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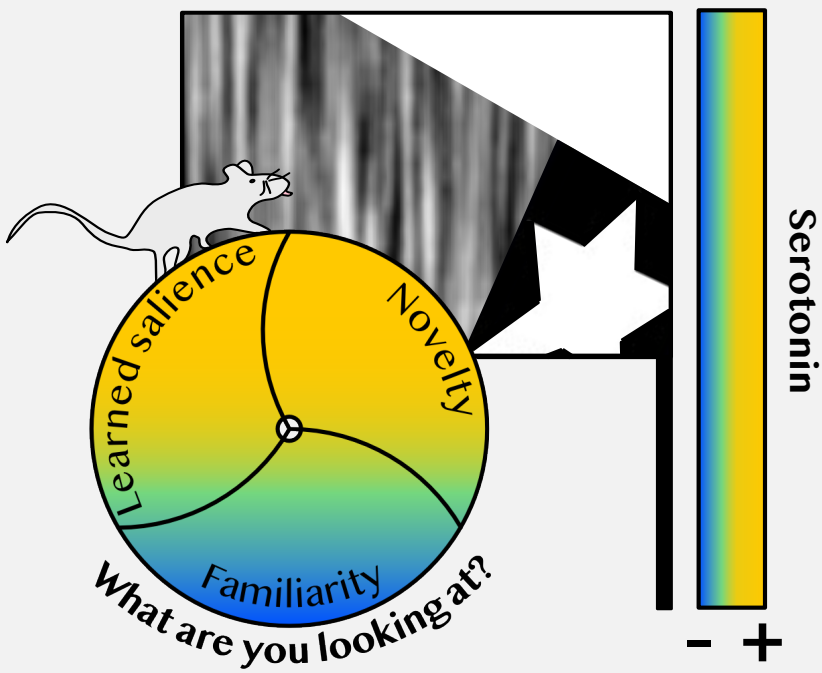
Evolution of Serotonin Responses to Visual Stimuli

Novelty, Familiarity and Learned Salience

Solène Sautory



Dissertation presented to obtain the **Ph.D degree in Neuroscience**

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INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA ANTÓNIO XAVIER | UNIVERSIDADE NOVA DE LISBOA

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Instituto de Tecnologia Química e Biológica | Universidade de Lisboa

Research work coordinated by:

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EVOLUTION OF SEROTONIN RESPONSES TO VISUAL STIMULI

NOVELTY, FAMILIARITY AND LEARNED SALIENCE

SOLÈNE SUZANNE CÉCILE JOËLLE MARIE SAUTORY

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Abstract

Neuromodulatory systems in the brain play a pivotal role in processing behaviourally relevant cues, such as rewards, punishments, and intense sensory stimuli. As such, the context in which stimuli are presented can greatly influence these responses. The neuromodulator serotonin (5-HT) from the dorsal raphe nucleus (DRN), amongst others, has been shown to respond to salient sensory stimuli. Familiar stimuli encountered unexpectedly in the environment also induce serotonin release, interpreted as prediction error signals, broadcasted throughout the brain. As such, serotonin has been identified as a signal for surprise in tasks involving abrupt reversals of learned associations. However, limited research was carried out to understand how DRN serotonin neurons respond to novel cues that become relevant after associative learning.

Our study investigates how serotonin neurons respond to novel visual cues and during the process of learning. We created a virtual reality (VR) setup where head-fixed mice running on a wheel controlled the flow of the virtual environment. Mice learned to associate pairs of images on corridor walls with rewards across ten consecutive days. The corridors either contained fixed or ambiguous image pairs, and these images were novel on the first day of the task. Using fiber photometry, we recorded serotonin neuron activity in the DRN throughout the task. Serotonin neurons responded strongly to novel and unexpected images on the first day, but this response adapted across image repetition. Over the first few days, while the images remained non-informative for rewards, serotonin responses stayed in this adapted state. Their responses recovered in the later stages of learning, correlating with the animals' task performance.

To assess whether learning was the key factor influencing the recovery of these responses to images, we ran a control task where rewards were delivered randomly, without requiring associative learning. In this control group, serotonin neurons responded to novel images and adapted across image repetition but did not show response recovery across days, suggesting that learning is crucial for the recovery of serotonin responses to images. We also separated serotonin signals into motion-dependent and motion-independent components based on their correlation with locomotion, finding that learning-related signals were predominantly present in MI serotonin activity.

Serotonin neurons also differentiated rewarded and unrewarded images in the task, with MI signals responding selectively to reward-predicting cues. In contrast, MD signals were more responsive to unrewarded images. In a preliminary reversal task, serotonin neurons' responses reduced during relearning but regained selectivity for reward-predictability once learning stabilized. Additionally, serotonin neurons responded most strongly to large, unexpected stimuli, adapting across repetitions.

Overall, our findings reveal that serotonin neurons respond to novel and unexpected stimuli, whilst also signalling behaviourally relevant cues. As such, the activity of serotonin neurons appears sensitive to contextual information of sensory cues, namely, predictability and learning, whilst keeping track of the animal's internal and behavioural states. This flexible modulation could allow the serotonergic system to optimize behaviour in dynamic environments.

Título

Evolução das Respostas da Serotonina a Estímulos Visuais:

Novidade, Familiaridade e Saliência Aprendida

Resumo

Os sistemas neuromoduladores do cérebro desempenham um papel fundamental no processamento de sinais relevantes para o comportamento, tais como recompensas, castigos e estímulos sensoriais intensos. Como tal, o contexto em que os estímulos são apresentados pode influenciar grandemente estas respostas. Foi demonstrado que a serotonina (5-HT), entre outros, responde a estímulos sensoriais quando associados a resultados comportamentais relevantes. Os estímulos familiares encontrados inesperadamente no ambiente também induzem a libertação de serotonina, interpretada como sinais de erros de previsão transmitidos por todo o cérebro. Como tal, a serotonina foi identificada como um sinal de surpresa em tarefas que envolvem reversões abruptas de associações aprendidas. No entanto, foram realizados poucos estudos para compreender como os neurónios serotoninérgicos respondem durante a aprendizagem associativa de novos estímulos a resultados relevantes para o comportamento.

O nosso estudo centra-se na forma como os neurónios serotoninérgicos respondem às pistas visuais durante o processo de aprendizagem. Criámos uma configuração de realidade virtual (RV) em que os ratos fixados à cabeça, correndo numa roda, controlavam o ambiente virtual. Durante dez dias, os

ratinhos aprenderam a associar pares de imagens nas paredes dos corredores a recompensas. Os corredores continham pares de imagens fixas ou ambíguas, com imagens novas apresentadas no primeiro dia. Utilizando fotometria de fibra, registámos a atividade dos neurónios serotoninérgicos no núcleo dorsal da rafe (DRN) ao longo da tarefa. Os neurónios serotoninérgicos responderam fortemente a imagens novas e inesperadas no primeiro dia, mas esta resposta adaptou-se com a repetição. Durante os primeiros dias, enquanto as imagens permaneciam não informativas para as recompensas, as respostas da serotonina mantiveram-se neste estado adaptado, mas recuperaram nas fases posteriores da aprendizagem, correlacionando-se com o desempenho dos animais na tarefa.

Para avaliar se a aprendizagem era o fator-chave que influenciava estas respostas, realizámos uma tarefa de controlo em que as recompensas eram entregues aleatoriamente, sem necessidade de aprendizagem. Neste grupo de controlo, os neurónios de serotonina adaptaram-se a novas imagens, mas não mostraram recuperação ao longo dos dias, o que sugere que a aprendizagem é crucial para a recuperação das respostas da serotonina. Também separámos os sinais serotoninérgico em componentes dependentes do movimento (MD) e independentes do movimento (MI), descobrindo que os sinais relacionados com a aprendizagem estavam predominantemente presentes na atividade da serotonina MI.

Os neurónios de serotonina também diferenciaram entre imagens recompensadas e não recompensadas na tarefa, com os sinais MI a responderem seletivamente a pistas que preveem recompensas. Em contrapartida, os sinais MD respondiam mais às imagens não recompensadas. Numa tarefa de reversão preliminar, as respostas dos neurónios serotoninérgicos diminuíram durante a reaprendizagem, mas recuperaram a seletividade para a previsibilidade da recompensa quando a aprendizagem estabilizou. Além disso, os neurónios serotoninérgicos

responderam mais fortemente a estímulos grandes e inesperados, adaptando-se às repetições.

De um modo geral, os nossos resultados revelam que os neurónios serotoninérgicos respondem a estímulos novos e inesperados, enquanto sinalizam pistas comportamentais relevantes. Como tal, a atividade dos neurónios serotoninérgicos parece ser sensível à informação contextual das pistas sensoriais, nomeadamente, à previsibilidade e à aprendizagem, enquanto acompanha os estados internos do animal. Esta modulação flexível poderá permitir ao sistema serotoninérgico otimizar o comportamento em ambientes dinâmicos.

Keywords

- Serotonin
- Learning
- Surprise
- Novelty
- Familiarity
- Learned salience
- Reward
- Uncertainty
- Prediction error
- Information and meaning
- Virtual reality
- Mice
- Fiber photometry

Author Contributions

This work was carried out at the Fundação Champalimaud (CF), under the International Neuroscience Doctoral Programme (INDP) of the year 2017/2018. This thesis was written by Solène Sautory. The task was designed in collaboration with Dr Leopoldo Petreanu and Dr Zachary F. Mainen. All experimental work, including building the virtual reality setup, performing surgeries on mice and running the experiments, was performed by Solène. All datasets were analysed by Solène. The analyses presented in section 7 were done in collaboration with Stefan Hadjuk. Detailed contribution is provided within the section.

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List of Abbreviations

5-HT	Serotonin
AI	Artificial intelligence
DRN	Dorsal raphe nucleus
LTP	Long-term plasticity
MRN	Median raphe nucleus
RPE	Reward prediction error
VR	Virtual reality
SEM	Standard error of the mean
MI	Motion-independent
MD	Motion-dependent

1. General Introduction

1.1. Serotonin, a “Feel-Good Hormone”?

With the recent development of machine learning and artificial intelligence (AI), key concepts such as "neural networks" and "reinforcement learning" have spread outside of the scientific orbit and into general knowledge (Pasick, 2023). It would be interesting to explore how these AI advancements might influence the general public's understanding of the brain. Media has long shaped societal views of neuroscience, and since the late 20th century, neuromodulators like dopamine and serotonin have been popularly dubbed as "feel-good hormones" (Watson, 2024). You can easily find online tips for naturally “boosting” these neuromodulators in your brain, advertised to enhance your mood and improve overall well-being (Raypole, 2022). The reasons why dopamine and serotonin have been classified as such differ greatly but share similar media “short-circuits”.

Dopamine's association with rewards was first established in 1954 as researchers demonstrated that stimulating dopamine neurons could reinforce behaviour (Olds & Milner, 1954). Subsequent discoveries about reward prediction errors and the development of reward-related models in the field of reinforcement learning soon led to dopamine being classified as the brain's "pleasure chemical" in the general population (Schultz et al., 1997; Kringelbach & Berridge, 2010). Despite consensus within the scientific community on dopamine's link to reward, this connection has clearly been oversimplified. Conversely, the association between serotonin and *happiness* largely takes roots in the serotonin hypothesis of depression, proposed by

British psychiatrist Alec Coppen in 1967 (Coppen, 1967). He suggested that depression might result from low levels of serotonin in the brain. The development of selective serotonin reuptake inhibitors (SSRIs) as medication to treat depression further consolidated this idea (Healy, 2015). SSRIs are thought to increase baseline serotonin levels by enhancing serotonin release at the synapses (Sharp & Collins, 2023). In a 2011 Australian survey, about 80% of participants believed that low levels of serotonin would cause depression (Pilkington et al., 2013). Today, this link is highly debated as emerging research challenges this assumption (Moncrieff et al., 2023). It is also often overlooked that only about 60 to 70% of people with depression do respond to treatments with SSRIs, amongst which around 15% experience only partial recovery (Al-Harbi, 2012). Of those who show no signs of improvement, around 10 to 30% are considered treatment-resistant, with no improvement whatsoever from any medication. The reason for this remains unknown, and debate continues in the field, with voices supporting both sides of the arguments (Jauhar et al., 2023).

Whilst the primary focus of this work is not to resolve serotonin's role in depression, this topic illustrates the complexity of serotonin research. Disagreements and varying opinions are common, not only in understanding serotonin's role in mood regulation. As noted in the 1968 book "Serotonin" by Irvine Page, an American physiologist who initially identified serotonin as a vasoconstrictor, "The great variety of suggested roles (for serotonin) can be said to be a tribute to man's ingenuity and his unquestionable willingness to write papers" (Rapport et al., 1948; Page, 1968).

Since the time of Page, techniques have improved immensely, and scientific knowledge has become more and more disconnected from these folk

representations. Our work aims to bring more light into serotonin's involvement in motion and cognition, specifically in novelty, learning and surprise. We will start by briefly introducing the serotonergic system, from cells to pathways, and explore the topics of motion, learning and surprise by presenting agreements and contradictions within the field. This introduction will conclude with an overview of our work and its potential to bring new light on how novelty, familiarity and learned salience could be dynamically represented within the serotonergic system.

1.2. The Serotonergic System

Before Irvine Page’s contributions, serotonin was identified in the gut, where it was found to increase smooth muscle tone (Vialli & Erspamer, 1937). Initially called “enteramine”, Page later coined the term "serotonin", which is still used today. Serotonin is synthesized from the essential amino acid L-tryptophan, giving it its molecular designation, 5-HT, or 5-hydroxytryptamine (Clark et al., 1954). Approximately 95% of serotonin-expressing neurons reside in the gut, and it was not until 1953 that serotonin was discovered in the mammalian brainstem and later classified as a neurotransmitter (Terry & Margolis, 2017; Twarog & Page, 1953; Brodie & Shore, 1957). Serotonin is now recognized as a prominent neuromodulator, capable of modulating the intrinsic firing properties of neurons within neural circuits through synaptic and volumetric release at synapses (Marder, 2012). Although serotonin-expressing axons are found throughout the brain, neuromodulation is believed to be highly specific, involving unique pathways and receptor subtypes at each synapse.

Serotonergic neurons are located within nine nuclei in the pons and midbrain (Dahlstroem & Fuxe, 1964). The caudal nuclei primarily target the spinal cord and brainstem, while the rostral group, including the raphe nuclei, serves as the primary source of serotonergic neurons for the forebrain and midbrain (Azmitia & Segal, 1978). The dorsal and median raphe nuclei (DRN and MRN respectively) together account for more than 80% of forebrain innervation. Recent genetic advances, such as rabies tracing and single-cell transcriptomics, have allowed for precise mapping of the DRN and MRN's input-output connections. Both nuclei receive substantial projections from the prefrontal cortex, lateral habenula, basal ganglia, and amygdala, with the MRN also receiving inputs from the septo-hippocampal region (Dorocic *et al.*, 2014; Zhou *et al.*, 2017). The MRN preferentially projects back to the hippocampus and septum, while specific subgroups of DRN neurons project to the cortex, amygdala, striatum, hypothalamus, thalamus, brainstem, and cerebellum (Ren *et al.*, 2019).

Despite their extensive projection patterns, serotonin release modulates postsynaptic cells through at least 14 different serotonin receptor subtypes, enhancing precision (Sharp & Barnes, 2020). Presynaptic modulation also occurs via the 5-HT1A receptor, which controls 5-HT release as a local feedback mechanism. Most receptors are G-protein-coupled receptors (GPCRs), characterized by slower modulatory dynamics compared to the 5-HT3 receptor, a ligand-gated ion channel preferentially expressed on interneurons (Engel *et al.,* 2013). Ongoing research seeks to map specific serotonin functions to unique pathways or receptors, continually revealing the complexity of the serotonergic system (Okaty et al., 2019; Sharp & Barnes, 2020; Salvan *et al.*, 2023).

1.3. The Relationship Between Serotonin and Body Motion

The first body of work related to the function of serotonin we will introduce for this thesis is the field of body motion, as serotonin has been implicated in both the activation and the inhibition of movement. Studies across species, including zebrafish, mice, and invertebrates like drosophila melanogaster, have demonstrated a strong relationship between the serotonergic system and the sleep-wake cycle (Lai & Siegel, 1988; Oikonomou et al., 2019; Liu et al., 2019). Notably, serotonin activity ceases during REM sleep, a period marked by immobility. Disruptions in serotonergic function can impact sleep; for example, tonic stimulation can induce sleep, while burst stimulation can promote wakefulness. Early studies demonstrated that systemic injection of 5-hydroxytryptophan (5-HTP), an intermediate metabolite in the synthesis pathway of serotonin, could evoke locomotor-like activity in anesthetized rabbits (Viala & Buser, 1969). It was then suggested that serotonin neurons could facilitate motor output by activating the spinal cord's central pattern generator, important for rhythmic movements (Jacobs & Fornal, 1993; Flaive et al., 2020). Sequentially, Fornal observed positive correlations between orofacial movements (chewing, biting, licking, grooming) in cats and serotonin neurons' activity levels in the DRN (Fornal et al., 1996). These findings have been extended in studies linking serotonin to whisking in mice (Hattox et al., 2003), thus implying a role of 5-HT in movement generation.

Other studies present a contrasting view. For instance, increasing serotonin levels using SSRIs in isolated spinal cord preparations from salamanders has been shown to destabilize fictive locomotion induced by bath-applied glutamatergic agonists (Flaive et al., 2020). In drosophila melanogaster, serotonin stimulation induces behavioural quiescence (Pooryasin & Fiala, 2015). In mice, in the context of a waiting task, stimulating serotonin neurons

increases waiting (Fonseca et al, 2015). Additional studies have suggested that lower serotonin levels could correlate with increased exploration and locomotor activity (Gately et al., 1985) and heightened startle responses (Davis & Sheard, 1974; Davis et al., 1980). These results have suggested a role for serotonin in behavioural inhibition, a popular theory of serotonin even today (Soubrié, 1986; Colwell et al., 2024).

More recently, two experimental papers have been able to reconcile both perspectives. Research from the Mainen laboratory showed that activating serotonin neurons in mice using optogenetics could induce an acute reduction in running speed as mice ran freely in an open field (Correia et al., 2017). Interestingly, baseline running speed increased over days, a potential expression of learning or adaptation of the system to the recurrent stimulation. Stimulation of these serotonin neurons in mice running in a linear track for reward did not have any effect on locomotion speed. Overall, this work suggests a highly context-dependent modulation of the serotonergic system on locomotion.

More recently, a study recording the activity of serotonin neurons with light thanks to fiber photometry reinforced this context-dependent relationship of serotonin with movement (Seo et al., 2019). Indeed, researchers showed that in low-threat environments such as an open field, the activity of serotonin expressing neurons would decrease with movement onset. Conversely, they showed that in high-threat environments like the tail suspension test, the activity of these neurons would increase when escape movements are initiated. Stimulating optogenetically these neurons in this latter context would lead to enhanced mobility. Overall, the relationship between the serotonergic system and behaviour is unlike an *on-off* switch but rather seems to depend

on the context of the event. Our next section will thus explore advances in our understanding of how serotonin neurons learn from experience.

1.4. The Serotonergic System Learns from Salient Events

In line with the behavioural inhibition view of serotonin, researchers suggested that the serotonergic system could function in opposition to the one of dopamine in motivation and behavioural control (Deakin & Graeff, 1991; Daw et al., 2002; Cools et al., 2011). In this framework, serotonin neurons would promote learning from negative outcomes to avoid harmful actions by signalling aversive prediction errors, as opposed to reward predicting ones signalled within the dopaminergic system (Daw et al., 2002; Wise et al., 1973). Unlike dopamine, which can induce conditioned place preference when stimulated in an open field, stimulating serotonin neurons of the DRN does not have this effect, supporting this view (Tsai et al, 2009; Correia et al., 2017). Stimulating the DRN to VTA (ventral tegmental area, a nucleus for dopamine neurons) pathway does induce place preference, but this effect seems mainly driven by the release of glutamate from DRN neurons, and not by serotonin release itself (Liu et al, 2014; McDevitt et al, 2014). Moreover, as stimulating serotonin neurons in mice running in an open field makes them slow down, this behavioural effect might be misinterpreted as a preference to stay in this context (Correia et al, 2017). Along this line, researchers recently showed that serotonin neurons' terminals in the prefrontal cortex could be critical for punishment-based learning in mice, and that levels of serotonin in the brain could positively correlate with impulse control and aversive learning in humans (Yoshida et al., 2019; Colwell et al., 2024).

Since 2011, this opponency theory has evolved towards presenting serotonin and dopamine as cooperating rather than competing (Boureau & Dayan, 2011). The reciprocal connection pathways between serotonin-expressing neurons in the DRN and dopamine neurons of the VTA have suggested information about rewards for learning could in truth be shared in both neuromodulatory systems (Ogawa et al., 2014; Cools et al., 2011). Indeed, stimulating serotonin neurons increases certain behaviours associated to reward consumption, such as increasing the number of nose pokes performed in a probabilistic reward task in mice (Lottem et al, 2018). Similarly, optogenetic activation of these neurons changes the learning rate involved in processing delayed rewards in a waiting task, making the mice wait longer for collecting the reward (Miyazaki et al, 2014; Iigaya et al., 2018). These experimental results correspond to theoretical work on serotonin's involvement in reinforcement learning through temporal discounting and value encoding (Doya, 2002; Grossman, 2022). Specifically, serotonin neurons, through tonic signalling, could be signalling “beneficialness” as the encoding of how beneficial the environment is for the animal (Luo et al, 2016). This tonic firing of serotonin neurons would keep track of value in the environment, contrasting with their phasic firing properties which could signal prediction error for future punishments (Khalighinejad et al, 2022; Daw et al, 2002). Overall, the modulation of serotonin neurons’ activity appears greatly modulated by learning, integrating contextual information to shape its response to stimuli in the environment.

At a cellular level, serotonin release has been conceived as a powerful modulator of synaptic weights that stimulates neural plasticity and regulates the development and the wiring of brain circuits, where dysregulations could be linked to mental conditions such as autism (Higa et al, 2024; Gutierrez-Castellanos et al, 2024; McEntee & Crook, 1991; Yang et al, 2014). We

explore in the next section where and how this contextual learning takes place in the serotonergic circuitry.

1.5. Serotonin Neurons' Responses to Sensory Stimuli

Electrophysiological recordings of serotonin neurons have revealed selective responses of these neurons to both rewards and punishments in classical conditioning tasks (Bromberg-Martin et al., 2010; Cohen et al., 2015). Importantly, the activity of serotonin neurons is not only reactive in response to the event, but the shape of the response changes with learning (Zhong et al, 2017). Specifically, learning induces ramping up serotonin neurons' activity from reward predicting cues until the reward delivery, also reflecting the magnitude of reward and reward likelihood (Miyazaki et al, 2011; Nakamura et al., 2008; Bromberg-Martin et al., 2010) whilst stressful conditions could also influence these responses (Inaba et al., 2013). Like dopaminergic neurons, serotonin neurons respond to reward-predicting cues, whether olfactory or auditory cues (Ranade & Mainen, 2009; Hurley & Hall, 2011; Cohen et al., 2015; Hayashi et al., 2015). Thus, the system appears to learn an association between sensory stimuli to specific rewarding outcomes, signalling preferentially the reward-predicting cue after learning than the outcome itself.

Crucially, researchers showed that in a reversal task where mice had to re-learn new cue-outcome associations after a reversal, serotonin neurons responded to the unexpected reward delivery in the reversed context (Matias et al, 2017). Importantly, serotonin neurons also responded to unexpected reward omissions. Whilst the former observation stands in line with reward prediction errors as seen in the dopaminergic system, the latter has

suggested a more general role of the system to signal unexpected events irrespective of their affective valence (or unsigned prediction errors). In general, serotonin neurons appeared to respond more vigorously to unexpected stimuli when out of context, presenting a novel framework for serotonin neurons to learn from, and signal surprise (Matias et al., 2017; Ranade and Mainen, 2009). At the theoretical level, researchers have hypothesized serotonin could signal uncertainty and adjust information processing accordingly (Grossman et al., 2022, Harkin et al., 2023).

Revisiting and updating earlier hypotheses linking serotonin and depression, recent clinical trials have tested the efficiency of psychedelic drugs in relieving symptoms in depression (Reiff et al, 2020). Psilocybin and lysergic acid diethylamide, more commonly known as LSD, two major psychedelic drugs, interact with the serotonergic system (Nichols, 2016). Research has shown that these drugs could enhance neural plasticity via the 5-HT2A receptors (Vargas et al, 2023). At the experiential level, psychedelic drugs appear to alter sensory perceptions of the world in the acute phase of drug consumption (Kometer & Vollenweider, 2016; Barrett et al, 2020). Importantly, their effects outlast their physiological presence, and long-lasting changes such as reduced negative mood and anxiety are commonly reported. A recent article has associated these effects to a generation of “synthetic surprise” from the drug itself (De Filippo & Schmitz, 2024). By acting on the serotonergic system, psychedelic drugs could down-modulate the weight of prior information (acquired through learning) in perception and decision-making and enhance the creation and perception of novel sensory experiences (Carhart-Harris & Friston, 2019). In the long run, this hypothetical mechanism might dampen the weight of negative associations and help balance the affective meaning of events. In the cortex, expected unsurprising stimuli tend to elicit lower cortical responses (Bastos et al, 2023), in line with predictive coding theories. Endogenous serotonin release typically modulates sensory

responses by reducing cortical responses to stimuli, though the effect can vary depending on the brain region (Lottem et al., 2018; Azimi et al., 2020; Seillier et al, 2017). As such, serotonin release in the cortex could modulate the top-down and bottom-up information streams existing within defined networks. Psychedelics, acting on the serotonergic system, may induce a general state where prior knowledge is minimized, allowing for unfiltered sensory inputs to integrate the neural wirings, altering perception of familiar stimuli (Wießner et al, 2022).

1.6. Novelty Processing by Serotonin

If the activity of serotonin neurons is sensitive to the learned context and the surprise of sensory stimuli, and that serotonergic drugs can alter how familiar stimuli are perceived, how do these neurons respond to novel stimuli? After a reversal in a reversal task, the surprise is caused by the presence of a familiar outcome in the context that was never associated to it (Matias et al., 2017). As such, one can compare the response to a reward or an air-puff when predictable or unpredictable due to the sensory cues that precede its presence. Truly novel stimuli, which have never been encountered before, share similar principles as error signals but also present another dimension of surprise (Wessel et al, 2012). Whilst there could be a prediction of what should’ve happened next that is broken as with familiar events in unexpected contexts, the novel stimulus remains unknown until experienced. As there should be no prior experiences or expectations to draw upon, novel stimuli are not only surprising in the spatial and temporal domains ("when" and "where"), but also with respect to their identity (“what”). Importantly, novelty processing has been tightly linked to adaptive behaviours such as threat avoidance and curiosity, crucial for the survival of species (Tapper & Molas, 2020).

There is limited research on serotonin neuron's responses to novel stimuli compared to other neuromodulators, though 5-HT receptor-specific pharmacological studies have claimed its involvement in the process (Rangel-Gomez & Meeter, 2016; Bouet et al, 2018). Both dopamine and acetylcholine pathways have been more studied in relation to novelty processing (Tapper & Molas, 2020). In a battery of behavioural tests including social interaction and reward consumption, it was shown that serotonin neurons showed increased activity upon investigation of a novel object in mice (Li et al, 2016). Interestingly, this result was framed not in the framework of serotonin neurons being sensitive to predictions, but rather to rewarding events, as novelty processing could be rewarding per se (Bevins & Besheer, 2005). Moreover, studies on genetic mutations affecting the 5-HT2A receptor reveal memory impairments in mice, with affected mice judging novel stimuli as more familiar than controls (Schott et al., 2011). Another study found that mice lacking the 5-HT4 receptor displayed less novelty-seeking behaviour due to increased stress, and the 5-HT7 receptor has also been implicated in novelty processing (Compan et al., 2004; Ballaz et al, 2007). Similarly, male mice with neonatal serotonin depletion explored novel stimuli less than controls, a finding that links serotonin's role in anxiety and mood disorders (Hohmann et al., 2004).

In head-fixed behaviour, the oddball task is a widely used paradigm to study contextual modulation of sensory processing (Bastos et al, 2023). In this task, a deviant stimulus appears on the screen 10% of the time, as opposed to the familiar one that repeats the rest of the session. The response to the deviant stimulus in humans has been associated to a novelty processing signal, associated to a unique event-related brain potential (Polich & Criado, 2006). Across stimulus repetition, neurons in the primary visual cortex (V1) adapt in response to the familiar stimulus and respond selectively to the deviant one (Bastos et al, 2023). These responses have been characterised as cortical prediction error signals, signalling the deviation of the sensory input from the

predicted one. Analysis of the effect of acute tryptophan depletion (ATD) in the cortex with EEG in humans revealed no modulation of the responses to deviant stimuli in an oddball task, suggesting that the serotonergic system might not be required for this type of novelty processing (Caldenhove et al, 2017). Importantly in this experiment, participants were instructed to ignore these visual cues. In another variant of the oddball task, the local-global paradigm, participants were instructed to count the number of deviant patterns presented to them (Mazancieux et al, 2023). In this context, the serotonergic raphe nucleus responded significantly more to the deviant patterns compared to the familiar ones. As such, the serotonergic response could keep track of stimuli based on their predictability, as novel stimuli, and behaviourally relevant contexts. It is thus crucial to understand the evolution of serotonin neurons' responses to sensory stimuli during learning and monitor how these responses keep track of contextual changes in the environment.

1.7. A Novel Task to Examine the Many Faces of Serotonin

Overall, serotonin appears as a multifunctional neuromodulator that can influence behaviour and cognition in different ways depending on the context. It plays a complex role in signalling both uncertainty and expectation, complementary aspects of learning and adaptive behaviour.

With this in mind, we introduce the project of this PhD thesis. Our aim is to explore how the activity of DRN serotonin neurons represents motion and cognition, specifically in learning and surprise. All experiments were performed in head-fixed transgenic mice, running in a virtual reality (VR) corridor. We recorded the activity of DRN serotonin neurons with fiber

photometry, giving us a clear indication of DRN population dynamics in the task. Using one VR environment, we developed two tasks – an associative-learning task, in which mice are trained to learn to form cue-outcome associations across days to collect rewards, and one that did not require learning of such pairing for reward delivery. Importantly, visual cues were novel and unexpected for the animals on the first day of the task. We recorded the activity of DRN serotonin neurons across days of the tasks and report the evolution of the responses as stimuli, originally novel to the animals, become familiar and relevant or not for behaviour. As such, our work aims to explore how the DRN serotonin neurons respond to stimuli as their context changes over time. Overall, the dynamic modulation of serotonin neurons underscores its involvement in both flexible learning and adapting to changing environments.

2. Materials and Methods

2.1. Animals

All procedures were reviewed and approved by the Champalimaud Centre for the Unknown Ethics Committee and performed in accordance with the Portuguese Direcção Geral de Veterinária. Mice used in this study were all transgenic animals, bread in house of the line Ai148 (TIT2L-GC6f-ICL-tTA2)-D Slc6a4-Cre, expressing GcAMP6s in serotonin transporter positive neurons. All Cre drive lines were backcrossed with C57BL/6J mice and bread in house. Male and female mice were used in this study (9 and 6 respectively). After fiber implantation, mice were single housed in a 12-hour normal light-dark cycle. They had access to food ad-libitum and had water restriction starting the first day of handling for the task. The weight of the mice was monitored daily and could not go below 80% of the initial weight on the first day of handling.

2.2. Surgical Procedures

Surgery was performed on mice between 7 and 8 weeks of age to chronically implant an optic fiber over the DRN. Mice were first anaesthetised with isoflurane at 2% with a constant oxygen flow at 0.8%. The fur above the skull was trimmed and the mice were placed in a stereotaxic frame with the head holding thanks to two ear bars left and right (Kopf Instruments, Tujunga, CA). Mice were placed above a heating pad to prevent the body from reaching hypothermia. Eyes of the mice were kept hydrated by application of an eye ointment cream (Visidic). An injection of carprofen (50 mg/ml) and

dexamethasone (0.5 mg/ml) for analgesia and anti-inflammation were performed before starting the surgery. The shaved skin above the skull of the animal was cleaned and disinfected with ethanol and betadine. An injection of bupivacaine (100 µl) below the skin on the skull was performed as a local analgesic. A longitudinal cut of the skin was made on top of the skull and parts of the skin was removed to have a wide and clear access to the mice' skull. The skull was cleaned with a drop of hydrogen peroxide (H_2O_2) that facilitated the removal of the viscous membrane and the detachment of neck muscles from the skull. The skull was dried, and the skin was glued on the sides of the skull with super glue. Scratches on the skull were performed with the scalpel to enhance attachment of the glue and later dental cement. Bregma was found as the meeting point of the coronal and sagittal sutures, between the frontal and two parietal bones. Adjustments of the head tilt in the antero-posterior and medio-lateral axes with respect to bregma were performed to ensure the head of mouse was aligned to the stereotaxic apparatus and perpendicular to the floor. Optic fibers from Doric were used in this experiment (Doric Lenses). They were made of borosilicate, flat end of 0.66 NA, 4 mm long. Fiber implantation targeted over the DRN, located at -4.5 antero-posterior, 0.0 medio-lateral and -3.0 dorso-ventral, was performed by inserting the fiber at an angle of 32 degrees, through the cerebellum. The following coordinates were thus used to implant the fiber over the DRN; antero-posterior: -6.6 mm from bregma, medio-lateral: 0 from bregma, dorso-ventral: -3 mm from the top of the skull, with an angle of 32 degrees. A small craniotomy was drilled at this location, large enough to let an optic fiber of 400 µm diameter pass through. The craniotomy was maintained humid with an NaCl solution whilst the fiber was mounted on a fiber holder. Before proceeding to the implantation, an injection of 100 µl buprenorphine (0.3 ml/ml) was given to insure pain reduction for the mouse during the recovery period. The implantation of the fiber was performed whilst recording live signals through the fiber, connecting the fiber to the Doric photometry apparatus and to the computer. As mice were transgenic mice already

expressing the GCaMP6s calcium indicator, it was possible to record fluorescence signal by switching on the blue LED during fiber insertion inside the brain. This technique was optimal to target fiber depth position optimally above the DRN for each mouse. Serotonin DRN neurons showed a response to tail pinch, when mice are anesthetized, and this was used as a landmark for refining fiber position. The target implantation depth was -3 mm from the top of the skull and across all mice, depths ranged between -2.7 and -3.2 mm overall. The fiber was slowly implanted inside the brain, making sure the whole process would take at least 10 minutes, with 5 steps with 2 minutes waiting in between fiber descents. Upon reaching the target location, the recording was stopped, and the skull was dried one last time. A drop of super glue was placed around the fiber above the craniotomy, as well as on the skull to enhance attachment of the dental cement on the skull. A thick layer of black dental cement was then placed over the fiber and covering the entire skull. A long period of around 15 minutes was necessary to wait for the cement to dry and for the fiber to be fixed in position. Once the cement dry, the fiber holder was gently removed from the fiber holding position and the fiber held into place. With the use of a headpost holder, a headpost was positioned horizontally above the front centre of the skull, above the dental cement. A thick layer of black dental cement was put above the headpost to attach the headpost to the skull. Once the cement dried again, the headpost holder was removed and mice were displaced from the anaesthesia mask and the stereotaxic frame. Monitoring of the mice's well-being throughout recovery was performed for 15 minutes and up to 2 hours and the surgery day. Mice were then checked for three consecutive days post-surgery, until full recovery.

At the end of the experiment, a perfusion procedure was performed to collect their brain and confirm fiber location and GCaMP6s expression in cells of the DRN. Mice were anesthetized at a non-recovery dose with an injection of ketamine/ xylazine. The perfusion procedure was performed by replacing the

blood from the blood vessels with phosphate buffered saline (PBS) and fixing the tissue with paraformaldehyde (PFA). Brains were then collected and stored in PFA and PBS with 10% azide.

2.3. Processing of Brain Tissue

Post perfusion, brains were sliced sagittally along the midline, where the DRN is located. Brain slices were stained with DAPI to label cell nuclei. An additional procedure to enhance the green fluorescent protein (GFP) signal from the GCaMP6s protein was also performed (anti-GFP rabbit polyclonal antibody, Alexa Fluor 488 conjugate, Invitrogen). Brain slices were mounted on slides and then imaged with a confocal microscope (VS200, Olympus).

2.4. The Experimental Setup

We built a novel virtual reality setup in the lab to carry out VR experiments in head-fixed mice. The virtual environment was displayed on two LCD screens (iPad retina display and external bare driver, Adafruit), on both sides of a custom 3D printed running wheel. The screens were positioned more forward of the wheel, still within the monocular field of views of the animal. The rotations of the running wheel produced by movements of the animal during the experiment were measured and recorded thanks to a rotary encoder (MAE3, US digital). The encoder was itself connected to a behaviour board from HARP, a product from the hardware platform at the Champalimaud Foundation, built in house. The behaviour board allowed us to connect data from the running wheel to the computer. We ran the VR task with the BonVision VR engine from Bonsai. All experiments were performed in closed

loop, such that the running speed of the animal would define the speed of the movement of the corridors as mice ran through this virtual environment. The behaviour board was also connected to a camera (point grey, FLEA3) that was used to record face movements and licks of the animal. The camera was recording the face of the animal via a reflective glass that would reflect infrared light. We used two infrared lights positioned above the LCD screens to illuminate the face of the animal for better recording quality. The camera frame rate was fixed by the behaviour board at 30 Hz. The behaviour board also relayed the reward delivery signals from the VR task engine to a water valve, then connected to a lick-port positioned at reaching distance from the tongue of the mouse, in front. The size of the reward drops was fixed to 4µl and was calibrated at least once a week throughout the experiments. Finally, we positioned a photodiode on the top right corner of the right screen, itself also connected to the HARP behaviour board, to relay information of screen brightness throughout the task.

2.5. The Photometry Apparatus

A Doric Lenses photometry system was used to measure fluorescence signals in head-fixed mice in the VR setup during the task (Doric Lenses, Quebec, Canada). Power intensity of both 465 nm and 405 nm LED lights was tailored for each mouse. Reference frequencies were fixed at 208 Hz and 572 Hz respectively. Light was coupled to a filter cube (FMC4, Doric Lenses), and was connected to the optic fiber implanted in the animal via a patch cord (NA 0.57, core 400 µm). This collection of fluorescence signals from GcaMp6s and isosbestic was performed via the same patch cord, transmitted back to a photodetector. The signal was then relayed via the Doric Lenses Photometry Console to the computer that ran the Doric Neuroscience Studio software. The signal of the two excitation channels were demodulated. These two

datasets, as well as the TTLs sent from the HARP behaviour board and received by the photometry console via BNC cables, were ultimately saved in a csv file and later pre-processed before running all task-related analyses.

2.6. Behavioural Protocols

2.6.1. The training protocol

We present s a detailed protocol on how mice were trained to run head-fixed on the wheel, prior to starting the experiment. Over weekends, we added 2% citric acid to the water in the mice's water bottles, to enhance the drive for performing the task and collect normal water on Mondays.

Day 1 - Weight, remove water access from the mouse, place hand in home cage for 2/3 minutes, handle the mouse briefly. Maintain water restriction on all consecutive days of handling and training, building the association between the experimental setup and water consumption for the animal.

Day 2 - Weight, handle the mouse for 5 minutes and give water to the mouse with a syringe, holding the mouse in your hand. Deliver maximally 0.7ml of water to the mouse. If the mouse doesn't drink, put the rest in a well in the home cage.

