Distributed cortical recordings to explore the role of cortex in freely moving rats playing a Videogame

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Declaration

I, Lorenza Calcaterra, confirm that the work in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed:

Abstract

An organism's behaviour is a continuous stream of actions and reactions to the changing demands of a complex, unpredictable environment. We are able to approximate different levels of complexity within the laboratory by using a set-up that we call *Videogame,* a closed-loop back-projection display assay that engages freely moving Lister Hooded rats in increasingly complex and unpredictable tasks. Using a reactive data stream processing framework (Bonsai), we are able to control, in closed-loop many parameters of the environment in response to the rat's behaviour, thus generating a rich-yet-controlled dataset for quantitative behavioural analysis. Rats successfully and quickly learn to 'play' different levels of the Videogame in a naturalistic fashion, foraging and hunting for virtual objects randomly appearing on a floor projection screen. Rats' performance in each level, provides us with evidence that they develop a deep understanding of the proprieties of the virtual objects. In fact, they adapt and change their speed of approach to the virtual object, whether it is to forage or a moving prey to pursue. Overall, this assay proved to be an extremely flexible tool that allowed us to explore a wide range of behavioural repertoire and was used as substrate to investigate the role of cortex in solving rich and complex tasks like foraging and hunting. To fully interrogate the scope of neural activity underlying complex behaviours, we must record from multiple areas simultaneously. We therefore designed a new 11 shanks, 121 channel silicon probe for distributed cortical recording.

We target the entire thickness of the rat cerebral cortex spanning from pre-motor to visual cortex. Each cortical layer can be monitored with an unprecedented distribution of recording sites to provide a unique picture of the cortical dynamics ongoing during complex visual motor tasks. Preliminary electrophysiological analysis of local field potential (LFP) does not show any LFP modulation around event of interest such as the touch of the virtual object.

Interestingly, multi-unit activity (MUA) analysis shows clear event-related modulation that varies according to the brain region considered. These preliminary results will be presented along with corresponding behavioural analysis. As proof of concept, we were able to reveal both behavioural and cortical dynamics that occur during more naturalistic behaviours. As future perspectives, the Videogame proved to be a flexible tool for the development of a variety of complex behavioural tasks bridging real and augmented world while the 11 shanks probe gave us the opportunity to showcase the cortical activity under a different light providing proof and reasoning for more experiments of this kind to happen.

Impact statements

Over the past years, interest in more naturalistic behaviour rose within the neuroscience community. Being able to explore the neural dynamics in a setting that resembles the one that brains evolved in and adapted to seems like the best approach to gather precious and relevant insights both about when a behaviour take place as well as how brains 'behave'. The work undertaken in Chapter 2 showed that we can elicit complex behaviour using a novel augmented reality assay that we call *Videogame*. Rats engage with a virtual object, in foraging and hunting behaviour while, as in nature, unexpected events occur. We can monitor their behaviour to a great extent and gather a rich dataset for quantitative analysis. Great interest in high resolution dense recordings has taken place in the past decade due to technological advances and the advent of CMOS technology. Alongside the behavioural complexity, we designed a new custom 11 shanks 121 channels silicon probe for distributed recordings in the cortex (Chapter 3). The unprecedented layout of this probe allows for the simultaneous monitoring of cortical dynamics from pre-motor areas to visual areas providing a novel picture of cortical function.

This work not only provides a proof of principle that this kind of experiment is possible, but it also lays out the foundation for further exploration and optimisation for even higher resolution recordings while animals behave in increasingly complex tasks.

Acknowledgements

I would like to thank my beloved *Italy*, my home that I left 8 years ago. I have been on a journey from NYC to Lisbon, and now ultimately London, yet my family and my oldest friends were there for me every step of the way. For this I am grateful. You have supported my choices even if leaving security and departing to follow my dreams was not always easy to comprehend.

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Last but not least, I would also like to thank myself for having chosen this path allowing for all this beauty to happen.

Grazie di cuore to you all.

Figure 0 The best abstract

Drawing showing a hungry rat staring at a smiley chocolate bar. Adapted from Jessica's card after a lab tour. She made my day back then and she still does every time I come across her card. Thanks to you!

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Anima bianco corvino

Chapter 1

Introduction

1.1 The vision

It is not true that "the laboratory can never be like life."

The laboratory must be like life!

James J. Gibson

James J. Gibson's quote from his book *'The Ecological Approach to Visual Perception'* (1979) summarises the main motive of this dissertation.

The context in which a behaviour takes place has an essential role in shaping the behaviour itself and each one of its variables and components. The *laboratory* is clean, neat, and limited, but it requires removing natural context to focus on the detailed and controlled behavioural variables of interest. The *field*, on the contrary, is authentic, raw, and unfiltered making it challenging to parse apart each variable from the context with which it is so tightly entangled. The natural world, as we know it, is inherently more complex than a laboratory, yet we use the latter setting in an attempt to explore and study behaviour and brain function. During an organism's lifespan, behaviours evolve in, and because of, the natural environment and laboratory experiments can only sample a reduced subset of behaviourally relevant environments (Gomez-Marin et al., 2014).

Researchers have generally opted for simple behaviours and studies typically consider only single variables in order to distil animal behaviour to its basic components. Inevitably, this approach comes with caveats. A minimalistic approach has yielded fundamental insights into functional specialisation in the brain and brain-behaviour relationship, but its ethological validity remains uncertain. Repeatedly presenting the same input signals in a temporally

structured manner in order to repeatedly produce the same motor output obscures the fact that in the real world, no such a thing could ever occur (Gomez-Marin & Ghazanfar, 2019). Highly constrained and simplified experimental preparations allow researchers to focus on the complexity of the brain, but this approach does not account for the complexity of behaviour (Gomez-Marin et al., 2014).

The breadth of behaviours elicited by the environment's richness, dynamics, and uncertainties could be missed by solely using a simplistic approach, ergo the study of behaviour in the laboratory feels, at times, incomplete and unsatisfactory.

Nonetheless, it is fair to say that it is impossible to study the full extent of most organisms' behavioural repertoire in the laboratory. In an ideal scenario, brains should be studied in an environment that matches closely the one they were built for. Brains grew increasingly complex by adapting to the changing environmental requirements. Can we study them if they are removed from their natural environment and placed in a laboratory? As per behaviour, it is also fair to say that it is impossible to study the full extent of the neural activity of the whole brain. In fact, focusing on one small portion of the brain has been the researcher's preferred approach and it has provided an enormous amount of knowledge about brains for many different species. How could we study the brain as a whole? This question is rather utopic, yet with the technological advances, made in recent years, we can now measure the activity of thousands of neurons simultaneously in behaving animals (Jun et al., 2017). This could surely be an incentive for some of us to step away from the intensive, yet important, study of one single area and attempt to study the brain with a multi area, distributed approach. Can we design more naturalistic tasks that can depict more realistic and complete explanations of the brain's functions? Can we use the technological advances in the field and borrow features from the natural environment to design more ethological inspired experiments while recording from large portions of the brain? Yes, we can try, and we will.

This dissertation will walk you through the steps taken to tackle the above questions, describing successes, failures and highlighting the importance and feasibility for this new approach to neuroscience.

1.2 The study of animal behaviour

Historically, the study and interpretation of the natural behaviour of animals has developed along two different, yet complementary, lines: ethology and psychology.

Ethology has its roots in the study of animal behaviour displayed in the natural environment. The principal emphasis of ethological work is upon the behaviour of the animal, considered as a part of nature, which the ethologist wishes ultimately to understand. The research is oriented primarily towards gaining understanding of the origin and nature of the behaviour patterns that characterise an animal's life in its natural environment. Ethologists, step into the natural world and devote their studies to extended and patient observations of a wide range of behaviours and species in the wild. The ultimate result of such an approach is a detailed description, almost like an inventory, called an "Ethogram" of each behaviour observed. The main principle is that one needs to know in detail what sorts of things animals do before one can begin any serious attempt to understand their behaviour (Purton, 1978).

By contrast, the focus of most work in animal psychology was primarily on fewer animal species studied exclusively and extensively in the laboratory. The interest was not on aspects of the life of the animal but instead, the animal became a tool for investigating a general concept in psychology that could be representative of the animal beings in general, including humans (Lehrman, 1961). They prefer to treat the organisms as a "black box" and to study descriptively the condition under which behavioural changes occur before proposing mechanisms to account for the changes. The approach of experimental psychologists was to

design experimental apparatus and mazes to test hypotheses and theories in the laboratory with great focus on the study of learning.

In this thesis, we will argue that blending ethologically relevant aspects of an animal's behaviour together with semi-controlled laboratory settings could be the right compromise to gain a more complete picture of an animal's behaviour.

1.2.1 Ethological approach

In the middle of 20th century, in Europe, the study of animal behaviour became an independent scientific discipline called 'Ethology', mainly through the efforts of Konrad Lorenz (1903-1989) and Niko Tinbergen (1907-1988). In 1973, together with Karl von Frisch (1886-1982), they were awarded the Nobel Prize in Physiology or Medicine for their studies of behaviour. Among others, the studies conducted by these ethologists has led to major discoveries in the field that could only be made by an attentive and detailed observation of animals in their natural habitat.

Frisch's work focused on the sensory perception of honeybees, and he was one of the first to translate the meaning of their waggle dance. Honeybees can communicate foraging locations to nest mates via a peculiar stereotyped 'language' (Ai et al., 2019). In the waggle dance, a returned forager bee advances linearly on the comb, quickly vibrating the abdomen from side to side. After the waggle, the bee circles back (return phase) by turning, alternating from the left or right, until returning approximately to the start position (Figure 1.1B) (Couvillon, 2012). Distance from the source of food is encoded in the waggle phase while the direction is encoded by the orientation relative to vertical (De Marco & Menzel, 2005). As per the genuine ethological approach, while Frisch was conducting studies on bees' colour vision, he witnessed the bees dance for the first time, defining it as "the most fateful observation of his life" (Munz, 2005). Being in the field was key to observing a peculiar

behaviour not yet discovered which would have been difficult to witness and ultimately interpret, studying bees in a laboratory.

Referred to as "the father of ethology and the foster-mother of ducks", Lorenz was responsible of laying the fundamentals of the Ethology field framework in the 1930s. In some of his work, he focused on instinctive behaviour in birds and he was the first to highlight the scientific significance of the phenomenon of imprinting, whereby some species of birds would follow and become attached to the first moving object they encounter after hatching (Vicedo, 2009). To study the mechanism of imprinting, he separated a nest of goose eggs into an experimental group and a control group. He left the control group to hatch naturally with their mother while the experimental group hatched in an incubator and Lorenz was the first "object" encountered by the goslings after birth. To test his hypothesis, that goslings would form a bond with the first moving object that they meet, he marked the two groups and put them together under a box. Once the box was lifted the control group started following their mother, but the experimental group would only follow him (Figure 1.1A). Lorenz also noticed that the process of imprinting occurred only in a short period of time after birth leading to the notion that there are critical periods in the development of the brain and behaviour.

The aim to understand why animals behave the way they do, brought ethologists not only to observe their behaviour in nature, but also to conduct field and laboratory experiments. Tinbergen designed simple experiments to tease apart particular behaviours displayed by animals (Figure 1.1C) (Tinbergen, 1948, Tinbergen, 1952).

Tinbergen is best remembered for his formulation of the four questions of ethology in his paper 'On aims and methods of ethology' published in 1963. To understand behaviour, one must ask four main questions: 1) what is it for? 2) how did it develop during the lifetime of the individual? 3) how did it evolve over the history of the species? 4) how does it work? (Burkhardt, 2014).

By laying out survival value, ontogeny, evolution, and causation as the main principles in the study of behaviour, Tinbergen provided biologists with a clear framework for research. The core message was that there was need for careful studies, which build up evidence through careful observations and experiments, to justify any conclusion (Bateson & Laland, 2013).

Despite his emphasis on the need for an integrated understanding given by the joint answers to his four questions, researchers have largely been addressing them independently. The study of song-birds has been one of a few areas in which the behaviour has been extensively explored and understood, largely answering each one of Tinbergen's four questions (Spector, 1994; Hogan & Bolhuis, 2005).

Integration of different aspects of the same behaviour is the key to progress in the understanding and successful explanation of a certain behaviour of interest.

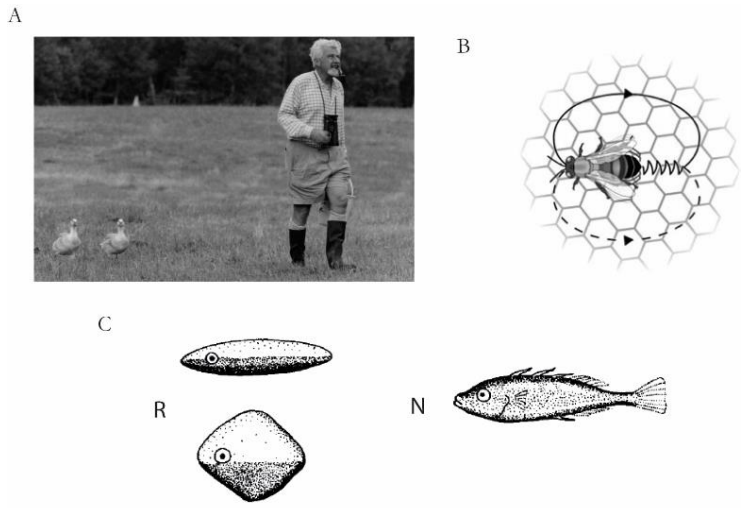


Figure 1.1 Ethological approach

A) Iconic picture of Konrad Lorenz followed by geese that had imprinted on him. Picture from Vicedo, 2009. B) Drawing of a honeybee (forager) performing the waggle dance. The dancer performs a waggle run followed by a turn to one side circling back to the starting point of the waggle (return phase). She starts another waggle run and turn on the opposite side. This dance can be repeated up to 100 times depending on the quality of the food source. Drawing adapted from Couvillon et al., 2012. C) One of numerous experiments and observations made by Tinbergen on the releaser concept (sensory stimuli necessary to release innate responses) on the three spined sticklebacks. In mating season, male stickleback, dress a brilliant red on throat and belly while the back is white. The red belly acts as a releaser for males (competitor/enemy) and females (mate). In a series of experiments to test the releasing power of the red belly, Tinbergen created two series of models (dummies): R red on the ventral side) and N of neutral colours. Models with red belly were attacked more often compared to N, confirming his hypothesis. Adapted from Tinbergen 1948.

1.2.2 Psychological approach

The emphasis of the North American psychologists on learning was manifested by the rise of behaviourism in the 1930s. Behaviourism refers to the study of trained behavioural responses in a laboratory context and it is based on the idea that *it is not possible to objectively study the mind, therefore psychologists should limit their attention to the study of behaviour itself*. Behaviourism was a very influential school of thought initiated by the American psychologist John B. Watson (1878-1958) with his book called *Behaviourism* (1924). According to Watson, psychologists can only investigate the physical manifestations that we can observe in the form of behaviour. Much of his theory was based on the classical conditioning work of the Russian psychologist Ivan Pavlov's (1849-1936). Classical conditioning involves the pairing of two different stimuli that elicit a learned response in the experimental subject. Pavlov discovered that dogs would salivate at the sound of a tone that had previously been associated with the presentation of food. The stimulus corresponded to the food or, after learning, to the tone that produce the response of salivation in dogs (Cambiaghi & Sacchetti, 2015). Watson began to use these ideas to explain how events that humans and other organisms experienced in their environment (stimuli) could produce specific behaviours (responses) (John B . Watson, 1926). The Little Albert experiment was one of Watson's best-known experiments to prove his claims despite being considered, nowadays, ethically controversial. He found that systematically exposing a child to fearful stimuli (loud noise) in the presence of objects (rat), which did not themselves elicit fear, could lead the child to respond with a fearful behaviour to the presence of the objects (Watson & Rayner, 1920). In line with the behaviourist approach, the boy had learned to associate the rat with the loud noise, resulting in crying (Beck et al., 2009). Another behaviourist who used the ideas of stimulus and response, along with the application of rewards or reinforcements to train pigeons and rats was Watson's student Burrhus Frederick Skinner (1904-1990). Skinner developed the

concept of operant conditioning that gave him great prominence in the psychology field. According to Skinner a behaviour that is reinforced (rewarded) has increased probability of recurrence while a behaviour that is not reinforced tends to weaken or be extinguished. Skinner studied operant conditioning by conducting experiments using animals (rats and pigeons) that he would place in a controlled environment known as an operant conditioning chamber or "Skinner box".

In its most common form, the box consists of a closed space in which the animal can move freely, and it is usually equipped with a lever for rats, or a small, illuminated disk upon which pigeons can peck, and with a food dispenser for delivering a calibrated amount of food. By exploring this environment spontaneously, the subject will eventually discover the basic relation between a defined response (pressing the lever or pecking the disk) and the presentation of a reinforcing stimulus (food reward). The consequence of receiving food if they pressed the lever ensured that they would repeat the action again and again. The basic relation is between an operant response (i.e., an instrumental response to produce a subsequent event) and its consequence (i.e., the reinforcement), rather than between a stimulus and a response elicited by it, as in the Pavlovian conditioning. Skinner also believed that when and how often a behaviour is reinforced will have a great impact on the strength and rate of the response. Skinner recorded the animals' responses, either lever pressing or disc pecking, on a device that he created called the cumulative recorder, thus allowing for the precise quantification of behaviour.

In the 1930s, Edward Tolman suggested a cognitive approach to learning. His theory allowed for the possibility that learning could be latent and that it involves an unobservable stimulus-stimulus association without the presence of a reinforcement as theorised by Watson and Skinner. This association would only manifest itself when a reward is available providing the organism with a reason to demonstrate what it has learned (Zentall, 2002). The association between stimuli could result in the formation of a cognitive map. Despite allowing for

mechanisms and mental processes that cannot be seen directly, Tolman's approach was still very similar to a typical psychologist in observing behaviour obtained from carefully controlled experiments such as mazes. In 1948, he wrote an article entitled *"Cognitive maps in rats and men"*, summarising a number of studies on five different topics: latent learning, vicarious trial and error, searching for the stimulus, hypothesis experiments, and spatial orientation experiments (Carroll, 2017).

In one of many experiments mentioned in his paper he suggested that animals would acquire a means to achieve a goal despite the previously learned path being blocked. Rats were presented with a simple maze (Figure 1.2A) and they had to run from the start box (A) through an open circular area (B), through an alley (C), and finally to the goal box (G). After three days of trials in which the animals ran directly and without hesitation from A to G, they were presented with the sunburst maze shown in Figure 1.2B. A and G stayed the same, but a series of radial arms were added, replacing the original maze. The animals ran across B but suddenly found themselves blocked in the alley. After exploring various paths and despite paths 9 and 10 were closest to the path they had been trained on (i.e., the path from C to D, Figure 1.2), there was a tendency to choose path 5, which ran to a point very close in front of the goal box (Tolman et al., 1946). Based on this and other experiments he concluded that animals do not merely base their actions on specific associations but that they also internally reorganize acquired spatial information to form cognitive representations of the environment. One important property of such representations is that they allow animals to react to stimuli that are not immediately present, because the relationship of such stimuli to those actually perceived is maintained in a cognitive representation, that is, a map (Ellen & Anschel, 1981). Importantly, a major consequence of such a representation is that it bridges informational gaps about the environment, thus conferring greater flexibility and efficacy to the animal's behaviour. Nonetheless the cognitive map hypothesis met great resistance at the time it was proposed, because of the dominant stimulus-response approach to understanding

behaviour. However, O'Keefe and Nadel's 1978 influential book, "*The Hippocampus as a Cognitive Map"*, resurrected Tolman's hypothesis, suggesting that not only was Tolman right about the existence of a cognitive map, but that such a map might occupy a specific region of the brain, such as the hippocampus. This finding emerged from the recording of neural activity in the hippocampus while rats navigated an open space. Psychological approaches, now supported by neurophysiology, became prominent in neuroscience and the ease of combining them in laboratory settings explains their current dominance over the ethological approaches.

However, the framework outlined by Tinbergen is still fundamental if we want to understand how the brain produces behaviour. The neurophysiological and psychological studies, that dominate the era, focus, and provide some insight into his question on how the behaviour happens. Instead, among psychologists, other approaches have focused on why a behaviour happens. These studies use lesions as predominant technique, and they offer an interesting demonstration of why ethological and psychological studies of animal behaviour should not remain separated but find the right balance in order to maximise our understanding of animal behaviour.

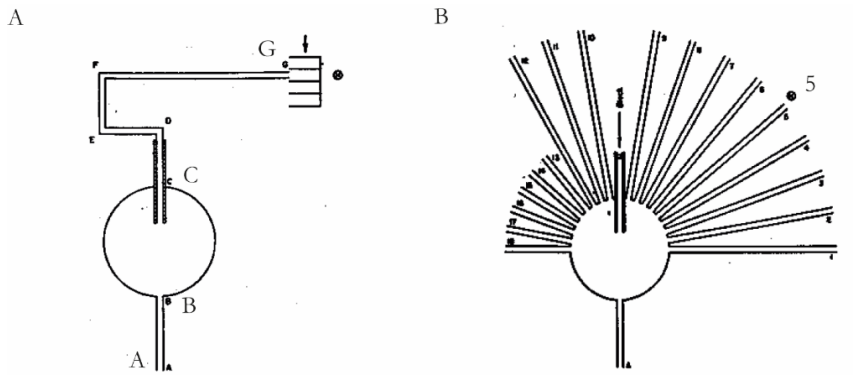


Figure 1.2 Sunburst maze

A) Elevated maze for preliminary training where rats had to reach the food source (G) running across A through a circular table (B) and through C to F. B) Sunburst maze with similar but with radiating paths added to the circular table. The old path via C is blocked. Rats predominantly chose path 5 which is the closest to where the food box was located. Figure adapted from Tolman 1946.

1.2.3 Understanding the brain through lesions

Studying the brain and understanding how it ultimately generates and controls behaviour using a lesion approach has been a method extensively used in neuroscience. Psychologists used lesions to study their effects on behaviour, and in so doing, they hoped to draw inferences about the likely functions of the damaged brain. While other manipulations of brain activity were temporary and reversible, their short-term behavioural deficits would often quickly recover. Chronic lesions are very effective and definitive but their implementation can be difficult and often lack precision, making the interpretation of the results harder (Vaidya et al., 2019). Lesion studies have also been a way in which the behavioural complexity studied could be increased. In fact, scientists tested lesioned animals in a huge variety of behavioural tasks, from simple to complex, in an attempt to find when and how the capability to perform certain tasks would be disrupted due to lesion (Kolb & Whishaw, 1985; Whishaw et al., 1981; Whishaw & Kolb, 1983; Whishaw & Kolb, 1984; Oakley & Russell, 1979; P Terry & Oakley, 1990) .

Creating permanent lesions through aspiration or excision is perhaps the oldest documented lesion technique, beginning with the work of researchers like Jean Pierre Flourens (1794-1867) in the 1820's (Pearce, 2009). Among others, pial stripping (Metz & Whishaw, 2002), thermocoagulation (Napieralski et al., 1998), and excitotoxic methods (Lipska et al., 1998) were also utilised to remove portions of the brain. They all require a period of post-operative recovery before the animal could be tested. This period ranges from a few days to a few weeks depending on the brain area, lesion method, and nature of the research (Bell & Bultitude, 2018). Nonetheless, ultimately it can be difficult to precisely estimate the extent of the lesion.

Great focus and interest was given to cortex, as a target for lesions studies, as it was thought to be involved and necessary for accomplishing various behavioural tasks and was long

proposed to be the structure responsible for integrating a representation of the world (H. B. Barlow, 1986). Any harm to the brain will first become evident in changes to behaviour (Strassmann, 2014). However, proving this statement has not always been as easy and straightforward as initially thought, and in fact, this does not seem to be true when cortex is partially or fully missing in some mammals. The necessity of the cortex for behaviour has been investigated in experimental psychology for over a century, including the fundamental work of Karl Lashley and his students. Lashley was in search of an "engram" which he thought to be located in the cortex (Josselyn et al., 2017). A typical experiment involved navigate mazes for food before and after ablating different parts of the cortex suspected to be associated with memory and/or the ability to learn (Lashley, 1950). The logic underlying these experiments was simple, if the ablated cortex contains the engram supporting the memory for maze learning than the rats would show a memory deficit upon testing. Lashley observed that rats' ability to learn the task was not disrupted by any specific lesion site but only the larger the amount of cortex missing or damaged, the more errors the rats would make (Lashley, 1950). Controversially, he concluded that the engram would have to be distributed throughout cortex rather than spatially localised, giving rise to the principles of mass function and equipotentiality (Thomas, 1970).

David Oakley was another influential scientist who focused a great part of his career on studying the effect of cortical lesions on behaviour. He performed much larger cortical lesions up to its full extent which is referred to as decortication (Figure 1.3A). After extensive lesion studies, was clear that complete removal of neocortex in rats and rabbits, does not affect the acquisition, differentiation or reversal of Pavlovian behaviour (D. Oakley, 1979). Nonetheless, decorticates performance in instrumental learning during operant behaviours was inferior, but still present, requiring special training procedures (D. Oakley & Russell, 1972). This was an incentive to investigate their performance in even more complex tasks (D. A. Oakley, 1981; D. A. Oakley, 1979). With this aim, Oakley tested the behavioural

flexibility of decorticated rats. The capacity of quickly learning a novel behaviour, when a previous learned action is prevented, is a complex adaptive ability that one might think to be disrupted in an animal lacking all of their cortex. Rats were divided into sham operated and decorticated with an estimated mean extent of cortex removed of 96.7% ±1.4 % standard deviation (Philip Terry et al., 1989). After water restriction, rats were tested in an alleyway obstructed by a ball (Figure 1.3B) and required to push the ball into goal box to earn a water reward. Subsequently, metal pegs were inserted to determine which action, push or pull, the rats had to use to clear the path from the obstructing ball and reach the reward in the goal box. Surprisingly, decorticated rats were much quicker to learn to pull the ball back into their start box when the peg prevented the previously learned pushing response (Philip Terry et al., 1989). This finding suggests that decorticates may have a greater capacity for behavioural flexibility than had previously been predicted.

Oakley's study demonstrates that challenging animals in increasingly complex tasks can lead to unexpected insights and challenge long held assumptions (assumptions based primarily on generalizations from simple laboratory experiments). Lesion studies have long highlighted the need for more complexity in the assays used by psychologists for studying behaviour, and a shift towards a more ethological approach is desirable, if proven to be technically feasible.

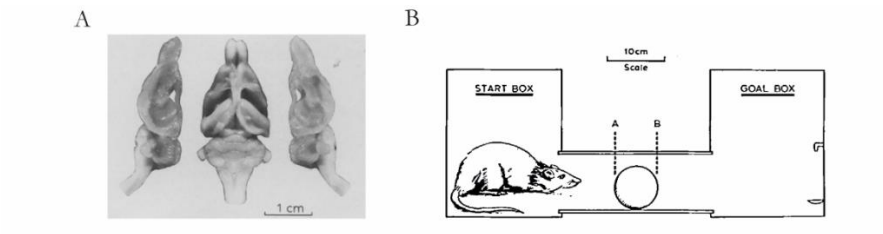


Figure 1.3 Lesion studies

A) Black and white picture of a decorticated rat brain. Picture from Oakley 1978. B) Side view of the obstructed alleyway apparatus showing the ball placed midway along the alleyway. The water spout and drinking cup are visible in the goal box. A and B are the location of the pegs that prevent the ball movement. Figure from Oakley 1989.

1.2.4 What does the cortex do?

At the beginning of the nineteenth century, neuroscientists were already aware that regions with specialized functions could be identified on the surface of the cerebral cortex. This led to the theory of functional localization, which preceded the identification of the first functional cortical area by neuroanatomist Paul Broca (1865), who demonstrated that speech was localized in a specific region of the frontal lobe. The mammalian neocortex is an extremely complex, highly organized, six-layered structure that is tangentially parcellated into many functional areas with different cytoarchitectonic features, neural types and specialized patterns of afferent-efferent connectivity (Molyneaux et al., 2007; Lodato & Arlotta, 2015). Functional columns were first defined in the cortex by Mountcastle (1957), who proposed the columnar hypothesis, which states that the cortex is composed of discrete, modular columns of neurons, characterized by a consistent connectivity profile. This discovery, originally built on data from electrophysiological recordings in the somatosensory cortex of monkeys, was a turning point in the understanding of neocortical organization (Mountcastle, 1997; Da Costa et al., 2010). The columnar theory has been prominent in the field for more than 50 years, although investigation is still ongoing to determine whether columnar organization applies across the entirety of the neocortex (Payne et al., 2001).

The neocortex is the part of the brain responsible for execution of higher-order brain functions, including cognition, sensory perception, and sophisticated motor control (Shipp et al., 2007; Rubenstein et al., 2011). We have extensive knowledge about cortex, its structure, development, circuitry, and its implication in several neural processes from vision to memory, from decision making to audition, but there seem to be missing puzzle pieces once it comes to answer an apparently simple question: what does the cortex do? Lesion studies, as highlighted in the previous paragraph, were not satisfactory in clearly elucidating the role of cortex. In fact, often, animals would retain most of the previously learned behaviours

while missing a substantial portion of their neocortex. Cortex has long been proposed to be the structure responsible for integrating our presentation of the world and improving the predictive power of this representation with experience (Barlow, 1986; Barlow, 1987). However, studies of cortex have largely focused on specific regions rather than their collective function. Here we propose to record from multiple cortical areas simultaneously, but will first introduce each individual area that will targeted by using a novel multi-shanks probe.

1.2.4.1 Cortical Areas

Visual Cortex

The visual cortex is the primary cortical region of the brain that receives, integrates, and processes visual information relayed from the retinas. The largest visual area, known as the primary visual cortex (V1), has greatly contributed to the current understanding of mammalian and human visual pathways and their role in visual perception (Glickfeld et al., 2017). In 1959, the pioneering study by Hubel and Wiesel found that individual neurons in V1 of anesthetized cats fire action potentials when oriented bars of light are presented in specific locations within the visual field (Hubel and Wiesel, 1959). Since then, it has been widely studied, ultimately leading to the discovery of cortical maps, in which neurons in V1 are organized in a retinotopic arrangement, presenting a topographic mapping from the retina to the cortex and show tuning identified for a variety of features like position, orientation, direction, spatial frequency, and colour. (Van Essen et al., 1984; Xing et al., 2004; Ohki et al., 2005; Nassi & Callaway, 2009; Van Den Bergh et al., 2010).

More recently, the development of awake, behaving assays for neural electrical and optical recording has found responses in visual cortex to "unexpected" visual input, such as aberrant optical flow during self-motion. The role of this prediction error signal, now identified in

many cortical areas, in learning is an area of active and intense investigation (Keller et. al, 2018).

Parietal Cortex

While the primary sensory cortices are efficient at processing their respective sensory modality, higher-order sensory association cortices contain neurons that are multimodal and respond to auditory, visual, somatosensory stimuli or to any of their combinations (Lyamzin & Benucci, 2019). This allows cortex to piece together multiple sensory inputs to generate a more comprehensive representation of the outside world, as well as an appropriate motor output. In rodents, the posterior parietal cortex (PPC) is one such association area (Mohan et al., 2018). PPC acts as interface between sensation and action, and it has been found to take part in numerous aspects of animal behaviours. Amongst others, PPC has been implicated in navigation, in the control of movement and self-body motion, and in visually guided decision making (Nitz et al., 2006, Whitlock et.al., 2014, Krumin et al., 2018).

Unlike other areas, the parietal cortex affords to be studied and better understood using experimental protocols that allow cross-modal integration to take place. Virtual reality, due to its flexibility and ease in incorporating different modalities, as well as variations of the 2AFC tasks have in fact helped elucidating several aspects of the multimodal nature of this brain area (Harvey et al., 2012, Raposo et al., 2014, Akrami et al., 2018).

Somatosensory cortex

Tactile feedback from the body is received by primary sensory cortex. In rodents, a large portion of literature focuses on investigating state-dependent neural processing by studying whisking, considered an indicator of active state (Busse et al., 2017).

Each individual whisker is known to sends signals to specific, isolated column of cells in cortex (Adibi, 2019). This organization provided the "name" barrel cortex and provided

some of the first evidence for the dominant columnar organization of cortex (Mountcastle, 1997). Furthermore, by depriving one barrel from sensory input (by trimming the corresponding whisker), the plasticity of surrounding cortical areas has been extensively studied (Diamond & Arabzadeh, 2013; Feldmeyer et al., 2013; Staiger & Petersen, 2021).

In our assay, our electrode shanks primarily target an area in somatosensory cortex receiving sensory feedback from the trunk/flank of the animal. This feedback has not been extensively studied; however, we would expect responses related to interaction of the animal and the walls/corridors of our complex maze (Menzel & Barth, 2005).

Motor Cortex

Finally, let us focus on motor cortex, extensively explored by our laboratory. Motor cortex is widely believed to underlie the acquisition and execution of motor skills, yet its contributions to these processes are not fully understood (Kawai et al., 2015). Motor cortex is still broadly defined as the region of the cerebral hemispheres from which movements can be evoked by low-current stimulation, following Fritsch and Hitzig's original experiments in 1870 (Fritsch and Hitzig, 1870; Taylor & Gross, 2016). Remarkably, motor cortex can be heavily lesioned in rodents and yet complex behaviour is retained. Quite the contrary is true for primates and human that in fact have unique pathways and specializations of motor cortex that seem to be absent in other mammals (Omrani et al., 2017).

The activity in rodent motor cortex has been correlated with movements in every part of the body (Erlich et al., 2011) but the high degree of connectivity with structures like the spinal cord, the basal ganglia, the thalamus, the cerebellum and the brainstem provide us with a clue on the broader functions of this cortical area (Graziano et al., 2002; Ebbesen & Brecht, 2017; Heindorf et al., 2018)

To be able to understand further the role of motor cortex, we need to design the right behavioural tasks, the one that would exploit its full potential.

Goncalo Lopes, former Kampff Lab member, investigated whether the intact rat motor cortex is required for the robust control of movement in response to unexpected perturbations. He designed a shuttle task where rats had to cross a dynamic obstacle course with stable and/or unstable steps to earn water reward. He tested rats with and without motor cortical (forelimb area) lesion finding that basic motor performance was retained but, when the steps were suddenly made unstable, lesioned rats showed incapacity of deploying an efficient strategy to respond to it. Lesioned rats could not respond to a motor challenge that did not conform with their expectations.

Ultimately, Goncalo suggested that motor cortex is primarily responsible for extending the robustness of the subcortical movement systems specifically when animals face unexpected events. Recent work using optogenetic manipulations has confirmed this hypothesis, finding selective impairments only when motor cortex was inactivated while responding to unpredicted sensory feedback (Heindorf et al., 2018). Motor Cortex does not seem to be required in stable, predictable, and unperturbed environments. These findings were possible only by using an exploratory approach to the study of behaviour. Real world complexity and events were brought into the laboratory, and this is the only way we could come across this discovery. This approach to introducing controlled complexity into assays used to investigate the role of cortex was the primary motivation for embarking on the challenging project extensively described in this dissertation.

Cortex as a whole

One or two cortical areas can be studied simultaneously based on the questions asked by the experimenters, but from how many areas are sufficient to gather data to start addressing our questions in a satisfactorily? We think "the more the better", but we are not the only ones

thinking this way. Studying each of the cortical areas in a compartmentalised way allowed us to gain key insights on each one of them lacking the overall dynamics during the tasks. In recent years, thanks to technological advances as well as a general shift within the scientific community, things are starting to change in the direction of considering the cortex as a whole rather than a compartmentalised entity.

Together with calcium imaging, 2-photon microscopy has been routinely used to examine behaviour-related activity in populations of neurons (Denk & Svoboda, 1997; Peron et al., 2015). Nonetheless, to be able to target many brain regions at the same time, large field of view 2-photon microscopes are required (Goard et al., 2016; Sofroniew et al., 2016).

Others have attempted to record from multiple brain regions using silicone probes implanted in various locations simultaneously (Chung et al., 2019; Luo et al., 2020; Steinmetz et al., 2020) and we will also contribute to this effort by targeting several cortical regions with a multi-shank probe.

As a side note, the opening of these new venues in the study of the brain comes with caveats that we must keep in mind at all times: *larger observations bring more nuance, but also lay bare gaps in our tools for interpreting brain-wide neural dynamics* (Urai et al., 2021).

1.3 New technology for the study of animal behaviour

Creating the complexity of a natural environment in a controlled laboratory setting is a technical challenge. The 20th century has seen tremendous progress in the technologies for building complex "artificial" environments, which was largely driven by advances in computing and a desire for increasingly realistic (i.e.,"natural") videogames. These technologies have matured in the 21st century to the point where this complexity has become more manageable so much so that scientists can start thinking of new experimental designs to bridge the gap between nature and laboratory. Complex and "natural" environments can be more easily created allowing for a convergence of ethological and psychological paradigms.

