

Pharmacogenetic excitation of the median raphe region affects social and depressive-like behavior and core body temperature in male mice

Csilla Lea Fazekas^{a,b,*}, Manon Bellardie^{a,c}, Bibiána Török^{a,b}, Eszter Sipos^a, Blanka Tóth^d,
Mária Baranyi^a, Beáta Sperlág^a, Mihály Dobos-Kovács^a, Elodie Chaillou^c, Dóra Zelena^{a,e}

^a Institute of Experimental Medicine, Budapest, Hungary

^b János Szentágothai Doctoral School of Neurosciences, Semmelweis University, Budapest, Hungary

^c INRAE Centre Val de Loire, CNRS, IFCE, Université de Tours, UMR 85 Physiologie de la Reproduction et des Comportements, France

^d Budapest University of Technology and Economics, Faculty of Chemical Technology and Biotechnology, Department of Inorganic and Analytical Chemistry, Budapest, Hungary

^e Centre for Neuroscience, Szentágothai Research Centre, Institute of Physiology, Medical School, University of Pécs, Pécs, Hungary

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ABSTRACT

Aims: Median raphe region (MRR) is an important bottom-up regulatory center for various behaviors as well as vegetative functions, but detailed descriptions and links between the two are still largely unexplored.

Methods: Pharmacogenetics was used to study the role of MRR in social (sociability, social interaction, resident intruder test) and emotional behavior (forced swim test) parallel with some vegetative changes (biotelemetry: core body temperature). Additionally, to validate pharmacogenetics, the effect of clozapine-N-oxide (CNO), the ligand of the artificial receptor, was studied by measuring (i) serum and brainstem concentrations of CNO and clozapine; (ii) MRR stimulation induced neurotransmitter release in hippocampus; (iii) CNO induced changes in body temperature and locomotor activity.

Key findings: MRR stimulation decreased locomotion, increased friendly social behavior in the resident intruder test and enhanced depressive-like behavior. The latter was accompanied by diminished decrease in core body temperature. Thirty minutes after CNO injection clozapine was predominant in the brainstem. Nonetheless, peripheral CNO injection was able to induce glutamate release in the hippocampus. CNO had no immediate (<30 min) or chronic (repeated injections) effect on the body temperature or locomotion.

Significance: We confirmed the role of MRR in locomotion, social and depressive-like behavior. Most interestingly, only depressive-like behavior was accompanied by changed body temperature regulation, which was also observed in human depressive disorders previously. This indicates clinical relevance of our findings. Despite low penetration, CNO acts centrally, but does not influence the examined basic parameters, being suitable for repeated behavioral testing.

1. Introduction

Social interactions are essential for life in many species including humans. Their abnormalities are widespread and commonly present in different mental illnesses (e.g. depression, schizophrenia, autism) [1]. Moreover, these pathological conditions are often accompanied by changes in vegetative functions, such as disturbed stress reactivity [2], cardiac autonomic dysfunction [3] or body temperature regulation. Interestingly, there seems to be a connection between depression and disturbed body temperature regulation: depressed patients have higher body temperature and sweat less [4,5], a process mediated by the

serotonergic (5-HT) system [6].

The midbrain median raphe region (MRR) is a well-known nucleus that provides significant serotonergic projections to the forebrain [7]. The MRR is known to have a role in various behaviors, among others in stress adaptation [8–10]. Due to its 5-HT positive cells, it has also been linked to depressive behavior [11–15]. In our previous study we found that optogenetic stimulation of MRR decreased aggressive behavior [16], which is a well-known symptom of depression [17]. Its contribution to vegetative functions was also suggested, since its manipulation may influence the sleep-wake cycle [18] and body temperature [19,20]. However, the results are contradictory on many areas (e.g. circadian

* Corresponding author at: 1083 Budapest, Szigony utca 43., Hungary.

E-mail address: fazekas.csilla@koki.hu (C.L. Fazekas).

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rhythm [21] or body temperature [22]).

With the help of pharmacogenetics it is possible to manipulate neuronal activity in a less invasive manner compared to other possibilities (e.g. intracerebral local injections, electric stimulation, ablation or even optogenetics) [23]. The virally introduced designer receptors exclusively activated by designer drugs (DREADDs) utilize modified human muscarinic receptors in order to excite or inhibit neurons [24]. Their artificial ligand, which cannot be found in mammalian body under

physiological conditions, the clozapine-N-oxide (CNO) can be applied both locally and systematically. However, CNO may back-metabolizes to a well-known atypical antipsychotic drug, clozapine, which may confound any obtainable results [24–26].

We had dual aims. (1) Regarding the effects of MRR activation, (i) we wanted to confirm our previous findings on the role of MRR in social behavior obtained by optogenetics [27]. However, to distinguish between different aspects of social behavior, we used an extended test