Day 3 - Weight, put the mouse on the wheel for 5 to 8 minutes without head-fixing the animal. Move the wheel with your hands so the mouse can move on the wheel without falling, whilst already experiencing the movement of the wheel. Deliver water from the lick-port at small regular distances (5 to 10 cm), so the mouse can lick for water and get accustomed to lick-port and water delivery. Give 0.6 ml of water to the mouse in the home cage.

Day 4 - Weight, put the mouse on the wheel for 5 to 8 minutes without being head fixed, same as day 3. Then, head-fix the mouse for the first time for 2 minutes, bringing the lick-port very close to the mouse to facilitate licking to collect rewards. If the mouse did not drink 0.7 ml of water in the experimental setup, supplement with water in the home cage.

Day 5 - Weight, put the mouse on the wheel for 5 minutes without being head fixed, then head-fix the mouse for 5 minutes. The lick-port should always be delivering water very regularly (every 10-20 cm) and give extra water droplets if the mouse is not moving/ looks stressed.

Day 6 - Weight, put the mouse on the wheel for 10 minutes head-fixed and make sure the lick-port is easily accessible, give 0.5ml of water in home cage.

Day 7 - Turn on the LCD screens and display the VR environment before putting the mouse on the setup. Put the mouse 12 minutes head-fixed on the wheel with a distance between rewards of 30 cm. Supplement with water in the home cage making sure the mouse could drink 0.7ml of water in total on that day.

Day 8 – Again, have the LCD screens display the VR environment before head-fixing the mouse on the setup. Put the mouse 20 minutes head-fixed on the wheel running for rewards with a distance between rewards of 30 to 50 cm. If the mouse shows signs of discomfort (tries to get out of the head fixation, does not move), remove the mouse from the setup before the end of the 20 minutes. Supplement with water in the home cage the amount of water the mouse needs to drink to have drunk 0.7ml on that day.

Day 9 until day X - Repeat day 8, with increasing distances between rewards adjusting per each individual mouse. If the mouse looks stressed, remove it from the setup earlier. If the mouse isn't moving much, give additional rewards to get the incentive to run. Also, once the distances between rewards becomes larger, increase the reward size so the mouse gets enough water per day on the setup without getting frustrated. Slowly, as the mouse becomes

more eager and comfortable to run, reduce the reward size to the desired size for the task (4 microliter per drop). When the mouse shows an engaged state in running, head-fix the mouse first before turning on the LCD screens with the VR environment. This will be crucial to record serotonin neurons' activity in response to this task event.

To prepare the mice for my task, where rewards are delivered on 50% of the trials at a unique reward location (360 cm down a corridor of 400 cm), I trained the mice to collect rewards at 360 cm on 50% of the trials on the training days. The reward zone texture was already present on the wall of the habituation corridor. Mice had to run at least 100 corridors at 50% chance of reward delivery to start the task on the next day.

2.6.2. The learning task

The task is a conditioning task where mice had to learn the association between reward contingencies and corridors by running in a virtual reality environment. Corridors consisted of 2 images fixed at 2 different locations along the track, as well as a reward zone texture. All corridors were identical except from the identity of the images at the two locations. Corridors were either rewarded or not (50% distribution). Four corridors were considered "fixed" corridors, such that the images in the first position always preceded images in the second position, and the reward contingency was fixed (2 rewarded corridors, 2 non-rewarded corridors). Four other corridors were "ambiguous" corridors such that all four corridors shared the same image in the first position. All second images were either one of the 4 images in the second position of fixed corridors. The reward contingency was bound to the identity of the second image. Hence two ambiguous corridors were rewarded

and two were not. Fixed corridors made up of 80% of the corridors' distribution and ambiguous corridors made up the last 20%, with each ambiguous corridor being randomly chosen 1/4th of the time for these 20%. Within these constraints, corridors were randomly distributed during the session. Additionally, images were semi-randomly assigned to locations and corridors, making sure no image was associated to any corridor or location across mice.

A session consisted of head-fixing the animal on the VR setup, starting the photometry recording apparatus to record 5-HT neurons' activity in the task and launching the behavioural protocol. Mice had to continuously run during the session to collect rewards. In rewarded corridors, mice had to run below an average of 30 cm/s in the last 8 cm window before the reward delivery location to trigger the reward delivery. If mice ran too fast in this spatial window, rewarded corridors were not rewarded. The session ended after a maximum of 1h30, if the animal ran more than 300 corridors or repeatedly stopped running for bouts longer than 5 minutes. Mice ran in the task for 10 consecutive days and only mice that ran at least 50 corridors per day were kept for analysis.

2.6.3. The control task

Mice ran through identical corridors, but corridors were never associated to reward outcome. To restrict mice running speeds around similar values than. In the learning task, we fixed a running speed threshold at 40 cm/s to trigger reward delivery. Mice running above that running speed would not receive rewards. As rewards were never cued in this control task, mice maintained a

running speed on average slightly below 40 cm/s along the track. Mice in the control task were trained before the experiment in two different ways.

One batch of mice was trained to run in the identical habituation corridor as in the learning task but never associated the reward delivery zone to reward deliveries. Rewards were delivered randomly throughout the habituation corridor track. Upon starting the task, rewards were delivered in random corridors, 50% of the time, and never more than one reward per corridor. Rewarded corridors were never associated to corridor identities, determined by images on the corridor walls.

The second batch of mice were trained in an identical way to the mice in the learning task. Mice learned to run for rewards at regular intervals and on the last couple of training days, rewards came half of the time in the reward zone (360 cm from corridor start), same as the learning task. On the first day of the experiment, rewards were delivered in random locations in the corridors, pooling from an exponential distribution (days 1-4: mean = 865 cm between rewards and days 5-10: mean = 840 cm). So multiple rewards could be delivered in the same corridor, making the visual information of the corridor completely irrelevant for the animal. So, this controls for images as spatial landmarks for reward delivery.

2.6.4. The off-on experiment

Four mice from the learning group and all 6 mice from the control group experienced an additional small experiment called the off-on experiment. The experiment consisted of 12 to 18 repetitions of VR screens off-on events,

where the screens would stop to display the virtual world whilst mice were still running in the same corridors, performing the task. Screens turned off for a period of either 500 ms, 1 second or 2 seconds, every 2 to 6 minutes randomly. Events happened at random epochs in the task. The task lasted for 3 consecutive days. **Table 1** represents the number of repetitions and duration of off-events for each mouse group per day.

day	**Mice group 1, n=4**			**Mice group 2, n=6**		
	Number of repetitions	**Stimulus Duration (seconds)**		**Number of repetitions**	**Stimulus Duration (seconds)**	
1	12	2		12	1	
2	12	1		18	1	0.5
3	18	2	0.5	18	0.5	1

Table 1. *The off-on protocols for all mice.*

2.7. Analyses of Behavioural and Neural Data

2.7.1. Extracting behaviour, face motion and pupil data

Behavioural readouts from the rig were extracted using the HARP system from Champalimaud hardware platform, as explained above. Running speed was extracted from the rotary encoder connected to the running wheel. The

rotary encoder measured a rotation of the wheel as an analog signal from 0 to 5V that could then be calculated into position using the perimeter of the wheel (62 cm). Facial data including face motion, pupil movement and size and lick extensions to collect the rewards were recorded using a point grey camera and a combination of an IR reflective mirror and IR light.

Running speed and position across trials and sessions were extracted from the HARP csv file analog rotary encoder data with custom MATLAB scripts. Extraction of face motion and pupil data were performed using the python software Facemap from Carsen Stringer. In short, videos were loaded into the software and one region of interest was drawn around the whiskers and the nose of the animal to record face motion. Another region of interest was drawn to track the motion and size of the pupil. All extracted data was then z-scored for further analyses in relation to serotonin neurons' transients in the task.

2.7.2. Photometry data pre-processing

MATLAB custom scripts were written to extract the photometry data from the raw traces recorded by the Doric photometry apparatus and saved in a csv file. Raw data consisted of a GCaMP6s signal recorded with 465 nm light illumination, and an isosbestic signal recorded with 405 nm light illumination. These datasets will then on be called serotonin trace and isosbestic trace. The isosbestic signal is a measurement of the brain fluorescence that does not vary with calcium influx inside the cell. It is used to correct for motion artefacts and other types of signals that could be represent in the GCaMP6s dataset but that would be independent from the activity of the cells.

Pre-processing steps were adapted from the published methods paper of Simpson et al, 2024. A first low pass filter with a 10 Hz cutoff was applied on the raw serotonin and isosbestic traces, to improve signal-to-noise ratio of the data. Then, both serotonin and isosbestic traces were fit with a double exponential curve, to correct for bleaching effects happening throughout the session. Movement artefacts were then corrected by running a linear regression on the serotonin and the isosbestic detrended datasets and subtracting the residuals from the serotonin trace. A moving mean of 25 frames was ran to smoothen the serotonin and isosbestic traces. Finally, to compare image responses across multiple days of learning and control tasks, we concatenated all smoothed serotonin traces all 10 consecutive days. We z-scored this trace and separated back the serotonin activity traces within their respective day matrices.

2.7.3. Behavioural Analysis

We developed a learning index to characterize how well the mice learned the task. We used a combination of the running speed and the lick-rate data to assess how well the animals discriminate rewarded from unrewarded corridors across days. We took data from a window of 2 seconds before the reward delivery location. We took the difference in means of rewarded corridors minus unrewarded corridors for each day in speed and lick-rate and z-scored the data across days for each mouse. We then calculated the learning index as follows:

Learning index = sum (-z-score (delta speed), z-score (delta lick-rate))

This index is used throughout the thesis to characterize how well mice show they learned the task.

We employed a linear regression model to classify fixed rewarded from unrewarded corridors daily based on the running speed of the animals along the corridor track. We split the corridor into 20 bins of 20 cm, covering the entire 400 cm long track. For each spatial bin, we calculated mice average running speeds in fixed rewarded and unrewarded corridors respectively. We ran a logistic regression model at each of these bins, aimed at predicting and classifying corridor category. We ran these models for every mouse, every day of the task. The model was as follows:

$$y=\beta 0+\beta 1 \cdot x1+\beta 2 \cdot x2+...+\beta n \cdot xn+\epsilon$$

where y represents the response variable, $x1,x2,\ldots,xn$ are the predictor variables, $\beta 0$ is the intercept, and $\beta 1,\ldots,\beta n$ are the coefficients for each predictor. The model was fitted using MATLAB built in functions, using the ordinary least squares method to estimate the parameters. We ran 5-fold cross-validation on the data, training the model on 4/5th of the data and assessed model accuracy on the test dataset. The resulting coefficients were then used to classify the input data into two categories based on model predictions. We report as mean scores the fraction of correctly classified corridor identities in the session. To examine how this model classifies rewarded from unrewarded corridors at image locations and in the anticipatory reward zone, we average the mean scores for the first two bins of each image (80 – 120 cm and 220 – 260 cm for the first and second image locations respectively), as well as the two bins before the reward delivery location (320 – 360 cm).

For the reversal analysis, we performed a similar analysis using linear regression modelling to classify rewarded from unrewarded images across the session. We concatenated all trials after reversal and ran the logistic

regression model we acquired on day 10 to perform the classification. We convert fraction correctly classified corridors to percentages.

2.7.4. Event analysis

For image responses, I average the serotonin signal in a 1 second window once the animal is at the image location and normalise it to a baseline which is -2 to -0.5 seconds before the image location (of the leading image). In the corridor, images in the leading location are at 80 cm from the corridor start and images in the second location are at 220 cm from corridor start. For the VR onset and offset responses, as well as for the off-on responses during the off-on experiment, I analyse the serotonin activity in a 1 second window from the time the screens turn off or on. Image responses are normalised to a baseline window before entering the image location (-2 to -0.5 seconds before image location).

We analysed the decay of serotonin neurons' image responses over time using an exponential decay model in two occasions. First, across the first 10 image repetitions of the first and second days of the learning and control tasks. Secondly, across all serotonin image responses for all trials of each day of the task for mice in the learning task.

The exponential decay model was specified as follows:

$$y=A\cdot e-k\cdot t+C$$

where y represents the measured response variable, A is the initial amplitude, k is the decay constant, and C is the horizontal asymptote. The model was

fitted to the data using the nonlinear least squares method in MATLAB. Initial parameter estimates for A, k and C were based on visual inspection of the data. We assessed the significance of the fit with a p<0.05.

2.7.5. Extraction of locomotion correlates in serotonin neurons

The analysis based on extracting locomotion correlated activity from serotonin neurons' fluorescence profiles was performed by a visiting student in the Mainen laboratory, Stefan Hadjuk.

To investigate the relationship between locomotion speed and serotonin levels, and to control for the potential confounding effects of speed on serotonin measurements, we employed a systematic approach to normalize serotonin levels relative to speed. First, we segmented locomotion speed into discrete bins with a resolution of 1 cm/s, ranging from 0 to 60 cm/s. For each speed bin, we extracted all corresponding serotonin measurements, sampled at a frequency of 100Hz, and calculated the average serotonin level within that bin. This process was repeated across all speed bins to construct a profile of average serotonin levels as a function of speed. Next, to create a continuous representation of this relationship, we interpolated these average values to form a function, denoted as f, which returns the average serotonin level, s1, for any given speed, v1 (i.e., *f(v1) = s1*). Finally, to obtain a serotonin trace that is corrected for the influence of speed, we subtracted the expected serotonin level, based on our function f, from the observed serotonin levels at each corresponding speed. Specifically, for a given speed v1, we adjusted the serotonin measurement by subtracting f(v1) from the observed value. This adjustment allows us to analyse variations in serotonin levels independent of speed effects, thereby isolating other factors

that may influence serotonin dynamics. We thus called to motion-dependent and motion-independent serotonin signals that we acquired thanks to this analysis.

2.7.6. Statistical analyses

Statistical analyses were performed using MATLAB to evaluate the effects of serotonin neurons' image responses across trials and days, between experiment types, corridor identity, MD and MI serotonin signals, as well as on the mice locomotion patterns across these same patterns.

Thus, we ran one-way repeated measures ANOVA, two-way repeated measures ANOVA and mixed model ANOVA comparisons. We systematically ran t-tests following significant ANOVA results by correcting for multiple comparison to control for false discovery rate (FDR). This was implemented using built-in MATLAB functions. Data were presented as means ± standard error of the mean (SEM), and all statistical tests were considered significant at p<0.05.

We ran multiple linear regression models on the datasets at various occurrences using built-in MATLAB functions. We assessed goodness of fit with r squared values and p-values for significance of the model on the data. We looked at the sign of the slope if the p-value was significant, at p<0.05.

3. A Novel VR Paradigm for Learning in Mice

3.1. Background and Motivation

This project aims to bring novel insights on how neural activity from DRN serotonin neurons evolve during associative learning in response to behaviourally relevant cues. Serotonin neurons have been shown to respond to both reward predicting cues and to unexpected reward deliveries, as well as to ramp up in firing rates until reward delivery in predictable reward delivery contexts (Matias et al., 2017; Zhou et al, 2017). How these responses evolve across trials and days when mice get presented to novel cues that will become associated to different reward-predicting contexts during learning has not yet been fully described. To capture this evolution, we developed a novel virtual reality task for mice in which visual cues, at first novel to the animals, became informative contextual cues for reward delivery timing. We recorded neural activity from the DRN with fiber photometry.

3.2. Description of the Novel VR Paradigm

The novel virtual reality task for mice we developed consists of a virtual world, made up of eight distinct corridors, in which mice had to run to collect rewards. We present in **Figure 1.A.** an example corridor from this world. The VR environment consisted of 8 different corridors 400 cm long, each identified by two unique 70 cm long images on the corridor walls, fixed at distinct locations on the corridor track (80 cm and 220 cm from the start). A reward delivery

zone was marked by a 20 cm long grey cue on the walls of the virtual corridor at 350 cm along the track, and reward delivery happened in the middle of this cue only in rewarded corridors.

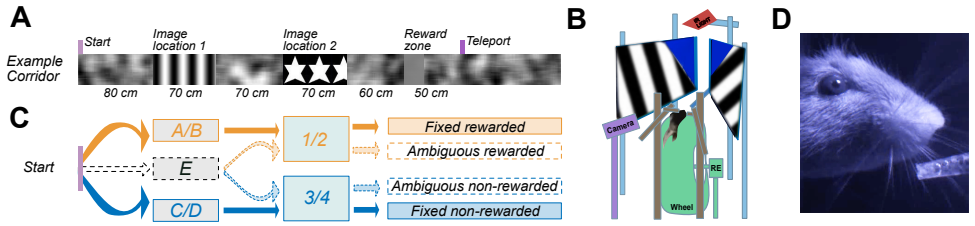

Figure 1. *Mice associate corridors to reward contingencies in a new VR environment.*

A. Example corridor with two image locations, a reward delivery zone and a reward contingency. **B.** Diagram of the experimental setup – two LCD screens, a running wheel attached to a rotary encoder, a camera with a mirror to record the face of the animal, infrared (IR) lights. **C.** Structure of the corridors in the task. **D.** Picture of the field of view from the camera of a mouse's face in the task.

Figure 1.B illustrates the setup on which the experiments were ran. Mice were head-fixed and ran on a 3D printed running wheel, connected in closed loop to the VR engine via a rotary encoder (see Materials section for more information). The virtual world was displayed on two LCD screens located one on each side of the animal's face. We recorded face motion, pupil and lick-rate thanks to that camera (**Figure 1.D.**).

Corridors could be classified into categories by the following properties – reward contingency or image and reward predictability (**Figure 1.C**.). Firstly,

half of the corridors contained images predictive of rewards, and half did not contain any reward predicting images (respectively illustrated in orange and blue). Secondly, half of the corridors were categorised as “fixed” corridors, where the identity of the images at the first and the second locations were fixed (illustrated with solid lines). The other half of the corridors were categorised as “ambiguous” corridors, where corridors shared the same image in the first location, but the second image could consist of either one of the four second images of the fixed corridors (illustrated with dotted lines). In the case of “fixed” corridors, the first image systematically predicted the second image, whilst in the “ambiguous” case, the first image predicted either one of four images in the second location. Importantly, the corridor’s reward contingency was bound to the identity of the second image, where two images predicted reward delivery at the reward delivery zone, whilst the other two images did not. Hence, corridors could either be fixed rewarded, fixed unrewarded, ambiguous rewarded or ambiguous unrewarded. In fixed rewarded corridors, reward information was already present at the location of the first image. In ambiguous corridors, information of reward delivery was only available from the second image.

Before starting the experiment, mice were trained to run head-fixed on the wheel and collect rewards at increasing distances for 10 to 30 days. The training corridor was identical to the background texture of the experimental one with the reward delivery cue, but without any images on the corridor walls. Mice were considered ready for the experiment once they could run for rewards delivered at the reward location (360 cm along the track) in half of the corridors for at least 100 consecutive corridors, hereafter also referred to as trials. Afterwards, mice navigated in the experimental environment for 10 consecutive days, where corridors were randomly alternating within the session. Importantly, corridors were seemingly infinite as there was no cue signalling corridor change.

3.3. Mice Differentiate Rewarded from Unrewarded Corridors

Mice learned to differentiate rewarded from unrewarded corridors. To collect rewards in rewarded corridors, mice had to run slower than 30 cm/s in an 8 cm window before the reward delivery location. Failure to run below this running speed threshold prevented reward delivery. We thus measured how well mice learned the corridor-reward associations by comparing their behaviour in rewarded and unrewarded corridors for both fixed and ambiguous corridor types. Data in **Figure 2.A.** and **B.** shows behavioural traces of locomotion (cm/s) and lick-rate (licks/s) of an example mouse in position along the corridor track on experimental days 1 and 10 for the four corridor types described above.

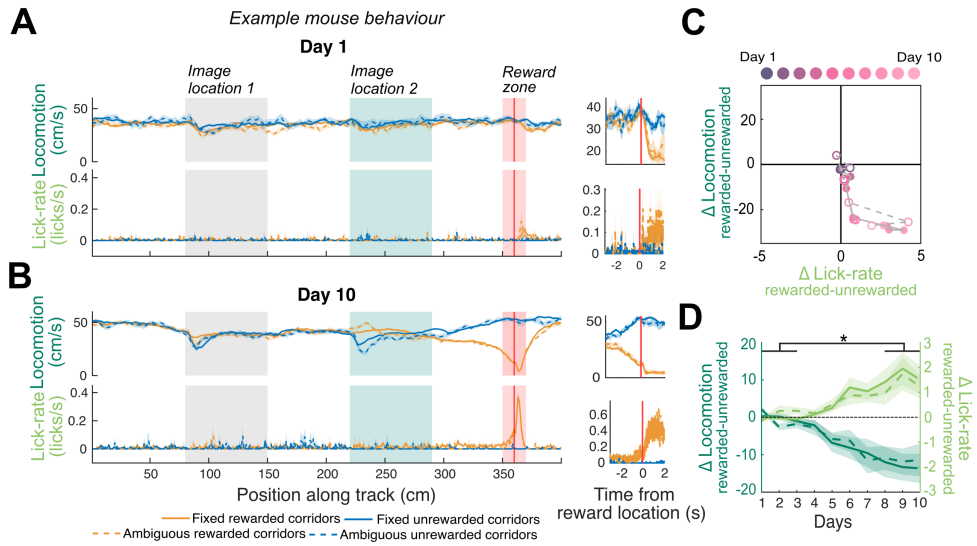

Figure 2. *Mice learn to discriminate rewarded from unrewarded corridors.*

A. & B. Example mouse behaviour in 4 corridor types on day 1 and day 10. Running speed (cm/s) and lick-rate in space and time (mean and SEM across trials). **C.** Evolution of the delta running speed and delta lick-rate in rewarded

minus unrewarded corridors across days for the example mouse in **A.** and **B**. Full circles represent fixed contexts while dotted circles represent ambiguous contexts (n=1). **D.** Average of all mice delta running speed and delta lick-rate across days (n=9, mean and SEM across mice).

Whilst the locomotion and lick-rates in space were indistinguishable for all corridor types until the reward delivery location on day 1, these behavioural readouts in rewarded and unrewarded corridors differed by the 10th day. **Figure 2.C.** illustrates the evolution of learning as the delta running speed and delta lick-rate in rewarded minus unrewarded corridors across days in a 2 second window before the reward delivery location for this example mouse, for both fixed and ambiguous corridors (solid and dotted lines respectively).

Across mice, both behaviours gradually diverged between corridor types from day 5 onward, reflecting learning of the corridor-reward association (**Figure 2.D.**). Indeed, mice started to run slower in rewarded corridors before the reward delivery zone as compared to unrewarded corridors, revealed by a decrease in the delta running speed across days (paired t-test, days 1-3 vs days 8-10, fixed corridors p= 0.0037, ambiguous corridors, p=0.0137). In parallel, mice increased their lick-rate in anticipation to the reward, shown as an increase in the delta lick-rate across days (paired t-test, days 1-3 vs days 8-10, fixed corridors p= 0.0108, ambiguous corridors, p=0.0260). Anticipatory licking has been shown to occur as reward expectancy increases, thus becoming a valuable readout of learning in the task (e.g., Yamamoto et al, 2022). Importantly, animals showed identical behaviour in both fixed and ambiguous corridors of the same reward contingency before the reward zone, suggesting no asymmetry in the learning of the structure of the environment between these corridor types (2-way rmANOVA, locomotion before the reward zone across days and corridor predictability type: day, p=2.872e-08;

corridor predictability type, p=0.4987; interaction, p=0.0934; 2-way rmANOVA, lick-rate before the reward zone across days and corridor predictability type: day, p=2.0293e-05; predictability corridor type, p=0.3044; interaction, p=0.0822).

3.4. Mice Learn to Associate Images at Both Locations to Reward Contingencies

We define learning indices to assess mouse performance across days. To integrate both the locomotion and the lick-rate changes across days, we calculate a learning index as the sum of the negative delta running speed and the positive delta lick-rate metrics between rewarded and unrewarded corridors, z-scored across days for each mouse (**Figure 3.A**.). Whilst all learning indices increased across days, only 6 mice out of 9 who ran the task significantly differentiated rewarded from unrewarded corridors in both fixed and ambiguous categories on the 10th day in either locomotion or lick-rate before the reward zone (paired t-test across mice, day 1 vs day 10, p= 6.4993e-05).

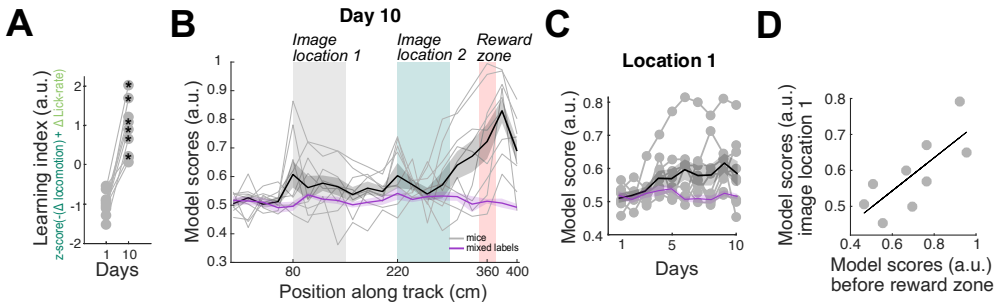

Figure 3. *Learning indices for corridor discriminability across mice.*

A. Learning index, calculated as the sum of the negative delta locomotion and delta lick-rate for rewarded minus unrewarded corridors, each z-scored across days (n=9). **B.** Logistic regression model for classifying fixed rewarded from fixed unrewarded corridors across days using running speed at image locations per mouse on day 10 (black line: mean and SEM of modelled data, n=9; Purple line: mean and SEM of shuffled data, n=9). **C.** Evolution of model score across days of learning for location 1 for all mice. Purple line is average of location 1 scores from shuffled data (mean and SEM, n=9). **D.** Correlation of model score on location 1 and pre-reward delivery location on day 10 for all mice.

This behavioural analysis reveals that mice learn to discriminate rewarded from unrewarded corridors by performing significantly different behaviours before the reward zone in late days of the task in both corridor types. As the behaviour is identical for both fixed and ambiguous corridors, this suggests that mice learned the informative reward value of at least images in the second location of each corridor, as these images were present in all corridor types.

To further understand if mice also associated the images in the first location to those in the second location and to the reward contingencies, we ran a logistic regression model using the locomotion of each mouse in location bins of 20 cm along the track on each day. This allowed us to assess how well a model could classify rewarded from unrewarded corridors using this behavioural readout. We find a significant interaction between model scores at each location across days (2-way rmANOVA, location on track across days: location, p=4.8980e-27; days, p=1.2694e-06; interaction, p=1.9684e-14). **Figure 3.B**. shows the model scores across location bins for all mice on day 10. When comparing model scores between the real data and the shuffled

corridor labels for each mouse, we find significant differences at the first image location with a similar trend for the second location (2-way rmANOVA, location in real or shuffled data: location, p=3.7287e-15; group, p=0.0045; interaction, p=5.47938e-13; from adjusted paired t-test, image location 1 (80 cm), p=0.0304; image location 2 (240 cm), p= 0.0564). All locations in the anticipatory reward zone were also significantly different from the shuffled data (340 cm until 400 cm in the track, p-values below 0.03).

To see how this discriminability of corridor types evolved with learning for images in the first location, we plotted the evolution of each mouse's model mean scores across days of learning for the average of the first 2 bins (40 cm) of these images in the first location (**Figure 3.C**.). On average, mean scores of all mice increased across days, suggesting that discriminability of rewarded and unrewarded corridors from mice locomotion evolved alongside learning (1-way rmANOVA, days, p=0.0058, days 1-3 vs 8-10, paired t-test, p=0.0418). We ran the same analysis for behavioural classification of images in the second location and see that this increase across days holds as well (1-way rmANOVA, days, p=3.9837e-04, days 1-3 vs 8-10, paired t-test, p=4.3847e-04, **Figure 4.A.**). However, testing model mean scores for the first and second image locations on day 10 against the shuffled labelled dataset revealed no significant effect per image but a significant difference between datasets overall (adjusted paired t-test from 2-way rmANOVA testing model mean scores on day 10 at image locations 1 and 2 for real and shuffled data: real or shuffled, p=0.0494; image location, p=0.4676; interaction, p=0.7854; paired t-test for image location 1, p= 0.0831; location 2, p=0.1280). This suggests that not all mice discriminated rewarded from unrewarded corridors at each location but information about corridors' reward predictabilities was present when combining both images on average.

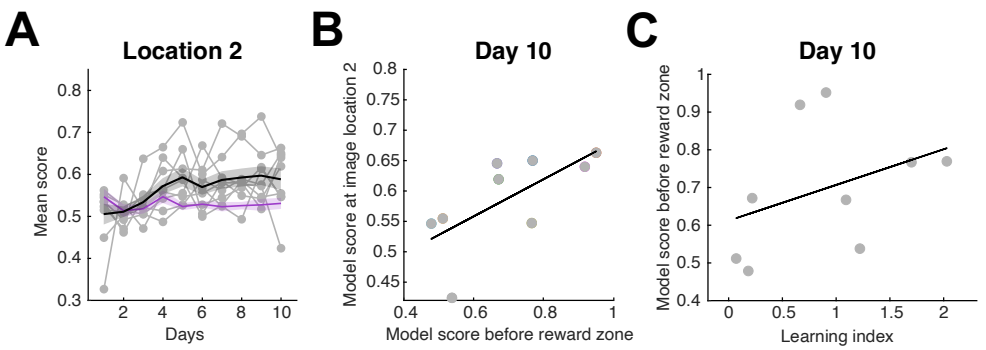

Figure 4*. Model scores before the reward zone correlate with the score at the second location but not with the learning index on day 10.*

A. Evolution of model score across days of learning for location 2 for all mice. **B.** Correlation of model score on location 1 and anticipatory reward delivery location on day 10 for all mice. **C.** Correlation of learning index and anticipatory reward zone model score.

We asked how the model scores at each image location were related to the behavioural performance before the reward zone. We found that mice that discriminated best the corridors in anticipation to the reward zone also discriminated best the corridors at the first image location on the last day, suggesting learning influenced behavioural discriminability of the images in the first location (fit coefficient, p=0.0052, **Figure 3.D**). This was also true when analysing the model scores of the second image (fit coefficient p=0.0468, **Figure 4.A.** and **B.**).

Finally, we aimed to assure that our learning indices were capturing similar trends in performance across mice. We find a non-significant trend in the correlation between the learning index calculated in **Figure 3.A.** and model scores on day 10 in the anticipatory reward zone locations (fit coefficient, p=0.3119, **Figure 4.C.**). The trend does reflect a relationship between both

behavioural metrics, but also suggests they capture different aspects of mouse behaviour. Indeed, mice could be differentiating rewarded from unrewarded corridors in a reliable way but with a small absolute difference in running speed or lick-rate before the reward zone. This could result in a smaller learning index but an accurate modelling of the behaviour.

3.5. Discussion and Interpretation

We describe a novel VR experiment for mice where mice have to run through linear corridors to collect rewards (**Figure 1**). The analysis of mouse behaviour in the task, namely of locomotion and lick-rate before the reward delivery zone, reveals that mice learn to process images as informative cues of the corridors to predict reward delivery (**Figure 2**). Importantly, mice are only required to run below a fixed running speed threshold in anticipation to the reward. All behavioural differences that they perform between corridor types are strategies they choose to perform. By analysing the running speed of mice at both image locations, we can infer that all images become informative of reward delivery across days of learning in the task (**Figure 3**, **Figure 4**). We will discuss in later sections the importance of this difference in the behaviour at image locations between corridor types.

Overall, this task is well suited to allow us to examine, in the next chapters, how serotonin neurons from the DRN respond to novel, unexpected cues that become relevant during learning.

4. Serotonin Neurons' Responses to Novel Visual Cues

4.1. Background and Motivation

The link between the serotonergic system and novelty processing remains undefined. As introduced above, novel stimuli, with novelty defined as "something that has not been experienced before and so is interesting" by the Cambridge Dictionary, differ from familiar stimuli, "well known or quickly recognized" in terms of their relation to the observer (Cambridge dictionary, n.d). A traditional task in mice to assess novelty processing is the novel object recognition (NOR) task (Antunes & Biala, 2012). Mice freely explore an open field in which a familiar and a novel object are placed. Behavioural readouts associated to novelty processing are defined as time exploring the novel object. In a more simple version of the task, Li et al report that serotonin neurons increase their responses when approaching a novel object placed in an open field arena (Li et al, 2016). The unfortunate absence of a familiar object in the open field to assess whether this effect was truly driven by the exploration of a novel object and not any object leaves the results open for intepretation.

Thanks to our task design, we were able to record the activity of serotonin DRN neurons to novel, unexpected visual stimuli in mice. Importantly, as mice perform more trials by running through virtual corridors, images become more predictable and more familiar, changing their contextual setting. We thus explore in this section the dynamics of DRN serotonin neurons to novel stimuli as they become familiar for the animals in the task.

4.2. Serotonin Neurons Respond to Novel Images and Adapt Across Repetition

Mice used in this experiment were double transgenic mice expressing the CRE-dependent fluorescent protein GCaMP6s in CRE-positive cells expressing the serotonin transporter gene (SERT-GCaMP6s) (schematic diagram taken from Fonseca et al, 2015, **Figure 5.A**). Imaging of these serotonin neurons in the dorsal raphe nucleus (DRN) was done by inserting an optic fiber through the cerebellum and placed above the region of interest to collect fluorescence signals from these cells with fiber photometry (**Figure 5.B.**, see Methods section for more details). In brief, this technique allows us to measure bulk activity from cells in the region of interest below the optic fiber, without single cell resolution. We report serotonin activity as z-scores of fluorescence values, z-scored across days of the task. Mice were on average 8 weeks old at the date of the surgery, and 12 weeks old at the start of the experiment.

We took advantage of the first day of the task, where novel images are presented unexpectedly to the animal and repeat across trials, to study how serotonin neurons would respond to novel, unexpected stimuli and as they became familiar. **Figure 5.C.** illustrates with two example raw traces from two mice the bulk activity of serotonin neurons in the first 60 seconds around the start of the experiment on day 1. For all mice in the experiment, serotonin neurons respond strongly to the start of the experiment at time 0 seconds, where the two LCD screens, at first black, start to display the virtual world. This response was present on every consecutive experimental day and is discussed later in the thesis results section 11.

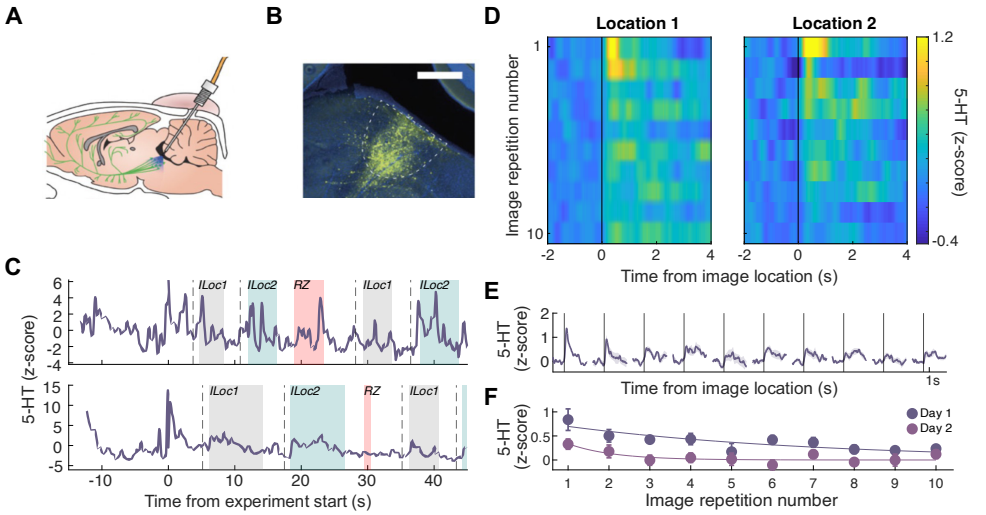

Figure 5. *Serotonin neurons respond to novel images and adapt across repetition.*

A. Schematic of DRN targeting with fiber photometry, taken from Fonseca et al, 2015. **B.** Example mouse brain slice from sagittal cut through DRN. Serotonergic neurons labelled with GCaMP6s, stained with GFP, in a mouse that did the task. **C.** Raw data from two mice of the first 45 seconds from the start of the session on day 1. **D.** First ten image repetitions for all mice, split into location 1 and location 2, z-score, baseline normalised (mean across mice, n=9). **E.** Average of all mice first 10 image repetitions for all 9 images (mean and SEM across mice). **F.** One second average after image location, in the first 10 repetitions of each image of day 1 and day 2 and their respective exponential fits (mean and SEM across all 9 mice).

In the raw data presented in **Figure 5.C.**, it is also possible to notice transient activity from serotonin neurons for both animals upon entering image locations (illustrated with the grey and blue shaded areas). To assess how serotonin neurons respond to such novel stimuli and adapt across repetition, we accounted for image identity by averaging the first 10 image repetitions

across all nine images present in the task and across mice on the first day. We tested whether image location influenced the serotonin neurons' responses to images across repetition by averaging image responses in both locations respectively (**Figure 5.D.**). We find that for both image locations, serotonin neurons of the DRN are significantly modulated by image repetition but find no effect nor interaction of repetitions with image location (2-way rmANOVA, repetition and image location: repetition, p= 0.0080; image location, p=0.5227; interaction, p=0.2622). Thus, we average all images together across all mice to capture, by fitting an exponential decay, how the serotonin response to images evolves across repetition (***Figure 1*****Figure 5.E.** and **F.**). We do so by averaging the serotonin response in a 1 second window from the time the animal entered the image location in the VR world and report a time constant for the decay rate of 1.8 repetitions, consistent across mice (1-way rmANOVA, repetitions, p=0.0089; exponential decay, r^2=0.7516, p=0.0031).

We repeat this analysis on the second day to understand how this effect was specific to novel and unexpected images (**Figure 5.F.**). On the second day, serotonin neurons' responses to the first 10 image presentations of the day were significantly different than those of day 1, with the first image repetition being significantly smaller on the second day than on the first (2-way rmANOVA, repetitions and days, repetition, p=0.0047; days, p=0.0007; interaction, p=0.3653, adjusted paired t-test first repetition, day 1 vs day 2, p=0.0313). Moreover, we find that the serotonin response decays with a decay constant of 1.1 repetitions, acquired from the fit of an exponential decay (r^2 =0.7116, p=0.0097). Hence, this response to the first image presentation and the following decay across repetition is different across the first two days of the task. A hypothesis could be that serotonin responses to images are higher when images are novel and unexpected. The signal could then adapt

and not fully recover on the second day as images become familiar to the animal.

4.3. This Adaptation Is Specific to Image Repetitions and Is not an Effect of Time or Trial Number

Such a decay of the serotonin signal on the first day in response to images could not only be due to the repetition of unique novel images across trials. The decay could also be due to a decay of the overall serotonin activity in time as the experiment takes place. Another explanation could be that the simple presentation and repetition of any images across trials, not specific to image identity, could be responsible for the decay of the signal across repetition. We overlay these three possible adaptation types in **Figure 6.A**.

To account for time, we average for each mouse the overall fluorescence serotonin activity spanning the amount of time it took for each mouse to be presented with all 10 repetitions of each image (dotted line). To account for image presentations, we average 9 consecutive image presentations throughout the first 45 trials of day 1 (90 images presented), which accounts for the same number of images than when averaging them while maintaining the repetition order of each image identity (dashed line).