1.3.1 Videogames

Early videogames were limited by the available computers, and thus resembled the simplistic tasks that behavioural psychologists asked their animal subjects to perform. However, computers experienced an exponential increase in performance over the past 50 years, which has made possible games with ever more realistic environments and actions.

In the 1980s, the development of specialized hardware and software, allowed the first simulated 3-D environments in which a player's actions (turns, movement) are quickly translated into a change of view, simulating their motion through a space. Although these first 3D games were presented on a stationary monitor, it was soon realized that by wearing goggles (with screens) and updating them based on the real-world actions of the user, one could create a "virtual reality".

1.3.2 Virtual reality and neuroscience

The concept of Virtual Reality (VR) was formulated in the 1960s while the first commercial VR tools only appeared in the late 1980s. Ivan Sutherland attempted to describe VR as a window through which a user perceives the virtual world as if it looked, felt, and sounded real and in which the user could act realistically (Sutherland, 1965). In the last 20 years, VR attracted researchers from different scientific fields, and it quickly became a valuable research tool to investigate a wide spectrum of behaviours from sensorimotor interaction to spatial navigation and cognition. VR offers a way to simulate reality and in principle, anything that can happen in reality can be programmed to happen "virtually" (Slater & Sanchez-Vives, 2016). Experiments are usually designed such that the presentation of the stimuli are often limited to simplistic text, static graphics, or computer based abstractions of real world objects and situations, usually presented on screens (Naik et al., 2020). Different versions allow the presentation of the stimuli on the floor, accounting for the tendency of rodents to attend to objects in their lower visual hemi-field (Furtak et al., 2009; Scaplen et al., 2014; Yang et al., 2017) or using touch screens for the animals to directly interact with (Cook et al., 2004; Horner et al., 2013; Heath et al., 2016).

This highly controlled but contextually impoverished stimuli (static pictures, shapes or movies) simplify the world, again leaving doubts about the generalizability of the results and their ecological validity, given that the relevance of the stimuli to the subject's natural behaviour is unclear (Bohil et al., 2011; Doucet et al., 2016). An advantageous feature of VR is the possibility of developing closed-loop experiments where the sensory stimulation is constantly updated by the animal's actions. This is of great importance to bring the animal experience one step closer to the real world. Additionally, the use and implementation of videogame graphics, animations and rendering allow for a more realistic visual experience for the animals (Naik et al., 2020) by adding complexity, but in a controlled manner.

The first VR system for rodents was successfully developed by Hölscher et al. (2005) taking inspiration from a smaller version of a VR flight simulator designed for insects (J. R. Gray et al., 2002). Rats were placed onto a large air-cushioned Styrofoam sphere surrounded by a 360° toroidal screen. Rats could navigate the virtual environment while wearing a little harness to prevent them from jumping off the rotating ball. The virtual environment was updated in real time with the movement of the rat and for the first time it was proven that rats can navigate virtual environments to earn a reward (Hölscher et al., 2005).

Since its introduction to the rodent neuroscience community in 2005 a wide variety of VR systems have been developed, enabling sophisticated experimental paradigms like randomized arena layouts (Aronov and Tank, 2014), objects that appear and disappear over time (Harvey, Coen, and Tank, 2012; Pinto et al., 2018), and complex and dynamic labyrinths (Thurley et al., 2014).

VR set-ups have become popular to address questions that would be difficult to tackle in the wild. In fact, while naturalistic behaviours take place, the brain activity can be precisely monitored with imaging (Dombeck et al., 2010; Harvey et al., 2012) or neural recording (Harvey et al., 2009; Fournier et al., 2020). However, the degree of stability required by the technique in use, influences the degrees of freedom of the animal during the experiment.

The need to restrain rodents within VR setups, leads to restrictions in the sensory information that is available to the animal. The natural head movements are impeded, removing the vestibular inputs important in spatial navigation tasks. Inevitably, both head and body fixation result in mismatches between vestibular, proprioceptive, and visual inputs (Thurley & Ayaz, 2017a) which could lead to perturbation in the neural processing under study (Aghajan et al., 2015).

Although, in some cases, mismatches and conflict are incorporated into the experiments by introducing brief perturbations between optical flow and locomotion (Keller et al., 2012).

Technical advancements try to overcome VR limitations by allowing the animals to be less restricted. However, this comes at the expense of reduced stimulus control and fewer options for recording neural activity (Del Grosso et al., 2017).

Despite these limitations, virtual reality seems to be the best compromise for combining precise experimental control with more natural sensory environments and behaviours.

1.4 The study of neural (electro)physiology

In the past centuries, various electrophysiological recording techniques were developed for measuring the voltage change or electrical current on a wide variety of scales. Recording techniques are divided into two main categories: intracellular recording and extracellular recording. Intracellular recording allows us to make direct measurements of intracellular electrical activity of a single neuron using techniques like voltage clamp, current clamp or patch clamp. These techniques usually require small electrodes with micro-meters in tip diameter which are inserted directly into or attached to a single cell (Huang, 2016). Nevertheless, the difficulty of intracellular recording severely limits its use for awake freely moving experiments. For the purpose of this dissertation, we are only focusing on extracellular techniques.

Extracellular recording techniques require the electrode tip to be left in continuity with the extracellular space. These techniques, based on the preparation, the electrode sizes, and its placement, allow sensing action potentials (spikes) from a single cell (single unit activity) or the activity of several nearby cells simultaneously, called multi-unit activity (MUA).

Extracellular neural recordings are typically performed by inserting micro electrodes insulated everywhere except one or more small recording sites directly into brain tissue. This recording approach is invasive but does not require the same level of stability needed for intracellular recording, making it possible to use in awake and freely moving animals. Large electrodes, placed on the scalp (EEG) or on the dura mater (EcoG) are unable to resolve

single neurons, but they can cover much larger areas of the brain, integrating the activity of many cells to generate a local field potential (LFP).

Electrophysiological *in vivo* recordings of neural activity have greatly helped elucidate aspects of brain function and understand the underlying complex interaction among neurons and between areas (Ruther & Paul, 2014).

1.5 New technology for the study of electrophysiology

Technological advances have impacted not only the study of animal behaviour, but they have also helped revolutionize neural recordings. There is the need for electrophysiological recordings with the high spatiotemporal resolution of extracellular methods together with the coverage extent of surface recording techniques (EEG/EcoG). Both for improving the scale of physiology data that can be acquired, but also changing the kinds of questions that can be asked.

The pace of technological development in neuroscience has been accelerating in the past decades and we are now in a better position to start deciphering how groups of neurons in many different regions work together to drive behaviour (Altimus et al., 2020). Understanding how the brain operates implies the simultaneous observation and monitoring of many neurons and their dynamics across multiple brain regions. Additionally, it would be crucial to do so in freely behaving subjects while performing ethologically relevant tasks for an extensive period.

Large scale distributed neural recordings using CMOS probes

The electric nature of neurophysiology was first revealed by Luigi Galvani and Alessandro Volta in the 1700s. In the following centuries, scientists began to ask how the sensory information is transmitted by electric signals. It was not until the 1920s when Edgar Adrian could record, for the first time, the activity of the nervous system at a cellular level (Adrian, 1928). In 1957, after Hodgkin and Huxley studied the giant squid axon, an important step forward was taken by fabricating sharpened tungsten electrodes with tip sizes capable of recording from much smaller neurons in the brain (Hubel, 1957). Microwires evolved into tetrodes formed by four microwires twisted and closely spaced together. Tetrodes have become one of the most used tools in electrophysiology, due to their low cost, stability, the possibility of distinguishing one neuron from another (i.e., spike sorting) and their usability in *vivo* (C. M. Gray et al., 1995; Emondi et al., 2004).

Recent, technological progress has contributed to advance beyond microwires/tetrodes to microfabricated silicon probes. The development of silicon probes extended the function of the classic extracellular tetrode (with only 4 channels), to devices containing initially 16 to 64 separate channels (polytrodes). However, these first silicon devices were still limited by the number of wires that could be connected to a thin shaft that was implanted into the brain tissue (Blanche, 2005).

It is crucial to increase the number of neurons that can be simultaneously monitored and this requires large dense arrays of recording sites on a probe, ideally compatible with freely moving animals (Dutta et al., 2019). Advances in complementary metal oxide semiconductor (CMOS) technology has made this possible, enabling incorporation of recording electrodes with integrated multiplexing circuits directly into the probe shaft (Najafi & Wise, 1986; Hong & Lieber, 2019). Now, the size of the shaft no longer limited the number of electrodes.

CMOS circuitry has resulted in the creation of probes with thousands of individual sites (Neuropixel, NeuroSeeker) (J. J. Jun et al., 2017; Dimitriadis et al., 2018; Steinmetz et al., 2018; Dutta et al., 2019). These CMOS probes open the possibility of distributing an exceptionally large number of small electrodes throughout the brain allowing for high resolution local recordings at a global scale. As circuit fabrication technologies improve, the devices sizes tend to shrink. By employing CMOS technology, these probes have been designed to overcome the geometric restrictions on the number and density of electrodes. Despite their density, electrodes are located in one shaft only. It has been shown that up to eight Neuropixels probes could be implanted into the same mouse brain to provide large-scale mapping of activity in the forebrain in conjunction with calcium imaging (Sringer et al., 2018).

However, the true potential of large-scale distributed recording will only be achieved with the development of multi-shank CMOS probes, in which tens of thousands of microelectrodes are fabricated on a device with multiple individual needles (shanks) that target different brain areas. While these devices are currently being manufactured (Steinmetz et al., 2020), this dissertation will explore the potential of multi-shank silicon probes as a tool for recording from many sites throughout the brain during unrestrained behaviour.

1.6 Goal and thesis outline

Historically, neuroscientists have taken a reductionist approach to understanding both brain functions and animal behaviour. Technological advances allow us to bring experiments one step further in both fields. In this dissertation we report our attempt to study behaviour in a semi-naturalistic and loosely constrained manner while investigating the neural activity of multiple cortical areas simultaneously.

We are neither proposing a fully ethological approach nor a purely Skinner-like psychological approach to the study of behaviour but instead, we designed a new behavioural set-up taking into consideration the type of behaviours rats would do in the wild. Our behavioural assay of choice is a videogame with increasingly complex levels, which we developed using augmented reality (AR). AR, relative to VR, is a newer technological system in which virtual objects are added to the real world in real time (Cipresso et al., 2018). According to Azuma et al. (2001) an AR system should: (1) combine real and virtual objects in a real environment; (2) run interactively and in real-time; (3) register real and virtual objects with each other. This tool allows us to combine the virtual and real world in an attempt to create engaging and life-like tasks for our rats.

We ask rats, without any prior food or water restriction (motivational aid) to forage, hunt, and deal with unexpected events requiring quick adaptation of their behavioural strategies. We monitor their behaviour and control the stimulus in closed-loop so that their actions have consequences in the augmented reality world. We also record their brain activity, focusing on a large portion of their cortex. Combing the large-scale high-resolution recordings with a more naturalistic behaviour will be the central goal of this thesis.

In Chapter 2 we provide a detailed description of the steps required to build our Videogame assay for rats and implement a behavioural protocol that exploits the rats' natural behavioural repertoire in absence of food or water restriction. Additionally, we report and discuss the

rats' performance throughout the training and how they react to unexpected changes encountered.

In Chapter 3 we report a detailed design description of a novel 121 channels silicon probe for distributed recording in the cortex. Probe and implant design, assembly, and surgical implantation are also meticulously described. Featuring 11 shanks, this probe allows us to gather information simultaneously from different cortical areas up to 1 cm apart from one another. Furthermore, we describe the rats' behavioural performance post-surgery and assess the probe quality. Finally, we approach the data analysis in an exploratory manner, reporting and discussing our preliminary findings.

Finally, in Chapter 4 we summarise the overall results and purpose of our work. We discuss the impact of stepping outside the box and how this approach could give insights and open new avenues for future experiments.

The project presented in this dissertation started in Lisbon where I was awarded a fellowship from the Fundação para a Ciência e Tecnologia, (SFRH/BD/52445/2013) to join the International Neuroscience Doctoral Program at the Champalimaud centre for the Unknown, Lisbon, Portugal. In 2015 the Kampff Lab moved to London, and I transitioned to the Neuroscience PhD program at the Sainsbury Wellcome Centre for Neural circuits and behaviour, University College London, London, United Kingdom.

Chapter 2

Videogame Assay

2.1 Abstract

The laboratory limitations can be overcome by systematically introducing complexity in our experimental pipelines. We designed, prototyped, and built a novel behavioural assay, the Videogame, that allows us to create increasingly complex levels for rats to learn using augmented reality. We attempted to mimic naturalistic behaviours such as foraging and hunting including unexpected events typical of the natural environment and that require high flexibility and adaptive skills. We tested 12 Lister Hooded rats in four levels monitoring and recording their behaviour successfully gathering a rich dataset for quantitative behavioural analysis. In the following chapter we present the Videogame assay and describe in detail how to build it and implement the behavioural protocol. Additionally, we analysed several aspects of rat's behaviour and learning, and we report preliminary behavioural findings.

2.2 Introduction

The natural world is a sophisticated continuum of events, expected and unexpected, that is far more demanding than the laboratory settings our animals live in. How can we attempt to recreate such complexity? How can we access and explore the behavioural repertoire our animals have not yet had the chance to express with conventional laboratory tasks? The Kampff Lab has attempted to answer these questions. Playgrounds and motor cortex challenges for rats (Lopes et al., 2016) have been developed as well as the 'Cuttle Shuttle' to study hunting behaviour in cuttlefish (http://www.danbeekim.org/open-lab-notebook/cuttlefish-hunting-behavior/). Complexity has been introduced and constraints have been released while still maintaining control over the behavioural variables of interest.

Taking deep inspiration by this, one of the purposes of the work presented in this manuscript was to develop a complex and flexible behavioural set-up, inspired by the natural world and the challenges faced by animals (e.g., rats) in the wild. The behavioural set-up of choice was a *Videogame*, where each level was an attempt to mimic naturalistic behaviours and adapt them to a laboratory context. Why a Videogame? We can think of videogames as a rough simulation of the real world. As players, we need to interact with objects presented on a screen, learn the rules and challenges of a specific game and master them to achieve a goal or an ultimate victory. This process requires the ability to flexibly adapt to an ever-changing environment, where unexpected events can and do occur. Thus, a Videogame seemed the ideal substrate for creating a complex environment for rats and their brains to experience. The Burwell Laboratory, at Brown University, developed the Floor Projection Maze, an apparatus for presenting 2D visual stimuli directly on a screen floor, exploiting the natural tendency of rats to attend to items located on or close to the ground (Furtak et al., 2009a). This setting has been used to explore a variety of visual psychophysics tasks for rats and they have shown that they can acquire visual discrimination tasks more rapidly when presented on a floor compared to vertically on a monitor (Furtak et al., 2012; Scaplen et al., 2014; Jacobson et al., 2014; Scaplen et al., 2017). The Videogame, has a similar structure to the Floor Projection Maze but we developed levels in which rats, not only are presented with 2D visual stimuli but can interact with them in a closed-loop manner. Freely moving rats were trained to interact with projected objects (yellow spots) whose behaviours were changing according to the level they were on. We attempted to mimic behaviour like foraging and hunting and included unexpected events and challenges (see Videogame protocol). Using a reactive data stream processing tool, developed in our laboratory (Bonsai, see Videogame software), we could precisely control a large variety of parameters in the environment, relative to the animal's behaviour, generating a rich dataset for quantitative analysis (see Videogame software). Both hardware and software made the Videogame a flexible set-up for

exploring and prototyping a wide range of complex semi-naturalistic behavioural paradigms. Ultimately, we integrated a recording system to allow for chronic electrophysiological recordings while rats played the Videogame.

2.3 Methods

2.3.1 Videogame Design

The Videogame prototype was initially designed using a 3D mechanical design software called Autodesk Inventor (Autodesk, 2016). Each component was created separately and then assembled to generate a 3D model of the structure. This step gave us insight into what could have worked or failed, and importantly, it gave us an estimate of how much material we should purchase for each component. The physical assembly of the videogame was facilitated by the presence of a very resourceful Maker Space at the Sainsbury Wellcome Centre (SWC), providing the machinery and the expertise to make the Videogame mechanical design possible.

2.3.2 Videogame Hardware

The skeleton of the Videogame was built out of a combination of different sizes of clear anodized aluminium extrusions (5 Series, HFS5- 2020, MISUMI Europe). The frames were cut at the appropriate size, based on the 3D model, using a metal circular saw (PANDA 400). Post assembly insertion spring nuts (HNTP5-4, MISUMI Europe), stopper nuts (MISUMI Europe) and 90° aluminium tabbed reversal brackets (HBLFSN5-4, MISUMI Europe) were used to fasten the frames together. These configurable components allowed for an easy and flexible prototyping approach for building custom behavioural set-ups. The Videogame

featured four main components: a projection screen table, a modular arena, a projector holder and a vertical enclosure (Figure 2.1A-B).

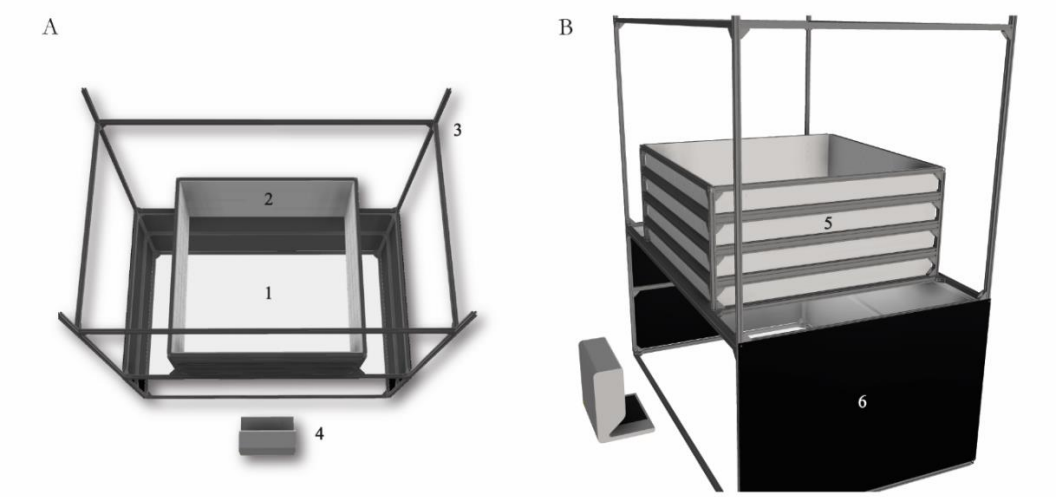


Figure 2.1 Videogame Autodesk Inventor 3D model

A) Videogame top view. 1 - Projector screen table, 2 - Modular arena, 3 - Vertical enclosure, 4 - Projector. B) Videogame side view. 5 - Modular arena, emphasis on the modularity facilitated by the equally spaced aluminium frames. 6 - Dibond panels, covering the outer part of the structure to provide darkness and shielding from the room electrical noise.

2.3.2.1 Projection Screen Table

The projection screen table is composed of an aluminium base and a glass digital rear projection screen (PRODISPLAY Innovative Display Solutions, 1050 mm x 1590 mm x 8 mm thick) placed on top. The base (1050 mm x 1600 mm x 700 mm) is the main support for all the Videogame' components, therefore, the frames combination must create a strong and stable structure. The top of the table-like base is covered with a foam adhesive tape (RS PRO, #619-1786, UK), to create a soft surface for the glass screen to rest on. The screen is gently placed onto the foam and held in position with additional frames, also covered with foam. After being sandwiched between soft frames, the screen is additionally covered by a 2 mm thin clear polycarbonate anti-reflective sheet (Cut Plastic Sheeting, UK) for protection against abrasions due to rat's scratches and cleaning agents, used to sanitize the arena after each behavioural session (Figure 2.3A). Three of the sides of the table are covered with panels

of black Dibond aluminium composite (Amari plastics PLC, I-bond UK). Dibond is a lightweight material and consists of a flexible polyethylene core covered with a thin aluminium sheet on each side and finished with black glossy and matte lacquer. The matte side is placed facing the inner part of the table to prevent reflection given by the projector light. This material is not suitable for laser cutting, so cut to size orders or hand saw cutting is required. Holes are drilled into the panels, and they are screwed directly onto the frames with post insertion nuts. The choice of this material was made to ensure darkness and the ability to ground the structure, decreasing ambient noise during chronic behavioural recordings. If a smaller size videogame is required, it is possible to build a cheaper, yet efficient, option by using a rear projection diffusive fabric (Rosco, twin white screen, UK) sandwiched between a clear anti-reflective acrylic sheet (5 mm thick) and a polycarbonate sheet (2 mm thick), to protect the fabric from abrasion. This option proved to fail for big size videogames because the thin acrylic sheets would bend in the middle, distorting the projected images. Thicker acrylic options did not bend but instead, they gave light aberrations when images were projected onto them.

2.3.2.2 Modular Arena

The modular arena is the enclosure where the behavioural paradigm takes place, and it is fastened directly onto the projector screen table with aluminium brackets (Figure 2.3B). The arena has a rectangular shape (970 mm x 1210 mm) and it is built using regularly spaced anodised aluminium extrusions, which allow for its modularity. Black matte acrylic (Amari plastics PLC, Polarlite Black #8881, UK) tiles (12 cm x 12 cm, 5 mm thick) are laser cut (Trotec Speedy 300 Flexx) and they constitute the basic modules of the walls of the arena. Multiples of this size can be cut and used to create any desired wall configuration. The final modules are assembled by fitting and gluing (RS, Araldite Rapid Epoxy Adhesive, #756-0111, UK) four neodymium magnets (First4magnets, #F306-25, 6 mm Ø x 3 mm thick, UK)

into four 3 mm deep partial holes engraved in proximity of the corners of each tile. Post insertion nuts are also equipped with a neodymium magnet (First4magnets, #F643-256, 6 mm Ø x 1 mm thick) and slid into the aluminium extrusion's grooves. This magnetised structure allows for easy attachment of the magnetic tiles which can slide across the grooves thus allowing for a quick rearrangement of the configuration (Figure 2.3C).

Reward delivery module

Functional wall modules can be created depending on the behavioural paradigm of choice. In our case a 12 cm x 24 cm tile is created to allocate the reward port for pellet delivery. The tile is laser cut and magnets are inserted as for the basic module. The centre of the tile is additionally laser cut to insert a custom 3D printed (Stratasys J750 Polyjet - Vero white; Stratasys Mc 450 FDM - ASA white) reward port module designed using Autodesk Inventor. The module can be magnetically secured to the tile (Figure 2.2). On the front side, the reward port featured a collection chamber, to contain the pellets when delivered. An emitter and a receiver of an infrared beam break sensor (RS, Sharp wide gap photo interrupter, #GP1A57HR, UK) are also placed at each side of the chamber. When the infrared beam is interrupted by the rat's snout, the pellet delivery is triggered. The chamber is designed such that it would extrude enough into the arena to allow for a comfortable reward collection, by the rat, while carrying a chronic implant. A shorter chamber could create discomfort and force the rat to change the strategy to collect the reward after surgical procedure, impacting its performance. A piezoelectric speaker (RS, Kingstate KPEG110, UK) was inserted within the 3D model to generate the reward availability and reward tone played during the task. The back side of the reward port featured an opening, connected to the chamber for the pellet dispenser tube, and a holder to support an Arduino UNO, an Arduino relay shield (https://store.arduino.cc/) and a prototyping breadboard (RS, 100-4097). The module is connected via the tube to a pellet dispenser (model 80209-45, Cambridge Instruments Ltd,

UK) directly fastened onto the projection screen table. The dispenser is equipped with a 45 mg interchangeable pellet size wheel. As an alternative, if required by the experiment, a water delivery module could be easily integrated instead of the pellet dispenser, and magnetically attached at the back of the tile.

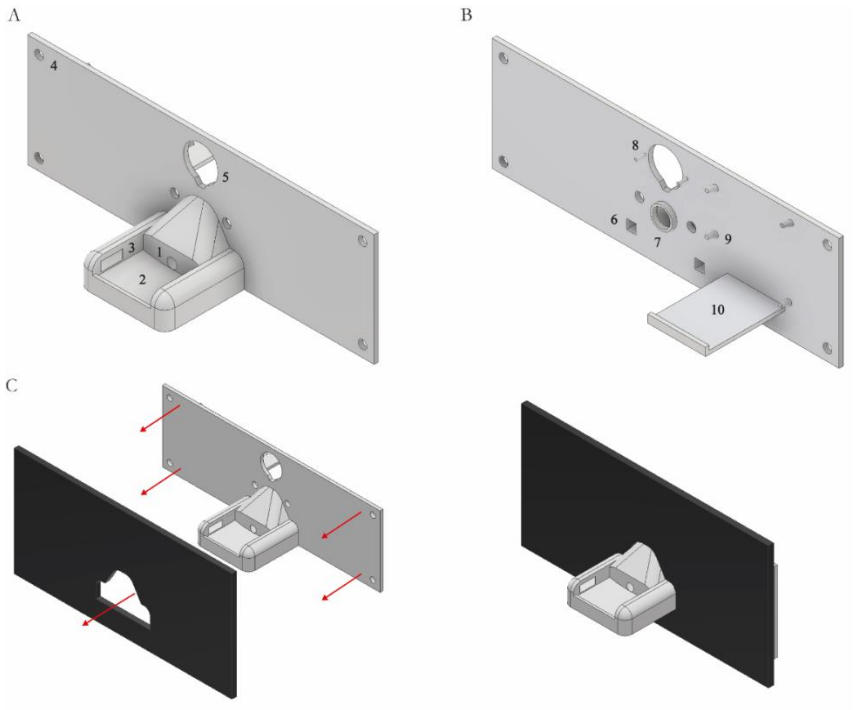


Figure 2.2 Reward port module Autodesk Inventor 3D model

A) Reward port front view. 1 - Pellet delivery exit. 2 - Pellet collection chamber. 3 - Infrared beam sensor slot. 4 - Insertion holes for the magnets mating with the magnets placed on the back side of the tile. 5 - Piezoelectric speaker slot. B) Reward port back view. 6 - Infrared beam sensor slot. 7- Pellet dispenser tube entry. 8 - Piezoelectric speaker holding pins. 9- Arduino board holding pins. 10 - Arduino and Arduino shield supporting platform. C) On the left, a representation of the motion to assemble the modular tile with the 3D printed reward poke module. On the right, the final reward delivery module as it appears from the modular arena.

2.3.2.3 Projector Holder

Images are back projected from underneath the table by an Ultra Short Throw projector (UST projector, MW843UST, BenQ, UK) positioned on the back side of the Videogame projection screen table. The projector native resolution is 1280 x 800 pixels at 120 Hz. It is held in place by an enclosure made of anodised aluminium frames and surrounded by

Dibond. The projector is supported upside down to allow a direct projection onto the rear projection screen. UST projectors have lenses that can create a large image from shorter distances allowing to position the projector close by the screen, reducing the set-up's overall size. Our assay required a wide projection, at least as big as the projection table screen (1050 mm x 1600 mm). Based on the UST specifications and its native aspect ratio of 16:10, the projector was placed with an offset of circa 25 cm away from and 60 cm below the screen.

2.3.2.4 Vertical Enclosure

To create a dark enclosed environment and hold both camera and recording hardware, a vertical aluminium structure is built and fastened onto the projection table. The enclosure is surrounded by Dibond panels screwed directly onto the aluminium frames (Figure 2.3D). To ensure access to the modular arena, only the upper portion of the enclosure is covered by the panels while the lower one is covered with blackout matte fabric sheets (Thor Labs, BK5, USA), acting as convenient curtains to access the arena at any time. The fabric is cut to size and neodymium flexible magnetic adhesive strips (First4magnets, NeoFlex #NF19, UK) are glued along each side as well as on the aluminium frames. The curtains can be magnetically secured onto the sides. To easily lift the curtains, plastic rings are inserted at the edges and metal hooks attached to the frames (Figure 2.3D). An aluminium frame is setup as a beam across the top of the enclosure, supporting a high-speed, high-resolution overhead camera (Grasshopper3 Point Grey U3, 120 FPS, USA). The camera is equipped with an 8 pins general purpose I/O connector (GPIO connector) and a C-mount lens (Fujinon DV3.4x3.8SA-1 lens, 1:1.4/3.8 mm-13 mm, ½", C mount varifocal lens). The camera is set in trigger mode, and it is connected, via a GPIO Hirose connector cable, to a photodiode (Thor Labs, PDA100A, USA), facing the projection screen table, outside the modular arena. Underneath the photodiode, a pure blue colour patch is projected. Taking advantage of the projector colour wheel sequence (Blue Green Red), the photodiode acts as trigger to open

the camera shutter every 8.3 ms (1/120FPS), which corresponds to every time it detects a blue frame coming from the projector. Across the enclosure, near the camera, a nylon fishing wire is used to support a commutator for electrophysiological recordings. The electrophysiology apparatus is held by a Dibond shelf-like structure fastened to the back of the enclosure.

Figure 2.3 Videogame assembly

A) Projection screen table and partial vertical enclosure. B) Modular arena skeleton fastened onto the table and highspeed camera positioned at the top of the enclosure. C) Fully assembled modular arena with magnetic modular tiles and partial Dibond panelling. D) Fully assembled Videogame. Blackout curtains are lifted to access the arena. On the back, visible Dibond extrusion where the projector is held and fully enclosed.

2.3.3 Videogame Protocol

A vast range of behavioural tasks could be explored with the flexibility of both the Videogame hardware and software. During the prototyping and testing phase, before finalising the protocol presented in this study, a variety of tasks have been developed (e.g., avoidance, shuttling, looming) with different projected objects and background colours (Figure 2.4).

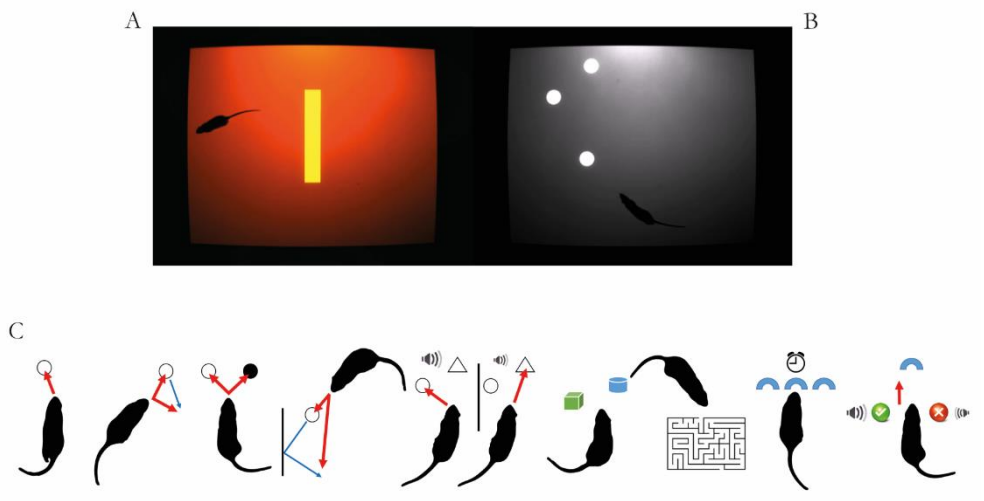


Figure 2.4 Videogame variants

A) Shuttling task. The rats need to shuttle between the opposite side of the arena to collect reward while avoiding a moving yellow bar projected in the centre. If the bar is touched the reward is omitted. B) Pac-Man with 3 moving balls to catch. C) Diagram showing a variety of task which could be implemented in the Videogame, from multiple choice tasks to mazes to go/no go tasks.

Ultimately, we wanted to create a behavioural protocol organised in levels of increasing complexity, like a videogame, in which the rats could learn the rules of the task while performing in each level and encounter unexpected events. Initially, a water restriction protocol was used but, in the work presented, the rat's behaviour is assessed without utilising any kind of restriction, food or water (see Restriction paragraph). During early stages, experiments were conducted in complete darkness with a black background and white virtual objects presented. Despite being successful, those experiments required a colour camera to record the projection table screen and a camera with an infrared filter, to capture and track the rat's behaviour. To overcome the need of having to control and align two cameras, and the requirement for infrared lighting, all the levels, are now played while a uniform red colour is projected onto the projection table screen (Figure 2.5). Thus, only one camera and no additional lightings was needed. The choice of the red colour was made taking advantage of the assumption that rats do not see red because, unlike humans, they have a dichromatic vision and a rod-dominant retina (Szél & Röhlich, 1992; Deegan & Jacobs, 1993). Red lights

and filters are also widely used in rodent husbandry and during experiments, allowing researchers to observe animals in an environment that is expected to appear dark to rodents. Over the years, different studies have been contradicting and challenging this assumption, showing changes in plasma melatonin levels (Sun et al., 1993) and circadian rhythm (Dauchy et al., 2015) in rats and mice when exposed to red light. Interestingly, a recent study found that, in both pigmented (Brown Norway) and albino (Wistar) rats, there is a significant scotopic and photopic response (measured using electrorenitography) to red light, even at low intensities (Niklaus et al., 2020). Despite these findings, for the purposes of this study, we would like the rat to be able to discriminate the 2D virtual objects presented on the floor and interact with them in different ways. Yellow objects projected onto a red background have been sufficient for the rats to successfully learn the task and for us to be able to track and record their behaviour. The Videogame behavioural protocol is composed of five levels. Level 0 and level 1 are the key levels to habituate and train rats and they provide the basics rules to perform in the subsequent levels. Level 2, or foraging level is simulating a foraging task where virtual objects (yellow circles/balls) are projected on the red floor background and appear at random times and locations. The goal of this level is earning chocolate reward by collecting the yellow balls. From foraging balls to hunting for them, is learnt in Level 3, or hunting level. The transition to Level 3 includes an unexpected moment where, for the first time, rats realize that the rules of the world have changed, from a static ball to a dynamic one that they must chase like prey. Initially, the prey moves with a quite simple physics resembling a bouncing ball, but in level 4, or challenge level, the ball movement is controlled by a human subject with a joystick. Every session takes place in the dark. The curtains are lowered, and the room lights are turned off. In the Videogame version presented in this work, the modular arena is enriched with interior walls, to create a maze for the rat to explore and with the aim to enhance a more diversified behavioural outcome. Laser cut acrylic walls, of different sizes (14 cm tall x 5 mm thick), are glued together and temporarily attached onto

the projection screen using micro suction tape (Sewell, AirStick, SW-30418, 0.8 mm thick, USA). The structure combines black matte and transparent acrylic walls to occlude or facilitate the view of the yellow balls, promoting the use of different strategies to solve the task.

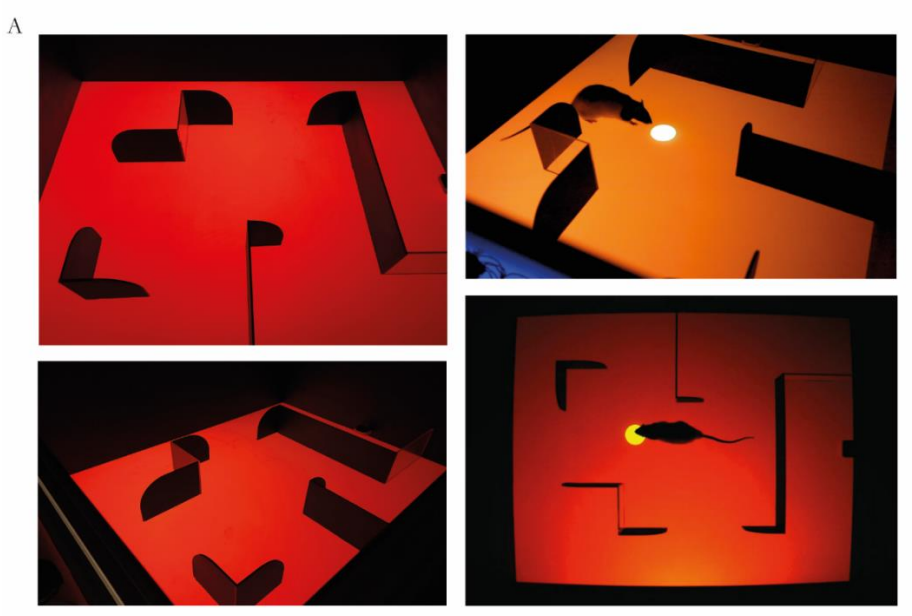


Figure 2.5 Modular arena layout

A) Overview of the setting where the Videogame levels take place. A red background is projected, and the arena is enriched with walls, creating a maze. While the rats explore the maze, a yellow spot of light appears at random time and location. Depending on the level, rats need to either forage or pursue the spot. The snout partially covering the spot is defined as 'touch' of the spot and it triggers the disappearance of the spot itself and the onset of the reward availability tone.

