If I Only Had A Brain: Memory Retention After Decapitation and Regeneration in Planaria

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Introduction

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- What I found
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Background

A brain in isolation is just a clump of extravagant cells. A brain earns its keep through interacting with the body and the external world. It is among these brain-environment interactions that an organism can set and acheive goals and, ulitimately, carve a pathway to survival. But brains operate in the dark. Their only insight into the on-goings of the world is through delicately placed sensory organs such as eyes, nose and ears. The sensory technology that each organisms possess', what pilosophers call its sensorium, differs across species. Some

build a picture of the world by capturing light using light sensitive proteins. Others live where no light can penetrate and so must form their worldview using other sensory modalities like exholocation. Notwithstanding these differences, neuroscience (in collaboration with biology) seeks to understand the suite of abilities each organism possesses, the neuronal and molecular mechanisms which underpin these, and the factors that determine when and why an organism deploys the behaviours in its arsenal. When we use non-human organisms during scinetific inquiry, we often do so while hopeing the knowledge gleaned will teach us something about our own brains and bodies.

We now have at hand a broad tool set for inspecting the brain across different time spans and at different levels of analysis. From looking at activity within a single dendritic spine over microseconds to looking at connectivity between different brain structures over many seconds. We can even track changes in the size and number of individual spines on a single dendrite over time – impressive given the width of a spine is 100 times smaller than the thickness of a human hair. At the network level, we are able to identify groups of neurons (ensembles) involved in encoding and storing memory, and can use precise tools to excite or inhibit those networks to alter an animals behaviour.

Our current experimental competency arose from many small steps. Before we had the means to manipulate neurons and undestand their role in memory, we had to make do with simple procedures like learning lists of nonsense syllables or simple motor tasks. This research often utilised patients who had conditions like amnesia or who had suffered some kind of brain injury.This early research helped answer the question whether memory is a unitary system or a suite of seperate systems which can be dissociated. Distinctions such as episodic and procedural, as well as short- and long-term emerged. Theoretical progress provided the foundation upon which specialsied tools and procedures could be developed to manipulate and characterise the biology of memory in its different forms.

Overview of key concepts in the field of learning and memory

The meaning of memory and the different forms it takes

Generally speaking, memory is the embodiment of past experience which shapes our future behaviour. Learning is the process of memory acquisition. Though, it must be said that there may be as many different definitions of learning and memory as there are papers published on the topic. Barron et al. (**?**) surveyed the various uses of the term "learning" across disciplines such as cognitive psychology, behavioural ecology, and machine learning and identified at least 50 definitions (albeit with a lot of overlap). Memory has been parceled into several distinct categories based on the content of the information being stored. The major distinction is between explicit (or declarative) and implicit (or procedural) memory (**?**; **?**) as shown in Figure 1 below. Explicit memories are those accessible to consciousness. Implicit memories refer to information that you cannot consciously access. Explicit memory has been further subdivided into episodic and semantic memory (Tulving, 1972). With episodic referring to the rich experiential quality of personal memories, while semantic relates to things that you know, but that lack an experiential component (e.g. facts about the world).

Memories can also be categorised on the temporal dimension. Atkinson and Shiffrin (1968) proposed three memory stores: a sensory register, a short term, and a long term store. This division is still usefully applied in the field of learning and memory (e.g, **?**). A temporal framing of memory reflects the learning process itself. Learning is normally broken up into several stages, each building on those before to further engrain the memory and expand its longevity (see Section **??** below) . Initially, information enters a short term state and may alter behaviour and decision making in the immediate future. However, most of the information that our brains initially perceive – all the light hitting our eyes, and all the vibrations in the air registered by our ears – is not retained. To successfully retain this information it must be actively incorporated into our biology and maintained so that it can be accessed or recalled

in perpetuity. To put it another way, the Information that is taken on board but must undergo specific processes (known as consolidation) to stave off the forgetting process.

These distinctions make a lot of sense when talking about memory in humans. But it may not be immediately clear what relevance these distinctions have when looking at other organisms. Most people do not attribute rich episodic (experiential) memories to rodents, let alone invertebrates. Yet, it is clear that even very simple organisms have the capacity to learn and store varied types of memory; Ranging from basic changes in a reflexes (habituation and sensitisation) to more complex operant behaviours. Such distinctions may be particlalry relevent for the phenomena of memory storage outside of the brain, as is the topic pursued in this project. if, as the literature suggests, some memories are able to be retained outside the brain (and perhaps outside the CNS) these concepts may help us derive the bounds of which memory types hold this property and which do not. It may be that procedural memories such as non-associative responses are stored outside of neural networks. But memories which are consciously accessible are only able to be stored among complex ensembles of neurons in the brain. We do not yet know the bounds of non-neuronal memory storage, if it truly exists. But armed with these conceptual distinctions between types of memory, we can apply tailored training techniques to find out what forms of learning persist outside the brain and which do not.

Associative and non-associative learning

Associative learning is learning the relationship between two stimuli. One stimulus reliably precedes another, or a behaviour reliably precedes a reward. Non-associative forms of learning captures learning about a stimulus itself, but not in relation to other stimuli. This typically takes the form of behavioural sensitization or habituation. If you were to mildly shock my hand, I would withdraw it reflexively as it would be startling. But with repeated administration of the shock over time, I may come to learn that the shock is actually not that painful after all. The size of my startle response decreased over time with repeated exposure (habituation). I have learned something about

the shock, but have learned nothing about its relationship to other stimuli. To extend this example to an associative form of learning, the shock could be delivered after being shown a picture of a certain flower. I would therefore associate (likely subconsciously) the flower imagery with a negative experience, and would eventually display a fear startle to presentations of the flower (tense up, squint my eyes, dip my head). This captures the fact that I have learned a temporal association between the flower and the shock. My body learns to prepare for the before it arrives.

Classical conditioning and operant conditioning are two frequently used forms of associative learning. Classical conditioning involves learning an association between two or more stimuli, as in the flower/shock example above. Operant conditioning differs from classical conditioning in that rather than a stimulus (e.g. shock) being paired with another stimulus (flower), a behaviour comes to be associated with a desirable outcome. For example, I learn that signing up to psychology experiments often invovles being exposed to (mildly) irratting stimuli. This changes the likelihood of that response being produced in the future. In other words, I stop signing up as a participant. Good for me, not so good for postgraduate students trying to complete their research.

Maladaptive learning

The examples outline above cover adpative learning. preparing for shocks in the case of classical conditioning reduces the painfulness of the stimuli. Avoiding experiments means that I am exposed to less unnecessary irritants. These are of course simple toy examples, but real life examples abound whereby our wellbeing and longevity is greatly enhanced by learning from past experiences. A close call when crossing a road (and the negative phsyiology experience that ensues) may increase the likelihood of diligently looking both ways in the future before crossing a road. These learning experiences allow us to persist in a world full of novel and ancient dangers. But the capacity for learning raises several vulnerabilities.

