Behavioural Testing for the Study of Impulsivity in Rats

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MASTER THESIS

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The present work aims to prepare a study that will investigate the role of serotonin in respect to impulsive behaviour in rats. The methodology followed employs behavioural testing, based on operant conditioning, which will be later combined with optogenetic stimulations. The stimulations will be applied to the animals in order to influence their serotonergic system. For the implementation of this project, an experimental set up was built that consists of an operant behavioural box, connected through microcontrollers with a computer and a laser. Also, three different behavioural protocols were designed: Autoshaping, Cue Matching and Peak Interval. Autoshaping has a training character, whereas the other two tasks are used in order to study the different aspects of impulsive behaviour. The results without the laser stimulations that will be obtained from these experimental tasks will be used as control, in order to allow the comparison with the results from the experiments employing the laser stimulations.

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Chapter 1. Introduction

Decision making can be defined as the cognitive process of making a choice among several alternative options [1]. This process always results in a final choice that can be either an action or an opinion [2]. A branch of decision making is temporal discounting, that is the tendency characterizing both people and animals to prefer gain that is available in the present or near future, disregarding greater benefits offered after a larger time period. The behaviour that is characterized by the choice of smaller immediate rewards over the larger delayed rewards can be considered as impulsive behaviour [3].

Impulsivity is described as the tendency to act with low or even insufficient degree of forethought or control [4, 5, 6, 7]. A basic characteristic of impulsive behaviour is the lack of significant consideration of the consequences following the action. This absence of appropriate forethought enhances the potential for negative consequences following the actions in impulsive behaviour. However, an impulsive style of responding may sometimes have positive effects. Under time pressure conditions, it has been observed that a high level of impulsivity could outperform low impulsivity in some occasions [8]. This led to the classification of impulsivity into functional and dysfunctional [9].

Impulsive behaviour has been shown to be a complex construct that arises from numerous different mechanisms, controlled by neural/cognitive system [10, 11, 12, 13]. The multi-diversity of impulsive behaviour is due to the fact that it consists of several independent components, while its exact nature has been found difficult to determine. Studies indicated that an important component of impulsivity is impulsive choice which can be generally defined as an abnormal aversion for the delay of reward. Impulsive choice is characterized by an increased preference for smaller short-term gain over more beneficial long-term gain [9, 10, 14, 15]. A second widely recognized component of impulsive behaviour is impulsive action, which is the inability to withhold from a response in the presence of prepotent stimuli [10, 14, 15].

Although impulsive behaviour is commonly regarded as a feature of a normal personality both in humans and animals, it is also considered as a strong evidence of various neuropsychiatric disorders and psychiatric disturbances. Attention deficit hyperactivity disorder (ADHD) [16], bipolar disorder [17], substance abuse [18], pathological gambling [19, 20, 21] and suicidal behaviours [22] are some of the disorders in which impulsivity is a core feature. Thorough understanding of the underlying mechanisms that control impulsive behaviour is crucial in order to gain knowledge and insight into these types of disorders and disturbances.

Several behavioural experiments in laboratory animals have been performed in order to study impulsive decision making and temporal discounting. Various forms of such tasks have been specifically designed to investigate the several aspects of impulsive behaviour and measure impulsivity.

The behavioural paradigms performed in laboratory animals can be grouped into three categories, based on the kind of response/behaviour that is considered as impulsive at each time. The first category contains the response perseveration tasks which consist of punished and/or extinction paradigms. Impulsivity is defined here as the perseverance of a response that is either punished or not rewarded [23]. In the second category are the reward - choice tasks, in which impulsive behaviour is defined as the
abnormal preference for a small reward that is offered immediately over a larger but delayed reward [24]. The third category consists of the response disinhibition/attentional paradigms, in which impulsive behaviour is defined either as making responses that are consider as premature or as the inability to withhold a response [25, 26].

Impulsive choice and impulsive action can be studied in different behavioural tasks. For impulsive choice, tasks that belong in the second category as Delay Discounting tasks [27, 28, 29], the Marshmallow test [30] and the Iowa Gambling task (IGT) [31] are employed in order to investigate impulsive choice. For the study of impulsive action tasks such as the go – no go task [32, 33, 34], the stop signal reaction time task [35] and the 5 – choice serial reaction time task (premature responding) [36] are used. For the majority of these behavioural paradigms similar versions exist for both humans and animals.

All the experimental tasks arising from the three behavioural models of impulsivity employ operant learning. Operant conditioning is a type of associative learning that has been studied extensively by Skinner [37, 38, 39]. Skinner believed that the comprehension of certain behaviour is achieved through the investigation of its causes and consequences. Skinner’s work was based on Thorndike’s Law of Effect that governs all processes in operant conditioning. According to this law, a behaviour that is reinforced is strengthened and will be more likely to be repeated, whereas a behaviour that is punished will be weakened and therefore decrease in likelihood [40, 41]. In order to achieve the desired behaviour, reinforcers or punishers are used that will tend to increase or decrease respectively the likelihood of the behaviour that they follow. Note here that Skinner believed that there is no significant difference between the way that humans and animals (e.g. laboratory dogs, pigeons and rats) learn behaviour.

Both reinforcers and punishers are defined by the effect and not by their intent. There are two types of reinforcement that can be employed in order to reward a desired response: positive and negative. These two different types apply to the case of punishment as well (punishment: positive and negative). Positive reinforcement is the addition of something desirable, whereas negative reinforcement is the subtraction of something undesired. The type of reinforcement that is chosen during a behavioural task is applied right after a wanted response is performed, aiming to increase the probability of this behaviour to happen more often [37, 38]. As for the two types of punishment, positive punishment is the addition of something undesired, while negative is the subtraction of something desirable. Again, the selected type punisher is applied after an aversive or unwanted response, but in this case the aim is to decrease the likelihood that this behaviour happens more often [37, 38].

As in classical conditioning, the procedure followed in operant learning is the repeated pairings of an unconditioned stimulus that elicits an unconditioned response, with a neutral stimulus that elicits no response, in order to become a conditioned stimulus that produces the conditioned response. This process represents a learned association, since a natural stimulus that causes a natural and unlearned response is turned into a conditioned stimulus that causes the desired learned response/behaviour. The basic difference in operant learning is the use of reinforcers or punishers in order to reach acquisition, which is the initial stage of learning. During the stage of acquisition, a response is first established and then gradually strengthened.

In operant behavioural tasks, there are two technical features that require special attention since they have an impact on the results. First, the presentation of the neutral stimulus should immediately precede the presentation of the unconditioned stimulus. It is very important for the time elapse
between those two stimuli to be really small, since acquisition occurs faster and better in this case. Second, the neutral stimulus should always precede the unconditioned one in order to predict it. The opposite case, called backwards conditioning, is shown not to work well.

The use of operant conditioning chamber, known also as Skinner box, is of great importance for the control of the experimental environments of the animals subjected to behavioural testing [37]. A Skinner box is a simple box in which the subject is enclosed and a variety of stimuli can be applied to it. The stimulus or stimuli that are employed during a behavioural task can be visual, auditory, electrical and stimulus from laser. For the stimulus delivery, the chamber may be equipped with lights, sound generators and visual displays. The box also allows the delivery of reinforcement and/or punishment [39]. Mechanisms for producing reward are usually pellet dispensers.

The control of the experimental chamber is performed through the computer (at the beginning it was controlled manually), according to a pre-designed behavioural protocol that can employ different complex stimuli and apply the chosen form of reinforcement or punishment. This automation ensures that the stimuli and reinforcement employed in each protocol are presented at the appropriate time point. For the data-acquisition hardware and software, various different devices and programs can be used depending on the nature and demands of each behavioural experiment.

Studies in neuropharmacology that were performed in both human and animal subjects have linked impulsivity with the neurotransmitter serotonin (or 5-HydroxyTryptamine), indicating the later as one of the two major neurotransmitters that is involved in impulsive behaviour [42]. Serotonin has also been associated with a variety of psychiatric diseases like depression, mania, attention deficit hyperactivity disorder (ADHD) and substance abuse [43]. In fact, low levels in the serotonin have been found in people with impulsive behaviour, depression and suicidal behaviour as well. As mention before, many of the patients suffering from disorders like these showed deficits in temporal discount and impulsive related tasks. Under stressful conditions or situations involving punishment, depletion in the serotonin brain levels have been shown to be a promoting agent for impulsive behaviour, although the exact opposite result was expected [44, 45, 46].

