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Median raphe region GABAergic neurons contribute to social interest in mouse

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ABSTRACT

Gamma-aminobutyric acid (GABA) is a well-known inhibitory neurotransmitter implicated in numerous physiological and pathological behaviors including social interest. Dysregulation of the median raphe region (MRR), a main serotoninergic nucleus, is also characterized by increased social problems. As the majority of MRR cells are GABAergic, we aimed to reveal the social role of these cells.

Chemogenetic techniques were used in vesicular GABA transporter Cre mice and with the help of adeno-associated virus vectors artificial receptors (DREADDs, stimulatory, inhibitory or control, containing only a fluorophore) were expressed in MRR GABAergic cells confirmed by immunohistochemistry. Four weeks after viral injection a behavioral test battery (sociability; social interaction; resident-intruder) was conducted. The artificial ligand (clozapine-N-oxide, 1 mg/10 ml/kg) was administrated 30 min before the tests. As possible confounding factors, locomotion (open field/OF), anxiety-like behavior (elevated plus maze/EPM), and short-term memory (Y-maze) were also evaluated.

Stimulation of the GABAergic cells in MRR had no effect on locomotion or working and social memory; however, it increased social interest during sociability and social interaction but not in resident-intruder tests. Accordingly, c-Fos elevation in MRR-GABAergic cells was detected after sociability, but not resident-intruder tests. In the EPM test, the inhibitory group entered into the open arms later, suggesting an anxiogenic-like tendency.

We confirmed the role of MRR-GABAergic cells in promoting social interest. However, different subpopulations (e.g. long vs short projecting, various neuropeptide containing) might have divergent roles, which might remain hidden and requires further studies.

1. Introduction

Understanding the neurobiology of social behavior has been a key to comprehend mental disorders, as most of them are related to its dysregulation [1]. Among many other brain areas, the median raphe region (MRR) is also implicated in social behavior [2,3]. Studies dating back to the 70's suggested that MRR is involved in controlling aggression [4,5], which was later confirmed by optogenetic [2] and chemogenetic [3] experiments in mice. These studies were concentrating on serotonin, as raphe nuclei are known as the main source of this neurotransmitter in the brain. Manipulating the serotoninergic system is beneficial in various psychiatric disorders [6] suggesting that neurochemical imbalances in MRR might be associated with mental disorders such as depression [7,8] and schizophrenia [6]. Indeed, MRR innervates the medial prefrontal cortex (mPFC) [9], and the dysfunction of this brain area is associated with the development of different mental illnesses [10]. However, MRR is also involved in regulation of fear behavior [11] as well as memory consolidation [12].

We have to emphasize that the serotoninergic neurons make up only 5–6% of the total neuronal population of the MRR, whereas the gamma-amino-butyric acid (GABAergic) containing ones represent at least 61% [13]. GABAergic cells can be interneurons or may have long range

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projections [14]. However, it is still unknown if MRR-GABAergic neurons act only locally, or project directly to remote brain areas.

Nevertheless, on other brain areas GABA is widely known for its role in the regulation of social behavior, such as aggressive, sexual and parenting behavior in the medial amygdala [15–17], social withdrawal in the prefrontal cortex and basolateral amygdala [18], and social play in the lateral septum [19]. Yet, despite the large predominance of GABAergic neurons in the MRR, their role is not yet understood in divergent behaviors including social behavior. Due to its small molecular size, it is not easy to detect neurons utilizing GABA as neurotransmitter [20]. Vesicular GABA transporter (VGAT) packs GABA into synaptic vesicles, and thus, it is an excellent marker of GABAergic neurons [21]. Expressing the Cre recombinase enzyme under VGAT promoter (e.g. VGAT-Cre mouse strain) enables cell-type specific manipulations of specific brain areas (e.g. MRR), therefore we used this model.

We hypothesized that GABAergic neurons of the MRR are involved in the dysfunction of social behavior [22]. Chemogenetic technique (designer receptor exclusively activated by designer drug (DREADD) and its artificial ligand, the clozapine-N-oxide (CNO)) was used in VGAT-Cre mice and a behavioral test battery was conducted to describe in details the effects of the manipulation (stimulation and inhibition) of the MRR-GABAergic cells on social behavior. As locomotion is the basis of almost all behavioral tests and anxiety and cognitive performance might strongly influence social interest, we briefly examined these aspects as well.

2. Methods

2.1. Animals

The colony of the VGAT-iRES-Cre (VGAT-Cre) mice (C57BL/6J background) were obtained from The Jackson Laboratory (USA; Stock No.: 016962), and local colonies were maintained at the Institute of Experimental Medicine, Budapest, Hungary in homozygous mating pairs. For a subsequent experiments VGAT-Cre mice were crossbred with Gt(ROSA)26Sor-CAG/LSL-ZsGreen1 (ZsGreen) mice (The Jackson Laboratory, USA; Stock No.: 007906) that express enhanced ZsGreen fluorescent protein in all GABAergic cells following Cre-mediated recombination ensuring easy detection.

Adult male mice (8–10-week-old) were housed in Macrolon cages (40 cm × 25 cm × 26 cm) under a standard 12-h light–dark cycle (21 ± 1 °C, 50–60% humidity), with food (standard mice chow, Charles River, Hungary) and tap-water available ad libitum. Animals were habituated to a reversed cycle (lights on at 19:00 h) for 2 weeks before starting the behavioral examination. Tests were conducted during the early dark phase (9:00–13:00 h). Animals were housed 2–3/cage until the start of the test battery and were isolated at the time of the first intraperitoneal (i.p.) injection with CNO (i.e. 30 min before the first test) to enhance social interest.

All tests were approved by the local committee of animal health and care (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. Stereotaxic surgery for virus injection

VGAT-Cre mice underwent stereotaxic surgery as described earlier [2,11]. First, animals were anesthetized i.p. with a ketamine-xylazine solution (0.5 ml ketamine (Medicus Partner, Hungary), 0.1 ml xylazine-hydrochloride (Medicus Partner, Hungary), 2.4 ml 0.9% saline and administered in 0.1 ml/10 g body weight concentration). Mice were placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) and adeno-associated viral vectors (20nl) were injected into the MRR (anteroposterior: −4.1 mm; mediolateral: 0.0 mm; dorsoventral: −4.6 mm from Bregma) through a glass capillary (tip diameter: 20–30 μm) at a rate of 100 nl/min by a Nanoject II precision microinjector pump (Drummond, Broomall, PA, USA). The capillary was left on the coordinates for an additional 3 min to ensure diffusion before slow extraction. Animals had 28 days to recover after surgery, which is more than enough for DREADD expression [23]. During the last two weeks of recovery the animals were habituated to reversed lighting cycle.

2.3. Viral vectors

Adeno-associated viruses (AAVs) carrying Cre-inducible (double-inverse orientation; DIO) transgenes were purchased from Addgene (Watertown, MA, USA). Three subgroups were formed based on the injected AAVs containing different DREADD sequences: control (no DREADD sequence, only red fluorophore, AAV8-hSyn::DIO-mCherry, 4.1e12 GC/ml titer, #50459), stimulatory (AAV8-hSyn::DIO-hM3Dq-mCherry, 4.0e12 GC/ml titer, #44361) or inhibitory (AAV8-hSyn::DIO-hM4Di-mCherry, 1.9e13 GC/ml titer, #44362).