Learning has a darker side. Consider Rebecca's first experience with heroine. Prior to consumption, Rebecca has heard about herione, but only in the sense that she knows it is a harmful drug, that similar drugs are used in a medical setting, and so on. She has no prior subjective epxerince of its effects. But after several experiences with the drug at parties, she learns about the intense sense of euphoria that comes from taking the drug. Later on, Sarah starts to feel a strong motivation to take the drug again, especially when she sees cues associated with the drug (syringes, white powder). Without the capacity to form such associations, addiction would not be an issue. Sarah would fail to remember what actions led to such euphoric experiences, and no memories would be recalled at the sight of drug paraphenalia. we can see that addiction arises from the often useful ability to remember what past actions and events resulted in positive and negative experiences. In Rebecca's case, the euphoria that was experienced as positive in the moment led to neurological rewiring, hijacking the cognitive system to pursue herione in the face of adverse consequences in the future. This is an unfortunate but common case of maladaptive learning.

Many aspects of poor mental health conditions, including aspects of depression, PTSD, and anxiety, similarly rely on memory systems for their development. Anxiety is especially relevant, as it involves the worry or concern over some perceived threat. The threat has not yet occurred, but to a person with anxiety, memory of past experience of similar circumstances creates excessive fear and a sense of unease as they imagine a similar negative outcome occurring in the future. By understanding what leads to the creation of these associations between a context and a negative outcome, memory research can then help us explore ways to prevent anxiety-like responses in the future, and identify methods to unlearn the initial negative association. But to understand

Mechanisms of memory storage

As described earlier, the process of acquiring a memory involves transferring information from a short term store to a long term store. The default process for all organisms is to forget information. In fact, we probably forget most of the information we

encounter. This is evolutionarily sensible. Storing information takes energy, and attaining energy comes at a price. Every organism therefore has a small number of things about which it must dedicate a lot of attention towards to track and understand. But most of the information it encounters, be it visual, tactile, or olfactory, does not matter to it. A penguin cares about the scent of a polar bear, but not about the small bug being crushed under its foot. While a penguin's brain would process both to some degree, it is not equally likely to remember both experiences and the contexts in which they took place.

When discussing how information is stored, one becomes steeped in complex biological pathways. Such pathways involve proteins interacting with other proteins, proteins interacting with DNA, and the production of new proteins. In the neurobiological literature, much of the discussion around learning takes place at the level of synapses – synapses being the points where two neurons interact. The axon from neuron A attaches to a dendrite from neuron B and thus form a synapse. Synapses are the locus of neuronal communication in the brain. At an abstract level, learning is realised by information affecting neurons such that they progress through a sequence of stages: acquisition, stabalisation, consolidation and maintenance (see Rudy 2014 for a digestible overview of each stage). There are complicated mechanisms involved in each stage of learning, and not all the components are fully understood. Despite that, the literature provides a good sense of many important molecular events that are involved in the process of storing information in the brain and being able to act on that information at a later time point.

When some new bit of information has been processed by the brain, the physical embodiment in the brain is often described as the "memory trace" (Asok et al., 2019; Robins, 2023; Semon, 1921). An influx of calcium (resulting from stimulation by an upstream neuron A) is necessary for the initial **generation** of the memory trace. The calcium interacts within the post-synaptic dendritic spine on neuron B and leads to actin being broken down into smaller chunks. Actin helps give structure to the spine. Breaking it down is a necessary step to create room to fit more receptors into the post-synaptic membrane of neuron B – the membrane acts as the skin between the cell and

the watery world outside of the cell. Adding receptors to the membrane makes the spine on neuron B more sensitive to the upstream firing of neuron A, and thus more likely to itself fire an action potential in response. But this rapid change in sensitivity (potentiation) is short lived. Without additional actions, this potentiation will revert back to baseline. **Stabalisation** of the trace requires expanding and strengthening the actin network in the dendritic spine. The spine head is enlarged, and the actin scaffolding is changes to make it less vulnerable to being broken down (depolymerated). Furthermore, proteins in the cell membrane called adhesion molecules help to couple the pre- and post-synaptic neurons, improving the effectiveness of neurotransmission. These first two stages, generation and stabalisation, establishthe memory in the curreny of biologically material, but are not themselves enough to ensure the memory stands the test of time. Much like a digital file that you create and store on your local drive. It has been written and stored in a meanignful way, but it can be easily lost if the hard drive is damaged or the file is deleted by someone freeing up space for their own documents. It is not until you back your files up to a network that you can be confident in their future accessibility.

Consolidation is a unique step in the process of learning where proteins enter the nucleus and establish a chain of events leading to the productiong of new proteins. After entering the nucleus, the proteins bind to DNA signal to the genome which leads to transcription of new molecules (mRNA) that will then be turned into proteins, such as receptors that can be inserted in the membrane. These instructions to the genome to continually mint new proteins ensures a sufficient pool of receptors and other elements are avaliable to keep the spine in a potentiated (more sensitive) state. Finally, during the **maintenance** stage this supply of membrane receptors is produced and inserted into the membrane. and the typical dynamics of receptor removal, where receptors are removed from the membrane and recycled, is dampened. More excitatory receptors remain on the cell membrane which ensures heightened sensitivity to signals from presynaptic neurons both now and in the future.

Memory research in animals and invertebrates

Many organisms have found themselves being poked, prodded and leered at as we attempted to understand the underlying mechanisms of memory. Scrub jays, a bird sporting a bold blue coat and pointed black beak, have long been subjects in studies investigating spatial memory because of their food caching expertiese (Shettleworth & Krebs, 1982). Rather sophisticated methods have been used to study changes in hippocampal volume with caching and the possibility of season-dependent changes in hippocampal neurogenesis in caching birds (reviewed in Pravosudov, 2007). Rodents have also featured heavily in the experimental memory literature (Ghafarimoghadam et al., 2022). Recent advances in stimulation (e.g. optogenetics; Goshen, 2014) and imaging (e.g. two-photon microscopy; Kawakam et al., 2015) have enabled us to study representation of different types of memory at levels ranging from individual synapses to neuronal ensembles.

Rodent models have been developed for tapping into spatial memory, working memory, associative memory, recognition, and long term memory. Most strikingly, the last two decades saw an intense exploration of episodic memory in rodents. Episodic memory, at its essence, is the ability to represent the past and draw on specific encoded events in a manner akin to mental time travel (Tulving, 2002; Eacott & Easton, 2007; Crystal, 2022). Intricate tasks have been developed which enable rats to demonstrate memory for the context in which a stimulus had been previously presented, and to disentangled this from mere familiarity with the stimuli due to temporal proximity (Panoz-Brown et al., 2016). However, the existence of episodic-like memory in non-human animals remains debated (Tulving (**?**); Hoerl and McCormack (**?**)). The establishment of procedures for identifying and manipulating complex episodic memory, by optogenetic and other means, may help inform us on the type of mechanisms (synaptic or molecular) likely to support episodic memory in humans.

