UNIVERSIDADE DE SÃO PAULO
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Neurofisiologia da predição baseada em memórias sobre regularidades passadas

Neurophysiology of prediction based on memories of past regularities

São Paulo
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Versão Corrigida

Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências

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SUMMARY

RESUMO

SILVA, D. G. **Neurofisiologia da predição baseada em memórias sobre regularidades passadas**. 2022. Tese (Doutorado em Ciências) – Instituto de Biociências, Universidade de São Paulo, São Paulo, 2022.

O sistema nervoso monitora o ambiente continuamente, comparando previsões geradas por memórias sobre regularidades passadas e informações sensoriais atuais. Quando o conteúdo previsto corresponde à informação sensorial, o comportamento em curso continua sem interferência. Porém, quando o conteúdo previsto difere da informação sensorial, a ação em andamento é interrompida e uma atividade exploratória é gerada para investigar a origem da discrepância. Isso possibilita obter mais informações para criar novas memórias, resultando em melhores previsões no futuro. O sistema septo-hipocampal compara estímulos presentes com informações previstas. As informações atuais são recebidas por aferências neocorticais, via córtex entorrinal, e as informações previstas são fornecidas por um sistema gerador de previsões, formado pelo subículo, corpos mamilares, tálamo anteroventral e córtex cingulado. A tarefa de extrapolação a partir de padrões seriais de estímulos parece permitir a avaliação de respostas antecipatórias. Porém, restrições dessa tarefa estão relacionadas ao número de sessões de treinamento necessárias para que os sujeitos possam gerar uma previsão. Assim, o objetivo desse trabalho foi aprimorar a tarefa de extrapolação a partir de padrões seriais de estímulos, tanto para reduzir a fase de treinamento, quanto para aumentar a magnitude dos efeitos da previsão. Visto que a citocromo C oxidase é uma enzima mitocondrial da cadeia de transporte de elétrons e seu aumento indica maior atividade celular, um objetivo adicional foi avaliar a hipótese de que a expressão do citocromo C oxidase aumentaria no subículo e no tálamo anteroventral de sujeitos treinados na tarefa de extrapolação a partir de padrões seriais de estímulos, em comparação a controles não-treinados. Ratos Wistar machos, foram treinados a correr em uma pista reta para receberem reforço ao seu final. Em cada sessão (uma por dia), os animais correram 4 tentativas sucessivas, recebendo quantidades diferentes de sementes de girassol em cada tentativa. No padrão monotônico os sujeitos receberam 14, 7, 3 e 1 sementes de girassol, enquanto os sujeitos expostos ao padrão não-monotônico receberam 14, 3, 7 e 1 sementes de girassol. Os animais foram treinados ao longo de 20 sessões. Na 21ª sessão do experimento, uma quinta tentativa, nunca antes experienciada pelos animais, foi adicionada à sessão. Como controle, um grupo adicional, não exposto ao treinamento, foi usado na avaliação de expressão de citocromo C oxidase. A evolução do desempenho dos sujeitos expostos aos padrões monotônicos e não-monotônicos, ao longo de vinte sessões de treinamento, bem como na sessão de teste, corroboram dados de estudos anteriores relatando extrapolação após um número maior de sessões de treinamento. Isso indica que a modificação do aparato experimental e no procedimento de treinamento para realizar a tarefa foram efetivos. Ainda, análise da expressão de citocromo C oxidase mostrou aumento da atividade do tálamo anteroventral e redução da atividade especificamente no subículo dorsal, no grupo não-monotônico e o inverso no grupo monotônico. Em conclusão, esses dados sugerem que o tálamo anteroventral pode estar envolvido em processos de aprendizagem de informações posteriormente utilizadas na previsão, e que o subículo dorsal pode estar envolvido na recuperação de informações necessárias para a geração de previsão.

Palavras-chave: Comportamento Antecipatório. Padrões Seriais de Estímulos. Tálamo Anteroventral. Subículo. Citocromo C Oxidase.

ABSTRACT

SILVA, D. G. **Neurophysiology of prediction based on memories of past regularities**. 2022. Tese (Doutorado em Ciências) – Instituto de Biociências, Universidade de São Paulo, São Paulo, 2022.

The nervous system continuously monitors the environment, comparing predictions generated by memories of past regularities and current sensory information. When the predicted content matches the sensory information, the ongoing behavior continues without interference. However, when the predicted content differs from the sensory information, the ongoing action is interrupted and an exploratory activity is generated to investigate the source of discrepancy. This makes it possible to obtain more information to create new memories, resulting in better predictions in the future. The septo-hippocampal system compares present stimuli with predicted information. The current information is received by neocortical afferents, via the entorhinal cortex, and the predicted information is provided by a generator of predictions system, composed by the subiculum, mammillary bodies, anteroventral thalamus and cingulate cortex. The extrapolation of serial stimulus patterns task seems to allow the evaluation of anticipatory responses. However, serious restrictions on this task are related to the number of training sessions required for subjects to generate a prediction. Thus, the objective of this work was to improve the extrapolation of serial stimulus patterns task, both to reduce the training phase and to increase the magnitude of the prediction effects. Since cytochrome C oxidase is a mitochondrial enzyme of the electron transport chain and its increase indicates greater cellular activity, an additional objective was to evaluate the hypothesis that cytochrome C oxidase expression would increase in the subiculum and anteroventral thalamus of subjects trained in the extrapolation of serial stimulus patterns task, compared to untrained controls. Male Wistar rats were trained to run on a straight alleyway to receive reinforcement at the end. In each session (one per day), the animals ran 4 successive trials, receiving different amounts of sunflower seeds in each trial. In the monotonic pattern subjects received 14, 7, 3 and 1 sunflower seeds, while subjects exposed to the non-monotonic pattern received 14, 3, 7 and 1 sunflower seeds. The animals were trained over 20 sessions. In the 21st session of the experiment, a fifth trial, never experienced before by the animals, was added to the session. As control, an additional group, not exposed to training, was used in the evaluation of cytochrome C oxidase expression. The evolution of the performance of subjects exposed to monotonic and non-monotonic patterns, over twenty training sessions, as well as in the test session, corroborates data from previous studies reporting extrapolation after a greater number of training sessions. This indicates that the modification of the experimental apparatus and the training procedure to perform the task were effective. Furthermore, analysis of cytochrome C oxidase expression showed increased activity in the anteroventral thalamus and reduced activity specifically in the dorsal subiculum, in the non-monotonic group and the opposite in the monotonic group. In conclusion, these data suggest that the anteroventral thalamus may be involved in learning processes of information later used for prediction, and that the dorsal subiculum may be involved in retrieving information necessary for prediction generation.

Keywords: Anticipatory Behavior. Serial Stimulus Patterns. Anteroventral thalamus. Subiculum. Cytochrome C Oxidase.

LIST OF ABBREVIATIONS

AD Anterodorsal thalamus

AM Anteromedial thalamus

ATN Anterior thalamic nuclei

AVT Anteroventral thalamus

C Control

CM Sham-operated monotonic control

CNM Sham-operated non-monotonic control

COX Cytochrome C oxidase

CxSS1 Primary somatosensory cortex

CxSS2 Secondary somatosensory cortex

CxVS1 Primary visual cortex

CxVS2 Secondary visual cortex

CxAUD Auditory cortex

DAB 3,3'-Diaminobenzidine

Dsub Dorsal subiculum

GPS Generator of prediction system

HDC Head Direction Cells

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

L Lesioned

LM Lesioned monotonic

LNM Lesioned non-monotonic

M Monotonic serial pattern

N Non-monotonic

NM Non-Monotonic serial pattern

PBS Phosphate Buffered Saline

S Strongly Monotonic

Vsub Ventral subiculum

V1 Visual area 1

V2 Second-order visual area

W Weakly Monotonic

1. Introduction

One of the most remarkable characteristics of the nervous system is its ability to anticipate relying on memories of past regularities. The nervous system is constantly comparing present sensory stimuli with information stored in memory, in order to generate predictions. Such capacity allows generating behaviors modulated by pending events, making it one of the fundamental characteristics of intentional behavior (Campos, Santos & Xavier, 1997). It is interesting to note that once the individual behaves in an anticipatory way, he is adapting his present behavior to deal with situations that may occur in the future (Poli, 2010). It is, then, an extremely important mechanism, the result of the evolution of the nervous system, which allowed to direct attention to relevant aspects of the environment (Helene & Xavier, 2003).

Therefore, anticipation is a widely studied ability, especially in biology and neuroscience. For instance, Pavlov (1927) already mentioned anticipatory behavior in his studies on conditioning. By repeatedly pairing a conditioned stimulus, such as light, to an unconditioned stimulus, such as food, the animal will begin to anticipate the presentation of food, as soon as it receives the light. That is, light will become an anticipatory signal for the food. Not only that, but Krushinsky (1990) also studied the ability to generate prediction in a wide range of wild and laboratory animals. The method used by the author consisted in the animal determining the future direction of a food that moved in a straight rail at a constant speed. At a given moment, the food leaves the animal's sight, and the subjects would need to define the place where this food would appear again. The author demonstrated that animals such as rodents, dogs and crows were able to determine the future and unknown direction of the food, based on the known trajectory that they saw before (Krushinsky,1990).

There is also a wide range of studies demonstrating anticipatory ability in rodents. In contrast of incentive gain, fasting rats learn to decrease consumption of a first presented solution containing 0.15% saccharin, by anticipating the presentation of a second preferred solution, containing 30% sucrose, minutes later. Interestingly, when the animals were part of a group in which they also received 0.15% saccharin as a second solution minutes after the first one, they do not show the same decrease in ingestion of the first solution (e.g., Flaherty & Checke, 1982; Onishi & Xavier, 2011). In addition, several studies involving serial stimulus patterns were able to demonstrate the predictive capacity of rodents. In these studies, rodents abstracted rules out of sequences of stimuli and from them predicted not only the reappearance of constant stimuli, but also the outcome of stimuli never seen before (Fountain & Hulse, 1981; Kundey & Fountain, 2011).

1.1. Serial stimulus pattern

Learning from serial stimulus patterns involves abstracting and applying identifiable rules from sequences of stimuli. Being able to learn these rules is beneficial for an individual's survival, as it can guide actions through time in an organized manner, even in unprecedented circumstances (Vassena *et al*., 2014; Garlick, Fountain & Blaisdell, 2017; Geddes, Li & Jin, 2018), preparing better and responding faster and in a more refined way to upcoming stimuli. The importance of serial stimulus learning is even seen in areas of intelligent systems, such as inference, planning, reasoning, robotics, natural language processing, speech recognition, time series prediction and financial engineering (Sun & Giles, 2001).

Human beings tend to abstract rules to facilitate the understanding of sequences of stimuli as a strategy (Fountain, 1990; Loffing, Stern & Hagemann, 2015). Thus, humans can divide a long sequence of stimuli, therefore more complex, into smaller subcomponents by applying simple rules to aid their learning (a process known as *chunking*) (Wallace, Rowan & Fountain, 2008; Muller & Fountain, 2016). Humans benefit from this strategy to create lyrics, write speeches (which require generating a logical series of words to make sense), or memorize and reproduce serial numbers such as telephone numbers (Garlick, Fountain & Blaisdell, 2017). Learning of serial stimulus patterns has also been observed in many other groups of animals (Sun & Giles, 2001; Rowan *et al*., 2001; Fountain, 2008; Rowan, Fountain & Kundey, 2021). Such learning seems similar to those seen in humans (Sands & Wright, 1982; Terrace & McGonigle, 1994; Fountain, 2006), being found in rodents (Fountain, 1990; Murphy, Mondragón & Murphy, 2008; Kundey *et al.,* 2019; Caglayan, Stumpenhorst & Winter, 2021), cetaceans (Mercado *et al*., 2000) and pigeons (Blaisdell & Cook, 2005; Garlick, Fountain & Blaisdell, 2017), for instance.

By learning the rules that identify a sequence of stimuli, the individual is able to predict when each item will likely occur again and generate behavioral responses in accordance with the serial pattern. Predicting a given item in the repetitive sequence of stimuli is known as anticipation (Haggbloom & Brooks, 1985; Fountain, 1990). Furthermore, these animals are able to extend the rules of a learned sequence and predict events never experienced before – as long as they are congruent with the original sequence – a process called extrapolation (Krushinsky, 1990; Poletaeva, Popova & Romanova, 1993; Poletaeva & Zorina, 2015). Extrapolating demands complex cognitive

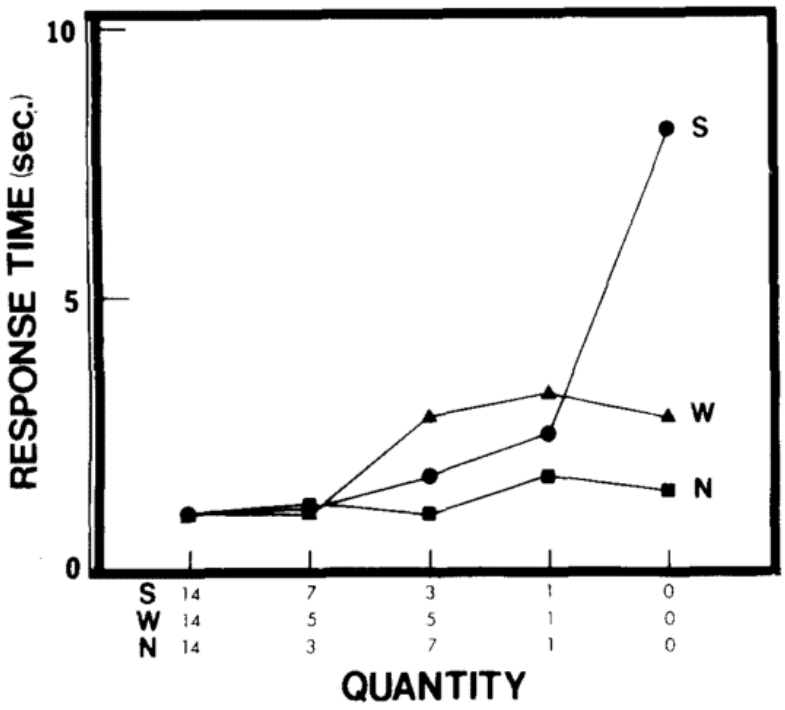
performance, as the organism uses past experiences to predict the possible consequences of novel events, never experienced before, constituting an adaptive advantage (Guigon, 2004). Therefore, it is not surprising that extrapolation has been reported not only in humans (Srinivas & Schwoebel, 1998; Schlag *et al*., 2000; Murphy, Mondragón & Murphy, 2008), but in lemurs (Merritt *et al*., 2011), dogs (Sjölander, 1995), rodents (Fountain & Hulse, 1981, Silva & Xavier, 2021), corvids (Wilson, Mackintosh & Boakes, 1985) and bees (Howard *et al*., 2017), among others.

Fountain and Hulse (1981) showed that rats are able to extrapolate from serial stimulus patterns. According to these authors, the subjects abstract and apply rules that describe the sequence of stimuli. These authors trained rats to run through a straight alleyway to receive different amounts of reward at the end of each of four consecutive trials. Independent groups of subjects were exposed to serial patterns involving 4 items presented in defined sequences. One of the groups, the Strongly Monotonic (“S”) received 14, 7, 3, and 1 food pellets along 4 trials, respectively. Note that this group was exposed to a decreasing amount of food pellets from trial to trial (i.e., 14-7, 7-3, and 3-1). A second group, the Weakly Monotonic (“W”) received 14, 5, 5 and 1 food pellets. In this group there are two decreasing transitions (14-5 and 5-1) and one transition without any change in the amount of food pellets (5-5). A third group, the non-monotonic (“N”) received 14, 3, 7 and 1 food pellets. That is, for this group there are two decreasing transitions (14-3 and 7-1) and one increasing transition (3-7). On the first day of training, the animals were exposed to two training sessions. From days two to thirteen they were exposed to four sessions per day, each session with 4 trials. Along training the animals expressed their ability to anticipate the

next item in the sequence by running faster on trials that provided greater reinforcement and slower on trials with smaller reinforcement. On day fourteen, the animals were exposed to a regular training session followed by an additional session that included a fifth trial just after the fourth trial. Note that this fifth trial had never been experienced by any of the groups. The speed of the animals on the fifth trial was consistent with the logically possible extrapolation from the serial stimulus pattern. That is, the animals exposed to the S pattern substantially reduced their running speeds on the fifth trial, as if they were expecting an amount of food pellets smaller than the one received in the last fourth trial **(Figure 1)**. Animals exposed to the W pattern exhibited running speeds consistent with the expectation of 1 reinforcement. Finally, animals exposed to the N pattern exhibited running speeds congruent with the expectation that they would receive a greater amount of reinforcement as compared to the last trial **(Figure 1)**.

As the fifth trial was new for all subjects and the total amount of food pellets received in the previous trials within the session was the same for all groups, differences in running speeds could not be ascribed to either novelty or motivation. Thus, the authors ascribed this result to the extrapolation relying on the serial pattern to which each group of subjects was exposed to. The authors' interpretation for the running times in the fifth trial by subjects of the S group was that they identified a simple "less than rule". Data of the N subjects were interpreted as "lack of extrapolation". That is, subjects would have not been able to learn the rule of the serial pattern they were exposed to, because it would have been more complex (i.e., a decreasing transition, an ascending and another decreasing one).