According to behavioural studies, low levels of serotonin in the Central Nervous System boost impulsivity in choice during tasks in which subjects preferred the small but immediate rewards over the larger delayed ones [10, 47]. Other behavioural experiments in rats indicate an increased activity of the serotonin neurons in dorsal raphe nucleus (DRN) when there is a delay in reward delivery [48]. The dorsal raphe nucleus is the major origin of serotonergic projections to the forebrain. During the waiting period for the reward delivery, the DRN serotonergic neurons increased firing, whereas they stopped their activity before the animals give up waiting for the long delayed rewards [49]. These findings are evidence for the existence of a correlation between the activity of serotonergic neurons in dorsal raphe serotonin and waiting behaviour for delayed rewards. Further research in rats with inhibited serotonin neural activity, revealed that the animals were almost incapable of waiting for long delayed rewards, indicating that the activity of dorsal raphe serotonin neurons is essential for waiting for the long delayed rewards [50].

Therefore, the forebrain serotonergic system has been proven to be of great importance in the control of impulsive behaviour. The motivation is to causally test the role of serotonin, mostly sitting in raphe nucleus in the brain, in intertemporal choice behaviour. The aim is to influence or modulate the serotonergic system and investigate if the selective activation/inhibition of 5-HT neurons changes any
aspect of impulsive behaviour in rats. The method that will be followed employs behavioural testing, which will be later combined with optogenetics.

Within the scope of this study, an experimental set up will be built that consists of an operant behavioural box, connected through microcontrollers with a computer and a laser. Three separate behavioural tasks were designed, each one following a different protocol: Autoshaping, Cue Matching and Peak Interval. Note here that Cue Matching protocol will be altered to a more complex protocol (New Cue Matching). All tasks have common features, but also possess unique characteristics that are not met in the other two. According to the corresponding protocols, the tasks were planned to take place during daily sessions. Each session may contain a different number of trials, since the trial duration depends on the animals’ acquisition and learning ability. A different number of animals may participate in each task. Positive reinforcement is employed in the form of reward delivery after each successful trial completed by the animals. The reward used is sucrose liquid (positive reinforcement).

Initially, the animals will be subjected in behavioural testing according to the Autoshaping protocol. Autoshaping has a training character, aiming to introduce the animals to the experimental box and familiarize them with the concept of reward following a certain response. After a series of trials, when a satisfactory level of training will be reached, a number of these animals will continue the experiment according to Cue Matching and the rest according to Peak Interval protocol.

Cue Matching is a task employed in order to study impulsivity in respect to impulsive choice. New Cue Matching is the altered version of the initially designed protocol (Cue Matching) and describes a delay discounting task, suitable for the study of impulsive choice. The reason of not employing New Cue Matching from the beginning of the behavioural testing is because the animals needed testing in a less complex task before subjected to a delay discounting paradigm. Only the animals that demonstrated a good performance in Cue Matching and achieved the goals of this task will continue the testing according to the New Cue Matching protocol. Peak Interval on the other hand aims to study impulsive behaviour in respect to impulsive action.

When adequate learning is achieved by the animals, fibers will be implanted in their dorsal raphe nucleus for the stimulation of the DRN serotonergic neurons with light. The implanted animals will continue participating at the same behavioural testing as they did before, with the only difference that the protocols will also include laser stimulations. The results from the experiment without the light stimulations are meant to serve as the base line, allowing the comparison with the data that will be recorded from the tasks employing the laser stimulations.

The experimental results obtained from each trial day (session) will be treated as raw data. The reason behind this choice for the analysis of data is that the point of interest is the progress in the animals’ learning and response according to the purposes of each behavioural task. The programming platform employed for the analysis of the recorded data is MatLab R2012a.

This report consists of four chapters, including the Introduction. Chapter 2 gives a full description of the experimental set up that was built for the needs of the behavioural testing. The behavioural tasks along with the experimental results that were obtained from the animals without the laser stimulations are presented and analyzed in Chapter 3. The last chapter of the report discusses the conclusions and the outcomes of the present study. The future work arising from this study, including also the scheme for the optogenetic stimulations in the animals’ dorsal raphe, is discussed in the fourth chapter of the report.
Chapter 2. Method and Experimental Setup

In order to subject the animals to the behavioural testing, it is of great importance to build a computer controlled experimental setup, suitable for delivering the stimuli and reward, as well as to record the behavioural responses with absolute precision. For behavioural experiments, it is also crucial that the rewarding events are presented in close temporal proximity to sensory stimuli in order to establish a successful association between the target stimulus and the animal’s response. The computer software being developed for this project will control other behavioural parameters as well, such as time delays and reward magnitude.

2.1 Hardware, Software development and Integration

The experimental setup that was built and used for all three experimental tasks is demonstrated in Figure 1 and consists of:

- Operant conditioning chamber, designed and built in the lab
- chipKIT™ Uno 32 Board, manufactured by Digilent [51]
- Computer
- Arduino™ Board Leonardo [52]
- Digital to Analog (DAC) Breakout Board, manufactured by adafruit [53]
- Cobolt MLD™ 473 nm diode laser module [54]

Figure 1: The experimental setup built for the behavioural testing: (a) the operant conditioning chamber, (b) the chipKIT™ Uno 32 Board, (c) the computer controlling the experiment, (d) the Arduino™ Board Leonardo, (e) Digital to Analog (DAC) Breakout Board and (f) Cobolt MLD™ laser
The last three parts of the setup (Figure 1: d, e, f) are used only during the experiments that employ optogenetics, since they are necessary for the delivery of the laser stimulations to the animals.

For the tasks in Autoshaping and Cue Matching (and New Cue Matching), the same operant conditioning chamber was used. One of these chambers is illustrated in Figure 2. For the needs of Peak Interval task, slight modifications were performed in the box, mostly regarding the hoppers. The middle hopper was made exactly the same with the left and right hoppers in order to serve as a third feeder. Furthermore, a fourth hopper, called the back hopper, was built in the box below the house light (Figure 2: Image 1). All the experimental boxes are equipped with LED lights for the delivery of visual stimuli, pumps for the delivery of reward and sensors (infrared beams) in the hoppers for the detection of the animal responses. All LEDs, pumps and sensors are connected to the chipKIT™ Uno 32 Board, enabling the communication between the board and the box (Figure 2: Image 3).

ChipKIT™ Uno 32 Board is coded with MPIDE (Multi-Platform Integrated Development Environment) interface which is compatible with the microcontroller of the Uno 32 [55]. This board receives and sends signals from and to the behavioural box. The Uno 32 Board is connected via a connector (the mini-B connector on board) to an available port on the computer controlling the experiment.

The Arduino™ Board Leonardo is used in order to control the digital to analog (DAC) Breakout Board and therefore to provide the proper analog input to the Cobolt™ laser. Leonardo Board receives its input from Uno 32 board at appropriate time points, which is a digital ON/OFF. The program controlling the laser is coded in Leonardo Board with arbitrary frequencies, such as 10, 20, 60 Hz, and its digital output is passed to the DAC Breakout Board. The output of the DAC gives finally input to the Cobolt laser, signaling the laser on or off. The Cobolt laser takes 0-1 V analog input for regulating output power.

The behavioural protocol, which has to be translated into a sketch in MPIDE, is loaded onto the chipKIT UNO 32 board’s Microcontroller through the computer, where it is compiled and then executed according to the inputs and outputs of the box. In MPIDE serial monitor (on the computer screen), the
data events are displayed throughout the duration of the experiment. At the end of each experiment, the computer interface prints out the trial data and also saves it in a formatted text file.

One or more data events are generated each time a different event occurs. The computer interface records the time stamp and the type of every event. All the onsets and offsets of the various lights that are employed to deliver the visual stimuli to the animals are defined as events in the software. Also, the delivery and the no delivery of reward are two additional distinct events. Certain actions of the animals, which are their nose entries into the hoppers under different stimulus, are defined as events as well. All these events are sent to the recording hardware and used as input/output to the MPIDE interface. The distinct behavioural coded events defined for every protocol are the following:

For Autoshaping task:
- House Light turns OFF (HL: OFF)
- Nose poke in the middle hopper (CNP)
- Trial start
- Nose poke in the middle hopper: before the CNP or during the time delay or ITI
- Nose poke in the left hopper: before the CNP or during the time delay or ITI
- Nose poke in the right hopper: before the CNP or during the time delay or ITI
- Nose poke in the left hopper after the time delay (final response)
- Nose poke in the right hopper after the time delay (final response)
- Reward delivery
- House Light turns ON (HL: ON)

ITI and CNP are the abbreviations used to denote Inter-Trial Interval and Central Nose Poke, respectively.

For Cue Matching and New Cue Matching tasks:
- House Light turns OFF (HL: OFF)
- Nose poke in the middle hopper (CNP)
- Trial start
- Conditioned Stimulus turns ON to left target with 1 led on (single left CS: ON)
- Conditioned Stimulus turns ON to left target with 2 led on (double left CS: ON)
- Conditioned Stimulus turns ON to right target with 1 led on (single right CS: ON)
- Conditioned Stimulus turns ON to right target with 2 led on (double right CS: ON)
- Nose poke in the middle hopper: before the CNP or during the time delay or during ITI
- Nose poke in the left hopper: before the CNP or during the time delay or during ITI
- Nose poke in the right hopper: before the CNP or during the time delay or during ITI
- Exit the middle hopper
- Exit the left hopper
- Exit the right hopper
- Nose poke in the left hopper with correct response to the CS
- Nose poke in the left hopper with incorrect response to the CS
- Nose poke in the right hopper with correct response to the CS
- Nose poke in the right hopper with incorrect response to the CS
- Conditioned Stimulus turns OFF (CS: OFF)
- Reward delivery
- House Light turns ON (HL: ON)

CS is the abbreviation for Conditioned Stimulus.

