INSTITUTO DE NEUROCIENCIAS

NEURONAL CODING AND INTEGRATION OF TEMPORAL PATTERNS IN THE BARREL CORTEX

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San Juan de Alicante, 2016

Acknowledgements

This thesis is not just a pure lab work. It´s only a tip of an iceberg, something that everyone sees but misses what lies underneath.

More than anything it is an outcome of a very challenging time, both professional and personal, when I constantly had to confront myself, reorganize my ideas, learn perseverance, especially when everything was against, and stand for myself. Even though I tend to be very independent, without certain people in my life getting through this period would be impossible.

I'm grateful to my parents, who gave me the best start in life ever, freedom, wisdom and responsibility. Who has always been there for me and supported me in every single moment, both success and breakdowns… and never tried to change my mind, neither to discourage me from achieving my goals. Without their encouragement this thesis would not exist. I am grateful to my brother, who I can always count on.

I wish to thank my supervisor Prof. Miguel Maravall, for giving me the precious opportunity of working and carrying out the doctoral thesis in his lab; I wouldn't be where I am now and I wouldn't have learned as much without this opportunity. Thank you for support, giving me independence, your never-ending patience and understanding (including the understanding for my need to travel, the two internships abroad were worth it).

I wish to acknowledge the support of my colleagues: Michael Bale, Matina Bitzidou, Giovanni Ferrati, Fran Martini, Ana Lía Albarracín and Javier Alegre who has always been of great help and who were great companion and source of knowledge and experience. I am grateful for the assistance of animal unit staff (especially Lorena and Sonia, always very kind, helpful and understanding).

And, in the end, I wish to thank to my friends, those that I met years ago, the new ones and those who has been in my life very briefly but stayed in my mind. The friendship with some of them provided support and company (very often during never-ending hours and in very difficult moments) and with others was an eye-opening and life-changing experience. Special thanks to: Alutka Wosz and Tomasz Kuśnierz, Kasia Pietrajtis, Stefanie Subczinski, Anton Filipchuk, Danny Florez Paz, Nacho Brocal, Isabel Benjumeda-Wijnhoven, Manu Molano-Mazón, Graciela Navarro Mora, Ana Carmena's lab: Alyona Keder, Janka Slovakova, Noemí Rives Quinto and Stephan Speicher, Mária Jusková, the INA rowing girls: Auxi Casanova, Berta Sánchez-Laorden and Aida Arcas, the English team: María Martínez-López, Philip Vassilev, Marie Decol and Léa Darnet, who made my stay in Brighton very homy and cosy, the Belgian-Dutch team: Wiola Walentowska, Ewelina Bartoszek & Steffen Kandler, João Santos, Andrea Alamia and Emanuel van den Broeke… and many others…

Thank you!!!

And thank God that rowing exists: xD

…oh wait, this one's better (in the end it's team work):

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Abstract

In order to make sense of the world living organisms have to perceive and interpret sensory stimuli and compute the parameters that describe them (such as velocity or acceleration). Those stimuli typically follow a distinct pattern over time (such as in birdsongs, speech or passages of melody). In the latter case, only integrating the information about the whole pattern of stimuli can give the organism a fully meaningful idea about the environment, enabling a correct behavioral response.

The capacity to integrate sequences over time is crucial for sequence selectivity and correct behavior. Despite the importance of this phenomenon, not much is known about the sites where sensory temporal patterns are integrated and sequences represented. In experiments requiring behavior in a tactile, whisker-mediated sensory discrimination task, it has been observed that rodents can integrate tactile information over time (up to hundreds of milliseconds) and that barrel cortex (vibrissal primary somatosensory cortex) neurons can adjust their responses over long timescales (tens of seconds) as a function of changes in stimulation context. Nevertheless it is still unknown how single neurons in the whisker system convey integrated information about sequences of whisker deflections over time.

In order to map cortical signatures of sequence sensitivity in the rodent tactile system, I performed juxtacellular recordings of single neurons in the barrel cortex of anesthetized and awake mice. I applied stimulation consisting of sequences of whisker deflections applied at pseudo-random intervals. My colleagues and I compared the information carried by individual neurons about single intervals (reflecting sensitivity to instantaneous frequency) and about sets of several successive intervals (reflecting integration over time). The result indicated that individual neurons in the barrel cortex carry significantly more information about the latest interval than about previous ones. Some neurons however stood out from the main group: neurons in layer 4 and deeper layers were notable for integrating little, i.e. carrying particularly low information about previous intervals, while a subset of neurons in supragranular layer 3 integrated most strongly. Experiments carried out in awake animals confirmed that neurons in the barrel cortex of behaving and alert animals display similar levels of integration. These results place limits on the amount of temporal integration carried out by neurons in the primary somatosensory cortex of

rodents and support the notion that primary sensory cortex is principally an encoder of current stimulation values.

To further investigate sequence selectivity, we developed a sensory discrimination task in a Go/No-Go paradigm. Mice were trained to discriminate between Go and No-Go sequences consisting of noisy vibrations delivered to the whiskers; sequences differed only in that temporal patterns were scrambled on a 100 ms timescale. Mice were able to discriminate between the two sequences at around 70% success rate.

Resumen

Para entender el mundo, los organismos vivientes han de percibir e interpretar los estímulos sensoriales y determinar los parámetros físicos que les definen (como velocidad o aceleración). Normalmente, estos estímulos se producen con un patrón temporal específico (observado por ejemplo en el canto de los pájaros, el habla o la música). En este último caso, sólo integrando la información sobre el patrón de estímulos puede alcanzarse una interpretación apropiada sobre el entorno, dando lugar a una respuesta conductual correcta.

La capacidad de integrar secuencias de estímulos a lo largo del tiempo es, por lo tanto, imprescindible para el reconocimiento de dichas secuencias y la generación de respuestas correctas. A pesar de la importancia de este mecanismo, se sabe poco acerca de cómo se integran las secuencias de estímulos sensoriales. En los experimentos de comportamiento basados en tareas de discriminación sensorial, se ha observado que los roedores son capaces de integrar información táctil (mediada por las vibrisas) a lo largo del tiempo (cientos de milisegundos) y que las neuronas de la corteza de barriles (la corteza somatosensorial vibrisal primaria) pueden ajustar su actividad en función de cambios contextuales en la estimulación a lo largo de decenas de segundos. Sin embargo, aún no se sabe cómo las neuronas individuales en el sistema de vibrisas transmiten la información integrada sobre secuencias de movimientos de vibrisas.

Con el fin de establecer la base del mecanismo de la sensibilidad frente las secuencias de los estímulos en el sistema táctil de los roedores, he realizado registros de la actividad eléctrica de neuronas individuales en la corteza de barriles de ratones tanto anestesiados como despiertos. La estimulación consistió en deflexiones de vibrisas generadas a intervalos aleatorios. Mis colaboradores y yo comparamos la información que las respuestas neuronales proporcionaban sobre intervalos únicos (es decir, información correspondiente a la frecuencia instantánea de estimulación) y sobre conjuntos de intervalos (información correspondiente al patrón de estímulos integrado en el tiempo). El resultado indicó que las neuronas individuales en la corteza de barriles proporcionan significativamente más información sobre el intervalo más reciente que sobre intervalos anteriores. Sin embargo algunas neuronas destacaron con respecto al grupo principal: las neuronas en la capa 4 y en las capas profundas integraron débilmente, mientras que las neuronas en la capa superficial 3 integraron más fuertemente a lo largo del tiempo. Los

experimentos realizados en animales despiertos confirmaron que las neuronas en la corteza de barriles de dichos animales muestran un nivel de integración parecido a los animales anestesiados. Estos resultados limitan la cantidad de integración temporal que llevan a cabo las neuronas de la corteza somatosensorial primaria y apoyan el concepto de que la corteza sensorial primaria representa principalmente la estimulación actual.

Para investigar la integración temporal con más profundidad, hemos desarrollado una tarea de discriminación sensorial en un paradigma Go/No-Go. Los dos patrones consisten en vibraciones aplicadas a las vibrisas de los ratones y se diferencian sólo en su estructura temporal. Los ratones son capaces de discriminar entre los dos patrones con 70% de éxito.

1. Introduction

1.1. Preface

Functioning in the world implies perceiving it and reacting to it in an appropriate way. Successful perception is built upon the capacity to determine and extract physical parameters that describe the environment (for example the speed and direction of moving objects) as well as to interpret them relatively to their context in order to produce an appropriate behavior (for instance moving away if an object is approaching quickly).

As any environment comprises a set of dynamically changing conditions, its parameters are not constant but continuously fluctuating over time. They are often arranged in patterns and sequences with no clear boundaries in between, as in the case of human speech or passages of melody. Moreover some sequences of stimuli become more meaningful to the organism, whereas others are irrelevant and, in consequence, are ignored or evoke a much weaker reaction. Intuitively such recognition of sequences unfolding over the timescales of milliseconds to seconds is crucial in the auditory system, where it allows us to make sense of speech and enjoy music (Brosch & Schreiner, 2000). Sequence recognition also plays an important role in other modalities, such as visual (Gavornik & Bear, 2014) or tactile, and supposedly is involved in information processing across the brain with the purpose of object perception and recognition influencing behavior.

It has been known for decades that neurons encode the information about stimuli in their spiking activity – patterns of action potentials. Adrian et al. discovered in 1926 that the magnitude of stimulus parameters is encoded by spike count, i.e. the number of action potentials fired in a certain time window (Adrian, 1926). Later, knowledge on stimulus encoding through temporal patterns of action potentials expanded. Segundo et al. (Segundo et al., 1963) were the first to observe that if a cell is presented with two stimuli of the same mean firing rate, but different second order statistics, it displays different activity. The outcome of this experiment suggests that the decision of neurons as to whether to generate a response depends on the temporal pattern of the input. The study therefore suggests that already single neurons are able to distinguish between temporal patterns (Figure 1.1).
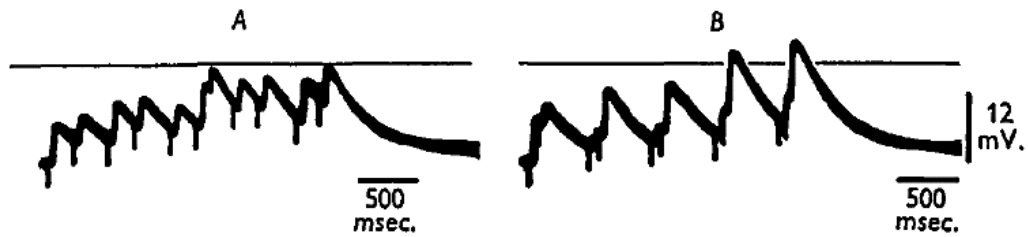
Figure 1.1. **Sequence selectivity in isolated visceral ganglia of *Aplysia californica*** (most observations carried out in so-called giant nerve cell): arriving input consisted of bursts of pulses delivered at different times but characterized by the same mean frequency; different inputs evoked responses of different magnitudes; observed differences in membrane voltage to different types of stimulation suggests that neurons are sensitive to the temporal pattern of arriving input. Adapted from (Segundo et al., 1963)

How this process takes place and how neurons represent sequences of stimuli in the intact mammalian brain, still remains unknown: if understanding a neuronal representation of the world means that we should be able to decode the activity of nervous cells and reproduce the aspect of the animal's surroundings that they encode, then by that standard we still do not understand how neurons represent sequences of stimuli.

Necessary for sequence recognition is the ability to integrate information over time. Neurons capable of integrating over time would be able to combine information about the present and the past (Moore, 2003), and their response might not be evoked purely by the instantaneous value of the stimulus, but by the history of stimulation over a certain time course.

The present study aimed to investigate temporal integration and sequence representation in a well-known model system, thus increasing understanding of how sensory systems represent information about the world.

