Grant Writing / 18 – 23 April 2024

Methodological workshop in evolutionary biology

DO DIURNAL AND NOCTURNAL RODENTS RESPOND DIFFERENTLY TO BLUE LIGHT TREATMENT?

The research aims to compare these responses, considering the diurnal nature of humans.

BEHAVIOURS RELATED TO PHEROMONE TRAILING IN *Daboia russelii*

Confronting superstitions and unveiling novel insights into their communication.

HUMAN ASSOCIATED ARCHAEA

Their interaction with other microbes and their role in human disease.

INTERACTIONS BETWEEN RLN3 AND THE vHPC IN PTSD

Unraveling the underlying neurobiological mechanisms of the disorder.

JAGIELLONIAN UNIVERSITY IN KRAKÓW

Contents

General information about the workshop

Doctoral students work in small teams to develop projects (formulating scientific hypotheses, developing a plan of a study, and preparing its budget) based on their own ideas. Each stage of creating projects is discussed by all participants. The role of the instructor is to coordinate activities, stimulate discussion, give advice and choose the reviewers among the course participants and from outside.

After this course, the PhD students are able to prepare a research project proposal to funding agencies, e.g. Praeludium contest of the National Science Center, and can write a review of the project.

More information about this workshop can be found at:
https://www.usosweb.uj.edu.pl/kontroler.php?_action=katalog2/przedmioty/pokazPrzedmiot&kod=WB.SDSP.B%2FPhD-4

Participants

Organizer/Instructor	
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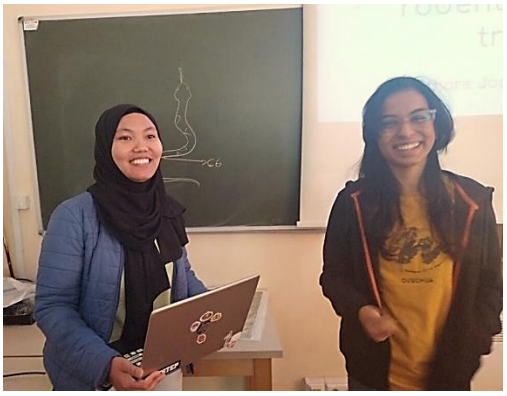
Navina Francis	Institute of Environmental Sciences, JU
Payal Dash	Institute of Zoology and Biomedical Research, JU

Photos of the teams during the trip

Group photo. Front row: Prof. Joanna Rutkowska, Navina, Payal (left to right). Back row: Diana, Kutlu, Afni, Karolina, Kinga, Joanna (left to right).



Snake Team. Afni (left) and Navina (right) after drawing a snake on the blackboard.

Stress Team. Kinga (left) and Payal (right) after submitting the final version of their proposal.

Topics initially suggested by course participants

1. What mechanisms underlie the protective effects of high-fat diet on the effects of chronic stress in rodents? Are there gender differences? (JR)
2. Can soil invertebrate communities indicate the level of disturbance in forests? (PD)
3. Are the microbes associated to mosquitoes symbionts or frequent visitors? (DR)
4. The impact of green and blue space exposure on impact child development, mental well-being and cognition (KP)
5. Can fish become companion animals for human? (KA)
6. Can human activities shape natural selection on example of brown bears? (KC)
7. Exploring the Therapeutic Potential of Ibu Ida Dayak's Bintang oil: Bridging Science and Indigenous Knowledge (AM)
8. Exploring Midges as an Alternative Protein Source: A Nutritional and Social Analysis. Can biting midges being a part of human diet solve the problem of it's over proliferation? (NF)
9. Do countries that have implemented bans or restrictions on plastic bags have lower plastic pollution indicators compared to countries that have not taken such actions? (KC)
10. Reconstructing the Vegetation History of Riau Peatlands for future Conservation (AM)
11. What are the cultural, psychological, and biological factors contributing to the perpetuation of myths and misconceptions about snake behaviour seeking revenge for the death of a related snake? (NF)
12. What are the neurobiological mechanisms underlying the sex differences in the response to traumatic stress/anxiety? (KP)
13. How does exposure to blue light influence circadian activity of the suprachiasmatic nucleus? (JR)
14. What are the differences that people from different ethnicities have with respect to thermotolerance? (PD)
15. Are archaea relevant to host health? (DR)
16. How can we tackle the problem of implementation edible algae to be grown in the human operated space vehicles to reach further destinations with genetics? (KA)

Title: The neurobiological mechanisms underlying the sex differences in the response to traumatic stress.

Authors: Kinga Przybylska, Payal Dash

Summary

Post-traumatic stress disorder (PTSD) is a severe mental health condition, with a higher prevalence in women than men, yet neural mechanisms of this disorder as well as the neurobiology of sex differences in PTSD remains poorly understood. In this project, we aim to explore the neurobiological basis for sex differences in PTSD using a PTSD rat model. We will focus on relaxin-3 (RLN3) signaling in the ventral hippocampus (vHPC), as both RLN3 and the vHPC are implicated in anxiety regulation. We plan to compare anatomical, electrophysiological, and behavioral aspects between male and female rats, as well as different estrous stages in females, and rats prone and resistant to PTSD. Our study hypothesizes that there will be differences in the number of neurons containing the RXFP3 receptor in various groups, with males having fewer neurons containing RXFP3 receptors. Electrophysiologically, we expect fewer neurons to respond to RLN3 administration in the vHPC network in males and certain female groups. Behaviorally, we expect RLN3 to exacerbate fear extinction impairment after conditioning in all groups.

By examining the interactions between RLN3 and the vHPC in PTSD, we aim to unravel the underlying neurobiological mechanisms of the disorder, particularly regarding sex differences. Understanding these mechanisms will lead to better understanding of this disorder and could pave the way for more targeted treatments for PTSD in the future.

1) Scientific goal of the project

Post-traumatic stress disorder (PTSD) is a severe psychiatric condition, characterized by clinical features such as fear and anxiety symptoms following exposure to trauma (American Psychiatric Association, 2013; Nemeroff et al., 2006; Bystritsky, A. et al., 2013). The signs and symptoms of PTSD, suggest a lasting, abnormal adaptation of neurobiological systems to the stress induced by witnessed trauma (Sherin & Nemeroff, 2011). Interestingly, although the vast majority of people are exposed to traumas at some time in their life only a small minority of people (approximately 10%) of the population develop PTSD, which suggests **individual differences in psychological vulnerability** (Kessler et al., 2017; Bisson et al., 2015). It is known that the pathophysiology of PTSD is associated with the circuitry of stress, context processing, and anxiety control that involve brain regions like the hippocampus, prefrontal cortex, thalamus, and amygdala (Sherin & Nemeroff, 2011). **However, the neural mechanisms of this disorder remain largely unknown.** Furthermore, despite the higher prevalence of PTSD in women compared to men, **the neurobiology of sex differences in PTSD remains poorly understood.** This knowledge gap is largely compounded by the avoidance of using female models in PTSD research due to their variable hormone profiles (Christiansen & Hansen, 2015)**.**

The scientific goal of this project is to elucidate the neurobiological mechanisms underlying sex differences in PTSD, particularly focusing on the role of relaxin-3 (RLN3) signaling in the ventral hippocampus (vHPC) in the rat model. Importantly, **both the RLN3 signaling pathway and the vHPC are linked to the regulation of anxiety-related behaviors**. Furthermore, chronic activation of the RLN3 receptor, RXFP3, in the vHPC of rats increases anxiety and social avoidance (Rytova et al., 2019), **yet the precise underlying neuronal mechanisms, specifically in the pathophysiology of PTSD, remain unknown.**

The major objective is to uncover the sex differences in the interplay between RLN3 and vHPC in the PTSD rat models. Therefore, we will conduct **anatomical, electrophysiological, and behavioral studies** and compare the results between rats **(1)** of 2 sexes **(2),** in 4 estrous stages of females, and **(3)** in animals prone and resistant to PTSD.

These three comparisons will allow testing the following hypotheses:

Anatomy. We hypothesize that there will be a lower number of neurons containing RXFP3 in males in (1) and (3) comparison, during proestrus in the (2) comparison and in rats resistant to PTSD in (3) comparison.

Electrophysiology. We hypothesize that the number of neurons responsive to RLN3 administration on the vHPC network activity will be the lowest in males in the (1) and (3) comparison, during proestrus in the (2) comparison and in rats resistant to PTSD in the (3) comparison.

Behavior. We hypothesize that RLN3 will increase the impairment of fear extinction after fear conditioning in all groups **of animals prone to PTSD**. Animals **resistant to PTSD will be excluded** from the experiments.

2) Significance of the project

PTSD (Post Traumatic Stress Disorder)

Post-traumatic stress disorder (PTSD) is an impairing mental health condition that can occur in individuals who have experienced or witnessed a traumatic event. According to DSM-5 by APA, PTSD individuals show intrusion symptoms (flashbacks, nightmares, emotional distress), avoidance, alterations in trauma-associated cognitions and mood, as well as arousal and reactivity (American Psychiatry Association, 2013; Quinones et al., 2020). These symptoms have a negative effect on the quality of life of PTSD-affected individuals, as they often experience higher rates of comorbidity with other psychological conditions, including depression, anxiety disorders and phobias (Spinhoven et al., 2014; Quinones et al., 2020). Since men and women experience trauma differently in their lifetime, there have been perspectives on possible sex differences in PTSD. Despite men facing more trauma in their lifetime, **women are twice as likely to develop PTSD,** possibly due to events of rape and sexual abuse (Christiansen & Hansen, 2015; Kessler et al., 1995) Despite the trauma type and prevalence, **women experience more chronic PTSD symptoms and comorbidities** like panic disorder and agoraphobia (Nemeroff et al., 2006). The neurobiology of PTSD is complex and involves dysregulation in various neural circuits, and neurotransmitter systems **implicated in fear, stress, and emotion processing.** Animal models, **particularly rodent models**, have been instrumental in studying the neurobiological underpinnings, allowing researchers to investigate the effects of trauma and potential therapeutic measures (Verbitsky et al., 2020). Some commonly used sensitization-based **PTSD models are SPS (Single Prolonged Stress)** (Bienvenu et al., 2021). Furthermore, there are other condition-based models in PTSD research, such as fear conditioning, which involves pairing a neutral stimulus (sound), with an aversive event (electric shock), to elicit a fear response when the stimulus is provided. However, exposing the stimulus alone repeatedly without the aversive event reduces or diminishes the conditioned fear response (Johnson et al., 2012, Bienvenu et al., 2021). This phenomenon is called **fear extinction**, and its mechanism

is often investigated in the context of PTSD. Current studies focus on understanding the molecular and cellular mechanisms, identifying biomarkers, and developing targeted interventions. **However, despite significant progress, there are still gaps in our understanding of PTSD. Further research is needed to explore the heterogeneity of PTSD,** the interplay between genetic and environmental factors, and the long-term effects of trauma. Additionally, more studies are required to **optimize treatment approaches and enhance the translation of research findings into clinical practice.** PTSD research contributes to our broader understanding of **human cognition, emotion, and resilience.** This knowledge can have implications beyond PTSD and help inform research in related areas of mental health and well-being.

The role of the ventral hippocampus in PTSD

The ventral hippocampus (vHPC) is mainly responsible for emotional processing, affect, stress, and anxiety (Leuner & Gould, 2010; Moser & Moser, 1998). It plays a critical role in regulating the body's response to stress (Phillips et al., 2006) and has been linked to the inhibition of the hypothalamic–pituitary–adrenal (HPA) axis (Ulrich-Lai & Herman, 2009), the primary system that moderates the physiological response to stressors in mammals (Jacobson & Sapolsky, 1991; Walker & Diforio, 1997). Importantly, the vHPC exhibits heightened sensitivity to anxiety-inducing stimuli and chronic stress, i.e., factors that can cause morphological changes in its structure, as many studies have reported decreased HPC density and volume in various anxiety disorders (Kim et al., 2015). Furthermore, traumatic or prolonged stressors can cause alterations in the HPC's synaptic plasticity (e.g., dendritic debranching and spine loss in excitatory neurons) and a reduced number of interneurons, disrupting the balance between excitation and inhibition signals (Shi et al., 2023). Rats with lesions specifically in the vHPC exhibit decreased anxiety-based behaviors (Bannerman et al., 2002; Kjelstrup et al., 2002). Moreover, lesions of specific ventral subfields revealed their individual functions (Weeden et al., 2015). Notably, the ventral CA3 is essential for the retrieval of contextual fear memory, whereas the ventral CA1 region is critical for the retention of trace fear conditioning (Hunsaker & Kesner, 2008). Furthermore, anxiety-like behaviors were shown to be suppressed by the activation of granule cells in the ventral dentate gyrus. Furthermore, stress has been identified as a factor that impairs memory retrieval processes linked to the hippocampus in humans. **Individuals diagnosed with PTSD often present volume reduction in the anterior hippocampus, which is equivalent to the rodent vHPC**, and difficulties in performing tasks dependent on hippocampal function (Logue et al., 2018). Among combat-exposed US Veterans diagnosed with PTSD, investigations have unveiled anatomical and functional disruptions in the anterior hippocampus (Abdallah et al., 2017). Although growing evidence supports the involvement of the vHPC, in anxiety, stress adaptation, and disorders like PTSD, there is still much to uncover regarding the underlying mechanisms. **A promising area of research in this field, which has yet to be explored, is the interaction between the vHPC and the RLN3 signaling pathway and their impact on PTSD symptoms.**

The RLN3 signaling pathway in vHPC

Relaxin-3 (RLN3) is a highly conserved neuropeptide of the relaxin superfamily (Smith et al., 2014). It's a selective ligand for the cognate Gi/o-protein-coupled receptor - RXFP3. RLN3 is primarily expressed in the nervous system, particularly in four brain regions: the nucleus incertus (NI), pontine raphe nucleus, periaqueductal grey and an area dorsal to the substantia nigra (Kania et al., 2014). Neurons containing RLN3 have extensive projections that are widely spread throughout the brain (Nasirova et al., 2020). Importantly, they strongly innervate among other structures involved in anxiety and fear processing, the vHPC. In addition, there is a significant amount of RXFP3 receptors in the vHPC (Bathgate et al., 2013; Olucha-Bordonau et al., 2003), which are distributed in a similar pattern as RLN3-positive fibers, with the highest concentration located in the ventral DG (Goto et al., 2001; Ma et al., 2007)

RLN3/RXFP3 signaling is mostly engaged in the regulation of physiological and behavioral stress responses, anxiety, depression, arousal, cognition, memory and sensory input integration (Kumar et al., 2015; J. H. Lee et al., 2016; Ma et al., 2007; Kumar et al., 2017). Furthermore, there is growing evidence that RLN3 may play a role in the regulation of the HPA axis (B. M. McGowan et al., 2014). Importantly, it is postulated that **RLN3-containing neurons in the NI are highly sensitive to stressors** (Ryan et al., 2013). Notably, studies have shown that **chronic stimulation of RXFP3 receptors in the vHPC neurons promotes anxiety and social avoidance** (Rytova et al., 2019).

In conclusion, the comparable distribution and prevalence of RLN3-positive fibers and RXFP3 receptors in the vHPC, along with the similar involvement of the vHPC and RLN3/RXFP3 signaling in stress and anxiety, **emphasize the significance of exploring the mechanisms that underlie the RLN3/RXFP3 signaling pathway in the vHPC.**

Sex Differences

While it's recognized that the **prevalence and symptoms of PTSD differ between sexes**, the **neurobiological mechanisms underlying these distinctions remain elusive**. For instance, in one study, male and female rats exposed to trauma exhibited sex-specific responses that mirrored differences in PTSD between men and women. Furthermore, male rats displayed a hyper-responsive phenotype which matched with well-established characteristics of PTSD in males. In contrast, the same traumatic event had minimal effects on these measures in female rats (Pooley et al. 2018). Moreover, the **effects of RLN3 also appear to be sex-specific** (De Ávila et al., 2021). For instance, behavioral phenotyping of rats revealed that female RLN3 knock-out (KO) mice showed hypoactivity and reduced social interactions, while male KOs exhibited increased stress sensitivity, in response to chronic stress (Smith et. al, 2009). Importantly, the **RLN3/RXFP3 system also seems to be influenced by the estrous cycle**. A study showed fluctuations in RXFP3 mRNA levels across the estrous cycle, while **RLN3 mRNA levels in the NI were lowest during proestrus** (De Ávila et al., 2021).

3) Concept and work plan

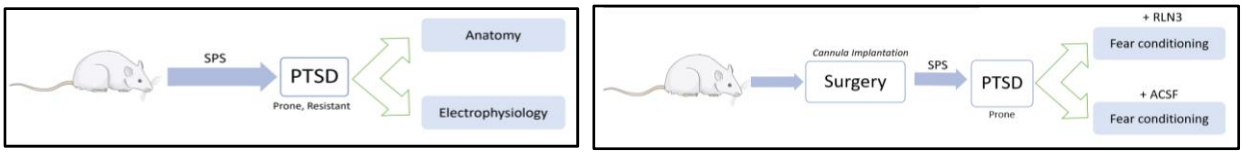


Fig. 1 *Experimental design for anatomical, electrophysiological (left) and behavioral experiments (right) For anatomical and electrophysiological experiments, the rats will be subject to SPS treatment for modeling PTSD. Anatomy and electrophysiology tests will be done in both PTSD-prone and resistant individuals. (B) For behavioral experiments, cannula implantation surgery would be conducted on rats, followed by SPS treatment for modeling PTSD. Behavioral tests would be continued in only PTSD prone individuals, by dividing them into two groups and injecting RLN3 or ACSF (control), followed by fear conditioning.*

	Comparisons				Project year			Personel				
	C1. Two sexes (M vs F)											
	C2. Four estrous stage (Pr, Es, Me, Di)											
	C3. Prone vs Resistant (Pro vs Res)											
	Research Area	**Predictions**				**2025**	**2026**	**2027**	**PI**	**TA1**	**TA2**	**TA3**	**TA4**
PTSD modeling (SPS protocol)	**Anatomy (*in situ* hybridization studies)**	**Parameter**	**C1**	**C2**	**C3**								
	To examine number of neurons containing RXDF3 mRNA in vHPC	Neurons containing RXFP3 mRNA	M < F	Pr < others	M < F, Res < Pro	X	X		X	X	X		
	Electrophysiology (*ex vivo* experiments)	**Parameter**	**C1**	**C2**	**C3**								
	To verify effects of RLN3 on neuronal activity in the vHPC	Neurons responsive to RLN3 administration	M < F	Pr < others	M < F, Res < Pro	X	X		X			X	X
	Behaviour (contextual fear test)	**Parameter**	**C1**	**C2**	**C3**								
	To examine the influence of RLN3 on the impairment of fear extinction	Fear extinction impairment	Increased in all rats prone to PTSD		Not tested		X	X	X	X	X	X	X

Fig. 2: *Table summarizing the work plan with a division into research areas, tasks, comparisons that will be carried out, parameters measured, predictions and timetable for allocating main tasks for all team members.* ***All studies will be conducted on animals after PTSD modeling (using the SPS protocol).*** *TA - technical assistant; PI - principle investigator.*

Anatomy

1) Characterization of vHPC neurons containing RXFP3 mRNA - multiplex *in situ* hybridization

To examine the number of neurons containing RXFP3 mRNA in the vHPC and verify if they differ between groups in all 3 of our comparisons multiplex *in situ* hybridization studies will be conducted. Moreover, potential differences in the distribution of these neurons will also be verified. Importantly, our preliminary findings confirmed that RXFP3 mRNA is expressed in the vHPC interneurons in naive male rats (without PTSD modeling) (Fig.3).

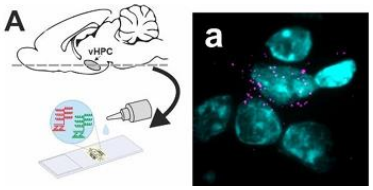


Fig. 3: *RXFP3 mRNA is expressed in the rat vHPC A, Experimental procedure. a, Representative images of RXFP3 (magenta) mRNA-expressing neurons. DAPI-stained nuclei (blue). Scale bar: 20 µm.*

Electrophysiology

1) Verification of the effects of RLN3 on neuronal activity in the vHPC – electrophysiological *ex vivo* experiments.

To investigate the effect of RLN3 on the neuronal activity of the rat vHPC, electrophysiological recordings using multielectrode arrays (MEA) will be conducted in all groups of PTSD model. The responses to RLN3 will be compared within all comparisons. Importantly, our preliminary data in naive male rats revealed that neurons in the vHPC are sensitive to activation of the receptor for RLN3, with the majority of responses being inhibitory (Fig.4).

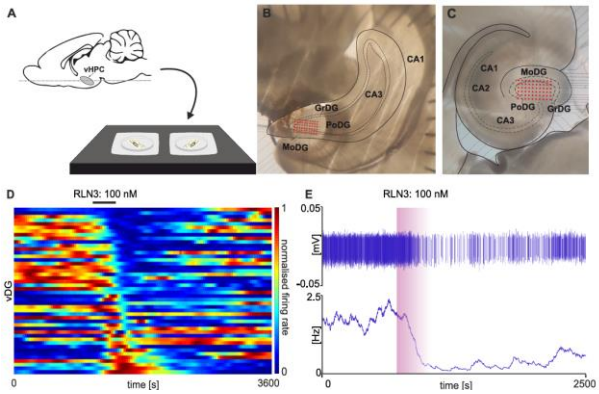


Fig. 4 - *Ex vivo multielectrode array recordings of the activity of vHPC neurons in* ***naive male rats.*** *A – Schematic illustration of the experimental procedure. B, C – Exemplary placements of coronal (B) and horizontal sections (C) of brain slices on multielectrode arrays. Red dots indicate single recording spots. D -Temporal heatmaps encoding the single-unit activity (SUA) of all recorded neurons (A; n = 197) and only RLN3-responsive neurons (C; n = 25) E - Exemplary DG neurons recorded during RLN3 administration. Top panel – raw signal, middle – separated spikes of a single neuron, bottom – corresponding frequency histogram. The pink rectangle indicates the duration of RLN3 action.*

Behavior

1) Characterization of the role of RLN3/RXFP3 signaling pathway in the vHPC in contextual fear processing.

Behavioral studies will be conducted to **examine the influence of RLN3 on the impairment of fear extinction** after fear conditioning across all experimental groups. Initially, rats will undergo surgical implantation of a cannula targeting the vHPC, followed by the induction of PTSD. After 7 days, rats will undergo contextual fear conditioning, with injections of RLN3 or artificial cerebrospinal fluid (ACSF) administered prior to the test, which in turn will **verify whether the fear response persists longer in the experimental group receiving RLN3 injections** compared to the control group receiving ACSF injections.

Risk assessment

While the research team's expertise and preliminary investigations instill confidence in the project's success, certain elements pose higher risks. **Challenges with the PTSD model** are minimized by using well-established behavioral procedures. Additionally, **analyzing substantial amounts of electrophysiological and behavioral data poses potential challenges.** To address this, existing MatLab scripts for analyzing data will be utilized.

4) Research methodology

Animals

All experiments will take place at the Department of Neurophysiology and Chronobiology at the Institute of Zoology and Biomedical Research, Jagiellonian University. **Lewis rats will be used due to their heightened vulnerability in PTSD models.** They demonstrate PTSD-like symptoms in 50% of cases, which is double the incidence observed in Sprague-Dawley rats (Cohen et al., 2006). **All experiments will be conducted on the Single Prolonged Stress (SPS) model of PTSD**, a well-documented approach in the literature (Souza et al., 2017).

Overall approx. **300 animals** will be used in this project:

Anatomical studies

SEX	Female				Male	Total
STAGE	Proestrus	Estrus	Metestrus	Diestrus	-	
Prone	5	5	5	5	5	50
Resistant	5	5	5	5	5	

Electrophysiological studies

SEX	Female				Male	Total
STAGE	Proestrus	Estrus	Metestrus	Diestrus	-	
Prone	10	10	10	10	10	100
Resistant	10	10	10	10	10	

Behavioural studies

SEX	Female				Male	Total
STAGE	Proestrus	Estrus	Metestrus	Diestrus	-	
Prone (+RLN3)	15	15	15	15	15	150
Prone (+ACSF)	15	15	15	15	15	

Fig. 5: *Table summarizing the number of animals used in each research area, divided by gender, stage of the estrous cycle, and susceptibility to PTSD.*

Multiplex *in situ* hybridization

To examine the number of neurons containing RXFP3 mRNA in the vHPC *in situ* hybridization by HiPlex RNAscope will be performed. The animals will be deeply anesthetized with pentobarbital, and the brains will be rapidly isolated and frozen on dry ice and stored at -80°C. The vHPC will be cut into coronal slices (16 µm) at -20°C using a cryostat (Cryocut CM 1800, Leica), placed on primary slides, and subjected to a hybridization procedure according to the manufacturer's protocol (Advanced Cell Diagnostics, ACD). Subsequently, the RXFP3 probes will be used to **detect RXFP3 mRNA**. Afterward, the sections will be covered with coverslips containing VectaMount medium with DAPI and imaged using a Zeiss fluorescence microscope (Axio Imager M2). **The results will be compared across all groups in all comparisons.**

Preparation of the vHPC for electrophysiological studies

For the preparation of brain tissue animals will be deeply anesthetized with isoflurane (Baxter) and subsequently decapitated. The brain will be rapidly removed from the skull and placed into ice-cold, carbogenated (5% CO2, 95% O2) artificial cerebrospinal fluid (ACSF) containing 185 mM sucrose, 10 mM glucose, 25 mM NaHCO3, 3 mM KCl, 1.2 mM NaH2PO4, 2 mM CaCl2, 10 mM MgSO4 * 7H2O. 20:6960789693 20 To minimize excitotoxicity and the generation of AP after preparation stress, ACSF did not include Na+ (NaCl). Subsequently, the tissue will be cut into 250 µm brain slices using a Leica VT 1000S vibrating microtome (Leica). The obtained vHPC brain slices will be incubated for 90 min in carbogenated, preheated (32 °C) recording ACSF, containing: 123 mM NaCl, 25 mM NaHCO3, 3 mM KCl, 1.4 mM NaH2PO4, 2 mM CaCl2, 2 mM MgSO4, 5 mM glucose, 0.01 g/L phenol red.

