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Modulating Neuronal Activity: Copper, Isoproterenol, and Beta-Blockers on the Brain

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Neuromodulation, or modulation of neurons, can be done by several molecules, some that are natural, and some that may need to be taken in the form of a drug. In turn, these molecules can alter the activity of neurons within the brain, such as changing the behavior, the rhythmicity, the functionality, and even the rate of activity. Some examples of neuromodulators include copper and isoproterenol, a synthetic analog of adrenaline.

In this thesis, I will discuss how these neuromodulators can affect the brain in different ways. In the first part of my thesis, "Dynamic Neuronal Activity: Circadian Rhythms and Copper", I will confirm previous results by showing circadian rhythmicity, and I will also discuss copper and its effects on neurons associated with circadian rhythms. This research was done in Dr. Prosser's lab at the University of Tennessee, Knoxville.

In the second part of my thesis, called "Effects of peripheral beta blockers on isoproterenol-induced fear conditioned rats", I will explain how I used neuropharmacology to induced anxiety using a synthetic form of adrenaline (isoproterenol) to confirm previous studies that show that isoproterenol is a valid way to create fear responses in rat. In addition, I will also explain how I utilized peripheral beta-blockers to reduce isoproterenol-associated fear responses. This research was done through an internship called Minority Health and Health Disparities International Training (MHIRT), where I did research at Universidad Federal de Santa Catarina in Florianopolis, Brazil.

Dynamic Neuronal Activity: Circadian Rhythms and Copper

Veronica Go, Yuki Yamada, Rebecca Prosser

Abstract

Circadian rhythms, or biological day-to-day rhythms, are stimulated and regulated by different factors within and outside the body. These circadian rhythms are controlled by a specialized set of pacemaker cells in an area of the brain called the Suprachiasmatic Nucleus (SCN). If the circadian pacemaker is functioning properly, these cells will exhibit neuronal firing that will peak around mid-day. In this study, we confirm circadian rhythmicity and show that highest neuronal firing rates occur during mid-day.

I. Introduction

Circadian rhythms, or behavioral and physiological expressions of an internal "clock", encompass many functions in the body, such as sleep-wake cycles, hormone secretion, body temperature, and other bodily functions that work on a cyclical pattern (Repert and Weaver 2001, Waterhouse 1993). In mammals, a set of specialized pacemaker cells within the Suprachiasmatic Nucleus (SCN) are primarily responsible for generating circadian rhythms (Figure 1) (Turek 1983). In normal conditions, the SCN pacemaker is entrained, or synchronized to external time signals, with light as the dominant entraining signal (Ashcoff 1965). Light must first enter the eye, and undergo neuronal processing before it reaches the SCN (Bersen et al. 2002), where the input stimulates the release of glutamate. Light stimulation at night alters normal entrainment, causing phase advances if it is presented early in the night or phase delays if it is presented late in the night (Daan et al. 1976).

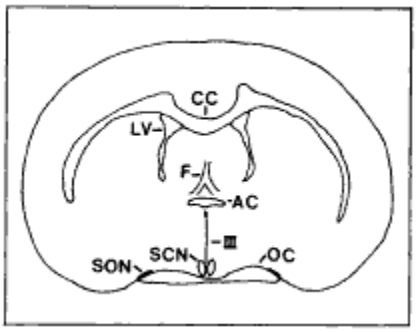

Figure 1: *The location of the Superchiasmatic Nucleus lies just above the chiasm, towards the ventral side of the brain.*

The neuronal firing and physiological activity of the Suprachiasmatic Nucleus can be observed in vitro through electrophysiological experiments. If the organism is healthy and does not have circadian rhythm abnormalities, there will be circadian rhythmicity and high neuronal activity mid-day. In this study, we will use single unit recordings to confirm circadian rhythmicity in the SCN.

II. Materials and Methods

A. Experimental Protocols

Coronal brain slices are collected from adult C57BL/6Nhsd male mice (Harlan Laboratories) that are housed in a 12:12 light/dark cycle. Two brain slices are placed in a continuous perfusion of Earle's Balanced Salt Solution (EBSS), with additions of gentamicin, glucose, and bicarbonate at the pH of 7.4 in a Hatton-style brain slice chamber (Prosser 1998, Hatton et al. 1980). The perfusion of EBSS is then kept warm and oxygenated throughout the course of each experiment (95% O2/5% CO2) at a temperature of 37º F.

B. Single-Unit Recordings

Recordings of single-units (cells) began on the second day in vitro. Glass capillary electrodes filled with 3 M NaCl were used to detect the spontaneous action potentials of single SCN neurons . Upon detecting the action potentials from a neuron, the cell signal is recorded for 5 minutes (Prosser 1998). The neuronal signals are sampled over an 8-12 hour period by moving the electrode to variable areas and depths (using a microtome) of the SCN and recording the neuronal signals found. The movement to different areas and depths of the SCN produces as little sampling bias as possible (Heinricher 2000).

C. Data Analysis

The data is collected using a Data-Wave system and is analyzed using cluster analysis, which separates noise and neuronal signals. Generally speaking, each hour consisted of approximately four to seven single-cell recordings. These individual firing rates were then combined to calculate 2-hour running averages, which have been lagged by 1 hour (±SEM) to determine the overall pattern of neuronal activity. Once the data are analyzed, the peak of the neuronal activity is determined visually by assessing the time of the highest symmetrical activity. For instance, if the two highest 2-hour means are roughly equal, the time of the peak is said to occur halfway between them. (Prosser 1998).

III. Results

A. Control Slices and the Circadian Clock

In the SCN slice recordings, the population neuronal firing rates of untreated control slices peaked during mid-subjective day on 2 d *in vitro* with a mean (± SEM) time-of-peak of

zeitgeber time (ZT) 6.75 ± 0.07 (n=4; Fig. 2). These results are consistent with previous studies that show peak activity occurring around midday on days 1-3 *in vitro* (Gillette and Prosser 1988, Prosser et al. 1989).