### For Peak Interval task:
- House Light turns OFF (HL: OFF)
- Nose poke in the back hopper (BNP)
- Trial start
- Conditioned Stimulus turns ON to the left hopper (left CS: ON)
- Conditioned Stimulus (CS) turns ON to the right hopper (right left CS: ON)
- Conditioned Stimulus (CS) turns ON to the middle hopper (middle CS: ON)
- Conditioned Stimulus (CS) turns ON to the left hopper, probe trial (probe left CS: ON)
- Conditioned Stimulus (CS) turns ON to the right hopper, probe trial (probe right CS: ON)
- Conditioned Stimulus (CS) turns ON to the middle hopper, probe trial (probe middle CS: ON)
- Nose poke in the middle hopper: before the BNP or during the time delay or during ITI
- Nose poke in the left hopper: before the BNP or during the time delay or during ITI
- Nose poke in the right hopper: before the BNP or during the time delay or during ITI
- Nose poke in the back hopper: during the time delay or after time delay or during ITI
- Exit the middle hopper
- Exit the left hopper
- Exit the right hopper
- Exit the back hopper
- Nose poke in the middle hopper with response to the rewarded hopper
- Nose poke in the left hopper with response to the rewarded hopper
- Nose poke in the right hopper with response to the rewarded hopper
- Conditioned Stimulus turns OFF (CS: OFF)
- No Reward delivery, probe trial
- Reward delivery
- House Light turns ON (HL: ON)

BNP is the abbreviation used to denote Back Nose Poke.

Less behavioural events are coded for Autosahping protocol due to the simplicity of this task. In contrast, for the other tasks that are more complex (Cue Matching, New Cue Matching and Peak Interval) the number of coded events employed is two times more.

This method of coding allowed the calculation of the time difference between the events of interest with a low computational cost. For example, the animal response times to the central nose poke (CNP) can be measured by subtracting all the timestamps corresponding to the event ‘Nose poke in the middle hopper (CNP)’ from all the timestamps that correspond to the event ‘House Light turns ON (HL: On)’. Furthermore, through this way of coding numerous different data regarding the animal responses or performance can be obtained. Some of them are for example the number of: correct or incorrect trials,
correct responses during single or double trials, responses to the middle, left or right hopper during the delay time or ITI, etc... The extraction and the analysis of the recorded data events from the formatted text file are performed in MatLab 2012a.
Chapter 3. Experimental Results

3.1 Introduction

This chapter includes the description of the protocols and the experimental findings from the three behavioural tasks: Autoshaping, Cue Matching and Peak Interval. As mentioned in Chapter 1, changes in the protocol were performed in the Cue Matching task, which was renamed to New Cue Matching. For each task, the corresponding protocol is described at first and then the data obtained from the animals are presented and analyzed. All protocols share common characteristics, but also possess unique characteristics that are not met in the other. All tasks are planned to take place in daily sessions. The number of trials in each session may vary, since the trial duration depends on the animals’ acquisition and learning ability. A different number of animals may be subjected to testing in each task. Positive reinforcement is employed in the form of reward delivery. The reward is always offered to the animals after a successful trial termination. The reward that was chosen is sucrose liquid.

3.2 Autoshaping task

Six animals were employed for the free choice behavioural experiment, two female and four male rats: a1, a2, a3, a4, a5 and a6. The animals performed their tasks separately in six identical operant chambers. The protocol employed for the sessions is Autoshaping and has a training character. The aim is to familiarize the animals with the experimental box and introduce them to some basic rules of the behavioural testing, preparing them for the more complicated forced choice tasks. Each chamber was equipped with a house lamp and three hoppers: one left, one right and a central. Two dispensers were connected to the left and right hoppers (feeders) in order to deliver reward after a respond to the corresponding hopper. All the hoppers were equipped with an infrared beam that detected the animal’s nose entries. A drawing of the chamber used is illustrated together with a description of the protocol in Figure 3.

3.2.1 Description of Autoshaping protocol

The protocol begins with the offset of the house light and the onset of a LED light above the middle hopper (HL: Off / middle LED: On). The illumination of the middle LED prompts the animal to initiate the trial start. This stimulus remains present until the animal makes a nose entry (nose poke) in the middle hopper of the box. At this phase, the animal can poke numerous times into either the left or right hopper, but only the first central nose poke (CNP) will start the trial.

Following the occurrence of the central entry, the middle LED turns off (middle LED: Off) and the peripheral LEDs above the left and right hoppers turn on. Also, an eight second delay period starts. During this time, the animal is allowed to poke into any of the three hoppers. After the end of the delay period, only the first entry either into the left or right hopper will terminate the trial with the delivery of sucrose liquid reward in the corresponding hopper. Any occurrence of nose pokes into the central hopper will not affect the trial termination. Depending on the final choice of the animal, the trial is classified as a left or right.
After the reward delivery, the house light is turned on again to indicate the trial termination. The time period between the end of each trial (Reward: 1 / HL: On) and the start of the next trial (middle LED: Off) is the Inter-Trial Interval (ITI) and it is not of fixed duration.

Figure 3: The Autoshaping task: in the left is the drawing of the experimental box used and in the right is the description of the protocol.

The absence of correct/incorrect response and the offer of reward that follows always the animal's final choice are the two characteristic features of this protocol. These features come in agreement with the training purpose of the task.

3.2.2 Experimental results of Autoshaping task

The proportion of trials based on the first and final choices for the six animals in Autoshaping tasks is presented in Figure 4. The red colored bars indicate the percentage of trials in which the first response to the offset of the middle light stimulus was an entry into the right or left hopper. The black colored bars indicate the percentage of trials according to the final response to the left or right hopper. Since only the final response is responsible for the classification of trials as left or right, the black colored bars are also indicative of the number of left and right trials.

Figure 4: Proportion of left and right trials according to the first choice during the 8 second delay (red) and the final choice after the 8 second delay (black) for the six animals in Autoshaping.
For each animal, the difference in height between the red and black bars indicates the percentage of trials that started as left/right, but ended as right/left. As illustrated in Figure 4, for all the animals in Autoshaping this difference is almost negligible. This means that only a small number of trials is characterized from a change in the animal's first response/choice.

The animals’ learning curves of the response rates during trial and ITI are demonstrated in Figure 5. For the calculation of the response rates during trial and ITI, the number of left and right nose pokes was counted separately during each trial and during each ITI, respectively. The number of trial nose pokes was then divided by the trial duration, whereas the number of ITI nose pokes was divided by the ITI duration. The cumulative sum of the calculated response rates was employed in order to illustrate the obtained results. In the plots of Figure 5, a vertical line to the x axis is indicative of no change in the behaviour, since it is equivalent to the absence of entries in the left and right hoppers. Such behaviour is desirable during the ITI, in which animals are required to stop their response in the absence of stimulus.

![Figure 5: The cumulative response rates during ITI and trial for the six animals in Autoshaping. The dashed horizontal lines within the graphs indicate the session boundaries. Note the different scales used in the plots, due to the large difference among the data obtained from each animal.](image-url)
For all animals in Autoshaping, the cumulative rates during ITI are lower than the rates during trial. As the number of completed trials increases, the cumulative entries are much more during trial time and less during ITI. It is apparent that the animals tend to poke less in the absence of stimulus which occurs between two successive trials and to respond with more intensity during each trial. These findings are evidence of learning, indicating that the animals acquired some insight of how to respond during trial and during ITI. Among all animals, a1 and a2 demonstrate the better results.

According to the Autoshaping protocol, the animals must make an entry to the central hopper after the onset of the middle light in order to initiate a trial. This part of the task aims to introduce the animals to how to start a trial. The animals are required to associate the offset of the house lamp and the illumination of the middle light, with the opportunity to make a central nose poke and trigger a trial initialization. The latency to the central entry is therefore of great importance, as it shows whether or not learning has been achieved. The learning curves of the latency in CNP are illustrated in Figure 6.