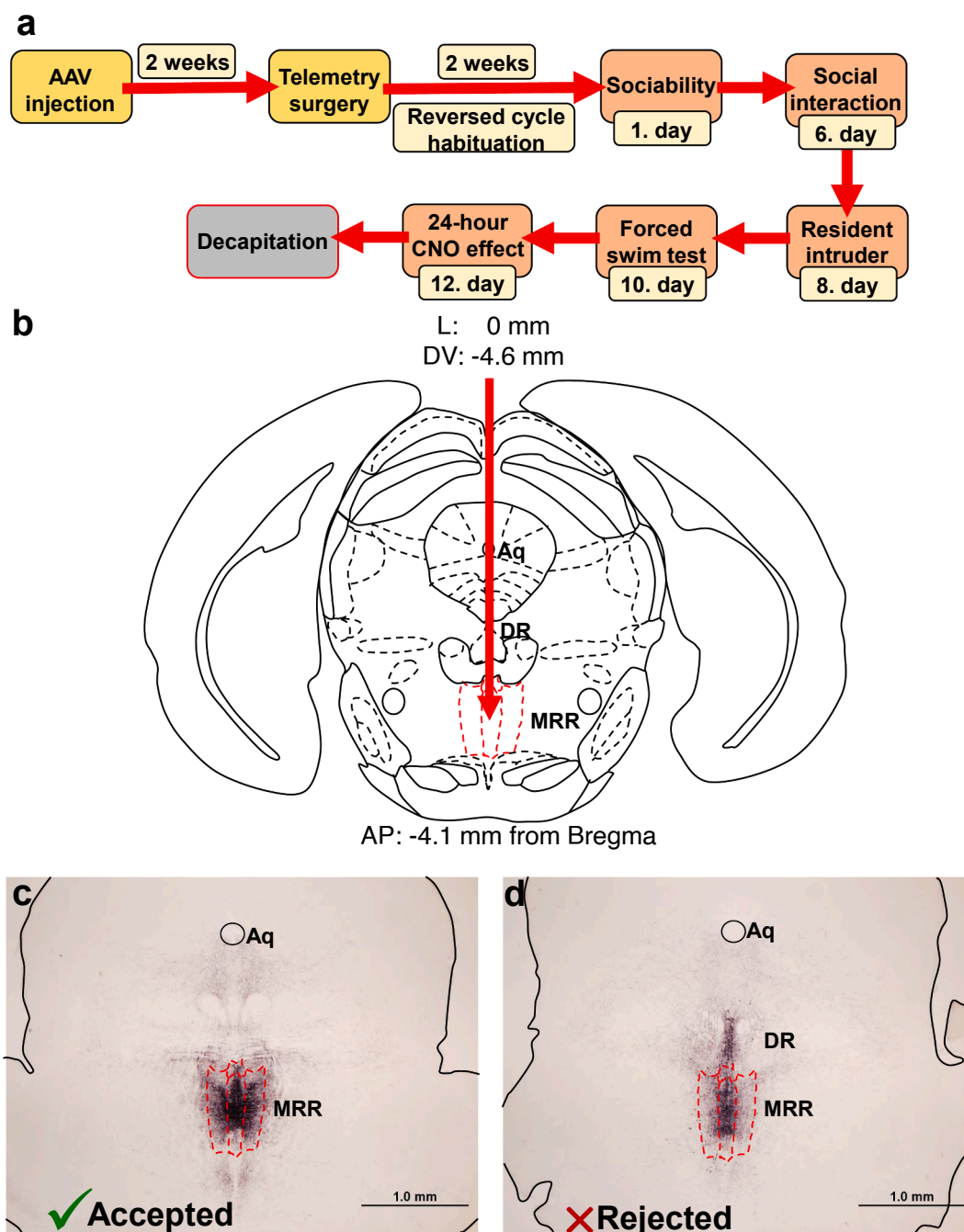


Fig. 1. Timeline of the behavioral test battery and virus injection details. (a) Protocol of the behavioral experiments starting from virus injection. Thirty minutes before all experiments the test animals were injected intraperitoneal with clozapine-N-oxide (CNO, 1 mg/10 mL/kg diluted in saline). Trunk blood was taken after decapitation and stress hormones (corticosterone and adrenocorticotropic hormone [ACTH]) were measured. (b) Schematic representation of the targeted mouse median raphe region (MRR) in coronal section according to the Mouse Brain in Stereotaxic Coordinates [68]. (c) Representative figure of an appropriate virus expression (visualized by Ni-DAB staining for the reporter protein red fluorescent protein [RFP]) in the MRR. (d) Representative figure of a rejected virus expression, which appears both in the dorsal raphe (DR) and MRR.

AP: anterior-posterior coordinate; Aq: aqueductus cerebri; DR: dorsal raphe; DV: dorso-ventral coordinate; L: lateral coordinate; MRR: median raphe region; Ni-DAB: nickel-3,3'-diaminobenzidine.

battery that contained sociability, social interaction (SIT) and resident intruder tests (RIT). (ii) Additionally, we wanted to see if vegetative alterations (i.e. core body temperature studied by biotelemetry) may corroborate behavioral changes. (iii) Due to the possible connection between body temperature and depression [4–6], we also investigated a depression-related behavior, namely, floating in the forced swim test (FST).

(2) Secondly, we aimed to validate the effectiveness of CNO, a widely discussed topic in pharmacogenetics. To do so, (i) the distribution of the drug and its metabolite, clozapine was measured both in serum and brainstem tissue in a separate batch of male mice. (ii) In order to see that neurotransmitters are released due to DREADD activation and not because of the CNO injection, neurotransmitter release was measured in the hippocampus - a well-known projection area of the MRR [7,17,28–30] - of mice expressing excitatory or control DREADD in the MRR in a third group of animals. (iii) Information on short- and long-term influence of peripheral CNO injections on core body temperature and locomotion were extracted from data collected during the behavioral test battery.

2. Experimental procedures

2.1. Animals

Adult male mice (C57Bl/6J, 14–15-week-old) were bred at the Institute of Experimental Medicine, Budapest, Hungary, in Macrolon cages (40 cm×25 cm×26 cm) under a 12 h light–dark cycle, $21 \pm 1^\circ\text{C}$, 50–60% humidity, with food (standard mice chow, Charles River, Hungary) and tap-water available ad libitum. After surgery the animals were kept one per cage for individual telemetric recordings. Behavior was tested during the dark, active period (between 9:00 h and 13:00 h, lights on at 18:00 h) under dim light, except for SIT and FST, during which lights were on.

All tests were presented to, approved and supervised first by the local (Animal Welfare Committee of the Institute of Experimental Medicine, Budapest, Hungary), then the regional committee of animal health and care (Government Office for Pest County, PEI/001/33-4/2013, PE/EA/254-7/2019) and performed according to the European Communities Council Directive recommendations for the care and use of laboratory animals (2010/63/EU).

2.2. Surgery

Mice were anesthetized with intraperitoneal (ip.) cocktail (0.1 mL/10 g mixture of 0.5 mL ketamine, 0.1 mL xylazine and 2.4 mL saline) and fixed in a stereotaxic device. Recombinant adeno-associated viral vector (rAAV) containing excitatory (hM3Dq) or inhibitory (hM4Di) DREADD and/or red fluorescent protein (RFP; control) gene sequences were injected (20 nL; Addgene) into the MRR (AP:−4.1 mm; L:0.0 mm; DV:4.6 mm from Bregma; Fig. 1b). For description of the viral vectors see Suppl. Materials 2.1.

The animals had 14 days to recover, then a telemetric device was implanted into the abdominal cavity under the same anesthesia [31]. During the next 14 days, mice were accustomed to reversed light–dark cycle. During the whole observation period their weight increased ($F_{(5,80)} = 2.995$; $p < 0.050$) (from average of 27.365 ± 0.395 g to 28.171 ± 0.276 g), without any difference between the groups. A total of 35 animals participated in the telemetric measurements ($N_{\text{control}} = 13$, $N_{\text{excitatory}} = 9$, $N_{\text{inhibitory}} = 13$).

2.3. Behavioral testing

The experiments were recorded and the videos were analyzed later by computer-based event recorders (H77, Budapest, Hungary; Solomon Coder [https://solomoncoder.com]) by experimenters blind to the treatment (virus type). Thirty minutes before the experiments all

animals (control, excitatory and inhibitory) were injected ip. with CNO (1 mg/10 mL/kg in saline, Tocris). The following behaviors were measured:

1. social interest with sociability;
2. social behavior with SIT and RIT;
3. depressive-like behavior with FST.

All behavioral tests were conducted on telemetry platforms. Right before the tests, the home cages were swapped to the respective experimental cages and animals were immediately placed into them. After the end of the experiments mice were returned to their home cages and were placed back onto the telemetry platforms.

A subset of animals ($N_{\text{control}} = 5$, $N_{\text{excitatory}} = 4$) was injected with CNO several days after completion of all behavioral tests. The effect of MRR stimulation was examined for 24 h via the telemetric system.

To ensure that CNO cleared out from the body, at least 48–72 h were kept between subsequent behavioral tests (Fig. 1a). At the termination of the experimental test battery, animals were decapitated and trunk blood was collected for corticosterone and adrenocorticotropin (ACTH) measured 30 min after ip. CNO injection. Brains were immersed in 4% paraformaldehyde (PFA) until further processing.