Figure 1. Mean of running times (sec) of the subjects exposed to the Strongly monotonic (S), Weakly monotonic (W) and Non-monotonic (N) serial patterns along the five trials of the testing session, as a function of the amount of food pellets received in corresponding trials of previous sessions and along trials in the present session (Quantity).



Source: Fountain & Hulse, 1981.

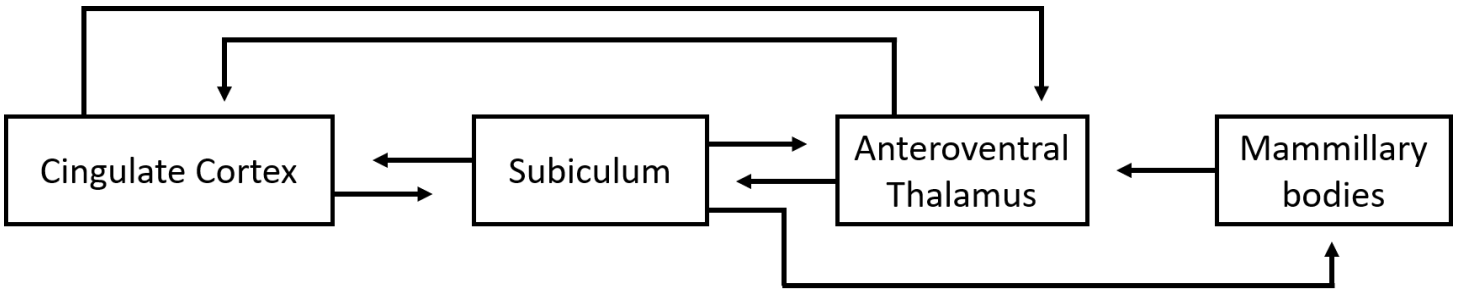
Even though different species can learn rules embedded in sequences of stimuli and generate predictions relying on them, little is known about the neural substrates underlying generation of predictions. Gray (1982) proposed that the nervous system continuously monitors the environment, comparing predictions generated from memories of past regularities in the same context of current sensory information (Henke, 1982; Stolar *et al*., 1989; Brod, Werkle-Bergner & Shing, 2013). When the predicted content corresponds to the sensory information, monitoring continues without interference of the ongoing behavior. However, when the predicted content differs from sensory information, the action in progress is interrupted and exploratory activity is generated to investigate the possible origin of the discrepancy. This renders possible to

obtain new information and to create new memories, resulting in better predictions in future occasions.

1.2. Neural Substrates Underlying Generation of Predictions

According to Gray (1982), part of the hippocampal system, specifically the subiculum, would compare the content of predictions and present sensory information. Present information would be received from neocortical afferents, via entorhinal cortex, and predicted information would be provided by a generator of prediction system (GPS), constituted of both (1) a long loop, including the subiculum, mammillary bodies, anteroventral thalamus (AVT) and cingulate cortex, and (2) a short loop, involving direct and reciprocal projections between the subiculum and the AVT **(Figure 2)**. Thus, the GPS would have access to current sensory information, information stored in memory and the motor plans of the individual **(Figure 3)**. The subiculum would also be the comparator in both loops.

Figure 2. Schematic representation of the structures and projections that form the Generator of Prediction System (GPS).

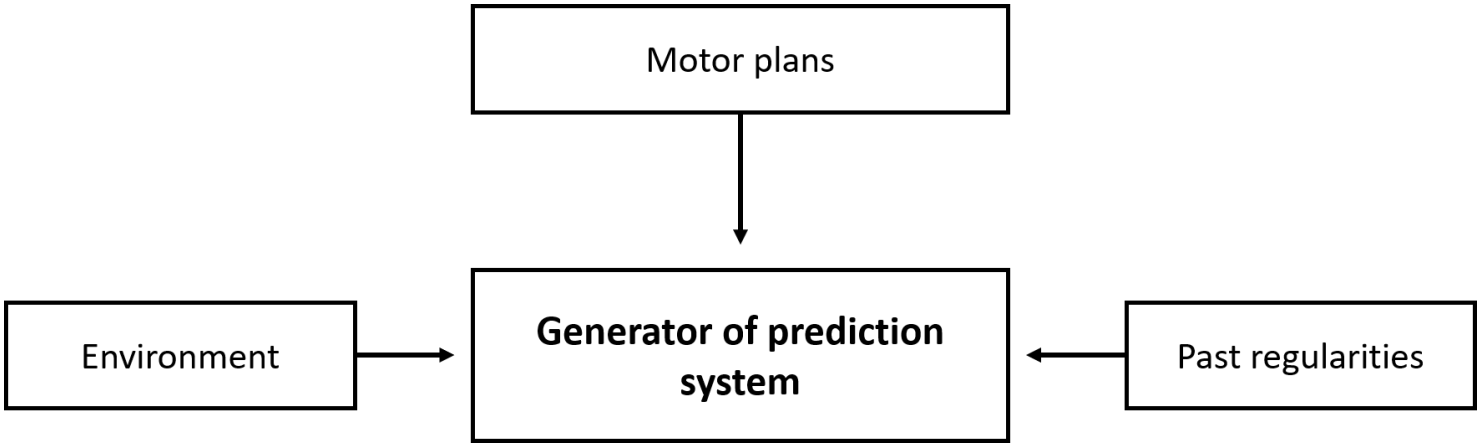


Source: Gray, 1982.

From the experimental point of view, among the structures that participate in the GPS, the AVT is in a strategic position to investigate this

hypothesis. First because it would participate in both the long and the short loops. Second, because it is relatively far from the other structures postulated to be involved in the system, thus rendering possible, for instance, to induce damage in it without reaching other constituents of the system. Furthermore, there have been reports that the AVT plays an active role in the processing information coming from the subiculum (Vinogradova, 2001), as it receives direct and indirect projections from this structure (Dillingham *et al*., 2015; Aggleton & Christiansen, 2015; Christiansen *et al*., 2016). In addition, the AVT receives indirect projections from the CA1 subfield, bringing processed current sensory information from the entorhinal cortex (Gray *et al*., 1991; Gigg, 2006). Finally, according to Stolar and colleagues (1989), the AVT reacts to the probability of pending stimuli. Together these data emphasize the key position of the AVT to investigate this postulated GPS.

Figure 3. Schematic representation of the basic functioning of the Generator of Prediction System (GPS).



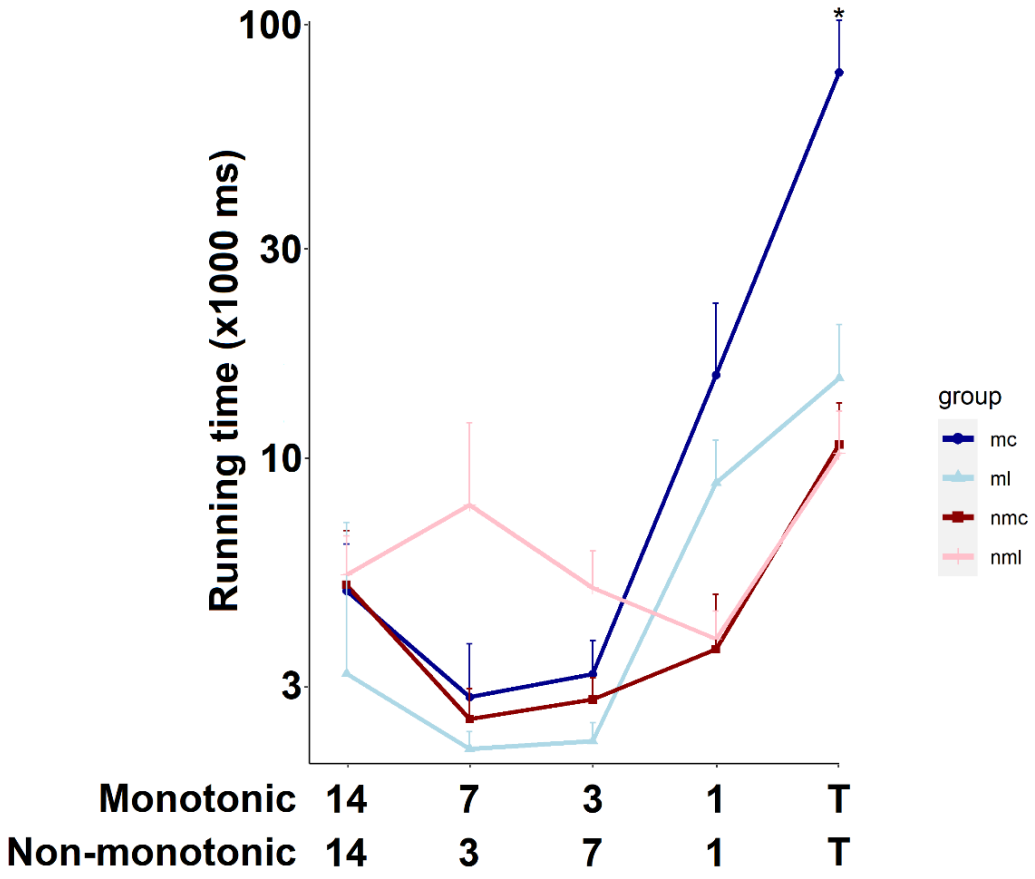
Source: Gray, 1982.

Silva and Xavier (2021) submitted rats with selective damage in the AVT (L Group) and respective sham-operated controls (C Group) to an extrapolation of serial stimulus pattern task. Part of the subjects in each group was trained using a strongly monotonic schedule (hereafter referred to as monotonic - M) and the other part using a non-monotonic schedule (NM). Therefore, there were four groups: LM, LNM, CM and CNM. The subjects were trained along 31 sessions in their respective serial patterns, one session per day. On the 32nd day, a never experienced fifth trial was added to the session soon after the fourth trial. As expected, the running times in the fifth trial of control animals exposed to the monotonic pattern (CM) were substantially longer when compared to control animals exposed to the non-monotonic pattern (CNM), indicating the occurrence of extrapolation **(Figure 4)**. In contrast, lesioned subjects exposed to the monotonic pattern did not exhibit such increase in latency (LM), indicating that these animals did not extrapolate **(Figure 4)**. These results indicate that extrapolation is impaired following selective lesion of the AVT, corroborating Gray's proposal (1982) about the participation of this neural structure in a GPS. Silva and Xavier (2021) report constitutes the first consistent demonstration that the AVT integrity is required for generating predictions, thus stimulating further investigation of the subiculum and AVT involvement in a GPS, as postulated by Gray (1982).

The AVT is one of the components of the anterior thalamic nuclei (ATN). The ATN include two other nuclei (Aggleton *et al*., 2010), namely, the anterodorsal thalamus (AD) and the anteromedial thalamus (AM). Evidence in scientific literature shows that the AD is related to the propagation of signals from Head Direction Cells (HDC) (Clark & Taube, 2011), the AVT would act as

a return loop that modulates theta rhythm (Vertes *et al*., 2001), thus, assisting spatial and non-spatial functions in the hippocampus (Buzsaki, 2005), and the AM would form a connection network between hippocampal-diencephalic and prefrontal areas (Jankowski *et al*., 2013). In fact, evidence shows that the ATN helps in distinct components of learning. Not surprising, humans with ATN injury or atrophy show symptoms similar to those seen in Korsakoff syndrome (Harding *et al*., 2000; Tsivilis *et al*., 2008; Carlesimo *et al*., 2011; De Lima, Baldo & Canteras, 2017).

Figure 4. Mean (+ S.E.M.) of the running times (x 1000 ms) of control (C) and lesioned (L) subjects exposed to training either with the monotonic (M) or the non-monotonic (NM) serial patterns, and subjected to Testing (T) by introducing a fifth trial (never experienced before) in the 32nd session.



Source: Silva and Xavier (2021).

Vinogradova (2001) reported that signals from the subiculum reach structures of the limbic system through the fornix. Such structures constitute the ATN, mainly the AVT (Aggleton *et al*., 2010; Christiansen *et al*., 2016), the mammillary bodies (Irle & Markowitsch, 1982; Christiansen *et al*., 2016), through its direct and reciprocal projections with the subiculum (Vann & Aggleton, 2004), and the cingulate cortex (mainly its posterior area) (Irle & Markowitsch, 1982; Wolff & Vann, 2019). It is interesting to note that Vinogradova (2001) emphasized that information coming from the subiculum receives additional processing when passing through these structures. In turn, the mammillary bodies have extensive connections with the AVT, through the mammillothalamic tract (Vann & Aggleton, 2004). As for the posterior region of the cingulate cortex in rats, designated retrosplenial cortex, because it does not have the equivalents of the areas 23 and 31 of primates, is the cortical target of the ATN, specially the AVT (Shibata, 1993; Van Groen & Wiss, 1995; Wolff *et al*., 2008; Vann, Aggleton & Maguire, 2009; Shibata & Honda, 2015). In fact, rodent with ATN lesions exhibit disruption of activity in the retrosplenial cortex (Dupire *et al*., 2013; Aggleton & Nelson, 2015). Wolff and Vann (2019) called attention to the functional relationship involving the ATN, the hippocampal formation – of which the subiculum is part – and the retrosplenial cortex, where the ATN would play a role in synchronizing these areas and updating representations of existing information (Corcoran *et al*., 2016; Eichenbaum, 2017).

The current literature refers to the structures integrating Gray's (1982) GPS system as "extended hippocampal system" (Aggleton & Brown, 1999, 2006; Wright *et al*., 2013; Carlesimo *et al*., 2015). Differently, however, the

extended hippocampal system has been related to learning and memory, including spatial memory in rodents and episodic memory in humans (Byatt & Dalrymple-Alford 1996; Aggleton *et al*., 2010; Jankowski *et al*., 2013; Marchand, *et al.,* 2014; Dillingham *et al*., 2015; Dumont *et al*., 2015; Milczarek & Vann, 2020).

Conejo and colleagues (2010) trained rats in the Morris water maze and accompanied the evolution of both the hippocampal system and limbic structures activities using cytochrome C oxidase. The authors reported that distinct groups of rats trained for one, three or five days in the water maze exhibited greater neural activity of the AVT in the first day of training, while hippocampal formation structures presented activity from day 1 of the spatial memory task up to day 5. The authors interpreted these results in terms of the contribution of the “extended hippocampal system” for spatial learning and memory.

Although it seems clear that the structures composing the “extended hippocampal system” play a critical role in spatial learning and declarative memory, one should not ignore evidence that they are not restricted only to this role (e.g., Carlesimo *et al*., 2015; Wolff *et al.,* 2015). For example, it has been shown that these structures participate in attentional set-shifting (Wright *et al.,* 2015; Bubb *et al.,* 2021), contextual fear memory (Dupire *et al*., 2013; Marchand *et al*., 2014) and fear conditioning promoted by predator threats (Carvalho-Netto *et al.,* 2010; De Lima, Baldo & Canteras, 2017).

In discriminative avoidance conditioning task, rabbits learned to avoid a shock by moving in a wheel at a specific moment, electrophysiological recordings of the subiculum and AVT were performed during the task. The data

showed that the subiculum exhibited greater activity in the early learning stages of the task, in other words, aiding to gather information that the animals will need to predict when they should move in the wheel, in order to avoid the shock. On the other hand, the AVT was more active during more advanced stages of the behavioral acquisition, guiding behavior after the information about the task rule had already been well acquired (Gabriel, Sparenborg & Stolar, 1987).

1.3. Possible neural changes associated with generation of prediction

Cytochrome C oxidase (COX – also known as Complex IV) is an enzyme that forms the last step in the mitochondrial electron transport chain to produce ATP (Wong-Riley, 2012). Measurement of COX activity can be obtained by histochemistry, acting as a marker of neural metabolism by revealing the energy demand of neurons. Highly metabolic brain regions usually show high expression of COX activity in histochemical assays and vice versa (Mendelez-Ferro *et al*., 2013). For instance, COX histochemistry helped to understand the functional organization of parallel visual pathways in primates (Peres *et al*., 2019), and provided evidence for delineating boundaries of cortical neurons in layers and areas (Balaram, Young & Kaas, 2014), and allowed identification of human visual area 1 (V1) cortical areas related to processing of information from the left and the right eyes (Lingley *et al*., 2018). Furthermore, COX marking helped to reveal that the primate second-order visual area (V2) shows compartmental organization based on bands that run orthogonal to the limits between V1 and V2 (Wong-Riley & Carroll, 1984; DeYoe & Van Essen, 1985; Zeki & Shipp, 1989; Gattass *et al*., 1990). These studies helped to understand

how the modular architecture of areas V1 and V2 is associated with parallel pathways originating in the retina and relayed through the lateral geniculate nucleus (Levitt, Kiper & Movshon, 1994; Gattass *et al*., 1997; Federer *et al*., 2009).

Expression of COX has aided to identify energy demand of neurons during prolonged stimulation or repetitive performance of behavioral tasks (Luo, Hevner & Wong-Riley, 1989; Gonzalez-Lima & Cada, 1994), reflecting the degree of neural activity of cells involved in the task performance (Divac *et al*., 1995). For this reason, it has been used in studies involving learning and memory (Poremba, Jones & Gonzalez-Lima, 1997; Conejo *et al*., 2004; Conejo *et al*., 2007). Furthermore, it is notable that results obtained through COX reflect a stable state of metabolic capacity of the neurons of interest, which occurs over hours (Conejo *et al*., 2010). Thus, the use of COX seems interesting in prospective approaches to the study of the neural circuitry underlying a given function. In other words, given that the use of COX histochemistry was successful in the aforementioned behavioral systems and tasks, it seems plausible to assume that it is adequate to evaluate the activity of structures comprising the GPS during performance of serial learning, anticipation and extrapolation tasks.