- Paragrapgh on primates in memory research?

These odd collection of experimental subjects supplied us with decades of insights into learning processes and the nature of

memory. But when seeking to understand the precise molecular and structural changes that underpin memory, the field first looked to invertebrates. The simplicity of invertebrate neural architecture allowed researchers to account for and track the entirety of the nervous system, and to observe functional specificity of neurons. But simplicity is not the only benefit. By using simpler organisms costs can be significantly reduced and environmental variables can be more easily controlled. Moreover, ethical concerns are relieved (albeit not completely removed) due to a lower porbability of quantity and/or quality of sentience at this level.

While perhaps largely unknown outside of the sciences, Aplysia is a celebrity among the invertebrates for its contribution to the neurobiology of learning. Aplysia is a marine snail with a simple nervous system. The abdominal ganglion (collection of neurons) of the aplysia is home to the largest known neurons in nature (Moroz et al.,2014). This makes it an ideal candidate for electrophysiology – the approach where neural activity is recorded by inserting electrodes into cells or in the space surrounding cells. In aplysia. stimulation of the siphon (used for transporting water in the body) leads to a defensive retraction of the gill. Repeated stimulation leads to a decrease in the intensity and length of the response, a simple form of non-associative memory called habituation. This simple form of learning is not something we typically care about . Yet, even this basic for of learning in aplysia served as a useful platform for understanding the stages of learning from acquisition through to consolidation described above, which likely translate to and underpin more complex forms of memory that we do care about in humans.

C. elegans is another organism which towers above most invertebrates in its popularity. C. elegans gained prestige after it was the first organisms to have its connectome mapped (White, Thomson & Brenner, 1986). Understating the wiring of all 302 neurons in C. elegans provided systems level insight into how an central nerveous system coordinates a body to perform a suite of actions like movement, digestion, and defensive behaviours. Though the scale of the nerveous system is small compared to mammals, it revealed principles of neuronal organisation which persist across brains of all sizes, principles such as reciprocal inhibition to facilitate movement, computing at the level of the

cell for efficiency, and minimising the total length of neuronal wire (Sterling & Laughlin, 2015).

One of the most surprising findings from the study of learning and behaviour in invertebrates is the complexity that can arise from a paucity of neural cells. Consider that C. elegans has less connections in its entire nervous system (~7000) than a single mamillian pyramidal neuron has (Cook et al., 2019; Sterling & Laughlin, 2016; Megías et al., 2001). Yet, this small neuronal setup is sufficient to detect a variety of chemical and olfactory cues, navigate an environment, escape threats and detect dynamic environmental signals such as temperature and social crowding. Moreover, several thousand connections can support a variety of learning including classical and operant conditioning. And as we climb the ladder of neural complexity from C. elegans to invertebrates like planaria, the cognitive capabilities and potential insights multiply in suit.

Planaria as a model organism

Planaria are a broad group of invertebrates which have become the centerpiece for a number of scientific investigations. Planariar are being used to investigate topics including regenerative biology (Karami et al. (**?**)), toxicology (Li (**?**); Hagstrom et al. (**?**)), radioprotective materials (Ermakov et al. (**?**)) addiction (Raffa (**?**)), and the effect of zero gravity environments on morphology (Vista SSEP Mission 11 Team et al. (**?**)). Planaria have built niches across many ecological contexts and can be found across salt-water, fresh-water and land environments. Land dwelling planaria span up to half a meter long (Esser, 1981), whereas freshwater planaria, which are more commonly used in behavioural research, are less than a centimetre in length (Vásquez-Doorman, Escobedo & Allende, 2022).

Planaria are bilaterians, they display bilateral symmetry across their left and right sides (Sluys & Riutort, 2018). Planaria exhibit anterior-posterior polarity, such that their head can be distinguised from the tail in both its structure and its behavioural repertoire. While the tail end of a planarian rather uninteresting, the head encompasses many useful features. Auricles, the sensory organs capable of chemoreception, are what give

the head of many planarian species its triangular shape. These provide planaria with their ability to detect food and noxious chemicals in the immediate environment. Eyespots, which are the most discernible feature of planaria, sit atop the dorsal surface of the head. These light-sensitive cell clusters enable planaria to detect light intensity and its direction. Of particular interest to neruoscientists, the planarian head encases a bilobed symmetrical brain which coordinates activity throughout the body. This simple neural structure is of special evolutionary significance as planaria are thought to be the oldest organism to house an organised central nervous system, or what we might label as a true brain (Pagán, 2014; Sarnat & Netsky 1985). In real terms, a planarian brain is drab compared to the exuberance of the mammalian brain. But in relative terms, the brain-to-body mass ratio of planarians is similar to that of a rat (Best, 1983).

The planarian brain resembles an inverted U-shape (Agata et. al, 1998; Sarnat & Netsky, 1985) and has been estimated to contain between twenty to thirty thousand neurons (Inoue, 2017, p.82). The brain exhibits nine branches on each side which radiate out from the center. The lobes of the brain form thin nerve cords at the posterior end. These chords extend down the length of the body towards the tail, and together with the brain, comprise the central nervous system. The left and right nerve cords are connected by commissures which form a ladder-like structure (Sluys & Riutort, 2018). At first glance, the nerve chords do not have the organisational compexity of the plaanrian brain. Judging purely from apperances, the nerve chords should not be capable of performing complex behaviour. However, It has been shown that even cells in the nerve chords can take on the behavioural profile of a head, indicating that the nerve chords are not simply axons innervating muscle (Le et al., 2021).

Planarian neurons appear more similar in structure to those of vertebrates than of other invertebrates. They feature spine-like protrusions on dendrites (Sarnat & Netsky, 1985; Petralia et al., 2016). Just as in the CNS of vertebrates, multipolar neurons containing many dendritic branches but a singular axon are common in the planarian. Zooming in further, planarian neurons contain a variety of synaptic vesicles, such as clear and

dense-core variations, which resemble those seen in vertebrate neurons (Oosaki & Ishii, 1965). Most relevant to the research described in this project, planaria produce many of the same neurotransmitters and neuromodulators that we humans posses. These include serotonin, dopamine, epinephrine, acetylcholine, GABA, glutamate and opioid peptides (Sarnat & Netsky, 1985; Welsh & Williams, 1970; Rawls et al., 2006; for a of planarian neurochemistry see Buttarelli et al., 2008).