2.3.1. Sociability test

It measures the interest of the tested animals towards conspecifics [32]. Mice were placed in empty, white plexiglass boxes covered by transparent plexiglass for 5 min (open field phase; OF). Right after this, two identical wired containers were placed into the test arena for 5 min (habituation phase). Then an unknown, juvenile conspecific was placed under one of the wired containers for 5 min (Fig. 2a). The animals could

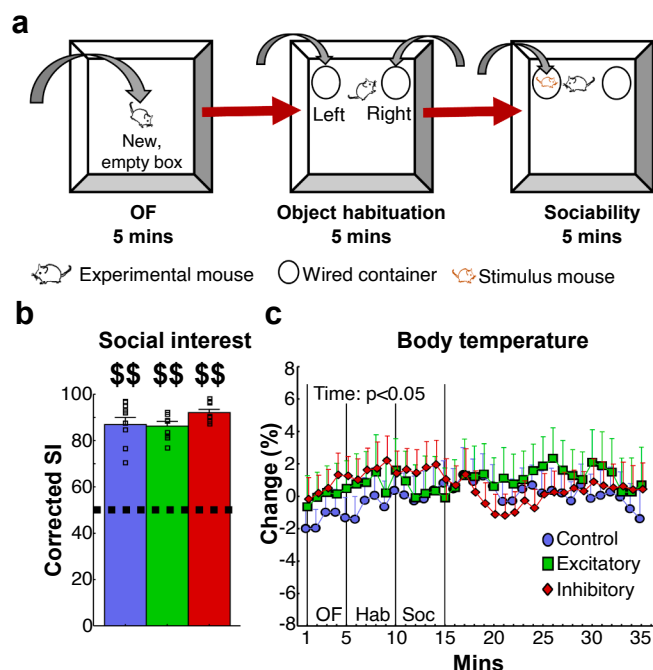


Fig. 2. Results of the sociability test. (a) Protocol of the sociability test. The 15-minute-long test consisted of three, 5 min long phases: open field (OF), object habituation and sociability. (b) Social preference index (SI), corrected by side preference, showed intact social interest (above random 50%, as indicated by the dotted black line, single sample *t*-test) in all three groups. (c) Change in body temperature showed an increase during the behavioral test, but there was no difference between the groups (repeated-measures ANOVA). Data are expressed as average±SEM. Empty squares (□) represent individual values. \$\$\$ $p < 0.01$ vs random 50.

not contact physically, but were able to see, hear and smell each other (sociability phase). The sides of the stimulus animals (either under the right or left container) were interchanged between animals.

During habituation the time spent with the two containers (indicated as 'objects' in Suppl. Table S1) were measured to analyze place preference. During sociability the frequency and time spent with conspecific was measured to reflect social interest. Social preference index (SI) was calculated as follows: $SI = t_{\text{mouse}} / [t_{\text{mouse}} + t_{\text{container}}] \times 100$, where t_{mouse} was the percentage of time spent sniffing the container with the stimulus mouse; $t_{\text{container}}$ was the percentage of time spent with sniffing the empty container.

Due to the animals showing place preference during habituation (effect of choice: $F_{(1,27)} = 7.451$; $p < 0.05$; Suppl. Table S1), the raw data of sociability phase had to be corrected. Sociability data was adjusted by dividing the percentage of time spent with given container (containing a conspecific or being empty, respectively) by the total time spent with both containers: $(t\%_{\text{side}} / (t\%_{\text{left}} + t\%_{\text{right}}))$, where $t\%_{\text{side}}$ was the percentage of time spent with the respective container during habituation; $t\%_{\text{left}+\text{right}}$ was the percentage of time spent with both containers during habituation (see Suppl. Raw Data excel file).

2.3.2. Social interaction test (SIT)

SIT investigates anxiety-driven social behavior [16]. The test was conducted in a transparent plexiglass cage (35 cm × 20 cm × 25 cm) with bedding on the bottom. The day before the experiment animals were habituated to the test arena for 2 × 15 min, 4 h apart. During test an unfamiliar stimulus mouse, roughly the same age and bodyweight was put into the arena together with the test subject. They could freely interact for 10 min in normal light (Fig. 3a).

Friendly (e.g.: sniffing), aggressive (e.g.: biting, aggressive dominance) and defensive (e.g.: running away) behaviors of the test animals were analyzed (frequencies and time spent).

2.3.3. Resident intruder test (RIT)

RIT measures the territorial aggression [33]. An unfamiliar, smaller, but sexually mature conspecific was put into the home cage of the test animals for 10 min (Fig. 4a). Only the behavior of the test animal was analyzed with the same parameters as in SIT.

2.3.4. Forced swim test (FST)

FST measures depressive-like behavior [34]. Mice were put in a 2 L-glass cylinders filled with 1.5 L, $24 \pm 1^\circ\text{C}$ water for 6 min. After removal they were dried with paper towel and the water was changed between animals. Three behavioral elements were analyzed: floating (no movements except for balancing), which reflects depressive-like behavior; struggling (climbing) and swimming (vertical movement [35]), which reflect active-coping (frequencies and time spent).

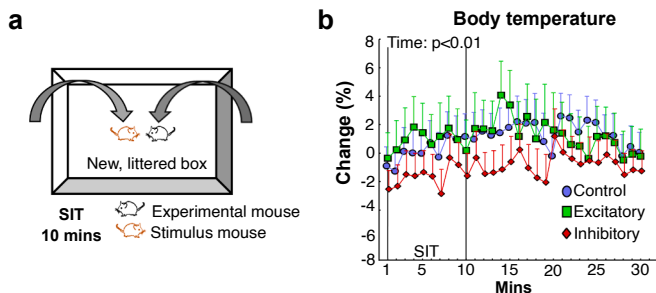


Fig. 3. Results of social interaction test. (a) Protocol of the social interaction test (SIT). For 10 min, the test animals could freely interact with a physically similar, but unknown conspecific in a previously habituated cage in light. (b) Change in body temperature showed an increase during the behavioral test, regardless of group (repeated-measures ANOVA).

2.4. Stress hormone measurements

Blood samples were collected 30 min after CNO injections and were kept on water ice until they were centrifuged on 4°C , 2000g for 30 min. Serum was kept at -20°C until processed. Hormone levels were measured by radioimmunoassay in unextracted serum (ACTH: 50 μL ; corticosterone: 10 μL), both using specific antibody developed at the Institute of Experimental Medicine (Budapest, Hungary) [36]. The intraassay coefficient of variation was 7.5% and 4.7%, respectively. All samples from a particular experiment were measured in one session. All the animals took part in the behavioral test battery described above ($N_{\text{total}} = 32$, $N_{\text{control}} = 11$, $N_{\text{excitatory}} = 9$, $N_{\text{inhibitory}} = 12$).

