Functional and connectivity changes in the Amygdala underlie fear-related behavior

Master Thesis

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Index

Chapter 1. Introduction

Memory is the cognitive process through which we learn, encode, and retrieve information (Davelaar, 2013). Often, memory is understood as a process made up of two main branches, the short-term memory, which mainly involves the sensory input acquisition and "retaining" but is not mainly involved in processing (Atkinson and Shiffrin, 1971). On the other side, the Long-term memory is mainly involved in processing the information as useful outcomes and encoding it for future retrievals (Atkinson and Shiffrin, 1968).

Much has evolved from the classical view of memory; nevertheless, the original learning paradigms and methodology still can be used as a tool for understanding how our brains treat the external information and how they regulate our cortical output.

Still after all this years, classical fear conditioning (Carew *et al.*, 1981) is still recognized as the best model to elucidate the neurobiological mechanisms of learning and memory and to understand the relation between memory and other brain processes such as risk assessment, attention and cognitive biases (Weidenheim, 2001). In this work we compare behaviors and responses between groups trained with a Fear-Conditioning protocol and non-trained Control subjects. Furthermore, we analyze the physiological responses to the training in the form of Skin Conductance Response (SCR), Electroencephalographic records (EEG) and Functional Magnetic Resonance Imaging (fMRI) to understand which areas, when, and where, are involved in the information processing and behavior response.

1.1. Classical Conditioning

Our brain and our senses allow us to interact with the world outside of our own body, and we can receive, store and generate responses to the different stimuli of our day-by-day life. The process of learning is a powerful tool: we generate a response to each event, and if this is followed by a successful outcome, we can keep using the same technique every time we encounter those events.

Over time, our brains learn a wide spectrum of responses that we will apply automatically with each and every stimulus. Some can be obviously adequate: no one would doubt that for the early humans, the idea that fire burns so you should not touch it, was useful, but others, like the act of “freezing” or staying very still when we hear a loud sound, seem to be less obviously useful, especially in a modern society (Illeris, 2018).

The study of human behavior is not something new, but in the early 20th century, pioneers in the subject like Pavlov and Watson started working with models (animal and later on humans), to elucidate the steps and components of behavior, and one of the first attempts to explain the phenomena was Pavlov’s learning, or now called “Classical Conditioning” (Watson, 1913; Pavlov, 1938; Rescorla, 1988).

Pavlov’s learning paradigm is based on the ability of our brains to relate two different stimuli, one called a “Conditioned Stimulus” and another one called “Un conditioned Stimulus”. In his experiments, he rang a bell (CS) and immediately after, he fed a dog (US)(Bouton and Meyers-manor, 2016). The natural response of the dog in front of the food is salivation. After a couple of trials, Pavlov started noticing that after he rang the bell, the dog would start drooling, this shift of the natural response from the US to the CS means that in the dog’s brain, the previously different stimuli, now are a pair CS-US and two unrelated events, now have become one unique thing (Rescorla, 1988; Douglas L. Medin, Brian H. Ross, 2005).

This association was shown later to have the same principle in human beings, along with a variation of the paradigm that includes what is now called “Operant Conditioning”; together they are the backbone of the learning mechanisms.

Since its first publication, there are number of hypotheses of why or how this occurs, one proposed by Pavlov himself is the Stimulus-substitution theory (Hilgard, 1936), in this scenario, there is no “new” response learned, only it is an adaptation of an old response to a new stimuli.

This has opened the field to a technique called “Counter conditioning” which involves the conditioning of an unwanted or unhelpful behavior by association with a second set of stimuli (Blaisdell *et al.*, 2000).

For example, let us imagine a person with a fear of dogs, in his youth he had been bitten by a dog, so by now, he learned the association DOG-BITE and since he does not like to be bitten, he does not like dogs. Now, we let this person eat candy in a room where a dog is, and slowly sit him closer to the animal, eventually we tell him that he has to touch the dog and every time he pets it he eats candy.

With enough time, the person will generate a new DOG-CANDY pair, which will take the Unconditioned Stimulus (US) “Dog” and will pair it with the new Conditioned Stimulus (CS) “Candy”.

This technique does not erase the previous memory, but depending on the context (Bouton and Moody, 2004), (other stimuli that can be in the environment, like a room or a smell), one memory pair should be prevalent over the other one and therefore, we should see the behavior change accordingly.

Over the years, several experiments expanded the idea of the pairing of the CS-US. Part of Rescorla et al. work resides in the idea of the results of a new procedure called “Zero contingency procedure”(Rescorla, 1967) , in which the CS-US are paired, but the US also appears without the CS. Conditioning is successful when the Conditioned Response (The drooling for example), precedes the US.

With this scenario in mind, we see that instead of pairing between the CS and US, the key for conditioning relies in how well the CS predicts the US. These findings take into account the effect of the context, in which certain surroundings or environment; increase the predictive power of the CS.

A good example of this is in Pavlov’s experiments itself, in several of them, the Conditioned response (drooling) came when the dog saw the experimental equipment, even before the bell was ringing, by context alone, the dog was able to predict that the US would come.

The Prediction based hypothesis allowed Rescorla and Wagner to generate what it is now known as "Rescorla-Wagner model" in which learning is taken as the strength of association between a CS and a US (Rescorla and Wagner, 1972). The Rescorla-Wagner Model (RW-Model) is one of the first ones to tackle the CS-US pairing as a measure of how well the CS predicts the appearance of the US, before the training, the subject is surprised by the US, since there is no connection between it and anything, but through the trainings, the CS starts acting as a predictor of the US.

The strength of the prediction, called by the RW-Model as "Association Value" (Symbolize by "β") can range from zero to one, depending if it does not predict at all or predicts 100% of the US respectively (Glaze, 1928). The value of this prediction can be represented as the summed associative strengths of the previous CSs.

This Model represented major advancements and allowed more simple and accurate explanations of the experimental phenomena seen by the scientists at the time, but it had its limitations that eventually led to modifications and new models that could fit the newly obtained data.

One of the biggest limitations of the RW-Model and that we address in our research involves the "Extinction training". This training consists in, once the CS is accurately predicting the US (The conditioning was successful), the CS is shown without any US. In this scenario, the lack of US starts being a surprise, but after enough trials, the CS no longer predicts the US (Myers and Davis, 2007; Michael B. VanElzakkera, 2008) .

There are some discrepancies over if the Extinction training consist in the unlearning of a CS-US pairing/prediction, such as the RW-Model would suggest by the "weakening" of the association value, or if it involves a new kind of learning that competes with the first one, as researchers (Konorsky, 1969; Pearce and Hall, 1980) later theorize.

When using the RW-Model to account for the Extinction training, we fail to explain the spontaneous recovery of an extinct memory, as can be seen in Post-Traumatic Stress Disorder (PSTD) patients or even in experiments done in animals, meaning that the first CS-US pairing was not “unlearned” but is still there.

In this scenario, it seems that there are in fact several memories competing with each other, depending of time, context or external stimuli, and the one that stronger fits the current events, is the one that “leads” the behavior.

The Pearce and Hall model (PH-Model) is better than the RW-model to predict the data obtained by the Extinction trainings, the spontaneous recovery and is still able to explain the CS-US pairing proposed by the latter, its strength relies on the idea that the CS has an associability property (Or how much information concedes or is paid attention). This property would decrease if the CS successfully predicts the US and increase when the outcome is uncertain (Hall and Pearce, 1979; Swan, J. A.; Pearce, 1988).

This explains why an animal needs to pay full attention and fully process an event when this has an unpredicted consequence (Like a CS-US pair where the Associative strength is close to 0), but not so much when the CS can already predict the US.

Nowadays there is still controversy about the subject, and there are several theories that try to explain the accelerating increase of data obtained, not only because of the increasing number of methods and techniques that are being used, like fMRI, EEG, PET Scans and others, but because there was an increase of the number of scientists interested in the subject.

We based our work mostly sharing Bouton’s interpretation of learning and memory (Bouton and Moody, 2004), since we used not only the classical conditioning and extinction trainings, but the “Context dependent phenomena” of extinction training called “Reinstatement”. This last part of our work is focused on the “recovery” of a previously learned and extinct memory, meaning that a CS-US pair was formed to the point where the CS successfully predicted the US, then an extinction training until the CS no longer predicted the US, and in this point, the CS-US pair was “brought back” with a reinstatement procedure (Buchsbaum *et al.*, 2012).

In this procedure, when the animal or subject is presented with the US alone, because they were part of the conditioned environment, this can bring back the CS-US pairing. When the US is presented after the extinction, the subject associates the US with the context, and it is this association CS-Context and US-context that brings back the CS-US pairing (Smith, 1985). This means that if the US is presented in another environment, and therefore there is no context to associate, the US alone is not enough to generate a Reinstatement.

In our work, we decided to use faces as conditioned stimuli (CSs) and sound as an unconditioned one (US). We took the faces from a list of characterized stimuli from the Karolinska Institute in Stockholm, Sweden, these faces are divided regarding sex (Men and Women) and regarding the "Valence", that can be positive when the face is smiling or doing a characteristically positive gesture, negative when the face is frowning or doing aversive gestures, or neutral when it does not involve any of the previous (Huys *et al.*, 2011).

We decided to use three male faces, one neutral, one aversive and one aversive that would be paired with the US, and we call them CSn, CSa and CS+ respectively. The reason for this is that the stimuli are easy to recognize, are available and both aversive faces, due to sociological and psychological context, are "more negative" than the CSn.

To avoid that one of the aversive faces may be more negative than the other, we had two versions of the pavlovian training, where one of the faces would be CS+ and the other CSa in one version, and switched in the other. Half of the participants used the first version and half of the participants used the second.

1.2. Anxiety-Scores

Anxiety is a complex and broad umbrella of behavior, signs and emotions that can be summarized as an unpleasant feeling of fear, worrying and nervousness over things or scenarios that are yet to happen or will never be (Myers and Davis, 2007). It is important to remark the difference between an anxiety response and a fearful one.

These two responses might share some symptoms and behaviors, but the key difference relies on the fact that a fear response and fear-related behaviors are the response of the brain to an aversive or dangerous stimuli, while an anxiety response is due to the idea that a stimulus might come, it is a pre-emptive response that does not involve the stimulus per se but the expectation of it.

Because of the nature of our research, we need to be sure that the responses and behavior we see are due to the fearful stimuli and are not being masked or increased by an anxiety response. Therefore, we propose the use of Anxiety-Scores, based on the State-Trait Anxiety Inventory (STAI) and the Beck-Anxiety Inventory (BAI), as an exclusion criterion for our analysis (Creamer, Foran and Bell, 1995; Roberts *et al.*, 2016).

The STAI test is divided into two main branches, the “State” part of the STAI test consist in a series of questions which can be answered by filling a multiple-choice style sheet, in which the answer goes from No / Never (or 1) to Yes / Always (or 4). These questions focus on the emotion and mental state of the person at the moment of the taking, and can be both positive and negative questions (i.e. “Are you well rested?” “Do you feel anxious about the test?”)

Both positive and negative scores are computed and the final score is obtained by an equation:

$$\text{STAI Score} = (\text{Positive} - \text{Negative}) + 50$$

Score values can range from 20 to 80, with higher values correlating with higher levels of general anxiety. Based on the literature, any score over 45 was considered a high anxiety trait and was used as an exclusion criterion for our experiment (Spielberger, 1989).

The State test was performed twice per subject, once before the training and once after the whole experiment to assess the anxiogenic effects of the pavlovian training.

The other branch of the STAI test is the “Trait” which like the other consist in a series of questions regarding the emotion and mental state of the subject, but in this case the participant is told to answer about his day to day life and general state, as the one above, the answers range from No / Never (or 1) to Yes / Always (or 4), and can also represent both positive and negative questions (i.e. “Do you feel happy?” “Do you think you take things too seriously?”).

The analysis of the responses is similar, with the same equation and the same interpretation of the scores, any STAI-T score over 45 is considered too high for our experiment and therefore is taken as an exclusion criterion, and in this case, though, the test is taken only once, before the training starts.

The other test involved in the analysis of the participant’s anxiety state is the Beck-Anxiety index (BAI), this test designed by Beck et al. (Aaron T Beck; Steer Robert A, 1993)consists of 21 questions that can be ranked from “Not at all” (0) to “severely” (3). This test differs from the STAI test in that these questions correspond to more Anxiety-like signs and symptoms (i.e. “Do you feel numbness” or “Do you usually fear that the worst would happen?”).

This test acts as a complementary test for our work, along with both the STAI tests, because the questions focus on the events of the week prior, we together have an anxiety-score overview for the same day (STAI-S), week (BAI) and life in general (STAI-T).

The score obtained for the test is then compared with a scale provided by Beck et al.

0– 9 : Normal anxiety

10–18 : Mild anxiety

19–29 : Moderate anxiety

30–63 : Severe anxiety

For our purposes, a BAI score over 30 was considered too high and was used as an exclusion criterion.

1.3. Adaptive Responses to Threat Uncertainty

Uncertainty can be described as the situation in which there are insecurities about or lack of information. When we are in an unknown situation, we cannot predict the outcome of the events, since of all the possible ones, there is none that "jumps out" as the most likely (Grupe and Nitschke, 2013a).

In this sense, learning increases our ability of predicting and reacting to the most likely scenarios, so for example, learning that red in a traffic light means that you cannot cross the street, increase our prediction of what might be the outcome of crossing the street anyway.

In our research, we tackle this matter by using a fear conditioning protocol. Our idea is that once learned, all the mechanisms involved in the threat response are put into motion and we see some degree of behavioral changes.

These responses can be adaptive or maladaptive depending on context, we can assess this by proposing a thought example.