1.4. Rational for the proposed experiments

The extrapolation of serial stimulus pattern task may help to investigate the GPS because it allows revealing at least two different forms of predictions. One can evaluate anticipation relying on "reconstruction from memory", that is, when within the same situation involving a sequence of events, the subject

predicts the next event relying of its previous memories for that experience, like it occurs along training in this task. One can also evaluate extrapolation of a novel (never experience before) event relying on the memories of past regularities, like it occurs during the fifth trial of the testing session.

It seems important to emphasize that although the extrapolation of serial stimulus patterns task usually produce clear results, it demands too many training sessions, but allows only one convincing extrapolation testing session, where the individual generates an extrapolation relying on the rules learned previously. Such a long experimental design can be risky, since if something goes wrong in the testing session this part of the experiment could be lost. Thus, to amplify scores expressing anticipatory effects during performance of this task, a longer straight alleyway with higher walls was employed. Because performance of the task is evaluated based on running times in each trial, a longer alleyway should amplify possible differences. On the other hand, higher walls should avoid possible distracting extra-maze stimuli thus helping the subject to focus on the performance of the serial learning task. These changes aimed at fewer training sessions for learning the rule to be used to generate a prediction in the testing session.

In addition, independent groups of subjects exposed to training using either the Monotonic or the Non-monotonic serial patterns provided brains for COX histochemistry, in order to evaluate COX expression in the AVT and subiculum, critical brain structures involved with the GPS.

2. Objectives

The present study aimed at improving the behavioral task for studies of extrapolation of serial stimulus patterns in order to both reduce the training phase and increase magnitude of the extrapolation effects.

An additional aim was to evaluate the hypothesis, directly derived from Gray's (1982) proposal, about the brain structures involved in the GPS, that the COX activity in the subiculum and the AVT in subjects trained in the extrapolation of serial stimulus pattern task would be increased as compared to that seen in control untrained subjects.

3. Extrapolation of serial stimulus pattern

This experiment represents an attempt to reduce the amount of training the subjects were exposed to before the extrapolation testing session. And possibly amplifying scores expressing anticipatory effects along learning of the serial stimulus pattern task and extrapolation in rats. A longer straight alleyway with higher walls was employed. The rationale was that running times in each trial are used to reveal the serial pattern learning and, therefore, a longer alleyway should amplify possible differences, depending on the pending amount of reward. In addition, higher walls in the corridor were used in order to avoid possible distracting extra-maze stimuli thus helping the subject to focus on performance of the serial learning task, and learning the serial task quicker.

3.1. Materials e Methods

3.1.1. Subjects, food deprivation and groups

Seventy five 3-month-old male Wistar rats (Institute of Biosciences, University of São Paulo), kept in standard polypropylene cages (3 animals per cage), on a 12h/12h light/dark cycle (lights on at 0600), with temperature maintained at 23°C $\pm$ 3, were used. The experiments were carried out during the light phase.

Ten days before starting the training sessions, the animals were subjected to a food deprivation schedule, involving exposure to food (Nuvilab® chow) for three hours a day soon after the training session, maintained until the end of the experiment. The rats' weigh was monitored to ensure that they maintained at least 85% of the weight recorded in animals of the same age that had *ad libitum* access to food.

The animals were organized in 2 groups, one exposed to the Monotonic serial pattern (M) scheme (i.e., received 14, 7, 3 and 1 sunflower seeds over 4 successive training trials) and one exposed to the Non-Monotonic serial pattern (NM) scheme (i.e., 14, 3, 7 and 1 sunflower seeds over 4 successive training trials). 35 subjects were trained per group (M and NM). After the training sessions, 20 trained rats of each group, randomly chosen, were exposed to the extrapolation session (in which a fifth trial was introduced right after the fourth), corresponding to the extrapolation groups. An additional group, not exposed to training, was the untrained control group (C). The C group was used for the histochemical experiment, see Neural activity experiment. At the end of the experiment, the animals were euthanized following the recommendations of the Conselho Nacional de Controle de Experimento Animal. All procedures and care followed the guidelines of the Laboratory of Neuroscience and Behavior of the Institute of Biosciences of the University of São Paulo, which follows national and international norms and standards of ethics in the use of animals in research. The protocol was approved by the Ethics Committee of the Institute of Biosciences of the University of São Paulo (process number 347/2019).

3.1.2. Straight alleyway used in the behavioral training and testing

The device **(Figure 5)** consists of a straight alleyway built in grey acrylic and composed of two triangle-shaped boxes, with the sides and bottom measuring 30 cm and the connection to the corridor measuring 11 cm, connected to each other by a straight corridor measuring 200 cm in length, 11 cm wide and 30 cm high. The size of the walls was made to reduce the rats' contact with the external environment during training and testing. The boxes are

connected to the corridor by guillotine-type doors. The doors were controlled by the experimenter remotely, via a nylon wire. In each of the boxes, 25 cm away from the doors, next to the wall opposite to the door, there is a depression on the floor measuring 4 cm in diameter and 1 cm in depth, in which sunflower seeds were placed as reinforcement. Its content is not visible from the corridor, so, one must enter the box to view it. **Figure 6** consists of actual photos of the linear corridor.

Six photocells arranged in one of the corridor's side walls connected to a microprocessor, aligned with infrared light emitters installed in the opposite wall, allowed to record the passage of the animal and the time spent in each part of the corridor. That is, whenever the animal interrupts the light beam when moving down the corridor, the photocell corresponding to the place in which the animal is located is stimulated by the interruption of the infrared beam. The microprocessor monitors the interruption of stimulation, recording in real time, with precision of milliseconds, the time spent by the animal in each part of the corridor.

Figure 5. Schematic representation of the straight alleyway used in the experiment.

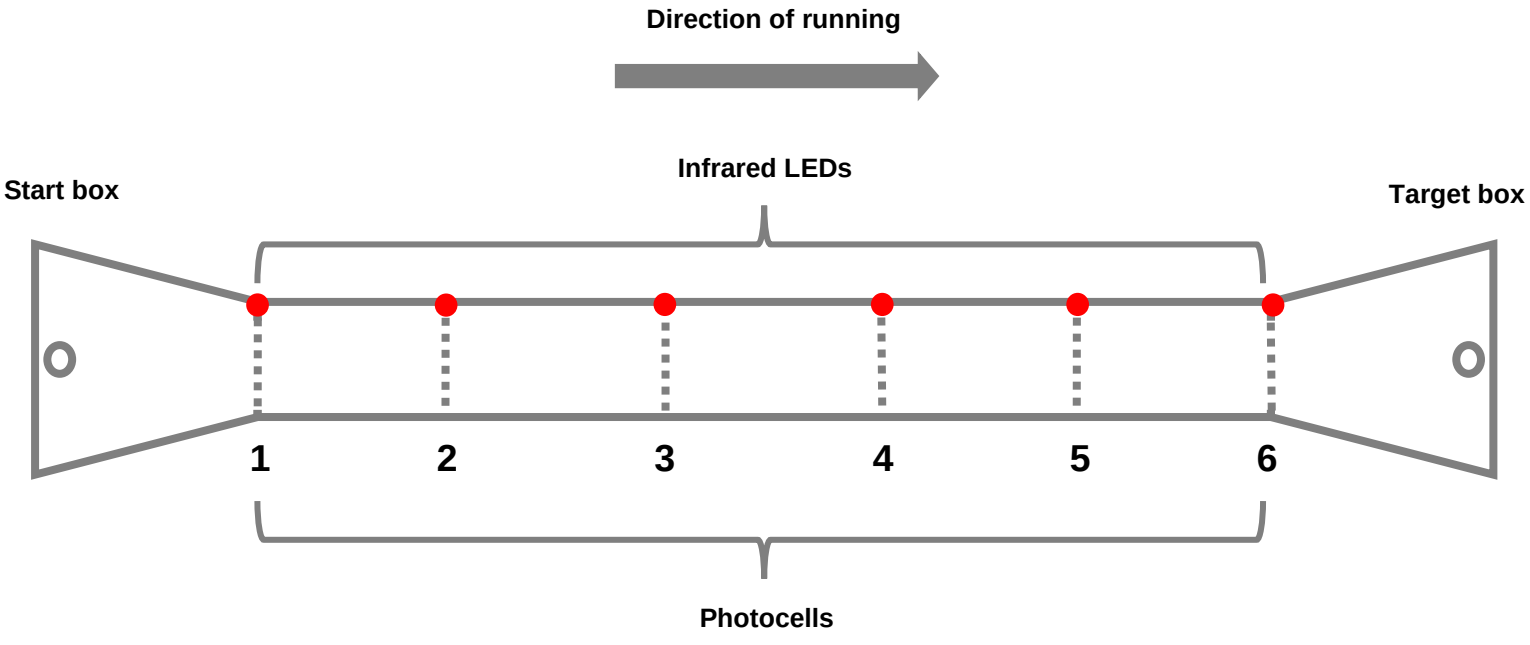


Figure 6. Photographs of the straight alleyway used in the experiment.

3.1.3. Extrapolation of serial stimulus patterns task – training and testing

The extrapolation of serial stimulus patterns task employed in the present study followed the guidelines of Fountain and Hulse's (1981) and Silva and Xavier's (2021) experiments.

Before starting the training sessions, the animals were placed individually in the linear corridor, with the doors open, so that they could walk freely throughout the device for 10 minutes. After this time, the rats were removed from the device and returned to their cages. Each animal realized this habituation procedure for five successive days.

After the habituation to the straight alleyway, the rats were trained to run in it to receive reinforcement (sunflower seeds). In each day, the animals were submitted to one training session consisting of 4 successive trials, with no time

interval between them. Each run consisted of placing the animal in the “start box” with the doors to the corridor closed. The doors were then opened and the animal was able to move freely along the corridor, until it found the reinforcement offered in the “target box”. The time elapsed from the opening of the start box door until the animal (with all four legs) entered the target box was recorded as the latency in each trial, for further analysis. At the end of each trial, the animal was kept in the target box until all seeds were consumed; then, it was placed back in the start box, beginning the next trial. This procedure was repeated until the 4 trials of the day were completed. At the end of the 4 trials, the rats were placed back in their cages.

The number of sunflower seeds varies according to the trial and group, forming two distinct serial reinforcement patterns. In the monotonic pattern, the animals received 14, 7, 3 and 1 sunflower seeds, respectively, in the first, second, third and fourth trials. In the non-monotonic pattern, the animals received 14, 3, 7 and 1 seeds, respectively. Thus, the only difference between the monotonic and non-monotonic patterns is the inversion of the sequence of trials in which 3 and 7 seeds were offered, in the second and third trials.

The animals in C group were not submitted to training or testing sessions. The animals in M and NM groups were trained for 20 sessions, in the 21st session the test was performed, which consisted of introducing a fifth trial similar to the previous ones, however, never experienced before by the animals, performed immediately after the fourth trial.

3.1.4. Behavioral Results Analysis

Regarding to the training sessions data, means of running times per trial across five blocks of four sessions each were calculated. These scores were

then compared using repeated measures Analysis of Variance (ANOVA), having serial pattern (either M or NM) as between-subjects factors, and blocks and trials as within-subjects factors. Tukey-Kramer multiple comparison's tests and contrast analyses were performed *a posteriori*, when required.

Relative to the data of the testing session, running times across trials were compared using ANOVA, having Serial Pattern (either M or NM as between-subjects factors, and Trials as within-subjects factor. Tukey-Kramer multiple comparison's tests and contrast analyses were performed *a posteriori*, when required.

Differences were considered significant when P-values were less than 0.05. The possibility of the existence of marginally significant P-values, when greater than 0.05 and smaller than 0.1, was also considered.

3.2. Results

3.2.1. Training sessions

Figure 7 shows the mean latencies of M and NM groups as a function of the number of sunflower seeds received by each group along trials, along 5 Blocks of training (means of 4 sessions per block, per trial). Note that the running times of the Y axis of the blocks 1 and 2 goes from 0 to 80 (in milliseconds x 1000), and the running times of the Y axis of the blocks 3 to 5 goes from 0 to 20 (in milliseconds x 1000). It is due to the rats taking longer time to finish the trials in the initial stages of training.

The ANOVA revealed a significant main Block effect ($F_{(4, 64)} = 8.84$; $P < 0.0001$) and lack of Trial ($F_{(3, 48)} = 0.58$; $P = 0.63$) and Group ($F_{(1, 16)} = 2.01$; $P = 0.17$) main effects. Furthermore, the ANOVA revealed a significant Trial x

Group interaction effect ($F_{(3, 48)} = 3.48$; $P = 0.02$) and lack of Block x Group ($F_{(4, 64)} = 0.80$; $P = 0.5$), Block x Trial ($F_{(12,192)} = 1.61$; $P = 0.09$) and Block x Trial x Group ($F_{(12, 192)} = 1.27$; $P = 0.24$) interaction effects.

Tukey-Kramer *post hoc* analysis did not reveal significant differences in scores on trial 1 when compared to trials 2 and 3 for both M and NM subjects. In contrast, there was a marginally significant difference between the scores on trial 1 when compared to trial 4, for both M and NM subjects ($F_{(1,16)} = 3.88$; $P = 0.06$). In fact, as **Figure 7** shows, latencies of M subjects are usually longer on trial 4 when compared to their own scores on trial 1; the reverse condition is observed for NM subjects.

3.2.2. Test sessions

Figure 8 shows latency scores for M and NM subjects as a function of the trials of the testing session.

ANOVA revealed lack of Group main effect for the scores of the testing session ($F_{(1, 29)} = 1.64$; $P = 0.21$). In contrast, ANOVA did reveal significant main Trial ($F_{(4, 116)} = 5.08$; $P < 0.01$) and Trial x Group interaction ($F_{(4, 116)} = 4.18$; $P < 0.01$) effects for scores of the testing session. An additional analysis including only the latency scores in the fifth trial revealed a significant Group difference ($F_{(1, 29)} = 4.22$; $P = 0.049$).

Figure 7. Mean (+ S.E.M.) of the running times (in milliseconds) in each trial of the training sessions, over blocks 1 to 5 (containing 4 sessions each), for animals exposed to the Monotonic or Non-Monotonic patterns. Note that blocks 1 and 2 have Y axis values going from 0 to 80 (in milliseconds x 1000) and blocks 3 to 5 (Blocks C, D and E) have Y axis values going from 0 to 20 (in milliseconds x 1000).