2.5. HPLC/MS/MS for CNO distribution

A second batch of mice ($N = 5$) were injected ip. with CNO (1 mg/10 mL/kg solved in saline) and decapitated 30 min later. Blood samples and brainstem tissue (containing the MRR) were collected in sterile Eppendorf tubes. Brains were frozen on dry ice. Blood was centrifuged (2500 g for 30 min) and the serum was isolated. Both serum and brain samples were kept on -80°C until further measurements. Concentrations of CNO and clozapine were measured by high pressure liquid chromatography/tandem mass spectrometry (HPLC/MS/MS). For details see Suppl. Materials 2.2.

2.6. MRR neurotransmitter release in hippocampus

A third group of mice ($N_{\text{total}} = 7$, $N_{\text{control}} = 3$, $N_{\text{excitatory}} = 4$) were injected with control or stimulatory DREADD containing virus and 4 weeks later were used for collecting microdialysis samples [16,37]. A microdialysis probe (EICOM CX-I Brain Probe [membrane: artificial cellulose, molecular weight cut off: 50,000 Da, OD: 0.22 mm, length: 2 mm]) was placed into the dorsal hippocampus (AP: -2.4 mm; L: -2.2 mm; DV: 2.5 mm from Bregma). Sample collection started after 2 h equilibrium. Samples were collected every 30 min for 3 h. After 1 h all animals received ip. CNO injection (1 mg/10 mL/kg solved in saline) and for another 1.5 h samples were collected every 30 min. Before the start of the last 30 min, Ringer's solution was exchanged to KCl (100 mM in Ringer's solution) for 5 min in order to validate the viability/reactivity of the cells around the microdialysis probe. The placement of the probe was verified on coronal sections of the dorsal hippocampus by using C2 confocal laser-scanning microscope (Nikon Europe; 4× objective). The following neurotransmitter concentrations were measured: serotonin (5-HT), glutamate (Glu) and γ -aminobutyric acid (GABA). For detailed description see Suppl. Materials 2.3.

2.7. Immunohistochemistry

Appropriate virus infection and expression site was validated by nickel-3,3'-diaminobenzidine (Ni-DAB) immunohistochemistry [38]. After immersion of the brains in 4% PFA for one day, then in phosphate saline buffer (PBS) containing azide (1 g/L). The solution was changed to 10% sucrose-PBS for cryoprotection for a day, then the MRR region was cut by a sliding microtome (30 μm) and the slides were kept at -20°C in cryoprotective solution till further processing. The primary antibody targeted RFP (1:4000, rabbit, Rockland Inc.; 2% BSA; 0.1% TXT diluted in PBS), while the secondary antibody was biotinylated anti-rabbit (1:100, donkey, Jackson; 2% BSA diluted in PBS).

The Ni-DAB stained slices were evaluated with Olympus DP70 light microscope (4× objective). The virus expression was examined from -4.04 mm to -4.96 mm from Bregma. If there was no staining, or it was unilateral, or other brain regions (e.g.: dorsal raphe) were also stained (see Fig. 1c, d), the animal and the data belonging to it (15/50 animals, 30% in total) were excluded from statistical analysis.

2.8. Telemetric data handling

Telemetric data of body temperature ($^{\circ}\text{C}$) and locomotion (distant moved in counts/min) were collected by an automatic system (Vital-View® Telemetry Software, Starr Life Sciences, Oakmont, USA) every minute from the end of the telemetric surgery until the decapitation of the animals. Unfortunately, not all telemetric devices were working properly and due to technical reasons, not all behavioral experiments were analyzed for all animals. The actual sample sizes are shown in Suppl. Table S7.

Body temperature was expressed as change [39,40] compared to baseline: $\text{change (\%)} = (T_k/T_{\text{average}} - 1) \times 100$, where T_k stands for temperature ($^{\circ}\text{C}$) at the given time point; and T_{average} stands for the average of body temperature before. During the behavior tests the average of 10 min before CNO injections was used. For the circadian rhythm, the data points were normalized to the average body temperature of the first full (24 h) day of the telemetric measurement. Then, these were averaged for every 4 h. For the 24 h effect of MRR manipulation, the comparison was made to the average body temperature 1 h before the CNO injections. These were averaged for every hour.

Locomotion data were expressed as a sum of data points (5 min for sociability and FST, while 10 min for SIT and RIT). The change of locomotion was expressed similarly to body temperature, but the averages were calculated for 30 min before injection due to the frequent immobility.

2.9. Statistical analysis

We used StatSoft 13.4 software (StatSoft, Inc., TIBCO, USA). Outliers were defined as group mean $\pm$ 2SD for the given parameter, and thus, were excluded from the statistical analysis.

The behavioral parameters, hormone levels and average body temperatures before behavioral tests, area under the curve (AUC) and circadian rhythmicity were analyzed by one-way analysis of variance (ANOVA; effect of virus). SI in sociability was tested by single-sample *t*-test compared to 50%. The comparison of CNO and clozapine levels and average neurotransmitter levels were done by *t*-test (CNO vs clozapine; control vs excitatory). The comparison of side during object habituation phase (left vs right) and social preferences in sociability (mouse vs object) test were analyzed by repeated-measures ANOVA (effect of virus, effect of choice). Weight change, body temperature, locomotion and microdialysis data were also analyzed by repeated-measures ANOVA (effect of virus and effect of time). Fisher LSD was used as posthoc analysis. Fourier transformation was used on the 4 h telemetric data to calculate the circadian rhythmicity. Data are expressed as mean $\pm$ SEM and $p < 0.05$ was considered statistically significant, while $0.1 < p < 0.05$ was accepted as trend.

3. Results

3.1. Effects of MRR activity

3.1.1. Sociability (Fig. 2a)

All animals had intact social preference as all groups had significantly higher than 50% corrected SI (control: $t_{(9)} = 12.349$; $p < 0.001$; excitatory: $t_{(8)} = 19.797$; $p < 0.001$; inhibitory: $t_{(10)} = 35.436$; $p < 0.001$) (Fig. 2b, Suppl. Table S1). Stimulation or inhibition of MRR had no effect.

During the sociability test the body temperature increased (time: $F_{(14,364)} = 2.289$; $p < 0.001$) regardless of the groups and normalized 20 min after the behavioral test (Fig. 2c).

There was a marginal virus $\times$ time interaction ($F_{(12,132)} = 1.776$; $p = 0.058$) in the locomotor activity, but no effect of virus or time (Table 1). Post hoc test showed that compared to the control, excitatory group moved less during sociability phase ($p = 0.048$).

3.1.2. Social interaction test (Fig. 3a)

There was a marginal effect of virus in the case of friendly social behavior ($F_{(2,24)} = 3082$; $p = 0.064$) (see Suppl. Table S2). There were no differences in other parameters.

Body temperature increased with time during the test ($F_{(29,725)} = 1.797$; $p = 0.007$) and normalized after 20 min without differences between groups (Fig. 3b).

Change in locomotion only showed weak differences between the groups: both the effect of time ($F_{(2,44)} = 3.105$; $p = 0.054$) and virus $\times$ time interaction ($F_{(4,44)} = 2.147$; $p = 0.091$) had a marginal effect (Table 1). Post hoc comparison of effect of time showed that after the test, mice moved more, regardless of the groups.