1.2. The model

1.2.1. Why barrel cortex?

Rodents as nocturnal animals with poor vision do not rely on visual cues (Prusky et al., 2000) to the same extent as humans, other primates or carnivores in order to build an idea of the external world. Instead, predominant channels for information are the olfactory and tactile systems. The main channel for fine tactile information is provided by the vibrissae ("whiskers") growing on the rodent's snout, which are regarded as a major somatosensory organ equivalent in function to human fingertips (Carvell & Simons, 1990; Fassihi at al.,

2014). The cortical representation of whiskers occupies around 69% of the surface of somatosensory cortex in mouse (Lee & Erzurumlu, 2005), consistent with the major innervation received by whisker follicles.

Whisker system is regarded as an "expert" sensory processing system, one that maintains high reliability in representing the environment and overall enables quick and accurate reaction (Diamond & Arabzadeh, 2013b). In 1912 it was proved that rats require their whiskers in order to navigate through a labyrinth (Vincent, 1912). The barrel cortex, the area of primary somatosensory cortex that receives whisker information was first examined in the early twentieth century by Lorente de Nó, who described cortical circuit anatomy (Lorente de Nó, 1922). The distinctive "barrel" anatomy was described in 1970 by Woolsey and Van der Loos (Woolsey & Van der Loos, 1970). Since then it has been well investigated in various research projects. Nowadays barrel cortex is a popular model in the research of somatosensation and cortical plasticity, and is relatively well documented at the cellular and synaptic level.

The somatosensory system, analogously to the auditory one, continuously processes information about sequences of stimuli extended over time and encodes them in patterns of action potentials (Salinas et al., 2000). This information, having reached the cortex, has to be decoded in order to produce correct behavior. A convenient example is Braille alphabet, where the subject has to recognize a spatial pattern of coarsely spaced dots embossed on a surface and, based on the pattern of action potentials evoked by this tactile stimulus, assign them a meaning (an appropriate letter of the alphabet) (Phillips et al., 1990). Mice, analogously to primates, while examining a rough surface, perceive grooves and the spatial intervals between them through changes in the movement parameters of the whiskers. Changes in whisker motion over time are encoded by receptors in the whiskers follicles. Tactile information, both in the case of rodents and primates, travels upstream in the form of action potentials arranged in time, and contains information about the spatial frequency of the events (Diamond, 2010; Jadhav & Feldman, 2010; Mackevicius, 2012).

The whisker system, as a system that processes temporally patterned information about the environment, is therefore an appropriate model for studying sequence encoding, recognition and temporal integration (Simons, 1985), especially given the existing knowledge about its architecture as well as cellular and synaptic mechanisms, which is crucial for understanding more complex neural processes.

1.2.2. Whisking movement

Environment exploration via whiskers is a well-controlled process based on somatosensory and motor feedback loops and is actively regulated by the animal itself depending on the environmental context and input.

Whiskers explore the environment in two different manners: passively, when they sweep objects as a consequence of head and body movement, and actively, when they either immobilize whiskers in order to sense vibration of an object or perform rhythmical horizontal movement forwards (this movement is termed protraction) and backwards (retraction) at the frequency of 5 to 25 Hz (Kleinfeld et al., 2006). This motion of vibrissae is called "whisking" and gives the animals significant opportunities to perceive the environment and collect information enabling them to e.g. move around avoiding obstacles, distinguish objects or discriminate textures. Rodents, contrary to early assumptions, are able to whisk bilaterally asymmetrically (Towal & Hartmann, 2006) and asynchronously as well as modulate both the frequency and the amplitude of whisker movements. Therefore they control whisking and switch between its different modes, depending on what kind of information they are searching for, suggesting participation of motor system in the acquisition of sensory knowledge (Berg & Kleinfeld, 2003; Grinevich et al., 2005; Petreanu et al., 2012).

We can distinguish two modes of active sensing via vibrissae: generative, divided into exploratory and foveal, as well as receptive. The first one is used when the animal scans the environment for an object and, once found, "palpates" it in order to build up a percept. It is characterized by whisking, i.e. scanning movements of the whiskers. Within this class we can identify exploratory whisking, when the animal looks for contact with the objects. It is characterized by whisker motion of frequency of 5 to 15 Hz and large amplitude. Foveal whisking, on the other hand, takes place when whiskers are directed rather to the front, and the movements tend to be more frequent (15-25 Hz), but also of smaller amplitude (Kleinfeld et al., 2006). It increases the density of possible touch events enabling an effective build-up of the neuronal representation of the object. Generative whisking also refers to texture discrimination, when whiskers sweep over a surface with high velocity in order to discriminate its roughness (Figure 1.2). During this specific movement they slow down whenever they get stuck and accelerate when they come free. Such irregularities in whisker motion, sudden acceleration or slowing down, are called "stick-and-slip" events and give rise to "kinetic signature" of a texture (Ritt et al., 2008;

Wolfe et al., 2008). In other words: as every surface is slightly different, it gives rise to a unique sequence of stick-and-slip events and subsequently a unique neuronal representation of its roughness (Arabzadeh et al., 2005). A second mode of whisker sensing, called receptive, takes place when the object itself is moving or vibrating. In order to collect sufficient amount of information about it, the animal puts whiskers in contact with the object or a surface and immobilizes them enabling nearly instantaneous frequency or vibration discrimination (Diamond & Arabzadeh, 2013).
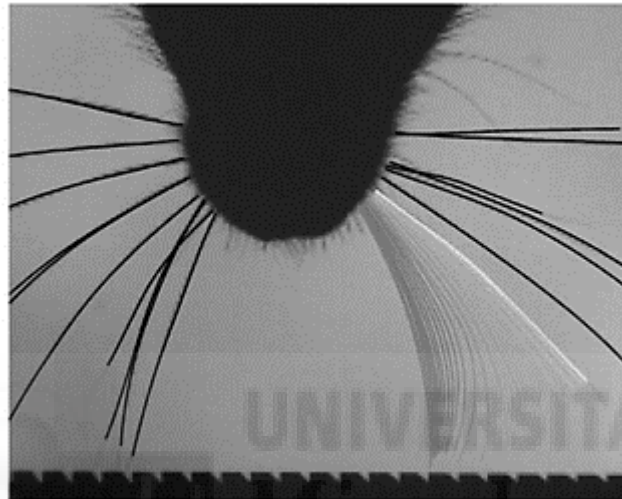
Figure 1.2. **A rat sweeping its whiskers over a rough surface.** Each surface evokes a different sequence of whisker motion, a so-called "kinetic signature" of the surface. A mouse or a rat drives its whiskers with determined parameters (such as velocity and acceleration); sudden changes in those parameters evoked by surface irregularities give rise to a neuronal percept of a given texture. Adapted from: (Diamond & Arabzadeh, 2013a)

Particularly it has been proposed that macrovibrissae are primarily responsible for navigation and coarse object detection whereas microvibrissae for finer texture discrimination (Brecht et al., 1997).

1.2.3. Anatomy of the whisker system
a. Arrangement of whiskers and barrels

Whisker system includes a three-synapse pathway where neuronal terminals at each processing stage, including cortex, are organized into cytoarchitectonic units. Each of those units receives input from the corresponding whisker. Such type of one-to-one organization (Brecht et al., 1997; Chen-Bee et al., 2012), which makes it predictable which regions of the brain will respond to the stimulation of a particular whiskers on the animal's snout, is confirmed both by histological (Woolsey & Van der Loos, 1970) and

electrophysiological (Welker & Woolsey, 1974) studies and makes this system a convenient model for research.

Whiskers are arranged in a specific manner – they protrude from the mystacial pad and are arranged in 5 rows (A-E) and 4 columns (1-4), where the uppermost row is named by the first letter of the alphabet and the columns are assigned numbers increasing towards the nose. The most posterior columns are not exactly aligned with the anterior ones and are usually named after Greek letters (α, β, γ, δ) (Figure 1.3).
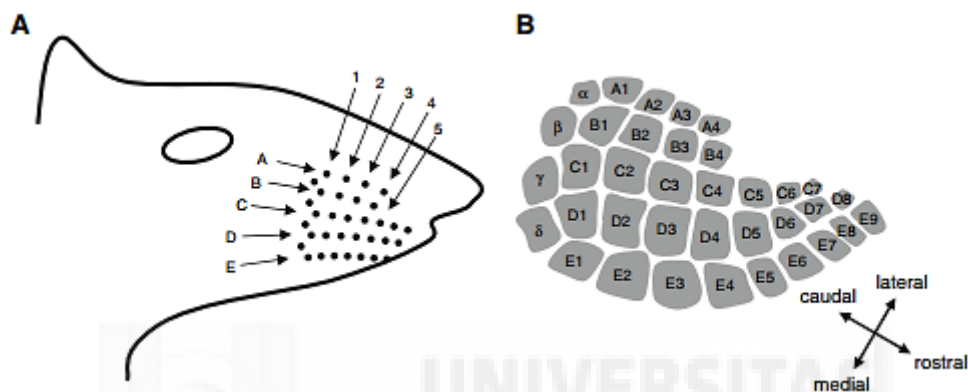
Figure 1.3. **Spatial distribution of whiskers and corresponding barrels**. Vibrissae are located on the mystacial pad (A) and are connected with corresponding barrels in the vibrissal somatosensory cortex (barrel cortex) (B). Each whisker is indicated with a Latin or Greek letter (coordinate of the row) and a digit (coordinate of the column). The same coordinates are assigned for each barrel in the final stage of the whisker pathway.
Adapted from: (Petersen et al., 2009)

Therefore upon stimulating a whisker in the row C and column 2, one could restrict investigation to neurons of C2 barrel of the contralateral hemisphere (Simons, 1978). Further, barrel field – the cortical stage of the whisker system, is very easy to localize. It is visible *in vitro* in bright field microscopy or can be found *in vivo* via intrinsic imaging or voltage-sensitive dye imaging (Petersen, 2007) as well as upon applying specific coordinates in relation to Bregma (mice: AP 1.5, L 3mm) (Paxinos, 2012).

b. Hierarchical organization of the whisker pathway

Somatosensory pathways start with an array of mechanoreceptors that collects signals from the external world and encodes them in the form of a characteristic pattern of responses depending on the interaction between the stimulus parameters and the preference of the receptor for a certain set of stimulus properties (this features is termed: receptive field). The pathway must convey this message, which constitutes the totality of sensory information collected at the receptors, to the cortex, where the information is extracted and given a meaning.

In the whisker system sensory information is collected at the base of whisker shaft by several types of mechanoreceptors. Each whisker follicle is innervated by up to 200 axons (Lee & Woolsey, 1975) classified as slowly adapting (displaying tonic activity evoked by constant whisker displacement) or rapidly adapting (firing briefly at the onset of the movement), which all form the infraorbital nerve.