***Ex vivo* multielectrode array recordings**

Following a 90-minute incubation period,the tissue slices were transferred into the recording wells of the MEA2100-System (Multi Channel Systems GmbH), and the vHPC DG was carefully situated above the recording electrodes of the 6 × 10 perforated multi-electrode array (MEA; 100 µm spacing; 60pMEA100/30iR-Ti, Multi Channel Systems. The brain slices were then perfused, with the same ACSF as the one used for incubation, at a rate of 2 ml per minute, while maintaining a temperature of 32°C and were given an hour to settle before recording. The experimental procedure began with a 30 min baseline recording needed to assess spontaneous neuronal activity, before drug administration. The raw signal was sampled at a frequency of 20 kHz and recorded using Multi Channel Experimenter (Multi Channel Systems). RLN3 (100 nM; 5/10 ml; Phoenix Pharmaceuticals, Inc.) will be freshly diluted in the recording ACSF before application and will be delivered via bath perfusion.

Behavioral studies

To examine the influence of RLN3 on fear extinction following fear conditioning, a contextual fear conditioning test will be conducted in all animals prone to PTSD. Initially, rats will undergo stereotaxic surgery to implant a guide cannula (PlasticsOne) into the vHPC and fixed to the skull. Following surgery, each rat will receive an anti-inflammatory and analgesic drug (Tolfedine 4%, i.p., Vetoquinol, Biowet) and glucose (5 ml, 5% in saline, i.p.) to prevent dehydration during the post-surgery recovery period. Additionally, antibiotics will be added to the drinking water (Sul-Tridin 24%, Biowet) for the first five days post-surgery. Three weeks post-injection, rats will undergo the Single Prolonged Stress (SPS) protocol, and those demonstrating resistance to PTSD will be excluded. Rats prone to PTSD will then be divided into groups receiving either intra-brain injections of RLN3 (5 nmol in 5 µl) or artificial cerebrospinal fluid (ACSF, 5 µl) using a Hamilton syringe connected to a microinjection syringe pump, prior to the fear conditioning test. For contextual fear conditioning, a 3-day protocol will be implemented with two days of conditioning sessions followed by a recall session. Rats will be placed in a fear conditioning chamber with a metal grid to administer electrical foot shocks. During the first two days, rats will receive 7 foot shocks within a 20-minute session, each shock lasting for 10 seconds, delivered at 1-min intervals. The following day, rats will be re-exposed to the training context without receiving foot shocks. Freezing levels will be automatically quantified using image tracking software.

Data analysis

Electrophysiological data will be analyzed using custom MATLAB scripts and Kilosort will be used for spike-sorting. Data will be analyzed using NeuroExplorer. Freezing behavior will be analyzed using Anymaze software. All results will undergo statistical analysis, with significance determined at $p < 0.05$. Statistical tests and calculations will be conducted using Statistica software. Images of brain slices will be processed and analyzed by ImageJ software.

5) Project literature

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6. Table with budget of the project.

	Amount in PLN
Direct costs, including	428 400 PLN
- personnel costs and scholarships	252 000 PLN
- research equipment/device/software cost	36 400 PLN
- other direct costs	140 000 PLN
Indirect costs, including:	94 248 PLN
- indirect costs of OA	8 568 PLN
- other indirect costs	85 680 PLN
Total costs	522 648 PLN

7. Breakdown of project costs including justification and relevance for the tasks in the project.

Category	Description	Amount
Direct costs, including:		
Salaries	• 1 Principal Investigator (PI) • 4 Technical Assistants (TA) • 4 TAs for the project timeframe (Refer to Fig. 2) • PI Salary (5000 PLN/month) • TA Salary (1500 PLN/month)	PI (5000 PLN/month) x 36 months = 180000 PLN TA (1500 PLN/month) x 12 months x 4 = 72000 PLN
Equipment	• **4 portable computers:** Essential for storing and analyzing experimental data. Large data files and specialized programs require powerful computers for efficient processing. • **4 Portable external drives:** Crucial for transferring and storing large amounts of experimental data that surpass the capacity of PC storage. Ensures data security and accessibility. • **5 multi-electrode array chips**: Allows recording of extracellular action potentials from vHPC Essential for capturing neural activity, enabling insights into brain function and response to RLN3.	6000 PLN x 4 = 24 000 PLN 600 PLN x 4 = 2400 PLN 2000 PLN x 5 = 10 000 PLN
Others	**<u>Research Materials</u>** • **RNAScope kit with RXFP3 probe**: Used for molecular analysis to investigate gene expression of RXFP3 mRNA • **Reagents for PBS, recording ACSF, and protective cutting ACSF solution:** Ensure preservation and vitality of brain slices during *ex vivo* experiments, maintaining physiological conditions necessary for accurate data collection. • **Anesthetic, analgesic and anti-inflammatory drugs** (Ketamine, Xylazine, Isoflurane, Pentobarbital,	50 000 PLN 10 000 PLN

	Tolfedine): Crucial for performing stereotactic operations on rats, ensuring humane treatment and minimizing distress during experimental procedures. ● **Expendable, small laboratory supplies** needed in research (e.g. eppendorf tubes, pipette tips, well-plates)	5 000 PLN 10 000 PLN
	<u>Miscellaneous</u> ● **Food and cages** for the study of animals ● **Bio-hazard waste management**: Covers expenses related to animal welfare, housing, and disposal of biological waste generated during experiments.	20 000 PLN
	<u>Travel costs</u> ● **FENS, IBRO International conferences** ● Conferences will allow data presentation and the chance to discuss results with other field specialists. ● Approximate cost (per person, per conference) includes Registration fee + tickets + daily allowance + accommodation = 7500 PLN ● 4 TA x 1 conference each = 7500 PLN x 4 = 30000 PLN ● PI, 2 conferences = 7500 PLN x 2 = 15000 PLN	45 000 PLN
Indirect costs, including:		
- indirect costs of OA	Publication of results (open access journals)	8 568 PLN
- other indirect costs	Host institute	85 680 PLN

*All equipment, materials and travel costs include VAT

8. Reviews of grant proposal
8.1 Review No. 1

Title: The neurobiological mechanisms underlying the sex differences in the response to traumatic stress.

Authors: Kinga Przybylska, Payal Dash

1. Assessment of scientific quality of the research project (scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context)
Questions asked form coherent research proposal. They are well supported by the existing data, or lack of them. The problem itself is scientifically interesting. It may be completely novel but the idea of concentrating on rat females instead of women is a new approach.

2. Assessment of potential impact of the research project (the potential for substantial international impact on the research field(s) and for high quality research publications and other research outputs, taking into account the specifics of the research field and the variety of forms of impact and output; impact ought to be evaluated using an international context)
Results could be important enough to enable publication(s) in very good journals. Potential applications in treatments would extend their impact beyond basic research.

3. Assessment of feasibility of the research project (the feasibility of the proposed project, including the appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), other factors affecting the feasibility of the project)
The authors have both knowledge and enough experience to succeed. The anatomical, electrophysiological and behavioral components on the planned research appear well devised with good chances of being successfully applied.

4. Are the costs to be incurred well justified with regards to the subject and scope of the research?
Material costs are justified very well.

5. Strengths of the proposal
Excellent knowledge of the research connected with this project. The authors build on that and appear to know very well what needs to be done next. Was the project really developed within as little as 48 h. It may be a real Preludium stuff (under preparation?). Looks already as a nearly ready to submit. Good luck.

6. Weaknesses of the proposal
Risk assessment is poorly done. The authors apparently realize that inferring human psychic disorders from rat behavior may have serious limitations. This should be explained in more detail.

8.2 Review No. 2

Title of the project: The neurobiological mechanisms underlying the sex differences in the response to traumatic stress.

1. Assessment of scientific quality of the research project

I highly value the significance, originality, and novelty of the research proposed by the authors. The research project carries significant scientific relevance as it aims to explain the neurobiological mechanisms underlying sex differences in PTSD, with a specific focus on the role of relaxin-3 (RLN3) signaling in the ventral hippocampus (vHPC) using a rat model. This investigation is important because both the RLN3 signaling pathway and the vHPC are associated with regulating anxiety-related behaviors. The primary objective is to uncover sex differences in the interplay between RLN3 and vHPC in PTSD rat models. The proposed approach brings a very interesting point of view to this type of research.

2. Assessment of potential impact of the research project

The researchers' directions are according to aims as well as conference presentations. The impact of this study can be considered substantial, in the area of neurobiology and mental health research, in particular. This project works to demonstrate the variants of the male and female brain functionality as it supports PTSD, which as a result will result in unearthing unknown mechanisms involved in this health problem. It may have a lot of impact on scientific research dedicated to PTSD and could be a vehicle for developing more innovative and precise treatment creating positive effects for those with PTSD. Furthermore, the authors consider the delivery of project outcomes at the international conference among the major steps towards its breakthrough. The project is making an impact by sharing its findings, which can stimulate discussions, collaboration, and future studies in the fields of PTSD and mental health.

3. Assessment of feasibility of the research project

The study is able to use major methods such as molecular, electrophysiological, and behavioral techniques that are aligned to its goals in order to investigate the proposed hypotheses by thoroughly examining them. The team's knowledge, along with suppression of essential facilities and equipment, guarantee that every experiment is efficiently and well done. The implementation of a strong risk management strategy that includes human behavioral procedures and data analysis overcomes all difficulties among which the research system may stumble, hence making the research process more powerful and effective. Also, having research facilities and instruments on site is important because of the project's feasibility and the ability to conduct experiments in a feasible way and get the right results. Similarly, the international cooperation ability which is reflected by their attendance of international conferences can be learned by other authors and resources shared among members, in turn, can improve the feasibility of the whole project and extend its impact.

4. Are the costs to be incurred well justified with regards to the subject and scope of the research?

Overall, the relevant allocation of the budget to the parts of the research project seems justified and nicely done. The direct costs including personnel, research equipment, consumables, and other direct expenses connected to the project are allocated in an approach based on the project's goals and tasks. However, it may be a good choice to re-examine your budget in the context of business

travel. The budget allocated for technicians assistants conferences appears to be an unnecessary idea. At the same time, the salaries of technicians' seems to be too small.

5. Strengths of the proposal

5.1. The current project is designed to take up crucially important hole concerning the sexual discrepancies for PTSD. The discovery is extraordinarily interesting as it presents a different direction for research in the field of the vHPC activity. By focusing on this area of research, the study might reveal authentic uncovers into the neurobiological mechanisms. This could allow for progress towards the neurobiological mechanisms underlying the disease and generally in the field of research. In the ongoing scientific exploration of human health, the study of PTSD contexts is promising. Addressing gender differences in this context is not entirely overlooked, and including females is a significant consideration.

5.2. Multiple-pronged experimental approach employed. Multiple experimental techniques may be used that include molecular, electrophysiological, and behavioral techniques; which is a great feature that makes the proposed study credible. This extensive tool makes the campaign more resilient and comprehensive as it gives more grounds for exploration of the hypothesis.

5.3. Very well planned methodology. The proposal benefits from including research facilities, equipment, and funding. The proposed use of experimental techniques was very well explained, planned and timed. Utilizing proposed methodology it enables to conduct effective experiments and meet the project's goals within the designated time frame.

6. Weaknesses of the proposal

6.1. Lack of coherence and inconsistent font use. The proposal lacks coherence due to the excessive use of bold font and inconsistent font use (different fonts used for Fig. 2 and line numbering). This detracts from the readability and professional presentation of the document.

6.2. Illegibility of some figures. Figure 1 is illegible, which makes it difficult to understand the key information presented in the proposal. The font should be bigger so that the reader does not have problems getting to know what it is representing. In Figure 2, the text is overly lengthy, which may overwhelm reviewers and detract from the clarity and conciseness of the research plan.

6.3. Insufficient sample size. The proposed sample size (only 5 individuals) may be insufficient to yield statistically significant results, raising concerns about the robustness and reliability of the findings.

6.4. Incorrectly assessed budget allocation. There are unnecessary expenses in conference costs. Sending 4 Technical Assistants to conferences may be unnecessary and not cost-effective. Given their role in the project, it is questionable whether all technical assistants need to attend conferences. Considering the budget, it is also unclear whether you are planning for 4 or 8 technical assistants? Also, the salary of technical analysts seems to be quite low.

8.3 Review No. 3

Title of the project: The neurobiological mechanisms underlying the sex differences in the response to traumatic stress

Project's main aims and focus points are clearly stated and well described from the beginning of the proposal's section 1 to the end of section 2. From line 58 to line 62, PTSD is clearly defined which helps with understanding the whole context of the project proposal better. The writers of the proposal clearly stated the neural mechanism of the disease in different gender remains unknown in line 67 to 70 despite the previous studies mentioned especially in lines 107 to 109 and 115. But in line 115 the biomarkers and the main inspected locations in brain structure could be explained extensively to support the presence of the gap as later explained for RLN3 and RXFP3 in the locations, nucleus incertus (NI) and vHPC in lines 152-157, 162-165. Overall, in sections 1 and 2, writers explained status of our knowledge and research progress to understand effects of RLN3/RXFP3 system in vHPC clearly which shows necessity and importance of more information about RLN4/RXFP3 system. Besides, writers highlight the deficiency of previous PTSD research avoidance of female rat models due to certain reasons and they strongly emphasise the need of female rat model usage. Thus, including both genders in experiments and different estrous stages adds novelty and originality to the research proposal.

In section 4 lines 249-253 the reason behind the chosen Lewis rat and the protocol of PTSD modelling clearly mentioned. The documented rat and PTSD models shows, the animal and disease modelling has a lower risk which increases the feasibility of modelling part. Following steps of the methodology including well explained details but especially the details and reasonings in preparation of the vHPC step missing supporting references. In lines 273-276, it is mentioned that Na+ ions will not be included but the remaining substances such as NaHCO3 and NaH2PO4 has the potential of Na+ release. The reasoning should be supported by a protocol reference. Following, two steps also include details in protocol and equipment which shows it is well understood and common way to study ex vivo multielectrode array recordings and behavioural studies but including a standard protocol or consensus reference would be plus. Lastly, MATLAB and ImageJ are one of the most common software in data and image analysis together and represents no risk, but adds applicability in data analysis.

The costs of the equipment and reagents are reasonable and parallel with the described methodology. As it is a 3-year project with high potential of contribution in science and medicine with multiple aspects, salaries for PIs understandable but for TAs it is lower than minimal wage and it should be explained. Even though the potential impact of the project is well understood, regarding to conferences, the reason for TAs attendance should be justified.

The proposed project has many strengths as it aims to; demonstrate the need for including female rat usage with different estrous stages for a better implementation of research results on human health in perspective of female gender, construct a bridge in current literature to connect morphological and molecular studies on PTSD by filling the gap in RLN3/RXFP3 affects on PTSD in vHPC.

The only weakness in the project its work subjects; neural connections, projections, and brain structure. These subjects remain complex and even tough there are many studies with standardized protocols, it is still hard to conduct a research in practical and analytical perspective.

Title: The neurobiological mechanisms underlying the sex differences in the response to traumatic stress.

Authors: Kinga Przybylska, Payal Dash

Summary

Post-traumatic stress disorder (PTSD) is a severe mental health condition, with a higher prevalence in women than men. However, the neural mechanisms of this disorder as well as the neurobiology of sex differences are not fully understood. In this project, we aim to explore the neurobiological basis for sex differences in PTSD using a PTSD rat model. Specifically, we will focus on relaxin-3 and a brain region called the ventral hippocampus, which are both involved in regulating anxiety. By comparing males and female rats in different estrous stages, and rats that are more or less prone to developing PTSD, we hope to uncover the biological reasons behind these gender differences. Specifically, we hypothesize that there will be differences in the number of neurons containing relaxin-3 receptors with males having fewer neurons containing these receptors. Additionally, we expect fewer neurons to respond to relaxin-3 administration in the ventral hippocampus network in males. Finally, we expect relaxin-3 to decrease fear extinction after fear conditioning in all groups. This knowledge could help us understand the underlying neurobiological mechanisms of PTSD, particularly regarding sex differences and could pave the way for more targeted treatments for this disorder in the future.

1) Scientific goal of the project

Post-traumatic stress disorder (PTSD) is a severe psychiatric condition, characterized by clinical features such as fear and anxiety symptoms following exposure to trauma (American Psychiatric Association, 2013; Nemeroff et al., 2006; Bystritsky, A. et al., 2013). The signs and symptoms of PTSD, suggest a lasting, abnormal adaptation of neurobiological systems to the stress induced by witnessed trauma (Sherin & Nemeroff, 2011). Interestingly, although the vast majority of people are exposed to traumas at some time in their life only a small minority of people (approximately 10%) of the population develop PTSD, which suggests individual differences in psychological vulnerability (Kessler et al., 2017; Bisson et al., 2015). It is known that the pathophysiology of PTSD is associated with the circuitry of stress, context processing, and anxiety control that involve brain regions like the hippocampus, prefrontal cortex, thalamus, and amygdala (Sherin & Nemeroff, 2011). **However, the neural mechanisms of this disorder remain largely unknown.** Furthermore, despite the higher prevalence of PTSD in women compared to men, **the neurobiology of sex differences in PTSD remains poorly understood.** This knowledge gap is largely compounded by the avoidance of using female models in PTSD research due to their variable hormone profiles (Christiansen & Hansen, 2015)**.**

The scientific goal of this project is to elucidate the neurobiological mechanisms underlying sex differences in PTSD, particularly focusing on the role of relaxin-3 (RLN3) signaling in the ventral hippocampus (vHPC) in the rat model. Importantly, **both the RLN3 signaling pathway and the vHPC are linked to the regulation of anxiety-related behaviors**. Furthermore, chronic activation of the RLN3 receptor, RXFP3, in the vHPC of rats increases anxiety and social avoidance (Rytova et al., 2019), **yet the precise underlying neuronal mechanisms, specifically in the pathophysiology of PTSD, remain unknown.**

The major objective is to uncover the sex differences in the interplay between RLN3 and vHPC in the PTSD rat models. Therefore, we will conduct **anatomical, electrophysiological, and behavioral studies** and compare the results between rats **(1)** of 2 sexes (male and female in 4 estrous stages) and **(2)** in animals prone and resistant to PTSD.

These two comparisons will allow testing the following hypotheses:

Anatomy. We hypothesize that there will be the lowest number of neurons containing RXFP3 in males in both comparisons and in rats resistant to PTSD in the (2) comparison.

Electrophysiology. We hypothesize that the number of neurons responsive to RLN3 administration on the vHPC network activity will be the lowest in males in both comparisons and in rats resistant to PTSD in the (2) comparison.

Behavior. We hypothesize that RLN3 will increase the impairment of fear extinction after fear conditioning in all groups of animals prone to PTSD. Animals resistant to PTSD will be excluded from the experiments.

2) Significance of the project

PTSD (Post Traumatic Stress Disorder)

Post-traumatic stress disorder (PTSD) is an impairing mental health condition that can occur in individuals who have experienced or witnessed a traumatic event. According to DSM-5 by APA, PTSD individuals show intrusion symptoms (flashbacks, nightmares, emotional distress), avoidance, alterations in trauma-associated cognitions and mood, as well as arousal and reactivity (American Psychiatry Association, 2013; Quinones et al., 2020). These symptoms have a negative effect on the quality of life of PTSD-affected individuals, as they often experience higher rates of comorbidity with other psychological conditions, including depression, anxiety disorders and phobias (Spinhoven et al., 2014; Quinones et al., 2020). Since men and women experience trauma differently in their lifetime, there have been perspectives on possible sex differences in PTSD. Despite men facing more trauma in their lifetime, **women are twice as likely to develop PTSD,** possibly due to events of rape and sexual abuse (Christiansen & Hansen, 2015; Kessler et al., 1995). Despite the trauma type and prevalence, women experience more chronic PTSD symptoms and comorbidities like panic disorder and agoraphobia (Nemeroff et al., 2006). The neurobiology of PTSD is complex and involves dysregulation in various neural circuits, and neurotransmitter systems implicated in fear, stress, and emotion processing. Animal models, **particularly rodent models**, have been instrumental in studying the neurobiological underpinnings, allowing researchers to investigate the effects of trauma and potential therapeutic measures (Verbitsky et al., 2020). Some commonly used sensitization-based **PTSD models are SPS (Single Prolonged Stress)** (Bienvenu et al., 2021). Furthermore, there are other condition-based models in PTSD research, such as fear conditioning, which involves pairing a neutral stimulus (sound), with an aversive event (electric shock), to elicit a fear response when the stimulus is provided. However, exposing the stimulus alone repeatedly without the aversive event reduces or diminishes the conditioned fear response (Johnson et al., 2012, Bienvenu et al., 2021). This phenomenon is called **fear extinction**, and its mechanism

is often investigated in the context of PTSD. Current studies focus on understanding the molecular and cellular mechanisms, identifying biomarkers, and developing targeted interventions. **However, despite significant progress, there are still gaps in our understanding of PTSD.** Further research is needed to explore the heterogeneity of PTSD, the interplay between genetic and environmental factors, and the long-term effects of trauma. Additionally, more studies are required to optimize treatment approaches and enhance the translation of research findings into clinical practice. PTSD research contributes to our broader understanding of human cognition, emotion, and resilience. This knowledge can have implications in **molecular and behavioral neuroscience, neuropsychology, medicine and psychotherapy.**

The role of the ventral hippocampus in PTSD

The ventral hippocampus (vHPC) is mainly responsible for emotional processing, affect, stress, and anxiety (Leuner & Gould, 2010; Moser & Moser, 1998). It plays a critical role in regulating the body's response to stress (Phillips et al., 2006) and has been linked to the inhibition of the hypothalamic–pituitary–adrenal (HPA) axis (Ulrich-Lai & Herman, 2009), the primary system that moderates the physiological response to stressors in mammals (Jacobson & Sapolsky, 1991; Walker & Diforio, 1997). Importantly, the vHPC exhibits heightened sensitivity to anxiety-inducing stimuli and chronic stress, i.e., factors that can cause morphological changes in its structure, as many studies have reported decreased HPC density and volume in various anxiety disorders (Kim et al., 2015). Furthermore, traumatic or prolonged stressors can cause alterations in the HPC's synaptic plasticity (e.g., dendritic debranching and spine loss in excitatory neurons) and a reduced number of interneurons, disrupting the balance between excitation and inhibition signals (Shi et al., 2023). Rats with lesions specifically in the vHPC exhibit decreased anxiety-based behaviors (Bannerman et al., 2002; Kjelstrup et al., 2002). Moreover, lesions of specific ventral subfields revealed their individual functions (Weeden et al., 2015). Notably, the ventral CA3 is essential for the retrieval of contextual fear memory, whereas the ventral CA1 region is critical for the retention of trace fear conditioning (Hunsaker & Kesner, 2008). Furthermore, anxiety-like behaviors were shown to be suppressed by the activation of granule cells in the ventral dentate gyrus. Furthermore, stress has been identified as a factor that impairs memory retrieval processes linked to the hippocampus in humans. **Individuals diagnosed with PTSD often present volume reduction in the anterior hippocampus, which is equivalent to the rodent vHPC**, and difficulties in performing tasks dependent on hippocampal function (Logue et al., 2018). Among combat-exposed US Veterans diagnosed with PTSD, investigations have unveiled anatomical and functional disruptions in the anterior hippocampus (Abdallah et al., 2017). Although growing evidence supports the involvement of the vHPC, in anxiety, stress adaptation, and disorders like PTSD, there is still much to uncover regarding the underlying mechanisms. **A promising area of research in this field, which has yet to be explored, is the interaction between the vHPC and the RLN3 signaling pathway and their impact on PTSD symptoms.**

The RLN3 signaling pathway in vHPC

Relaxin-3 (RLN3) is a highly conserved neuropeptide of the relaxin superfamily (Smith et al., 2014). It's a selective ligand for the cognate Gi/o-protein-coupled receptor - RXFP3. RLN3 is primarily expressed in the nervous system, particularly in four brain regions: the nucleus incertus (NI), pontine raphe nucleus, periaqueductal grey and an area dorsal to the substantia nigra (Kania et al., 2014). Neurons containing RLN3 have extensive projections that are widely spread throughout the brain (Nasirova et al., 2020). Importantly, they strongly innervate among other structures involved in anxiety and fear processing, the vHPC. In addition, there is a significant amount of RXFP3 receptors in the vHPC (Bathgate et al., 2013; Olucha-Bordonau et al., 2003), which are distributed in a similar pattern as RLN3-positive fibers, with the highest concentration located in the ventral dentate gyrus (Goto et al., 2001; Ma et al., 2007). RLN3/RXFP3 signaling is mostly engaged in the regulation of physiological and behavioral stress responses, anxiety, depression, arousal, cognition, memory and sensory input integration (Kumar et al., 2015; J. H. Lee et al., 2016; Ma et al., 2007; Kumar et al., 2017). Furthermore, there is growing evidence that RLN3 may play a role in the regulation of the HPA axis (B. M. McGowan et al., 2014). Importantly, it is postulated that RLN3-containing neurons in the NI are highly sensitive to stressors (Ryan et al., 2013). Notably, studies have shown that **chronic stimulation of RXFP3 receptors in the vHPC neurons promotes anxiety and social avoidance** (Rytova et al., 2019).

In conclusion, the comparable distribution and prevalence of RLN3-positive fibers and RXFP3 receptors in the vHPC, along with the similar involvement of the vHPC and RLN3/RXFP3 signaling in stress and anxiety, **emphasize the significance of exploring the mechanisms that underlie the RLN3/RXFP3 signaling pathway in the vHPC.**

Sex Differences

While it's recognized that the **prevalence and symptoms of PTSD differ between sexes**, the **neurobiological mechanisms underlying these distinctions remain elusive**. For instance, in one study, male and female rats exposed to trauma exhibited sex-specific responses that mirrored differences in PTSD between men and women. Furthermore, male rats displayed a hyper-responsive phenotype which matched with well-established characteristics of PTSD in males. In contrast, the same traumatic event had minimal effects on these measures in female rats (Pooley et al. 2018). Moreover, the **effects of RLN3 also appear to be sex-specific** (De Ávila et al., 2021). For instance, behavioral phenotyping of rats revealed that female RLN3 knock-out (KO) mice showed hypoactivity and reduced social interactions, while male KOs exhibited increased stress sensitivity, in response to chronic stress (Smith et. al, 2009). Importantly, the **RLN3/RXFP3 system also seems to be influenced by the estrous cycle**. A study showed fluctuations in RXFP3 mRNA levels across the estrous cycle, while RLN3 mRNA levels in the NI were lowest during proestrus (De Ávila et al., 2021).