3.1.3. Resident intruder test (Fig. 4a)

There was a significant main effect of virus for percentage of time spent with friendly social behavior ($F_{(2,26)} = 3.949$; $p = 0.032$; Fig. 4b, Suppl. Table S3). Post hoc test showed that excitatory group spent more time with this behavior compared to both controls ($p = 0.016$) and inhibited ones ($p = 0.024$). Frequency of friendly social behavior only showed a marginal effect ($F_{(2,26)} = 2.746$; $p = 0.083$). Other parameters did not differ between groups (Suppl. Table S3).

The body temperature increased for all three groups during RIT ($F_{(29,725)} = 1.532$; $p = 0.037$) and normalized 20 min later (Fig. 4c).

There was no difference in the locomotion of the animals (Table 1).

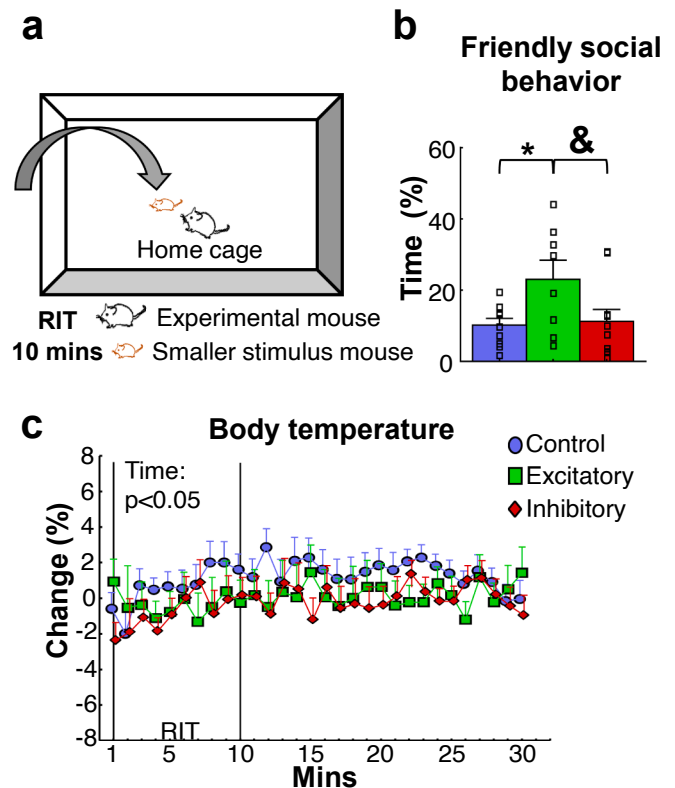


Fig. 4. Results of resident intruder test. (a) Protocol of the resident intruder test (RIT). For 10 min the test animals could freely interact with an unknown, smaller stimulus animal in their home cage (territorial aggression). (b) The excitation of the median raphe region (MRR) resulted in an increase of friendly social behavior (one-way ANOVA). (c) Body temperature increased during the observed time in all three groups, without any difference between them (repeated-measures ANOVA). Data are expressed as average $\pm$ SEM. Empty squares ($\square$) represent individual values.

* $p < 0.05$ vs control; & $p < 0.05$ vs inhibitory.

Table 1

Change of locomotion for each behavioral test. In sociability test, sociability phase, the excitatory group moved less compared to the control group. Locomotion increased after SIT (time: $p = 0.05$ SIT vs 20 min after the test), but there was no difference between the groups. There were no changes in the locomotion during and after RIT. The locomotion of the animals decreased after FST regardless of groups. Repeated-measures ANOVA; * $p < 0.05$ vs control.

Test		Virus		
		Average change of locomotion (counts/min)±SEM		
		Control	Excitatory	Inhibitory
Sociability test	OF phase	5.067±3.453	-1.667±1.860	-4.600±1.685
	Habituation phase	7.967±6.387	-3.167±2.937	0.500±2.133
	Sociability phase	10.867±7.266	-3.167±1.296 *	-1.700±1.630
SIT	20 min after	5.192±3.890	-2.229±2.083	0.400±1.133
	During the test	10.567±8.002	-8.500±3.158	-2.300±2.899
	20 min after	5.267±1.968	3.625±4.695	11.200±7.813
RIT	During the test	6.926±8.003	6.917±5.570	6.367±4.682
	20 min after	6.315±8.128	6.417±4.919	5.717±8.157
FST	During the test	5.130±2.918	6.000±0.589	2.037±2.092
	1 h after	0.766±0.967	-0.292±1.894	-3.084±1.515

3.1.4. Forced swim test

Both frequency ($F_{(2,15)} = 4.005$; $p = 0.040$) and percentage of time ($F_{(2,15)} = 4.638$; $p = 0.027$) of floating were significantly influenced by MRR manipulation. Post hoc test showed that the excitatory group spent more time with this behavior than the control (frequency: $p = 0.026$; percentage: $p = 0.015$) and inhibitory (frequency: $p = 0.023$; percentage: $p = 0.018$) groups (Fig. 5a, Suppl. Table S4). There was a marginal difference in struggling (see Suppl. Table S4) as well, supporting that the excitatory group displayed less active coping in the FST.

As a result of FST, body temperature immediately decreased and slowly normalized in the time course of 1 h (time: $F_{(90,2160)} = 47.988$; $p < 0.001$) (Fig. 5b). Our pharmacogenetic treatment significantly influenced this time-course (virus×time interaction: $F_{(180,2160)} = 1.486$; $p < 0.001$). If only the 6 min of the FST was analyzed, there was a significant effect of virus ($F_{(2,25)} = 3.373$; $p = 0.050$). Post hoc test showed that the change of body temperature was less in the excitatory group compared to both control ($p = 0.025$) and inhibitory ($p = 0.028$) groups. The areas under the curves (AUC; calculated for the total of 90 min, starting from CNO injection) values were also different between groups ($F_{(2,25)} = 4.362$; $p = 0.024$), being smaller in excitatory group (AUC = 5.507 ± 1.886) compared to the control (AUC = 10.808 ± 1.176 ; $p = 0.001$) or inhibitory (AUC = 9.809 ± 0.826 ; $p = 0.024$) groups. The 6-minute FST significantly elevated locomotion (time: $F_{(17,340)} = 3.643$; $p = 0.001$) (horizontal movement in water [35]), while it was decreased after the test ($p = 0.003$) regardless of the virus treatment (Table 1).

