# Heart Rate Variability over the Lifespan and its Potential as a Peripheral Biomarker for Cognitive Decline in Long-Evans Rats

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# *Abstract*

This study investigates the effects of pharmacological cholinergic manipulation and aging on heart rate variability (HRV) and performance during an odor discrimination task in Long-Evans rats. Rats from distinct age cohorts ("Adult," "Older Adult," and "Elderly") completed proactive interference (PI) odor-detection tasks using an olfactometer. Proactive interference refers to the phenomenon where previously learned information impedes the acquisition of new, related material. Pharmacological interventions involved systemic intraperitoneal (IP) injections of normal saline (0.9% sterile medical grade), methylscopolamine (0.25 mg/kg), or scopolamine (0.25 mg/kg). The ability to overcome PI was evaluated for each injection type. HRV data were collected via surgically implanted telemeters recording electrocardiogram (ECG) data. Results revealed no significant age-related differences in HRV between the "Elderly" and "Adult" cohorts. Pharmacological manipulation affected PI resolution differently between age cohorts, with adult rats showing impaired PI resolution under scopolamine and methylscopolamine compared to saline controls, while older adult rats behaved comparably across each injection type. These data suggest that cholinergic drugs may affect the body differently in naturally aged subjects. Unexpectedly, rats also experienced declines in baseline and new learning accuracy from cholinergic manipulation, which may be due to learning odor discriminations at an older age compared to previous studies that used younger animals. Rats experienced a drop in HRV after injection, suggesting a reduction in vagal tone due to reduced cardiac parasympathetic regulation.

# *Introduction*

### *Cognitive Decline and Disease in the Aging Brain*

Neurodegenerative diseases such as Alzheimer's disease, Lewy body dementia, and Parkinson's disease are common among those over the age of 65 and lead to great amounts of disability and suffering, however their causes are not fully understood.[1] Alzheimer's disease (AD), the most common form of dementia, initially manifests as mild memory impairments and eventually progresses into a severe neurodegenerative state, causing severe disability and dependency for those affected. As the world's population ages, the number of people affected by AD is already rising and is expected to increase, emphasizing the need for more extensive research on early detection, preventative measures, and treatments. Research suggests that complex brain changes seen in AD can begin several years before problems with memory and thinking arise,[2] potentially providing opportunity for early intervention and prevention of significant cognitive decline. This research is essential not only because of the physical and emotional impact on patients and their families but also because of the significant economic and healthcare burdens it imposes on societies globally.[3]

Even in the absence of disease, our brains undergo continuous transformation as we age. It is known that many cognitive abilities become less efficient with aging, although some functions may decline more noticeably than others. Among the functions that typically decline are processing speed, working memory, and episodic memory. Older adults may be slower in recalling names, have problems with multitasking, and experience slight decreases in attentional capacity.[4] Another change in the aging brain is a reduction in cortical acetylcholine, an important neurotransmitter involved in learning and memory processes. Connections between lower levels

of certain cortical cholinergic markers and clinical dementia ratings suggests a link between reduced cholinergic function and the cognitive impairments seen in dementia. In AD, loss of cholinergic cells in a specific region of the basal forebrain results in decreased cortical acetylcholine levels.[3,5] The cholinergic hypothesis, which has laid the groundwork for understanding AD, postulates that a reduction in acetylcholine is responsible for a significant portion of the cognitive decline observed in AD, such as impairments in cognitive processes, memory, and attention.[3]

In their earliest and mildest stages, neurodegenerative diseases may be confused with normal aging, preventing quick and accurate diagnoses. Although diminishing efficiency of certain brain functions and a loss of acetylcholine are present in normal aging and disease, the memory loss and cognitive decline associated with diseases such as AD are not a part of normal, healthy aging.[3] This poses a challenge in distinguishing between cognitive decline due to age and cognitive decline due to disease.

***Acetylcholine and Proactive Interference***

Difficulty overcoming a memory phenomenon known as proactive interference (PI) may be an early cognitive feature detected in those with MCI and mild AD. Proactive interference occurs when previously learned information impedes the acquisition of new material.[5] Understanding proactive interference is crucial for gaining insights into memory encoding, storage, and retrieval. Overcoming PI has been shown by previous studies to depend on the basal forebrain, the main source of acetylcholine in the central nervous system.[6,7] Inhibition of muscarinic cholinergic receptors has been shown to impair interference learning and increase proactive

interference. When the brain is presented with novel tasks, cholinergic activity suppresses excitatory feedback from old memories to aid in learning new information. Blocking this suppression enhances excitatory feedback, increasing proactive interference.[8] A study by Loewenstein et al. investigated the susceptibility to semantic proactive interference in individuals with mild AD, mild cognitive impairment (MCI), and healthy older adults. The researchers found that mild AD patients demonstrated greater PI compared to MCI patients, and healthy older adults were the least inhibited by PI.[9] These findings are consistent with previous research that has demonstrated that PI is related to damage in specific areas of the brain, including impairment of the cholinergic basal forebrain, a structure known to be involved in the early stages of AD.[5,9] Another study by Dewer et al. used a minimal interference/interference paradigm on patients with AD and those with MCI without AD. The researchers found that memory retention is much higher in these groups when there is minimal interference compared to a normal interference paradigm. Their paradigm also strongly predicted which patients with MCI would progress to AD within 2 years.[10,11] These findings are consistent with previous studies indicating that memory problems in individuals with AD are associated with an increased likelihood of experiencing memory interference.[11]

***The Effect of Systemic Pharmacology on Proactive Interference***

To better understand the role of acetylcholine on PI, researchers have used anticholinergic drugs to inhibit its action. For example, scopolamine and methylscopolamine are muscarinic receptor antagonists that inhibit the activity of the parasympathetic nervous system, which uses acetylcholine as its primary neurotransmitter. This approach approximates “turning off” the system to observe the resulting effects. Scopolamine, a central-acting competitive muscarinic

cholinergic receptor antagonist,[5] is often used in neuroscience-related research to induce cognitive disorders because of its ability to permeate the blood-brain barrier. In the context of AD research, scopolamine causes cholinergic dysfunction, a trait previously highlighted as inherent to the condition.[12] Methylscopolamine is a peripheral muscarinic receptor antagonist and does not pass through the blood-brain barrier, therefore acting as a control for the effects of scopolamine in the peripheral nervous system. Previous studies have investigated the effects of scopolamine and methylscopolamine on proactive interference resolution in awake, behaving rats.[5,6,13] For example, in an associative learning task, rats receiving scopolamine were more impaired at acquiring overlapping (PI) than nonoverlapping odor pairs compared to methylscopolamine and normal saline controls, suggesting a selective decrease in the resolution of PI.[13] In another study similar, researchers examined the effect of scopolamine on memory for word-pair associates in humans. They found a significant decrease in the recall of overlapping word pairs in subjects injected with scopolamine compared to controls, supporting the conclusion that acetylcholine plays an important role in associative learning by decreasing PI.[8]