2.3.4 Restriction

The Three Rs (3Rs) are basic principles of human experimental techniques that were first set out by Russel and Burch in 1959 (Russell & Burch, 1959). They are now widely accepted and used within the international scientific community as means of avoiding or reducing animal use and suffering, aiming to improve the quality of scientific research. The NC3Rs is the UK national organisation for the 3Rs that stands for Replacement, Reduction and Refinement. Replacement involves the adoption of alternatives (e.g., computer modelling, tissue cultures, human studies) to animal research, when possible. As for The Krogh Principle: 'For a large

number of problems there will be some animals of choice or a few such animals on which it can be most conveniently studied' (Krogh, 1929). Reduction refers to the effort in minimising the number of animals used to achieve the experiment goals. Refinement involves either reducing the invasiveness of a technique or improving animal welfare and health during scientific studies (Sneddon et al., 2017). Although replacement can be considered the ultimate goal, as long as animals are used in scientific research, refinement is the 'R' with the greatest potential impact. It improves the life of the animal subject, but also influences the quality of the science. Thanks to the collaboration and feedback of the Neurobiological Research Facility at the SWC, we tried to apply the concept of refinement to different phases of the study presented in this thesis. Especially, we focused our effort onto the design of a lightweight implant to be carried by the rats chronically after surgery (see Probe holder design) and in the design of a behavioural protocol without utilising any kind of food or water restriction. Food or water are widely used as rewards to train laboratory animals on a variety of sensory, motor, and cognitive tasks. Restriction protocols are used to initiate or maintain a motivational state required to accomplish the task. The degree of restriction that is necessary to promote consistent behavioural performance is influenced by the difficulty of the task, the experience of each animal and its individual coping mechanism (Toth et al., 2000). Despite the use of a water restriction regime in the early development stage, we decided to pursue a restriction free approach. High palatable food (flavoured pellets: banana, chocolate or sucrose flavoured) or drink (Ribena, blackcurrant flavour) was delivered at the reward port to test whether rats could accomplish the task by simply providing an appetitive reward. Surprisingly, rats showed high interest and motivation in doing the task when presented with flavoured pellets. Ultimately, chocolate pellet purified diet (Rodent purified diet, 45mg dustless precision pellets, 1024030, LBS (Serving Biotechnology) LTD, UK), proved to be the favourite and was chosen as reward to earn during the Videogame levels.

2.3.5 Videogame Levels

12 adult Lister Hooded male rats, 6 months old, were trained in the Videogame. Rats were pair housed in individually ventilated cages (IVC), on a 12:12 hours reversed light-dark cycle (10am light onset) with controlled temperature (19-23 degrees) and humidity (50-70%), water and food were supplied ad libitum. Prior to the start of the behavioural protocol, rats are handled for 3 to 5 days in their home cage. Handling gradually helps the animals to familiarise with the experimenter and treats (mini yogurt drops, 1024099, LBS (Serving Biotechnology) LTD, UK) are also used as positive reinforcement to help the process. Rats do not undergo any type of food restrictions to perform in the Videogame. The same chocolate diet pellets, used as reward to earn, are provided during this phase, to habituate them to the taste. During handling, rats are also exposed to the weighing scale which will be used daily to record their weight before starting the behavioural session. Based on the animal personal stress level, handling can be prolonged until good level of trust and cooperation is reached. In Appendix A Table A.1, a collection of videoclips for each of the level described below.

Level 0 – HABITUATION

Rats are gently placed into the modular arena, and they are allowed to freely explore for about 20 to 30 minutes. The projection floor is turned on and red colour is projected but no other stimuli are present during this initial session (Figure 2.6A). This level is essential in helping the animals to familiarise with the arena and become comfortable with the steps required by the experimenter to start and end each behavioural session (e.g., weighing, placement and removal from the arena, curtain noise, changes in light intensity).

Level 1 – TRAINING

Level 1 provides the animals with the basic knowledge to be used in subsequent more complex levels. Each training session last about 45 minutes during which the rat needs to learn the association between a tone playing and the availability of a chocolate pellet at the reward delivery port (Figure 2.6B). At the beginning of each trial, a tone (availability tone, 80 pulses of 350 ms at 2Hz) is played for a maximum of 40 seconds during which the rat can collect chocolate. The pellet delivery is triggered if the rat places its snout in the delivery port, breaking the infrared beam sensor. The beam breaking is detected, the pellet dispenser is triggered, and the pellet delivered in the collection chamber. A second shorter tone (reward tone, 100 pulses of 100 ms at 5Hz), is played to reinforce the correct behaviour of the rat. A trial, in Level 1, is defined from the start of the availability tone to the end of it. Trials can be classified into reward trial (shorter than 40 s), where the rats successfully collect the reward, or missed trial, when the rats fail to collect the reward within 40 s. The inter trial interval (ITI) varies each trial according to an exponential delay, to prevent the rats from learning and anticipating the trial onset. Initially, the ITI is randomised within a short pre-established exponential delay (mean: 6 s – offset: 3 s, minimum ITI: 3 s and maximum ITI: 9 s). For instance, during short exponential delay trials, the trial onset is on average 6 s from the end of the previous trial, but it can vary from 3 to 9 s. When rats learn the tone-reward association they tend to spend more time around the reward delivery area, waiting for the availability tone to play. Despite this being a sign that rats can engage in the task without food restriction, we would like to encourage them to explore the rest of the arena. To enable this, in later sessions, we increase the ITI to try to elicit a more exploratory behaviour. Level 1 usually last for 8 days, 5 of which at a short ITI and the last 3 at longer ITIs (medium exponential delay: mean: 10 s – offset: 6 s, long exponential delay: mean: 10 s – offset: 20 s). The protocol for this level can be subject to change based on the rat performance.

Level 2 – FORAGING

Level 2 requires the rats to learn a foraging task making use of the association between tone and reward, previously learned in the training sessions. Each session usually lasts from 45 minutes to 1 hour. While exploring the arena, rats encounter a yellow circle (ball of light to forage) projected onto the red floor (Figure 2.6C). Foraging for a ball means partially covering it with their body, usually the snout. This event triggers the light to disappear and the reward tone to start playing for 40s, similarly, to Level 1. As previously learnt, a pellet is now available at the reward port. Rats quickly learn, within a few days, to associate the 'touch' of the ball with the pellet availability. A trial, in Level 2, is defined from the appearance of the ball on the floor to the successful or missed reward collection. Different from Level 1, trials no longer have a fixed maximum length, but they vary based on the animal learning curve and engagement in foraging. The ball's location changes every trial to allow rats to explore the arena in search of them. The ITI (appearance of the ball on the floor), is randomised within a pre-stablished exponential delay (short) which does not change throughout Level 2. The length of this level is flexible and can vary based on rat performance.

Level 3 – HUNTING

The transition to Level 3 or hunting level, provide both increased complexity and capture the instance in which the rules of the Videogame change, resembling an unexpected sudden event that often occur in nature. Usually, each session lasts from 45 minutes to 1 hour. During the first day, rats are presented with five foraging trials at the start. The sixth trial is the unexpected trial. Only for this particular trial and for all the rats, the ball, now a 'prey', appears always in the centre of the arena to maximize the chances of being noticed. From this trial onward the spot acts as a prey and when approached it moves away from the animals with basic physical rules. The prey has a simple bouncing ball behaviour and passively bounces off of the walls, decelerating over time due to virtual friction (Figure 2.6D). The

reward availability tone is triggered when the rats successfully cover the moving prey with a portion of the body, like in Level 2. A trial, in Level 3, is defined from the onset of prey motion to the successful or missed reward collection. Like in Level 2, the prey location changes every trial allowing rats to explore in search of it. The ITI is randomised in the same way than for Level 2. The length of Level 3 is flexible and can vary based on rat performance.

Level 4 – CHALLENGE

The last level developed, is a more advanced hunting task where the movement of the prey is controlled by a human subject using an XBOX wireless controller (Figure 2.6E). Each session usually lasts from 45 minutes to 1 hour. The physics of the prey is defined by the skills of the subject using the joystick. Like in Level 3, the rats need to chase and cover partially the prey to trigger the reward availability tone. The goal of the subject is to avoid the availability tone to be triggered. A trial is defined as in Level 3 and the rats can earn or miss the reward. The ITI is randomised as Level 2 and 3. One of the many variants to explore could be, for instance, removing the physical walls but maintaining the virtual wall outline for the human subject to navigate. This would create an interesting dynamic where rats can now pursue the spot more easily, but the human subject needs to memorize where the walls were located to perform efficiently.

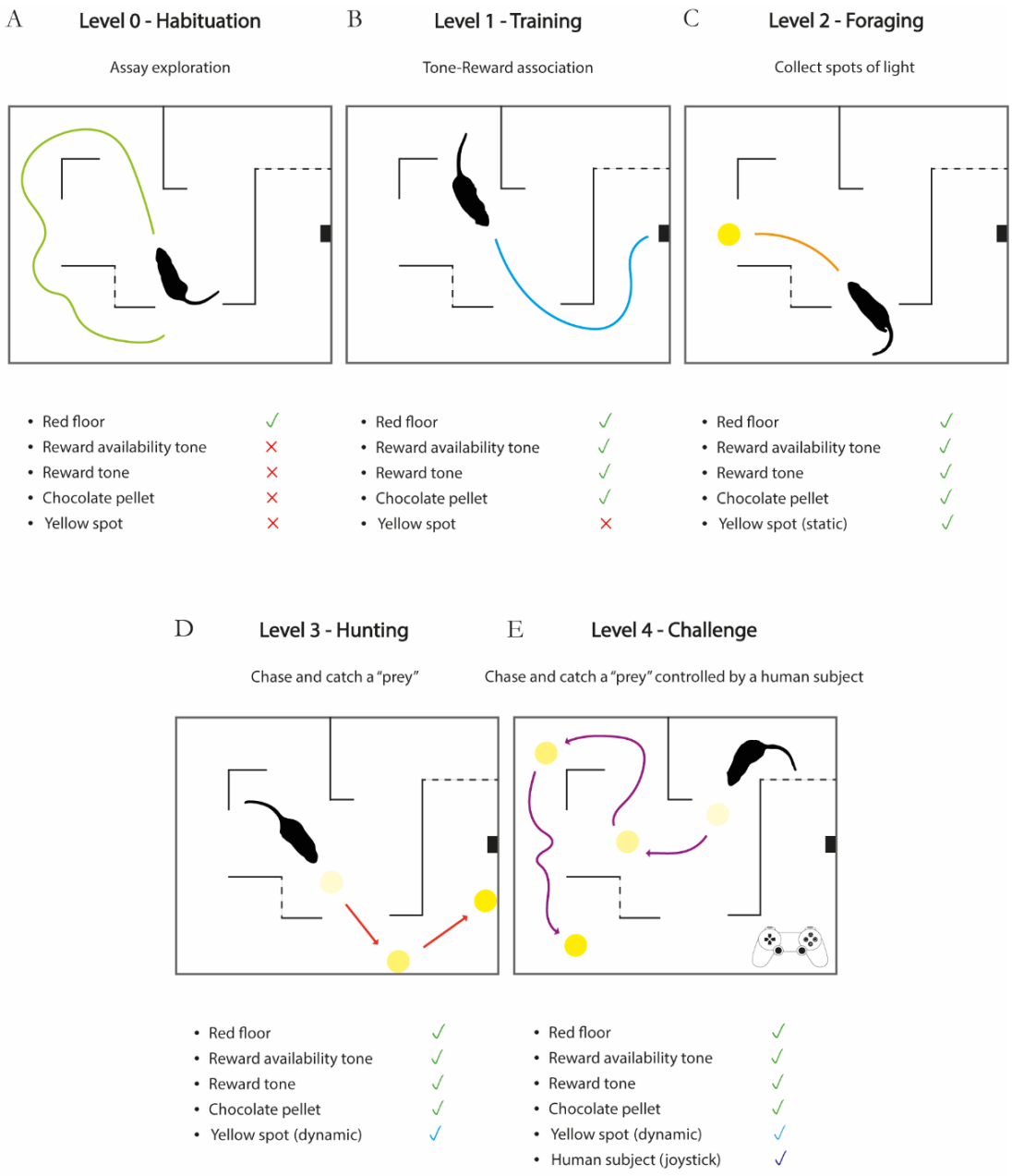


Figure 2.6 Levels visualisation diagrams

A) Visual summary of the Videogame levels. The dashed lines represent the transparent walls in the maze. The black rectangular shape at the right side of the arena represents the reward delivery port. Clips of each level are listed in Table A.1 (Appendix A).

2.3.6 Videogame Software

Despite the behavioural complexity, given by both Videogame hardware and behavioural protocol, we do not want to lose control over parameters of interest, which could provide insights into how our rats and their cortex are playing the Videogame. Our assay posed the challenge of having to control and monitor a variety of different devices and asynchronous incoming data streams. To integrate this heterogeneity in both devices (e.g. high speed camera, projector, sensors and electrophysiology apparatus) and data streams, we used Bonsai, a visual programming language developed within the Kampff Lab. Bonsai was developed to aid scientific research, simplifying and accelerating the development of state of the art experimental designs and to encourage the exploration of innovative and even more challenging paradigms (Lopes et al., 2016). Bonsai is free and open source and can be downloaded at https://bitbucket.org/horizongir/bonsai/. Bonsai allows creating and manipulating graphically each element of the data stream. Each node of the dataflow can be classified as source or combinator. Sources allow access to the raw data streams (e.g., cameras, microphones) while combinators can manipulate the input coming from the sources. To facilitate the process of creating a dataflow (connecting nodes), Bonsai includes visualizers to access the data at each step and debug the flow. Calibration of some of the experimental parameters (e.g., threshold, filtering) is also possible online together with the development of ad hoc nodes matching one's experimental needs. The Videogame, not only required a coordination of multiple sensors and devices but also the development of a trial structure where the visual stimuli, appearing on the projection floor, are controlled by the animal behaviour in closed-loop. In more detail, in Level 2 a projected yellow circle must appear at a random location each trial and disappear when the rat covers it partially with its body, in Level 3 the yellow circle must move away from the rat when the rat is located in its proximity and finally, in Level 4 the yellow circle must be controlled by a human via a joystick.

To implement the levels, frame by frame monitoring of the rat-visual stimulus interaction is required. Despite the high degrees of freedom, using Bonsai, we can have control over the behavioural parameters of interest, which are saved each session of each level.

Input and implementation of the Bonsai workflow was provided by Goncalo Lopes, former Kampff Lab member and developer of Bonsai.

2.3.6.1 Image processing

The raw image (1600 x 1200 pixels) from the 120 FPS overhead camera is initially cropped to only include the red projection floor and a small portion of the black arena outer walls to monitor the rats when rearing. The cropped image is additionally resized to half the cropped size. Using a python transform, a node which allow one to write custom python code (IronPython), the image's channels are split, and the green channel is selected. The choice of working with a cropped, resized, and single channel image was done to facilitate the computation and increase the computer performance needed to record a video at high speed while implementing online closed-loop dynamics and controlling a variety of different hardware simultaneously. The image is converted into a binary image and with a set threshold the rat silhouette can be highlighted, appearing white on a black background. Using a threshold can be noisy and other areas could appear white. Therefore, to guarantee the reliable detection of the rat shape, a minimum area of connected white pixels is set, so that smaller white areas completely disappear. The largest region, the rat, is detected and parameters of interest like centroid, area, orientation, and contour can be externalised, saved for post hoc analysis or used elsewhere in the workflow. The centroid coordinates allow us to track the animal movements during each session while the contour is used by the physic engine (see Videogame Physics) to create a colliding object. The raw full-resolution video is compressed (H.264 codec) and stored in each session folder together with a file containing

the timestamps of each frame and the camera frame counter to detect the loss of frames. Missing entries in the frame counter correspond to the skipped frames.

2.3.6.2 Videogame graphics

Images projected onto the projection floor were generated in Bonsai using the GLSL package. GLSL is the shader language used by the OpenGL graphics driver to leverage the parallel processing pipeline available on modern video cards to render rich and dynamic 3D content in real time. Although the stimuli in this assay were visually simple, the use of shaders for graphics generation and animation allowed for much lower latency, and thus more responsive interactions during closed-loop experiments.

2.3.6.3 Videogame physics

The physical dynamics of the spot of light during the hunting level was generated using a physic engine (ODE) included as an external package in Bonsai. ODE is an open source, high performance library for simulating rigid body dynamics. It has advanced joint types and integrated collision detection with friction. Among other things, ODE is useful for simulating vehicles, objects in virtual reality environments and virtual creatures.

Using a physics simulation in our Videogame assay allows us to both mimic real-world physical behaviours, as well as produce virtual objects/prey with unnatural physics (Figure 2.7), which will ultimately allow to assess whether, and if so how, rats use intuitive physics when interacting with their environment.

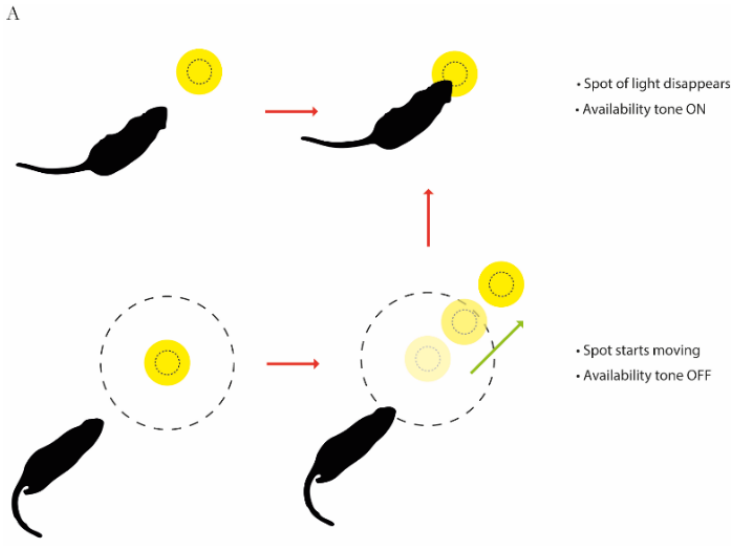


Figure 2.7 Videogame physics schematic

A) Schematic of the mechanism used to detect the position of the rat in respect to the ball and generate the appropriate outcome (contact with the ball or trigger of the ball movement). The dashed circles denote the radius used by the collision detector.

2.3.7 Data Analysis

For the analysis described in the following section, we used the parameters saved by Bonsai in each rat folder for every behavioural session. Custom Python code is implemented and available on GitHub (https://github.com/kampff-lab/Pac-Rat).

For most of the events considered (i.e., touch, catch, reward) we saved the time of occurrence (timestamps) while for others (trigger) we had to detect them post hoc. The tracked behaviour parameters (X and Y rat centroid for each video frame) were also saved, but in shader coordinate space (-1 to 1) so we converted the X and Y body centroid position to match the pixel space of the camera (1 shader unit ≈ 630 pixels). As shader space we refer to the normalised coordinates space that is the coordinate system for the actual imagine being rendered and this space encompasses a cube where the x, y and z components range from -1 to 1. To create the ethogram, we used a custom Bonsai workflow that displayed the recorded video of a session and assigned a keyboard key to each of the behaviours we wanted to annotate, such that by a key press we could directly mark the occurrence. When computing

the speed of the rat around salient trial events, we measured the total Euclidean distance of the rat centroid moved during each 120 Hz video frame (pixels per 8.3 ms) and converted this to millimetres per second in all reported results.

Adam Kampff input was fundamental for the discussion and implementation of the behavioural analysis presented in this chapter.

2.4 Results

Firstly, despite our goal and vision, we needed to assess whether rats could understand and perform any of the levels of the Videogame without food or water restriction. We specifically designed the assay such that the behaviour could be as 'natural' as possible while both monitoring rat behaviour and controlling stimuli in a closed-loop manner. Rats need to understand the rules of each level and adapt their behaviour with the final goal of earning appetizing chocolate pellets. From preliminary tests in a smaller arena, we knew that water restricted rats would learn and engage in the Videogame up to Level 3 or hunting level.

Can rats learn without any restriction? Can we motivate them enough by only providing chocolate pellets as reward? We have trained 12 Lister Hooded male rats (6 months old) and in this chapter we report a detailed description of their behaviour along with the quantification of a variety of behavioural parameters of interest.

2.4.1 Can rats learn to play a videogame?

Level 0

During the first session or level 0, rats have 20' to 30' to familiarize themselves with the assay. Initially, as expected, they tend to behave cautiously due to the novelty of the arena and the overall process of starting and ending a behavioural session (i.e., weighing, delivery and removal from the assay). For these reasons, they have the tendency to spend more time

on either the outer edges of the arena or in a corner as exemplified in Figure 2.8B for a representative sample (n = 3). Additionally, despite the presence of a built-in diffusive material in the arena's floor, it has proven difficult to fully avoid the halo left by the projector located on one side of the arena, resulting in an uneven red colour floor. This feature did not affect the task itself, but we could notice an initial avoidance of the lighter side supposedly perceived brighter and therefore less safe. Nonetheless, by the end of this level, rats explore the entire arena more comfortably, hopping over the maze's walls and marking the territory.

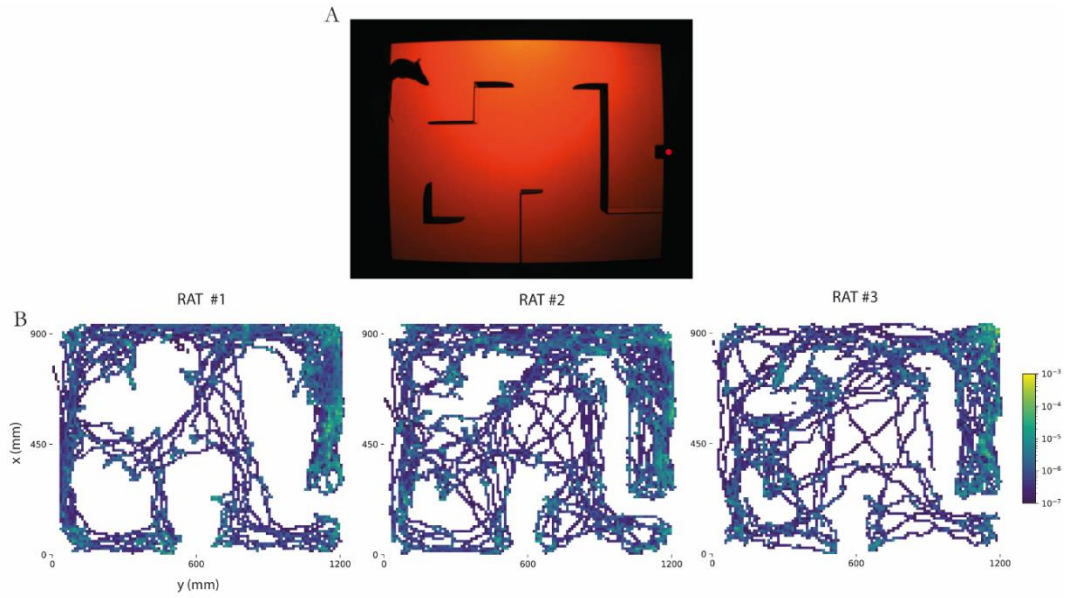


Figure 2.8 Representative tracking from 3 rats during Level 0

A) Top view of the modular arena scaled and oriented to match the tracking plots. The red dot highlights the location reward port for future reference. B) 2D Histogram of the rat x and y body centroid coordinates during Level 0. The bins size is 10 mm and for visualisation purposes the data are shown on a logarithmic scale. After binning the arena, owe report how much time the rat has spent in a bin (occupancy). Values are normalised to range between 0 and 1 on a logarithmic scale.

Before introducing the results of the subsequent levels, the following diagram (Figure 2.9) allows us to define the terminology that we will be using in the rest of the chapter.

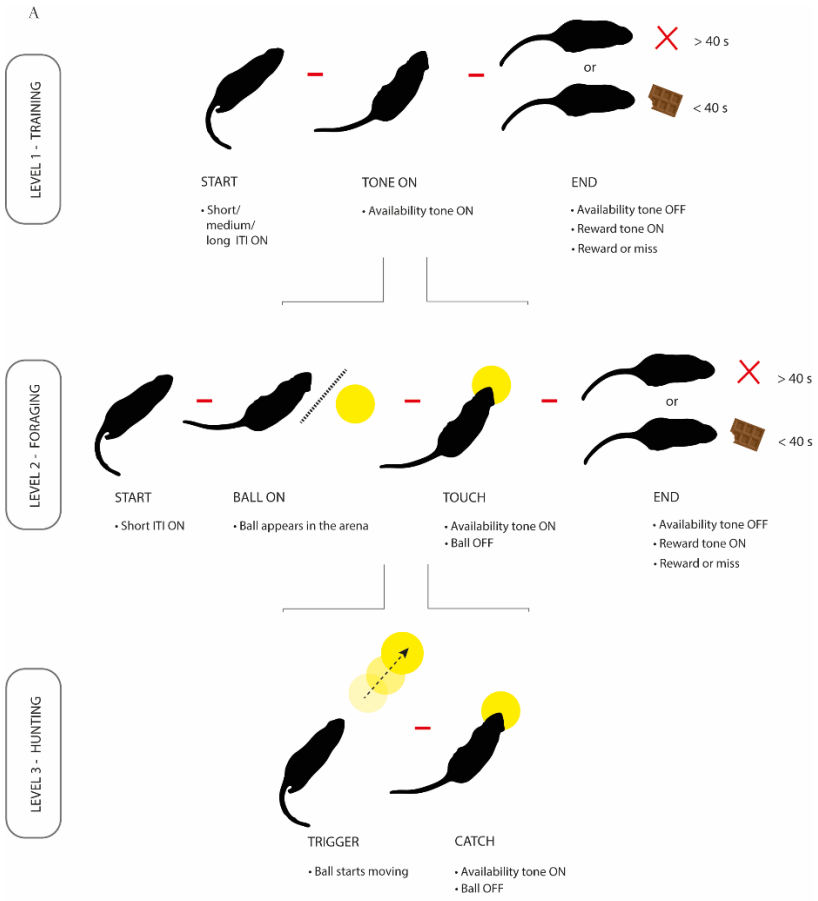


Figure 2.9 Diagram of the trial structure

A) Visual summary of the trial structure of Level 1, 2, and 3. In Level 2 we can identify four main events. Each trial starts with a short ITI after which a yellow ball appears on the floor of the arena in a random location. If the rat partially covers the ball with its body (usually the snout), the ball disappears triggering the reward availability tone. We call this a **touch**. The end of the trial can have two outcomes, either a successful trial with collection of a chocolate pellet or a missed trial if the reward port is not reached within 40 s following the touch. After the 40 s expire, the ITI resets and a new random position is chosen for the ball to appear. A Level 3 trial features an additional moment in which the approach to the ball **triggers** its movement away from the rat. Touch and **catch** are the same physical action, but we do consider them as different phases because they have different meanings due to the nature of the levels, they happen in. After catch the trial ends, as in Level 2.

Level 1

During level 1, rats need to learn the association between the reward availability tone and the presence of a chocolate pellet at the reward port. Each session lasts about 45' to 1h. In detail, while the tone is playing, rats have 40 s to reach the reward port and successfully break the infrared beam sensor which triggers the chocolate pellet delivery. If the 40 s expire and the

rat has not collected the reward, the trial is considered a missed trial. Understanding this step is key for the subsequent levels. Usually, during the first day, we leave one chocolate pellets at the reward port to lure the animals into its proximity. By chance, rats may start to investigate the reward port while the tone plays, which triggers the pellet to be delivered. Over trials this association is established and reinforced.

Surprisingly, despite the lack of motivational drive that could be induced by a regime of food restriction, rats showed a lot of interest in the chocolate reward spending a significant amount of time in the reward port location without exploring the rest of the environment, as highlighted in Figure 2.10 for 3 representative rats.

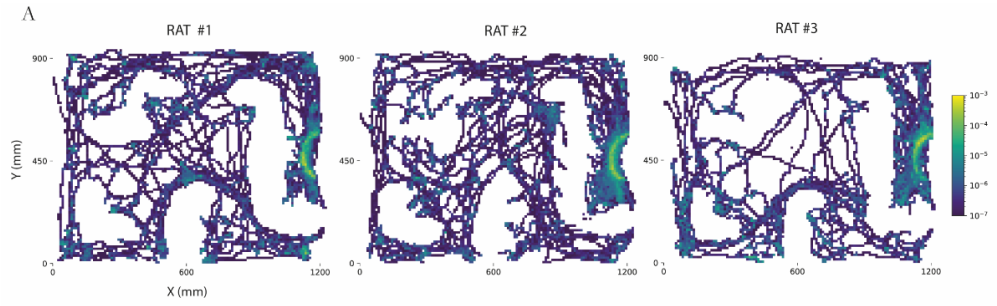


Figure 2.10 Representative tracking from 3 rats during Level 1

A)2D Histogram of the rat x and y body centroid coordinates during Level 1. The bins size is 10 mm and for visualisation purposes data are shown in a logarithmic scale. The lighter semi-circular shape indicates that rats spend more time in proximity of the reward port.

Exploration should facilitate learning of the upcoming levels, thus, in an attempt to limit port-waiting behaviour, we utilised a strategy that has proven successful in the preliminary prototyping phase with water restricted rats. After 4 or 5 sessions with a short ITI, we increased it to medium and long with the goal of reducing the frequency of the reward availability tone onset throughout the session. In so doing, we hoped to drive their exploratory behaviour during these long wait intervals.

To estimate the percentage of time spent at the reward port, we have drawn a ROI (region of interest) around it and counted how much time the rat spent within the boundaries (Figure

2.11A, green outline) during each session. The location was given by the tracking of the rat body centroid. Despite an initial success, this approach did not show the expected result, in fact, the percentage did increase significantly both between session 1 and 4 with short ITIs (p value < 0.05, paired t-test) and between session 4 and 5 with a medium and long ITIs (p value <0.001, paired t-test). Nonetheless this trend did not change during the session with longer ITIs (no statistical significance (n.s.), paired t-test) (Figure 2.11B).

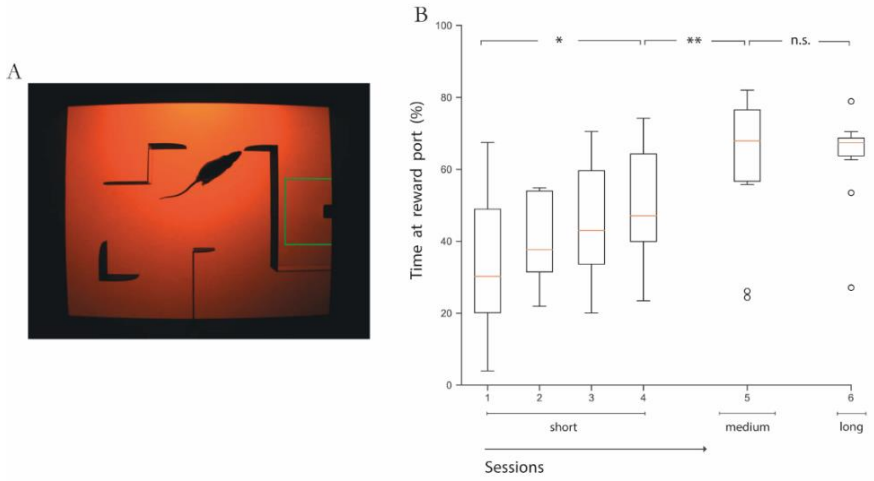


Figure 2.11 Summary of the percentage of time spent at the reward port during Level 1

A) Top view of the modular arena with highlighted the ROI (green outline) used to calculate the percentage of time spent in proximity of the reward port. B) Box and whisker plot shows the range of percentages of time spent within the green boundaries for all the rats (n = 12) and for 6 sessions of Level 1. Sessions 1-4 have a short ITI while session 5 and 6 have a medium and long ITI respectively. The plot shows the outliers beyond the ends of the whiskers. Note that we only show the results for 4 session with short ITI. Session 4, 5 and 6 are not consecutive days. P value < 0.05 (*), p value < 0.01 (**), p value < 0.001 (***), p value not significant (n.s.). Statistical test: paired t-test.

The number of trials accomplished by the rats during each session as well as the frequency are good indicators of engagement in the task.

The performance varies for each individual, but we usually see an increase in the number of completed trials over sessions. As expected, due to an increase in ITI, the trial number decreases during medium and long ITI sessions (Figure 2.12)

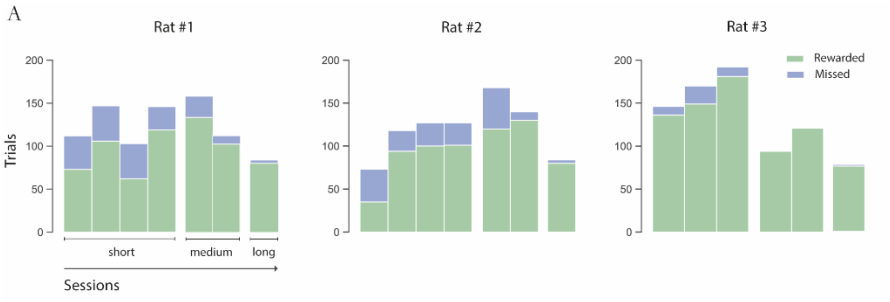


Figure 2.12 Representative trial count changes over sessions

Overview of the number of trials performed during Level 1 for three representative rats. Rewarded trials are shown in green and the missed trial in indigo. The total amount of trials per session is dictated by the ITI thus, a decrease in the number of trials is expected during sessions with longer ITI.

In Figure 2.13, we show a summary of the average trials (mean ± standard error of the mean (SEM)) performed by all the rats during the initial 4 days of Level 1 with short ITI. An overall significant (p value **, paired t-test) increase in the number of trials over sessions is noticeable (Figure 2.13A), from an average of 83.67 ± 13.46 trials of the first day to an average of 135.58 ± 15.61 trials of day 4. On the contrary, the number of missed trials tend to decrease significantly (p value*, paired t-test) (Figure 2.13B)

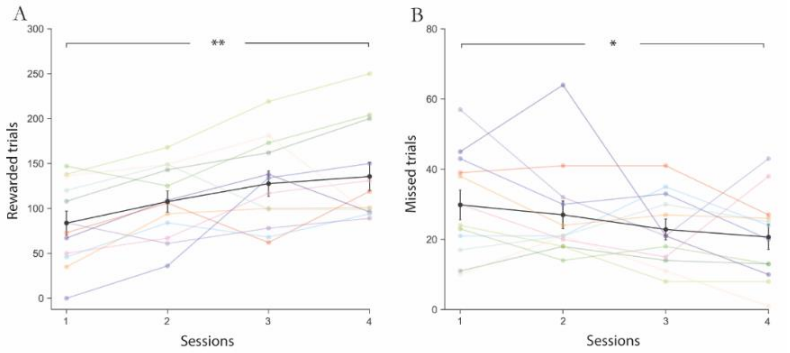


Figure 2.13 Successful and missed trials performed during Level 1

A) Average number of rewarded trials performed during the initial 4 sessions with short ITI for all the rats (n=12). B) Average missed trials (n=12). In colour are reported each individual performance. The results are shows as mean ± SEM. Statistical test: paired t-test.

Rats not only show interest in the reward and promising engagement in the task but over sessions they become significantly faster at collecting the reward after the reward availability tone onset (Figure 2.14).

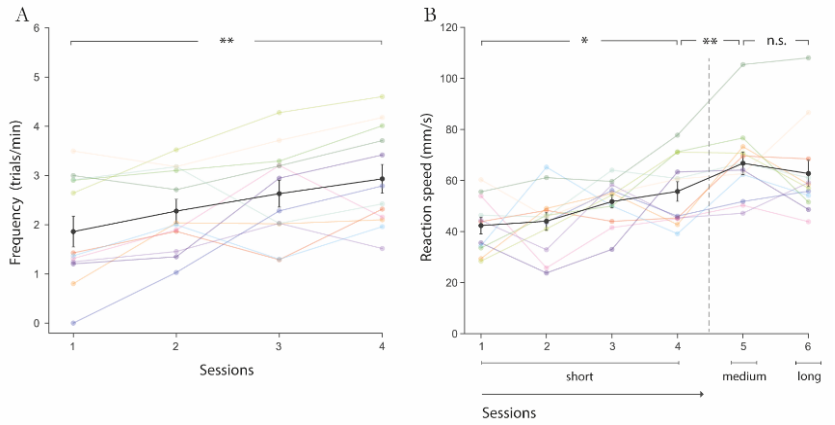


Figure 2.14 Rats' performance during Level 1

A) Average rate of successful trials performed during the initial 4 sessions with short ITI for all the rats (n = 12). B) Average speed to collect reward considering the distance of the rat from the reward port at the availability tone onset (n = 12) is defined as reaction speed. Trials in which the rat position is within the ROI boundaries at the tone onset are excluded. In colour are reported each individual performance. The results are shows as mean ± SEM. Statistical test: paired t-test.