![Figure 6](image-url)

Figure 6: Moving average of response time to the central nose poke (CNP) that triggers the trial initialization for the six animals in Autoshaping. 28, 34, 18, 13, 16 and 11 points were used for the moving average of a1, a2, a3, a4, a5 and a6, respectively. Note that different scales were used in the graphs for the x-axis, due to the large difference among the trials that each animal completed.
As seen in Figure 6, except from a4 and a5, the other four animals managed to reduce their response times to the onset of the middle light, showing a great improvement during their training. Once more, a1 and a2 had the best performance, since they both needed the least time to make the central nose poke. Despite some drops in their performance, after a number of trials, animals a3 and a6 succeed in reducing the time needed to start the trials. On the contrary, a4 and a5 were incapable of maintaining good response times to the central nose poke. The experimental findings for a1, a2, a3 and a6, suggest that the four animals learned that the offset of the house lamp and the onset of the middle light are the signal prompting them to initiate the trial.

In Autoshaping, the offset of the middle light is the cue stimulus used and the animals are required to response with a nose poke into either the left or right hopper. In order to assess if the animals have succeed in responding to the stimulus at each trial, the moving average of the latency in the first left or right hopper entry during the time delay was calculated (first response).

![Graphs showing response times for six animals](image)

Figure 7: Moving average of response time to the first and final choice for the six animals in Autoshaping. 28, 34, 18, 13, 16 and 11 points were used for the moving average of a1, a2, a3, a4, a5 and a6, respectively. Note that different scales were used in the graphs for the two axes, due to the large difference among the data obtained from each animal.
The same procedure was repeated but for the first entry after the time delay (final response). Low response times indicate that the animals are less likely to respond to the stimulus by pure luck. The learning curves of the latency in first and final responses for the six animals are demonstrated in Figure 7. Note that the response time to the final choice coincides with the trial duration. Furthermore, there are trials where the first response to the stimulus occurs after the time delay. In all these cases, the first response is considered also as the final, since it results in the trial termination and reward delivery. This is the reason why there is a similar pattern between the two graphs for all the six animals.

According to the graphs in Figure 7, all animals showed evidence of learning. From the beginning of their training, animals a1 and a2 achieved very low response times, both for first and final responses, showing the best performance like before. The other four animals managed initially to reduce their high latency in the stimulus and then, they completed the tasks with very good response times.

3.3 Cue Matching task

Four of the animals trained in Autoshaping were selected for the forced choice behavioural experiment. These animals were a1, a2, a3 and a4. The protocol employed for the experimental sessions is Cue Matching, according to which the animals are required to correctly match the central cue with one of the two possible peripheral target/distractor stimuli. As before, all the subjects performed the task separately in identical operant chambers. A drawing of the box used is illustrated in Figure 8. Each chamber was equipped with a house lamp and three hoppers. The position of the two hoppers in the box is below the “Target” and “Distractor” lights, respectively, and each of them was connected with a dispenser. The dispenser is used in order to deliver the reward after a correct response in the corresponding hopper. The third hopper is placed below the “Cue” lights. All hoppers were equipped with an infrared beam that detected the animal’s nose entries.

**Figure 8: The operant chamber used in Cue Matching task.**
3.3.1 Description of Cue Matching protocol

Cue Matching is illustrated in Figure 9. In this protocol, the offset of the house lamp prompts the animal to initiate the trial by making an entry into the middle hopper (below “Cue” light) of the experimental box. Like in Autoshaping protocol, the animal may poke into either the left or right hopper, but only a central nose poke (CNP) will start the trial.

Following the central nose poke, the illumination of the central cue light instructs the animal to the upcoming peripheral target (or conditioned) stimulus (CS: On). Two different types of cue stimulus are used during these tasks: one dim yellow LED light in single trials and two bright white LED’s lights in double trials.

At each trial, one of the two cue lights is combined with the corresponding peripheral target above either the left or right hopper, classifying the trials into four different categories: single left, single right, double left and double right target trials. The aim is to match the central cue stimulus with one of the two simultaneously presented peripheral stimuli. The target stimulus that is selected at each trial remains queued until a correct or incorrect response occurs. In Figure 9 a single left trial is demonstrated, since the single LED lights (green colored) are turned on above both the middle (Cue) and left hoppers and the double LED (red colored) is turned on above the right hopper. Therefore, the left hopper is selected here as the target and the right as the distractor.

After the presentation of target, a delay period with duration of one to eight seconds begins. During the delay, entries into either the central or the target hopper will not affect the trial termination and its outcome. In contrast, a poke into the hopper not indicated by the target is considered as incorrect choice, resulting in an unsuccessful and premature trial termination. In this case no reward is delivered to the animal.

![Figure 9: The Cue Matching protocol. A single left trial is illustrated in this figure. For demonstration purposes, the two stimuli are colored as green and red, yielding a single and a double trial, respectively. (The actual stimuli used are yellow and white, respectively.) The cue stimulus in this case is the single green light above the central panel and the correct response is an entry into the left hopper.](image)

After the delay period, the first poke into the hopper indicated by the target will be regarded as the correct response, terminating the trial successfully and resulting in reward delivery (liquid sucrose). On the other hand, an entry into the hopper not designated by the target (that is the distractor) triggers an incorrect response, ending the trial unsuccessfully and leading to no food reward. At this phase, the occurrences of central entries are simply being disregarded as before.
Following the final response, the conditioned stimulus disappears and the house light is turned on again, signaling the trial termination. The latency between the end of each trial (CS: Off / Reward: 0 or 1 / HL: On) and the start of the next one (CS: On) is the intertrial interval (ITI) and it is not of fixed duration. Like in Autosshaping protocol, ITI duration varies since it depends on the time that the animal will make the central nose poke in order to cue the target stimulus and initiate the trial.

3.3.2 Experimental results of Cue Matching task

The data obtained from the experimental tasks in Cue Matching for the four animals are demonstrated in Table 1.

<table>
<thead>
<tr>
<th>animal</th>
<th>a1</th>
<th>a2</th>
<th>a3</th>
<th>a4</th>
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<td>total trials</td>
<td>776</td>
<td>705</td>
<td>132</td>
<td>150</td>
</tr>
<tr>
<td>single trials</td>
<td>369</td>
<td>334</td>
<td>59</td>
<td>69</td>
</tr>
<tr>
<td>double trials</td>
<td>407</td>
<td>371</td>
<td>73</td>
<td>81</td>
</tr>
<tr>
<td>correct trials</td>
<td>398</td>
<td>360</td>
<td>71</td>
<td>61</td>
</tr>
<tr>
<td>incorrect trials</td>
<td>378</td>
<td>345</td>
<td>61</td>
<td>89</td>
</tr>
<tr>
<td>single correct trials</td>
<td>181</td>
<td>157</td>
<td>34</td>
<td>22</td>
</tr>
<tr>
<td>single incorrect trials</td>
<td>188</td>
<td>177</td>
<td>25</td>
<td>47</td>
</tr>
<tr>
<td>double correct trials</td>
<td>217</td>
<td>203</td>
<td>37</td>
<td>39</td>
</tr>
<tr>
<td>double incorrect trials</td>
<td>190</td>
<td>168</td>
<td>36</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 1: The trial data obtained from the four animals in Cue Matching.

As can be seen from the table above, the number of the single trials that the animals completed in this task is not significantly different from the number of double trials. The program used to control the experiment was set so that each kind of trial run in blocks, with the length of the blocks being equal to 10 throughout all the sessions. The choice of blocks throughout a session contributes to the animals’ learning through the adequate repetitions of the same kind of trial – either single or double. Also, in order to avoid or at least reduce the undesired phenomenon of conditioning in the hopper, which is the animals’ preference to the left or right feeder, the target was indicating the left or the right hopper with equal probabilities at each trial, independently of the kind of trial (single or double).
For each animal in Cue Matching, the normalized difference between the correct and incorrect final responses in single and double trials was calculated according to the formula:

\[ ND = \frac{\text{number of Correct trials} - \text{number of Incorrect}}{\text{number of Correct trials} + \text{number of Incorrect}} \]

The normalized difference indicates the balance between the correct and incorrect responses for each type of trial and is illustrated in Figure 10. Since each trial in Cue Matching is characterized as correct or incorrect based on the animal’s final response to the central cue stimulus, the normalized difference is also indicative of the balance between correct and incorrect trials, in single and double cases. A negative proportion of choices corresponds to more incorrect choices/trials, whereas a positive proportion corresponds to more correct choices/trials. Note here that the animal’s first and final responses are the same during a correct trial, whereas in incorrect trials these responses differ. This means that if the animal made its first choice towards the e.g. left hopper, then if the trial is classified as correct the final response is also into the left hopper. Of course, if the trial is incorrect the animal made the final choice in favor of the right hopper.

![Figure 10: The normalized difference between correct and incorrect responses in single and double trials for the four animals in Cue Matching.](image)

As shown in Figure 10, the first two animals, a1 and a2, achieved a higher success ratio in double target trials, exhibiting more correct responses with respect to the incorrect ones. In contrast, they both completed the single trials with more incorrect responses. The third animal, a3, managed to maintain a higher percentage of correct trials in both cases, showing a better performance in single target trials. On the other hand, the fourth animal completed all kind of trials with low percentages of success. Especially in single target trials for a4, the number of incorrect is two times more than the correct. These data could be assessed in order to investigate whether or not a more illuminated target has an effect on the correct response.