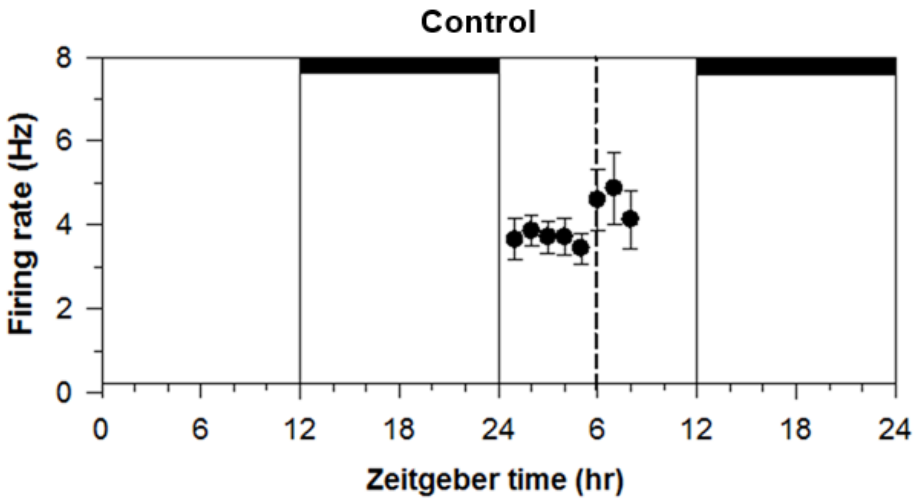

Figure 2: *This is the 2-h means ± SEM of SCN neuronal activity that was recorded in untreated control slices on day 2 in vitro. This is a control experiment with no drug treatment displaying a normal peak around midday, at Zeitgeiber time (6.75 ZT).*

IV. Discussion

A. Implications

This experiment's results confirm previous results of neuronal firing in the SCN. While there was a peak that occurred at 6.75, the time of peak could be clearer if the recording was extended by 1-2 hours (resulting in the experiment ending at the 10^{th} ZT hour). By doing this, we could determine whether or not firing of the neuron was actually declining at the 9^{th} or 10^{th} ZT hour.

While the results of these experiments are consistent with previous reports (REFS), the recordings do not show the sinusoidal pattern typically seen. Several factors may have affected the quality of my results. For instance, conservative analysis – analysis that includes minimal noise and enough signal without including too much noise – was done in Data-Wave, which can result in seemingly low firing rates. However, because this style of analysis was done throughout all experiments, it resulted in consistent data analysis throughout all experiments and small peaks for each experiment.

Other factors that affected the quality of the recordings are biological aspects, such as the health of the slice or the biological needs of the slice. For example, there were some days in which the oxygen was not being perfused into the solution because of a faulty seal that allowed oxygen to leak into the outside environment instead of directly going into the Hatton style dish. This could have negatively affected the health of the tissue. Other issues that arose were technical errors that affected the recording quality. For example, there were many experiments that had circuit issues (i.e. DC shifting and 60 cycle). To account for these electrical issues, most lights were turned off and all electronic devices that could transmit signal – such as laptops and cell phones – were removed from the general area to prevent extraneous electrical signal from infiltrating the recording. However, noise was still an issue even after removing all electronic devices and turning off the lights, which may have been a result of external factors (such as a storm) that resulted in inevitable noise in the recording. Other issues that arose during the experiments were problems with the electrode, in which there were experiments in which there was salt leaking from the electrode or there was salt accumulation on the electrode. Both of these issues can result in seemingly small amplitude action potentials when recording, even though the neurons may actually be exhibiting large action potentials. To account for this, we changed the

electrode every few hours and broke a piece off of the electrode as minimally as possible to keep the electrode sharp and able to record good data and to disallow the salt from leaking and infiltrating the brain slice.

Finally, there are many different neurons within the SCN. For example, previous studies have shown that there are neurons that can differ in neuropeptide concentration, have different expressions based on environmental cues, and even have disparities in the rhythms they control (Antle and Silver 2005). In fact, there are some cells that can exhibit "clock" or pacemaker genes that can control the rhythmicity of the circadian rhythms, while some neurons can only express physiological response when experiencing photic stimulation, and some neurons that can allow for the connection between these two large subgroups of cells (Antle and Silver 2005). In addition, there are some neurons that have different expressions during the day vs. during the night (Colwell 2011). For instance, some cells, such as calcium dependent neurons, require light to have depolarizing activity (Colwell 2011). This results in highest activity during the day and low activity at night. Conversely, some cells, such as cells that are heavily engrained with potassium channels are more likely to have higher activity at night through repolarization of the neurons, but ultimately have low activity during the day. Such differences in neuron types can result in different characteristics, such as different firing rates throughout the day (Colwell 2011). Based on this information, although there are many different neurons and firing rates from each of the subsets of neurons, the rhythmicity of the experimental data is not affected, since previous results have shown similar outcomes.

B. Future Directions

As previously stated, future directions would include extending the recordings an additional 1 to 2 hours to more accurately determine the time of peak in the firing rates. Another future direction would involve the use of drug treatments on *in vitro* brain slices to observe changes in neuronal firing rates, such as using copper drug treatments. For example, previous studies have shown that copper can affect periodicity of circadian rhythms, and has the ability to affect overall health in organisms as well. (Yamada and Prosser 2014).

C. Copper Level Disturbances and Health

Because copper is an essential component within many active sites of enzymes, it has an important role in biological equilibrium and overall health. Hence, maintaining a stable amount of copper within an organism is extremely critical for maintaining proper health. In fact abnormalities in copper levels can result in diseases (Uriu-Adams and Keen 2005). For instance, copper deficiency, usually caused by a lack of copper in nutrition can result in various coronary heart diseases, such as arrhythmias, as well as possible osteoporosis (Klevay 1998). Other issues that can occur as a result of copper deficiency can result in anemia and the inability to form proper musculature (Danks 1998).

Conversely, while there are many ailments that can arise due to the lack of copper there are also many pathologies that can arise as a result of copper excess, or copper toxicity (Uriu-Adams and Keen 2005). This condition of having too much copper within an organism usually occurs due to genetic abnormalities, age, and diet (Bremmer 1998). For instance, there are several diseases within the liver (such as cirrhosis due to copper build up) and gastrointestinal tract that can arise as a result of an excess in copper (Barceloux 1999). In addition, a copper

excess can also result in weakness and lethargy (Semple et al. 1960). Finally, because of copper's ability to produce free radicals, it can cause oxidative stress and damage DNA within an organism, such as causing double-stranded breaks (Kawanishi et al. 1989).

While there are a variety of pathologies that can occur at the larger scale, such as damage to the liver or the disruption of proper muscle development, it can also affect smaller, individual cells, such as neurons within the brain. In fact, there are several diseases that can stem from an anomaly in copper levels. For instance, there are several diseases that are associated with copper anomalies. For instance, some of the most prominent diseases in the brain include Menke's disease, Wilson disease, and several neurodegenerative diseases, such as Alzheimer's disease, sclerosis, and Parkinson's disease (Madsen and Gitlin 2007, Gaetke and Chow 2003). While Menke's disease occurs due to a copper deficiency, Wilson's disease occurs due to a copper excess (Madsen and Gitlin 2007). These diseases are both due to improper ATP-dependent copper transport. Specifically in Wilson's disease, there is a mutation in the pWD gene, which results in the inability to export excess copper from the cell (Tanzi et al. 1993). Through the use of BLAST sequences, previous studies showed that the pWD gene responsible for Wilson's disease has sequence homologies that are identical to the gene *Mc1,* a gene that is also responsible for copper transport (Tanzi et al. 1993). However, a mutation in the *Mc1* gene results in the inability for the cell to import proper amounts of copper into the cell, resulting in a copper deficiency. Ultimately, this copper deficiency results in Menke's disease (Tanzi et al. 1993).