2.4. Drugs

Half an hour before the start of most behavioral tests (except phase IV. of sociability, the social discrimination) VGAT-Cre mice were injected i.p. with 1 mg/kg clozapine-N-oxide (CNO, 0.1 mg/ml in 0.9% saline, Tocris Bioscience; 4936, CAS No: 34233-69-7), the artificial ligand of the designer receptors. All animals (including control vector injected) always got CNO, to control any possible side effect of this drug [24].

2.5. Behavioral testing

Fig. 1 illustrates the timeline of the test battery.
Four aspects of the behavior were measured:

1. Mobility in open field (OF), elevated plus maze (EPM) and Y-maze tests;
2. Social behavior with sociability, social interaction (SIT) and resident intruder (RIT) tests;
3. Anxiety-like behavior during OF and EPM tests;
4. Short-term memory by Y-maze and social discrimination (SDT) tests.

The experimental room was dark, only lit by 20 lx infrared light during the experiments (except for SIT and EPM). Experiments were recorded from above by ceiling-mounted camera (Samsung SNB 7000; OF, sociability, SDT, EPM and Y-maze) or from the side by hand-cameras (SIT, RIT) and analyzed later by computer-based event recorders (H77, Budapest, Hungary; Solomon Coder, Hungary, https://solomoncoder.com; EthoVision XT 15, Noldus Information Technology, Wageningen, Netherlands). The test apparatuses were cleaned with 20% Et-OH between animals except SIT and RIT (when bedding was used).

To ensure elimination of previously injected CNO, behavioral tests followed each other with 48-72 h intervals (except for SDT, where only 24 h passed, but the animals received no CNO injection in this case).

2.5.1. Sociability test

2.5.1.1. Phase I.: open field (OF). The first 5 min was for the habituation to the box. It is practically an OF test, which measures the locomotor activity and anxiety-like behavior of mice (Fig. 1c**)**. Animals were put into an empty white plastic box (40 cm × 36 cm × 15 cm) without bedding for 15 min. Four empty white boxes were placed in 2 × 2 position, and four experimental animals were tested simultaneously. Recordings were analyzed automatically by Ethovision XT 15 software. 'Distance moved' was measured as a parameter of mobility. The arena was virtually divided into two compartments: periphery and centrum (75% of the arena). The time spent in each compartment and number of

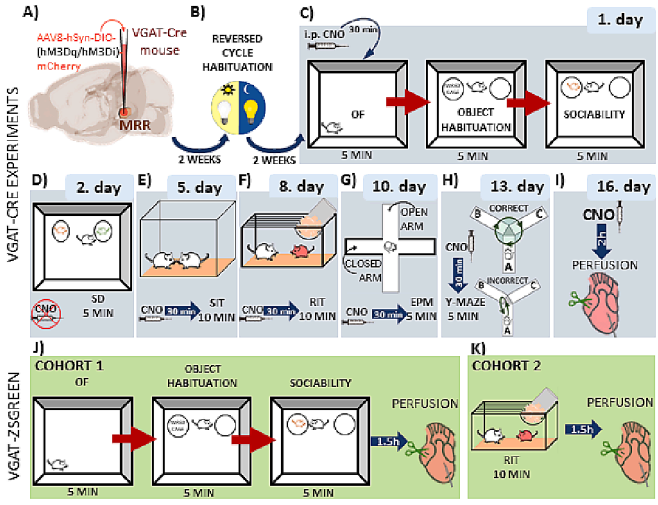

Fig. 1. Timeline of the behavioral test battery. A–I: Procedures with VGAT-Cre mice. Thirty minutes before all experiments (except social discrimination /SD/ test) animals were injected intraperitoneal with clozapine-N-oxide (CNO, 1 mg/10 ml/kg diluted in saline). A) Control, stimulatory or inhibitory adeno-associated viral vectors (20 nl) were microinjected into the MRR (anteroposterior: −4.1 mm; mediolateral: 0 mm; dorsoventral: −4.6 mm from Bregma). For details see Methods section. B) Animals had 14 days to recover after surgery and an additional 14 days to habituate to reversed light-dark cycle. This 28-day incubation time is also enough for DREADD expression. C) On the first test day, 5 min open field (OF), 5 min object habituation, then 5 min sociability test was conducted. D) On Day 2, SD test was performed for 5 min to measure social memory. E) On Day 5, social behavior was measured by social interaction test (SIT) for 10 min. F) Resident-intruder test (RIT) was performed for 10 min to investigate aggressive behavior on the 8th day. G) On Day 10, to study anxiety-like behavior, elevated plus maze (EPM) test was used for 5 min. H) On day 13, Y-maze test was conducted for 5 min to measure working memory. I) On the 16th experimental day, animals were transcardially perfused 2 h after CNO injection.
J–K: Procedure with VGAT-Cre-ZsGreen mice. J) In the first cohort, 5 min OF, 5 min object habituation, then 5 min sociability test was conducted. 1.5 h after the end of the sociability test, animals were transcardially perfused. K) In the second cohort, RIT was performed for 10 min. 1.5 h after the end of RIT, animals were transcardially perfused. For detailed description of the behavioral tests, see Methods section.

entries into the centrum (frequency) was measured, reflecting anxiety-like behavior, because rodents tend to avoid open areas even in natural environments.

2.5.1.2. Phase II.: side preference. During the second 5 min of the sociability test, two small identical wired cages (with identical weights on them in order not to be moved by the animals) were placed into the white plastic boxes for object habituation (Fig. 1c). Based on their location they were either labelled as 'left' or 'right' and the frequency and time the test mice spent investigating the two wired cages were measured to analyze object and place preference.

2.5.1.3. Phase III.: sociability. The third 5 min was the sociability phase, which measures the incline of the animals to behave socially (Fig. 1c). During this phase an unknown, smaller, juvenile male conspecific was placed under one of the wired cages. Animals could not contact physically, but were able to see, smell and hear each other. Frequency and time spent with conspecific under the wired cage vs. empty wired cage were measured to reflect social interest. Any other type of behavior was labelled as 'other'. Social preference index (SI) was calculated based on the following equation:

$$SI = \frac{t_{mouse}}{t_{mouse} + t_{cage}} \times 100,$$

where t_{mouse} stands for the time spent with sniffing the cage containing the juvenile mouse during sociability; t_{cage} stands for the time spent with sniffing the empty cage during sociability. SI ≤ 50% was considered as inappropriate social interest.

2.5.1.4. Phase IV.: social discrimination test (SDT). Twenty-four hours after the sociability test an SDT was conducted (Fig. 1d). SDT is based on the innate preference for novelty of mice. The experimental setting was similar to that of sociability: in an empty white plastic box two wired cages with weights on were placed. Under each of them one conspecific was placed. One was the same as during sociability (called juvenile 1, 'J1' mouse), thus, J1 is already known for experimental mice. The other one was an unknown (called 'J2') mouse. The position of the J1 mouse was interchanged compared to previous day to avoid place preference. The experimental animal is expected to spend more time with an unknown conspecific than with an already familiar one. The experimental animal could freely behave for 5 min. Before this test, animals did not get CNO injection. Thus, the influence on memory consolidation and not on recall was tested.