***Heart-Brain Axis***

Deactivating the parasympathetic nervous system by inhibiting the action of acetylcholine also hinders heart-brain communication. Our understanding of the heart and brain's communication has deepened over time, moving from a simplistic view of the heart solely as a pump to a recognition of its complex relationship with the brain. While before, it was understood that the brain was the center of intelligence and control, we now know that the heart contains its own internal nervous system, known as the "intrinsic cardiac nervous system,"[14] that allows the heart to send signals to the brain and modulate its activity. Further research on heart-brain

communication "[has] confirmed that the neural interactions between the heart and brain are more complex than thought [of] in the past",[14] and suggests bidirectional flow of information between the heart and brain.

The heart's internal nervous system allows it to generate and modulate its own electrical signals that can be sent to the brain through neural pathways, such as the vagus nerve. The vagus nerve is the longest cranial nerve in the body and plays a vital role in the parasympathetic nervous system. Upon activation of the vagus nerve, the neurotransmitter acetylcholine is released and binds to muscarinic receptors on the heart, causing a decrease in heart rate. This process helps the body maintain a healthy autonomic balance and is an important component of the vagus-parasympathetic regulation of heart rate.[15,16] Scopolamine impairs this balance by blocking acetylcholine receptors on postganglionic neurons, disrupting heart-brain communication and decreasing vagal tone. The vagus nerve serves as an important connector in the heart-brain axis. Low vagal tone, or low activity of the vagus nerve, has been associated with mental and neurological conditions such as depression, epilepsy, and Parkinson's disease.[17]

***Heart Rate Variability***

The vagus nerve is important in regulating heart rate variability, which reflects the activity of the parasympathetic and sympathetic nervous systems.[18] Heart rate variability (HRV) describes the "changes in the time intervals between consecutive heartbeats called interbeat intervals (IBIs)."[19] It is an "indicator of psychological resiliency and behavioral flexibility, reflecting the individual's capacity to adapt effectively to changing social or environmental demands."[14] A healthy heart typically exhibits complex patterns of heart rate variability; the IBIs will constantly

change.[19] HRV can also tell us how well our brain and body work together when it comes to emotions and thinking. Research has shown that people with higher HRV tend to have improved emotional regulation, and people with lower HRV may have more difficulty regulating their emotions and coping with stress.[20]

It has been generally observed that HRV naturally declines with age due to a variety of factors including changes in the autonomic nervous system, structural and functional changes in the cardiovascular system, and hormonal shifts. This decline may be influenced by the natural changes and reductions in acetylcholine with aging. A large longitudinal UK population‐based study from 2016 showed that "HRV decreased with aging independent of pathological conditions or medication use."[21] However, it has been found that lower HRV may also be a "predisposing factor that increases the susceptibility of developing emotional and psychiatric problems."[20]

***HRV as a Potential Biomarker for Cognitive Decline***

HRV reflects "an estimation of autonomic control of the heart"[22] and may have close associations with the development of dementia. Low HRV has been a common finding in many mental disorders such as depression, anxiety, and schizophrenia, but its role in dementia patients is still not completely understood. However, low HRV reflects a reduction in vagal activity, which can be associated with dementia. HRV has the potential to act as a peripheral biomarker for cognitive impairment, but more research on this metric across the lifespan is needed so that changes in HRV due to natural aging can be distinguished from changes in HRV due to cognitive decline.

Biomarkers such as neuroimaging and cerebrospinal fluid examinations are important in diagnosing dementia, but they are expensive, invasive processes that are not equally accessible to all.[22] Cognitive impairment such as dementia is also difficult to diagnose, especially in the early stages of disease due to the compensatory mechanisms of the brain. HRV could act as a cheaper, non-invasive method that could potentially function as an earlier biomarker to cognitive decline.

# *Methods*

## *Subjects*

Male and female Long-Evans rats (n=14, male=13) were obtained from Charles River Laboratories and used across two studies. Rats were named into three distinct age cohorts: "Adult" (n=4, male=4, mean age=7 mo), "Older Adult" (n=7, male=6, mean age=21.5 mo), and "Elderly" (n=3, male=3, mean age=31 mo). As done in similar previous studies,[5,6,13] rats were housed individually and kept on a 12-hour light-dark cycle. The rats were allowed one week for acclimatization to the humidity- and temperature-controlled vivarium in the Weill Conventional facility of Cornell University. The following week, rats were acclimated to human handling by lab members for 15 minutes each day, and then acclimated to the experimental apparatus for 15 minutes each day during the next week. To ensure motivation to perform the task, access to water was restricted for 24 hours prior to their initial training. After each session, the rats were given ad-lib access to water for 15 minutes; otherwise, they were only provided water as a reward during experiment sessions. Previous research in the lab has shown that this post-experiment water time is adequate for hydration, and rats do not typically experience adverse outcomes. Veterinary staff and lab members periodically monitor the rats for signs of poor health such as ungroomed hair and porphyrin staining.

## *General Paradigm*

### *Olfactometer*

The following description of the olfactometer is based on previous similar studies.[5,6,13] An olfactometer is an odor generator and test chamber designed to deliver odorants in a precise and controlled manner to test subjects. Clean air flows through a series of channels controlled by flow meters and two-way solenoid valves at 1.5 liters per minute (LPM). After being dehydrated, filtered, and rehydrated, clean air passes through vials containing odorants before being directed to the odor ports as designated by the specific task.[6] A detailed description of the olfactometer construction can be found in a previous study by De Rosa and Hasselmo (2000).[13] Infrared (IR) photobeams span across the openings of two odor ports, a water port, and the width of the box to act as a "trial initiation" beam. A green LED and a bright yellow LED positioned above the water well signaled "correct" and "incorrect" trials, respectively. Between each trial, air directed into the experimental apparatus was cleared by a vacuum pump. A custom Python program was used to operate each task and record progress by test subjects.[6] A schematic of the experimental apparatus can be found in **Figure 1a.**

