnocellular OT neurons form neurohemal contacts important for peripheral OT supply, we quantified OT plasma levels before and after DREADD activation. Indeed, chemogenetic activation of hypothalamic OT neurons induced a prominent rise in plasma OT concentration indicative for axonal release from neurohypophysial terminals. Most stimuli that trigger OT secretion into peripheral blood flow also evoke central OT release (Jurek and Neumann, 2018). To test whether chemogenetic activation also affects secretory activity of OT neurons within the PVN, we performed intracerebral microdialysis. Here, we identified reliably increased OT content in microdialysates indicative for increased OT release in somato-dendritic fashion peaking during the second sampling period after CNO injection, which is in line with in vivo Ca^{2+} responses. Although the duration and intensity of CNO-induced activation of DREADD-expressing cells is controversially discussed (Alexander et al., 2009), we have to keep in mind that OT neurons belong to a highly specialized and complex type of neuroendocrine cells capable of propagating action potentials as typical neurons, but also to synthesize and release OT from various parts of the neuronal membrane independent of synaptic inputs. In that context, Ca^{2+} release specifically from intracellular stores has been shown to trigger dendritic OT release, which can become self-sustaining and long lasting due to autocrine

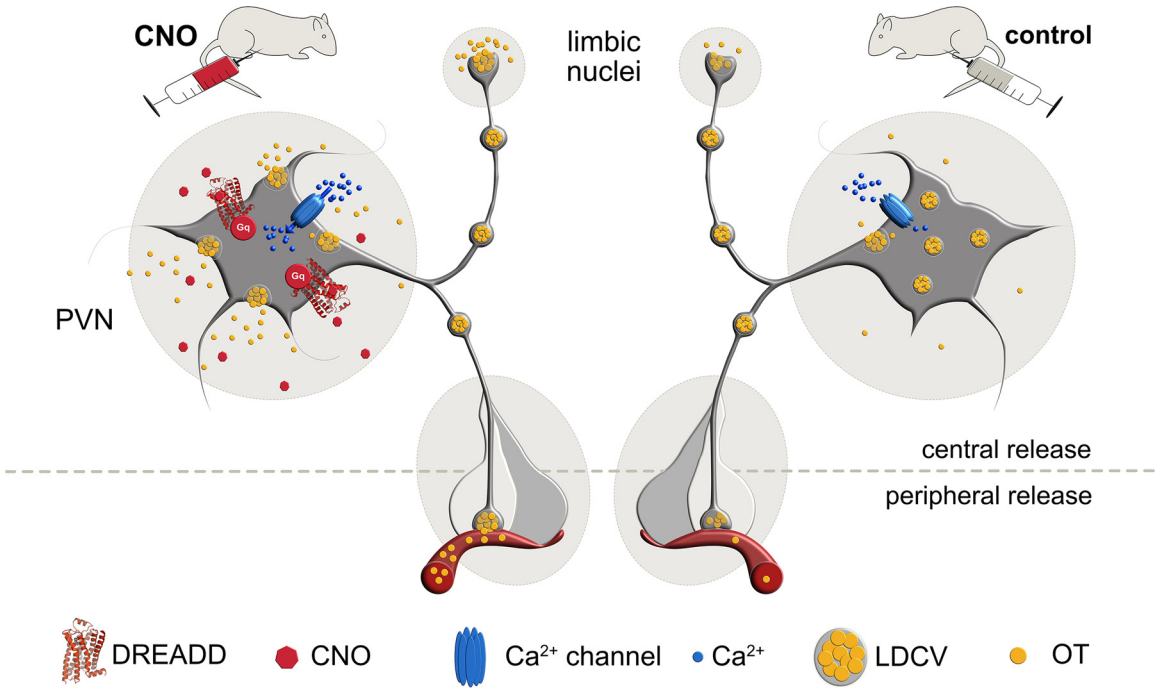

Fig. 4. Cell type-specific activation of neuroendocrine cells by chemogenetics evokes increased OT release (left panel) compared to control (right panel). The designer receptor (DREADD) present at plasma membrane of transfected OT neurons is activated by i.p. injection of the DREADD agonist clozapine N-oxide (CNO). In turn, this promotes a G_q-mediated Ca^{2+} influx from intra- and extracellular stores. The rise in intracellular Ca^{2+} levels evokes the fusion of OT-containing large dense-core vesicles (LDCV) to the plasma membrane and OT release in somato-dendritic fashion within the PVN, from axons in distant limbic brain regions and/or from neurohypophysial terminals into the periphery.