Firstly, when plotting the general temporal decay of the serotonin activity in the same temporal span as the 10 image repetitions, we find the serotonin average fluorescence value does not adapt when fitting a linear regression (slope coefficient = -0.0050, p =0.7654). Thus, the decay of the serotonin

neurons' responses to images is specific to these images presented in the corridors for the first time on day 1 of the task.

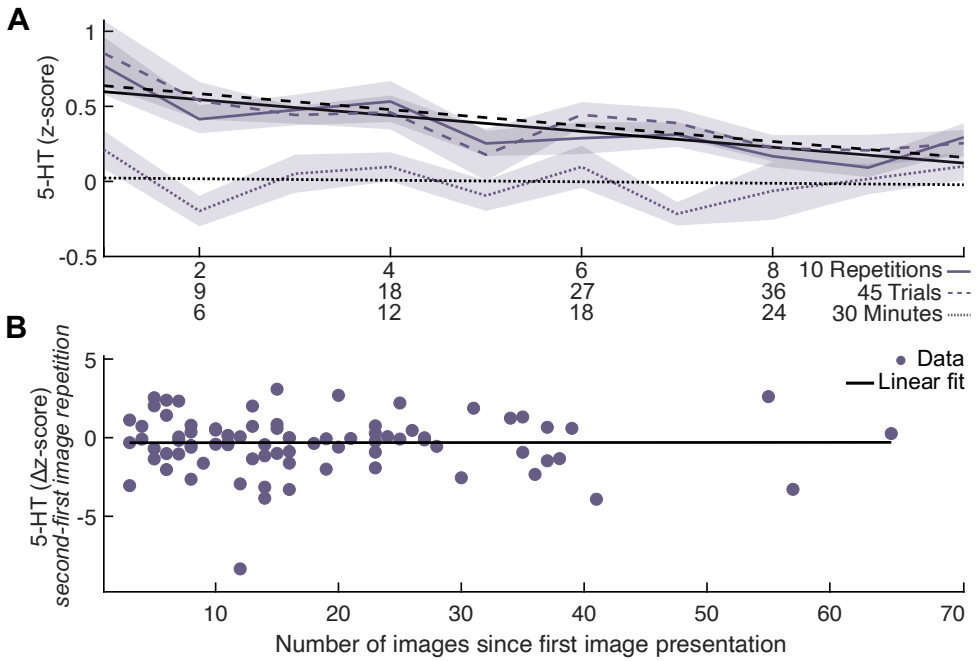

Figure 6. *Serotonin neuron's adaptation across image repetition is specific to images and not a general temporal decay of the signal.*

A. Average of serotonin neurons' image responses across the first 10 repetitions (solid line) and the first 45 trials (dashed line). Average of the general serotonin activity over the same amount of time (dotted line) (mean and SEM across mice, n=9). Black lines are the respective linear regression fits. **B.** Difference in the serotonin image response for first minus second image presentation for all images and all mice, plotted as a function of number of images between the first and the second image presentation (9 images, 9 mice). Black line is linear regression fit.

Secondly, the decays of the serotonin image responses ordered by image repetition or by any trial number adapt at a very similar rate (adaptation by image repetition, slope=-0.0531, p=0.0065; adaptation by trials, slope =-0.0528, p=0.0041). Due to the structure of the task and the random distribution of all corridors across trials on the first day, image repetition is tightly correlated with trial number. Nonetheless, the protocol allows us to have a clearer understanding of the interaction between both factors. We plot the difference in the serotonin image response for the second minus the first image repetition for all images as a function of the number of images between them (9 images, 9 mice) (**Figure 6.B.**). If the number of images between image repetition would be most relevant for the decay of the serotonin neurons' image responses, then second image repetitions with less image trial numbers between these repetitions should induce a higher serotonin response than images that repeat later in the session. In the latter case, the animal ran in front of more images than in the former case, making their presence on the corridor wall less surprising.

We find no linear relationship between the delta z-score for first minus second image presentations of the same images and the number of image trials presented in between them (linear regression, slope coefficient =3.7350e-04; p=0.9818). Hence, neither the temporal decay of the serotonin signal nor the number of images presented within the first trials are sufficient to explain the initial response to the first image presentation and the adaptation of the signal across repetition. Overall, this suggests that serotonin neurons respond to novel images and the response adapts across repetition.

4.4. The Serotonin Novelty Response and Adaptation Are Image Sensitive

As the same 9 images in the task were presented to all mice in different orders, we asked if serotonin responses to images and the subsequent adaptation of the signal were sensitive to image identity. **Figure 7.A.** presents the 9 different images used in the task. We tested the interaction between images and repetition number for each 9 images' first 10 repetitions and find significant effects for image identity, repetitions and the interaction of both across mice (2-way rmANOVA, repetition and image identity: repetition, p=0.0086; image identity, p=0.0061; interaction, p=0.0003, **Figure 7.B.**).

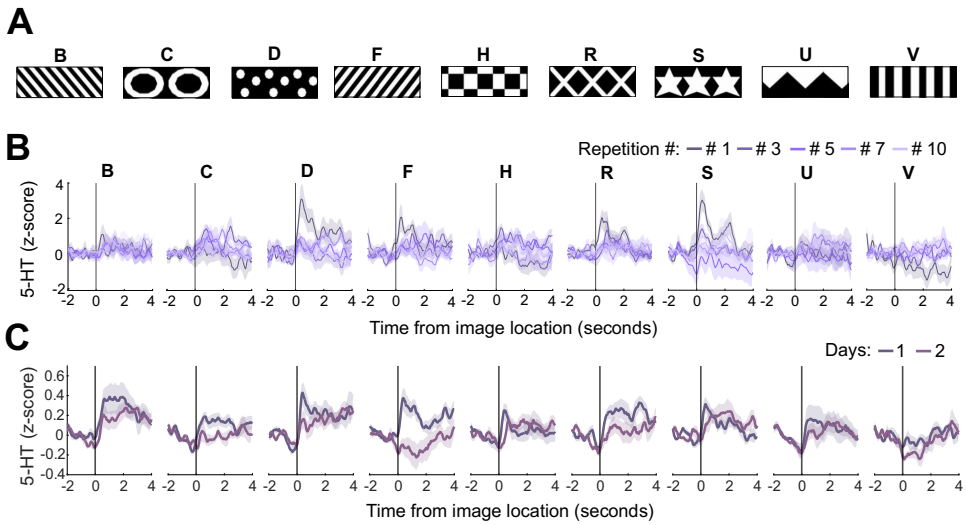

Figure 7. *Serotonin responses to novel images is image specific across repetition but not across days.*

A. All the images in the task. **B.** Image specific first 5 image repetition responses averaged across all mice (mean and SEM, n=9). **C.** All image repetitions averaged for each image on days 1 and 2 (mean and SEM, n=9).

This suggests that serotonin transients respond differently to all images presented in the task even when novel, and the adaptation is specific to each image. This could be due to the nature of each image, as the images creating the most contrast with the background texture of the corridors, or interfering with the optic flow, would induce more surprise and be more noticeable. Nonetheless, averaging each image's response across all repetitions on day 1 reveals that there is no preferred image across mice overall (1-way RM ANOVA, image identity averaged response in 1 second window from image location, p= 0.0790, **Figure 7.C**.). Images also induce higher responses on the first day compared to the second day (paired t-test, day 1 vs day 2, p=0.0030). Hence serotonin neurons appear sensitive to image identity, but overall, serotonin neurons respond most to images once novel and unexpected and adapt as images become familiar across the first two days of the task.

4.5. Discussion and Interpretation

The paradigm we developed and describe in the first chapter allowed us to assess whether serotonin neurons would respond to unexpected novel stimuli. Thanks to working with headfixed mice running in a virtual corridor, we were able to precisely monitor mouse location along the track with respect to image locations. When aligning serotonin neurons' activity to image locations, we report that serotonin neurons resond to the first repetition of novel, unexpected images (**Figure 5**). These responses adapt quickly across the first 10 image repetitions. We were able to show that this adaptation is not an effect of time, neither of the number of images seen in the task (**Figure 6**). Averaging all image responses for each of the 9 images individually on the first day revealed some specificity in serotonin neurons' responses to images (**Figure 7**). Image responses did not recover on the second day of the task.

The adaptation of serotonin neurons' repsonses to images across the first two days of the task could arise from the change in state of the images for the observer. Indeed, at this stage of the task, mice have not yet learned to differentiate rewarded from unrewarded corridors thanks to images on the corridor walls. Through corridor repetition, images become familiar to the mice running in the task, but images carry no informative value of the task, yet. The combination of the images being familiar and irrelevent to the mice in these first two days of the task could explain the rapid adaptation of the serotonin image response across repetition. As we presented in the introduciton, serotonin neurons appear to signal both unexpected and behaviourally relevant and salient cues (Matias et al, 2017, Paquelet et al, 2022). A novel stimulus, as our novel images, could exist within all three categories – unexpected, behaviourally relevant and salient.

It will be important to examine how serotonin neurons in response to other sitmuli in the task also respond across trials. In our analysis, we have averaged the entire serotonin trace across the same duration of time that mice took to run past the first 10 repetition of each image. As such, this average response might not reflect if serotonin responses to other stimuli adapt across time. A future analysis could be to examine how serotonin neurons' reward responses adapt with the session (Paquelet et al, 2022). We could then compare the decay rates of serotonin reward and image responses and assess whether the adaptation we report here is specific to the novel images in the task.

The unexpected state of these image presentations triggering the serotonin neurons' response aligns well with the unsigned prediction error hypothesis of serotonin, signalling surprise in the brain (Ligneul et al, 2023). The need to understand "what" is present in the environment could be interpreted as a

relevant behaviour to perform, ethologically for survival (Tapper & Molas, 2020). Uninformative, familiar cues that are not very inherently salient could thus not be the correct stimuli to induce activity of serotonin neurons (Caldenhove et al, 2017). Our results align with fiber photometry recording results of mice discovering a novel object in the open field, where serotonin transients were locked to approaches to the novel object (Li et al, 2016). For future experiments, one could introduce a novel image in late trials of day 1 and measure serotonin image responses in response to this image, compared to familiar images introduced in the early trials.

The task we developed in this VR environment allows mice to learn to associate combinations of images within corridors to specific reward contingencies. Hence during learning, images become behaviourally relevant as informative of reward delivery across days. In the next section, we will explore how this learning affects the serotonin neurons' responses to images within and across sessions.

5. Evolution of Serotonin Neuron's Responses to Visual Cues Across Trials and Days During Learning

5.1. Background and Motivation

The influence of contextual information on the response of serotonin neurons to sensory stimuli in the environment is now well established (Sizemore et al, 2020). As discussed in the previous section, that serotonin neurons respond to novel cues and adapt as they become familiar is an example of this flexible signalling. Serotonin neurons have also been shown to release more serotonin in the auditory cortex and inferior colliculus in mice during social or stressful events as opposed to when in control environments, supporting this body of work (Hurley & Hall, 2011).

In the context of learning, papers have shown that serotonin neurons modulate their firing activity with information about future outcomes such as rewards or punishments (Matias et al, 2017; Cohen et al, 2015). However, most of the research has been focused on recording activity of serotonin neurons once learning has been acquired, and not during the learning process itself. Zhong *et al* recorded the activity of serotonin neurons with fiber photometry as mice learned to associate an auditory cue to a reward delivery (Zhong et al, 2017). This work reveals a gradual shift in the serotonin response from the reward time to a ramping up of activity from the cue until the reward. This dynamic of serotonin activity was recorded across trials and 4 consecutive days in the task.

We developed our task with the intention to bring more insights on how serotonin neurons evolve during learning of cue associations and reward contingencies. Before separating images based on their corridor value, we explore how serotonin neurons' responses to all images evolve across trials and days. From the previous section, we report a specific adaptation of the serotonin image response for images in the task on the first and second days, as novel images become familiar. During learning, familiar images become predictive of other images and of reward location, changing their behavioural relevance across trials and days. We thus first explore how this adaptation within the session is affected as animals learn these associations. We then ask how the activity of serotonin neurons in response to images evolves across days on average, and if these responses are correlated to gradients of learning.

5.2. Serotonin Neurons' Responses to Images Adapt Within Sessions as a Function of Learning

We start by examining how serotonin neurons' image response adaptation that we reported in the previous section changes across days. We define early and late trials as the first 30 trials and last 30 trials of each day and plot an example mouse serotonin image responses for first and last trials across location and days, aligned to the image location on the track (**Figure 8.A.**). At first sight, it is possible to observe that serotonin neurons' image responses are always greater on the first trials compared to the last trials. The overall image responses also seem to increase across days. To acquire a general understanding of how the serotonin image response evolves across trials within a session, we average all mice's averaged image responses across trials and days (**Figure 8.B.**). We average specifically the serotonin image response within a 1 second window after image location, normalised to the

baseline serotonin activity in a 1.5 second bin before the image. We average serotonin activity in 10 consecutive trials up to 200 trials for all mice for both image locations respectively. We find a general adaptation of the serotonin image response across trials for all mice and days averaged, with no effect or interaction of image location (2-way rmANOVA, trials and location: trials, p=1.9699e-16; location, p=0.38236; interaction, p=0.41293). This result suggests that image responses at both locations are similarly modulated across trials. Specifically, comparing the first 30 trials with the last 30 trials averaged for all images together reveals a significant decrease in the image response across trials (paired t-test, p=0.0005).

As this adaptation of the serotonin image response across trials appears to gradually decay, we fit exponential decay curves to each averaged image responses across trials of each day (dotted lines in **Figure 8.B.**). We find that exponential decays significantly represent this averaged data (location 1, p=1.33e-06; location 2, p=2.84e-10). For images in the first location, serotonin neurons' image responses decay by 88 images, whilst they decay by 37 images for images in the second location. We show in **Figure 8.C.** six days' image responses across trials for an example mouse, with their associated exponential fits calculated with our exponential decay model (days 1, 3, 5, 6, 8, 10). We average across mice these exponential fits for all days across the first 200 trials for both image locations (**Figure 8.D.**).

We quantify the evolution of these decay constant, the initial value and the asymptote of these exponential decays for each mouse across days. Importantly, in our exponential decay model, we fixed an upper bound of the decay constant to 150 image repetitions. We chose this value as it is greater than the average decay constants found in **Figure 8.B.**

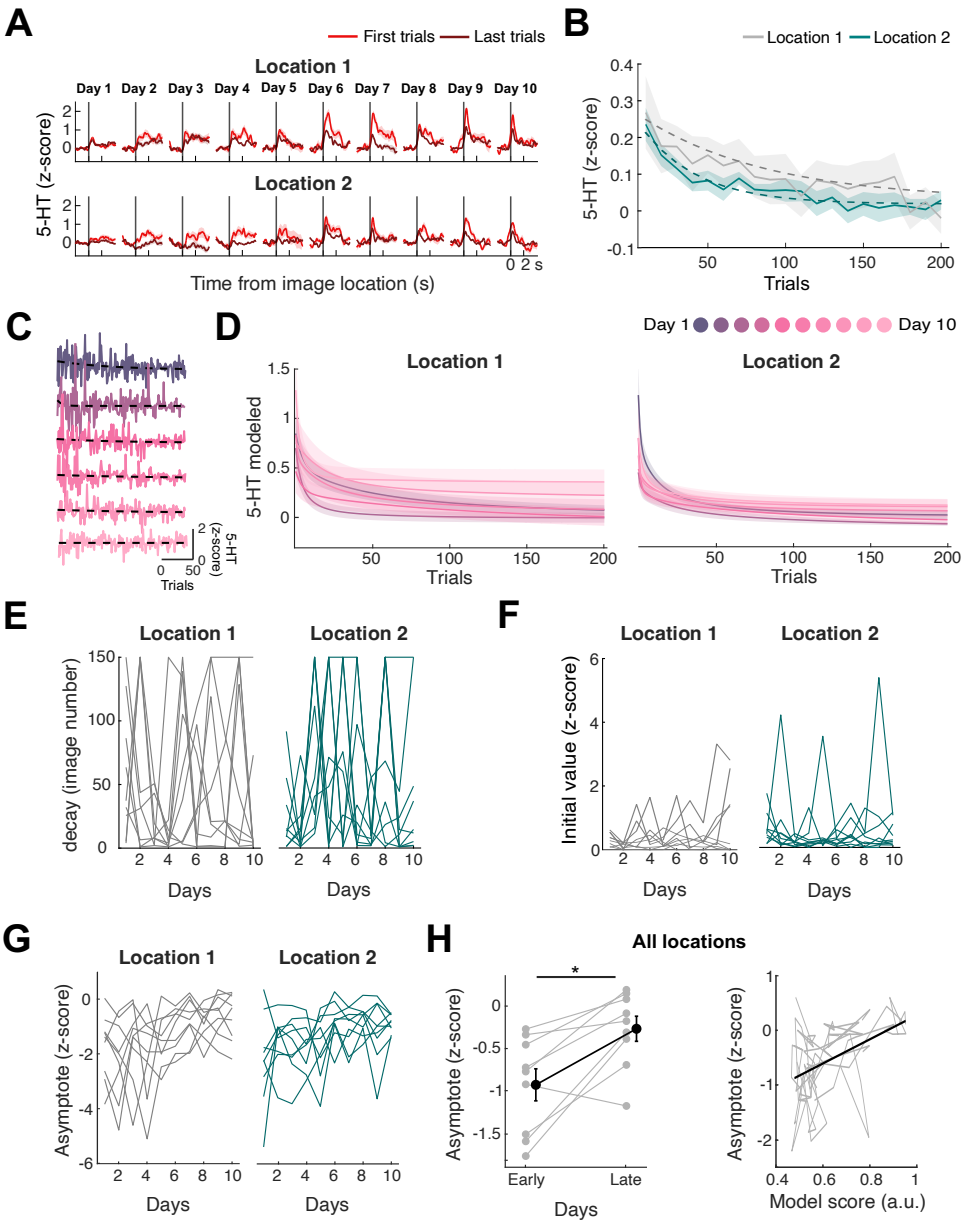

Figure 8. *Serotonin responses to images dynamically change across trials during learning.*

A. Example mouse serotonin response in first and last trials across days for both image locations (mean and SEM across trials). **B.** Serotonin image responses across 10 trials averaged until the 200th trial for all mice in locations

1 and 2 (mean and SEM across mice, n=9). **C.** Example mouse traces of 1 second averages from image location 1 serotonin neurons' image responses across trials for 6 days in the task. Black lines are exponential decay fits. **D.** Average of all exponential decay fits across mice for each day at both image locations respectively, plotted over the first 200 trials of each day (mean and SEM across mice, n=9). **E.** Decay coefficients from exponential decay fits for each mouse and each day for image location 1 and 2 respectively (n=9). **F.** Initial value of exponential decay fits for each mouse and each day for image location 1 and 2 respectively (n=9). **G.** Asymptote from exponential decay fits for each mouse and each day for image location 1 and 2 respectively (n=9). **H.** left: average for each mouse and across mice of asymptote values for early (2-4) and late (8-10) days (n=9, grey are individual mice, averaged across days, black is average across mice and SEM); right: asymptote values as a function of model scores (n=9).

When plotting the evolution of the decay constants for all mice across days for each image location, multiple daily decay constants reach this upper decay constant bound (**Figure 8.E.**). This result suggests that this adaptation across trials is not present for all mice and days. A proper analysis of this decay would consist of performing a model comparison analysis. A linear model would be compared to an exponential decay model for each mouse and each dataset from each day and each location. For lack of time to perform this analysis before submitting this thesis, we rely on this exponential decay analysis, without interpreting the evolution of the decay constants across days. The initial value of the model and the asymptote remain interpretable. We find no significant organisation across mice of the evolution of the initial value across days of the task and across location (2-way rmANOVA, days and location: days, $p=0.6949$; location, $p=0.6071$; interaction, $p=0.1088$; **Figure 8.F.**).

We plot the evolution of the asymptote acquired from the exponential decay models for each mouse across days of the task for both image locations (**Figure 8.G.**). We find a significant organisation of the asymptote predicted by the model across days and mice for both image locations (2-way rmANOVA, days and location: days, p= 8.2341e-06; location, p= 0.8636; interaction, p=0.3549). Importantly, there is no interaction between image locations and the evolution of the asymptote across days. To capture how this asymptote changes across days in the task for all mice, we compare average asymptote values for all mice in early and late days (left, **Figure 8.H.**). Due to the unique status of images on day 1 being novel and unexpected, we consider early days of the task as days 2 until 4. Throughout these days, mice show also no sign of discriminability of rewarded and unrewarded corridors (**Figure 2**). Mice do show corridor discriminability on the last 3 days of the task. We thus define late days of the task as days 8 until 10. Comparing the average asymptote values of modelled serotonin image responses across all image locations across mice in early versus late days, we find a significant increase in the asymptote across days (paired t-test, p=0.0054).

We ask how the evolution of the asymptote across days for all mice is related to learning. We make us of the logistic regression modelling presented in **Figure 3** to explore this relationship. Specifically, we take the model scores for each mouse and each day averaged in the 40 cm window leading up to the reward delivery zone. As previously explained, model scores are high when the logistic regression model is able to make accurate predictions on corridor identity based on the running speed of the animals in this bin. In the right panel of **Figure 8.H.**, we plot for each mouse the evolution of the asymptote values on each day as a function of daily model scores. Running a linear regression on the data for all mice reveals a very strong positive relationship between asymptote values and performance (slope coefficient =2.2063; p=1.7628e-0.6). Hence, good behavioural performance correlate

with higher asymptote values, suggesting the serotonin response does not decay as much across trials as it does for mice and days with less good performance.

We then wanted to explore whether this model prediction could be found in the data. As not all mice run the same number of trials daily, we focus our analysis on the seven mice that ran more than 100 trials each day. We chose this cutoff at 100 trials thanks to the average decay constant of the image responses across trials measured from the exponential fit in **Figure 8.B.**

We average for every mouse the first 30 and last 30 trials daily as in **Figure 8.A.** and plot the evolution of these responses across mice and days for both locations respectively (**Figure 9.A.**). We test independently the evolution of first and last trials for both image locations and find that image locations do not affect the responses across days (2-way rmANOVA, first trials across days per image location: days, p=0.0026; image location, p=0.6914; interaction, p=0.9296; 2-way rmANOVA, last trials across days per image location: days, p=0.0109; image location, p=0.8146; interaction, p=0.8195). Thus, averaged across image locations, we find a significant modulation of the serotonin image responses across days for both first and last trials but no interaction (2-way rmANOVA, early and late trials across days: trials, p=0.0001; days, p=0.0002; interaction, p=0.2273). With a significant difference between first and last trials, serotonin neurons in response to images appear significantly modulated across trials and days of the task.

To better understand how this adaptation evolves across days of the task, we compare these averaged image responses in early and late days of the task, as defined in the previous analysis (**Figure 9.B.**). Across the seven mice, we

find a significant adaptation of the serotonin image responses across trials only in early days of the task (Early vs late days, first vs last trials, 2-way rmANOVA, trials, p= 0.0113; days, p= 0.0553; interaction, p= 0.4644, adjusted paired t-test, first vs last trials: early days, p= 0.0147; late days, p= 0.1689). Moreover, the responses on late days of the task adapt less than those on early days, with late trials image responses on early days being significantly smaller than those on later days (early vs late days: first trials, p= 0.1786; late trials, p= 0.0278). Hence, it appears that the adaptation to images changes across days of behaving in the task, as found when analysing exponential decays (**Figure 8**).

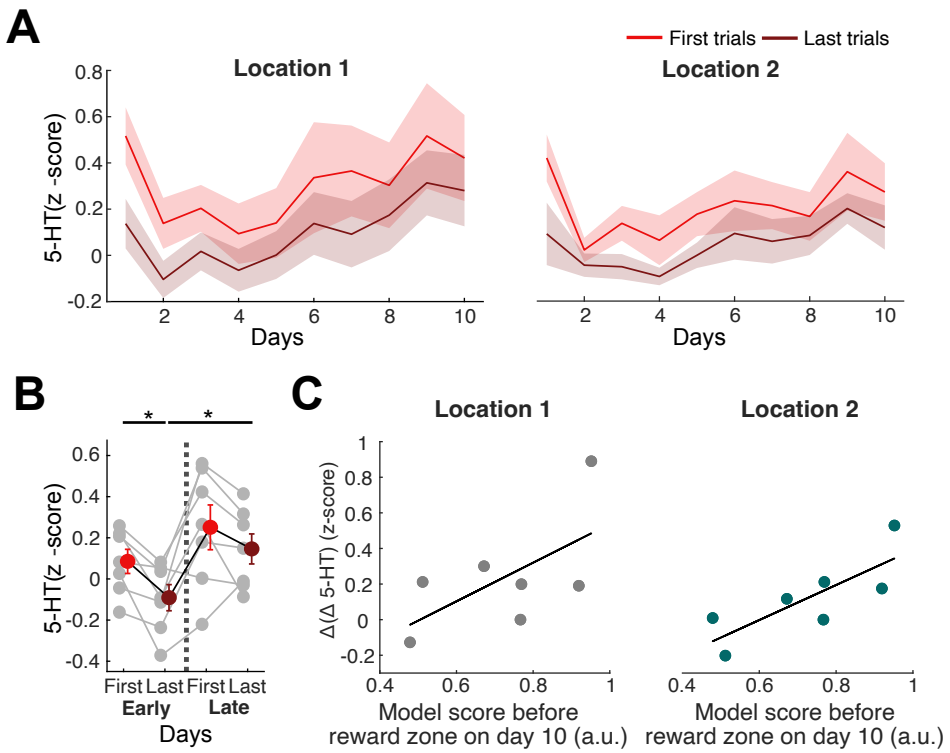

Figure 9. *Serotonin neurons respond differently to images within sessions across learning.*

A. First and last trials across days for images in the first location (left) and second location (right) across mice and days (mean and SEM across mice, n=7). **B.** Average of first and last 30 trials in early and late days of the task for all mice (mean and SEM across mice, n=7). **C.** Adaptation across trials of image responses for all days averaged as the delta z-score of first minus last trials of the delta z-score of early minus late days for images in location 1 (left) or 2 (right) as a function of model score before the reward zone on the 10th day of the task (n=7).

Our final aim in this section is to capture how the evolution of serotonin image responses across trials and days is related to learning and performance (**Figure 9.C.**). To do so, we calculate the difference of early minus late days for first and last trials respectively per mouse and subtract these first minus last trials' deltas for each mouse. We plot this delta-delta serotonin response per mouse as a function of each mouse's model score on the last day of the task (day 10). We find a significant positive relationship between model performance on day 10 and this metric of serotonin neurons' adaption across trials and days for images in the second location ($p=0.0326$). We find a similar but not significant trend for images in the first location (location 1, $p= 0.1393$). In line with the asymptote analysis from **Figure 8**, this result suggests that mice with better behavioural distinction between rewarded and unrewarded corridors before the reward delivery zone show less serotonin neurons' image response adaptations across days. Mice with stronger adaptation as the difference between first and last trials evolved across days of learning discriminate corridors less well with locomotion.

All together, these results suggest that serotonin neurons' responses adapt across image repetition during learning. As the images gain in meaning and

behavioural performance increases, image adaptation gets weaker. We ask how the averaged image responses evolve across days of learning.

5.3. Serotonin Image Responses Increase Across Days of Learning

To explore the evolution of serotonin neuron's responses to images throughout days of the task, we average for each mouse all image responses of all corridors ran per day, maintaining both image locations separate. Image responses are again defined as the 1 second window from image location. **Figure 10.A.** shows the evolution of these responses for an example mouse in both image locations across the 10 days of the task.

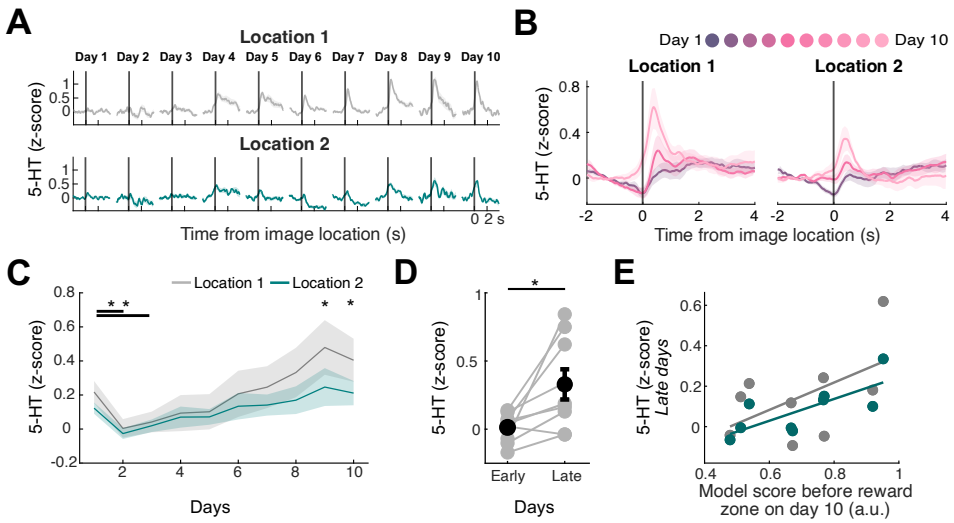

Figure 10. *Serotonin neurons' responses to images increase across days.*

A. Example mouse of serotonin responses in time for all images at image locations 1 and 2 respectively, averaged across trials (mean and SEM across

trials). **B.** Serotonin responses around image locations for days 2, 5 and 10, all mice averaged, location 1 and location 2, (n=9, SEM across mice). **C.** Averaged serotonin image responses across days in a 1 second window from image location for both locations separately. **D.** Comparison of early days (2-4) and late days (8-10) days for all mice and all images. **E.** Serotonin image responses on late days as a function of learning on day 10 for images in the first and second location (n=9).

It is possible to note an increase in the serotonin activity aligned to both image locations across days, which remains present when averaging across all 9 mice that performed the task (**Figure 10.B.**).

We find a significant interaction between image location and the response to these images across days, with a significant effect of days alone modulating the serotonin response for both image locations independently (2-way rmANOVA, days and location: days, p=0.0001; location, p=0.1393; interaction, p=0.0045, **Figure 10.C.**). Specifically, for both image locations, serotonin image responses are higher on day 1 than on day 2, reflecting the sensitivity of serotonin neurons for signalling novel stimuli and adapting as these become familiar, as reported in the previous section (adjusted paired t-test, day 1 vs day 2; location 1, p= 0.0139; location 2, p= 0.0259). Moreover, serotonin neuron's responses to images on days 9 and 10 are higher for the first location compared to the second (adjusted paired t-test, location 1 vs location 2; day 9, p=0.0246; day 10, p=0.0144).

When comparing the serotonin image responses in early days and late days of the task for both image locations averaged together, we find a significant increase in the serotonin transient amplitude in late days of the task compared

to the first days (paired t-test, p=0.0276, **Figure 10.D.**). We ask again whether this general increase in serotonin neurons' image responses on late days of the task is related to performance in the task. We plot the average serotonin image response across mice on late days of the task averaged for both image locations respectively as a function of model scores on the last, 10^{th}, day of the task (**Figure 10.E.**). We find a significant relationship between the average serotonin neuron's responses to images in the second location in late days of the experiment with performance on the last day (slope coefficient = 0.5391; p=0.0197). For images in the first location, the slope was of a similar trend but not significant (slope coefficient =0.1349; p=0.1349). This analysis supports the results presented in the previous figures **Figure 8** and **Figure 9**. Whilst all mice show an increase in the serotonin neurons' image responses across days, this magnitude of the response is correlated with the overall performance of the animal on the last day of the task.

5.4. Revealing Serotonin Neurons' Dynamics in Response to Images Across Trials and Days of Learning

Overall, we present evidence that the responses of serotonin neurons aligned to images at both locations in the corridors are modulated across trials and days in the task. To capture the entire dynamic of the transients in response to images across trials and days, we plot a matrix of serotonin image responses for up to 200 trials on each session, as averages of 10 consecutive trials, in the 10 days of the learning task, averaged across mice and split between image location (location 1 and location 2, left and right, **Figure 11.A.** and **11.B.**).

We normalise all mice serotonin image responses to the maximum value across all trials and all days before averaging all mice together. It is possible to see this pronounced serotonin response to novel images in the task across the fist 30 trials of day 1. This response adapts across trials of this first day and within the first 4 days of the task.

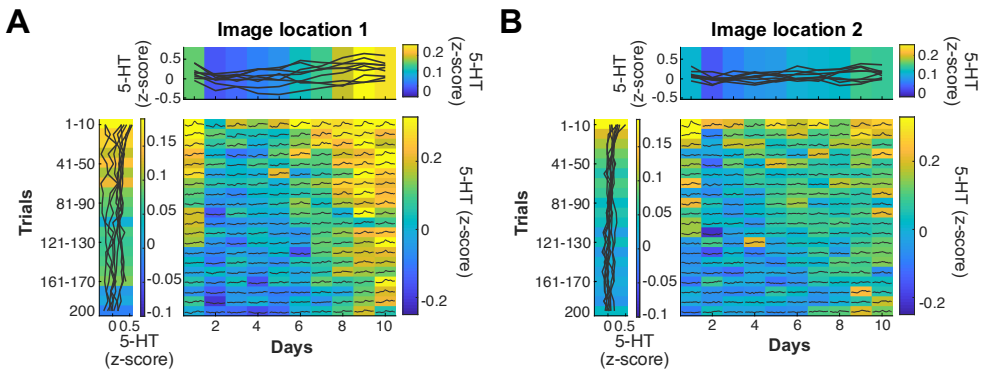

Figure 11. *Evolution of serotonin neuron's image responses across trials and days.*

A. Matrix of serotonin image responses across days and trials for images in the first location. Projections of serotonin image responses across days (top rows) and across trials (left columns) for all mice (n=9). **B.** The same plot for images in the second location.

On later days, serotonin neurons' image responses increase and adapt less across trials. This effect is more pronounced for images at the first location. Plotting the projection of the serotonin image responses across days (top rows) reproduces this increase in image responses across days, whilst the projection of serotonin image responses across trials reflects again the adaptation within the sessions (left columns). Thus, within and across days, serotonin image responses are dynamically modulated.

5.5. Discussion and Interpretation

In this section, we report that the learning of the association of visual cues with reward contingencies in mice dynamically changes the responses of serotonin neurons across trials and days. Serotonin image responses adapt across trials, and this adaptation is modulated by learning (**Figure 8** and **Figure 9**). Specifically, mice that differ best rewarded from unrewarded corridors in their running speeds before the reward delivery location show less adaptation of serotonin neurons across trials, as opposed to those with less good behaviour. In line with this result, average daily image responses increase across days and correlate with performance on late days (**Figure 10**). By plotting the serotonin neurons' image responses across trials and days of the task, we show how these responses are dynamically modulated as images change their contextual information – from novel to familiar and irrelevant to learned salient as relevant for behaviour (**Figure 11**).

It is possible to combine these results with the perspective of serotonin neurons behaving as unsigned prediction error signals throughout the task (Matias et al, 2017). As mice run through corridors daily, corridor distribution throughout the session is always random. Images in the first position are thus always $1/5^{th}$ predictable. The increase in the image responses across days could thus represent this inherent unpredictable context of first images being present on the corridor wall, enhanced by the relevance of the cues in the task. Indeed, errors could be larger for stimuli for which the subject is more attentive to, either as informative of a future state or behaviour or purely surprising and unexpected. This would place inherently salient stimuli and learned salient stimuli on a unique axis to which serotonin neurons would be modulated by. Looping back to our novelty response analysis, this could relate

to the “what” question regarding stimulus identity. This would be true for all mice regardless of how well they learned the task.

That serotonin image responses are smaller in late days of the task for images in the second location compared to images in the first location could reinforce the idea that the predictability context influences serotonin responses to learned salient stimuli. Indeed, images in the second location in fixed contexts are fully predictable, whilst 1/4th predictable for ambiguous corridors. As the ambiguous corridors make up of only 1/5th of the corridors of the environment, their specific response could be masked when averaging all images in the second location together. We will explore in section 10 these specific image responses. In our case, as both images are introduced simultaneously in the corridors to be associated with the reward contingency, the smaller serotonin image response to these images in the second location cannot be due to a classical conditioning blocking effect of the second image with the reward outcome by the first image (Sanderson et al, 2016). For future experiments, it could be useful to introduce trials with only one of the two images in the corridor, to assess if mice can perform correct behaviour with single images alone.

Thanks to our behaviour allowing for mice to collect rewards with different strategies, we were able to look at how gradients of learning are reflected in the serotonin image response. With better performance correlating with more serotonin responses to images, it seems plausible to associate these responses to attentional processes (Thiele & Bellgrove, 2018). It was shown in cortical areas that attention modulates visual responsive neurons in a learning task (Poort et al, 2022). More adaptation of image responses within the session could represent a more disengaged behaviour of mice in the task (Ortiz et al, 2020). Thus, an important future analysis to perform is to assess

whether this adaptation is correlated with engagement in the task across trials of a given session.

Though we have presented clear relationships between the modulation of serotonin neuron's image responses across trials and days with learning, we cannot claim that other factors could not be responsible for this increase in the serotonin image response across days. Experience of the images in the VR corridor across days alone could turn them behaviourally relevant and salient for mice in the task, as landmarks for navigation and changes in optic flow that mice would pay attention to. Moreover, as mice change their locomotion across days along the corridor, these image responses could be correlated with locomotion in the task. Hence in the next two section, we present our work aimed at disentangling the serotonin image responses from these hypotheses.

6. Serotonin Activity in the Absence of Learning

6.1. Background and Motivation

Serotonin neurons have been shown to be involved in a plethora of behaviours, responding to stimuli depending on contextual information. The dynamics we report in our task of serotonin neurons' responses aligned to images could arise not only from learning but from other contextual factors. As previously mentioned, the activity of serotonin neurons has been associated to attentional processes (Thiele & Bellgrove, 2018). Injections of a 5-HT2A agonist in rats performing a reaction time task was reported to increase impulsivity and reduce accuracy (attention) but stimulating serotonin neurons with light increased patience for delayed rewards (Koskinen et al., 2000; Fonseca et al, 2015). Injecting 5-HTP in rhesus macaques when viewing social and non-social images affected attentional processes bidirectionally (Weinberg-Wolf et al, 2018). Macaques already attentive at baseline showed a reduction in looking duration of images in the task, whilst macaques looking for short periods of time at baseline revealed more attention with prolonged looking durations after injection.

In our task, attentional processes targeted towards these images could arise without learning of the task, as they become visual landmarks in the corridor track, for example (Saleem et al, 2018). As the visual cortex encodes visual cues differently based on their position in the linear track, it could be that serotonin neurons behave the same. In our VR experiment, mice have the tendency to increase their running speeds across days. This consequential

change in optic flow, combined with attention, could become responsible for serotonin responses (Horrocks et al, 2023).

To tackle this issue, we developed a second virtual reality task where mice ran in identical corridors as in the learning task, but without learning any association between corridor identity and reward delivery. We ran the task on 6 SERT-GCaMP6s mice of identical genotype as the mice in the learning task, expressing the calcium fluorescence sensor in serotonin neurons. We recorded serotonin neuron's activity in this control task with fiber photometry across 10 days.

6.2. Mice Learn to Run for Randomly Delivered Rewards

Corridors in the control task were identical to those in the learning task in terms of visual cues on the corridor walls (**Figure 12.A.**). Corridors were also either fixed or ambiguous based on image predictability, but no corridor was defined rewarded or not. Rewards were delivered randomly in the corridors, with an average of 650 cm of distance between each reward, slightly shorter than the average distance between rewards in the learning task (800 cm). We fixed a running speed threshold for reward delivery at 40 cm/s to enforce mice to run at similar speeds than in the learning task. As such, mice had to run below this threshold throughout the entire session, as reward delivery could never be predicted. Hence, in this task design, images in the corridors and the reward location zone never gained any informative value of reward delivery. Any serotonin activity in response to images across days could thus not be due to any form of reward-based associative learning.