Let us imagine we are in a plane going through the ocean on our way to London. At some point, we start feeling that the plane is moving abruptly and we hear over the speaker that the pilot says we will be experiencing some turbulence. We start getting nervous about what might happen and how likely that this is normally happening in a long flight, etc. We start paying more attention to what the flight attendant is saying or doing and if we can, what is happening outside the windows.

When we see that the people look relaxed, that the stewardess are talking calmly to each other, and we start remembering that is no more than a little normal situation, we calm ourselves and after a couple of minutes, we hear the pilot saying that the turbulence is over, and we can order another coffee.

All these responses involve a large spectrum of interconnected behavior, but for our study and easiness of understanding, we divide them into three big categories.

Risk Assessment:

When we talk about risk assessment we refer to the ability of the subject to estimate the risk involved in a defined situation with a known threat, in the example above, the risk of course is the idea of the plane falling down, that would be the end point or consequence of the "Threat", which in this case would be the turbulence. The idea of the turbulence would eventually lead to the fall of the plane and that is what makes it a threat, as a mean from which an ultimate bad or aversive outcome will appear (Nees, Heinrich and Flor, 2015). The defined situation is the plane and all the surroundings of our main character, and it is this context that will help to assess the level of risk involved in the situation.

In our research, we used this pavlovian training precisely to test the changes in the perceived risk of certain stimuli. We called this experiment "Discriminatory Index" and it was performed once before and once after the Pavlovian Training.

Using the ideas described previously as a template, when we first show the different faces, there are eight faces mixed between having aversive and neutral expressions, there is no significant threat, since those faces don't represent anything yet, the surroundings are also new, the room, computer and faces are shown for the first time here in all cases (Let us remember that having been part of a previous or similar experiment was an exclusion criteria).

Therefore, the results obtained by this stimuli, depend exclusively on the subjective perception of the subject. After the training, when the subjects are asked again to perform the "Discriminatory Index" experience, they are shown the same eight faces, but the main difference is that now, the CS+ (Aversive face with conditioning training) represents a real threat, and that means that is related to a risk, which in this case is the sound used in the conditioning training.

After this, the result obtained no longer depends only on the subjective perception of each subject but also on the success of the pavlovian training.

Increased threat attention and hypervigilance:

All the time we are bombarded with thousands of stimuli from all types and places, the sounds of the street, the light in our rooms, the feeling of our clothes on our skin. If we were to analyze every single stimulus that come to us, we could not even get up from the bed.

The process of “choosing” which stimuli to analyze and work with is called attention, a more formal definition would be that is the cognitive process of concentrating in a selected piece of information ignoring the rest.

We cannot voluntarily choose to what part of the information we pay attention, but this is instead done by our brain in response to what is considered vital for our survival or our task at the moment.

Let’s consider what our imaginary character in the plane was doing before, we could have been listening to music or looking out the window, reading a book, etcetera, but almost for sure was oblivious to the sounds of the other passengers and crew.

When the turbulence started this was taken as a threat, as explained above, and there was a significant increase in attention, no longer the music or book was enough for the brain, but all the surroundings had to be taken into account, this is when in our scene this person starts paying attention to the faces of the other passengers, the sounds of the crew and even the wings of the plane through the window.

In most cases, this is also accompanied by an increase in threat perception of those new stimuli, they all seem to be more dangerous that would seem in any other situation and until it is resolved, we run the risk of a “snowball effect” in which the threat creates another threat and this in turn creates more threats (Kimble *et al.*, 2014).

Our work tries to assess this increase of threat perception using the “Dot-Probe” paradigm (Koster *et al.*, 2004). This test allows us to see the selective attention of different stimuli, in our case we compare the three faces used in the Pavlovian training and see how the attention is more focused on the “threatening” face than the others.

The reason for which we expect that the threatening face consumes more resources and takes the attention from the other ones (Attentional bias) is that since this face is now considered a threat, it is related to a risk, as explained above, and therefore constitutes a menace to our survival.

Inflated estimates of threat cost and probability:

There is some confusion regarding what we refer to when we speak about costs and probability of certain events. It is of course a topic of debate between neuroscientists even to this day.

When we study behavior we need to understand not only how our brain behaves and the mechanisms involved, but also why do we behave the way we do.

Every living sentient being must think about the ramification of its actions, to know what to do, what can be eaten or where to hide, and this comes with the analysis of what is called “Expected Value”.

This as its names suggest is a measurement of what kind of outcome we expect by using the information we have about the object and its and our surroundings (Grupe and Nitschke, 2013b). The Expected value of each stimulus therefore can be said to consist of two different but related components.

First, we have the probability of the outcome actually happening, this is calculated by our brains using what we know of this situation, and the history of the outcomes and in which context we are in.

If one of those things change, the whole probability calculation changes, lets for instance use the example we talked about before. If we do not know what a plane is, or how gravity works we cannot calculate the probability of the plane falling down, and the more we know, like the route, or the flying history of the plane and pilot and company, or the plane model, the more we can create a more realistic idea of what are the chances of the outcome happening. Of course, that is only half of the picture, the context in which we are or we acquire the information gives rise to the probability. As an example of the latter, we can imagine that we know everything about a plane, its pilot, its company, and its route and flight history. The chances of being in a plane accident go down dramatically if we are not in the plane to begin with.

The other side of the coin regarding the expected value is the "cost" or intensity of the outcome per se, and as it was with the case before, the ability of our brain to calculate the cost or intensity of an outcome is related to the amount of information we have about both itself (the stimulus or situation) and the context.

In our example of the plane, and putting all the information about the expected value all together, the perceived outcome is of course, a plane accident. The probability of the outcome increases when information related to accidents start to come, so the turbulence in this scenario would be information that increases the probability of the plane crashing. Besides, the context in which we are also follows the idea that an accident might be possible. On the other hand, the cost of the accident is very high, since it involves a serious threat to be in a plane crash.

In our work though, what we do is to pair a stimulus (CS+) with a sound (US) and therefore, as said when we look at risk assessment, this CS+ is now a threat. To evaluate the Expected Value linked to the different stimuli, we perform a test in which different faces in different scenarios are involved, both positive and negative, so for instance a question asking how likely is that the conditioned face gives you a present (Dorfman *et al.*, 2016).

The scenarios proposed involve calculating the probability of low and high cost situations, and the cost of rare and likely scenarios. And with that, the goal is to see the differences between the perceived threat of each stimulus, so we would expect to have answers saying that negative scenarios are more likely than positive scenarios with the conditioned face (Since it is a threat) compared to the other stimuli.

Combining the three of them, we can get a picture of how the behavior changes when confronted with a threat, even in a neutral environment. Of course, we have to mention that the real behavior cannot be compartmentalized as easily, and each part depends and affects the others.

1.4. Skin Conductance Response

It is widely known by now that the skin is the largest organ in the human body. The skin, being part of the integumentary system, acts as a barrier between the external world and our bodies with its underlying muscles, bones and internal organs.

This distribution (As an outer cover of the whole body) means that the skin not only acts as a protector from, but also interacts with, the world around us. This is why our skin is full of receptors, glands, hairs, they help us interact and convey information.

The skin has more functions that we could focus on our research, from immunity up to vitamin production, but regarding our research, the main function of the skin is the temperature regulation and water loss.

The outer layers of the skin consist mostly of keratin fibers and dead cells, this oily-protein barrier has a very low permeability, this means that most external substances cannot enter or diffuse through the skin.

One of the consequences of this low permeability barrier is that our skin has normally a very low conductance (or high resistance), and this can be measured with two electrodes, placed on the skin of the subject without touching each other, a technique that is called Electro-Dermal Activity (EDA).

This EDA is constantly changing, because our skin is a very dynamic organ, but the most noticeable changes comes from the amount of sweat that is being produced (Benedek and Kaernbach, 2010). Sweat is the way our body has to decrease our body temperature, through the activation of the sweat glands that populate the layers of the skin; an adult human can produce up to 12 liters per day. Because it is mainly composed of water and ions, the evaporation of it at the surface of the skin creates an “evaporating cooling” effect thus lowering the air temperature around the body.

We cannot consciously control the rate of sweating or the activation of our glands, this is part of the realm of the “Autonomous Nervous System” (ANS), which controls the bodily functions that keep us alive and breathing, thermoregulated and inside the “normal” range, in a process called Homeostasis.

Let us briefly explain what we mean when we refer to the ANS. It is a control system that acts unconsciously and regulates most of the internal organs bodily functions, like heart rate, digestion, breathing, thermoregulation, pupil dilation, between many others (Schmidt, A; Thews, 1989).

The ANS can be divided into three main branches, the enteric nervous system, the sympathetic nervous system and the parasympathetic nervous system. We will focus on the latter two.

It is of course a complex subject, with many sides and still things to research, but these two systems can be grossly said to be involved in the management of energy and energy reserves.

The Sympathetic system is commonly associated with the fight or flight response, in which our body gets ready for action, this means that the pupils get dilated, our heart and breathing rate increases, there is an increase of blood flow, temperature (and sweating), reserves becomes available and our whole body expects to start spending energy.

On the other hand, to counteract the previous one, the Parasympathetic system is associated with digestion and rest, this means that our body is in an energy saving mode. The pupils contract, the breathing and heart rate diminish, and the blood flow is focused in the digestive track, where the peristalsis and absorption is increased, the food is transformed into reserves and they accumulate for the next time they are needed.

The “head” of the ANS is an area at the base of the brain called Hypothalamus, which keeps a balance between all the parts of the ANS, this is at the end what gives this system the function of homeostasis, the equilibrium of energy obtained and expended is what keeps the body normally functioning.

The increased activation of our sweat-glands relies on the activity of the sympathetic part of the ANS, and this activation is not easily divided, meaning that we usually cannot activate some part of the sympathetic response without activating the others, so a side effect of an increase in the sympathetic response, whichever the reason, will be an increase in sweat activity.

Emotional arousal such as anxiety, nervousness, pain or fear, and sympathetic activity are deeply connected. There is no way of discerning which it is by the EDA activity alone, but it is known that sympathetic activity is involved in a "fight or flight" response to threat (Critchley, 2002).

This fits in our work perfectly, for what we explained before of our training protocol. We have three faces that are used for the Pavlovian training, in which one of them is paired with the US and now it is taken as a threat (CS+).

The idea behind the Skin Conductance Response (SCR) is that the EDA measured by the electrodes, one placed on the second phalanx of the index finger and the other on the second phalanx of the middle one, changes from stimulus to stimulus, meaning the difference between a trained and non-trained face (i.e. CS+ Vs CSa) and also the progression of the training when we compare the firsts trials of the conditioned stimulus with the last ones.

1.5. Electroencephalography

There has been an increase in understanding about our brain, how it is composed and how it works in the last decades, but perhaps one of the most important understanding regarding this organ was the "Neuron Doctrine" by the end of the 19th century which states that the whole nervous system consist of a special type of cells called "Neurons" (Shepherd, 1991).

This doctrine shifted the focus from the anatomy to the histology of brain functions for decades to come and opened the field to the same rules that were being applied to the (In that times, new) cell theory.

The neurons are not limited to the brain alone, although we are going to focus only on the brain activity, they make up the whole Central Nervous System (CNS) including the Brainstem, Nerves, Ganglia, etc.

These millions and millions of neurons communicate with each other by synapses, which can be because they contact each other directly (Electrical synapses) or they are close enough to communicate through Neurotransmitters

(Chemical synapses). In any case, the information is conveyed in the form of electrical changes between both sides of the cell membrane.

The polarization or changes in the electrical charge on the membrane of the cell are induced by the opening or closing of different ion channels, which underlies the core of the neuronal synapses.

Keeping in tune with the electrophysiological records, besides the electrode rmal activity we measured the Electroencephalographic Records (EEG) of our subjects during our research.

Despite both analyses consisting in the recording of electrical activity, they are very different from each other, starting with the fact that the EDA does not record the actual electrical activity of the skin, but records the changes in conductance, therefore it does not give information about the intrinsic activity of the skin but indicates how well the skin allows current to pass through it.

Contrarily, the EEG measures the intrinsic activity of the brain through the skin. To measure the EEG we place electrodes on the scalp, being careful that there is no hair or obstruction between the electrode and the skin and measure the changes in voltage resulting from the underlying ionic currents in the brain, as described above (Niedermeyer, F.L; Da Silva, 2004).

In our research, as is usually done, we diminish the normal skin resistance by soaking the electrodes (That come with a sponge attached) in salty water and a dash of detergent. The chemical properties of the detergent “wash away” quite literally the oily residues on the skin and hairs, allowing the electrode to be in direct contact with the layers of the skin.

Now in contact, the ionic solution in which the sponges were soaked comes as an advantage, the salty water decreases the resistance several times and the electrical signal detected becomes stronger and clearer.

The placing of the electrodes is of course not random, the international 10-20 system (Oostenveld and Praamstra, 2001) is the widely used standard method of electrode placing regarding the EEG exams. The system takes an image of the head from above, with the nose on the anterior side (Nasion) and the occipital on the posterior (Inion), and the ears (Tragus) marking the left and right sides.

The number of electrodes depends on the equipment involved, but standard naming is maintained. The name of the electrodes consists of a letter, representing one of each lobe of the brain: Frontal (F), Temporal (T), Parietal (P) and Occipital (O), with in some cases frontopolar (Fp). Also there is a Central (C) which does not represent a lobe per se, but a central position, so that can be combined with the other letters to indicate where the electrode is, for example, “F5” represents the frontal lobe, “FC5” also represents the frontal lobe but is more posterior (More to the middle of the brain) than the other one.

As mentioned in the example, the electrodes also are accompanied by a number. These numbers represent the distance from the sagittal middle line, so for example “F8” is more lateral than “F2”. The numbers also represent the side, with even numbers going to the right hemisphere (2,4,6,8) and odd numbers in the left one (1,3,5,7).