Another important parameter which helped us understanding whether rats did learn an association between tone/reward was given by carefully watching them and monitoring their behaviour during every session. Especially during the first session, it was clear that most animals were just exploring the arena without any knowledge that the tone playing was associated with a reward being available for collection. The more they explored the reward port the higher the chances to break the infrared beam triggering the pellet delivery. Despite preferring to stay nearby the port to have a quicker access to the pellet, rats showed clear interruptions of ongoing behaviours at the onset of the reward availability tone which led to a quick return to the port. These observations were key to comfortably move them to the next level.

Level 2

Contrary to Level 1, during Level 2 the reward availability tone does not play unless the rats engage with a virtual object appearing at unpredictable times and locations on the floor of the arena. As per the diagram in Figure 2.9, this object is called "ball". Earning chocolate

pellets require understanding of the new rule. The sessions are usually 45' to 1 hour long. At the beginning of the first session, despite having learned the tone/reward association, rats usually spent time at the reward port attempting to activate it even in the absence of the availability tone. At times, they also displayed inquisitive and aggressive behaviour toward the port that no longer behaved according to their expectations. After this early phase, they actively explore the arena and by chance, they happen to come across a yellow ball projected on the floor which, if partially covered, disappears, triggering the reward availability tone. Level 2 requires more exploration to forage and collect the balls as shown for 3 representative rats in Figure 2.15.

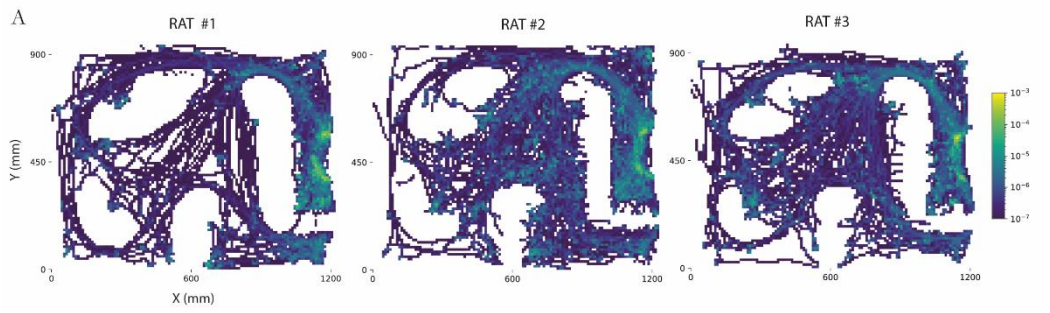


Figure 2.15 Representative tracking from 3 rats during Level 2

2D Histogram of the rat x and y body centroid coordinates during Level 2. The bins size is 10 mm and for visualisation purposes data are shown in a logarithmic scale. Rats spend more time through the only free path that connect the reward port to the arena (top right).

As shown in Figure 2.16A the percentage of time spent at the reward port is less compared to Level 1 and it gradually decreases over sessions. This trend might be linked to a significant (p value***, paired t-test) increase in successful trials from an average of 31.50 ± 3.74 during the first session to 74.33 ± 8.17 of session 5 (mean ± SEM) (Figure 2.16B). Missed trials are very few compared to Level 1 which suggests commitment to collect the reward once the availability tone has been triggered (Figure 2.16C). By the second session (first session for some animals) all the rats show understanding of the rules and engagement in the task.

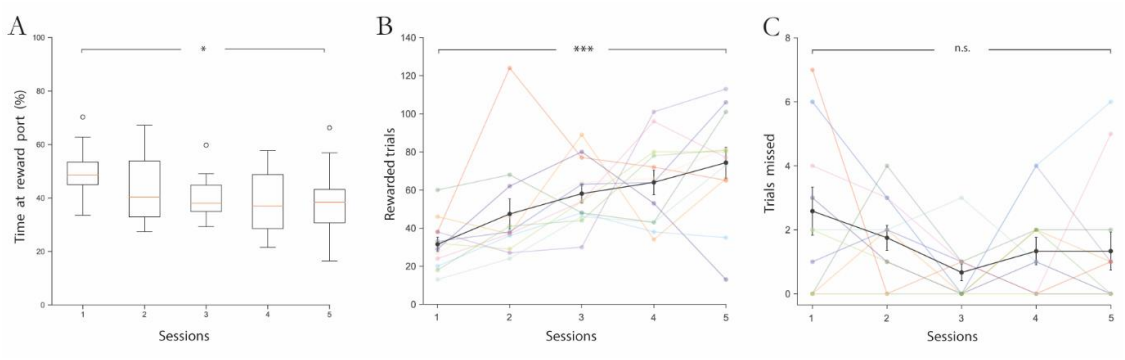


Figure 2.16 Rat performance during Level 2

A) Box and whisker plot shows the range of percentages of time spent within the green boundaries of the ROI for all the rats (n = 4). Outliers are shown beyond the ends of the whiskers. B) Average number of rewarded trials performed by all the rats (n=4). C) Average missed trials (n=4). In colour are reported each individual performance. The results are shows as mean ± standard error or the mean (SEM). Statistical test: paired t-test.

Overtime, along with an increase in rewarded trials performed, rats also significantly increase the frequency at which they collect the reward (Figure 2.17A) and the speed utilised to go back to the reward port after triggering the reward availability tone (Figure 2.17B).

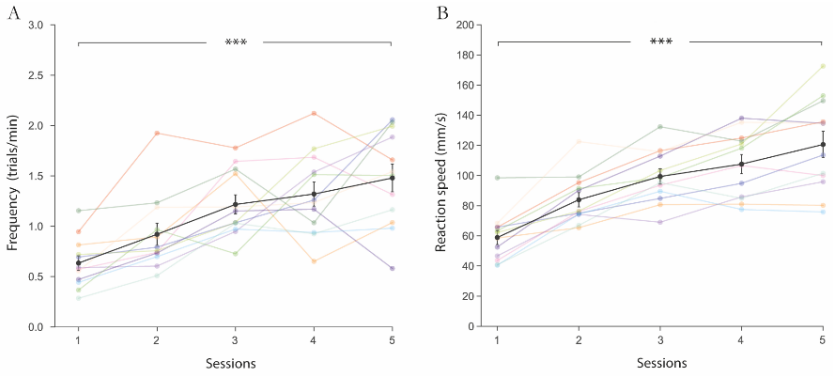


Figure 2.17 Rats' performance during Level 2

A) Average rate of successful trials performed during the initial 5 sessions (n=12). B) Average reaction speed to collect reward (n=12). In colour are reported each individual performance. The results are shows as mean ± SEM. Statistical test: paired t-test.

Failed ball touch trials, in which rats mistakenly think they have covered enough of the stimulus and begin heading to the reward port, only to interrupt their run and go back to the ball, were key moments to ensure that they were learning how to interact with a virtual object.

Level 3

Level 3 features a sudden change in the rules, from searching and "touching" a static yellow ball, to searching and triggering the movemet of the ball that rats must then chase and catch. We developed this level in order to capture the moment of a sudden change in the rules. In fact, during the first session, rats are presented with 5 trials belonging to level 2 paradigm to give the impression that they are performing the level that they are used to play in. We positioned the sixth ball in the centre of the arena to facilititate the recording of this moment both from a camera persptective as well as for the rats to fully experience this event (i.e., the ball has room to move away from the animal without directly encountering a wall and bouncing back).

From this trial onward, each approach to the ball triggers its movement in the direction away from the rat. The ball starts moving away from the animal, bouncing off of the 'physical world' if an obstable is encountered in its path. The speed of the ball decreases over time due to 'virtual' friction.

Exposure to unexpected events triggers behavioural changes

We trained 10 rats in this Level, 6 of which were carrying an 11 shank probe (see Chapter 3, Results section). In this chapter we are only reporting the behaviour of the 4 rats that did not undergo surgery. Strikingly, rats show a common and distinct reaction when they encounter a moving virtual object for the first time. In particular, once the movement is noticed, rats interrupt any ongoing behaviour and remain still in the same position. While the body is immobile, they move their head and ears to follow the ball movement. They show surprise and in some cases they look frightned by this change in the rules of the augmented environment. This seems to be in line with the findings of a former labmate (Lopes et al., 2016) which took place in the physical world. In his shuttling task, rats had to cross an obstacle course to collect water rewards. The sudden change in the physical state of the

obstacle course (from stable to movable steps) was enough to elicit surprise and haulting behaviour in the animals that had to face and deal with an unexpected new rule in the enviromnent. Despite being a visually striking event, it proved to be difficult to visualise in a graph. Therefore, we have made video clips capturing this moment which are listed in Table B.1 (Appendix B) and a simple ethogram for each rat (Figure 2.18).

Firstly, we decided to include only the first 15 trials of the first session, 5 of which have a static ball to touch. For clarity, the choice of having only 5 Level 2 type trials was influenced by a decrease in the rat's behavioural performance post surgery, which we will elaborate on in the next chapter. For consistency in our dataset, we decided to adopt the same protocol for not implanted rats. To be noted that, at the beginning of each session, rats tend to engage in exploratory behaviour with the goal or marking the territory, being temporarely distracted from the task (i.e. ignoring the ball). The marking is accompanied by sniffing, rearing, hopping and rubbing over and against walls to leave their scent. Once this phase ends, rats show committment and engagment in the task that wears off towards the end of the session, probably due to satiation. This is true for each of the levels and could act as a confound in our attempt to create an ethogram. Ideally, to properly capture this event, a choice of about 15-20 Level 2 type trials would be best.

We decided to manually annotate 9 behaviours/events that often appears during each session and they are summarised in Table 1.

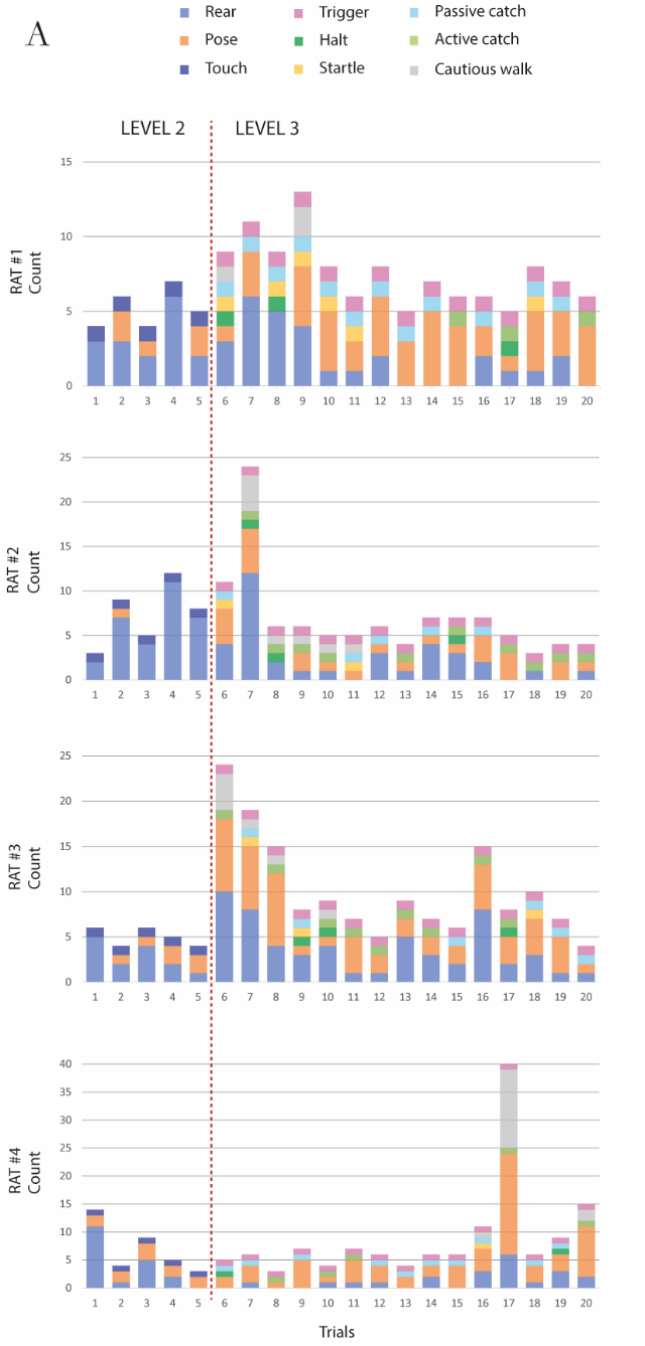
POSE	**Sustained stillness (at least 3 s)**
TOUCH	**Ball disappearance after rat body contact (Level 2)**
REAR	**Upright position or upright leaning on the arena's walls. Only hind paws in contact with the floor**
HALT	**Sudden interruption of any ongoing movement/behaviour**
STARTLE	**Sudden involuntary shock of surprise or alarm**
PASSIVE CATCH	**Ball bounces against the rat (contact and disappearance)**
ACTIVE CATCH	**Ball chased and followed by the rat (contact and disappearance)**
CAUTIOUS WALK	**Slow walk and prudent movement. The body is stretched/elongated and closer to the floor while slowly walking.**
TRIGGER	**Instance at which the ball starts moving**

Table 1 Description of the behaviours/events included in the ethogram

The table provide a guideline on how we defined each of the behaviours/events we looked for while annoating the videos.

Additionally, we thought that having a visual aid could also help, therefore we also added video clips links for each of them in Table B.1 (Appendix B). To create the ethogram, we have used a custom Bonsai workflow which allowed us to manually annotate each video by pressing keyboard keys paired with each of the behaviour.

Figure 2.18A shows our attempt to build an ethogram by counting how many times each of the behaviours of interest occured. We also show the display sequence of each behaviour (Figure 2.18B).


A
Rear
Pose
Touch
Trigger
Halt
Startle
Passive catch
Active catch
Cautious walk
LEVEL 2
LEVEL 3
RAT #1 Count
RAT #2 Count
RAT #3 Count
RAT #4 Count
Trials

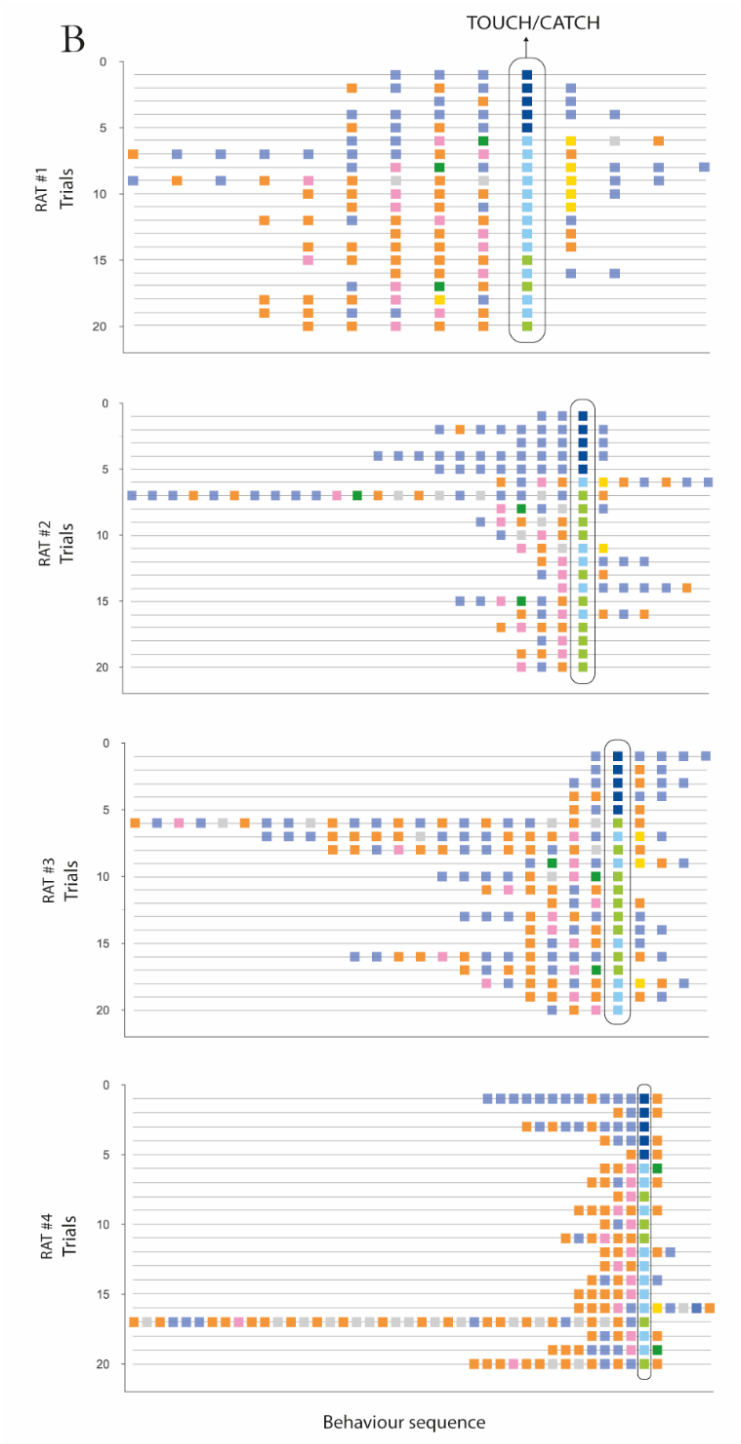


Figure 2.18 Ethogram

A) Ethogram of the first session of Level 3 for 4 rats. The barplots show how many times each of the 9 selected behaviour/event happens in the first 15 trials of Level 3, 5 of which are Level 2 type trials. The red dashed line across the plots highlight the transition from Level 2 to Level 3 trials. From this moment onward the rules change and the ball starts moving away from the rat when approached. At the top of the figure a color coded legend helps navigate throught the plots. B) Behaviour sequence aligned to touch/catch depending on which trial type was performed by all the 4 rats.

Generally, rats show a much more diverse range of behaviour right after the 5th trial. This ethogram does not show how long each of the behaviours lasted for, but, from watching the video, we could notice longer posing and halting at the trigger of the ball. Occasionally, the posing lasted from trigger of the ball movemet until it slowly bounced back at them. Only rat #4 showed a late reaction (trial 17) to the ball when it started to move without being approached, due to a bug in the Level 3 workflow. We also noticed a qualitative difference between the rearing prior to Level 3 trials. In fact, as expected, the initial rearing is predominantly due to exploration and marking of the territory while after the ball starts moving, rats tend to rear or lean on walls to inspect and monitor its movement.

As described earlier, when noticing the ball moving for the first time, rats start behaving differently and act more alarmed and cautious around the arena. For several subsequent trials, rats still display an inquisitive and assertive behaviour. Nonetheless after a few trials, rats seem to adapt to the new rules and engage with the moving object displaying pursuit behaviour. A full list of clips of the first encounter with the moving ball and 2 subsequent trials can be found in Appendix C (Table C.1) for all the rats.

It was important for us to tackle the behavioural analysis from a purely ethological perspective especially to investigate such obvious changes in behaviour that would be difficult to capture otherwise. Nonetheless, manual annotation remains a very labour intensive and time consuming approach and it was not possible for us to analyse any further session out of about 300 hours of videos. Steps towards a more automatised annotation strategy combined with the quantification of several parameters of interest could definitely reveal more insights and test further hyphothesis.

We have evaluated the performace of the rats also with paramenters previously explored for Level 1 and 2 (Figure 2.19).

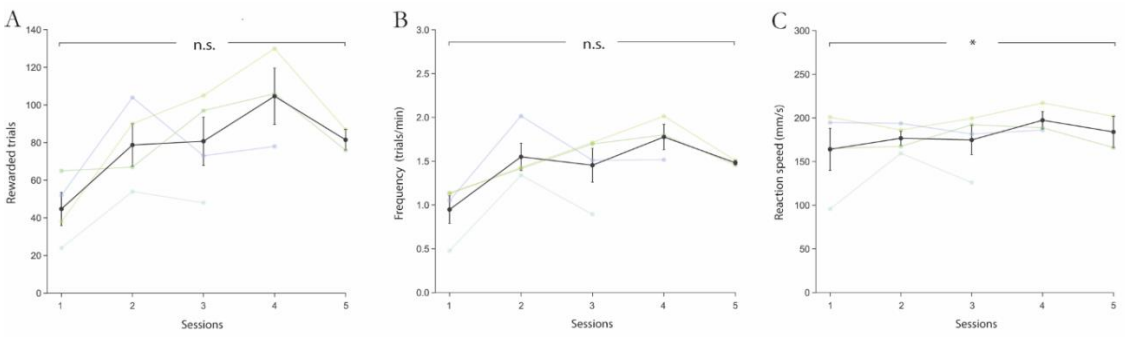


Figure 2.19 Level 3 performance

A) Average number of rewarded trials performed by all the rats (n=12). Rats performed different number of sessions. B) Average rate of successful trials performed during Level 3 (n=12). C) Average reaction speed to collect reward (n=12). In colour are reported each individual performance. The results are shown as mean ± SEM. Statistical test: paired t-test.

Figure 2.19A shows a gradual increase of the number of trials performed over sessions and a similar trend can be noticed for the average rate of successful trials (Figure 2.19B). Interestingly, during the first day, in which rats need to adapt and understand the new rules, trials are almost halved compared to the second session.

Nonetheless the reaction speed remains stable over sessions (Figure 2.19C), but overall and already from the first session, rats are faster to go back to the reward port compared to Level 2 and Level 1. This might be due to the ongoing fast pace of the trial due to the chasing behaviour.

2.4.2 How do rats play the videogame?

We next looked in more detail into different phases within the trial structure to address whether rats could engage with a virtual object, and if so, in which ways. The ball plays a key role in each level and its change in behaviour dictates new rules in the environment. The rats' behaviour and performance are inevitably influenced by these changes, which require understanding and flexibility to adapt to the new circumstances. Can a rat learn to interact with a virtual object? Can a rat change their strategies based on changes in the "behaviour"

of a virtual object? We have explored several aspects of their behaviour in search of the answers.

Firstly, we have divided each trial into different, discrete phases. As per diagram (Figure 2.9), Level 2 trials can be sub-divided into four phases (start, ball on, touch, end), and Level 3 trials into five phases (start, ball on, trigger, catch, end). The time to accomplish each of the phases varies not only according to the rats' behaviour, but also based on the nature of the phase itself. Phases prior to touch or catch do not have a time limit while the time to collect reward is set to a maximum of 40 s. In Figure 2.20A we show the distributions of times for each phase sampling only times lower than 40 s for all the phases. Note that there are instances in which rats can take up to a few minutes to interact with the ball but for purposes of visualisation we only show a subset. The time taken by the rats to touch or trigger the ball seems to have a much broader distribution compared to any other phase. After trigger, rats tend to quickly catch the moving ball. This could be partially due to the arena wall structure that limits the ball movements making it easier for it to bounce back to the rat triggering the availability tone as well as a higher pace due to chasing behaviour. The end phase tends to be faster during Level 3.

We then selected the three main events which occur in both levels: touch, trigger, and catch. Their commonality lays in the fact that in each of them, rats are interacting with a virtual object that happens to have different meaning/consequences. Do rats behave differently with the ball based on which Level and which phase they encounter it?

To attempt to answer this question, we explored their behaviour in close proximity to these interactions. We collected 5702 trials from 12 rats during Level 2 and 1302 trials from 4 of the 12 rats during Level 3. Figure 2.20B shows the change in rat movement speed (delta speed) 1 s before and after the interaction with the ball for all the rats, all their trials and all the events of interest.

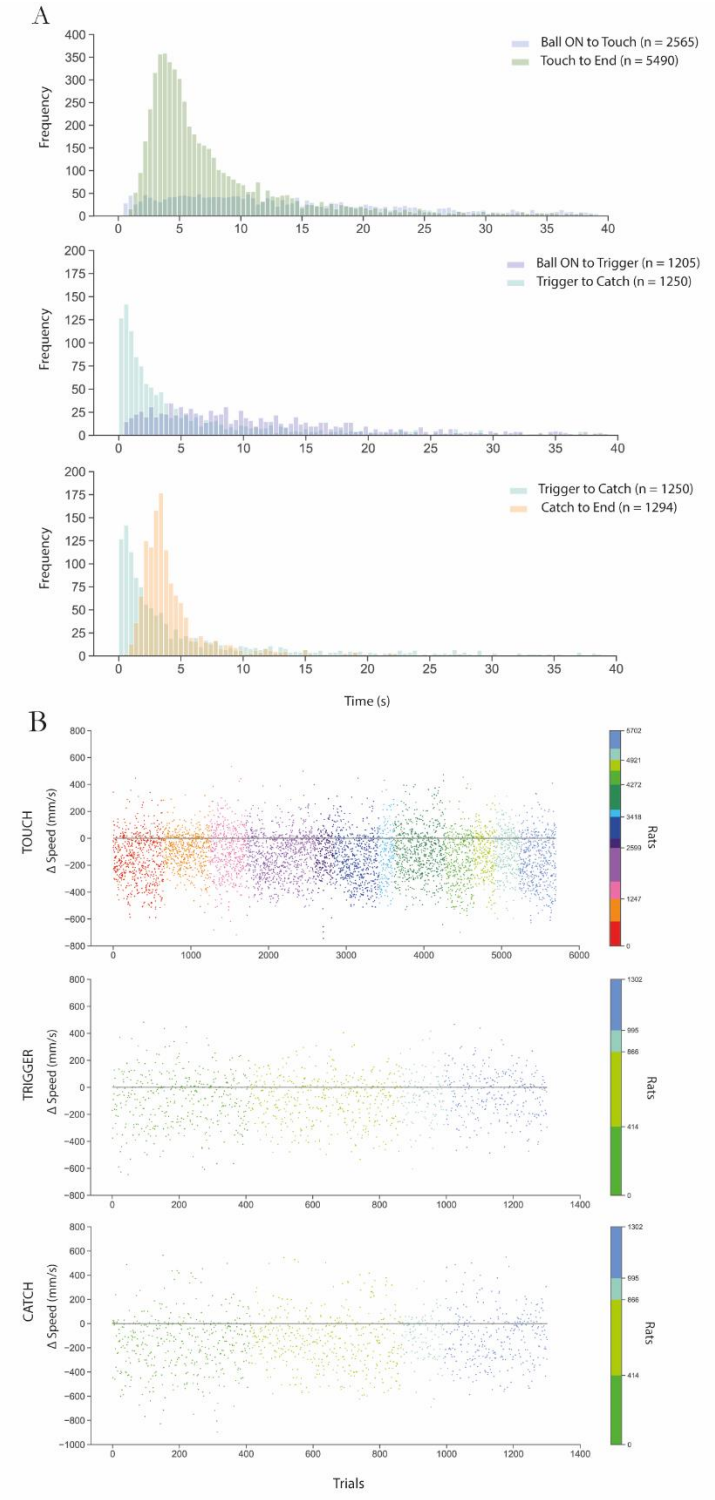


Figure 2.20 Summary speed during different phases of Level 2 and 3

A) Histograms of the time distribution to accomplish different trial phases. Reported only phases with a maximum time of 40 s. B) Scatter plots of the delta (Δ) speed around three main events during the trial: touch, trigger, catch. Delta speed values are obtained by subtracting the distance of the rat from the ball 1 s before and after the event. Level 2: we pooled all the trials (n = 5702) performed by all the rats (n = 12). Level 3: we pooled all the trials (n = 1302) performed by all the rats (n = 4). Each dot represents one trial and each colour a different rat.

The scatter plots suggest that the speed of the rats after the interaction with the ball decreases (halves) irrespective to the event. Trigger and catch belong to the same trial, but they retain a quite different meaning. The first causes the ball to escape from the rat, while the second has a similarity to the touch, but while the ball is still in movement. Therefore, we compared their distributions and rats are moving significantly faster after a trigger, than after a catch (p value***, independent-samples t-test), as noticeable by the higher density of trials above the zero line.

Speed profile adaptation to virtual object properties

We also investigated their movement speed time course around the event of interest, and we show a summary of the findings (median ± SEM) in Figure 2.21.

Firstly, to obtain the speed profile we filtered and smoothed the speed data from each trial to remove outliers and failures in the tracking of the body centroid of the rats during each session. In row #1 we report the average speed profile around touch (n = 5702), trigger (n = 1302) and catch (n = 1302) for all the trials. To be noted, the similarity between touch and catch profiles in contrast to the higher speed of approach and lack of deceleration prior to triggering the ball movement.

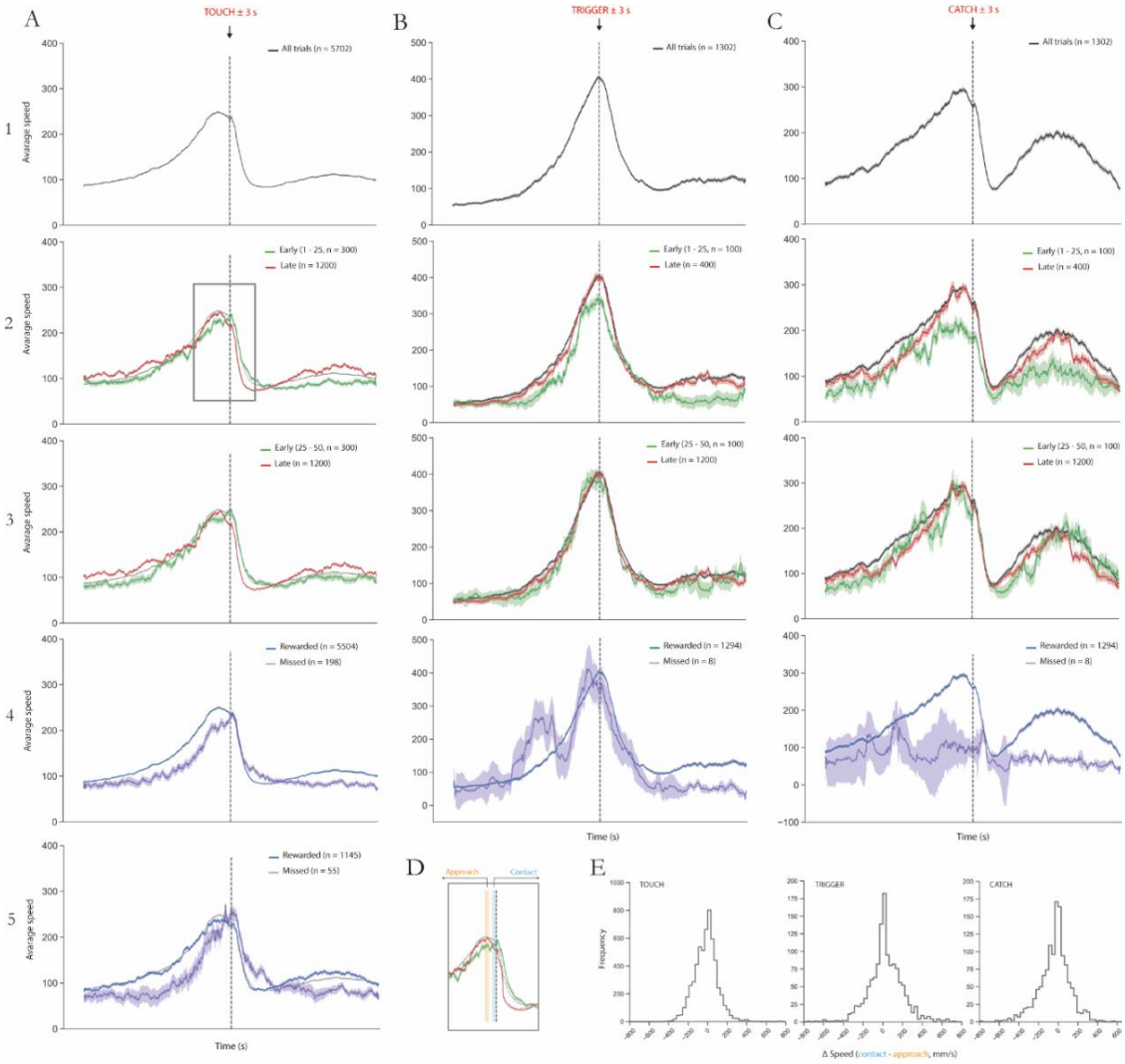


Figure 2.21 Speed profile summary around touch, trigger and catch

A) Average speed profile around touch (Level 2). A1: all the trials performed by 12 rats (n = 5702, black). A2: speed profile comparison between the first 25 trials (green) and the last 100 (red) for all the rats. A3: speed profile comparison between trial 25 to 50 and the last 100 trials. A4: speed profile comparison between successful (blue) and missed trials (violet). A5: speed profile comparison of the successful and missed trials performed in the last 100 trials for each rat. B) Average speed profile around trigger (Level 3). The same comparisons with the same colour scheme than Level 2 are presented. Due to a lack of missed trials the last comparison is not shown. C) Average speed profile around catch (Level 3). The same comparisons with the same colour scheme are presented. Due to a lack of missed trials the last comparison is not shown. D) Enlarged detail of plot A2 highlighting approach (orange) and contact (turquoise). E) Δ speed distribution for all the trials and for all the events of interest. Δ speed has been computed by subtracting the average speed at contact by the average speed at approach.

All the speed profiles are shown as median ± SEM while the Δ speed as mean ± SEM.

Note that the small speed increase visible in most speed profile plots right after the event (right after the dashed line) is an artifact which corresponds to the disappearance of the ball from the floor which momentarily affects the tracking quality, and it is not fully filtered out by threshold and smoothing.

One of the main differences between the selected events lays in the fact that touch and catch require a 'virtual interaction' with the ball causing its disappearance and the reward availability tone onset, while during trigger, the rat proximity to the ball is enough to start its movement. This could justify the lack of slow-down seen during trigger as well as the quicker decrease in speed after touch/catch. Rats also need to orient their body position from the location of touch/catch to efficiently go back to the reward port within 40 s, which intrinsically can require a decrease in speed. Additionally, accuracy in touching the ball guarantees the onset of the availability tone, therefore, a rat might opt to decrease its speed to make the touch more efficient and not having to attempt multiple times.

Furthermore, we wanted to investigate whether in early trials the interaction with the ball would be different, and if over time rats would learn and adopt a more efficient strategy, which does seem to be the case. Figure 2.21 row #2 shows the comparison between early (first 25 trials performed by each rat) and late trials (last 100 trials for each rat on that level). Interestingly, rats tend to approach the ball at a slower pace and without slowing down (touch and trigger). A more prominent decrease of speed is noticeable during catch which is maintained during later trials. In row #3 we show the same profiles but for a different subset of early trials (25-50). By the 50^{th} trial the speed profiles resemble the one of late trials suggesting that rats might have already adapted their behaviour and found their strategy to interact with the ball. This also suggests a deeper understanding of the 'virtual properties' of the ball which change based on Level and trial phase.

Additionally, we looked at the difference of speed profiles based on the trial outcome. Our behavioural protocol does not include a time out for touching or triggering the ball therefore, rats can take all the time they wish to approach the ball that remains in the same position. Row #4 and #5 show that speed profiles differ if a trial will subsequently be missed or rewarded. Due to the low sample number of missed trials in Level 3, we only discuss the results for Level 2 (touch).

During missed trials, rats do approach the ball increasing their speed, but they do not slow down before the virtual contact. It appears that they do not adapt their speed to optimise the contact but instead overshoot the ball. The speed post-touch tends to decay slower compared to trials in which rats quickly go back to the reward port. To confirm this hypothesis, we also show the last 100 trials for each rat whether they were missed or successful. Despite an increase in pace, the profile of late missed trials does remain different in proximity of the touch suggesting that rat might not be as engaged, or we can speculate that they might have decided to not collect the reward even before the touch was made.

Ultimately, we have compared two segments of the speed profiles prior to each event to verify whether there are significant differences in the way rats interact with the stimulus in the various combinations considered (early/late, rewarded/missed). We measure "approach" speed in a 50 ms window 250 ms prior to the event, and the "contact" speed in a 50 ms window immediately prior to the touch, trigger or catch (Figure 2.21D). We computed the change in speed as the difference between the approach and the contact speeds (delta speed = contact – approach), and we show three representative histograms of the delta speed distribution for all trials and all the events. The same results with all the additional combinations are reported in Table 2 as mean ± SEM with their statistical significance (one sample t-test against zero mean).

	ALL TRIALS	EARLY (25)	LATE (100)	REWARDED	MISSED
TOUCH	-16.26 ± 1.60 ***	0.21 ± 6.71 n.s.	-25.75 ± 3.51 ***	-17.04 ± 1.63 ***	6.26 ± 8.25 n.s.
TRIGGER	31.35 ± 4.86 ***	-2.38 ± 13.09 n.s.	31.22 ± 8.52 ***	31.83 ± 4.88 ***	-39.49 ± 30.35 n.s.
CATCH	-38.78 ± 4.53 ***	-30.59 ± 12.55 *	-48.41 ± 7.66 ***	-38.68 ± 4.56 ***	-56.16 ± 27.81 n.s.