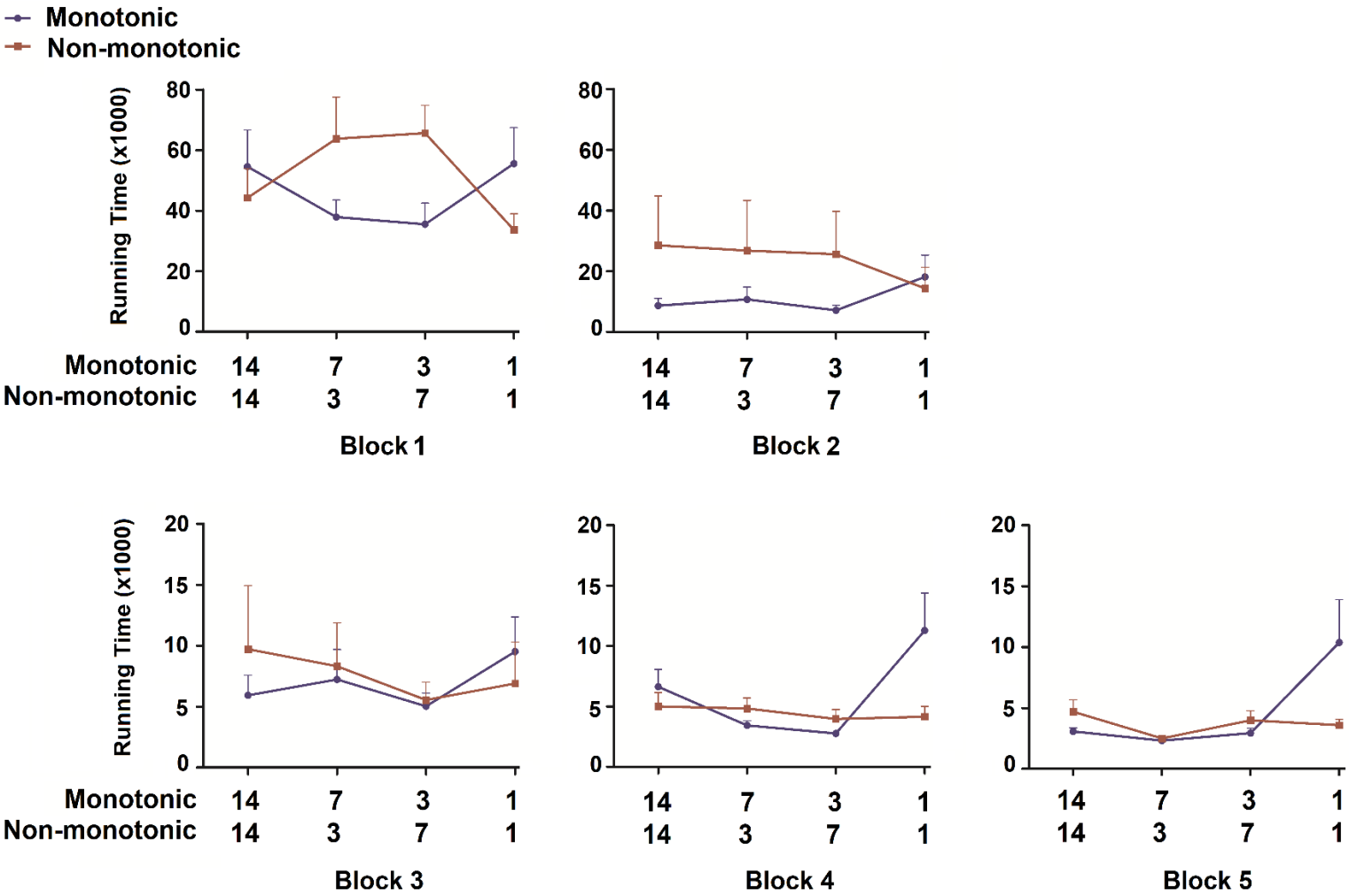
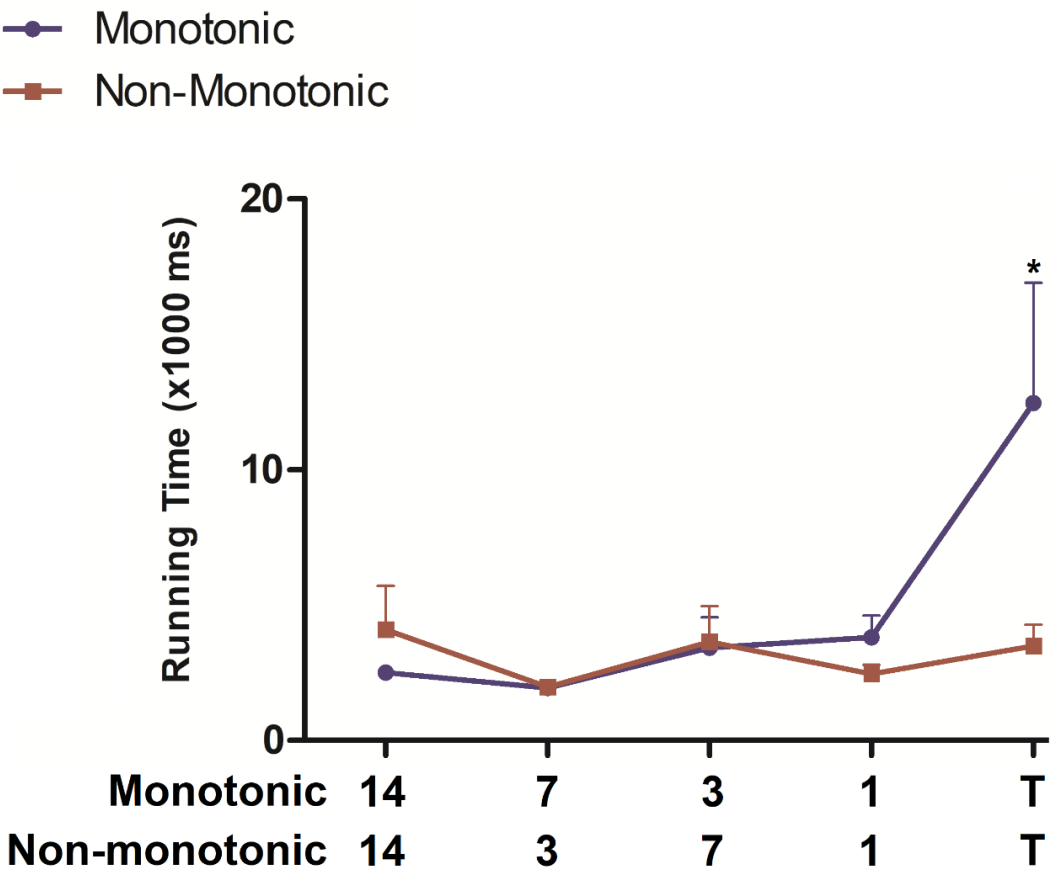


Figure 8. Means (+ S.E.M.) of latencies (in milliseconds x 1000) as a function of trial in the testing session, for animals exposed to the Monotonic (N = 17) or Non-Monotonic (N = 16) patterns. *P < 0.02.



3.3. Discussion

The most striking result of the present experiment relates to the distinct effects of the M and NM serial patterns, which only difference relates to the inversion of the number of sunflower seeds offered on trials 2 and 3 – respectively 7 and 3 for M subjects, and 3 and 7 for NM subjects – on performance along both training and testing. That is, running times along repeated training reflected the serial pattern to which the subjects were exposed to, being relatively stable along trials for NM subjects and substantially increasing, particularly on the last trial, for M subjects **(Figure 7)**. This difference was statistically confirmed by a significant Group x Trial interaction

effect. These figures indicate that the subjects were sensitive to the amounts of food received along the four trials of training, and used this information do modulate their behavior.

Therefore, it seems plausible to admit that subjects trained with either M or NM serial patterns were able to abstract the respective serial pattern they were exposed to, thus reflecting their expectancy in the running times of the fourth trial **(Figure 7)**, despite the smaller amount of training sessions (21 in the present study) relative to previous studies [e.g., 51 training sessions (Fountain & Hulse, 1981) and 31 training sessions (Silva & Xavier, 2021)]. Therefore, even though a direct between-study comparison is not possible, the apparatus changes including alleyway length, wall's height, boxes form, and particularly the reduction of the number of training sessions, did not disturbed acquisition of the task.

This interpretation was further confirmed by testing data. That is, while subjects exposed to the M schedule of reward exhibited a substantial increase in running times in the testing session, thus indicating the expectation of a smaller reward in this novel 5^{th}, never experienced, trial, as compared to previous trials, the NM subjects maintained their running times relatively stable **(Figure 8)**. Therefore, it seems plausible that subjects trained with the M serial pattern learned it and used this information to extrapolate that the amount of reward to be received in the novel, fifth trial, would be smaller as compared to those received in the previous trials.

Alternative explanations ascribe this latency increase on the fifth trial either to the novelty represented by the addition of this novel trial or to motivational factors. However, both animals exposed to M and NM serial

patterns were subject to this same novelty, and only animals in the M group exhibited such increase (Fountain & Hulse, 1981; Silva & Xavier, 2021). Therefore, this novelty interpretation can be discarded. Another explanation ascribes the testing times difference to motivational factors since the number of seeds received in intermediate trials were different. However, on the fifth trial, the total amount of sunflower seeds received by the subjects of both M and NM groups in previous trials was exactly the same, i.e., 25 seeds. Therefore, one cannot ascribe the running times on the fifth trial to motivational factors. These results seem more parsimoniously interpreted as an effect of extrapolation relying on the learned serial stimulus pattern. That is, because the subjects were consistently exposed to a decreasing amount of reward on previous trials, on the fifth trial they would expect an even smaller amount of sunflower seeds as compared to the fourth trial.

In contrast, running times on the fifth trial by subjects exposed to the NM serial pattern did not differ to their own performance on previous trials. Wallace and Fountain (2002) hypothesize that the non-monotonic pattern is more complex, since it decreases from trial 1 to 2, increases from trial 2 to 3 and then decreases again from trial 3 to 4, thus exhibiting more elements to be learned. According to these authors, this would render these animals unable to learn the rule. However, it seems also possible to hypothesize that the animals trained in the NM serial pattern were able to learn it and to extrapolate the pending result on the fifth trial. That is, since the NM serial pattern intercalates decreasing (1st to 2nd trials and 3rd to 4th trials) and increasing (2nd to 3rd trials) amounts of reward, the content of the extrapolation possible in the fifth trial should be an

increase in the number of sunflower seeds, which should render a short running time.

Together, the results of the present experiment confirm findings of Fountain and Hulse (1981) and Silva and Xavier (2021) relative to extrapolation of serial stimulus patterns, whilst employing a much smaller number of training sessions.

4. Expression of Cytochrome C Oxidase in the Subiculum and the AVT/AD following training and testing in the Extrapolation task

As discussed above, according to Gray (1982) the subiculum and the AVT are critical components of the GPS. In addition to participating in the GPS, the subiculum would also play a critical role in comparing predicted and actual information. On the other hand, the AVT would participate in both, the short and the long loops of the GPS.

Silva and Xavier (2021) showed that damage to the AVT disrupts rats' ability to extrapolate relying on serial stimulus patterns, thus rendering support to the proposal that the AVT may participate in the GPS.

The present experiment evaluated the postulate that the subiculum and the AVT do participate in the GPS, by analyzing the expression of COX in these structures following training and testing rats in the extrapolation of serial stimulus pattern.

4.1. Materials and Methods

4.1.1. Histochemical procedures

At the end of the behavioral testing, 5 animals of the M group, 5 animals of the NM group and 3 animals of the C group, randomly chosen, were deeply anesthetized with Ketamine and Xylazine, 200 mg/kg and 20 mg/kg, respectively. The brains of the animals were removed from the skulls, and stored at -80°C. Frontal 30 µm thick sections at the level of the subiculum and the AVT, were taken in cryostat. While subiculum sections were taken at intervals of 120 µm, AVT sections were taken at intervals of 60 µm. The slides were then placed in a laboratory oven at 37 ºC for 75 minutes in a solution containing: 22.4 mg of COX, 115.23 mg of DAB, 4.5 g of sucrose and 12.51 ml of 1% nickel sulfate and 100 ml of 0.1 M HEPES (pH 7.4) for marking

mitochondrial expression of COX. Interruption of the enzymatic reaction, was achieved by immersing the slides in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for one hour at room temperature.

4.1.2. Optical Densitometry

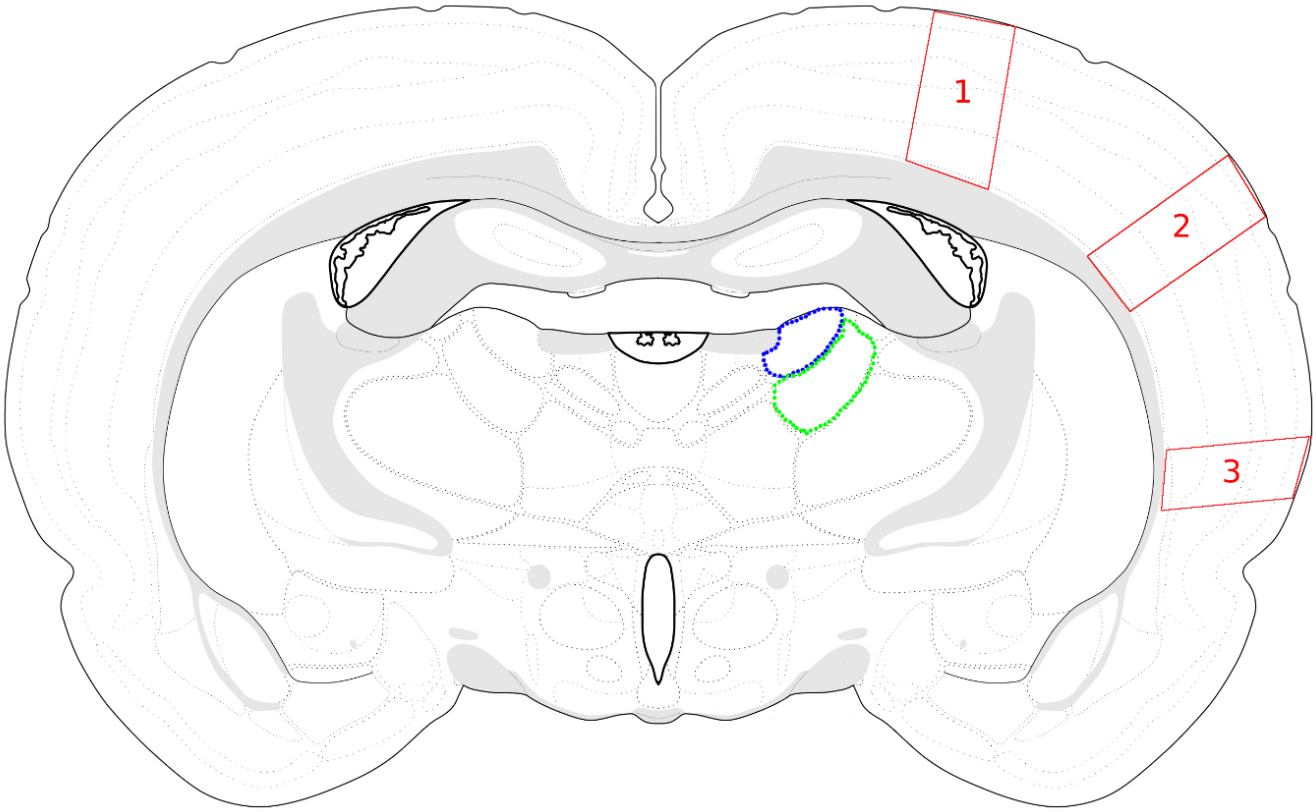
Photomicrographs of brain sections were taken under a light microscope for optical densitometric analysis. The microscope camera parameters were kept the same for all photomicrographs: objective, 2.5; exposure, 5.22 ms; gain, 2.2x; saturation, 0.80 and gamma, 0.75. The optical densitometry of the photomicrographs was then measured in ImageJ 1.53e software following the steps ahead: 1. the photomicrographs were converted to 16 bits; 2. before measuring optical densitometry, background was subtracted for all images; 3. a rectangular selection tool was then used to define the region of interest and finally; 4. optical densitometry values were extracted from the regions of interest.

Photomicrographs of five distinct individual sections, per animal, along the AVT and AD, were taken between anteroposterior -1.20 and -1.92 mm coordinates of the Paxinos and Watson (2004) atlas (Sections 1 to 5). For each photomicrograph of the AVT and AD (regions of interest), three additional photomicrographs of the cortical areas at the same section (therefore, likely exposed to very similar staining conditions) were taken **(Figure 9)**. Two of these cortical areas included the primary somatosensory cortex (CxSS1) **(Figure 9, CxSS1.1 and CxSS1.2)** and the third included the secondary somatosensory cortex (CxSS2) **(Figure 9, CxSS2.3)**. These cortical measurements were used as an “internal control”, that is, brain regions which activities were supposed not

to be changed by training and testing in the extrapolation task. The AVT and AD densitometric scores of each section were individually divided by densitometric scores of each cortical area appearing at the same section, thus resulting in the following densitometric ratios: AVT/CxSS1.1, AVT/CxSS1.2, AVT/CxSS2.3, AD/CxSS1.1, AD/CxSS1.2 and AD/CxSS2.3. These ratio scores allowed comparison of the expression of COX activity in both the AVT and AD, following M and NM training and testing.

Similarly, photomicrographs of five distinct individual sections per animal, along the dorsal (Dsub) and the ventral (Vsub) subiculum, were taken between anteroposterior -4,92 and -6.84 mm coordinates of the Paxinos and Watson (2004) atlas. Each photomicrograph of the Dsub was taken at the same section as that of the Vsub. In addition, three photomicrographs of cortical areas appearing in the same section **(Figure 10)** were taken as “internal controls”. These cortical areas included the primary visual cortex (CxVS1) **(Figure 10, CxVS1.1)**, the secondary visual cortex (CxVS2) **(Figure 10, CxVS2.2)** and the auditory cortex (CxAUD) **(Figure 10, CxAUD.3)**. The Dsub and Vsub densitometric scores of each section were individually divided by densitometric scores of each cortical area appearing at the same section, thus resulting in the following densitometric ratios: Dsub/CxVS1.1, Dsub/CxVS2.2, Dsub/CxAUD.3, Vsub/CxVS1.1, Vsub/CxVS2.2 and Vsub/CxAUD.3. These ratio scores allowed comparison of the expression of COX activity in the Dsub and Vsub following M and NM training and testing.

Figure 9. Illustration of the approximate level where AVT (green dotted lines) and AD (blue dotted lines) optical density were measured and representation of the three cortical areas adopted as internal controls for optical densitometry for AVT and AD regions. Cortical areas 1 and 2 correspond to the primary somatosensory cortex (CxSS1.1 and CxSS1.2, respectively) and area 3 corresponds to the secondary somatosensory cortex (CxSS2.3). Measurements were taken from 5 sections at distinct levels (Sections 1 to 5) between anteroposterior -1,20 and -1,92 mm coordinates of Paxinos and Watson (2004) atlas.