3) Concept and work plan

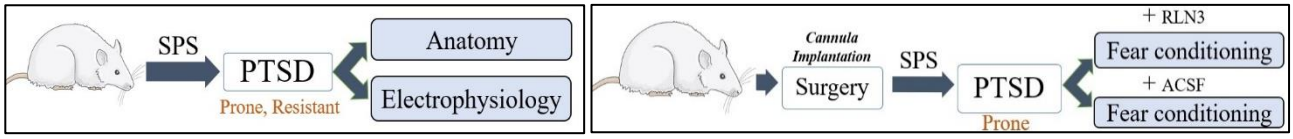

Fig. 1: *Experimental design for anatomical, electrophysiological (left) and behavioral experiments (right). For anatomical and electrophysiological experiments, the rats will be subject to SPS treatment for modeling PTSD (in both PTSD-prone and resistant individuals). For behavioral experiments, cannula implantation will be conducted, followed by SPS treatment. Behavioral tests will be continued in only PTSD-prone individuals, with either RLN3 or ACSF (control) injection followed by fear conditioning.*

PTSD modeling (SPS protocol)	Comparisons: C1. Two sexes M vs F (F in all 4 estrous stages); C2. Prone vs Resistant (Pro vs Res)				Project year			Personnel				
	Research Area	**Predictions**			**2025**	**2026**	**2027**	**PI**	**TA1**	**TA2**	**TA3**	**TA4**
	Anatomy (*in situ* hybridization studies)	**Parameter**	**C1**	**C2**								
		Neurons containing RXFP3 mRNA	M < F	M < F, Res < Pro	X	X		X	X	X		
	Electrophysiology (*ex vivo* experiments)	**Parameter**	**C1**	**C2**								
		Neurons responsive to RLN3 administration	M < F	M < F, Res < Pro	X	X		X			X	X
	Behaviour (contextual fear test)	**Parameter**	**C1**	**C2**								
		Fear extinction impairment	Increased in all rats prone to PTSD	Not tested		X	X	X	X	X	X	X

Fig. 2: *Table summarizing the work plan with a division into research areas, comparisons that will be carried out, parameters measured, predictions and timetable for allocating main tasks for all team members.* ***All studies will be conducted on animals after PTSD modeling*** *(using the SPS protocol**).** TA - technical assistant; PI - principal investigator; M – male; F – female; Pro – prone; Res – resistant.*

Anatomy

Characterization of vHPC neurons containing RXFP3 mRNA - multiplex *in situ* hybridization

To examine the number of neurons containing RXFP3 mRNA in the vHPC and verify if they differ between groups in both comparisons, multiplex *in situ* hybridization studies will be conducted. Moreover, potential differences in the distribution of these neurons will also be verified. Importantly, our preliminary findings confirmed that RXFP3 mRNA is expressed in the vHPC interneurons in naive male rats (without PTSD modeling) (Fig.3).

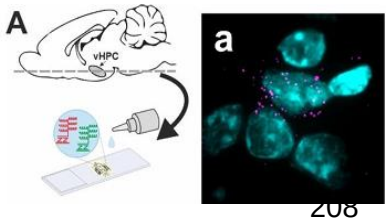

Fig. 3: *RXFP3 mRNA is expressed in the rat vHPC A, Experimental procedure. a, Representative images of RXFP3 (magenta) mRNA-expressing neurons. DAPI-stained nuclei (blue). Scale bar: 20 µm.*

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Electrophysiology

Verification of the effects of RLN3 on neuronal activity in the vHPC – electrophysiological *ex vivo* experiments.

To investigate the effect of RLN3 on the neuronal activity of the rat vHPC, electrophysiological recordings using multielectrode arrays (MEA) will be conducted in all groups of PTSD model. The responses to RLN3 will be compared in both comparisons. Importantly, our preliminary data in naive male rats revealed that neurons in the vHPC are sensitive to activation of the receptor for RLN3, with the majority of responses being inhibitory (Fig.4).

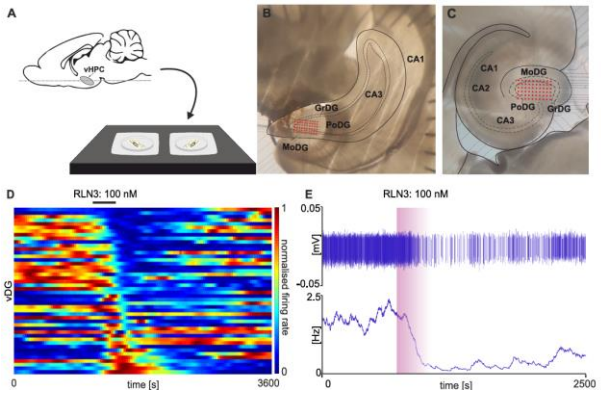

Fig. 4: - *Ex vivo multielectrode array recordings of the activity of vHPC neurons in* ***naive male rats.*** *A – Schematic illustration of the experimental procedure. B, C – Exemplary placements of coronal (B) and horizontal sections (C) of brain slices on multielectrode arrays. Red dots indicate single recording spots. D -Temporal heatmaps encoding the single-unit activity (SUA) of all recorded neurons (A; n = 197) and only RLN3-responsive neurons (C; n = 25) E – Exemplary vHPC neurons recorded during RLN3 administration. Top panel – raw signal, middle – separated spikes of a single neuron, bottom – corresponding frequency histogram. The pink rectangle indicates the duration of RLN3 action.*

Behavior

Characterization of the role of RLN3/RXFP3 signaling pathway in the vHPC in contextual fear processing.

Behavioral studies will be conducted to **examine the influence of RLN3 on the impairment of fear extinction** after fear conditioning across all experimental groups. Initially, rats will undergo surgical implantation of a cannula targeting the vHPC, followed by the induction of PTSD. After 7 days, rats will undergo contextual fear conditioning, with injections of RLN3 or artificial cerebrospinal fluid (ACSF) administered prior to the test, which in turn will **verify whether the fear response persists longer in the experimental group receiving RLN3 injections** compared to the control group receiving ACSF injections.

Risk assessment

While the research team's expertise and preliminary investigations instill confidence in the project's success, certain elements pose higher risks. **Challenges with the PTSD model** are minimized by using well-established behavioral procedures. The number of animals planned is overstated, due to **potential unforeseen errors**. Additionally, **analyzing substantial amounts of electrophysiological and behavioral data** poses potential challenges. To address this, existing MATLAB scripts for analyzing data will be utilized.

4) Research methodology

Animals

All experiments will take place at the Department of Neurophysiology and Chronobiology at the Institute of Zoology and Biomedical Research, Jagiellonian University. **Lewis rats will be used due to their heightened vulnerability in PTSD models.** They demonstrate PTSD-like symptoms in 50% of cases, which is double the incidence observed in Sprague-Dawley rats (Cohen et al., 2006). **All experiments will be conducted on the Single Prolonged Stress (SPS) model of PTSD**, a well-documented approach in the literature (Souza et al., 2017). Overall, approx. **350 animals** will be used in this project:

Anatomical studies

SEX	Female				Male	Total
STAGE	Proestrus	Estrus	Metestrus	Diestrus	-	
Prone	10	10	10	10	10	100
Resistant	10	10	10	10	10	

Electrophysiological studies

SEX	Female				Male	Total
STAGE	Proestrus	Estrus	Metestrus	Diestrus	-	
Prone	10	10	10	10	10	100
Resistant	10	10	10	10	10	

Behavioural studies

SEX	Female				Male	Total
STAGE	Proestrus	Estrus	Metestrus	Diestrus	-	
Prone (+RLN3)	15	15	15	15	15	150
Prone (+ACSF)	15	15	15	15	15	

Fig. 5: *Table summarizing the number of animals used in each research area, divided by gender, stage of the estrous cycle, and susceptibility to PTSD.*

Multiplex *in situ* hybridization

To examine the number of neurons containing RXFP3 mRNA in the vHPC *in situ* hybridization by HiPlex RNAscope will be performed. The animals will be deeply anesthetized with pentobarbital, and the brains will be rapidly isolated and frozen on dry ice and stored at -80°C. The vHPC will be cut into coronal slices (16 µm) at -20°C using a cryostat (Cryocut CM 1800, Leica), placed on primary slides, and subjected to a hybridization procedure according to the manufacturer's protocol (Advanced Cell Diagnostics, ACD). Subsequently, the RXFP3 probes will be used to detect RXFP3 mRNA. Afterward, the sections will be covered with coverslips containing VectaMount medium with DAPI and imaged using a Zeiss fluorescence microscope (Axio Imager M2). The results will be compared across all groups in both comparisons.

Preparation of the vHPC for electrophysiological studies

For the preparation of brain tissue animals will be deeply anesthetized with isoflurane (Baxter) and subsequently decapitated. The brain will be rapidly removed from the skull and placed into ice-cold, carbogenated (5% CO2, 95% O2) artificial cerebrospinal fluid (ACSF) containing 185 mM sucrose, 10 mM glucose, 25 mM NaHCO3, 3 mM KCl, 1.2 mM NaH2PO4, 2 mM CaCl2, 10 mM MgSO4 * 7H2O. To minimize excitotoxicity and the generation of action potentials after preparation stress, ACSF included limited Na+. Subsequently, the tissue will be cut into 250 µm brain slices using a Leica VT 1000S vibrating microtome (Leica). The obtained vHPC brain slices will be incubated for 90 min in carbogenated, preheated (32 °C) recording ACSF, containing: 123 mM NaCl, 25 mM NaHCO3, 3 mM KCl, 1.4 mM NaH2PO4, 2 mM CaCl2, 2 mM MgSO4, 5 mM glucose, 0.01 g/L phenol red.

***Ex vivo* multielectrode array recordings**

Following a 90-minute incubation period, the tissue slices will be transferred into the recording wells of the MEA2100-System (Multi Channel Systems GmbH), and the vHPC will be carefully situated above the recording electrodes of the 6 × 10 perforated multi-electrode array (MEA; 100 µm spacing; 60pMEA100/30iR-Ti, Multi Channel Systems. The brain slices will then be perfused, with the same ACSF as the one used for incubation, at a rate of 2 ml per minute, while maintaining a temperature of 32°C and will be given an hour to settle before recording. The experimental procedure begins with a 30 min baseline recording needed to assess spontaneous neuronal activity, before drug administration. The raw signal will be sampled at a frequency of 20 kHz and recorded using Multi Channel Experimenter (Multi Channel Systems). RLN3 (100 nM; 5/10 ml; Phoenix Pharmaceuticals, Inc.) will be freshly diluted in the recording ACSF before application and will be delivered via bath perfusion.

Behavioral studies

To examine the influence of RLN3 on fear extinction following fear conditioning, a contextual fear conditioning test will be conducted in all animals prone to PTSD. Initially, rats will undergo stereotaxic surgery to implant a guide cannula (PlasticsOne) into the vHPC and fixed to the skull. Following surgery, each rat will receive an anti-inflammatory and analgesic drug (Tolfedine 4%, i.p., Vetoquinol, Biowet) and glucose (5 ml, 5% in saline, i.p.) to prevent dehydration during the post-surgery recovery period. Additionally, antibiotics will be added to the drinking water (Sul-Tridin 24%, Biowet) for the first five days post-surgery. Three weeks post-injection, rats will undergo the Single Prolonged Stress (SPS) protocol, and those demonstrating resistance to PTSD will be excluded. Rats prone to PTSD will then be divided into groups receiving either intra-brain injections of RLN3 (5 nmol in 5 µl) or artificial cerebrospinal fluid (ACSF, 5 µl) using a Hamilton syringe connected to a microinjection syringe pump, prior to the fear conditioning test. For contextual fear conditioning, a 3-day protocol will be implemented with two days of conditioning sessions followed by a recall session. Rats will be placed in a fear conditioning chamber with a metal grid to administer electrical foot shocks. During the first two days, rats will receive 7 foot shocks within a 20-minute session, each shock lasting for 10 seconds, delivered at 1-min intervals. The following day, rats will be re-exposed to the training context without receiving foot shocks. Freezing levels will be automatically quantified using image tracking software.

Data analysis

Electrophysiological data will be analyzed using custom MATLAB scripts and Kilosort will be used for spike-sorting. Data will be analyzed using NeuroExplorer. Freezing behavior will be analyzed using Anymaze software. All results will undergo statistical analysis, with significance determined at $p < 0.05$. Statistical tests and calculations will be conducted using Statistica software. Images of brain slices will be processed and analyzed by ImageJ software.

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6. Table with budget of the project.

	Amount in PLN
Direct costs, including	499 400 PLN
- personnel costs and scholarships	324 000 PLN
- research equipment/device/software cost	36 400 PLN
- other direct costs	139 000 PLN
Indirect costs, including:	109 868 PLN
- indirect costs of OA	9 988 PLN
- other indirect costs	99 880 PLN
Total costs	609 268 PLN

7. Breakdown of project costs including justification and relevance for the tasks in the project.

Category	Description	Amount
Direct costs, including:		
Salaries	• 1 Principal Investigator (PI) • 4 Technical Assistants (TA) (for the project timeframe (refer to fig. 2))	PI (5000 PLN/month) x 36 months = 180 000 PLN TA (3 000 PLN/month) x 12 months x 4 = 144 000 PLN
Equipment	• **4 portable computers:** Essential for storing and analyzing experimental data. Large data files and specialized programs require powerful computers for efficient processing. • **4 Portable external drives:** Crucial for transferring and storing large amounts of experimental data that surpass the capacity of PC storage. Ensures data security and accessibility. • **5 multi-electrode array chips**: Allows recording of extracellular action potentials from vHPC Essential for capturing neural activity, enabling insights into brain function and response to RLN3.	6000 PLN x 4 = 24 000 PLN 600 PLN x 4 = 2 400 PLN 2000 PLN x 5 = 10 000 PLN
Others	**<u>Research Materials</u>** • **RNAScope kit with RXFP3 probe**: Used for molecular analysis to investigate gene expression of RXFP3 mRNA • **Reagents for PBS, recording ACSF, and protective cutting ACSF solution:** Ensure preservation and vitality of brain slices during *ex vivo* experiments, maintaining physiological conditions necessary for accurate data collection. • **Anesthetic, analgesic and anti-inflammatory drugs** (Ketamine, Xylazine, Isoflurane, Pentobarbital, Tolfedine): Crucial for performing	50 000 PLN 10 000 PLN 5 000 PLN

	stereotactic operations on rats, ensuring humane treatment and minimizing distress during experimental procedures. ● **Expendable, small laboratory supplies** needed in research (e.g. eppendorf tubes, pipette tips, well-plates)	10 000 PLN
	<u>Miscellaneous</u> ● **Food and cages** for the study of animals ● **Bio-hazard waste management**: Covers expenses related to animal welfare, housing, and disposal of biological waste generated during experiments.	40 000 PLN
	<u>Travel costs</u> ● **FENS, IBRO International conferences** ● Conferences will allow data presentation and the chance to discuss results with other field specialists. ● Approximate cost (per person, per conference) includes Registration fee + tickets + daily allowance + accommodation	PI (12 000 PLN/conference) x 2 = 24 000 PLN
Indirect costs, including:		
- indirect costs of OA	Publication of results (open access journals)	9 988 PLN
- other indirect costs	Host institute	99 880 PLN

*All equipment, materials and travel costs include VAT

Title: The effects of manipulations of the archaeal community on gut microbiota diversity and human health

Authors: M.Sc. Kutlu Alkan, M.Sc. Diana Rojas

Summary

Archaea are so frequently found among the members of the gut microbiota and have been linked to diseases such as obesity, irritable bowel syndrome and cancer. However, current research on archaea lacks resolution and quality and fails to address their relevance in human health or within the gut microbiota itself. Here, we propose to characterize the complexity of the dynamics and roles of the archaeal members of the human microbiome in a rat model and their impact on other members of the microbiome as well as their overall effects on host health, using high-throughput amplicon sequencing and chromatography focused on microbial metabolites, as well as bioinformatic and statistical approaches to weight the significance of the shifts within the microbiome. Our findings will provide a high-resolution taxonomic catalog of gut-associated archaeal species and describe the dynamics of the gut microbiota, and the specific role of archaea in human health. The project will elucidate specific multi-kingdom interactions of relevance to obesity that may be used as innovative strategies of clinical treatment.

1. Scientific goal of the project

Archaea are poorly understood core members of the human microbiota(Chibani et al., 2021)**.** They were thought to inhabit only extreme environments until they were isolated from vertebrate feces, and later were confirmed to inhabit the human intestine. Although archaea have been sampled from fecal samples across worldwide populations(Chibani et al., 2021; Maya-Lucas et al., 2019; Rani et al., 2017; Wampach et al., 2017), they remain the unpopular prokaryotes due to their lack of clinical relevance. However, in recent years, different studies have provided evidence that archaea may have tissue tropism within the gastrointestinal tract(Koskinen et al., 2017), that they interact with the immune system(Bang et al., 2014, 2017) and that they are live components of breast-milk(Togo et al., 2019): hinting that their role in human health and development might be greater that we suspect. However, their impact on health remains controversial due to contradictory results(Bang & Schmitz, 2018; Geesink & Ettema, 2021).
The human microbiome composition has been widely linked to health status(Hou et al., 2022), and throughout the years many correlations have been determined between specific bacteria and specific diseases, neglecting the fact that bacteria are not the sole inhabitants of the human gastrointestinal tract. Archaea have been shown to form a syntrophic relationship with bacteria in the colon where they maintain the microenvironment, by taking up bacterial metabolites, in optimal conditions for bacterial fermentation of dietary fibers(Bang & Schmitz, 2018; Morris et al., 2013). Such relationships could be indicators that archaea are key organisms in the preservation of the gut homeostasis. **A holistic and systematic approach to the identification of archaeal diversity and their interaction within the microbiome and with the host will substantially change our view on archaea as members of the human microbiome**. However, the reliability of the currently available data is limited, since the methods used for the screening of archaea usually have low resolution or do not account for the complexity of the communities in which archaea reside.

The overarching goal of this project is to comprehensively survey the dynamics of archaea within the human microbiome to understand their role in host health. The diversity of archaea in the human gut has been addressed several times, and results show the prevalence of *Methanobrevibacter smithii* in healthy individuals, while the diversity of the less abundant archaea remains poorly understood. Furthermore, the shifts in abundance of *M. smthii* that have been linked to differences in human body mass show contradictory results(Geesink & Ettema, 2021; Mafra et al., 2022). Whether these issues are a consequence of biological processes or an artifact caused by methodological pitfalls is unknown. **Therefore we will address this with qualitative and quantitative methods that will provide higher resolution regarding the identity of human-associated archaea. Additionally, we will manipulate the composition of the archaeal communities in rats to understand the inter-kingdom interactions that shape the gut microbiome. Understanding the nature of these dynamics is critical for assessing the impact of archaea in human health.** These approaches will allows us to address the following questions:

Question 1. Determine the composition of the gut microbiota of healthy and obese individuals. In addressing this question, we will increase the efforts in the sampling of archaea since identifying them by molecular markers becomes tricky considering their phylogenetic relationship with bacteria. The outcome will allow us to address the following hypotheses:

H1.1. *There is a core "archaeome" within the gut microbiota composed of highly abundant and rare species.*

H1.2. *The microbiome of obese individuals is dominated by a strain of* M. smithii *that differs from the strain prevalent in microbiota of healthy individuals.*

Question 2. Establish a rat model with humanized human microbiota characteristic of healthy and obese individuals. This will allow us to test whether our model is reliable in comparison with the

human microbiota, while also evaluating the shifts in the microbiota diversity and metabolic production during the development of obesity induced by a high-fat diet. We hypothesize that:

H2.1. *The whole community of human microbiota can survive as a whole in a new host, as long as the diversity is maintained.*

H2.2. *There are archaea that can be used as indicators of the development of obesity.*

H2.3*. The changes in production of bacterial byproducts caused by obesity can be used to predict shifts of archaeal species.*

Question 3. Evaluate the impact of the variation of archaeal composition on other members of the microbiome and on host health. In addressing this goal, we will manipulate the archaeome to address the relevance of specific species on the whole microbial community dynamics and on the development of obesity.

H3.1. *Methanogenic archaeal abundance can be manipulated without affecting bacterial and fungal members of the microbiota by inducing targeted viral infections.*

H3.2. *Reduction of methanogens within the microbial community is accompanied by decrease of fermenting bacteria.*

H3.3. *Reduction of methanogens will lead to the increase of other archaeal groups in a short period of time.*

H3.4. *Alteration of archaeal diversity will lead to the development of obesity in a shorter time frame.*

H3.5. *Alteration of archaeal composition will eventually lead to the reduction of bacterial and fungal diversity.*

2) Significance of the project

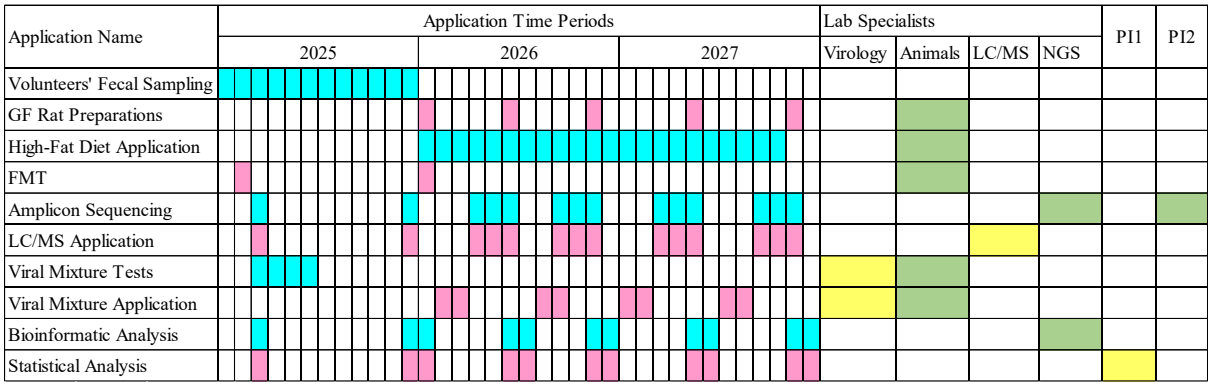
Over the past few years, the composition of the human microbiome has been strongly linked to host health and susceptibility to develop metabolic diseases, such as obesity, diabetes, and irritable bowel syndrome(Hou et al., 2022). However, most of these studies have focused on the dynamics of bacterial members of the microbiota, neglecting less abundant microbes such as fungi and archaea. Nevertheless, the diversity of gut-associated archaea has also been sampled in healthy individuals across multiple populations and in people of different ages, suggesting that archaea are residents of the human gut. Most agree that methanogenic archaea are the most abundant and prevalent group among other archaeal members of the human microbiota, and thus have been documented to vary in association to human disease, making them the sole focus of many studies. However, most studies focusing on 16S rRNA-based characterization of archaea lack sensitivity. On the other hand, whenever quantitative methodologies or highly specific probes are used for their detection, they usually target one or two prevalent species, neglecting the relevance of less-known members of the human archaeome. Furthermore, methanogenic archaea depend on byproducts of bacterial fermentation in the colon while fermenting bacteria rely on the metabolism of archaea to maintain the adequate conditions of their microenvironment. However, whether these archaea interact with non-fermenting bacteria, fungi or other archaea remains unexplored in healthy and non dysbiotic individuals. Additionally, there is an abysmal knowledge gap regarding gut-associated archaea biology; whereas we know that methanogenic archaea produce methane as a byproduct of their metabolism, the biological relevance of this compound is unknown, leaving open the question if it could be used by other microbes, such as methanotrophic archaea. Due to these issues, **we have not been able to understand the dynamics of archaeal populations within the gut microbiota community**, such as metabolite production and take up, as well as the potential syntrophic, antagonistic and/or mutualistic relationships that exist at the level of intra or inter-kingdom and that are relevant for maintaining gut homeostasis. Understanding these dynamics holds promise for the future implementation of archaea as regulators of the human microbiome. Here**, we propose an approach to sensitively screen for archaea and study their**

interaction with other microbes, as well as their relevance in disease progression. Specifically, we address obesity as an archaea-linked metabolic disease that is responsible for millions of premature deaths each year worldwide.

This project will be the first effort to describe quantitatively the diversity of the whole community of microbes in the human gut, while also providing a high-resolution taxonomic catalog of gut-associated archaeal species that will form a valuable resource and tool for the international research community as it expands efforts to comprehend and describe the dynamics of the gut microbiota, and the specific role of archaea in human health. The project will elucidate specific multi-kingdom interactions of relevance to obesity that may be used as innovative strategies of clinical treatment. The novelty of the approaches and questions the project addresses to understand the significance of archaea are likely to be published in high-impact journals.

3) Concept and work plan

General work plan. This project encompasses a suite of methodologies to measure and analyze microbiota and metabolites at determined time intervals. It will start with mapping the human microbiota for obese and normal-weight individuals of different sexes and ages between 20 and 40 to address **Question1.** Simultaneously we will perform fecal microbiota transplantation (FMT) from volunteers to germ-free (GF) rats in sterile laboratory conditions. We will use, amplicon sequencing techniques to assess and map human microbiota and GF rat microbiota in time intervals to see the effects of subsequent applications, such as diet type, virus injection, and recipient profiles. With different diet applications, we plan to observe different microbiota effects on inducing or reducing the obesity process (**Question2**), and with virus injection, we plan to assess the role of archaeome on host health and microbiome composition (**Question3**). To analyze the amplicon sequencing results, we plan to use specific bioinformatics tools. Additionally to amplicon sequencing, we will use LC/MS to measure the initial and changing abundance of gaseous byproducts and metabolites. Lastly, we will apply statistical methods to define condition- application- and caused changes in microbiota, archaeome, and metabolome.