The middle line in the sagittal plane is represented by the letter “z”, so the electrodes “Fpz”, “Fz”, etc. are all placed on the sagittal middle line. Since anatomically speaking they are over the corpus callosum, usually these electrodes are used more as reference, with “Cz” being the “ground” or main reference for being the electrode placed in the exact middle of the head.

The measurement of the activity is obtained by measuring the voltage at a particular channel and comparing it with a reference electrode, in most cases “Cz”, this is one of the several montages that allow us to compute the increase or decrease brain activity of a particular area.

The data obtained reflect the whole activity of the brain with no regards for the frequency or origin of the signal, so that has to be determined from a post-hoc analysis. For the frequency analysis, which is one of the most common ways of analyzing the EEG data, the signal can be decomposed into its original frequencies (or range of frequencies) by a Fourier transformation. The localization of the signal is more difficult because of the way EEG works (Armando Freitas da Rochaa and Jr, 2015).

As mentioned before, the electrodes are located on the scalp in contact with the skin on top of the head, this means that the more superficial the activity (and therefore closer to the electrode), the stronger the signal. That being said, the EEG has a very low sensitivity to very deep structures. In general, the EEG cannot easily locate a given source, but it can show a gradient of activation in which the center, with a higher signal value, can be inferred as being closer to the signal source.

In fact, what we just mentioned is one of the key limitations of the EEG, the spatial resolution is very low, since we are limited to the amount of the channels available, the position of the electrodes on the scalp, the small differences in the resistance throughout the scalp, and even so, the signal can only be roughly localized into an area where the signal is stronger.

In opposite to that, the temporal resolution of the EEG is what makes this technique so useful. The temporal analysis of when the signal came and the changes before, after and even through the event potential is very accurate.

We used this EEG analysis in our research to see the changes before, during and after the different stimuli, we compared the changes in the signal of the conditioned face and the non-conditioned faces (CS+ Vs CSa and CS+ Vs CSn), and again as we did in the previous case, the changes between the different trials of the conditioning training.

This strong temporal resolution also allows us to see difference between a selected range of frequencies (The most commonly used according to the literature), and even between different time windows, ranging from 250 msec to 1000 msec after the stimuli was presented.

As we said before, this technique has its limitations, which is why EEG analysis works best when combined with a high spatial resolution technique, such as the Functional Resonance Magnetic Imaging (fMRI).

1.6. Functional Magnetic Resonance Imaging

The advantage of using different methods is that we can take the strengths and limitations and combine them in such a way that we can overcome these problems. This, as mentioned above, is what we tried to do when we combined the high temporal resolution of the EEG with the high spatial resolution of a brain imaging technique.

There are several methods that can be used to obtain an image of the brain, and all of them have some advantage over the other, we choose to use the Magnetic Resonance Imaging (MRI) (Ogawa *et al.*, 1990). There were several reasons for that choice, but mainly, it was because the MRI allows us to perform more easily functional analyses as we will be explaining later.

It starts with the generation of a strong oscillating magnetic field by the scanner, which consist of a series of magnets and coils (Bandettini *et al.*, 2018). With the subject inside the scanner, this magnetic field is set to oscillate at the resonance frequency of the sample (Usually hydrogen atoms in the water molecules of the tissue), thus exciting it.

After the excitation, each tissue returns to its non-excited equilibrium state, a process known as relaxation and occurring over a time period known as the relaxation time. In any case, each type of tissue (Fat, gray matter, fluids, etc.) will have a different relaxation time, allowing us to differentiate them to create an anatomical representation of the brain and its different tissues.

During relaxation, the tissue sample emits signals that can be received by a receiving coil, and by continuously switching spatially varying magnetic fields on and off, it becomes possible to decode the position of any signal within the sample.

But having the image of the brain is only half of the picture, what we really needed was to obtain a functional representation of the brain, meaning that not only we needed the anatomy, but we also require a technique to assess which particular area of the brain was active in which moment.

This is why the MRI was our method of choice; using the same principle as MRI, we can assess the Functional Magnetic Resonance Imaging (fMRI), which focuses on changes in blood flow, called hemodynamic response (Ogawa *et al.*, 1990). More precisely, fMRI can measure concentrations of deoxyhemoglobin, which is a paramagnetic substance that affects MRI relaxation times.

The physiological basis of the hemodynamic response assessment is that our neurons do not have the ability to keep glucose reserves (or Oxygen) for when they are needed, therefore, whenever the cell is in a very active state (like when it is firing or involved in information processing), it needs more blood flow to synthesize the correct amount of ATP.

This method is called Blood-Oxygen Level Dependent (BOLD) signaling (Kwong *et al.*, 1992; Frahm *et al.*, 2018), a now widespread technique that can localize the activity source within a millimeter resolution.

We can infer that the amount of BOLD-signal correlates with a stronger activation, since it “needs” more blood and oxygen, this allows us to represent brain activation, normally with a color-coding representing which areas are involved in a corresponding task.

Besides the direct measurement of BOLD activations, it is also possible to correlate BOLD activity time series between different areas to infer the strength of the connections between them. This can be performed even in the absence of a specific task paradigm. In other words, the method can use a Resting State or Task-Negative State (A period of time with no particular stimuli nor action) to evaluate the intrinsic activity of the areas of the brain.

In some cases, the activation of different areas in the brain, happens at the same time in a synchronous fashion, this gave rise to the study of functional connectivity or networks, where parts of the brain are not necessarily anatomically connected, but fire at the same time and are involved in the same information and coding processes.

In both cases, we depend on the BOLD-signal; its main limitation is that the hemodynamic response is very slow, up to 10 seconds, so the temporal resolution of the method is not so strong.

Here we see again the advantage of combining methods with “opposite” strengths so in the end, the limitations of one is being compensated by the strengths of the other.

We use both types of analysis to get a wider picture of the effects of the trainings, both Pavlovian and Extinction (Lally *et al.*, 2017). In both cases, during the training we analyze the BOLD-Signal and recover information over the areas involved in face recognition, emotional processes, decision, among others. Besides, we were able to compare the activity in the resting states from before and after the trainings, so we could spot changes in the functional connectivity inside the brain as a consequence of the learning protocols.

Chapter 2. Materials and Methods

2.1. Sample population

Sixteen volunteers (eight males and eight females; mean age = 27, SD = 5.3) from the Universitätklinikum Freiburg and Freiburg University were recruited to participate in the experiment. The study was performed with the approval of the Ethik-Kommission der Albert-Ludwigs-Universität Freiburg (EKFR).

All participants were healthy volunteers between 18 and 45 years, with no regards between genders, they were subject to the following criteria of exclusion. Age: Participants outside the range of 18 to 45 Years were not allowed to perform the experiments, it is widely known that with age, the memory functions deteriorate and change, therefore the results in memory performance could not be reliable.

Previous experiences: Any participant who had previously done an experiment regarding memory, learning or facial recognition was not allowed since previous memories could interfere with the results of the actual experiment.

Psychopharmacology: Regarding psychiatric treatment, all participants with a clinically known psychiatric disease were excluded. Also those without diagnosis, but under a psychiatric treatment that could interfere directly with perception, awareness or behavior, were excluded from participating in the experiments.

Anxiety Statement: The STAI (State Trait Anxiety Inventory) and BAI (Beck's Anxiety Inventory) tests were performed according to the Research Domain Criteria (Rdoc) (National Advisory Mental Health Council Workgroup on Tasks and Measures for Research Domain Criteria, 2016) from NIH mental health department, United States. A value of STAI > 45 or BAI > 35 indicates a self-report higher than normal anxiety, these subjects are allowed to perform the experiment but the data was not used for the analysis in the end.

Functional Imaging: metal prosthesis, pacemakers, and dental implants are not allowed inside the Scanner because of its strong magnetic field, subjects with any of the latter or who display some discomfort because of the enclosed space of the scanner or the magnetic field were excluded from this experiment.

2.2. Pavlovian Training

The Pavlovian training consists in the presentation of three faces, these faces were obtained from the Karolinska Institute (KI) in Stockholm, Sweden (Lundqvist, D.; Flykt, A.; Öhman, 1998).

The faces are ranked by the Karolinska Institute (KI) from None-Mild to Highly aggressive, and from a pool of ranked faces, 3 were chosen, one was a non-aggressive one (CSn), other one was an aggressive one (CSa) and the other one was an aggressive one that later will be associated with a disturbing sound (CS+).

The sound generated corresponded to a high frequency beeping sound during 1.5 seconds given through a speaker integrated in the MR-Scanner. The CS+ and CSa were assigned randomly between the two aggressive faces so for half of the subjects one of the aggressive faces was without the sound (CSa) and half of them with the sound (CS+), and vice-versa for the other aggressive face.

The Pavlovian training based on previous works (Fernández *et al.*, 2018), the protocol consisted of a black screen, at which the volunteer would stare until after some pseudo-random interval (either eight, ten or twelve seconds) a face would appear. The face stays on the screen for 6 seconds total and then disappears to a black screen again. This is repeated 24 times, 8 for each of the three selected faces, in the case of the conditioned faces, during the 6 seconds that the faces stays on screen, at second 4.5 and until second 6 there is the Unconditioned Stimulus (US) that will appear 75% of the time, the US consisting in an intense 800 Hz beeping sound. The effectiveness of the training is measured by changes in the Skin conductance response (SCR), which is related to the activation of the sympathetic nervous system. When exposed to fear or a dangerous situation, the Autonomous nervous system (ANS) leans towards a sympathetic response, which includes dilation of the pupils, increase in blood pressure and sweating. The latter is responsible for the increase in the SCR. The SCR is obtained by skin electrodes on the second phalange of the middle and ring fingers, with a pair of surface electrodes (Silver/Silver chloride) from Braintools Equipment's and with Spectra 360 electrode hypoallergenic salt-free gel from Parker (Benedek and Kaernbach, 2010).

The SCR was obtained inside the MR-Scanner, during MR-imaging; there is a continuous change of the magnetic field inside the scanner due to the switching of the gradients every time an image is acquired. This induces strong so-called gradient artifacts that can completely obscure any electrical signals recorded in the scanner.

The most used method to remove gradient artifacts from electrical signals collected during fMRI is the average artifact subtraction (AAS), which uses the repetitiveness of the artifact shape to form an average and therefore an artifact template, which is then subtracted from the raw signal.

The Complete Training includes an Extinction training, in this case, and always after the Pavlovian experiment, the same faces are shown but no sound comes out. As before the faces appear over a black screen for 6 seconds, but this time no sound appears. The Extinction training lasts 36 trials, 12 with each face.

2.3. Cognitive Response

The Cognitive tests are performed also according to the Research Domain Criteria (Rdoc) from the NIH in the United States (National Advisory Mental Health Council Workgroup on Tasks and Measures for Research Domain Criteria, 2016), this consist in two different experiments, each one assessing different behavioral patterns (Lissek *et al.*, 2005).

Discriminatory Index: This experiment shows eight faces from the KI pool, over a black screen (with the three faces that will be included in the pavlovian training included), ranking from none to highly aggressive. Below this face, a scale ranging from 0 with the label “none” to 8 with the label “Extremely” was shown and the participants were asked to report how ugly/unlikeable the face was, using that scale. This experiment is done twice, once before the Pavlovian training and the other one after, the changes of perceived ugliness due to the training was assessed as the difference between after and before.

Probability and Cost: In this case, only two of the faces are shown, the CSa and the CS+ over a black screen, with a question/statement written over the picture. This question/statement could be positive (How likely is that this person helps you? Or how happy would you be that this person is around you?) Or negative and subjects are asked to answer the question from one (No-Not likely) to eight (Yes-Very likely).

The questions were divided in four categories:

- Probability of a positive outcome
- Probability of a negative outcome
- Cost (Intensity) of the positive outcome
- Cost (Intensity) of the negative outcome

Twelve questions/statements from each of the categories were presented in a pseudorandom order for both faces, with a total number of 96 for each subject.

This test falls under the umbrella of the Cognitive effort discounting (*COGED*) which is used to assess evaluation of cognitive effort costs, balanced against rewards. Cognitive effort can impact task performance in a wide variety of tasks, ranging from arithmetic to political attitude formation, decision-making and features prominently among the symptomatology of motivational and mood disorders and schizophrenia (Westbrook and Braver, 2015). We use an adaptation of the COGED protocol described by Westbrook et al. (Westbrook, Kester and Braver, 2013) to view the expected values (Probability and cost) of the negative/positive outcome, instead of focusing on the decision making process.

2.4. Attentional Bias

To assess the Attentional Bias we use the DOT-Probe paradigm developed previously by Halkiopolus and Macleod in the 1980s (MacLeod, Mathews and Tata, 1986), and it has been widely used to examine selective attention to different stimuli.

During this task, the subjects is placed in front of a black screen, which has a white fixation cross in the middle for 1 second, then the fixation cross will disappear and two faces would appear, one on the left side and one on the right one, the faces were presented for 600 msec.

The selection of the faces was the same as the ones chosen for the pavlovian training, the combination of the three presented faces was structured in a way that every face was presented against each other, and both on the right and left sides, so for instance the presentation of CS+ Vs CSa was performed 40 times with 20 of them with the CS+ on the right and 20 of them with the CS+ on the left.

After the 600 msec, the faces would disappear and a white dot would stay either on the left or on right where the faces were. The trials were arranged in a pseudorandom order, giving half of the trials with the dot on the side with the most aversive face (CS+ or CSa in the case of CSa Vs CSn), and half of them otherwise.

The trials where the dot was on the "Aversive" side, are consider congruent trials, meanwhile, when it was on the other side, the trial is considered incongruent. The faces stayed on the screen for 1 second, before the next trial begins.

During the dot presentation, the subject was told to press a button in a response box to indicate which side of the screen the dot was on as fast as it was possible, for subject's ease two response boxes were given, one for each hand.

