REGULATORY EFFECTS OF MEDIAN RAPHE GABAERGIC AND DOPAMINERGIC NEURONS ON SOCIAL BEHAVIOR AND REINFORCEMENT- BASED LEARNING

PhD Thesis in Neuroscience

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Introduction

Mental illnesses, including anxiety disorders and depression, significantly impact global well-being. Their complex neurobiological bases, often involving dysregulation of serotonergic (5-HT), GABAergic, and dopaminergic (DA) systems, have made therapeutic advancements challenging. These neurotransmitter systems are fundamental to mood, anxiety, reward, and cognition.

The Median Raphe Region (MRR), a brainstem nucleus, is a key modulator of these functions. While historically known for its serotonergic neurons, the MRR is neurochemically diverse, also containing substantial GABAergic and glutamatergic populations, alongside a less-characterized dopaminergic (DAT-expressing) contingent. This heterogeneity suggests a multifaceted role for the MRR beyond its classical serotonergic actions.

Recent conceptualizations, such as the MRR functioning as a 'subcortical switchboard' orchestrating behavioral states, highlight the coordinated action of its varied neuronal types. However, the specific contributions of non-serotonergic MRR populations, particularly GABAergic and dopaminergic neurons, to complex behaviors remain insufficiently understood, as previous research often employed non-specific manipulations.

This PhD research aimed to dissect the distinct roles of MRR GABAergic and MRR dopaminergic neurons. Utilizing advanced chemogenetic techniques (DREADDs: hM3Dq for excitation, hM4Di for inhibition, activated by CNO) combined with Cre-loxP genetic strategies in transgenic mice (VGAT-Cre for GABAergic neurons, DAT-Cre for dopaminergic neurons), this work investigated the causal links between the activity of these defined MRR neuronal populations and their impact on reinforcement-based learning, social interaction, anxiety-related behaviors, and memory.

Objectives

The main objectives of this PhD research were structured into three distinct projects:

Project I: To determine the role of MRR GABAergic neurons in reinforcement-based learning, using both nonspecific whole-MRR manipulation and targeted GABAergic neuron manipulation to assess learning, reversal learning, and impulsivity.

Project II: To investigate the influence of MRR GABAergic neurons on social and emotional behaviors, through targeted chemogenetic manipulation, comprehensive behavioral testing (social behavior, locomotion, anxiety, memory), and examination of neuronal activation (c-Fos) patterns following social tasks.

Project III: To characterize the presence and investigate the functional role of DAT-expressing (dopaminergic) neurons within the MRR, by confirming their localization, performing targeted chemogenetic manipulation, and conducting behavioral testing analogous to Project II.

This multi-project approach aimed to elucidate the functional contributions of these distinct MRR neuronal populations.

Methods

All experimental procedures were approved by relevant Hungarian ethics committees and complied with EU and ARRIVE guidelines.

Animals: Adult male C57BL/6J, VGAT-Cre (JAX#016962), and DAT-Cre (JAX#006660) mice were used. For some experiments, VGAT-Cre mice were crossed with ZsGreen reporter mice (JAX#007906). Animals were group-housed (unless specified for single housing post-manipulation) under standard or reversed 12-hour light/dark cycles with ad libitum food and water. Behavioral testing occurred during the early dark (active) phase.

Stereotaxic Surgery and Viral Vector Delivery: Mice were anesthetized (ketamine/xylazine) and received stereotaxic injections of AAV2 (nonspecific: AAV2-hSyn-hM3Dq-mCherry, Addgene#50474, 10 nl) or AAV8 (Cre-dependent, 20 nl) vectors encoding hM3Dq-mCherry (stimulatory, Addgene#44361), hM4Di-mCherry (inhibitory, Addgene#44362), or mCherry (control, Addgene#50459) into the MRR (AP: -4.1, ML: 0, DV: -4.6 mm from bregma). Animals recovered for 4 weeks. Injection accuracy was histologically verified.

Drug Administration: Clozapine-N-oxide (CNO; 1 mg/kg, i.p.) was administered 30 minutes before behavioral tests (except SDT in Project II & III, which was CNO-free). Control animals also received CNO (or saline in one Project I experiment).