### *Shaping Stages*

After being acclimated to human handling and the experimental apparatus, rats performed a series of "shaping" tasks in order to build the skills necessary for the final proactive interference (PI) odor-detection task. The shaping stages taught rats to "sit" in front of an IR beam to initiate a trial, "poke" an odor port by sticking their nose past an IR beam, and receive water from the water well when a green light turns on. These stages prepared rats for the "Odor no Odor" task, the first stage that utilizes specific odorants in the experiment. In this stage, rats learned to

determine which of two odor ports in the olfactometer is releasing an odorant; they must "poke" their nose across the IR beam in the port releasing an odor to receive a "correct" response. Following the successful completion of 64 trials of "Odor no Odor," demonstrating high accuracy across various scents, rats advanced to the "AB" stage. The goal of this stage was to overlearn a baseline odor pair, the "AB" odor pair, where odor "A" was the "correct" odor and odor "B" was the "incorrect" odor. Upon mastery of "AB," rats advanced to "Full Task," where they must resolve proactive interference by overcoming past learning of the previous target stimulus, odor "A". Rats must learn that when odor "A" is paired with odor "B," odor "A" is the correct response, but when odor "A" is paired with a new odor "C," odor "C" is the new correct target. A novel odor pair, "DE" is also included in "Full Task" as a nonoverlapping odor set to represent "new learning." For any stage of the experiment, rats receive 0.05 ml of water as a reward for "correct" responses. Behavioral data was analyzed using R statistical software (v4.3.3; R Core Team 2024) to determine when the rats were prepared to advance to each stage.

***General Full PI Task Paradigm***

Successful completion of "Full Task" required rats to complete 80 trials of odor discriminations. Each odor set used in this task contained five perceptually distinct odorants, two of which were used and learned in the preceding "AB" task. In the beginning of "Full Task," rats were presented with 16 trials of the previously learned "AB" odor set (A+B-). The "+" following a letter indicates the target odor ("correct" response) within the particular odor pair, and the "-" indicates the distractor odor ("incorrect" response). The following 64 trials randomly presented either an "AC" odor pair (A-C+) or a "DE" odor pair (D+E-), where the "A" odor was the same in the "AB" and "AC" odor sets. A schematic of the final PI task can be found in **Figure 1b**. In

each trial, two distinct odorants were simultaneously released from separate odor ports, with the presentation of each odor from a particular port being counterbalanced across trials.[5] Average accuracy during "Full Task" was analyzed using R statistical software (v4.3.3; R Core Team 2024) to determine the number of "correct" responses out of trials completed.

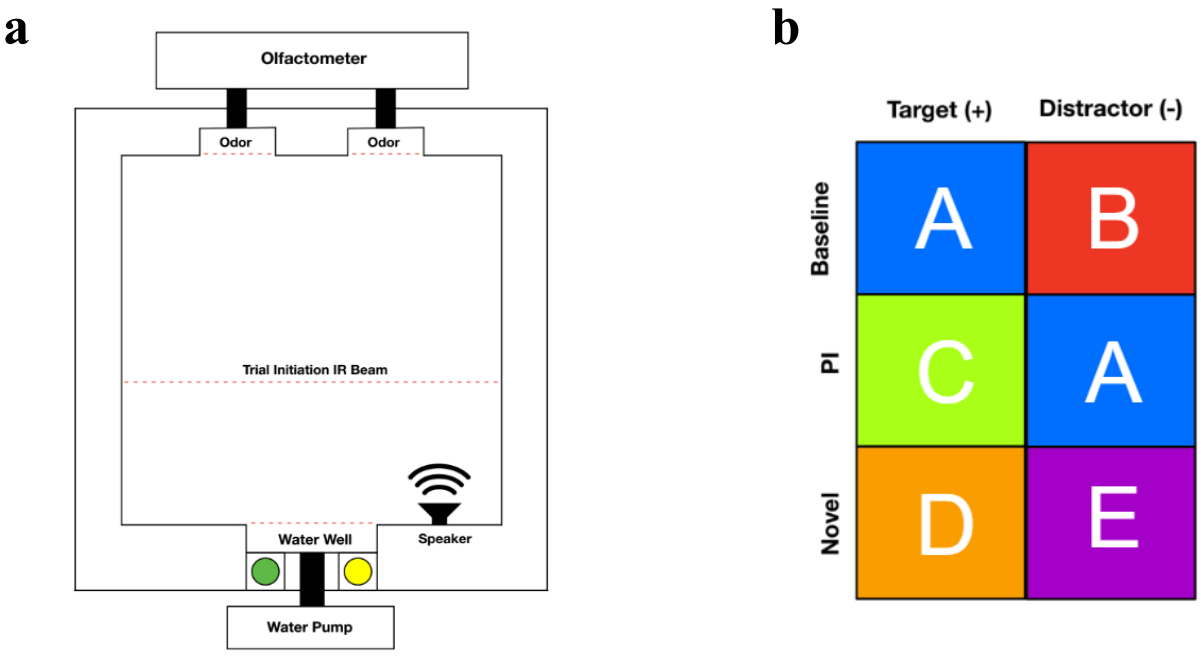



**Figure 1.** *Experimental apparatus and "Full Task" schematic. Figures were developed from Cammarata & De Rosa (2022)* ***(a)*** *Operant chamber with the olfactometer and water pump attached. Infrared (IR) beams are represented by dashed red lines. A green light signals a "correct" response while a yellow light and loud tone from a speaker signal an "incorrect" response.* ***(b)*** *Schematic of the final proactive interference task. Odors are represented by different colors and letters. Target ("+" signifies a correct response) odors are represented in the left column and distractor ("-" signifies an incorrect response) odors are represented in the right column.*

***Heart Rate Variability (HRV) Data Collection Across the Lifespan***

Situational heart rate variability (HRV) data was collected from the "Adult" and "Elderly" cohorts from surgically implanted wireless telemeters (TR50BB, ADInstruments) that recorded electrocardiogram (ECG) data. A telemeter is a device that measures and transmits data over a particular distance. The telemeters used in the study had a pair of biopotential leads that were placed in the lead II configuration. One lead was placed on top of the xiphoid process and the second was tunneled to the neck and placed parallel to the trachea and sutured to the sternomastoid muscle, with a final placement next to the right atrium. This positioning optimized signal quality and reduced potential interference while rats were conscious and moving around. The electrodes produced a voltage signal, measured by an ECG. Data was recorded at 1000 Hz and filtered using a 200 Hz low-pass filter. ECG data was recorded for 20 minutes of "sleep"and 10 minutes of "baseline" activity. ECG data was also recorded during "Full Task," however due to data dropout and signal noise, this data was not analyzed for the present study. The "baseline" data was recorded immediately before rats completed odor detection tasks. ECG data from the "Adult" cohort was analyzed over part of their lifetime, from 7 to 17 months of age, for a within-subjects comparison. ECG data from the "Elderly" cohort at 31 months was compared to ECG data from the "Adult" cohort at 7 months of age, for a between-subjects comparison. In future studies, ECG data from the "Older Adult" cohort will also be analyzed, however due to time constraints and resource limitations, telemeters were not implanted in this cohort for the present study.