Risk analysis. We believe the use of viral injections to eliminate archaea in microbiota will provide target-specific elimination to observe indirect reduction or elimination in bacteria abundance. The trials of viral injections to archaea eliminations were practiced in recent years but it has never been tried and tested for complex microbial structures like microbiota. Therefore the uncertainty in its success represents a risk factor for our research. Notwithstanding, its application has great potential to surmount one of the biggest problems in understanding the dependencies in microbiota.

4) Research methodology

Fecal Microbiota Transplantation (FMT). We plan to start by obtaining fecal material usage consent of obese and healthy-weight volunteers of different sex groups between ages 20 and 40. With the consent, volunteers will accept no antibiotic usage for 3 months unless it is not needed for their health situations. We aim to reach 100 volunteers for each of the 4 subgroups in one month. Obtained samples will be preserved at -80C with cryopreservation protocol for up to 3 months(Bircher et al., 2018). For FMT, the administration, equipment, feeding-fasting, and volume of oral gavage will be applied according to rat-specific procedures(Turner et al., 2011).

Germ-Free Rats (GF rats) as Laboratory Model and Recipient Groups Types. Germ-free rats are organisms raised in sterile conditions to maintain no microbiota in them. GF rats will be used as recipients for laboratory applications of FMT. Therefore, housing, FMT, sample collection, and diet applications will be operated in the ISOcage P System to maintain isolation from outer factors(Dremova et al., 2023). There will be 8 group types of 20 GF rats; male and female supergroups, high-fat diet-induced obesity, healthy-diet mid-groups under each supergroup, and archaea-eliminating virus (AEV) injection and no-injection subgroups under each mid-group of 3-6 month age rats. These 8 group types will be used for the combinations of, female donor to female and male recipient and male donor to female and male recipient.

Amplicon Sequencing. For each step, we plan to use amplicon sequencing to quantify microbiome composition. The first will be directly applied to samples from all volunteers to map donor microbiota in conditions of sex, health (obesity), and age. The second and others will be applied with the fecal samples collected each week from receiver rats to represent short-term, mid-term, and long-term composition and changes in the microbiota depending on all group objectives. Microbial DNA will be extracted from fecal samples with a DNA Fecal/Soil Microbe Kit. Subsequently, extracted DNA will be purified and unwanted short sequences in the isolate will be eliminated by a short-read eliminator. Pure and ready DNA will be amplified with different combinations of 16S, 18S, ITS, and rRNA operon primers. Each target and different primer pairs will contribute to covering more taxonomic units and enhancing the taxonomic resolution(Kinoshita et al., 2021). Amplicons will be purified before library preparation and the library will be formed by Illumina index adding PCR. After, PCR products will be purified again before Illumina sequencing. The sequencing will be performed by the MiSeq platform. Read outputs from the MiSeq platform will be analyzed by enrolling bioinformatic tools such as QIIME, mothur, and DADA2 to obtain the taxonomic unit composition of each sample(Koskinen et al., 2017).

Liquid Chromatography - Mass Spectrometry (LC/MS). For each step, LC/MS will be applied to detect and quantify the proportion of gaseous byproducts such as methane and H2, and metabolites such as SCFAs related to dysbiosis and obesity. The first will be directly applied to samples from all volunteers for quantitative analysis of gaseous byproducts and metabolites. The second and others will be applied with the fecal samples collected each week from receiver rats to analyze the compositions and track short-term, mid-term, and long-term changes in the abundance of gaseous byproducts and metabolites. Collected samples will be pretreated before LC/MS application. For this, according to the storage state of the sample, additional pretreatment steps such as freeze-thaw cycles, will be included. During LC/MS application, online metabolite databases such as the Human Metabolome Database and METLIN will be used to obtain better metabolite coverage(Xu et al., 2019).

Viral Injection to the Recipient Groups. Different mixtures of Caudoviricetes class viruses will be prepared in saline solution and injected into the rat model groups by oral gavage to see the effect of each viral mixture on the *archaeome* of the rats. For this, 3 virus mixtures are planned; Sulfolobus spindle-shaped viruses (SSVs), pleolipoviridae, and archaeal-tailed viruses (arTVs). Each mixture will be applied to the individuals of each group type and combination. We expect that initial tests of virus injections will provide a profile of viral infection's effect on the *archaeome* and so, related microbiota. We plan to use this profile to set a final mixture of the mentioned viruses(Sensevdi et al., 2024).

Statistics for Microbiome and Metabolome Change Analysis. We are planning to apply combinations of different statistics tools to analyze all of our bioinformatics data obtained from all mentioned applications. For this, 3 main applications of statistics will be combined; correlation-based methods, condition dependence models, and network-based methods. Multiple packages are present for the metabolite/metabolome, microbiota, and trans-kingdom analysis. From correlation-based methods; SparCC, Meta-Network, Correlation-Centric Network, from condition dependence models; MDiNE and MixMPLN, from network-based methods; Multi-Omics Factor Analysis will be used(Matchado et al., 2021).

5) Project literature

1. Chibani, C. M. *et al.* A catalogue of 1,167 genomes from the human gut archaeome. *Nat. Microbiol.* **7**, 48–61 (2021).
2. Rani, S. B., Balamurugan, R. & Ramakrishna, B. S. Molecular analysis of the human faecal archaea in a southern Indian population. *J. Biosci.* **42**, 113–119 (2017).
3. Maya-Lucas, O. *et al.* The gut microbiome of Mexican children affected by obesity. *Anaerobe* **55**, 11–23 (2019).
4. Wampach, L. *et al.* Colonization and Succession within the Human Gut Microbiome by Archaea, Bacteria, and Microeukaryotes during the First Year of Life. *Front. Microbiol.* **8**, 738 (2017).
5. Koskinen, K. *et al.* First Insights into the Diverse Human Archaeome: Specific Detection of Archaea in the Gastrointestinal Tract, Lung, and Nose and on Skin. *mBio* **8**, e00824-17 (2017).
6. Bang, C. *et al.* Biofilm formation of mucosa-associated methanoarchaeal strains. *Front. Microbiol.* **5**, (2014).
7. Bang, C., Vierbuchen, T., Gutsmann, T., Heine, H. & Schmitz, R. A. Immunogenic properties of the human gut-associated archaeon Methanomassiliicoccus luminyensis and its susceptibility to antimicrobial peptides. *PLOS ONE* **12**, e0185919 (2017).
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10. Bang, C. & Schmitz, R. A. Archaea: forgotten players in the microbiome. *Emerg. Top. Life Sci.* **2**, 459–468 (2018).
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14. Bircher, L., Schwab, C., Geirnaert, A. & Lacroix, C. Cryopreservation of artificial gut microbiota produced with *in vitro* fermentation technology. *Microb. Biotechnol.* **11**, 163–175 (2018).
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19. Sensevdi, E. R., Sourrouille, Z. A. & Quax, T. E. Host range and cell recognition of archaeal

viruses. *Curr. Opin. Microbiol.* **77**, 102423 (2024).
20. Matchado, M. S. *et al.* Network analysis methods for studying microbial communities: A mini review. *Comput. Struct. Biotechnol. J.* **19**, 2687–2698 (2021).

6) Budget of the project

7) Breakdown of project costs including justification and relevance for the tasks in the project.

8. Reviews of grant proposal
8.1 Review No. 1

Title: The effects of manipulations of the archaeal community on gut microbiota diversity and human health

Authors: M.Sc. Kutlu Alkan, M.Sc. Diana Rojas

1. Assessment of scientific quality of the research project (scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context)
Questions are generally well selected. They are of high scientific relevance and very important for practical reasons. It is undoubtedly worth to learn about Archea within microbiota.

2. Assessment of potential impact of the research project (the potential for substantial international impact on the research field(s) and for high quality research publications and other research outputs, taking into account the specifics of the research field and the variety of forms of impact and output; impact ought to be evaluated using an international context) Results would be likely interesting and potentially very impactful. Cataloguing Archea would be in itself worthwhile, finding a solid link to obesity would be widely recognized as a breakthrough.

3. Assessment of feasibility of the research project (the feasibility of the proposed project, including the appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), other factors affecting the feasibility of the project)
Some elements are feasible, such as sampling and taxonomic identification. Inoculating or sterile rats with human samples appear ok, too. Killing of Archea with viruses appears very unsure, perhaps not worth trying. Not explained what for are LC/MS analyses.

4. Are the costs to be incurred well justified with regards to the subject and scope of the research?
Do not see costs.

5. Strengths of the proposal
Authors found interesting questions. Indeed, one can wonder why they were studied so little up to now. Even partial success would likely end up in good, possibly very good, journals.

6. Weaknesses of the proposal
Too many goals, meeting them would require much larger project and team. It would be ambitious enough to provide a high-resolution taxonomic catalog of gut-associated archaeal species. But, I was not convinced that bioinformatics was well planned: “To analyze the amplicon sequencing results, we plan to use specific bioinformatics tools - different combinations of 16S, 18S, ITS, and rRNA operon”, fine, but it not enough explained. Chemical analyses not described at al. Also, statistics linking archeal species with humans or rats will be very demanding, no reliable plans how to do it. And it will have to be done at the initial stages because there is no reason to proceed with rats experiments without knowing that human phenotypes correlate with archean diversity.

8.2 Review No. 2

Title of the project
The title of the project could be a bit catchier and more concise.

1. Assessment of scientific quality of the research project
The main goal of the project is to explore the dynamics of archaea community with respect to human microbiome and to understand their effect on host health. Based on previous literature, the authors believe that the archaeal diversity can be vital to maintaining gut homeostasis. Although the lack of availability of reliable evidence seems to be the drive towards designing this project. The authors aim to use higher resolution screening methods and address the complexity of the system which is lacking in previous studies. The idea is interesting and ambitious in the context that it is directly related to exploring new realms of predicting and improving human health and has a futuristic potential to it.

Few questions regarding specific methodologies:

a) Authors do not mention how they are planning to control for the temperature and metabolic activity differences in human and rat model while they are transferring the microbiome and trying to maintain it in the model. I feel it will great effects on the dynamics of the microbiome.
b) The authors could mention how they are planning on viral doses (e.g. TCID50) for the viral injection experiment.
c) The authors may also consider having controls for viral injection experiments i.e., sham injected individuals to see any potential changes due to the procedure.
d) I feel like there are too many hypotheses to address.

2. Assessment of potential impact of the research project
The authors have very clearly mentioned the relevance of the idea and its potential impact on the field. They have efficiently highlighted valid points regarding the application of the outcomes of the project in a futuristic sense. They have addressed the knowledge gaps based on existing studies and have tried to include few resolutions in their own.

3. Assessment of feasibility of the research project
The authors have clearly explained their methodology with valid justifications as to why they are choosing to do so. They have given an elaborate about the plan of action of the project. However, in the risk assessment part, I would also expect an objective based assessment risk along with an overall analysis. However, the authors have not mentioned when, where and how they are going to maintain the lab rat colony or which facilities they are going to use for the experiments like liquid chromatography/mass spectrometry, amplicon sequencing etc. They have also not mentioned what facilities are readily available to them and what needs to be procured from elsewhere. I am also not sure how they will be able to achieve all the objectives in a span of three years. Hence, it is impossible to completely assess the actual feasibility of the proposed project.

4. Are the costs to be incurred well justified with regards to the subject and scope of the research?
Unfortunately, the authors have failed to present a budget and justification for their proposed study.

5. Strengths of the proposal

The project is well thought of and has an application-level approach to it. The authors show justice to their claim of aiming to use higher resolution analysis methods to conduct the study. They have expressed their objectives very clearly and have justified methodologies to fulfil them.

6. Weaknesses of the proposal

Lack of information on logistics/procurement and valid justifications for the same. Lack of information on use of controls in the experiments they have proposed.

8.3 Review No. 3

Title of the project: The effects of manipulations of the archaeal community on gut microbiota diversity and human health

1. Assessment of scientific quality of the research project

The project covers an important, under-researched aspect of archaea in microbiome studies. The proposal highlights and discusses the role of archaea in the gut microbiome and their relationship to human health. This issue is highly relevant and raises the subject of human diseases such as obesity and irritable bowel syndrome. I am not an expert in this field, however I believe that the research on such a specific topic is truly lacking and should be acknowledged. Thereupon, the research is innovative and the potential results are very promising.

The description of the problem is very detailed and the subject is discussed extensively. This is a strong point because the complexity of the research significance is explained clearly to a reader without direct experience in the field. However, considering that the length of the short description slightly exceeds the limit of 5 pages, the authors should consider compressing the text. There is a lot of relevant background information, however it should be possible to shorten the description without omitting any crucial aspects.

The summary is well-written, however it is a bit shorter than the lower word limit.

2. Assessment of potential impact of the research project

As stated by the authors, the relevance of archaea to human health is an unrecognised issue worldwide. The project is interdisciplinary, as it covers the subject of microbiology/molecular biology and addresses a highly important human health problem with the application of a wellestablished animal model. One thing that could be clarified is what kind of contradictory results are the authors referring to in line 57.

All research questions and hypotheses are explicitly provided and plainly introduced. A strong point is that the authors are aware of many possible outcomes of the study and propose multiple hypotheses. The proposal indicates a good familiarity with the topic and I believe that the authors take into consideration many potential findings that may emerge during the process.

3. Assessment of feasibility of the research project

The work plan is clearly written, every planned methodology references a specific research question which leaves no doubt about the research plan. The methodologies chosen to address the research questions are appropriate and I believe they will provide substantial information on the subject.

The description of experimental groups including germ-free rats is quite complicated, therefore a graphical representation of the plan representing all group types would be helpful for the reader.

The provided risk analysis is reasonable. The authors are aware of the fact that the application

of viral injections may yield unexpected results and testing of this method is included in the timeline. Another potential risk that could be included arises from including volunteers in the study – many

factors that people are potentially not aware of can affect the results. This differentiation however cannot be excluded but should be kept in mind.

As the study plans to include animal model, one statement regarding ethical considerations, e.g. ensuring animal welfare and the awareness of legal regulations could be included.

4. Are the costs to be incurred well justified with regards to the subject and scope of the research?
Unfortunately the budget of the project has not been provided.

5. Strengths of the proposal
A significant gap is addressed by the research proposal, the idea is very innovative and related directly to human health. If successful, the project may lead to a breakthrough in medical treatment of diseases of affluence.

The authors plan to use various scientific methodologies, which makes the approach really comprehensive. This increases the probability of successfully completing the research tasks and verification of the research questions.

The use of germ-free rats as a model is a very good approach. Many potential variable factors are thus eliminated which makes the obtained results more reliable.

Graphical representation of the study timeline provides very detailed information, at the same time being readable and understandable – including more figures like that could be beneficial.

Overall, the project proposal is very well-written, all aspects are clearly explained. The undertaken subject is very ambitious.

6. Weaknesses of the proposal
Lack of budget is the biggest issue in this project proposal. This fact makes it hard to assess some aspects of the project's feasibility, especially that some of the planned methods seem advanced.

Visual representations of some aspects, as mentioned before, would be helpful to reduce the long paragraphs of text.

Some minor editorial mistakes occur and adjustments should be incorporated in order to improve the quality of the proposal:

- the font color is inconsistent in paragraph 2 ("Significance of the project"),
- there should be spaces before parentheses with cited literature, starting from line 48,
- in line 76 the word "pitfalls" is highlighted in green,
- in hypothesis 1.2., a Latin name of species occurs and should be written in italics as well,
- in line 222 a class of viruses is mentioned, I might be wrong but this should probably be written in italics as well,
- I assume one word is missing in line 168 – the sentence is not clear.

Title: The effects of manipulation of the archaeal community on gut microbiota diversity and human health

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Summary

Archaea are so frequently found among the members of the gut microbiota and have been linked to diseases such as obesity, irritable bowel syndrome, and cancer. However, current research on archaea lacks resolution and quality and fails to address their relevance in human health or within the gut microbiota itself. Here, we propose to characterize the dynamics and roles of the archaeal members of the human microbiome in a rat model. Additionally, we will assess their relationship with the development of obesity and their impact on bacterial and fungal members of the human gut microbiome. We will use high-throughput amplicon sequencing, chromatography focused on microbial metabolites, and bioinformatic approaches to address this goal. Our findings will provide a high-resolution taxonomic catalog of gut-associated archaeal species and describe the dynamics of the gut microbiota, and the specific role of archaea in human health. The project will elucidate specific multi-kingdom interactions of relevance to obesity that may be used as innovative strategies for clinical treatment.

1. Scientific goal of the project

Archaea are poorly understood core members of the human microbiota (Chibani et al., 2021)**.** They were thought to inhabit only extreme environments until they were isolated from vertebrate feces, and later were confirmed to inhabit the human intestine. Although archaea have been sampled from fecal samples across worldwide populations (Maya-Lucas et al., 2019; Rani et al., 2017), they remain the unpopular prokaryotes due to their lack of clinical relevance. However, in recent years, different studies have provided evidence that archaea may have tissue tropism within the gastrointestinal tract (Koskinen et al., 2017), that they interact with the immune system (Bang et al., 2014, 2017) and that they are live components of breast-milk (Togo et al., 2019): hinting that their role in human health and development might be greater that we suspect. However, their impact on health remains controversial due to contradictory results.

The human microbiome composition has been widely linked to health status (Hou et al., 2022), and throughout the years many correlations have been determined between specific bacteria and specific diseases, neglecting the fact that bacteria are not the sole inhabitants of the human gastrointestinal tract. Archaea have been shown to form a syntrophic relationship with bacteria in the colon where they maintain the microenvironment, by taking up bacterial metabolites, in optimal conditions for bacterial fermentation of dietary fibers (Bang & Schmitz, 2018; Morris et al., 2013). Such relationships could be indicators that archaea are key organisms in the preservation of the gut homeostasis. **A holistic and systematic approach to the identification of archaeal diversity and their interaction within the microbiome and with the host will substantially change our view on archaea as members of the human microbiome**. However, the reliability of the currently available data is limited, since the methods used for the screening of archaea usually have low resolution or do not account for the complexity of the communities in which archaea reside.

The overarching goal of this project is to comprehensively survey the dynamics of archaea within the human microbiome to understand their role in host health. Multiple studies demonstrate the high prevalence of *Methanobrevibacter smithii* in healthy individuals, while the diversity of the less abundant archaea remains poorly understood. Furthermore, the increase in abundance of *M. smithii* has been linked to both obesity and malnutrition, making any associations between this species and human health controversial (Geesink & Ettema, 2021; Mafra et al., 2022). Whether these issues are a consequence of biological processes, or an artifact caused by methodological pitfalls is unknown. **Therefore we will address this with qualitative and quantitative methods that will provide higher resolution regarding the identity of human-associated archaea. Additionally, we will manipulate the composition of the archaeal communities in rats to understand the inter-kingdom interactions that shape the gut microbiome. Understanding the nature of these dynamics is critical for assessing the impact of archaea in human health.** These approaches will allow us to address the following questions:

Question 1. Determine the composition of the gut microbiota of healthy and obese individuals. In addressing this question, we will increase the efforts in the sampling of archaea since identifying them by molecular markers becomes tricky considering their phylogenetic relationship with bacteria. The outcome will allow us to address the following hypotheses:

H1.1. *There is a core "archaeome" within the gut microbiota composed of highly abundant and rare species.*

H1.2. *The microbiome of obese individuals is dominated by a strain of M. smithii that differs from the strain prevalent in microbiota of healthy individuals.*

Question 2. Establish a rat model with humanized human microbiota characteristic of healthy and obese individuals. This will allow us to test whether our model is reliable in comparison with the human microbiota, while also evaluating the shifts in the microbiota diversity and metabolic production during the development of obesity induced by a high-fat diet. We hypothesize that:

H2.1. *The whole community of human microbiota can survive as a whole in a new host, as long as diversity is maintained.*

H2.2. *There are archaea that can be used as indicators of the development of obesity.*

H2.3*. The changes in production of bacterial byproducts caused by obesity can be used to predict shifts of archaeal species.*

Question 3. Evaluate the impact of the variation of archaeal composition on other members of the microbiome and on host health. In addressing this goal, we will manipulate the archaeome to address the relevance of specific species on the whole microbial community dynamics and on the development of obesity.

H3.1. *Methanogenic archaeal abundance can be manipulated without affecting bacterial and fungal members of the microbiota by inducing targeted viral infections.*

H3.2. *Reduction of methanogens within the microbial community is accompanied by decrease of fermenting bacteria.*

H3.3. *Reduction of methanogens will lead to the increase of other archaeal groups in a short period of time.*

H3.4. *Alteration of archaeal diversity will lead to the development of obesity in a shorter time frame.*

H3.5. *Alteration of archaeal composition will eventually lead to the reduction of bacterial and fungal diversity.*

2) Significance of the project

Over the past few years, the composition of the human microbiome has been strongly linked to host health and susceptibility to develop metabolic diseases, such as obesity, diabetes, and irritable bowel syndrome (Hou et al., 2022). However, most of these studies have focused on the dynamics of bacterial members of the microbiota, neglecting less abundant microbes such as fungi and archaea. Nevertheless, the diversity of gut-associated archaea has also been sampled in healthy individuals across multiple populations and in people of different ages, suggesting that archaea are residents of the human gut (Chibani et al., 2021). Most agree that methanogenic archaea are the most abundant and prevalent group among other archaeal members of the human microbiota, and thus have been documented to vary in association to human disease, making them the sole focus of many studies. Furthermore, most studies focusing on 16S rRNA-based characterization of archaea lack sensitivity or usually target only methanogenic species, neglecting the relevance of less-known members of the human archaeome (Geesink & Ettema, 2021). Whether these archaea interact with non-fermenting bacteria, fungi, or other archaea remains unexplored in healthy and dysbiotic individuals. Additionally, there is an abysmal knowledge gap regarding gut-associated archaea biology; whereas we know that methanogenic archaea produce methane as a byproduct of their metabolism, the biological relevance of this compound is unknown, leaving open the question if it could be used by other microbes, such as methanotrophic archaea (Chibani et al., 2021). Due to these issues, **we have not been able to understand the dynamics of archaeal populations within the gut microbiota community**, such as metabolite production and take up, as well as the potential syntrophic, antagonistic and/or mutualistic relationships that exist at the level of intra or inter-kingdom and that are relevant for maintaining gut homeostasis. Understanding these dynamics holds promise for the future implementation of archaea as regulators of the human microbiome. Here**, we propose an approach to sensitively screen for archaea and study their interaction with other microbes, as well as their relevance in disease progression.** Specifically**,** we address obesity as an archaea-linked metabolic disease that is responsible for millions of premature deaths each year worldwide. This project will be the first effort to describe quantitatively the diversity of the whole community of microbes in the human gut, while also providing a high-resolution taxonomic catalog of gut-associated archaeal species that will form a valuable resource and tool for the international research community as it expands efforts to comprehend and describe the dynamics of the gut microbiota, and the specific role of archaea in human health. The project will elucidate specific multi-kingdom interactions of relevance to obesity that may be used as innovative strategies of clinical treatment. The novelty of the approaches and questions the project addresses to understand the significance of archaea are likely to be published in high-impact journals.

3) Concept and work plan

General work plan. This project encompasses a suite of methodologies to measure and analyze microbiota and metabolites at determined time intervals (Table 1). It will start with mapping the human microbiota for obese and normal-weight individuals of different sexes and ages between 20 and 40 to address **Question1.** Simultaneously we will perform fecal microbiota transplantation (FMT) from volunteers to germ-free (GF) rats in sterile laboratory conditions. We will use, amplicon sequencing techniques to assess and map human

microbiota and GF rat microbiota in time intervals to see the effects of subsequent applications, such as diet type, virus injection, and recipient profiles. With different diet applications, we plan to observe different microbiota effects on inducing or reducing the obesity process (**Question2**), and with virus injection, we plan to assess the role of archaeome on host health and microbiome composition (**Question3**). To analyze the amplicon sequencing results, we plan to use specific bioinformatics tools. Additionally to amplicon sequencing, we will use LC/MS to measure the initial and changing abundance of gaseous byproducts and metabolites. Lastly, we will apply statistical methods to define condition- application- and caused changes in microbiota, archaeome, and metabolome.

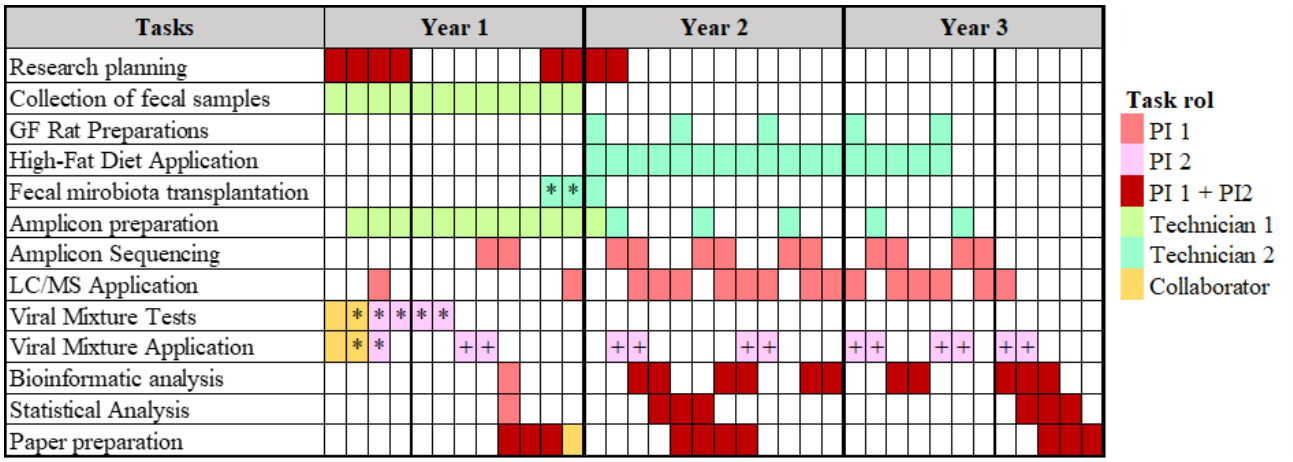

Table 1. Project timeline and research tasks assigned to each team member indicated by color code. "*" indicate trial tests for different experiments; "+" indicate instead vancomycin treatment if necessary.