The physiologist August Krogh posited that "You will find in the lower animals mechanisms and adaptations of exquisite beauty and the most surprising character" (1929, p.203). The conservation of neurochemistry in planaria is intriguing. But the primary feature which embodies Krogh's dictum is the regenerative capability of planaria. Planaria undergo a natural form of fission as part of their reproductive cycle. They tear themselves in half, with each half then regrowing all the necessary parts of its basic body plan to form a complete planarian again. A form of reproductive immortality. Regeneration is not completely novel in nature. Humans can regrow skin, and salamanders can regrow amputated limbs. But what sets planaria apart from the rest of the natural world is its ability to regrow its brain and central nervous system. This regeneration is facilitated by adult pluripotent neoblast cells which are found throughout the body (Neuhof et al., 2016). After significant injury, these cells proliferate and undergo differentiation, providing the cell types needed to restore organs, membranes, and neural networks in the brain. This capability has drawn interest from medical researchers for more than a century (Morgan, 1898; Child, 1941; Reddien, 2018), who realised that by understanding the factors that control planarian regeneration, we may be able to artificially simulate these processes in humans to restore limbs or neural structures after injury. We have now reached the stage where business ventures like the company Morphoceuticals are being established to apply lessons learnt from planarian regeneration to rodents and, pending preclinical success, eventually humans.

Review of planarian memory literature

In the early 1900's, a common belief was that invertebrates did not have the cognitive means needed to learn. People conceded that perhaps local tissue change may allow some acute form of memory. But this would inevitably be erased or overwritten due to subsequent changes. Invertebrates were thus thought to lack permanent memory systems. This view is evident in the strong statement provide by Donald Jensen in the 1970s that "no invertebrate, no matter how complex is capable of showing 'true learning' " (quoted in Rilling p. 591). This view established an artifical floor seperating the types of animals that are suitable models of human cognition from those that are not. Because invertebrates were off the table, researchers had to try and make progress on the neurobiology of memory by starting with extremely complicated animals like rodents. After many years in search of the engram (the collection of neurons underlying a specific learning event), this venture unearthed little if anything of value. A group of psychologists including James McConnell in the 1970s were aware that little progress was being made in this endevour. The group then moved away from rodents and, looking past the anthropocentric restirctions imposed on cognition, drifted towards invertebrates. Starting with much simpler organisms would allow more insightful discoveries, as small neural systems could be mapped and understood as a first step, while complicated mammalian nervous systems could not. The situation was akin to assembling a rocketship for exploring space before figuring out commercial aviation within the earths atmosphere.

At first, McConnell and colleagues completed basic experiments showing that planaria could learn to associate a light with a shock. Compared to control subjects, trained planaria would exhibit more body contractions in response to light (a conditioned stimulus) and also perform more changes of direction. But criticism arose over the lack of controls in these experiments. Later follow ups included blinding the experimenter and testing for confounding factors such as pseudoconditioning (the eliciting of the unconditioned response by other stimuli despite there being no relationship between them) and sensitisation (an increase in responding to a CS due to repeated

presentation, rather than because of its association with a US). Contrary to the expectations of psychologists at the time, evidence for learning in invetebrates accrued study after study. It was eventually impossible to deny the capacity for stable associative memories to these (admittedly) primitive looking creatures. McConnell and others such as Eric Kandel established definitively that invertebrates are capable of learning, retaining, and acting on information.

The ability to form associative memories is an impressive feat given the bare-bones neural networks of the planarian brain. But learning research could be carried out in thousands of other organisms. It was the pairing of the capacity to learn with the rare ability for regeneration that sprouted one of the most peculiar branches of scientific endevour to date - investigating the retention of memory after decapitation and regeneration of the brain. Because planaria could learn simple associations, researchers wee able to ask the question: What happens if you teach a planarian, then cut it in half? Does the tail, which needs to regeenrate its head and central nerveous system, retain any of that prior learning? James McConnell was one of the first people to pose and pursue the answer to this question. Across a range of different training procedures, McConnell found that planarian tails indeed retain information, suggesting the intuition that memories must be stored in the brain may be mistaken, at least in some cases. Through some mechanism, memories are stored or backed up outside the brain and can be reinstantiated in the new brain during regeneration.

After the McConnell chronicles of the early 60s, invertebrate memory research saw its modern resurgence in the work of Shomrat and Levin (2013). The authors published an important paper which utilised an automated training protocol for planaria. Planaria, like rodents, are hesitant to approach food in the center of a novel environment. They will first explore the territory, and only then engage in consumption. As planaria become familiar with the environment through repeated trials, they begin to approach the food more quickly, demonstrating a form of recognition memory. The authors wanted to know whether this type of memory persists in the tails of trained planaria after they have been cut in half and had to regenerat a brain from scratch.

Planaria can detect and distinguish surface textures – an ability referred to as thigmotaxis. Shomrat and Levin's study therefore used smooth and rough surface textures for the conditioned stimuli. Over ten consecutive days, half of the planaria were fed on the target rough surface ("familiar" planaria) while the other half were only fed on a smooth surface ("naeive" planaria). At the end of the training period, the familiar group took a significantly shorter amount of time to approach and consume the food in the rough environment. Both groups were then bisected into head and tail halves ("offspring") and left to regenerate for 10-14 days. The authors then looked at whether the tail offspring of familiar planaria retained familiarity of the rough environment and thus approached food relatively quickly compared to the naive tail offspring. The data suggested that regenerated fragments from familiar planaria did approach food more quickly, but this did not reach statistical significance. Whats more, after u ndergoing the same training procedure as the oroginal planaria, the regenerated tail fragments from familiar planaria demonstrated a form of memory savings. The familiar tail sections became accustomed to the rough environment faster than tail sections of control (unfamiliar) planaria. This hints that some memory trace from prior training survived brain regeneration, but required repetition of the training process for the memory savings to be expressed.

More recently, Samuel and colleagues (2021) corroborated this puzzling memory retention effect. The authors used sucrose to shift the surface preference of some planaria from their innate preference for a smooth surface to the sucrose-paired rough surface. After amputating the planaria and allowing time for head regeneration, it was observed that the tail halves retained the sucrose-paired rough preference, despite the newly regenerated brain never having been exposed to the rough surface. In contrast, the tail halves of control planaria – which were exposed to the rough surface but did not receive sucrose in this environment – showed the expected initial preference for the smooth surface.

One assumption implicit in planaria experiments to date is that, while a brain is not needed for storage of all memories, it is necessary to act upon the memories. For this reason, sufficient time is given for the brain to regenerate, enabling the planarian

to swim and feed. However, a 2022 preprint by Shimojo and colleagues is at odds with this assertion. They sought to test whether dissected tail halves of planaria can display a conditioned response (which was learnt before dissection) before the bran has a chance to regenerate. In this study, planaria were trained to associate a neutral weak UV light (conditioned stimulus) with an aversive shock (unconditioned stimulus). The shock typically causes planaria to twist their body – an unconditioned contortion response. After pairing the light with the shock, planaria will display a conditioned contortion response to the UV light alone. On the second and third day after dissection, well before the brain is thought to be reformed, the tail halves were exposed to the conditioned stimulus over a number of trials and their responses were recorded. The authors analysed the data using a deep neural network to classify behaviour. They found that most responses from the tail halves were similar to those produced by an electric shock rather than those produced by a neutral ultraviolet light. Ultimately, this suggests the tail halves retained the conditioned behaviour and were able to act on it, despite lacking a brain at the time.