For the data representation and the analysis that follows, the moving average algorithm was employed as well as before, in order to smooth the data obtained and capture the important trends.
The first essential feature to investigate in this behavioural task is the latency to the central entry. As described, in order to initiate a trial start in Cue Matching, the animals must make an entry to the central hopper after the offset of the house lamp. This is the reason why the response time to the central nose poke has a special interest. The learning curves of the latency in the central nose poke for the four animals are presented in Figure 11.

Except from a4, all the other animals showed an improvement in their performance, as illustrated in Figure 11. Despite a rapid but temporal increase, after a number of trials the three animals succeed in reducing the time needed to start the trials. During the last sessions, the latency in the central nose poke was less than 10 seconds for both a1 and a2. Animal a3 managed to set its response times low, although at the last tasks its performance dropped and its response time rose again up to 60 seconds. What is interesting in this data is the performance of the fourth animal, a4. Although the animal starts with the smallest latency in the central entry, it seems incapable of continuing the rest trials with such good reaction time. In fact, during the last session, a4 needs more than 70 seconds to initiate the trials. The experimental findings for the three animals, a1, a2 and a3, suggest that they managed to associate the offset of the house lamp with the opportunity to trigger a trial initiation.

![Graphs showing response time to the central nose poke for four animals.](image)

**Figure 11:** Moving average of response time to the central nose poke that triggers the trial initialization for the four animals in Cue Matching. 77 and 71 points were used for the moving average of a1 and a2, respectively, where the number of trials was large. 13 and 15 points were used for the moving average of a3 and a4, respectively, as the number of trials was smaller. Note that different scales were used in the graphs for the x-axis, due to the large difference among the trials that each animal completed.

In order to assess if the animals had succeed in responding to the stimulus queued at each trial, the moving average of the latency in first response was calculated, but only for the correct trials, both single and double. The learning curves of the response time to the conditioned stimulus are displayed in Figure 12. Like in the response time to the central nose poke, after an initial rapid increase in the time needed
to respond to the target, animals a1 and a3 managed to set an average of two seconds in their response time during the last sessions. The same applies also for a2, with the only difference that it has two more drops before being able to maintain a good performance. As for animal a3, although its latency to the first response is short, during the final session where all the other animals achieved the lowest response times and showed evidence of learning, it had the worst performance.

According to the Cue Matching protocol, a trial is regarded as incorrect if an entry in the hopper not indicated by the target is made either during or after the time delay. A simple statistical analysis of the data obtained from incorrect trials reveals that the incorrect response occurred almost always during the time delay. In fact, the four animals made the error at their first attempt to respond to the conditioned stimulus with percentages of 97.62%, 93.91%, 100% and 93.26% that correspond to a1, a2, a3 and a4, respectively.

Since an incorrect first response to the conditioned stimulus is still regarded as a first attempt of the animals to respond to the target, the latency was grouped also in four different histograms for each animal in Cue Matching. In this graphical representation, the data obtained from the trials have been classified as single correct and incorrect, as well as double correct and incorrect. The distribution of the time needed for the animals to respond to the conditioned stimulus in each case is displayed in Figure 13. The first two histograms for every animal is simply an alternative representation of the moving average displayed in Figure 12, with the difference that the response times are grouped also according to single and double.

From the data in Figure 13, it is apparent that there is a clear trend of decreasing number of trials that correspond to greater time responses. In all cases, the great majority of responses to target,
regardless if they are correct or incorrect, occur within the first two seconds from the moment that the conditioned stimulus is queued. Trials with response times greater than thirty seconds are not displayed in the histograms. The reason is that the number of these trials is very small (up to three for each kind of trials) and can be considered as insignificant. The mean value of reaction time, illustrated in each plot with the thin vertical line, has been affected from the extreme reaction times to target.

For each animal, the latency in the final response for all kind of trials is presented in Figure 14. What is interesting in this data and should not be misinterpreted is that all the animals have very small response time during the incorrect trials, both single and double. This is due to the fact that the vast majority of the incorrect entries in the distractor occur within the time delay, terminating the trial prematurely. As mentioned before, the animals made almost always the incorrect choice at their first attempt to respond to the conditioned stimulus during the incorrect trials.

The amount of time needed for the final response to the target coincides with the duration of the trial and reward delivery as well. Like in the case of histograms, trials with final response times/duration greater than thirty seconds are not included in the graphs of Figure 14. Apart from the fact that this trial
number is small and can be neglected, grate response times to the stimulus can be considered as a loss in the purpose of the task. The animals during these trials might have lost their attention, forgetting to respond. Therefore, high response times to any cue stimulus could be indicating that the corresponding response is not due to the animal’s learning, but occurred at some point by pure luck. Note that Figure 14 illustrates the evolution in the animals’ performance. For all the four animals in Cue matching, the data obtained from all the correct trials, single and double, show a clear trend of decreasing trial duration.

![Figure 14](image)

Figure 14: Response time to the target stimulus in single correct and incorrect, as well as in double correct and incorrect trials for the four animals in Cue Matching. Note that x-axis denotes the trial number that each trial has out of the total number of trials for each animal.

A final essential feature to investigate in Cue Matching is the trial outcome from all the experimental sessions per animal. The moving average was employed in this case as well. The data demonstrated in Figure 15, indicate that the first two animals, a1 and a2, showed a progress in learning. Initially, the percentage between correct and incorrect trials is almost the same. This can be interpreted as guessing, meaning that their response to target stimulus seems to occur at random. But as the number of trials increases, the percentage of correct trials rises as well. During their final session, both animals are completing the trials with 90% of success (Figure 15: a1, a2). This is clear evidence that they became able to distinguish the target stimulus from the distractor. Note that the rapid drop in their
performance, showing between trials 300 to 500, is due to a technical malfunction of a component in the experimental box.

As for the other two animals, a3 and a4, it would not be safe to conclude that they became capable of discriminating between the target and the distractor. Their performance is shown to suffer from constant drawbacks and a low percentage of correct trials (Figure 15: a3, a4). In fact, at the end of testing the percentage of correct trials is below 50% for both a3 and a4.

![Graphs of animal performance](image)

*Figure 15: Moving average of the trial outcome for the four animals in Cue Matching. 77 and 71 points were used for the moving average of a1 and a2, respectively, where the number of trials was large. 13 and 15 points were used for the moving average of a3 and a4, respectively, where the number of trials was small. Note that different scales were used in the graphs for the x-axis, due to the large difference among the trials that each animal completed.*

### 3.4 New Cue Matching task

Cue Matching protocol was modified in respect to the magnitude of the offered reward and the time delay. Up to now, the same amount of reward was given during both single and double trials. The time delay was also the same in the two kinds of trial, taking values from 1 to 8 seconds. The New Cue Matching protocol incorporates all the characteristic features of a delay discounting task that aims to study impulsive choice. From all the animals that were subjected to Cue Matching task, only the ones that were able to establish an association between the central cue stimulus and the target were selected to continue with the more complex force choice task. This is the reason why only two animals, a1 and a2, participated in New Cue Matching task. The chambers used during New Cue Matching are the same with the ones used in Cue Matching (Figure 8).
3.4.1 Description of New Cue Matching protocol

In the modified version, a correct choice of the double target stimulus was paired with a larger reward than the one offered after a correct response to the single target. The reward delivered after a correct double trial was 50% larger than the one offered after a correct single trial. Further, the double target trials were paired with a larger time delay, referred as long time delay. During the first half of the total number of sessions, the long time delay employed for the task was set constant to 3 seconds. For the next sessions that followed, the long time delay varied but remained the same from the beginning up to the end of each session. The values employed for the long time delay were: 1, 5, 6, 9, 13, 21 and 41. For the last three sessions, there was a variation in the long time delay from 1 to 41 seconds every 15 repetitions of the task. The values were set randomly from the program controlling the task. The time delay during all single trials was set to one second (short time delay = 1 sec). Finally, the block length of the trials did not remain constant throughout all the sessions like before, but started as 10 and for the last three sessions was set to 15. Everything else remains the same as in Cue Matching protocol.

3.4.2 Experimental results of New Cue Matching task

The data obtained from the experimental tasks in New Cue Matching for the two animals, a1 and a2, are demonstrated in Table 2.