Risk analysis. This project will address a series of bold research questions using sequencing based approaches, and propones innovative designs to manipulate the human microbiome. Since transplantation of human microbiota into laboratory mice has been done before, our biggest challenge is the depletion of archaea in a live system. The trials of viral injections to achieve archaea elimination were practiced in recent years but it has never been tried and tested for complex microbial structures like the gut microbiota. However, **we believe that the use of viral injections with archaea-infecting phages will successfully provide target-specific elimination of archaea with minimal effect on bacterial communities.** Therefore the uncertainty in its success represents a risk factor for our research. Notwithstanding, its application has great potential to surmount one of the biggest problems in understanding the dependencies in microbiota. Alternatively, if trial infection and depletion of archaea will not yield the expected results, the rats will be treated with antibiotics, such as vancomycin, to alter the diversity of methanogenic archaea, specifically. Immediate administration of probiotics will restore the bacterial community, allowing us to assess shifts within microbes of the gut and disease progression under disturbance of the archaeal community.

4) Research methodology

Fecal Microbiota Transplantation (FMT). We plan to start by obtaining fecal material usage consent of obese and healthy-weight volunteers of different sex groups between ages 20 and 40. With the consent, volunteers will accept no antibiotic usage for 3 months unless it is not needed for their health situations. After medical evaluation by a hired medical practitioner, volunteers will be assigned to the "healthy group" if they are declared clinically healthy and their body max index is between 18.5 - 24.9. Volunteers with body max index above the mentioned range will be considered for the "obese group". We aim to reach 100 volunteers for each of the 4 subgroups in one month. Obtained samples will be preserved at -80°C with cryopreservation protocol for up to 3 months (Bircher et al., 2018). For FMT, the administration, equipment, feeding-fasting, and volume of oral gavage will be applied according to rat-specific procedures (Turner et al., 2011).

Germ-Free Rats (GF rats) as Laboratory Model and Recipient Groups Types. Germ-free rats are organisms raised in sterile conditions to maintain no microbiota in them. GF rats will be used as recipients for laboratory applications of FMT. Therefore, housing, FMT, sample collection, and diet applications will be

operated in the ISOcage P System, under room temperature, to maintain isolation from outer factors and avoid stressful conditions (Dremova et al., 2023). There will be 8 group types of 20 GF rats; male and female supergroups, high-fat diet-induced obesity, healthy-diet mid-groups under each supergroup, and archaea-eliminating virus (AEV) injection and no-injection subgroups under each mid-group of 3-6 month age rats. These 8 group types will be used for the combinations of, female donor to female and male recipient and male donor to female and male recipient.

Amplicon Sequencing. For each step, we plan to use amplicon sequencing to quantify microbiome composition. The first will be directly applied to samples from all volunteers to map donor microbiota in conditions of sex, health (obesity), and age. The second and others will be applied with the fecal samples collected each week from receiver rats to represent short-term, mid-term, and long-term composition and changes in the microbiota depending on all group objectives. Microbial DNA will be extracted from fecal samples with a DNA Fecal/Soil Microbe Kit. Subsequently, extracted DNA will be purified and unwanted short sequences in the isolate will be eliminated by a short-read eliminator. Pure and ready DNA will be amplified with different combinations of primers targeting the V3 and V4 hypervariable regions of bacterial 16S rRNA, 16S-ITS-23S rRNA operons for the identification of archaea and the fungal marker 18S rRNA. Each target and different primer pairs will contribute to covering more taxonomic units and enhancing the taxonomic resolution (Kinoshita et al., 2021). Amplicons will be purified before library preparation and the library will be formed by Illumina indexing PCR. After, PCR products will be purified again before Illumina sequencing. The sequencing will be performed by the MiSeq platform. Raw reads corresponding to short amplicons (i.e. 16S and 18S) from the MiSeq platform will be analyzed using bioinformatic tools such as QIIME, mothur, and DADA2 to obtain the taxonomic unit composition of each sample. Long reads corresponding to archaeal operons will be concatenated and quality filtered using the CCMetagen v. 1.2.2 and trimmomatic v. 03.9 softwares, respectively (Koskinen et al., 2017, Kinoshita., 2021).

Liquid Chromatography - Mass Spectrometry (LC/MS). For each step, LC/MS will be applied to detect and quantify the proportion of gaseous byproducts such as methane and H2, and metabolites such as SCFAs related to dysbiosis and obesity. The first will be directly applied to samples from all volunteers for quantitative analysis of gaseous byproducts and metabolites. The second and others will be applied with the fecal samples collected each week from receiver rats to analyze the compositions and track short-term, mid-term, and long-term changes in the abundance of gaseous byproducts and metabolites. Collected samples will be pretreated before LC/MS application. For this, according to the storage state of the sample, additional pretreatment steps such as freeze-thaw cycles, will be included. During LC/MS application, online metabolite databases such as the Human Metabolome Database and METLIN will be used to obtain better metabolite coverage (Xu et al., 2019).

Viral Injection to the Recipient Groups. Different mixtures of Caudoviricetes class viruses will be prepared in saline solution and injected into the rat model groups by oral gavage to see the effect of each viral mixture on the archaeome of the rats. For this, 3 virus mixtures are planned; *Sulfolobus* spindle-shaped viruses (SSVs), *Pleolipoviridae*, and archaeal-tailed viruses (arTVs). Each mixture will be applied to the individuals of each group type and combination. We expect that initial tests of virus injections will provide a profile of viral infection's effect on the archaeome and so, related microbiota. We plan to use this profile to set a final mixture of the mentioned viruses (Sensevdi et al., 2024). In case of a possible failure in viral injection tests, vancomycin treatment will be applied. It is shown that vancomycin is effective on *M. smithii* but not *Lactobacillus reuteri* and *Escherichia coli*. Even though the specificity of vancomycin treatment on whole microbiota remains unknown, it possibly has a broader effect on microbiota due to its known gram-positive eliminating speciality (Million et al., 2013).

Statistics for Microbiome and Metabolome Change Analysis. We are planning to apply combinations of different statistics tools to analyze all of our bioinformatics data obtained from all mentioned applications. For this, 3 main applications of statistics will be combined; correlation-based methods, condition dependence models, and network-based methods. Multiple packages are present for the metabolite/metabolome, microbiota, and trans-kingdom analysis. From correlation-based methods; SparCC, Meta-Network, Correlation-Centric Network, from condition dependence models; MDiNE and MixMPLN, from network-based methods; Multi-Omics Factor Analysis will be used (Matchado et al., 2021).

5) Project literature

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6) Table with budget of the project

	Year 1	**Year 2**	**Year 3**	**Total**
Direct costs	**313,520**	**190,450**	**41,400**	
Personnel	53,800	47,700	41,400	142,900
Research equipment	60,000	128,000	-	188,000
Reagents, chemicals	194,520	14,750	-	209,270
Additional laboratory supplies	16,000	-	-	16,000
Indirect costs	**17,000**	**14,000**	**14,000**	**45,000**
Total				**582,380**

7) Breakdown of project costs including justification and relevance for the tasks in the project.

7.1 Direct costs

All costs and salaries are indicated in PLN. Costs of equipment, reagents and other supplies are approximated.

a. Personnel salaries

Position/Contribution	**Salary per month**	**Months paid in year 1**	**Months paid in year 2**	**Months paid in year 3**	**Total**
PI 1	1,200	x 12 = 14,400	x 12 = 14,400	x 12 = 14,400	43,200
Contribution to the project:	Research planning, sequencing, bioinformatic analysis, biophysical analyses (i.e. chromatography), statistical inference, and preparation of conference presentations and papers.				
PI 2	1,200	x 12 = 14,400	x 12 = 14,400	x12 = 14,400	43,200
Contribution to the project:	Research planning, manipulation of virus and infection of archaea, bioinformatic analysis, statistical inference, preparation of conference presentations and papers.				
Collaborator	800	x 2 = 1,600	-	-	1,600
Contribution to the project:	Guidance and instruction (to PI 2) on viral infection of archaea.				
Laboratory technician 1	900	x 12 = 10,800	x 1 = 900	-	11,700
Contribution to the project:	Collecting human fecal samples and processing for sequencing.				
Laboratory technician 2	900	x 2 = 1,800	x 12 = 10,800	x 6 = 5,400	18,000
Contribution to the project:	Fecal transplantation, implementation of diet to rats, fecal sampling, and processing for sequencing.				
Research assistant	600	x 12 = 7,200	x 12 = 7,200	x 12 = 7,200	21,600

Contribution to the project:	Administrative paperwork, placing orders and management of laboratory supplies, maintenance of lab rats.				
Medical practitioner	300	x 12 = 3,600	-	-	3,600
Contribution to the project:	Clinical assessment of volunteers.				
				Total:	142,900

b) Research equipment and reagents.

Application: Human Fecal Sample Collection		**Units x Cost**	**Total**
Equipment	Fridge	1 x 15,000	15,000
	-80°C Freezer	1 x 45,000	45,000
Reagents and chemicals	Protease Inhibitor (1.5mL)	3 x 490	1,470
	Cryoprotectans	2 x 350	700
Additional supplies	Sample collection kit (bags, sanitizer, gloves, tongue depressor, sample container)	150 x 40	6,000
Justification	The listed equipment above is needed to increase sample collection in our laboratory. The reagents are specific to preserve microbiota in fecal samples. The sample collection kit is a general standard for the applicability of sample collection.		
Application: Germ-Free Rat Preparation and FMT			
Equipment	ISOcage P	640 x 200	128,000
Reagents and chemicals	Microbiome-free nutrients (5kg) and water (5L)	100 x 50	5,000
	High-fat microbiome-free nutrients (5kg)	70 x 50	3,500
Additional supplies	Oral gavages	1000 x 10	10,000
Justification	The listed equipment is one of the latest technologies for microbiota research with rodents and provides a sterile environment which is a must in our project. The listed reagents above are routines for animal handling. The additional supply mentioned is the crucial for FMT application.		
Application: Amplicon Sequencing			
Reagents and chemical	Maxwell® RSC Fecal Microbiome DNA Kit (50prp.)	30 x 200	6,000

	HotStarTaq Master Mix Kit (2500 U)	2 x 2,500	5,000
	Nuclease-free water (1L)	5 x 630	3,150
	Agarose (500g)	5 x 5,700	28,500
	SPRIselect Magnetic Bead (60 mL)	1 x 5,300	5,300
	NextSeq 500/550 High Output Kit (300 Cycles)	10 x 6,000	60,000
	NextSeq 500/550 FlowCell	6 x 10,000	60,000
	1X TE buffer (5L)	10 x 1,200	12,000
Justification	The listed reagents and chemicals above are specific to amplicon sequencing steps for microbial organisms and to obtaining the most accurate genetical information		
Application: LC/MS			
Reagents and Chemicals	Sample Extraction Kits	5 x 900	4,500
	Sample Cleanup Kits	5 x 850	4,250
	Internal Standard Kits	5 x 500	2,500
	Quality Control Kits	5 x 700	3,500
Justification	The listed reagents above are routinely used for LC/MS applications. Among them, the Internal Standard Kit is specific to the metabolites mentioned in the methodology.		
Application: Viral Mixture Tests and Use			
Reagents and Chemical	SSVs cultures	1 x 1,000	1,000
	Pleolipoviridae cultures	1 x 1,000	1,000
	and arTVs cultures	1 x 1,000	1,000
	Viral Transport Media (500 mL)	1 x 900	900
Justification	Testing Viral Injection Method is a revolutionary step for microbiota studies and vital for on of the research questions. The usage of viral cultures above is unrivaled, therefore it adds a high-point novelty to this research.		

c) Readily available equipment, facilities and reagents:

The equipment required to prepare amplicon libraries, such as: thermocyclers, shaker with adaptors for 96 well plate, centrifuges, thermoblocks, bioanalyzer / nanodrop for DNA quantification, sequencing platform, are readily available for our team at the Jagiellonian University.

Laboratory rats will be kept in a facility belonging to the Jagiellonian University, where we will also conduct the fecal transplantation.

7.2. Indirect costs

	Justification	**Units x Costs**	**Total**
Book and journal licenses	Access to the newest research and other literature materials.	3,000	3,000
Scientific article publication	Yearly publication of findings in high profile scientific journals.	3 x 14,000	42,000
		Total	**45,000**

Title: Pheromone trailing in *Daboia russelii* among kins

Authors: Navina Francis, Afni Marpaung

Summary

Snakes are often perceived as solitary creatures due to their secretive nature and the challenges of studying them in their natural environment. However, contrary to this belief, many snake species do exhibit group behaviours such as aggregations for various purposes like caring for young, defending against threats, and recognizing kin. When snakes move, they leave behind chemical traces including integumental lipids and elements like ketones, which play a role in chemical communication, including the transmission of pheromones. Pheromones are chemical signals emitted by organisms to convey information about gender, age, size, reproductive status, location, and general health. While multiple studies have investigated pheromone trailing for aggregation in squamates (including snakes), little to no information exists about social behaviours like pheromone trailing in the Russell's viper (*Daboia russelii*). We aim to explore the genetic basis and external factors like temperature and stress influencing this behaviour. Temperature fluctuations and signals from stressed individuals are thought to be underlying factors in phenomena like aggregation and kin selection, respectively. To study the role of pheromone trailing, we will conduct validation experiments combining chemical analysis and behavioural experiments on closely related individuals and individuals raised together from a young age (cross-fostering). Environmental conditions are also known to affect pheromone trailing behaviour in snakes. Y-maze based behavioural experiments will be used to test the trailing behaviour of cross-fostered and sibling snakes, while also investigating the impact of external factors such as temperature and stress on pheromone trailing behaviour.

1. Scientific Goal

The belief of killing *D. russelii* will attract other snake is a superstition without any scientific science basis so far. Snakes are commonly seen as the least sociable among vertebrates, primarily because they are secretive and hard to study in their natural habitat, rather than because there's direct proof of their unsociability (Clark, 2004). Aggregations or gathering in groups occur among many snake species and in a variety of contexts in nature (Gillingham, 1987). Animals aggregate for active allo-parenting, group defence, and kin recognition (Vonhof et al. 2004). Snakes demonstrate the ability to find fellow members of their closely related species by following pheromone trails. When snakes shed their skins, they leave behind chemical traces like integumental lipids, aiding juvenile in returning to the den for aggregating. *D. russelii* is known to possess chemical elements like ketones in its skin and cloacal scent gland, which contribute to chemical communication such as pheromones Andonov et al. (2023). Pheromones are chemical signals emitted by organisms to communicate various information, including gender, age, size, reproductive status, location, and general health (Ford, 1981; Mason et al., 1989; Wilmes et al., 2012). Snakes exhibit chemosensory behaviours to perceive their surroundings and differentiate between scents from closely related conspecifics, which offers significant advantages for their aggregation and survival. The concept of kin-based groups in lizards and snakes is widely observed. An individual snake can increase its fitness by helping relatives. Multiple studies exist investigating pheromone trailing for aggregating in squamates, most of the research has focused on garter snakes (*Thamnophis* sp. and brown tree snakes (*Boiga irregularis).* However, we have little to no information about the social behaviour like pheromone trailing of *D. russelii* or the basis of exhibiting this behaviour i.e., if the behaviour will be the same if they were related individuals or fostered together and hence, they are considered as a very secretive group. We want to explore the genetic basis and other external factors like temperature and stress affecting this behaviour as temperature fluctuations and signals from distressed individuals are thought to be basis phenomena like aggregation and kin selection respectively. Hence, we hypothesise that *D. russelii* use pheromone trailing behaviour to track down its kin.

2. Significance

Snakes are currently still viewed negatively in society. Venomous snakes such as the *D. russelii* have been widely studied but generally focus on their venom content for health. The myth about killing a *D. russelii* can bring in other snakes has not yet been scientifically proven. Understanding snake behaviour allows us

to dispel myths and misconceptions about these animals. This myth motivates our exploration into the social behaviour of *D. russelii*. Prior studies indicate that snakes exhibit social connections as well. They do aggregation with closely related conspecifics, a phenomenon with the purpose of collective defence, particularly among the related individual. Snakes utilize chemical signals like pheromones for communication, a trait also observed in *D. russelii* snakes. The study of behaviours related to pheromone trailing in *D. russelii* not only confronts entrenched superstitions but also to unveil novel insights into their communication behaviour.

3. Concept and Work Plan

Animal

The research will be performed on one species of viper - Russell's viper (*Daboia russelii*). We have chosen this species as it has not been widely studied for its pheromone trailing behaviour and we would like to explore this niche. We will be establishing a laboratory colony of the species at the Institute of Environmental Sciences following acquirement of necessary ethical and legal permissions from the Regional Directorate of Environmental Protection and Local Ethical Committee.

Objective 1: Validation experiment to confirm the role of pheromone trailing using secretion extracted from cloacal scent gland.

In this objective, we would like to confirm the role of pheromone in the phenomenon of pheromone trailing in *D. russelii* by using a combination of chemical analysis and behavioural experiments on closely related individual, preferably from the same litter. Hence these set of experiments are designed to validate the presence of pheromones in the context of pheromone trailing and to extract this substance for further investigation.

Obj.1.1: Chemical analysis of pheromones found in skin sheds and precloacal scent gland.

This experiment is designed to extract and analyse the components of pheromone secretions from precloacal scent gland of the snake and compare the results. Samples will be collected from the cloacal scent glands and skin sheds of the individuals. The samples from the precloacal scent glands and skin sheds will be marked with individual IDs and will be grouped as coming from kinship and into males and females.

Secretions from precloacal scent glands will be obtained manually by gently pressing the area with forceps and collecting the samples in a glass vial. Pheromone samples from skin sheds will be obtained by extraction of lipid fraction wherein skin sheds will be soaked in *n*-hexane for 24 hours to get the extract. The secretion samples from both the techniques will be analysed using gas chromatography/mass spectrometry.

From this, we will separate out the aromatic pheromone components ie, specific ketones and aldehydes that are characterised for further investigation.

Obj.1.2: Chemical analysis of pheromone found in the soil after trailing.

We will again use gas chromatography/mass spectrometry to test the presence of previously identified pheromone components from trail soil samples to confirm the secretion of these components during the act of trailing. We will let a snake pass through a track based with soil and collect the soil sample from the trail made by the individual's passage. We will then test the presence of the pheromone compounds extracted in obj.1.1 in the soil samples collected. This will confirm the presence the characterised pheromone compounds in the trail created by the snake.

Obj.1.3: Validation experiment to confirm the role of pheromone in pheromone trailing using secretion extracted from cloacal scent gland.

In this part we will verify that the individuals following the trails of their conspecifics are doing so due to the presence of pheromone compounds in these trails. This will act as preliminary study on which we will build the rest of the study. For this experiment, we will use a standard Y-shaped maze commonly used to

study squamate behaviour. There will be 2 conditions that will be tested: a) choice of arm of an individual following trail making of another individual and b) choice of arm of an individual following the application of traces of pheromone compound on one of the arms of the maze. We expect that in both cases they will choose treatment arm over control arm as we assume traces of pheromone and pheromone trail will have the same effect. As these set of objectives are backed by literature and it is a validation of existing data, there are no risks associated with this part.

Objective 2: Test for the effect of genetic background on pheromone trailing in *D. russelii.*

In this objective, we want to assess the role of genetic background on the pheromone trailing behaviour in the species and would like to see if there is any significance difference in this behaviour based on if they are siblings or fostered siblings. To fulfil this objective, we will take 6 pregnant mothers in the same phase of gestation period. As soon as the mothers give birth, we will reciprocally cross-foster half of each litter with non-siblings from another litter. We will have 6 such groups with a set of males and females who are related and unrelated. To confirm the unrelatedness among the siblings of these 6 mothers, we will conduct a genetic screening based on allele sharing approach (allele matching cutoff score, AMCOS, Kakkar. S et al. 2021). We will be sending our samples to Department of forensic sciences, PGIMR Chandigarh to conduct this analysis.

With these groups, we will make combinations of pairs of male and female individuals separately. The combinations are as follows: a) familiar siblings b) unfamiliar siblings c) familiar unrelated individuals and d) unfamiliar unrelated individuals. These pairs will be subjected to the Y-maze experiment where the first partner of the pair will be let to trail along through the arm of our choice while the other arm is blocked for control and the second partner of the pair is left to trail along the arm of choice while the control arm is also open. The same experiment will be done in 25 replicates and the treatment arm will be changed alternatively. In this part, we expect from the consolidated result that they incline more towards choosing related individuals. The risk associated with this part is that they respond equally to the partners pheromones irrespective of their genetic background, in that case we will analyse the results combining all data and move on to look for effect of external factors.

Objective 3: Test for the effect of external factors on pheromone trailing in *D. russelii.*

In this part we will check for the effect of external factors and in specific, temperature and stress on the pheromone trailing behaviour in the species. Temperature fluctuations are common in the environment and have been known to be linked with aggregation in snakes and we expect that they use pheromone trailing to track down their conspecifics and if given a choice, related individuals over unrelated individuals. Stress is an important external factor as they are subjected to constant interactions with potential predators and adverse climatic conditions. Evidence suggest that they release chemical cues during these situations of distress. Hence, we want to check the pheromone trailing behaviour of this species in response to a sibling or an unrelated individual in distress.

Obj.3.1: Testing the effect of temperature on pheromone trailing in *D. russelii.*

For this experiment, we will be using the same combinations and experimental setup as described in obj.2 but with 2 sets of temperature conditions. As they are comfortably kept captive within a temperature range of 28-31ºC, we will choose cold treatments of 20ºC and 24ºC and warm treatments of 35ºC and 39ºC to record their behaviour. These temperature treatments will not harm them as they are known to be found in different and extreme climatic conditions around the Indian subcontinent (Stidworthy, 1974). To fulfil this objective, we will conduct the same experiment described in obj.2 but with different temperature treatments mentioned above. The experiment from obj.2 will act as a control for this as it is the same experimental setup but with normal temperature conditions.

Obj.3.1: Testing the effect of stress on pheromone trailing in *D. russelii.*

We will use the same combinations and experimental setup in this part as well but with the first individual of each pair being stressed induced (distressed partner). This species is known to respond violently to even small disturbances or interference (Whitaker, 1989). Hence, introduction of a foreign body and in this case a 3D model of a mongoose which is its most natural predator (Cyriac, 2022). We expect that the partners of distressed individuals avoid the arm towards them as they sense chemical cues from these distressed individuals indicating danger.

We will have a treatment and control group here, treatment being distressed individuals and control being individuals with no external stress stimuli. In both treatment and control, the first individual of the pair will be let to trail through the Y-maze first while one of the arms will be blocked as control. As they reach the collection box, the individuals from treatment group will be exposed to the external stimuli and control will be exposed to none. Post exposure, the first individual will be removed from the collection box and second individual will be allowed to choose the arm while the control arm is also open.

4. Methods

4.1 Access to the equipment

All the experiments will be conducted in the Institute of Environmental Sciences as we have access to multiple climatic chambers. These are suitable for conducting all the Y-maze based behavioural studies including the temperature mediated one. The chemical analyses including gas chromatography and mass spectrometry will be carried out in the Faculty of Chemistry, Jagiellonian University. The animals will be transported from India with the help of Dr. Chirag Jyoti Roy.

4.2 Maintenance of the animals

a) Enclosure/Housing requirements: We will house the snake in a terrarium with a minimum size of 4ft x 2ft x 1.6ft (or 2ft height for some species). The base will be lined with loose soil to provide a burrowing substrate and hiding places. We will also install an artificial termite mound made from plaster for further enrichment. To accommodate the snake's active nature, the enclosure will be landscaped to create basking areas. As basking is crucial for this species, we will maintain a warm zone within the terrarium at 28-31°C. Additionally, the humidity will be controlled between 70-80%.

b) Feeding: We will be feeding this species a diet consisting exclusively of rodents in captivity. Lab rats are a suitable option for this purpose. To ensure animal welfare, the rats will be humanely euthanized before their gastrointestinal systems are carefully removed to minimize disease risk. The amount and frequency of feedings will be determined on an individual basis, considering the activity level and health of each animal.

4.3 Extraction of pheromones from skin sheds and soil

Lipid fractions from skin sheds will be extracted following protocol from Baedke et al (briefly explained inobj.1.1). The samples will be extracted from soil in a similar way by soaking soil in *n*-hexane for 48 hours instead of 24.

4.4 Chemical analyses of pheromones

Lipid fractions extracted from the cloacal secretions, skin sheds and soil will be analysed using gas chromatography and mass spectrometry following the protocol from Andonov, et al. (2020).

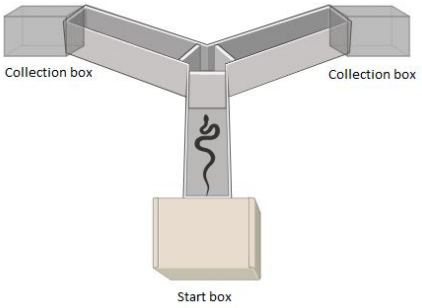
We will be using an Agilent 7820A GC System Plus gas chromatograph coupled with a 5977B Mass Selective detector and flame ionization detector for the GC/MS analysis. The system will be equipped with a mid-polar capillary column DB-17HT (J&W Scientific) with the following specifications: 60 meters in

length, 0.25 mm inner diameter, and 0.25 µm film thickness. The oven temperature will follow a programmed ramp: starting at 75°C and holding for 5 minutes, then increasing to 150°C at a rate of 10°C/min. From 150°C, the temperature will rise to 250°C at a rate of 5°C/min, followed by another increase to 275°C at a rate of 2.5°C/min. Finally, the temperature will climb to 325°C at a rate of 10°C/min and hold for 30 minutes at that final temperature. Helium (99.999%) will be used as the carrier gas with a constant flow rate of 0.8 mL/min. For detection, we will use electron ionization (EI) mode at 70 eV electron energy. The ion source temperature will be set to 230°C, and the quadrupole temperature will be 150°C. The mass selective detector will operate in a scan range of 45 to 1000 m/z. MassHunter Workstation Software (Revision B.06.07, Agilent Technologies) will be used for instrument control and data collection.