However, there are other aspects of copper, such as the ability to create radicals which can cause oxidative damage that can ultimately giving rise to neurodegenerative diseases like Parkinson's and Alzheimer's (Gaetke and Chow 2003). For instance, previous studies have shown that increased oxidation within nucleotides can result in oxidative mitochondrial DNA

and nuclear DNA that ultimately gives rise to Alzheimer's disease (Mecocci et al. 2004). Similarly, previous studies have shown that Parkinson's disease have a deficiency in Complex I, which gives rise to a malfunctioning Electron Transport Chain within the mitochondria (Beal 2003). Ultimately, the inability to have a fully functional ETC that can prevent free radical damage results in oxidative damage and gives rise to the progression of Parkinson's disease (Beal 2003).

D. Electrophysiology and Ion Channels

The use of electrophysiology is purposeful because of the inherent qualities of neurons and electrical signaling. Within the SCN and physiological structure of the brain, there are many ion channels that contribute to both the rhythmicity and the neuronal firing of these neurons. For instance, there are many channels, which include, but are not limited to sodium, potassium, and calcium channels, GABA dependent channels, VIP dependent channels, and also cyclic nucleotide gated channels, and ATP purinergic channels (Colwell 2011). Each of the different channels contributes a unique characteristic that can give rise to different event in the cells, such as neuronal firing (depolarization), rhythmicity and periodicity, hyperpolarization of the neurons, and electrical circuits that connect all pieces together, forming a network (Colwell 2011).

For instance, sodium channels are extremely critical for allowing daytime depolarization and neuronal firing. Previous studies have used the drug Tetradotoxin (TTX), a pharmacological blocker that blocks sodium channel activity (Colwell 2011). As a result, these cells that did not have functional sodium channels were unable to depolarize, and thus, were unable to create action potentials. In addition, because the sodium is unable to initiate the action potential,

potassium is no longer regulated or balanced anymore, since it has a role in action potential generation as well.

Another ionic channel that is important to the overall neural network is calcium channels, which has a large role in activity regulation and acting a as a secondary messenger to allow for other channels to open and release more neuropeptides (Colwell 2011). Through the use of L-type calcium channels, neurons are able to generate spontaneous activity and mediate responses to glutamate in the brain (Colwell 2011). For example, in previous studies, pharmacological blockade of ryanodine (calcium stores) was done, which resulted in dysregulation and a lack spontaneous firing (Colwell 2011). This shows the importance of calcium channels.

Finally, another key ion channel that has great importance is the use of potassium channels, which are extremely important for repolarizing the neuron (Colwell 2011). While these channels are important for use of generating action potentials, these are also important for repolarizing the neurons at night as well, in conjunction with the use of calcium ions (Colwell 2011). Within the subgroup of potassium channels, there are leak channels that can undergo the entire voltage range, and can both silence and hyperpolarize the cells during the night (hence being inactive channels during the day) and can also help regulate spontaneous SCN neurons during the day as well (Colwell 2011).

Through the use of the various ions as mentioned above, as well as other channels such as VIP channels, which are able to provide rhythmicity, or BDNF channels, which respond to light from external environments, the channels can provide a plethora of different activities, such as spontaneous generation of action potentials, regulation of the action potentials, activity at night, activity during the day, and even rhythmicity for the channels as well (Colwell 2011).

E. Copper and Electrophysiology

As previously mentioned, copper is an important trace element because of its essentialness to all tissues, such as the liver, the brain, and the pancreas. However, it also has a major role in modulation of ion channels within nervous tissue. In reality, copper is able to inhibit calcium-potassium channels, as well as GABA within the neuron. In addition, it can affect protein at the synapse and alter vesicle trafficking between synapses. As a result, further research with copper in nervous tissue is important to understand because of the importance and versatility of copper, and its ability to give rise to diseases when there is too little or too much of it within cells.

F. Use of Single-Unit Extracellular Electrophysiology and Circadian Rhythms

Single unit extracellular recordings within brain slices allow for the researcher to observe the activity of individual neurons without damaging them (Heinricher 2000). Because single neurons can display action potentials for hours, even days, they can be generalized to explain the overall rhythmic or arrhythmic behavior of the SCN (Heinricher 2000). Hence, by using the single-unit electrophysiology techniques, one is able to study the behavior of an individual neuron and ultimately understand the physiological responses within the region of interest.

While the use of electrophysiology can be useful for understanding circadian rhythms and observing neurological behavior, they can be useful for understanding other nerves and neurons as well. For instance, it can be used to understand and observe cardiac dynamics and cardiac rhythm abnormalities, cortical auditory potentials, and even help in observing whether or not a neuron is responding to a drug (Heger et al. 1981, Billings 2013, Bennet and Guthrie 2003).

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Effects of peripheral beta blockers on isoprenaline-induced fear conditioned rats

Veronica Go, Caroline Ferraz Webber, Eloisa Pavesi, Antônio de Padua Carobrez

Abstract

Anxiety disorders are the most prominent mental illness in the United States, affecting 18% of the population. Aversive memories, such as traumatic events, are associated with unconditioned stimuli. Few studies have shown that cardiovascular dysfunctions can be associated with aversive contexts or cues. However, preliminary results demonstrate that the beta-adrenergic agonist isoprenaline can induce defensive reactions in rats, which can promote aversive learning to the odors. The purpose of this study was to investigate whether aversive associations were prompted by activation of peripheral receptors. To test this, beta-blockers were injected 15 minutes before isoprenaline to induce fear. Male wistar rats (280-350g) were trained using an olfactory fear conditioning paradigm (OFC), consisting of 5 minute training and test sessions. Isoprenaline was used to arouse fear (unconditioned stimulus) and the neutral odor amyl acetate was used as the conditioned stimulus in an acquisition chamber, 24 h after a 3 min familiarization session. In the first experiment (during the training session), rats were injected with either saline or isoprenaline 15 min before conditioning to investigate whether isoprenaline promoted defensive behaviors (crouch sniffing, freezing and stretch ahead). Once isoprenaline was confirmed to promote fear, the second experiment used a pre-treatment of β-blockers nadolol (10 mg/Kg, a peripheral non-selective beta-blocker) or atenolol (10 mg/Kg and 20 mg/Kg, a peripheral selective beta-1 blocker), which was injected 15 minutes before the isoprenaline treatment. The fear association was analyzed 24h later during the test session within

a different context (odor box) composed of an enclosed and roofed zone as opposed to an open compartment where the odor was released after a minute of habituation. Defensive behaviors, as well as total time active, approach time, hide time, and crossings were observed in the odor box.