The first relay station for any sensory message is the brainstem or spinal cord and so it is in the whisker system. The infraorbital nerve axons correspond to neurons which have their somas in the trigeminal ganglion (TG) and innervate the trigeminal nuclei (TN) in the brainstem: principalis (SpV), oralis (SpVo), interpolaris (SpVi) and caudalis (SpVc). Infraorbital nerve endings in the brainstem form barrelettes - the first representation of the mystacial pad (see Figure 1.5). There exists a rich connection network between the trigeminal nuclei themselves as well as feedback loops between them and the corresponding regions of the thalamus and external to it – ventroposterior medial nucleus (VPm), posterior medial nucleus (POm) and reticular nucleus (TRN) (Figure 1.4).
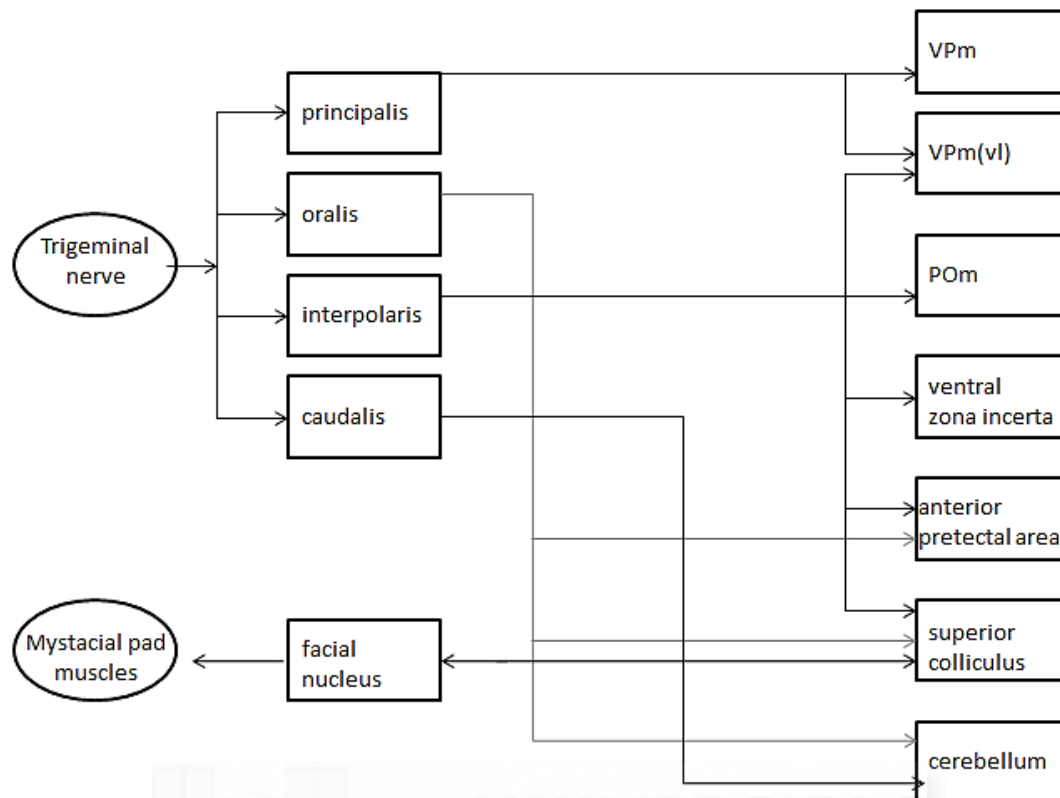
Figure 1.4. **Projections from brainstem trigeminal nuclei to the thalamus and other higher order areas**. Projections to and from zona incerta, pretectal area, superior colliculus and cerebellum are not described.

Adapted from: (Fox, 2008)

The reticular nucleus contains mostly inhibitory cells that, once excited by thalamocortical or corticothalamic fibers, provide inhibitory feedback to the thalamus. VPm is a main relay station for vibrissae tactile information from the brainstem to the cortex, where it sends projections to layer 4 and, to a lesser extent, to the layer 5 – layer 6 border (Constantinople & Bruno, 2013). POm complements projections sent from VPm to the cortex, but unlike VPm its nerves terminate especially in layer 1 and 5A, both in the barrel columns and in septa, as well as in septa of layer 4 (Ohno et al., 2012). Whereas POm does not have histological units relevant to each whisker, VPm contains a complete representation of the mystacial pad (Diamond et al., 1992). Those cytoarchitectonic units found in VPm, termed barreloids (Figure 1.5), consist of around 250 neurons (Montemurro et al., 2007) and are formed in three distinguishable parts: head, core and tail. Each of those parts is a relay station for one of three ascending pathways (Furuta et al., 2009; Urbain & Deschênes, 2007). Having reached specific layers of the cortex, the signal spreads above and below layer 4 forming cortical columns – functional and

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anatomical units being able to transfer the tactile information. Barrel columns are able to transmit the information along (throughout the subsequent layers of the columns), but they also "talk to each other", due to rich horizontal projections. Pyramidal cells in layer 6A sending feedback projections back to the thalamus become a foundation of a system that's a gatekeeper to its own input (Olsen et al., 2012).
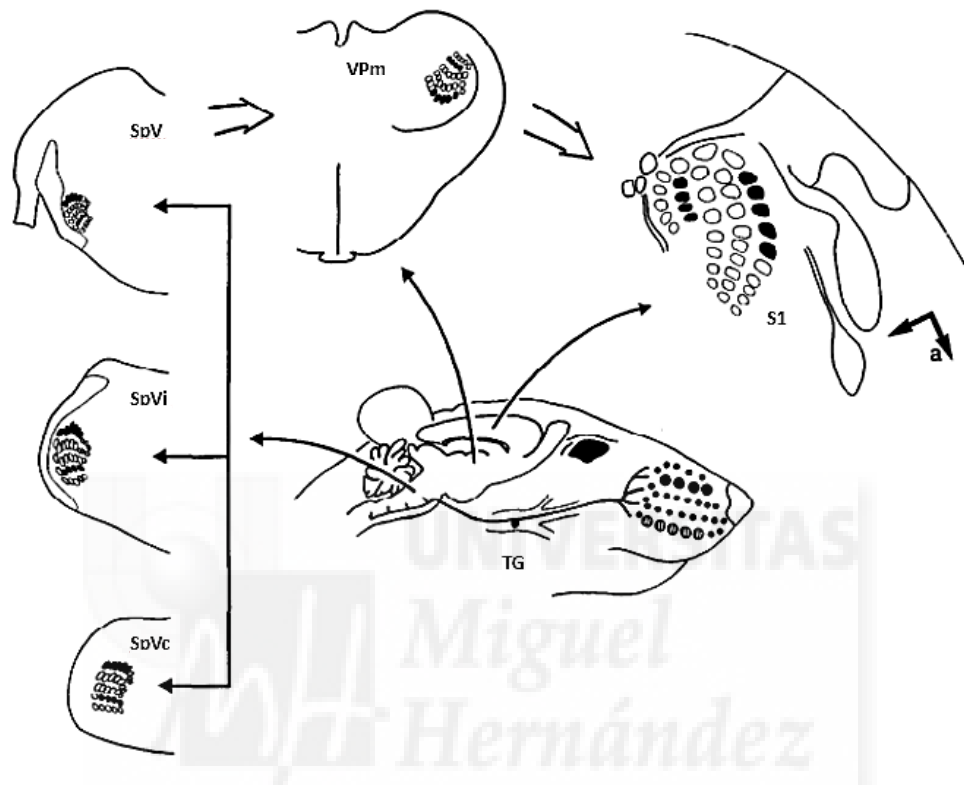
Figure 1.5. **Representation of the mystacial pad on subsequent levels of the nervous system.**
Somatotopic maps on the level of trigeminal nuclei (SpVc, SpVi, Spv), thalamus (VPm) and primary somatosensory cortex (S1) being a representation of the mystacial pad.
Adapted from (Killackey et al., 1995)

In the whisker system however a certain complication takes place: as mentioned above, the information proceeds in parallel through three different tracts: lemniscal, paralemniscal and extralemniscal pathway (Figure 1.6). Lemniscal pathway divides into two subtracts: the first conveys information through mono-whisker cells in barrelettes in SpV and barreloids' cores in VPm until reaching layer 4, basal layer 3, layer 5B and layer 6A within corresponding barrel columns (Henderson & Jacquin, 1995). The second lemniscal subtract conveys multi-whisker information through SpV and barreloids' heads until septal columns separating the barrels (Furuta et al., 2009; Veinante & Deschênes, 1999). Extralemniscal pathway projects to multi-whisker cells in SpVi, subsequently to barreloids' tails and reaches dysgranular primary and secondary somatosensory cortex,

which surrounds the barrel field, in which layer 4 displays low cytochrome oxidase (CO) activity suggesting low synaptic density (Pierret et al., 2000; Veinante et al., 2000). Finally paralemniscal pathway relays multi-whisker information through caudal SpVi to anterior part of POm and layer 1 and layer 5A of the primary somatosensory cortex (Ohno et al., 2012; Veinante et al., 2000; Wimmer et al., 2010). POm also sends projections to S2 and motor cortex (M1) (Deschênes et al., 1998).
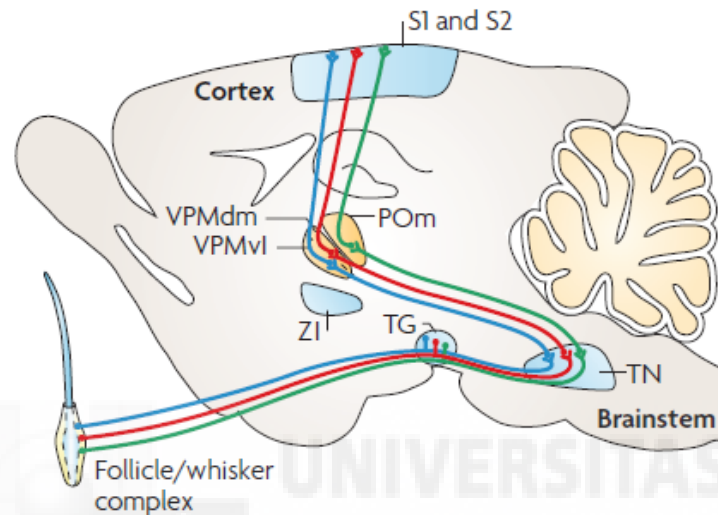
Figure 1.6. **Three pathways forming a connection between the whisker follicles and cortical barrels**. Neurons in the trigeminal ganglion (TG) send their projections to whisker follicles. The signal is projected to the trigeminal nuclei (TN). From there each pathway takes slightly different course: through ventro-posterior medial nucleus in the thalamus (VPm) or medial area of the posterior nucleus (POm) until arriving to primary and secondary somatosensory cortex (S1 and S2 respectively).
Adapted from (Diamond et al., 2008)

c. Barrel cortex and its connectivity

Barrels themselves, thanks to their structure, are easily distinguishable even in bright field microscopy, and enhance their visibility upon carrying out specific staining procedures on fixed tissue. They consist of several thousands of neurons that group on the borders of the barrel-shaped units, leaving their centers filled with neurites – both their own, as well as thalamic projections (especially in layer 4) (Figure 1.7). That difference in soma density across the barrels makes it possible to localize them easily. Specific types of staining such as the one for cytochrome oxidase (CO) make them even more visible. Cytochrome oxidase is a mitochondrial enzyme, present mostly in synapses, which marks metabolic activity of the cells in the fixed tissue. Due to barrel architecture and rich

synaptic density of barrels, staining for cytochrome oxidase produces a powerful marking of the barrels (see Figure 2.11 in Materials and Methods) (Wong-Riley & Welt, 1980).
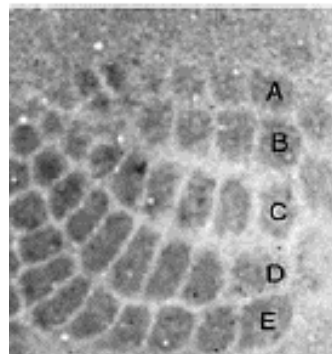
Figure 1.7. **Somatotopic organization of the barrel cortex.** A section through layer 4 of the barrel cortex reveals barrels arranged in analogous way as whiskers on the mystacial pad. Tissue labeled with cytochrome oxidase enhances the visibility of the barrels.
Adapted from (Fox, 2008)

In terms of self-connectivity, barrel cortex (or vibrissal somatosensory cortex) is characterized by high number of neural connections within cylindrical barrel columns and, to a lesser degree, across them. Barrel columns include the layers above and below the barrels themselves (which are located in layer 4) and those, sending horizontal projections, are highly interconnected with the neighboring ones (Fox et al., 2003). As barrels receive exclusively mono-whisker information such trans-columnar cross-talk allows processing information not only from a principal whisker (PW) but also from the neighboring ones and helps creating multi-whisker representations (Simons, 1978).

Layer 4 is the main receiver of thalamocortical input within the barrel cortex and therefore one of its roles is to push the information further within the circuit. It also receives very little cortical input. Supragranular layers (those above layer 4 and closer to the brain surface) connect to other columns (layer 3) and cortical areas (layer 2), both sending and receiving information. Infragranular layers, on the other hand, are located below layer 4 and receive input from neighboring supragranular ones as well as project to extracortical structures (e.g. layer 6 projects to the thalamus).