Following a similar procedure, Rhodes and Vierick (2024) used classical conditioning to establish conditioned negative phototaxis (moving away from the conditioned light stimulus). Typically, planaria are strongly averse to blue light, mildly averse to green light, and show no natural aversion (or attraction) to red light (Paskin et al., 2014). Planaria were trained to associate a neutral red light with an aversive green light across 5 days. After conditioning, some planaria were bisected into head and tail halves. Three weeks later, the head and tails were tested for retention of the memory – negative phototaxis to red light. The results indicate that the bisected halves retained the conditioned memory just as well as intact planaria. Moreover, memory retention was not statistically different for regenerated heads and tails. This suggests planaria can retain a conditioned response for at least three weeks. What's more, the tail halves which lost their head and therefore had to regenerate a head and brain also retained and acted on this memory. One major issue with this study was that the number of planaria per group was very small, most containing just four to six subjects. Another key issue is it was not clear how much

movement was necessary to support negative phototaxis on a given trial.

Although classical conditioning procedures are common in the planarian literature, some experimenters have employed operant conditioning methods (Crawford & Skeen, 1967; Chicas-Mosier & Abramson, 2015; see Best 1967 for a review of early studies). A simple learning procedure known as the Van Oye was one of the first forms of reinforcement learning in planaia (van Oye, 1920; Wells, 1967; Nicolas, Abramson, & Levin, 2008). In the typical setup, planaria are housed in a beaker and a fishing line with food attached is placed near the surface of the water. Planaria can detect the presence of food through chemosensory receptors. Planaria must navigate up the wall, across the surface and down the line to reach the food. This is a low probability behaviour, but a small to medium percentage of planaria will eventually do this given enough trials. The rod is lowered gradually across training trials, so that planaria will learn to descend to lower depths on the line to reach the food. Control planaria undergo similar methods but without the food reward attached. At test, food is not placed on the rod, but is instead dissolved in the water beforehand. The dissolved food is a cue that food is available. Trained planaria are subsequently found in much greater numbers on the suspended line compared to control subjects. Across five experiments performed by Wells, an average of ~17 trained subjects were found on the line at test compared to an average of ~3 experimental subjects (reviewed in Corning & Riccio, 1970). This procedure demonstrates that planaria can be trained using reinforcement learning.

Another operant conditioning study was conducted by Corning (1966) during the zenith of planaria fame. Corning wondered whether planaria can retain an operantly conditioned behaviour after bisection and regeneration. Using a T-maze apparatus, planaria were trained to select their less preferred side. Being returned to their home cage for 10 minutes was used as a positive reinforcer for the correct choice. Otherwise, they were taken to the start of the maze for another trial. Corning decided on a threshold for successful learning of nine out of ten consecutive correct choices across trials. After being dissected and left to regenerate for two to three weeks, the offspring (heads

and tails) were given a baseline preference test and were subsequently conditioned to criterion. Corning found that the baseline of trained offspring differed significantly from the baseline of the original planaria, while untrained planaria offspring did not differ from the original subjects. This suggested operant conditioned behaviour can be retianed outside of the planarian brain. Extending this, the regenerates of trained planaria could also be conditioned to threshold faster than regenerates of untrained planaria. A demonstration of direct memory recall and memory savings.

Cocaine as a positive reinforcer for Planaria

Investigators have used many different stimuli, both aversive and appetitive, in their behavioural studies. One of the most common appetitive stimuli in the planarian literature is cocaine. Cocaine is an ideal candidate drug for behavioural studies given it acts on dopamine receptors which are abundant in planaria (Algeri et al. (**?**); Buttarelli, Pellicano, and Pontieri (**?**)). Moreover, cocaine can be easily dissolved in water at low cost given the small quantitiy needed to reward planaria. The literature shows that cocaine induces strong effects on locomotion and atypical behaviours when given at high doses (Rawls et al., 2010, Pagan et al., 2013).

Cocaine is a stimulant which exerts it agonistic effects by blocking reuptake of dopamine through the dopamine transporters. In humans, this results in more dopamine activity in the synapse and therefore more neural activity in downstream neurons, particularly in the meso-limbic pathway connecting the ventral tegmental area to the nucleus accumbens (Nestler, 2009). This drug effect is linked to the rush or high that users experience, and is common to most if not all drugs of abuse. Cocaine also acts on serotonergic and noradrenergic transmission by blocking their respective transporters. The noradrenergic effects are thought to stimulate the sympathetic nervous system by blocking reuptake of norepinephrine and an decreased sympathetic nerve discharge, resulting in effects such as increased blood pressure and heart rate (Jacobson et al., 1997; Freye, 2009; Nestler, 2009).

Amaning-Kwarteng et al., (2017) explored the establishment and extinction of a drug reinforced preference, and the ability to reinstate this preference after extinction. They found that planaria can be conditioned using cocaine to shift their surface texture preference (e.g from smooth to rough), and that this preference can be extinguished (reverted back to the original preference) after repeated exposure without reinforcement. Subsequently, exposure to a bath of cocaine was enough to reinstate the conditioned preference when provided with both textures and given the choice of which surface it spends its time. Building on this research, Jawad, Hutchinson & Prados (2019) investigated addiction like learning and memory behaviour using sucrose. As is common in the planarian literature, the authors employed a conditioned place preference paradigm and measured conditioning, extinction, and tolerance. Conditioned place preference is commonly used to assess the motivational strength of drugs (Milton & Everitt, 2012). A novel aspect of the work by Jawad and colleagues was the demonstration that appetitive learning in planarians requires dopaminergic activity, evidenced by a dopamine D1 antagonist blocking acquisition of a CPP but not interfering with tolerance.The use of planaria to understand addiction related phenomena such as tolerance and extinction has benefited from a recent resurgence. But its roots date back to at least the 1960's (Needleman, 1967).

Understanding the molecular changes and circuit dynamics underlying the establishment of addiction may illuminate a path towards chemical or behaviour therapies to change the brain and thus change behaviour. Currently, therapies focus on top down strategies. People are coached to recognise their thoughts and emotions related to drugs, and to manage them rather than simply act on them. However, if the chemistry and structural wiring of the brain change during the acquisition of an addiction, bottom up therapies may assist in reverting these maladaptive neuroadaptations (Chodkiewicz, 2023). And although we may not be in a position to attempt extreme interventions in humans or other mammals, planaria enable us to pursue these questions and progress our understanding of how bottom up changes may reduce drug seeking behaviour.