Our results showed that nadolol attenuated isoprenaline-induced fear, while atenolol rats continued to display signs of fear during the training session. During the test, isoprenaline promoted aversive memories to odor, which was seen by increased defensive time during the odor test, while nadolol impaired this aversive association. Conversely, atenolol did not block the OFC induced by isoprenaline. These results suggest that fear memory can be induced by the peripheral beta receptor activation. While the beta-1 blocker was not able to prevent the odor-association, the peripheral non-selective blocker could impair cardiovascular mal-dysfunctions responsible in the fear mechanisms, suggesting a potential anxiolytic compound.

I. Introduction

Anxiety disorders are the most prevalent mental disorder in the United States, affecting approximately 18% of the population (Kroon and Carobrez 2009). Evolutionarily, fear and anxiety was used as a defense mechanism that would allow for the sensing of a potential threat or danger (Kroon and Carobrez 2009). Once such threats were detected, the organism would be able to respond to these dangers using various defense mechanisms, such as the fight-or-flight response. In today's society, fear and anxiety still exists, however, it often appears in social settings rather than a way to defend against a predator or possible danger. While minor anxiety can heighten focus, allow for the best decisions to be made, or produce the best performance an employee may give (Berglass 2013), it can also be harmful in excessive amounts. For instance, too much fear and anxiety in a college student can be associated with lower test scores (Cassady

and Johnson 2002). Another example in which fear and anxiety could be harmful is in a public speaking situation, where the speaker may be so fearful that he stumbles over all of his words and is unable to give a clear presentation (Katz 2000). In both of these cases, the fear is no longer for their own survival as seen in the past, but rather, it is perceived fear, hence becoming a pathological disorder.

Though a variety of tests and examinations have been developed for studying the behavior of fearful rats, the Pavlovian classical conditioning paradigm has been shown to provide a simple, yet effective way of understanding the processes of fear and anxiety in the animal model (Kim and Jung 2006).

In the classical fear conditioning paradigm, a neutral stimulus is paired with an unconditioned stimulus that would normally cause fear. After several pairings between the fear-inducing unconditioned stimulus and the neutral stimulus, the stimulus will eventually become a conditioned stimulus (CS1) even though the unconditioned stimulus is no longer being presented to the animal (LeDoux 2000). However, the conditioned fear does not always terminate after the first conditioning. In fact, animals can remain fearful of the conditioned stimulus, and associate it with a new neutral stimulus, allowing for a second conditioning (CS2) (Gerwitz and Davis 2000). Thus, the animal will exhibit fear in the first conditioning session, but it will also experience fear to the second conditioned stimulus (CS2). In the past, studies have used auditory signals (Debiec and LeDoux 2004, Gravius et al 2006, etc.), visual cues (Campeau and Davis 1995, Shi and Daus 2001, Fanselow 2000), and electrical footshock to induce fear into the animal. However, few studies have begun to use olfactory cues in the animal model, because olfaction is considered a critical sense, as it allows the animal to smell danger, choose a potential mate, and even aid in feeding (Brennan and Keverne 1997, Kroon and Carobrez 2009).

Using aversive conditioning to induce fear appears to activate neural circuitry that is also associated with interactions between emotional behavior and cognitive function (thus creating a fear association with something that was originally neutral) (Ledoux 2000, Maren 2001). Furthermore, in addition to olfaction aiding in survival, olfaction and different scents (especially those that promote fear) appear to activate the medial hypothalamic defensive circuit, which is commonly associated with natural fear responses (Canteras 2002, Canteras and Branchard 2008). Finally, using odor has been shown to serve as a good conditioned stimulus, given that it is a neutral odor and one that the animal has not smelled before (Kroon and Carobrez 2009, Otto et al. 2000). It was also shown in several experiments that the olfactory cues were robust in the sense that they would produce good fear association (Paschall and Davis 2002).

In the past, different techniques have been used to induce fear – the most common being the electrical footshock to the animal – in which the animal is placed in a cage that can produce electrical pain as the unconditioned stimulus (Kroon and Carobrez 2009, Paschall and Davis 2002). However, in newer studies, the novel concept of using synthetic adrenaline, isoprenaline, to induce anxiety-like effects has been introduced into fear conditioning paradigms (Králová et al 2008). This isoprenaline has shown to be a non-selective β-adrenergic agonist, which can affect the heart as well as arterioles and blood vessels (Králová et al 2008). Within the heart, there appears to be an increased heart rate (which can also be seen in anxiety stricken situations), as well as a change in blood pressure (also seen in anxious situations) (Králová et al 2008). In a clinical study, Pohl et al. demonstrated that isoprenaline appeared to induce panic attacks and anxiety-like behavior in their subjects (Pohl et al. 1998).

Through γ-aminobutyric acid (GABA), as well as catecholamines, different subjects are able to be observed in the process of fear and anxiety, as well as learning and memory behaviors

(Bertoglio and Carobrez, 2004, Pohl et al. 1998). In previous studies using isoprenaline, there appeared to be a change in the heart beat, shortness of breath, chest discomfort, panic-like behavior, etc., which not only indicates a promotion of anxiety-like behavior, but also indicates that the β-adrenergic system appears to have a main role in inducing the anxiogenic effects (Pohl et al. 1998). Hence, the use of isoprenaline is a robust method to pharmacologically induce anxiety and anxiety-like behavior to study the process of learning and memory within a subject. Finally, previous studies have indicated that the use of β-antagonists seem to mitigate these effects, and also appears to impair the continuation of fear associations (Anagnostaras et al. 1995).

As mentioned before, some signs of anxiety and fear include shortness of breath, rapid heartbeat, chest discomfort, and overall panic-like behavior (Pohl et al. 1998). This shows an activation of the β-adrenergic system (Pohl et al. 1998). In fact, even the smallest amount of acute emotional stress appears to increase nervous system activity (Hoehn-Saric and McLeod 1988). Specifically, in subjects with existing anxiety, there was an increase in the sympathetic nervous system as well as increased autonomic system activity (Hoehn-Saric and McLeod 1988), both parts of the peripheral nervous system. While the central nervous system may have a role, previous studies have indicated that the peripheral nervous system is more important in the reduction of anxiety (Hoehn-Saric and McLeod 1988).