The HRV metrics extracted from ECG data included average RR and RMSSD. The RR interval—also referred to as the interbeat interval (IBI)—is the time elapsed between consecutive

heart beats, from one R peak to the next in a QRS complex **(Fig. 2)**. A smaller RR interval corresponds to a faster heart rate. RMSSD is the root mean square of successive differences between normal heartbeats and is obtained by squaring the successive time differences between heartbeats in milliseconds, averaging the squared values, and taking the square root of the total. RMSSD reflects beat-to-beat variance in heart rate and is commonly used to estimate vagally mediated changes in HRV.[19]

The HRV module of LabChart Pro (ADInstruments) was used to exclude beats where RR intervals were outside the range of 100-200ms, the standard range used for rats in the module. When necessary, parameters were adjusted based on each individual rat's data. The HRV beat detection algorithm was used to identify and analyze RR intervals. Data were visually inspected to remove sections of data with large amounts of high frequency noise or data dropout, including ectopic beats. The LabChart HRV module (ADInstruments) was used to calculate RMSSD (ms) and average RR (ms) intervals of 2 minute clips of each day of "sleep" and "baseline" data for each rat. Data was further analyzed using R statistical software (v4.3.3; R Core Team 2024).

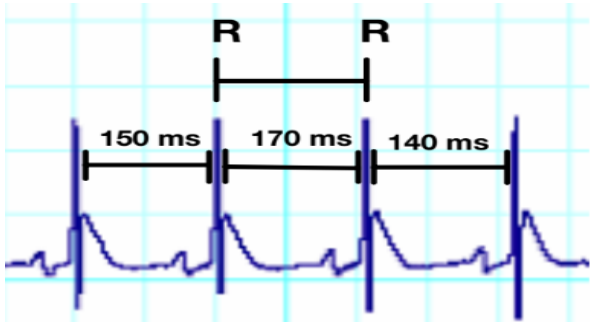



**Figure 2.** *An example of the QRS complex from sleep ECG data from rat 198, recorded by LabChart Pro (AD Instruments). The RR interval is shown with example measurements between each R peak.*

***Systemic Pharmacological Intervention***

The "Adult" (n=4, male=4) and "Older Adult" (n=7, male=6) cohorts were systemically administered IP injections of either normal saline (0.9% sterile medical grade given at the same volume as the calculated anticholinergic volumes), methylscopolamine (0.25 mg/kg), or scopolamine (0.25 mg/kg). The dosage of 0.25 mg/kg was based on previous research that showed selective impairment of PI resolution for scopolamine; lower doses did not have an effect on PI resolution, and higher doses indiscriminately affected both new learning and learning with interference.[6] An overview of the following description of the injection regimen for the study can be found in **Table 1**. During the first two weeks of injections, all rats received saline injections as a control for cholinergic manipulation and to allow for the measurement of heart rate and HRV changes due to the stress of the injection itself. A second week of saline injections was added to acclimate rats to the stress of injections. During the first week of anticholinergic injections, rats received either methylscopolamine or scopolamine. The order of administration was randomized between rats, so that half received scopolamine first, and the other half received methylscopolamine first. Lab members were also blind to drug assignments. During the last week of injections, the two treatment groups were flipped, so that each rat received both methylscopolamine and scopolamine injections. This last week of injections occurred 2 weeks later than originally intended due to poor rat performance on "Full Task" during the second week of anticholinergic injections. During non-injection weeks, rats learned the "AB" odor set to be used in the following injection week for "Full Task." A new odor set was used for each drug condition.

Rats were administered injections 20 minutes before beginning “Full Task.” ECG data from the “Adult” cohort was collected for 10 minutes of “baseline” before injection and 20 minutes of “baseline” post injection. When permitted, the first 2 minutes of the “baseline” pre-injection ECG and the last 2 minutes of the “baseline” post-injection ECG were used for RMSSD (ms) analysis using LabChart Pro (ADInstruments). If data dropout or signal noise within these windows did not allow for proper analysis, alternative 2 minute clips in the ECG recording were used. Average accuracy of each odor pair during “Full Task” was analyzed for all rats for each drug condition using R statistical software (v4.3.3; R Core Team 2024). This was run as a within-subjects manipulation to reduce the impact of individual differences between rats.

**Table 1.** *Overview of Injection Regimen. The experiment was run as a crossover design, and researchers were blind to cholinergic injection type. “Methyl” is used to abbreviate methylscopolamine and “scop” is used to abbreviate scopolamine. Odors A-E are specified for each week. The “AB” stages in between each “PI” week were necessary to learn baseline odor pairs and to allow for drugs to completely clear from rat systems. The first week of saline injections is not shown, as this was an acclimation week and thus data was not analyzed.*

| Week | Injections | Stage | Odors |
|---|---|---|---|
| 1 | n/a | AB | Sandalwood (A) vs. Chamomile (B) |
| 2 | saline | PI | Sandalwood (A) vs. Chamomile (B)<br>Fennel (C) vs. Sandalwood (A)<br>Ylang Ylang (D) vs. Clary Sage (E) |
| 3 | n/a | AB | Allspice (A) vs. Litsea (B) |
| 4 | methyl/scop | PI | Allspice (A) vs. Litsea (B)<br>Parsley (C) vs. Allspice (A)<br>Cajeput (D) vs. Myrrh (E) |
| 5 | n/a | AB | Myrtle (A) vs. Calamus (B) |

| | | | |
|---|---|---|---|
| 6 | methyl/scop | PI | Myrtle (A) vs. Calamus (B)<br>Coriander (C) vs. Myrtle (A)<br>Bay (D) vs. Catnip (E) |

***Statistical Analysis***

All statistical analyses were performed using R statistical software (v4.3.3; R Core Team 2024). Outliers in RR intervals (ms) were identified and removed from the data separately for "Sleep" and "Baseline" trials within the "Elderly" and "Adult" cohorts. Due to a non-normal distribution and based off of previous HRV studies,[19] RMSSD was log-transformed before outliers were excluded. Specifically, RR (ms) and RMSSD (ms) values that were more than 3 standard deviations from the mean were considered outliers, and were not included in analysis. Line graphs for average RR (ms) and RMSSD (ms) were separately plotted over the course of 10 months to visualize general trends over the lifespan of 2 rats from the "Adult" cohort. HRV between the "Adult" and "Elderly" cohorts was assessed via linear mixed-effects models[23] with RR (ms) or RMSSD (ms) as the dependent measure, rat SID as random effects, and session (day 1-6), trial type ("baseline" or "sleep"), cohort ("Adult" or "Elderly"), and the interaction of trial type and cohort as fixed effects. Outputs of the mixed models were used to calculate estimated marginal means and conduct pairwise comparisons[24] with Kenward Roger estimation of degrees of freedom.[25] Normality of residuals was calculated using the DHARMa package[26] to test assumptions of the model.