The frequency and time spent with each conspecific were measured. Any other type of behavior was labelled as 'other'. Social discrimination index (SD) was calculated based on this equation:

$$SD = \frac{t_{J1'} - t_{J2'}}{t_{J1'} + t_{J2'}} \times 100,$$

where $t_{J1'}$ stands for the time spent with sniffing the cage containing the familiar stimulus mouse during SDT; $t_{J2'}$ stands for the time spent with sniffing the cage containing the unfamiliar stimulus mouse during SDT. A value higher than 0 reflects intact social memory.

2.5.2. Social interaction (SIT)

SIT is used to investigate the social behavior influenced by anxiety, therefore the test was conducted in bright light (120 lx). As rodents are nocturnal animals, an arena illuminated by white light can induce anxiety-like behavior in them.

The day before the test animals were put into a transparent Plexiglass aquarium (35 cm× 20 cm × 25 cm) in the experimental room with bedding on the bottom one by one for 2×15 min, 4 h between the two session, for habituation. During the test two animals from the same group (e.g.: control vs control, inhibitory vs inhibitory) with similar weights were put together into the aquarium (Fig. 1e). The similar body weight was sought for the pursuit of hierarchy equality to minimize the appearance of unilateral territorial aggressive behavior. Mice could freely behave for 10 min.

Friendly social (e.g.: sniffing), aggressive (e.g.: biting, aggressive dominance) and defensive behaviors were analyzed (frequencies and time spent) for both test animals. Any other type of behavior was labelled as 'other'. Friendly Social index (FI) was calculated based on the following equation:

$$FI = \frac{t_{social}}{t_{social} + t_{aggressive}} \times 100,$$

where a value higher than 50% represents friendly rather than aggressive phenotype.

2.5.3. Resident intruder (RIT)

RIT measures the aggressive behavior of rodents while protecting their own territory. An unfamiliar, smaller, but adult conspecific was put into the home cage of the test animals (Fig. 1f).

For this experiment, the test mice (resident) were isolated in a cage for a week. During this period the home cages were not cleaned as olfactory cues help to determine the territoriality of the animal [25]. The test started when one stimulus mouse (intruder) was introduced into the cage of the test mice. We prioritized younger and smaller stimulus mice, as they tend to be submissive to older/bigger mice. Our intent with this

was to observe only the aggression coming from the dominance of the test mice. Animals could freely behave for 10 min. Only the behavior of the test mice (resident) was analyzed with the same parameters as in the SIT.

2.5.4. Elevated-plus maze (EPM)

EPM is designed to assess the anxiety-like behavior of rodents. Animals were placed onto the middle, central zone of the EPM apparatus (67 cm × 7 cm × 30 cm). EPM has two open and two arms enclosed by a wall (Fig. 1g). Mice prefer to stay in the closed arms, feeling safer there, but animals are driven by natural curiosity to explore unknown territory, therefore a healthy animal often venture out into the open arms. Mice could freely explore the apparatus for 5 min. The test was conducted in bright light (120 lx).

The number of open and closed arm entries, and time spent in each compartment were measured. Open arm entries independent from mobility were calculated based on this equation:

$$\frac{open}{total}(\%) = \frac{number\ of\ open\ arm\ entries}{sum\ of\ open + closed\ arm\ entries}.$$

The frequency of risk assessment behavior (such as head dipping, stretched attend posture [SAP] and rearing) and grooming were also analyzed.

2.5.5. Y-maze

The Y-maze is a test used to detect the ability of short-term, working memory of rodents, reflected by spontaneous alternation. The Y-maze apparatus consists of three arms (A, B and C; 25 cm × 5 cm × 21 cm) at 120°, connected by a central zone (Fig. 1h). Consecutive arm entries reflect intact short-term memory of the animals. Mice were placed at the end of arm 'A' and were allowed to explore the maze freely for 5 min.

Spontaneous alternation was calculated based on this equation:

$$Spontaneous\ alternation = \frac{'correct'\ alternation}{sum\ of\ all\ arm\ entries - 2} \times 100.$$

'Correct' alternation means entry into all three arms on consecutive choices (i.e. ABC, BCA, or CAB, but not CAC, BAB, or ABA) [26].

2.6. ZsGreen mice

Sociability (Fig. 1j) and resident-intruder tests (Fig. 1k) were performed on ZsGreen mice in two separate cohorts (see the previous section for a detailed description of the tests). Animals in both sociability test (control: $n = 7$, SIT: $n = 9$) and RIT (control: $n = 5$, RIT: n = 7) cohorts were transcardially perfused 90 min after the end of the behavioral test for c-Fos immunohistochemistry, which indicates neuronal activity. Control animals in these cohorts were not put into any experiments.

2.7. Immunohistochemistry and microscopy

2.7.1. Tissue processing

At the end of the test battery, 2 h after a single CNO injection, VGAT-Cre animals were anesthetized i.p. with ketamine-xylazine solution and transcardially perfused with phosphate-buffered saline (PBS) followed by paraformaldehyde (PFA; 4% in PBS) (Fig. 1i). ZsGreen mice were anesthetized 1.5 h after sociability test or RIT and perfused in the same way (Fig. 1j,k). Brains were removed and postfixed in 4% PFA in PBS for 24 h at 4 °C. For cryoprotection, brains were immersed in 30% sucrose-azide-PBS solution for 24 h. Thirty μm thick coronal sections were cut with sliding microtome and stored in a cryoprotectant solution (containing 20% glycerine and 30% ethylene glycol) at −20 °C until immunostaining. Immunohistochemistry staining was done to validate the virus expression and cell specificity, and to investigate neuronal activity changes in MRR after chemogenetic manipulation in VGAT-Cre mice, and to study neuronal activity changes after social tests in ZsGreen animals.

2.7.2. Virus extension investigation (Fig. 2a)

In case of VGAT-Cre mice appropriate virus infection and expression had to be validated. To check exactly what brain areas were infected, mCherry (fluorophore of the injected virus constructs) was stained with a primary antibody against red fluorescent protein (RFP).

Slices were washed with PBS for 3×10 min, then membranes were permeabilized by adding 0.5% Triton X-100 (TXT, Sigma-Aldrich) and 0.3% hydrogen peroxide (H_2O_2) diluted in PBS. After 2 additional PBS washing, blocking was done with 2% bovine serum albumin (BSA, Sigma-Aldrich) diluted in PBS for 1 h. Slices were then incubated in anti-RFP primary antibody solution (1:4000 rabbit anti-RFP, #600–401-379, Rockland, Limerick, PA, USA; 2% BSA; 0.1% TXT diluted in PBS) for 2 nights at 4 °C. After 3×10 min of PBS washing, they were incubated in biotin-conjugated (biotin-SP) donkey anti-rabbit secondary antibody solution (1:1000 in 2% BSA and PBS, #711–065-152, Jackson ImmunoResearch, Cambridgeshire, UK). After 2 × 10 min PBS, then 10 minutes TRIS washing slices were placed into avidin-biotin complex (ABC, 1:1000; Vector Laboratories, Burlingame, CA, US) diluted in TRIS buffer for 1 h. Peroxidase reaction was developed in the presence of diaminobenzidine tetrahydrochloride (DAB), nickel–ammonium sulphate ($NiNH_4SO_4$), and H_2O_2 dissolved in TRIS buffer. Slices were pre-incubated in the dilution of TRIS buffer, DAB (0.3 $\frac{mg}{ml}$) and 0.2%