<table>
<thead>
<tr>
<th>animal</th>
<th>a1</th>
<th>a2</th>
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</thead>
<tbody>
<tr>
<td>total trials</td>
<td>4145</td>
<td>4612</td>
</tr>
<tr>
<td>single trials</td>
<td>2066</td>
<td>2224</td>
</tr>
<tr>
<td>double trials</td>
<td>2079</td>
<td>2388</td>
</tr>
<tr>
<td>correct trials</td>
<td>2392</td>
<td>3219</td>
</tr>
<tr>
<td>incorrect trials</td>
<td>1753</td>
<td>1393</td>
</tr>
<tr>
<td>single correct trials</td>
<td>1200</td>
<td>1613</td>
</tr>
<tr>
<td>single incorrect trials</td>
<td>866</td>
<td>611</td>
</tr>
<tr>
<td>double correct trials</td>
<td>1192</td>
<td>1606</td>
</tr>
<tr>
<td>double incorrect trials</td>
<td>887</td>
<td>582</td>
</tr>
</tbody>
</table>

Table 2: The trial data obtained from the two animals in New Cue Matching.

As can been seen from the table above, the number of the single trials that the animals completed in this task is not significantly different from the number of double trials.

Like in Autoshaping and Cue Matching tasks, the latency to the central nose poke that elicits the trial initialization is the first important feature to investigate here. The learning curves of the latency in the
central entry for the two animals are presented in Figure 16. Since the animals participating in this task had already been trained in how to initiate a trial during both Autoshaping and Cue Matching tasks, their performance in this phase is expected to be really good.

As illustrated in Figure 16, animal a1 managed to keep its latency below 20 seconds. Animal a2 although initially responded with large latency to the offset of the house lamp, it managed to decrease its response times and maintain a good performance as well. Both animals achieved low response times to the central nose poke, despite the drop in their performance during the last sessions. This drop might be a sign of loss of attention due to overtraining. Therefore, the experimental findings for the two animals, a1 and a2, indicate that the association between the offset of the house lamp with the opportunity to trigger a trial initialization is successfully established in this altered task as well.

Following the central entry, the target was queued and the animals had to make a choice. A correct response to the cue target resulted in the delivery of reward but after the time delay period of each trial, contrary to the initial Cue Matching. The introduction of the long time delay paired with all the double target trials is expected to affect the animals’ behaviour in this task. The long delay may cause confusion to the animals, since the animals had learned to expect reward after completing their trials successfully. Therefore, employing long delays may have an impact on the outcome of the trials that will follow (next trial effect). Of course, the long delay influence on the trial outcome is expected to be reduced as the trial number increases. The proportion of correct trials is shown in Figure 17.

---

**Figure 16:** Moving average of the response time to central nose poke that triggers the trial initialization for the two animals in New Cue Matching. 415 and 461 points were used for a1 and a2, respectively.

**Figure 17:** Moving average of the trial outcome for the two animals in New Cue Matching. 415 and 461 points were used for a1 and a2, respectively.
According to the trial outcome presented in Figure 17, both animals start the first session with low percentages of correct trials that can be interpreted as guessing. But as the sessions continue, the number of correct trials increases. The drop in their performance and the rise that follows after is due to the alteration in the value of the long time delay. The last session is finished with little more than 60% of successful trials for a1 and 70% for a2. These results may not be as high as the ones obtained from the initial Cue Matching, where during the final session both animals completed the trials with 90% of success (Figure 15: a1, a2). But despite this, a1 and a2, made a progress in learning and became able to respond correctly to the target paired with different time delays for the reward delivery.

A graphical representation of the animals’ responses into the target hopper during the trials classified as left and right, as well as single and double is illustrated in the four following heat maps (Figure 18 - Figure 23). This representation provides information about the duration of the trials and the intensity of the animals’ response in each case.

In order to create the heat maps the number of nose pokes into the target hopper per second was measured. The values of these response rates range from 0 to 2 and are mapped through a grey scale in the maps. The black areas of the heat maps correspond to increased response rates. In all graphs, the black horizontal lines in the y – axis separate the trials into the different sessions. In all heat maps, the first session is mapped at the top and the last at the bottom of the graph. Each row of data in the heat maps corresponds to a trial.

An interesting observation that can be made from all heat maps is how close to the time delay is the animal’s final response to the target. Since the time of the final nose poke coincides with the trial...
duration, information about the trial duration in respect to the time delay employed at each trial. The similarity between the two patterns resulting from the correct responses to the left and right target is evidence that there is no bias in the animals’ response during the left and right trials. Both animals did not show any preference towards any of the two feeders, as seen in Figure 18 and Figure 19. More, these patterns obtained from the classification of the trials as left and right prove that the hoppers are unbiased.

From the heat map of the incorrect responses to the target hopper during the single trials, it is apparent that as the number of sessions increases, the number of nose pokes into the distractor decreases. This can been seen from the color and density of the rates in the right heat maps of Figure 20 and Figure 21, in which the responses to the incorrect hopper go from black to grey for a1 and almost white for a2. This behaviour is also consistent with the fact that in the last sessions the responses occurred faster and the trial duration showed a tendency to last for a shorter time. This is shown from the dense rates colored with black at the bottom of the left heat maps for the correct responses in Figure 20 and Figure 21. All these findings suggest that the repetitions of the same task with constant time delay of 1 second resulted in the animals’ learning.

Through the right heat maps of the correct responses to the double target shown in Figure 22 and Figure 23, an essential feature to examine is the intensity in the animals’ response during the trials in relation to the waiting period. In the trials that correspond to sessions where a relatively small value was set for the long time delay, there is an increased constant poking into the feeder in order to get the reward. In the rest sessions, where large values for the long delay were used, the response rates are not
that high as the waiting period is prolonged. These findings need further investigation since they could be indicating two different behaviours. The first is that the animals may have given up waiting as the delay in the delivery of reward is extended. The second behaviour is that learning was achieved, therefore the animals reduce their response rates to the target and simply wait for the delivery of reward.

The results obtained from the incorrect double trials could be used in order to gain more insight for the animals’ behaviour during the waiting period. But before that, some more information about the right graphs of Figure 22 and Figure 23 should be given at this point. According to the protocol followed in this task, a trial is terminated if a response towards the distractor occurs while the target is queued. Such trial is classified as incorrect. This means that during the incorrect trials only the last response is incorrect, causing the unsuccessful trial termination. So, all the responses made before the incorrect one are towards the target and therefore characterized as correct. The results from the incorrect trials indicate that in the majority of these trials the incorrect response occurred within a short period after the target was queued. Of course, the long delays employed in the reward delivery affected the trials in the sense that the animals, after responding to the target and not receiving the reward as was the case before, were also poking into the distractor to check if their delayed reward was offered there instead. But this response is considered incorrect in Cue Matching and the animals soon learned to wait.

A conclusion that can be made out of the experimental findings obtained from the double target trials is that the animals were able to handle the incorporation of the long delay in the tasks and associate the double target (2 LEDs: On) with the delayed reward delivery.

Figure 22: Responses to the double target during the correct and incorrect trials in New Cue Matching for animal a1.

Figure 23: Responses to the double target during the correct and incorrect trials in New Cue Matching for animal a2.
The final essential feature to investigate in this delay discounting task is the effect of the time delay in the trial outcome. A graphical representation of the percentage of correct trials in respect to the magnitude of the time delay till the larger reward is illustrated in Figure 24 and Figure 25, for animals a1 and a2 respectively. The percentage of correct trials decreases as longer time delays are employed in the double target trials, as can been seen in both Figure 24 and Figure 25. These findings indicate that the two animals have a tendency not to wait during trials in which reward is delivered with large delay. From the two animals, a2 showed the best performance during double target animals since it had a higher percentage of success for all the different time delays.

**Figure 24:** Percentage of correct double target trials for animal a1. During these trials, the amount of the offered reward is 50% more than the reward in single correct trials.

**Figure 25:** Percentage of correct double target trials for animal a2. During these trials, the amount of the offered reward is 50% more than the reward in single correct trials.
3.5 Peak Interval task

The four animals, two female and two male rats, performed the free choice behavioural tasks in four identical operant chambers. These animals were a5, a6, a7 and a8. The protocol employed for the experimental sessions is Peak Interval. The aim is to test whether or not the animals are influenced by the delay in reward delivery in their choice. All the animals that participated in Peak Interval task received training according to Autoshaping protocol. The results from Autoshaping tasks for the two of the four animals used in this kind of behavioural testing, a5 and a6, are demonstrated in Chapter 2.

The drawing of the chamber used for Peak Interval task is illustrated in Figure 26. Each chamber was equipped with a house lamp and four hoppers, named as back, left, middle and right hopper according to their corresponding position in the experimental box. The back hopper is used for the trial initialization, whereas the rest three are associated with reward. Before the start of the task, the left, middle and right hoppers are matched to three different time delays that remain the same until the end of all trials. The time delays employed are 8, 16 and 32 seconds. Depending on the time delay that will be chosen, the trial is classified as a 8, 16 or 32 – second trial. Three dispensers were also connected to the three hoppers in order to deliver reward with the corresponding delay after the target presentation. All the hoppers were equipped with an infrared beam that detected the animal’s nose entries.