Behavioral Experiments: A battery of tests was employed:

- **Operant Conditioning (Project I)**: Automated chambers, FR1 schedule for food pellets, with learning (10-14 days) and reversal (7 days) phases. Measured total responses, reward preference, timeout responses.
- **Active Avoidance (Shuttle-Box) (Project I)**: Automated learning to avoid/escape 0.15mA footshocks cued by light/tone, with learning (5-7 days) and reversal (3-5 days) phases. Measured escapes (EDST, EDFS) and failures (ESFL).
- **Sociability and Social Discrimination Tests (SDT) (Project II & III)**: Included Open Field (OF) for locomotion/anxiety, habituation to empty cages, sociability (conspecific vs. empty cage; SI calculated), and SDT (novel vs. familiar conspecific 24h later; SD calculated).
- **Social Interaction Test (SIT) (Project II & III)**: Free interaction between two mice in a novel arena under bright light (120 lx). Scored prosocial, aggressive, defensive behaviors; PI calculated.
- **Resident Intruder Test (RIT) (Project II & III)**: Unfamiliar intruder in resident's home cage. Scored resident's aggression.
- **Elevated Plus Maze (EPM) (Project II & III)**: Assessed anxiety (time/entries in open/closed arms, risk assessment) under bright light (120 lx).

- **Y-Maze (Project II & III)**: Assessed spatial working memory (spontaneous alternation).

Immunohistochemistry (IHC) and Tissue Processing: Brains were perfused (PBS, 4% PFA), sectioned (30 µm), and stained.

- **Viral Targeting**: RFP staining (Ni-DAB or fluorescence) using anti-RFP (Rockland #600-401-379).
- **Neuronal Phenotype**: Double/triple immunofluorescence for RFP with anti-GABA (Sigma #A2052), anti-TPH (Sigma #T0678), anti-VGluT3 (Synaptic Systems #135203) (Project I nonspecific); RFP with anti-GAD67 (Millipore #MAB5406), anti-GABA (Project II VGAT-Cre); RFP with anti-TH (DiaSorin or Cell Signaling #45648), anti-DBH (Invitrogen #PA1-18314 or Abcam EPR20385), anti-DAT (Sigma MAB369) (Project III DAT-Cre).
- **Neuronal Activation (c-Fos)**: c-Fos (Synaptic Systems #226004 or Santa Cruz #sc-52) /RFP colocalization post-CNO (Projects I & II). c-Fos in ZsGreen+ neurons post-social tasks (Project II). Imaging via confocal microscopy (Nikon C2) or slide scanner (3DHISTECH).

Molecular Biology Methods (RT-PCR for Project III): RNA extracted (RNeasy Mini Kit) from mouse (DRN, MRR) and human post-mortem brainstem samples (pontine raphe). RT-PCR for *Dat/DAT*, *Th*, *Dbh*, and *Gapdh/GAPDH*. Human *DAT* primers: Fwd: GTCTCTTTGGATTGACGCGG, Rev: ACTGTGCTTCTGTGCCATGT. Products verified by gel electrophoresis.

Statistical Analysis: Data (mean ± SEM) analyzed using Statistica. Tests included t-tests, ANOVAs (one-way, repeated-measures), with Fisher's LSD or Bonferroni post-hoc tests. $p < 0.05$ considered significant.

Results

Project I: The role of the GABAergic cells of the MRR in reinforcement-based learning: Nonspecific chemogenetic stimulation of the entire MRR (transducing GABAergic (45%), VGluT3+ (6%), TPH+ (0.3%) neurons) produced only marginal effects on operant conditioning and active avoidance tasks, suggesting complex interactions within the MRR. CNO administration was confirmed to induce c-Fos in transduced neurons. In contrast, targeted manipulation of MRR GABAergic neurons in VGAT-Cre mice revealed clearer contributions. Stimulation of these neurons led to impaired behavioral flexibility in operant conditioning, evidenced by increased total responses and more responses during timeout periods, particularly during the reversal learning phase. In the active avoidance task, stimulation of MRR GABAergic neurons increased the number of escapes made during the presentation of the conditioned stimulus (EDST) in the initial learning phase, suggesting an enhancement of proactive avoidance. Inhibition of MRR GABAergic neurons did not lead to significant alterations in these learning paradigms compared to controls.