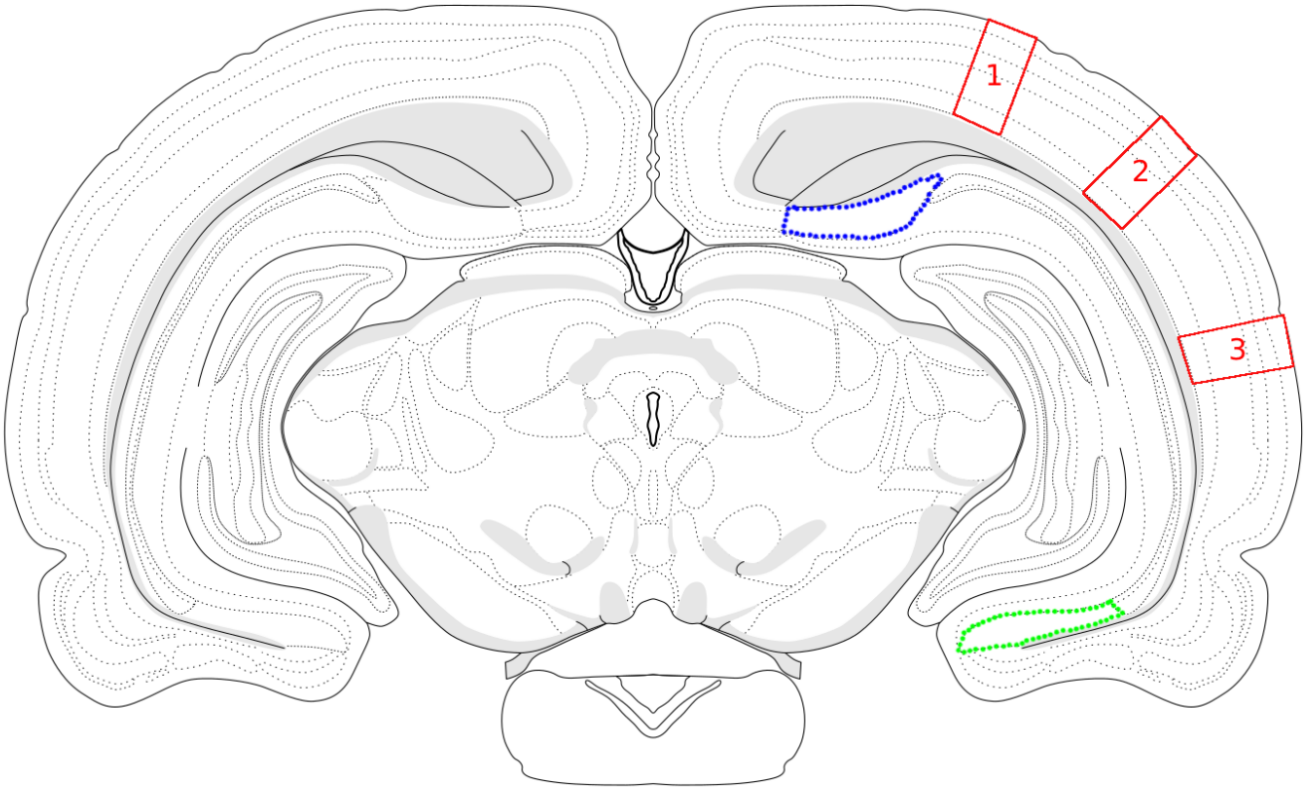


4.1.3. Analysis of data from optical densitometry

Densitometric ratios including AVT/CxSS1.1, AVT/CxSS1.2, AVT/CxSS2.3, AD/CxSS1.1, AD/CxSS1.2, AD/CxSS2.3, Dsub/CxVS1.1, Dsub/CxVS2.2, Dsub/CxAUD.3, Vsub/CxVS1.1, Vsub/CxVS2.2 and Vsub/CxAUD.3, were individually subjected to repeated measures Analysis of Variance (ANOVA), having groups (C, M and NM) as between-subjects factor, and section as within-subjects factor. Tukey-Kramer multiple comparison's tests and contrast analyses were performed *a posteriori*, when required.

Differences were considered significant when P-values were less than 0.05. The possibility of the existence of marginally significant P-values, when greater than 0.05 and less than 0.1, was also considered.

Figure 10. Illustration of the approximate level where Dsub (blue dotted lines) and Vsub (green dotted lines) optical density were measured and representation of the three cortical areas adopted as internal controls for optical densitometry for Dsub and Vsub. Area 1 corresponds to the primary visual cortex (CxVS1.1), area 2 to the secondary visual cortex (CxVS2.2) and area 3 to the auditory cortex (CxAUD.3). Measurements were taken between anteroposterior -4.92 and -6.84 mm coordinates of Paxinos and Watson (2004) atlas.



4.2. Results

4.2.1. COX histochemistry

Figure 11 shows a representative photomicrograph of AVT/AD region stained with COX. **Figure 12** presents the region of the primary somatosensory cortex (CxSS1), as an example of the cortical regions used as an internal

control for performing densitometry of the AVT/AD. In turn, **Figure 13** shows a photomicrograph of the Vsub and **Figure 14** shows a photomicrograph of the Dsub, both stained with COX. Finally, **Figure 15** presents the region of the auditory cortex (CxAUD), as an example of the cortical regions used as an internal control to perform densitometry of the Dsub and Vsub.

Figure 11. Representative photomicrograph of a coronal brain section stained for cytochrome C oxidase histochemistry, showing the AVT and the AD nuclei (green dotted lines represent the boundaries of the AVT and blue dotted lines represent the boundaries of the AD).

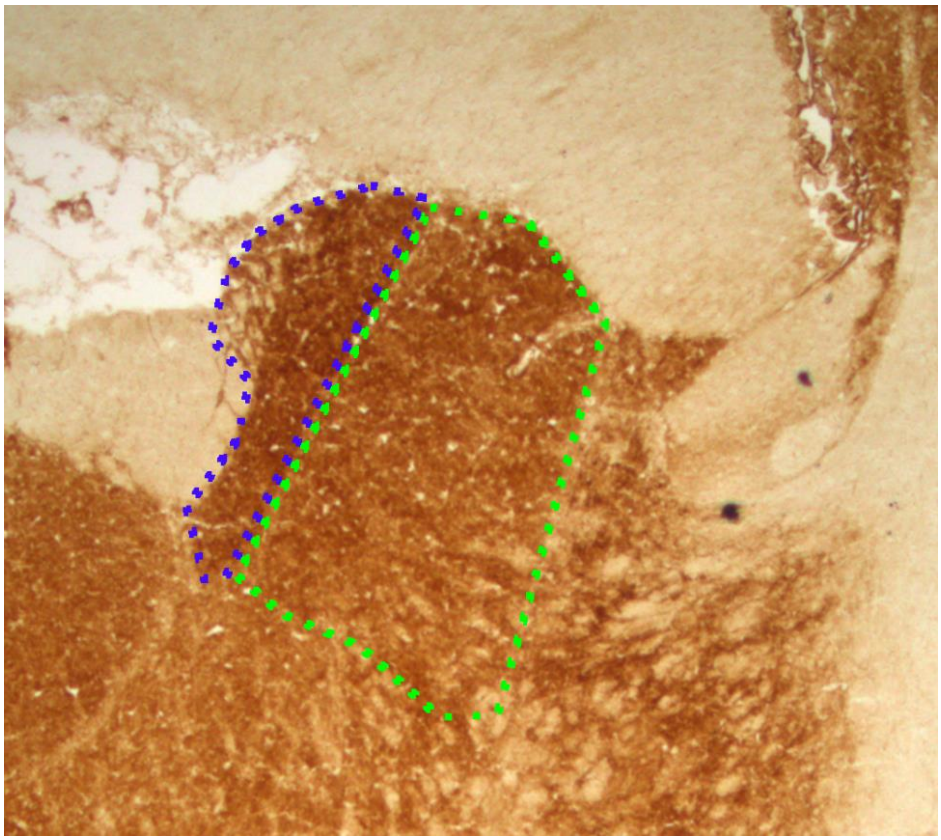


Figure 12. Representative photomicrograph of a coronal brain section stained for cytochrome C oxidase histochemistry, showing the primary somatosensorial cortex (CxSS1), as an example of cortical area used as internal control for the AVT and AD nuclei (green dotted lines represent the primary somatosensorial cortex).

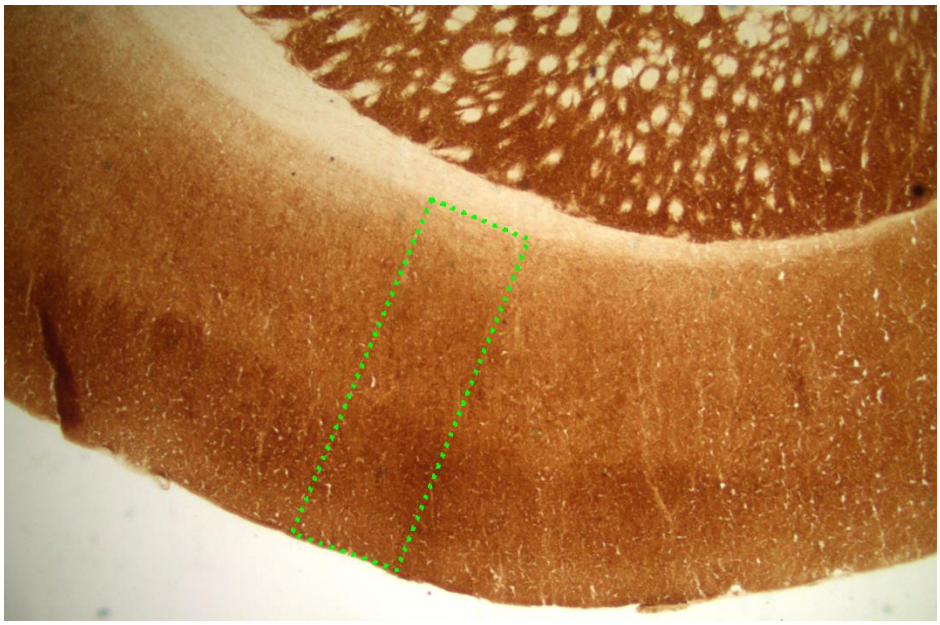


Figure 13. Representative photomicrograph of a coronal brain section stained for cytochrome C oxidase histochemistry, showing the Vsub (green dotted lines represent the boundaries of the Vsub).

Figure 14. Representative photomicrograph of a coronal brain section stained for cytochrome C oxidase histochemistry, showing the Dsub (green dotted lines represent the boundaries of the Dsub).

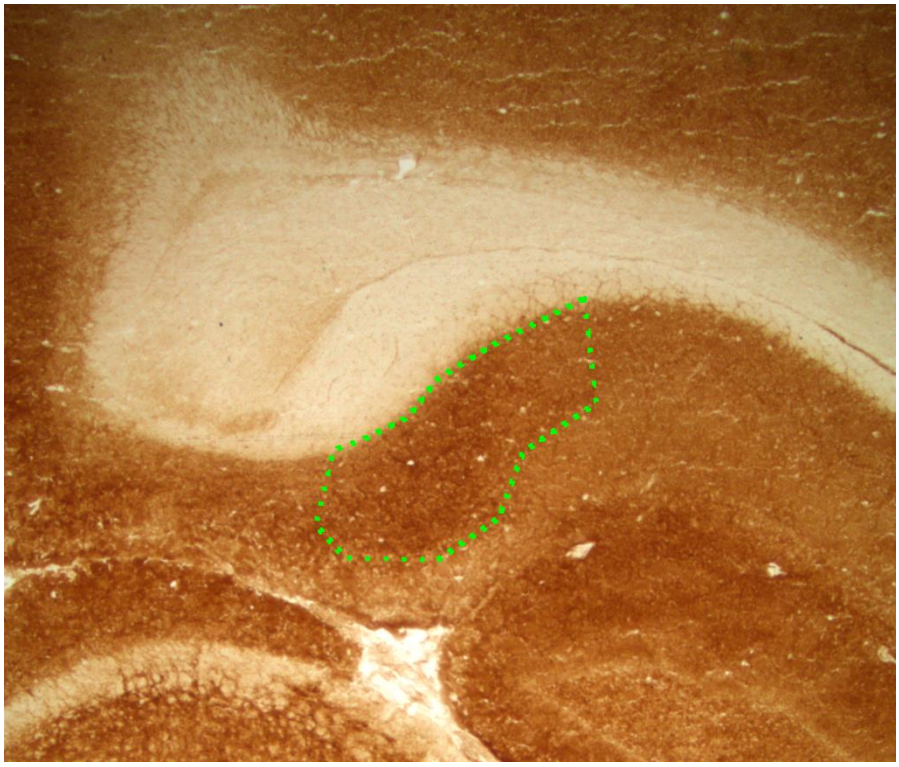
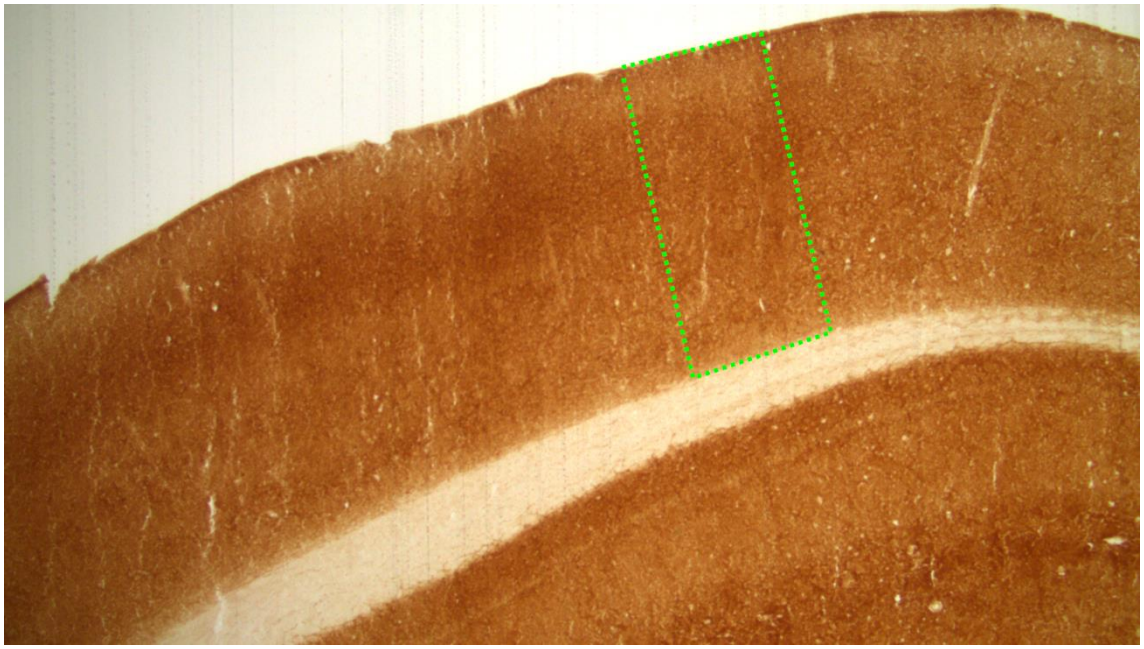


Figure 15. Representative photomicrograph of a coronal brain section stained for cytochrome C oxidase histochemistry, showing the auditory cortex (CxAUD), as an example of cortical area used as internal control for the Vsub and Dsub (green dotted lines represent the auditory cortex).



4.2.2. Optical densitometry results

Regions of interest (AVT, AD, Dsub and Vsub) brain slices of subjects exposed to either M or NM serial patterns along 20 sessions of training and one session of testing, and of untrained control subjects (C), were processed for revealing COX. Then, these slices were subjected to optical densitometry. Only data which ANOVA revealed either significant or marginally significant main Group effects were presented in **Figures 16 to 19**.

To verify the control consistency a set of ANOVAs were ran in the AVT/AD internal controls and for the dorsal and ventral subiculi internal controls. If any significant effect were found between any of these internal controls, the data using that control would not be considered. A significant effect was found for CxVS2, therefore data using CxVS2 as an internal control were disregarded.

Optical densitometry scores for the AVT are presented in **Figure 16**. This figure also shows relevant statistical comparisons for each ratio presented **(Figures 16A, 16B, 16C, 16D and 16E)** (see below).

The ANOVA revealed main significant Group effects for AVT/CxSS1.2, section 3 **(Figure 16A)** and AVT/CxSS1.2, section 5 **(Figure 16B)**, and marginally significant Group effects which additional *post hoc* analyses revealed significant effects for AVT/CxSS1.1, section 3 **(Figure 16C)**, AVT/CxSS2.3, section 5 **(Figure 16D)** and AVT/CxSS1.1, section 1 **(Figure 16E)**.

Optical densitometry scores for the AD are presented in **Figure 17**. The ANOVA revealed a main significant Group effect for AD/CxSS1.2, section 5 (**Figure 17** shows relevant statistical comparisons).

Optical densitometry scores for the Dsub are presented in **Figure 18**. This figure also shows relevant statistical comparisons for each ratio presented **(Figures 18A, 18B and 18C)** (see below).

The ANOVA revealed main significant Group effects for Dsub/CxAUD.3, section 1 **(Figure 18A)** and Dsub/CxVS1.1, section 5 **(Figure 18B)**. ANOVA also revealed marginally significant Group effects for Dsub/CxAUD.3, section 5 **(Figure 18C)**.

Optical densitometry scores for the Vsub are presented in **Figure 19**. This figure also shows relevant statistical comparisons for each ratio presented **(Figures 19A, 19B, 19C and 19D)** (see above).

The ANOVA revealed a main significant Group effect for Vsub/CxVS1.1, section 4 **(Figure 19A)**. The ANOVA also revealed marginally significant Group effects for Vsub/CxAUD.3, section 4 **(Figure 19B)**, Vsub/CxVS1.1, section 5 **(Figure 19C)** and Vsub/CxAUD.3, section 5 **(Figure 19D)**.