Accuracy and HRV during injection weeks were assessed via linear mixed-effects models[23] with average accuracy (% correct) or log(RMSSD (ms)) as the dependent measure. "Full Task" data with less than 48 trials completed were excluded from analysis to allow for an approximate equal

number of odor pair trials ("AB," "CA," and "DE") performed. Rats who did not achieve a behavioral criterion of 70% or greater on the last 2 days of "AB" learning were also excluded from analysis. For the HRV model, log(RMSSD (ms)) was the dependent measure, rat SID was included as a random effect, and session, drug (saline, methylscopolamine, or scopolamine), type (pre-injection or post-injection), and the interaction of drug and type were included as fixed effects. For the accuracy model, average accuracy (% correct) for each trial type ("AB," "CA," or "DE") was the dependent measure, rat SID was included as a random effect, and session, sex, cohort, drug, and the interaction of drug and cohort were included as fixed effects. Outputs of the mixed models were used to calculate estimated marginal means and conduct pairwise comparisons[24] with Kenward Roger estimation of degrees of freedom.[25] Normality of residuals was calculated using the DHARMa package[26] to test assumptions of the model.

## *Results and Discussion*

### *HRV Over the Lifetime: Within-Subjects*

The changes in RR interval (ms) and RMSSD (ms) over time for "Sleep" and "Baseline" conditions were first compared across the lifespan of the "Adult" cohort. Over a period of approximately 7 months of age to 17 months of age, the respective "Sleep" and "Baseline RR (ms) averages remained stable. Average RR (ms) during sleep was consistently higher compared to average RR (ms) during baseline activity (**Fig. 3a**). Since the RR interval is inversely related to heart rate, this means that heart rate was consistently lower when rats were sleeping versus awake across the lifespan. This is logical as the heart is generally under less stress during sleep.

These data are comparable to other studies evaluating heart rate across the rat lifespan, which also did not find significant differences in heart rate between 6 and 28 months of age.[27]

Over the same time period, the respective "Sleep" and "Baseline" log(RMSSD (ms)) averages were not as clearly distinguished in the first few months of recordings. However, after 11 months, there is a clear separation between trial types, with "Sleep" log(RMSSD (ms)) being higher than "Baseline" log(RMSSD (ms)) (**Fig. 3b**). Overall, there is no clear pattern of general increases or decreases across the lifespan. These results conflict with previous studies that have shown significant rapid declines in RMSSD in healthy aging humans[28] and "U-shaped" patterns for RMSSD with aging, decreasing from 40 to 60 years and increasing again after age 70.[21] Reduction in RMSSD over time suggests reduced autonomic regulation of heart as individuals age.[29] Based on available literature, it seems that previous studies have not yet investigated metrics in HRV in aging rats. Perhaps the pattern of RMSSD (ms) changes in aging humans differs in aging rats.

Faster heart rates, and therefore shorter RR intervals, reflect a reduction of time between successive beats as well as the opportunity for the interbeat intervals (IBIs) to vary, lowering HRV. Slower heart rates, and therefore longer RR intervals, reflect an increase in time between successive beats and the chance for IBIs to vary, increasing HRV.[19] Separation of baseline and sleep RMSSD (ms) after 11 months of age could potentially be due to increased RR intervals during sleep compared to baseline, however this pattern is not seen before 11 months. It is also crucial to note that these findings are based on a sample size of 2. The original sample included 3 rats, but unfortunately, one of the telemeters malfunctioned during data collection. Given the

costliness of wireless telemeters and the potential delays in rat recovery post-implantation due to surgical infections, it's important for future research to prioritize increasing sample sizes and devising strategies to minimize data dropout.