In more detail, as layer 4 receives direct thalamic input carrying a message about whisker deflections, these intracortical connections are likely to facilitate immediate flow of information related to ongoing sensory stimulation from layer 4 to supra- and infragranular ones. Moreover the tendency observed in supragranular layers is to send

stronger input from deeper to superficial layers with the reverse trend observed in infragranular layers. There is also a strong excitatory connectivity between supra- and infragranular layers with the strongest input from layer 2 to layer 5A and from layer 3 to layer 5B. Overall the most significant excitatory inputs originate and terminate within the same layer or connect supragranular layers to the infragranular ones (Lefort et al., 2009):

- ✓ L3 → L3
- ✓ L4 → L4
- ✓ L5A → L5A
- ✓ L4 → L2
- ✓ L3 → L5B.

Layer 5 is the main target of strong feed-forward projections sent from other layers of the barrel column (Feldmeyer et al., 2013).

Connectivity between the barrel cortex and other cortical and subcortical areas can be well described by the order in which different brain regions become activated after stimulus onset: in quiet wakefulness state deflecting a whisker would evoke a response in contralateral corresponding barrel within 10 ms. Subsequently the rest of the barrel column and, after that, neighboring barrel columns, as well as secondary somatosensory cortex become activated. Around 8 ms after the initial response, an increased activity is observed in motor cortex and surrounding regions. Ipsilateral cortex (frontal cortex, M1 and S2) as well as, at the very end, posterior parietal association cortex become activated in this cascade evoked by a single whisker deflection. This final activation takes place within first 100 ms after the single whisker deflection (Aronoff et al., 2010).

Long-range projections of barrel cortex are diverse and reach both other cortical areas as well as subcortical ones. Layers 2/3 send excitatory projections to the cortical areas external to barrel cortex such as: motor cortex, mostly to its infragranular layers (Aronoff et al., 2010), or secondary somatosensory cortex (Sato & Svoboda, 2010; Yamashita et al., 2013). Layers 5 and 6, on the other hand, innervate rather supragranular layers of M1 (Aronoff et al., 2010). Layer 1 is a main target for cortical-feedback projections from S2 or M1 (Petreanu et al., 2012) acting as an integrator of sensory and motor signals. Layers 5 and 6 also send their projections to subcortical structures: layer 6 to barreloids in VPm, whereas POm receives input mainly from layer 5B. This way, deep layers, contribute to

corticothalamic feedback loop providing regulatory information for the thalamus and incoming tactile information.

Above mentioned layers comprise mostly pyramidal neurons, whereas layer 4 contains a specific type of excitatory neurons – spiny stellate cells.

1.3. Information processing over time: temporal integration

Living organisms are able to perceive and recognize stimuli arriving from the external world. Very often those stimuli are extended over time and the brain has to perform various computational operations in order to create a percept of the world valid both in its spatial and temporal aspect. The first question refers to how time is perceived. Intuitively, culturally and historically humans perceive it as a linear or circular continuity. However, in terms of encoding sensory, including tactile information, the brain understands the time as a series of chunks extending over different timescales – for instance from milliseconds to seconds. Each chunk would contain some part of the sensory information. The operations of processing multiple sensory signals, extended over different timescales, take place in the brain simultaneously. Another aspect of time comprehension comes down to the fact that sensory stimuli are often organized in particular patterns and only perceiving the whole pattern (not just a discrete pulse) allows to extract meaningful information and create complete percepts of objects that surround us (Dehaene et al., 2015; Griffiths & Warren, 2004). We can come across this situation for instance when perceiving sounds (speech, melody, natural songs and vocalizations etc.) (Telkemeyer et al., 2009) or touch (texture, vibration) (Diamond et al.,2008). Even though sequence recognition is also one of the main research topics of cognitive science (Dehaene et al., 2015), not much is known about its cellular and circuit mechanisms.

Supposedly temporal integration performed by neurons over several hierarchical stages is what underlies sequence recognition (Gao & Wehr, 2015; Wong et al., 2007). This process involves combining current and past information in order to improve detection and discrimination of patterned stimulation. In other words, integration comes down to collection of serial sensory events by neurons. This process leads to response being a function of that accumulated information, not only a single pulse. A capacity of a neuron to integrate over time would give an idea of how close in time two serial events have to occur to be sensed as part of the same percept (event or object). Therefore we would like

to know if neurons performing the operation of integration over time are present in the nervous system and where exactly they are located.

Certain experiments suggest the existence of sensitivity of neurons to particular sequences of intervals. For instance Carlson's group work shows that fish midbrain neurons are classified into three groups, where each of them is sensitive to different length of the inter-stimulus intervals (George et al., 2011). In the primary auditory cortex of primates it has been observed that neuronal activity is not just a simple representation of pure tone or vocalization parameters. Instead spectrotemporal characteristics of natural songs are integrated over time and this way represented in the neuronal activity (Wang et al., 1995). Similar observation was obtained in the ferret primary auditory cortex, where neuronal activity represented not only the current tone but also a preceding one (Klampfl et al., 2012). Sequence recognition and learning has been reported in several higher order areas in primates as well as in primary visual cortex of mice. Alterations in neuronal activity, as a correlate of sequence learning and sequence prediction, appear already in the thalamorecipient layer 4 of primary visual cortex (V1) (Gavornik & Bear, 2014). Furthermore recordings in cat primary visual cortex demonstrated that neurons in the early processing stages not only respond to instantaneous (current) stimulation but also encode the visual stimuli several hundreds of milliseconds in the past: current stimulation did not clear the memory of the previous stimulus and supported the hypothesis about the existence of fading memory in contrast to frame-by-frame coding (Nikolić et al., 2009).

1.3.1. Temporal integration in the tactile system

The tactile information encoded in highly temporally reliable manner (Jones et al., 2004) is sent from barrelettes to thalamic barreloids. In the neocortex neurons tend to respond with high trial-to-trial variability which is mainly an effect of interaction of incoming information contained in spiking response of neocortical neurons with ongoing spontaneous and state-dependent activity (Petersen et al., 2003; Sachdev et al., 2004). Even though spike trains in the cortical stage appear to be noisy, it has been shown that layer 4 neurons respond with millisecond accuracy to tactile stimulus (Hires et al., 2015). Overall, it has been shown that neurons in the early processing stages specialize in creating faithful representations of individual "events" (such as "slips" during texture exploration). However, those individual events has to be integrated in order to build a meaningful representation of the stimuli evolving in time.

In this thesis I focused on single neurons in the barrel cortex and questioned if and to what extent tactile information undergoes integration over time in this early cortical level, with a special focus on the problem if barrel cortex neurons are able to encode information about the inter-stimulus intervals in patterned stimuli.

Exploration of an object by whiskers produces a 200 - 300 ms sequence of stimuli (Von Heimendahl et al. , 2007). These stimulus sequences are not regular but fluctuating over time in a manner depending on the object or texture properties (Jadhav & Feldman, 2010; Wolfe et al., 2008). Therefore the intervals between the subsequent pulses would be of variable length throughout the whole sequence and recognizing their particular pattern may be of interest.

According to one study, where rats were supposed to solve vibrotactile task using their whiskers, temporal integration in the barrel cortex was limited to short timescales, 25 ms (Stüttgen & Schwarz, 2010). Researchers working in the primary somatosensory cortex of primates have also found this area to be involved in coding of ongoing, rather than previous, stimulus properties and claim that the role of primary somatosensory cortex, as the receiver of thalamic input, is mainly to generate a reliable neuronal representation of the stimulus which is ready for further processing in higher-order sensory areas (Romo & de Lafuente, 2013; Salinas et al., 2000).

In sum, even though there is research supporting temporal integration up to hundreds of milliseconds in primary sensory cortical areas, no such evidence has been found in primary somatosensory cortex.

1.4. Aim of the study

The aim of this thesis project was to investigate the ability of single neurons to integrate tactile information over time *in vivo*. If a barrel cortex neuron was sensitive to sequences of stimuli, it would be tuned to sets of several individual whisker stimuli (or the pattern of time intervals dividing them) rather than just to a single stimulus or interval. The neuron's spiking activity would then contain information about the entire sequence.

To test this I carried out patch-clamp recordings *in vivo*, mostly in anesthetized mice, while applying strictly controlled stimulation to the whiskers. Subsequently, in order to exclude the effect of anesthesia, the results were confirmed in awake naïve animals. Together with my colleagues, I then reasoned that it would be necessary to investigate if

temporal integration in awake animals participating in a relevant sensory discrimination task is increased compared to naïve animals, and developed a task to address this.

1.4.1. Objectives

The detailed objectives are:

1. To analyze, in anesthetized animals, the ability of single neurons in the primary vibrissal somatosensory cortex (barrel cortex) to integrate over time, that is, to response to temporal patterns of pulses of stimulation applied to whiskers, or else simply to the individual intervals between stimulation pulses.
2. To study if the ability to integrate over time (if present) was correlated with:
    a. A neuron's responsiveness (firing rate) and overall ability to carry information about the stimulus
    b. The location of a neuron within the circuit (indicated by the recording depth)
3. To investigate if temporal integration occurred similarly in awake animals, without the effect of anesthesia.
4. The final goal of the study was to design a paradigm for a behavioral task, to investigate if behaving animals are able to learn and distinguish between two stimuli of different temporal structure as well as report their decision.

2. Materials and methods

2.1. Preface

All experiments were carried out in accordance with European Union and institutional standards for the care and use of animals in research.

This thesis incorporates two types of experiments. The first type were electrophysiological experiments designed to quantify the information conveyed by the spiking responses of cortical neurons about interval sequences. These were first carried out in anesthetized animals and, once the results were acquired, an analogous design was implemented in awake animals to exclude the effect of the anesthetic on the observed tendency. The second type of experiments involved searching for a paradigm where we could train mice to recognize predetermined sequences of whisker deflections. Recording neuronal activity throughout the task allows not only linking it to a specific behavioral output but also investigating the sensitivity of neurons to specific sequences of pulses extended over time in an animal performing in a task.

Until the day when electrophysiological recordings were performed (anesthetized experiments) or until the behavioral protocol started (awake, including behavioral, experiments), all animals were housed in cages with bedding material, at normal light conditions (12:12 LD) and providing relative humidity level of 55 ± 10 % and temperature of $22°C \pm 2°C$. Water and food were available *ad libitum.*

2.2.   Overview of electrophysiology experiments: anesthetized animals

2.2.1. Experimental subjects

All animals used in experiments with use of anesthesia were female ICR mice (age 4-9 weeks old; mean weight 23.6 g, SD 4.8 g). Total number of mice used in these experiments was 35.

2.2.2. Preparation
   a. Anesthesia

Animals were anesthetized with the combination of ketamine and xylazine in a single intraperitoneal injection (100 mg/kg, 10 mg/kg, i.p.). The combination was prepared at least 30 min prior to the experiment and was stored no longer than two days in 4°C.

Ketamine is antagonist of NMDA (N-methyl-D-aspartate) receptors. As a dissociative anesthetic, it produces sedation with loss of the awareness of the surrounding (White, 1982) (Flecknell, 2009, pp. 68–69). It provides deep sedation and mild to moderate analgesia. Other effects are immobilization, slight respiratory depression and increased blood pressure. One of its disadvantageous effects is muscle rigidity: therefore it is often necessary to combine it with other sedatives in order to eliminate this effect. In the present study I used xylazine (Flecknell, 2009, p. 24).

Xylazine is an agonist of alfa-2-adrenergic receptors. It is a strong sedative drug that significantly potentiates the action of other anesthetic drugs (Flecknell, 2009, p. 23).

Both substances provide sedation and mild to moderate analgesia. Their mixture provides surgical anesthesia lasting from 20 to 30 minutes and sleeping time of 60 to 120 minutes (Flecknell, 2009, pp. 121, 193). Hence it is necessary to monitor the depth of anesthesia by checking regularly e.g. the hindpaw reflex as well as breathing pattern, and injecting extra doses if required. In the present study 20 - 30% of the initial dose was injected, approximately every 30 min.

b. Craniotomy

Upon inducing anesthesia the animal was placed in the stereotaxic apparatus (Narishige, Scientific Instruments) enabling stabilization of the skull by using ear bars and a bite bar. Eyes were covered with a layer of ophthalmic gel (Viscotears, Alcon) in order to protect the cornea against drying. Body temperature was maintained at 37°C using a homoeothermic heating pad (FHC). During the experiment the level of anesthesia was monitored regularly.