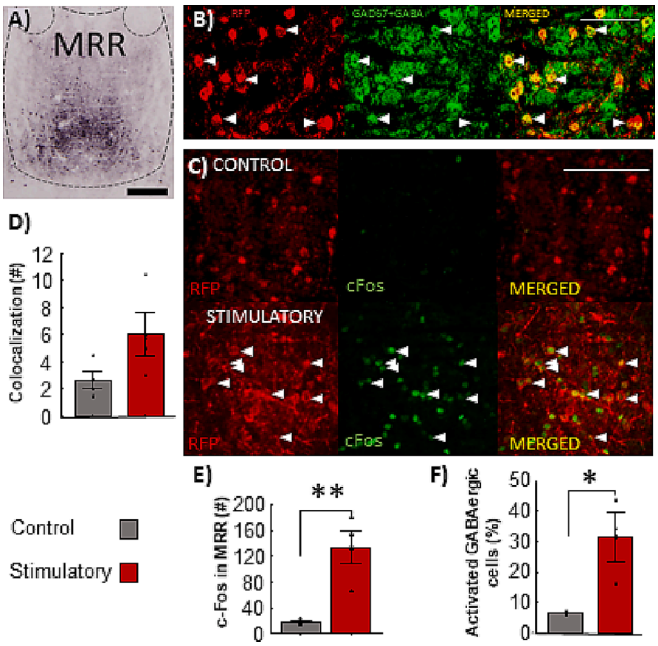

Fig. 2. Confirmation of the chemogenetic technique by immunohistochemistry A) A typical example for an accepted viral extension in the median raphe region (MRR) in VGAT-Cre mouse. Scale bar: 250 μm B) The RFP was expressed in GABAergic neuron of the MRR of the VGAT-Cre mouse. Red fluorescent protein (RFP; mCherry, the fluorophore of DREADD, red) as well as GABA (with a mixture of GABA and GAD67 antibodies, green) were labelled with double immunofluorescent staining and the last panel shows their overlap (yellow) indicated by arrows. Scale bar: 50 μm. C) Representative image of immunofluorescence labeling of RFP (red) and c-Fos (green) as well as their colocalization (yellow) 90 min after CNO injection in the MRR of VGAT-Cre mice. Representative cells are indicated by arrows. Scale bar: 100 μm D) Number of colocalizations between c-Fos and RFP positive cells in control and stimulatory DREADD containing virus injected VGAT-Cre mice. E) Number of c-Fos expressing cells in the MRR. F) Percentage of GABAergic neurons activated after CNO injection in the MRR. Statistical significance is represented by asterisks (*$p < 0.05$, **$p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$NiNH_4SO_4$ for 10 min. After adding 0.003% H_2O_2 and waiting for precipitation, slices were washed with TRIS buffer for 10 min. They were mounted with gelatine, dehydrated in xylol and covered with DPX Mountant (Sigma-Aldrich).

The RFP-stained slices were digitalized with Olympus DP70 light microscope CCD camera system (4× objective). RFP expression, though to resemble DREADD, was examined antero-posterior from −4.04 mm to −5.20 mm according to Bregma, concentrating on the centrum of the injection. In case of unlabelled neurons in the region of interest or labelling outside of the MRR animals were excluded from the analysis. Out of the 58 injected mice 31 was a correct hit (53% success rate). Finally, we compared 12 control mice with 9 stimulatory DREADD-injected and 10 inhibitory DREADD-injected animals.

2.7.3. Identification of virus-infected cells (Fig. 2b)

To validate that the virus-infected cells were GABAergic, double immunofluorescent staining was done against RFP and GABA (due to the difficulty in detecting GABA, we have also stained glutamate decarboxylase 67 (GAD67) enzyme on the same channel).

Slices were washed with PBS for 4×10 min. Then they were incubated for 2 nights at 4 °C in a solution containing anti-RFP (1:1000, host: rat; #5f8-100, ChromoTek GmbH, Planegg, Germany), anti-GAD67 (1:200, host: mouse; #MAB5406, Merck Millipore KGaA, Darmstadt, Germany), anti-GABA (1:500, host: rabbit; #A2052, Sigma-Aldrich), 0.5% BSA, 0.25% TXT and PBS. After PBS washing for 3×10 min, slices were incubated for 1.5 h in a fluorescent secondary antibody solution of anti-rat conjugated with Alexa-594 (1:200, host: goat; #ab150160, Abcam plc, Cambridge, UK), anti-mouse conjugated with Alexa-488 (1:200, host: goat; #A11029, Invitrogen, Waltham, MA, US), and anti-rabbit conjugated with Alexa-488 (1:200, host: goat; #111-545-003, Jackson ImmunoResearch, Cambridgeshire, UK) diluted in PBS. After 3×10 min PBS washing, slices were mounted with gelatine, and covered with Mowiol 4–88 (Sigma-Aldrich/Merck). The double immunofluorescent staining was imaged by C2 Confocal Laser-Scanning Microscope (Nikon CFI Plan Apo VC20X/N.A. 0.75, xy:0.62 μm/pixel, Nikon Europe, Amsterdam, The Netherlands).

2.7.4. c-Fos immunohistochemistry

2.7.4.1. VGAT-Cre mice (Fig. 2c–f). c-Fos immunohistochemistry was applied to assess possible chemogenetic manipulation-induced neuronal activity in MRR (control: $n = 4$; stimulatory: $n = 4$). After 3 × 10 min PBS washing and 30 min incubation in 10% normal goat serum (NGS; #31873, Thermo Fisher Scientific, Waltham, MA, US), fluorescent immunolabeling was used against c-Fos and RFP (1:2000 guinea-pig polyclonal anti-c-Fos IgG, #226004, Synaptic Systems with monoclonal rabbit anti-RFP IgG 1:4000, #600-401-379, Rockland) diluted in 2% NGS with 0.1% TXT in PBS overnight at 4 °C. Primary antibodies were detected by fluorescent-conjugated antibodies (1:500 Alexa-488 conjugated donkey anti-guinea-pig, #S32354, ThermoFisher Scientific, Waltham, MA, USA, and 1:500 A-594 conjugated goat anti-rabbit, #ab150160, Abcam plc, Cambridge, UK). c-Fos-RFP immunohistochemistry was imaged by C2 Confocal Laser-Scanning Microscope (Nikon CFI Plan Apo VC20X/N.A. 0.75, xy:0.62 μm/pixel, Nikon Europe, Amsterdam, The Netherlands). Quantitative analysis of the colocalizations was done with the NIS Elements software (Nikon Europe, Amsterdam, Netherlands).

2.7.4.2. ZsGreen mice (Fig. 3a–d, i–l). Possible behavioral test-induced neuronal activity changes in MRR-GABA were investigated by c-Fos immunohistochemistry. First, 3x10min PBS washing was applied. After, slices were incubated in 0.3% TXT solution in PBS for 30 min, then 2.5% BSA solution in PBS for additional 30 min. Primary antibody against c-Fos (1:1000, host: rabbit; #sc-52, Santa-Cruz Biotechnology, Dallas, TX, US) was applied in PBS solution with 2.5% BSA for 3 days at 4 °C.