The reaction time was obtained by a Matlab script which allowed us to compute the difference between when the trial was presented and when the response box was activated.

The congruent trials were the mean reaction time of the trial where the dot position matched the aversive face, and the incongruent trials the mean reaction time where the dot position matched the least aversive face. The attentional bias is the difference between the incongruent trials and the congruent one.

2.5. Electroencephalographic record

Electroencephalography refers to the non-invasive monitoring method of recording the electrical activity of the brain. It works by placing electrodes along the scalp of the subject, this electrodes measure voltage fluctuations resulting from the ionic current that underlie the neuron circuitry of the brain.

The brain is composed of a network of billions of neurons; these cells are constantly exchanging ions with the extracellular matrix, creating a difference of charge between the two sides of the cellular lipid-membrane. This is known as the resting potential, which can be actively modified to polarize the cell and increase or decrease this difference.

The resulting changes can induce an “action potential” that will propagate through the neuron and in some cases, induce the release of neurotransmitters and the ulterior new action potentials. This flow of action potentials from neuron to neuron is the basis of brain activity and it is what is being measured by the electrodes on the scalp.

The firing pattern can change from neuron to neuron and from time to time, because of this we can see different rates of firing, commonly divided between frequencies into 5 groups. Delta waves (0-4 Hz), Theta waves (4-8 Hz), Alpha waves (8-13 Hz), Beta waves (13-30 Hz) and Gamma waves (>30 Hz) (Armando Freitas da Rochaa and Jr, 2015).

For this, we used an EEG MR-compatible system from Electrical Geodesic Inc. (EGI) with 256 channels, the cap diameter was adjusted to each of the subjects before the recording.

Each channel connects to the scalp through a hypoallergenic sponge which once soaked would contact with the skin. To decrease the resistance and therefore increase the EEG signal, we soaked the sponges in a saline solution of Potassium Chloride (KCl) and detergent.

For the data acquisition, the 256-channel system included 70 electrodes from the international 10-10 system (Oostenveld and Praamstra, 2001)(Fig. 1a) and we got one long single recording for the whole experiment, once the data was collected, we divided the single recording into all the experiments and trials.

The EEG recordings were performed inside the MR-Scanner, and in the same fashion as with the Skin Conductance Response, an artifact was generated due to the switching of magnetic field gradients every time a new image was acquired.

Two different fields are applied: RF and gradients. Artifacts induced by RF have a much higher frequency than EEG and hence can be reduced effectively by low-pass filtering applied before the EEG amplifier front end. This is not the case for Gradient Artifact (GRA), which has a frequency in the range of the EEG measurement, so it cannot be filtered as the previous one, but its amplitude is usually around 100 times higher than the EEG signal.

Besides the latter, there is also an artifact in the EEG acquisition called the Ballisto-Cardiogram Artifact (BCG), this is due to the pulsation of the scalp and blood movements under the electrodes caused by the cardiac cycle. The amplitude and frequency of this artifact is considerably less than the GRA artifact. To avoid the contamination of the signal with this artifact, a simultaneous electrocardiogram recording (ECG) is performed.

To surpass the problem of both the GRA and BCG artifacts, the collected data was pre-processed, and artifact removal was performed using the AAS method implemented in Matlab (Fig. 1b) and the “Clean” signal was used for the analysis.

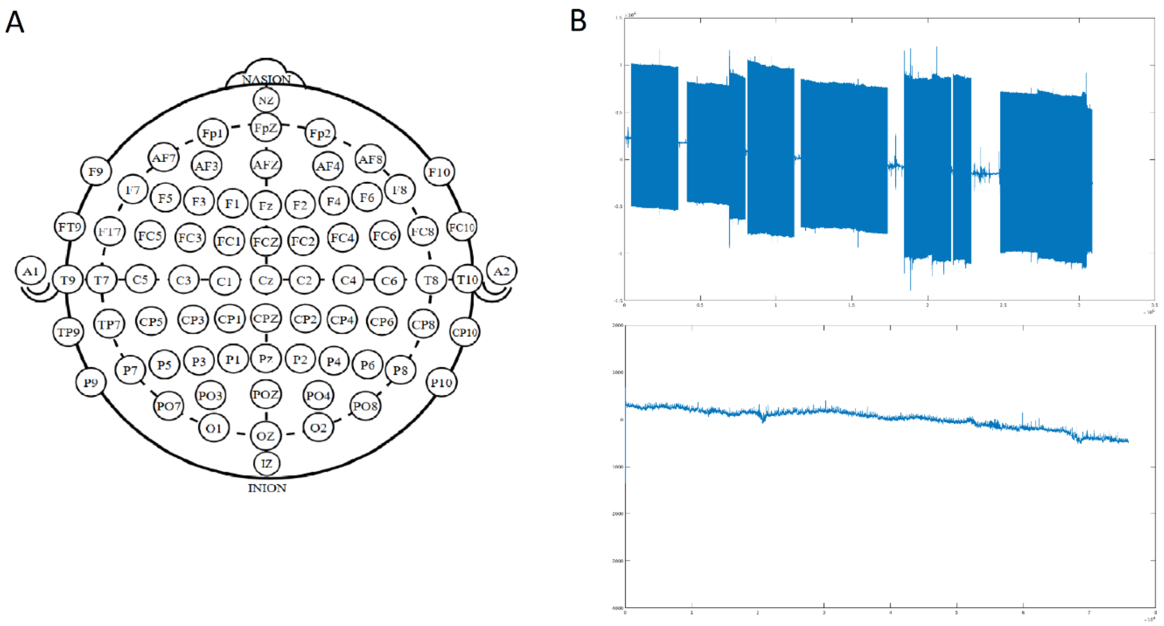

Fig. 1. The International 10-20 system (A) of the electrodes placement in the scalp. The site "Cz" is placed in the midline sagittal plane and represents the reference point for the measurements. In (B) we see the EEG signal obtained through the whole experiment (Up), each "Block" correspond to a 100x artifact due to the imaging acquisition, the cleaned signal (Down) was obtained once the artifacts were removed.

Once the artifacts were removed and the signal could be analyzed, we used the timing of the artifact generated by the fMRI acquisition to segment the whole EEG recording into its corresponding experiments (Either Pavlovian or Extinction, and all the corresponding Resting states) (Abreu, Leal and Figueiredo, 2018).

For the Analysis of the Pavlovian training, we took a 2 second window of each of the face presentations, and ordered them according to its corresponding valence (CS+, CSa and CSn). The 2 second window consist of a first second before the presentation of the stimuli and one second after, therefore the first half of the window was used as a baseline for the analysis of the second half. To avoid the possibility of the subject learning and predicting when the stimulus may come, the inter-stimuli interval (ISI) was pseudorandom generated between eight, 10 and 12 seconds.

Once done, we end up with eight windows of 2 seconds each, each one of the previous corresponding to the number and order of stimuli presentation. The windows were divided, from the first second, a mean was computed and a baseline value was obtained, then the other second was divided in four 250 msec windows from 0 (Stimulus presentation) until 1000 msec.

From these data we computed a Power Spectral Density (PSD), this analysis describes the intensity (Power) in each of a discrete number of the frequency components of a signal. As stated by the Fourier Analysis, any signal can be divided into its frequency components, in our case, we focus the analysis into five common known frequency ranges, Delta (1–4 Hz), Theta (4–8 Hz), Alpha (8-13 Hz) Beta (13–30 Hz) and Gamma (Low-Gamma (30–70 Hz) and High-Gamma (70–150 Hz)) (Hall and River, 2009).

Based on the literature we focus mainly on the analysis of Alpha-Wave power in 250 msec windows when comparing the Conditioned Vs Unconditioned stimuli (Thompson and Westwater, 2017).

2.6. Brain Imaging

Neuroimaging is the use of various techniques to measure, directly or indirectly the structure, function and metabolism of the nervous system. The field of neuroimaging can be then divided into two big branches.

- Structural imaging, which deals with the anatomy of the gray and white matter, the nerves, ventricles and all the structures that make the nervous system, and is mainly used for the diagnosis of gross lesions, injuries and masses (i.e.: Tumors).
- Functional imaging, which deals mainly with changes in the metabolism and activity of areas or nuclei of the nervous system.

We focus our study on the Functional imaging, all our volunteers were healthy and Young-Adults so we were not searching for differences in the anatomy or disease diagnosis, but were looking for which areas and nuclei were active or inactive across volunteers when performing the experiment.

For the brain imaging we used a technique called Magnetic Resonance Imaging (MRI), the MR-Scanner consist in a series of magnets and coils which can create a strong magnetic field. When applied to water-containing molecules, the protons (Hydrogen Atoms) create a signal, which in return is measured by a receiving coil (McRobbie *et al.*, 2006). Varying the magnetic field using another type of coil (Gradient coils), the position of the signal can be encoded.

The different concentration of water molecules and densities of the different tissues generates different contrasts and the overall information can be processed to form an image of the whole brain.

We used a method called Blood-Oxygen-Level depending contrast imaging (BOLD-Contrast imaging) or simply BOLD-signal. This method relies in the fact that neurons do not have internal reserves of glucose, the main substrate to produce energy along with oxygen; therefore, they have to obtain it from the blood vessels.

The hemodynamic response, in which the neuron takes the oxygen (and sugar) from the arterial blood, can be seen as a balance between the oxygenated hemoglobin (Oxyhemoglobin) and non-oxygenated one (Deoxyhemoglobin) (Ogawa *et al.*, 1990).

In active neurons, this balance favors the Deoxyhemoglobin, since more oxygen is being taken from the arterial blood; as deoxyhemoglobin is a paramagnetic substance, this can be measured by the MR-Scanner, therefore, we can infer the relative activity of a group of neurons or brain area.

This BOLD-signaling is the most common type of Functional Magnetic Resonance Imaging (fMRI), in our case, we performed the analysis of the BOLD-signal to see which areas were involved in the Pavlovian and Extinction trainings, and therefore, we performed all the trainings inside the MR-Scanner.

With the images reconstructed into a 4-D file (3D image and time) for each of the subjects, we segmented it, making a 10 second window for each of the stimuli presentations. This leaves the Pavlovian training with 24 windows, which are then ordered between the different stimulus (CS+, CSa and CSn) and in order of appearance (1 to 8), and for the Extinction training, 36 windows divided into CS+, CSa and CSn and in order of appearance from 1 to 12.

Because we wanted to see the difference that the training generates in the perception of the stimuli, we performed a Generalized Linear Model (GLM) to analyze the changes from the first to the last trial in each of the three stimuli.

The GLM consists in modeling the expected hemodynamic response to each trial and fitting this model to the measured fMRI time series at each voxel by linear regression, allowing the identification of voxels showing an activation following each trial.

In the ordinary GLM, each trial is assumed to contribute equally to the final model. In our case, we generated an additional regressor in which the modeled hemodynamic responses were modulated by a linear trend to specifically identify voxels that showed a gradual increase in activity over the course of the conditioning process. Additionally, for the second-level group analysis, subject-specific activations were weighted by the change of the corresponding Skin Conductance Response (SCR) as a measure of how much the Pavlovian training was successfully being learned by each subject. Therefore, subjects with a higher SCR response (considered as "Responders") will contribute more to the final group results than subjects with a low SCR response (or "Non-Responders").

Besides the latter, we performed another type of analysis, in which we looked at changes in functional connectivity before and after the trainings.

In between the trainings, we measured basal activity during 5 minutes without any stimuli and a black screen, the subject was told not to fall asleep but not to perform any task whatsoever. This Baseline without any task is called "Resting State".

The connectivity in the resting states is measured by the synchronization between the activity within a seed (area of interest) and the rest of the brain (or another area in particular). In our case, we choose the Amygdala as a seed. Since we did this for all the resting states, we could then compute the difference in amygdala connectivity after and before the conditionings.

We acquired four resting states, one before the training and one after, therefore we had four Resting States, Pre-Pav (Before the Pavlovian training), Post-Pav

(After the Pavlovian training), Pre-Ext (Before the Extinction training) and Post-Ext (After the extinction training) (Abreu, Leal and Figueiredo, 2018).

We repeated the analysis for Left and Right Amygdala separately to see the contributions of each of the amygdala separately. To avoid detecting areas associated with activity common to both amygdalae, we regressed out the contribution of one amygdala from the other, so that each result was showing the exclusive contribution of only one of the amygdalae.

Prior to the GLM analyses, the fMRI data were preprocessed using the FSL toolbox. Preprocessing steps consisted of image realignment to correct for subject motion, co-registration to normalized MNI space, and spatial smoothing with a 6 mm Gaussian kernel.

Chapter 3. Results

3.1. Anxiety scores

The mean and standard deviation of the State-Trait Anxiety Inventory (STAI) and Beck's Anxiety inventory (BAI) was computed across subjects, the STAI-Trait (mean = 33, SD = 1.96) and BAI (mean = 10.3, SD = 1.88) was performed before the training and was a self –reported measurement of anxiety.

STAI-States were performed before and after the Pavlovian training and the rest of the experiments, we obtained the difference between the State after the training STAI-S_{post} and the one before STAI-S_{pre} (STAI-S_{post} mean = 33.6, SD = 2.17; STAI-S_{pre} mean = 30.46, SD = 1.88; STAI-S_{diff} mean = 3.13, SD = 1.34; $p < 0.05$).

3.2. Cognitive responses

As part of the behavioral assessments described by the Rdoc, we chose to analyze the negative valence system and its enhancement or modification by the pavlovian training. With that goal in mind, we chose two different tests.

As described, the Discriminatory Index, in which a series of eight faces appear on a screen and the participant has to evaluate them according to its own subjective idea of how Ugly/Aversive do they perceive each face to be. This is repeated by the end of the whole experiment. These two results are saved under the $Eval_{pre}$ and $Eval_{post}$, and we compute the difference.