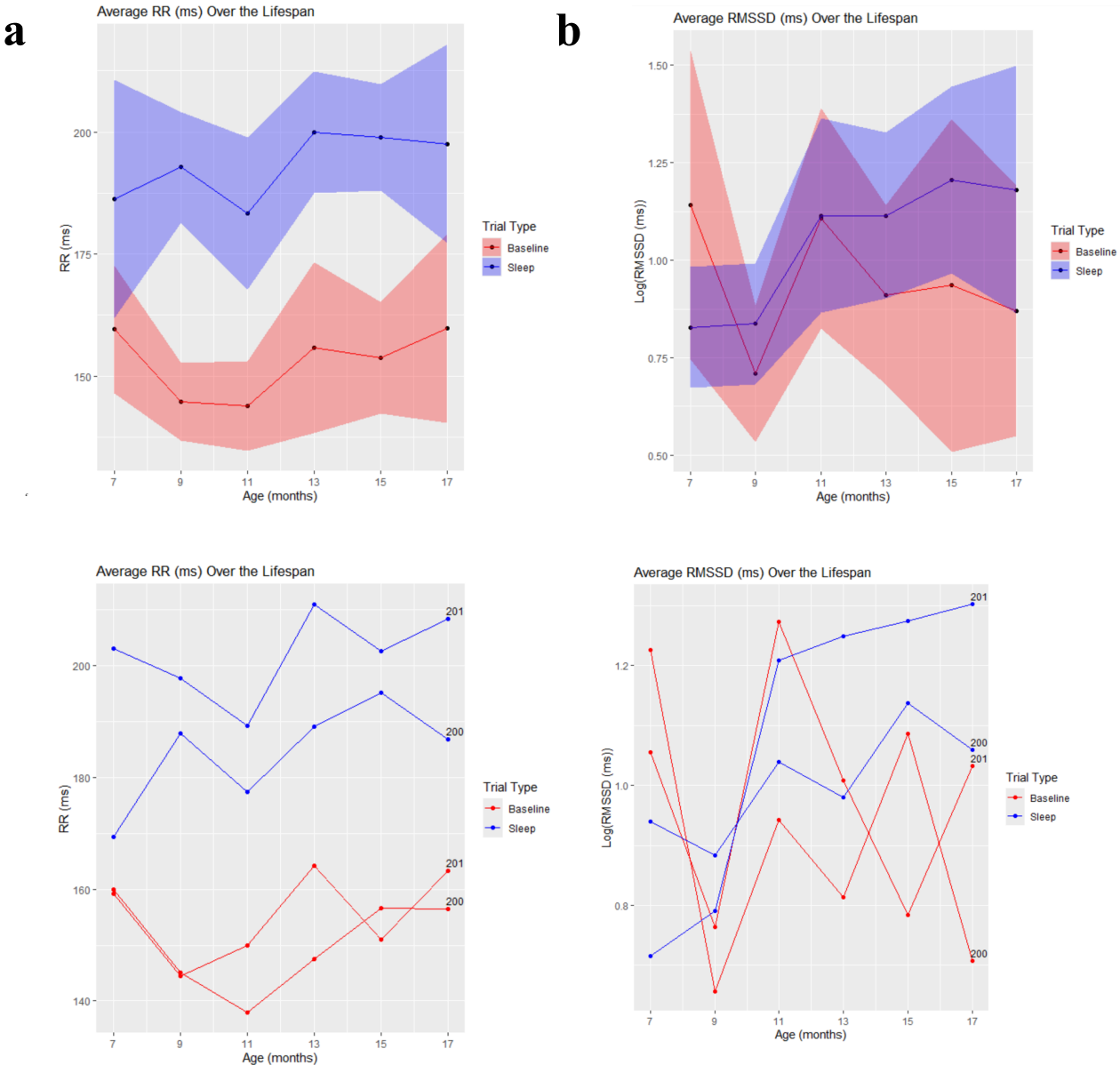


**Figure 3.** *Average RR intervals (ms) and RMSSD (ms) over the lifespan of two male Long-Evans rats (SID 200 and 201) during sleep and baseline conditions.* ***(a)*** *The top plot shows mean and standard deviations of RR intervals (ms) during sleep and baseline conditions across subjects over time, and the bottom shows individual subject averages over time.* ***(b)*** *The same visualization but for RMSSD (ms).*

***HRV Over the Lifetime: Between-Subjects***

The average RR intervals (ms) and RMSSD (ms) for "Sleep" and "Baseline" conditions were compared between the "Adult" cohort at 7 months of age and the "Elderly" cohort at 31 months of age. Analysis revealed that RR (ms) during sleep and baseline activity between age groups was not statistically significant (**Fig. 4a & 4b**). Again, these results are comparable to heart rate studies on rats over the lifespan that have indicated no significant changes in heart rate from 6 to 28 months,[27] which is a similar age difference to that of the present study. This is not surprising, as resting heart rate does not change significantly with normal aging.[30] Average RMSSD (ms) between cohorts was not statistically significant for either sleep or baseline conditions **(Fig. 4c & 4d)**. Notably, there was increased spread in RMSSD (ms) in the "Elderly" cohort compared to the "Adult" cohort during sleep conditions (**Fig. 4c**), which may hint towards a differentiation in HRV between cohorts. A few studies have observed "U-shaped" patterns in RMSSD over the lifetime,[19,31] and it is possible the data reflect this trend. One rat month is approximately equal to three human years,[32] thus the "Adult" cohort at 7 months of age is comparable to 21 human years, and the "Elderly" cohort at 31 months of age is comparable to 93 human years. A study in humans found that individuals in the 20-29 age group have similar RMSSD to those in the 80-99 age group. The authors noted that the decrease in HRV-parasympathetic function seen after the

second decade of life reached its lowest point in the eighth decade of life, but then progressively increased to higher levels typical of a younger population. They concluded that healthy longevity depends on preservation of HRV-parasympathetic function, despite the observed age-related decreases early on in life.[31] The RMSSD of the "Elderly" cohort may reflect preserved HRV-parasympathetic function, however, HRV data from earlier in their lifespan is needed to confirm this trend.