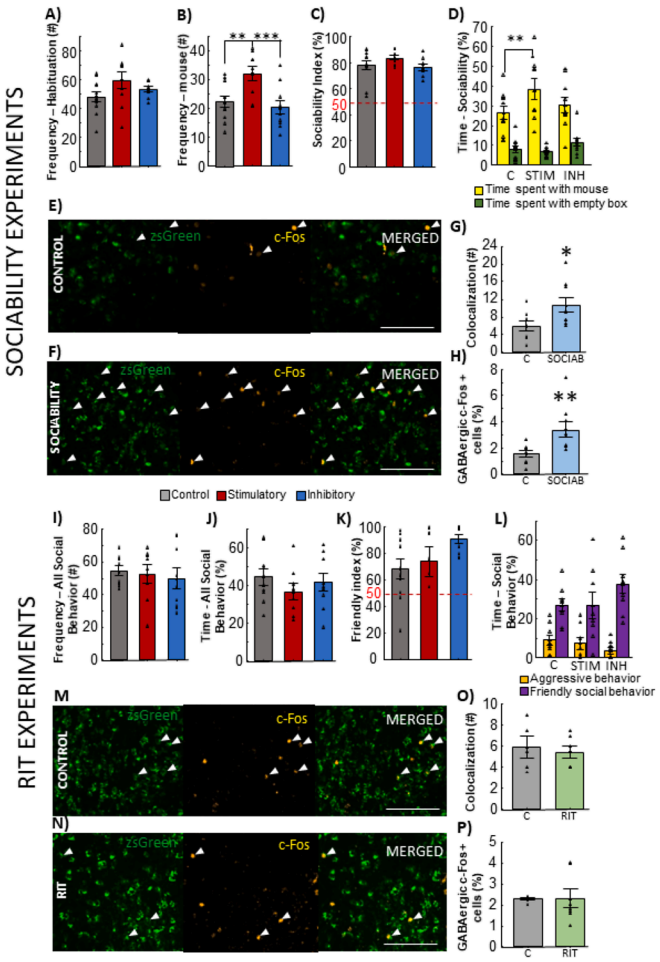

Fig. 3. Sociability and resident intruder tests. A–D: Sociability results in VGAT-Cre mice. I–L:. RIT results in VGAT-Cre mice E–H, M-P: ZsGreen (GABA) c-Fos colocalization in the median raphe region (MRR) after sociability (E–H) and resident intruder test (RIT) (M–P).
A) The frequency of the object visits (left and right together) for each group during habituation phase. B) The frequency of the visits of the stimulus mouse (J1) for each group during sociability phase. C) Sociability index during the third phase of the sociability test. All animals performed above threshold (50%), displaying intact social preference. D) Each group spent more time with the stimulus mouse than the empty box during sociability phase, however, this was significantly higher in MRR-GABA stimulated than control VGAT-Cre mice. E) Merged photos of c-Fos and ZsGreen (GABA) in the MRR in control, home cage animals. Scale bar: 100 μm F) Merged photos of c-Fos and ZsGreen (GABA) in the MRR after the sociability test. Scale bar: 100 μm G) Number of colocalized of c-Fos and ZsGreen positive cells in the MRR. H) Percentage of GABAergic neurons among c-Fos positive MRR cells after sociability test.
I) Frequency of all (friendly+aggressive) social behavior that each group displayed. J) Percentage of time spent with any kind of social behavior that each group displayed. K) Friendly behavior index. All the animals performed above threshold (50%) displaying preference for friendly behavior over aggressive. L) Time spent with friendly or aggressive social interaction during the 10 min RIT. Statistical significance is represented by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). M) Merged photos of c-Fos and ZsGreen (GABA) in the MRR in control* animals. Scale bar: 100 μm N) Merged photos of c-Fos and ZsGreen (GABA) in the MRR after the RIT. Scale bar: 100 μm O) Number of colocalizations of c-Fos and ZsGreen positive cells in the MRR. P) Percentage of GABAergic neurons among activated cells in the MRR.
* As the resident intruder and sociability tests with the ZsGreen animals were conducted on different days, we created two sets of control animals. Each set was used as baseline for each experiment.

Primary antibody was detected by A-594-conjugated secondary antibody (1:500, goat anti-rabbit, #ab150160, Abcam plc, Cambridge, UK).

Immunohistochemistry against c-Fos and ZsGreen fluorophore were imaged using a Panoramic Digital Slide Scanner (Zeiss, Plan-Apochromat 10×/NA 0.45, xy: 0.65 μm/pixel, Pannoramic MIDI II; 3DHISTECH, Budapest, Hungary) equipped with LED (Lumencor, SPECTRA X light engine). Red and green fluorescent nuclei/cells were counted with the help of NIS Elements software (Nikon Europe, Amsterdam, Netherlands).

In both VGAT-Cre and ZsGreen c-Fos investigation, 3 sagittal sections of the MRR/mice were examined to count the average number of colocalization.

2.8. Statistical analysis

For evaluation of the data StatSoft 13.4 (Tulsa, USA) software was used. Outliers were defined as data points which were not in the interval of group mean ± 2×SD for the given parameter and thus, were excluded from the statistical analysis.

For comparing groups one-way analysis of variance (ANOVA; effect of virus) was used. SI and spontaneous alteration were tested with single-sample *t*-test against 50% (difference from random choice was measured), while SD against 0% (this value or below this value represents lack of memory). The comparison of sides (left vs right), social preferences in sociability (mouse vs cage) and social discrimination (old vs new) tests were analyzed by repeated-measures ANOVA (effect of group as between factor and effect of choice as within factor) followed by Fisher LSD as a post hoc analysis. Data are expressed as mean ± SEM and $p < 0.05$ was considered statistically significant, while $0.07 < p < 0.05$ is mentioned as a marginal difference.

3. Results

3.1. Confirming the chemogenetic technique

We successfully confirmed that GABAergic cells of the MRR express the RFP suggesting that they also express the DREADD receptor (Fig. 2b).

Intraperitoneal CNO injection increased the activated (c-Fos positive) cell numbers in the MRR of VGAT-Cre animals in case the AAV contained not only the RFP (control virus), but also a stimulatory DREADD sequence ($_{F(1,6)} = 22.370$, $p = 0.003$) (Fig. 2e). Although the number of colocalized cells increased only marginally during this manipulation ($_{F(1,6)} = 3.927$, $p = 0.095$) (Fig. 2d), but this was significantly higher portion of GABAergic cells in the case of stimulation compared to control virus injected mice ($_{F(1,5)} = 13.583$, $p = 0.014$) (Fig. 2f).

The correctness of the virus injection was also confirmed and mice with correct hits were only included in the further analysis (for a representative slide see Fig. 2a).

3.2. Social behavior

For detailed analysis of social behavior our animals underwent a behavioral test battery.

3.2.1. Sociability

During the habituation phase (introduction the animals to two empty cages), there was no side preference neither in frequency ($_{F(1,27)} = 0.452$, $p = 0.507$), nor in the time spent with the objects ($_{F(1,27)} = 0.846$, $p = 0.366$) and the different treatments had no influence at all (Fig. 3a). Thus, the manipulation of MRR-GABAergic cells did not induce any object aversion/preference as well as the results of later phases of the test was nor confounded by side-preference.

Introduction of a juvenile mouse (J1) under one of the wired cages significantly increased the interest toward this cage (sniffing frequency: $_{F(1,27)} = 45.574$, $p < 0.001$). Moreover, there was a significant difference between the groups ($_{F(2,27)} = 3.564$, $p = 0.042$) and the treatment significantly influenced the mouse preference (interaction: $_{F(2,27)} = 6.905$, $p = 0.004$) (Fig. 3b, for simplicity only the frequency sniffing the mouse-containing cage was shown). Post hoc analysis showed that stimulation of MRR-GABAergic cells significantly enhanced the frequency investigating the mouse-containing cage both compared to control ($p = 0.001$) and inhibitory ($p < 0.001$) groups. The index of social interest was above 50% chance level in all studied groups, suggesting intact social preference (single sample *t*-test: Control: $_{t(11)} = 7.626$, $p < 0.001$; Stimulatory: $_{t(9)} = 18.514$, $p < 0.001$; Inhibitory: $_{t(9)} = 11.400$, $p < 0.001$), however, there were no differences between the groups (Fig. 3c).