Table 2 Table Δ speed summary

Summary of the Δ speed for touch, trigger and catch. Results are shown as mean ± SEM. Statistical test: one sample t-test.

Table 2 confirms that rats have the tendency to interact with the virtual ball decreasing their speed prior to touch and catch while the opposite is true for trigger. It appears that this behaviour is learned over time and does not occur within the first 25 trials performed by all rats. Together, these results suggest that rats learn to anticipate the virtual ball contact by slowing down prior to receiving any external cue (reward availability tone) that contact was made. Furthermore, when the rules of the Videogame change from Level 2 to Level 3, rats quickly relearn a new behaviour for trigger events, better adapted to the task.

2.5 Discussion

We think that the ability to bring controlled complexity into the laboratory is key for the study of behaviour. There are many ways in which one could achieve this goal. We opted to use a videogame. We took inspiration from a quite common feature of many videogames that have levels of varying difficulties, rules to learn, and sudden events that require a quick change in behaviour and strategy. This resembles the demanding and complex tasks animals face daily in their environment. We tried to incorporate this concept into our set-up and developed a series of levels to challenge our animals. In this dissertation we particularly focused on designing a Videogame with levels to mimic foraging and hunting behaviour using augmented reality.

As proof of principle, we are able to show that rats can engage and learn a variety of tasks and we can monitor their behaviour in detail, gathering a rich behavioural dataset. In this section we will be discussing the important outcomes of this experience and provide suggestions for future improvements.

Videogame assay: a robust and flexible tool for probing rats' behaviour

We initially prototyped a small version of the videogame to test whether we could implement any of the goals we set out to achieve. The initial experiments proved to be successful, and we decided to step up the videogame assay. Despite initial challenges in scaling up the sizes of the screen the set up resulted in a robust but flexible assay for quickly prototyping a huge variety of behavioural protocols. Its characteristic modularity is key for easily changing configurations and testing new ideas. We equipped it with a bigger arena and the addition of a maze structure proved to be a more engaging and interesting setting for our rats, allowing for a wider range of behaviours to take place. Our choice of sensors was tailored to our needs, but any modular custom tile can be designed and easily incorporated into the arena's walls. Ideally one could add different reward ports (water or food), servo motors with platforms to hop over or odour ports. The floor could also be enriched with a variety of mazes and a mixture of real and augmented objects to interact with or avoid. We are satisfied with the level of complexity we could achieve, and the potential shown by the Videogame hardware and software. Bonsai was key for the development of the augmented reality, which also give us endless opportunities to develop new ideas and tasks. Additionally, Bonsai also coordinates all the sensors that we used, and it is able to interface with many others if needed.

Videogame assay: what could we improve?

The overall Videogame structure allowed us to implement the behavioural complexity we wanted to achieve, but there are certainly changes one could do to improve some aspects or ease its use. The main change would be in the projection floor. We have mentioned how to build a small and big projection table, but nonetheless both retain the same issues with light diffusion. The presence of the projector does affect the overall floor projection creating a halo on one side of the arena that is visible from the camera view. This could be addressed by using more modern screen options or sandwich diffusive material to help diffusing the

light even further. Adding more cameras could give different points of view and insights into how the rat performs the behaviour. Interestingly, we noticed a change in whisker rate in proximity of the projected object, but it was not possible to capture this event from the point of view of our overhead camera but adding different camera views could help elucidate how they interact with virtual and real objects. Furthermore, we could gain more information to add and enrich our ethograms.

Videogame levels: software defined behavioural tasks

As mentioned, Bonsai not only has coordinated the camera and sensors in our assay but also was responsible for the projection dynamics and closed-loop features of each of the levels designed. Importantly, Bonsai adds the flexibility in terms of tasks that we can develop without having to change the physical structure of the assay. Rats showed us that they can quickly learn the core rules of the task (i.e., Level 1 availability tone-reward association) as well as the interaction with the virtual object projected on the floor. Rats not only learn how to interact with a virtual object, but also adapt their interaction based on the object's behaviour (speed profiles). The only drive to play is earning a chocolate pellet for every trial accomplished until satiation. The lack of water or food restriction was an important step that we decided to take towards promoting a more naturalistic behaviour, and we were surprised to see some of our rats performing 80-100 trials per sessions. It would be interesting to compare the rats' performance with and without restrictions, however, the lack of restriction has also proven useful in transitioning rats to neural recording sessions following surgery (see Chapter 3).

Interestingly, the unexpected transition of the ball behaviour from level 2 to level 3 elicited very striking bodily reactions. This was true for most of our rats that showed temporary signs of surprise and general alert and cautious behaviour. Including other "unexpected" events in our tasks is easily accomplished with a small modification of the software.

Rats not only can play all the levels up to hunting level, but they can also play Level 4 or challenge. However, we have not expanded on the findings for Level 4 throughout the dissertation. The reason for this choice, was that we used this level to further experiment and explore what rats could do. During this level, a human subject was responsible for the ball dynamics via an XBOX controller. The reasoning behind this level was to try to explore how rats would adapt their strategy to a smarter prey compared to the simple bouncing behaviour of the level 3 prey. At the same time, this level was also designed as a challenge for humans, learning to navigate a maze with a joystick with or without physical walls while escaping from a rat. This level lacks the design rigor compared to the other levels as we did not have a cohort of human subjects and a proper protocol for it. I, lab members or curious colleagues tried to play 'against' the rats and did not always succeed. We added a few representative video clips to show this level (Appendix A, Table A.1). As per the video clips, it is clear how engaged and excited rats are to play, as well as how difficult manoeuvring the ball is for the experimenter. It is definitely worth expanding this level in a rigorous setting, as the behavioural interactions displayed by the rats were quite different than with Level 3's simpler prey.

Videogame levels: what could we improve?

Our protocol design together with the outcome of our rats' behavioural performance, are promising and proved that we can engage rats without restrictions in playing levels of different difficulties. Several changes could improve upon these results. Firstly, to increase the trial number per session, we could halve the pellet size to delay satiation. Additionally, we were bound to work with 6 months old rats, due to the layout of our multi-shank silicon probe (which required a large, mature skull surface, see Chapter 3), but younger rats are a good alternative to increase the trial number as they are generally more active. Designing social or cooperative tasks with conspecific or with an artificial agent (i.e., robot) could also

be key for maintaining the rats' engagement longer in the task and to explore interesting behavioural dynamics. Finally, a mixture of both real objects and virtual objects, with altered closed-loop dynamics could increase the controlled complexity of the tasks.

The powerful combination of videogame hardware and software can open endless routes to explore behaviour with increasing, yet controlled, complexity at many levels gradually approaching the rich diversity of the natural environment for which these animals' behaviour (and brains) evolved.

2.6 Conclusions

We successfully built and tested a novel assay for rats to engage them in complex tasks. We used augmented reality to project a yellow ball to forage or hunt for. The behavioural protocol used, allowed rats to learn, within a week, the basic rules of the task without any motivational aid. We assessed their behavioural performance through key parameters like trial count and trial frequency and both suggest successful learning of each level.

Additionally, we analysed the speed profile at key events during the levels (i.e., interaction with virtual object presented), and discovered that rats adapt their behavioural outcome according to the level they are in. Rats tend to slow down before contacting the ball when it is to forage while they do not when is to hunt. Reliably, a successful catch, requiring contact with the ball, show a speed profile like in the foraging task. Rats adapt their behaviour to the level they are playing in suggesting understanding of the properties of the virtual object in that level. Moreover, a simple ethogram highlighted interesting behavioural changes due to the first encounter with an unexpected event, the virtual object moving for the first time.

Overall, Videogame hardware, software and behavioural protocol were successfully designed and tested, and a comprehensive preliminary behavioural analysis and anecdotes are reported in this chapter.

Chapter 3

Cortical recording during complex behaviour

3.1 Abstract

The desire to record from many neurons led to the development of increasingly dense probes. Such recordings gather a large amount of data, but usually only allow us to investigate a few brain areas at once, usually in a columnar, linear fashion. There is a need for extending this approach to be more distributed to reach a broader range of areas. Towards this goal, we designed, fabricated, and implanted a custom 11 shanks passive silicon probe spanning a large portion of the cortex and were able to sample different cortical areas simultaneously. Despite the challenges concerning the probe holder and the surgical procedure itself, here we have shown that it is possible to implant 11 shanks in the cortex and have presented preliminary findings that provide and intriguing overview of the distributed cortical activity of rats playing the videogame levels.

3.2 Introduction

To gather insights and understanding into how the brain works, we have to observe neural activity at many different scales, ranging from individual neurons to brain areas (Ma et al., 2019). In vertebrates, cognitive functions are likely to be mediated by networks of many interconnected neurons and thus should be addressed with adequate methods. An ideal method to observe the brain dynamics would have to monitor many neurons, have a high spatial and temporal resolution, enable access to multiple regions across the brain, and be compatible with awake freely behaving subjects (Chung et al., 2019). As previously mentioned in Chapter 1, great effort and technological advances have been made in the past

decade towards this common goal. Highly dense, single shank, silicon probes (Neuropixel, Jun et al., 2017) have been developed and are now widely used by the neuroscientific community. Despite allowing to record the activity of hundreds of different neurons, located in different areas, the majority of these devices access the brain in a columnar fashion (Juavinett et al., 2019; Liu et al., 2020). In addition to exploring the brain activity of vertically aligned neurons, there is the need to also spatially distribute electrodes horizontally. Our goal is to try to investigate the cortical involvement and dynamics while rats perform new complex Videogame tasks. How can we access and monitor a large portion of the rat cortex? The tool we developed to try and achieve this goal, is a custom 11 shanks, 121 channels silicon probe that allows for recordings at a transcortical scale. The probe spans 1 cm of the cortex, anterior to posterior, it conforms to its anatomical thickness and can record from every cortical layer simultaneously. The length of the probe allows us to target and sample the neural activity from visual, parietal, motor, and frontal cortex. Conveniently, we think that these cortices play a key role in the Videogame's levels that we designed, them being visual-motor tasks with auditory and visual cues where rats need to understand rules and make the right actions to earn a reward.

The volume of the cerebral cortex of a rat is about a hundred times smaller than that of the cerebral cortex of macaques, and about a thousand times smaller than that of humans (Uylings et al., 2003). To briefly touch on the cortical areas aforementioned, the frontal cortex is especially important for planning appropriate behavioural responses to external and internal stimuli. The frontal cortex integrates complex perceptual information from sensory and motor cortices as well as from the parietal and temporal association cortices to perform cognitive tasks (Miller, 2000; Carlén, 2017). In humans, injury to the frontal cortex or deficits in its function are associated with impairment in planning and executive functions, personality changes and reduced creativity. Additionally, several psychiatric diagnoses have

been associated with alterations in frontal cortical function, such as schizophrenia, depression, and obsessive-compulsive disorder (Buchsbaum, 2004; Siddiqui et al., 2008).

The visual cortex is the primary cortical region of the brain that receives, integrates, and processes visual information relayed from the retina. Monkeys and cats have been the preferred animal model to study vision and more than 30 separate visual areas with many functional differences have been defined (Felleman et al., 1991). These areas are organized in a hierarchical way, so that information that enters cortex in primary visual cortex (V1) is processed in multiple steps. Hierarchical streams exist in the primate, such as the ventral pathway, important for object recognition, and the dorsal pathway, critical for the link between perception and action (Mishkin et al., 1983; Sheth & Young, 2016). Subsequent work has shown that also rodent V1 shares many of the features found studying monkeys and cats. The mouse, in particular, has emerged as a model system in which the modern genetic toolkit can be leveraged to probe the mechanistic basis of the visual system. For instance, like cats and monkeys, mouse V1 contains a topographic mapping of visual space (Wang et al., 2007; Smith et al, 2010) and has orientation-selective cells (Ohki et al., 2005; Niell et al., 2008; Niell, 2015).

As mentioned in Chapter 1, motor cortex function has been at the centre of debate. In 1870, physicians Fritsch and Hitzig electrically stimulated, what we nowadays know being the motor cortex, in awake dogs, eliciting different movements based on the location of the stimulation (Lazar, 2009; Hagner et al., 2012). The importance of this discovery was threefold: it was the first demonstration of cortex devoted to motor function, the first indication that the cortex was electrically excitable, and the first evidence of a topographically organized representation in the brain (Gross, 2007; Taylor & Gross, 2016). Motor cortex is widely believed to play a central role in both motor skill learning and execution but, in most mammals, the behavioural effects of motor cortical lesions are remarkably subtle and simple

behaviours (e.g., locomotion) persist even after partial or total removal of motor cortex (Kawai et al., 2015; Lopes et al., 2016; Ebbesen et all., 2017) .

Finally, since mid-90s, a parietal cortex was described in the rat based on neuroanatomical and cytoarchitectonic characteristics (Krieg, 1946). Parietal cortex occupies a unique position in the brain at the interface of perception and representation. It plays a role in multimodal processing and, as a result, it is essential for many cognitive processes (Save & Poucet, 2009; Fitzgerald et al., 2011). Its associative function is provided by the pattern of corticocortical connections, in fact, the parietal cortex receives inputs from various sensory regions including the somatosensory cortex, primary and secondary visual cortex, and the auditory cortex. It is also connected to cortical regions involved in goal-directed behaviour such as the orbitofrontal, and medial prefrontal cortices (Andersen et. al, 2009; Akrami et al., 2018; Lyamzin et al., 2019).

This brief description interlude on cortical areas is to provide an idea of the variety of information that we can gather by targeting such extensive portion of cortex. Nonetheless, we emphasise that the nature of this project remains exploratory, and we believe that being able to sample the cortical activities from many different cortical regions simultaneously could enable a more detailed description of the role and contribution of this structure to behaviour. This unusual probe layout does come with challenges. In this chapter we present an overview of how we tried to tackle them and successfully record from a large portion of cortex in freely moving rats playing the Videogame.

3.3 Methods

3.3.1 Transcortical 11 shanks probe

Simultaneously recording from different cortical areas required a brand-new custom passive silicon probe design. Firstly, an 11 shanks, 121 channels probe 3D model was created using Autodesk Inventor. The aim of this design was to guarantee that the length of each shaft would fit within the cortical thickness and in so doing at least one to two electrodes would ultimately be placed in each cortical layer. To precisely target the cortical regions of interest, a 3D model rat brain, described by a colleague (Dimitriadis et al., 2014), was used as reference. The probe layout and size were designed to be implanted into fully grown adult rat brains. The target coordinates were decided in order to maximize the number of cortical areas from which to record from. Ultimately, the probe was inserted in the right hemisphere circa 2.9 mm mediolaterally (ML) and the first shank was placed circa 2 mm anteroposterior (AP). By doing so, based on the rat brain Atlas (Paxinos G.; Watson C., 2007, 6th Edition), we could aim to record from 7 different cortical areas simultaneously (Table 3). The shank's pitch (centre to centre) was 1 mm for a total probe length of circa 1 cm (Figure 3.1). To follow the cortical outline, the 11 electrodes on each shank, had to be positioned in a linear fashion but at different distance from each other, making each shank unique for its target coordinates. For details on shanks lengths and pitch of the electrodes per shank see Table 3.

Shank (AP)	1	2	3	4	5	6	7	8	9	10	11
Cortical area	M1	M1/S1FL	S1HL	S1HL	S1HL	S1Tr	LPtA/MPtA	V2ML	V2ML	V1M	V1M
Shank length (µm)	4808	4276	4108	3971	3483	3127	2874	2831	2989	3004	3198
Pitch (µm)	240	210	200	200	170	160	140	120	140	140	140

Table 3 Probe specifications

From left to right (rostro caudal) a summary of each shank target cortical area, total length and electrodes pitch. The longest, with bigger electrodes distance is the frontal most shank while the shortest (thinnest cortical area) is shank 8. Estimated target areas: Primary motor cortex (M1); Primary motor cortex/ Primary somatosensory cortex, forelimb (M1/S1FL); Primary somatosensory cortex, hind limb (S1HL); Primary somatosensory cortex, trunk (S1Tr); Medial/Lateral parietal association cortex (MPtA/LPtA); Secondary visual cortex, mediolateral area (V2ML); Primary visual cortex, monocular area (V1M).

The probes were built and assembled by ATLAS Neuroengineering (Leuven, Belgium) and featured 11 equally spaced shanks, as per design, 4 ultra-flexible polyimide cables interfacing with 4, 36 pin Omnetics connectors (Herwik et al., 2011) (Figure 3.1A). Each pair of connectors mated with one 64 channel Intan Headstage (Intan Technologies, #C3315 and #C3325 with accelerometer, US) for a total of 128 channels, seven of which were not connected. In this work 4 probes with Platinum (Pt) electrodes and 2 probes with Iridium Oxide (IrO_2) electrodes were chronically implanted in freely moving rats. The length of the surgical procedure together with the overall probe size and custom 3D printed implant (see Probe holder design), influenced the preferred gender and age of the rats. Older and bigger male rats (about 500 g) could more comfortably undergo a long surgical procedure (6-7 hours) and carry a 3D implant with two wearable headstages embedded in it. Additionally, a combination of longitudinal MRI measurements and histological tissue characterization has shown, in male Wistar rats, that the cortex final thickness and brain expansion are reached within 2 months from birth, but full neuro development is reached around 6 months of age due to late cortical myelination (Mengler et al., 2014). Taking all these factors into consideration we decided to use adult male Lister Hooded rats of about 6 months old for our experiments.

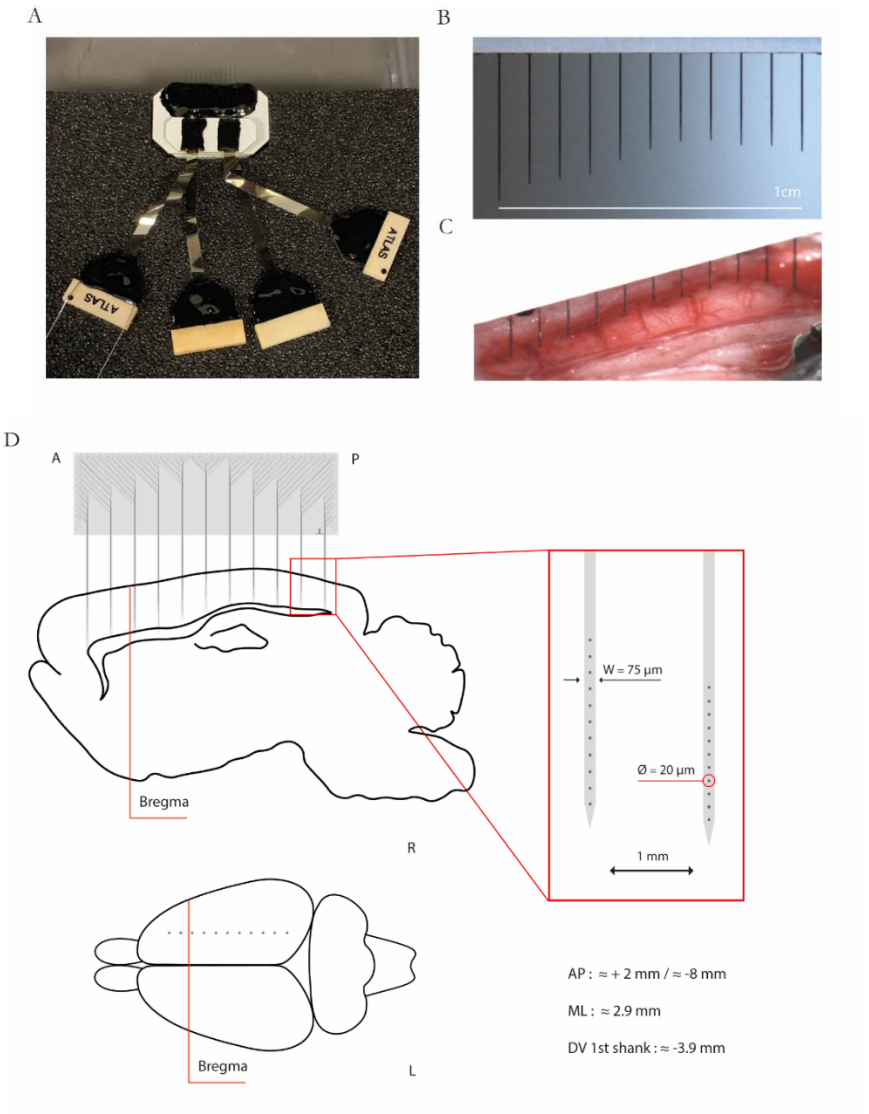


Figure 3.1 11 shanks probe

A) Probe main body with 4 polymide cables and 4 omnetics (32 pins each). Reference wire visible on the leftmost omnetics. B) Picture of the 11 shanks probe layout. C) Example of complete probe insertion trying to avoid bursting superficial cortical blood vessels. Visible: a long (>1 cm) craniotomy. D) Diagram illustrating the probe insertion target (sagittal and dorsal view). Two representative shanks are highlighted in the red box.

3.3.2 Probe coating

Despite the substantial technological advances in hardware and design, chronic probe applications remain challenging. The reliability and stability of the recordings tend to deteriorate over time due to a variety of factors, from the quality of the surgical and implantation procedures to the body reactions after insertion. As the neural probe is inserted

in the brain, a progressive inflammatory tissue response takes place and it is considered one of the main reason for a fluctuation and increase in impedance over time (Kook et al., 2016; Kozai et al., 2015). Impedance is a measure of the ability of a circuit to resist the flow of charges across the electrode-solution interface, in this case electrode-tissue interface. After implantation, the measured impedance on all electrodes usually increases after the third day due to immune response of cortical tissue to the implanted silicon probe (Kook et al., 2016; Ludwig et al., 2006). The impedance of the electrodes at 1 kHz is considered biologically relevant and often used as performance metric in detecting the activity of individual neurons (Ehrlich, 2015; Won et al., 2018). High electrode impedance may affect data quality in extracellular recordings. If silicon probes with high impedance electrodes (> 2 MΩ at 1 kHz) are used, a voltage divider will be created when using a differential amplifier system, as the one from Intan Technologies, which has an input impedance of 13 MΩ at 1 kHz. For example, assuming electrode impedances of 2 MΩ and 100 kΩ, the signal loss is around 15 % and 1 %, respectively. In our experiments, the electrode impedance is decreased by using conductive polymers before implantation to prevent this phenomenon. Biocompatible conductive polymer coatings can be used to modify the electrode recording sites improving their long-term performance after chronic brain implantation. The microelectrode sites on the neural probes are the actual interface to communicate with neurons. The goal is to create an interface that can optimise and facilitate the signal transport at the neuron-electrode level (Cui & Martin, 2003). Prior to surgery the 11 shanks probe was coated with a solution of poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS). The PEDOT:PSS electrodeposition protocol is a simple, stable, and reliable method for decreasing the impedance of a microelectrode up to tenfold (Neto et al., 2018). PEDOT:PSS is electrodeposited onto electrodes due to its chemical stability and mechanical integrity when implanted in the brain (Ludwig et al., 2006). Moreover, when compared to metals, these polymers are typically softer materials offering a more intimate contact between the electrode

surface and brain tissue (Green et al., 2008). The coating was deposited using a galvanostatic electropolymerization process with a Pt wire as reference electrode. The reference wire was placed around the edge of the deposition chamber and the probe shanks were positioned in the centre. The deposition was performed, 32 channels at the time, using Nano-Z kit and software (Neuralynx, USA). The deposition solution consisted of 0.01 M EDOT (Sigma-Aldrich, 97 %, M_w = 142.18) and 0.1 M of PSS (Sigma-Aldrich, M_w = 1000000) dissolved in deionised water. At first, the shanks were immersed in the chamber filled with saline solution (0.9% NaCl) and one omnetics was connected to the Nano-Z. Baseline impedance measurements at 1 kHz were recorded and the shanks thoroughly cleaned with deionised water before coating. The shanks were coated in 'fixed plating time' mode with the following optimal parameters: 1004 Hz, 30 nA, 5 s duration, 2 s pause. After the PEDOT:PSS deposition, the final impedance was measured in saline and the probe cleaned with deionized water. The same procedure was repeated for all the omnetics. The impedance magnitude was routinely measured using a protocol implemented in the Intan evaluation board (Intan Technologies, RHD200, US). The impedance was recorded right before implantation, directly after surgery and daily, before each behavioural assessment.

3.3.3 Probe holder design

The 11 shanks probe design required a custom-made holder to be easily carried by freely moving rats performing in the Videogame arena. The 3D model was designed in Autodesk Inventor and printed (Stratasys J750 poly-jet, Vero white). The final design allowed for two 64 channels Intan headstages to be temporarily embedded within the structure and retrieved at the end of each experiment. The choice of integrating portable headstages, as a refinement method, aimed to ease the tethering experience at the beginning of each behavioural session both for the rats and the experimenter. In our design, we reached a balance between limiting a stressful process for the animals and to avoid adding too much weight on their head. In

doing so we created a lightweight holder featuring three main components: a base, a movable arm and two headstage cases (Figure 3.2). To guarantee a good alignment of the probe, we used three different tools, two of which included in the 3D printed design. All the part numbers highlighted in this section refer to Figure 3.2. The base is composed of five parts, four of which (#1 to #4) have accessory functions and will ultimately be removed from the final implant. In detail, a thin rectangular surface (#1) act as first implant alignment tool. The holder is attached to the stereotaxic arm by inserting two optical posts in the 3D printed post holder (#3). The posts are connected with a compact kinematic mirror mount (Thor Labs, #KMS/M, USA) used as additional alignment tool. The probe is easily slid and locked into the guiding grooves designed in #5, which is the main body of the base, ultimately carried by the rat. The movable arm, host the paired omnetics and provide support to the headstage cases. Pivoting around a pin, inserted in the base (#7), the arm also allows to stretch the ribbon cables of the probe during surgery to easily access the probe main body while building the implant with cement. At the end of the surgery the arm is fully moved medially, close to the probe, and cemented in position. The headstage case protects the Intan headstages and sit on movable arm. They feature a thin (0.5 mm thick) sleeve (#9) with guiding grooves to allow the headstages to slide in straight and a cap (#11) to secure them with a nylon screw (RS, 527-971). The connection between headstages and acquisition board is facilitated by a custom-made Y shaped adapter built following the Open Ephys protocol (https://open-ephys.atlassian.net/). The Y cable is connected, on one side, to the headstage 12-pin Omnetics PZN-12-AA connector (#10) and kept in position by a slot into the cap. On the other side, it connects to one ultra-thin SPI (Serial Peripheral Interface) cable (Intan Technologies, #C3216, 1.8 m, US). This design allows us to use only one thin SPI cable which facilitated the recording process, and it has a reduced probability to entangle while the rats perform behaviour. The SPI cable is adapted to be wired to a commutator which then connect to the Intan acquisition board via a shorter SPI cable. Ultimately, once the

headstages are enclosed in the cases, they can be firmly attached to the arm with dental cement (Henry Schein, light curable flowable composite, #199950, UK).

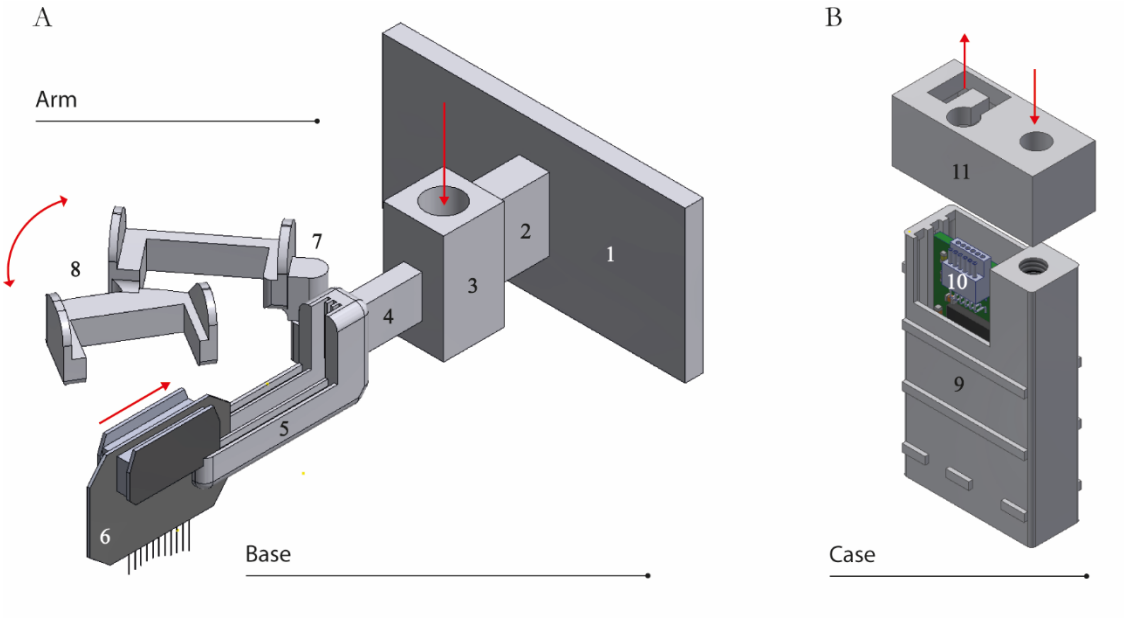


Figure 3.2 Implant Autodesk Inventor 3D model

A) Probe holder base and arm. Base. #1 Alignment tool; #2 Accessory, to be cut after alignment using #1; #3 Post holder, insertion of 2 posts and a compact kinematic mirror mount (alignment tool) directly connected to the stereotaxic frame. #4 Accessory, to be cut after surgery; #5 Main body, it holds the probe in position and is cemented directly onto the skull during surgery; #6 11 shank probe sliding into #5 grooves which tighten it in place and guarantee straightness. Arm. #7 Arm pin, it is inserted in a hole designed in the base and pivot around it allowing the arm to move; #8 Headstages holder, the paired omnetics and headstages are cemented in place once inserted in their cases. Case. #9 Case sleeve, it covers the headstages and keep them straight in position thanks to lateral guiding grooves; #10 12 pin omnetics connector, it connects to one end of the Y cable; # 11 Cap, the 12 pin / Y cable connection is kept in place by the cap tighten to the sleeve with a plastic screw.

3.3.3.1 Probe preparation and assembly overview

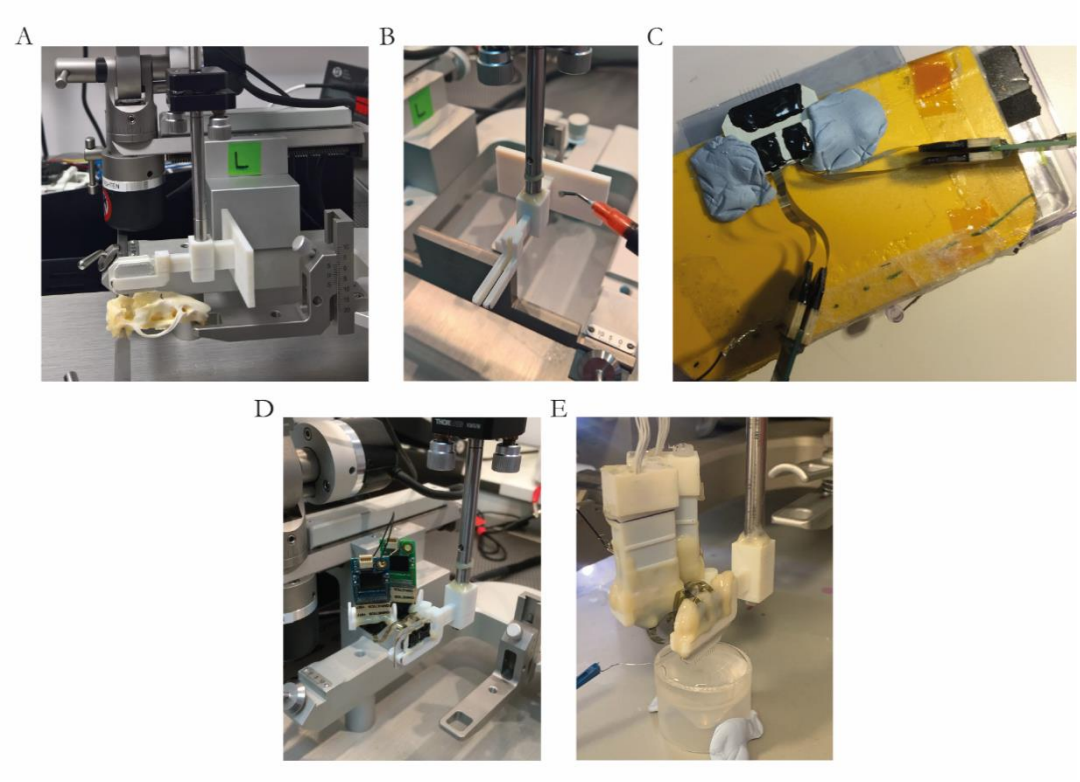


Figure 3.3 Probe assembly

A) Manual alignment using a rat skull and a 3D printed probe mock. Visible the 3D probe holder held by the posts and attached to the stereotaxic frame. B) First Alignment using the flat 3D printer rectangular surface and the stereotaxic alignment tool. The posts are secured in place with cement to provide additional protection from sudden movements. The alignment portion of the implant is removed after this step C) Probe secured in place onto a custom-made surface to carefully mate the omnetics and headstages. Visible at the bottom left side, the probe reference wire soldered to a kynar wire D) Headstages cemented onto the probe holder arm. E) Complete implant assembly during impedance testing in saline.

Several steps are required for both the 11 shanks probe and the 3D probe holder before proceeding with the chronic implant. After PEDOT:PSS coating and prior to insertion in the holder, the exposed metallic parts on the surface of the probe were coated using a transparent flexible silicone coating (RS, Electrolube, #535-525,UK), to ensure protection against moisture and saline drops during surgery. The probe was secured onto a custom-made surface (Figure 3.3C), the omnetics were paired, the headstages were mated and a short kynar wire (RS PRO, #209-4849, 0.05 mm^2, UK) was soldered to the reference wire of the

probe. This wire will be soldered to the reference screw wire during surgery. To guarantee a good alignment of the probe, before each probe assembly, the stereotaxic frame was aligned using a dial. The empty holder is attached to the frames (Figure 3.3A) and roughly aligned by hand. A more precise alignment was done by mating the holder flat rectangular surface with the stereotaxic flat alignment tool (Figure 3.3B). The post is secured in position with cement and the flat surface cut with a drill. The probe can now be inserted in the holder base grooves which provide additional alignment, and the probe is cemented in place. The paired omnetics with the headstages were attached to the movable arm (Figure 3.3D). Finally, the case sleeves were positioned onto each headstage, the Y cable ends were connected to the headstages and inserted into the caps. The plastic screws were tightened in place and both cases were attached with dental cement onto the movable arm. Impedance was tested to evaluate the probe quality (Figure 3.3E). An additional alignment was required to guarantee the probe straightness before implantation. Using a 10X objective (Computar, MLH-10X macro zoom lens, C-mount, USA) mounted onto a camera (Grasshopper3 Point Grey U3, 120 FPS, USA) and connected to a laptop we could zoom in into the front and lateral side of the probe, magnifying the shanks. With the help of a marker positioned onto the laptop we could follow the probe travel while moving the stereotaxic arms. If the travel differed from the marker, the probe position was adjusted rotating the nobs of the compact kinematic mirror mount. Those micro movements together with the previously used alignment tools allowed us to reach a satisfactory straightness of the implant prior to surgery.

3.3.4 Surgical protocol

Six Lister Hooded male rats, 6 months of age, were chronically implanted with an 11 shanks transcortical probe in the right hemisphere. Rats showing a good and reliable engagement level and trial number, while performing in Level 2, where chosen for chronic probe implantation.

Pre-surgical care

Rats undergoing a surgical procedure were single housed at least 2 days prior to surgery, to habituate them to isolation. This step was necessary to prevent the risk of post-surgery injury and implant damage caused by the interaction with a conspecific. Implant safe enrichment and treats were provided. Furthermore, rats were exposed to the same raspberry gelatine (Hartley's raspberry jelly, UK) which will be use as vehicle to administer post-operative medications.