Figure 16. Boxplot of the optical densitometry ratio for the anteroventral thalamus (AVT) and the **(A and B)** second area of the primary somatosensory cortex (SS1); **(C and D)** first area of the SS1; and **(E)** secondary somatosensory cortex (SS2), for Control (C), Monotonic (M) or Non-Monotonic (NM) subjects. *P < 0.05.

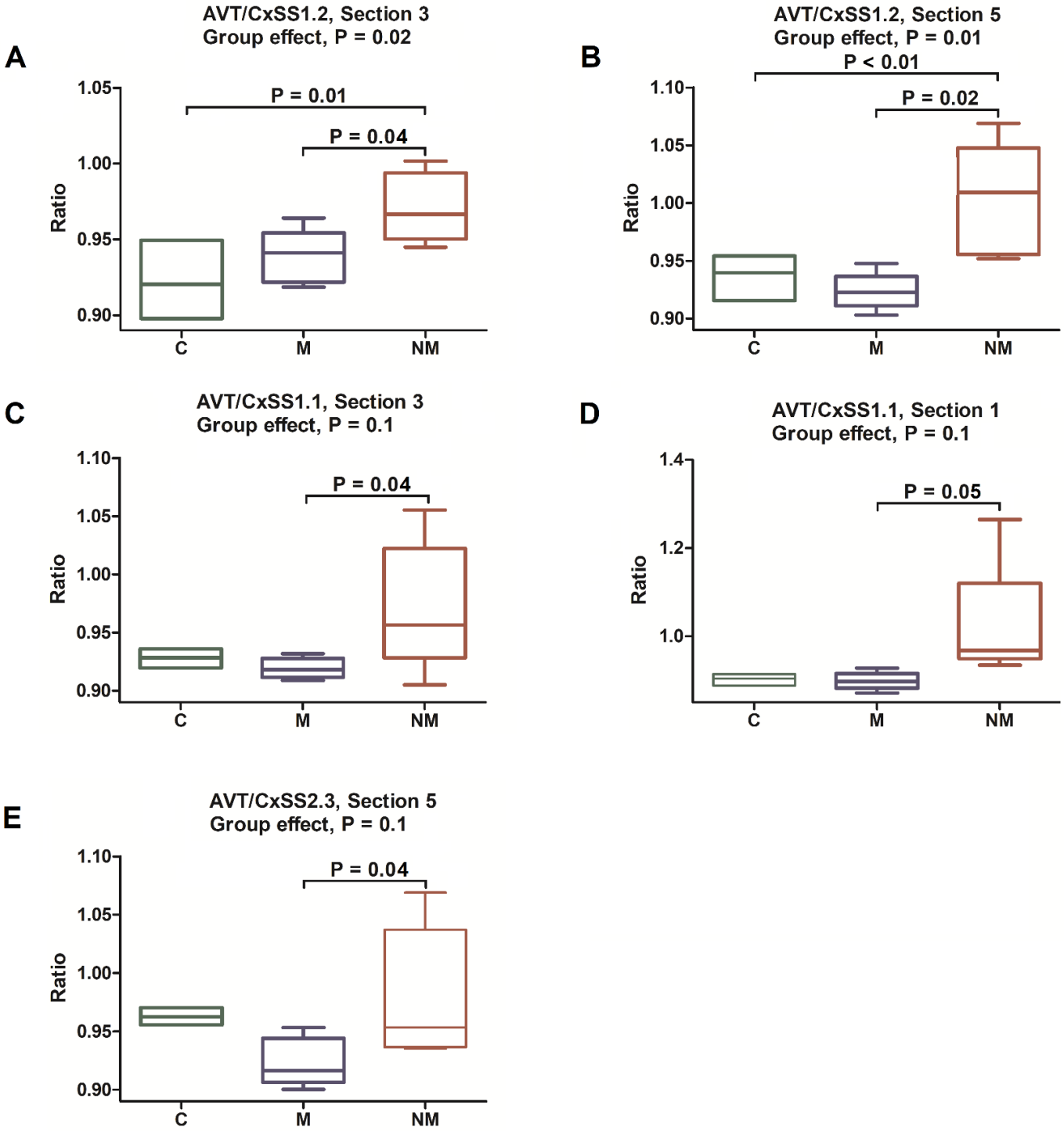


Figure 17. Boxplot of the optical densitometry ratio for the anterodorsal thalamus (AD) and the second area of the primary somatosensory cortex (SS1), for Control (C), Monotonic (M) or Non-Monotonic (NM) subjects. *P < 0.05.

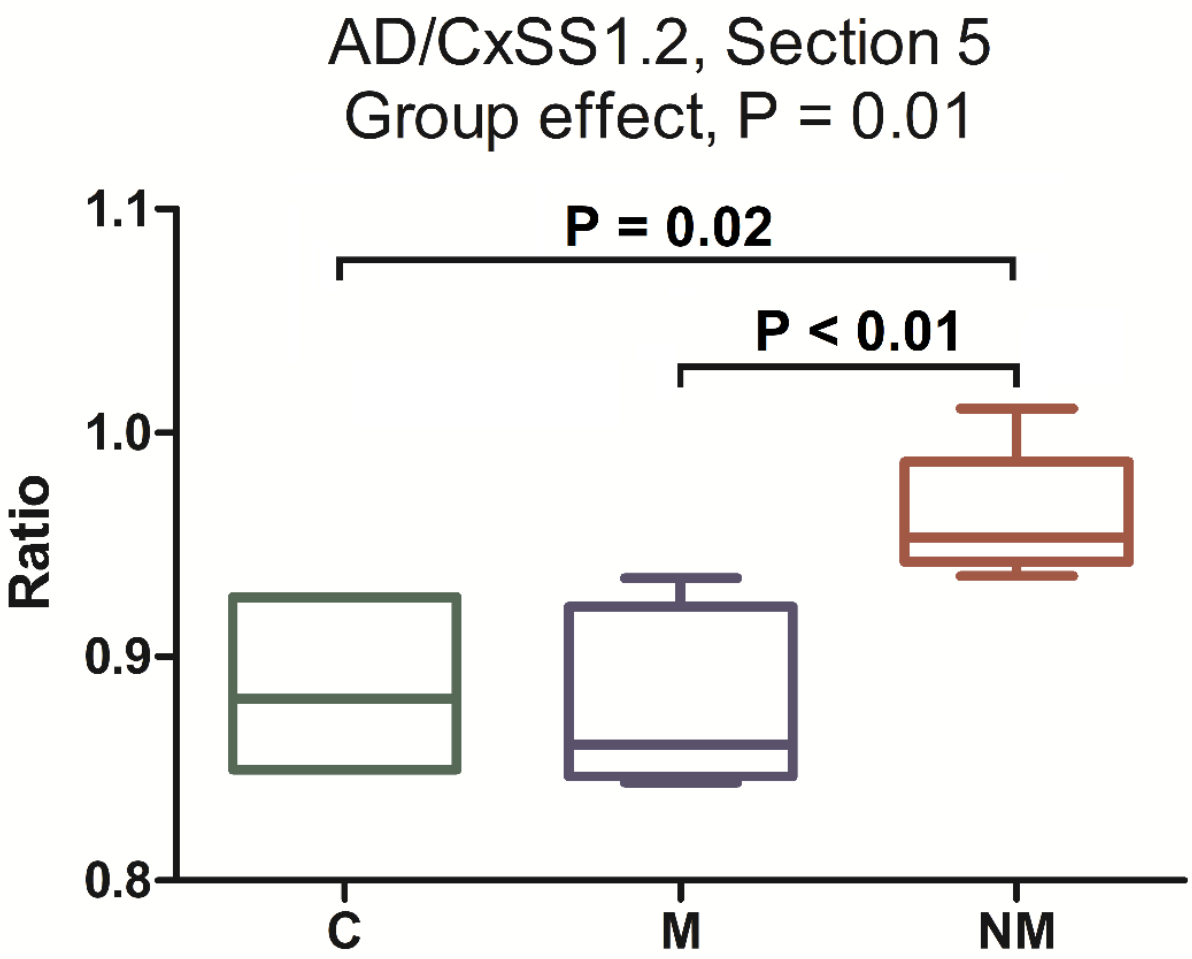


Figure 18. Boxplot of the optical densitometry ratio for the dorsal subiculum (Dsub) and the **(A and C)** auditory cortex (AUD); and **(B)** primary visual cortex (VS1), for Control (C), Monotonic (M) or Non-Monotonic (NM) subjects. *P < 0.05.

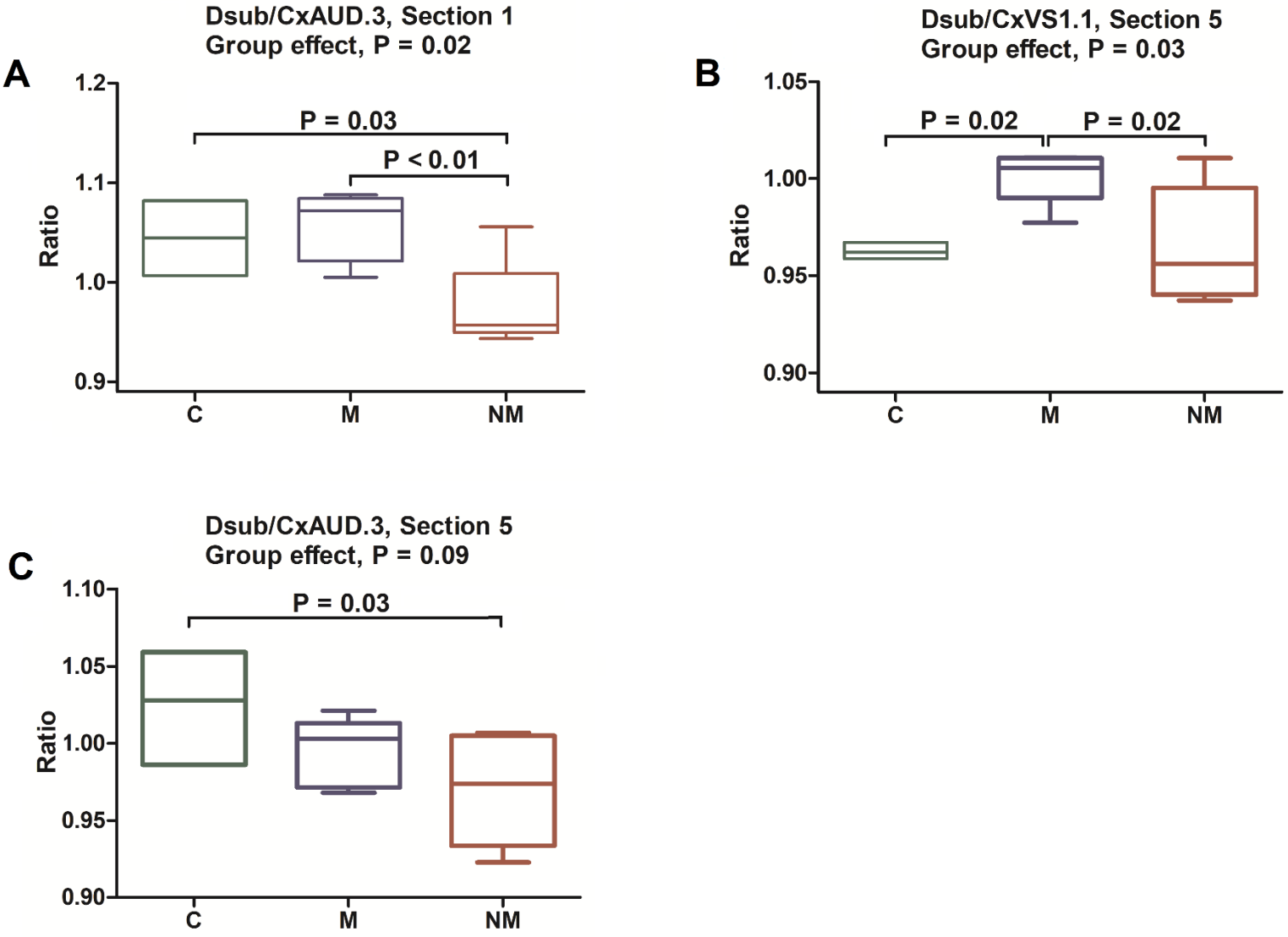
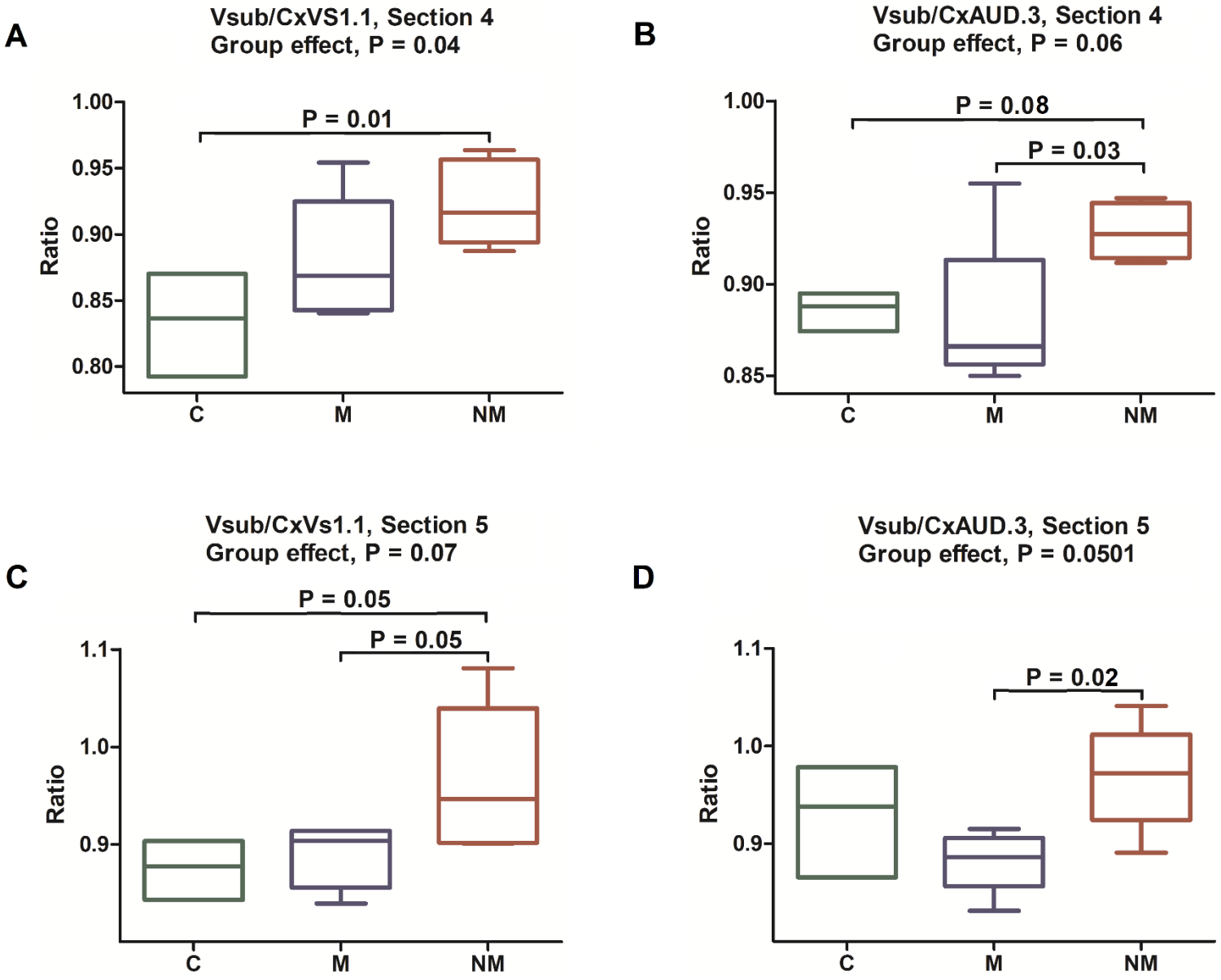


Figure 19. Boxplot of the optical densitometry ratio for the ventral subiculum (Vsub) and the **(A and C)** primary visual cortex (VS1); and **(B and D)** auditory cortex (AUD), for Control (C), Monotonic (M) or Non-Monotonic (NM) subjects. *P < 0.05.



4.3. Discussion

Optical density for cytochrome C oxidase stained sections focusing on the AVT, AD, Dsub and Vsub following training (20 sessions) and testing (21st session) in the extrapolation of serial stimulus pattern task revealed interesting

and intriguing results when comparing scores of C, M and NM subjects **(Figures 16, 17, 18 and 19)**.

In a general overview, NM subjects, as compared to C and M subjects, exhibited both greater optical density scores in the AVT **(Figure 16)**, AD **(Figure 17)** and Vsub **(Figure 19)** brain regions, and smaller scores in the Dsub region **(Figure 18)**. These differences seem consistent since they sustained despite the use of optical density scores involving distinct internal control cortical areas.

Surprisingly, the AVT, AD and Vsub increase in optical density scores seen for NM subjects was not observed for M subjects which density scores were similar to those observed in untrained C subjects. In this context, it seems important to note that behavioral performance in both M and NM subjects had already established by the fourth block of training, maintaining the same basic performance pattern up to the end of the fifth block of training **(Figure 7)**. In addition, performance of these groups did differ in the testing session, when the fifth extrapolation testing trial was introduced **(Figure 8)**.