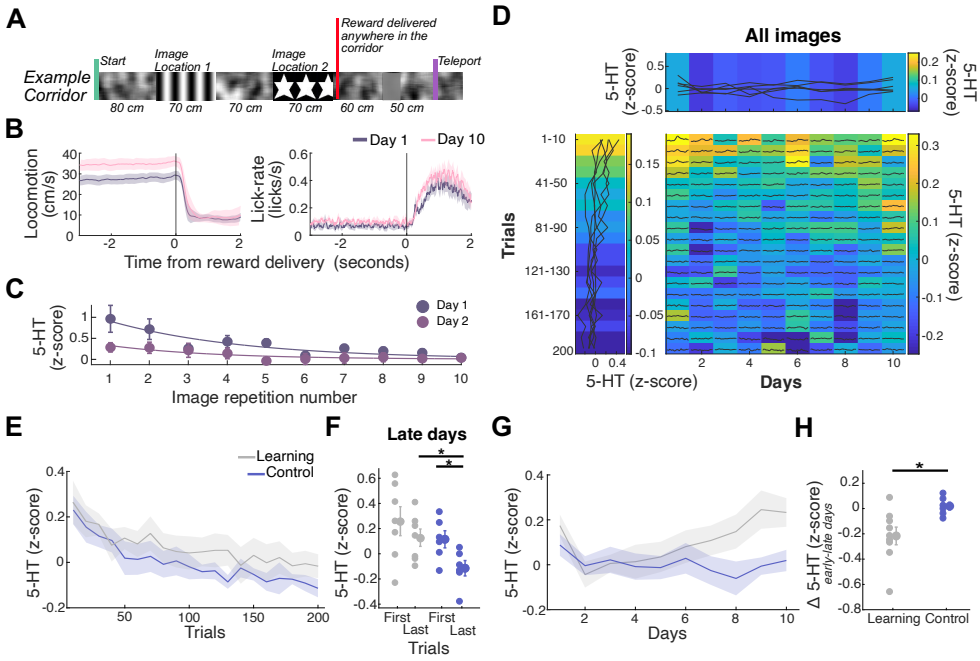

Figure 12. *In a control task without learning, serotonin transients respond to novel images but are not recovered across days.*

A. Schematic of the corridors, rewards are delivered at random locations, and we kept the same reward rate. **B.** Locomotion and lick-rate around reward delivery events in days 1 and 10 (mean and SEM, n=6). **C.** Novel image responses and adaptation across repetition number for all control mice. **D.** Serotonin image responses across days and trials for all mice at both locations (mean and SEM, n=6). **E.** Evolution of serotonin image responses across days for the learning group (mean and SEM, n=7) and the control group (mean and SEM, n=6). **F.** Comparison of the delta z-score early minus late days for the learning group vs control mice (individual mice data points and mean across mice with SEM). **G.** Adaptation across trials for the learning and control groups, averaged across all 10 days of each task (mean and SEM, n=9 and n=6). **H.** First and last 30 trials for all mice in the learning and control groups, only in late days of the tasks (individual mice data points and mean across mice with SEM).

To examine the evolution of mice behaviours across days of the task, we plot, in time, the locomotion and lick-rate around the reward delivery periods for both days 1 and 10, averaged across mice (**Figure 12.B.**). We find that behaviours on both days are not significantly different, with mice running from 28 cm/s until 35 cm/s before the reward delivery location and slowing down only after the reward has been delivered to 8.4 cm/s on average on days 1 and 10 (locomotion: day 1 vs 10, paired t-test, average 2 seconds before reward delivery, p=0.0908; after reward delivery, p=0.8353). In parallel, mice had a constant baseline lick-rate throughout the corridor track of on average 0.08 licks/s, and only after reward delivery did the lick-rate increase to 0.35 licks/s on average to consume the reward (lick-rate: day 1 vs 10, paired t-test, average 2 seconds, before the reward delivery, p=0.2074; after reward delivery, p=0.4284).

6.3. Serotonin Neurons' Transients in the Control Task Reproduce the Novel Image Response

We first asked if we could reproduce in this experiment the novel image results of the learning task presented in section 2. Indeed, control mice were also trained to run for rewards in a corridor with only the background and reward delivery zone textures. Thus, images of the corridors in the first trials of day 1 were also new and unexpected to these mice. Thus, for all mice, we averaged the first 10 repetitions of each image and averaged the mice together, as done in **Figure 5.F.** (**Figure 12.C.**). We find once more that the serotonin image responses adapt on the first day across image repetitions after fitting an exponential decay (1-way rmANOVA, repetition, p=0.0006, exponential decay fit on mean, p=0.0008). Moreover, the adaptation of the serotonin image response on the second day is different from the one of the first day, with a decay constant on day 1 of 2.1718 images and of 2.9538 on

the second day (2-way rmANOVA repetition and days; repetition, p=0.0003; days, p=0.0488; interaction, p=0.0798; exponential fit day 2, p=0.0032). First repetition serotonin image responses on the first day are however not different from those of the second day (day 1 vs day 2 first repetition, p=0.1158). Running a mixed model comparison between learning and control groups on the evolution of serotonin neurons in these first 10 image repetitions on the first day confirmed no significant difference between groups (Mixed model ANOVA, repetitions and experiment group: repetitions, p=4.6137e-0.7; group, p=0.5660; interaction, p=0.5194). Thus, this experimental group reproduces the novelty response of the learning group and is a good candidate to explore how serotonin neuron's responses to images in the task evolve across days when there is no associative learning.

6.4. Serotonin Neuron's Image Responses Adapt Most Without Learning

We asked how the dynamic of serotonin neuron's image responses within each day was evolving for all mice in the control task. Averaging the consecutive 10 trial averages across days per mouse and across mice reflects a strong adaptation of the serotonin signal across trials (1-way rmANOVA, trials, p=2.3567e-11, left column, **Figure 12.D.**). When comparing with the learning group, we find that the image response is significantly modulated by trials for all groups and find no group or interaction effect between trials and experimental group (mixed model ANOVA, trials and group; trials, p=0.0046; group, p=0.0949; interaction, p=0.3524, **Figure 12.E.**). When comparing first and last trials specifically in late days of the task, when animals have learned the associations in the learning task, we find no difference between groups in the first trials response amplitudes, but a significant difference in the last trials between groups (first trials, t-test,

p=0.3153; last trials, t-test, p=0.0124). Moreover, whilst there is no significant difference in the learning group for first vs last trials on late days, we find a significant difference in the control group (first vs last trials, learning group, t-test, p=0.1476; control group, p=0.0124, **Figure 12.F.**). Hence, this suggests that serotonin transients aligned to images adapt more for the control group compared to the learning group specifically in late days of the task, when learning happened in the learning group.

6.5. Serotonin Neurons' Image Responses Are Not Recovered Across Days Without Learning

To capture how the serotonin neuron's responses to images evolve across days of the task, we plot the evolution of the serotonin transients in response to images across days for all images combined in the control task (top row, **Figure 12.D.**). We find no significant trend in how the signal evolves across days (1-way rmANOVA, days, p=0.4426). When comparing the serotonin image responses across days between this control group and the learning group, we find a significant interaction between experimental group and days, as well as an effect of days alone, suggesting that the context of the task influences the dynamics of serotonin image responses across days (mixed model ANOVA, days and group; days, p= 0.0023; group, p= 0.2080; interaction, p=0.0096, **Figure 12.G.**). We calculate the delta serotonin image responses for early minus late days and compare the two groups (**Figure 12.H.**). We find a significant difference between groups, where control mice's serotonin activity in response to images does not increase as those of the learning group mice, with a mean delta z-score close to zero (t-test, p=0.021).

To summarise these results, we plot a similar matrix of serotonin image responses across trials and days as in **Figure 11** for the learning mice group in **Figure 12.D.** for our control experiment. In this matrix, we find the novel image response of the first trials of day 1, as well as the subsequent adaptation, as for the learning group. However, serotonin activity in response to images is not recovered across days of the task.

6.6. Discussion and Interpretation

We provide evidence that serotonin transients in response to images in the control and learning groups are differently affected across trials and days of the tasks (**Figure 12**). Whilst in both groups, serotonin neurons respond to novel and unexpected images on day 1 and adapt within the session, serotonin transients in response to images across days are only recovered in the context of learning. Specifically in late days of the task, serotonin neurons' adaptation to images in the control group is more pronounced than for the learning group.

The adaptation of serotonin neurons' responses to images across days of the control task could support once more the result that the response of serotonin neurons is modulated by stimulus predictability (Matias et al, 2017). Though the images are not novel anymore to the mice on late days of the task, the environment might never be completely predictable. This argument has been used when explaining the remaining firing of dopamine neurons in response to rewards, even when these are fully predictable (Fiorillo et al, 2003). The response diminishes with learning, but never disappears fully. In our case, serotonin neurons could be responding to images in the first couple of trials each day as a sign of this remaining error signal that exists even when

environments are seemingly always predictable. It will be important to analyse how the decay of the serotonin image response across days of the control task evolves, as performed for the learning group in **Figure 8**. A hypothesis could be that the decay constant of the serotonin image response in control mice would occur on earlier trials on late days of the task, as images are familiar and more predictable.

Serotonin neuron's image responses being only recovered in the learning group suggests that learning is critical for this dynamic. Indeed, as images in the control task never become informative of future reward locations, they remain familiar and irrelevant for behaviour even on late days of the task. Images in the second location of fixed corridors are, as in the learning task, more predictable than images in the first location in the control task. However, we do not report an interaction between image location and serotonin response across trials and days. Thus, that images are irrelevant for behaviour appears as a dominating contextual influence driving serotonin neurons' image response, whilst image predictability plays a lesser role. This result hints again towards a bidirectional modulation of serotonin neurons along stimulus predictability and relevance that we will discuss in the discussion (Zhong et al, 2016). Overall, though images on the corridor walls can remain relevant visual landmarks in the corridors for navigation, this is not sufficient to recover the serotonin response (Fischer et al, 2020).

Another interpretation for the small but sustained serotonin response across days of the task in the first trials of each session could correspond to the general behavioural state of the animals. As mentioned in the introduction, activity of the serotoninergic pathway has been shown to be correlated with locomotion in freely moving mice (Correia et al, 2017; Seo et al, 2019). As mice are continuously running in a VR environment in both learning and

control tasks, it is important to assess whether the DRN serotonin activity we report is not also modulated by locomotion. In the next section, we will explore the relationship between the activity of DRN serotonin neurons and the locomotor behaviour of mice running in the task. By extracting motion-dependent and motion-independent signals from the serotonin photometry activity trace, we will examine if and how these signals in both tasks represent task-related effects we have reported in sections 4, 5 and 6.

7. Motion-Independent and Motion-Dependent Serotonin Signals in the Tasks

7.1. Author Contributions

The work in this section was carried out in collaboration with a visiting student in the laboratory of Dr Zachary F. Mainen, Stefan Hadjuk. Together we discussed about the data and analyses to be performed. Stefan wrote the codes for the analyses, as well as for plotting **Figure 13**.

7.2. Background and Motivation

In the last decade, a lot of effort in neuroscience has tried to capture the relation between animal movement and neuronal activity across brain areas in mice (Busse, 2018). Specifically in cortical areas, originally thought to be involved in purely sensory processing, researchers have found uninstructed movement-related information in the activity patterns of sensory neurons (Musall et al, 2019). To understand the best way how neural activity is related to processing information of the environment and higher cognitive variables, it has become crucial to separate neural activity based on its correlation with animal movement. Analysing both signals separately can reveal how motion influences neural activity, and which signal remain.

It is important to remember that the relationship between serotonin neurons and locomotion is not unidirectional. Stimulating serotonin neurons with optogenetics in mice running in an open field induces mice to decelerate (Correia et al, 2017). Correlation analyses of locomotion onset and serotonin neurons' activity has also shown that serotonin activity precedes changes in locomotion (Seo et al, 2019). In contrast, 5-HT levels have been shown to increase after physical exercise (Dey et al, 1992). Thus, activity from serotonin neurons can either lead or follow locomotion. As such, it is worth to not disregard the motion-correlated serotonin activity in our task. As locomotion is the main criterion for good performance in the task, serotonin motion-related activity has the potential to change with learning of the task. These changes could occur from attentional processes or behavioural control through inhibition, two states that have been shown to influence serotonin activity (Homberg, 2012; Boureau & Dayan, 2011).

Therefore, it is important to understand first if the activity of serotonin neurons is correlated with locomotion of mice running in the tasks, and secondly how the motion-dependent and motion independent signals vary across trials and days in different contexts.

7.3. Investigating the Locomotion and Serotonin Correlation

7.3.1. Locomotion and serotonin activity are negatively correlated

To visualise the relationship between serotonin DRN activity and the locomotion of mice running in the virtual environments, we overlay two raw traces of the same time window of the serotonin z-scored activity extracted

from fiber photometry and running speed of an example mouse (**Figure 13.A.**). It is already possible to observe a relationship between the dynamics the serotonin activity across time and the mouse's running speed, with serotonin transients increasing when the mouse decelerates. To explore how serotonin neurons are correlated with running speed, we plot in **Figure 13.B.** the serotonin response averaged in consecutive 1 cm windows along the corridor track, selected only from the first 60 cm on each day and the first five days of the tasks. We selected this restricted dataset to avoid capturing the influence of task-related signals that could correlate with a specific locomotion state. An example of such correlation would be serotonin neurons' responses to rewards, only delivered and consumed when mice are close to being still on the wheel. We combine data from mice across both experiments and plot only the positive averaged running speed values. From this scatter plot, we find a negative relationship between the DRN serotonin neuron's activity with the animals' running speeds. When fitting a linear regression on all the data points from all mice combined, we find a highly significant, negative, linear fit (intercept, p=0.2363, 95% CI: 0.2319,0.2407; slope coefficient= -0.0121, 95% CI: -0.0122, -0.0120; p=0), revealing a negative correlation between the serotonin neurons' activity and locomotion.

We further explore the relationship between serotonin and running speed by binning the data of **Figure 13.B.** in two ways along the locomotion values. First, we separate the data by maintaining an identical number of datapoints per each speed bin per mouse, averaging all serotonin activity values in these bins (grey lines are individual mice, black is average for all mice, **Figure 13.C.**). As mice run on average around 35 cm/s along the corridor track, this binning method allows us to capture evenly distributed serotonin fluorescence values across running speeds. In doing so, we present again the strong negative relationship between serotonin activity and locomotion. The activity

of serotonin neurons once mice run faster than 40 cm/s plateaus, suggesting that serotonin activity could be inhibited at high speeds, or vice-versa.

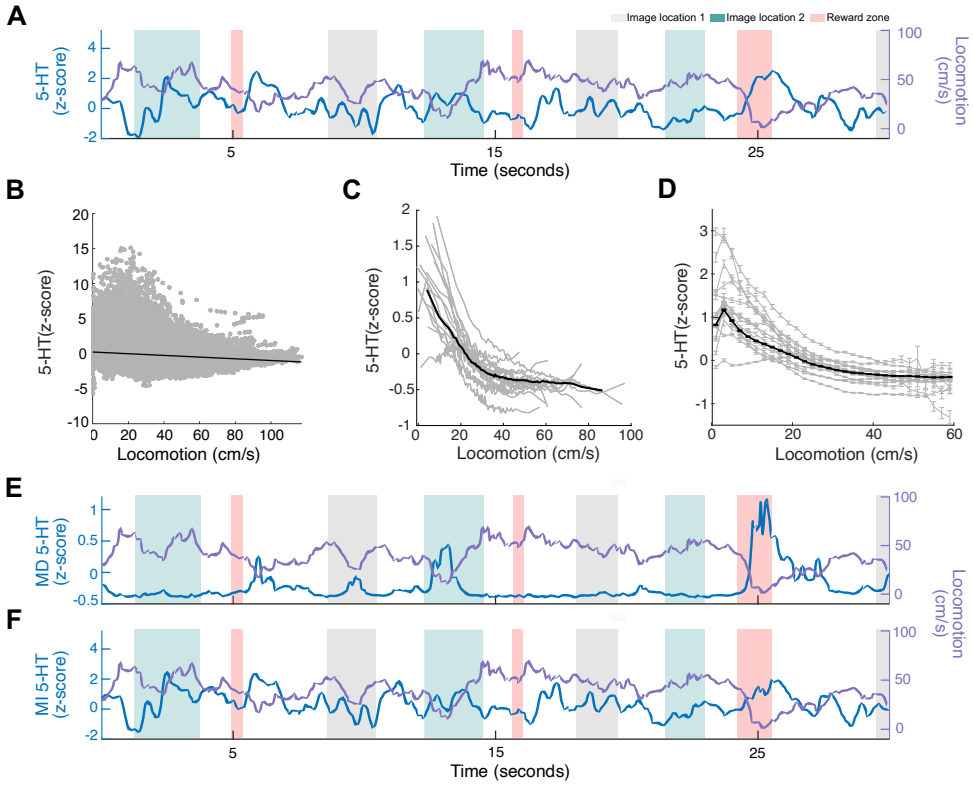

Figure 13. *Locomotion and serotonin activity are negatively correlated.*

A. Example trace in time of serotonin z-score and locomotion (cm/s) along the corridor track. **B.** Scatter plot from all mice serotonin neurons' fluorescence activity in a 1 cm window as a function of the average running speed of that window (n=15). **C.** Identical 1 cm averaged serotonin fluorescence values binned in evenly distributed bins of data as a function of running speed (grey lines are individual mice, black is the mean combining all mice datasets). **D.** Identical 1 cm averaged serotonin fluorescence values binned across average running speed in the 1 cm bin, from 1 to 60 cm/s, bin size of 2 cm/s (grey lines are individual mice, black is the mean combining all

mice datasets). **E.** Same snippet of time as in **A.**, running speed and motion-dependent model extracted serotonin signal in time. **F.** Same as in **E.** but showing the serotonin motion-independent extracted signal.

Secondly, we took for each mouse the average serotonin fluorescence signals in speed bins of 2 cm/s and plotted the relationship of serotonin activity as a function of speed for each mouse, ranging from 0 to 60 cm/s (grey lines, **Figure 13.D.**). Whilst presenting again the clear negative relationship between serotonin activity and running speeds, this analysis reveals an opposite relationship between serotonin and locomotion for speeds below 4 cm/s (1-way rmANOVA, speed bins, p=8.1957e-140). As such, the relationship between serotonin neurons and locomotion might be dependent on the instantaneous running speed, reflecting a possible link between serotonin neurons' selective responses across internal states (Anderson & Adolphs, 2014).

Based on this analysis, we built a function that would incorporate the relationship between serotonin and locomotion using the averaged serotonin values per speed bin, using data from all mice combined (black trace, **Figure 13.D**). We applied this unique function on all running speed datasets for each mouse across days of the tasks to acquire a locomotion-predicted serotonin trace. **Figure 13.E.** represents this modelled z-score trace in the same time window as the raw data shown above. We label this newly extracted serotonin trace and the motion-dependent (MD) serotonin activity. It is possible to see that the predicted serotonin trace varies with locomotion changes, mainly increasing when the animal decelerates. An example of this dynamic in this plot is the deceleration of the mouse at the second image location (light blue) before the 15th second.

To capture the serotonin activity independent from serotonin-correlated locomotion activity, we subtract this MD serotonin activity from the full serotonin z-scored trace we obtained from our photometry recordings. As such, we obtain a motion-independent (MI) serotonin activity trace, that we exemplify in **Figure 13.F.** From this example, it is already possible to observe differences between the MD and MI serotonin signals, and that the MI serotonin signal remains richly modulated across time.

7.3.2. Serotonin neurons' activity tracks locomotion transitions

Locomotor behaviours are continuous processes, where every timepoint is related the previous one. We explore how the full and MD serotonin signals capture locomotion dynamics as changes in running speeds over time (**Figure 14.A.**). We create running-speed transition matrices by finding initial and final running speeds for bouts of locomotion and calculate the delta full and MD serotonin z-scores between these timepoints (left, full serotonin trace; right, MD serotonin trace). Timepoints are defined as all local maxima and local minima extracted over time within the running speed datasets.

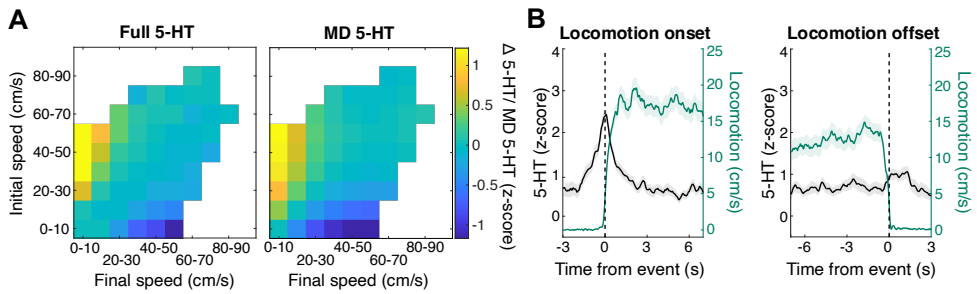

Figure 14. *Serotonin neurons can keep track of locomotion transitions.*

A. Delta serotonin activity between initial speed and final speed bins in a transition matrix for both full serotonin data (left) and MD serotonin data (right) (n=15). **B.** Average serotonin responses upon locomotion onset and offset (mean and SEM across events, n=101).

We find that both full and MD serotonin activity traces appear anticorrelated with acceleration and deceleration (**Figure 14.A.**). Upon deceleration, serotonin transients increase, as the delta serotonin response between initial and final speeds is positive. Contrarily, negative delta serotonin responses are observed as mice accelerate, going from low running speeds to high running speeds. Importantly, these effects are mainly observed at extreme running speed transitions, when the delta running speeds are the biggest. We find no consistent running speed influences on full and MD serotonin signals when mice run at constant, medium speeds. Statistical tests are crucially required to ensure the significance of these dynamics. Nonetheless, these results suggest that serotonin neurons could track deviations in the running-speed of the animals, alongside being sensitive to the instantaneous running speeds of mice in the tasks.

As a final exploration of the serotonin and locomotion relationship, we explore the dynamics of serotonin neurons upon locomotion onset and offset (**Figure 14.B.**) We define periods of stillness as periods longer than 5 seconds during which mice average running speeds are below 2 cm/s. We find that upon locomotion onset, serotonin transients peak and drop around the event time (1-way rmANOVA, time windows of 1 second before, around and after locomotion onset, p=6.2118e-12; pre vs around, p=9.5781e-10; around vs post, p=1.8237e-08). Thus, just before locomotion onset, serotonin activity is systematically high, and drops as the behaviour starts. This result aligns with our serotonin and locomotion correlation plot from **Figure 13.D.**, where bins

of low speeds below 5 cm/s are, on average, the highest serotonin activity values. In parallel, a small increase in serotonin activity appears locked to locomotion offset, suggesting that serotonin neurons could signal locomotor state transitions (1 second before vs 1 second after, paired t-test, p=0.0046).

Overall, we find a strong negative correlation between the activity pattern of serotonin neurons and locomotion of mice running in our VR setup, that keeps track of locomotion transitions. Before analysing how the MD and MI serotonin signals behave in the task in response to images, we ask how the locomotion of mice in both tasks evolve across trials and days at both image locations. This will inform us on the possible influence of locomotion modulations within our serotonin neurons' image response analyses.

7.3.3. Mice in the learning and control groups differently change their running behaviours around image locations across days of the tasks

We plot the average running speeds at both image locations across days for all mice in both learning and control tasks to ask whether absolute running speeds at image locations change across days (**Figure 15**). We find a significant organisation of mice running speeds across days for all mice in the learning task, without any interaction with image location (2-way rmANOVA, days and location, days, p=0.0431; location, p=0.2363; interaction, p=0.0739, **Figure 15.A.**). Importantly, this effect does is not caused by changes in running speeds between early and late days of the task (paired t-test, all images combined, p=0.3840). For control mice, running speeds are differently modulated across days and image location (2-way rmANOVA, days and location, days, p=0.2575; location, p=0.5392; interaction, p=0.0266, **Figure 15.B.**). However, running speeds across mice are also not different when

comparing early and late days of the task for either image location (adjusted paired t-test, early vs late days: location 1, p=0.4446; location 2, p=0.3101). Thus, instantaneous running speeds at image locations do not change across days of running in the learning or control tasks.

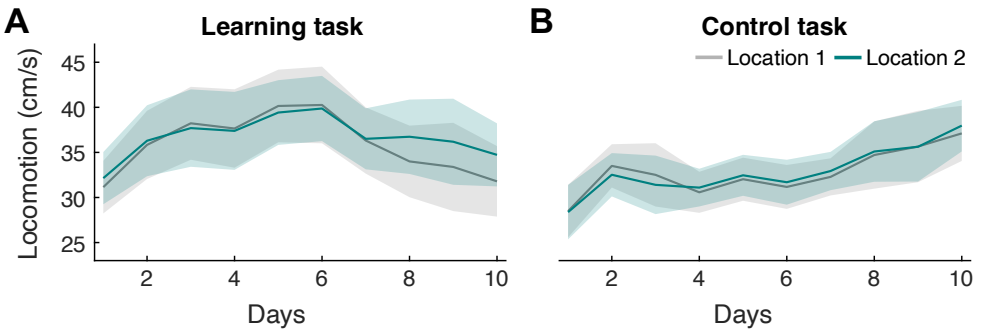

Figure 15. *Locomotion at image locations does not change across days for mice in the learning and control tasks.*

A. Average running speeds (locomotion) across days and mice in the learning task (mean and SEM, n=9). **B.** Same analysis but for mice in the control group (mean and SEM, n=6).

As shown in **Figure 14**, serotonin neurons are not only correlated with instantaneous running speeds but also capture running speed decelerations and accelerations. As such, we plot the matrices of running speeds across trials and days normalised to the baseline average running speed before image location, averaged across mice for both image locations in both tasks respectively (minus 2-0.5 seconds before the image location) (**Figure 16**). Before averaging all mice together, we normalise for each mouse all baseline corrected running speed values in the matrix to its maximum across trials and days. Negative normalised running speed values reflect a deceleration from the baseline upon entering image location. Positive normalised running

speeds reveal accelerations from baseline. We plot all matrices and projections across trials and days along the same axes, to facilitate comparisons between plots.

We first compare the dynamics of running speeds at both image locations in the learning task (**Figure 16.A.**). We find no systematic relationship between running speeds across trials and mice when averaging both image locations (2-way rmANOVA, trials and location: trials, p=0.6294; location, p=0.1678; interaction, p=0.9987). This suggests that mice behave differently at image locations across trials of the task. In contrast, we find that normalized running speeds at image locations are differently modulated across days (2-way rmANOVA, days and location: days, p=0.0816; location, p=0.1140; interaction, p=0.0026). Indeed, daily average running speeds are only significantly different across days for images in the first location (1-way rmANOVA, days: location 1, p=0.0014, left top row, **Figure 16.A.**; location 2, p=0.9823, right top row, **Figure 16.A.**). Mice slow down more in late days of the task compared to early days (adjusted paired t-test, early vs late days, location 1, t-test, p=0.0488). We also find that normalized running speeds in late days are smaller at the first image location compared to the second image location (late days, comparison normalized running speeds in location 1 vs location 2, t-test, p=0.0154). Thus, across days of learning in the task, mice start to slow down more in front of images in the first location, and not for second image locations. This running speed modulation could potentially be represented in our analysis of serotonin neurons' image response dynamics across trials and days as decelerations are often correlated with increases in serotonin activity (**Figure 14**).

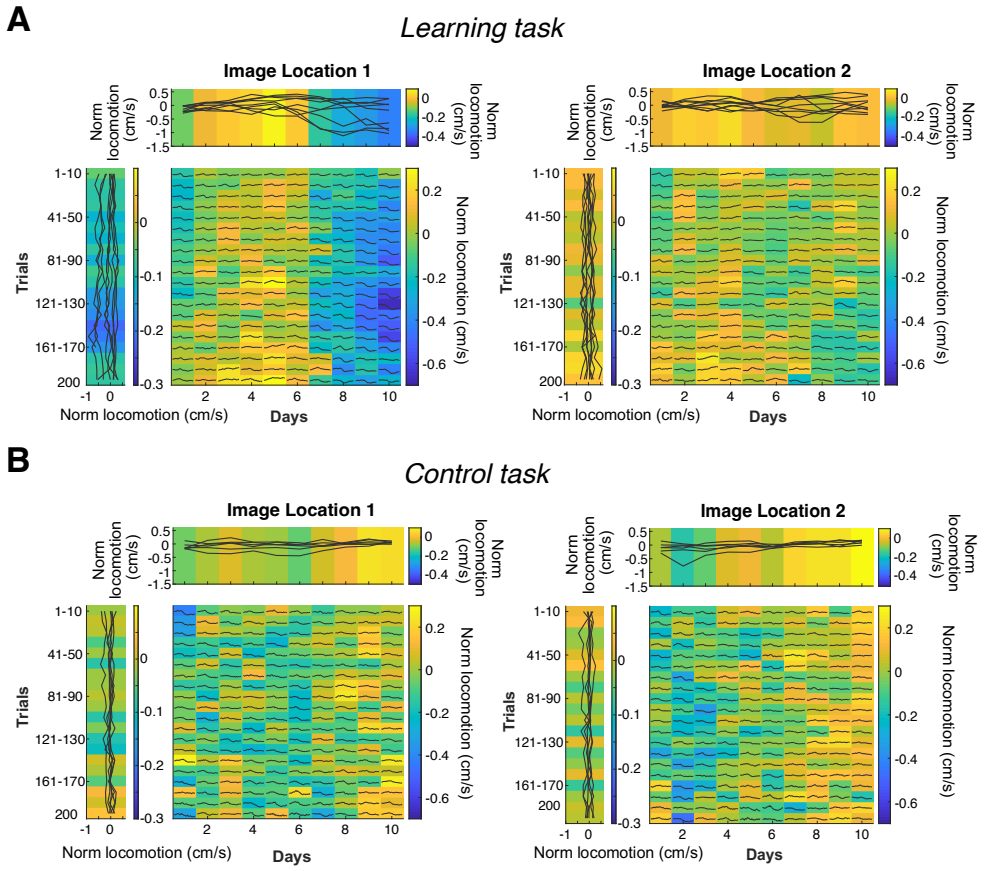

Figure 16. *Locomotion changes across trials and days for the learning and control tasks.*

A. Matrix of normalised running speeds at image locations across trials and days for all mice in the task. Projections of averaged normalised running speeds for all trials (left column) and all days (top row). Right: image location 1; left: image location 2 (n=9). **B.** Same analysis but for mice running in the control task (n=6).

For mice running in the control task, we find again no significant organisation across mice for normalised running speeds to be dynamically modulated across trials and image locations (2-way rmANOVA, trials and image location:

trials, p=0.7022; location, p=0.6342; interaction, p=0.7993, **Figure 16.B.**). Normalised running speeds are, however, differently modulated across days of the task and across image locations (2-way rmANOVA, days and image location: days, p=0.0033; location, p=0.4335; interaction, p=0.0376). When comparing these normalised running speeds in early and late days of the task at both image locations, mice in the control group run faster in late days of the task at the second image location, compared to both normalised running speeds on early days and to late days at the first image location (adjusted paired t-tests, early vs late days, location 2, p=0.0441; late days, location 1 vs location 2, p=0.0414). Contrary to mice in the learning task, they do not slow down upon arrival at the first image location in late days (p=0.2756). Thus, mice in the control task run faster past the second image location across days of the task, when normalised to the baseline running speeds.

Overall, these results reveal that mice behaving in the learning and control tasks run past image locations on average at the same running speeds across days (**Figure 15**). However, mice in the learning group slow down more at the first image location in later days of the task, suggesting they run faster leading up to the image in late days (**Figure 16**). In contrast, mice in the control group run faster at the second image location compared to baseline on late days on average (**Figure 16**).

Knowing now that the activity of serotonin neurons in our task is significantly correlated with locomotion and that mice change their locomotion behaviours around image locations across days of the task, we explore how these locomotion changes influence the serotonin image dynamics we described in sections 4, 5 and 6. We make use of the MI and MD serotonin signals calculated above to assess this possible interaction. Specifically, we ask whether the response to novel images, the adaptation of the signal across

trials or the increase of image responses across days, correlate with mice's running behaviours in the learning task. We also question whether MI or MD serotonin image responses are different between learning and control contexts across novelty, trials and days.

7.4. MI Serotonin Image Responses in the Tasks

7.4.1. MI serotonin activity captures image response dynamics in the learning task

We first examine if the modulation of serotonin neurons' responses to images across trials and days in the learning task are still present in the motion-independent (MI) serotonin activity signal. We plot the matrix presenting the evolution of the MI serotonin image responses across trials and days for all mice and all images averaged in the learning task in **Figure 17.A.** We find that MI serotonin image responses are also modulated across trials and days of the task, and that image location does not influence these dynamics (2-way rmANOVA, trials and location: trials, p=4.6450e-14; location, p=0.7881; interaction, p=0.8478; 2-way rmANOVA, days and location: days, p=0.0003; location, p=0.8125; interaction, p=0.2543).

MI serotonin image responses also respond to the first five repetition of every image on day 1 (**Figure 17.B.**). We find that this activity adapts across the first 10 image repetitions on day 1 (1-way rmANOVA, repetitions, p=0.0040). Importantly, these responses are not different from those of the full serotonin image responses (2-way rmANOVA, repetitions on day 1 MI vs full serotonin data types: repetition, p=0.0050, data type, p=0.0049; interaction, p=0.3106).

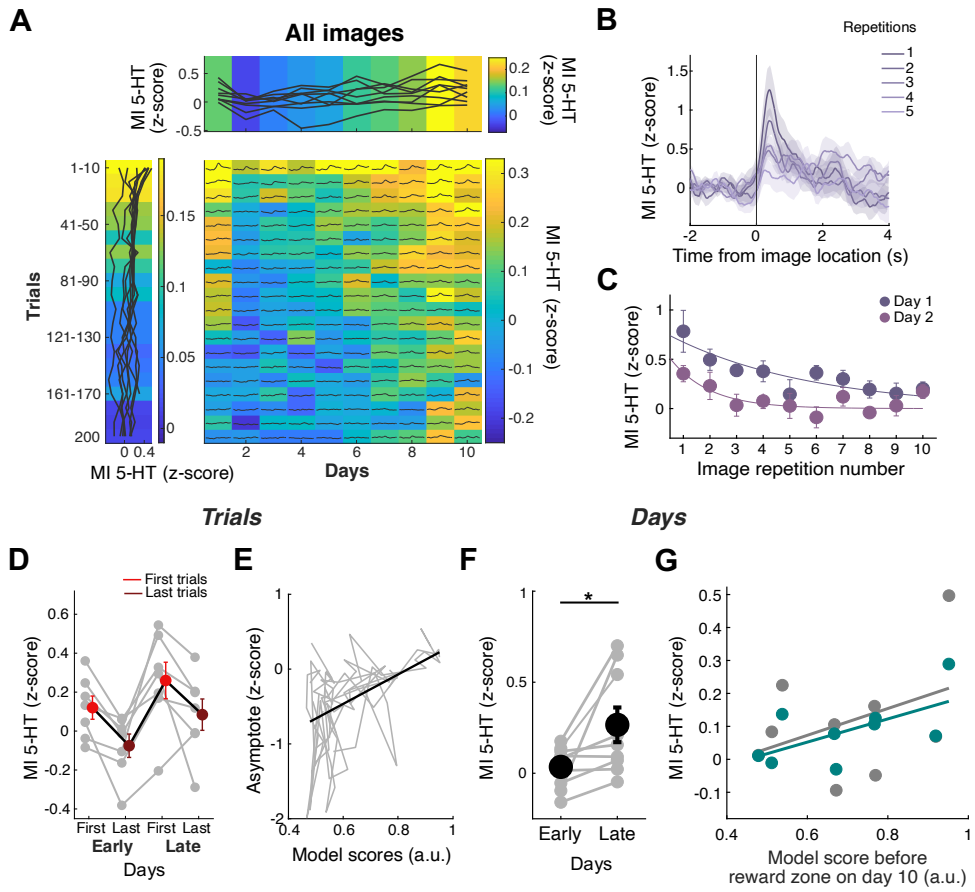

Figure 17. *Motion-independent serotonin activity responds to novel images, adapts across repetitions and increases across days of learning.*

A. The matrix of MI serotonin responses for all mice in the learning task across days and trials (n=9). **B.** MI serotonin responses to all images' first to 5th first occurrences on day 1, averaged across all learning mice (mean and SEM, n=9). **C.** Adaptation of this MI serotonin response to images across the first 10 repetitions on day 1 and day 2, fit with exponential decays, averaged across all learning mice (mean and SEM, n=9). **D.** Average MI serotonin of first and last 30 trials in early and late days of the task for all mice (mean and SEM across mice, n=7). **E.** Asymptote MI serotonin values from exponential decays fitted across each mouse and day as a function of model scores (n=9). **F.** Average MI serotonin neurons' responses to all images in early and late

days of the task (n=9). **G.** MI serotonin image responses on late days as a function of learning on day 10 for images in the first and second location (n=9).

The MI serotonin image responses are also still modulated across repetitions on the second day of the task but are not different from those of the first day (2-way rmANOVA, repetition and days 1 & 2: repetition, p=0.0012; days, p=0.0007; interaction, p=0.3156; from adjusted paired t-test, first repetition day 1 vs day 2, p=0.0498). Fitting exponential decays on the average MI serotonin neurons' responses for these 10 image repetitions on both days reveals that responses decay with a decay constant of 1.7884 image repetition on day 1, and of 1.1363 image repetition on day 2 (decay constant of day 1, p=0.0008; decay constant of day 2, p=0.0261). Hence, the MI serotonin activity responds to novel images and adapts across repetitions.

We reproduce our analyses examining how serotonin neurons' responses evolve across trials and days of learning in the task with the MI serotonin signal. We average the MI serotonin response to all images in the first and last 30 trials on each day of the task for the 7 mice that ran more that 100 trials daily (**Figure 17.D.**). We find a significant modulation of days and trial number on the MI serotonin image response, but no interaction between both factors (2-way rmANOVA, first and last trials across days; first and last trials, p=0.0013; days, p=0.0006; interaction, p=0.4995). When specifically comparing first and last trials in early and late days, we find a significant adaptation of the MI image response in early days only (adjusted paired t-test, first vs last trials: early days, p=0.0098; late days, p=0.0979). First and last trials are not significantly different between early and late days (adjusted paired t-test, early vs late days: first trials, p=0.2192; last trials, p=0.1581). However, the trend for MI serotonin image responses for last trials to be greater in late days compared to early days is present.

We asked whether asymptote values from exponential decay models applied on the MI serotonin image responses averaged across all locations on each day were related to learning. After acquiring asymptote values similarly as for **Figure 6**, we plot the daily asymptote value per mouse as a function of the model score of that given day (**Figure 17.E.**). We find a significant positive relationship between the asymptote of the serotonin MI signal in response to all images and the behavioural performance per day for all mice (slope coefficient=1.9619; p=7.9873e-07; asymptote in early vs late days of the task for all images, p=0.0077). This suggests that the MI signal from the serotonin activity recorded for fiber photometry adapts less for mice that show good behaviour, suggesting this dynamic is not solely due to locomotion effects in the serotonin trace.