Surgery

Prior to surgery, surgical instruments and saline solution were sterilised in autoclave. Surgical area, stereotaxic apparatus (Leica biosystems) and dissection microscope were also disinfected to meet aseptic standards. The rat weight was annotated, and the appropriate doses of medications were calculated. Anaesthesia was induced using a mixture of isoflurane and oxygen in an induction chamber (2% v/v). Once the rat was anaesthetised, analgesic (Carprofen 5 mg/kg, SC), antibiotics (Baytril, 0.4ml/kg, SC), and atropine (0.05 mg/kg, IM) were administered. The fur on the scalp was carefully shaven using an electric razor. The rat was placed in the stereotaxic frame on a heating pad (World Precision Instruments, ATC-2000, UK) and the mouth was secured on the mouth bar. The ear bars were placed into the ear canals and adjusted to the correct location (equal reading on both sides of the bars) before tightening into place. The tongue was gently pulled out with a small forceps to avoid

breathing complications during surgery. The air mask was placed on the rat snout to additionally stabilise the head and to ensure delivery of the anaesthetic. Rat's reflexes (tail reflex or toe-pinch reflex) were checked to ensure that an adequate level of anaesthesia was reached. A rectal temperature probe was inserted to continuously monitor the body temperature in closed-loop with the heating pad. Eye ointment (Lubrithal eye gel) was applied on the corneas to prevent dehydration. The surgery was undertaken with the help of the dissecting microscope changing the magnification as needed. Prior to incision of the skin with a scalpel, the scalp was cleaned with chlorhexidine gel applied with sterilised cotton swabs in circular motion to better disinfect the area. Sterilised hemostats were used to pinch off the skin and keep the incision open. The skull soft tissue was carefully removed with a spatula and the surface was kept as dry as possible using cotton swabs. After the skull was exposed, Bregma and Lambda were marked. To confirm that the head was levelled, a needle was placed onto both Bregma and Lambda touching the skull, and the dorso-ventral coordinated were automatically recorded using the computer Atlas integration of the stereotax, which reads the stereotaxic arms exact location. If the two coordinates were more than 100 μm apart the mouth bar was adjusted to correct the head position. This procedure was repeated until the animal was correctly positioned. The interaural ratio (Paxinos interaural distance / measured interaural distance) was also calculated to verify the full growth of the skull and thus making adjustments in the target coordinated for probe insertion, if needed. To support and stabilise the implant, 4 sterile screws were inserted in the skull. Three 4 mm screws were place on the left frontal, parietal and interparietal bone and a 3 mm screw was placed on the right parietal bone after gently detaching the muscle from the bone with a spatula. Before tightening the screws to the skull, 4 small holes are created with a drill bit, preserving the meningeal membrane layers intact. The screws are placed in the holes and tightened with a screwdriver, turning the screw enough to be rigidly attached to the bone without penetrating the brain. The parietal screw hole was completely

opened, exposing the dura mater, so that the screw could be in contact with the brain surface and used as reference. A short kynar wire was soldered to the reference screw prior to surgery. Before performing the craniotomy, an additional step is taken to ensure stability. A self-cure dental adhesive cement (Super-bond C&B) was applied on the left hemisphere bones and around the screws without covering Bregma. Craniotomy was performed on the right hemisphere using a diamond tip drill (Mectron, OT5B). The craniotomy margins extended from + 3.5 mm to - 9.5 mm AP relative to Bregma. Care was taken to avoid the drill bit to penetrate through the dura mater or blood vessel preserving the cortical surface. The exposed brain tissue was kept hydrated with warm saline (36-37°), body temperature and breathing were monitored closely throughout each step. Durotomy was performed by carefully lifting the meningeal layers with a small surgical spatula or tweezers and cutting with a scalpel or surgical scissors. The exposed brain was kept hydrated with warm saline and covered with haemostatic collagen sponge (Spongostan). The probe holder was attached to the stereotaxic frame, the probe most anterior shank was aligned to Bregma and brought to the target coordinates of insertion. Additional adjustments were made if there was any possibility that the slow insertion of the shanks would pierce one or more main vessels visible on the cortical surface. Those adjustments were made to preserve the tissue health as much as possible after insertion of the probe. The slow probe insertion was monitored via the microscope as well as the same camera, with high magnification, used for probe alignment placed on the surgical table and connected to a computer screen. After each shank was successfully inserted in the cortex, Duragel (Cambridge NeuroTech, UK) was used to fill the craniotomy and help maintaining healthy tissue. Light curable Composite Flowable resin (Henry Schein) was used to build the implant structure embedding the 3D printed probe holder within it. The reference screw wire was soldered to the probe reference wire to ensure grounding. To complete the implant, #4 is cut, to release the implant main body (#5) from

the stereotaxic frames (Figure 3.2). The wound was cleaned with warm saline and sutures were used once the implant was finished.

Post-surgical care

Upon completion of the surgery the rat was rehydrated with saline injection (1-1.5 ml, SC). Before removal from the stereotaxic frame, a second analgesic injection was given, and the nails were trimmed. While waiting for the animal to wake up, a brief impedance test and recording were performed to check both data quality and the success of the surgery. The rat was placed on a heating pad and closely monitored until it became conscious and an attempt to eat soft diet or gelatine was made. Analgesic (Metacam, 1.5 mg/ml oral suspension) and antibiotic (Baytril 2.5% oral solution) were given post-surgery, for 2 and 4 days respectively mixed with raspberry gelatine. The rat was allowed to start behaviour only 48 hours after surgery. Soft diet and plain gelatine were provided for the subsequent 48 hours to help food and water intake during recovery.

Animal experiments were approved by the local ethical review committee and conducted in accordance with Home Office personal and project (I0592BA9A; 70/8116) licenses under the UK Animals (Scientific Procedures) 1986 Act.

All surgical procedure were conducted by me, and technical support was provided by Joana Nogueira, Joana Neto and George Dimitriadis.

3.3.5 Videogame Protocol for chronic recording

Chronic recordings were performed starting from level 2, learnt before surgery. Prior to each behavioural session rat weight was recorded and the impedance at 1 kHz was measured using the Intan evaluation board (RHD2000, Intan Technologies, US). To allow this measurement, the rat was tethered to the SPI cable, temporarily glued to the Y cable (Figure 3.4A). A spring system allowed the cable length to adjust according to the rat position in the arena. Across the top of the Videogame enclosure, a commutator, connected to the SPI cable, was hooked to the nylon fishing wire. Both commutator and spring system facilitated the movement of the rat across the modular arena and helped preventing the entanglement of the cable. Rats soon habituated to the tethering and to play the Videogame under these new circumstances. The daily recording lasted from 3 to 4 weeks depending on the animal and included the transition to Level 3 and 4. During each session, the recording of the neural activity could be saved and monitored online through Bonsai. The neural data were sampled at 30 kHz with 16-bit resolution. Additionally, camera, infrared beam sensor and piezo were synched with the neural data via the acquisition board. Precisely aligning the neural activity with events of interest was crucial for subsequent analysis.

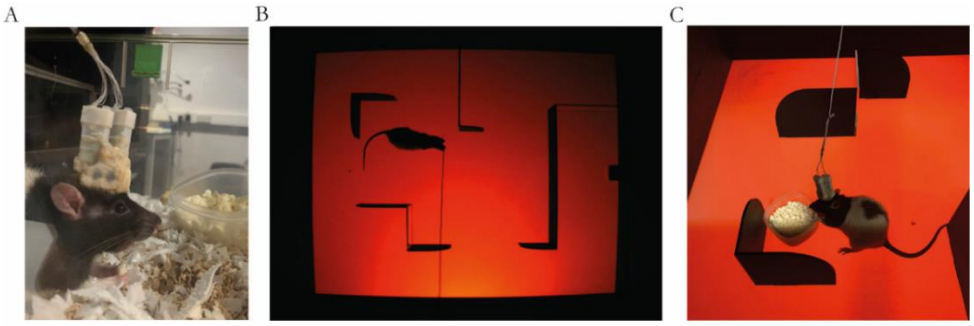


Figure 3.4 Rat during chronic recording

A) Side view of a rat carrying an 11 shanks probe implant during impedance measurement in its home cage, right before starting the daily behavioural session. B) Top view of the modular arena while a rat is performing the task. Frames taken by the daily raw movie saved at the end of each session. C) Detail of a rat enjoying a well-deserved treat at the end of the session.

3.3.6 Histology

After completion of the recording sessions the rats were anesthetized with isoflurane (2 % v/v) and injected with sodium pentobarbital (Euthatal, 150 mg/kg). After sedation, the animal toe-pinch and tail-pinch reflexes were tested to check for signs of pain. Once a deep sedation was reached a transcardial perfusion was performed with saline (NaCl 0.9%) and 4% paraformaldehyde (PFA, 28908, Thermo scientific) (Figure 3.5A). Prior to brain dissection, the wearable headstages and Y cable connector were safely retrieved and removed from the cases. Brains were kept in vials containing PFA at least 24 hours at 4 °C to ensure a homogeneous fixation of the tissue before starting the immunostaining protocol. Sagittal sections (50 μm thick) were sliced using a vibratome (Leica, VT1000S) (Figure 3.5A). On the sections of interest, where the probe was inserted, glial fibrillary acidic protein (GFAP) staining was performed using free floating method. GFAP is the principal intermediate filament of mature astrocytes, and it is considered an astrocyte specific cell marker. The activation of the astrocytes by injury (e.g. probe insertion) leads to upregulation of this protein (Polikov et al., 2005). GFAP immunostaining is the most common method for astrocyte identification and for determining the extent of the 'reactive gliosis', which describes the activation, hypertrophy and proliferation of astrocytes in response to an injury (Bignami et al., 1980; Eng, 1985). DAPI was used as nuclear counterstaining (Vectashield hard set with DAPI, Vectors Laboratories, USA) (Figure 3.5C). This staining was used to qualitatively detect the shank traces and estimate their position within the cortical tissue. Images were acquired at 10X using an Axio Imager 2 microscope (ZEISS, Germany) and were post processed using the software ZEN Blue 2.3 pro.

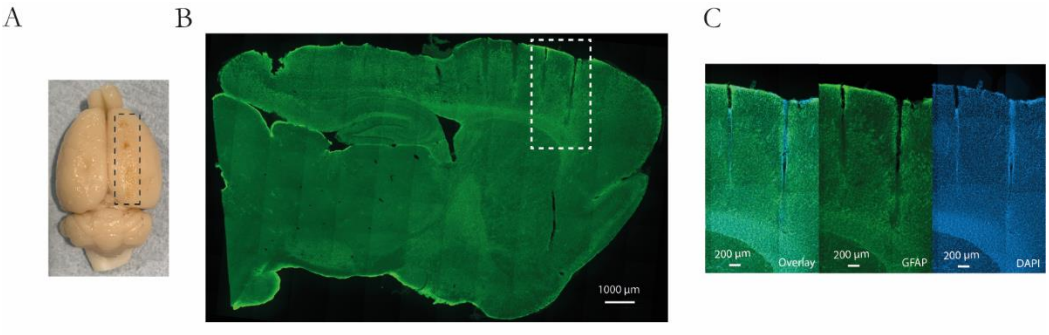


Figure 3.5 Histology

A) Picture of a brain prior to dissection with the vibratome. The black dashed box, on the right hemisphere, highlight the probe insertion site. B) Representative sagittal brain slice (GFAP) showing the traces left by the probe insertion. C) Detail of two shanks' traces (dashed white box) shown in both channels and the overlay (GFAP + DAPI).

3.3.7 Data analysis

The rats' behaviour was analysed as per Chapter 2 methods and results section. For the electrophysiology analysis we used different approaches depending on the frequency range considered. For LFP we firstly lowpass filtered and downsampled the raw signal (30 kHz) to obtain a 1 kHz signal.

For the cluster analysis, we selected snippets of downsampled data for all the trials performed by all rats with the best performing probes (n = 4) before and after the events (± 1.5 s). The events considered are touch and successful reward for level 2 and trigger, catch, and successful reward for level 3. We calculated the power spectrum of the snippets with a multitaper approach (MNE library, https://mne.tools/stable/index.html), which applies several tapers, with slightly different temporal characteristics, to the signal. This approach increases the signal-to-noise ratio of the frequency representation. In brief, the data from each snippet are multiplied by a series of tapers resulting in several tapered time series. Different tapers concentrate the signal in different regions of time. Next, the Fast Fourier Transform of each tapered time series is taken and the resulting spectra are averaged together (Mitra & Pesaran, 1999). There is a trade off on how many tapers one should use. Too many

tapers would smooth the signal too much resulting in the loss of accuracy of the frequencies' features. On the contrary, too few tapers reduce the signal-to-noise and hide brain dynamics. After testing different parameters, we decided to use 14 tapers to extract frequencies up to 300 Hz. The MNE function that we used had the following parameters: sampling frequency/sfreq = 1000, minimum frequency of the power spectrum/fmin = 1, maximum frequency of the power spectrum/fmax = 300, bandwidth = 10, number of concurrent jobs/n_jobs = 8. After applying multitapers to both before and after snippets we calculated the average power (all the trials) of the sum of each frequency band considered (delta = 1-4 Hz, theta = 4-8 Hz, alpha = 8-12 Hz, beta = 12-30 Hz, gamma = 30-100 Hz, high gamma = 100-300 Hz). We calculated the normalised difference around the events ((power after – power before)/power before) and plotted in custom probe shaped plots for better visualising the real channel layout. We then looked for clusters of channels that could be responsible for the change in power before or after an event, if any. In doing so we applied the non-parametric statistical test described by Maris & Oostenveld (2007).

For the Inter Trial Phase Consistency (ITPC) analysis, we took inspiration from: https://mark-kramer.github.io/Case-Studies-Python/05.html. Instead of selecting snippets of data before and after each event, we took the 3 s around it. We then created 32 groups of channels to compare. In order to generate new grouped channels, we averaged the downsampled signal over a number (3 or 4) neighbouring channels belonging to the same shank (see Chapter 3 Results section). We calculated the ITPC by first calculating the phase difference (angles in radians) for every trial and all the pairs of channel groups and subsequently averaging those phase differences over all the trials and all the animals (n = 4). We used the standard deviation of this grand average as the measure for the inter trial phase consistency. The smaller the ITPC, the more synchronised (in phase) the pairs of channel groups are.

In order to analyse the multi-unit spiking activity (MUA), we cleaned the raw signal by re-referencing each channel by the mean voltage coming from the corresponding headstage. Data were high-pass filtered with a third order Butterworth filter with a band-pass of 500 or 14250 Hz (95 % of the Nyquist frequency). The magnitude of the background noise was estimated both by calculating the absolute median of the signal (σ_{Median} = median(|signal|/0.6745) or simply by the standard deviation of the signal (root-mean-square, σ_{RMS}). The events (spikes) were detected using dual threshold crossing (2σ lower and 4σ upper). Each event was assigned to the corresponding 10 ms bins to obtain a 100 Hz MUA signal. Events appearing in more than a quarter of the channels were considered artifacts and the containing bin excluded from analysis. All the data are analysed using custom Python 3.7.1 scripts and for plotting we used matplotlib and seaborn libraries.

Significant input on the LFP analysis was provided by George Dimitriadis who assisted me during the Python code implementation as well as he provided the code for the cluster analysis. Adam Kampff' input was fundamental for the implementation of the MUA analysis.

3.4 Results

In the previous chapter we have shown that rats can play the Videogame, learning how to interact with a virtual object and adapting to its behavioural changes. The best performing rats in Level 2 were chosen to undergo the surgical implantation of the 11 shanks probe. Prior to surgery, the probe was coated with PEDOT:PSS and tested to guarantee its quality while rats were single housed to familiarise them with living alone in the cage. Rats undergoing surgery were given 48 hours to recover before resuming the daily behavioural assessment starting from Level 2. This timing was the minimum allowed by the guidelines of the Home Office. Rats were usually tested for about 3 weeks, depending on their performance and the probe/implant stability over time.

Can rats play the Videogame after surgery? Can we successfully implant and record from a large portion of the cortex while the rats engage in the levels? We have implanted 6 out of the 12 rats previously included in the Chapter 2 Results section and tested them from Level 2 up to Level 4. In this chapter we report a behavioural comparison between the rats' performance before and after surgery together with exploratory analysis of the rich electrophysiology dataset acquired from our 11 shanks probe. We focus our analysis on levels 2 and 3.

3.4.1 Rats and Videogame post-surgery

The Videogame assay was equipped with the necessary tools to allow for chronic recordings (see Method section for details). At the beginning of each behavioural session, rats were weighed and tethered in the cage (Figure 3.6A). Furthermore, the impedance magnitude of each electrode was measured (2-3 repetitions for a better estimate) and recorded. Once gently placed in the Videogame arena, the spring system attached to the SPI cable facilitated their movement without discomfort, adapting the cable length based on the position of the rat in

the arena (Figure 3.6B). The arena's walls were also designed with smooth edges so to avoid entangling of the cable and easing locomotion during the session.

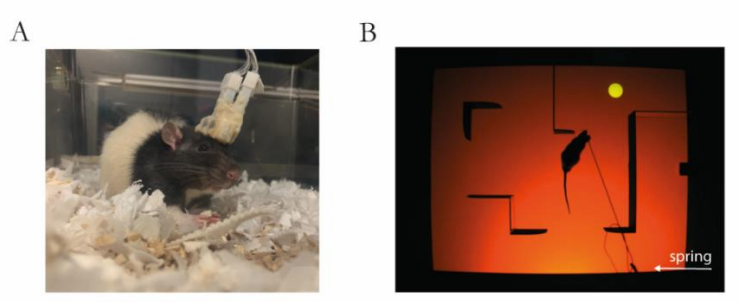


Figure 3.6 Chronic 11 shanks recordings

A) Front view of a rat carrying the 11 shanks probe during routine impedance measurement. Visible the 2 headstage' cases and the Y cable connected to the acquisition board via an SPI cable. B) Top view (camera view) of a tethered rat during level 2, highlighting the spring mechanism that stretches the SPI cable according to the rat's location in the arena.

Rats perform fewer trials post-surgery but retain commitment to the task

During the first session post-surgery rats are still recovering and they are under post-surgical medications. Therefore, despite looking bright, they might still be affected by the procedure. Usually, a couple of days are required for the rats to adapt to the tethering prior to the start of the session and to adjust to the new experience of being in the arena.

Nonetheless, rats exhibit a decrease of performance during both level 2 and 3 post surgery (Figure 3.7). We compared the performance of the same rats before and after surgery during Level 2. The frequency at which rats accomplish a successful trial is the parameter that has been affected the most, with less than 1 rewarded trial performed per minute (Figure 3.7B). Furthermore, the total number of rewarded trials performed during Level 2 appears to halve for some of the implanted rats (Figure 3.7A). Interestingly, the speed at which rats travel to collect the reward, after the reward availability tone onset, does not seem to be as affected, but instead, some rats tend to be quicker in reaching the reward port (Figure 3.7C). This result confirms that rats commit to collect the chocolate pellet once they decide to touch the ball. Summarizing, despite knowing the rules of Level 2, the new setting (including the

implant) is influencing the performance of most rats. This seems to be true also for Level 3. We compared 4 rats that were tested only behaviourally and 6 rats that underwent surgery. These results resemble those obtained for Level 2. The performance is disrupted post-surgery in all the parameters considered compared to the rats assessed without carrying an implant. Overall, rats tend to be slower at performing successful trials, which impacts the total number of trials per session (Figure 3.7D-E). After catching the ball, they reach the reward port at a slower pace (Figure 3.7F).

Beside the first session post-surgery, which comes with the novelty of the setting, rats do not show signs of discomfort in exploring the arena while tethered. Occasionally, some rats more than others, show stress signs that we noticed due to an excessive need to scratch their body. We also noticed that rats tend to have resting bursts in which they rest or sleep in the corner of the arena before then resuming, or not, the task. This behaviour was rare, if not absent, prior to surgery. Despite knowing the task, they do not seem to be as motivated as they were prior to surgery.

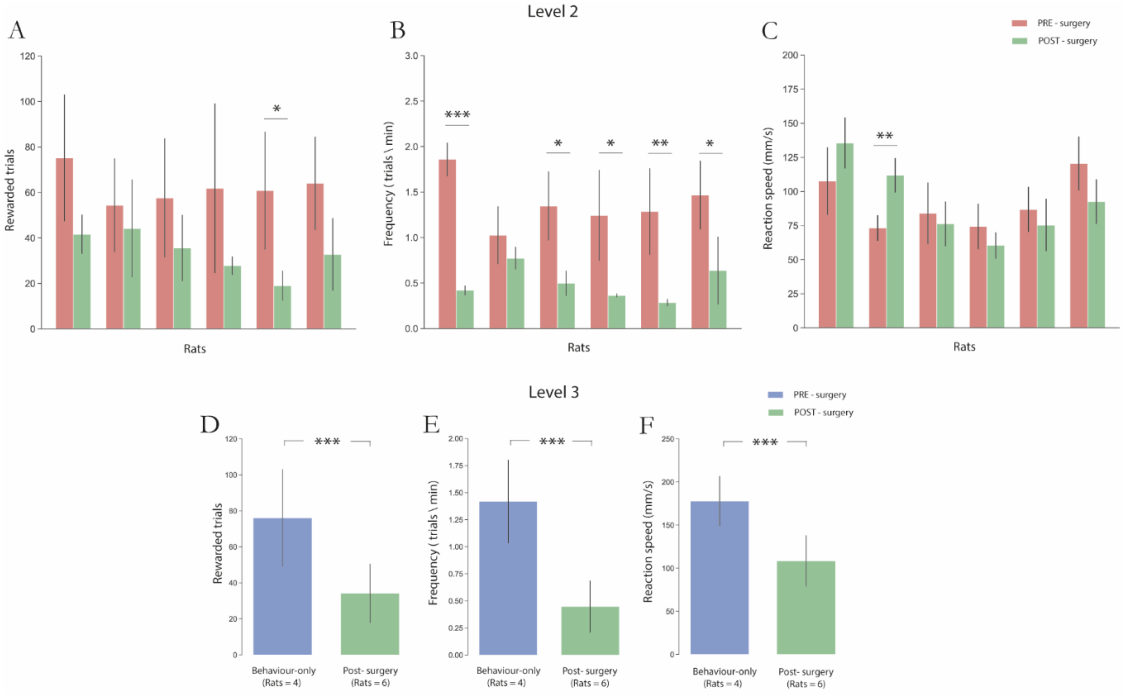


Figure 3.7 Behaviour performance comparison pre/post-surgery

A) Comparison of the average number of rewarded trials performed by each rat before (red) and after surgery (green) during level 2. B) Comparison of the average number of rewarded trials performed per minute by each rat before (red) and after surgery (green) during level 2. C) Average reaction speed to collect reward before (red) and after surgery (green) during level 2. D) Level 3 Comparison of the average number of rewarded trials for 4 rats performing without implant (blue) and 6 different rats that underwent surgery (green). E) Level 3 average frequency of rewarded trial compared between implanted (green, n= 6) and not implanted rats (blue, n= 4). F) Level 3 average reaction speed to collect reward compared between implanted (green, n= 4) and not implanted rats (blue, n= 6).
All the results are shown as mean ± STD. Statistical test: paired t-test. Level 2 results compare 5 selected sessions before and after surgery. Level 3 results compare 5 sessions selected from implanted rats and 5 from not implanted. Two out of four not implanted rats had only 3 and 4 sessions performed in this level.

Similarly to the analysis shown in Chapter 2, we explored the rats speed profiles around the events: touch, trigger and catch (Figure 3.8). Considering all the trials performed by the 6 rats, we immediately noticed an overall decrease in the speed around all the events as well as a change in the speed profile around touch and catch. Interestingly, the speed to trigger the ball movements is lower, but the shape of the profile is retained post-surgery. We think that this might be caused by the fact that, for events like catch and touch, the rats need to 'physically' come into contact with the ball, while for trigger, it is enough to be in its proximity. Inevitably, carrying an implant on the head causes a change in the rat's silhouette detected using the computer vision pipeline of the Bonsai workflow (binary contour). This

can have an impact on when the overlap of rat and ball is detected as sufficient to trigger the availability tone. We did try to overcome this by eroding the rat silhouette in an attempt to remove the implant from being detected, which definitely helped but did not solve the problem. Unfortunately, the shape of the probe implant is not a symmetric shape, and it is shifted to one side of the head. Additionally, rats interact with the ball in a variety of different ways that are too many to predict by setting a threshold and partially eroding. We think that this change in the interaction with the ball can be seen in the lack of anticipatory slowdown that rats had shown prior to surgery when touching or catching the ball. If we inspect early versus late trials, then we can only see a slight improvement in their approach before touching the ball, but no change in catch. Unexpectedly, rats trigger the ball at a faster pace in early trials compared to late trials. Missed trials follow a similar speed profile to rewarded trials, but this differs more dramatically during catch, where miss trials are much slower. Averaging only the last 100 trials performed by each rat, whether missed or rewarded, highlights again a bigger difference in the catch profile compared to the other events. Rats are much faster to catch the ball if they commit in going to the port to collect the chocolate. Finally, we computed the Δ speed, as previously shown in the Chapter 2 Results section, which confirm that rats do not touch the ball while decreasing their speed, while the contrary is true for trigger and catch despite a difference in the profile shape for the latter (Figure 3.8D-E).

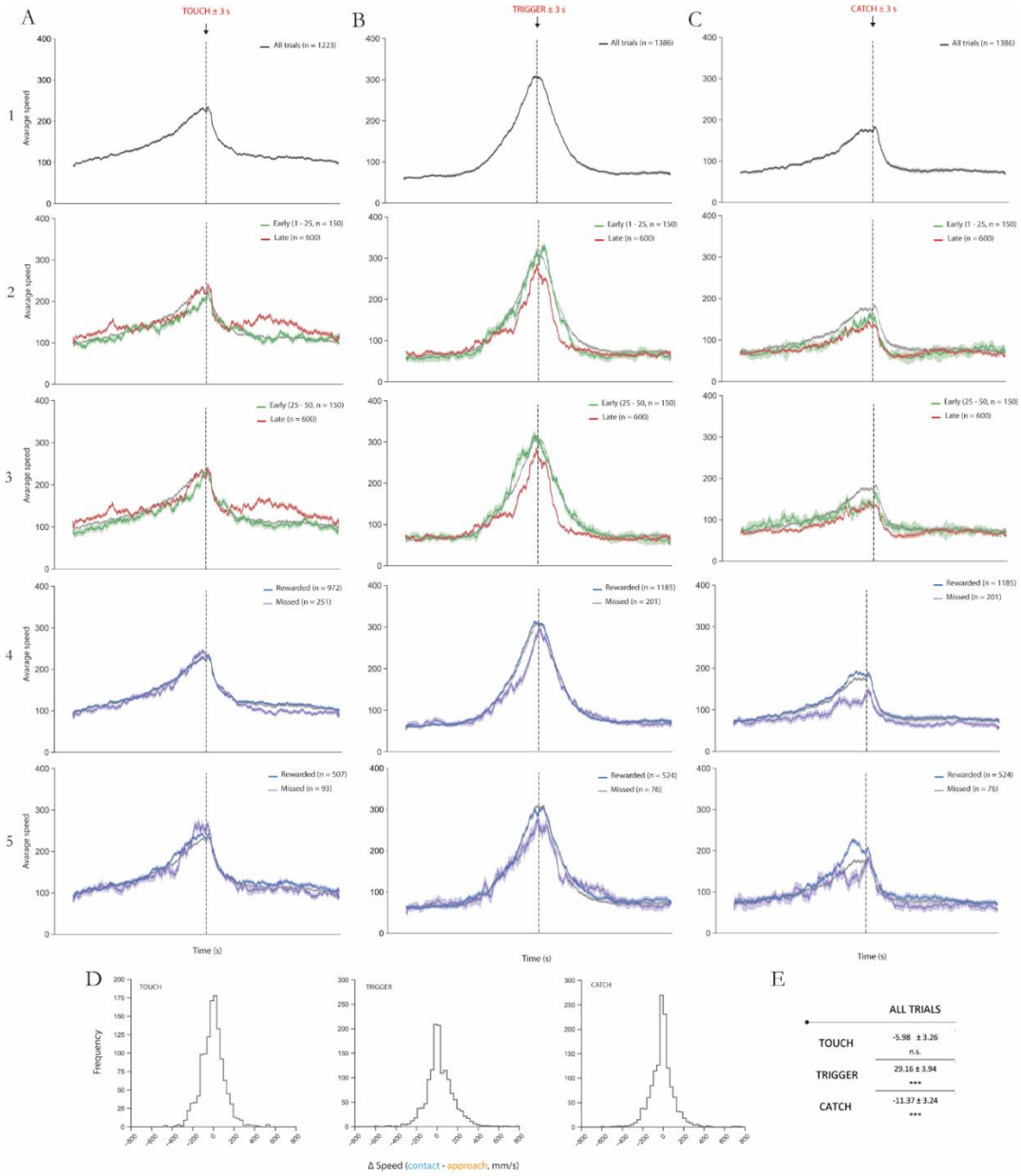


Figure 3.8 Post-surgery speed profile summary around touch, trigger and catch.

A) Average speed profile around touch (Level 2 post-surgery). A1: all the trials performed by 6 rats (n = 1223, black). A2: speed profile comparison between the first 25 trials (green) and the last 100 (red) for all the rats. A3: speed profile comparison between trial 25 to 50 and the last 100 trials. A4: speed profile comparison between successful (blue) and missed trials (violet). A5: speed profile comparison of the successful and missed trials performed in the last 100 trials for each rat. B) Average speed profile around trigger (Level 3 post-surgery). C) Average speed profile around catch (Level 3 post-surgery). D) Δ speed distribution for all the trials and for all the events of interest. Δ speed has been computed by subtracting the average speed at contact by the average speed at approach. E) Table summarising the Δ speed distribution for all the trials. All the speed profiles are shown as median ± SEM while the Δ speed as mean ± SEM.

To conclude the behavioural comparison pre/post-surgery, we would like to emphasize that implanted rats also show a striking surprised and alert behaviour when first encountering a moving ball. The transition from level 2 to 3 happens in the same way as for the not implanted rats described in Chapter 2. As previously mentioned, we did not manually annotate these sessions, but we do have a list of video clips to show the behaviour of each of the implanted animals at the first moving ball trial and for the subsequent two trials (Appendix C, Table C.1).

3.4.2 Probe characterization: impedance and noise measurements

Before being able to implant the 11 shanks probe chronically, a few steps are necessary to characterise and improve the quality of the probe and increase the chances of a long and stable recording. The main step is to decrease the impedance values by coating each individual electrode with the polymer PEDOT:PSS. We investigated the effect of this procedure on the 11 shanks probe by simply comparing the impedance measurements before and after coating. For this analysis, we separated the results by electrode material (Pt and IrO_2). We would like to clarify that due to our limited sample size (4 Pt probes and 2 IrO_2 probes) we are not able to draw any overall conclusion about these materials' performance.

In Figure 3.9A-B, we pooled all the electrodes of each probe used in this study and we show the impedance changes from pristine to coated electrodes. The values reported in these figures are acquired during the Nano-Z protocol before and immediately after coating.

Each data point corresponds to one electrode and in red we show the impedance values measured in saline solution prior to coating while in green, the same electrode's impedance values measured in saline, but after the coating deposition protocol.

It is evident how the PEDOT coating can reduce the impedance (up to tenfold) for most electrodes despite the material they have been fabricated with. The increase in impedance for

a few Pt electrodes is due to an error in the deposition protocol that was unfortunately not reversible.

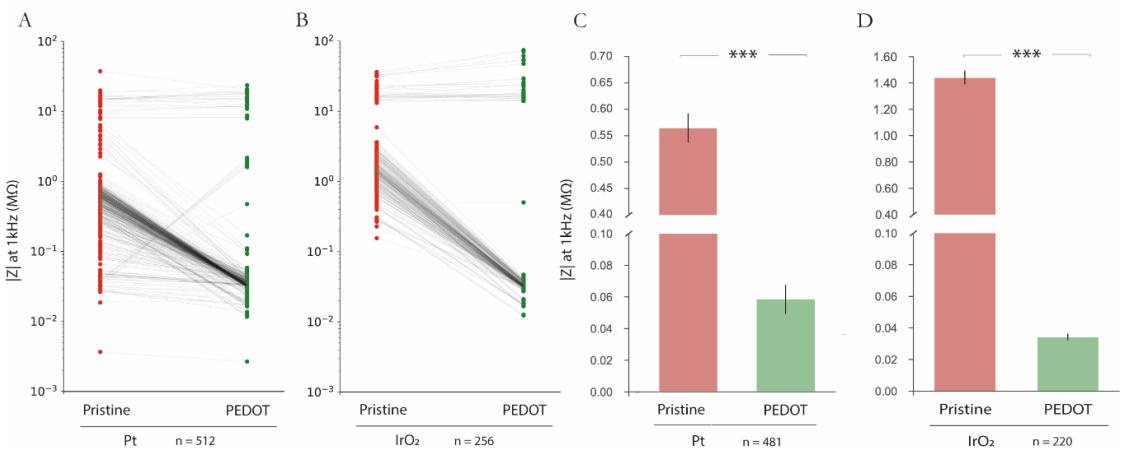


Figure 3.9 Impact of PEDOT coating on impedance measurement

A) Comparison of each Pt electrode impedance value measured in saline pristine (red) and in saline after PEDOT coating (green). B) Comparison of each IrO_2 electrode impedance value measured in saline pristine and in saline after PEDOT coating. C) Average comparison of the impedance magnitude for all the Pt electrodes before and after coating. Excluded all the channels with an initial impedance higher than 6 MΩ and 0.01 MΩ. D) Average comparison of the impedance magnitude for all the IrO_2 electrodes before and after coating. Excluded all the channels with an initial impedance higher than 6 MΩ and 0.01 MΩ.
All the results are shown as mean ± SEM. Statistical test: paired t-test.

Interestingly, PEDOT fails to decrease the impedance of electrodes with an initial impedance greater than circa 10 MΩ, perhaps because the electrical connectivity of these channels was compromised during fabrication. Figure 3.9C and D compare the average impedance before and after coating for both electrode's types. Coated electrodes show a significant decrease in impedance values (paired t-test) for both electrode material. Overall, we also noticed that pristine IrO_2 probes show a higher average impedance to start with compared to Pt probes. Nonetheless, the coating impressively reduces the impedance from 1.44 MΩ ± 0.028 MΩ to 0.034 MΩ ± 0.002 MΩ. After coating and as per the current analysis, we can speculate that IrO_2 probe seem to be better for implantation, having a lower impedance despite the initial higher measurements. Pt probes also show good impedance values for chronic implantation.

Additionally, we wanted to monitor the impedance magnitude and stability throughout the length of the behavioural assessment by measuring it daily prior to the start of the Videogame session. The values reported in Figure 3.10 are measured (at 1 kHz) using a protocol implemented by the RHD2000 series chip (InTan Technologies). For each probe, we also show the impedance measured in saline prior to surgery (quality check) as well as the values measured right after the surgical procedure.

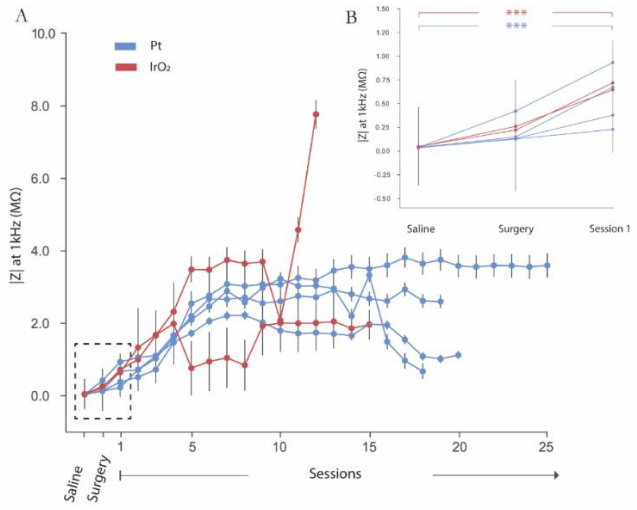


Figure 3.10 Impedance measurement summary

A) Impedance trend for each probe from before implantation until the last day of behavioural assessment which varies according to the rat, implant and recording stability. B) Zoomed in visualisation of the impedance in saline prior to surgery, right after surgery and the first day of behaviour post-surgery (dashed box). The colours refer to the nature of the electrode material. The impedance values are reported as median ± SEM.

The Pt probes show an increase in impedance values over time before reaching a semi-plateau after day 7 of behavioural assessment. We noticed a marked increase for all the probes from day 3 to 4. This event was reliable and consistent for all the 4 Pt probes.

A faster rise in impedance is evident for the IrO_2 probes which start having erratic behaviour after day 4. This unpredictability of the IrO_2 had a big impact on the quality of the recordings throughout the behavioural assessment.

Finally, we have estimated the background noise for each probe and monitored it over time (Figure 3.11). The magnitude of the background noise can be estimated from the median

absolute high-pass filtered signal, assuming a normal noise distribution (σ_{Median} = median(|signal(t)|/0.6745)), to avoid contamination by spike waveforms (Quiroga et al., 2004) or it can simply be defined as the standard deviation (root-mean-square noise, σ_{RMS}) of the signal (Scott et al., 2012).

Figure 3.11A shows a more coherent and homogeneous noise profile for Pt probes compared to IrO_2, despite being noisier overall.