At first the hair on the dorsal part of the skull was depilated and both the scalp and periosteum were removed. After cleaning and drying the surface of the cranium, the coordinates for barrel cortex were marked (L 3 mm, AP 1.5 mm relative to Bregma) and the craniotomy was opened with a dental drill (Foredom, Electric Company) equipped with Tungsten carbide bur (Heger & Meisinger GmbH), taking care not to overheat the brain by stopping and washing the skull frequently with cooled sterile saline solution. After exposing the cortex, the skull, apart from the craniotomy site, was covered with 2% agarose (Sigma). The well over barrel cortex was filled with sterile saline solution which prevented the brain surface from drying and a consequent damage. This way the animal

was prepared for undergoing the experiment (see Figure 2.2, an example in an awake naïve animal).

2.3. Overview of electrophysiology experiments: awake animals

2.3.1. Experimental subjects and housing

All animals used in the awake experiments (without the use of anesthesia) were female C57BL/6J mice (n = 17, medium weight = 21.6 g, SD = 1.1 g). The age varied between 7 and 12 weeks at the moment of implanting the head-post (more mature animals were used to set up and calibrate the method).

C57BL/6J strain was chosen for awake experiments because, even though these mice display high locomotor activity, their body size, muscle strength and weight gain are lower in comparison to ICR mice. Therefore the overall power applied to the junction between the dental cement and the headbar, while the animal is being trained under head-fixation, is lower, diminishing the probability of detaching the headbar during behavioral training. It has also been observed that C57BL/6J mice are quieter and express lower level of conditional fear, which is why they are more likely to spend longer time head-fixed and licking water (Bryant et al., 2008).

Upon implanting the head bar, mice were housed singly in separate cages containing bedding material and tunnels, in a reversed light cycle cabinet (12:12 LD). Behavioral training and the experiments themselves took part in the dark phase which is referred to as an active phase characterized by higher locomotor activity (Tankersley et al., 2002). Each animal underwent a maximum of three recording sessions on subsequent days, depending on overall health and tissue status.

2.3.2. Preparation
   a. Anesthesia

All mice assigned to awake experiments were implanted with a small aluminum headbar (19.1 mm x 3.2 mm), required for fixing the animal's head steadily in the behavioral apparatus and enabling carrying out the experiment (see Figure 2.1 and Figure 2.2). Its implantation is a simple surgical procedure that does not require craniotomy. For that purpose animals were anaesthetized with isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane) (IsoFlo, Abbott). This is a strongly volatile substance and its concentration both in oxygen, a carrier gas, and in blood stream can be easily controlled

by a vaporizing device (Harvard Apparatus). Concentration of isoflurane used most usually is 5% for induction and 1.5 - 2% for maintenance of surgical anesthesia (Flecknell, 2009, p. 54). Isoflurane hardly undergoes any biotransformation in the organism, overall making the depth of anesthesia even easier to manipulate. It produces muscle relaxation and analgesia sufficient for surgical procedures (Flecknell, 2009, p. 52).

b. Head-post (headbar) implantation and recovery

After anesthetizing the animal and mounting it in the stereotaxic apparatus (Narishige, Scientific Instruments), eyes were covered with ophthalmic gel (Viscotears, Alcon) and body temperature was maintained at 37°C using a homoeothermic heating pad (FHC). Upon hair removal and skin disinfection (3 passes of 70% ethanol and povidone-iodine [Videne, Ecolab Ltd.] in alternation), the scalp and periosteum were removed and the bone surface was cleaned with sterile saline solution and dried. The edges of the skin were glued to the bone with cyanoacrylate adhesive (Vetbond, 3M Animal Care Products). At this stage future craniotomy site was marked (L 3 mm, AP 1.5 mm, relative to Bregma) with a surgical marker. A thin layer of cyanoacrylate adhesive (Loctite Super Glue, Henkel Ltd) was applied to the skull and covered with dental acrylic once dried (Ortho Jet, Lang Dental), providing strong fixation for the headbar. To ensure good adhesion of the implant, the skull surface and the layer of cyanoacrylate adhesive were scratched with a scalpel providing more bonding surface. The headbar was attached to the skull, once the fresh layer of dental acrylic was applied.
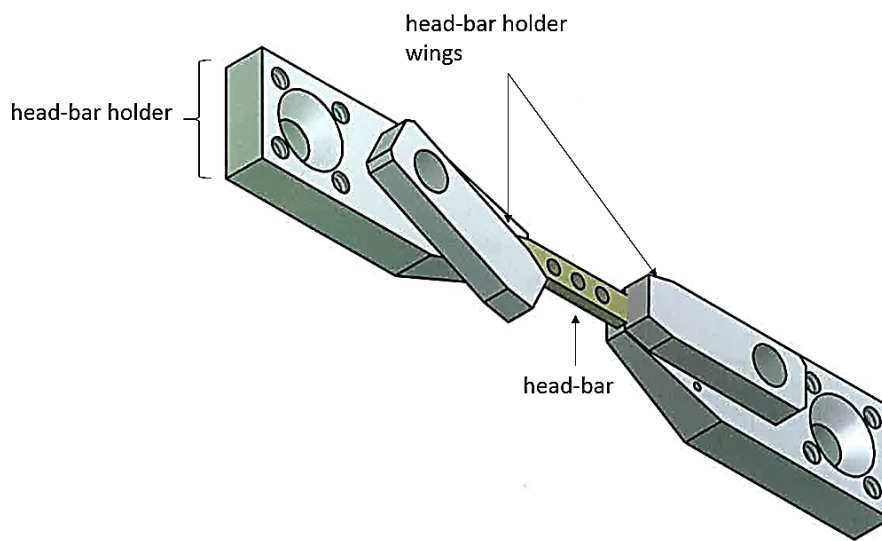
Figure 2.1. **Headbar and headbar holder configuration**. Headbar, which is cemented to animal's head, is placed in the headbar holder and clamped with headbar holder wings in order to disable movements of the animal's head. Compare with Figure 2.2.

Thanks to the courtesy of Karel Svoboda (Janelia Farm, Virginia, USA).

Mice were given buprenorphine hydrochloride (Grape & Tramèr, 2007) delivered in subcutaneous injection (0.1 mg/kg, s.c.) for postoperative analgesia and were left to recover for 5-7 days. During this period their health was monitored daily for locomotor activity, posture and grooming, eating and drinking, and hydration (Guo et al., 2014).

c. Licking and water deprivation

Licking is considered a highly stereotyped behavior consisting of repetitive jaw and tongue movements leading to consumption of water. This motion is under control of a central pattern generator (Hayar et al., 2006) – an ensemble of motor neurons sending a rhythmic output in the absence of sensory input.

Licking itself requires no training and can be easily quantified. Moreover rodents can produce a large number of licks per experiment. Taken together, these properties make licking an easily-controlled behavior appropriate for combining with head-fixation and whisker stimulation. In such configuration, conditioned licking serves as an instrumental response in a psychophysical task reporting a decision made by an animal (Schwarz et al., 2010). As a result of making the right decision and licking the lickport at appropriate time points, the animal is allowed to consume water reward (Stüttgen & Schwarz, 2010). In these awake experiments licking behavior was not conditioned, however the training

session and the actual experiments were the only time during the day when the animals were allowed to consume water. Therefore water here served as a motivation for the deprived animals to stay head-fixed in the behavioral apparatus with minimal or no signs of stress.

Daily water intake for mice amounts to 3-7 ml depending on their weight, age and strain (Schwarz et al., 2010). Adult C57BL/6J mice typically consume around 3 ml of water per day (Guo et al., 2014; O'Connor et al., 2010) and due to their physiology they are able to maintain stable body weight if restricted, even to as little as 12.5% of its daily supply (Haines et al., 1973). Thus it is a safe method to motivate animals to stay head-fixed, provided that the weight and health of the animal are controlled every day (Guo et al., 2014). If any signs of stress were observed or if the weight dropped below 70% of the initial value (Guo et al., 2014; O'Connor et al., 2010), the animal was withdrawn from the water restriction protocol until recovery.

Mice were water deprived from 8 to 16 days before performing the craniotomy. The duration of this time interval depended on the time mice needed to become habituated to the experimenter (handling) as well as their performance in the training (the poorer performance was, the longer it took and therefore the longer was the period the animal was on water restriction). Mice were limited to 0.5 ml of water per day (Mouse Phenome Database from the Jackson Laboratory, www.jax.org/phenome). The consumed amount of water was estimated by weighing the animal before and after the training session (including excrements); the difference in weight stood for consumed amount of water. In case the animal drank less than 0.5 ml of liquid, the missing amount was provided in the cage after the end of the session.

   d. Behavioral training

The training itself started the day after water delivery was restricted and consisted of subsequent phases:

   → Handling (2-3 days);
       This procedure consisted of habituating mice to the experimenter's hands, as well
       as the experimental rig: once the mouse did not show any signs of stress while
       being held, it was allowed to explore and enter the tube, where it was restrained;
       upon entering the tube, it was given small amount of water delivered from 1 ml

syringe; finally the animal was head-fixed and the mouse was presented with a lickport as a source of water;

→ Head-fixation for increasing amount of time on subsequent days; Water was presented to water-deprived animals during fixation sessions; it motivated them to stay in the apparatus for extending periods of time, as well as decreased levels of stress caused by fixation of the head. If signs of stress, excessive movement or discomfort were observed before the session ended, the amount of water was increased; if the symptoms persisted the mouse was removed from the apparatus and the same session was repeated the following day (Tab. 2.1);

Day	Head fixation time [min]	Water valve open time [ms]
1	1	50
2	10	40
3	25	25
4	40	20
5	60	15

Table 2.1. **Summary of the protocol for desensitization to head-fixation, and water delivery.** On subsequent days the animal was head-fixed for increasing amounts of time and was provided with a decreasing amount of water (to maintain the motivation for staying head-fixed for longer amount of time without getting sated too early). The animal was passed to the next step only if no symptoms of stress were observed.

→ Performing a sham experiment including actions such as e.g. whisker stimulation (different than the experimental one) with the piezoelectric actuator, which served for habituating the animals to the noise and objects present in the proper experiment.

e. Craniotomy

Once the goal of the behavioral training was achieved, mice were anesthetized with isoflurane (IsoFlo, Abbott) and placed in the stereotaxic apparatus (Narishige). The surface of dental acrylic was disinfected with 70% ethanol and its portion over the

previously marked craniotomy site was thinned down with a dental drill equipped with Tungsten carbide bur (Heger & Meisinger GmbH) to uncover the skull surface. Once the craniotomy (Figure 2.2) was performed, the cortex was covered with a thin layer of 2% agarose and silicone elastomer (Kwik Cast, World Precision Instruments) providing protection against damage and infection until the next day, when the experiment was carried out.