As shown in Fig. 2 we focus on the difference of the aversive faces that were involved in the Pavlovian training, we compare two of the "Aversive" faces according to the Karolinska Institute own index, one of them was the one trained (CS+) while the other one was not (CSa) and see the change in the "Aversiveness". (CS+ $mean_{diff}$ = 0.733, SD = 0.41; CS- + $mean_{diff}$ = -0.20, SD = 0.33; $p < 0.01$).

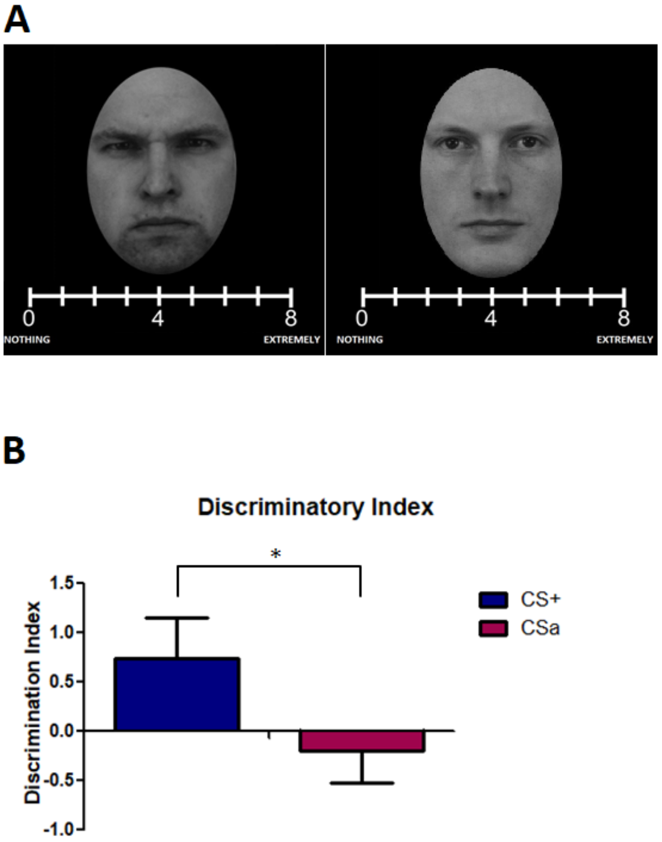

Figure 2: Discriminatory Index difference between the trained and non-trained faces. **(A)** We see an example of how the experiment is done; the face change once is ranked from 0-8. The order is randomized to avoid any kind of pattern learning.

(B) The difference between the index after and before the training for both the trained (CS+) and non-trained (CSa) shows a statistically significant increased report of "Aversiveness" for the trained face, increasing around 10% after one training session

The discriminatory index serves as a direct measurement of the change in valence of a stimulus after a training, but only involves one side of cognitive systems. To see both the negative and positive valence systems, we adapt another of the recommended paradigm, called Cognitive Effort Discounting (COGED) task, which proposes a reward scenario (Positive valence) and a punishment one (Negative valence).

To measure the probability and cost bias consequence of the training we compared the probability (chance) and cost (intensity) of a scenario and related them to a face. As before, we compared the trained face (CS+) and the non-trained aversive face (CSa) once the pavlovian training was finished. To compare, we divided the instances in two, positive scenarios, in which you can assess the probability of that scenario happening and the cost or intensity of it, and negative scenarios divided in the same way. With the trained face (CS+), the probability of a positive scenario (mean = 3.20, SD = 0.16) see a decrease compared to the non-trained one (CSa) (mean = 3.97, SD = 0.15).

Surprisingly the intensity of this positive action (CS+ mean = 4.78, SD = 0.20; CSa mean = 4.58, SD = 0.16) was higher in the trained face. Following the same trend, when compared with the negative scenario, with the CS+ the probability (mean = 3.86, SD = 0.14) sees an increase compared to the non-trained one (CSa) (mean = 3.01, SD = 0.18). This trend remains for the intensity of the scenario, being higher for the CS+ (mean = 5.03, SD = 0.15) and lower for the CSa (mean = 4.56, SD = 0.20). As shown in Fig. 3, the trend is maintained across subjects but it is not enough to be statistically significant (p >0.05 in all cases)

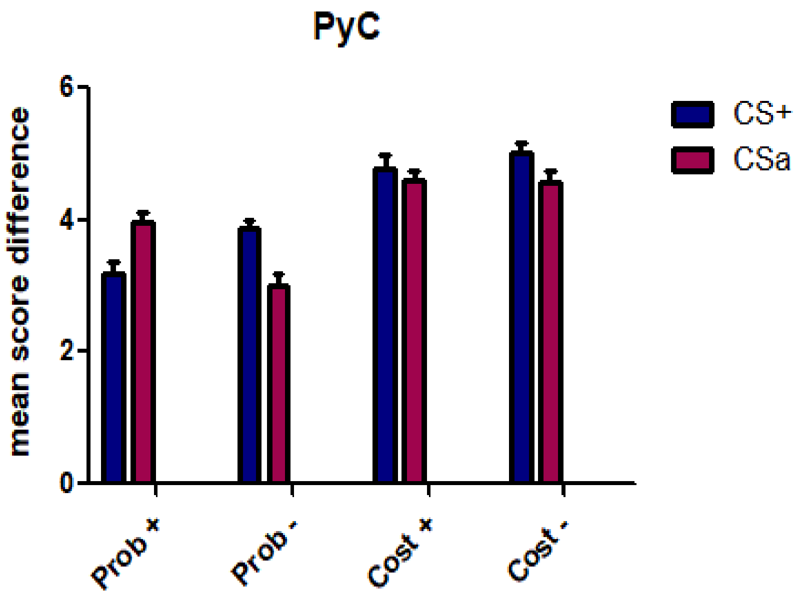

Figure 3: The difference between the perceived cost and possibility of both positive and negative outcomes for CS+ and CSa faces after and before the training. There is a trend of increasing results for negative outcomes for the CS+

3.3. Attentional Bias

The other system, as part of the behavioral assessments, was the attentional system regarding external stimuli. The vigilance state changes when in presence of threat, although, these tasks do not directly measure individual's response to a sustained threat, but rather assess consequences of having previously experienced a sustained threat.

The difference between the incongruent and the congruent trials will give the attentional bias measured in milliseconds.

When we draw the comparisons between the faces like is shown in Fig. 4, we see that CSa Vs CSn shows little to no difference, but when compared CS+ Vs CSn an increase in the time response becomes clear (CSa Vs CSn mean = 1.17 msec, SD = 0.03; CS+ Vs CSn mean = 27.4 msec, SD = 4.34; $p < 0.001$) and shows an attentional bias toward the threat that the CS+ imposes.

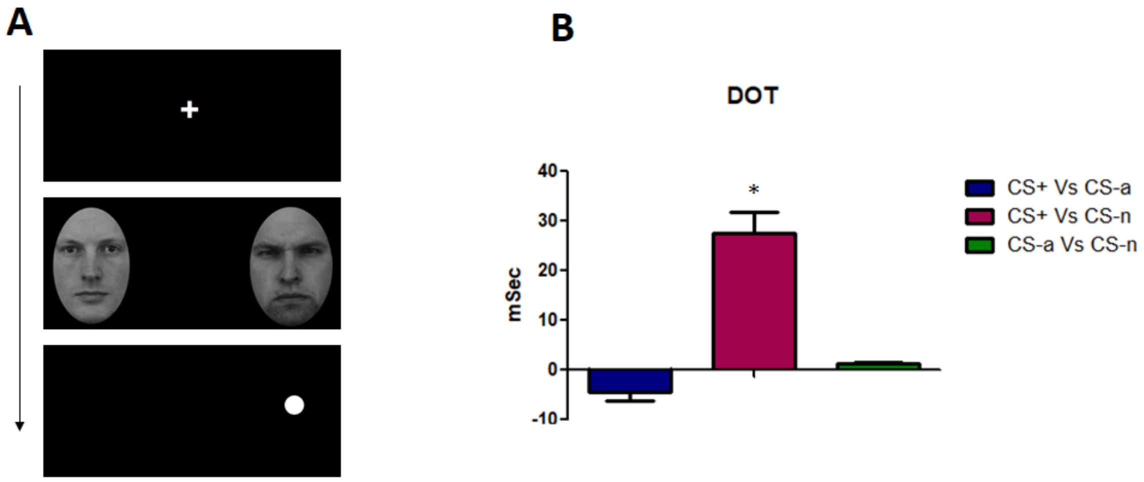

Figure 4: Attentional Bias measured in DOT-Probe task paradigm, **(A)** shows the progression of the task, the faces appear in a random order and the DOT switches between left and right side of the screen in a random fashion. **(B)** We see the difference between the attentional bias of the CS+ with both the CSa and CSn and the CSa and CSn between each other.

3.4. Skin Conductance Response

As mentioned before, an increase in the sweating as a consequence of the sympathetic activation of the SNA decreases the resistance of the skin where the electrodes are placed, therefore increasing the measured voltage.

Once the signal is cleaned of artifacts, we obtain a single recording for the length of the trial. For the analysis, we took a 6-second window from the moment when the stimulus appears.

Each of the three faces chosen for the pavlovian training was presented 8 times in a pseudorandom order, therefore we have 24 windows. The windows were split between each stimulus and sorted in order of appearance from 1 to 8

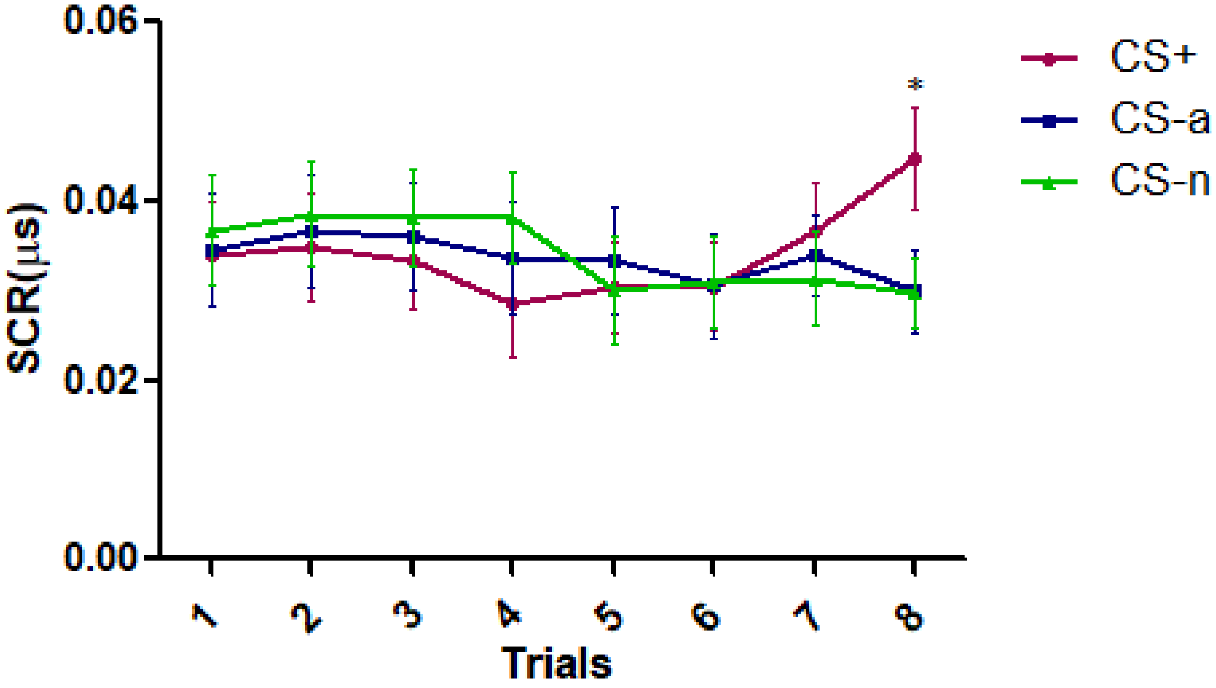

Figure 5: Skin Conductance response in the Pavlovian training during the 8 trials for each one of the trained faces, the SCR of all the faces start with similar values, since before training neither of this faces were shown before, and start to differ until the last trial when the CS+ becomes significantly higher that both non conditioned faces

As shown in Fig. 5 there is an increase of the SCR for the CS+ when comparing the last trial with the first one. For the statistical analysis we performed a Two-Way ANOVA between the 3 stimuli and the 8 trials comparing them to CS+, shown in Table 1, the last trial was significantly higher ($p<0.001$).

These points show a statistical significant increase for the last point on the CS+, compared both to its own first point and with the last points of both CSa and CSn. From this, we can infer an increase of the Sympathetic Nervous System, and therefore the CS+ is being treated as a threat.

Table 1:

CS+ Vs CSa			
Trials	Difference	P-Value	Summary
1	0.000	$P > 0.05$	ns
2	0.002	$P > 0.05$	ns
3	0.003	$P > 0.05$	ns
4	0.005	$P > 0.05$	ns
5	0.003	$P > 0.05$	ns
6	0.000	$P > 0.05$	ns
7	-0.003	$P > 0.05$	ns
8	-0.015	$P<0.001$	***

CS+ Vs CSn			
Trials	Difference	P-Value	Summary
1	0.003	$P > 0.05$	ns
2	0.004	$P > 0.05$	ns
3	0.005	$P > 0.05$	ns
4	0.010	$P<0.001$	***
5	0.000	$P > 0.05$	ns
6	0.001	$P > 0.05$	ns
7	-0.005	$P > 0.05$	ns
8	-0.015	$P<0.001$	***

In the same manner that we applied the SCR to the Pavlovian training, we did it to the Extinction training.