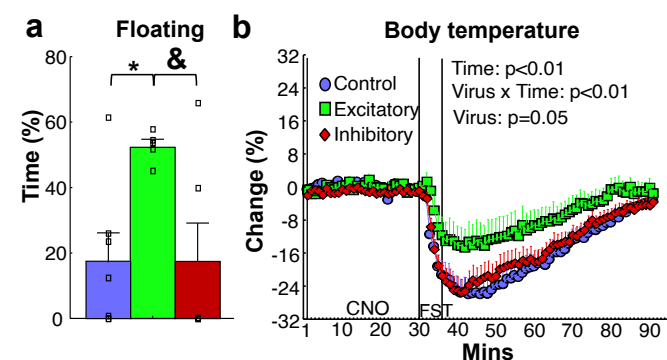


Fig. 5. Results of forced swim test (FST). (a) The excitation of the median raphe region (MRR) facilitated the time spent with floating, an indicator of depressive-like behavior (one-way ANOVA). (b) In parallel to exhibiting depressive-like behavior, the excitatory group showed a diminished decrease in body temperature during and after FST compared to both control and inhibitory groups (repeated-measures ANOVA). Data are expressed as average±SEM. Empty squares (□) represent individual values.

* $p < 0.05$ vs control; ** $p < 0.01$ vs control; & $p < 0.05$ vs inhibitory.

3.1.5. Stress hormone measurements

There were no differences between the treatment groups in ACTH or corticosterone levels 30 min after ip. CNO injection (Table 2).

3.2. Effects of CNO

3.2.1. CNO distribution

Thirty minutes following ip. 1 mg/10 mL/kg CNO injection (the same timing and dosage used for behavioral studies) resulted in unequal distribution of CNO and its metabolite, clozapine, in the body and brainstem. CNO serum concentration levels (5.055 ± 0.436 ng/mL) were significantly higher than clozapine levels (0.376 ± 0.029 ng/mL) ($t_{(8)} = 10.707$; $p < 0.001$) (Fig. 6a). Contrary to this, in the brainstem CNO levels (0.001 ± 0.0007 ng/mg tissue) were remarkably lower (but still well detectable) compared to clozapine levels (0.007 ± 0.001 ng/mg tissue) ($t_{(7)} = -3.595$; $p = 0.009$) (Fig. 6b).

3.2.2. Neurotransmitter release in hippocampus

The extracellular concentrations of measured substances varied with time, being the highest in the 3rd sample, just after the ip. CNO injection also for 5-HT ($F_{(4,20)} = 4.909$; $p = 0.006$) as well as for Glu ($F_{(4,20)} = 3.303$; $p = 0.031$) (Fig. 6c and d, Suppl. Table S5). However, Glu levels were increased only in the group containing stimulatory DREADD construct in their MRR, but not in animals treated with control virus (time×virus interaction: $F_{(4,20)} = 2.976$; $p = 0.044$). In all cases local KCl administration in sample 6. induced significant elevation of the measured neurotransmitter (in all cases $p < 0.001$ vs sample 1.), confirming the viability of the neurons even at the last timepoint of the

Table 2

Stress hormone levels after decapitation and circadian rhythmicity measured for two weeks. Degree of freedom for ACTH is (2,28) and for corticosterone is (2,29). Degree of freedom (df) for virus effect is (2,26) in case of BT change and (2,20) for locomotion parameters. F- and p-values show the effect of virus in one-way ANOVA.

Parameter	Virus			F- value	p- value
	Average hormone levels±SEM				
	Control	Excitatory	Inhibitory		
ACTH (fmol/mL)	102.991± 15.031	116.989± 25.738	133.958± 31.511	1.134	0.335
Corticosterone (pmol/mL)	239.436± 57.030	271.078± 60.406	346.958± 48.426	0.785	0.466
Average circadian rhythmicity (hours)±SEM					
BT change	23.638± 0.247	23.267± 0.029	23.477± 0.657	0.093	0.911
Locomotion change	23.015± 0.928	23.317± 0.027	23.442± 0.070	0.136	0.874

ACTH: adrenocorticotrophic hormone; BT: body temperature.

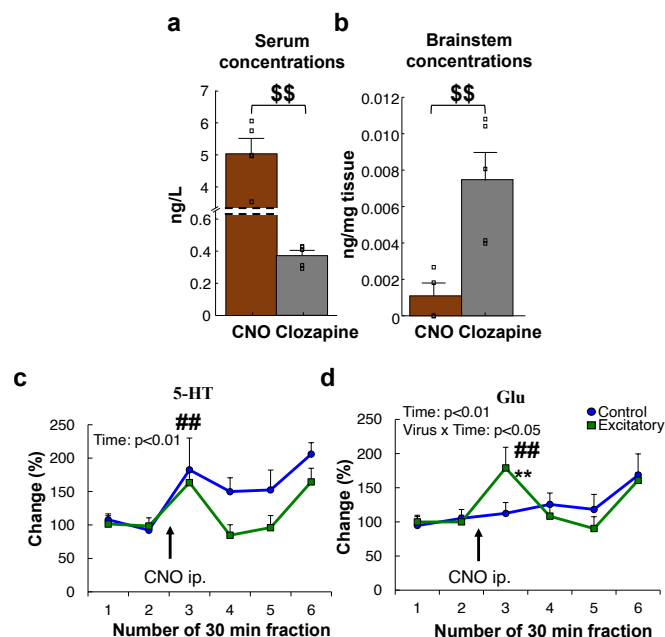


Fig. 6. Effects of clozapine-N-oxide (CNO) injection. (a) Serum and (b) brain-stem concentrations of CNO and clozapine 30 min after single intraperitoneal (ip.) CNO injection (1 mg/10 mL/kg) ($N = 5$, t -test). (a) In the serum, CNO was predominantly present, while (b) in the brainstem, clozapine concentrations were prevalent. (c) Extracellular concentrations of serotonin (5-HT) in the hippocampus of mice with control or excitatory virus in their median raphe region (MRR) after single ip. CNO injection. 5-HT concentrations increased in the third fraction, regardless of groups (repeated-measures ANOVA). (d) Extracellular concentrations of glutamate (Glu) in the hippocampus of mice with control or excitatory virus in their MRR after single ip. CNO injection. Glu concentration of the excitatory group increased significantly in the third fraction (repeated-measures ANOVA). Neurotransmitter levels are expressed as changes in percentage compared to the average of the first two fractions. Data are expressed as average $\pm$ SEM. Empty squares ($\square$) represent individual values.

$\$p < 0.01$ vs CNO concentration; $\#\#p < 0.01$ vs fraction 1; $\#\#p < 0.01$ vs control.

experiment. GABA levels were increased due to local KCl administration only in sample 6. (Suppl. Table S5).

3.2.3. Short-term effects of CNO administration and MRR manipulation

According to literature, CNO/clozapine concentrations appear and peak 15–30 min after ip. injection [26,41,42]. Thus, we investigated the widely used 30-minute incubation time regarding body temperature change and locomotion.

There was no difference in the average body temperature or locomotion counts before different tests, indicating that repeated CNO injection did not induce long-term alterations in these parameters (Suppl. Table S6). The only change we observed was the drop in body temperature right after CNO injections (effect of time: $F_{(29,2784)} = 3.062$; $p < 0.001$), which quickly normalized after a few minutes. Interestingly, this appeared only during the injection before SIT and RIT and mostly, but not exclusively controls were affected (Fig. 7a). We could not detect any group difference, either. The CNO had no immediate effect on locomotion: it did not differ before and after injection and there were no differences between the groups (Fig. 7b).