Project II: Median raphe region GABAergic neurons contribute to social interest in mice: Chemogenetic stimulation of MRR GABAergic neurons in VGAT-Cre mice effectively increased c-Fos expression in the targeted neurons. Behaviorally, this stimulation enhanced social investigation: mice showed increased frequency and duration of sniffing towards a conspecific in the sociability test and an increased overall frequency of social interactions in the SIT. Conversely, inhibition of these neurons did not produce clear opposing effects on these social approach measures. Neither stimulation nor inhibition significantly affected territorial aggression in the RIT.

Corroborating these findings, c-Fos mapping in VGAT-Cre x ZsGreen mice showed that MRR GABAergic neurons were significantly activated during the sociability test, but not during the RIT, when compared to home-cage controls. This supports their involvement in general social interest rather than aggression.

Locomotor activity was not significantly affected by either manipulation of MRR GABAergic neurons. However, their inhibition induced a mild anxiogenic effect, reflected by an increased latency to first enter an open arm of the EPM. No significant effects of MRR GABAergic manipulation were observed on social recognition memory in the SDT or on spatial working memory in the Y-maze; performance in the SDT was at chance level for all groups.

Project III: The Dopaminergic Cells in the MRR Regulate Social Behavior in Male Mice: The existence of a dopaminergic neuronal population in the MRR was confirmed. Immunohistochemistry in DAT-Cre mice showed virally transduced (RFP-positive) neurons that were positive for TH and DAT, but negative for DBH, indicating a dopaminergic phenotype. Furthermore, RT-PCR analysis detected *Dat* mRNA in the mouse MRR and, significantly, *DAT* mRNA in human post-mortem pontine raphe samples (the human MRR equivalent).

Chemogenetic manipulation of these DAT-MRR neurons specifically modulated social behavior. Stimulation of DAT-MRR neurons resulted in a significant decrease in the total time spent investigating both familiar and novel mice during the SDT, which was assessed 24 hours after the CNO administration. In contrast, acute inhibition of DAT-MRR neurons led to a significant increase in the time spent engaged in prosocial interactions during the SIT. Neither manipulation significantly affected territorial aggression in the RIT. Manipulation of DAT-MRR neurons did not produce significant effects on locomotor activity, anxiety-like behavior, social recognition memory (SDT, where all groups performed at chance), or spatial working memory (Y-maze).

Conclusions

This PhD research elucidated distinct and context-dependent roles for GABAergic and dopaminergic neurons within the MRR in modulating complex behaviors, expanding our understanding beyond its traditional serotonergic functions.

The findings from Project I demonstrated that MRR GABAergic neurons are critically involved in reinforcement-based learning. Specifically, their stimulation impaired behavioral flexibility and increased response disinhibition in operant tasks, and enhanced proactive avoidance learning. Project II further revealed that these MRR GABAergic neurons promote social investigation and are endogenously activated during social encounters, though not during territorial aggression. Their inhibition induced a mild anxiogenic effect. These neurons did not appear to be primary modulators of the short-term memory paradigms tested.

Project III successfully characterized a DAT-expressing (dopaminergic) neuronal population in the mouse MRR and confirmed the presence of *DAT* mRNA in the human MRR-equivalent. These MRR DAT neurons were found to specifically modulate social behavior: their stimulation reduced subsequent social investigation, while their inhibition enhanced ongoing prosocial interactions. This suggests a fine-tuning, context-dependent role in social engagement. These neurons did not significantly influence locomotion, anxiety, or the memory tasks employed, indicating functional specificity.

Collectively, this work underscores the MRR as a heterogeneous nucleus where distinct non-serotonergic populations differentially contribute to behavioral regulation. MRR GABAergic neurons appear to broadly influence behavioral engagement and flexibility across learning and social domains. In contrast, MRR dopaminergic neurons exert more selective effects on social interaction. The lack of robust memory effects for both populations in the specific tasks used suggests other MRR cell types or brain regions may be more critical for these cognitive functions.

This research highlights the importance of cell-type-specific investigations in complex brain regions like the MRR and provides a foundation for future studies into the circuit mechanisms underlying these behaviors and their potential dysregulation in neuropsychiatric disorders.

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