When we analyzed the time spent with the containers the mice-containing cage was preferred above the empty cage ($_{F(1,27)} = 82.808$, $p < 0.001$) (Fig. 3d). The genotype could marginally influence this preference ($_{F(2,27)} = 2.808$, $p = 0.078$). Post hoc analysis showed that stimulation of MRR-GABAergic cells significantly enhanced the time spent with mouse-containing cage compared to controls ($p = 0.007$) with a marginal difference from the inhibitory group ($p = 0.076$).

The sociability test significantly activated the VGAT positive cells of the MRR in VGAT-Cre x ZsGreen crossbred animals represented by an increase in c-Fos colocalization in GABAergic cells ($_{F(1,14)} = 7.234$, $p = 0.017$), as well as by the increase percentage of GABAergic cells among the c-Fos positive neurons ($_{F(1,14)} = 8.170$, $p = 0.012$) (Fig. 3e–h).

Thus, stimulation of MRR-GABAergic cells promoted social interest.

3.2.2. Social interaction

During the 10 min of the anxiogenic SIT, animals initiated more friendly than aggressive encounters with each other (frequency: $_{F(1,26)} = 90.310$, $p < 0.001$) (Fig. 4a). This was reflected by higher that 50% Friendly Social Index equation in each groups (single sample *t*-test: Control: $_{t(11)} = 2.99$, $p = 0.013$; Stimulatory: $_{t(7)} = 2.820$, $p = 0.030$; Inhibitory: $_{t(10)} = 265.710$, $p < 0.001$) with a tendency of higher friendly behavior in the inhibitory group ($_{F(2,26)} = 2.827$ $p = 0.077$) (Fig. 4b). When we compared the frequency of all type of social behavior, we saw a significant difference between groups ($_{F(2,26)} = 7.443$ $p = 0.003$) (Fig. 4c). Post hoc analysis showed that stimulatory group investigated the conspecific more often than the control ($p = 0.004$) and inhibitory groups ($p = 0.001$). No difference was observed in the time spent with any studied behavior (Fig. 4d,e). These results further confirmed the role of MRR-GABAergic cells in promoting social interest.

3.2.3. Resident intruder

As expected, the mice initiated aggressive behavior more often and spent more time with aggression during this test than during the SIT (controls frequency of aggression: 3.182 ± 1.174 for SIT and 11.200 ± 1.744 for RIT; time%: 6.535 ± 3.029 for SIT and 13.200 ± 4.665 for RIT; direct statistical comparison cannot be conducted as they were done on separate days). However, there was no significant difference between groups in the investigated parameters (Fig. 3i–l, Table 1).

In line with the above mentioned results no change was observed in the number of activated MRR-GABAergic cells after RIT compared to cage controls ($_{F(1,10)} = 0.186$, $p = 0.648$) (Fig. 3m–p).

3.2.3.1. Locomotion. Manipulation of the GABAergic cells of MRR had no effect on locomotion in none of the studied tests (distance travelled in the OF, closed arm entries in EPM, total arm entries in Y-maze) (see Table 2). Thus, locomotory changes could not confound the results of other tests.

3.2.3.2. Anxiety. As anxiety may influence the social behavior, especially during the social interaction test, we analyzed this parameter in the OF and EPM tests.

In the OF (first phase of sociability), there was no difference between

FIG. 4

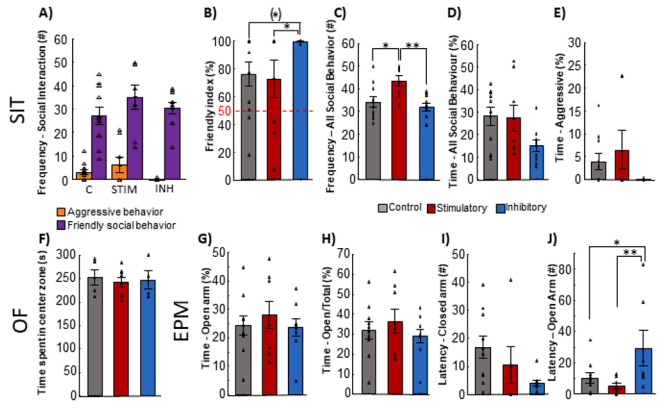

Fig. 4. Anxiety after manipulating the MRR-GABAergic cells in VGAT-Cre mice. A–E: Social interaction test (SIT) reflects both social interest as well as anxiety. F: The open field (OF) test is the first 5 min of the sociability test. Here, beside locomotion, the anxiety could be also addressed. G–J: The classical anxiety test is the elevated plus maze (EPM).

A) All groups showed more social than aggressive behavior frequency. B) Friendly behavior index. All the animals performed above threshold (50%) displaying preference for friendly behavior over aggressive one, the inhibitory group being the friendliest. C) However, in case of all number of social interaction (Friendly + Aggressive + Defensive) the stimulated group showed the highest level. D) In case of the time engaged in social behavior there were no differences between groups. E) No difference were observed between the groups in the time spent with aggressive interaction as well. F) No difference were observed between the groups in time spent in the center zone of the OF test. G) Similarly, treatment had no effect on time spent in the open arm of the EPM. H) The locomotion independent measure of anxiety, the open arm frequency (Open Arm Time / Open Arm Time + Closed arm Time *100) was also similar in each group. I) The inhibitory groups revealed marginally lower latency to entering the closed arm of the EPM, J) with significantly higher latency in entering the open arm.

Statistical significance is represented by asterisks ((*)$p < 0.07$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table 1

Locomotion in VGAT-Cre mice, injected with control, excitatory or inhibitory DREADD (designer receptor exclusively activated by designer drug) sequence containing adenoassociated virus vector into the median raphe region studied 30 min after an intraperitoneal injection of 1 mg/kg clozapine-N-oxide. During the open field test (the first 5 min of sociability) the distance travelled was analyzed by Ethovision in centimeters (cm), during the elevated plus maze test (EPM) the number of closed arm entries served as a measure of locomotion, while during the y-maze test the number of all arm entries reflected the locomotion. No significant difference was found between groups.

DREADD type	Control ($N = 12$)	Excitatory ($N = 9$)	Inhibitory ($N = 10$)	F-value	*p*-Value
Open field - distance (cm)	1979±228	2105±132	2317±268	0.556	0.580
EPM – number of closed arm entries (count)	13.7±1.1	17.2±2.0	15.5±0.8	2.649	0.091
Y-maze – number of all arm entries (count)	27.9±2	27.8±5	22.0±2.1	1.188	0.321

groups in the time spent in the central zone (Fig. 4f).