MI serotonin image responses also increase across days of learning in the task on average, with transients on late days being significantly higher than those of early days of the task (paired t-test, p=0.0278, **Figure 17.F.**). To better understand if this increase across days is related to learning, we average the MI image response for each mouse in late days of the task and plot these values as a function of learning on the last day of the task (**Figure 17.G.**). We find a non-significant trend in the relationship between MI second image response and behavioural performance in the task, whilst MI serotonin responses at the first image appear uncorrelated with behavioural performance (location 1, slope coefficient = 0.4065; p= 0.2893; location 2, slope coefficient = 0.3521; p=0.0732).

Overall, MI serotonin image responses recover task-related signals across trials and days of the learning task. This suggests that such dynamics in the task are independent from behavioural effects and that the learning process could be responsible for these serotoninergic dynamics across days.

7.4.2. MI serotonin activity is different in learning and control tasks across trials and days

Finally, we analyse the MI serotonin signal dynamics across trials and days of the control task and compare these results to those of the learning task, to control for other effects such as attention without associative learning. We plot the matrix of MI serotonin neurons' responses to all images combined across days and trials, averaged across all mice (**Figure 18.A.**) MI serotonin transients are not influenced by days of running in the task (1-way rmANOVA, days, p=0.7152). However, they do adapt across trials (1-way rmANOVA, trials, p=6.2082e-12).

We perform a similar analysis as in **Figure 12** to assess whether MI serotonin signals in the control task also respond to novel, unexpected images. We find that MI serotonin signals in response to novel images respond and adapt across the first 5 repetitions on day 1 of the task (1-way rmANOVA, first 10 repetitions, p=0.0055, **Figure 18.B.**). MI serotonin image responses also adapt on the second day but show only an insignificant trend as being different from those of the first day (2-way rmANOVA, repetition and days 1 & 2: repetition, p= 0.0025; days, p=0.0992; interaction, p=0.1087; and **Figure 16.C.**).

Comparing MI serotonin image responses across the first 10 image repetitions on day 1 in the learning and control groups reveals no significant difference between groups (mixed model 2-way rmANOVA, repetitions and experiment group: repetitions, p=2.9189e-06; group, p=0.5365; interaction, p=0.7669). Thus, MI serotonin signals from serotonin neurons respond to novel, unexpected images in the control task.

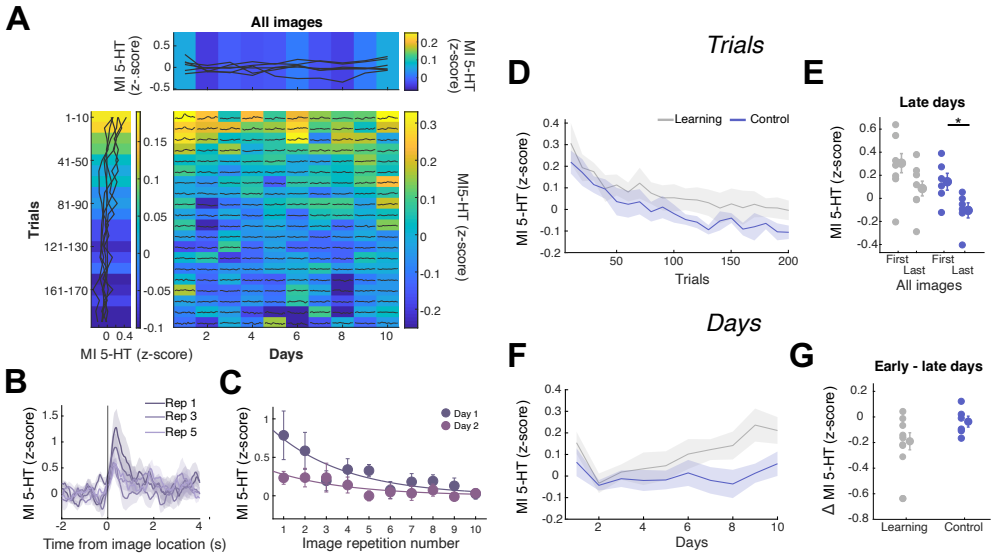

Figure 18. *Motion-independent serotonin responses to images in the control task also respond to novel images, adapt across trials but do not increase across days.*

A. The matrix of MI serotonin responses for all mice in the learning task across days and trials (n=6). **B.** MI serotonin responses to all images' first to 5th first occurrences on day 1 for all mice (mean and SEM across mice, n=6). **C.** Adaptation of this signal across the first 10 repetitions on days 1 and day 2, fitted with an exponential decay (mean and SEM, n=6). **D.** Evolution of the MI serotonin image responses across trials averaged across days and mice for the learning group and the control group (mean and SEM, n=7, n=6). **E.** Comparison of MI serotonin image responses for early and late days image responses for first and last trials of both the learning and control groups (mean and SEM, n=7, n=6). **F.** Evolution of MI serotonin image responses across days averaged across all trials in the tasks for the learning and control groups (mean and SEM, n=9, n=6). **G.** Delta MI serotonin image responses for early minus late days of all mice in the learning and control groups (mean and SEM, n=9, n=6).

Exponential decay models also significantly fit the MI serotonin image response adaptation for both days of the task, with responses decaying by 2.1 images repetitions on the first day and by 4.6 repetitions on the second day (exponential decay, day 1, p=0.0012; day 2, p=0.0053, **Figure 16.C.**).

When comparing MI serotonin image responses at all locations from the control group with the learning group across trials collapsed across days, we recover the strong modulation of trials on image responses for both groups, and find no difference between groups (mixed model 2-way rmANOVA, trials and groups, trials, p=0.0031; group, p=0.1438; interaction, p=0.6274, **Figure 18.D.**). On late days, however, we find that last trials are close to but not significantly different between learning and groups (t-test, control vs learning, first trials, p= 0.3210; last trials p= 0.0981). Trends in the data suggest that serotonin neurons' image responses for last trials of late days in the control group could be smaller than those of the learning group. Moreover, MI image responses only in the control group are different between first and last trials on late days (first vs last trials, learning group, paired t-test, p= 0.0893; control group, p= 0.0090, **Figure 18.E.**).

Across days, the MI serotonin image responses are not different between groups, but all groups are significantly modulated across days (mixed model 2-way rmANOVA, days and groups, day, p=0.0103; group, p=0.2806, interaction, p=0.3978, **Figure 18.F.**). We compare the evolution of MI serotonin image responses between early and late days for the control mice and the learning mice in **Figure 18.G**. We find a non-significant trend for mice in the learning group to increase more across days than those in the control group (t-test, p= 0.19).

Overall, MI serotonin signals in the task appear to respond to images as the full serotonin neurons' activity trace across trials and days in the learning and control tasks. Comparisons of learning and control task MI signals reveal a trend for MI serotonin image responses to behave as the full serotonin trace. This suggests that the modulation of serotonin neurons across trials and days in the task is independent from locomotion effects. We will now explore how the motion-dependent (MD) serotonin signal evolves in the learning task in response to images, as well as in comparison with the control task.

7.5. MD Serotonin Image Responses in the Tasks

7.5.1. MD serotonin image responses are not modulated across trials and days but correlate with performance during learning

We analyse first MD serotonin signal dynamics across novel images, trials and days of the learning task in **Figure 19**. We plot the evolution of MD serotonin image responses for all image locations averaged across trials and days to understand if and how these responses dynamically evolve in the task (**Figure 19.A.**). We find no general effect of trials and days on MD serotonin image responses for all images combined (1-way rmANOVA, trials, p= 0.9989; 1-way rmANOVA days, p= 0.8718). However, we find a significant interaction across days of the task between image locations, suggesting MD serotonin image responses evolve differently across location and days of the task (2-way rmANOVA, days and image location: days, p=0.9710; image location, p=0.0218; interaction, p=0.0027).

We plot the MD serotonin image responses aligned to the first 5 image repetitions averaged across all images and mice of day 1 in the task (**Figure 19.B.**). MD serotonin neurons respond to novel and unexpected images, but all responses to image repetitions appear to overlap. When plotting the average image response in a 1 second widow from image location across all images and mice for the first 10 image repetitions, we find no significant modulation of the MD serotonin response by image repetition (1-way rmANOVA, repetition, p=0.2326, **Figure 19.C.**). When comparing with the first 10 repetitions of the second day, we find that these first 10 image responses are significantly different across days (2-way rmANOVA, repetitions and days: repetitions, p=0.6983; days, p=0.0180; interaction, p=0.5755). MD serotonin image responses averaged across all first 10 repetitions are greater on the first day compared to the second day. As the MD serotonin responses to these first image repetitions are not modulated across repetition, trying to fit an exponential decay as done for the full and MI serotonin image responses yields in an insignificant fit (exponential decay: day 1, p=0.755; day 2, p=0.166). This suggests that MD serotonin activity does respond to novel images but only adapts by the second day of the task and not along the first 10 repetitions of day 1.

Next, we analyse how MD serotonin image responses evolve between the first and last 30 trials averaged for each mouse on each session across days of the task. We find that these responses to first and last trials are not differently modulated across days, though both factors interact (2-way rmANOVA, first and last trials across days: trials, p=0.6079; days, p=0.4785; interaction, p=0.0481). Specific comparison of first and last trials averaged in early and late days of the task reveals that first and last trials are not significantly different for either early or late days (adjusted paired t-test, first versus last trials: early days, p=0.6166; late days, p=0.2331, **Figure 19.D.**).

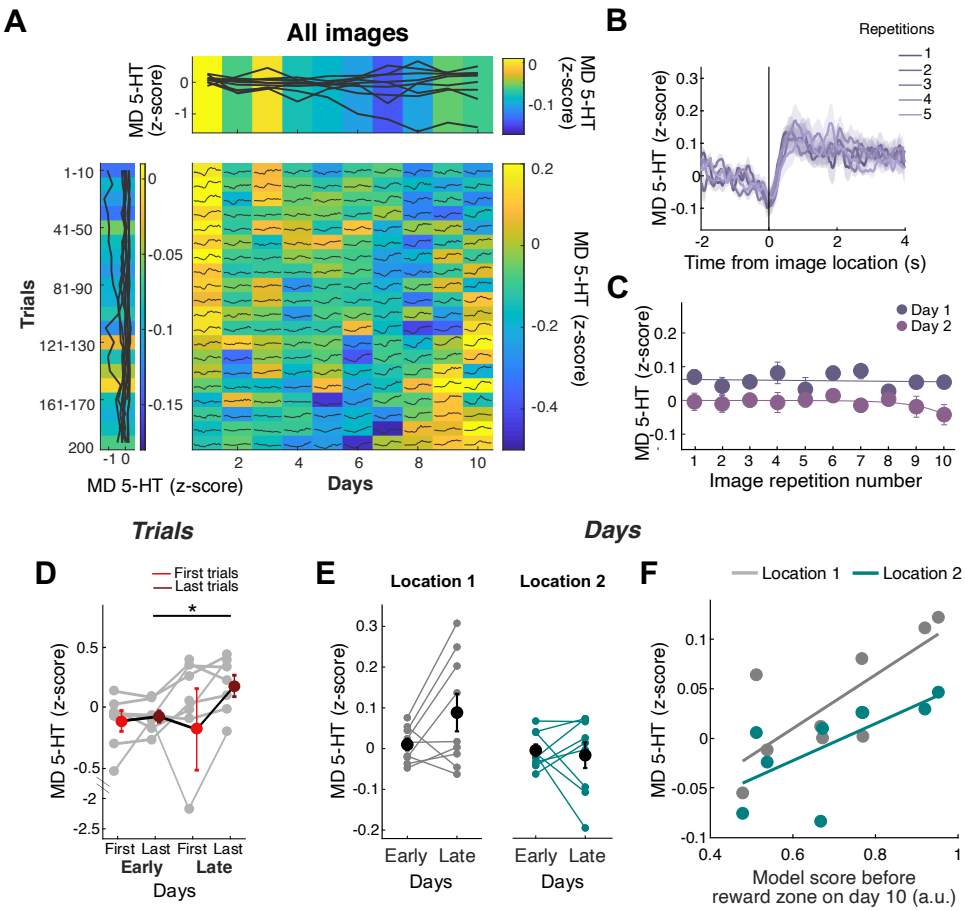

Figure 19. *Motion-dependent signals in the learning task are not significantly modulated across trials and days for all mice.*

A. The matrix of MD serotonin responses for all mice in the learning task across days and trials (n=9). **B.** MD serotonin responses to all images' first to 5th first occurrences on day 1, averaged across all learning mice (mean and SEM, n=9). **C.** Adaptation of this MD serotonin response to images across the first 10 repetitions on day 1 and day 2, fit with exponential decays, averaged across all learning mice (mean and SEM, n=9). **D.** Average MD serotonin of first and last 30 trials in early and late days of the task for all mice (mean and SEM across mice, n=7). **E.** Average MD serotonin neurons' responses to images in the first (left) and second (right) image locations in early and late

days of the task (n=9). **F.** MD serotonin image responses on late days as a function of learning on day 10 for images in the first and second location (n=9).

Last trials across all mice are, however, significantly greater in late days compared to early days of the task (p=0.0288, **Figure 19.D.**). We find no organisation across mice for early trials' responses between early and late days (p=0.8039). This suggests that, for all images in the task, the motion-dependent serotonin neurons' responses increase in late days.

As the MD serotonin activity trace is differently modulated across days of the task per image location, we compare MD serotonin image responses for early and late days of the task for each image location respectively (**Figure 19.E.**). We find an insignificant trend for MD serotonin image responses at the first location to increase across days of the task (2-way rmANOVA, days and location: days, p=0.3497; location, p=0.0111; interaction, p=0.0136; adjusted paired t-test, early vs late days: location 1, p=0.0818; location 2, p=0.7287). It appears that this difference between early and late days is very strong for 4 mice in the task, whilst it is not present or very slim for the other 5 mice. This increase in MD serotonin responses for images in the first location of late days of the task aligns with the locomotion behaviour of mice at these images, decelerating there on late days of the task (**Figure 16**).

Thus, we ask whether this increase in the MD serotonin image response across days in the task is related to learning. We plot the average MD serotonin image response for late days (the last 3 days) of the task for all mice for image locations 1 and 2 respectively, as a function of our behavioural model score on the last day of the task (**Figure 19.F.**). We find, for both image locations, a significant, positive, relationship between the MD serotonin image

response in late days of the task and behavioural performance (location 1, slope coefficient =0.2710, p=0.0168; location 2, slope coefficient =0.1870, p=0.0439). This result suggests that mice with better performance have a higher MD serotonin image response on late days of the task, possibly due to stronger decelerations at these images. Overall, MD serotonin image responses differ from the results of the full serotonin trace, suggesting that what we observe in the full serotonin trace is not fully locomotion dependent.

7.5.2. MD serotonin activity does not differentiate learning from control contexts across trials and days

We ask how the MD serotonin image response evolves across trials and days for mice performing the control task. We first plot the matrix of averaged MD serotonin image responses across the first 200 trials of each session and the 10 days of the task (**Figure 20.A.**). We find that neither trials nor days of mice running in the task significantly modulate MD serotonin neurons' activity across mice (1-way rmANOVA, trials, p=0.1692; 1-way rmANOVA, days, p=0.1683).

To assess whether the MD serotonin signal responds to novel images, we plot the first five image repetitions of day 1, averaged across all images and all mice in the control task (**Figure 20.B.**). Across the first 10 image repetitions on day 1, we find that MD serotonin image responses are significantly modulated by repetitions (1-way rmANOVA, repetitions on day 1, p=0.0068, **Figure 20.C.**). This result is different from the learning group, where we found no adaptation of the MD serotonin image response across image repetitions of day 1 (mixed model rmANOVA, repetitions on day 1 and experimental

group, repetitions, p=0.0015, group, p=0.8282; interaction, p=0.0129, see **Figure 19.C.** for learning group novelty responses).

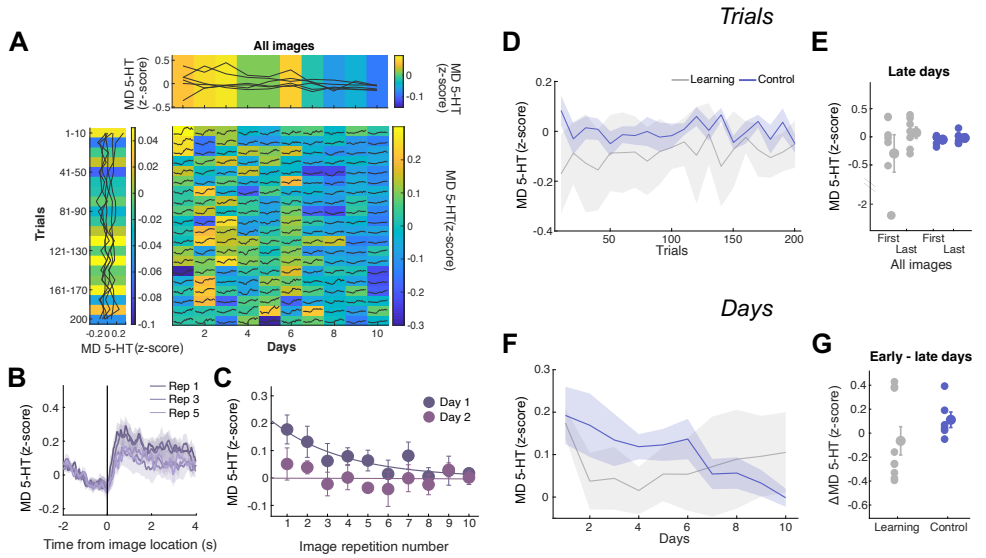

Figure 20. *Serotonin motion-dependent activity in response to images is not different between control and learning mice.*

A. The matrix of MD serotonin responses for all mice in the learning task across days and trials (n=6). **B.** MD serotonin responses to all images' first to 5th first occurrences on day 1 for all mice (mean and SEM across mice, n=6). **C.** Adaptation of this signal across the first 10 repetitions on days 1 and day 2, fitted with an exponential decay (mean and SEM, n=6). **D.** Evolution of the MD serotonin image responses across trials averaged across days and mice for the learning group and the control group (mean and SEM, n=7, n=6). **E.** Comparison of MD serotonin image responses for early and late days image responses for first and last trials of both the learning and control groups (mean and SEM, n=7, n=6). **F.** Evolution of MD serotonin image responses across days averaged across all trials in the tasks for the learning and control groups (mean and SEM, n=9, n=6). **G.** Delta MD serotonin image responses for early

minus late days of all mice in the learning and control groups (mean and SEM, n=9, n=6).

When analysing the evolution of these first 10 image responses across the first two days of the task, we find that MD serotonin neurons' image responses are also significantly different across the first two days of the task (2-way rmANOVA, repetitions across days 1 and 2: repetitions, p=0.0313; days, p=0.0361; interaction, p=0.4872, **Figure 20.C.**). Days do not interact with the MD serotonin neurons' responses across repetitions. However, when fitting an exponential decay to these first 10 image repetitions for each day respectively, we find that only image responses on the first day follow such adaptation, with a decay constant of 2.47 images (exponential decays: day 1, p= 0.0020, day 2, p=0.11). Thus, in the control task, MD serotonin activity responds to novel images on day 1 and adapt across image repetition.

Though neither MD serotonin image responses in the learning experiment nor the control task are significantly modulated across trials in the task, we ask if these responses are still different from each other (**Figure 20.D.**). We find no significant difference in learning and control group MD serotonin image responses across trials in the task (mixed model ANOVA, trials and experimental groups: trials, p=0.9922; group, p=0.5282; interaction, p=0.4647). Moreover, there are no specific differences between the first and last 30 trials of late days for both experimental groups (**Figure 20.E.**).

Across days, MD serotonin image responses are also not differently modulated between both experimental groups (mixed model ANOVA, days and experimental groups: days, p=0.6233; experimental group, p=0.5151; interaction, p=0.9818, **Figure 20.F.**). When analysing the evolution of the MD

serotonin image responses as the difference between early and late days of the task, learning and control mice are not significantly different from each other (paired t-test, p=0.7497, **Figure 20.G.**). It is clear, however, that the distribution of delta MD serotonin image responses is broader in the context of learning compared to the control group. Thus, between learning and control groups, MD serotonin image responses are not different across trials and days of the task on average but differ in their responses to novel images. As MD serotonin image responses in the learning task are different across mice based on their performance on the last day, averaging all mice together might hide more subtle, learning-dependent, differences.

7.6. Discussion and Interpretation

In this section, we present our work focused on understanding the relationship between serotonin activity and locomotion. We also explore how this relationship could influence the serotonin neurons' image response dynamics across trials and days of the tasks we reported in the previous sections. Firstly, we report that serotonin neurons are negatively correlated with locomotion, and that their activity can also track dynamic locomotion transitions (**Figure 13**, **Figure 14**). From this relationship, we generated a serotonin-locomotion function that we used to predict motion-dependent (MD) serotonin activity from running speeds in mice across trials and days of both tasks (**Figure 13**). We acquired motion-independent (MI) signals in the task as the residuals from the subtraction of the MD serotonin trace from the full serotonin activity trace for each mouse and day. We made use of these complementary serotonin traces to disentangle whether our reported serotonin neurons' dynamics in response to images in the tasks across trials and days were only representative of evolving locomotor behaviours that we find in **Figure 15** and **Figure 16**. We find that the MI serotonin signal in the

task maintains all task-related dynamics we reported in the previous sections 4,5 and 6. Namely, the novelty response, the adaptation across trials and the increase across days (**Figure 17**). We find non-significant trends differentiating MI serotonin signals across trials and days between the learning and control tasks (**Figure 18**). In contrast, MD serotonin neurons' image responses respond to novel images but show no adaptation across trials (**Figure 19**). The increase across days is positively correlated with mouse behaviour. Finally, MD serotonin image responses appear different between learning and control mice when responding to novel images on day 1, but groups do not differ across trials and days (**Figure 20**).

We start our discussion by presenting current limitations of our methodology used to extract MD and MI serotonin signals across mice. The method we used applies a general function on the running speed data of all mice to predict the MD serotonin activity from locomotion on each experimental day. We created the function by averaging the serotonin and locomotion data in 1 cm bins from all mice in both tasks. Thus, applying this general function to each individual mouse potentially masks unique serotonin-locomotion dynamics that could exist at the mouse level. Though it appears that the serotonin-locomotion relationship is very stereotypical across mice (**Figure 13**), it is plausible that the learning process in our task could influence this relationship. As mice need to monitor their running speeds to collect rewards, this acquired control of motor behaviour across days of the task could be represented within the serotonin activity signal. This could align with the activity of serotonin neurons having been repeatedly linked to behavioural inhibition, sensitive to context (Correia et al, 2017). Mice could also pay more attention to the speed at which they run on late days of the task as they become proficient, possibly modulating the relationship between both factors across days (Thiele & Bellgrove, 2018; Abdolrahmani et al, 2021).

An important future direction for this project is then to use other mathematical methods such as traditional linear regressions using locomotion as a regressor for serotonin activity. These models would be performed on every session for all mice independently to extract MD and MI serotonin signals. As such, we could better capture how the relationship between serotonin and locomotion could be affected by trials and days in the task. However, with this method, the risk that other corelated signals in the task could influence the serotonin predictions exists. An example of a correlated signal would be the consumption of rewards. It was shown that serotonin neurons respond to rewards, and, in our task, this behaviour happens only at low running speeds (Cohen et al, 2015). To create our function, we prevented to incorporate these signals by selecting data bins from the first 60 cm only of each corridor and for the first 5 days of the task. Thus, using another modelling technique with multiple predictors used to predict the serotonin activity across time might be needed to extract MD and MI serotonin responses most accurately.

Nonetheless, with our current function at hand and our MD and MI extracted signals, we found that MI serotonin image responses were also dynamically modulated by novelty and learning, as the full serotonin trace. This result reinforces the view that serotonin neurons can respond to task variables necessary for cognition and that they are not only representing locomotion in the task (Bacqué-Cazenave et al, 2020). Serotonin neurons appear to learn to respond selectively to cues depending on context, flexibly adapting over time.

Our results also suggest that the MD component of the serotonin activity trace in response to images in late days of the task is also related to learning. This suggests that mice learn to perform a unique self-generated behaviour at image locations that could possibly be part of their strategy to perform the

task. Serotonin neurons could thus be relevant for linking correct motor behaviours to unique environmental contexts (Homberg et al, 2012). This could therefore explain the wide distribution of MD serotonin responses for images across trials for mice behaving in the learning task. As mice are free to run as they desire along the corridor track, the MD serotonin response correlates with the diversity of locomotor behaviours at these locations.

Interestingly, we found that the MD serotonin signal between learning and control mice only differ in the adaptation to the novel image (**Figure 20**). This could reflect that mice in both groups run differently in front of these images on the first day of the task. We hypothesise that this difference arises from the different relevance of visual cues on the corridor walls in both tasks. Whilst in the learning task, reward delivery systematically happens within the reward delivery zone, marked by a grey texture on the wall, rewards are delivered randomly throughout the corridor for control mice. It could thus be that already from the first day, mice learn to relate to images differently, by different attentional processes for example (Wang & Krauzlis, 2018). Consequentially, mice from different tasks could perform different behaviours at image locations after novel images, which would be reflected in the MD serotonin signal.

Overall, it appears clear that the activity of serotonin neurons can be separated along a motion correlation axis. As previously introduced, serotonin neurons have also been shown to respond differently to reward-predicting and unrewarded cues. We will explore in the next section how the full serotonin trace, as well as the MI and MD components respectively, represent reward predictability at image locations during learning.

8. Serotonin Image Responses and Value

8.1. Background and Motivation

Similarly to dopamine neurons, serotonin neurons have been shown to respond to reward delivery at the beginning of learning in classical conditioning tasks (Zhong et al, 2017). During learning, the reward response was shown to dynamically start to respond in anticipation to the reward and to reward predicting cues (Zhong et al, 2017; Matias et al, 2017). These reports suggest that serotonin neurons adjust their firing patterns as animals learn to associate certain stimuli with rewards (Luo et al, 2019). It was also shown that activity from a small population of serotonin neurons could track negative outcomes prior to and during the event (Cohen et al, 2015). In most reports, cues predicting the absence of reward are not associated with responses from serotonin neurons. Therefore, serotonin neurons could be selectively responding to reward predicting cues during learning.

Throughout the previous sections of this thesis analysing image responses across trials and days of the learning task, we have systematically combined images from both reward-predictability contexts. We now aim to compare how serotonin neurons respond to reward-predicting and unrewarded images. Importantly, images in the task were assigned to rewarded or unrewarded corridors ensuring that, across mice, corridor category type could not be confounded with image identity. This would ensure that signals recorded in both corridor types arise from their value contexts and not from visual

processes. Thus, we analyse in this section the evolution of DRN serotonin neurons' responses to images with opposing reward predictabilities.

As we've shown in **Figure 3** and **Figure 4**, information about the reward-predictability state of corridors can be extracted from the analysis of locomotor behaviours of mice running past image locations. This suggests that mice locomotor behaviours could differ between rewarded and unrewarded images. To better understand how serotonin neurons' responses differ between image value categories, we thus also explore MI and MD serotonin neurons' image responses respectively for both image types in the task. We also ask how these responses differ from image responses in the control task where images are not associated to reward delivery.

8.2. Serotonin Neurons' Image Responses for Rewarded and Unrewarded Images in the Learning Task

8.2.1. Full serotonin transients respond to both rewarded and unrewarded images after learning

We start by plotting the matrix representing the evolution of serotonin transients in response to rewarded and unrewarded images at both locations using the full serotonin activity trace in 100 consecutive trials for all 10 days of the task (**Figure 21.A.** for rewarded images and **21.B.** for unrewarded images). We selected images from only fixed corridors to avoid capturing potential modulation of the second image location from ambiguous contexts. We find a significant organisation across mice for the evolution of serotonin image responses across trials for both image reward contingencies,

regardless of image location (2-way rmANOVA, rewarded images across trials and location: trials, p=0.0124; location, p=0.8106; interaction, p=0.6828, left columns, **Figure 21.A.**; 2-way rmANOVA, unrewarded images across trials and location: trials, p=0.0021; location, p=0.5352; interaction, p=0.3793 left columns, **Figure 21.B.**).

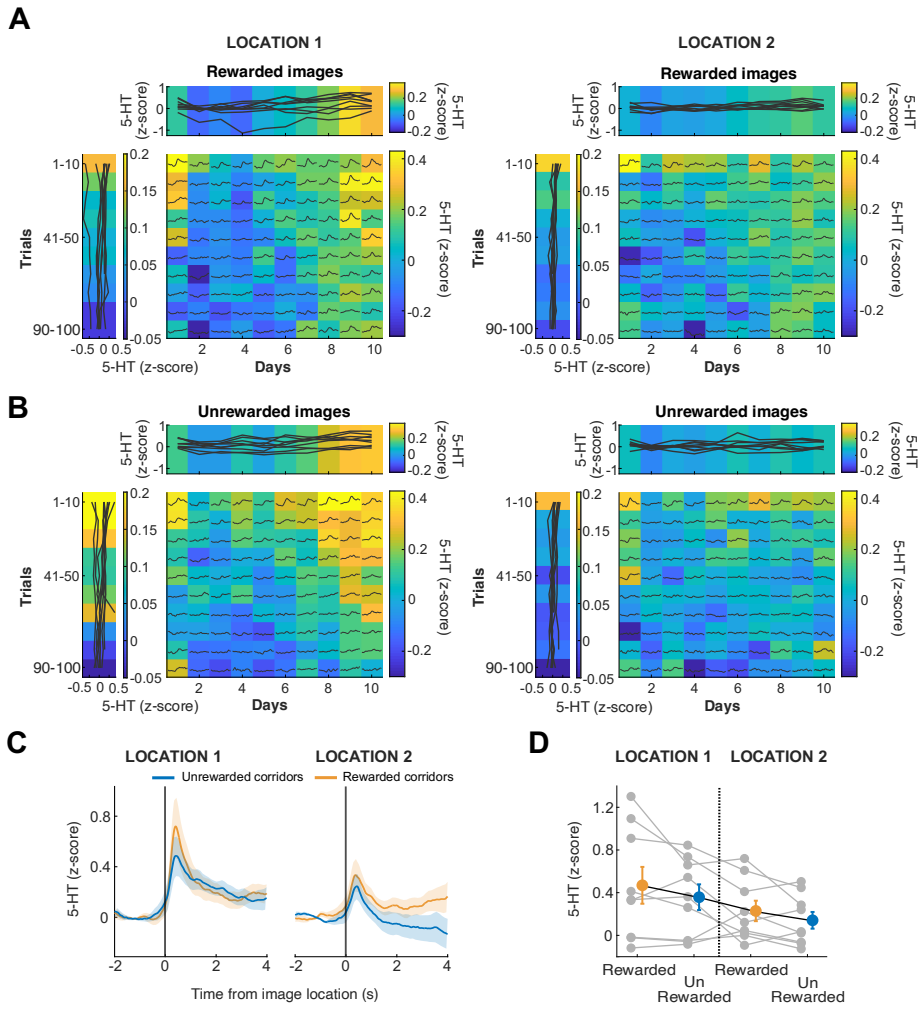

Figure 21. *Full serotonin responses do not differentiate rewarded from unrewarded images.*

A. and **B.** The matrices for all rewarded images in location 1 and location 2 respectively. Projections of averaged trials and days for each image category (n=9, respectively: top row, left column). **C.** and **D.** The same plot for all unrewarded images at location 1 and location 2. **E.** Average of late days rewarded and unrewarded images at location 1 and location 2 (mean and SEM, n=9). **F.** Stats of late days serotonin responses for rewarded vs. unrewarded images (grey, individual mice; mean and SEM, n=9).

For all image locations averaged together, we find serotonin neurons' responses to rewarded and unrewarded images are modulated across trials indistinguishably (2-way rmANOVA, trials and image value: trials, p=0.0009; value, p=0.7139, interaction, p=0.7977). Thus, the reported serotonin image adaptation in section 6 is not value dependent.

Across days, we find that serotonin neurons' responses to both rewarded and unrewarded images are modulated for all mice, with however a strong interaction between image location and value only for unrewarded images (2-way rmANOVA, rewarded images across days and location: days, p=2.5060e-06; location, p=0.6926; interaction, p=0.0856, top rows, **Figure 21.A.**; 2-way rmANOVA, unrewarded images across days and location: days, p=0.0111; location, p=0.1284; interaction, p=0.0003, top rows, **Figure 21.B.**). Unrewarded images in the first location evolve similarly across days for all mice (1-way rmANOVA, days: location 1, p=0.0001; 1-way rmANOVA, days: location 2, p=0.5258). Comparing the evolution of serotonin neurons' responses to rewarded and unrewarded images across days for all image locations combined reveals that all responses to images are significantly modulated across days (2-way rmANOVA, images across days and image value; days, p=0.0001; value, p=0.9988; interaction, p=0.0222). Moreover, image value does not differentiate the serotonin responses to images overall,

though it does interact with the evolution of the response. Thus, serotonin image responses to rewarded and unrewarded images are both modulated across trials and only unrewarded images in the second location are not modulated across days.

To understand whether serotonin neurons' image responses are sensitive to image value once the animal has learned the behavioural relevance of the visual cues, we plot the serotonin responses for rewarded and unrewarded images in both locations respectively on late days of the task (days 8 to 10, **Figure 21.C.**). Comparing the mean responses in a 1 second window after each image location reveals no significant difference in serotonin neurons' response amplitude for images of different values across image locations (2-way rmANOVA, value and location: value, p=0.1625; location, p=0.0218, interaction, p=0.7390; adjusted paired t-test, rewarded vs unrewarded images: location 1, p=0.2208, location 2, p= 0.1837, **Figure 21.D.**).

We do find that both serotonin neurons' image responses to rewarded and unrewarded images are higher for images in the first location compared to those in the second location. This aligns with previous results revealing that serotonin neurons' image responses for all image types are different between image locations on late days of the task (**Figure 10**). Thus, when examining the full photometry trace, serotonin neurons do not differentiate image value across trials but are less modulated across days for unrewarded images in the second location specifically. On late days, when the animals have learned the task, serotonin neurons' response amplitudes are the same for rewarded and unrewarded images.

8.2.2. MI serotonin activity selectively responds to reward-predicting cues

In the previous chapters, we have shown that information about corridor value can already be extracted from the running speed at both image locations for mice that have learned the task (**Figure 3.C.** and **4.B.**). **Figure 22.A.** presents the average running speed at both image locations respectively, normalised to the baseline running speed of each mouse before entering the image location. We find that mice run slower in unrewarded images, significantly at the first location, compared to rewarded images in a 1 second window from image location (2-way RM ANOVA: value, p=0.0385; location, p=0.0201; interaction, p= 0.0991; adjusted paired t-test, rewarded vs unrewarded images: location 1, p=0.0275; location 2, p=0.3002, **Figure 22.B.**).

We plot the MI serotonin neurons' responses for both rewarded and unrewarded images at both locations to first assess how the motion-independent serotonin signal keeps track of reward-predictability (**Figure 22.C.**). When averaging the responses in a one second window from image location, we find that serotonin transients in response to rewarded images are significantly greater compared to unrewarded images in late days of the task for both image locations (2-way rmANOVA, value and location: value, p=0.0243; location, p=0.1394; interaction, p=0.2724, **Figure 22.D.**).

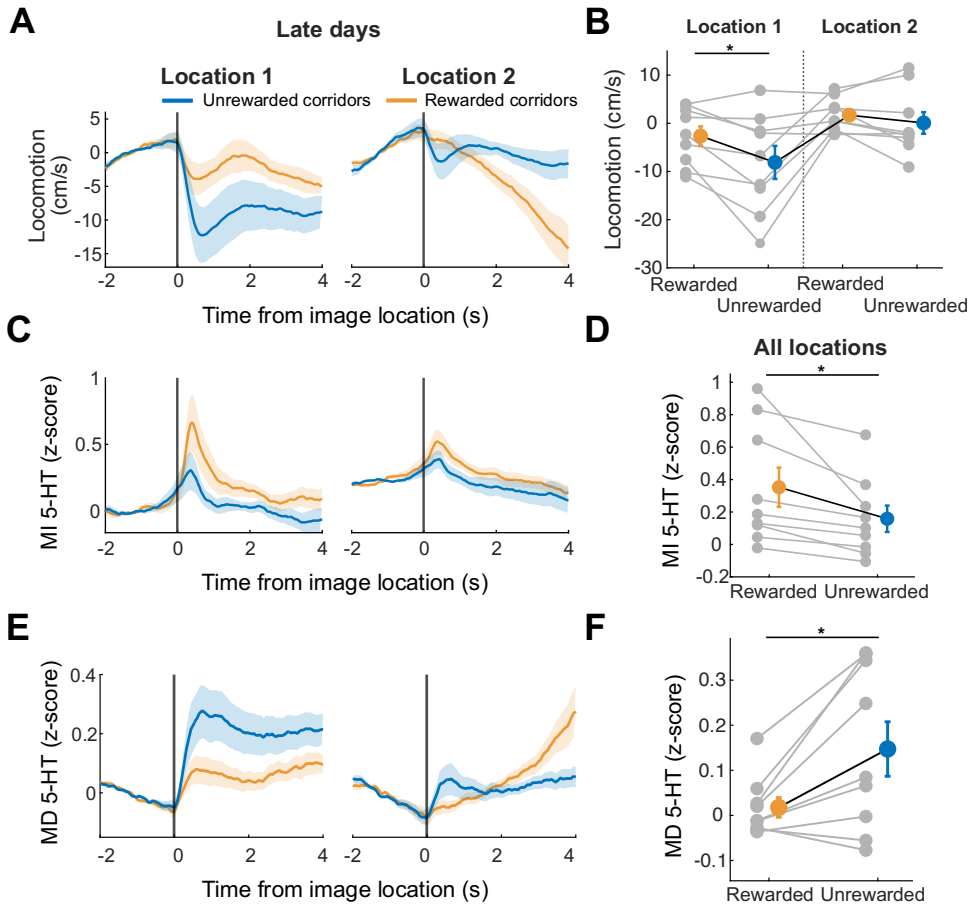

Figure 22. *Serotonin motion-independent and motion-dependent signals differently care about image value.*

A. Locomotion on learned days, normalised to average locomotion before the image location, for both image locations and rewarded and unrewarded images, averaged across mice (mean and SEM across mice, n=9). **B.** Correlations between serotonin and MI serotonin image responses for rewarded and unrewarded images at the first and second image locations (solid and dotted lines respectively). **C.** Motion-independent serotonin activity averaged across mice and baseline normalised for rewarded and unrewarded images at both locations 1 and 2. **D.** Comparisons of average image responses of motion-independent serotonin activity for rewarded and

unrewarded images at both locations in a 1 second window after the image location. **E.** and **F.**, same as **C.** and **D.** but for motion-dependent serotonin activity.

Specifically, MI serotonin image responses are significantly greater for rewarded images compared to unrewarded images in the first location, whilst the trend is close to significant at the second location (adjusted paired t-test, location 1, p= 0.0231; location 2, p=0.0843). Hence, contrary to the full serotonin trace, MI serotonin neurons' responses respond selectively more for reward-predicting cues once mice have learned the task.