This protective cap was removed upon fixing the animal's head in the behavioral apparatus and just before introducing a recording electrode into the tissue. The cap was then replaced with a new layer of 2% agarose which served to minimize brain motions produced by movement of the animal's body and, in lower degree, by heart and pulmonary pulsations. It also protected the cortex against drying throughout the experiment.
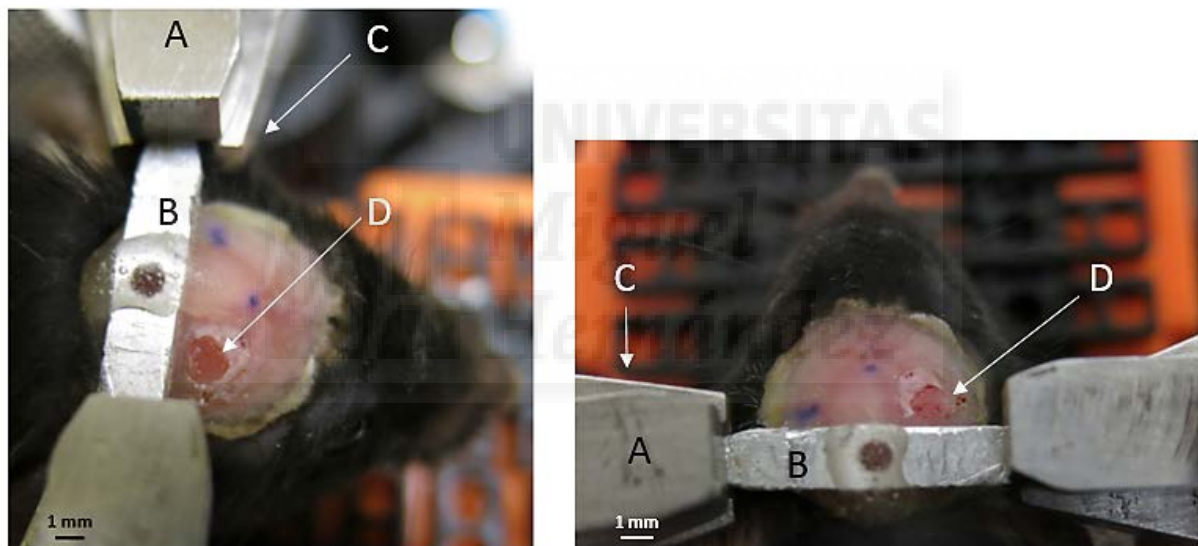
Figure 2.2. **Example of a craniotomy performed in an implanted animal**; images taken in the same animal, from different angles. The animal is placed in a behavioral apparatus: headbar holder wings (A) fixate the headbar (B) to the headbar holder (C) disabling the movement of the head of the animal. Before performing the craniotomy the layer of the cement over the recording site was thinned down, in order to expose the surface of the skull. The surface of the brain is visible (D), the pipette was inserted in a reasonable distance from major blood vessels in order to prevent bleeding.

Recording sessions lasted from 1.5 to 2 hours and each animal underwent the experiment up to a maximum of three times on subsequent days, depending on overall health and brain tissue status.

2.4. Electrophysiology configuration: theory

2.4.1. Principle of patch-clamp technique

Electrophysiological techniques in use can be divided into those that record ion flux across the cellular membrane in an indirect way (e.g. through the skin and skull as in EEG - electroencephalography) or in a direct one, as when a micropipette is inserted into the neuronal tissue in order to make a direct physical contact with the cell. If the latter option is chosen, it is possible to chronically implant the electrodes in order to record activity of many neurons throughout prolonged periods of time in a behaving animal. Despite its obvious advantages, due to pulsating movement of the tissue, electrodes permanently placed in the brain might cause irreversible lesions, which affect the quality of collected data and the health of the tissue. Moreover, certain regions of the brain *in vivo* are not easily accessible to the chronically implanted recording electrode. The patch-clamp method of recording of neuronal activity provides a measure of a single neuron's electrical state either *in vitro* (in cultured cells and in slices) or *in vivo* (in an intact animal).

The principal differences between *in vivo* and *in vitro* modes are (DeWeese, 2007):

→ In *in vitro* mode differential interface contrast optics helps to visualize the electrode immersed in the tissue as well as a neuron being recorded;

→ In *in vivo* mode there is no possibility of controlling the content of external solution – only the internal solution ingredients can be controlled;

→ In *in vivo* mode introduction of a pipette into the tissue might provoke its dimpling or compression. The tissue in intact animal displays microscopic motions because of cardiac and pulmonary pulsations, or animal movement in case of awake experiments, which might result in an increased difficulty to patch a neuron.

In vivo patch-clamp recordings can be carried out either in an anesthetized or awake animals (Long & Lee, 2012). Electrophysiological recordings in anesthetized animals are easier to perform, as there are fewer mechanical disturbances occurring during the experiment. Nevertheless anesthesia can affect in a significant way various aspects of neural coding, which has to be then investigated in awake preparations. In our project, awake recordings were necessary to check whether temporal integration is affected by

anesthesia and whether the observation made in awake animals is similar to the one in anesthetized animals.

2.4.2. Recording technique

Achieving a patch-clamp recording *in vivo* requires four stages (Figure 2.3) (Kodandaramaiah et al., 2012a):

→ Pipette localization – pipette is placed over a chosen brain region and, under a positive pressure, rapidly lowered to a desired depth;

→ Neuron hunting when the pipette already is at certain depth and is advanced slowly in order to detect a neuron;

→ Gigaseal formation when a tight seal, of at least 1 GΩ resistance, between the pipette and the cell membrane is formed

→ Optionally, break-in during which a patched piece of membrane breaks, due to negative pressure applied to the pipette, providing an access to the cytoplasm (this step takes place only if one decides to perform recordings in whole-cell configuration)
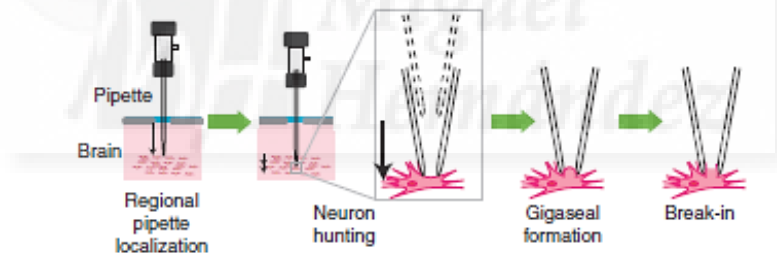
Figure 2.3. **Four stages of *in vivo* patch-clamp procedure**: pipette localization, neuron hunting, gigaseal formation, break-in.
Adapted from (Kodandaramaiah et al., 2012b)

Before inserting a recording electrode to the tissue positive pressure was applied to the glass pipette (around 120-150 mmHg). This procedure prevented debris from clogging the pipette tip as well as limiting their introduction into the cortex. The pipette filled with an intracellular solution was placed right over the barrel cortex keeping a reasonable distance from major blood vessels. Pipette offset was set to zero, which nulled the voltage difference between the bath electrode and the patch electrode. This step was always repeated after the pressure was decreased as well as before patching a cell. Pipette was introduced into the tissue and positive pressure was slightly decreased (to around 70-80

mmHg). Once the pipette reached around 100 µm of depth indicated on the micromanipulator reader, the pressure was decreased to 30 mmHg and maintained at this level. Beginning at that depth the pipette was slowly advanced through the cortex (1-3 µm/sec) to hunt for cells.

Neurons were searched for in voltage clamp mode. In this mode voltage pulses of 5 mV and 5 Hz (200 ms) were applied and the evoked changes in current were monitored (DeWeese, 2007). According to Ohm's law (Eq. 2.1) resistance can be estimated by dividing voltage of applied pulse by the height of the current response observed on the computer screen (Signal) or oscilloscope. The proximity of a cell would be indicated by sudden increase in resistance, i.e. a decrease in current response. Therefore the closer the pipette was to the cell membrane or an obstacle, the higher was the resistance and the lower was the registered current. At this point the pipette was advanced very carefully and the positive pressure was released slowly. Once the further increase in resistance reached around 20-30 MΩ, the pressure was further decreased and nulled.

$$R = \frac{V}{I}$$

Equation 2.1. **Ohm´s law.** According to Ohm's law, the closer the pipette is to the cell membrane the higher the resistance, the higher the voltage between the two points of reference and the lower the recorded current. Applying this law in the first stage of the experiment (neuron hunting) is essential for evaluating the closeness of the pipette to the cell membrane.

Electrophysiological recordings always start from the cell-attached configuration (also termed juxtacellular) which provides a loose seal between the pipette and the cell membrane. The resistance can increase roughly to 15-20 MΩ meaning that the pipette tip is very close to the cell membrane but does not form a seal yet (Figure 2.4 and Figure 2.5). This configuration is sufficient for well-isolated recordings of single neurons' action potentials, providing good signal to noise ratio, and therefore it was considered suitable for the present work (DeWeese, 2007; Hromádka, 2007)
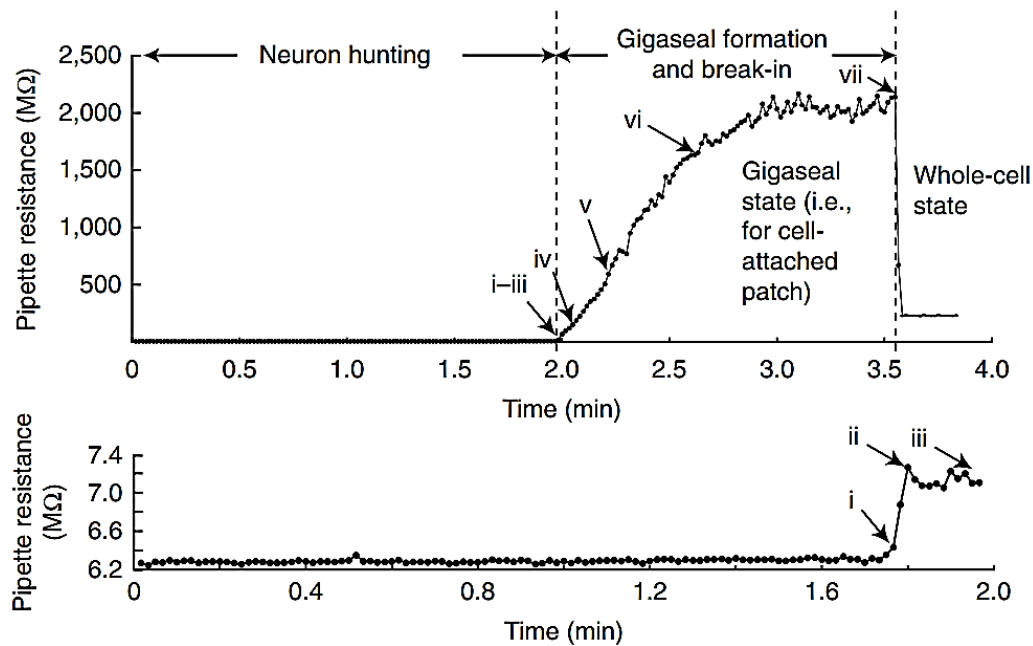
Figure 2.4. **Mechanics of patching a neuron from the point of view of the experimenter.** Observing temporal fluctuations in pipette resistance helps implying subsequent steps from the neuron hunting until forming a gigaseal (top panel). Bottom panel shows neuron hunting phase (i-iii) in magnification. At phase (iii) positive pressure is slightly decreased and the pipette is carefully advanced, whereas at stage (iv) and (v) suction and gradual hyperpolarization of the cell membrane up to -65 mV (vi) are implied. When the break-in takes place, pipette resistance drastically drops down to lower values (vii). For information on membrane current at each of those stages see Figure 2.5.

Adapted from (Kodandaramaiah et al., 2012b)
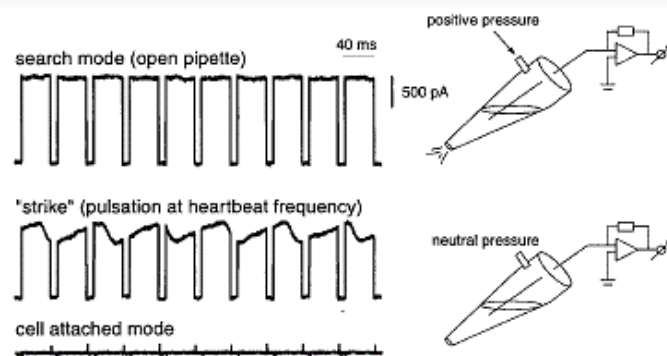
Figure 2.5. **Current flowing through patch pipette throughout subsequent stages of patch-clamp procedure:** here from the stage of neuron hunting up to cell-attached mode while applying square pulse (5 Hz, 5 mV). Pipette was advanced in the brain tissue in steps of 1-3 µm/s. Pulsations synchronized with heartbeat frequency visible in phasic shift in electrode impedance are the sign of neuron's proximity. According to Ohm's law, the closer the pipette is to the cell or an obstacle (such as a blood vessel) the higher the resistance. Nulling the pressure in the pipette and hyperpolarizing the cell membrane should ensure increases in resistance.