Gabriel and colleagues have related anterior thalamic nuclei to learning of non-spatial information (Gabriel *et al*., 1983; Gabriel, Sparenborg & Stolar, 1987; Gabriel, 1993; Kang & Gabriel, 1998). According to these authors, the AVT would participate in the final processes of acquiring information, when the individual is already becoming proficient in the task (Gabriel, Sparenborg & Stolar, 1987; Shibata & Honda, 2015). In this context, it seems plausible to hypothesize that NM subjects, being confronted with a possibly more complex serial pattern as compared to M subjects [see Wallace and Fountain (2002)], could still be in the process of acquiring information about the serial pattern,

thus imposing a greater demand to the AVT as compared to M subjects. This would explain the greater COX optical density observed in NM subjects and the lack of difference between M and C subjects **(Figures 16A, B, C, D and E)**. In other words, M subjects, exposed to a simpler rule as compared to NM subjects, had already mastered the acquisition of task performance and thus would not engage the AVT any longer. Therefore, their pattern of COX activity, as revealed by COX optical density, would be similar to that seen in C subjects. A similar rationale could explain the increased COX optical density in the AD for NM, but not for M and C subjects **(Figure 17)**. Shibata and Honda (2012) demonstrated that the AD has greater activity in the initial stages of acquiring information. Such evidence corroborates the idea that the NM subjects are still in process of acquiring the pattern in which they were exposed, as opposed to the M subjects. This also raises an interesting point regarding the stage of learning in which the rats of the NM group are. A hypothesis to be considered is that if the complexity of the NM pattern is too high, it can still engage the activity of structures related to the beginning of acquiring information, even when these animals have already been exposed to several training sessions. That is, it is possible to consider that certain aspects of the NM pattern are still in the initial stages of acquiring the rules related to it, because of its complexity.

In relation to the subiculum, COX optical density revealed distinct staining patterns for its dorsal and ventral subcomponents **(Figures 18 and 19)**. That is, while NM subjects exhibited relatively lower COX expression in the Dsub as compared to both C and M subjects **(Figure 18)**, the reverse was observed for the VSub (**Figures 19B and C**, greater than both C and M groups; **Figure A**, greater than C group; **Figure D**, greater than M group). In this

context, it seems important to note that the internal controls for the Vsub activity were the auditory and primary visual cortices, components that are not determinant for performing the extrapolation of serial stimulus pattern task, corroborating the interpretation that this increase in Vsub COX activity relates to the task acquisition or performance.

There have been reports that the ventral subiculum controls stress when the animal is exposed to novelty (Shane O'Mara, 2005; Herman & Mueller, 2006). It is tempting to hypothesize that NM subjects, exposed to a greater uncertainty because of the complexity of their serial pattern as compared to M and C subjects, were exposed to a more stressful condition. Another possible related explanation, based on Fountain and Hulse (1981) views (see in 3.3. Discussion), would be that NM subjects did not completely learn the serial pattern to which they were exposed to, and incomplete learning would render training and testing with this serial pattern more stressful. This could explain the greater COX optical density for NM subjects as compared to M and C subjects. The result observed for M subjects can also be explained by these ideas. That is, because M subjects would have been exposed to a simpler serial pattern [see Fountain and Hulse (1981) and Wallace and Fountain (2002)], therefore easier to be learned and consequently predicted (Hulse & Dorsky, 1977; Rowan *et al*., 2001; Wallace, Rowan & Fountain, 2008; Kundey *et al*., 2019), thus generating less uncertainty, they would exhibit lower Vsub activity. In other words, M subjects were able to learn the serial pattern they were exposed to and thus could anticipate recurring events, even extrapolating the serial pattern, a situation which would be less stressful, thus with a smaller engagement of the Vsub.

In relation to the dorsal subiculum, NM subjects exhibited smaller COX optical density as compared to both C **(Figures 18A and C)** and M **(Figures 18A and B)** subjects, independently on using distinct internal controls, i.e., either primary visual cortex **(Figure 18B)** or the auditory cortex **(Figure 18A and C)**, for calculating optical density ratio. Reports indicate that Dsub is related to retrieving information already acquired (Roy *et al*., 2017; Melo, Favaro & Oliveira, 2020). It is then possible to raise the hypothesis that since the M group was exposed to a simpler pattern and was capable of fully learning it, the Dsub of this group would be more active, retrieving the acquired information to deal with the anticipation and extrapolation of the task. On the other hand, since the NM group was still in process of learning its more complex pattern, it seems reasonable to predict that the Dsub optical density of this group would be smaller, because probably there is no fully acquired information to be retrieved, hence, no engagement of the Dsub.

The greater optical density for NM subjects following COX staining for AVT and AD regions **(Figures 16 and 17)** seem to favor the interpretation that these animals, assumed to have been exposed to a more complex and harder-to-learn serial pattern task [see Fountain and Hulse (1981); Wallace and Fountain (2002)], were still engaging these brain structures for performance of the task by the end of training and testing sessions (see Gabriel, Sparenborg & Stolar, 1987; Herman & Mueller, 2006; Aggleton & Christiansen, 2015; Melo, Favaro & Oliveira, 2020). Following these guidelines, M subjects, assumed to have been exposed to a simpler and easier-to-learn serial pattern, had already completed acquisition of the task, thus prescinding of these brain structures to

perform the task. This would explain why their optical density scores did not differ in relation to those seen for C subjects **(Figure 16 and 17)**.

In its turn, just as it happened with the AVT and AD regions, there was greater optical density for NM subjects following COX staining for Vsub region **(Figures 19).** It seems to agree with the statement that these animals were exposed to a more stressful situation, due to the higher complexity of their pattern. That is, the smaller the domain of the task, the greater the stress and, therefore, the greater the engagement of the Vsub to deal with it. On the other hand, M subjects, trained in an assumed easier serial pattern, that would generate less uncertainty to be learned and consequently less stress, would present a lower activity of the Vsub. Explaining why there was also no optical density difference in this structure in relation to the C subjects **(Figure 19)**.

Finally, regarding the Dsub, NM group smaller COX optical density, in relation to M and C group, agrees to the hypothesis that the NM subjects were still in process of acquiring information in order to learn a more complex pattern **(Figure 18)**. It would also explain why M group has a higher COX optical density, since this group has already learned its pattern and, thus, is engaging the Dsub to retrive the abstracted information to deal with future exposure of the task **(Figure 18)**. M group even presents higher COX optical density in comparison not only to NM group, but also to C group in **Figure 18B**. These data are in accordance to records that states the Dsub's role in retrieving acquired information (Roy *et al.*, 2017; Melo, Favaro & Oliveira, 2020) to solve different tasks.

5. General Discussion

This study aimed at both improving extrapolation relying on serial stimulus patterns in association with a reduction of the amount of training sessions required for task acquisition. Such shorter training phase would be useful in future studies of brain structures involved in extrapolation. An additional aim was to evaluate COX expression, as a marker for activity in brain regions possibly involved in a postulated GPS after training and testing in this behavioral task. The present experiments focused on the AVT, AD and dorsal and ventral subiculum.

5.1. On the serial stimulus pattern

The results obtained during training and testing in the extrapolation of serial stimulus pattern task demonstrated that the rats were able to learn about the serial pattern they were exposed to, i.e., either M or NM, and abstract the respective rules, even when employing only 20 sessions. Previous studies had reported extrapolation effects after 51 (Fountain & Hulse, 1981) and 32 (Silva & Xavier, 2021) training sessions. This reduction in training phase associated with the maintenance of the extrapolation effect brings important practical advantage for those interested in studying neural substrates of anticipation and extrapolation. There have been reports that introduction of improvements in the apparatus and in the experimental methodology speeds up learning, as well as improves performance of rodents in training and testing cognitive tasks (Caglayan, Stumpenhorst & Winter, 2021).

Studies on serial pattern learning have shown that rodents are sensitive to structural rules of how the environment works, making them able to identify logical or repetitive sequences of events (Hulse, 1978; Hulse & Dorsky, 1977,

1979; Rowan *et al.*, 2001). Therefore, they are able to encode and store in memory different forms of information about serial stimulus patterns, such as rule learning, stimulus-response learning, serial position learning, timing and spatial learning (Stempowski, Carman & Fountain, 1999; Sharp *et al.*, 2018; Fountain, Rowan & Wollan, 2013; Muller & Fountain, 2010, 2016; Wallace, Rowan & Fountain, 2008). Simpler rules would be more easily coded. On the other hand, more complex rules would be more difficult to be represented by the organism, making its learning harder (Hulse & Dorsky, 1977, 1979; Fountain, Evensen & Hulse, 1983; Wallace & Fountain, 2002). Like humans, rodents seem to actively search for serial patterns, making this an important factor in abstracting rules about the environment (Kundey & Fountain, 2011).

For example, Fountain and Rowan (1995) exposed rats to a serial multiple choice procedure task. The animals were introduced to an octagonal chamber with retractable levers on each of the walls. In each trial the animal should choose a lever in the correct wall, forming a sequence that the animal should abstract. Choices of the correct lever led to a hypothalamic brain-stimulation reward. After the correct choice, the levers retracted, a 3-second interval was imposed and then the levers would be presented again, proceeding to the next trial. According to the number of the walls, the correct sequence of choices was as it follows:

123 234 345 456 567 678 781 812.

The animals were able to abstract the two rules of the task. The first would be the rule “+1” (proceed to the next clockwise lever) for 3 successive choices. The second rule, “-1” (anticlockwise), must be repeated only once, then returning to the previous rule. Then there are 3 new choices following the

“+1” rule, followed by a new choice adopting the “-1” rule. The results do not conflict with those of the present experiment, that is, rats can learn about sequences of events that constitute a pattern and use this learning to guide their behaviour. Analogously, the subjects of the present study trained with the monotonic pattern abstracted the rule they were exposed to, that is, that the next trial would offer fewer sunflower seeds at each trial per session. Likewise, behavioral results seem to suggest that the animals trained in the non-monotonic pattern also abstracted a rule and relied on it to guide their behavior during the extrapolation of serial stimulus pattern task.

As a matter of fact, information about sequences of events stored in memory is used to guide behaviour. Past experience helps to build “schema” employed for prompt reaction to environmental stimulation. They render learning about new related practices faster as these latter are incorporated into pre-existing schemas (Tse *et al*., 2007; Baker *et al*., 2014; Redish, 2016). For instance, Tse and colleagues (2007) introduced rats to an arena containing several sand wells, in fixed locations. Some of these sand wells contained food buried in it. The animal was exposed to an aroma immediately before entering the arena, such as the aroma of banana, and later finding in the arena the sand well that contained the banana, which was used as a reward. After a month of training the animals were introduced to a different arena, containing additional sand wells. According to the authors, since they already had a consolidated memory of the initial arena, the rats were able to learn the new conformation, with more elements added, after just one training session. This result shows that rats incorporate new information into previously stored representations of related experiences. This allows to build and associate memories for past

experiences rendering learning more efficient, thus helping to guide behavior, which constitutes an advanced cognitive skill (Shettleworth, 2010; Thornton & Lukas, 2012; Boussard *et al*., 2020).

5.2. On the generation of predictions

The understanding that animals are able to use rules abstracted from serial patterns to predict sequences of stimuli is a well-established concept (Rowan *et al.,* 2001). As expected, the rats of the present study seem to have abstracted a rule from the serial pattern to which they were exposed. Thus, it seems possible to claim that once this rule was established, the animals were able to anticipate the reinforcement of each run in a session, based on what was previously experienced. This hypothesis is evidenced by the behavior presented by the animals, particularly the rats of the M group, that increased the latency throughout the four trials of a session, as the reinforcement decreased.

Several studies have contributed for understanding the rodents' ability to anticipate events in different situations (e.g., Flaherty & Checke, 1982; Flaherty & Rowan, 1985; Onishi & Xavier, 2011). For example, in the incentive contrast situation (Flaherty & Checke, 1982), while one group of rats received access to a 0.15% saccharin solution (first drinker) followed, minutes later, by a 30% sucrose solution (second drinker) (Group 0.15-30), another group of rats received access to a 0.15% saccharin solution (first drinker) followed by a 0.15% saccharin solution (second drinker) (Group 0.15-0.15). This procedure is repeated for both groups over several training days. Therefore, the two groups receive the same solution, 0.15% saccharin in the first drinker, but different solutions in the second drinker. The animals' preference for 30% sucrose is

evident, thus reflecting in a higher consumption of this solution. Although the first solution, 0.15% saccharin, is identical for both groups, its consumption along successive days strongly differ between groups. That is, while subjects 0.15-0.15 exhibited a substantial increase in the consumption of the first solution, subjects 0.15-30 exhibited a substantial reduction of consumption of the first solution. In other words, the pending offer of a preferred, 30% sucrose, second solution leads the animals to consume less 0.15% saccharin offered as first solution, as if they were “reserving stomach space” for the consumption of the preferred solution. This behavior, named anticipatory contrast, together with results of other studies (see Flaherty, 1996) have added to evidence that rats anticipate the future occurrence of an event by exhibiting behaviors consistent with that anticipation (Flaherty & Checke, 1982; Flaherty & Rowan, 1985; Onishi & Xavier, 2011). It is important to emphasize that these anticipatory behaviors involving rodents seems a “memory-based reconstruction of previous experiences” about either the environment or the environmental contingencies, since the animals were exposed, along training, to the pairs of solutions within the same experimental condition.

There have been alternative explanations for the anticipatory contrast phenomenon. For instance, that saccharin decrease in consumption in the 0.15-30 group is not related to anticipation, but instead because the animals remember the last solution received the day before (30% sucrose); thus, the animals would compare it with the first solution received at the moment (saccharin 0.15%) and therefore reduce consumption.

To test this hypothesis, Flaherty and Rowan (1985) trained rats by offering pairs of solutions (e.g., 0.15% saccharin and 30% sucrose) that were

alternated each day for several successive days. Therefore, each animal received the 0.15-0.15 pair on one day and 0.15-30 on the other. Each pair of solutions was associated to different stimuli, which served as cues for the pair that would be used every day. Since the pairs of solutions were presented on alternate days, on the day the animals received the 0.15-30 pair, the second solution from the previous day had been 0.15 (0.15-0.15 pair). Thus, if the hypothesis that the rats reduce consumption because they would compare the last solution received the day before with the one received at the moment was correct, there should be no reduction in consumption on the days of 0.15-30, as the last solution in the previous day was equal to the first solution of the day under test. But results demonstrated that saccharin consumption decreased only when the associated stimuli indicated that the second solution would be 30% sucrose, and not when they indicated that the second solution would be 0.15% saccharin. The authors point to these results as further evidence that the contrast is based on anticipation of the pending preferred stimulus, 30% sucrose, and not on a comparison with the last solution consumed the day before. Together with evidence from other studies (Flaherty & Checke, 1982; Onishi & Xavier, 2011), these results have led to the conclusion that rats anticipate the future occurrence of an event by exhibiting behaviors consistent with that anticipation.

Data of the present study are congruent with Hulse and Campbell's (1975) proposal that accurate animal response to a serial pattern of stimuli is related to anticipation of the amount of reinforcements to be received. This requires quantification of each reward, order the different amounts and store

this information about the sequence in memory, thus allowing to use it for generating the anticipation of a pending reward.

Rodents' ability to generate predictions based on acquired rules seems not limited to recurring events. The results of the present study indicate that predictions can also be generated by extrapolation, relying on learned rules, to novel unexperienced events (see Silva & Xavier, 2021). The results in the testing session, involving addition of a fifth, extrapolation trial, immediately after the fourth trial, in the 21st session, indicates that rodents are able to extrapolate unprecedented events based on information stored in memory. These results are in line with data reporting the ability of different species to extrapolate learned rules to events never experienced before (Mercado *et al*., 2000; Spetch & Friedman, 2003; Howard *et al*., 2017), a cognitive ability considered advanced in humans and non-human primates (Srinivas & Schwoebel, 1998; Wallis, Anderson & Miller, 2001; Spetch & Friedman, 2003; Merritt *et al*., 2011). They also resemble data observed for humans, where the prediction generated by volunteers about an athlete's next unprecedent moves is influenced by their pattern of previous moves (Gray, 2002; Loffing, Stern & Hagemann, 2015). For example, volunteers exposed to a pattern of four series of similar moves made by a volleyball player tend to generate the expectation that the fifth move will correspond to the previous pattern, that is, that the athlete will perform an action similar to the preceding ones (Loffing, Stern & Hagemann, 2015). Thus, extrapolation is characterized by comparing current information (the athlete's movement) and expectations (the rule abstracted through the pattern of previous events), based on the volunteers' prior knowledge of previous moves (Körding, 2007).

There have been alternative interpretations for the behavior exhibited by rats during the extrapolation of serial stimulus pattern. For instance, Capaldi and colleagues (1980) proposed that a discrimination learning by the subjects would explain the results. That is, each amount of reinforcement in a sequence of stimuli would form a distinct memory that would serve as a cue for the reinforcement that would follow in the next element of the sequence. This memory would then be used to cause an excitatory or inhibitory response, stimulating the rat to run faster or slower on the next trial (Capaldi, Verry & Davidson, 1980; Capaldi & Verry, 1981; Capaldi & Miller, 2003). So, instead of learning a general rule regarding the entire sequence of stimuli, the animals would form a specific memory for each element of the sequence, that would allow it to generate a proper behavior for the next element of the trial. Thus, in Capaldi and colleagues' view, in a reinforced series with 14-7-3-1 elements, the reward of the first trial (14 food pellets) would be remembered in the second trial (7 pellets), becoming a signal to anticipate the reinforcement of 7 pellets in future sessions and so on (Capaldi, Verry & Davidson, 1980).