Knowing that simple classical conditioning can be used to have a fear association to a neutral stimulus, and knowing that olfaction can be extremely important and have a lot of relevance in learning and memory, this study was created to first reproduce isoprenaline-induced anxiety and anxiety-like behavior and induce an aversive conditioning to the neutral amyl acetate odor. The next stage of the study was to determine whether anxiety was actually caused by the

activation of the peripheral beta-adrenergic receptors, and to examine potential β-blockers that could prevent isoprenaline-induced anxiety as well as reduce fear associations. In this study, when the CS1 was presented, two different drugs – atenolol (a selective β1-antagonist) in doses of 10 mg/kg as well as 20 mg/kg and nadolol (a non-selective β-antagonist) in the 10 mg/kg dose were used to determine whether or not they could attenuate the effects of anxiety.

II. Materials and Methods

A. Materials

B. Subjects

Adult, 12-16 weeks, male Wistar rats (n=49) obtained from the Universidade Federal de Santa Catarina, with the weight of approximately 280 – 350 g were used for the purpose of this study. The animals were stored in cages (50 cm x 30 cm x 15 cm), in groups containing three or four rats. They were kept in a 12 hour light, 12 hour dark cycle in a temperature controlled environment (23 ± 1·C) with water and food available at all times.

C. Drugs

(±)-Isoprenaline (Sigma-Aldrich, USA), Nadolol (Sigma-Aldrich, USA), and (RS)-Atenolol (Sigma-Aldrich, USA) were dissolved into 0.9% saline, which served as a vehicle control. The first solution of saline and isoproterenol had a 10 mg/1 mL concentration. The nadolol solution was also created using a 10 mg/1 mL concentration, but the pH had to be corrected using hydrochloric acid and sodium hydroxide to make it pH 7. Finally, two doses of atenolol were created – the first dose was 10 mg/1 mL, and the second concentration was 20 mg/1 mL within saline. These atenolol solutions also had to be corrected using HCl and NaOH to pH 7. These drug solutions were injected intraperitoneally using a 10 mg/kg dose (nadolol and

atenolol) and 20 mg/kg (atenolol only). A 25% solution of amyl acetate in propylene glycol was used as an odor source during the study.

D. Apparatuses and behavioral measures

Each experiment consisted of two different apparatuses: a conditioning box and an odor box. During both uses of the boxes, the boxes were placed within a fume hood with 4 lux lighting. The conditioning box (24.5 cm x 26 cm x 25 cm) was constructed with grey plexiglass and one clear side (for video recordings). It also had parallel steel bars as the floor, which allowed for odors to be delivered when necessary. An olfactometer that was connected to tubes was used to deliver the odor in 1 ml/min flow into each of the boxes during the experiments.

The odor box was constructed out of black plexiglass and had two different compartments – an open compartment (40 cm x 26 cm x 40 cm) and an enclosed compartment, which had a "roof" on it (20 cm x 26 cm x 40 cm). Again, there was one side that had clear plexiglass, which allowed for video recordings to be taken. A 6 cm x 6 cm opening between the two chambers of the odor box served as a "door", which allowed the rats to move freely between the open compartment and the enclosed compartment. In the corner farthest away from the enclosed compartment of the odor box, the odor tube from the olfameter was placed with tape above it to secure it (CS1). During the experimental study, several behaviors were observed and recorded: the amount of time and number of times the rat would spend close to the odor tube (approach time), the amount of time and number of times the rat spent in the enclosed area (hide time), and defensive behaviors: the amount of time the rat would spend stretching ahead from the enclosed space (stretch ahead), the amount of time the rat's head was out of the enclosed space but the front legs were not out of the hide zone (head out), the amount of time the rat was immobile (freezing), and the amount of time the rat was immobile but the nose was moving

(crouch sniffing). Between each day and between every subject, a 10% alcohol-water solution was used to clean the apparatus used.

E. General Procedures

Two experiments were done during the study. The first was done in order to determine whether or not isoprenaline could actually induce anxiety-like effects in rats, and the second experiment was done to test for pharmacological compounds that could block the effects of the isoprenaline-induced anxiety.

F. Experiment 1

The first experimental design had two phases: conditioning (2 days) and the odor test (2 days). Each phase had 2 sessions, resulting in a total of 4 overall sessions. Each session was spaced 24 hours apart, and habituation was always done 30 minutes before each of the sessions.

The conditioning phase was done in the conditioning chamber, with the first session being 3 minutes, and the second session set for 5 minutes. On the first day, the rats were placed into the conditioning chamber with no odor and no injections, and are allowed to freely explore the cage – a session called "familiarization". On day 2, the "conditioning" session, the rats were injected with either saline (control) or isoprenaline (1.0 ml/kg), which was paired with the amyl acetate odor in the conditioning chamber for 5 minutes.

During the odor test phase, the rats were tested to determine whether or not the rats were successfully conditioned to fear the amyl acetate odor. This was done in two sessions on two separate days: CS1 test (day 3) (1 or more minutes of habituation and 5 minutes of odor test) and CS2 test (day 4). During the CS1 test, there was a habituation session that consisted of the rats walking into the enclosed space and walking out at least once. The rats had to be habituated for

at least a minute, but if the rat took longer than a minute to walk in and out of the enclosed space at least one, the rat was allotted the time. No odors were presented at this time, and the purpose of the habituation was to allow the rats to explore the new odor box before any stimulus was presented. After the habituation section of the CS1 test, the rats were presented with the same amyl acetate odor for 5 minutes using the olfactometer. During the CS2 test (day 4), the rats are placed into the odor chamber again, but there are no injections or odors presented. This test was used to observe whether or not the rats had aversive learning and would approach the area where the odor was presented originally (in the open area of the odor box). This allows for a contextual test. (Figure 1, Kroon and Carobrez 2009).

Experiment 2

During experiment two, the familiarization, CS1 test, and CS2 test are all exactly the same. During the study, four different treatments were tested: saline + isoprenaline (control), nadolol (10 mg/kg) + isoprenaline, atenolol (10 mg/kg) + isoprenaline, and atenolol (20 mg/kg) + isoprenaline. The pre-treatment drugs (saline, nadolol, atenolol) were injected 15 minutes before the isoprenaline. This was done in order to determine whether or not the drugs would have be able to prevent the effects of isoprenaline and impair the acquisition and consolidation of aversive events. Two different concentrations of atenolol were used to test possible dose-dependent effects.