Adapted from (Margrie et al., 2002)

Once the desired cell-attached configuration was obtained I switched from voltage clamp to current clamp mode. Thus I changed from the state when membrane potential was clamped (in other words: compared to holding potential set by the experimenter and any difference in potentials was corrected by compensatory current injections) to the state which involves setting the amount of injected current manually, without voltage control. Switching to current clamp mode allowed me to record the membrane potential of the patch. The configuration is most frequently used to check the quality of the patch in whole-cell configuration (it measures the membrane potential) or to record action potentials (as in the present study) (Figure 2.6).
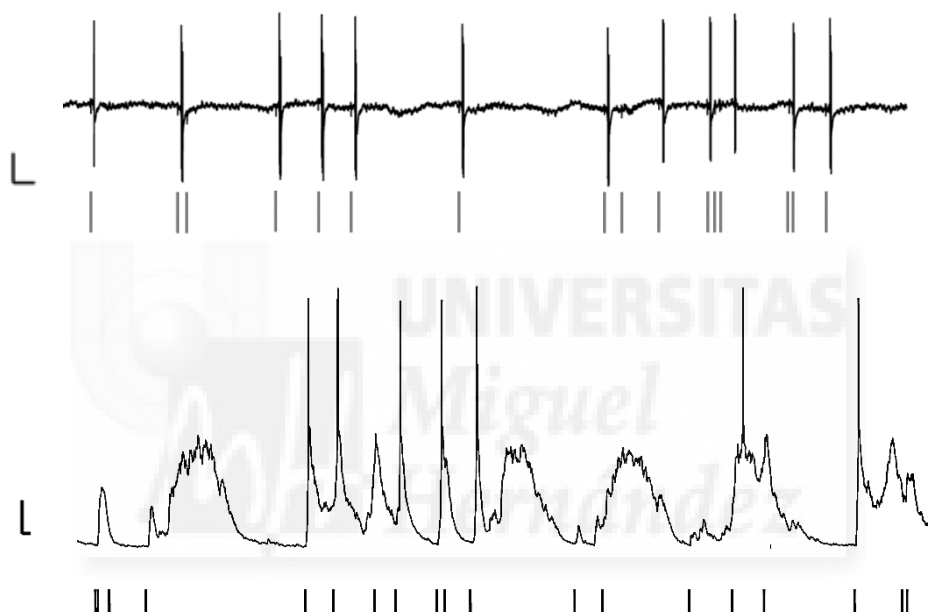
Figure 2.6. **Comparison of the neuronal activity recorded in cell-attached and whole-cell configuration**. Both traces show membrane potential of an example neuron in the barrel cortex; the ticks below correspond to pulses of stimulation applied to the whiskers. The upper plot shows a neuron's membrane potential recorded in cell-attached configuration, scale bars: 100 ms, 2 mV. The bottom one shows a different recording in whole-cell configuration, scale bars: 40 ms, 10 mV. Cell-attached configuration is sufficient for recoding action potentials as it provides good signal to noise ratio. Note the difference in the value of recorded voltage between both configurations.

2.5. Electrophysiology: set up and data acquisition

Here I describe the specific aspects of the awake behavioral setup used for training and experimental sessions (for anesthetized experiments mice were placed in a stereotaxic apparatus, see paragraph 2.2.2.b) and then proceed to the electrophysiological

configuration in detail. The configuration for the recordings themselves did not differ between anesthetized and awake experiments.

2.5.1. Behavioral setup for awake experiments

a. The apparatus: the tube and the head-holder

Mice were placed in an acrylic tube (length: 78 mm shorter edge, 89 mm longer edge, 39 mm outer diameter, 30 mm inner diameter), covering the whole body but not the head. Animals were head-fixed by mounting the surgically implanted headbar to the headbar holder extending to both sides of the mouse' head (see Figure 2.1 and Figure 2.2). Overall this design provided loose body restraint but did not impair the natural crouching position: the animal was able to rest its front paws comfortably on the front edge of the tube. It also permitted stable immobilization of the head and did not limit the movement of whiskers, as well as provided easy access of the piezoelectric wafer to the whisker pad (Figure 2.7).
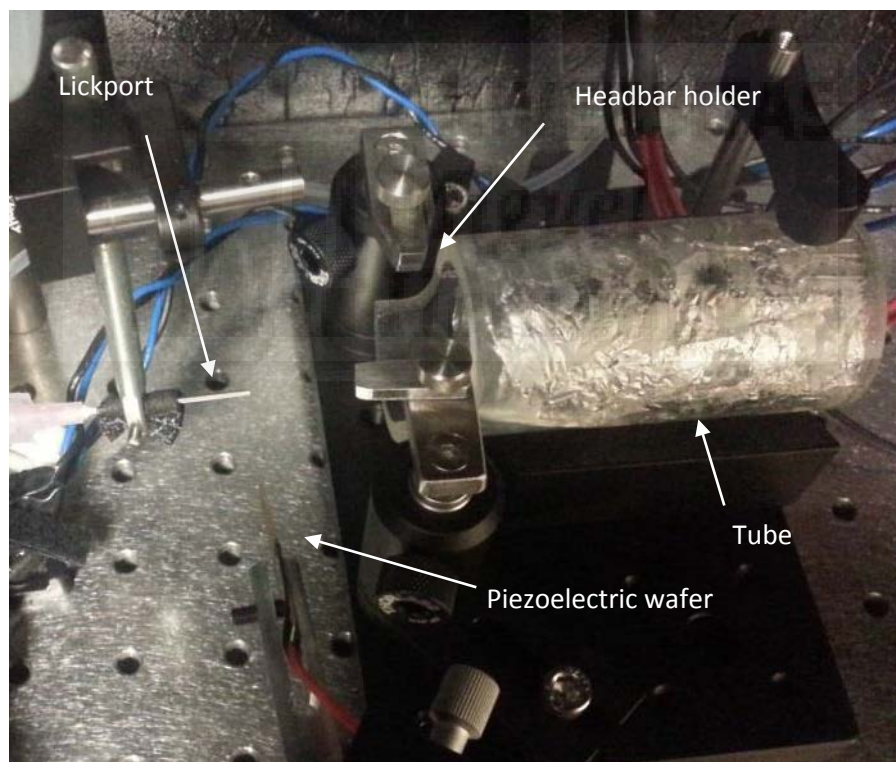
Figure 2.7. **An actual behavioral setup**. The mouse was inserted into the tube and head-fixed via headbar implanted on the top of its head (see Figure 2.2) and the headbar holder forming part of the behavioral set up. Piezoelectric wafer which was in direct contact with the whiskers on the left side of the snout delivered horizontal pulses at variable intervals. The mouse could drink water delivered via lickport, at the front of its head.

b. The apparatus: the lickport

A lickport made of a needle with blunted tip was placed in front of the mouse within the reach of its tongue in order to deliver water drops as a reward. Water was delivered regularly (every 5 s) and directed to the lickport by gravity through a solenoid valve (The Lee Co., Essex, UK). Water was available throughout the whole training and experiment session, and its delivery was not conditional on actions of the animal. To avoid pooling and decrease the time water was present on the lickport, the excess water was pumped out by a peristaltic pump (Harvard Apparatus, MP II) through a blunt needle placed right below the lickport.

c. Whisker stimulation

For stimulation in anesthetized experiments three glass pipettes were glued to the piezoelectric bender (Physik Instrumente). The pipettes were brought proximal to the whisker pad (1-3 mm) and all visible macrovibrissae were introduced to the pipettes. For stimulation in awake experiments the piezoelectric wafer was elongated (so that its length was equal to the length of the wafer and pipettes used in the anesthetized protocol) and wrapped with double-sided adhesive tape. All visible macrovibrissae were stuck to the elongated part of the wafer.

2.5.2. Experimental setup and data acquisition

Upon performing a craniotomy animals designated for anesthetized experiments were kept in the same position within the stereotaxic apparatus (Narishige) in order to perform electrophysiological recordings. In awake experiments animals were placed in the behavioral apparatus described above (see paragraph 2.5.1.a).

Pipettes for patch-clamp recordings were fixed in a pipette holder attached to a headstage mounted on a micromanipulator. The depth of recorded neurons was controlled through the micromanipulator reading (Sutter MP-225, Sutter Instrument Company) and ranged between 120 and 1039 µm (mean 574 µm). The surface of the cortex was viewed via dissection microscope (Olympus SZ51) enabling the correct positioning of the pipette.

For data acquisition I used a CED micro 1401 acquisition unit (Cambridge Electronic Design) together with Signal and Spike 2 software: the former for hunting for neurons in voltage clamp mode and the latter for outputting stimulation while recording responses in current clamp mode. Recordings were digitized at 10 kHz; a MultiClamp 700B

amplifier (Axon Instruments) was used to amplify the signal; stimulus output voltage was recorded simultaneously with the patch clamp signal (Figure 2.8).
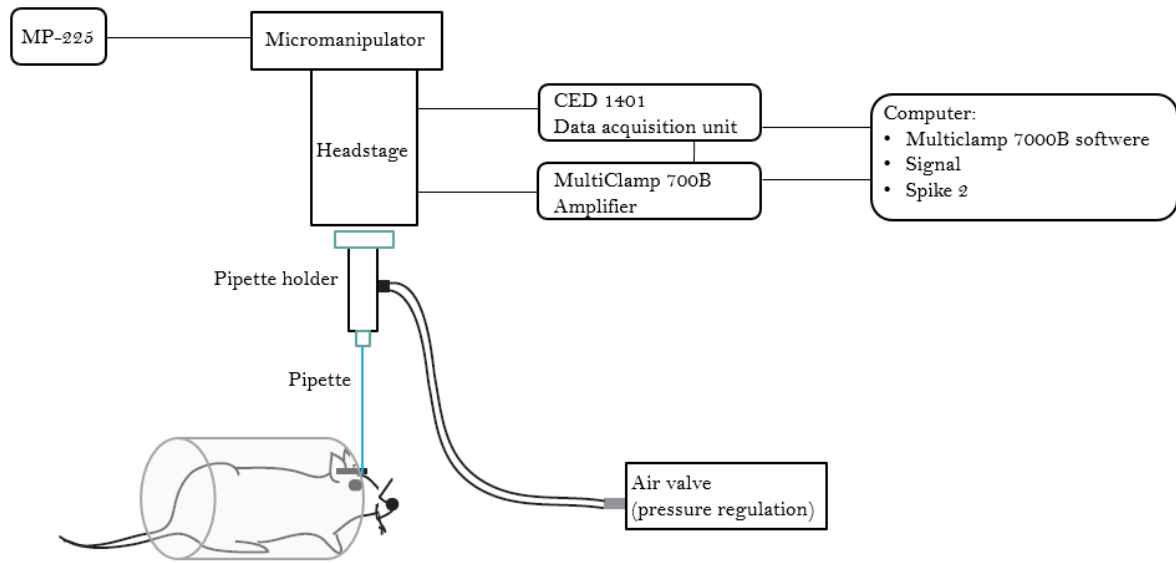
Figure 2.8. **Schematic of a simple setup for patch-clamp recordings** consisting of: MP-225 micromanipulator, CED micro 140 data acquisition unit, MultiClamp 700B Axon Instruments amplifier connected both to the headstage and the computer operating on software including: MultiClamp 700B (to set the parameters of registering neuronal activity), Signal (for monitoring neuronal activity together with oscilloscope) and Spike 2 (recording of current traces flowing throughout the membrane of recorded neurons as well as applied stimulation, allowing for real-time following of spontaneous and evoked neuronal activity).
Adapted from (Kodandaramaiah et al., 2012b)

a. Pipettes and intracellular solution

Pipettes were prepared from borosilicate glass (Harvard Apparatus: 1.5 mm outer diameter, 0.86 mm inner diameter) in a vertical two-stage puller (PC-10, Narishige). Resistance was 5-7 MΩ before entering the brain tissue. Prior to the experiment pipettes were fire-polished and partially filled with intracellular solution making sure no air bubbles blocked the pipette. The internal solution contained (in mM): 130 K-methylsulfonate, 10 Na-phosphocreatine, 10 HEPES, 4 MgCl, 4 Na2-ATP, 3 Na-ascorbate, and 0.4 Na2-GTP. The osmolarity of the solution was 287-303 mOsm and pH 7.33. For the experiments involving cell-attached labelling, 1 mg/ml biocytin (Sigma) was added to the intracellular solution and filtered prior to loading the solution to the pipette.

b. Stimulus design

Stimulation consisted of sequences of whisker deflections. Each deflection was a stereotypical waveform: a Gaussian-filtered differential filter (Petersen et al., 2008). Each deflection had a dynamic range of 400 µm and was powered by a purpose-built amplifier (Physik Instrumente). Whiskers were deflected jointly (in fully coherent manner). Joint stimulation of whiskers with a common waveform simplified the parameter space, as responses did not depend on the relative motion of whiskers, and instead depended only on the temporal structure of the stimulus. Neurons sensitive to correlated joint whisker motion are readily found in barrel cortex (Estebanez et al., 2012). We checked that the piezoelectric wafer followed the deflection waveform by optical monitoring with a custom LED-phototransistor circuit. The direction of whisker deflections was manually adjusted at the start of each recording to produce the clearest onset response from the neuron.