With this experiment, the same three faces were presented 12 times in a pseudorandom order. We took a 6-second window for each case leaving us with 36 windows that were split between each stimulus and sorted in order of appearance from one to 12.

In the case of the Pavlovian training, we expected the first appearances to be similar since neither of the faces had been trained before. In this case, we expected the first points to be utterly different, with the CS+ already being perceived as a threat, as can be seen in Fig. 6

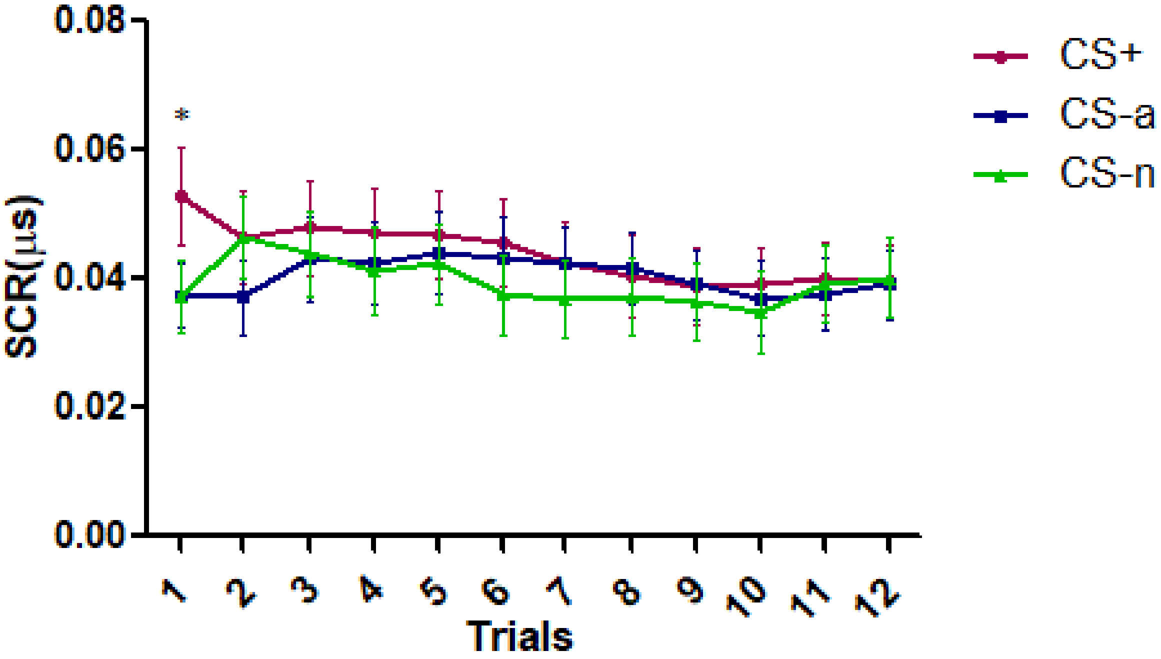

Figure 6: Skin Conductance response in the Extinction training during the 12 trials for each one of the trained faces, the SCR of all the faces start in a different place, since the CS+ was previously trained to be consider a threat, but the SCR slowly declines until reaches the baseline established by the CSa and CSn

Through the course of the 12 trials we can see how the trained CS+ face is slowly decreasing the SCR signal, we performed a Two-Way ANOVA for the statistical analysis again comparing every face to the CS+ and by the results obtained in Table 2 we can infer that is no longer consider a threat and it returns to values similar to the ones of the CSa and CSn

Table 2:

CS+ Vs CSa			
Trials	Difference	P-Value	Summary
1	-0.015	P<0.001	***
2	-0.009	P<0.001	***
3	-0.005	$P > 0.05$	ns
4	-0.005	$P > 0.05$	ns
5	-0.003	$P > 0.05$	ns
6	-0.002	$P > 0.05$	ns
7	0.000	$P > 0.05$	ns
8	0.001	$P > 0.05$	ns
9	0.000	$P > 0.05$	ns
10	-0.002	$P > 0.05$	ns
11	-0.002	$P > 0.05$	ns
12	0.000	$P > 0.05$	ns

CS+ Vs CSn			
Trials	Difference	P-Value	Summary
1	-0.016	P<0.001	***
2	0.000	$P > 0.05$	ns
3	-0.004	$P > 0.05$	ns
4	-0.006	$P > 0.05$	ns
5	-0.005	$P > 0.05$	ns
6	-0.008	P<0.01	**
7	-0.005	$P > 0.05$	ns
8	-0.003	$P > 0.05$	ns
9	-0.002	$P > 0.05$	ns
10	-0.004	$P > 0.05$	ns
11	-0.001	$P > 0.05$	ns
12	0.001	$P > 0.05$	ns

3.5. Electroencephalographic record

In the same fashion as the SCR, each of the electrodes placed on the scalp gives us a continuous signal of the changes in brain activity. This signal is composed of a sum of all the frequencies and noise from the surrounding structures.

After cleaning the signal and extracting the frequency spectrum, we quantified the power of Alpha waves that are neural oscillations in the range of 7.5–12.5 Hz.

Similar to the approach we use to the SCR, we measure the EEG during the Pavlovian and extinction training; therefore, we obtained for each of the stimulus 8 and 12 time-windows respectively. In this scenario, the windows were two seconds long, with the first second set before the stimulus and used as a baseline to calculate stimulus-related spectral power changes.

In Fig. 7 we see the progression of the Alpha power distribution for the CS+, from 250 msec to 1000 msec, moving from a frontotemporal location to a prefrontal one.

We focus on the frontotemporal location, therefore the window chosen for the analysis was from 250-500 msec.

In this case, we performed a statistical analysis with a Two-Way ANOVA in which we compared the 70 electrodes and the Time-windows with a baseline ("F3" electrode, $p<0.05$; "AF3" electrode, $p<0.05$).

There is a significant increase in alpha power at the F3-AF3 electrodes, that in the 10-20 international system correspond to a left frontotemporal location, the significant increase was accompanied by a trend of increased values at surrounding electrodes as seen in Fig. 7.

When comparing with the other stimulus, we again chose the 250-500 msec window and in this case, we compute the difference between the last trial and the first one. With this, we aim to see the difference in activation in the frontotemporal area between the first trial, which is close to baseline, to the last trial where we see the action of the Pavlovian conditioning, as shown in Fig. 8(A).

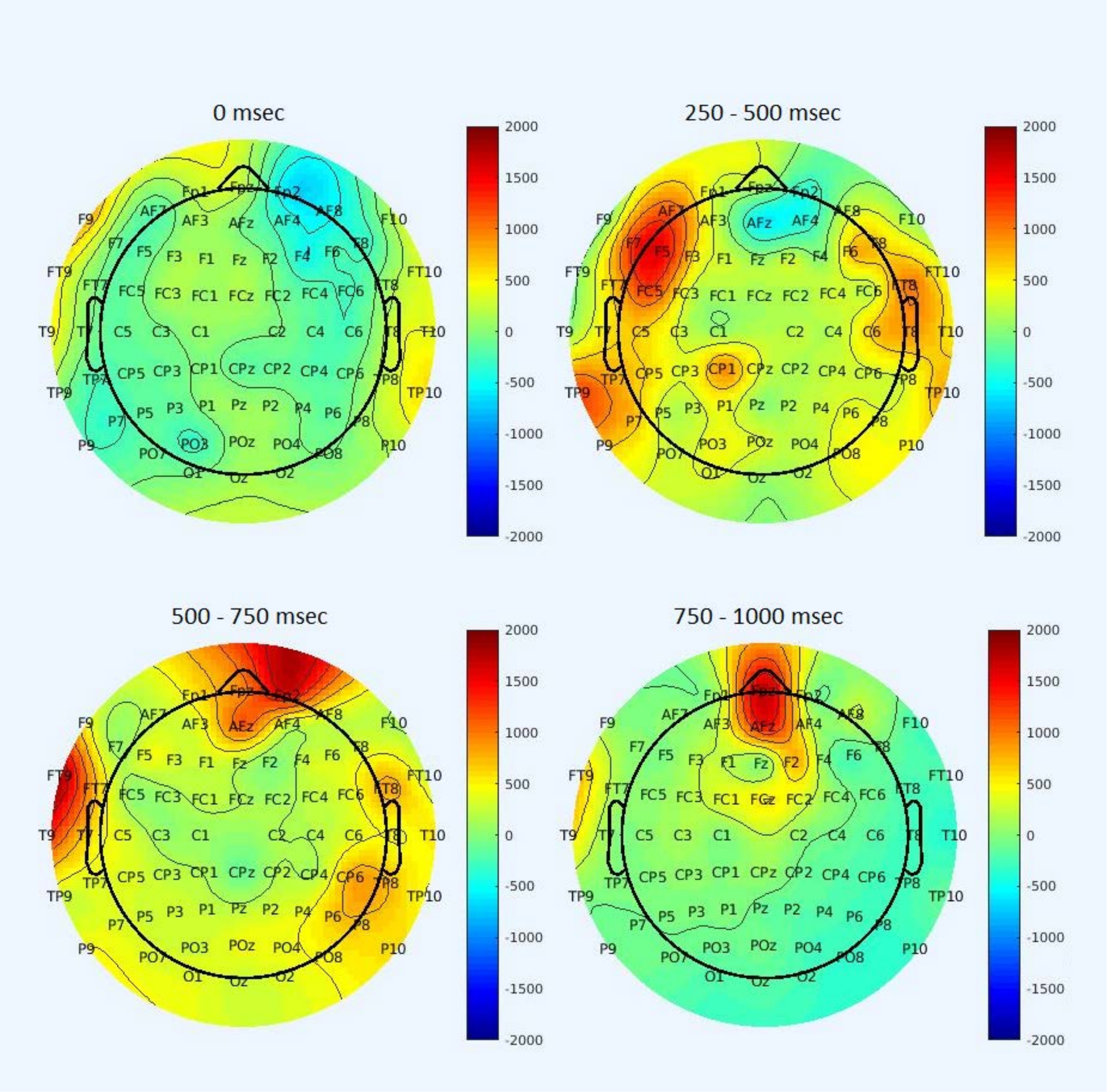

Figure 7: Alpha power distribution following the presentation of the Conditioned stimulus in four Time-windows starting with the presentation of the CS+ and up to one second after.

Moreover, the other two faces were also analyzed and we performed a Two-Way ANOVA of all the electrodes and the three faces. In CS+ Vs CSn (“AF8” electrode, $p<0.05$; “AF7” electrode, $p<0.05$) There is again a significant increase of the left and right frontotemporal areas, meanwhile CSa and CSn show no statistical difference with baseline or each other.

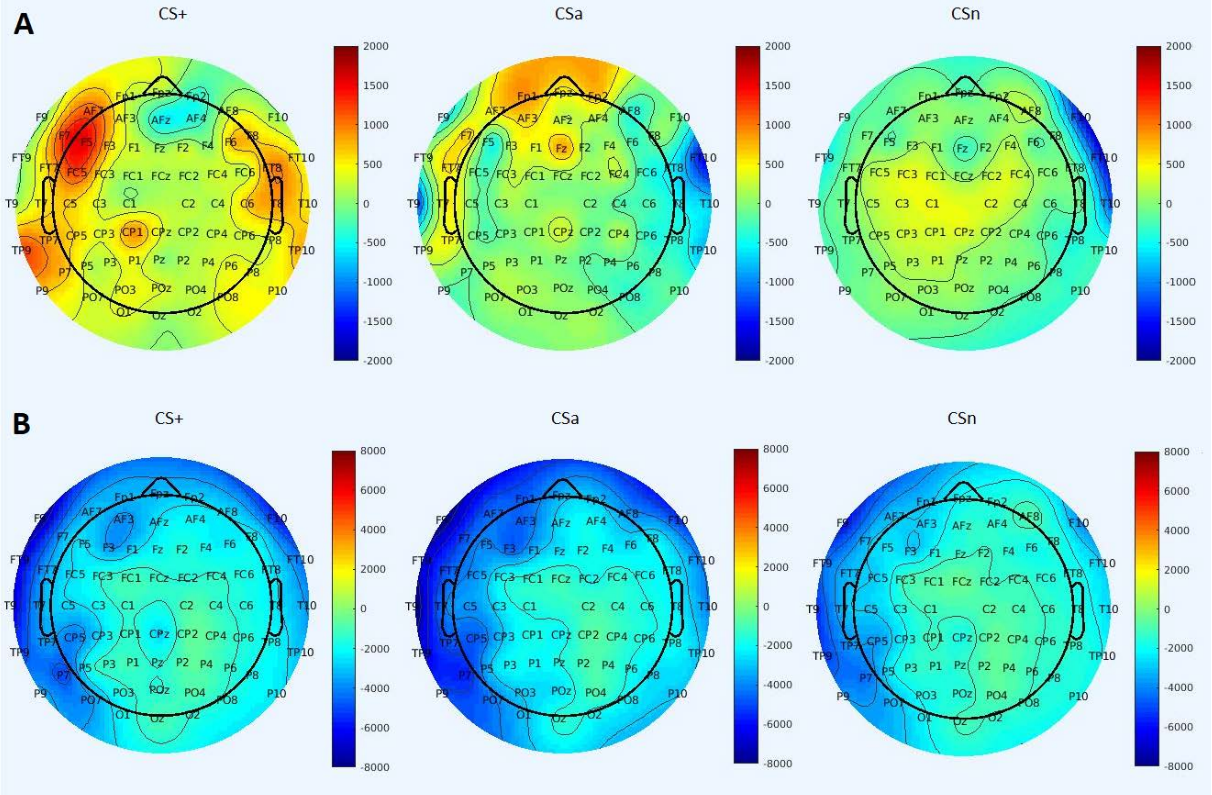

Figure 8: (**A**) Alpha power distribution in the 250-500 msec window of the three trained faces, here we see the increase in activation in the CS+ but very low activation of the other two compared with baseline. **(B)** Alpha power distribution in the 250-500 msec window of the same three trained faces after the extinction training, where we can see the deactivation in darker blue, comparing the last to the first trial.