G. Pharmacological analysis

The pharmacological efficacy was determined by using the odor pair of amyl acetate and the aforementioned drugs (isoprenaline, nadolol, and atenolol). Both the control groups, as well as the experimental groups underwent the same procedures to assess fear memory: CS1 test and

CS2 test to measure the success of fear conditioning and memory retention of the learned fear. In experiment 1, isoprenaline was compared to saline alone to determine if isoprenaline could induce anxiety-like effects, such as freezing and increased hide time. In experiment 2, saline (control), nadolol (a β-adrenergic receptor), or atenolol (10 mg/mL or 20 mg/mL, also a β-adrenergic receptor) was injected 15 minutes before the isoprenaline on day 2 (conditioning day) of the experimental procedure. On day 3 and day 4 (CS1 and CS2), the fear retention and conditioning was observed and noted.

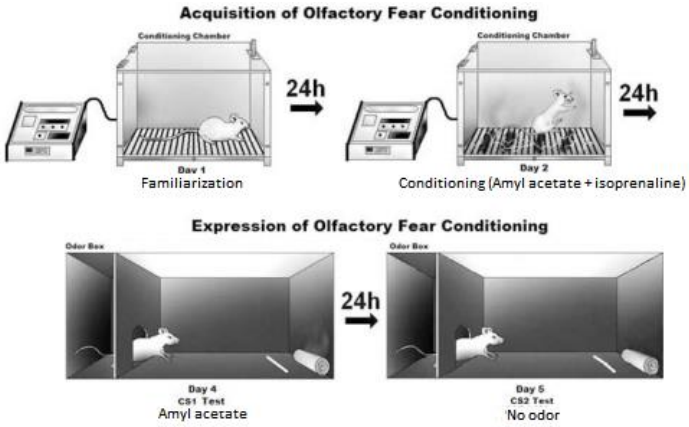

Fig. 1. Schematic procedures of olfactory fear conditioning.

H. Statistics

Data that was collected from the olfactory fear conditioning had a two part analysis. Within each experiment, there was a training session as well as the (odor) test data (as seen in the figure above). With each experiment, in the training conditioning section of the experiment, a one factor analysis of variance (ANOVA) was used. Next, the data for the test sets of data within each experiment was analyzed using a one factor one-way ANOVA test. All data from both phases of the study were compiled and analyzed together. Pharmacological investigation was analyzed by repeating the experiments multiple times (in order to standardize, determine whether or not there was a difference between control and experimental subjects, etc.). The statistical

significance level that was used was $p < 0.05$ for the purpose of analysis. All analyses were completed using the Statistica software package.

III. Results

A. Olfactory Conditioning as Fear Conditioning Paradigm

B. Behavior of rats when exposed to neutral stimulus, amyl acetate

In preliminary studies, the rats did not exhibit any symptoms of fear towards the amyl acetate odor, indicating that amyl acetate functions well as a neutral stimulus for the rats. Only when the amyl acetate was paired with the isoprenaline did the rats exhibit fear (Figure 2).

C. Olfactory fear conditioning using pairings of amyl acetate and isoprenaline

According to Figure 2, the rats did not exhibit any defensive behavior (freezing (when the rat is fully immobile) and crouch sniffing (when the rat is immobile but sniffing the air)) to the neutral odor amyl acetate during the familiarization session in which the rat is able to explore freely. However, during the conditioning session, in which the amyl acetate odor and the isoprenaline (or isoprenaline + compound (nadolol or atenolol)) are paired, the rats in the saline + isoprenaline group, the rats in the atenolol (10 mg/kg) + isoprenaline group, and the rats in the atenolol (20 mg/kg) + isoprenaline group all exhibited higher levels of defensive behaviors to the amyl acetate odor than the other rat groups ($p < 0.05$). The exploration time, however, was not different in any of the rat groups in neither the familiarization session nor the training (CS1)

session.

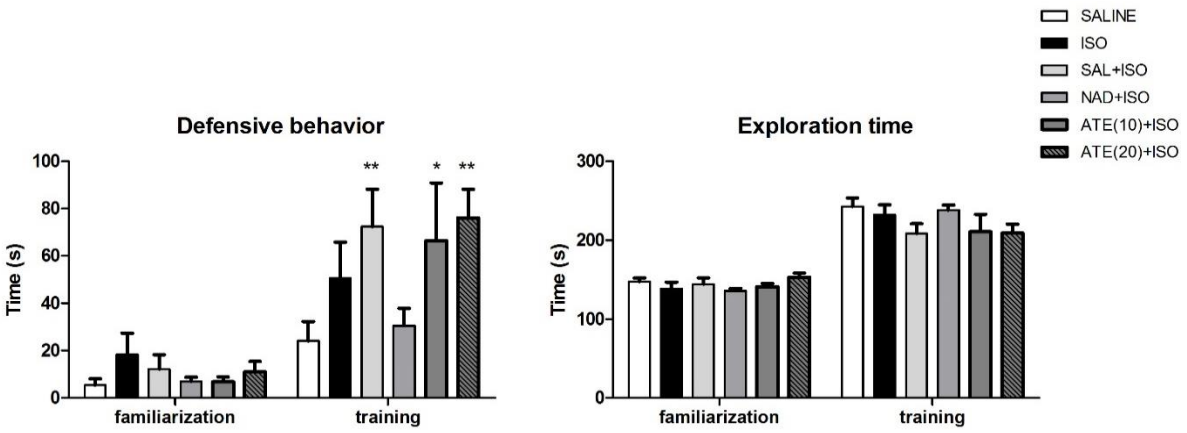

Figure 2: *This figure shows the time per second of defensive behavior within the familiarization period as well as the training period. Here, it is clear that there is no difference between the rat groups of defensive behavior in the familiarization session. However, in the training (conditioning) session, there appears to be higher rates of defensive behavior in saline + isoprenaline rats, atenolol (10) + isoprenaline group, and atenolol (20) + isoprenaline group. In the exploration time for the rats, there are no differences in any of the groups.*

D. Pharmacological Analysis

E. Effects of nadolol, atenolol (10 mg/kg), and atenolol (20 mg/kg) on acquisition and expression of fear conditioned responses

As stated before, nadolol, atenolol (10 mg/kg) and atenolol (20 mg/kg) treatments were injected 15 minutes before the injection of isoprenaline during the conditioning (day 2) session. On day 3, the CS1 test day, the rats are allowed to explore a new cage that has an enclosed section, as well as an open, unroofed section. In addition, the odor is presented a minute after habituation. Based on Figure 3, there was a higher amount of fear-like behavior in response to the odor in the saline + isoprenaline, atenolol (10 mg/kg) + isoprenaline and atenolol (20 mg/kg)

+ isoprenaline groups (Figure 3). However, when the test crossing was observed (the amount of times rats will enter the enclosed area into the open area and vice versa), the rat groups were not different.

Furthermore, the approach time percentage, as well as the hide time percentage were both collected, and was also analyzed. In the CS1 and CS2 tests, the approach percentages were different in the isoprenaline rat group, the saline + isoprenaline rat group, the atenolol (10 mg/kg) rat group, as well as the atenolol (20 mg/kg) rat group (Figure 4). In the hide time percentages, there was only a difference in the atenolol (20 mg/kg) rat group (Figure 4).