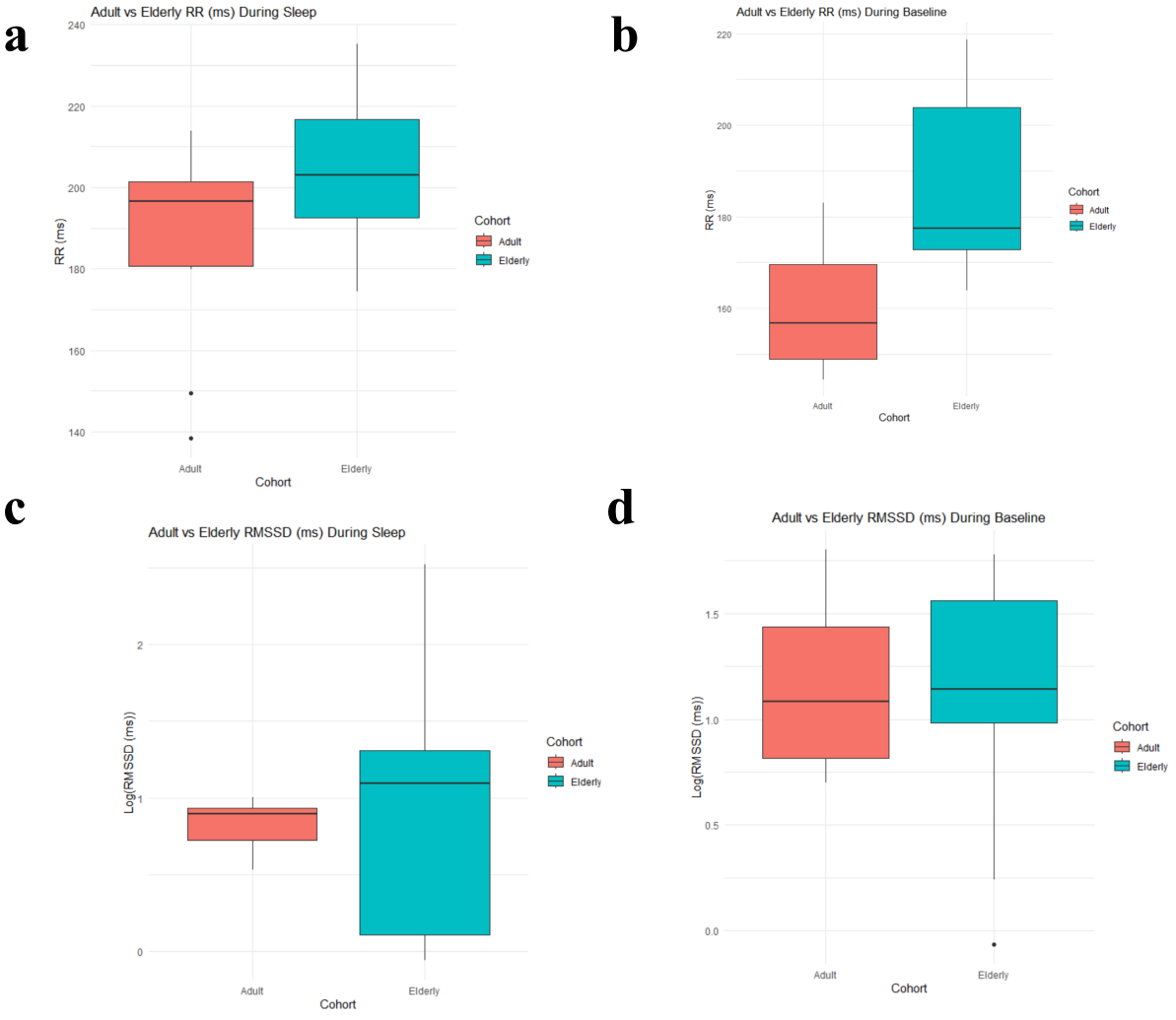


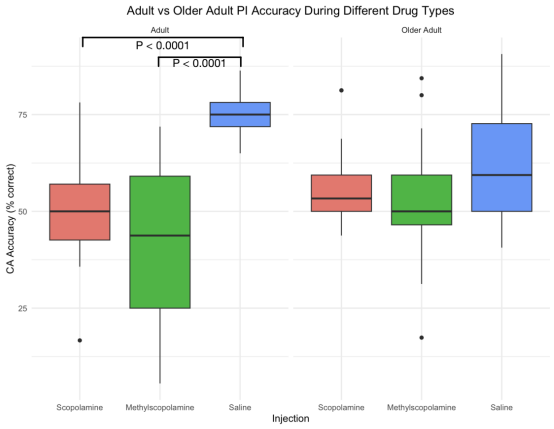
**Figure 4.** *Average RR intervals (ms) and RMSSD (ms) during sleep and baseline conditions between "Adult" (n=2) and "Elderly" (n=3) cohorts at 7 months and 31 months of age, respectively.* ***(a)*** *The average RR (ms) over 5 days of data collection between cohorts during sleep.* ***(b)*** *The same visualization but for baseline conditions.* ***(c)*** *The average RMSSD (ms) over 5 days of data collection between cohorts during sleep.* ***(d)*** *The same visualization but for baseline conditions.*

***Impact of Pharmacological Manipulation on Learning***

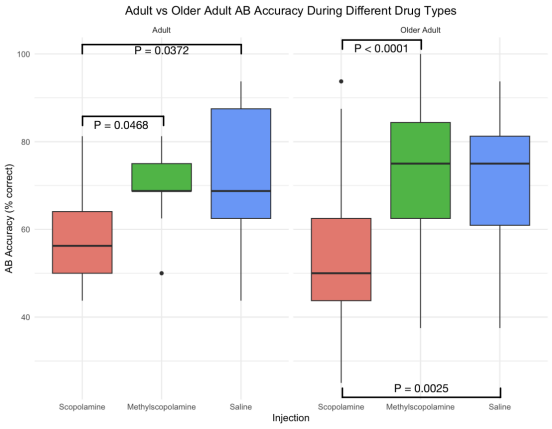
The average accuracy of each odor trial type ("AB," "CA," and "DE") was compared across injection types for the "Adult" and "Older Adult" cohorts. Adult rats receiving either scopolamine (0.25 mg/kg) or methylscopolamine (0.25 mg/kg) were significantly impaired at overcoming PI compared to saline controls (**Fig. 5a**). While previous studies have shown that scopolamine selectively increases PI, and methylscopolamine performance is comparable to saline controls,[5] the present study does not show this selectivity. This data shows that there is not a significant difference in ability to overcome PI whether adult rats receive methylscopolamine or scopolamine (**Fig. 5a)**. These results may be due to the older age of rats in the present study compared to previous studies that have studied drug effects on PI in younger rats. In a recent study, researchers found that aged rats (20.7 ± 0.5 months) were less able to resolve PI compared to their adult (9.8 ± 1.3 months) counterparts, but scopolamine did not have an additional effect on PI accuracy as was seen in the adult group.[6] At the time of "Full Task" data collection, the "Adult" cohort in the present study was around 18 months of age, which may explain why their behavior is more reflective of the "aged" group in the previous study. This apparent "aging effect" continues with the "Older Adult" cohort of the present study, who were around 21 months

of age at the time of data collection. The difference in their PI performance between scopolamine and methylscopolamine conditions is even less significant compared to that of the "Adult" cohort. This data suggests that as individuals age, there appears to be a modification in how cholinergic drugs influence the efficiency of encoding new information, particularly when prior learning interferes with this process.[6]

**a**




**b**




**c**

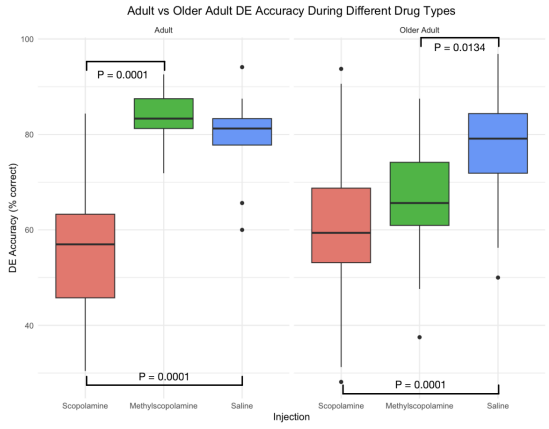


**Figure 5.** *Accuracy during "Full Task" of each odor trial type of "Adult" (n=4) vs "Older Adult" (n=9) cohorts for each drug manipulation. Data with less than 48 trials of "Full Task" completed were excluded from analysis. Rats who did not achieve an average accuracy of 70% or greater on the last 2 days of "AB" before the week of "Full Task" were also left out of analysis.* ***(a)*** *Average PI (CA) accuracy from one week of each Injection type of the "Adult" and "Older Adult" cohorts.* ***(b)*** *Same visualization but for AB accuracy.* ***(c)*** *Same visualization but for DE accuracy.*

Both adult and older adult rats receiving scopolamine (0.25 mg/kg) were significantly impaired in their response to the previously learned AB odor pair compared to those receiving either methylscopolamine (0.25 mg/kg) or saline injections (**Fig. 5b**). Based on previous studies, cholinergic manipulation should not impact previously learned information.[5, 13] Specifically, scopolamine should not have a meaningful impact on well-practiced odor discriminations.[33] One possible explanation for this unexpected result could be that the AB odor pair was not completely mastered during the baseline week leading up to "Full Task." Rats who did not achieve an average accuracy of 70% or greater on the last two days of "AB" before each "Full Task" week were excluded from analysis. Accuracy thresholds of 80% or greater were too stringent and would've reduced the sample size by 75% in some cases. The 70% accuracy threshold may not have been reflective of a heavily learned odor discrimination, leaving the AB odor pair susceptible to influence by scopolamine. Under methylscopolamine conditions, both cohorts behaved comparably to saline controls, which was expected.