![Figure 26: The operant chamber used in Peak Interval task. The left hopper with the yellow LED light is indicated in this figure as the rewarded hopper, whereas the other two are the non-rewarded ones.](image)

3.5.1 Description of Peak Interval protocol

In Peak Interval protocol, the offset of the house lamp (HL: Off) prompts the animal to initiate the trial by making an entry into the back hopper of the experimental box. The animals may poke into any of the rest three hoppers, but only a back nose poke (BNP) will start the trial.

Following the entry in the back hopper, one of the three lights above the three hoppers turns on, indicating the target. Depending on the hopper selected randomly by the software, a delay period of 8, 16 or 32 seconds starts. The target hopper is the rewarded hopper, while the other two are the
unrewarded ones. During this time, the animal is allowed to poke into any of the four hoppers, but only a response into the hopper indicated by the illuminated light will be rewarded. After the end of the delay period, if an entry into the rewarded hopper has occurred the trial will be terminated with the delivery of sucrose liquid reward. The duration of the trials in this case equals to the time delay.

If the entries that occurred during the delay correspond only to the two unrewarded hoppers, the trial is continued until the animal makes a nose poke into the target hopper. The duration of those trials is equal to the time delay plus the extra time needed for the animal to perform the rewarded response. The characteristic features of this task are the absence of incorrect responses and the offer of reward with three different time delays, paired with the hoppers.

After the reward delivery, the house lamp is turned on to indicate the trial termination. The time period between the end of each trial (Reward: 1 / HL: On) and the start of the next trial (HL: Off) is the intertrial interval (ITI) and it is not of fixed duration, since it depends on the animal’s back hopper entry.

A fifteen percent of the total number of trials corresponds to probe trials. The significant difference of the probe trials is that no reward is offered to the animals after a response in the rewarded hopper.

### 3.5.2 Experimental results of Peak Interval task

The data obtained for the four animals in Peak Interval task are demonstrated in Table 3.

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>total trials</td>
<td>2.042</td>
<td>3.153</td>
<td>1.416</td>
<td>1.765</td>
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<tr>
<td>rewarded trials</td>
<td>1827</td>
<td>2826</td>
<td>1251</td>
<td>1555</td>
</tr>
<tr>
<td>8-second delay rewarded trials</td>
<td>550</td>
<td>935</td>
<td>385</td>
<td>532</td>
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<tr>
<td>16-second delay rewarded trials</td>
<td>689</td>
<td>821</td>
<td>443</td>
<td>450</td>
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<tr>
<td>32-second delay rewarded trials</td>
<td>588</td>
<td>1070</td>
<td>423</td>
<td>573</td>
</tr>
<tr>
<td>probe trials</td>
<td>215</td>
<td>327</td>
<td>165</td>
<td>210</td>
</tr>
<tr>
<td>8-second delay probe trials</td>
<td>59</td>
<td>138</td>
<td>46</td>
<td>72</td>
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<tr>
<td>16-second delay probe trials</td>
<td>85</td>
<td>94</td>
<td>75</td>
<td>62</td>
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<td>32-second delay probe trials</td>
<td>71</td>
<td>95</td>
<td>44</td>
<td>76</td>
</tr>
</tbody>
</table>

*Table 3: The trial data obtained from the four animals in Peak Interval.*
As done in the previous tasks, the first important feature to study in Peak Interval protocol is the latency to the back nose poke that triggers the trial initialization after the offset of the house lamp. The learning curves of the latency in the back nose poke for the four animals are presented in Figure 27.

![Figure 27: Moving average of the response time to the back nose poke that triggers the trial initialization for the four animals in Peak Interval. 204, 315, 142 and 177 points were used for a5, a6, a7 and a8, respectively. Note that different scales were used in the graphs for the x-axis.](image)

As can be seen from Figure 27, the animals were able to establish an association between the offset of the house lamp and the opportunity to start a trial by making a nose entry in the back hopper. Animal a6 exhibited the best results, since it needed less than 40 seconds until the back nose poke in the vast majority of all trials. The other animals, a5, a7 and a8, also achieved low response times to the back nose poke, despite the rapid increase during the last sessions. A possible explanation for the drop in the animals’ performance might be the existence of the probe trials. As reward is not offered after the termination of a probe trial, animals may have lost their interest or attention.

The scope of this task is to manipulate the animals’ response according to the time delay. The optimum result here is a state where the animals respond around the time delay, by poking only into the hopper being queued at each trial. In order to evaluate if the animals had succeed in responding to the cue stimulus, the moving average of the latency in first response to the rewarded hopper was calculated, from all rewarded and probe trials. The learning curves of the response time to the conditioned stimulus are displayed in Figure 28.

In Peak Interval task, it is expected that the response time to the conditioned stimulus will vary depending on the time delay. Therefore, during the 8-second trials the latency in the animals’ first response should be around 8 seconds. The same applies for the other two types of trials: the 16-second and the 32-second trials. The latency during those trials is thus expected to be around 16 and 32 seconds, respectively.
In contrast to the results obtained from both Cue Matching and Autoshaping, note that in this task an increase in the response time to the conditioned stimulus is considered as evidence of the animals’ learning.

Figure 28: Moving average of the response time to the target/rewarded hopper for the four animals in Peak Interval. Black color indicates the 8 second delay trials, blue the 16 second delay trials and red the 32 second delay trials. 10% smoothing was performed in all kind of trials. Note that different scales were used in the graphs for the x-axis.

As seen in Figure 28, the results obtained from the latency to the conditioned stimulus are in agreement with the theoretical assumptions. The response times of all animals during the 32 – second trials, indicated in the graph by the red line, tend to increase in order to reach the value of the time delay employed. The same also happens in the case of the 16 – second trials, where the response times are mostly above the 10 seconds and below the 20 seconds. Finally, in almost all 8 - second trials, the response times to the rewarded hopper are below the 10 seconds and of course lower than the latency in the other two cases. As an overall conclusion from the animals’ response time to the conditioned stimulus, the pattern occurring in each graph is consistent with the rest and is indicative of the learning that took place during the task.

The heat maps of the tree types of trials employed in Peak Interval task are demonstrated for all the animals in Figure 29 - Figure 33. For the heat map representation, the number of nose pokes was measured per second for a duration of 60 seconds. Like before, this one minute interval was selected for illustration purposes as the vast majority of the trials is up to 60 seconds, whereas the results of the significantly few trials that last more may be considered as outliers.

The experimental results from the rewarded trials indicate that the animals responded to the delayed reward delivery. During the first sessions, poking into the rewarded hopper occurs at any time from the trial initialization to the trial termination.
Figure 29: Heat map of rewarded responses for animal a5 during 8, 16 and 32-second delay trials.

Figure 30: Heat map of rewarded responses for animal a6 during 8, 16 and 32-second delay trials.

Figure 31: Heat map of rewarded responses for animal a7 during 8, 16 and 32-second delay trials.

Figure 32: Heat map of rewarded responses for animal a8 during 8, 16 and 32-second delay trials.
This means that the animals respond to the rewarded hopper from the beginning of each trial, without accounting for the time delay. In fact, as it is illustrated in all the heat maps, the animals keep poking, as they consider that this will accelerate the reward delivery. But after some sessions, the animals learn that there is a delay in the reward delivery and therefore, they reduce their poking and start waiting for the appropriate time in order to perform a nose poke.

This progress in learning is reflected through the observation that the heat maps go sparser from top to bottom. In each type of trial, during the last sessions there is a clear response to the rewarded hopper around the corresponding delay. The findings presented in the heat maps support the results presented previously.

The animals’ response to the unrewarded hoppers was also investigated for the three types of trials. Again, during the first sessions, the animals were poking into the unrewarded hoppers as well. The reason for that behaviour is to check if the reward might be there due to the delay in the delivery. But as the sessions were carried on, the number of these unrewarded responses was significantly decreased. Through the repetitions of the task, the animals were able to learn that responding in the unrewarded hoppers would not have an impact in the task, like accelerating the reward delivery.

In order to assess whether or not learning occurred, the animals’ response to all the three rewarded hoppers was also examined during the ITIs. According to the Peak Interval protocol, from the trial termination until the beginning of the next, the only effective response for the animals is a nose poke into the back hopper in order to signal the trial initiation. Any other action would elicit no result. So, poking into any of the other hoppers would not trigger anything at this phase.

The animals’ behaviour here was as expected. During the first ITIs, there was some poking in the feeding hoppers, but as the trials were continued, this undesired responding was significantly reduced. This is evidence of the animals’ learning that allowed them to distinguish the difference between trial time and ITI time. Additionally, the results from the responses during the ITIs indicate that the association between the offset of the house light and the trial initialization was made successfully in this task as well as in the previous ones. This natural stimulus became conditioned, eliciting the desired response.

The last feature to study in Peak Interval is the data obtained from the duration of the rewarded trials for the four animals. The trial duration coincides with the time of the animal’s final response to the rewarded hopper. As mentioned in the case of the latency in the animals’ first response to the target hopper, the duration of a trial should coincide with the time delay used for the trial. Ideally, this means that the duration of each trial type is either 8, 16 or 32 seconds. Figure 33 demonstrates the four histograms of the trial durations together with the corresponding fitted distributions.