We see in Fig. 8 (B) that, using the same window as before, there is a clear deactivation in the frontotemporal areas when we compare the different faces in the Extinction training.

When statistical analysis was performed, the comparison between CS+ with CSn and CSa shows a significant decrease ("AF8" electrode, $p<0.05$; "AF7" electrode, $p<0.05$) of the EEG signal. As above, we see that the trend is maintained across subjects and faces in the surrounding areas of the before mentioned electrodes.

To go deeper in the analysis of this activation with the pavlovian training, we choose an area corresponding to the left and right amygdala complex and analyze that separated from the rest. The left amygdala complex consists in a circular area on the Fronto-Temporal left side of the scalp and involves the electrodes

AF7, AF3, FP3, F3, F5 and F7. On the other side, the right amygdala complex was composed of AF8, AF4, FP2, F4, F6 and F8 electrodes.

We focus only on the CS+ face since it was the only one significantly activated, but instead of computing the difference, we analyze each trial, in Fig. 9, we see the increase of the SPD of these particular areas through the trials.

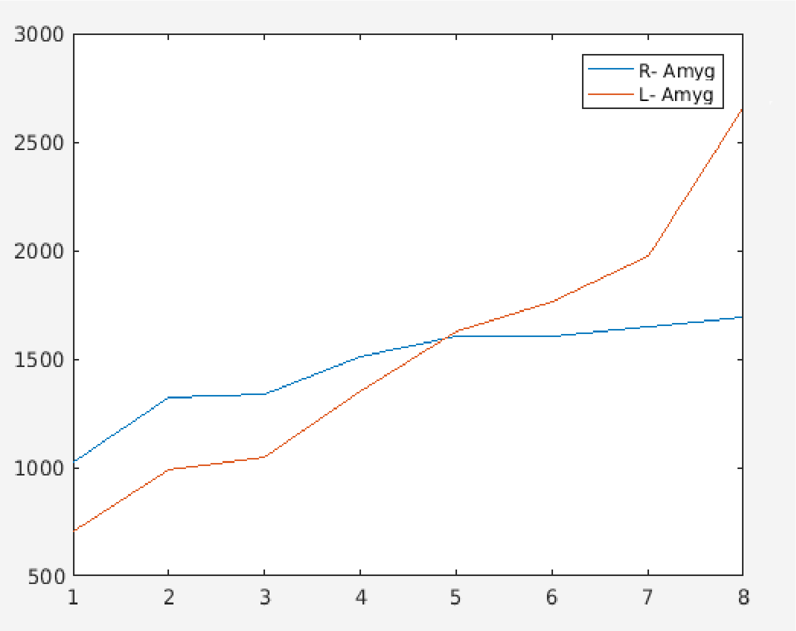

Figure 9: The graph shows the increase in alpha power in the areas corresponding to the Left and Right amygdala through the trials of the pavlovian training, for the conditioned stimulus. This result correlates to what is observed in the “Headplot” from previous figures.

The increase in the signal of the EEG corresponding to frontotemporal electrodes through the trials indicates an increase in the Amygdala activity and fear related areas. We can infer that the Pavlovian Training has been successful based on the formation of an aversive Face-Sound pair.

3.6. Functional Magnetic Resonance Imaging

For the imaging analysis, we used a general linear model (GLM) since we expected a continuous increase of the signal with each trial. As stated previously the SCR is used to check the response to the pavlovian training. In Fig. 10(A) we see the BOLD response across all subjects regardless of how they performed in the other test, there we can see in both cases little BOLD-signal difference with baseline. In Fig. 10(B) we used the SCR response as weight when generating our regressors; therefore, the signal was corrected according to how they respond to the pavlovian training.

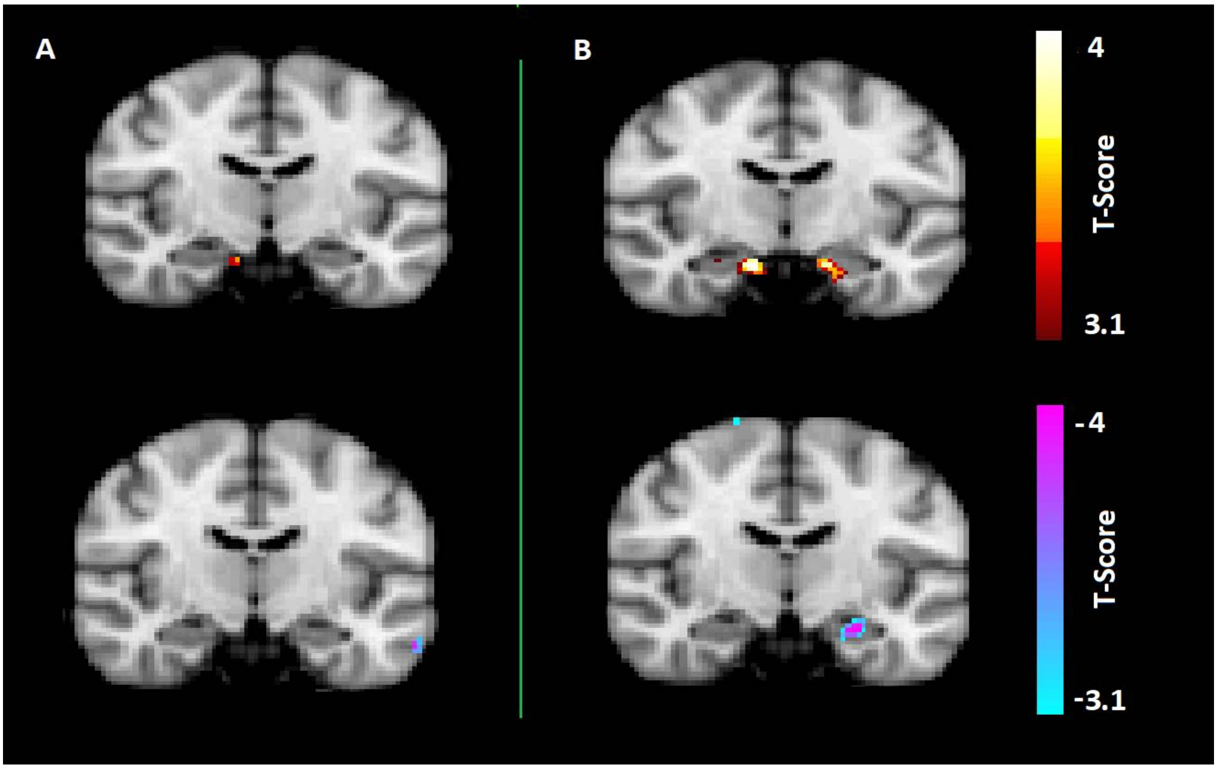

Figure 10: T-Map of the BOLD-Signal during the Pavlovian (top) and the Extinction (bottom) training, The T-Score represents consistent stimulus-related activations at the group level, here we see **(A)** the uncorrected (same weighting for all subjects) and **(B)** corrected (subjects weighted by SCR) activation maps.

The weighted map shows a strong T-score for areas corresponding to the amygdala both in the Pavlovian and in the Extinction trainings. The Pavlovian is associated with an increased activation of the amygdala once the CS+ starts being considered a threat, compared to a decrease in activation in the Extinction training, once the face is no longer threatening.

Once it is shown that the Amygdala was indeed involved in fear behavior, we wanted to analyze the connectivity between these nuclei and the rest of the brain. With this in mind, we performed a whole brain functional connectivity analysis during the resting states, and we compared the connectivity between the resting state before the pavlovian training and the one after, and the same for the Extinction.

When we check the connectivity in Fig. 11, we see that the combined amygdala activity has a strong synchronization with the Orbito-Frontal Cortex. Fig. 11(A) shows that after the pavlovian training, the amygdala-OFC circuit was increasingly active using the previous resting state as a baseline. When we perform the statistical analysis we see a significant increase in the medial Orbitofrontal cortex (mOFC).

In Fig. 11(B) we see the circuit decreasing its activity when compared to the previous resting state. This is expected with the extinction training since OFC is known for its role in emotional valence of the stimuli. The statistical analysis show again a decrease in the mOFC in pace with what was seen in the pavlovian training, moreover, there was a significant reduction in the connectivity with the inferior temporal gyrus, which is involved in facial recognition and object identification.

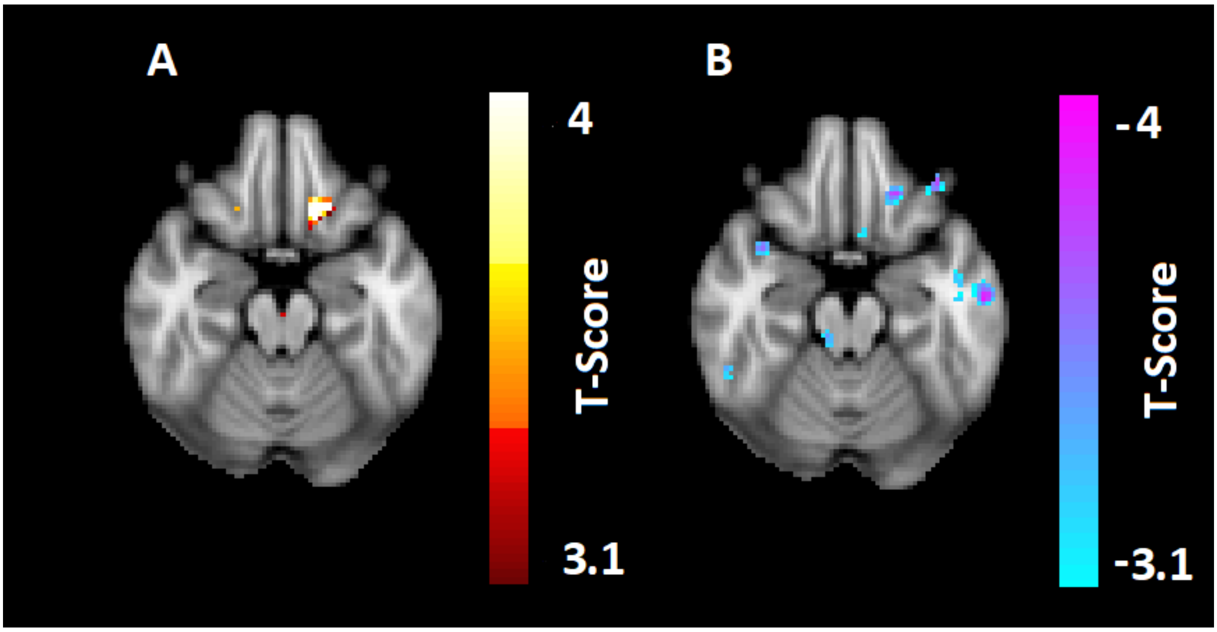

Figure 11: T-Map of the connectivity changes between the resting state before and after the (A) Pavlovian and (B) Extinction trainings. The areas in both cases are classically related to face recognition and emotion valence of stimuli

To differentiate the contribution of each of the amygdala, we repeated the analysis we did before but instead we took the whole brain connectivity of each individual amygdala and after regressing out the contribution of the other one, leaving us with the connectivity changes contributed only by the left amygdala and one only for the right.

As expected most changes were similar regarding which of the amygdala were being used as seed, the main difference came with lateralization, with left amygdala contributing more to left hemisphere changes, and right amygdala to the other one.

The main difference came regarding the trainings, in the pavlovian training, each of the amygdala contributed to an increase in connectivity to the dorsomedial prefrontal cortex (dmPFC $T > 3.80$, $p = 0.0015$) as seen in Fig. 12(A), and in the extinction training, the main connectivity changes were regarding the Insulate cortex, more specifically the anterior gyrus (ASIG $T < -4.015$, $p = 0.0013$) shown in Fig. 12(B).