4.5 Y-maze experiment

We will be conducting standard Y-maze (fig.1) based on Richard et al. (2020). The maze consists of a starting box (3 x 0.56 x 0.46 m) leading to a Y-junction, two arms (2 x 0.40 m each) leading to separate collection boxes (3 x 0.5 x 0.44 m each), walls made of PVC sides (2.5 x 15.2 cm) with a clear acrylic top for observation. The entire maze will be secured to a base (2.4 x 2.4 m). All components, including the top, sides, and boxes, will be meticulously cleaned with disinfectant (Micro® laboratory cleaner) and water between trials. We will air-dry them before reassembly. Disposable gloves will be worn during cleaning and setup. For each trial, the maze floor will be covered with fresh plastic sheeting and white Kraft paper. Finally, a layer of soil will be spread on top to provide a natural scent for the rats. The base will also receive new sheeting and paper before each use. The whole setup will be installed inside temperature and humidity-maintained chambers.

Figure 1. Y-maze box



4.6 Setting up temperature treatments for obj.3.1

For different temperature treatments, we will be regulating the temperature inside the chamber in which the setup is installed. After the temperature is changed for each trial, a buffer time of 1 hour will be given for the whole chamber to be of uniform temperature.

4.7 Inducing stress to first individual of each pair for obj.3.2

For inducing stress to first individual, we will be introducing a 3D model of mongoose which is its natural predator 5mins before recording the experiment. This experiment will be carried out based on the idea used in Clément et al. (2020).

4.8 4.8 Statistical analyses

We will be analysing the behavioural data using R statistical software (version 4.2.1). First, we will create contingency tables summarizing the choices made by individuals in each treatment group. These tables will show how many individuals chose the scent-marked side/arm and how many chose the control side/arm. Next, we will employ the binom.test function from the R stats package to conduct two-tailed binomial tests for each scent treatment. This will allow us to assess whether the number of individuals choosing a particular side/arm deviates significantly from what would be expected by chance (assuming a 50% probability of choosing either side).

We will analyse the relative concentrations of all detected compounds in the solution as our dependent variables. To identify significant differences between designated groups, we will first assess the normality of the chemical concentrations for each group separately.

Timeline

Project task	Semester					
	1	2	3	4	5	6
Purchase of necessary equipment and material						
Animal maintenance						
Chemical analysis of pheromones in skin sheds and precloacal scent gland						
Test for the effect of genetic background						
Testing the effect of temperature on pheromone trailing						
Testing the effect of stress on pheromone trailing						
Conference presentation and participation, and Article writing						

5. Reference

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6. Budget Plan

	Amount in PLN
Direct costs, including	179087
- personnel costs and scholarships	93000
- research equipment/device/software cost	30087
- other direct costs	56000
Indirect costs, including:	33881
- indirect costs of OA	0
- other indirect costs	33881
Total costs	211968

7. Budget plan justification

Type	Name of item	Justification	Person/ Unit	Months	Cost (PLN)	Total (PLN)
Direct cost						
Personal cost and scholarships	Principal investigator	Planning, preparing, and conducting experiments: Designing and creating Y-shape maze for experiments, preparing animal for the experiments (e.g. preparing meal, feeding, monitoring shedding cycle, collecting skin shed), maintaining the temperature of the cage, conducting the objective of experiments, data analysis and writing manuscripts	2	36	36000	
	Technical assistant	Assists with the purchase, transportation, and receipt of animals. keeps a sufficient supply of rat for foods.	2	36	57000	
Equipment	Y-shape maze	Behavioural test used to assess the pheromones trail of snake, made of plastic, easy to clean using ethanol 70%	8	36	25887	
	Camera	Recording snake behaviour pheromone testing	4	36	3200	
	Mongoose model	Natural predator model	4	12	1000	123087

Other direct cost	Animal maintenance	Boxes, water bowl, equipment for feeding, cleaning, paper towel, etc.		36	15000	
	Office supplies	Pencils, pen, notebook, printer papers, printer cartridges, etc		36	2000	
	Chemicals for GC/MC	chromatography column, the ferrules, 2 ml and 15 ml vials, 2 ml vials with septum, limited volume vials, hexane, etc.		6	8000	
	AMCOS analysis	Parentage analysis service, and sample shipping		6	30000	
	Laboratory consumables	Gloves, mask, alcohol, tapes, plastic container etc.		36	1000	56000
Indirect costs						
Other indirect costs	Cost in publication in Open Acces Journal	Journal APC with IF. 7 and score 200 points (e.g. Journal of Animal Science and Biotechnology)			10881	
	Book and journal	Material and reference for research and writing article			3000	
	Conference	Expenses related to traveling to participation to conferences, one conference per year for two members of team, For example, Societas Europea Herpetologia in 2013 cost around 1300 PLN for accommodation, 500 PLN for conference fee. Total cost will be different depending on location of the conference	2	36	2000	33881
Total (PLN)						211968

8. Reviews of grant proposal
8.1 Review No. 1

Title: Pheromone trailing in *Daboia russelii* among kins

Authors: Navina Francis, Afni Marpaung

1. Assessment of scientific quality of the research project (scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context)
The question is interesting, for zoologists but also for brad public. Its novelty and originality is somewhat unsure. I read: "Snakes utilize chemical signals like pheromones for communication, a trait also observed in *D. russelii* snakes." It sounds as research on the main question has been already done!! But, there is no citation after this claim. Perhaps something was done, not what the authors want to do, but they fail to explain what elements of their study would be truly original in relation to all snakes or this particular species.

2. Assessment of potential impact of the research project (the potential for substantial international impact on the research field(s) and for high quality research publications and other research outputs, taking into account the specifics of the research field and the variety of forms of impact and output; impact ought to be evaluated using an international context)
Reliable results could be well published in specialized journals. If kinship recognition were ascertained, even very good journals would pick a paper.

3. Assessment of feasibility of the research project (the feasibility of the proposed project, including the appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), other factors affecting the feasibility of the project)
Most of the planned research appears doable. I mean, the authors are probably correct in believing that they would manage to establish a colony of snakes, but I have no experience to judge it. I am more worried whether the chemical analyses can be so precise that differences between individuals will be discovered.

4. Are the costs to be incurred well justified with regards to the subject and scope of the research?
Rather yes, the costs of conferences is high, I do not know what are present standards.

5. Strengths of the proposal
The research can end up as a good behavioral study even if the chemical analyses fail. I mean that negative results, perhaps not completely negative, would also provide some answers. I like the idea of introducing the cold and predator stress.

6. Weaknesses of the proposal
Introduction, abstract and other parts of the text are not very well written, reader remains often unconvinced. More serious problem is in the study of relatedness. I would imagine, that a snake learn who are their kins by spending early life with them. How can it recognize smell if not by learning. Perhaps by comparing it to its own. Are there any indications of that. Furthermore, is there any other system that strict monogamy there. Under monogamy all little fellows would be kins, learning their smell would suffice. This is not explained, has it been considered?

8.2 Review No. 2

1. Assessment of scientific quality of the research project

Although the research question which the authors proposed is clear, the rationale behind undertaking such a project is not clear. In the section of 'Scientific Goal', they claim that there are "Multiple studies" on investigating pheromone trailing in aggregation behaviour, with a few examples in line 37, but they haven't cited any papers. Moreover, the claim that "Prior studies indicate that snakes exhibit social connections as well" in line 51 is not backed by any papers, even if it might be true. So as a reviewer, I'm unable to verify this.

Overall, the choosing an animal based on a superstition is questionable, and the authors have not totally convinced me why it is important to study the social behaviour of this snake. They should add existing literature on Russell's viper and mention the significance of conducting such research in a detailed manner. The authors claim that such a study has not been conducted in the past thus bring in the novelty of this research, but I don't see why this study can't be conducted on any other venomous snake which is easier to procure than a snake from India.

2. Assessment of potential impact of the research project

The authors haven't explicitly mentioned about the potential impact of their project in the 'Significance' section of the study. They do mention about the novel insights they will obtain from this project on the communication behaviour Russells' viper. Although they haven't mentioned, it seems that such a study could contribute to the field of animal behaviour, ecology, and human-wildlife interactions.

This study may also have an impact on the research projects investigating pheromone trailing behaviour in snakes from different ecosystems around the world. But the impact of this research might not be enough due to limited sample size and underrepresentation of diversity. Since this is a study is on a specific snake species from India, it might reduce its impact beyond the given study area and ecosystem. This might limit its international reach. It is also important for them to effectively plan how to communicate the findings of this research, as their goal suggests that they want to bust a myth related to these snakes.

3. Assessment of feasibility of the research project

The project is going to benefit from the access to specialized equipment for behavioural and chemical analyses, such as the availability of GC/MS facilities and climatic chambers. It will improve the reliability of the results. Specific housing requirements and feeding protocols reflect the goal of providing humane treatment of the snakes involved in the study.

However, for validation experiments choosing individuals from the same litter may not be diverse enough to accurately represent Russell's viper population, which could potentially limit the generalizability of the results. The sample size in the Objective 2 seems to be limited, as their research plan mentions using only six pregnant mothers to establish group of related and unrelated individuals. It may reduce the statistical vigour of the study. The method for extracting pheromones from skin shed and soil is briefly described, without any optimization steps, which may potentially affect the quality of pheromones.

I'm also not convinced about the quality of the research, as it is based on generalised assumptions about the effect of pheromones and expected behaviour in snake. It overlooks other complexities of social interaction and there is lack of evidence to support these assumptions.

Working with a highly venomous snake like Russell's vipers can be a risk factor for the researcher. But there is no mention of any safety protocol for the research team. A more comprehensive risk assessment might be needed for each of the objectives of the project since this is an exploratory project. The authors have considered obtaining ethical permissions, but it seems that they might have problems, considering they want to work with live specimens. They have not considered any alternative plans in case they don't obtain these permissions. Moreover, transporting live animals from India might be challenging, and may affect the feasibility of conducting this research. The section about risk assessment of conducting this project is entirely missing.

4. ***Are the costs to be incurred well justified with regards to the subject and scope of the research?***

The justification of the budget is quite detailed. The roles of project personnel clearly defined, but in my opinion the salaries of the Technical Assistants and the Principal Investigator are quite low. It is good to know that ethical considerations (animal housing, maintenance) are taken while allocating the budget for equipment.

Conference expenses should be added to direct costs. Moreover, the conference expenses do not add up according to the justification, and only one conference in a year for two team members can limit opportunities for presentation of results and knowledge exchange. There is no budget allocated for transporting live animals from India. All these factors could lead to budget constraints and difficulty to execute this project.

In Budget plan (line 263), the indirect costs of OA is 0, which does not tally with the budget justification (line 265). Moreover, in the budget justification, indirect costs of OA are way more than 2% of direct costs.

5. ***Strengths of the proposal***

- It has clear objectives, and a comprehensive approach to address various aspects of pheromone trailing behaviour such as genetic and environmental factors.
- The budget is detailed in terms of equipment
- Animal welfare is considered
- There is collaboration and access to research facilities, which could benefit the project.

6. ***Weaknesses of the proposal***

- Lack of justification for conducting this project.
- Lack of reliability and validity of experimental procedures.
- Limited sample sizes, representation
- Underestimated and missing costs in budget
- Lack of risk assessment and alternative plans
- Shortcomings in impact of this research

8.3 Review of No. 3

Title of the project: Pheromone trailing in *Daboia russelii* among kins

The proposal addresses the potential of chemoreception in the snake *Daboia russelii* for kin recognition.The authors ask about environmental and genetic factors that influence pheromone trailing in the snake.They address these questions using experimental and behavioral approaches.

1. **Assessment of scientific quality of the research project** ***(scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context)***

I consider the proposal original since it addresses a myth with a potential biological background. However, it does not propose a novel question, and it does not offer a strong scientific reason for conducting this research, despite recognizing the relevance of the species as a source of treatment for clinical applications. Aspects to take into consideration could be:

a. Does the myth really pose a threat to these species? – and if it does, then does it mean that itspopulations are so big that they are frequently encountered by humans?
b. Are there any wild populations of these species that are a highly sought source for healthapplications?
c. What is the ecological relevance of the species in its ecosystem? Does it contribute to the controlof populations of other species such as rodents, other snakes, etc.

2. **Assessment of potential impact of the research project** ***(the potential for substantial internationalimpact on the research field(s) and for high quality research publications and other research outputs,taking into account the specifics of the research field and the variety of forms of impact and output;impact ought to be evaluated using an international context)***

It is not clear what the concrete output of this project would be. It will certainly provide information on behavioral data on *D. russelii*. However, if the species lacks ecological/biological/economic relevance,the impact on the field of research might be low.

3. **Assessment of feasibility of the research project** ***(the feasibility of the proposed project, includingthe appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), otherfactors affecting the feasibility of the project)***

The project aims "to explore the genetic basis and other external factors like temperature and stress affecting" pheromone trailing in *D. russelii*. It is stated that these species communicate through chemicalsignals, and it is suggested that a snake would follow another for aggregation, which increases survivalfor alloparenting, group defense, or kin differentiation. However, the proposal only accounts for abiotic conditions (i.e. temperature, stress due to predation) and genetic background, leaving out that:

a. It is unknown if these species aggregate (for any given reason, such as group defense), which would be a cause for following a pheromone trail when the snake assumes itself in danger. Snakesof other species can aggregate, but if this species can do so too is not tested here.
b. Allo-parenting, as a reason to follow pheromone trails in looking for kin, calls for age-selected snakes. It is not clear if this is done in the experiments proposed here.

c. Sex is not tested as one of the factors affecting pheromone trailing. If the decision of a sexuallymature snake is based on sex, it might invalidate the effect of the genetic background.

All these omissions pose a risk to the interpretation and/or reliability of the results of the experiments.

4. Are the costs to be incurred well justified with regard to the subject and scope of the research?

Two PIs will carry out four experimental tasks. However, since the sample size is unclear and the study design is fairly simple. Therefore, unless the sample size is big enough to justify that completing each task will take one year, three years of research funding calls for a more complex study design. If not, reducing the length of the project is strongly recommended. One observation is that there is a discordance of 1,000 PLN in (6.)Budget plan.

5. Strengths of the proposal

I consider that the main strength of the proposal is that the researchers will test the link between behavior and genetics in snake biology. Additionally, the questions that they ask on kin recognition would provide results that could likely have more impact on population genetics and, potentially, higher significance for the conservation of endangered snake species or for advancing our understanding of the impact of inbreeding on genetic diversity if this is a frequent phenomenon in snake species. Potentially, asking questions pointing toward these directions might increase the strength of the proposal.

6. Weaknesses of the proposal

The overall aim of the project and potential results lack application or scientific/biological relevance. Asking an innovative question or using a novel method to address this issue more broadly would increase the chances of the results being applied to other species or the methods being applied as a standard in international research.

Title: Pheromone trailing in *Daboia russelii* among kins

Authors: Navina Francis, Afni Marpaung

Summary

Snakes, like other animals, have complex social behaviour that is influenced by kin or biological relative individual, past interactions, and environmental conditions. This study focuses on pheromone trailing among kin of the Russell's viper. There is little information about pheromone trailing in this species. This study's hypothesis is that *D. russelii* uses pheromones to find their kin. The aim of this project is to confirm the role of pheromones, evaluate the impact of kin, and investigate the effects of environmental factors on pheromone trailing. No previous studies have comprehensively analysed these aspects together. The findings will give new information on pheromone trailing behaviours of Russell's viper among kins, as well as potential applications for animal control during human-snake conflicts, contributing to biology field especially animal behaviour while also could be aiding in conflict resolution.

1. Scientific Goal

The belief of killing snake will attract other snake is a superstition without any scientific science basis so far. Snakes are commonly seen as the least sociable among vertebrates, primarily because they are secretive and hard to study in their natural habitat, rather than because there's direct proof of their unsociability (Clark, 2004). Aggregations or gathering in conspecifics occur among many snake species and in a variety of contexts in nature. It is often driven by a variety of factors, such as the mutual attraction of individuals and a beneficial habitat or condition (Gillingham, 1987). In these situations, snakes often use a variety of information to regulate their behaviour. For example, shed skin or scent and other sensory signals such as pheromone are crucial for attracting or locating a partner for mating and outside the context of mating (Gregory et al. 1987). However, the previous experience of snakes with conspecific also plays an important role (Hamilton, 1964). This experience allows animals to develop preferences based on familiarity and kinship, which can influence their social interactions (Brown and William, 1983). Regarding kinship, animals often prefer to gather with known or familiar individuals due to sense of trust and comfort in interactions with familiar individuals, as well as a possible greater sense of security in a social environment consisting of familiar individuals (Kristina et al., 2015; Keller et al., 2017). Thus, social behaviour in many animal species is often complex and can be influenced by a variety of factors, including previous experience, kinship, familiarity, and environmental conditions.

This project focus on the pheromone trailing among kin of the Russell's viper snake (*Daboia russelii*). This snake, ranging from medium to large size and venomous, is found across the Indian subcontinent, to China and Taiwan and southward to the Lesser Sunda islands (Daniel, 1983). Russell's viper exhibits aggregate behaviour not only during mating but also within nest sites among newborn siblings (Senji et al., 2021). This tendency towards aggregation is advantageous for their overall fitness, heightened vigilance, and defence. *D. russelii* is known to possess chemical compounds like ketones in its skin and cloacal scent glands (Andonov et al., 2023), which play a role in chemical communication, including the release of pheromones for mating. Many studies have primarily concentrated on examining sex pheromones in snakes such as *Thamnophis* sp., and *Boaedon fuliginosus* (Ford 1981; Wilmes et al. 2015). Investigations into pheromone trailing and kin recognition have been conducted on several species including *Agkistrodon piscivorus* and *Crotalus horridus*, (Clark 2004; Hoss et al. 2015). However, we have little to no information about the social behaviour like pheromone trailing of *D. russelii* or the basis of exhibiting this behaviour i.e., if the behaviour will be the same if they were related individuals or fostered together and hence, they are considered as a very secretive group. **We want to explore the genetic basis and other external factors like temperature and stress affecting this behaviour as temperature fluctuations and signals from distressed individuals are thought to be basis phenomena like aggregation and kin selection respectively. Hence, we hypothesise that *D. russelii* use pheromone trailing behaviour to track down its kin.**

Objective 1: Validate the role of pheromone in pheromone trailing behaviour of *D. russelii*.

Objective 2: Test for the effect of genetic background on pheromone trailing in *D. russelii.*

Objective 3: Test for the effect of external factors on pheromone trailing in *D. russelii.*

2. Significance

D. russelii is one of the so-called 'big four' deadly snakes in India. It is medically important for its venom potential (Suraweera et al. 2020). *D. russelii*. Many studies on Russell's viper only focus on the venom for health benefits. To our knowledge, research on the behavior of this snake has never been carried out. In this study, we tested the behavior of the Russell's viper in relation to its trailing pheromone towards Kin. Studies of snake behavior and trailing pheromone have only focused on ritualistic behaviors involved in reproduction. This project provides new information about the behavior of these snakes regarding trailing pheromones outside the mating context. Apart from that, we also tested the influence of genetic relationships and the environment, such as temperature and

stress, on snake aggregation behavior. No previous studies have comprehensively analysed both aspects together. Therefore, our results will give a better understanding of this snake behavior. In addition, this research can indirectly be used in animal control in cases of human-snake conflict. Sex pheromones have been studied and shown to be used to help control invasive brown tree snakes in Guam (Mathies et al. 2013). The same thing can also be done to overcome human conflicts with Russell's viper. As reported, this snake seems to be responsible for the most fatal snake bites and cases of long-term morbidity in India. The outcome of this research will contribute to the field of biology and animal behaviour and indirectly could help resolve human-snake conflict.

3. Concept and Work Plan

The research will be performed on one species of viper - Russell's viper (*Daboia russelii*). We have chosen this species as it has not been widely studied for its pheromone trailing behaviour and we would like to explore this niche. We will be establishing a laboratory colony of the species at the Institute of Environmental Sciences following acquirement of necessary ethical and legal permissions from the Regional Directorate of Environmental Protection and Local Ethical Committee. We will plan to maintain at least 10 separate litters, with at least 15 individuals each to conduct the experiments.

To address **objective 1** of our project, we will first chemical methods namely gas chromatography/mass spectrometry to analyse pheromone samples from cloacal secretions and skin sheds of the animals and compare them with traces of chemicals from the soil they trail through to confirm the presence of secretions while they trail. We will then validate the role pheromones in this behaviour by conducting behavioural experiments where we compare if they respond to actual trails of conspecifics and presence of traces of these chemical compounds we extracted previously in the same way. This will answer our first question of "are these animals following trails of conspecifics sensing compounds that are characterised as pheromones" or if it is an artefact.

In **objective 2**, we will explore how kinship affects their choice of following a conspecific's trail or in other words if they will identify their kins over unrelated individuals and follow their trail even if they have not been raised together. This will allow us to understand if pheromone trailing in these animals are governed by their genetic relatedness or solely being raised together. We will use the method of cross-fostering where individuals of different litters will be interchanged to have paired combinations of a) familiar siblings b) unfamiliar siblings c) familiar unrelated individuals and d) unfamiliar unrelated individuals. We will use Y-maze based behavioural experiments to compare their choices towards their pairs in each of these combinations. We expect from the consolidated result that they incline more towards choosing related individuals.

In **objective 3**, we are considering the effect of external factors namely temperature and stress. Temperature fluctuations are common in the environment and have been known to be linked with aggregation in snakes and we expect that they use pheromone trailing to track down their conspecifics and if given a choice, related individuals over unrelated individuals. Stress is an important external factor as they are subjected to constant interactions with potential predators and adverse climatic conditions. Evidence suggest that they release chemical cues during these situations of distress (Lind C, 2018). To study the effect of temperature, we will conduct experiment similar to the one in objective 2, but under 4 different temperature conditions (2 low and 2 high). To study the effect of stress (in this context, having a distressed individual in the vicinity), we will again conduct similar experiment but with first individual of every pair is induced stress by exposing it to artificial predation cue. This will give us information about how their choices will change under adverse conditions like temperature fluctuations or predation risk.

Risk assessment

We anticipate the possibility that they do not respond significantly different among the different combinations that we have designed. However, in that instance we will design the same experiments with male-female combinations from same and different litters and assess the effect of external factors

on those combinations as that is also a less explored niche. To tackle the risk of handling these venomous snakes, we will be appointing 3 persons who are experts in handling them with all necessary safety protocols.

Table 1. Project Timeline.

Project task	Timeline (semester)					
	1	2	3	4	5	6
Purchase of necessary equipment and material						
Animal maintenance						
Chemical analysis of pheromones in skin sheds and precloacal scent gland						
Test for the effect of genetic background						
Testing the effect of temperature on pheromone trailing						
Testing the effect of stress on pheromone trailing						
Conference presentation and participation, and Article writing						

4. Methods

4.1 Access to the equipment

All the experiments will be conducted in the Institute of Environmental Sciences as we have access to multiple climatic chambers for 3 years (Table 1). These are suitable for conducting all the Y-maze based behavioural studies including the temperature mediated one. The chemical analyses including gas chromatography and mass spectrometry will be carried out in the Faculty of Chemistry, Jagiellonian University. The animals will be transported from India with the help of a collaborator who has expertise in maintaining them.

4.2 Maintenance of the animals

a) Enclosure/Housing requirements: We will house the snake in a terrarium with a minimum size of 3m x 2m x 1m. The base will be lined with loose soil to provide a burrowing substrate and hiding places. We will also install an artificial termite mound made from plaster for further enrichment. To accommodate the snake's active nature, the enclosure will be landscaped to create basking areas. As basking is crucial for this species, we will maintain a warm zone within the terrarium at 28-31°C. Additionally, the humidity will be controlled between 70-80%.

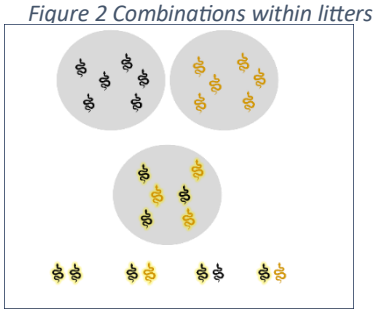
b) Feeding: We will be feeding this species a diet consisting exclusively of rodents in captivity. Lab rats are a suitable option for this purpose. To ensure animal welfare, the rats will be humanely euthanized before their gastrointestinal systems are carefully removed to minimize disease risk. The amount and frequency of feedings will be determined on an individual basis, considering the activity level and health of each animal.

4.3 Extraction of pheromones from skin sheds and soil

Lipid fractions from skin sheds will be extracted following protocol from Baedke et al using *n*-hexane method. Cloacal secretions will be collected by gently pressing the precloacal region of the animal with forceps and collecting it inside a vial. Samples from skin shed will be extracted by soaking the skin sheds in *n*-hexane for 24 hours. We will let a snake pass through a track based with soil and collect the soil sample from the trail made by the individual's passage. The samples will be extracted from soil in a similar way by soaking soil in *n*-hexane for 48 hours. The samples from the precloacal scent glands and skin sheds will be marked with individual IDs and will be grouped as coming from

kinship and into males and females separately. The secretion samples from both the techniques will be analysed using gas chromatography/mass spectrometry.

Figure 2 Combinations within litters



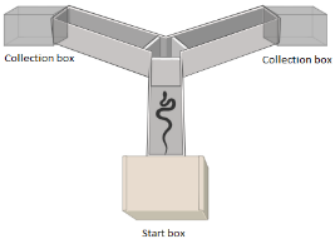
4.4 Chemical analyses of pheromones

Lipid fractions extracted from the cloacal secretions, skin sheds and soil will be analysed using gas chromatography and mass spectrometry following the protocol from Andonov, et al. (2020). We will be using an Agilent 7820A GC System Plus gas chromatograph coupled with a 5977B Mass Selective detector and flame ionization detector for the GC/MS analysis. The system will be equipped with a mid-polar capillary column DB-17HT (J&W Scientific) with the following specifications: 60 meters in length, 0.25 mm inner diameter, and 0.25 µm film thickness. The oven temperature will follow a programmed ramp: starting at 75°C and holding for 5 minutes, then increasing to 150°C at a rate of 10°C/min. From 150°C, the temperature will rise to 250°C at a rate of 5°C/min, followed by another increase to 275°C at a rate of 2.5°C/min. Helium (99.999%) will be used as the carrier gas with a constant flow rate of 0.8 mL/min. For detection, we will use electron ionization (EI) mode at 70 eV electron energy. MassHunter Workstation Software (Revision B.06.07, Agilent Technologies) will be used for instrument control and data collection.