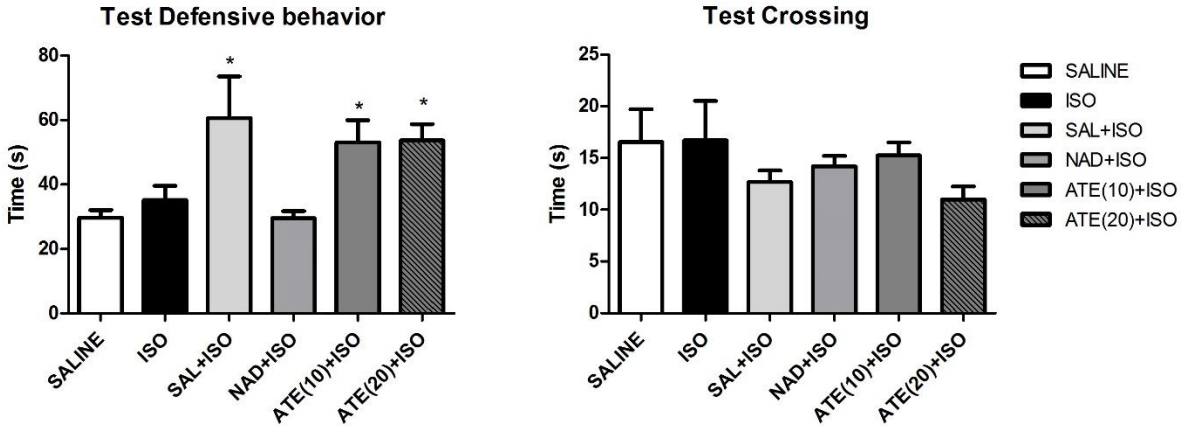

Figure 3: *This is the time that the rat was exhibiting defensive behavior and the amount of crossing during the CS1 and CS2 tests. There is a difference between the saline + isoprenaline, atenolol (10) + isoprenaline group, and atenolol (20) + isoprenaline groups in the amount of defensive behavior when compared to the other groups. When the test crossing was analyzed, there was no difference in any of the groups.*

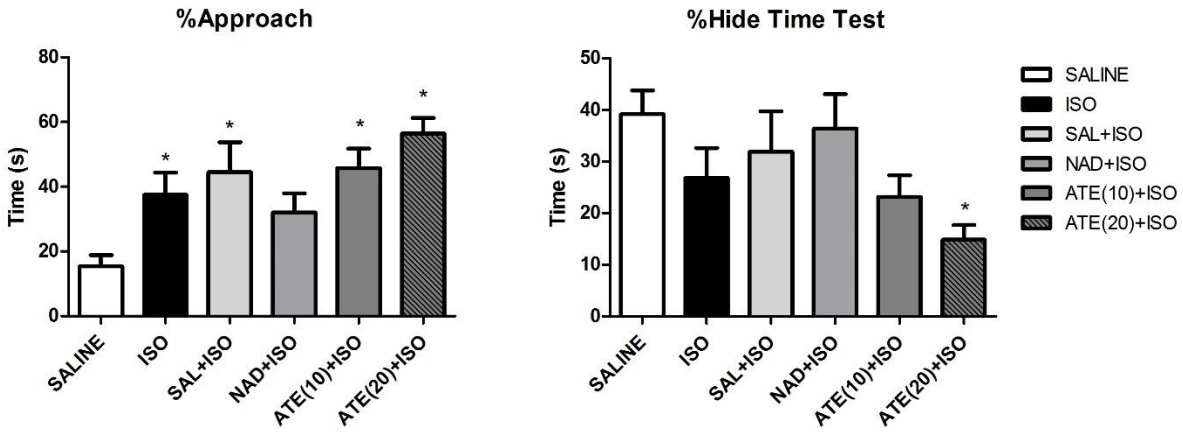

Figure 4: *This figure shows the percentage of time that the rats were in the hide zones as well as the percentage of time that the rats were in the approach zone. The isoprenaline, saline + isoprenaline, atenolol (10 mg/kg) + isoprenaline, and atenolol (20 mg/kg) + isoprenaline groups were different from the other groups. When the hide time percentages were analyzed, the atenolol (20 mg/kg) was the only group that was significantly different from the rest of the groups.*

F. Effects of nadolol and atenolol in the post-conditioning session

Upon statistical analysis ($p < 0,05$), there appears to be a reduction in fear-like behavior in only the nadolol group, as it decreases the amount of fear-like behavior, and has defense behavior times and approach times similar to rats with only saline injected. Conversely, atenolol, while an antagonist for the β-adrenergic receptor, does not reduce the amount of anxiolytic behavior in rats (Figures 2, 3, 4).

G. Evaluation of nadolol and atenolol treatments before the conditioning session

Prior to any of the pharmacological treatments, the rat groups did not have any statistical differences in any of the familiarization tests.

IV. Discussion

A. Implications

Past studies have indicated that using odor in a fear conditioning paradigms have provided robust results for understanding fear associations, behavior, learning and memory (Kroon and Carobrez 2009). In addition, previous studies have indicated that isoprenaline induces fear-like behavior, such as panic attacks, shortened breathing, chest pain, and rapid heartbeat (Pohl et al 1998). The pairing between isoprenaline and the neutral odor amyl acetate has shown interesting results in the pasts (Pavesi et al. 2011). Multiple odors have been used in the past to condition several subjects (Kilpatrick and Cahill 2003). In the present study, amyl acetate was employed because its ability to vaporize easily, as well as the ability to measure a known ratio of amyl acetate to propylene glycol, hence allowing for a reproducible experiment. In addition, studies have shown that rats do not have an underlying behavioral reaction to amyl acetate (Pavesi et al. 2011). The experimental results from the familiarization session of this study confirmed that there was no behavioral bias to amyl acetate.

Because olfaction is so important for the rat's survival and daily lifestyle, it was important to see if the rat would display a memory to a conditioned odor (amyl acetate) during the fear conditioning paradigm. Thus, the four day protocol was chosen to observe whether or not the memory of the conditioned stimulus would retain even two days after the conditioning session. Though the rats did not display any type of defensive behavior during familiarization, they eventually displayed such behavior after the conditioning session, indicating that there was no association at first, but that an association was built after the injections. Furthermore, there was no initial difference between the rat groups during the familiarization session. All together,

these data show the effectiveness of the conditioning with the odor. Upon exposure to the amyl acetate, there were two behavioral landmarks: in the CS1 test, there was the expression of defensive behaviors in rats to the amyl acetate, and in the CS2 test, there was an association between the odor box and the fear that was felt to the scent in the previous day. Though there was no odor being presented in the CS2 test, the rats continued to remain in the enclosed (hide) zone, and displayed anxiolytic behaviors. These results show the ability of rats creating associations between a conditioned stimulus and fear-inducing conditioned stimulus, as well as the ability to make further associations between an odor and a new cage (CS2).