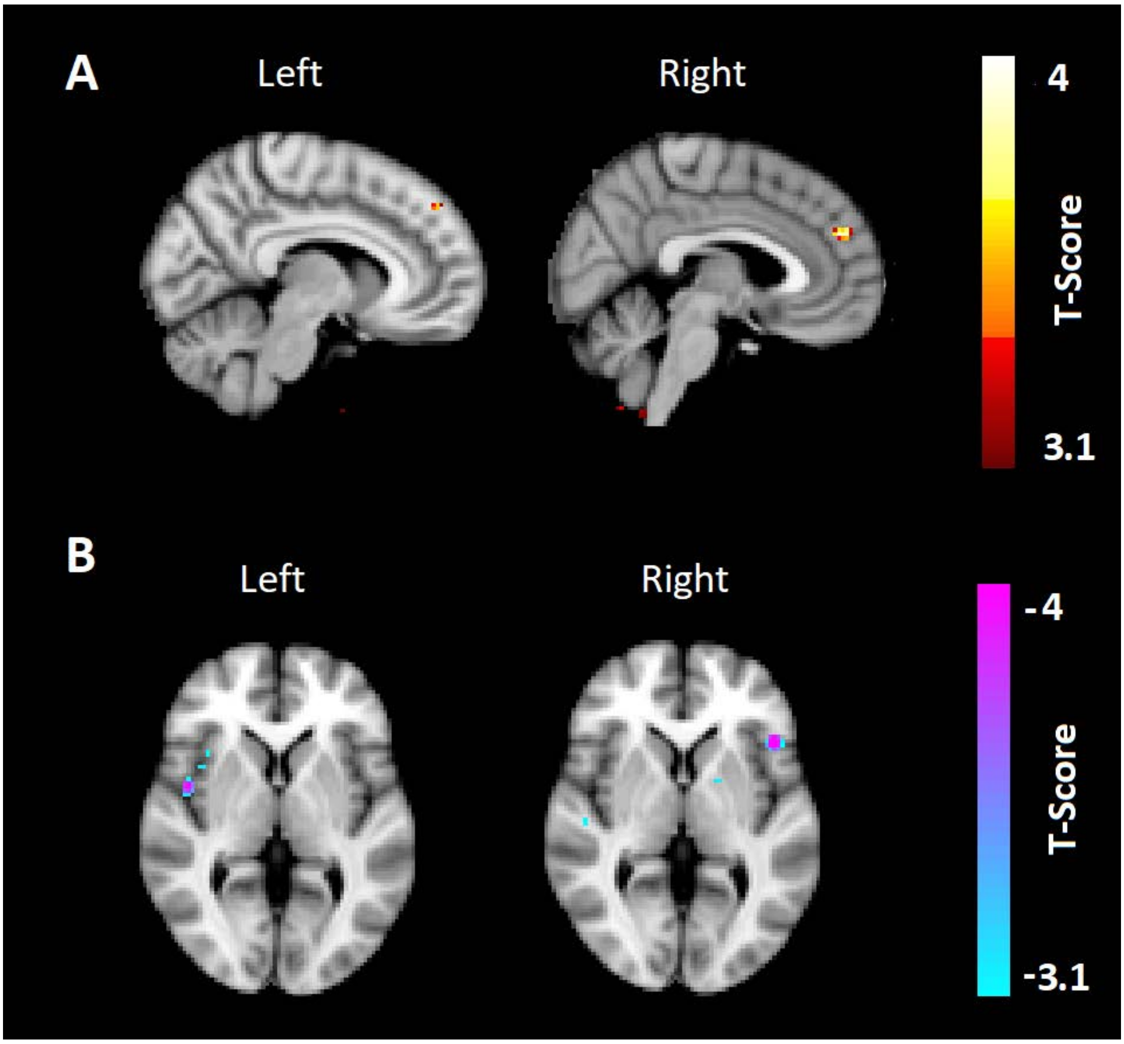

Figure 12: T-Map of the contribution of the left amygdala (left) and right amygdala (right) to connectivity changes between the resting state before and after the (A) Pavlovian and (B) Extinction trainings.

Chapter 4. Discussion

4.1. Fear-Induced Behavior

Throughout this work, we have seen how our conditioning training puts the participant in this scenario where it starts to learn to associate a face (CS+) to the annoying sound (US) and starts to consider this stimulus as a threat (Pavlov, 1938).

As we showed previously, this new aversive learning comes with a set of different changes in the way our participants react and respond to the tasks involved, some of this being general and noticeable enough to be considered as a change in general behavior. We englobe all these changes into what we call Fear-Induced Behavior, as we will refer to it from now on.

One of the first things to notice about these changes, comes from the STAI-State, this self-reporting inventory of anxiety was performed before and after the training. With the result of the Inventories, we can see that there is an increase of the anxiety scores after the Pavlovian training. It is important to mention that these are self-reported, meaning that the information obtained from these inventories speaks about how “Anxious” the subjects perceive themselves, but does not necessarily reflects the biological response to a threat.

We can actually assess the biological response to threat that correlates with the STAI scores, when we analyze the Skin Conductance Response (SCR). Let us remember that this particular analysis involves the increase of the Sympathetic response of the Autonomous Nervous System (ANS), this “Flight or Fight” behavior was a response to threats and, as a side effect, increased the activity of the sweat glands. This increase sweating was the direct responsible for the SCR increase, since a decrease in the skin resistance, increases its conductance (Dunn, Dalgleish and Lawrence, 2006).

Therefore, we can infer because of the increase SCR-signal that there was an activation of the sympathetic system, and with that, we get a clear picture there is in fact a biological activation that correlates with the self-reported anxiety inventory.

Included in the Fear-Induced behavior, is all the responses we mention in our introduction regarding the turbulence in a plane history.

In the Risk Assessment measurements, we compared the change in the self-reported dislike from after and before the conditioning training of one of the stimuli compared with the others.

As seen in the Results part, the discriminatory index was between 10-15% higher (1 point of 8) for the CS+ compared to the CSa (Aversive face without the US conditioning). The increase is related to the fact that the training involves the pairing between one of the stimulus and a "punishment" as it can be the US. The CS+ stimulus is now considered a threat, and is a fact that increases the perceived "probability" of a negative outcome.

Usually, as we mentioned before, there is no clear divisive line between the different parts of a cognitive behavior, the same rules that apply for the increase in risk assessment and that will transform this CS+ into a perceived threat, are the ones underlying the changes in the estimates of costs and probability of events when these stimuli are involved.

As seen in the "Expected Value" tasks, where our participants had to answer what they thought was the probability or chance of one outcome (that could be either positive or negative) when done by a particular stimulus (Knutson, 2005; Kurzban *et al.*, 2014). This meant that they were asked what were the chances of CS+, CSa and CSn of doing something positive or negative, and we compared these answers.

Despite not reaching the point of statistical significance, there was a clear trend in the results that indicate that the probability of a negative outcome was increased when it was related to the actions of the CS+ compared with the CSa, this is again a behavioral response of the perceived threat of the CS+ face.

Along with the other, there is another behavioral change that is related with the perceived threat of the stimuli, but in contrast with the last two, this does not represent a self-report or score, but represents reaction time and attention.

This hypervigilance state that the threat generates has its roots in our basic survival instincts, in the introduction we mention the definition of attention, we focus ourselves into perceiving more stimuli coming from one source or place that we consider a threat to our survival, this make us have a great detail about the information we are receiving but we are more oblivious about the sources around us.

In this case, the threat involves one of the faces; therefore, when we realize the “DOT-Probe” task, and we compare the reaction time when the different stimuli are involved, we can get a clear picture of how this is changing.

We already spoke about the analysis of the DOT-probe task; the time it takes to press the button when the dot is over the CS+ face (Congruent trial) is much lower than the time it takes when it is on the other side (Incongruent trial). It is the difference between these two that is taken as the reaction time, and it is this observation that we compare between the CS+ CSa and CSn stimuli.

The attentional bias consists in the increased reaction time regarding the CS+, meaning that the person “keeps looking” at the CS+ side even when the stimulus is gone, it agrees with the idea that the face is considered a threat and it will automatically “pay more attention” to it. We saw that the results yield a significant increase in this attentional bias or hypervigilance state regarding the CS+, when compared with both the CSa and CSn.

All these results point to the same direction, the Pavlovian conditioning training had as a result the perceived threat of one of the stimuli, and we see all the behavior responses that we would expect from an aversive (or dangerous) thing or situation. The fear of a negative outcome, the increased chance of it, the increased attention towards and the increase in sympathetic response, seem to indicate the success of the Fear-Conditioning and moreover, the behavior and physiological changes involved in preparation for a “Fight or Flight” response.

4.2. Amygdala as the main area in fear conditioning

When we started this work, the main goal we had in mind was to find a correlation between the distinct brain areas activation and the behavioral responses, with that in mind we decided on a protocol that involved not only the behavioral task, but also the Electroencephalographic record and the functional brain imaging.

We explained already both techniques, but we want to emphasize the advantage of recording both EEG and fMRI at the same time, this allowed us to overcome the weak points of both techniques, with the fMRIs high spatial resolution and EEGs high temporal resolution giving us a clearer picture of the events (Abreu, Leal and Figueiredo, 2018).

Our conclusion that the amygdala was the main driver of the fear-conditioning responses comes; first, from previous studies in animals and humans, there is increasing evidence in the literature that those areas are involved in emotional responses and memories (Maren, 1999; McGaugh, 2004). The main goal of our work was to assess which brain areas and connectivity changes are involved with the behavioral responses we obtained through our Fear-Conditioning training, in agreement to previous works on the subject (Fernández *et al.*, 2018).

Besides the latter, when we analyze the EEG response, during both the Pavlovian and Extinction trainings, we see that compared to the signal from the other stimuli (CSa and CSn), the CS+ signal was the strongest in the frontotemporal areas, during the whole 1 second window we took, although we focus our research on the 250-500 msec window.

In any case, the increase in the signal we saw during the Pavlovian was completely opposite to what we saw when analyzing the signal from the Extinction training, in this scenario, the frontotemporal areas were decreasing in a similar fashion to the increase in the previous training.

The strong temporal resolution of the EEG was key to see the fast response to the stimuli every time it appears, and how it was changing from trial to trial, as can be seen in Fig. 9 where we see both amygdala increasing their spectral power density (SPD) through the trials.

With the frontotemporal area analyzed, we shift our work to the Functional Magnetic Resonance Imaging (fMRI), and its strong spatial resolution. As we saw before, the analysis of our brains was mainly to see the changes between trials, meaning that the results we obtained were of the BOLD-signal after and before (the last trial and the first one), and it is these values that we use to compare between stimuli.

The non-conditioned stimuli showed no differences between the last and first trials, as expected, is only when we see the CS+ that we see changes, and in no brain area the changes are stronger than in the Amygdala.

In tune to what we saw for the EEG, the increase we saw during the Pavlovian training, was similar to the decrease we saw in the amygdala BOLD-signal during the Extinction training, in which the CS+ stops being perceived as a threat.

With this data, we would be able to say that the activation and deactivation of the amygdala is involved in the perceived threat of the stimuli, this would not only explain the self-reported scores, which would relate the amygdala to the cognitive responses in question, but also seems to indicate the strong correlation between these nuclei and the ANS sympathetic responses.

4.3. Other brain areas involved in fear conditioning

Other than the straight BOLD activation analysis in which we obtained the previous results we did another type of analysis, the Resting-State Analysis, in which the person has no particular task to solve and therefore the brain activity we detect, correspond to the internal connections and working of the brain while it is not performing a specific task.

The idea behind this analysis was already discussed, when several areas of the brain are involved in the information processing of a particular task (i.e. Visual Areas, Temporal-Auditory areas, etc.) they form a network in which the information “Flows” through the connections of these different parts. Despite this areas not being anatomically connected, they seem to respond in a similar fashion, as if they were (Lally *et al.*, 2017; Bandettini *et al.*, 2018).

This kind of network is known as functional network and is the one we studied with our fMRI analysis. As shown in results, there are some difference between the Pavlovian and Extinction trainings.

There were two different scenarios, in the first one; we analyze the connections between both amygdala and the rest of the brain. The comparison between the Resting state after the training and before, showed that there was an increase in connectivity between the Amygdala and the Orbitofrontal Cortex, in particular with the medial part (mOFC). As happened with the other comparisons, the Extinction training gave us the opposite connection signal, with a decrease in the mOFC connection.

Our results follow the current proposals that the mOFC is involved in sensory attributes of cue-response association and its proposed to be involved in stimulus-reward associations (Heimer and Van Hoesen, 2006). This would make our findings fit perfectly with the idea that the stronger the connection between the amygdala and the OFC, the stronger the “reward” associated with the stimulus (CS+). Association that seem to be lost, or at least diminish, with the extinction training.

In the other scenario, instead of taking both amygdala as a seed for the connections, we took one of the amygdala and subtract the involvement of the other one. With this, our results only represented the changes of connectivity consequence of one of the amygdala.

The results were as expected when it came to the different involvement of every amygdala, the left one yield more connectivity changes within the left hemisphere and the right one within the right one.

To our surprise though, the changes between the Pavlovian and Extinction training were not as before in the same place but opposite, but were stronger in different areas.

Pavlovian training showed an increase in connectivity between each amygdala and the dorsomedial prefrontal cortex (dmPFC), considered in the literature as an important part of the “Higher Cognitive Functions” (Lally *et al.*, 2017), being involved in risk and reward processing, with not only would fit with our training, but also could be one major player when the behavioral task was performed (Bornhövd *et al.*, 2002; Phelps, 2006).

The Extinction training on the other hand, showed a decrease in the connectivity between the amygdala and its corresponding Insulate Cortex (Van der Heiden *et al.*, 2014). The classical view of the Insular functions involves motor control and Interoception or “Self-Awareness”, but new research shows that the insular cortex is involved both in Audio-Visual integration tasks and social emotions (Quirk, Gregory J.; Paré, 2003).

Based on our research, we are inclined to agree with the last two, and it seems that the connectivity between amygdala and the Insular Cortex is key in processing the emotional response regarding these stimuli.

Chapter 5. Concluding remarks

The main goal of our work was to assess which brain areas and connectivity changes are involved with the behavioral responses we obtained through our Fear-Conditioning training.

We managed to obtain and explain a broad spectrum of cognitive responses related to this, which we called Fear-Induced behavior. From the increase in self-reported anxiety to the increase in sympathetic responses, and the hypervigilance states with increased threat perception.

With the help of EEG and fMRI, we manage to locate and pinpoint the brain areas involved and the time in which they were active.

We managed to see the activation and posterior deactivation of the amygdala with our training, with the strongest response being around 250-500 msec after the stimulus presentation, and the changes in connectivity between these nuclei and the Orbital Cortex and Prefrontal Cortex, with the Insular cortex being involved in the "loss" of threat related characteristics of the CS+.

We conclude that the amygdala is involved in the formation of a fear-induced memory and the "Value" of an emotional stimulus is computed by the mOFC, PFC and Insular cortexes, also, we theorize that the connectivity between the amygdala and the hypothalamus may be the responsible for the activation of the Autonomous Nervous System and the "Fight or Flight" responses.

We feel that the research can be expanded in the future to involve the decision-making modification involving the Prefrontal and Orbitofrontal Cortices, when this kind of training is done. Moreover, there is a substantial opportunity to expand the sample to people with PTSD (Post Traumatic Stress Disorder), OCD (Obsessive Compulsive Disorder) and other Anxiety Disorders, to assess if there are Electroencephalographic or Functional differences to a "Healthy" population.

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