Unresolved questions

In the first half of the 20th century, there was widespread doubt regarding whether invertebrates can learn. But as we look back nearly a centruy later, we have gathered ample evidence that planaria can learn (Wells, 1967; Samuel, 2021; Amaning-Kwarteng et al., 2017). In addition, Planaria serve as an especially useful organism due to their regenerative capabilities. Combined with conditioning procedures, there is now evidence that memory can be successfully retained in a planarian after having its head removed and thus having to regenerate a brain (Shomrat & Levin, 2013). It is an extraordinary finding that a simple preference or learned aversion can be maintained within the body despite complete devastation of the central nervous system. But even more incredible would be the persistence of complex behaviour.

Although a conditioned texture preference is a form of learning, it is very rudimentary and feels distant from the types of human memories that concern us in our day to day lives. Meanwhile, learning to perform some action to receive a reward, requiring both memory systems and their modulation by dopamine circuitry, is much closer to home for us humans. It is much closer to the forms of learning that we care about and value, and view as a sign of intelligence. If complex memories formed by operant conditioning can persist in spite of total brain loss, this may have profound implications for the way we view memory storage and retrieval in humans. With that said, to my knowldge there are no clear examples of retention of operantly conditioned behaviour through decapitation and regeneration in planaria. This project therefore attempts to generalise the principle of memory retention through regeneration shown for classical conditioning to an operant conditioning procedure.

A second uncertainty surrounbding the memory retention phenomena is the type of post-bisection exposure needed to reinstatiate a given memory. Shomrat and Levin (2013) observed that regenerated planaria tails from trained subjects did not initially differ in their performance compared to controls. However, it was clear that their performance on the task improved more rapidly than tail halves of control planaria. The memory seemed to lay dormant but could be reactivated to some extent

after exposure to the relevant training condition – a for of retention called "memory savings". This type of memory retention is similar to the procedure of reinstatement in addiction research. After successfully training an animal to lever press for a reward such as cocaine, extinction is achieved by allowing the animal to repeatedly engage in the lever pressing behaviour without being rewarded. Eventually, the animal will stop performing the conditioned response when the lever is presented. However, if the animal is exposed to the reinforcer before being placed back in the operant chamber, the lever pressing behaviour will spontaneously return.

With respect to both phenomena, the memory is not accessible or is not acted upon, and requires exposure to the right stimulus to be reactivated. Extinction and reinstatement of drug seeking behaviour has been shown in planaria (Amaning-Kwarteng et al., 2017). But what remains to be seen is whether reinstatement can be used to reactivate memories which are dormant after decapitation and regeneration. The phenomena of savings demonstrates some memory trace is retained in the brainless tail half. Perhaps this can be reactivated promptly by exposing the planarian to the reinforcing stimulus before assessing for memory retention – an invetebrate reinstatement procedure.

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Materials and Method

Colony maintenance

The species of planaria used in this research is not currently known. Due to restrictions on importing identified species such as *Schmidtea mediterranea* into New Zealand, local planria were sourced from two local streams within Wellington, New Zealand (X stream and Y stream). Given the basic characteristics of the planaria (colour, head shape etc.) it is thought that there is a combination of Cura and Neppia species – both of which are commonly found in New Zealand waterways. We intend to perform genomic analysis at a later date to confirm the species identity. Planaria were housed in a 50 liter glass aquarium with interal filtering and which contained a natural ecological environment (rocks,snails,algae etc.). The tank water was maintained with [describe water treatment protocol] – this water will be refered to as "planaria water" hereafter. The room containing the aquarium was maintained at 23°C. Planarians were fed between one and three times a week, with meals consisting of [describe food source]. The colony was maintained on a 12-hour light/dark cycle with lights on at 9:30am till 9:30pm.

Materials

Experiment 1 (dose-response analysis): plastic petri dishes with a diameter of 5.5cm were used to assess motility. Petri dishes contained a final solution of 8ml, made up of pure planaria water for control subjects and cocaine hydrochloride (Sigma Aldrich, United States??) mixed with planaria water for experimental subjects. Planaria locomotion was captured using an OPPO A17 smart phone and videos were imported into EthoVision (Noldus Information Technologies, Wageningen, the Netherlands) for motility tracking.

Experiment 2 (Y-maze conditioning): three custom Y-mazes were used. The mazes were identical, and were made from 80x80mm squares, with the compartments laser etched into the plastic. The mazes contain a 27mm long runway, and two arms each being 25mm long. All compartments were 6mm wide and

all walls were 6.5mm high. At the intersection between the runway and the arms, there is a small divot on the floor of the maze. This allows a plug 7mm in diameter to be inserted to trap liquid in the arms and enable controlled treatment with a drug. The maze floor contained subtle lines as a result of the etching process. At the base of the runway there is a small externally powered white light (~20 lux) which was fixed into the plastic. The light is a mild aversive stimulus intended to induce negative phototaxis and discourage the planarian from resting at the start of the runway.

Handling

Planaria were handled using several techniques based on the circumstances. When removing planaria from their 12-well-plate, a filbert paintbrush was prefered. However, when moving plannaria between petri dishes and the y-maze, a fine artist's paintbrush was prefered. In other cases, such as when planaria would sit in the middle of the y-maze divot, a plastic transfer pipette with the tip cut off was used. Planaria were handled very gently throughout their lifespan. Rough handling was suspected to have caused a very high mortality rate during pilot experiments. This was likely due to puncturing the planaria by pointing the bristles at the body on approach. For the studies reported here, a different technique was adopted whereby the side of the bristles was used to gently scoop the planaria up the side of the wall until they attached their cilia to the brush.

Experiment 1

Experiment 1 tested for an appropriate dose of cocaine which is rewarding to planaria while not drastically altering their their locomotive behaviour. Cocaine has been frequently used as a rewarding compound to classically condition planaria and to investigate its toxicity (Amaning-Kwarteng et al., 2017; Raffa & Desai, 2005; Tallarida et al., 2014a; Hutchinson et al., 2015; Pallidini et al., 1996). Doses used for conditioning have ranged from 1 M (Hutchinson, Prados & Davidson, 2015) to 90 M

(Raffa et al., 2005). Planaria species employed across conditioning experiments vary, including *Dugesia Tigrina*, *Dugesia gonocephala* and *Dugesia dorotocephala*.

Investigators have observed that some species are more amenable to conditioning procedures than others (Samuel et al., 2021; Mueller & Levin, 2002). Differences in the behaviour and responses of planarian species have been observed in response to several types of stimuli (Cochet-Escartin, Mickolajczyk, and Collins (**?**); DeBold, Thompson, and Landraitis (**?**)). The species used throughout this project likely differs from those used elsewhere in the literature and may in fact be a species indigenous to New Zealand. For this reason, it is important to identify a suitable dose of cocaine which is rewarding but does not significantly alter motility.