8.2.3. MD serotonin activity selectively responds to unrewarded cues

To capture how the MD serotonin signal responds to different image values, we plot the MD serotonin neurons' responses for both rewarded and unrewarded images at both locations in **Figure 22.E.** In contrast to MI serotonin responses, we find that MD serotonin transients in response to rewarded images are smaller than those for unrewarded images in late days of the task (2-way rmANOVA, value and location: value, p=0.0188; location, p=0.0137, interaction, p=0.1978; **Figure 22.F**). We find moreover a significant influence of image location on MD serotonin image responses when comparing rewarded and unrewarded images. Responses at the first image location only are significantly different from each other, whilst the trend is close to significant for image responses at the second location (adjusted paired t-test: location 1, p=0.0346; location 2, p=0.0579). Overall, MD serotonin neurons' responses are selective for unrewarded images compared to rewarded ones.

Overall, these results suggest that the serotonin image responses for rewarded and unrewarded images appear to be differently encoded within our locomotion-related signals. The responses to unrewarded images appear more driven by locomotion correlated signals, whilst those in response to rewarded images appear independent from locomotion.

In our sixth section, we described that serotonin neurons' responses to images differently evolve in trials and days when comparing image responses from the learning group and from the control group (**Figure 12**). We find that MI serotonin responses in particular appear to follow the same dynamics, but not the MD serotonin signal (**Figure 18**, **Figure 20**). We have now described that image responses are different for rewarded and unrewarded images for MI and MD serotonin signals in the learning task. Thus, we now ask how these image responses of respective reward predictability contexts from these two serotonin signals differ from mice running in the control mice.

8.3. MD and MI Serotonin Image Responses for Rewarded and Unrewarded Images in Comparison to Control

8.3.1. MI serotonin activity in response to rewarded images only is different from control group image responses across trials and days

We first evaluate how MI serotonin responses to rewarded and unrewarded images differ from MI image responses of mice running in the control task. We plot the response matrices of MI serotonin image responses for both rewarded and unrewarded images respectively to capture how transients evolve across trials and days within each value category (**Figure 23.A.** and

23.B.). We average both image locations within each image category, as we find no significant effect nor interaction of image location on the evolution of serotonin image responses across trials and days in the task (2-way rmANOVA, trials and location: rewarded images: trials, p=0.0160; location, p=0.7483; interaction, p=0.6051; unrewarded images: trials, p=0.0006; location, p=0.9495; interaction, p=0.5531; 2-way rmANOVA, days and location: rewarded images: days, p=2.3957e-06; location, p=0.7578; interaction, p=0.2258; unrewarded images: days, p=0.1690; location, p=0.7130; interaction, p=0.3975). We find that all images from both value categories are significantly modulated across trials, but responses to unrewarded images are not modulated across days for either image location.

When comparing the evolution of MI serotonin image responses for rewarded and unrewarded images, the transients only significantly differ across days, and not across trials (2-way RM ANOVA, trials, rewarded vs. unrewarded images: trials, p=2.3291e-05; value, p=0.0664; interaction, p=0.5368, left columns; 2-way RM ANOVA, days, rewarded vs. unrewarded images: days, p=0.0014; value, p=0.0286; interaction, p=0.0007, top rows, **Figure 23.A.** and **23.B.**). Hence, this suggests that MI serotonin transients evolve differently across days dependent on the value of the image, with image responses being selective for rewarded images in late days of the task (**Figure 22**).

On late days of the task selectively, however, image responses for rewarded images only are not significantly different between the first 30 and last 30 trials, and the responses on the last trials are significantly greater than those of the control group (adjusted t-tests, first vs last trials: control group, p=0.0297; rewarded images, p=0.7576; control vs rewarded images: first trials, p=0.2692; last trials, p=0.0091; right panel, **Figure 23.C.**).

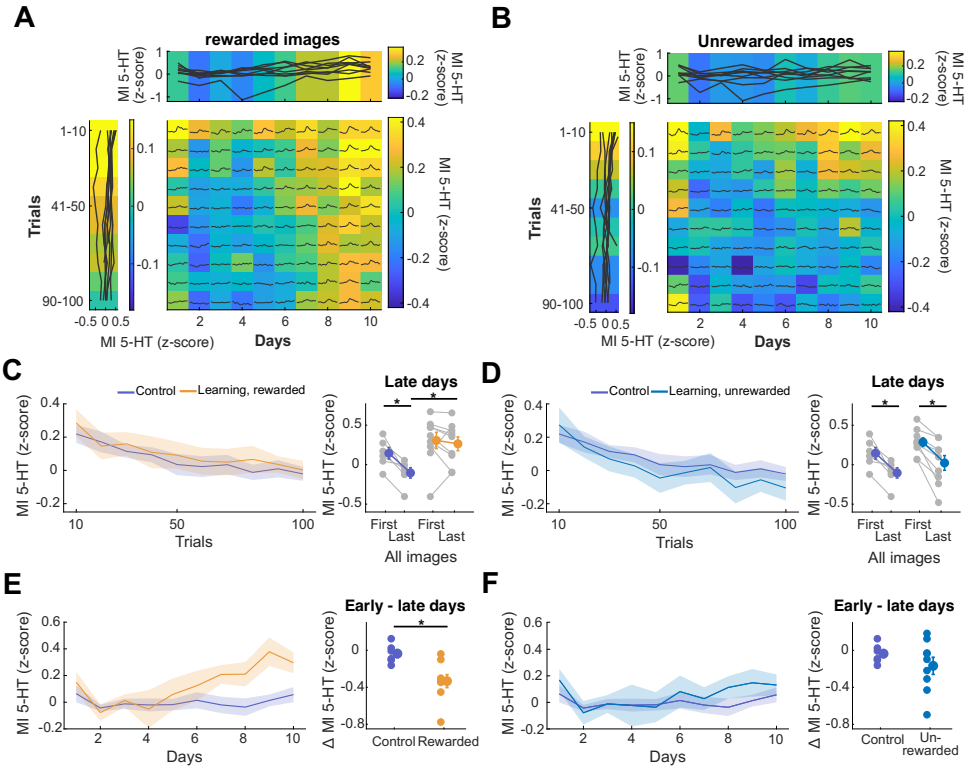

Figure 23. *Serotonin motion-independent activity is different for rewarded images from control mice and not for unrewarded ones.*

A. The matrix for MI serotonin neuron's responses to rewarded images at all locations mixed. Projections of averaged trials and days for each image category (n=9). **B.** The same matrix plot and projections for unrewarded images (n=9). **C.** Evolution of MI serotonin neurons' image responses across trials for control and rewarded image responses only; First and last trials for both groups in late days of the task (n=9 and n=6, mean and SEM across mice). **D.** Same as for **C.** but for control and unrewarded images only. **E.** Evolution of MI serotonin image responses across days of the task for control and rewarded images only; delta residuals z-score of early minus late days of the task for control and rewarded images (n=9 and n=6, mean and SEM across mice). **F.** Same as **E.** for control and unrewarded images only.

To compare these value-specific MI serotonin image responses with image responses from the control mice, we plot the evolution of either rewarded or unrewarded MI responses across trials alongside MI image responses from the control group, all locations combined (rewarded, **Figure 23.C.** and unrewarded, **23.D.**). For both image category, we find no difference in the evolution of the response across trials collapsed across days, compared to control mice (2-way mixed-model ANOVA, image category group/control group and trials: rewarded images: trials, p=1.6961e-07; group, p=0.8842; interaction, p=0.8420; unrewarded images: trials, p=3.9010e-10; group, p=0.2992; interaction, p=0.9715).

We find no difference across groups when comparing control mice with responses to unrewarded images selectively, and both groups adapt across trials on late days (adjusted t-tests, first vs last trials: control group, p=0.0297; unrewarded images, p=0.0270; control vs unrewarded images: first trials, p=0.0297; last trials, p=0.3377; right panel, **Figure 23.D.**). Thus, MI serotonin rewarded-only image responses on late days of the task appear to adapt less across trials compared to control.

Across days, we find again that MI serotonin responses to rewarded images evolve differently compared to control mice, but not for unrewarded images (2-way mixed-model ANOVA, image category group/control group and days: rewarded images: days, p=0.0001; group, p=0.2032; interaction, p=0.0080; unrewarded images: days, p=0.1396; group, p=0.6043; interaction, p=0.8897, **Figure 23.E.** and **23.F.**). Comparing the delta MI serotonin image responses as early minus late days for rewarded images and control group images reveals statistical difference, but not for unrewarded images (t-test, control vs rewarded, p=0.0071; control vs unrewarded, p=0.2929, right panels, **Figure 23.E.** and **23.F.**). Hence, MI serotonin image responses for reward-predicting

images differ from MI serotonin image responses from the control group, suggesting the gain in reward predictability is driving the sustained serotonin response in late days of the task.

8.3.2. MD serotonin image responses are not different between either rewarded or unrewarded images and control mice image responses

We perform a similar analysis to ask if and how MD serotonin image responses across trials and days of the task for rewarded and unrewarded images would differ from image responses from the control group. We plot the response matrices of MD serotonin neurons' image responses for both rewarded and unrewarded images respectively (**Figure 24.A.** and **24.B.**) As such, we capture how MD serotonin transients evolve across 100 trials on each day of the task within each reward category. We average here both image locations within each image category, though across image locations, we find a significant interaction between image location and days for unrewarded image responses only (2-way rmANOVA, trials and location: rewarded images: trials, p=0.6913; location, p=0.5931; interaction, p=0.5141; unrewarded images: trials, p=0.9636; location, p=0.1775; interaction, p=0.6265; 2-way rmANOVA, days and location: rewarded images: days, p=0.6462; location, p=0.2276; interaction, p=0.0633; unrewarded images: days, p=0.9301; location, p=0.0203; interaction, p=0.0010). However, neither for images in the first or second location, when averaging 100 trials each day, are MD serotonin image responses significantly modulated across days (1-way rmANOVA, days for images in the first location, p=0.1523; second location, p=0.6945).

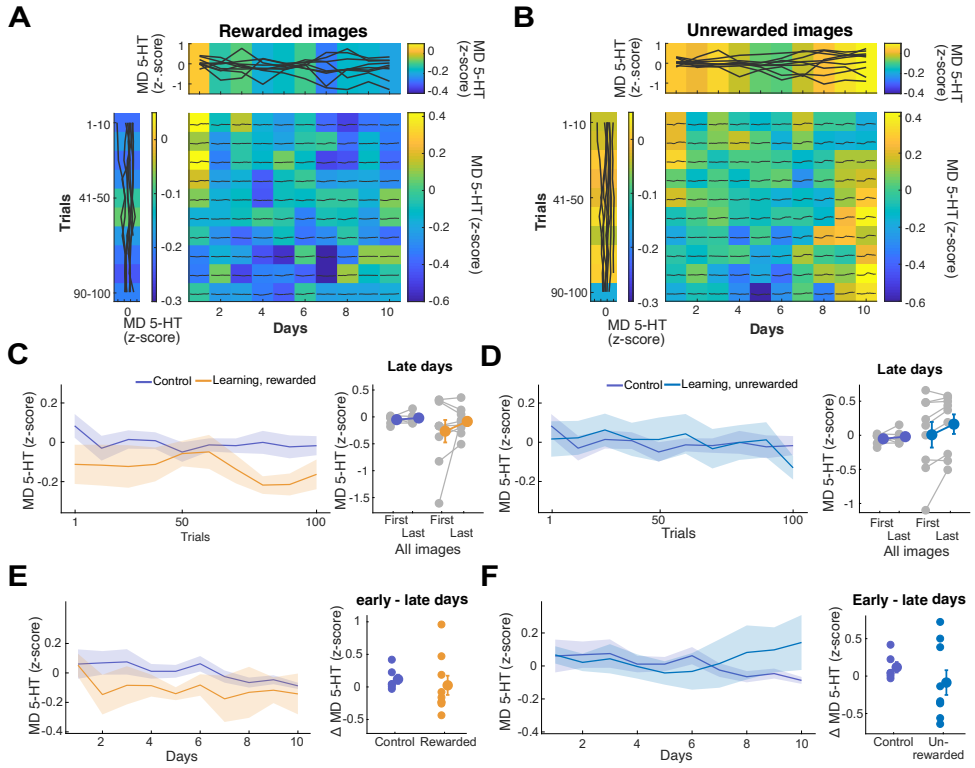

Figure 24. *Motion-dependent serotonin activity does not differentiate control mice image responses from either rewarded or unrewarded images.*

A. The matrix for MD serotonin neuron's responses to rewarded images at all locations mixed for mice in the learning group. Projections of averaged trials and days for each image category (n=9). **B.** The same matrix plot and projections for unrewarded images. **C.** Evolution of MD serotonin neurons' image responses across trials for control and rewarded image responses only; First and last trials for both groups in late days of the task (n=9 and n=6, mean and SEM across mice). **D.** Same as for **C.** but for control and unrewarded images only. **E.** Evolution of MD serotonin image responses across days of the task for control and rewarded images only; delta residuals z-score of early minus late days of the task for control and rewarded images (n=9 and n=6, mean and SEM across mice). **F.** Same as **E.** for control and unrewarded images only.

When comparing all MD serotonin image responses across days for rewarded and unrewarded images, we find a significant difference between both reward-predictability groups (2-way rmANOVA, days and value: days, p= 0.9166; value, p=0.0092, interaction, p=0.0721). That MD serotonin activity is modulated by reward predictability resonates with our behavioural analysis at image locations, with mice in the learning group running slower on late days for images in the first location, selectively more for unrewarded images (**Figure 16**, **Figure 22**). Thus, MD serotonin image responses are not modulated across trials and days, though their responses differ on average.

We find no significant difference across trials between MD serotonin neurons' image responses for either rewarded or unrewarded images in comparison with the MD serotonin image responses from the control mice (**Figure 24.C**.). The close to overlapping traces also when comparing first and last trials in **Figure 24.D.** reinforce the result that MD serotonin neurons' image responses do not adapt across trials.

Across days, MD serotonin responses for rewarded and unrewarded images are also not different from control mice's MD image responses (**Figure 24.E.**). Though we seem to find a trend in late days unrewarded image responses being significantly greater than for image responses from the control group, the widespread distribution of the data across mice appears to mask general effects (**Figure 24.F.**). Overall, this analysis focused on analysing MD serotonin image responses for rewarded and unrewarded images compared to control mice's image responses contrast greatly with the dynamics of the MI serotonin signal.

8.4. Discussion and Interpretation

We report in this section that serotonin neurons' responses to rewarded and unrewarded images do not evolve differently across trials for all mice and all image locations (**Figure 21**). Across days of the task, responses at the second image location differ, as only rewarded images are significantly modulated across days. However, on late days of the task, serotonin neurons' image responses do not differentiate rewarded from unrewarded images. Analysing the MI serotonin signal specifically in response to images with different reward-predictabilities yields contrasting results (**Figure 22**). MI serotonin neurons' activity is selective for reward-predicting images. When compared to MI image responses from control mice, only rewarded images show different adaptation across trials in late days of the task, and not unrewarded images (**Figure 23**). MI serotonin neuron's rewarded-only image responses increase across days compared to control. In contrast, MD serotonin activity appears selective for unrewarded images in late days of the task, and these image responses do not differ from image responses of control mice (**Figure 22**, **Figure 24**).

That serotonin neurons respond to reward-predicting images in our task corresponds well with the broad literature in the field of serotonin, linking its function to reward processing (Luo et al, 2015). Indeed, it was shown on multiple reports that serotonin neurons respond to reward predicting cues (Bromberg-Martin et al, 2010; Matias et al, 2017). In both cited papers, however, serotonin neurons appear not to respond to cues predicting the absence of reward. Negative value signals are found in the serotonergic system, but not aligned to the predicting stimulus (Cohen et al, 2015). Only thanks to our disentangling of the motion-dependent and motion-independent serotonin activity traces can we reconcile our work with theirs.

It is important to acknowledge again that our extraction of serotonin-locomotion correlates is one amongst multiple types of modelling that could be done. As mentioned previously, the relationship function we calculated across mice and that we applied on all mice's datasets to acquire the motion-dependent signal on all days could be masking more subtle serotonin-locomotion dynamics at the mouse level. Specifically in this analysis where we find that motion-dependent serotonin image responses are more selective to unrewarded images, it is crucial to explore, in other ways, our locomotion-serotonin correlation (**Figure 22**). Nonetheless, that motion-independent serotonin neurons' image responses are selective for reward-predicting images in our task suggests that our current analysis could be revealing true serotonin dynamics. A future experiment could be to record single cell activity of serotonin neurons in the DRN in this task with GRIN lenses to understand if these responses to rewarded and unrewarded images are from different populations of neurons or the same, and how these populations correlate with locomotion dynamics in mice (Paquelet et al, 2022).

We find that motion-dependent serotonin signals are responsible for the full serotonin neurons' responses to unrewarded images. As mice appear to slow down more in front of unrewarded images compared to rewarded ones, this result suggests that the serotonin response at these images reflects this specific deceleration. It could be tempting to conclude from this result that these responses are not dependent on learning, as they appear part of a more in-built relationship between both parts. An important analysis to perform, tailored to this, would be to analyse the relationship between late days unrewarded image serotonin, full and MD, responses as a function of our learning metric in the task. We have previously shown that MD serotonin image responses for all images at both image locations is correlated with learning (**Figure 19**). This result already suggests that mice that decelerate most at images could correspond to those that perform best. Previous work

from the lab has revealed that stimulating serotonin neurons prompted mice to decelerate (Correia et al, 2017). This was true only for mice running in an open field and not when performing a goal-directed task. If, in our experiment, the activity of serotonin neurons aligned to unrewarded images is responsible for the deceleration, and that the deceleration is strongest for mice that perform well the task, what is the serotonin system learning about the environment to induce this behaviour?

One hypothesis could be that decelerating at images could be beneficial to perceive better the image on the corridor wall. Decelerating specifically at unrewarded image locations to gain certainty about the future outcome could reduce the delay until the next reward. Indeed, mice could become more confident to run fast past the reward zone and quickly start the next trial. Moreover, it's been shown that serotonin neurons' responses in a visual task can enhance contrast discrimination (Sato et al, 2020). Thus, by decelerating at unrewarded images, mice could gain more confidence about which behaviour to perform next and avoid disappointment if slowing down at the reward delivery zone and not receiving rewards.

Another hypothesis could be that the deceleration at unrewarded images is a sign of disappointment, at the image. It has been reported that animals can show signs of frustration in the context of a reward omission (Papini et al, 2022). Whilst studied mainly in the context of unexpected absence of rewards, the behavioural readout could be applied here. The frustrated state arising from this expected absence of reward is thought to correlate with an aversive emotional state in the animal. The authors argue that this frustrative state could be an adaptive function that would promote search for new rewards elsewhere. As the mice that discriminate best rewarded from unrewarded corridors are also those that slow down most to unrewarded

images, it could be that this intermittent aversive state they are in is enhancing the fast run until the next corridor starts. As face motion has been used as a readout of emotional state in mice, it could be interesting to examine mice's face motion in the task, aligned to rewarded and unrewarded images. This result could give us insights on which state the animal is, and if an emotion of frustration could be responsible for the mice to slow down to unrewarded images. Finally, this hypothesis aligns with the view of serotonin neurons in opposition to dopamine, processing negative events with behavioural inhibition (Dayan & Huys, 2009).

Overall, it is unclear as of now why mice decelerate more when running past unrewarded images in late days of the task, compared to reward-predicting images. However, it appears clear that some component of the serotonin activity is related to this behaviour. As such, whilst our results support the claims that serotonin cares about value in the MI signal, we also bring to light another relationship between animal behaviour and sensory processing captured within the serotonergic system.

As introduced in our first section describing the behavioural paradigm, corridor types do not only differ based on reward predictability but also based on image predictability at the second location. As serotonin neurons haven been shown to be modulated by stimulus predictability context, we will look in the next section how the full, MI and MD serotonin signals respond to images within these different contexts (Matias et al, 2017).

9. Serotonin and Learned Uncertainty

9.1. Introduction and Motivation

The activity of serotonin neurons in response to sensory stimuli was reported to be greatly modulated by the stimulus' predictability context (Matias et al, 2017). Serotonin neurons recorded in mice respond to rewards differently whether delivered in a fully predictable context or unexpectedly. From these results, it was suggested that the activity of serotonin neurons could represent a global surprise signal in the brain. The surprise signal is not learned, as it is a response to the presence of the stimulus in the unexpected context.

It was also suggested that serotonin neurons could keep track of unpredictable contexts during learning, referred to as learning the expected uncertainty in the environment (Grossman et al, 2020). In their task, mice were trained to lick for rewards in response to an auditory cue. Rewards could be delivered either from a left or a right lick-port. The side of reward delivery was defined probabilistically, in blocks of consecutive trials where one side was more likely to deliver rewards than the other. DRN serotonin neurons' firing rates during inter-trial-intervals in this task were shown to correlate with a modelled signal of expected uncertainty across trials. This contrasted with unexpected uncertainty signals detected at the reward delivery when in the unexpected context. This latter response relates most to serotonin as the surprise signal, detecting sudden deviations from expected outcomes in the environment. Overall, these results suggest that serotonin neurons could encode both a slow, ongoing estimate of the uncertainty in the environment when expected, as well as instantaneous uncertainty at faster timescales.

We will explore in our learning experiment how serotonin neurons respond to both types of uncertainty. We first ask if the activity of serotonin neurons recorded in our task responds to stimuli with different predictability contexts after learning the structure of the environment. In ambiguous corridors, a single image in the first image location precedes either one of the four images of the fixed corridors. As reward delivery is contingent on the identity of the second image, both image identity and reward delivery are uncertain upon running past the first image of ambiguous trials, analogous to expected uncertainty. These corridors contrast with the fixed corridors, where the identity of the second image is fully predictable from the first one. Therefore, we will explore in this section how the full serotonin trace, as well as the MI and MD signals respectively, respond to images from these different contexts.

9.2. Serotonin Image Responses to Images in the Second Location of Ambiguous Corridors in Late Days

9.2.1. Full and MI serotonin neurons' image responses do not differentiate predictability contexts at the image location

We first ask whether the full serotonin signal recorded in the learning task differentiates image predictability contexts at the image once the task has been learned. We plot the serotonin neurons' responses to images in the second location of fixed and ambiguous contexts in rewarded and unrewarded corridors respectively in **Figure 25.A**. We select image responses from late days of the task only, once the animal has learned the structure of the environment (days 8-10). Importantly, as done throughout the previous analyses, we normalise the image response to the averaged baseline activity for 1.5 seconds before the image location.

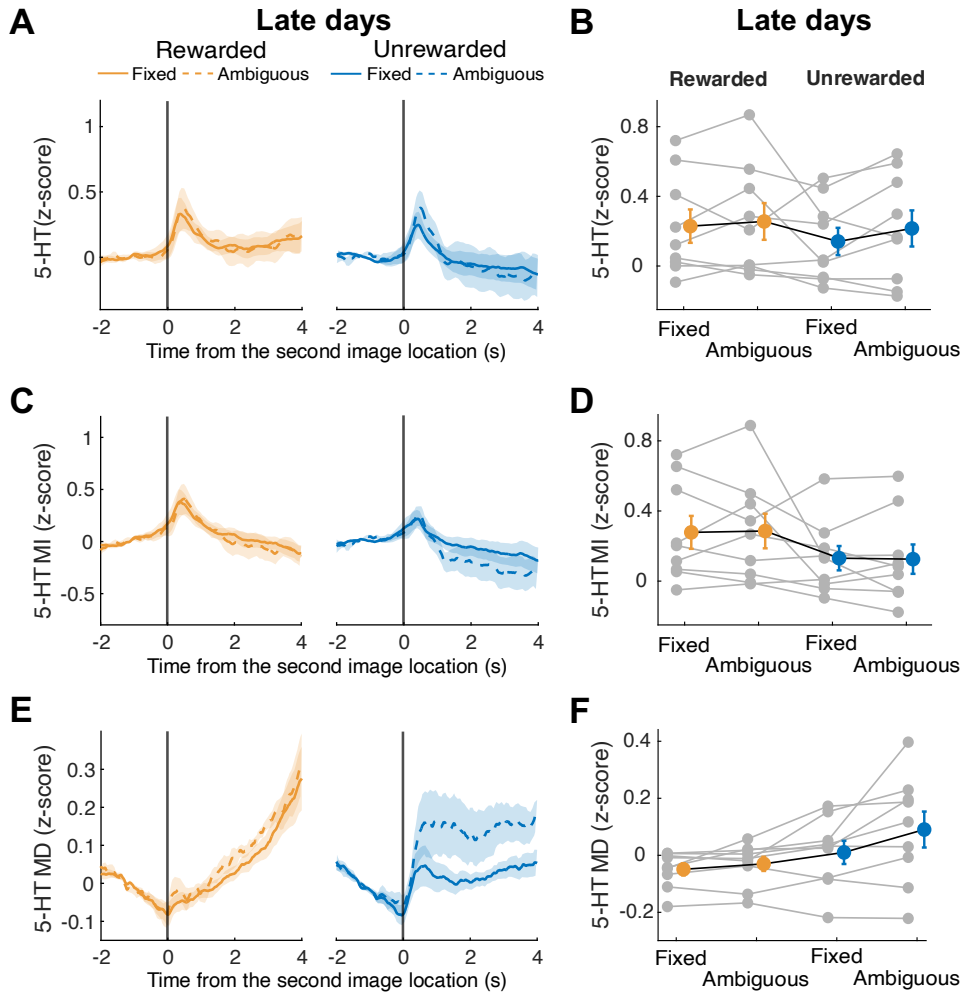

Figure 25. *MD serotonin signals only, in response to images in the second location, differentiate image predictability contexts.*

A. Serotonin neuron's full activity responses to image location 2 for fixed and ambiguous conditions in rewarded and unrewarded contexts (SEM, n=9). **B.** Comparisons of averaged image responses in a 1 second window from image location for all four corridor types for all mice (grey dots and lines) and averaged across mice (n=9, coloured dots, SEM). **C.** and **D.**, same as **A.** and **B.** for motion-independent serotonin activity. **E.** and **F.**, same as **A.** and **B.** for motion-dependent serotonin activity.

When averaging image responses in a 1 second window after image location, we find no difference between predictability contexts for either rewarded or unrewarded images across all mice (2-way rmANOVA, value and predictability context: value, p=0.4704; predictability context, p=0.2059; interaction, p=0.4359; **Figure 25.B.**).

For each value-based image category, we then plot the serotonin neurons' image responses from the MI signal only, averaged across mice, for both predictability contexts in late days of the task (**Figure 25.C**.). When averaging the response of each corridor type, we find again no significant difference between ambiguous and fixed corridors for both rewarded and unrewarded images across mice (2-way RM ANOVA, predictability and value; value, p=0.1299; predictability context, p=0.9620; interaction, p=0.8611; **Figure 25.D.**). Thus, with this analysis, it appears that neither the full nor the MI serotonin neurons' response are sensitive to the expected uncertainty context at the image location.

9.2.2. MD serotonin activity differentiates predictability contexts irrespective of reward-predictability

Finally, we ask whether the MD serotonin signal recorded in the task would carry information about expected uncertainty in the task. We plot the MD serotonin image response to rewarded and unrewarded second images in fixed and ambiguous contexts (**Figure 25.E.**). When averaging the MD serotonin response in a 1 second window from image location, we find no effect of predictability context within each value context but find effects for both value and context predictability (2-way rmANOVA, value and predictability context: value, p=0.0241; predictability context, p=0.0399;

interaction, p=0.1827; adjusted paired t-test, fixed vs ambiguous contexts: rewarded, p=0.2027; unrewarded, p=0.0728; **Figure 25.F.**). This suggests that serotonin signals correlated with locomotion in our task keep track of the predictability context of the stimuli.

9.3. Serotonin Neurons Respond to First Images of Ambiguous Corridors in Late Days of the Task

We chose to also explore how serotonin neurons respond to the image in the first location of this ambiguous context on late days of the task (**Figure 26**). This image shares the same predictability context as all other images in the first location. However, it carries information about this expected uncertainty of the second image and reward.

Specifically, first images in ambiguous corridors are 50% chance rewarded. We have seen in the previous section that serotonin neurons respond differently to images with different reward probability. Therefore, we first examine how the full activity of serotonin neurons responds to rewarded, unrewarded and ambiguous predicting images in **Figure 26.A.** We find no significant difference in first location image responses across all image categories across mice when averaging the responses in a 1 second window from image location (1-way rmANOVA, image categories, p=0.1035, **Figure 26.B.**).

When analysing the MI serotonin signal in response to these images in the first location, we find a significant organisation of these responses across mice (traces in **Figure 26.C.**, 1-way rmANOVA, image categories, p=0.0261,

Figure 26.D.). This effect is mainly driven by the close to significant difference in MI serotonin neurons' image response for rewarded and unrewarded images (p=0.0543). Neither rewarded image responses nor unrewarded image responses are different from the ambiguous image response (ambiguous vs. rewarded, p=0.1813; ambiguous vs. unrewarded, p=0.6159).

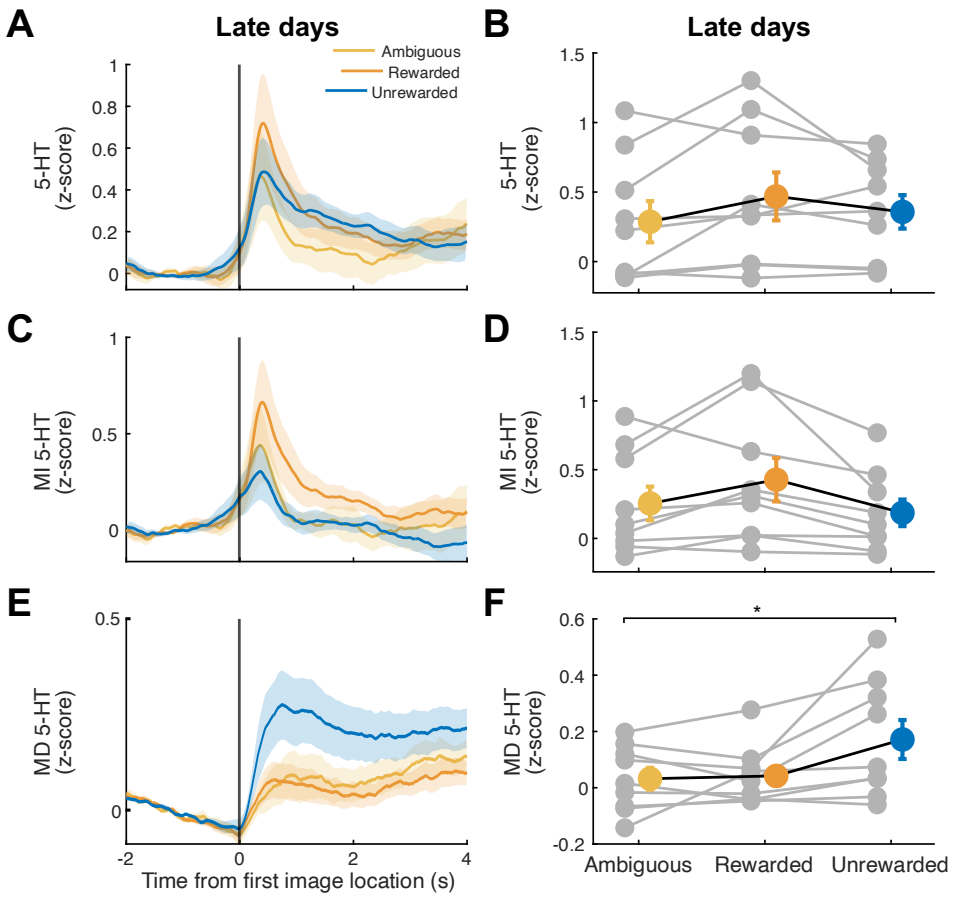

Figure 26. *Serotonin neurons respond to images in the first location of ambiguous corridors.*

A. Serotonin neurons' responses to ambiguous predicting, rewarded and unrewarded images in the first location (n=9, mean and SEM across mice). **B.** Average serotonin image responses in a 1 second window after image

location (grey, individual mice, colours are averages across mice and SEM). **D.** and **E.**, same and **A.** and **B.** for motion-independent serotonin activity. **E.** and **F.**, same and **A.** and **B.** for motion-dependent serotonin activity.

In contrast, we find a significant difference in the MD serotonin signal when comparing unrewarded first images from first images of ambiguous corridors (traces in **Figure 26.E.**, 1-way rmANOVA, image categories, p=0.0079, **Figure 26.F.**: ambiguous vs. unrewarded, p=0.0355). These first images in ambiguous corridors are, however, not significantly different from reward-predicting first images (p=0.9395).

9.4. Discussion and Interpretation

We explore in this section how serotonin neurons respond to images from different predictability contexts. These contexts were learned across days of the task as they defined the structure of the environment. Whilst the full and MI serotonin signals do not differentiate predictability contexts at the second image location, we find that the MD serotonin signal does respond differently (**Figure 25**). Serotonin neurons also respond to ambiguous leading images, and more to unrewarded images only for the MD serotonin signal (**Figure 26**).

Our negative result in this section leaves room for future analyses to be performed. It could be, in the first place, that mice did not learn the association between images in the first and second location of the corridors. As such, the second images in the ambiguous context would not be predictably different from those in the fixed corridors. Although we have shown hints, throughout these past sections, that mice have learned the predictability of images in

fixed corridors, we have never demonstrated it behaviourally (**Figure 3**, **Figure 4**, **Figure 10**, **Figure 21**). To test whether mice have associated the first and second images together would be to introduce catch trials, where we would omit the image in the second location. For ambiguous corridors, a hypothesis would be that mice would either slow down at the reward zone or not, as the chance of getting a reward is 50% (Kepecks et al, 2008). If mice behave in catch trials for rewarded and unrewarded as they would for corridors with all images, this would suggest that mice have learned that the first image of ambiguous corridors carries ambiguous information about reward delivery.

However, that serotonin neurons do respond to images in the first location of ambiguous corridors could suggest that mice have learned that these images are relevant for the task and predict ambiguity of reward delivery. Indeed, it would be plausible that if these images were truly uninformative of future outcomes for the animals, serotonin neurons would not respond at that location (Caldenhove et al, 2017). Instead, the response would be mainly driven by the predictability context of the image itself, being familiar and $1/5^{th}$ predictable from the structure of the task.

Moreover, our current methodology to average all image responses from respective value and predictability contexts during late days of the task to find signals of expected uncertainty might be not well suited for this question. At the image location, though the identity can be surprising as not fully predictable, the animal has learned from multiple days running in the task that the surprise will happen. This learning could possibly change the response of DRN serotonin neurons to the stimulus, as it's been sown that learning changes the response to rewards by anticipatory ramping of serotonin neurons' activity (Zhong et al, 2017). In our introduction of this section, we

presented a research paper focused on analysing signals of expected and unexpected uncertainty in a foraging task (Grossman, et al, 2020). The authors found that information about expected uncertainty in the DRN of mice was measured within inter-trial-intervals, and not at the stimulus itself. Therefore, in our task, serotonin DRN neurons might be tracking the ambiguity of the corridor from the first image location until they can perceive the second one. This is when the uncertainty happens.

It could also be that the difference between ambiguous and fixed corridors at the second image lies within the adaptation of the signal across trials. We have previously shown that stimulus adaptation in our task depends on the relevance of the image for the animal, and the animal's performance (**Figure 6**). MI serotonin responses to reward-predicting images adapt less than unrewarded images when compared to control (**Figure 18**). As images in the second location of ambiguous corridors only carry information about reward delivery, the response to these images does not adapt across trials, even for unrewarded images.

From our analysis, only the MD serotonin signal reflects the different image predictability contexts. This suggests that mice run differently past these images in the second location of ambiguous corridors compared to those of the fixed corridors. As the serotonin MD response is bigger for ambiguous corridors, mice might, in general, slow down more in these contexts. This result could align with both hypotheses presented in the previous section. Mice could slow down more at images in ambiguous contexts to perceive better the image and gain certainty of its identity, collecting more evidence (Brunton et al, 2013). Otherwise, as the response is even more pronounced at the unrewarded images, they might be more frustrated (Naik et al, 2024).

Taking these points altogether, future analyses focused on differentiating DRN serotonin neurons' responses to images in ambiguous and fixed corridors are needed. As introduced, this learning of uncertainty contrasts with uncertainty contexts where the stimulus is unexpected in the environment. A classical paradigm to assess how the brain computes unexpected events is the reversal learning task. In the next section, we present preliminary results of serotonin neurons' activity image and reward response dynamics in our continuation of our learning task with a reversal of learned cue-outcome associations.

10. Serotonin Neurons' Image and Reward Responses in a Reversal Task

10.1. Background and Motivation

The function of the serotonergic system has been deeply involved in facilitating cognitive functions such as cognitive flexibility in continuously changing environments (Homberg, 2012). Tasks that require adaptation and re-learning, such as reversal learning tasks, have revealed such functions of the system across species (Izquierdo et al, 2016). Importantly, reversals would correspond more to unexpected uncertainty, as opposed to the expected uncertainty of the previous section. Indeed, reversal learning tasks are a great tool to measure an organism's ability to adapt its behaviour when previously learned associations no longer apply, critical for flexible decision-making. Pioneering research performed in monkeys revealed that local depletion of serotonin in the prefrontal cortex (PFC) impaired performance in a reversal learning task (Clarke et al., 2004). Monkeys with serotonin PFC depletion could learn new stimulus-outcome associations in a classical conditioning task but showed increased number of preservative errors compared to controls upon reversal of this learned association. As such, serotonin depletion did not affect the learning of the new association but only the re-learning after reversal.

In mice, work performed in the Mainen laboratory focused on recording with fiber photometry the activity of DRN serotonin neurons during a reversal task

(Matias et al, 2017). The reversal task consisted of reversing the pairing of previously learned stimulus-outcome associations, specifically, of four odours to one of four outcomes being a large reward, a small reward, nothing or an aversive air puff. Importantly, serotonin neurons were shown to respond to reward-predicting odours, and not to odours that predicted negative outcomes. After re-learning of the new stimulus-outcome associations, serotonin neurons' responses to odours had also reversed, as serotonin started to respond to the odour it was previously not responding to. When comparing the response from DRN serotonin neurons to reward delivery before and after reversal, the authors also revealed that serotonin neurons responded most to unexpected rewards after reversal. Serotonin neurons were also shown to respond to the unexpected absence of reward, suggesting serotonin neurons could behave as unsigned prediction error signals in the brain.

Overall, these studies highlight a role of serotonin in facilitating cognitive flexibility and adaptive behaviours, enabling both humans and animals to re-learn and adjust their actions when faced with new or changing information. However, how the serotonergic system dynamically evolves as the animal relearns the task is not known, In our work, we have focused our analyses primarily on describing how the response of serotonin neurons to visual cues changes as the mice learn to associate them to specific outcomes. We present in this section our work that focuses on presenting dynamics of serotonin neurons specifically during the relearning process of new cue-outcome associations after reversal. We also ask if we can find similar results as in the previous work from the lab, namely, a switch in serotonin neurons' cue response that would track the new value of the image. We also examine how serotonin neurons respond to rewards and absence of rewards in the different reward predictability contexts.

Importantly, work in this section is very preliminary, as it has only been carried out in 5 mice, and 3 of which could relearn the task. More work should be done to confirm these results and interpretations.