During EPM there were no major differences in the classical parameters of anxiety between the groups (Fig. 4g, h, Table 3). Our treatment significantly influenced the latency to entering the open arm ($_{F(2,22)} = 4.096$, $p = 0.030$) (Fig. 4j). Post hoc results showed that the inhibitory group had a greater latency compared to both the control ($p = 0.030$) and stimulatory group ($p = 0.010$). Additionally, the latency of the closed arm entry showed a marginal effect of the virus ($_{F(2,24)} = 2.868$, $p = 0.070$) (Fig. 4i). There was no significant difference between groups in the frequency of risk assessment behavior (head dipping, SAP, rearing) and grooming (data not shown).

All in all, MRR-GABAergic inhibition had a mild impact on anxiety.

3.2.3.3. Memory. In order to assess whether the social recognition or social memory is affected by the stimulation of the GABAergic cells, we opted to use the social discrimination test, as this test is considered the most ethologically relevant [27]. The short-term memory may influence the time sniffing the stimulus mice (e.g. if the animals do not remember, it will go back more frequently). Therefore, we also tested short-term memory in the Y-maze.

During the social discrimination phase (24 h after the CNO injection) of the sociability test all animals spent similar and enough (around 30%) time with the stimulus animals, but none of the studied groups were able to distinguish between 'J1' and 'J2' test animals (single sample *t*-test: Control: $_{t(11)} = 3.958$, $p = 0.003$; Stimulatory: $_{t(6)} = 2.687$, $p = 0.043$; Inhibitory: $_{t(9)} = 4.466$, $p = 0.002$) (Table 4; Fig. 5a–b).

In the Y-maze test all animals entered considerable time into the arms (around 25 times), which is enough the detect alternation (Table 4; Fig. 5c–d). Indeed, all animal had intact memory (single sample t-test: Control: $_{t(11)} = -0.249$, $p = 0.808$; Stimulatory: $_{t(8)} = -0.234$, $p = 0.821$; Inhibitory: $_{t(10)} = -0.304$, $p = 0.767$), but there was no significant difference between the treatment groups (Fig. 5d).

4. Discussion

Our results suggest that manipulating the GABAergic cells of the MRR by chemogenetics is a successful approach: CNO-induced c-Fos elevation and co-localization with infected cells has been found in MRR in the stimulatory group. In ZsGreen cohorts the c-Fos elevation in GABAergic cells after social cue indicates that social behavior activates these cells. Manipulation of GABAergic neurons in the MRR influenced social behavior (stimulation increased frequency of mouse investigation during sociability and SIT) with a mild effect on anxiety (latencies in EPM) without influencing locomotion (OF, EPM, y-maze) and short-term memory (SD, Y-maze). Table 5 summarizes all observed changes.

Changes of locomotion may influence the outcome of behavioral tests, leading to misinterpretations. Indeed, previous studies showed that elevated GABA levels of the brain can decrease locomotion in rats. High level of GABA in the motor cortex and cerebellum might be responsible for the decrease in coordination of the hind limbs and in the performance of learned locomotor tasks [28]. In another experiment, microinjections of GABA into the nucleus accumbens septi decreased locomotion in OF, and administration of picrotoxin ($GABA_A$ receptor antagonist) increased locomotion [29]. Furthermore, it has been described that MRR plays an important role in the regulation of locomotion proved by lesion experiments [30,31] and pharmacological manipulations [32,33] in rats. For example, microinjection of baclofen ($GABA_B$ receptor agonist) induced hyperactivity, which was restored by co-injecting 2-hydroxysaclofen (a $GABA_B$ receptor antagonist) [32]. However, the abovementioned studies manipulated the GABA receptors, which might be expressed by numerous neuron types. We were focusing more on GABA producing cells and manipulated their activity on a small brain area, in the MRR. Our chemogenetic manipulation might affect the GABA signaling even on remote brain areas. Thus, conceptual differences might explain the discrepant results. Nevertheless, we confirmed that locomotor changes do not confound our interpretation on social behavior.

Our main focus was on social behavior as it is a fundamental property of daily interactions, and serves the basis of survival and reproduction [34]. During the habituation of the sociability test, we could not find difference between the studied groups, suggesting that activation or

Table 2
Resident Intruder (RI) test after manipulating GABAergic cells in the median raphe region. No significant differences were detected. Degree of freedom (df) for the one-way ANOVA was (2,24). Data are expressed in mean±SEM.

DREADD type		Control (N = 10)	Excitatory (*N* = 8)	Inhibitory (N = 9)	F-value	p-Value
Frequency (#)	Aggressive behavior	11.2 ± 1.7	8.8 ± 2.3	5.5 ± 2.2	1.897	0.171
	Social behavior	45.5 ± 4.4	41.5 ± 7.3	44.1 ± 5.0	0.130	0.878
	Social sum	57.4 ± 3.8	52.6 ± 5.8	49.8 ± 6.4	0.531	0.594
Time (%)	Aggressive behavior	13.2 ± 4.6	7.3 ± 3.0	3.6 ± 1.4	2.012	0.155
	Social behavior	30.7 ± 4.6	26.7 ± 6.8	37.6 ± 4.8	0.988	0.386
	Social sum	44.4 ± 4.2	36.6 ± 4.5	41.4 ± 4.7	0.729	0.492
Friendly social index		71.5 ± 7.4	74.1 ± 11.3	90.8 ± 3.5	1.835	0.181

Table 3
Elevated plus-maze (EPM) test after manipulating GABAergic cells in the median raphe region. All the values are based upon frequency. No significant differences were detected. Degree of freedom (df) for the one-way ANOVA was (2,23). Data are expressed in mean±SEM. SAP: stretched attend posture.

DREADD type		Control (N = 10)	Excitatory (N = 8)	Inhibitory (N = 8)	F-value	p-Value
Open/total		32.0±4.3	36.5±6.1	29.1±3.5	0.601	0.556
	Head dipping	16.3±2.4	15.5±1.5	11.1±2	2.900	0.235
Risk assessment frequency	SAP	61.7±7.9	48.3±6.2	37.6±1.6	2.900	0.235
	Rearing	18.8±1.9	22.3±2	20.0±2.5	2.340	0.310
Grooming		1.1±0.2	0.1±0.1	1.0±0.3	3.147	0.207

Table 4
Memory parameters after manipulating GABAergic cells of the median raphe region. The last row contains the results of the y-maze test, while all other rows represent the values of the social discrimination tests. No significant differences were detected. Degree of freedom (df) for one-way ANOVA was (2,26). Degree of freedom for the repeated-measures ANOVA was (1,26) for the effect of choice, while (2,26) for the effect of virus and virus×choice interaction. Data are expressed in mean±SEM.