60 planaria were used in this experiment and were spread across six conditions based on doses commonly used in the planarian conditioning literature. This included 0, 1, 5, 10, 20 and 100 M (n = 10 per condition). Subjects were run across twelve session. The first ten sessions each contained one subject from the 0 - 20 M conditions, whereas the last two session only contained subjects in the 100 M condition ^[initially only 0 - 20 M conditions were run. The 100 M condition was added after the initial data were analysed to ensure that the cocaine was having some effect on the planaria and was not inert]. Subjects were collected from the breeding tank on the day of data collection. Within each session, subjects were randomly allocated to their condition using a random number generator (https://stattrek.com/statistics/random-number-generator#table).

Each dose-response session lasted 15 minutes. Prior to the first recording session of the day, the drug concentrations were achieved by mixing cocaine (dissolved in distilled water) with planarian water to reach a final solution of 8ml. Each solution was mixed and allowed to sit for several minutes to ensure diffusion of the drug. Planaria were assigned to each condition from a large pool prior to the session. A planarian was picked up and a random number sequence was followed to determine which condition it was assigned to. The recording began once all five subjects were in their respective dishes. After completing a single trial, the planaria were rehoused in a large tank and were

not used for any subsequent experiments in this manuscript.

?@fig-Figure2 provides an overview of the apparatus setup. Five petri dishes were positioned on a white acrylic sheet. Recording sessions took place under red light, with the light positioned 36cm above the petri dishes. The dishes were aligned in a 2x3 grid, with a gap left in the top middle position which is where the light was focused to minimise shadows in the petri dishes; this was important for subsequent digital tracking. Each drug concentration was rotated across the 5 grid positions between trials to control for any effects of lighting angle.

Results

Figure **??** depicts the distance moved by planarians across the six conditions. Prior to performing any statistics, the assumptions of normality and homogeneity of variances were tested. Levene's test for homogeneity of variances suggests there were equal variances across conditions (F = 1.95, NA, $p = 0.101$, NA). The Shapiro-Wilk test results indicate that the data were not normally distributed (W = 0.935, $p = 0.00338$). Due to violation of the assumptions of ANOVA, a Kuskal test was performed. The results indicated that the effect of `Condition` on `Distance` is statistically significant (2 (5) = 11.3, $p = 0.0449$). An exploratory post-hoc Dunn's test was carried out to determine whether the 100 M group differed from other groups. The results indicated that the 100 M group differs significantly from several other groups: control ($p = 0.00627$), 5 M ($p = 0.00207$), 10 M ($p = 0.00252$), and 20 M ($p = 0.0157$).

The results in Figure **??** convey the variability of planarian behaviour. All conditions had at least one subject which moved less than 30cm over the 15 minute recording, and all groups had at least two subjects that moved more than 140cm. Experimenter observations indicate that when placed in the recording dish, some planaria – typically those visible at the bottom of Figure **??** – would move initially, and then come to rest within a few minutes at a spot on the wall. They would remain here without meaningful movement for the remainder of the recording. Although no statistically significant difference

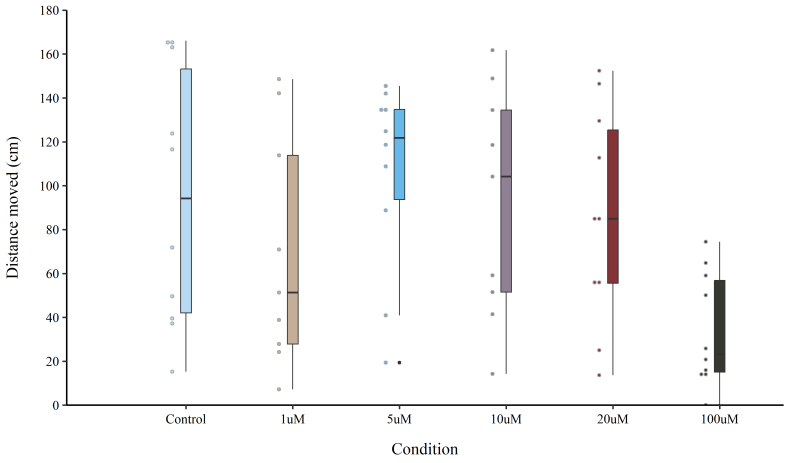

Figure 1: Plot of planarian motility by condition

was detected, there is a curious grouping of planaria in the 1 M condition below 60cm. Consistant with this, Hutchinson et al. (2015) observed a significant decrease in motility during exposure to 1 M of cocaine but not to 10 M when compared to a control group. No potential explanation was offered for this unusual dose-response effect. The most likely explanation may be a type 1 error.

The dose-response results suggest that any of the proposed doses would be appropriate for conditioning, in that they should not significantly alter planarian behaviour. Because planaria will be exposed to multiple trials within a short time interval, any effects on behaviour may mask evidence of learning. But given this null dose-response result, the primary selection criteria will now shift toward a focus on how rewarding (and therefore reinforcing) the dose is. A range of cocaine doses have been used to successfully condition planaria in CPP paradigms. These procedures typically involve low doses such as 1 M (Hutchinson et al., 2015), 5 M (Amaning-Kwarteng et al., 2017) or 10 M (Hutchinson et al., 2015). But in the case of CPP, exposure time per trial is relatively long, on the order of 15-20 minutes. In the operant conditioning paradigm proposed in Experiment 2, exposure time will be 3 minutes. Similar levels of absorbtion given the time constraint may be achieved by a higher concentration of the compound.

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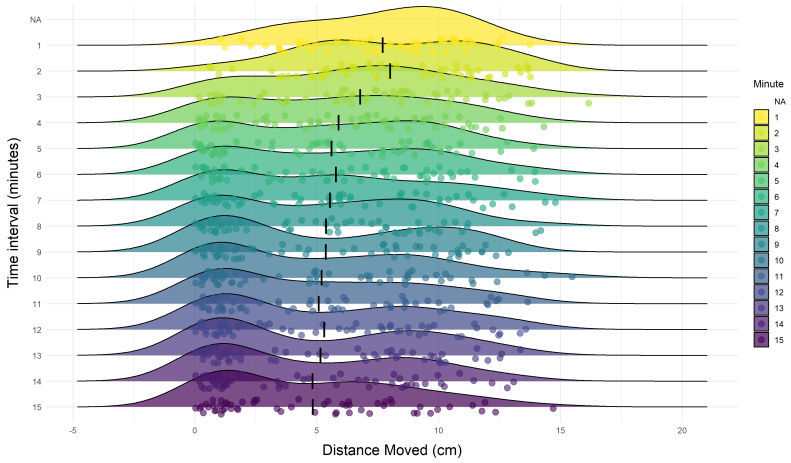