Fountain and Hulse (1981) showed that the interpretation of Capaldi and colleagues would not be the main strategy used by rats, and on the other hand, the animals would indeed abstract a rule regarding the sequence of stimuli. That is, in the experiment carried out by Fountain and Hulse (1981) (see 1. Introduction; 1.1. Serial Stimulus Pattern, for further details), the animals were trained to perform a series of 4 consecutive trials, i.e., 14-7-3-1. During the test, done in a single session, a fifth trial, never seen before, was added. According to Capaldi's interpretation, the rats would not be able to make any association between the previous trial and the fifth (unprecedented) one. The amount of 1

pellet, therefore, could not serve as a clue to the fifth trial, as it has never been experienced before by the rats. Thus, the animals should not be able to extrapolate the content of the fifth trial, which was not the case, since, for example, rats exposed to the monotonic pattern were able to extrapolate the event of the fifth trial, running slower. The present experiment then did confirm that rats are able of abstracting rules based on sequences of stimuli. Therefore, one cannot ascribe the extrapolation result to the introduction of a novel fifth trial, otherwise both M and NM subjects would have shown increments in their running times in the fifth trial, which was not the case. In other words, because only subjects of the M group exhibited such increase one can discard the possibility that the extrapolation effect has any relationship with the novel fifth trial.

5.3. On the brain structures related to generation of predictions

COX histochemistry revealed differences in optical densitometry between the NM, M and C groups. When observing the optical densitometry scores of the AVT and AD, the NM group has greater neural activity in this structure than the animals of the M and C groups. However, the M and C groups have no difference in densitometry between them **(Figures 16 and 17)**. Vsub presents similar results to AVT and AD **(Figures 19)**, in which the NM group has greater optical densitometry than the M and C groups. In turn, note that the Dsub presents an opposite pattern, that is, the M and C groups presented higher optical densitometry scores than the animals of the NM group **(Figure 18)**.

As mentioned above (1. Introduction; 1.2. Neural Substrates Underlying Generation of Predictions), Gray (1982) proposed that the septo-hippocampal

system would continuously monitor the environment. It would perform this function by comparing predictions relying on memories of past regularities (generated by a GPS that includes the subiculum, AVT, mammillary bodies and cingulate cortex) and current sensory information (provided by the entorhinal cortex). When current and predicted information match, the ongoing behavior is maintained. In contrast, when current and predicted events mismatch, the septo-hippocampal system interrupts the ongoing behavior and cause exploratory activity. This allows gathering additional environmental information that would support better predictions in the future (Schacter & Addis, 2007; Duncan *et al*., 2012; Sinclair & Barense, 2019; Bein, Duncan & Davachi, 2020; Quent, Henson & Greve, 2021; Sinclair *et al.,* 2021; Lee, Aly & Baldassano, 2021).

Silva and Xavier (2021) demonstrated that selective damage to AVT and AD impairs extrapolation of serial stimulus patterns, thus corroborating Gray's (1982) proposal involving the participation of the AVT in a generation of predictions system. Interestingly, this impairment was restricted to extrapolation, and spared for anticipation of recurring events. That is, even though the running times exhibited by lesioned subjects reflected the pending amount of reward to be received at the end of each trial, extrapolation in the novel fifth trial did not show up as it occurred for sham-operated controls. This result revealed that lesioned subjects not only discriminated the amount of food received at the end of each trial but also were able to rebuilt, from memory, the sequence of amounts of reward received. Congruent with these results, there have been reports that anticipation is a form of prediction distinct from extrapolation

(Haggbloom & Brooks, 1985; Fountain, 1990; Krushinsky, 1990; Poletaeva & Zorina, 2015).

Studies on the contribution of the ATN for orienting of attention also add to this discussion. For instance, Wright and colleagues (2015) showed that rats with ATN damaged exhibit impairments on the ability to orient attention to predictive stimuli in an attentional set-shifting task. Congruently, Bubb and colleagues (2021) reported similar results when interrupting projections from the anterior cingulate cortex to the ATN. The authors' interpretation was that the anterior cingulate cortex updates the ATN with current information stored in memory, thus modulating the ability of the ATN to turn attention to predictive stimuli (Bubb *et al.,* 2021; Wolff *et al.,* 2021). That is, events with predictive value would receive more attention.

Several studies have reported the existence of head direction cells, that fire action potentials only when the rat's head is pointing in a specific direction in the horizontal plane, in the ATN (Clark & Taube, 2011; Tsanov *et al*., 2011; Zirkelbach, Stemmler & Herz, 2019) and post-subiculum (Taube *et al*., 1990; Taube, 1995; Taube, 1998). Butler and colleagues (2017) showed that interference with the HDC normal function impairs spatial navigation in rodents. In the ATN, the HDC exhibit anticipatory activity, that is, they are better related to the future direction that the animal's head will point rather than the direction it is pointing in the present or it had pointed in the past (Taube, 1995; Blair & Sharp, 1995; Blair, Lipscomb & Sharp, 1997; Bassett *et al*., 2005; Zirkelbach, Stemmler & Herz, 2019). Thus, there seems to be a thalamic-cortical circuit, in which ATN HDC predictions project to the post-subiculum, providing future information for when it has to update its representation of the present state of

HDC (Blair & Sharp, 1995). In order to generate predictions, ATN HDCs would use information from the post-subiculum, which would provide ways to generate a given prediction (Blair & Sharp, 1995). In other words, HDC anticipatory activity allows to guide attention, by moving the head, to specific points in the environment, where predicted events are supposed to happen, an idea similar to Gray's proposal (1982).

When a rat is in a specific location of a maze, hippocampal place cells tend to fire in theta rhythm (6-10 HZ) (O'Keefe & Nadel, 1978). It seems important to note, however, the existence of evidence for predictive patterns in hippocampal place cells, as well. For instance, Redish (2016) have noted that this hippocampal theta rhythm is also seen when the animal is deliberating about where to go at intersection points in a maze. In other words, these fires seem to correspond to actions the animal will take in the future, instead of the behavior that it is presenting in the moment (Schmidt *et al*., 2009; Gupta *et al.*, 2012). Similar evidence has been reported for humans, in which the hippocampus is required for episodic future thinking (Buckner & Carroll, 2007; Redish, 2016). Altogether, these results strengthen the notion that hippocampal formation and ATN participate in the generation of predictions, contributing to build a representation of what is to come relying on past experiences (Eichenbaum, 1997; Eichenbaum & Fortin, 2009; Bubic, Cramon & Schubotz, 2010).

The subiculum and the anteroventral thalamus have been reported to be part of an "extended hippocampal system", involved with spatial memory in rodents (Aggleton & Brown, 1999; Aggleton *et al.,* 2010). Congruently, lesions either in the ATN or in the mammillary bodies cause different degrees of

impairment in tests of spatial learning in rats (Vann & Aggleton, 2004). Increased metabolic activity of the anterior thalamic nuclei, mammillary bodies and hippocampal formation is revealed by COX after the first day of training in tasks requiring learning of spatial memory. However, while ATN and mammillary bodies no longer exhibit such metabolic increase at later stages of testing, hippocampal formation maintained it increased together with an increased activity in the prefrontal cortex (Conejo *et al.,* 2010).

Evidence from scientific literature, using electrophysiological technique to record the hippocampal formation and the ATN, appears to present a different neural activity pattern of these structures when non-spatial tasks are involved. In discriminative avoidance conditioning, subjects learn to avoid shock by moving itself in a wheel at a precise moment. Gabriel, Sparenborg and Stolar (1987) recorded (using electrophysiology) specific regions of the hippocampal formation and the ATN during the performance of the task, respectively the subiculum and the AVT. The subiculum presented greater activity in the early learning stages of the task, when the subject is being presented to new information, in contrast, the AVT presented greater activity in the late stages of the task, when the subject is in the final stages of acquiring information about it.

According to these results, the authors (Gabriel, Sparenborg & Stolar, 1987) proposed a model, where the subiculum would deal with the detection of novelty and mismatch during the performance of the task, that is, it would support the animal to get new information that it may need to predict when to move in the wheel to avoid the shock. The AVT, in turn, would generate excitatory stimuli involved in the induction of behavioral conditioned response, in other words, it would act governing the behavior when the rules for the

realization of the discriminative avoidance have already been well established (e.g., criterial session, extinction, overtraining and reacquisition). So, based on this model, when external conditions are unprecedented, as seen for the first time or different from what was predicted (for example: first conditioning or first discrimination sessions), the subiculum would inhibit any conditioned behavior stimulated by the AVT activity, in order to deal with a possible mismatch and gather new information to generate more accurate behavior in the future (Gabriel, Foster & Orona, 1980; Gabriel *et al.,* 1983; Gabriel, Sparenborg & Stolar, 1987; Kang & Gabriel, 1998; Shibata & Honda, 2015). Note how the model concerning discriminative avoidance conditioning is similar to that proposed by Gray (1982), regarding the participation of the hippocampal formation in the detection of mismatches. Thus, structures of the hippocampal formation and the ATN, including the subiculum and the AVT, seem to participate in performance of distinct tasks, playing a role that involves the use of memories of past regularities for detection of mismatch and generation of predictions.

The COX optical density ratio analyses for the AVT and AD revealed increased activity after 20 training sessions and exposure to the extrapolation session in the NM subjects as compared to both C and M subjects **(Figures 16 and 17)**.

As discussed above, Gabriel, Sparenborg and Stolar (1987) proposed that the AVT participates in later stages of information acquisition, when learning is already well established. Following these guidelines, it seems plausible to hypothesize that because of the complexity of the NM schedule (see Fountain & Hulse, 1981), NM subjects were still going through the process

of learning the task after 20 training sessions. This would contrast with M subjects that had already learned it, because the M schedule would be relatively easier (see Fountain & Hulse, 1981). This would explain why NM subjects exhibited relatively greater COX density in the AD and AVT while M subjects did not.

The present study revealed differences in the relative COX density of the dorsal and ventral portions of the subiculum associated with the serial pattern employed along training and testing in the extrapolation task **(Figures 18 and 19)**. That is, while the dorsal subiculum exhibited, in general, a decrease in COX density in subjects exposed to the NM serial pattern of training **(Figure 18)**, the ventral subiculum exhibited in general a marginally significant increase in COX density in subjects exposed to the NM training **(Figure 19)**.

The ventral subiculum projects to the hypothalamus via the postcommissural fornix, the medial cortico-hypothalamic tract and the amygdala (Shane O'Mara, 2005) and appears to have an inhibitory function on the HPA axis, thereby exerting control over the stress response (Shane O'Mara, 2005; Herman & Mueller, 2006; Quintero *et al.,* 2011). Unexpected events, such as when an animal is introduced into an open field, can trigger innate stress responses in anticipation to hazard, such as a predator attack (Herman & Mueller, 2006). The ventral subiculum seems to control responses to potentially stressful events, by engaging previous experiences. Thus, greater ventral subiculum activity in NM subjects, could be related to the need for controlling less predictive and thus relatively more stressing contexts, since these subjects would have weaker predictions about the reward pending in the next trial (see Fountain & Hulse, 1981), both along training trials and during the fifth trial of the

testing session. Distinctively, subjects exposed to the “easier to learn” (see Fountain & Hulse, 1981) M serial pattern, would generate stronger predictions about the reward pending in the next trial, thus rendering the subject less stressed, therefore reducing the ventral subiculum activity.

The dorsal subiculum seems to be engaged in the processing of information about space, movement and memory (Shane O'Mara, 2005; Aggleton & Christiansen, 2015; Kitanishi, Umaba & Mizuseki, 2021). For instance, performance in spatial working memory tasks is disrupted following damage to the dorsal subiculum (Potvin, Doré & Goulet, 2007). In addition, c-fos expression is increased in the dorsal subiculum of rodents following performance of spatial working memory tasks (Vann *et al.,* 2000; Jenkins *et al.,* 2004; Aggleton & Christiansen, 2015). Furthermore, there have been demonstrations that this brain region is involved in retrieval of stored information, but not in the acquisition of new ones (Roy *et al.,* 2017; Melo, Favaro & Oliveira, 2020).

These observations add to the present discussion that M subjects have learned proficiently the pattern they had been exposed to, in contrast to NM subjects. That is, M subjects were using stored memories about learned and abstracted rules involving the monotonic pattern, to deal with the extrapolation task. In other words, M subjects became proficient in using stored information to generate predictions about the pending reward in the next trial, both in training trials and in the fifth never experienced testing trial. Thus, greater activity of the Dsub for M subjects during retrieval of fully acquired information, in order to generate an extrapolation, would be expected. On the other hand, since NM subjects were still in process of fully learning the pattern that they were

exposed, the role of the Dsub is not yet so prominent as it is for the M group, so it would also be expected that the Dsub for the NM group would not be so active. In other words, subjects of the NM group were probably generating predictions based on not so well stablished information.

Therefore, COX staining seems to have been an interesting initial approach to investigate activity of brain structures potentially involved in performance of behavioral tasks (Conejo *et al*., 2004; Conejo *et al*., 2007; Conejo *et al*., 2010). Through it, it was possible to obtain results that allowed to understand with further details the activity of structures that were proposed to participate in a GPS, mainly the subiculum and the AVT (Gray, 1982). Not only that, but it allowed better understand the activity of these structures in face of different degrees of complexity of serial stimulus patterns (monotonic and non-monotonic).

Taking into account the results obtained by COX histochemistry, the NM group seems to be still in the process of learning the pattern to which they were exposed, due to its higher degree of complexity, a result observed by the higher optical density of the AVT, AD and Vsub. In contrast, it is interesting to note that for M group, the animals were able to abstract their simpler rule and use it to extrapolate the result of the fifth trial of the test session, increasing latency by predicting that there would be a smaller amount of reinforcement at the end of this unprecedented trial **(Figure 8)**. This rapid acquisition of the animals in the M group is supported by the lower optical density of the AVT/AD and Vsub, which, as shown above, are related respectively to different phases of information acquisition (Gabriel, Sparenborg & Stolar, 1987; Shibata & Honda, 2012) and to the stress caused by novelties (Shane O'Mara, 2005). It was also

corroborated by the higher optical density of the Dsub, which is related to the retrieval of information stored in memory (Melo, Favaro & Oliveira, 2020). That is, it seems that these animals were already using information stored in memory to deal with the extrapolation of serial stimulus patterns task.

Finally, a hypothesis to deepen the contribution of the data obtained in this project would be to expose M group subjects to a smaller number of trainings, before performing the test. If rats trained in the monotonic pattern performed the extrapolation of serial stimulus patterns task after a smaller number of training sessions, there would probably be an increase in the optical density of AD, AVT and Vsub, and a decrease in the optical density of Dsub, since these animals would still be in the process of abstracting the monotonic pattern. Furthermore, changing the number of sessions before the test in M group could help to clarify the role of AD and AVT in the generation of prediction. In the present project, higher optical density was seen for both AD and AVT structures, in animals trained in a more complex NM pattern (for further details, see 4.3. Discussion). Possibly, it would be clearer to observe a difference in the moment of participation of each structure in animals trained in a simpler pattern, such as the M pattern. One hypothesis is that there would be greater optical density of the AD in the initial stages of the training, while the AVT would present higher optical density in the advanced stages of the training, in agreement with AD (Shibata & Honda, 2012) and AVT (Gabriel, Sparenborg & Stolar, 1987) reports from scientific literature.

6. Conclusion

Rodents are capable of using past experience to generate predictions about pending events. Such predictions may involve both recurring events, named anticipation, and never experienced events predicted by the application of rules relating serial patterns, named extrapolation.

Studies on generation of predictions are limited by the availability of tasks that provide unequivocal measures of anticipation. Extrapolation of serial stimulus patterns seems to allow evaluation of anticipatory responses about both recurrent and novel events.

Serious constraints involving the use of this task relate to the number of training sessions required for the subjects to anticipate events and the fact that extrapolation is evaluated in a single testing session.

One of the aims of this study was to modify the experimental apparatus and training procedure in order to perform the extrapolation testing after a smaller number of training sessions, whilst ensuring abstraction of the serial pattern rules and thus extrapolation. The evolution of performance by the subjects exposed to M and NM serial patterns, along twenty training sessions, as well as their performance in the extrapolation testing session, confirmed data of previous studies reporting extrapolation following a greater number of training sessions (Fountain & Hulse, 1981; Silva & Xavier, 2021). This indicates that the present version of the task allowed achieving that aim.

Another aim of the present study was to gather evidence that brain structures included in a postulated generator of predictions system, particularly the AVT and the subiculum (see Gray, 1982), would exhibit changes in their activity, as revealed by COX histochemistry, following training and testing in the extrapolation of serial stimulus pattern task. In general, results showed (1)

increased AVT and AD activity and (2) reduction of dorsal subiculum activity, in both cases in NM subjects, but not in M subjects. These figures, in association with relevant literature, suggests that the AVT may be involved in learning processes of information latter used in extrapolation, and that the dorsal subiculum may be involved in retrieval of information required for predicting.

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