10.2. Mice can Learn the New Structure of the Environment

Figure 27.A. illustrates the reversal paradigm performed on the 11th day for 5 mice of the learning task cohort. On this day, the first 45 trials maintained the previous corridor-reward contingencies and only upon entering the 46th trial were corridor and reward contingencies reversed for both fixed and ambiguous corridors. Importantly, image pairing within corridors did not change. We ran the mice in this new environment for 3 consecutive days. We present in **Figure 27.B.** the running speed and lick-rate around the reward delivery location for an example mouse in both rewarded and unrewarded fixed corridors during the reversal day. Pre-reversal, this example mouse shows accurate discrimination of corridor types by running in rewarded corridors at on average 9 cm/s in a 2 second window before the reward delivery location, significantly slower than in unrewarded ones, running at 42 cm/s (two-sampled t-test, p=2.484e-15). This animal was also licking significantly more in the anticipatory reward zone of rewarded corridors as compared to unrewarded ones (0.06 licks/s vs 0.003 licks/s respectively; two sampled t-test, p=3.2576e-05). On average, post-reversal, behavioural discrimination drops, as shown by the average running speed and lick-rate before the reward zone becoming indistinguishable between corridor types (two-sample t-test, running speed, p=0.6941; lick-rate, p=0.7463).

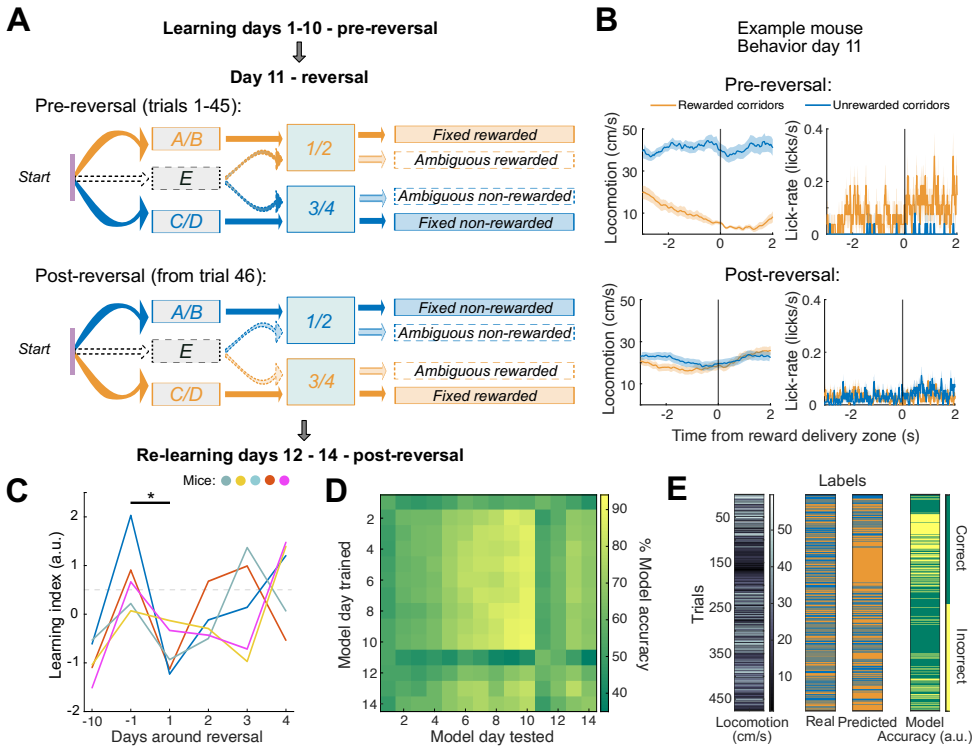

Figure 27. *Mice can learn the reversed corridor-reward contingencies.*

A. Schematic diagram of the reversal protocol of day 11. **B.** Example mouse behaviour around reward delivery zone on reversal day for running speed and lick-rate before and after the reversal on day 11. **C.** Learning index for day 1, day 10, reversal day and the 3 consecutive days. **D.** Matrix of model score results as percentage accuracy from model trained on all days and tested on all days (n=3; average 3 mice that at some point after the reversal, re-learned the task). **E.** Example locomotion, real labels (rewarded, orange or unrewarded, blue) and predicted labels from day 10 model prediction on all days from reversal onwards, model accuracy as correct or incorrect, across trials.

To capture the evolution of learning across days after reversal, we plot the learning index for each mouse on the first and last days of the learning task

(days -10 and -1), on the reversal day and on the consecutive 3 re-learning days (each colour is a mouse, **Figure 27.C.**). We find that the learning index drops on the reversal day for all mice and gradually increases again throughout the next days (1-way rmANOVA, learning index across days, p= 0.0065; t-test from multiple comparison correction, days -1 from reversal vs reversal day, p=0.0427). As it is important, when analysing the process of re-learning, that mice showed good behaviour in the task beforehand and similarly good behaviour afterwards, we selected mice that reached similar learning index scores pre- and post-reversal on either one of the following reversal days for the next analysis. We find that 3 mice out of 5 fit this criterion, all with learning index scores above 0.5.

To have insights on whether mice used similar behavioural strategies during learning and re-learning, we trained logistic regression models to classify rewarded and unrewarded corridors from their average running-speeds before the reward zone on every given day and tested corridor classification accuracy on all other days of the task for each mouse (**Figure 27.D.**). We plot the percentage accuracy of corridor classification of each model train/test combinations in a matrix averaged across mice and find that, from the second day onwards, models can already classify well corridors when using data from late days of the task. Moreover, model performance gradually increases across days trained. For all models trained, we find a drop in accuracy when testing the reversal day, as mice fail to discriminate the new corridor-reward contingencies, followed by a gentle recovery of accuracy on consecutive re-learning days. Thus, we make use of the model trained on day 10 to test how the behaviour evolves across trials and days after reversal for each mouse.

We present in **Figure 27.E.** the locomotion averaged in a 2 second window before the reward zone of all trials on the reversal day and consecutive days

concatenated for an example mouse, alongside the value identity of each corridor, the corridor predictions made by the model and the accuracy of the model on each trial as correct or incorrect predictions (orange, rewarded corridors; blue, unrewarded corridors). It is possible to observe a gradual shift in the proportion of correct trials to incorrect trials as the mouse experiences the reversal, followed by a recovery of correct trials, associated to re-learning.

10.3. Serotonin Transients in Response to Images in the Second Location Are Modulated During Re-learning

What happens to serotonin transients in response to images as mice learn the new images-reward contingencies? We explore serotonin neurons' responses to both image locations 1 and 2 on the reversal day and consecutive trials until 320 trials, capturing more than the gradual re-learning process (**Figure 28**). As a proxy for learning, we run a moving window of 20 consecutive trials on the incorrect and correct trial predictions for each mouse, as defined in **Figure 27.E.** (top rows of **Figure 28.A.**, **25.B.** and **25.C.**). This model accuracy plotted across trials ranges from 0% to 100% accuracy, where 100% corresponds to 20 consecutive, correctly labelled trials by the model. We find that each mouse, before reversal, peaks in model accuracy levels at 95%, 100% and 85% respectively. Percentage model accuracy for all mice drops as the moving window starts to incorporate trials post-reversal (dark green solid lines at trial 26). Minimum model accuracy scores for each mouse are labelled by the vertical solid purple lines, reaching, for some animals, well below chance level of 50%. We define the first bout of correct performance starting when the animal's percentage model accuracy reaches the maximal pre-reversal model scores within a 5% range (blue solid lines).

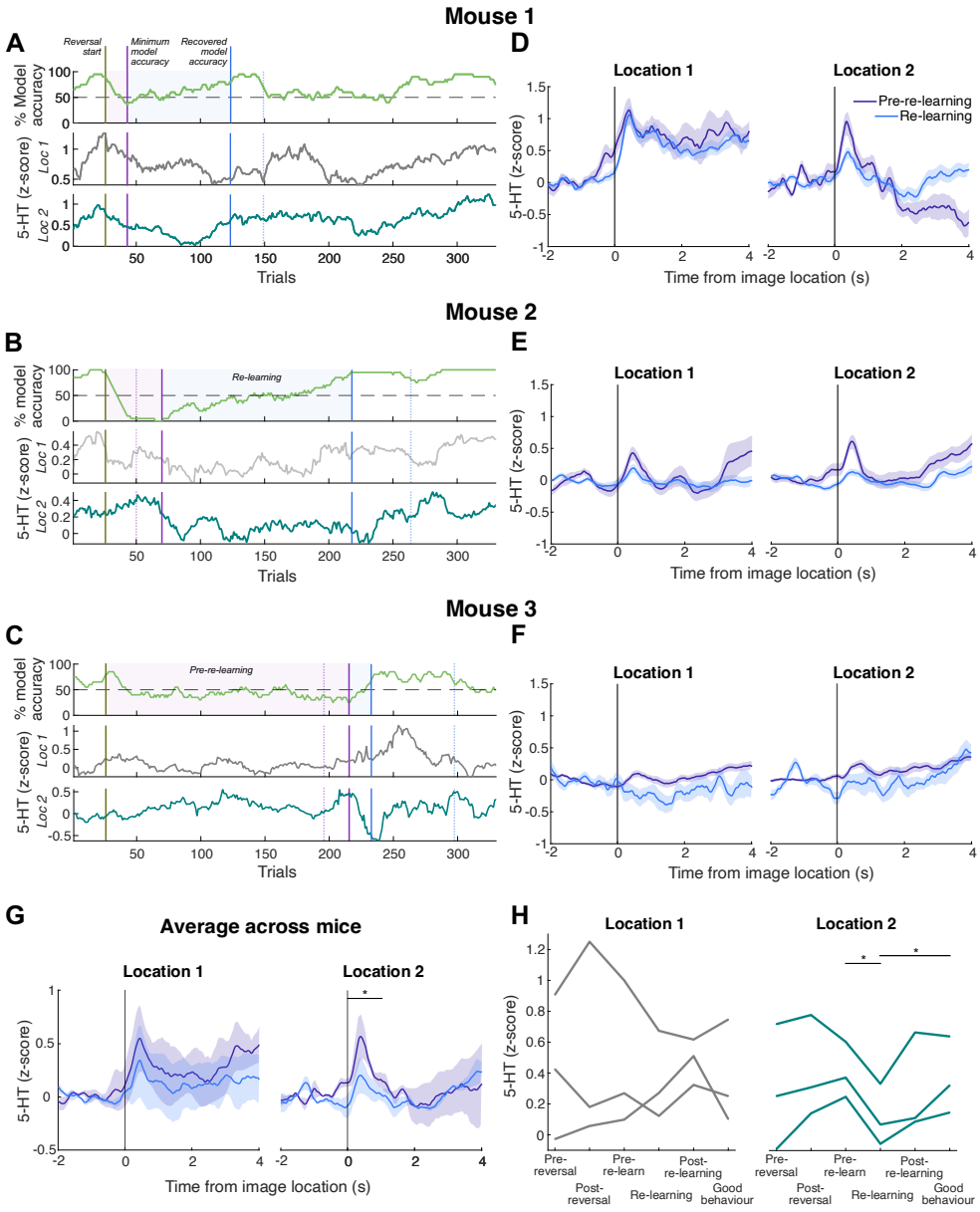

Figure 28. *Serotonin image responses during re-learning post reversal drop and increase again after the association has been learned.*

A. One mouse, 20 trials running averages of the model accuracy as percentage correct, the serotonin average image response to location 1 and lcoation 2 respectively, in 320 trials from the reversal day. **B.** and **C.** are

identical plot for the 2 other mice that ran the reversal task. **D.** through **F.** serotonin image responses for both image locations in the pre-learning and re-learning bouts of behaviour (shaded areas in the running average plots for each mouse respectively). **G.** Average of the 3 mice for both locations in these behavioural categories. **H.** Evolution of the serotonin response during re-learning vs pre-relearning and during re-learning differ from good behaviour.

Thanks to this classification, we can first observe that all 3 mice take very different numbers of trials until they start to learn the new contingencies, and different number of trials to re-learn. Whilst the first mouse has a gradual ascent in performance quickly after reversal, and re-learns within 130 trials, the second mouse maintains a performance score close to 0% for at least 20 more trials after reversal. This wrong classification for close to all trials in this bin suggests that the mouse persists on behaving according to the previous corridor-reward association after reversal, thus never collecting rewards. It then re-learns the task within 140 trials. In contrast, the third mouse behaves at chance level for more than 150 trials after reversal and eventually improves its behaviour very efficiently until good performance.

To make the link between behaviour and serotonin image responses in this preliminary analysis, we plot the average image responses in a one second window from image location running the same moving window across trials, aligned below all behavioural traces for each mouse (second and third rows respectively for each mouse, **Figure 28.A.**, **28.B**. and **28.C.**). To analyse re-learning specifically, we define two major bins: pre-re-learning and re-learning. The pre-relearning bin spans from the first trials in the model accuracy's trace that incorporates post-reversal trials until the minimum behavioural score of each mouse, essentially capturing the "persistent wrong behaviour" (purple shaded area). The re-learning bin consists of all

consecutive trials until the start of good performance (blue shaded area). We average the serotonin image responses per mouse and per location for both bins in **Figure 28.D.**, **28.E.** and **28.F.** Surprisingly, we find, for images in the second location, a selective reduction in serotonin image response amplitude for all mice during the re-learning process compared to the pre-re-learning state. We show the average serotonin neuron's image responses for both image locations across mice in **Figure 28.G.**, with a significant difference between bins in a 1 second window after the second image location alone (paired t-test, p=0.0353). Thus, this suggests that serotonin neurons' response to images in the second location drops as animals re-learns the task.

To capture the entire evolution of the serotonin activity pre and post reversal, we separate behaviour into 6 bins: pre-reversal, post-reversal, pre-relearn (last 20 trials post-reversal, before the minimum behaviour score; purple dotted lines indicate start of this bin), re-learning (as described above), post-relearning (from good performance until performance drops 20% from the maximal performance score, blue dotted lines) and later good behaviour (above 60%) until the end of the 3rd re-learning day (**Figure 28.H.**). We average serotonin neuron's image responses in these bins for each mouse and find a significant organisation across mice, solely for images in the second location (1-way rmANOVA, p= 0.0309). Specifically, serotonin neurons respond less during re-learning periods on average compared to pre-relearning periods (adjusted paired t-test, p=0.0047, **Figure 28.G.**). Re-learning responses are also smaller than future trials characterised as correct behaviours (adjusted paired t-test, p=0.0481). In parallel, we find no unique organisation across mice for first location image responses. It therefore appears that, for all mice, images in the second location have a drop in the serotonin transient amplitudes specifically when mice learn the novel,

reversed associations. Image responses are then recovered in late trials with correct behaviour.

Overall, we report preliminary results that serotonin neurons in response to images in the second location reduce in response amplitude during learning of the new cue-outcome associations. We find no specific organisation across mice for serotonin neurons' responses to the first image. Importantly, all image responses are recovered once the learning has been performed.

10.4. MI Serotonin Image Responses After Re-learning Are Sensitive to Reward Predictability

Finally, as serotonin neurons respond to images on late trials after the reversal, once animals have learned the new corridor-reward associations, we ask whether these responses re-acquire contextual modulation from value and image predictability. We average, across mice, the MI serotonin neurons' image responses for newly rewarded and unrewarded images on the last two days after reversal for both the first (left) and the second (right) image locations from fixed corridors alone (**Figure 29.A.**).

Though we find no significant organisation across mice for responses between image value and location being different from each other, responses to rewarded images in the first image location are greater than those for unrewarded ones, averaged across the three mice (2-way rmANOVA, value and location: value, p=0.0888; location, p=0.340; interaction, p=0.9970; from adjusted paired t-test analysis, rewarded vs unrewarded image responses: location 1, p=0.0406; location 2, p=0.2651, **Figure 29.B.**).

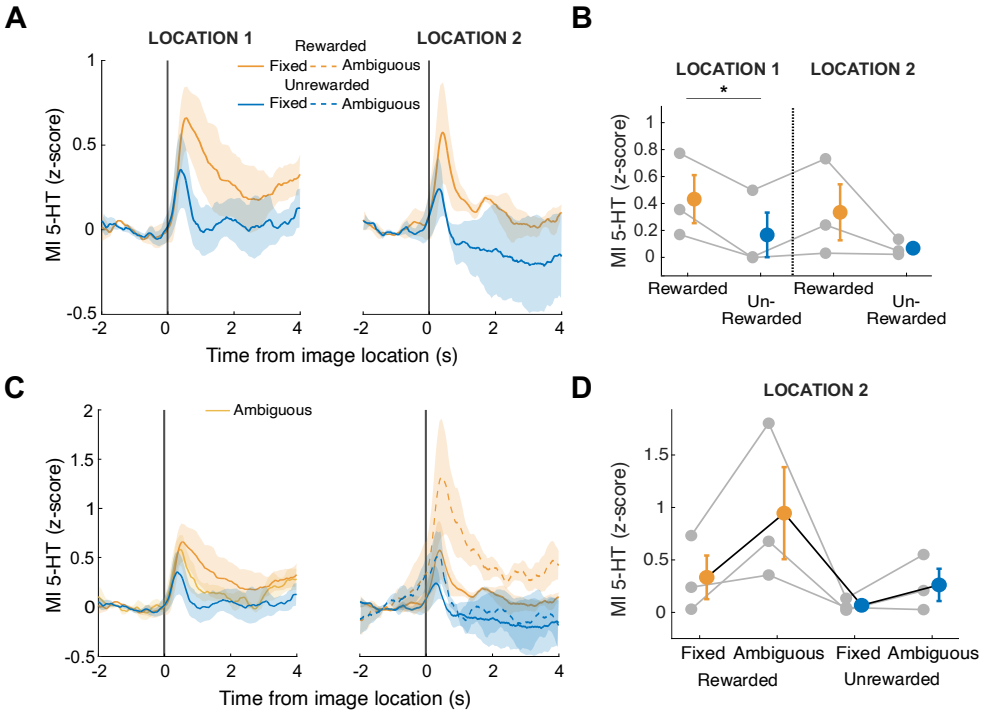

Figure 29. *Image responses regain sensitivity to image value after reversal.*

A. MI Serotonin image responses for rewarded and unrewarded images on days 3 and 4 post-reversal, averaged across mice (mean, SEM, n=3). **B.** Average serotonin response in a 1 second window for rewarded and unrewarded images in location 1 and 2 on days 3 and 4 post-reversal, per mouse and across mice. **C.** MI serotonin image responses for rewarded and unrewarded images in both fixed and ambiguous contexts on days 3 and 4 post-reversal, averaged across mice (mean, SEM, n=3). **D.** Average serotonin response in a 1 second window for rewarded and unrewarded location 2 images in both fixed and ambiguous contexts on days 3 and 4 post-reversal, per mouse and across mice.

To assess whether image predictability would be encoded within the MI serotonin neurons' image response, we plot the MI serotonin neurons'

responses to all second images for rewarded and unrewarded images in fixed and ambiguous corridors (right, **Figure 29.C.**). We find, as in the learning task, a non-significant trend for image responses in ambiguous contexts to be greater than for those in fixed contexts (2-way rmANOVA, value and predictability context: value, p=0.1830; predictability context, p=0.1691; interaction, p=0.109, **Figure 29.D.**). We also plot the MI responses to images in the first location and find once more that ambiguous leading images are not different from rewarded or unrewarded images (1-way rmANOVA, context, p=0.0804; ambiguous first image vs: rewarded, p=05957; unrewarded, p=0.4176, left, **Figure 29.C.**).

Thus, from these three mice that could re-learn the task after reversal of corridor-reward contingencies, MI serotonin neurons' image responses become contextually reversed within their new reward-predicting context. These results support a flexible and dynamic modulation of serotonin neurons' activity arising from contextual information of the stimulus.

A major finding from the Mainen lab is that serotonin neurons respond to unexpected reward deliveries after reversal (Matias et al, 2017). We will explore in the next sub-section these DRN serotonin neuron's reward responses across the different experiments performed in this work, namely, the learning task, the control task and the reversal task.

10.5. Serotonin Neuron's Reward Responses Are Modulated by Their Predictability Contexts

We plot the evolution of serotonin neuron's responses to rewards on days 1, 3, 5, 6, 8 and 10 averaged across mice for the learning and control groups, aligning the traces to the first lick after reward delivery location (**Figure 30.A.**). Whilst the shape of the response is seemingly identical for both groups on the first day, we find a gradual differentiation in the response shape and magnitude between both groups as we advance along days of the tasks.

To quantify this dynamic, we average the serotonin neuron's responses to rewards in a 2 second window after the first lick from reward delivery and average both groups across mice and days (**Figure 30.B.**). We find a strong, significant interaction and group effect of serotonin neurons' response evolution across days (2-way mixed model ANOVA, group and days; group, $p=0.0213$; days, $p=0.1742$; interaction, $p=0.0155$).

When averaging together the last 5 days of the tasks for both groups respectively, we find that the response to rewards is significantly higher in the control task than in the learning task (t-test, $p=0.0013$). This difference is not present when comparing the first 5 days of the tasks between groups (t-test, $p= 0.2766$). This reflects that reward responses gradually disappear across days as they become predictable in the learning task, and never adapt in the control task when they remain timely and spatially unexpected.

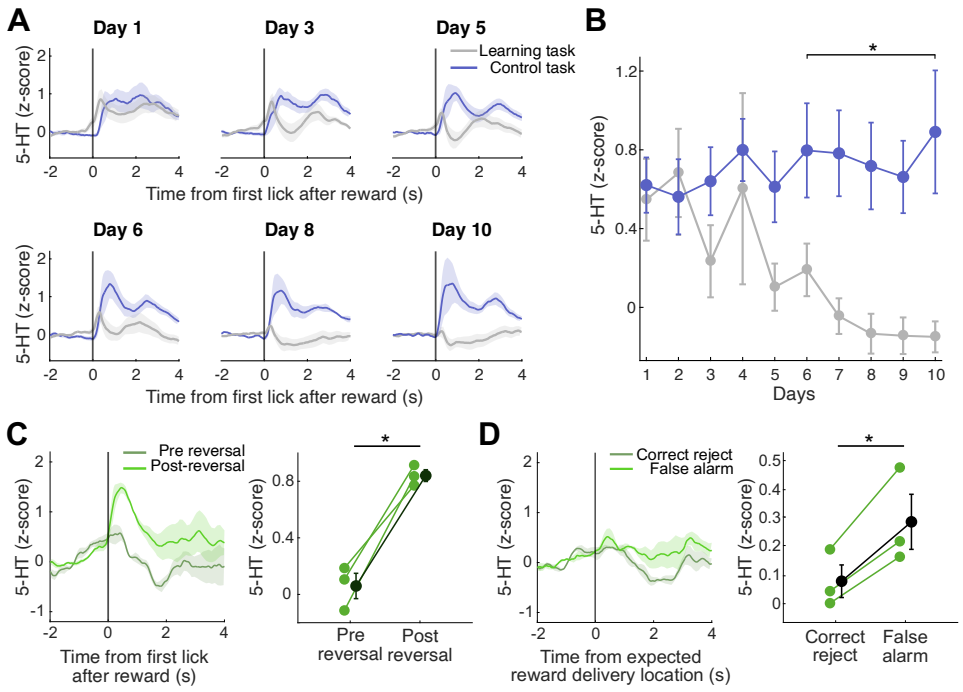

Figure 30. *Evolution of reward responses across days for the learning group, control group and the reversal.*

A. Reward responses in mice from the learning (n=9) and control (n=6) tasks in days 1, 3, 5, 6, 8 and 10 (mean and SEM). **B.** Average responses to rewards across days of experiment 1 to 10, 2 seconds window after first lick after reward for learning and control groups (mean and SEM). **C.** Reward responses before and after the reversal on day 11 (n=3, 20 trials averaged post-reversal) (mean and SEM), quantification of the 2 seconds average reward response from the first lick after reward delivery. **D.** Serotonin activity in unrewarded corridors aligned to the location where rewards would be delivered in rewarded corridors for correct rejects and false alarms, quantification of the 2 seconds average response from this location.

Together with the increase of visual cue responses across days and the selectivity of serotonin neurons' locomotion-corrected transients for reward-

predicting cues, the serotonergic system seems to behave as dopamine neurons would. This result also goes in line with previously published work that reports this gradual shift in the serotonergic reward response from unconditioned to conditioned stimulus (Zhong et al, 2017), which does not occur in the control task as rewards are not conditioned to any sensory cue.

Previous work from the Mainen lab has shown that serotonin neurons respond more to rewards after reversal compared to pre-reversal, as well as in the unexpected absence of reward in previously rewarding contexts (Matias et al, 2017). We asked if we could recover these results in our new reversal task. We average both the reward responses in the first 45 trials of the reversal day pre-reversal and the first 20 reward responses after reversal for each mouse we analysed the re-learning of (left panel, **Figure 30.C.**). We observe a clear distinction in the serotonin response shape pre and post reversal for all mice. When averaging the serotonin neuron's reward responses in a 2 second window after the first lick, we find a significant difference in the serotonin response, greater after reversal than before (t-test, $p = 0.0180$, right panel). Thus, we recover the positive prediction error signal previously reported and reinforce the concept of serotonin neurons being modulated by predictability contexts.

We can also examine in our task newly unrewarded trials after reversal, previously rewarded, when animals perform the reward-corridor specific behaviour of slowing down in anticipation to the reward before the reward delivery zone. In this new context, animals experience the absence of reward, a negative prediction error signal. We find these corridors on the reversal day post reversal by selecting unrewarded corridors wrongly classified by the logistic regression model of each mouse. We plot the serotonin neuron's responses for these corridors, analogous to false alarms, aligned to the

expected reward delivery location in the corridor track (left panel, **Figure 30.D.**). We compare these responses to correctly rejected corridors pre-reversal, selected as unrewarded corridors, correctly predicted by the model. After averaging a 2 second window post expected reward for each mouse and corridor type, we find a significantly smaller serotonin response to correctly rejected trials as opposed to false alarms (t-test, p=0.0342, right panel). When looking at the shape of the responses, this effect appears to be mainly driven not by a strong response to the absence of reward but by a reduction in the activity trace for correctly rejected corridors, 1 second after crossing the expected delivery location, leaving room for discussion and interpretation in the next sub-section.

10.6. Discussion and Interpretation

In this section, we analyse behavioural performance and serotonin neurons' responses to visual cues in the task as mice re-learn new cue-outcome associations after reversal. From the 5 mice that performed the reversal task as a continuation of the learning task, 3 mice could learn new cue-outcome associations after reversal and perform in the task as well as before the reversal (**Figure 27**). We separated trials after reversal based on how well our logistic regression model could classify, based on the locomotion of the mice before the reward zone, rewarded from unrewarded corridors. We find hints that, specifically during the re-learning period, serotonin neurons' responses to images in the second location reduce in amplitude compared to pre-reversal (**Figure 28**). Importantly, these responses are recovered as mice perform correct behavioural classification of corridor with new cue-outcome associations. The MI signal from serotonin neurons after reversal responds selectively to reward predicting cues (**Figure 29**). We do not find a significant modulation of corridor predictability in the MI serotonin neurons' image

responses. Finally, we report that serotonin neurons respond most to unexpected rewards in the control task, always temporally unexpected, and after reversal (**Figure 30**). As of now, it is not clear in our data whether serotonin neurons respond to unexpected reward omission. Importantly, these results were acquired only from 3 mice performing the reversal task. As such, all results and interpretations are preliminary, and more experiments are needed to confirm these results.

For the first time in this thesis, we present the evolution of serotonin neurons' responses to rewards in the learning and control tasks. We find a striking difference in the evolution of the serotonin neurons' reward response across days of the task between learning and control tasks. This result reinforces the importance of the predictability context on shaping this reward response (Li et al, 2016). Indeed, as the rewards are unpredictably delivered in time on the first days in both tasks, that reward responses do not differ suggests that serotonin neurons process these events in a similar way. Contrarily, on late days, that the response to predicted rewards decreases at the time of delivery could reflect the gain in predictability of the event during learning, as previously reported (Zhong et al, 2017). In contrast, reward responses in the control group do not change across days, suggesting they do remain unpredictable. These results contextualise well our measurements of serotonin neurons in response to the first rewards after reversal. Indeed, as previously reported in the lab, these reward responses could correspond to a positive reward prediction error signal, where the delivery of the reward in this context is unexpected (Matias et al, 2017).

Importantly, our results do not clearly present a response to the unexpected absence of reward in previously rewarded corridors. A hypothesis why this result differs from previously published work relies within the task structure,

and the possible unexpected reward omissions that mice experience during learning (Matias et al, 2017). The task we have developed is an operant conditioning task, where reward delivery is contingent on mice running below 30 cm/s before the reward delivery zone. As such, if mice run faster than this threshold throughout learning, they will miss receiving rewards in rewarded corridors. Therefore, unexpected reward omission at rewarded corridors might not be a surprising event, yielding possibly in an absence of response from the serotonergic system aligned to this event. Nonetheless, it will be important to examine better the dynamics of these responses across trials after reversal. We can also ask whether these responses are present during the learning process itself, when the mouse slows down to collect the reward, but not slow enough. In this context, rewards would be expected but missed.

We present a preliminary finding that serotonin neurons appear to reduce in image response amplitude as the animal learns the new cue-outcome associations. This result leaves room for creative interpretation, but is understandably, very preliminary and free from fixed statements. Within a short timeframe of a few hundred trials, the re-learning process post-reversal seems to capture similar dynamics as those during learning of the task in **Figure 11**. Serotonin image responses to the second cue are small at the beginning of learning, after the novelty response, and gradually increase as the associations between cues-outcomes are learned. Hypothetically, it could be that the reduction in the serotonin response to these images in the second location reflects the process of “losing” their contextual encoding, to regain a new one.

To test this hypothesis, It would be interesting to track the evolution of motion-independent signals after reversal for rewarded and unrewarded images specifically. We have shown that MI serotonin neurons responded to reward-

predicting images in late days of the task, and after reversal. However, we do not know how the response is transferred from one visual cue to the other. A hypothesis would be that serotonin neurons' responses to previously rewarded images decrease in amplitude, as the responses to the newly reward-predicting cue increase. This could create a period during learning in which the image responses are small, as we observe. Serotonin neurons' image responses remain sustained after reversal, before relearning, when mice keep on performing the previously associated behaviours. This could relate to a state of perseverance in producing old behavioural strategies as perceiving the cue with pre-reversal contextual information, or priors. Importantly, activating serotonin neurons have been shown to increase this active persistence state in mice performing a foraging task (Lottem et al, 2018). Therefore, in this context, the reduction in the serotonin neurons' responses to images in the second location could be reflective of this transition of contextual influences that the serotonergic system captures. As we do not find such an organisation for images in the first location, other processes might also be involved for influencing the responses of serotonin neurons to visual cues in this context.

The reduction in serotonin neurons' responses to second images could also come from a decreased attention towards that image by the animal. As we have mentioned in the previous sections, the activity of serotonin neurons could be reflective of attentional processes, responding to salient events (Thiele et al, 2018; Paquelet et al, 2022). Thus, a strategy for mice in our task to learn new cue-outcome contingencies might be to pay only attention to the first image of each corridor, and less to those in the second location. This could explain why we report a reduction of the serotonin image response only to the second image, and not to the first one. We could already examine our behavioural readouts such as locomotion, pupil and face motion to test this hypothesis, as they have been related to attention (Reimer et al, 2014).

Moreover, analysing respectively the MI and MD serotonin signals in the reversal task could also bring new inputs on the relation between this serotonin dynamic we report during relearning and the behavioural state of the animal.

The current literature focused on bridging the gap between serotonin neurons and reversal learning is not all consistent (Izquierdo et al, 2016). Though the most consistent result is that serotonin depletion in the cortex is associated with perseverance and impairments in reversal learning, studies examining receptor-specific effects have shown opposing results (Boulougouris et al, 2008). As such, it appears important to gain a better understanding of how serotonin neurons respond to cues for which the outcome has been reversed. As we have caried out this experiment on only 3 mice, more data should be acquired for us to pursue this analysis.

In this next and final section, we deviate away from analysing serotonin neurons within task related dynamics and explore a surprising feature of the experimental setup and design for which serotonin neurons are highly responsive.

11. Serotonin Neurons' Responses to Salient Stimuli

11.1. Background and Motivation

Throughout this thesis, we have explored the responses of serotonin neurons to visual stimuli, and how these responses are intricately modulated by contextual influences such as predictability and learned salience. It was recently suggested that serotonin neurons could specifically respond to emotionally salient stimuli in the environment (Paquelet et al, 2022). Serotonin neurons' response to rewards scales with reward magnitude and are affected by emotional components such anxiety-enhancing contexts. It was also shown that serotonin neurons respond to social investigations and by male ejaculation in female mice (Troconis et al, 2023). Even when emotionally salient stimuli repeat, such as foot-shocks, serotonin neurons were shown to adapt across stimulus repetition (Paquelet et al, 2022). This adaptation resonates with the adaptation we report in this work in section 2 when analysing serotonin neurons' responses to novel images that become familiar across repetition. This dynamic interaction between salience and predictability thus suggests that serotonin release could help organisms balance the need to react to important, unpredictable stimuli while reducing responses to expected ones that become less salient in time, optimizing behavioural and cognitive flexibility in changing environments.

We asked what was the most salient stimulus that mice experienced in our VR setup across days of the task, to understand whether this axis of inherent salience of the stimulus also scales serotonin neurons' responses. By chance,

we noticed that serotonin neurons respond to the switching-on and switching-off of the two LCD screens that display the task's virtual environment at the beginning and end of each session on each day. We analyse these VR on-off responses from serotonin neurons recorded in the task in this section. Furthermore, we examine if these responses also adapt as the events become more predictable by randomly repeating VR off-on events within the session across 3 consecutive days.

11.2. Serotonin Neurons Respond to Screen Onset and Offset at the Start and End of Each Experimental Day

On each experimental day, we head-fixed the mice in the setup on the running wheel whilst screens were displaying a black background. Only after launching the VR software would the first virtual corridor of the task appear on the screens and signal the start of the experiment. For accurate alignment, we chose to systematically start the recording of the serotonin photometry signal before launching the behavioural task, thus capturing in the data serotonin responses to this screen onset event. In reverse, at the end of the experiment, we first stopped the behavioural paradigm before, in turn, ending the photometry recordings. This allowed us to capture the responses of serotonin neurons as the screens turned back to black (**Figure 31.A.**). Though repeating daily, these familiar events were un-cued in time and remained informative for the animals as the start and end of the session.

We can align the photometry activity to the screen onset and offset events for most mice of both the learning and control groups thanks to configured hardware signals and a photodiode recording light intensity fixed on the right screen. We plot the VR screen onset and offset responses of an example

mouse throughout all ten days of the learning task in **Figure 31.B.** (top row, onset; bottom row, offset). We find very large transients in response to both screen onset and offset events, maintained throughout all mice and days (**Figure 31.C.**).

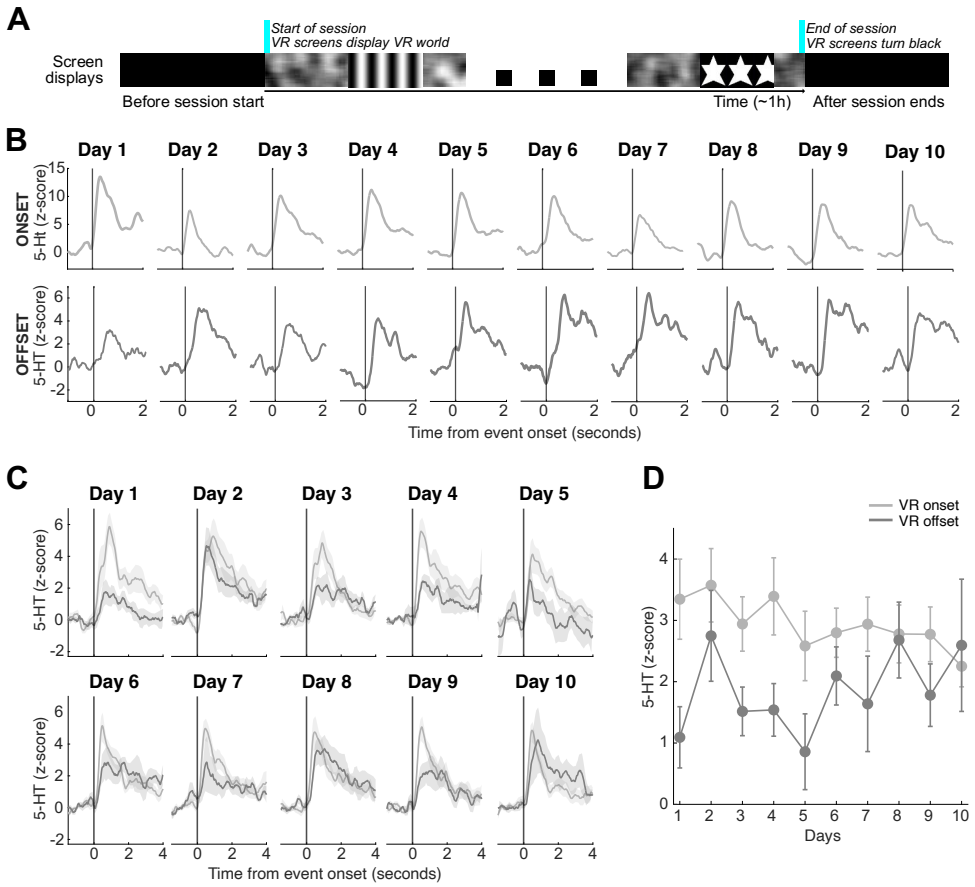

Figure 31. *VR onset and offset responses for all mice, every day of the task.*

A. Schematic diagram of every session, depicting the screens being off and black before the start of the protocol, then with the VR world and the screens turning off again once the session ends. **B.** Example single animal trace of the serotonin photometry traces aligned to the screen onsets and offsets across the 10 days of the task. **C.** Average across mice for the screen onset

(n=11) and screen offset (n=6) responses, in time for the 10 days of the task. **D.** Average response of the 10 days onset and offset responses across mice in a 2 second window after the event.

To quantify these responses, we average the transients in a 2 second window after each onset and offset event for each mouse (**Figure 31.D.**). Interestingly, we find no modulation of the serotonin neurons' responses to both event types across days, though on average, onset responses were greater than offset responses (2-way rmANOVA, screen on/ off events across days; days, p=0.3439; on or off, p=0.0186; interaction, p=0.2324).

Hence, serotonin neuron's responses to these events do not adapt across days, though they become familiar over time. We took advantage of these large responses to examine how serotonin neurons would respond to such salient events once they repeat multiple times across a given session and across days.

11.3. Serotonin Responses Adapt Across Repetition and Days to Very Salient, Surprising Events

We developed a protocol where, on three consecutive days, mice would experience 12 or 18 screen offset events within the session as they would run in either the learning or the control tasks (**Figure 32.A.**). These events happened at random times throughout the session and would last for either 500 milliseconds, 1 second or 2 seconds. A detailed table of the protocols per day and per mouse can be found in **Table 1.** The repetition of these screen offsets during the task would thus change their meaning – away from

signalling the end of the experiment, as the corridors would resume at the end of the event. As such, VR off-on events could become familiar and less surprising. We ran these protocols in 10 mice and show the responses to these events in an example mouse for all repetition number in three consecutive days (**Figure 32.B.**).