Figure 2: Plot of planarian motility across recording interval

X

Experiment 2

Next we sought to determine whether planaria can learn and retain an operant conditioned response. Prior research has demonstrated the capacity for learning by way of classical conditioning. And there is evidence that classically conditioned memories can be retained after decapitation and regeneration of the brain. But the capacity for complex memories shaped by operant conditioning to persist despite losing most of the central nervous system has not been definitively shown. As a first step towards testing retention of operant conditioned memory, we needed to first establish the capacity for operant learning in this species of planaria. This experiment was pre-registered prior to data collection and can be found online at Open Science Framework (https://osf.io/tq7u4/?view_only=9c794dd942fb4a54b6a986c0a893fe46) and at PsychArchives (https://www.psycharchives.org/en/item/d6109ed1-9aab-467b-b981-e009be95f308

Sixty planaria were used (cocaine group, $n = 30$, vehicle group, $n = 30$). This experiment had four stages: baseline, condi-

tioning, test, and priming. for baseline and conditioning two planaria were run concurrently in two separate Y-mazes. The maze was filled with 1.8ml of planaria water. The water was shaken so as to distribute evenly throughout the maze, with bubbles removed as needed. Six planaria were used per run, wherein they completed either 6 trials (baseline) or 4 trials per day (conditioning) with an intertrial interval of approximately 15 minutes. The six planaria were first moved into holding petri dishes, with the regular white room light on to encourage movement. At the start of a trial, two planaria were transferred to the middle of the runway using a paintbrush, and shaken loose into the water. Once the planaria had entered the runway, the timer was started. Planaria were given three minutes to enter one of the arms ^[If the planarian had some part of their body in an arm, they would be given up to an extra minute to make their decision]. Once a planarian had enterd an arm, the plug was inserted to stop liquid moving between compartments. Each arm contained 0.5ml after the plug was inserted. A planarian was considered to have entered the arm when the plug could be safely inserted without touching the planarian.

After plug insertion the timer was stopped, and the decision and time were recorded on a computer. For treatment subjects, if the active arm was selected, 43.5 L of cocaine in distilled water was pipetted near the body. If the inactive arm was selected, an identical volume of distilled water was pipetted near the body. After administration, the timer was restarted and three minutes were given for absorption. For control subjects, either arm resulted in plugging and then pipetting 43.5 L of distilled water into the arm. If a subject failed to enter an arm, the plug was inserted and 43.5 L of distilled water was pipetted near the subjects body while in the runway and then three minutes were given. The runway light was on throughout the duration of the trial. At the end of a trial, planaria were gently removed and placed back into their holding dish.

At test, six planaria were used per run. Three planaria were run concurrently in three separate Y-mazes. Planaria were given three minutes to make a decision. Once a decision was made, the plug was inserted and planaria were left for ~60 seconds before being moved back to the holding dish. No additional liquid was added to any compartment of the Y-maze during the test

trials. The next group of three planaria would then begin their first test trial. The inter trial interval was approximately six minutes and thirty seconds. For the priming stage, the procedure was identical to the test stage, with the added component of the drug exposure before the first trial. At the start of a run, the planaria were placed in a 8ml solution of cocaine diluted in planaria water for 10 minutes (staggered so that subejcts 1-3 had finished their first trial before the 10-minute period ended for subjects 4-6). At the end of the exposure interval, planaria were moved individually into a Y-maze to begin their first trial. Planaria were only exposed to cocaine before the first priming trial, but not before subsequent trials.

Results and disciussion

Figure **??** shows the average percentage of trials where subjects entered the active arm across the four time points. A generalised linear mixed effects model with family set to binomial was fitted in R. Subject ID was set as a random effect, with condition, time point and the interaction term as a fixed effects. Pairwise comparisons with a Bonferroni correction were carried out using the emmeans package in R. Type III Wald chi-square tests were carried out to identify whether there was a main effect of condition, time, and their interaction. The results did not find a significant effect of condition (2 (1) = 0.773, p 0.379). The results indicate a significant effect of time (2 (3) = 35.5, p = <.001) and a significant time*condition interaction (2 (3) = 10.2, p = 0.0171)

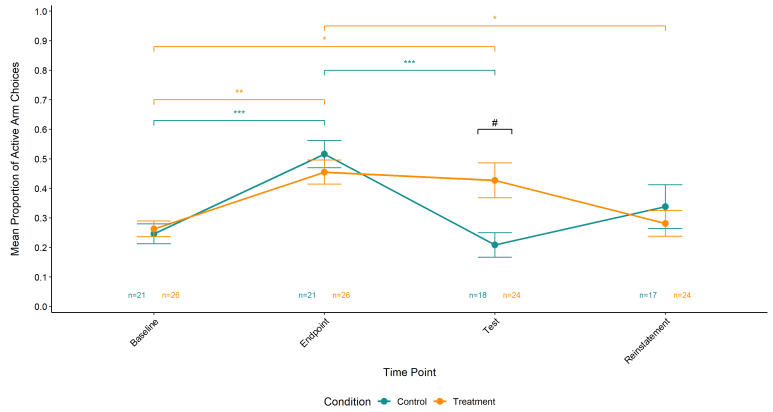

Figure 3: Mean percentage of choices for the active arm over time between conditions

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Details about writing headings with markdown in APA style are here.

Displaying Figures

A reference label for a figure must have the prefix `fig-`, and in a code chunk, the caption must be set with `fig-cap`. Captions are in title case.

To refer to any figure or table, use the `@` symbol followed by the reference label (e.g., **?@fig-myplot**).

Imported Graphics

One way to import an existing graphic as a figure is to use `knitr::include_graphics` in a code chunk. For example, **?@fig-import1** is an imported image. Note that in apaquarto-pdf documents, we can specify that that a figure or table

should span both columns when in journal mode by setting the `apa-twocolumn` chunk option to `true`. For other formats, this distinction does not matter.

Figure graphics can be imported directly with Markdown, as with Figure **??**.

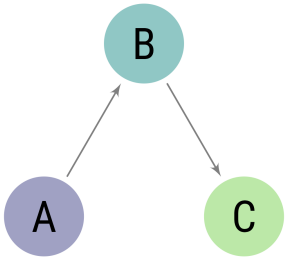

Figure 4: Another Way to Import Graphics

Which style of creating figures you choose depends on preference and need.

Displaying Tables

We can make a table the same way as a figure. Generating a table that conforms to APA format in all document formats can be tricky. When the table is simple, the `kable` function from knitr works well. Feel free to experiment with different methods, but I have found that David Gohel's flextable to be the best option when I need something more complex.

To refer to this table in text, use the `@` symbol followed by the reference label like so: As seen in Table **??**, the first few numbers and letters of the alphabet are displayed.

In Table **??**, there is an example of a plain markdown table with a note below it.

Table 1: Table Caption of a Markdown Table

Default	Left	Right	Center
12	12	12	12
123	123	123	123