3.2.4. Long-term effects of CNO administration and MRR manipulation

Two weeks repeated behavioral tests with CNO injections did not alter the natural circadian rhythm of body temperature (Fig. 7c) and locomotion (Fig. 7d) in neither the control, nor the excitatory or inhibitory groups (Table 2).

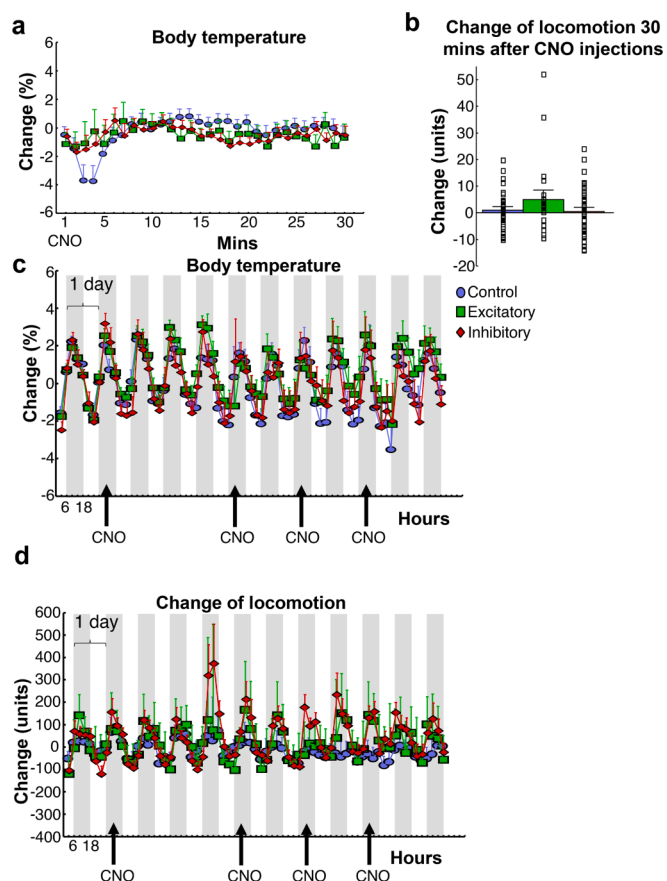


Fig. 7. Vegetative and locomotion effects of clozapine-N-oxide (CNO). (a) Body temperature changes 30 min after CNO injections (averaged data before sociability, social interaction, resident intruder and forced swim tests). The only observed difference was an acute (<5 min) drop in body temperature, most likely due to direct contact between the liquid and telemetric device (repeated-measures ANOVA). (b) Acute change in locomotion was not observed in either groups after CNO injection (one-way ANOVA). (c) Circadian rhythmicity was not altered in either groups by repeated CNO injections (Fourier transformation, one-way ANOVA). (d) There was no change in the circadian locomotion activity of groups due to repeated CNO injections (Fourier transformation, one-way ANOVA). On figure (c) and (d), the alternating day-night cycles (lights on at 18.00 h) are represented by white and grey columns, respectively. One column equals to 12 h. Data are expressed as average $\pm$ SEM. Empty squares ($\square$) represent individual values.

The 24-hour follow-up after the 5th CNO injection without any behavioral examination showed no difference between the body temperature of the control and excitatory group. There was only an effect of time ($F_{(23,161)} = 3.974$; $p < 0.001$), which shows the normal circadian changes in body temperature (data not shown). Change in locomotion ($F_{(23,138)} = 1.961$; $p = 0.009$) demonstrated a significant effect of virus $\times$ time. Post hoc tests showed that while the controls only had normal circadian decrease in locomotion, the excitatory group moved significantly less between 6 and 10 h after CNO injection ($p < 0.039$ for all time points; data not shown). Additionally, the change in locomotion was significantly higher compared to the control group ($0.01 < p < 0.1$ for all time points; data not shown).

4. Discussion

Our results covered two major topics: the effects of pharmacogenetic MRR manipulation on behavior, thermoregulation and locomotion, and the short-(<30 min) and long-term (days) effects of CNO. The main findings are summarized in [Table 3](#). In short, we found that the

Table 3
Summary of the results.

Test		Results
Aim 1.	Sociability	Effects of MRR activity
		Intact preference
		Friendly social behavior ↑ (marginal)
		E: friendly social behavior ↑
		E: depressive-like behavior ↑
	Stress hormones	E: reduced change in BT
		No change
		Circadian: no effect
		E: 24 h locomotion ↓
		Effects of CNO
Aim 2.	Aim 2/i. Distribution	Serum: CNO ↑
		Brainstem: clozapine ↑
		Increase in Glu
		Occasional acute drop in body temperature
		No effect

24 h: 24-hour effect; BT: body temperature; CNO: clozapine-N-oxide; E: excitatory group; FST: forced swim test; Glu: glutamate; MRR: median raphe region; NT: neurotransmitter; RIT: resident intruder test; SIT: social interaction test.

excitation of the MRR resulted in an increase of friendly social behavior in RIT and depressive-like behavior in FST. The depressive-like behavioral changes were accompanied by a diminished drop in body temperature. None of the social behaviors went parallel with body temperature changes. Validation of the pharmacogenetics showed that CNO could be detected 30 min after administration in both serum and brainstem. However, its serum concentration was higher, while its metabolite, clozapine, appeared more strongly in the brainstem. Nonetheless, CNO effectively stimulated extracellular glutamate release in the hippocampus in mice expressing stimulatory DREADD in their MRR, confirming the effectiveness of pharmacogenetics. Mice with control virus and injected with CNO did not show any changes in core body temperature and locomotion in a 30-minute time window or long afterwards. Moreover, the repeated CNO injection did not influence circadian rhythm.

Previously, in an optogenetic study we found that the stimulation of the whole MRR decreased aggressive behavior in mice in a RIT-like test [16]. Other researchers showed that pharmacological lesions of MRR in rats had no effect on aggressive behavior, but increased dominant behavior (e.g.: jumping or standing on the other animal) in RIT [43], which might also suggest that stimulation could decrease aggression. Nevertheless, our present results confirmed our previous findings. However, we should emphasize that MRR might influence special aspect of social behavior as there was no effect on behavior in sociability or in SIT (see Fig. 4b and Suppl. Table S3). Moreover, the observed change in social behavior was not accompanied by changes in temperature, suggesting that the effect of MRR is not influenced by its role in vegetative regulation in this case.