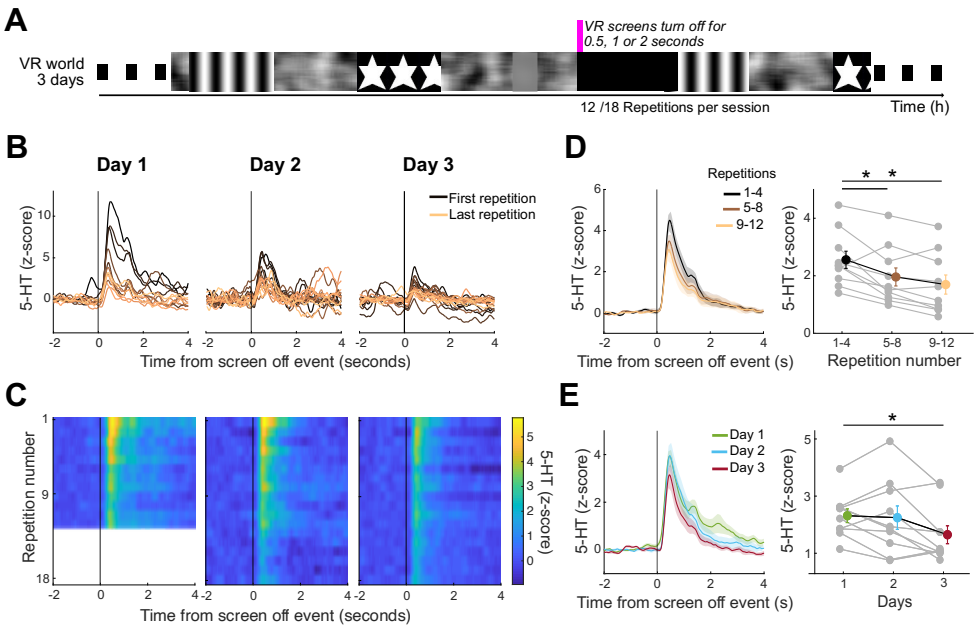

Figure 32. *Serotonin responds to screen off-on events and adapts across repetition and days.*

A. Protocol design of screen off events. **B.** Single mouse example of screen off serotonin responses across repetition and days. **C.** Average across mice of screen off events across days and repetitions (n=10). **D.** Adaptation across repetition for all days averaged, 1 to 12 repetitions for all mice. **E.** Adaptation across days for the first 12 repetitions for all mice (n=10).

It is possible to see a strong reduction in response amplitude of these VR screen off-on events across repetitions and days. We average all mice together and plot the responses in **Figure 32.C.** Interestingly, whilst we find significant effects across mice for repetition and days alone in modulating the DRN serotonin neurons' responses, we find no significant interaction between both factors, suggesting that the evolution of serotonin neurons' responses across repetition is not affected across days (2-way rmANOVA, repetition and day: repetition, p=0.0000; days, p=0.0214; interaction, p=0.8647).

Thus, to examine how serotonin signals adapt across repetition, we combine data from all mice and days (**Figure 32.D.**). We average the repetitions as three bins of four consecutive events and find a strong consistent effect of stimulus repetition across mice, with serotonin neurons' response amplitudes to offset events decreasing across repetition (1-way rmANOVA, repetitions, p=3.2017e-04; from adjusted paired t-test, repetitions 1-4 vs. 5-8, p=0.0434; repetitions 1-4 vs. 9-12, p=0.0035). Hence, serotonin neurons' responses adapt even to these very salient stimuli as they become familiar and irrelevant for behaviour.

To examine the adaptation of the signal across days, we average on each day the serotonin response to the first 12 event repetitions for each mouse and across mice (**Figure 32.E.**). We find a significant reduction of the offset response on the third day when compared to the first day of the task (1-way rmANOVA, days, p=0.0214; adjusted paired t-test, day 1 vs day 3, p=0.0385). Overall, these results reaffirm that serotonin neurons' activity is modulated by stimulus salience, predictability and relevance.

11.4. Adaptation From Serotonin Neurons Across Repetition is Not Caused by Bleaching

It is known that fluorescence signals bleach in time with prolonged exposure to light, consequentially risking to reduce the amplitude of the responses observed in the task (Simpson et al, 2024). To control for this effect of light on the within-day adaptation of the serotonin signal, we ran a similar protocol of repeating screen offset events in two groups of mice. In the first group, we maintained the photometry recording on for the whole session. In the other, we turned off the illuminating LED from the photometry rig for half of the session in the middle. We presented 30 screen-off events at random times and averaged serotonin event responses for the two groups presented in **Figure 33**.

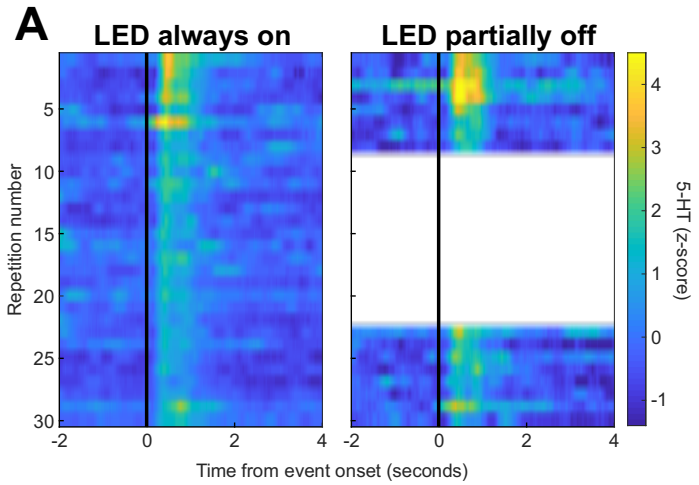

Figure 33. *30 repetitions of off on event adaptation of signal across repetition with and without recording in the middle.*

A. Serotonin response to 30 screen off-on repetitions for on and off photometry recording groups, averaged across mice (n=3 for both groups).

Importantly, we find no difference in the adaptation of the serotonin signal across mice between both conditions when looking at the first and last eight stimulus responses in a 2 second window after stimulus onset (2-way rmANOVA, repetition and condition; repetition, p=0.0001; condition, p=0.6016; interaction, p=0.0530). We can thus conclude that the adaptation we report throughout these experiments is real and not due to an artefact of the photometry technique.

11.5. Discussion and Interpretation

In this final section, we present our observation that serotonin neurons respond to very salient events, namely, the onset and offset of the VR screens at the beginning and end of the session (**Figure 31**). When we repeat the switching off and on of the two LCD screens multiple times across the session and across 3 days, we find that serotonin neurons' responses to these events adapt across repetitions and days (**Figure 32**). Importantly, this effect is not caused by the bleaching of the serotonin neurons throughout the session (**Figure 33**).

These DRN serotonin neurons' responses to screen onset and offset events are the largest responses we could find in the data aligned, to a stimulus we can control in the mice's environment. Image responses, when averaged across mice, reach 0.5 z-score (**Figure 10**), whilst the response to the onset of the LCD responses are on average 3 z-score, 6 fold greater (**Figure 31**). This could suggest that the DRN serotonin neurons' response amplitude scales with the intensity of the stimulus. Importantly, our recordings are of a population of DRN serotonin cells below the fiber tip. As such, the most plausible hypothesis for these different responses amplitudes is that these

salient events trigger more cells to respond than the images on the corridor walls. This could have the potential to broadcast a surprise signal more brain-wide, as opposed to more pathway-specific error signals, when the unexpected event is less inherently salient, such as images and rewards (Matias et al, 2017; Ren et al, 2018). Further experiments using single cell resolution in the DRN such as GRIN lenses could help us test this theory (Paquelet et al, 2022).

We find small trends with VR onset serotonin neurons' responses to decrease, and VR offset responses to increase across days of the task. Within our predictability and relevance framework, this small decrease of onset serotonin responses could arise from the stimulus becoming familiar and more predictable over time. However, as we have shown when analysing serotonin neurons' reward responses in the control task, this predictability context of the stimulus does not seem to modulate the response towards and adapted state (**Figure 30**). As such, a hypothesis for this sustained response could be that the unpredictability in time of these events, their very salient characteristics and their important behavioural relevance prevent the signal from adapting greatly.

In our follow up experiment, by repeating these off on events multiple times across trials and days, we were able to manipulate the predictability and relevance of these stimuli. Whilst they remained unpredictable as no other cue was associated to the event to inform of its occurrence, these events lost their informative value of signalling the end of the session. The strong adaptation of the DRN serotonin neurons' response to these repeating stimuli suggests therefore that the system is also flexible in integrating predictability and relevance contexts. By the third day, these VR off-on events, even if as salient as on the first day, could be ignored by the serotonergic system

because less surprising and irrelevant. This again contrasts with the reward responses in the control task that do not adapt across days. Even if the animal learns to predict that a reward will come if it keeps on running in the corridors, the event remains relevant for the animal's internal state. It would be important, as a future analysis, to explore how the serotonin neurons' reward responses adapt within the session, as the animal becomes more satiated.

The variability of these VR onset and offset responses from serotonin neurons could in turn be modulated by the internal state of the animal. Mice that are still very engaged in performing the task at the end of the session might correlate with higher VR offset responses as opposed to mice that have already disengaged at the end of the session (Oritz et al, 2020). It will be important to capture levels of engagement in the task thanks to our behavioural metrics to test this potential link. We can already start with analysing the serotonin MI and MD signals respectively aligned to these events. This should enlighten us on the potential motor-related component of the DRN serotonin signal that we are capturing, and if it is responsible for the adaptation across trials and days.

Finally, it was an important control to confirm that the adaptation we see in the serotonin responses to off-on events across repetitions are not related to the bleaching of the GCaMP6s fluorescent signal across time. It is of practice for all imaging techniques to correct for bleaching effects across time in the session, as we perform in this study (Simpson et al, 2024). Whilst the correction primarily corrects for a general decaying trend in the data, bleaching effects at the level of responses could still occur. This validation of our results opens the door to our next and final section, where we will discuss the work we report in this thesis in a global framework, as well as discussing limitations and possible future directions for this project.

12. General Discussion

Throughout this thesis, we have discussed every result within each section individually, aimed at building a continuous understanding of the responses of serotonin neurons within each dimension of our work. In this general discussion, we will attempt to integrate these findings within functional frameworks that exist in the field. We will pursue on discussing current limitations of our work, and present future directions for this project to bring more light on our understanding of the serotonergic system in the brain.

12.1. Stimulus in Context

12.1.1. One side: novelty and familiarity

The first result we report in this thesis when analysing responses of serotonin neurons to visual cues in our VR environment is the response to novel images in our learning and control tasks (section 4 and 6). Importantly, our work resides within a broad literature of rodent research that suggest an involvement of serotonin neurons in novelty processing (Bouet et al, 2018; Ballaz et al, 2007; Schott et al, 2011). These responses are particularly intriguing as they may play a key role in how the brain integrates novel stimuli into its existing model of the world. When an animal encounters a novel image, serotonin neurons respond, potentially signalling a latent space prediction error as something unexpected has occurred that contrasts with a prediction, possibly held within existing neural circuits.

This signal we record from serotonin neurons in response to novel images could thus be reflective of the general response of serotonin neurons to surprise (Matias et al, 2017). As we have seen when analysing the reward responses in mice running in the control task, serotonin neurons' responses to these stimuli also remain throughout days of the task (section 11). Whilst these responses differ from those of rewards being delivered in the learning task, when reward delivery is predicted by the identity of the images in the corridors, they resemble those post reversal. In this later context, reward delivery is unexpected, as occurring in previously unrewarded corridors. Combing these results presents once more the important contextual factor of the time at which the event takes place in shaping its serotonergic response.

Importantly, we have shown that serotonin neurons' responses to these images, after 10 repetitions, decay (section 4 and 6). The decay of these image responses is maintained throughout the second day of the learning and control tasks and is also observed throughout the entire sessions (section 4 and 6). A current interpretation of this result aligns with the contextual transition of these images for the observing mice - from novel to familiar. In our last results section (11), we report a similar effect when analysing the serotonin neurons' responses to the repetition of the VR screen off-on events. These very salient and unexpected events, switching the two LCD screens off for a period of maximally 2 seconds during the task, are also associated with an adaptation of the serotonin neurons' responses occurring across repetition. These events, though systematically identical, have changed context. It was reported in an experiment performed on humans that serotonin neurons did not respond to deviant stimuli in an oddball task (Caldenhove et al, 2017). In this task, the deviant visual stimulus appears less frequently on the screen than the familiar one. Its likelihood of appearing is therefore more unpredictable. However, that any image appears on the screen could have become predictable. Combined with its familiar attribute as repeating in time

across the session, these reasons could explain the adaptation of the serotonin response to this deviant stimulus.

It is therefore interesting to question why the serotonin neurons' responses to the LCD screens turning on daily, from a black background to starting to display the VR environment, do not adapt as much (section 11). Indeed, the animal is placed on the running wheel in the VR setup daily and experiences these events since the fourth day of training in the task. Therefore, a plausible prediction could have been that the serotonin neurons' response adapts across days of the task. Another stimulus that presents no apparent adaptation across days is, once more, the serotonin neurons' responses to rewards in the control task (section 11). To note, reward responses have been shown to adapt across reward delivery number within a session (Paquelet et al, 2022). In the current discussion, we are referring to an adaptation of the signal across days, that we do not observe in our task. Though we have just argued that their sustained response could be due to their unexpected timing of delivery, it is likely that mice have learned to expect reward delivery simply by running in the corridor.

A core attribute that separates reward delivery and screens turning on daily at the beginning of the session from the repeating images on the first two days of the learning task and the repetitions of the VR off-on events relies in the relevance of these events for the animals. Images on the corridor walls in the first days of the tasks, once familiar, remain, at this stage, irrelevant for mouse behaviour. In the learning task, we can infer this from the behaviour of the mice when comparing rewarded from unrewarded corridors (section 3). Indeed, by this stage, mice do not run differently in the anticipatory zone before the reward delivery when comparing corridors of different value. In the control task, images are never informative of reward location, and as such,

images never gain in prediction value for reward delivery. As such, they might not be so relevant for the mice in this task. The repetition of VR off-on events throughout the 3 consecutive days of this additional task has also the potential to alter the behavioural relevance of the VR offset event. Across days of the tasks, switching off the LCD screens would coincide with the end of the session. This information could be lost when these VR off-on events repeat. In both contexts, the familiarity of the stimulus across days could influence the adapted state of the serotonin neurons' responses aligned to them.

In contrast, that the VR screens turn on daily correlates with the start of the session, as we discussed above. This event could therefore remain relevant for the animals daily, though unexpectedly expected in some timeframe. Reward delivery in the control task aligns with this contextual setting as they remain salient and behaviourally relevant stimuli across days. This distinction of events that are accompanied by different dynamics of serotonin neurons in response to their reoccurrence across days in our tasks aligns with current work suggesting that serotonin neurons could respond to stimuli in emotionally salient behaviours (Paquelet et al, 2022). Stimuli that trigger such response in their task are social interactions, anxiety inducing contexts, reward delivery and foot shocks. As such, in our work, the absence of adaptation of the serotonin neurons' response to these stimuli across days could stem from their property as being relevant for animal behaviour.

12.1.2. Other side: learned salience

Importantly, the behavioural relevance of the VR screens turning on at the beginning of each session is learned as the animal is head-fixed daily on the VR setup. This reflects a second major driver of the shape of the serotonin

response that we have continuously observed and described during this thesis, namely, learning. The surprising characteristic of the VR screens turning on daily might reflect a more reflexive, bottom-up response from the serotonergic system. In contrast, the learned salience response might be more reflective of a top-down modulation of the serotonin response acquired during learning (Gacoin & Ben Hamed, 2023). Indeed, the DRN is widely interconnected with cortical areas (Ren et al, 2018; Zhou et al, 2017). The influence of cortical inputs on the DRN has been shown to affect behavioural performance in a foraging task in adolescent mice (Gutierrez-Castellanos et al, 2024). In reverse, ablation of serotonin neurons in the OFC impairs relearning in a reversal learning task (Clarke et al, 2004). As such, the modulation of the serotoninergic system on behaviour in the context of learning could be reflective of DRN-cortex interactions.

In our experiment, we presented evidence that serotonergic responses to visual cues in the learning task are modulated by learning (section 5). Images that were once previously novel and then familiar became informative of reward delivery across days of the task. In parallel, serotonin neuron's responses were recovered as the animal performed better across days, discriminating rewarded from unrewarded corridors with locomotion before the reward delivery zone. Importantly, this recovery of the response from serotonin neurons in late days of the task was not observed in the control task and was observed in the motion-independent serotonin signal (sections 6 and 7). Importantly, we found that this increase in the average serotonin response to these images in late days of learning was also reflective of the reduction in adaptation of the response across trials, compared to early days of the task (section 5 and 7). Good performance in the task was correlated with less adaptation of the serotonergic response.

This result suggests an interplay between two core features of the visual cues in the corridors to which the serotoninergic response is modulated by – their predictability and learning contexts. Another account in our results from the learning task that presents this interaction is the different response magnitudes of serotonin neurons aligned to images in the first and second locations (section 5 and 8). In late days of the task, we find that responses from serotonin DRN neurons are larger aligned to images in the first location compared to those in the second location. In the context of fixed corridors, the identity of the second image is predicted from the first, and so is the probability of reward delivery. However, which first image the animal will run in front of at each start of a new corridor is $1/5^{th}$ predictable due to the structure of the environment (section 3). As such, though serotonin neurons' responses increase across days at both image locations, reflecting the influence of learning on the response, the predictability context of the image might still play a role in shaping this response.

The absence of a difference of the serotonin neurons' responses to the second image of the learning task when comparing fixed and ambiguous contexts reflects that this interaction between learning and predictability might be more enigmatic (section 9). In the ambiguous context, the uncertainty of the identity of the second image on the corridor walls is learned across days of the task, as the animal learns to associate images at both locations together. Though we do not see, at the image location, a different response to the stimuli between predictability contexts, previously published work has suggested that serotonin neurons could keep track of this uncertainty at other time points in the task (Grossman et al, 2020). A more detailed analysis on this topic is present within the discussion of section 9. Overall, there is evidence that learning, and predictability contexts, interact in shaping the serotonergic responses to visual cues.

The learning of the informative value per se is also differently represented in the serotonin responses to images in the learning task. Indeed, we found that the motion-independent and motion-dependent signals within the serotonin response were respectively selective for reward-predicting and unrewarded images (section 8). Importantly, the MI reward response aligns with previously published work from the Mainen lab and others where serotonin neurons were shown to respond to reward-predicting cues (Matias et al, 2017; Cohen et al, 2015). That we could recover this distinction in the motion-independent signal after reversal suggests a flexible signalling the serotonin system even within the learning context (section 10). As old associations get overwritten by new ones, serotonin neurons might play a role in shaping the new connections inside the brain, keeping track of the new structure of the environment (Lesch & Waider, 2012). As such, both the predictability and learning modulations of the responses from the serotonergic system aligned to stimuli could reflect the flexibility of the system to keep track of changing environments.

12.2. Body and Internal State

We report in our task that serotonin neurons respond to unrewarded images in late days of the learning task (section 8). Specifically, these responses appear to be captured within the correlation between the activity of serotonin neurons and locomotion (section 7). In late days of the task, mice tend to slow down more at unrewarded images and this deceleration is correlated with the serotonergic response aligned to that location. Intriguingly, mice that decelerate most at all image locations are also those that perform best in the task (section 7). As such, the activity of serotonin neurons in the task goes beyond simply encoding reward expectation. Instead, it might also reflect a broader behavioural state within a given context, such as the need to slow down to enhance sensory discrimination, driven by attention, or represent an

emotional state of frustration when running past an unrewarded image. A critical paper in the field has put forward this relationship between locomotion and serotonin response, suggesting the direction of the correlation could be context specific (Seo et al, 2019). In low threat environments, initiation of locomotion was correlated with a reduction in serotonin activity. In high threat environments during escape, the activity of serotonin neurons increased with movement initiation. Therefore, in parallel with the flexible signalling of environmental cues in different contexts, serotonin neurons could also keep track of internal states within their responses to motor outputs.

We found multiple other events in our tasks that were correlated with a response from the MD serotonin signal. In our learning task, we found a significant difference in the MD serotonin response to images in the second location of ambiguous corridors compared to those of fixed corridors (section 9). The MD serotonin signal was shown to respond more to ambiguous images compared to fully predicted ones. As this response is mainly driven by ambiguous unrewarded images, this result could align with the interpretation abovementioned, with an additional contextual surprise component to the behavioural and neural responses. In both of our tasks, we also find MD serotonin responses aligned to the novel images (section 7). In this context, images are free from negative affect, but are surprising. The diversity of the VR screen onset and offset serotonin responses daily across days of the task could also arise from locomotion modulations of the response (section 11). Overall, the serotonergic system could reflect not only the context of the stimulus but also be modulated by the animal's behavioural state, correlated with the animal's action space.

Understanding locomotor behaviour in a task, alongside pupil size, face motion and others, has further been pursued in the attempt of getting insights

on the internal state of the animal (Anderson & Adolphs, 2014). We find a general, strong, correlation between locomotion and dynamics of serotonin neurons as mice run in our VR, not only restricted to image locations (section 7). As we have previously introduced, the relationship between serotonin and locomotion is likely bidirectional (Correia et al, 2017; Dey et al, 1992). With this in mind, and our discussion on the modulation of serotonin neurons' responses by stimuli in contexts, the serotonergic system is well placed to guide behavioural optimization of action and sensory processing by integrating contextual and internal state computations within its neural activity (Homberg, 2012). A prominent theory in the field on the functional role of serotonin in the brain is to promote behavioural inhibition and emotional affect, reflecting this link between serotonin, contextual motion and internal state (Boureau & Dayan, 2011).

Beyond locomotion, the broader behaviours an animal engages in, whether it is exploring, foraging, or even performing aggression or sexual behaviours, can be reflective of its internal motivational and emotional state. Serotonin neurons are shown to be involved in a plethora of innate and learned behaviours across species (Bacqué-Cazenave et al, 2020). Recently, an fMRI study performed in monkeys revealed that serotonin neurons of the DRN could specifically keep track of transitions in motivational states in a simple decision-making task (Priestley et al, 2024). In the zebrafish, the activity of serotonin neurons also appears to keep track of the behavioural state the animal is in, from exploration to exploitation during spontaneous foraging (Marques et al, 2019). As such, serotonin neurons could maintain information of internal states and respond to environmental cues through this lens. Overall, serotonin neurons could play a critical role in shaping behaviour by bidirectionally modulating its responses to external stimuli through internal states computations and state computations by external stimuli.

12.3. Axes of Modulation

We attempt to synthesise this discussion by referring to predictability and learning as two axes of modulation that could influence the serotoninergic response to stimuli in the environment. Importantly, this sub-section is not to be interpreted as a theory for the function of serotonin but is rather a simplified illustration of what we have discussed so far. It is also a game, to gain intuition on possible predictions of serotonin neurons' responses aligned to stimuli in our environment. From our two-dimensional space, predictability and learning, we incorporate a third axis, the one of inherent salience, as a core modulator of the response of serotonin neurons in our task. Highly salient stimuli such as turning the VR screens on and off during the task produce larger serotonin responses when surprising, compared to novel images on the corridor wall (sections 11 and 4). Therefore, the inherent salience of a given stimulus could play a role in shaping its associated serotonin response.

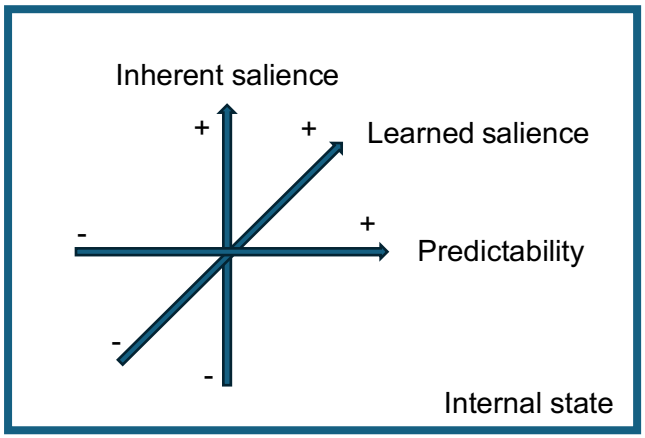

Figure 34. *Stimulus context: inherent salience, predictability, learned salience, within the internal state space.*

Figure 34 stands purely as a tool to try to capture and simplify the results in this thesis, for pleasure and not for instructive purposes. The figure illustrates three axes of contextual relevance that could modulate the serotonin response to sensory stimuli, as presented in our tasks.

Importantly, these axes stand within the *internal state* of the observer, restricting the space of these dynamics.

The first axis is the *inherent salience* of the stimulus, where inherently salient stimuli induce larger serotonin responses. This axis could correspond to the question “where”, as in, where is the stimulus in my environment, how much space does it take, how many sensory neurons will be activated, how much bottom-up inputs will the DRN receive.

The second axis is the *learned salience*. In this thesis we have presented evidence that serotonin neurons are sensitive to the behavioural relevance of stimuli for the animal. Meaningful or behaviourally relevant stimuli in the environment correlate with large serotonin responses. Irrelevant stimuli stand on the other end of this axis. This axis could correspond to the “what” question, in context. What is this stimulus in front of me?

The third and final axis represents *predictability*. Serotonin neurons respond differently to the same stimulus whether in predictable or unpredictable contexts. We report this modulation aligned to images on the corridor walls, rewards and VR off-on events. Within a representation of context, this axis could correspond to time, “when”, when does the stimulus or the event happen.

The thought experiment is now to place a stimulus, an event, within these three axes, within its context, and attempt to predict the sign or magnitude of the serotonin response – without forgetting to apply an overarching modulation of the response by the internal state of the observer as well.

We present two examples of a stimulus in context from the lens of an observer and infer a hypothetical response from serotonin neurons:

First, a large, unexpected stimulus, behaviourally irrelevant, but the observer is in a hurry. Imagine, one morning, the sky is pink. Very salient, very unexpected, but the observer is too busy running from A to B to avoid being late. The surprise could have been greater than it was, and so was the serotonin response smaller than it could have been, if time was taken to process this event.

Second example is, a small, very relevant stimulus, predictable, and the observer is fully present. Imagine a character from the movie Inception (Nolan, 2010). To make sure this is not a dream, the character takes out a coin. If it's upwards facing, it's a dream, if it faces downwards, it's reality. It is a small, yet extremely relevant object in the environment for the character, and serotonin could respond. For an external observer, this coin would be meaningless, irrelevant, a silent serotonin response.

These two examples are just food for thought, to think outside of the box of the task, to think about the brain, to think about us, in our world.

12.4. Limitations From Our Work

12.4.1. In behaviour

Our behavioural learning task design allows mice to make use of multiple different strategies to collected rewards, even after having learned the corridor-outcome contingencies (section 3). Indeed, as the only rule they must follow in the task is to decelerate in running speed in anticipation to the reward

to trigger reward delivery, mice can run freely run throughout the rest of the session. This variability in behaviour might influence our analyses of serotonin neurons' responses aligned to task events, as these responses are related to locomotion. Moreover, as mice are not actively punished for running below the running speed threshold in unrewarded corridors, some mice might have learned the image-outcome associations but not reflect it in their behaviour. As such, our analyses that examine dynamics of serotonin responses based on performance metrics that rely on the locomotion in rewarded and unrewarded corridors might be misleading.

In our learning task, we can only infer that mice have associated images at both locations together from their locomotion behaviour in the task. This point is especially relevant when it comes to analysing serotonin neurons' responses to second images in fixed and ambiguous corridors in late days of the task. Moreover, we performed no trials where we assessed whether mice had associated unique images with reward delivery probabilities. The use of catch trials could have allowed us to capture this, with the prediction that mice would behave the same in the anticipatory reward zone of corridors in trials with only one image as they would in normal trials.

Importantly, our protocol was not optimally suited to analyse serotonin neurons' novelty responses aligned to novel images on the first day of the task. Indeed, the appearance of novel images is correlated with the order of trials the animals ran in the task. Further control experiments where novel images are introduced later in the session would be key to disentangle image repetition and trial number effects.

In our control task, mice received rewards at any locations in the corridor, aimed to capture serotonin neurons' dynamics in response to visual cues that do not predict reward delivery (section 6). However, this task also changes the relation of the animal to the corridor walls, as reward delivery is not contingent on the reward delivery texture. As such, other control tasks could be helpful to accompany these results. One example could be that all corridors are rewarded, with reward delivery being contingent on running speed. Another example control task could be to maintain the random delivery of rewards along the corridor but always display the reward delivery texture on the walls aligned to the reward delivery location.

Finally, in our reversal task, mice might not understand that the reversal has happened directly (section 10). As mice run through previously unrewarded corridors, the likelihood that they decelerate before the reward delivery zone is low as they are not expecting any reward. As such, they will not receive rewards in these first trials. When they slow-down in previously rewarded corridors but fail to receive a reward, this event is also not directly informative of the reversal. Throughout learning, mice sometimes slow down to collect the reward, but still run above the running speed threshold, and miss collecting the reward. Therefore, the trial at which mice understand the reversal has happened is not so easy to infer, leaving room for variability in our reversal analyses.

12.4.2. In the technique

Fiber photometry, while a powerful tool for measuring population-level neural activity, has some limitations, particularly regarding the level of detail it provides (Simpson et al, 2024). Since fiber photometry records the combined activity of a large group of neurons, it offers a single, averaged activity trace

rather than capturing the specific dynamics of individual cells. This makes it impossible to distinguish the activity patterns of different neuron subtypes or isolate the contributions of specific cells to the overall signal. As a result, important details about how distinct neurons respond to certain stimuli or behaviours is lost.

12.4.3. In the analyses

It is important to acknowledge again the preliminary state of our analysis focused on understanding the relationship between serotonin and locomotion (section 7). Indeed, this function approach was carried out to attempt to capture the relationship between serotonin and locomotion without considering task dynamics. However, we are aware and have argued that the relationship between serotonin neurons and locomotion can be contextually modulated (Seo et al, 2019). As such, applying one function on all mice to extract motion-dependent signals in the task might create critical confounds and mask changes in how this relationship could be affected by learning, at the individual mouse level. As this serotonin-locomotion relationship is crucial for our work, it will be important to find other ways to extract motion-dependent and motion-independent signals within the serotonergic trace.

Another limitation from our analyses is our attempt to model the evolution of the serotonin neurons' image responses across trials on each day of the task (section 5). When performing these analyses, we came to understand that these responses do not always adapt. As such, fitting an exponential decay to these datapoints appears slightly inconclusive. Model comparison techniques could help us solve this issue.

12.5. Future Directions

12.5.1. In behaviour

Throughout all aspects of the task, it appears clear that mice can very dynamically run on the wheel head-fixed in our VR corridors. As such, it would be interesting to assess how serotonin neurons could keep track of escape responses as fast running bouts to avoid a negative outcome. Indeed, we could simply trigger the delivery of an aversive stimulus, like an air puff, when mice run below the running speed threshold, at the reward delivery zone, in unrewarded corridors. As such, the texture of the reward delivery zone would become informative of both positive and negative outcomes, and the unrewarded images would become informative of a future punishment. Timing of reversal would also become clearer for mice to infer in the reversal task. It would therefore be interesting to examine how serotonin neurons would respond to punishment predicting cues when mice are behaving in an active context, how they respond to aversive stimuli across days and how the serotonin-locomotion relationship would be represented in different contexts. Importantly, adding punishments to the task could also promote mice to perform more similar behaviours across trials and days, reducing the inter-mouse variability that is present in our current learning and reversal tasks.

Moreover, maintaining the same learning task structure as we have now, we could explore a reversal paradigm that changes the image pairing within the corridors. In our current reversal structure, we have reversed the reward contingency of the corridors, maintaining image associations the same as before reversal. It would be interesting to reverse, in fixed corridors, the second images associated with their reward pairing. We could do so by

reversing the reward contingencies of the first images or maintaining them the same. These different reversals could provide insights into how serotonin neurons respond to surprises of sensory stimuli of the same or of opposite reward prediction. In turn, this experiment could help disentangle the weights of predictability and learned salience on the serotonin neurons' image responses in the task.

As presented in the previous sub-section, there are multiple variants of behavioural tasks that we could perform to control for the interpretation of the serotonin neurons' results we have reported here. Examples of such tasks are to introduce novel images gradually throughout the first day of the task in both learning and control tasks and to introduce more novel images later on in later days of the tasks. These additional experiments could be relevant to explore in depth our novelty and adaptation analyses. We could also perform different control tasks that could help us better understand the interactions between different contextual influences in the task.

12.5.2. In the serotonin circuit

A future direction to explore deeper the responses of serotonin neurons in the task would be to record the activity of specific subpopulations of serotonergic cells in the DRN based on their projection patterns. There is already much published work going in this direction that suggests distinct roles for serotoninergic pathways in behaviour (Ren et al, 2019; Marcinkiewcz et al, 2016; Niederkofler et al, 2016). We could make use of rabies tracing to map the connections of serotonin neurons that project to areas like the visual or prefrontal cortices, and better capture which serotonin signals in our task are sent to these different areas. Moreover, it could help us understand if some

signals are shared between brain areas, such as reward or surprise responses.

Additionally, we could employ GRIN lenses to achieve single-cell resolution imaging of serotonin neurons and map the serotonin responses onto local ensembles, as done in Paquelet et al (2022). This approach would allow us to observe the responses of individual neurons in real time. As such, we could determine which specific cells respond to which stimuli or behavioural conditions, and again, if some information is shared within the local network. This could provide a more detailed understanding of the functional diversity within the serotonergic system. By combining these techniques, we could gain valuable insights into both the connectivity and functional responses of serotonin neurons across the brain, and how contextual information is encoded in the serotonin responses.

Crucially, manipulating the activity of serotonin neurons in the task would be informative of the functional relevance of the signals we report in this work. We could, for example, activate serotonergic neurons with optogenetics at image locations from the first day of task and examine if and how this manipulation would interfere with learning (Fonseca et al, 2015). It would also become informative of the direction of the serotonin-locomotion relationship, especially if we find animals slow down upon the stimulation, and if this effect changes across days (Correia et al, 2017). Finally, inhibiting the serotonin neurons' responses across days of the task with inhibitory opsins or DREADS would shed light on a causal, functional role for these serotonergic responses in learning in our tasks.

12.5.3. In the cortex

To further understand how the activity of serotonin neurons could influence cortical activity in our task, a future experiment could focus on imaging cortical neurons in regions like the visual and frontal cortices during task performance. This experiment could be performed either with classical two-photon microscopy or by using a mesoscope that could allow us to image multiple brain areas simultaneously. In the visual cortex, for example, we could focus on imaging both pyramidal neurons and 5-HT3 receptor-expressing VIP interneurons. This specific class of interneurons has been shown to be implicated in novelty detection, in the sharpening of sensory maps during brain development and responds to rewards and punishments (Garrett et al, 2020; Che et al, 2018; Szadai et al, 2022). As such, we could investigate how pyramidal neurons' responses to images evolve across days of the task, alongside the responses of VIP interneurons, and correlate these dynamics with our serotonin signals just presented in this work. In the motor cortex and prefrontal areas, we could examine how locomotor information and decision-making processes are also being represented in the task.

There are multiple techniques to image the release of serotonin in areas of choice, that could help us better understand where the serotonergic signals we recorded in our task modulate cortical activity. One technique would be to image directly serotonergic axon terminals in the cortex with two-photon microscopy (Broussard & Petreanu, 2021). We could make use of the same transgenic mice or inject specifically axon-targeted GCaMP viruses in the DRN of mice. New techniques have also emerged to record the release of serotonin neurons in the cortex with two-photon microscopy (Deng et al, 2024). The GRAB sensors are designed to increase in fluorescence upon the presence of the neuromodulator at the synapse. As such, we could record, in

the same experiment and the same field of view, neural dynamics of cortical neurons and the local release of serotonin. This experiment could bring new insights on the relationship between serotonin release and cortical responses during novelty, familiarity and learning.

12.6. Final Remarks

We close this discussion by connecting back to our introduction. How do predictability, learned salience and internal state relate to the association of the serotonergic system with depression?

As introduced, psychedelic drugs that specifically activate the 5HT-2A receptor of the serotonergic system have reached clinical trials for treatments against depression (Cavarra et al, 2022). Studies examining the effects of these drugs on animal models such as rodents have revealed their potential influence on brain networks (Ly et al, 2018). The presence of the drug in the brain was shown to promote dendritic arbour growth and induce synaptogenesis, hypothesised as bringing back a state of neural plasticity. It was suggested that this reopening of the plasticity period in the brain could generate important rewiring of neural networks that could have become malfunctioning or detrimental in depression (Carhart-Harris & Friston, 2019). For example, psychedelics could reopen the social reward learning period (Nardou et al, 2023). Adult mice spent more time in a space in the environment where they previously interacted with another mouse after receiving an injection of psylocibin or LSD compared to control mice. These drugs could therefore have the potential to change the weights or meanings of stimuli in the environment, alongside prior expectations that might be stored in these neural networks (Preller et al, 2017; Banks et al, 2021). As

such, where networks might overrepresent negative valence in depression, psychedelic drugs could relieve symptoms by triggering the formation of novel neural wirings.

The creation of new dendritic arbours does not mean that these will survive or be functionally used inside the brain. Clinical trials testing psychedelic drugs as treatments on depression are systematically accompanied by therapeutic sessions to integrate the experience (Raison et al, 2023). Another important, yet debated, aspect of the outcome of the psychedelic experience is the context in which the session takes place, also known as set and setting (Johnson et al, 2008; Borkel et al, 2024). Contexts in which the patients were most at ease, within natural settings or surrounded by significant others, had positive impact on the meaningfulness of the experience and future wellbeing. As such, the presence of the drug in the system might correspond to a “catalyst for change”, representing the tight relationship between the psychedelic experience and context, the long-term effects with a change in how we relate to stimuli in our environment and the meanings we’ve ascribed to them (Banks et al, 2021). Eventually, the long-term effects of psychedelic drugs could reflect this interconnected and bidirectional relationship between internal and external worlds existing within serotonergic dynamics in the brain.

13. Conclusion

To conclude, we present in this thesis our work that has focused on trying to understand how serotonin neurons respond to visual cues: from novelty to familiarity to learned salient. We report that the responses of serotonin neurons appear tightly modulated with the context in which the stimulus exists in the environment – namely in our experiment, predictability and learning, and the animal's internal state.

It is not the identity of the images on the walls of the corridors that change across days of the task, but it is how the animal relates to them within their new contextual space. The activity of serotonin neurons appears to keep track of these transitions, possibly to guide animal behaviour.

Serotonergic neurons also respond to novel stimuli in our task, and their activity resembles unsigned prediction error signals in the brain, signalling surprise. In these contexts, it is not the internal motivation of the animal that guides, but the external world that influences most the response.

These observations align with the many faces of serotonin. The activity of serotonin neurons could be bidirectionally influenced by the relationship between the animals' internal state and behaviours with the external environment and the stimuli present around them.

Whilst AI might be changing how we think about the brain, the field is still lagging when it comes to integrating neuromodulators within their system. Researchers are starting to build dopamine-like signals in neural networks to modulate learning with reinforcement (Vecoven et al, 2020). It might be a long way until serotonin neuromodulation makes its way into an AI network. What would be its purpose? To help a system become more flexible in applying correct "behaviours" in the right "context"? Another guess? We might just have to wait and see.

We transformed to remember not the name of every plant or every tree, not the constellations or python vocabulary. But throughout time there was always something to remember. Either these, or others. Not all of us cared about the same thing, learned the same way, remembered the same way. Our shared lives, our personal experiences. But the way that it is done – this we share. There is something that drives or helps us put attention on some aspects of the world today. The external world, or our inner world. And what is more relevant to you will grow, will take more space. It does, eventually, take space, in your inner world, in your shape. And your shape changes shape. It grows. It gets transformed – you transform it.

Not everything takes the same space, for you, for anyone. And that is – even what someone would consider small, can be immensely meaningful, to you. That is diversity.

So, what is surprising in this world? Only if you care, it is so.

What is meaningful, in this world? Only if you care.

What is new in this world? Only if you care to see it as new. Not distant, not blunt. But to care.

It might be all about – meaning. To give us feeling, surprise and growth. To give us care.

14. References

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