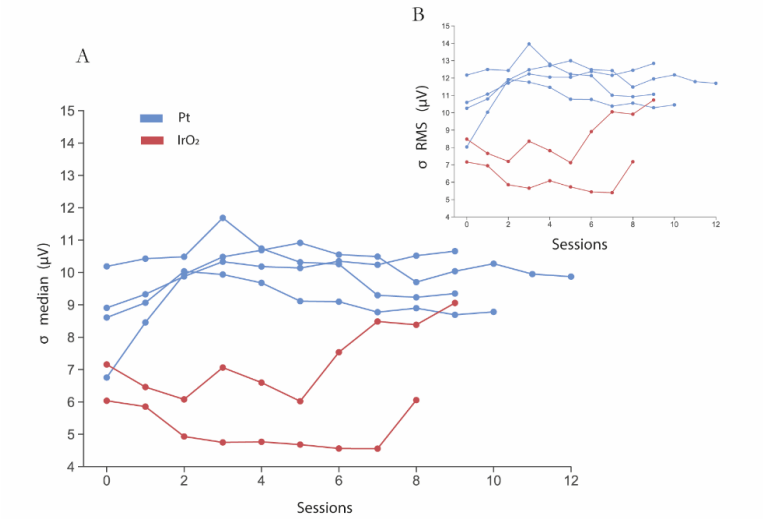


Figure 3.11 Noise summary

A) Noise trend for Pt and IrO_2 probes over time calculated as absolute median of the signal. B) Noise trend for Pt and IrO_2 probes over time calculated as absolute mean of the signal.
The colours refer to the nature of the electrode material. Selected only level 2 and level 3 sessions.

Higher values for the noise were found when taking into consideration σ_{RMS} values, probably due to a contribution of spikes (Figure 3.11B). The σ_{RMS} value is based on the standard deviation of the signal, which increases with the firing rate(Quiroga et al., 2004).

Pt probes not only show a consistent noise levels, but they remain stable overtime, which is an important feature for chronic recordings. Again, IrO_2 probes do show less noise, but also unusual and unstable profiles. For this reason, together with the impedance' results we have decided to exclude the 2 rats implanted with these probes from the results described in the remainder of this Chapter.

3.4.3 11 shanks probe: in vivo chronic recordings

After a successful coating and quality check in saline, the probe needs to be mounted in the customised probe holder and aligned. Implanting 11 shanks comes with challenges. As described in the methods section, we aimed to target at least 7 different cortical areas placing the 1st shank in motor cortex and the 11th in visual cortex (right hemisphere). This goal has been ambitious for a variety of reasons, from the probe alignment to the presence of main superficial blood vessels above the insertion path. Nonetheless, we successfully implanted a chronic 11 shanks probe in several rats, and we could record the cortical activity for several days while they were playing the Videogame. At the end of this section, we will show a sample slice for each rat for qualitative assessment of the coordinates of insertion.

After implantation and further impedance tests, the rats are allowed to recover and provided with post-surgical care for 48 hours before chronic recording can begin. As previously shown in Figure 3.10B, the impedance values at session 1 are significantly higher than before surgery, on the order of the hundreds of kΩ. Nonetheless, we can successfully record signal from the cortex of our rats. During each behavioural session we assessed the neural activity in real-time via Bonsai and thus monitored the data quality online. In Figure 3.12 we show a 2 s snippet of raw voltage traces for all 121 channels of the probe, which are subdivided in 11 shanks and spaced apart differently following the cortical silhouette (see Methods section for more details). The neural data were sampled at 30 kHz with 16-bit resolution.

As per methods section, rats chronically carry, two 64 channels InTan headstages embedded into the probe implant. For analysis purposes, we cleaned the raw data by re-referencing each channel by the mean voltage of the signal coming from the corresponding headstage (Figure 3.12B). Channels with too high (=> 6 MΩ) or too low (<= 0.010 MΩ, likely damaged) impedance measurements were excluded from the mean.

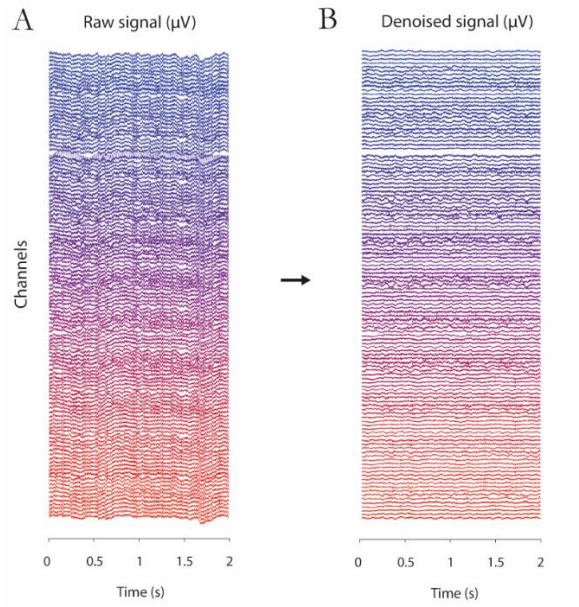


Figure 3.12 Representative raw and cleaned 121 channels signal

A) Snippets of 2 s raw 121 channels signal taken from the middle (± 1 s) of an early behavioural session. B) Same snippets of 2 s denoised 121 channels signal after headstage referencing. Data are converted and showed in μV.

Due to the probe layout and the unusual spacing of each electrode, this probe is not suitable for the conventional spike sorting pipeline. Normally, spike sorting algorithms are used to extract and classify the activity of individual neurons (spikes) from the recorded voltage traces. The first step is to high-pass filter the data and the spike detection can be done using a variety of different software (i.e., Kilosort, KlustaKwik). The process is not fully automated, thus, a manual intervention is still required as it has not yet proven possible to implement a fully automated algorithm that works robustly (Pachitariu et al., 2016). In Figure 3.13A we show a snippet (2 s) of high-passed voltage data for all the channels. Additionally, we highlight the cleaned and high-passed single channel traces for 2 channels that display spiking activity (Figure 3.13B and C). Applying a simple dual threshold crossing algorithm based on the median voltage value for each channel (3σ lower and 5σ upper) we are able to extract spike waveforms (Figure 3.13D). The purpose of this brief interlude into spike sorting was to show that we do see spiking activity in our dataset, and we can isolate putative single units,

but due to the physical spacing of the electrodes on our probe, we will only be focusing on multi-unit-activity (MUA) in our analysis.

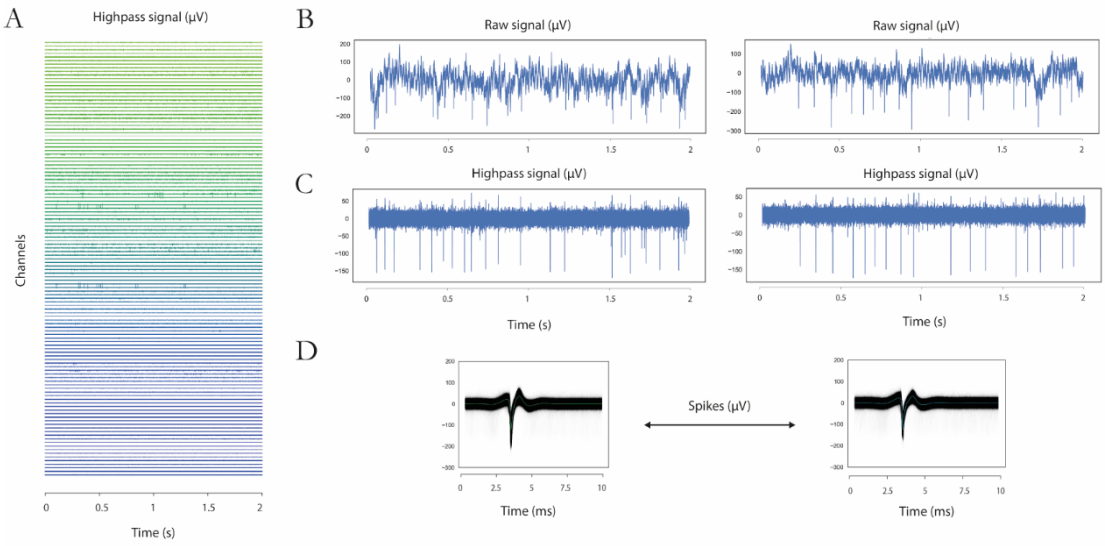


Figure 3.13 Highpass filtered signal overview

A) Snippets of 2 s highpassed 121 channels signal taken from the middle (± 1 s) of an early behavioural session. B) Cleaned 2 s traces of 2 representative channels displaying spiking activity. C) Highpassed filtered traces for the same representative channels. D) Average spike waveform after threshold crossing for both channels.

MUA refers to the simultaneous collective activity of numerous neurons surrounding each electrode. In our dataset, we detect MUA by using a threshold crossing algorithm but less stringent (2σ lower and 4σ upper) compared to the one used for putative single-unit detection. We refer to each spike found as an 'event'. Furthermore, we bin the signal (bin size = 10 ms), allocating the total count of events detected in each corresponding bin (100 Hz). We also have a criterion for artifact removal such that if an event is detected in the same bin in more than a quarter of the total probe channels (32), as occurs when electrical noise affects the entire probe, that bin is excluded from subsequent analysis.

The 11 shanks probe provides us with the advantageous opportunity to monitor the cortical activity sampled from many different areas at the same time. For these reasons we are going to present a preliminary overview of the different routes we took in exploring this dataset.

As per the behavioural analysis, we would like to investigate the brain activity around salient events during each level, touch and reward for level 2 and trigger, catch and reward for level 3. The colour coded probe schematics in Figure 3.14A, serves as guide through the colour schemes used in the Figure 3.14B. We averaged the MUA around each event (±3 s) for all the trials performed by all the rats during each Videogame level.

We first generated a global probe average of all channels (Figure 3.14B, black). This average of cortical activity revealed clear event-related modulation during level 2, around both touch events and reward events. Interestingly, the global average around the touch events followed the speed profile dynamics seen in the behaviour analysis, which is consistent with a broad correlation of large-scale brain activity with movement (Keller et al., 2012). Around reward events, which were aligned to the end of the trial signal, we found a clear artifact pulse corresponding to the activation of the pellet dispensing motors. Preceding pellet dispensing, we saw a global increase in MUA, consistent with anticipatory preparation of reward delivery (Schultz, 2000). In order to characterise where these global changes in brain activity were arising, we next compared shanks from the front of the brain with those in the back (Figure 3.14B, blue and red). Here we noticed different dynamics in different brain regions. Following touch, MUA activity decreases in the back of the cortex (visual cortex), while slowing increasing in the front (prefrontal cortex), possibly reflecting a shift from the more sensory guided foraging phase of the trial, to a more memory-guided reward collection phase. Similarly, around reward, we found a much larger anticipatory increase in MUA in the frontal cortex, also consistent with a hypothesized role in reward seeking behaviour.

We next compared upper vs lower electrodes across all 11 shanks (Figure 3.14B, green and light blue). While the dynamics of MUA detected in upper vs lower cortical layers were similar, there was generally higher activity detected in lower layers, possible reflecting a larger contribution of Layer 5 pyramidal cells to the MUA signal detected by our probe's electrodes.

For both levels, we also averaged random events (random snippets of signal during the session). This confirms that the dynamics noticed are specific to the event which they are aligned to. To further investigate the spatial differences in cortical activation around salient task events, we then averaged each shank and layer separately (Figure 3.15). Following touch, we found that different cortical areas undergo quite different activity dynamics, with the slow ramping activity changes appearing in frontal shanks, and more transient, event-related activity found in the back shanks, with some shanks showing no modulation at all. Prior to the reward event, we saw rich dynamic activity changes throughout frontal cortex, with each shank's activity ramping and peaking at different times relative to the reward delivery, whereas shanks in the rear of cortex were much less modulated.

These results from Level 2 are very encouraging and reveal the potential for large-scale electrophysiology during naturalistic tasks. However, the recordings from Level 3 show broadly similar activity modulation, but the quality of the probe signal had deteriorated and our MUA detection was greatly impaired. Further work on improving probe performance stability beyond the first week of implantation, as well as better algorithms for extracting MUA from noisy data will be necessary to extend these results into later Videogame levels. We are, nonetheless, very encouraged by this proof-of-principle experiment and analysis, which suggests that large-scale distributed recordings from freely moving animals are (finally) possible.

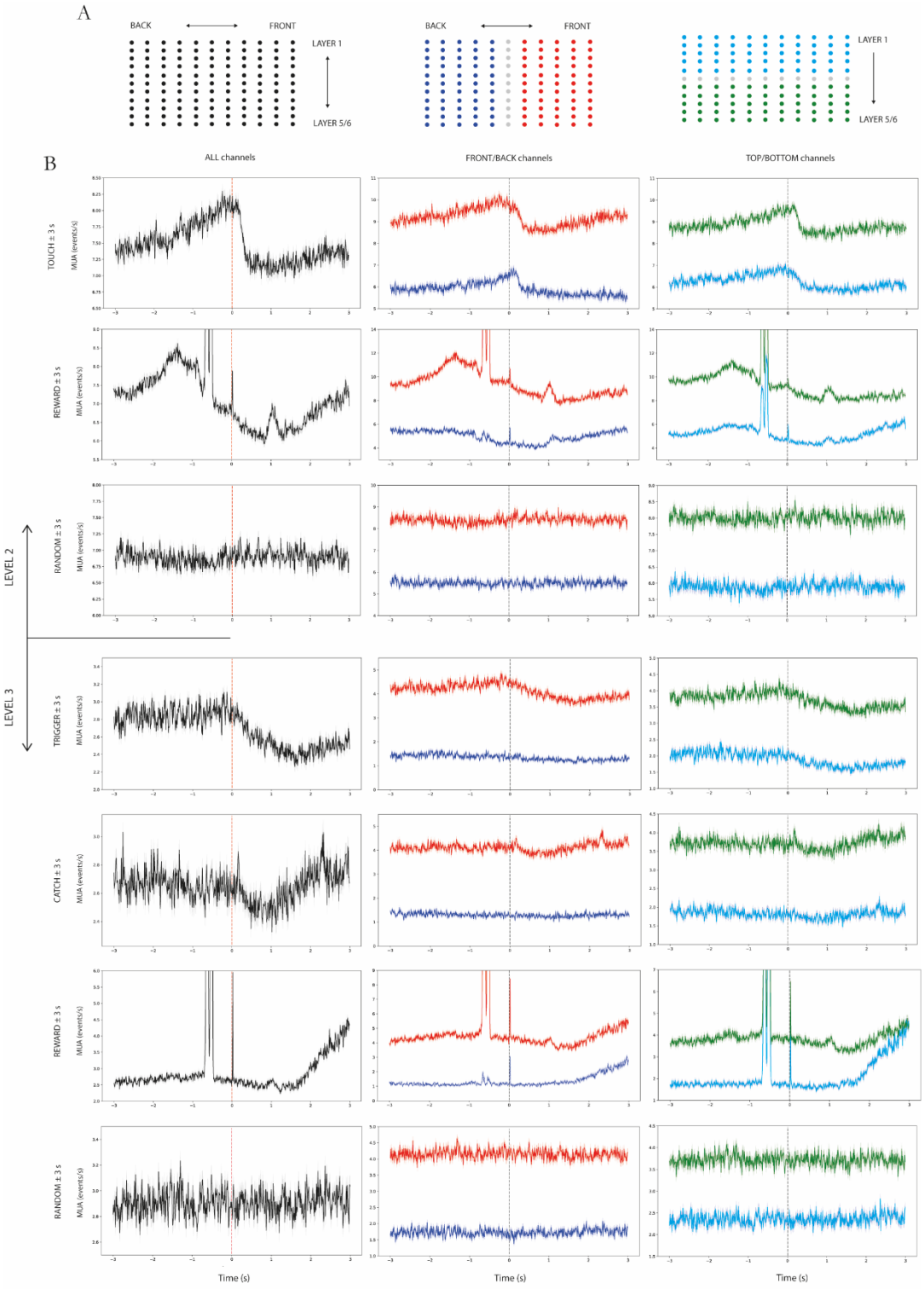
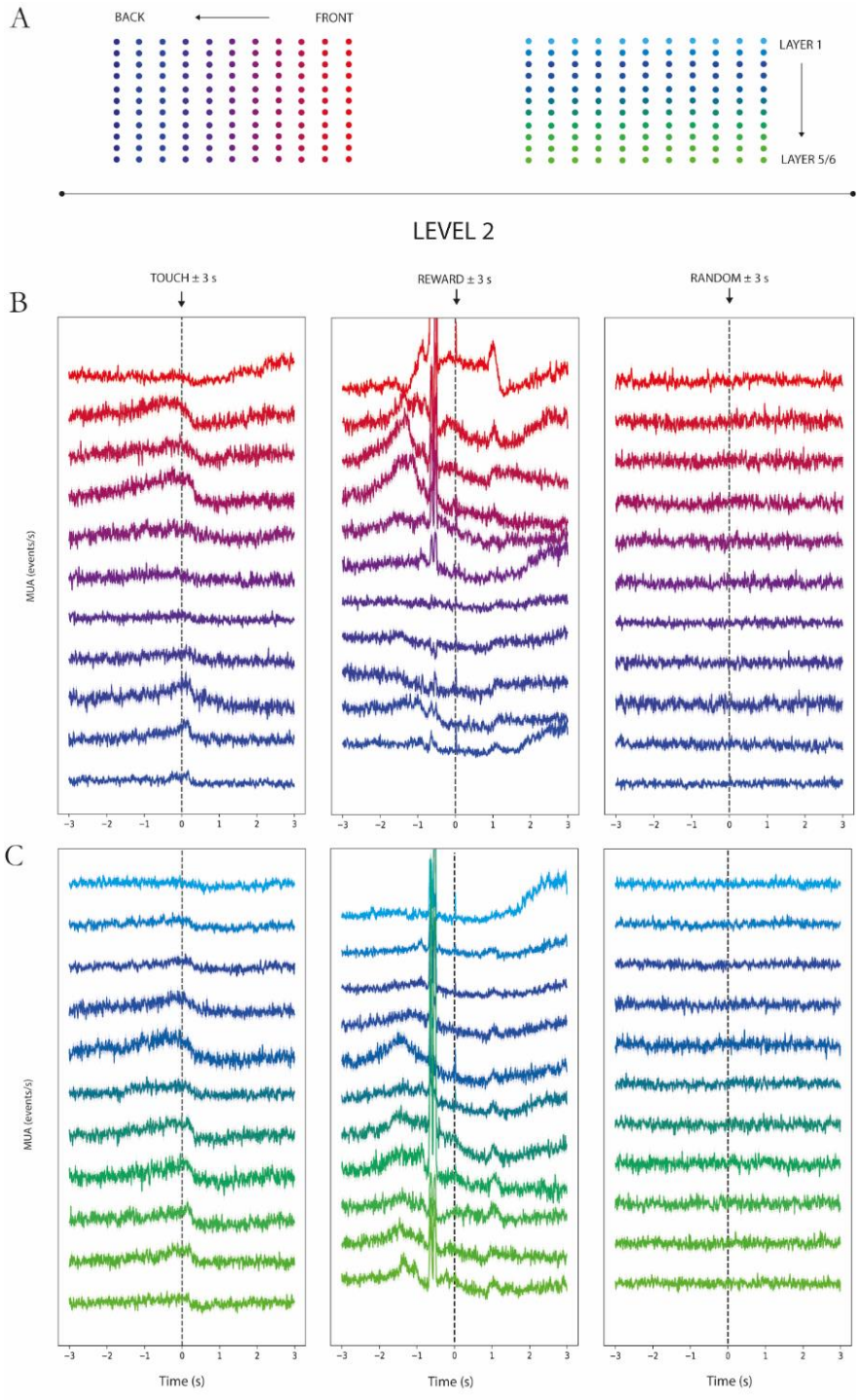


Figure 3.14 MUA dynamics

A) 11x11 colour scheme to help identify which channels are included in the averages. 121 channels average in black. Front and back channels in red and blue respectively. Top and bottom layers in light blue and green respectively. B) MUA activity around Level 2 and 3 events for all 3 comparisons. The event named random corresponds to the average of random snippets (equal to the trial count averaged for that event) of signal for both levels. Number of trials averaged: Level 2: touch n = 935), successful rewards (n = 726), random (n = 935); Level 3: catch (n = 661), trigger (n = 661), successful rewards (n = 522), random (n = 661). Data are shown as mean ± SEM.


A
BACK
FRONT
LAYER 1
LAYER 5/6
LEVEL 2
B
TOUCH ± 3 s
REWARD ± 3 s
RANDOM ± 3 s
MUA (events/s)
C
MUA (events/s)
Time (s)
Time (s)
Time (s)

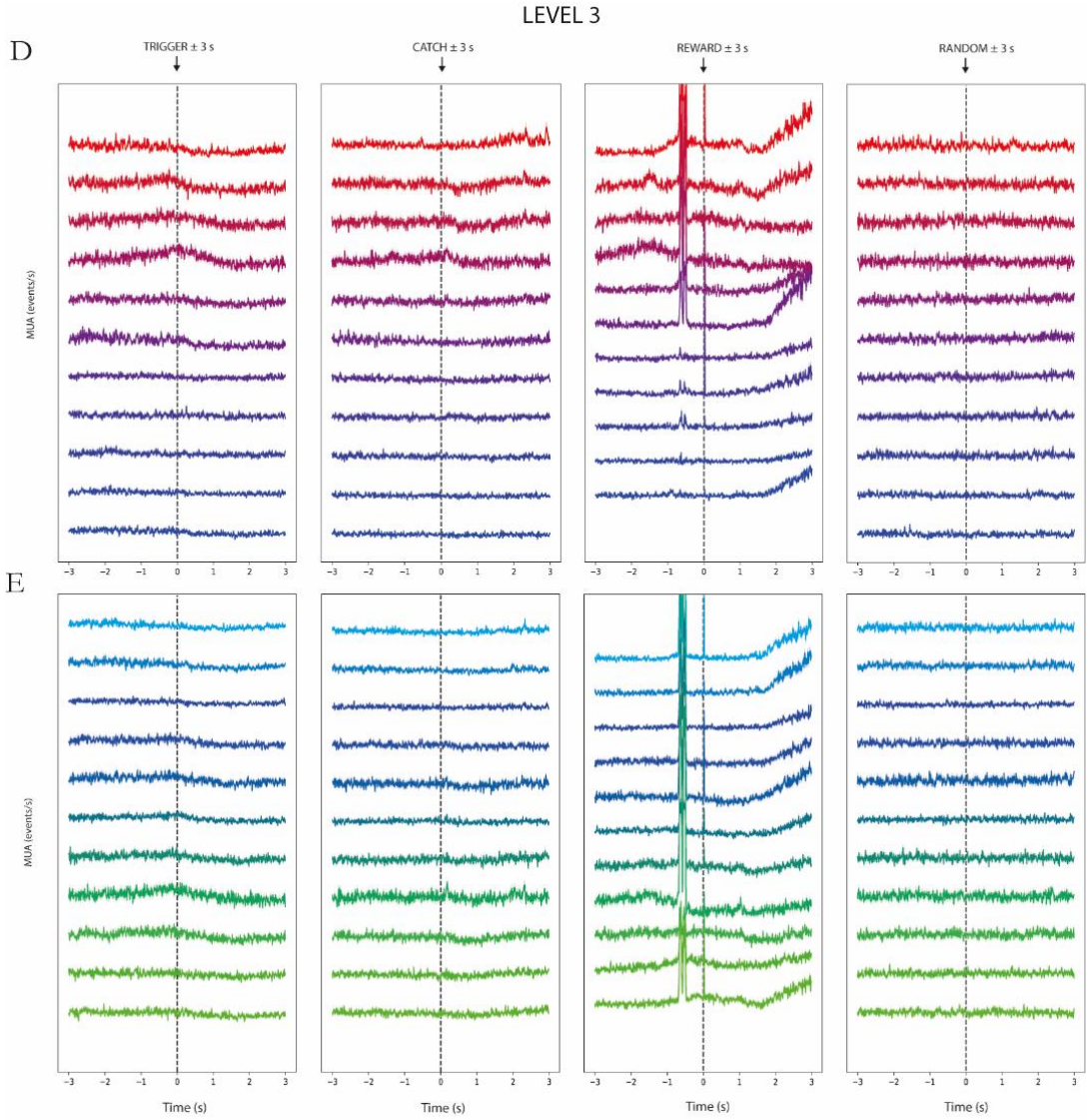


Figure 3.15 Shanks and layers MUA dynamics

A) 11x11 colour scheme to help identify which channels are included in the averages. Red to blue gradient: front to back shanks; light blue to light green gradient: top to bottom layers. B/C) Level 2 shanks and layers MUA dynamics for all the events including random. D/E) Level 3 shanks and layers MUA dynamics for all the events including random. Data are shown as mean ± SEM.

We also explored the low-frequency components of the raw brain signal, the LFP. LFP can be analysed both in the frequency and time domain. In this dissertation we report two exploratory analysis methods that tackle the frequency domain. Figure 3.16A shows a representative lowpass filtered snippet of signal (2 s) for all the 121 channels. A characteristic of the LFP signals is that their power spectrum exhibits 1/f behaviour (i.e., the power at each frequency decreases proportionally to the inverse of the frequency). The power spectrum is

a measure of the variance in the signal as a function of frequency. Figure 3.16B shows the frequency spectra of 1.5 s before and 1.5 s after touch, for two representative channels confirming that our data does follow the expected 1/f scaling. This quick comparison between pre-post event spectra shows differences in power over certain frequencies compared to others. Figure 3.16C empathizes a powerful feature of our probe's layout. In one image we can visualise and gather insights from a large portion of cortex at the same time. In this example, we can see how the power profile varies before and after touch between channels located in the front and back of the cortex and where instead, touching the ball does not affect the power of any bands.

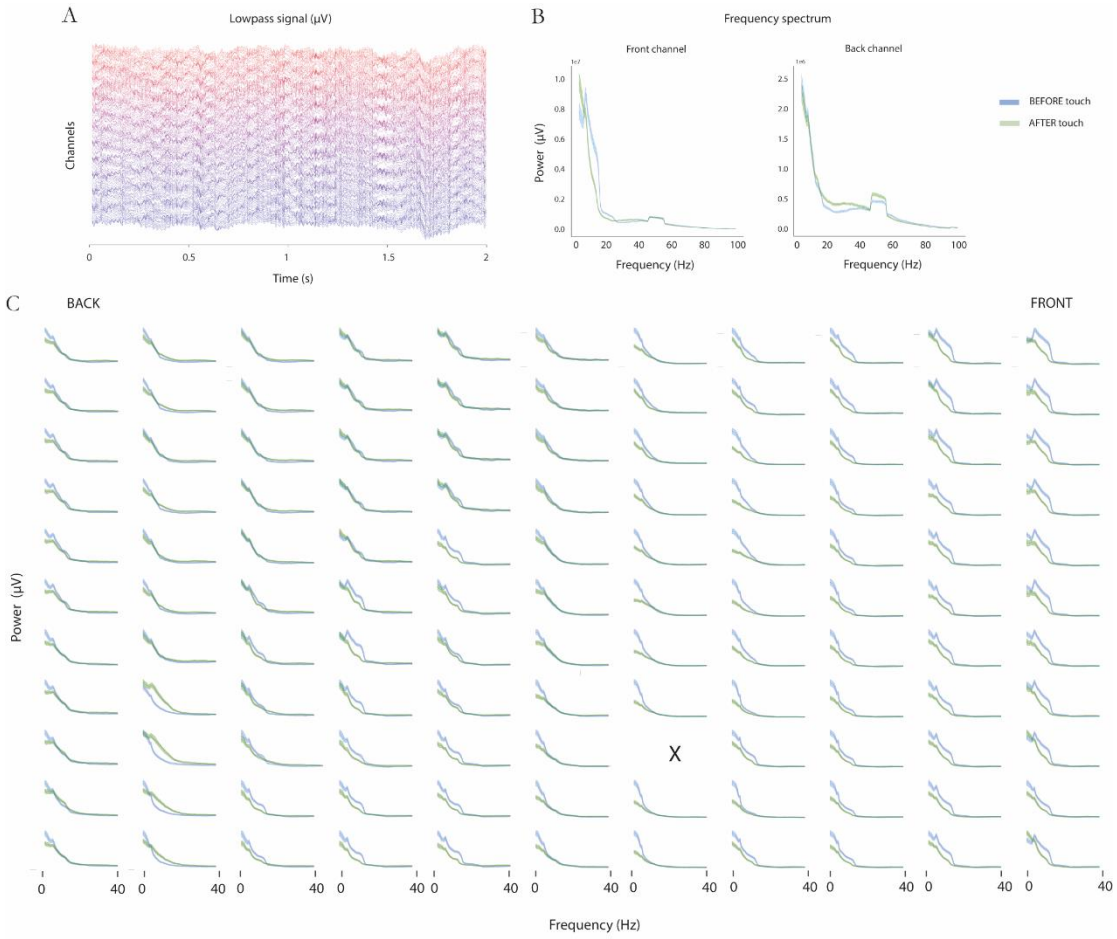


Figure 3.16 Lowpass filtered signal overview

A) Snippets of 2 s raw 121 channels signal taken from the middle (± 1 s) of an early behavioural session. B) Representative frequency spectra for 2 channels comparing the power before (blue) and after (green) touch for frequencies up to 100 Hz. C) 11x11 frequency spectra matrix representing all the channels in the probe. The broken channel is indicated with a x. For visualisation purposes we only show frequency up to 40 Hz and we do not fix the y axis maximum value.

Once again, we decided to focus our analysis on the 5 events of interest previously considered.

We have explored a range of frequency bands: delta (1-4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-30 Hz), gamma (30-100 Hz) and high gamma (100-300 Hz) and how their power changes before and after each event. We show the results in probe-shaped heatmaps to better visualise the real physical arrangement of the electrodes within the cortex (Figure 3.17)

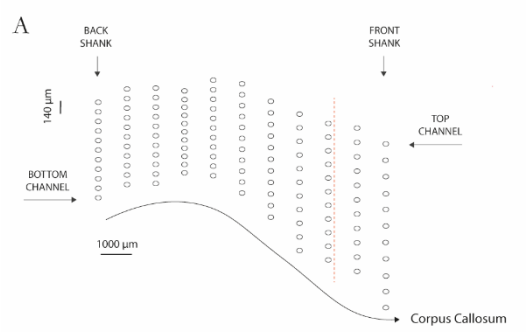


Figure 3.17 11 shanks probe-shape plot schematic

A) Schematic of a plot with 121 channels arrangement resembling the real probe layout. The red dashed line corresponds to the location of Bregma.

Figure 3.18 show the normalised power difference between before and after (± 1.5 s) each event (colour showing the (power after-power before)/power before).

For each frequency band, each channel's power was calculated by averaging over i) the power for all the frequencies in a specific band, ii) all the events of the same type in a session, iii) all the sessions of the correct level and iv) all the animals with good recordings on that level. This resulted in averaging the same total number of trials as for the MUA analysis over 4 animals. In order to ascertain whether the differences we saw were a phenomenon happening in the brain and not a random result due to the comparison of a large number of channels and events type, we first performed t-tests on all possible pairs (each channel/frequency band combination over the trials averaged) and then we applied a non-parametric statistic that allows for a valid correction of multiple comparison as well as clustering the channels

into groups (Maris & Oostenveld, 2007). The channels that are represented with a shape other than a circle are those clusters of electrodes found to be responsible for the statistically significant difference in power before and after the event of interest. Generally, we notice how the significance is predominantly driven by a decrease of the power of the oscillatory activity after the event regardless of the event (shades of blue). The major significant, but global (not localised in a brain area or layer) drop in power can be seen after reward for both levels and several frequencies. This means that most of the cortex is contributing to the drop in power after this event occurs.

Furthermore, with exception of the gamma and high gamma bands, we do see some increase in oscillatory activity in the front (probable motor area) and back of the probe (visual area). We can see this trend happening after reward, both for level 2 and 3.


-0.6 -0.4 -0.2 0.0 0.2 0.4 0.6
Normalised difference
A
Delta (1-4 Hz)
Theta (4-8 Hz)
Alpha (8-12 Hz)
BACK
FRONT
TOUCH
REWARD
Level 2
Level 3
TRIGGER
CATCH
REWARD

Figure 3.18 Power difference clusters heatmaps

A) Probe shaped heatmaps representing the power difference for 6 frequency bands and around touch and successful reward (level 2), trigger, catch and successful reward (level 2). The channels drawn as circles do not contribute to the difference in power before and after the event considered while the other channels' shapes corresponding to clusters found to be responsible for a statistically significant change in power around the event. Number of trials averaged: Level 2: touch n = 935), successful rewards (n = 726), random (n = 935); Level 3: catch (n = 661), trigger (n = 661), successful rewards (n = 522), random (n = 661). Data are shown as normalised difference.

Overall, it seems like lower frequency bands show more differences compared to the beta, gamma, and high gamma bands. Those differences appear to be localised in the central and deeper portion of the probe (probable associative areas and granular and sub granular layers). These results lead us to hypothesize that the increased power (for the alpha and theta bands) before events like touch, trigger and catch could indicate an increase in the animal 'cortical engagement' in the task, in the aforementioned areas, providing a first glimpse of overall cortical engagement surrounding time points that require a recalculation of near future behaviour. On the contrary, a drop of power in higher frequency after the reward has been successfully collected could indicate a global disengagement of the cortex.

Having shown that the cortex displays a rich "behaviour" of power changes surrounding the most salient behavioural events, the next step would be to calculate these power differences in much smaller but sliding time windows (time-frequency analysis). This would allow us to detect more specific temporal/ frequency patterns that would be more amenable for further analysis of correlations between the time and the frequency specific power shifts in certain parts of the brain.

To open the path to further generation of hypothesis, we have also analysed another aspect of the LFP: the Inter Trial Phase Consistency. More specifically we asked whether the oscillations in the same frequency band for different brain regions fall in or out of phase during the 5 events considered. Using ITCP measure, we aim to detect LFP synchronisation between the cortical regions recorded, we thus investigated the 'pairwise' phase differences for 33 clusters of channels which are highlighted by a pill shape outline in Figure 3.19A. The choice of the clusters took into consideration the distance between channels as we tried to maintain each cluster equidistant in both AP (shanks) and DV (functional layers). Neighbouring clusters that are in different shanks have a distance of 1 mm (AP) while clusters within the same shanks have a range of DV distances of 0.4 - 0.7 mm.

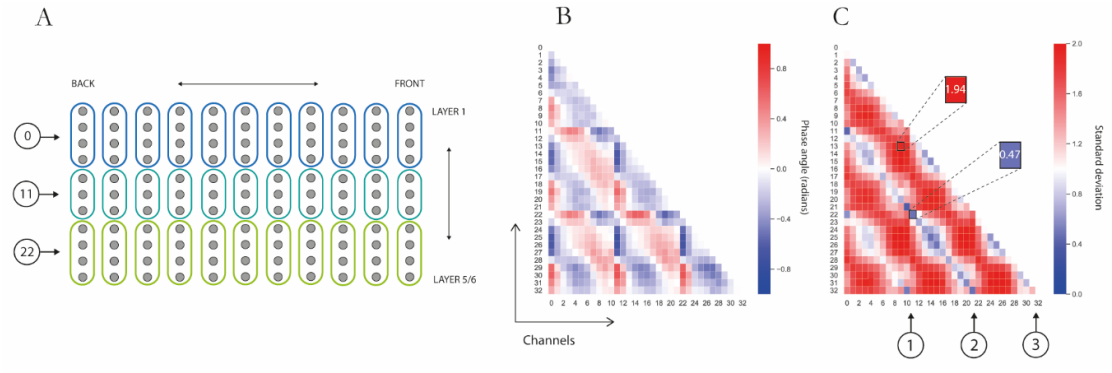


Figure 3.19 Inter trial phase consistency schematic

A) Simplified schematic of the 11x11 probe highlighting the subdivision into groups. Each pill shape outline surrounds each one of the 33 groups composed by 3 or 4 channels. The circled numbers indicate the clusters reference number. B) Representative correlation matrix showing the average (mean) over trials, phase difference (angle) for each group pair. Data are showed in radians. C) Representative correlation matrix showing the standard deviation of the phase angles over trials for each group pair. Highlighted two examples of low and high standard deviation (pairs: 13-9 and 22-11). The circled numbers indicate the 3 areas of higher phase coherence (shades of blue). The results shown here are for all the trials of one rat and refer to theta band around touch.