From this, we will separate out the aromatic pheromone components i.e., specific ketones and aldehydes that are characterised for further experiment purpose.

4.5 Y-maze experiment

Figure 1. Y-maze box



We will be conducting standard Y-maze (fig.1) based on Richard et al. (2020). The maze consists of a starting box (3 x 0.56 x 0.46 m) leading to a Y-junction, two arms (2 x 0.40 m each) leading to separate collection boxes (3 x 0.5 x 0.44 m each), walls made of PVC sides (2.5 x 15.2 cm) with a clear acrylic top for observation. The entire maze will be secured to a base (2.4 x 2.4 m). All components, including the top, sides, and boxes, will be meticulously cleaned with disinfectant (Micro® laboratory cleaner) and water between trials. We will air-dry them before reassembly. Disposable gloves will be worn during cleaning and setup. For each trial, the maze floor will be covered with fresh plastic sheeting and white Kraft paper. Finally, a layer of soil will be spread on top to provide a natural scent for the rats. The base will also receive new sheeting and paper before each use. The whole setup will be installed inside temperature and humidity-maintained chambers.

4.6 Validation experiment to confirm the role of pheromone in pheromone trailing using secretion extracted from cloacal scent gland.

For this experiment, we will use a standard Y-shaped maze described in point 4.5. There will be 2 conditions that will be tested: a) an individual following trail of another individual and b) an individual following the application of traces of pheromone compound on one of the arms of the maze. We expect that in both cases they will choose treatment arm over control arm as we assume traces of pheromone and pheromone trail will have the same effect.

4.7 Testing for the effect of genetic background on pheromone trailing in *D. russelii.*

As soon as the mothers give birth, we will reciprocally cross-foster half of each litter with non-siblings from another litter. We will have 6 such groups with a set of males and females who are related and unrelated. To confirm the unrelatedness among the siblings of these 6 mothers, we will conduct a genetic screening based on allele sharing approach (allele matching cutoff score, AMCOS, Kakkar. S et al. 2021). We will be sending our samples to Department of forensic sciences, PGIMR Chandigarh to conduct this analysis. With these groups, we will make combinations of pairs of male and female

individuals separately. The combinations are as follows: a) familiar siblings b) unfamiliar siblings c) familiar unrelated individuals and d) unfamiliar unrelated individuals. These pairs will be subjected to the Y-maze experiment where the first partner of the pair will be let to trail along through the arm of our choice while the other arm is blocked for control and the second partner of the pair is left to trail along the arm of choice while the control arm is also open. The same experiment will be done in 25 replicates and the treatment arm will be changed alternatively. In this part, we expect from the consolidated result that they incline more towards choosing related individuals.

4.8 Testing the effect of temperature on pheromone trailing in *D. russelii.*

For this experiment, we will be using the same combinations and experimental setup as described in point 4.7 but with 4 different temperature conditions (2 low and 2 high). As they are comfortably kept captive within a temperature range of 28-31ºC, we will choose cold treatments of 20ºC and 24ºC and warm treatments of 35ºC and 39ºC to record their behaviour. For different temperature treatments, we will be regulating the temperature inside the chamber in which the setup is installed. After the temperature is changed for each trial, a buffer time of 1 hour will be given for the whole chamber to be of uniform temperature. These temperature treatments will not harm them as they are known to be found in different and extreme climatic conditions around the Indian subcontinent (Stidworthy, 1974). The experiment from point 4.7 will act as a control for this as it is the same experimental setup but with normal temperature conditions.

4.9 Testing the effect of stress on pheromone trailing in *D. russelii.*

We will use the same combinations and experimental setup from point 4.7 in this part as well but with the first individual of each pair being stressed induced (distressed partner). This species is known to respond violently to even small disturbances or interference (Whitaker, 1989). Hence, we will be introducing a foreign body and in this case a 3D model of a mongoose which is its most natural predator (Cyriac, 2022) 5mins before recording the experiment. This experiment will be carried out based on the idea used in Clément et al. (2020). We will have a treatment and control group here, treatment being distressed individuals and control being individuals with no external stress stimuli. In both treatment and control, the first individual of the pair will be let to trail through the Y-maze first while one of the arms will be blocked as control. As they reach the collection box, the individuals from treatment group will be exposed to the external stimuli and control will be exposed to none. Post exposure, the first individual will be removed from the collection box and second individual will be allowed to choose the arm while the control arm is also open. We expect that the partners of distressed individuals avoid the arm towards them as they sense chemical cues from these distressed individuals indicating danger.

4.10 Statistical analyses

We will be analysing the behavioural data using R statistical software (version 4.2.1). First, we will create contingency tables summarizing the choices made by individuals in each treatment group. These tables will show how many individuals chose the scent-marked side/arm and how many chose the control side/arm. Next, we will employ the binom.test function from the R stats package to conduct two-tailed binomial tests for each scent treatment. This will allow us to assess whether the number of individuals choosing a particular side/arm deviates significantly from what would be expected by chance (assuming a 50% probability of choosing either side). We will analyse the relative concentrations of all detected compounds in the solution as our dependent variables. To identify significant differences between designated groups, we will first assess the normality of the chemical concentrations for each group separately.

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6. Budget Plan

	Amount in PLN
Direct costs, including	**217087**
- personnel costs and scholarships	93000
- research equipment/device/software cost	30087
- other direct costs	94000
Indirect costs, including:	**47758**
- indirect costs of OA	4341
- other indirect costs	43417
Total costs	**264845**

7. Budget plan justification

Type	Name of item	Description	Person /Unit	Months	Cost (PLN)	Total (PLN)
Direct cost						217087
Personal cost and scholarships	Principal investigator	Planning, preparing, and conducting experiments: Designing and creating Y-shape maze for experiments, preparing	2	36	36000	93000

		animal for the experiments (e.g. preparing meal, feeding, monitoring shedding cycle, collecting skin shed), maintaining the temperature of the cage, conducting the objective of experiments, data analysis and writing manuscripts				
	Technical assistant	Assists with the purchase, transportation/shipping, and receipt of animals. keeps a sufficient supply of rat for foods.	2	36	57000	
Equipment	Y-shape maze	Behavioural test used to assess the pheromones trail of snake, made of plastic, easy to clean using ethanol 70%	8	36	25887	
	Camera	Recording snake behaviour pheromone testing	4	36	3200	
	Mongoose model	Natural predator model	4	12	1000	30087
Other direct cost	Animal maintenance, and 3 snake handling experts.	Boxes, water bowl, equipment for feeding, equipment for protection against snake, bite cleaning, paper towel, etc.	3	36	35000	
	Office supplies	Pencils, pen, notebook, printer papers, printer cartridges, etc		36	2000	
	Chemicals for GC/MC	chromatography column, the ferrules, 2 ml and 15 ml vials, 2 ml vials with septum, limited volume vials, hexane, etc.		6	8000	
	AMCOS analysis	Parentage analysis service, and sample shipping		6	30000	
	Laboratory consumables	Gloves, mask, alcohol, tapes, plastic container etc.		36	1000	
	Conference	Expenses related to traveling to participation to conferences, for example Societas Europea Herpetologia in 2013 cost around 1300 PLN for accommodation, 500 PLN for conference fee. Total cost will be different depending on location of the conference		36	15000	94000
	Book and journal	Material and reference for research			3000	
Indirect costs						47758
	Indirect cost of OA	Cost of Open Access publication or research data			4341	
	Other indirect Cost	Publication Acces, or other information to support project			43417	
Total (PLN)						264845

308

Title: Illuminating breakthrough: a comparative study of diurnal and nocturnal rodents in blue light treatment

Authors: Joanna Roszkowska, Karolina Chuda

Summary

Depression, a widespread mental health issue, is often resistant to current pharmacological treatment methods. For this reason, alternative therapies such as blue-light treatment are applied. However the underlying mechanisms remain unclear with a potential mediator - melanopsin - located in the retina. Also the application of blue light in depression treatment is controversial due to inconsistent effects reported and non-standardised parameters. On the other hand, blue light can even cause harmful effects if applied improperly, for example due to the exposure to LED screens. Importantly, in the context of light, the choice of animal models for studying such cases is crucial. Traditionally used nocturnal rat models may not accurately reflect human responses to light exposure, which lead to the project's main concern: do diurnal and nocturnal rodents respond differently to blue light treatment? The research aims to compare these responses, considering the diurnal nature of humans. It will assess various aspects such as locomotor activity, depressive behaviour, hormone levels and melanopsin concentration. This innovative approach, comparing all the above factors in diurnal and nocturnal rodents with blue-light treatment at different parts of a day, will revolutionise our understanding of blue light's effects and its therapeutic potential in humans and standardise research methodologies.

1) Scientific goal of the project

Depressive disorders are among the most common psychological issues, affecting an increasing number of people all over the world. Due to the complexity of mechanisms underlying these conditions, the currently applied pharmacological treatments are often ineffective and unsuitable for many patients. This leads to interest in alternative therapies such as light therapy, particularly blue light treatment. A growing body of evidence suggests improvement and remission states in response to blue light exposure (Lam et al., 2016; Lam et al., 2020), yet the basis of these effects remains unclear. One of the factors identified as a potential mediator between the blue light and its therapeutic effects is melanopsin - a light-sensitive pigment present in a subpopulation of intrinsically photosensitive retinal ganglion cells (Dijk et al., 2009). Melanopsin is known for its function in mediating the effect of light on modulating the circadian rhythms and, importantly, the wavelength that it is most sensitive to is the blue light spectrum (Brainard et al., 2001).

Contrary to its possibly therapeutic effect, short wavelength blue light, when applied at unnatural times and intensity, can lead to harmful outcomes. This is highly relevant to yet another issue in human populations - exposure to LED screens, where the blue light prevails.

Another most important issue that we wish to address in this context is the choice of animal model for this type of study. An increasing number of reports argue whether the use of well-established nocturnal rat models in the research on the influence of light on animals is truly reliable and can be translated to humans (Verra et al., 2022; Shankar et al., 2021). This concern resulted in the emergence of the project's main research question as follows: **Are there differences between diurnal and nocturnal rodent models in response to blue light treatment?**

Thus, the primary scientific goal of the project is to investigate the differential responses of diurnal and nocturnal rodent models to blue light treatment. With this in mind, we plan to compare each of the experimental groups with the control groups, separately for diurnal and nocturnal rodents.

The following research questions were established:

Does the exposure of diurnal and nocturnal rats to blue light at two different periods (morning and evening) result in differences in:

Q1. locomotor activity,

Q2. depressive behaviour,

Q3. cortisol and serotonin levels,

Q4. melanopsin concentration.

Q5. Is melanopsin truly the mediator of the described therapeutic effect of exposure to blue light?

Q6. Does the impact of blue light exposure on the two different animal models differ based on the timing of exposure, with distinct effects observed for morning and evening protocol?

Based on the identified research questions, we present the following hypotheses:

Compared to nocturnal rodents, after blue light treatment, the diurnal rodents will display:

H1: increased locomotor activity,

H2: less depressive behaviour,

H3: higher levels of cortisol and lower levels of serotonin,

H4: higher concentration of melanopsin.

H5: The therapeutic effect of blue light exposure on depressive-like disorders in rodents is mediated by melanopsin.

H6: The impact of blue light exposure on the two different animal models will differ based on the timing of the exposure, with distinct effects observed for morning and evening protocols.

2) Significance of the project

However very promising, the idea of broadly applying blue light therapy during depression treatment seems controversial, as the effects reported by many studies are frequently inconsistent, the therapy parameters are not standardised and, most importantly, the underlying mechanism of the potential therapeutic effect remains unclear (Do et al., 2022). Accordingly, studies aimed at the clarification of the reason behind the phenomenon are highly important, also in respect of potential side effects of the treatment.

In addition to that, a significant part of research uses nocturnal rodents in laboratory tests, which have considerable influence on our understanding of human health. In this study, we aim to compare diurnal and nocturnal rodents to determine how differing daily cycles can affect physiological outcomes. Furthermore, given the diurnal nature of humans, it's crucial to identify and propose appropriate animal models for investigating the effects of blue light on behaviour. The existing literature highlights the advantages of using animals that are day time active as model organisms however there are few studies that directly compare experimental outcomes between diurnal and nocturnal rodents within a single investigation. The research planned will enable us to evaluate and compare locomotor activity, behaviour, hormone levels, and quantity of melanopsin in the retina as a mediator in exposure to the blue light. To our knowledge, no previous studies have comprehensively compared all these factors in both diurnal and nocturnal rodents. Therefore, this approach is innovative and promises to standardise experimental research methodologies and, indirectly, clinical approaches. The results obtained within the study will provide the validation for the use of diurnal animal models in research exploring the influence of light on mammals. The outcomes of this research can be interpreted with a higher degree of confidence and will allow us to make more assured predictions about the potential effects in humans. Such results will contribute to advancements in scientific fields such as behavioural neuroscience and comparative psychology.

3) Concept and work plan

The planned research assumes the use of two different species of rodents: diurnal Nile grass rat (*Arvicanthis niloticus*) and nocturnal Long-Evans.

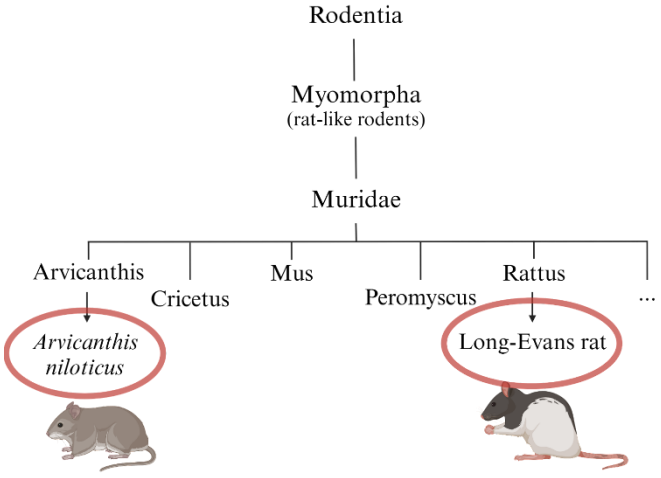

Figure 1. Phylogenetic tree representing evolutionary relationships between the chosen animals (based on Refinetti, 2004).

As the effects of blue light exposure vary greatly depending on many parameters such as exposure time, light intensity and, primarily, time of day when the treatment is applied, we have planned several experimental and control groups. The protocols will include exposure durations of 3 and 6 hours in either morning or evening, along with control groups exposed to white light for 12 hours and a group deprived of any source of blue light.

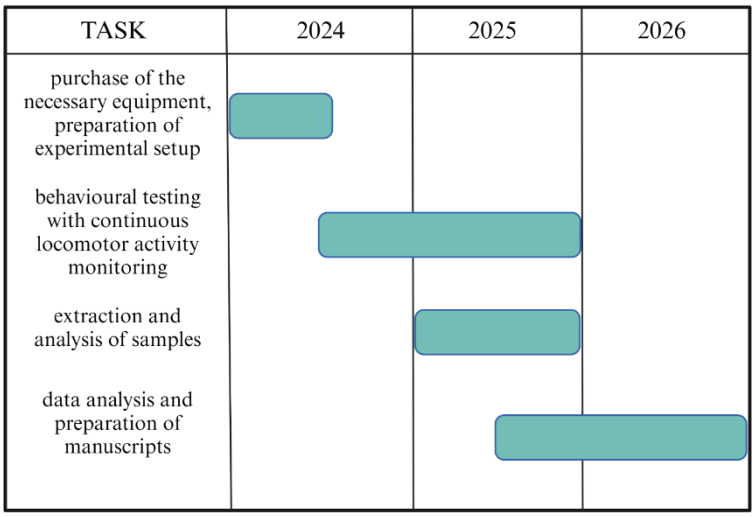

Figure 2. Project timeline including all the steps.

Risk assessment: The research methodology planned for this study is well-established and straightforward, which supports the likelihood of successful completion of the project. However, as with every experimental design using animals as a model, there is a risk factor concerning health conditions of the subjects. Also, human errors while conducting the procedures cannot be excluded. Due to the above unpredictable elements, the number of animals in each group is slightly overstated.

4) Research methodology

The research methodology for this study will incorporate a combination of precise, conventional methods and techniques to address the research questions and hypotheses. The experiment will be carried out under controlled conditions, in the Institute of Zoology and Biomedical Research, Jagiellonian University located in Cracow, Poland.

The methodology of our research consists of:

Animal models: diurnal Nile grass rats (*Arvicanthis niloticus*) and nocturnal Long-Evans rats were selected as the animal models for this study due to their opposite circadian activity patterns. Both species are well-established laboratory models described in scientific literature. Specifically, the Nile grass rat displays the lowest risk of shift in circadian activity under laboratory conditions, as opposed to other diurnal species of rodents. At the beginning of the experiments, the animals will be 10-12 weeks old. Both species will be bred and housed in standard rodent cages (5 rats in each cage) and fed with standard rodent chow, at the Institute of Zoology and Biomedical Research. Apart from the experimental protocol, the light dark cycle 12:12 will be maintained (lights on at 7:00 a.m., UTC+1). Food and water available *ad libitum*.

Blue light exposure protocols: various blue light exposure protocols will be implemented to investigate their effects on diurnal and nocturnal rodents. By 'morning' exposure we refer to the onset of the light phase (7:00 a.m. for every experimental group). End of the light phase is referred to as 'evening' exposure (1 p.m. in case of 6 hours exposure, 4 p.m. in case of 3 hours).

In the first step, we plan to expose 12 groups (6 diurnal and 6 nocturnal) to the following protocols:

- exposed to blue light for 3 hours in the morning,
- exposed to blue light for 3 hours in the evening,
- exposed to blue light for 6 hours in the morning,
- exposed to blue light for 6 hours in the evening,
- exposed to white light for 12 hours,
- group that will be deprived from any source of blue light.

Each protocol will be applied every day for 21 days, preceded and followed by 14 days of baseline observations.

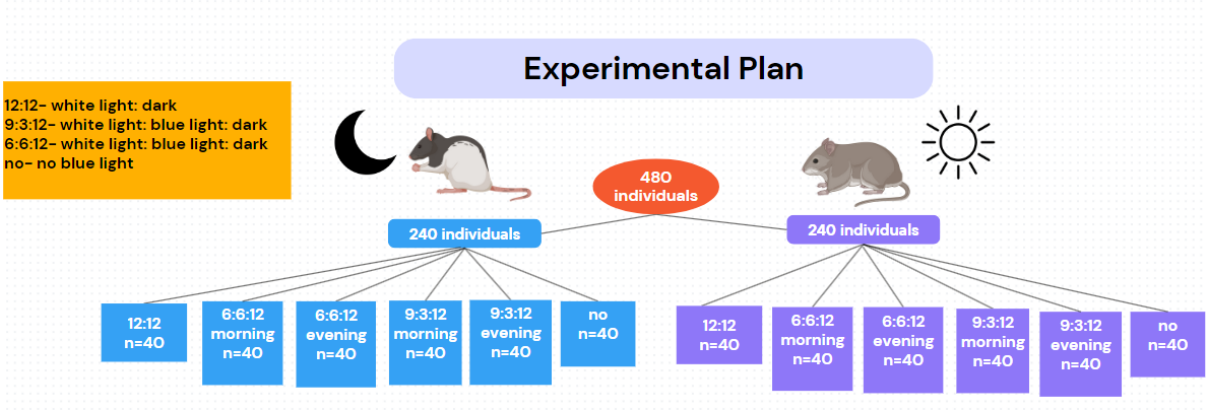

Figure 3. All experimental and control groups planned in the study, including sample sizes.

The next steps are included in the table below.

	objective of measurement	**method of measurement**	**measurement time**
1	locomotor activity assessment	infrared motion captor	monitored continuously
2	depressive behavior evaluation	standardised forced swim test	evaluated before and after the procedure
3	measuring levels of cortisol and serotonin	ELISA (Enzyme-Linked Immunosorbent Assay) technique	measured before and after the procedure
4	measuring the level of melanopsin in the retina	retinal tissue collected and analyzed using real-time qPCR	measured after the procedure

Table 1. Types of measurement methods used in experimental plans.

Data Analysis: for Statistical analysis we plan to use analysis of variance (ANOVA) or Generalized Linear Model (GLM) for comparing results between diurnal and nocturnal rodents across different experimental conditions.

Specific Tests:

For H1, H2, H3, H4: GLM models with appropriate covariates.

For H5: Mediation analysis to test if melanopsin mediates the therapeutic effect of blue light exposure on depressive-like disorders.

For H6: Interaction analysis between exposure timing and animal models.

All statistical analysis will be carried out with the R environment.

Equipment and devices: monochromatic blue lamps, polychromatic white lamps (all lamps will be positioned vertically on top of cages individually, at the same distance), blue light-blocking filters, behavioural testing setup, retinal tissue removal tools, blood sampling kits. Blood samples will be analysed by an analyst from the Institute of Zoology and Biomedical Research.

Ethical considerations: All experimental procedures will be conducted in accordance with ethical guidelines for animal research, ensuring the welfare of the animals.

5) Project literature

Brainard GC, Hanifin JP, Greeson JM, et al. Action Spectrum for Melatonin Regulation in Humans: Evidence for a Novel Circadian Photoreceptor. The Journal of Neuroscience. 2001, 21(16):6405-6412. doi:https://doi.org/10.1523/jneurosci.21-16-06405.2001

Daniela M. Verra, Benjamin S. Sajdak, Dana K. Merriman, David Hicks. Diurnal rodents as pertinent animal models of human retinal physiology and pathology. Progress in Retinal and Eye Research. 2020, 74, pp.100776 -. ff10.1016/j.preteyeres.2019.100776ff. ffhal-03489956f

Dijk DJ, Archer SN. Light, Sleep, and Circadian Rhythms: Together Again. PLoS Biology. 2009; 7(6):e1000145. doi:https://doi.org/10.1371/journal.pbio.1000145\

Do A, Li VW, Huang S, et al. Blue-Light Therapy for Seasonal and Non-Seasonal Depression: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. The Canadian Journal of Psychiatry. 2022; 67(10):745-754. doi:10.1177/07067437221097903

Lam RW, Levitt AJ, Levitan RD, et al. Efficacy of bright light treatment, fluoxetine, and the combination in patients with nonseasonal major depressive disorder: a randomized clinical trial. JAMA Psychiatry. 2016; 73(1):56-63

Lam RW, Teng MY, Jung YE, et al. Light therapy for patients with bipolar depression: systematic review and meta-analysis of randomized controlled trials. Can J Psychiatry. 2020; 65(5):290-300

Refinetti R. The Nile Grass Rat as a Laboratory Animal. Lab Animal. 2004; 33(9):54-57. doi:https://doi.org/10.1038/laban1004-54

Shankar A, Williams CT. The darkness and the light: diurnal rodent models for seasonal affective disorder. Disease Models & Mechanisms. 2021; 14(1). doi:https://doi.org/10.1242/dmm.047217

6) Table with budget of the project.

	Amount in PLN
Direct costs, including	**635 750**
- personnel costs and scholarships	412 000
- research equipment/device/software cost	169 750
- other direct costs	54 000
Indirect costs, including:	**139 865**
- indirect costs of OA	12 715
- other indirect costs	127 150
Total costs	**775 615**

7) Breakdown of project costs including justification and relevance for the tasks in the project.

1. **Personnel costs and scholarships**

- Two principal investigators, 36 months * 4 000 PLN = 144 000 PLN per person

justification: planning and conducting research tasks, statistical analyses and manuscripts writing

- Salaries for 2 technical assistants: 24 months x 2 000 PLN = 48 000 PLN per person

justification: caring for animal quarters and individuals

- Salary for laboratory blood sample analyst: 9 month x 2 000 PLN = 18 000 PLN

justification: analysing cortisol and serotonin levels in blood sample of individuals

- RT-qPCR training for the investigators: 2 * 5 000 PLN = 10 000 PLN

Sum = 412 000 PLN

2. **Equipment and devices**

- Computers and software: 2 laptops = 10 000 PLN, 2 hard drives (4 TB) = 2 000 PLN

justification: necessary for data storage and performing high-quality analysis

- Infrared motion captor + custom-made software: 16 * 150 PLN = 2 400 PLN
- Polychromatic LED white lamps: 4 * 200 PLN = 800 PLN
- Monochromatic LED blue lamps: 12 * 300 PLN = 3 600 PLN
- Filters for blue light: 4 * 150 PLN = 600 PLN
- Stoelting Porsolt Forced Swim Test: 2 * 5311 PLN + shipping + VAT = 12 000 PLN
- Shaker: 2 000 PLN
- Real-Time PCR System NEUPCR16E: 120 000 PLN (shipping and VAT included)

Sum = 153 400 PLN

justification: all equipment is necessary to carry out the planned experiments

3. **Materials and small equipment**

- Alcohol sanitizer spray - 10 bottles: 300 PLN

justification: necessary for disinfection of laboratory surface and behavioural test after each experiment

- needles: 1 000 * 0,9 = 900 PLN
- syringes: 1 000 * 0,5 = 500 PLN
- test tubes: 1 000 * 0,5 = 500 PLN
- protective gloves: 1000 * 0,1 = 100 PLN
- Distilled water: 50 * 1 = 50 PLN
- Biotinylated anti-cortisol Detection Antibody: 1* 2 000 = 2 000 PLN
- Biotinylated anti-serotonin Detection Antibody 1 * 2000 = 2 000 PLN
- DNA extraction kit: 2 000 PLN (shipping and VAT included)
- PCR reagents (DNA polymerase, dNTPs, primers, buffer): 8 000 PLN (shipping and VAT included)

justification: necessary for laboratory tests (ELISA, PCR)

Sum = 16 350 PLN

4. Business trips

- National conference: 2 * 5 000 PLN = 10 000 PLN
- International conference: 2 * 10 000 PLN = 20 000 PLN

justification: it is necessary to share the research results on international conferences, which is why at least two conferences are planned.