Under scopolamine (0.25 mg/kg) conditions, adult rats performed significantly lower on new learning ("DE" trials) compared to both methylscopolamine and saline conditions (**Fig. 5c**), which was not expected. It is possible that the dosage chosen for this study on older rats impacted new learning as previous studies have shown that higher doses of scopolamine impaired rats' ability to acquire novel odor pairs in younger rats.[13] A dose-response curve was not performed for this study and could aid in future dosage decisions in rat cohorts of various ages. A recent study has demonstrated that scopolamine selectively impairs the acquisition of PI trials (overlapping AC odor pairs) but not nonoverlapping DE odor pairs, regardless of age.[6] However, the animals in the present study were taught the "AB" learning task at an older age compared to previous studies[6] and therefore did not acquire the odor pairs to the high "over-learning" performance required to show robust PI. Under methylscopolamine conditions, adult rats behaved comparably to saline controls, which was expected. Under scopolamine (0.25 mg/kg) and methylscopolamine (0.25 mg/kg) conditions, older adult rats performed significantly lower on "DE" trials compared to saline controls (**Fig. 5c**), but their performance between scopolamine and methylscopolamine injections was comparable. It is expected that rats have comparable accuracy across all drug groups, and the discrepancy again may be due to later "AB" learning of the rats in the present study.

Unexpected "Full Task" results in the present study may also be due to the sample size of the adult (n=4) and older adult (n=7) cohorts that does not allow for substantial statistical power. Previous similar studies have suggested that a sample size of 9.6 animals allows detection of moderately sized drug effects,[6,13] therefore future research should focus on increasing sample size to strengthen conclusions of the study.

***HRV from Cholinergic Manipulation***

HRV was evaluated before and after injections of saline, methylscopolamine, or scopolamine. As expected, saline did not cause a significant change in HRV. The appearance of a drop in HRV after saline injection when looking at **Figure 6** may be due to the stress of the injection itself. Scopolamine and methylscopolamine both caused significant decreases in HRV, suggesting a reduction in vagal tone due to reduced cardiac parasympathetic regulation.[34] Scopolamine produces effects both in the body's peripheral systems and in the central nervous system, while methylscopolamine only affects the periphery. Peripheral effects involve blocking certain receptors sensitive to acetylcholine, which leads to actions like reducing muscle contractions, decreasing secretions from glands, and altering heart rate. Meanwhile, in the central nervous system, scopolamine induces sedation, helps control nausea, and causes temporary memory loss.[35] Acetylcholine is released by parasympathetic nerve fibers in the autonomic nervous system, where it functions as a neurotransmitter at various target organs such as the heart. It plays an important role in responding to stress,[36] thus muscarinic blockade by scopolamine may hinder an appropriate physiological response in stress-inducing situations by disrupting a healthy autonomic balance. Drops in HRV from cholinergic manipulation may be reflective of reduced ability to respond to the cognitive stress induced by "Full Task," leading to the subsequent impact on learning and memory. However, although HRV allows for the measurement of the influence of the parasympathetic nervous system on the heart, more research is warranted to provide a better understanding of the role of the cholinergic system in memory and cognitive aging.

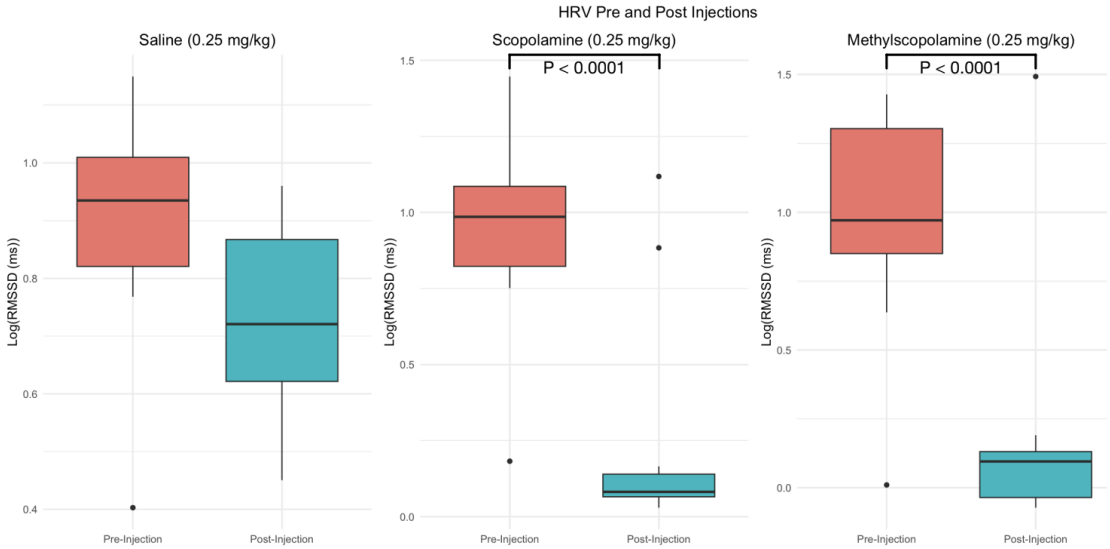



**Figure 6.** *Average log(RMSSD (ms)) before and after each injection type. This data represents an n = 2 from the adult cohort.*

Future research should focus on examining the relationship between PI accuracy and HRV, if such a relationship exists. The heart's connection with the brain is critical as it helps the body allocate its energy based on peripheral and central demands.[37] Not only is the heart responsible for responding to physical demands, but it also has a role in responding to cognitive and emotional needs. Understanding this interaction is crucial for comprehending how the body manages its resources in different situations and how disruptions in heart-brain communication may impact overall health and well-being.

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