DREADD type		Control (N = 12)	Excitatory (N = 9)	Inhibitory (N = 10)	F-values	p-Value
Frequency	'Old' mouse	24.5 ± 2.4	26.5 ± 2.6	23.6 ± 1.7	Virus:	0.265
	'New' mouse	23.1 ± 1.4	25.7 ± 2.4	21.2 ± 0.9	1.396	
					Choice:	0.311
					1.065	
					Virus×choice:	0.900
					0.1051	
	'Other' behavior	47.9 ± 2.8	51.1 ± 3.4	43.9 ± 2.5	1.411	0.261
Time (%)	'Old' mouse	14 ± 2.4	15.5 ± 2.7	19.4 ± 2.7	Virus:	0.113
	'New' mouse	12.1 ± 1.2	14.7 ± 2.6	17.4 ± 1.5	2.376	
					Choice:	0.316
					1.045	
					Virus×choice:	0.946
					0.055	
	'Other' behavior	73.8 ± 3.4	69.6 ± 4.5	63.1 ± 3.1	2.376	0.112
Discrimination index		49.1 ± 3.4	48.7 ± 5.1	48.8 ± 3.9	0.002	0.997
Y maze – spontaneous alteration		63.0±3.3	62.9±6.2	62±2.6	0.020	0.980

inhibition of MRR-GABA neurons did not result in object preference or avoidance. However, stimulation of these cells enhanced social interest both during the sociability as well as during the SIT tests. The imbalance between the effect of stimulation and ineffectiveness of inhibition might be explained by the fact that stimulatory DREADD activates the Gq-phospholipase C pathway, while the inhibitory DREADD acts through the inhibition of the adenylate cyclase [24]. The fact that sociability test activated MRR-GABA neurons further supports their involvement in social behavior. Our results relate with the previously described study by Sustkova-Fiserova et al. [35], where animals with high levels of GABA were more social. However, they were primarily targeting GABAergic neurons in the medial prefrontal cortex (mPFC). We can hypothesize that GABAergic neurons from the MRR have long projections to the mPFC as previous results confirmed a connection between these two areas [9]. We have to admit that most of the projecting MRR cells were glutamatergic and some serotoninergic. As the neurochemical identity of about 14% of the projections remained unknown, we might assume the existence of long projecting GABA innervation from the MRR to mPFC.

We did not observe any changes in aggressive behavior neither during SIT or RIT. As reported in a detailed review, the findings in the literature about the relationship between GABA and aggressive behavior are divergent [36]. The classic hypothesis suggested that there is a negative correlation between the neurotransmitter and aggressive behavior, but it has also shown that the usage of positive allosteric modulations of GABA (alcohol, benzodiazepines and neurosteroid allopregnanolone) can increase the occurrence of aggressive behavior [37]. These changes in behavior might be due to the role of GABA in the regulation of serotonin levels in the dorsal raphe nuclei [38,39]. However, similarly to our experiment, the GABA receptors in the MRR did not trigger any escalating effect on aggressive behavior [36]. This can be credited due to the different projections of the two raphe nuclei [40].

Although GABA is largely known for its sedative, anxiolytic and muscle relaxing effects [41–44], we failed to observe these during our experiments. The only difference was the increased latency to enter in the open arm in the inhibitory group. Beside low number of visits and short time spent in the open arm this might also reflect anxious phenotype [45], supporting a mild contribution of MRR-GABAergic neurons to the general anxiolytic effect of GABA.

GABAergic neurons in general have huge impact on learning and memory formation [46–48]. Due to its projections to the hippocampus, the MRR is also associated with the regulation of memory acquisition and consolidation [12,49], however, most studies focused on fear memories [11,50]. We did not observe any effect on short term social

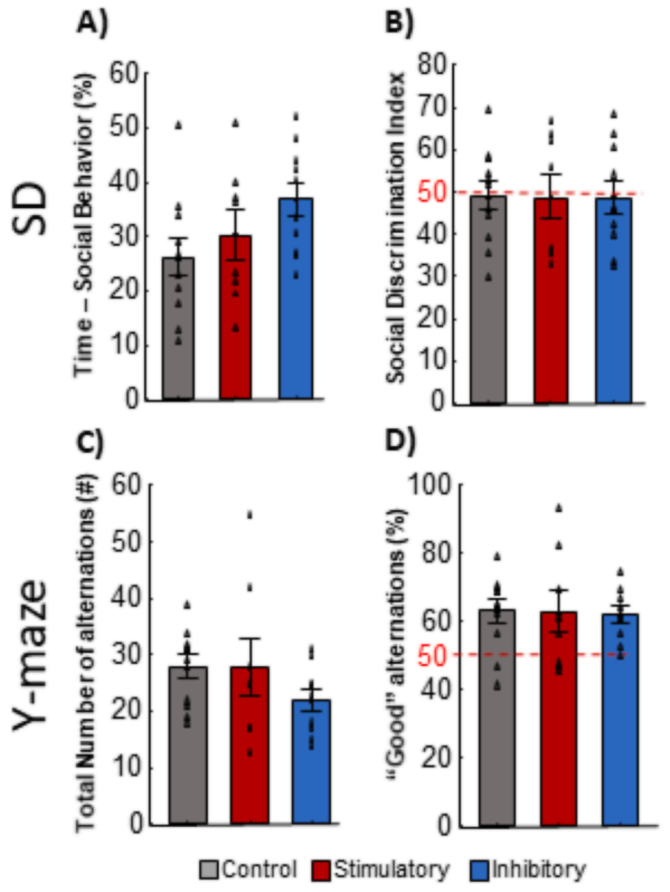

Fig. 5. Short term memory after manipulating the MRR-GABAergic cells in VGAT-Cre mice. A–B: Social discrimination (SD). C–D: Y-maze.
A) Percentage of time each group spent with both stimuli mice. B) Social discrimination index. All the animals performed below threshold (50%), displaying no preference for a new mouse without group differences. C) Total number of alternations on Y-maze reflecting locomotion. D) Total number of "good" alternations on Y-maze. All group had intact short-term memory in this test.

Table 5
Summary of the results.

Function	Test	Excitatory	Inhibitory
Locomotion	OF	Ø	Ø
	EPM	Ø	Ø
	Y maze	Ø	Ø
Social behavior	Sociability	↑ Frequency	Ø
	Social interaction	↑Frequency	Ø
	Resident intruder	Ø	Ø
Anxiety	EPM	Ø	(Anxiogen)
Memory	Social discrimination at 24 h	Ø	Ø
	Y maze	Ø	Ø

Ø no difference compared to control. Abbreviations: OF: open field, EPM: elevated plus maze.

and working memory after manipulating MRR-GABAergic cells. Controversially enough none of the groups had intact social memory. Might be that the 5 min "sampling" time during the sociability test was not enough for successful memory acquisition or 24 h was too long time to remember, however, several previous studies used even shorter (4 min) interaction (although mostly direct social investigation) and 24 h retention interval [51]. Strain differences as an explanation seems to be more plausible. Nevertheless, our treatment did not influence this parameter excluding a major role of MRR-GABA cells in social memory. Problems in short term memory might also influence the outcome of social tests (e.g. the animals do not remember the conspecific, therefore a constant interest is remained and they will appear as social), but we closed out the short-term memory influencing effect of MRR-GABAergic manipulation. Thus, memory disturbances are unlikely to alter the outcome of the social behavioral tests.

These results might further extend our understanding regarding the role of GABAergic neurons in fine-tuning the social behavior. This question has high translational value, as many human medications target GABA receptors for treatment of e.g. psychiatric disorders [52–54]. As MRR-GABA cells form a large, heterogenous neuron population [55], we might assume that different subgroups might have different roles. Neurochemical characterization of the subgroups (e.g., neuropeptide content) as well as elucidation their divergent innervation and projection patterns is still required to further understand all the functions of the MRR-GABA cells.

CRediT authorship contribution statement

TC and DZ conceptualized the work, BT, ES and DZ made the viral injections, TC, CsLF, PC and DE conducted the behavioral examinations, TC, BT, PC, DV, AH, DE analyzed the behavior, TC, BT, CsLF, PC, DV, AH and DE participated in immunohistochemical studies. TC and DZ wrote the original draft, while all other authors contributed to the review and editing process. TC, BT and DZ prepared the visualization, while DZ conducted the funding acquisition.

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.

Data availability

Data will be made available on request.

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