In contrast, depressive-like behavioral changes went parallel with changes in core body temperature. In accordance with our data (see Fig. 5a), MRR can facilitate the development of depressive-like behavior in rodents. For example, injection of 100 ng melanin-concentrating hormone into the MRR of rats increased time spent floating in FST – probably through the modulation of serotonergic neurons [13]. However, in a human study, researchers found that lesions (rather than stimulation) of the MRR due to stroke can result in depressive disorder, but these data could be confounded by stroke-induced general state [14]. Depression and stress go hand in hand, and numerous animal models of depression rely on chronic inescapable stress. It has been shown that MRR reacts to stress [8,10] and it has a role in behavioral changes in response to stressors [44]. Lesions of both dorsal raphe and MRR resulted in normal circadian rhythms of stress hormones, but decreased the amplitude and mean serum level of ACTH rhythm in rats [45]. In another study, injection of muscimol (GABA_A agonist) into the MRR increased the ACTH and corticosterone serum levels in rats, but the

effect was not mediated by 5-HT neurons [46]. However, in our study there was no difference in the serum levels of these stress related hormones after pharmacogenetic MRR manipulation in mice (see Table 2). We can assume that the selected timepoint (30 min) was not appropriate, or even prolonged manipulation (e.g. long lasting lesion and consequential redistribution of the network) is necessary for unmasking the MRR role in stress regulation. It is also possible that different cell-types affect the stress axis differently, and netto manipulation of the whole MRR leads to no overall effect.

The role of MRR in body temperature regulation has been investigated before. Some research did not show an effect [22], while others did. For example, the inhibition of the nucleus by tetrodotoxin resulted in decreased body temperature in normal (23 °C) and cold (5 °C) environment [19]. According to the authors this was due to increased heat loss and inadequate heat production [20]. Our data also support the involvement of the MRR in regulation of heat production and loss, as excitation of the MRR resulted in smaller reduction in body temperature but only in the cold environment of FST (see Fig. 5b). It has been shown that patients suffering from major depressive disorder have increased body temperature [47] and reduced cooling, which are affected by antidepressant treatment [48]. Our results suggest the involvement of MRR in connecting depression and body temperature regulation. Previously such connection was shown for the dorsal raphe and raphe pallidus [49], but – as far as we know – not for the MRR.

It is known that MRR influence locomotion. Its complete ablation resulted in hyperlocomotion [50–52], while its optogenetic stimulation caused hypolocomotion [27]. Both single optogenetic MRR [27] as well as DREADD-based [53] manipulations can have long-term – 7 days or 4 h, respectively – effect. In accordance with the literature, during a 24-hour follow-up the excitation of the nucleus resulted in hypolocomotion 6–10 h after injection. We have to emphasize that the effect of MRR manipulation on locomotion was not detectable during the behavioral tests.

The literature is controversial about the role of MRR in the circadian rhythm. The master clock is in the suprachiasmatic nucleus (SCN), which is innervated by the MRR [7,54]. However, species differences exist: in Syrian hamsters, the activation of MRR 5-HT_{1A} autoreceptors by BMY7378 resulted in a potentiated phase shift after light [18,55]. Also in hamsters, electrical stimulation of the MRR decreased light-induced c-Fos activation in the SCN [54]. However, in a comparative study, while in Syrian hamsters ip. 8-OH-DPAT (5-HT_{1A/7} agonist) injections induced phase shifts, it had no effect in C57BL/6J mice [21]. In our study we confirmed the latter finding: namely, MRR manipulation did not alter circadian rhythm in mice.

It has been shown that the CNO back-metabolizes to clozapine in rodents, too [26]. Moreover, CNO barely penetrates the blood-brain barrier [41]. Our results confirm these previous findings: although 30 min after single CNO injection CNO serum levels were higher compared to clozapine, in the brainstem tissue the opposite was true: CNO levels were significantly lower compared to clozapine (see Fig. 6a and b). Nevertheless, CNO was present in measurable concentration and – probably with the contribution of clozapine – was still able to activate DREADDs in vivo [56], confirmed also by our microdialysis experiments (see Fig. 6d). It is known that the MRR sends predominantly glutamatergic [17,57–60], but also serotonergic [61–63] and GABAergic [17] projections to the hippocampus. Thus, it is in line with the literature that both 5-HT and Glu elevations were detectable in the hippocampus after MRR stimulation. However, only Glu showed group specific effect. Indeed, around 8% of our virus infected, and thereby manipulated MRR cells were glutamatergic in contrast to the 1–2% serotonergic cells. Moreover, clozapine may act on the serotonergic system even in the absence of DREADD, thus, non-MRR originating 5-HT release might have masked group differences. Although more than 50% of RFP positive cells were GABAergic, it is highly possible that the majority of them are local interneurons and only a small population sends long projections to other brain areas, which remain undetected in our study.

Clozapine itself can induce hypothermia reaching the maximum at 1 h after injection [64]. We occasionally saw radical drop in core body temperature few minutes after injections regardless of groups (see Fig. 7a), but this normalized quickly. Thus, we believe this was neither MRR or CNO specific reaction, nor a peripheral thermoregulatory effect, but the consequence of direct contact between the injected cold liquid and the telemetric transmitter. Among control conditions CNO had no immediate effect on locomotion (see Fig. 7b). Moreover, we found no change in the circadian rhythm even after repeated CNO treatment neither in the case of body temperature, nor locomotion (Fig. 7c and d).

Previous studies showed that although CNO (and clozapine) have numerous molecular off-targets in the brain, behavioral changes only occur after injection of high concentrations (>10 mg/kg) even among within-subject control experimental designs [26,41,42,56]. At the same time, low concentrations (0.5 mg/kg) are already enough to activate DREADDs in the brain. Nevertheless, in order to avoid the possible confounding CNO influence on behavior, it is necessary to have CNO-injected controls. Thus, even if CNO injection influenced the overall behavior of the animals, it would appear in both control and manipulated (excitatory and/or inhibitory) groups. In our experiments we used a control group that differed from the others only by expressing a viral vector with no DREADD encoded in it, and were injected with CNO, as advised by the literature [24,41,65–67].

The use of pharmacogenetics and telemetry brought numerous limitations to our work. First, carefully planned timing and control groups were needed, considering the pharmacokinetic and pharmacodynamic properties of CNO [24,42,56,68]. Moreover, the stimulatory and inhibitory DREADD manipulate different signaling pathways (G_q versus G_i), which could lead to imbalance between the observed effectiveness of stimulation and ineffectiveness of inhibition. An alternative explanation is that activation of a relatively small number cells (as in case of MRR) may cause observable effects while inhibition of a small number may not. Second, due to technical reasons telemetric data can vary in great length, and thus, attentive data handling was necessary (e.g.: the use of relative change instead of the raw data).

5. Conclusion

Our results confirm previous findings on the behavioral effect of MRR. Namely, stimulation of MRR reduces locomotion, promotes friendly behavior and increases depressive-like behavior. However, we would like to emphasize that this is not generalized to all social interactions and goes parallel with vegetative changes only in case of depressive-like behavior. The latter was also found in human patients [47,48], thus, our results can have translational values: among other raphe nuclei [49], MRR also plays a role in changes in body temperature regulation during depressive disorders. However, whether this is linked to the MRR 5-HT neurons is still a question. Despite low penetration CNO might act centrally but does not influence several examined basic parameters. Thus, we believe that pharmacogenetics is suitable for repeated behavioral testing, during which animals receive injections multiple times.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Declaration of competing interest

We declare that there is no conflict of interest in the conduction and reporting of research on the behalf of the authors. The agencies had no further role in study design, in the collection, analysis, or interpretation of the data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lfs.2021.120037>.

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