Figure 3.19B and C show an example of the correlation matrices that compare the phase consistency of each group pairs (theta) for all the trials performed by one rat around touch. We report the mean phase difference angles (B) and corresponding standard deviations (C). The standard deviation gives a good estimate of how spread the ITPCs are for that pair of trials. As per the 2 pairs highlighted in C, a pair with low STD suggests that for that event and over all the trials the ITPC varies little while a larger STD suggests that the phase could be anything within $-\pi$ to π. We refer to small STD as the phase being coherent/consistent. For this analysis we pooled all the trials performed by all the rats ($n = 4$) for each level and event ($\pm$ 1.5 s).

Figure 3.20A does not show any change of ITPC in relation with any of the behavioural events as proven by comparison with the random events.

Nonetheless, 3 main areas (numbered in Figure 3.19C) of large ITPC (low STD) appears, and they seem to vary based on the frequency. Area 2 corresponds to the comparison of top and middle clusters, and of middle and bottom clusters suggesting higher synchronisation along cortical layers (DV). Area 1 refers to the comparison of top and bottom clusters confirming a consistency in the phase within the same shank. Area 3 compares neighbouring

clusters along shanks (AP) and shows a weaker phase coherence compared to areas 1 and 2. Furthermore, gamma frequencies seem to have the highest phase consistency amongst all the frequencies which is surprising as higher frequencies are not expected to synchronise as easily as lower bands.

Summarising, our analysis shows higher phase consistency between shanks than over functional regions, but further statistical analysis are required to confirm these findings. A next step in this analysis would again be the generation of phase difference temporal traces surrounding the behavioural event. A second step would be the detection of possible correlations between the phases of the detected oscillations and the spikes detected in the MUA signal, not only within the same channel but, between channels or clusters pairs.

Figure 3.20 Inter trial phase consistency

A) Correlations matrix heatmaps showing the pairwise standard deviation for all the 33 groups for all the frequencies and events considered. Blue patches indicate higher phase coherence. Number of trials averaged: Level 2: touch n = 935), successful rewards (n = 726), random (n = 935); Level 3: catch (n = 661), trigger (n = 661), successful rewards (n = 522), random (n = 661).

3.4.4 Verification of 11 shanks probe insertion

At the end of every experiment, we collected the brains and sagittal slices (50 μm) were sectioned using a vibratome and stained for GFAP and DAPI. In Figure 3.21A we present a selection of the best slices (visible shanks traces) for each of the rats included in the dataset. For technical reasons it proved to be difficult to visualise all the 11 shanks in one slice.

The cortical surface appears damaged in some of the slices, and this is due to the pressure applied to remove the 11 shanks from the brain when retrieving the headstages and the presence of scar tissue. In Figure 3.21B we enlarged the cortical view of the 11 traces left by the probe shanks. In this slice, we could capture all the traces and we can clearly see how most of the shanks sit nicely within the cortical layers.

As we can notice, the probe insertion varies across animals predominantly medio-laterally. This is due to a variety of reasons during surgery such as alignment adjustments, difficulty in piercing the dura in the target location, dura or blood vessels across the insertion sites. However, it is clear that locating the position of each shank is possible, and that validation of cortical targeting with post-hoc histology is possible with our multi-shank probe.

Details on surgery and target coordinates in Appendix D (Table D.1).

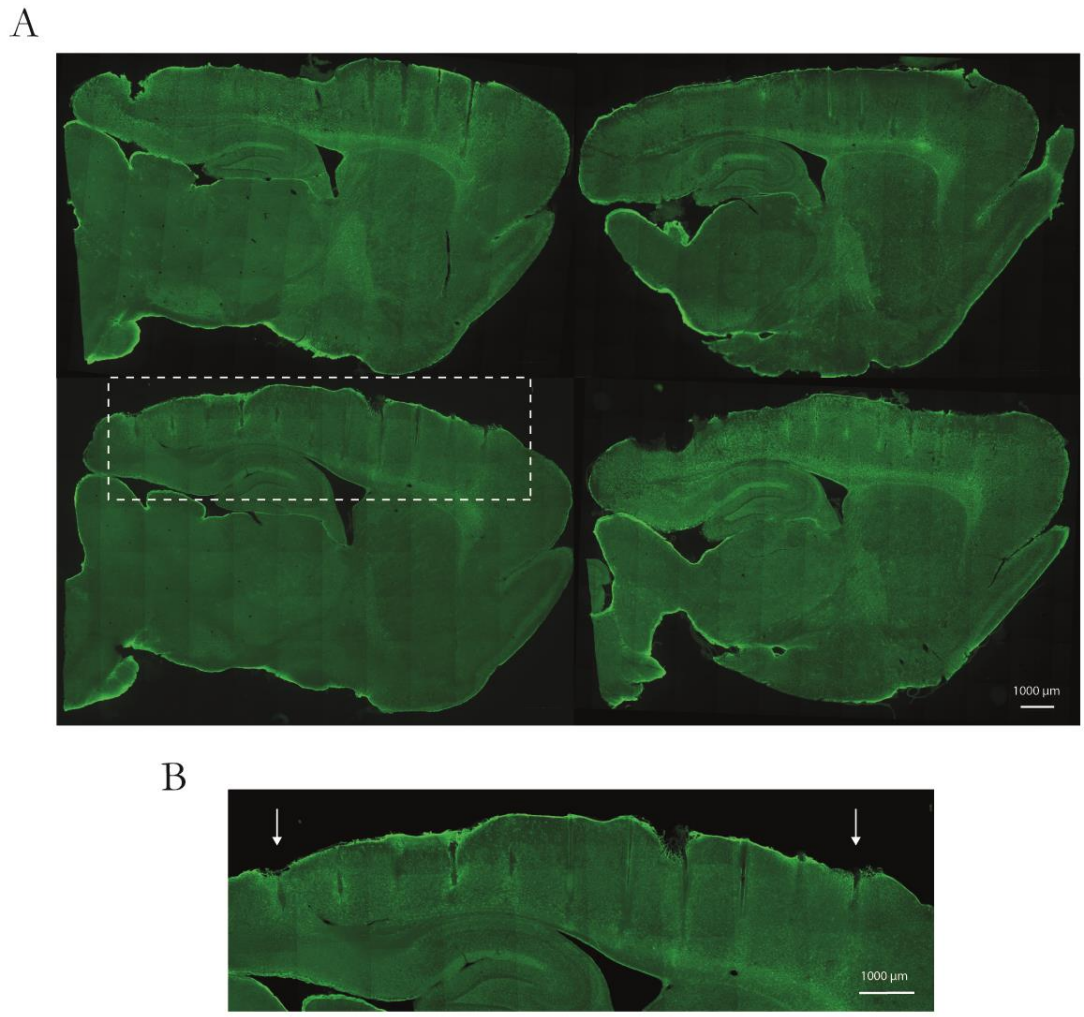


Figure 3.21 11 shanks probe histology

A) Sagittal view of the 4 best slices (GFAP) for all the rats included in the dataset presented.
B) Detail of the area within the white dashed box showing 11 traces detected in the cortical layers. Picture processed using ZEISS ZEN microscope software (Zen 2, blue edition).

3.5 Discussion

Videogame performance decline post-surgery

After surgery, rats do not seem to have the same drive and motivation in playing the Videogame despite knowing the rules of the task. From closely watching them, it does not seem likely to be due to the tethering because when they do engage, they run and hop over walls without discomfort or impairments. Nonetheless, we have tried to tackle this issue by changing both the implant, embedding the headstages to ease the daily tethering procedure, and the post-surgery care with administration of the medication via gelatine medium rather than injections to remove the stress induced by these procedures. Yet, we still see a reduction in their performance that is mainly reflected in a decrease of the daily trial number. Unfortunately, due to the quick decline in impedance and implant stability, we could not opt for a longer post-surgery recovery period which could have helped our rats to regain full strength before having to perform in the videogame. Being in isolation might also have affected their performance even though we did try to habituate them prior to surgery. We have attempted to test our rats in pairs, to explore whether this could help raise their motivation. This approach did not succeed due to the defensive and aggressive behaviour displayed by the implanted animals in response to being in a more vulnerable state. Obviously, cable and the implant are also more exposed to damage, therefore we do not think this could be a route to follow in our setup.

Interestingly, encountering a moving ball for the first time, evoked a similar response in rats' post-surgery compared to rat that did not undergo surgery, despite their reduced performance during the task. Due to the decline in data quality by level 3, however, we did could not analyse the neural correlate of this response.

11 shanks probe: a tool for a distributed exploration of the brain

The probe life proved to be quite short and did not allow for long lasting high quality chronic recordings. We tested two types of probes with channels made of different materials (Pt and IrO_2), both used previously. Unexpectedly, the IrO_2 channels showed a very unstable behaviour, and we could not include the results from those probes in our dataset. We do think that some problem in the fabrication and deposition might have occurred, but due to the limited amount of probes provided we could not further investigate the reason why we could not obtain long stable recording with IrO_2, but also more generally with the 11 shanks probes. The probe sizes and shape have dictated many of the choices we made due to the fact that the probe could not be scaled, and the shanks were designed ad hoc to fit a certain position within the cortical layers. For the future, having a more flexible design could be advantageous, either with a smaller inter shanks spacing or a much denser channels arrangement. Gathering data from the cortex at higher resolution, compared to the state of the art 11 shanks probes, would lead to more insightful data on the role of cortex during complex multi-modal behaviours. With better chronic recordings, one could also consider following the learning process starting from level 2, but instead we had to train rats on this level already prior to surgery.

As per the Chapter 3 Results section, we have explored the neural data in a variety of ways, both in the MUA and LFP frequencies, and showed encouraging preliminary findings. We gathered a global cortical view showing the presence of dynamics that are shank and layer specific which correlate with some of the behavioural events considered. One could think of starting to explore the LFP data in the time domain and not only in frequency. Comparison between different cortical regions and layers was also possible as well as applying the inter trial phase consistency approach to investigate power-phase and spike-phase coherence between areas. Regarding the MUA, we think that having a denser arrangement of electrodes, possible CMOS based, would allow for isolating single units with spike sorting methods.

Beyond improvements to the probe itself, we think improvement could also be made in the implant design to ensure more stability and longer recordings. In fact, after about 2-3 weeks from surgery, we noticed that the signal would be noisier if rats would hit the implant. The hardest part of the surgery was inserting the 11 shank, better ways of aligning the probe could improve, facilitate and make the insertion more reliable.

3.6 Conclusions

The aim of the work presented in this chapter was to determine if it was possible to implant and chronically record from cortex with a multi shanks silicon probe during complex behaviours. This was assessed by first designing a novel custom 11 shanks silicon probe featuring a peculiar shanks length matching the cortical thickness. While challenges were encountered with designing the ideal probe holder and surgical protocol, several changes and strategies were employed to successfully implant 11 shanks in cortex chronically for about 1 month. We recorded the cortical activity from 4 rats while performing level 2 to 4 of the Videogame. Overall, we noticed a decline in their behavioural performance despite knowing the rules of the tasks. Nonetheless rats engaged in the task and committed to it, just at a slower pace. We could record good quality neural data for about 10 days after implantation with subsequent quality decline due to an increase in impedance as well implant instability. We presented preliminary analysis of the he neural data both at low (LFP) and high (MUA) frequency bands. From the choice of analysis made it was not possible to extract any behavioural modulation in the LFP bands while the contrary was true for the MUA. Nonetheless, an unprecedented extent of cortical recording was achieved using the 11 shanks probe, but further in-depth analysis is required.

Further work should aim to increase the electrode density of the shanks to allow for spike sorting of the data as well as optimise the electrode design and impedance to guarantee long lasting chronic recordings.

Chapter 4

General discussion and conclusion

Study of complex behaviours through a Videogame

This dissertation describes our attempt to combine semi-naturalistic complex tasks with neural recording, not from isolated brain regions, but from the scale at which brains work. The main goal of this work was to prove that it is now possible, given recent advances in technology, to achieve such an ambitious experiment. "The raison d'être of the central nervous system is to optimize the organism's ability to interact with its environment" (Engel & Schneiderman, 1984). Animals and brains evolved for and in complex environments that have very little resemblance to our laboratory settings. In recent years, the need to expand and explore behaviour beyond the common simplified and reductionist approach is increasingly in vogue. Many scientists acknowledge the need for an increase in the complexity of neuroscience behaviour assays (Krakauer et al., 2017; Gomez-Marin et al., 2019). The Videogame fits well with the changing vision in the field because its flexibility allows one to easily introduce the complexity that a laboratory lacks and that brains are exposed to in the natural world.

These steps towards a more "natural" assay do not come without their challenges. Behaviourally, we are not only increasing complexity, but we are also introducing 'noise'. Unstructured and unrestrained tasks like the Videogame, contain a lot of insights mixed with confounding elements deriving from the way we set up the experiments. This is similar to what would occur in nature, and we did know this would happen and we did embrace it from the start. We can envision the Videogame as being a 'white behavioural canvas'. In fact, due

to its flexibility, it is a good substrate for the exploration of a multitude of different designs and ideas matching the needs of many neuroscientists.

Videogame and Virtual reality set ups

Virtual reality in rodents has emerged as a powerful tool that not only allows for the implementation of new behavioural tasks but also facilitates electrophysiological and optical measurements that benefit from restricting the animal's head movement providing precise control over sensory stimuli. Rodents VR applications thus far have been mostly limited to the visual modality and the behavioural tasks employed are usually on linear tracks (Dombeck et al., 2010; Harvey et al., 2012). Rodents have been trained to locomote toward distal virtual objects in one- and two-dimensional environments and stop upon reaching them (Hölscher et al., 2005; Kaupert et al., 2017). They can also learn to avoid virtual walls, which allows researchers to build virtual mazes, teach rodents to shuttle linear corridors (Driscoll et al., 2017; Sato et al., 2017) and make decisions in Y-mazes (Thurley et al.,; 2017, Pinto et al., 2019).

Nonetheless, the commonly used head fixation approach impedes the full extent of voluntary and natural behaviours, but several attempts to design more immersive and less constrained VR behavioural assay have been made. For instance, Cushman et. al, developed a non-invasive immersive VR maze modelled after the Morris water maze (Morris, 1984), without using head fixation, to study spatial navigation in rats (Cushman et al., 2013). Also, a VR treadmill for freely moving rodents has been designed to be integrated with the animals' group home cage so that each individual can voluntarily enter and initiate the behavioural session allowing for highly time-efficient experimentation in complex yet controllable virtual environment (Kaupert et al., 2017). Finally, rather than simulating movement, some VR setups allow full freedom of movement in space through head tracking. This can be found both in head-mounted VR setups and in freely moving CAVE (Computer-Assisted Virtual

Environment) setups, where the subject's head position is tracked continuously via accelerometers, gyroscopes, or multi-camera arrays (Del Grosso et al., 2017).

Another valuable but underutilized technique is the use of Augmented Reality that combines real-world features with virtual projections. By combining interactive virtual objects with real life objects, we can achieve higher level of complexity and elicit more interesting and rich behavioural outcomes with lower technological barriers to overcome compared to immersive VR assays. Examples of naturalistic behaviours elicited using Augmented reality have been studied in spiders and prey manties attacking 2D moving dots (Fenk et al., 2010; Nityananda et al., 2016) as well as in Zebrafish displaying social interactions once exposed to virtual models of conspecifics (Larsch et al., 2018). Our attempt to create a novel behavioural assay for implementing complex and naturalistic tasks using AR, resulted in the design of the Videogame. Combining a highly flexible hardware with Bonsai, we used AR in a novel and unprecedented way generating a variety of tasks for rats with ease and easy for them to successfully learn. Our assay fits well in the context of stepping forward in the design of tasks that account for the ethological relevance and complexity typical of the natural world.

What did we learn?

Several steps were necessary to develop what we called the Videogame. Taking inspiration from the Floor projection Maze developed at Brown University (Furtak et al., 2009) we designed and built a behavioural assay that would easily allow to implement and test several experimental protocols. In fact, a key feature of the Videogame is the ease at which a scientist can put into practice ideas and concepts, from the simplest to the most challenging. This, facilitates and speeds up the process of finding the right behavioural protocol needed to start answering a scientific question. In our project, the combination of a flexible hardware and software has made it possible to achieve the level of complexity that we desired our rats to experience and the one we think is appropriate to study in neuroscience. Rats not only

habituate to the assay but show high engagement and interest towards projected virtual objects which was something we did not take for granted at the very beginning of the project. Surprisingly their level of adaptation and understanding allowed us to explore Videogame's levels with various difficulties. Our behavioural approach was successful in allowing them to learn each one of the levels and accumulate enough knowledge to perform in the following more complex one. All the rats tested in this study, were successful in performing in the Videogame showing an increase in the number of trials performed each session as well as an increase in the rate of performance. Within a week, rats could learn the basic rules required in the task. Rats, within two days, could interact with a projected virtual object, never encountered before, to earn a reward. Impressively, they also shaped their interaction according to the level and meaning of the object itself.

The Videogame flexibility also allow to introduce unexpected events, typical of the unrestrained and unpredictable natural world. By solely watching our animals, a strong reaction to a change in the virtual object behaviours, was evident and we could capture it by using a simple ethogram. Finally, as a refinement based on the 3Rs, we showed that rat can perform the tasks without the need of motivational aid which suggest the high level of engagement induced by the tasks we designed alone. Thanks to the Videogames we could assemble the building blocks to create a controlled complexity.

11 shanks probe in the context of multi shanks and areas recordings

Technological developments now allow including naturalistic and complex nuances to the study of behaviour as well as gathering insights from incredibly large neural ensembles. To understand brain's dynamics, it is necessary to study the neural activity at local as well as global spatiotemporal scales.

The unprecedented layout of our 11 shanks probe was meant to serve as an example of what we could achieve by adding an extra dimension to the more conventional 1 shaft recordings.

Technological advances have allowed for enormous steps forward in the accessibility to the brain, but can we step further?

Unfortunately, there is not a "one-fit-for-all" solution for probe design but the probe fabrication process allows tailoring the size, shape and electrodes arrangement that best suits the area of interest to record from (Buzsáki et al., 2015). To monitor a large portion of the cortex, our strategy has been to customise a silicone probe with multiple shanks (11) to be able to reach as much cortical surface as possible (1 cm) while accounting for implant and surgeries feasibility. The study of the brain using multi shanks probes has already been attempted for diverse purposes, in different animal species and combined with other techniques, but the shanks were usually designed to be much closer to one another and to target a small portion of the brain (Royer et al., 2010; Buzsáki et al., 2015; Fiáth et al., 2016; Wang et al., 2020; Steinmetz et al., 2020; Novais et al., 2021). An entire brain structure that is especially suited for multi shanks approach is the hippocampus, as shanks can target different hippocampal structures simultaneously providing insights on the ongoing neural dynamics (Csicsvari et al., 2003). For decades, due to technical constraints, neuroscientist were limited in the kind of neural recordings they could perform but recent developments have brought a growth in experiments that gather large-scale surveys of the neural responses from a variety of brain areas including understudied, hard to access areas, as well as prompting new experimental design to elucidate neural flows across areas (Urai et al., 2021). Our probe not only allows to simultaneously record from neurons located 1 cm apart from one another but also from different cortical locations otherwise reachable only by implantation of several probes. The 11 shanks probe proves that this route might be one to take, because it provides an unprecedented and much needed view of the distributed neural dynamics, despite the (surmountable) challenges to assemble and implant.

What did we learn?

As proof-of-concept design, we demonstrated that it is possible to implant a large linear array of shanks in the cortex and records brain dynamics without damaging the brain tissue.

The 11 shanks can monitor and sample the activity of the same brain structure from multiple locations providing insights into cortical dynamics otherwise difficult to gather with different approaches. Several challenging steps were required to achieve satisfactory and successful chronic recordings. The design of the probe implant has required many iterations to be able not only to accommodate the probe but to be suitable for long lasting stable recordings in freely moving rats. Iterations were also needed to optimise the implant weight and size such that it could be carried with more ease. All the rats that underwent surgery could successfully perform in the Videogame for up to 3-4 weeks when a decrease in implant mechanical stability made it impossible to safely continue the behavioural assessment. Unfortunately, the extent of the surgical intervention was responsible for the decrease in stability seen over time. The big unilateral craniotomy, required for the successful insertion of the shanks, reduced the space available to add screws and cement, making the right side of the implant more unstable. Also, despite PEDOT coating, the probe quality showed signs of decline after about a week post-surgery making it difficult to gather good quality data especially for MUA analysis. Nonetheless, we gathered a rich dataset and we performed preliminary and exploratory LFP and MUA analysis. Higher electrodes density on each shank would have been beneficial to record from the brain at a higher resolution and apply spike sorting to the data to gather more insights on the source of the neural activity. This should inspire high shank count CMOS probes with sufficient electrode density to isolate single spiking units. The 11 shanks design is predominantly suited for exploratory studies, like ours, in which the involvement of different cortical areas, and their possible coordination in specific behavioural tasks is surveyed. Finally, being able to access different cortical areas simultaneously can be advantageous not only to understand cortical dynamics, but it can

serve the implementation of the 3RS principle of reduction. In fact, one experiment allows for the collection of extraordinarily complex and rich datasets that could be assessed and shared between laboratories surveying a larger community than the single isolated lab.

Videogame and 11 shanks probe: a hypothesis driven approach

Both Videogame and 11 shanks probe have being designed for an exploration driven approach. Nonetheless, often a hypothesis driven approach is the favourite choice of scientists. There are a variety of applications whereby both tools could be utilised in the former, more traditional approach. However, if one's interest lays in understanding in detail a cortical region of interest, its connectivity and neural activity, the 11 shanks probe would not be the ideal device of choice because it lacks enough fine resolution to achieve such specific goals. Currently, the assay is being used to study parametric spatial working memory in rats by colleagues at the Sainsbury Wellcome Centre. The developers of the Floor Projection Maze at Brown University have been using the assay to assess rats in visual discrimination, attention and navigation tasks (Furtak et al., 2009a; Jacobson et al., 2014; Scaplen et al., 2014). We propose the following hypothesis driven experiment that combines both Videogame and 11 shanks probe while maintaining ethological relevance, a key feature and rationale of the project described in this dissertation.

Rationale

In a complex world, a sensory cue may prompt different actions in different contexts. The nature of the Videogame assay, as well as the broad cortical reach achieved by our 11 shanks probe, could be used to study context dependent modulation in cortex during ethologically relevant tasks. We suggest the implementation of a predatory-prey behaviour where an auditory cue defines the meaning of the virtual object projected and we propose this to be either a prey to hunt or a predator to avoid. Rats would initially have to assess the virtual object identity (prey or predator) via the auditory cue and adapt their behaviour accordingly. Once the cue-identity association is learned, we would randomise the cues on a trial-by-trial basis to probe the rat's behavioural flexibility to the change in context.

Behaviourally, we expect rats to learn to escape from the predator or pursue the prey after identification. Escape is an instinctive defensive behaviour that has evolved to avoid harm from predators or threats from the environment.

At the neural level, we hypothesise that a context-dependent modulation of neural activity would flow systematically throughout cortex and, being able to target and monitor from frontal cortex to visual cortex, we expect to capture a cortical gradient of modulation. Due to the probe layout, we can also record the neural activity patterns across multiple cortical layers. This experiment would allow us to start answering to what degree is the neural activity localized or distributed and whether it is a continuum or relates to known boundaries between cortical areas.

The availability of increasingly large-scale, cellular recording techniques enables a thorough survey of the diversity of neuronal responses and the dynamic interactions that exist across cortical areas (Sofroniew et al., 2016, Jun et al., 2017). New evidence suggests that the encoding of task-related information can be highly distributed across related cortical areas (Steinmetz et al., 2018; Minderer et al., 2019). However, functional recordings alone do not provide insight as to whether such widespread signals are due to local processing within an area, or it results from other connected areas. For these reasons, the information flow between areas must also be tracked (Condylis et al., 2020).

Methods

To achieve this goal, we must train rats with a slightly different approach compared to the one applied for the Videogame protocol. During the prototyping phase, we noticed that projected objects are usually source of interest for rats, thus making it a challenge to design an effective avoidance protocol.

We would train rats up to level 2, as per protocol extensively described in Chapter 2, so that in two weeks rats can successfully interact with a static virtual object to ear reward. Differently from the previous protocol, we

would add an auditory cue at ball appearance when transitioning to Level 3. This to signal that a prey or a predator is in the arena.

We introduce the cue one at the time starting with the "prey-cue". This cue indicates that, as per original Level 3, rats need to learn to pursue and catch a moving virtual object or prey. Each trial lasts 20 s in which rats need to successfully contact the prey triggering the reward availability tone. If within 20 s rats cannot catch the prey, the trial is considered missed, and no reward is available. Once rats learn this level, we introduce a different auditory cue indicating a change of context, the prey is now a predator chasing the rat.

The trial length remains the same and if the rat is caught by the predator within 20 s (the virtual object chases the rat contacting its body) the reward is omitted, otherwise the trial is considered successful. If rats can understand this level, we would randomly present them with either of the two contexts by playing the cue at the beginning of the trial. The same visual cue represents opposite entity and rats need to flexibly change their strategy based on the contextual cue provided at the start of each trial. As per typical Kampff Lab approach we could introduce an unexpected switch of context within the trial to elicit interesting behavioural outcomes and monitor the cortical activity during these sudden events. Schematic summarising the behavioural protocol is shown in Figure 4.1.

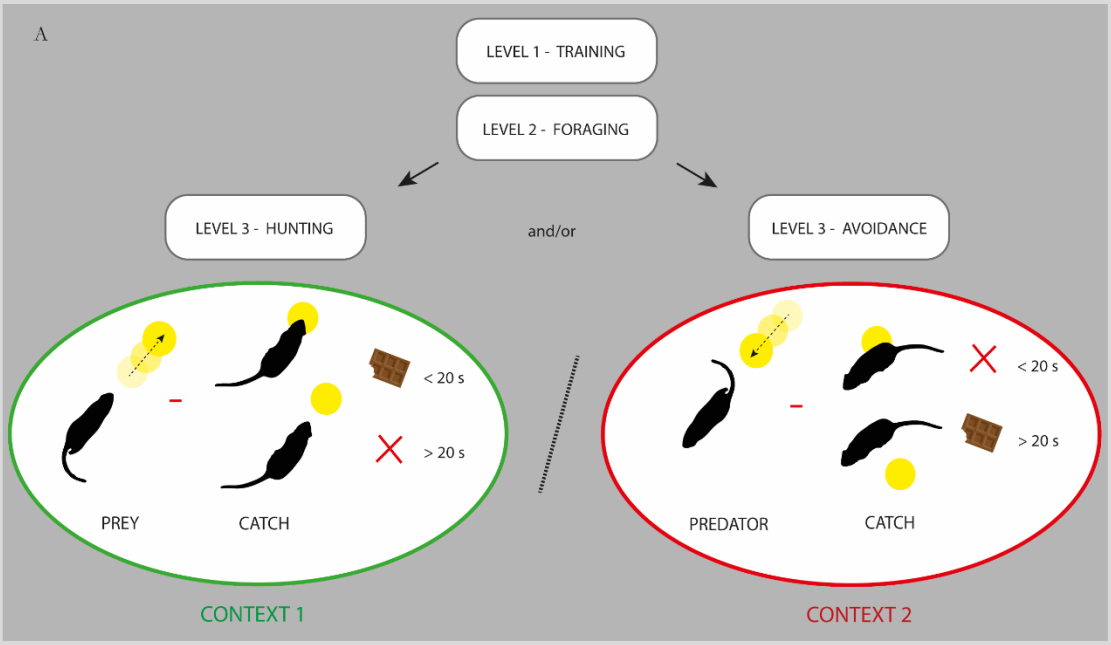


Figure 4.1 Example of hypothesis driven experiment using the Videogame

A) Schematic showing a possible behavioural protocol to be used for training rats in the proposed hypothesis driven experiment. In green one of the two contexts to learn where the ball is a prey and rats need to chase it and catch it, as per Level 3. In red, the second context in which rats become a prey and the ball the predator. Rats need to avoid being caught by the predator for at least 20 s to receive a reward.

There are a variety of ways in which we could generate the prey and a predator using Bonsai. Either we use a similar strategy to Level 4 where the virtual object movements are generated by a human subject via a joystick, or we could use simulations called Animats. Unlike standard artificial intelligence approach that aims at simulating the most elaborate faculties of the human brain (memory, decision making, language and logic), the Animat approach is based on the construction of simulated animals or robots capable of surviving in unpredictable environment (Meyer, 1996).

The surgical procedure to implant the 11 shanks probe would be identical because, due to the probe static and pre-defined layout we are limited in the position in which to insert it. Improvement can still be implemented in both probe holder and surgical procedure if needed. The surgery is performed after level 2, if successfully learnt, and after recovery, we assess rat's behaviour starting from the same level for at least one or two additional sessions before transitioning to Level 3 with contextual cues.

Expected results

From extended experience in designing behaviour training protocol as well as from insights acquired during the prototyping phase of the Videogame, we expect rats to quickly learn the different behaviour to adopt in each context. In accordance with the literature, we expect to observe task-related activity widely spread across the entire cortex rather than a more localised ramp in activity in specific locations (Pinto et al., 2019). We do not expect to see sharp boundaries between the cortical areas recorded but rather a gradual flow of information between them. We would search for the presence of neural cells that respond to the contextual cue, and we would investigate their modulation during the switch in contexts. Interestingly, we are also able to capture the cortical activity during sudden changes of context or mismatches between cue and expected virtual object behaviour.

We expect our neural responses to change as the rat learns the correct behavioural response to the auditory cued context switch (prey vs predator). Initially, we expect responses to the visual cue to be unaffected by the presence of the auditory cue, throughout cortex. However, after learning that the presence/absence of the auditory cue requires different behaviours, we expect to see the visual response modulated, albeit differently in different cortical areas.

In visual cortex, we expect the visual response to remain largely unaffected (unmodulated) after learning in *most* cells. However, visual responses in visual cortex have been shown to be modulated by movement (Saleem et al., 2013). Given that our animals will learn two different responses to the same visual cue, depending on the auditory context, we may find that the motor modulation of visual response in V1 is affected.

We will therefore pay particular attention to the movement dynamics resulting in the prey vs. predator trials, as any alterations in movement are likely to induce modulation in visual responses independent of the auditory context. Additionally, behavioural and lesion studies in rats, mice and primates suggested the involvement of visual areas in specific aspects of action guidance and object recognition during various natural behaviour, including navigation or escaping predators (Andermann et al., 2011). It has been shown in mice, how vision alone is sufficient to select between opposing freeze and flight behaviours (De Franceschi et al., 2016).

In parietal cortex, we expect multi-modal responses, and thus expect to find neurons that respond to auditory, visual, and/or somatosensory stimuli. It has also been shown how areas in PPC preferentially respond to fast moving objects (the prey) or are active only during task engagement. In rats, the activity of PPC neurons has been implicated in encoding movements before they occur (Lyamzin & Benucci, 2019). Given that the behaviour required depends on the coincidence of the auditory and visual cue, we expect parietal cortex to play a particularly important role in learning this task. We hypothesize, that after learning, the number of neurons that are auditory and visual will increase, and that they may represent a possible substrate of contextual modulation.

In motor cortex, we expect to see responses related to either behavioural response (hunt for prey vs escape for predator). While each of these responses requires movement driven by the visual stimulus. This movement is either an approach (hunt) or avoidance (escape) response. We hypothesize that these responses may be controlled by distinct sub-networks of neurons in motor cortex, and the appearance of these networks would occur gradually as the animal's learn the correct response to the auditory cued context.

Given the foundational nature of the behaviours under investigation, hunting and escape, then it is also possible that the behaviour is entirely controlled by sub-cortical structures. Given that we are unable to record from likely relevant areas, such as the lateral hypothalamus (LH) or the periaqueductal gray (PAG), that play a role in predation and evasion, we must be cautious in interpreting any activity observed in cortex as causally responsible for the behaviour (and learning) during our task. However, investigating how the more accessible areas of cortex are modulated by contextual cues, will provide important targets for future studies combining coincident sub-cortical recordings, ultimately helping understand how not only different cortical areas interact with each other, but also with the rest of the brain.

Overall, to achieve a more mechanistic understanding of these results we would require follow-up studies with denser silicone probes.

The future

Embarking in the exploratory project extensively described in this dissertation was a challenge and as such, numerous aspects of our behavioural assay and recording device can be tackled and improved. Suggestions for such improvements are described in the discussions paragraph of each chapter. Looking at the future we think that both approaches fit perfectly in the long-term scientific vision, combined, or deployed independently. The valuable and rich behavioural and neural datasets that raised from this project, not only were novel but also allowed to set the bases for an exciting research approach that prioritise the organism as a whole, embedded in a complex and raw environment and treats brains as those entities capable to embrace such complexity and organically make sense of it.

With this project, we hope to inspire more scientists to take similar steps to redefine what kind of neuroscience is possible in the future.

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Appendix A

Appendix A provides complementary information about the Videogame levels.

LEVEL	YouTube link
Level 0/Habituation	https://youtu.be/_FCnaDVp_BQ
Level 1/Training	https://youtu.be/62KY3MnCEnA
Level 2/Foraging	https://youtu.be/nooFjde0P-Y
Level 3/Hunting	https://youtu.be/AoBRL6_K3p4
Level 4/Challenge	https://youtu.be/RTl9QtVZxvw https://youtu.be/9I4bHG8jW-U https://youtu.be/T6oUOntI2Mg https://youtu.be/WfPuMluKtj8

Table A.1 Videogame levels videoclips

List of the YouTube videoclips for each of the levels described in Chapter 2, including Level 4.

Appendix B

Appendix B provides complementary information about the dataset used to generate the ethogram in Chapter 2.

BEHAVIOUR/ EVENT	YouTube link
POSE	▪ https://youtu.be/zddtKfmlKEo ▪ https://youtu.be/cG341RjMG-E ▪ https://youtu.be/S8UXAGe7z2g
TOUCH	▪ https://youtu.be/l8j3Etxc-7k ▪ https://youtu.be/Fq1oFhh3udY
REAR	▪ https://youtu.be/RuHpSj15vzU ▪ https://youtu.be/I0nloYFOJbI
HALT	▪ https://youtu.be/C71SdFgDZYk ▪ https://youtu.be/m6MrtCdwjHg
STARTLE	▪ https://youtu.be/CUBvS95-IUU ▪ https://youtu.be/jmkSEndB5to
PASSIVE CATCH	▪ https://youtu.be/iui8RJojSOc ▪ https://youtu.be/Wfgic4HX10M
ACTIVE CATCH	▪ https://youtu.be/Kf0pPLu5kZ0 ▪ https://youtu.be/A3q6YlVJ4mE
CAUTIOUS WALK	▪ https://youtu.be/bGTC3pLGUXI ▪ https://youtu.be/dEffux7hqvs
TRIGGER	▪ https://youtu.be/jfAobHW-FzE

Table B.1 Ethogram videoclips

List of the YouTube videoclips for each of the behaviour/event included in the ethogram and described in Table 1.

Appendix C

Appendix C provides complementary information about the first day of Level 3.

RAT #	YouTube link
1 - AK 49.1	https://youtu.be/LQkNPfJGw9k
2 - AK 49.2	https://youtu.be/MZQUOtxFudc
3 - AK 50.1	https://youtu.be/GbJV2nR3f9s
4 - AK 50.2	https://youtu.be/YA_PEC_mGDs
5 - AK 33.2	https://youtu.be/hF7wtNXHbO8
6 - AK 40.2	https://youtu.be/syq8aS3SsTg
7 - AK 41.1	https://youtu.be/v2qSNABo-Yw
8 - AK 41.2	https://youtu.be/mZMXLdKdzXI
9 - AK 48.1	https://youtu.be/roIe6OV-N4U
10 - AK 48.4	https://youtu.be/2rz9gygCRI8

Table C.1 First Level 3 session videoclips

List of the YouTube videoclips for each rat during the first day of Level 3. Each clip captures the first moving ball encountered and two subsequent trials. Rats are listed as RAT# - RAT ID. Highlighted in green the rats included in the ethogram.

Appendix D

Appendix D provides complementary information about the surgery and probe insertion for the 4 rats included in this dissertation.

RAT ID	Surgery date	Weight B (g)	Brain size (%)	Insertion AP (mm)	Insertion ML (mm)	Insertion DV (mm)	Weight A (g)
AK 33.2	25/04/2018	520	106	1.95	2.6	2.56	538
AK 40.2	23/11/2018	497	93	2.01	2.55	2.7	510
AK 41.1	01/02/2019	515	101	2.37	3	3.2	542
AK 41.2	13/02/2019	500	93	2.2	1.9	3.02	521

Table D.1 Surgery summary

List of the surgery and insertion parameters for each rat. Weight B refers to the weight prior to surgery while Weight A refers to the weight after surgery (the weight might have been measured on different weighing scales). AP: anteroposterior; ML: mediolateral; DV: dorsoventral. The brain size refers to the interaural calculation that allows us to estimate the skull growth and make adjustments in the target coordinates. ML adjustments were made for rat AK 40.2 and AK 41.2.