The histograms were generated from data that was right skewed, rather than symmetrical. Such a data distribution is reasonable, since it is impossible to have trial duration less than the time delay, which depending on the trial type may be either 8, 16 or 32 seconds. For this reason, the Weibull distribution was selected in order to model the data, as it is flexible and has the ability to assume the characteristics of many different types of distributions. Once more, results corresponding to trial duration more than 60 minutes were not encountered in the fitting and are not included in the graphs. The number of bins used in all histograms is 60; the same with the maximum selected trial duration for the data representation.
Figure 33: Histograms of the time duration to the final rewarded response for the four animals in Peak Interval. The time of the final rewarded response coincides with the duration of the trial. Grey, blue and red colored bars correspond to 8, 16 and 32-second delay trials, respectively.

The four graphs in Figure 33 show that the experimental results are in agreement with what was theoretically expected. The peak of each histogram is near the time delay in all cases for all animals. Only a significantly small number of trials have durations that are much greater than the time delay.
Chapter 4. Discussion and Future Work

As stated in the first chapter, the present work aims to study the role of the serotonergic neurons in the raphe nucleus in respect to impulsivity, by employing behavioural testing based on operant learning. Before the animals are implanted with fibers and subjected to testing with parallel laser stimulations, they received proper training and participated in the designed behavioural tasks. Each experimental task presented in this report has a certain scope to serve, contributing to the overall progress. The reason of decomposing the testing into smaller tasks is to isolate the components of interest in the animals’ behaviour and examine each one separately. This will provide with the necessary control data, as well as feedback for necessary improvements in the behavioural protocols.

The primary goal of the Autoshaping task was to familiarize the animals with some of the basic characteristics of the behavioural testing and prepare them for the upcoming more complex tasks. The rationale behind the design of Autoshaping was to introduce the concept of reward in the hoppers (feeders). Through the training received in Autoshaping, the animals were able to learn how to initiate the trials and get their reward from the two peripheral feeders. The unconditioned stimulus of the illumination of the LED above the middle hopper became conditioned, eliciting the wanted behaviour that was the trial initialization. The same occurred during trial time, in which the unconditioned stimulus of the middle LED offset and the illumination of the peripheral LEDs produced the conditioned response that was the animals’ nose entry into either the left or right feeder. The evidence for these two desired responses is the decrease in the latency to the central nose poke (CNP) and the conditioned stimulus (CS) shown in Figure 6 and Figure 7, respectively. Moreover, the animals became able to distinguish the difference between the inter trial interval and the trial time, and adjust their behaviour accordingly. This can be seen from the different response rates of the animals during these two different periods (Figure 5). Finally for Autoshaping, there was no record for bias in the animals’ choice between the two rewarded hoppers (Figure 4).

Cue Matching was the next protocol employed which shared a lot of common characteristics with Autoshaping. The elementary difference of Cue Matching was the fact that it was a forced choice task, including either correct or incorrect responses that were followed by the delivery or the absence of reward, respectively. The purpose of this task was to examine whether or not an association between the central cue stimulus and one of the two simultaneously presented peripheral stimuli could be established. In the beginning of the sessions, the animals had the same percentage between correct and incorrect responses. This behaviour suggests that the animals did not become aware of the changes in the new protocol and continued like they were performing a task according to Autoshaping. This was the reason why the correct trials have the same number as the incorrect ones. But as the number of trials increased, the percentage of correct trials also increased indicating that the animals were able to distinguish the target stimuli from the distractor. In fact, during the last sessions two out of the four animals had a high percentage of successful trials (Figure 15). The experimental results obtained from Cue Matching revealed that the pairing of the central cue stimulus with a response towards the queued hopper occurred.
Cue Matching task was continued with some elementary changes. The reason behind employing a new version of the task was to introduce the concept of temporal discounting to the animals, through combining a greater food reward with an increase in the delay period. The modifications performed in the original design of Cue Matching were required, since the task aimed to study impulsive choice. The forced choice periods are employed in order to acquire the reward and the delay differences that are associated to a given stimulus.

During New Cue Matching, the illumination of the two-light target was paired with a larger amount of reward offered with a longer delay. The choice of the double target stimulus was made in order for the animals to make the association that the more the lights in the target stimulus are, the greater will be their reward, but the delay as well. The single target trials remained the same as in the initial version of the protocol. According to the corresponding data, the long delay was successfully incorporated in the task and associated with a larger reward, despite the fall in the animals’ performance. More, the different values in the long delay were employed in the double target trials as a first attempt to study the animals’ behaviour during the waiting period. The increase and the various values in the long time delay had an impact in the outcome of the double trials (Figure 24 and Figure 25), but this finding needs further experimental investigation.

In Peak Interval protocol, the purpose was to test whether it is possible to manipulate the animals’ response in respect to time. For this reason, each of the three hoppers was linked to a fixed delay in the delivery of reward in order to have the animals’ responses close to the time delay. As mentioned before, an increase in the latency towards the rewarded hopper should not be interpreted in this task as evidence of not learning. The experimental results of the task indicated that it is possible to influence the response through the delay in reward, as presented in Figure 29 - Figure 32.

The behavioural testing according to Peak Interval that will incorporate laser stimulus in the implanted animals is proposed to be run in a block scheme, consisting of both rewarded and probe trials. This scheme design is presented in Figure 34. The proposed scheme consists of one block without the laser stimulation, in which the animals will participate in twelve rewarded and three non-rewarded trials according to the Peak Interval protocol. The experiment continues with the stimulating block that has the same number of rewarded and probe trials. In this block, the stimulation period will start immediately after the trial initialization by the animal and last till the delivery of reward. During the rewarded trials in both blocks, the rewarded hoppers will be selected in a randomized order according to the Latin square design [56], in order to ensure that at each new trial all time delays are equally probable to be selected. For the probe trials, all the three time delays (24 sec, 48 sec, 96 sec) will be employed per block.

In the experiment described above, it is expected that the inhibition of the 5-HT will increase the response rates of the animals, which equals with an increase in impulsivity. The exact opposite result is expected by the activation of the 5-HT. If these results were to be compared with the data demonstrated in Figure 33, then a shift in all peaks across the vertical direction would be observed. More analytically, an upward shift in the peaks of all curves should occur due to the depletion of the serotonin and a downward due to the increase in the serotonin.
The same experimental findings are expected to be obtained from the New Cue Matching task with the laser stimulations. In the case of inhibiting the serotonin, the increase in impulsive behaviour will be indicated by the low probability of choosing the long delayed rewards, since in this case the animals will become incapable of waiting for longer time. In fact, the longer the delay is in the reward, the less likely is the animal to wait for the delivery. Therefore, the animal is more likely to prefer the smaller but immediate reward, causing an incorrect response in this case. Accordingly, when the serotonin is activated, a decrease in their impulsive behaviour will be shown by an increase in the probability of choosing the long delayed rewards. The data demonstrated in Figure 24 and Figure 25 can be utilized as control data to account for the differences regarding the probability of choosing the long delayed rewards, with and without the variation of the DRN serotonin levels.

The experimental data obtained from this series of tasks will serve as a point of reference for the results that will be obtained from the laser stimulated animals in order to study the influence of the animals’ serotonergic system in impulsive behaviour. Limitations of this study need to be acknowledged, as well. In Peak Interval task the next trial effect is expected to occur after a probe trial, while in Cue Matching after an incorrect or a prolonged double - target trial. Unfortunately, the current research was not specifically designed to evaluate factors related to the next trial effect. To address this crucial matter...
and be able to investigate it, an improved design in the experimental protocols with more coded behavioural events is required.

For future testing, the time delay between the correct response and the reward delivery can be used as a cost in order to evaluate if the animals are willing to wait for a more desirable reward over the immediate delivery of a not so favorable. This can only be evaluated during free choice trials, since the forced choice trials are designated as reward scheme acquisition trials. Furthermore, in delay discounting tasks like Cue Matching and New Cue Matching, data from any time delay between the onset of the central cue light and the peripheral stimulus light could be potentially used to investigate attention.

Finally, it would be very interesting to explore how through the selective variation (activation/inhibition) of the serotonergic neurons in the DRN, perceived reward/cost can be manipulated in order to have the animals in three possible reward/cost states. The first state is the one where the animals are more likely to choose the less desired reward type which is offered immediately, but requires the more desirable reward to be very costly. The second state where the animals do not show a preference, since the cost of the desirable reward matches the less desirable immediately delivered reward. The third state where the animals are more likely to choose the highly desired reward, as the cost associated is low enough. The state of main interest is the second state, where it will hopefully be possible to shift animals to be either responding to high cost targets or mainly low cost targets.
References


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