The fear conditioning paradigm using odors has been shown to be consistent throughout many studies (Otto et al. 2000). Within these experiments, researchers have been able to promote defensive behaviors, such as freezing, crouch sniffing, and other behavioral responses associated with fear and anxiety (Kilpatrick and Cahill 2003). Also, different studies have shown that odors are good for longer experiments to test secondary associations, such as an odor and a new box or cage. Finally, the four day experimental protocol was a model that allowed for various pharmacological manipulations to be made at different time points (for this study, the second day). This concept allows for the observation of the effects of drugs on the acquisition of fear associations.

In the present study, nadolol (a peripheral, non-selective β-antagonist) and two different doses of atenolol (a peripheral, β1-antagonist) were used to attenuate isoprenaline induced fear and anxiety. In addition, because atenolol did not seem to work at the 10 mg/kg dose, the dose was increased to 20 mg/kg to observe whether or not it was a dose dependent effect. When the atenolol effects were analyzed, there was not a significant reduction in the isoprenaline-induced anxiety, even when the dose was increased. In fact, it appeared to have made the rats even more

immobile when the dose was increased. Clinical studies have shown that atenolol does not seem to reduce any type of phobia or any anxiety disorders (Liebowitz et al 1992). In reality, there seems to be no effect or any reduction in mitigating the effects of anxiety with atenolol use.

Conversely, when nadolol was tested as an anxiety reducing agent, it significantly decreased the amount of defensive behavior. Indeed, even during the conditioning session, the rats did not display as much anxiety as the rats with saline + isoprenaline. In addition, the fear associations were not carried for the following days as seen in the other groups. Several case studies have already studied the effect of nadolol on musicians, specifically, resulting in attenuation of a rising heart rate, as well as reducing overall performance anxiety (James and Savage 1984). Both the findings of nadolol and atenolol on the reduction of anxiety is in accordance with previous studies that have been done using human trials (James et al. 1984, Liebowitz et al 1992). The results observed in the present study imply the mitigation of defensive responses due to the injections of nadolol (10 mg/kg) 15 minutes before the isoprenaline-induced fear conditioning. This may be due to its ability to alter the fear-related emotions due to the aversive event, and its ability to block the association of fear with the event. Also, nadolol seemed to impair the initial association between the odor and the isoprenaline induced fear, hence the reduction of fear in CS1 (conditioned odor) and CS2 (context test).

Atenolol, on the other hand, did not seem to reduce the acquisition or retention of the aversive event, hence producing a lot of defensive behavior. In concordance with this study's results, clinical experiments have also shown the benefits of nadolol within human subjects, as well as the ineffectiveness of atenolol in reducing anxiety within human subjects (James et al. 1984, Liebowitz et al 1992). Furthermore, we can also suggest that isoprenaline-induced fear was promoted by the overall effect in beta-1 and beta-2 activated receptors, and once the selective

beta-1 blockade was unable to prevent the aversive conditioning, future studies with selective beta-2 antagonist would be addressed to indicate the role of each peripheral receptor during the fear-induced aversive learning.

Overall, the current study demonstrated the robustness of the simple Pavlovian fear conditioning paradigm using odors as the neutral stimulus. It showed that rats were able to create fear associations with odor tests, and it also showed the importance of olfaction in emotional memory and survival. In addition, the study showed the effectiveness of fear conditioning using isoprenaline as a drug to induce fear, which caused the subjects to exhibit defensive behavior. Furthermore, pairing isoprenaline to induce fear and odors as the conditioned stimulus provided robust results to demonstrate fear-like responses to an odor that originally did not promote any type of behavioral response. Finally, this study allowed for pharmacological manipulations during the experiment (15 minutes before the conditioning session). Using nadolol at 10 mg/kg, and two different doses of atenolol (10 mg/kg and 20 mg/kg), the study was able to show the different effects that each of these drugs had on impairing the acquisition of fear in response to an aversive event, as well as the ability to impair the consolidation of such events. The nadolol (a non-selective β-blocker) at the 10 mg/kg dose was clearly able to impair the acquisition and consolidation of the aversive events, while both doses of atenolol (a peripheral, selective β1-blocker) did not truly reduce the amount of anxiety during the conditioning session as well as the CS1 and CS2 tests. Because nadolol was far more effective in blocking defensive behavior and anxiety to the odor, it implies that isoprenaline-induced fear and anxiety is largely due to the β-adrenergic receptors in the peripheral nervous system. It also indicates that β1-receptor blockage is not enough to reduce anxiety-like behavior, and that aversive learning must be blocked by non-selective β-antagonists. These findings indicate the usefulness in the olfactory fear

conditioning paradigm, as well as the benefits of using pharmacological manipulation in an experiment.

B. Future work

In the future, more rats will be tested to see whether or not at the statistical tests and results still remain the same. Because there were only 49 rats that were used, there needs to be further testing to determine whether or not the results are consistent with the use of more subjects. Hence, more trials need to be done and the experimental procedures need to be continued to be replicated to get more accurate test data.

In addition, it would be interesting to try to lower the dose of nadolol. There is a strong suggestion that nadolol has the potential to block anxiety-like behaviors. However, pharmaceuticals are not always cheap to make and obtain, and have to be designed with careful precision. Hence, for future tests, testing for the minimal dose to block anxiety-like effects (with nadolol) would be beneficial to test – not only for patients, but also for pharmaceutical companies as well.

Because only atenolol (a non-selective β-blocker that does not cross the blood-brain barrier), and nadolol (a selective β-1 blocker that also does not cross the blood-brain barrier) were used, there was a lack of testing for β-2 blockers (such as butoxamine). Thus, it would be interesting to see the effects of β-2 blocking drugs and see whether or not it would significantly attenuate anxiety-like behavior. Alternatively, instead of using isoprenaline (a non-selective β agonist), a selective β-2 agonist could be used to see whether or not there are still anxiety-like effects.

Finally, another potential experiment would be to use a compound that could cross the blood-brain barrier but did not affect the peripheral nervous system. In this experiment, the central nervous system would be observed and analyzed to see whether or not anxiety-like behavior is reduced. For example, a drug that went straight to the brain and did not go into the rest of the nervous system to see if the isoprenaline-induced anxiety could be mitigated if only the central nervous system underwent altercations by the drug.

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