mechanisms (Lambert et al., 1994; Ludwig et al., 2002). It is therefore not surprising that the OT content within the third 30-min dialysate after CNO injection was still increased further supporting the idea of a long lasting stimulation of PVN-OT neurons. Based on previous data demonstrating paraventricular OT release in response to forced swim stress (Wigger and Neumann, 2002), we examined whether physical stress may further enhance chemogenetically-driven OT release. Whereas exposure to 5 min of forced swimming reliably increased the OT content in microdialysates of the control group, it failed to further enhance the OT content in microdialysates of DREADD-expressing rats treated with CNO probably due to a ceiling effect.

Since the stimulatory effects of an excitatory DREADD on the activity of OT neurons highlight potential consequences on different aspects of behavior, two strategies were used: First, we looked at DREADD-mediated effects on social motivation, and then for the effects on non-social behaviors. In an animal model of social defeat-induced lack of social preference (Lukas et al., 2011), chemogenetic excitation of hypothalamic OT neurons reinstated social preference of socially defeated rats. Besides pharmacological and optogenetic experiments, our DREADD-based results provide further direct evidence that OT exerts pro-social effects on male-male interactions. With respect to non-social behavior we revealed that a chemogenetically-driven increase in brain OT availability resulted in anxiolysis and increased self-grooming – two behaviors that were repeatedly shown to be regulated by OT (Blume et al., 2008; Drago et al., 1986; van den Burg et al., 2015).

Recent studies showed that clozapine, to which CNO rapidly converts in vivo, shows high DREADD affinity and potency, but has also other targets (Gomez et al., 2017; MacLaren et al., 2016). Therefore, the DREADD ligand CNO is controversially discussed (Mahler and Aston-Jones, 2018) and respective control groups (e.g. CNO-only, DREADD-free) are required to exclude possible side effects of CNO or its metabolites (Grund et al., 2017; Mahler and Aston-Jones, 2018; Manvich et al., 2018). To exclude unspecific outcomes of CNO administration demonstrated at > 5 mg/kg, we have applied CNO at a dose of 2 mg/kg to specifically activate the excitatory DREADD expressed in OT neurons. Importantly, CNO injection in DREADD-free rats did not alter central OT secretion, social motivation or anxiety-related behavior indicating a selective activation of OT neurons.

In conclusion, we employed a highly efficient and specific OT promoter, which allowed us to drive the expression of the stimulatory subunit hM3Dq of a modified human muscarinic receptor specifically in OT neurons of the rat hypothalamus. Based on in vivo fiber photometry and quantification of hormonal release, our findings demonstrate a convincing line of evidence that highlight the temporal dynamics and the physiological and behavioral consequences of chemogenetically-driven challenges in vivo that are schematically summarized in Fig. 4. Based on these findings, we advise to administer CNO or control 30–60 min before the start of the experiment – a time when chemogenetic excitation of OT neurons has been identified with increased Ca^{2+} dynamics, and a measurable OT secretion in axonal and somato-dendritic fashion. In addition, the efficiency of chemogenetically-driven activation of OT neurons was also demonstrated at the behavioral level, demonstrating increased social motivation in a psychopathological animal model and anxiolysis. Altogether, our results provide the basis for optimized experimental protocols that specifically focus on the physiology of neuroendocrine systems and peptidergic modulation of socio-emotional behavior.

Conflict of interest

None.

Contributors

TG, IDN and VG were responsible for the conception and design of the study. TG, YT, DB, FA, SP and LO contributed to data acquisition. TG, DB and FA analyzed data. TG and DB drafted the manuscript. VG, IDN and FA critically revised the manuscript. All authors have approved the final article.

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