Sum = 30 000 PLN

5. Other costs

- Maintenance of study animals (same for both species, 100 cm x 80 cm x 50 cm) - cages:

2 500 PLN, food: 3 200 kg for 3 years = 20 500 PLN, bedding: 1000 PLN

Sum = 24 000 PLN

Total = 635 750 PLN

8. Reviews of grant proposal
8.1 Review No. 1

Title: Illuminating breakthrough: a comparative study of diurnal and nocturnal rodents in bluelight treatment

Authors: Joanna Roszkowska, Karolina Chuda

1. Assessment of scientific quality of the research project (scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context)
Comparing diurnal and nocturnal rodents as a way to deal with unresolved questions of the UV role is a novel, original and interesting founding idea of this project. But, as I understand, only one species will represent every group. It means that the two species may differ in a number of ways and the diurnal/nocturnal dichotomy is just one and possibly not the most important one. So, multiple diurnal and nocturnal species would be advisable, ideally of different size, ecology, etc.

2. Assessment of potential impact of the research project (the potential for substantial international impact on the research field(s) and for high quality research publications and other research outputs, taking into account the specifics of the research field and the variety of forms of impact and output; impact ought to be evaluated using an international context)
The problem studied is important enough that any results, positive or negative, could be probably published in good journals. But, the data must be solid. The problem with depression is that any treatments, even the most commonly used pharmaceuticals, are often efficient for only a fraction of patients. UV is much more elusive than transmitter blockers. There is a real danger of getting rather weak and hard to interpret data. My advice would be to set stringent criteria to claim UV effects and be prepared to publish a paper with clear negative rather than unclear positive results.

3. Assessment of feasibility of the research project (the feasibility of the proposed project, including the appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), other factors affecting the feasibility of the project)
The experiments seem to be rather standard behavioral tests and there are no reasons they could not be done. The problem is with interpretation. I am not sure whether one can distinguish between “increased locomotor activity” and “less depressive behaviour”. Perhaps there are some differences regarded as standard in the field but they are not named here.

4. Are the costs to be incurred well justified with regards to the subject and scope of the research?
I have no present experience with Preludium grants but I am rather astonished with the slalries/stipends costs. Also, is there a need to buy the Real-Time PCR System NEUPCR16E for as much as 120 000 PLN, I mean can not an existing piece be used.

5. Strengths of the proposal
The general idea is worth considering. Perhaps more species with fewer specimens, if doable.

6. Weaknesses of the proposal
There is little, or no, information on the UV (light) research with rodents in aspects other than depression. So, it is hard to guess what is already known and what would constitute truly new insights. Planned behavioral tests are very briefly introduced. Nothing about blood sample analyses.

8.2 Review No. 2

Title of the project: lluminating breakthrough: a comparative study of diurnal and nocturnal rodents in blue light treatment

1. Assessment of scientific quality of the research project

The grant proposal addresses to main important scientific questions:

The first one is about understanding the underlying mechanisms of blue light as a treatment for depression, which is of very high relevance because depression is a profoundly serious illness and one of the most common, as stated in the text. I strongly support research in an area like this, specifically in treatments like blue light that have already been shown to have positive effects. However because of the contradictory results in research, it is important to further study this topic, that is why I think focusing on a mediator such as melanopsin could give straightforward systematizing results. Because melanopsin is most sensitive to blue light from the spectrum and mediates synchronizing the endogenous circadian clock, I feel that it is a very interesting and surprisingly understudied aspect in the context of blue light treatment. Therefore, I believe that this research project will provide novelties in the scientific and clinical field of studying and treating depression or depressive-like symptoms.

The second major scientific question addresses another very important issue, which is the difference in results between diurnal and nocturnal rat models. This is of high relevance because many circadian rhythm studies that are supposed to be comparative to humans use nocturnal animals, which may potentially not be the best model for such a topic. Questioning this shows the critical thinking of the authors.

2. Assessment of potential impact of the research project

This study has the potential to internationally revolutionize the way scientists approach and interpret their data in interdisciplinary fields using animal models and choose the adequate model. Therefore publications from this research could be published in a high-quality journal and be used as a reference for many scientists in not only circadian rhythm research but broader.

Furthermore, because blue light therapy is starting to be recognized worldwide, a multifaceted study with various measures including locomotor activity, depressive behaviour, cortisol and serotonin levels and melanopsin concentration can be very impactful.

3. Assesment of feasibility of the research project

I believe this project is fully feasible, however, I think the project description in the context of methodology sometimes lacks data from other articles. For example, it would be clearer if the authors addressed what time and intensity of light was harmful (line 53-54) in other articles. Moreover, an explanation of why particular parameters like locomotor activity, depressive behavior, cortisol and serotonin levels and melanopsin concentration will be measured would be a useful addition. Nevertheless, the methodology is well selected and I believe it will allow authors to obtain the results they are looking for. Facilities, equipment and data analysis seem to be appropriate for this project. Risk assessment is clearly stated in the text with a contingency plan.

4. Are the costs to be incurred well justified with regards to the subject and scope of the research?

Direct costs are well described and justified with regards to the research project. Indirect cost justification is missing, however, they are included in the budget and properly calculated.

5. Strengths of the proposal

This grant proposal is very well-written and well-structured. Visual representations of timeline, experimental design and animal models made it easier for the reader to understand the project. Furthermore, I appreciated the measurement methods being placed into a table. The objectives of the project are clearly stated with the importance highlighted.

6. Weaknesses of the proposal

Insufficient description explaining the parameters used, lack of hypotheses comparing diurnal and nocturnal rats within groups (e.g. effects on diurnal after 3-hour exposure to blue light vs no blue light). Furthermore, I think this is an unintentional error, but H3 is inconsistent with H2, the authors probably meant to write “lower levels of cortisol and higher levels of serotonin”.

8.3 Review No. 3

Title of the project:

1. Assessment of scientific quality of the research project (scientific relevance, importance, originality and novelty of research or tasks to be performed; quality ought to be evaluated in an international context)

This proposal fullfill the criterion of this funding. The project also meets the requirement. The main objective of this project is investigate the differential responses of diurnal and nocturnal rodents to blue light treatment. With the goal is to improve our understanding of blue light's effects and its potential as a treatment for depression in humans. This proposal addresses an interesting matter concerning depression treatment and the possibilities of blue light therapy. By comparing diurnal and nocturnal rodents respond to it, which hasn't been done much before. The result will contribute to make blue light therapy more effective and reliable for treating conditions like depression.

2. Assessment of potential impact of the research project (the potential for substantial international impact on the research field(s) and for high quality research publications and other research outputs, taking into account the specifics of the research field and the variety of forms of impact and output; impact ought to be evaluated using an international context)

This research project has the potential to make a big impact worldwide. By studying how different types of rodents respond to blue light therapy, it could help us understand how this therapy affects mental health. This could lead to better treatments for depression and other mental health issues. The project's innovative approach by comparing nocturnal and diurnal rodent could lead to important new information on these rodents behaviour toward blue light and used to future study for improvements in how we use light therapy to help people.

3. Assessment of feasibility of the research project (the feasibility of the proposed project, including the appropriateness of the research methodology to achieve the goals of the project, the risk management description, research facilities and equipment, international cooperation (if any), other factors affecting the feasibility of the project)

The research methodology section provides a clear description of the chosen animal models, blue light exposure protocols, measurement methods, data analysis plan, and ethical considerations. The use of established techniques and equipment adds credibility to the proposed study. The object in this reseach is two types of nocturnal rodent and diurnal rodent affect serotonin levels and behaviour, However, it should be noted that the sex of the rodent can influence the results of research concerning the behavioral and hormonal differences caused by light exposure. Therefore, mentioning the specific sex of the rodents studied in this research proposal would be beneficial for ensuring the comprehensiveness and validity of the research.

4. Are the costs to be incurred well justified with regards to the subject and scope of the research?

The costs incurred for the subject and scope of the research appear to be well-justified. Overall, each expense is directly related to the research objectives and is essential for conducting the study effectively. However, the indirect project in this proposal lacks a description and justification. Explanations and justifications should be provided for indirect project costs, which include open access, publication fees, of cost of research data. By incorporating these elements, the proposal's merit would be strengthened

5. Strengths of the proposal

The proposal clearly outlines the research questions and hypotheses, providing a solid foundation for the study. This clarity helps focus the research and ensures that the objectives are well-defined. The significance of the project shows its strong point in addressing current gaps in understanding the therapeutic effects of blue light therapy. The methodology section is detailed, covering various aspects such as animal models, blue light exposure protocols, measurement methods, data analysis plans, and ethical considerations. This project is innovative and will undoubtedly have a beneficial impact on human health therapy in the future.

6. Weaknesses of the proposal

The proposal notes that because of unexpected factors, the number of animals in each group is somewhat overstated. Providing a more detailed justification for the chosen sample sizes would strengthen the study's methodology and ensure adequate statistical power. Overall the project

Title: Illuminating breakthrough: a comparative study of diurnal and nocturnal rodents in blue light treatment

Authors: Joanna Roszkowska, Karolina Chuda

Summary

Depression, a widespread mental health issue, is often resistant to current pharmacological treatment methods. For this reason, alternative therapies such as blue-light treatment are applied. However the underlying mechanisms remain unclear with a potential mediator - melanopsin - located in the retina. Also the application of blue light in depression treatment is controversial due to inconsistent effects reported and non-standardised parameters. On the other hand, blue light can even cause harmful effects if applied improperly, for example due to the exposure to LED screens. Importantly, in the context of light, the choice of animal models for studying such cases is crucial. Traditionally used nocturnal rat models may not accurately reflect human responses to light exposure, which lead to the project's main concern: do diurnal and nocturnal rodents respond differently to blue light treatment? The research aims to compare these responses, considering the diurnal nature of humans. It will assess various aspects such as locomotor activity, depressive behaviour, hormone levels and melanopsin concentration. This innovative approach, comparing all the above factors in diurnal and nocturnal rodents with blue-light treatment at different parts of a day, will revolutionise our understanding of blue light's effects and its therapeutic potential in humans and standardise research methodologies.

1) Scientific goal of the project

Depressive disorders are among the most common psychological issues, affecting an increasing number of people all over the world. Due to the complexity of mechanisms underlying these conditions, the currently applied pharmacological treatments are often ineffective and unsuitable for many patients. This leads to interest in alternative therapies such as light therapy, particularly blue light treatment. A growing body of evidence suggests improvement and remission states in response to blue light exposure (Lam et al., 2016; Lam et al., 2020), yet the basis of these effects remains unclear. One of the factors identified as a potential mediator between the blue light and its therapeutic effects is melanopsin - a light-sensitive pigment present in a subpopulation of intrinsically photosensitive retinal ganglion cells (Dijk et al., 2009). Melanopsin is known for its function in mediating the effect of light on modulating the circadian rhythms and, importantly, the wavelength that it is most sensitive to is the blue light spectrum (Brainard et al., 2001).

Contrary to its possibly therapeutic effect, short wavelength blue light, when applied at unnatural times and high intensity, can lead to harmful outcomes, such as photothermal and photochemical damage of the retina (Tosini et al., 2016). This is highly relevant to yet another issue in human populations - exposure to LED screens, where the blue light prevails.

Another most important issue that we wish to address in this context is the choice of animal model for this type of study. An increasing number of reports argue whether the use of well-established nocturnal rat models in the research on the influence of light on animals is truly reliable and can be translated to humans (Verra et al., 2022; Shankar & Williams, 2021). This concern resulted in the emergence of the project's main research question as follows: **Are there differences between diurnal and nocturnal rodent models in response to blue light treatment?**

Thus, the primary scientific goal of the project is to investigate the differential responses of diurnal and nocturnal rodent models to blue light treatment. With this in mind, we plan to compare each of the experimental groups with the control groups, separately for diurnal and nocturnal rodents.

The following research questions were established:

Q1. Does the exposure of diurnal and nocturnal rodents to blue light at two different periods (morning and evening) result in differences in: locomotor activity, depressive behaviour, cortisol and serotonin levels, melanopsin concentration?

Q2. Is melanopsin truly the mediator of the described therapeutic effect of exposure to blue light?

Q3. Does the impact of blue light exposure on the two different animal models differ based on the timing of exposure, with distinct effects observed for morning and evening protocol?

Based on the identified research questions, we present the following hypotheses:

H1: Compared to nocturnal rodents, after blue light treatment in the morning, the diurnal rodents will display: increased locomotor activity, less depressive behaviour, lower levels of cortisol and higher levels of serotonin, higher concentration of melanopsin. Opposite effects are expected following blue light treatment in the evening.

H2: The therapeutic effect of blue light exposure on depressive-like disorders in rodents is mediated by melanopsin.

H3: The impact of blue light exposure on the two different animal models will differ based on the timing of the exposure, with distinct effects observed for morning and evening protocols.

2) Significance of the project

However very promising, the idea of broadly applying blue light therapy during depression treatment seems controversial, as the effects reported by many studies are frequently inconsistent, the therapy parameters are not standardised and, most importantly, the underlying mechanism of the potential therapeutic effect remains unclear (Do et al., 2022). Accordingly, studies aimed at the clarification of the reason behind the phenomenon are highly important, also in respect of potential side effects of the treatment.

In addition to that, a significant part of research uses nocturnal rodents in laboratory tests, which have considerable influence on our understanding of human health. In this study, we aim to compare diurnal and nocturnal rodents to determine how differing daily cycles can affect physiological outcomes. Furthermore, given the diurnal nature of humans, it's crucial to identify and propose appropriate animal models for investigating the effects of blue light on behaviour. The existing literature highlights the advantages of using animals that are day time active as model organisms (Refinetti, 2004; Verra et al., 2020), however there are few studies that directly compare experimental outcomes between diurnal and nocturnal rodents within a single investigation (Campi & Krubitzer, 2010). The research planned will enable us to evaluate and compare locomotor activity, behaviour, cortisol and serotonin levels, and quantity of melanopsin in the retina as a mediator in exposure to the blue light. The mentioned parameters will allow for understanding how blue light exposure influences overall locomotor activity, crucial for clarifying its effect on mood and energy levels, particularly in context of depressive behaviour. To our knowledge, no previous studies have comprehensively compared all these factors in both diurnal and nocturnal rodents. Therefore, this approach is innovative and promises to standardise experimental research methodologies and, indirectly, clinical approaches. The results obtained within the study will provide the validation for the use of diurnal animal models in research exploring the influence of light on mammals. The outcomes of this research can be interpreted with a higher degree of confidence and will allow us to make more assured predictions about the potential effects in humans. Such results will contribute to advancements in scientific fields such as behavioural neuroscience and comparative psychology.

3) Concept and work plan

The planned research assumes the use of two different species of rodents: diurnal Nile grass rat (*Arvicanthis niloticus*) and nocturnal Long-Evans.

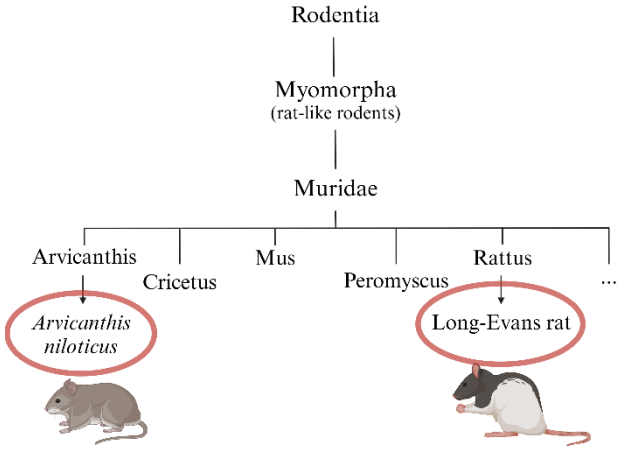

Figure 1. Phylogenetic tree representing evolutionary relationships between the chosen animals (based on Refinetti, 2004).

As the effects of blue light exposure vary greatly depending on many parameters such as exposure time, light intensity and, primarily, time of day when the treatment is applied, we have planned several experimental and control groups. The protocols will include exposure durations of 3 and 6 hours in either morning or evening, along with control groups exposed to white light for 12 hours and a group deprived of any source of blue light.

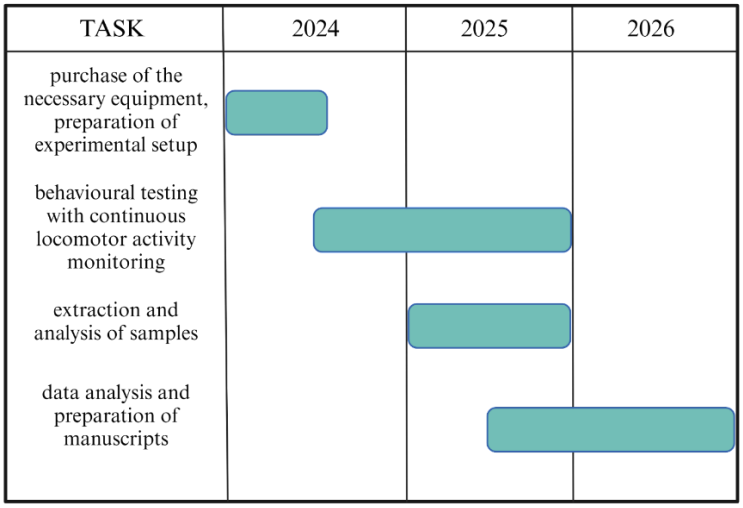

Figure 2. Project timeline including all the steps.

Risk assessment: The research methodology planned for this study is well-established and straightforward, which supports the likelihood of successful completion of the project. However, as with every experimental design using animals as a model, there is a risk factor concerning health conditions of the subjects. Also, human errors while conducting the procedures cannot be excluded. Due to the above unpredictable elements, the number of animals in each group is slightly overstated.

4) Research methodology

The research methodology for this study will incorporate a combination of precise, conventional methods and techniques to address the research questions and hypotheses. The experiment will be carried out under controlled conditions, in the Institute of Zoology and Biomedical Research, Jagiellonian University located in Cracow, Poland.

The methodology of our research consists of:

Animal models: diurnal Nile grass rats (*Arvicanthis niloticus*) and nocturnal Long-Evans rats were selected as the animal models for this study due to their opposite circadian activity patterns. Both species are well-established laboratory models described in scientific literature. Specifically, the Nile grass rat displays the lowest risk of shift in circadian activity under laboratory conditions, as opposed to other diurnal species of rodents (Refinetti, 2004; Shankar & Williams, 2021). At the beginning of the experiments, the animals will be 10-12 weeks old. Both species will be bred and housed in standard rodent cages (5 rats in each cage) and fed with standard rodent chow, at the Institute of Zoology and Biomedical Research. Apart from the experimental protocol, the light dark cycle 12:12 will be maintained (lights on at 7:00 a.m., UTC+1). Food and water available *ad libitum*.

The sample size is 480 individuals (240 Nile grass rats and 240 Long-Evans rats). This sample size will allow for obtaining satisfactory statistical power and is overstated due to the risk assessment.

We are aware of the fact that the study does not consider sex as a variable, which could influence the results. Also, other species of diurnal and nocturnal rodents could be analysed to include interspecies differences. However, we believe that both of these issues provide additional complexity and should be addressed in the future studies.

Blue light exposure protocols: various blue light exposure protocols will be implemented to investigate their effects on diurnal and nocturnal rodents. By 'morning' exposure we refer to the onset of the light phase (7:00 a.m. for every experimental group). End of the light phase is referred to as 'evening' exposure (1 p.m. in case of 6 hours exposure, 4 p.m. in case of 3 hours).

In the first step, we plan to expose 12 groups (6 diurnal and 6 nocturnal) to the following protocols:

- exposed to blue light for 3 hours in the morning,
- exposed to blue light for 3 hours in the evening,
- exposed to blue light for 6 hours in the morning,
- exposed to blue light for 6 hours in the evening,
- exposed to white light for 12 hours,
- group that will be deprived from any source of blue light.

Each protocol will be applied every day for 21 days, preceded and followed by 14 days of baseline observations. Light intensity will be 350 lux for white light and 500 lux for blue light (wavelength ~480 nm).

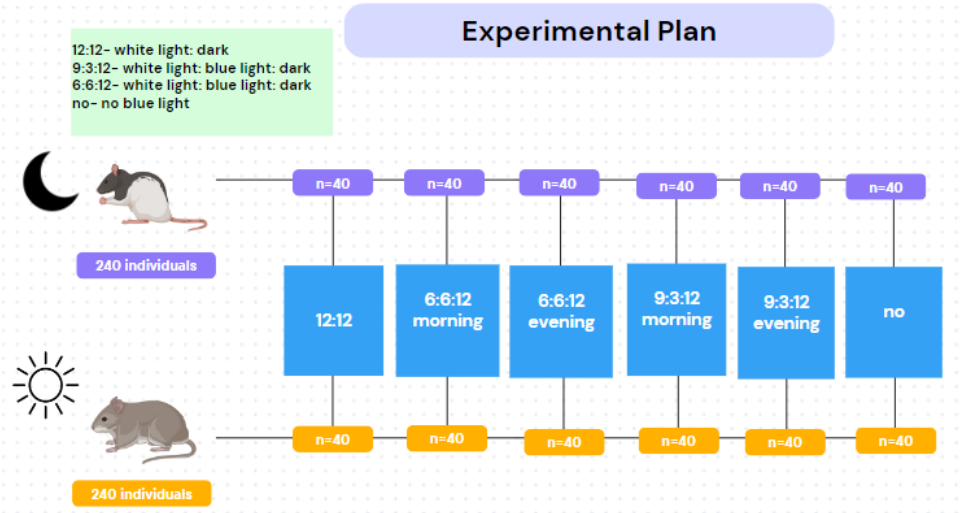

Figure 3. All experimental and control groups planned in the study, including sample sizes.

The next steps are included in the table below.

Table 1. Types of measurement methods used in experimental plans.

	objective of measurement	**method of measurement**	**measurement time**
1	locomotor activity assessment	infrared motion captor	monitored continuously
2	depressive behavior evaluation	standardised forced swim test	evaluated before and after the procedure
3	measuring levels of cortisol and serotonin	ELISA (Enzyme-Linked Immunosorbent Assay) technique	measured before and after the procedure
4	measuring the level of melanopsin in the retina	retinal tissue collected and analyzed using real-time qPCR	measured after the procedure

Data Analysis: for statistical analysis we plan to use analysis of variance (ANOVA) or Generalized Linear Model (GLM) for comparing results between diurnal and nocturnal rodents across different experimental conditions. We assume p value < 0.05.

All statistical analysis will be carried out with the R environment.

Equipment and devices: monochromatic blue lamps, polychromatic white lamps (all lamps will be positioned vertically on top of cages individually, at the same distance), blue light-blocking filters, behavioural testing setup, retinal tissue removal tools, blood sampling kits. Blood samples will be analysed by an analyst from the Institute of Zoology and Biomedical Research.

Ethical considerations: All experimental procedures will be conducted in accordance with ethical guidelines for animal research, ensuring the welfare of the animals.

5) Project literature

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6) Table with budget of the project.

	Amount in PLN
Direct costs, including	**650 750**
- personnel costs and scholarships	402 000
- research equipment/device/software cost	153 400
- other direct costs	95 350
Indirect costs, including:	**143 165**
- indirect costs of OA	13 015
- other indirect costs	130 150
Total costs	**793 915**

7) Breakdown of project costs including justification and relevance for the tasks in the project.

Direct costs include:

1. **Personnel costs and scholarships**

- Two principal investigators, 36 months * 4 000 PLN = 144 000 PLN per person

justification: planning and conducting research tasks, statistical analyses and manuscripts writing

- Salaries for 2 technical assistants: 24 months x 2 000 PLN = 48 000 PLN per person

justification: caring for animal quarters and individuals

- Salary for laboratory blood sample analyst: 9 months x 2 000 PLN = 18 000 PLN

justification: analysing cortisol and serotonin levels in blood sample of individuals

Sum = 402 000 PLN

2. **Equipment and devices**

- Computers and software: 2 laptops = 10 000 PLN, 2 hard drives (4 TB) = 2 000 PLN

justification: necessary for data storage and performing high-quality analysis

- Infrared motion captor + custom-made software: 16 * 150 PLN = 2 400 PLN
- Polychromatic LED white lamps: 4 * 200 PLN = 800 PLN
- Monochromatic LED blue lamps: 12 * 300 PLN = 3 600 PLN
- Filters for blue light: 4 * 150 PLN = 600 PLN
- Stoelting Porsolt Forced Swim Test: 2 * 5311 PLN + shipping + VAT = 12 000 PLN
- Shaker: 2 000 PLN
- Real-Time PCR System NEUPCR16E: 120 000 PLN (shipping and VAT included)

justification: all equipment is necessary to carry out the planned experiments

Sum = 153 400 PLN

3. Materials and small equipment

- Alcohol sanitizer spray – 10 bottles: 300 PLN

justification: necessary for disinfection of laboratory surface and behavioural test after each experiment

- needles: 1 000 * 0,9 = 900 PLN
- syringes: 1 000 * 0,5 = 500 PLN
- test tubes: 1 000 * 0,5 = 500 PLN
- protective gloves: 1000 * 0,1 = 100 PLN
- Distilled water: 50 * 1 = 50 PLN
- Biotinylated anti-cortisol Detection Antibody: 1* 2 000 = 2 000 PLN
- Biotinylated anti-serotonin Detection Antibody 1 * 2000 = 2 000 PLN
- DNA extraction kit: 2 000 PLN (shipping and VAT included)
- PCR reagents (DNA polymerase, dNTPs, primers, buffer) – 2 kits: 8 000 PLN (shipping and VAT included)

justification: necessary for laboratory tests (ELISA – 960 samples, PCR – 480 samples)

Sum = 16 350 PLN

4. Business trips

- National conference: 2 * 5 000 PLN = 10 000 PLN
- International conference: 2 * 10 000 PLN = 20 000 PLN

justification: it is necessary to share the research results on international conferences, which is why at least two conferences are planned.

Sum = 30 000 PLN

5. Other costs

- RT-qPCR training for the investigators: 2 * 5 000 PLN = 10 000 PLN
- Maintenance of study animals (same for both species, 100 cm x 80 cm x 50 cm) – cages: 2 500 PLN, food: 3 200 kg for 3 years = 20 500 PLN, bedding: 1000 PLN, biowaste disposal: 15 000 PLN

Sum = 49 000 PLN

Total direct costs = 650 750 PLN

Indirect costs include:

Publication of the results (open access cost) = 13 015 PLN

Host institute = 130 150 PLN

Total indirect costs = 143 165 PLN