Stimulation sequences satisfied the following design criteria: the range of intervals spanned physiologically feasible values, successive intervals were not significantly correlated (i.e. the distribution of values for each interval was drawn independently from that of its neighbor) and the ensemble of interval values permitted unbiased statistical analysis. In the initial design we implemented the first criterion by basing the sequence on a recording of thalamic spiking responses *in vivo* (mean interval 217 ms) (Petersen et al., 2008). We reasoned that the intervals contained in this pattern of thalamic spiking would be characteristic of temporal input patterns faced by cortical neurons. We then constructed additional stimulus patterns by shuffling (reordering) the initial sequence and by generating Poisson and log-normal distributed ensembles over a similar range of interval values. Stimulation protocols constructed in this way lasted 120 s and included an ensemble of around 400 usable intervals.

For the subset of experiments we constructed (represented in the figure 3.6.) an expanded stimulus set that included a larger (ca 3000) ensemble of independently distributed intervals, log-uniformly distributed over the range 40-500 ms (mean 182 s) to optimize equipopulated sampling (when each of bins contains the same number of samples; such binning helps to avoid biases in the information estimate (Panzeri et al., 2007)) (Figure 2.9) . This protocol lasted 586 s.
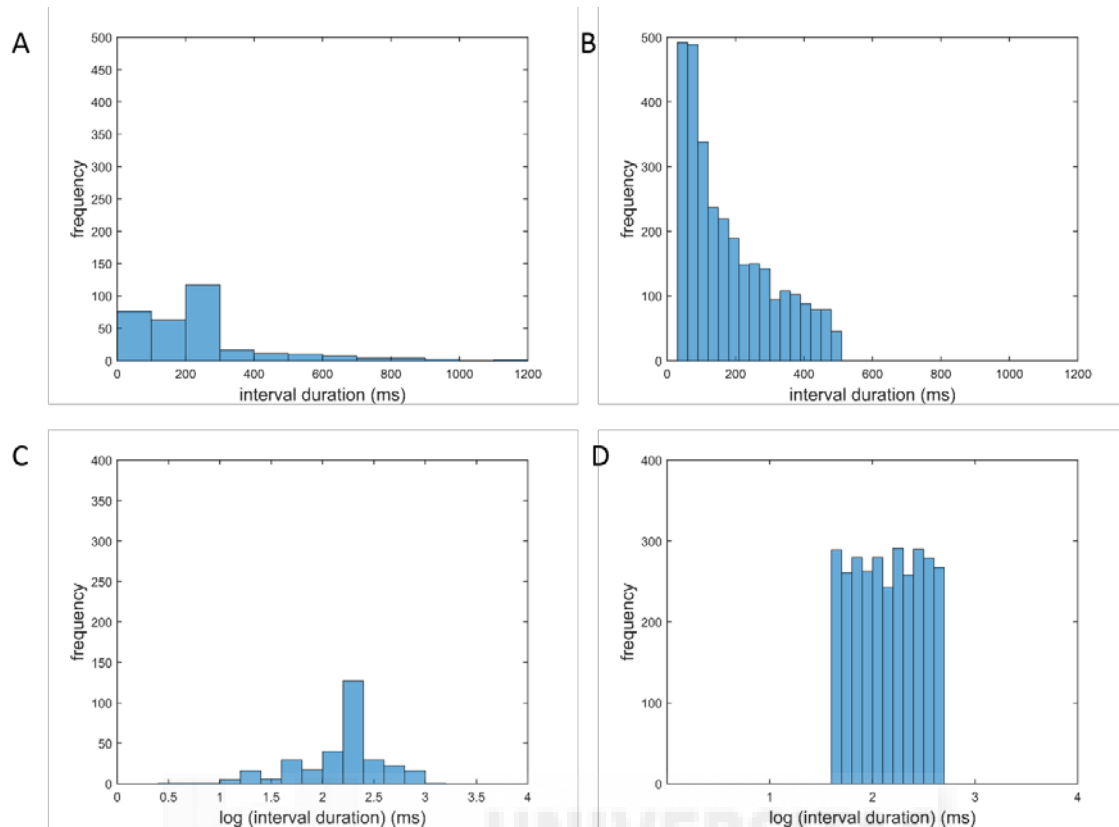
Figure 2.9. **Distribution of intervals**. The protocol for short stimulation of 120 s (A) included around 400 usable intervals, whereas the one for long stimulation (B) of 586 s contained around 3000 intervals. Bottom histograms show the distribution of logarithm of intervals subsequently for short (C) and long (D) stimulation. The latter one shows that the distribution of log(intervals) is flat, therefore providing equipopulated bins, when each of bins contains the same number of samples; such binning helps to avoid biases in the information estimate.

2.5.3. Histological procedures & morphological analysis

In a number of recordings the depth was verified by juxtacellular injections of 1% biocytin. Each injection lasted from 5 to 10 min.

a. Biocytin – basic information

Biocytin is a conjugate of D-biotin and L-lysine (Mishra et al., 2010). This popular neuroanatomical tracer, upon injection into the neuronal tissue, is taken up easily by neurons and undergoes both anterograde and retrograde transport. It is also characterized by high affinity to avidine: therefore if avidine-conjugated marker is used it is possible to visualize biocytin-labeled neurons (Horikawa & Armstrong, 1988). Once in the tissue however, biocytin is quickly degraded by biotinidase enzymes. This is the main practical disadvantage of biocytin, which makes it unsuitable for experiments with long post injection survival times (Hymes & Wolf, 1996).

b. Transcardial perfusion and labeling procedure

Animals injected with biocytin were perfused with fixative through the vascular system in order to preserve the structure of the tissue. Upon opening the ribcage a cannula was inserted into the posterior end of the left ventricle. In order to wash off blood cold 0.9% sodium chloride (Sigma) solution was injected using a peristaltic pump (Gilson, Manipuls 3) and the right atrium was cut to allow continuous flow through the vascular system and avoid rupturing the capillaries. Subsequently 4% paraformaldehyde (Panreac) solution was pumped in, providing tissue fixation. Finally the brain was extracted and placed in a fixative solution in 4°C for 24 hours. The following day the brain was removed from the solution, frontal and posterior parts were cut off leaving a thin portion of brain containing barrel cortex. It was embedded in 2% agarose solution; the block was cut horizontally into 60 µm coronal sections on a vibratome (Leica, VT 1000S) (the softer the tissue the higher the vibration and the lower the velocity of the blade; in the present study the forward velocity was set to intermediate values and vibration to high values in order to preserve the integrity of the tissue). In order to recover labeled neurons the slices were treated with biotin-horseradish peroxidase complex (ABC Kit, Vectastain) and diaminobenzidine DAB (Sigma) which is an enzyme substrate (Horikawa & Armstrong, 1988; Joshi & Hawken, 2006; Pinault, 1996) (Figure 2.10).

Figure 2.10. **Example image of two reconstructed neurons**. Neurons were stained with biocytin and visualized due to ABC Kit (Vectastain) and diaminobenzidine. Magnification 10x. The arrow shows the distance of the soma from the cortex surface. Subsequently this value was compared to the depth shown on micromanipulator reader.

In some slices additional Cytox staining was carried out (Figure 2.11): a double staining of biocytin and cytochrome oxidase aimed to visualize the position of single neurons in relation to the barrels, and not only to the brain surface. Slices assigned for double staining were rinsed with phosphate buffer solution (PB) and incubated in filtered solution of 5 mg of diaminobenzidine, 3 mg of cytochrome c (Sigma) and 2 mg of catalase (Sigma) in 10 ml of phosphate buffer solution (PB: 0.1 M, pH = 7.4). To obtain a good quality staining, incubation in 37°C was maintained up to 60-90 minutes. The reaction was stopped by washing the slices with PB.

Figure 2.11. **Example image of the brain slice upon cytochrome oxidase staining** (60 µm thickness, coronal plane, magnification 5x). Hollows of the barrels, due to large density of neurites and synapses, and therefore cytochrome oxidase enzyme, appear to be darker than the surrounding tissue.

c. Reconstruction

After washing and mounting the slices on the glasses (Fluoromount Aqueous Mounting Medium, Sigma), each of them was checked in the bright field microscope (Leica, DM 5000 B) for the presence of labeled neurons. In case of finding one, an image was taken. Subsequently the neuron's distance from the surface was calculated and compared to the depth value displayed on the micromanipulator reader. For the visualized neurons the depth discrepancy between the manipulator reading and the post-hoc measurement was 35 ± 8 µm (mean +/- SEM, n = 16 neurons, 10 mice) (for an image of reconstructed neurons see Figure 2.9).

2.6. Analysis: information theory

If a neuron is sensitive to a form of stimulation, its spiking response should correlate with (or "be tuned to") some stimulation parameter and convey information about the value of that parameter. In the case of our recordings of responses to stimulus trains at random intervals, the only parameter that varied over the course of a stimulation sequence and could modulate responses was the inter-deflection interval. To determine whether responses were modulated by and could discriminate between intervals we computed the mutual information between interval value and spiking responses.

2.6.1. Information theory: basic information

a. Probability distributions

There is an infinite number of possible stimuli and each of them has a certain probability of occurring, *P[s]*. There is also a certain variability in the response, so that a presentation of the same stimulus in different time points never evokes exactly the same response. Therefore, an organism as an observer of its own neuronal responses, relying on a single spike train, never has a total certainty on what information it conveys about the external world, even if the stimulus is repeated. If we reverse this correlation – we would not know what exactly stimulus evoked a given response. The observer can rely only on the probability correlation between the response and the stimulus that evoked it (Eq. 2.2).

$$\text{Eq. 2.2.1. } P\ (r\ |s)$$

$$\text{Eq. 2.2.2. } P\ (s\ |r)$$

$$\text{Eq. 2.2.3. } P\ [r,s] = P\ [s]P[r|s] = P[r]P[s|r]$$

Equation 2.2. **Probability correlation between the stimulus and the corresponding response**. Eq. 2.2.1. Encoding problem: conditional probability distribution of evoking response "r" given the stimulus "s" was presented. Eq. 1.2.2. Decoding problem: Describes the conditional probability distribution that the stimulus "s" was presented, given the response "r" was evoked. This distribution gives all the information that the observer could know about the stimulus observing a spike train, each information extracted from the spike train narrows the number of possible stimuli and so decreases the observer's uncertainty about the stimulus. Eq. 2.2.3. Shows a join probability distribution and gives the information about the probability of both a response being observed and a stimulus being presented.

b. Shannon's information theory

Shannon's information theory (Shannon, 1948) assumes that if the output *r* is predicted by the input *s* (in case of the present study *r* is a neural response to a stimulus *s*), than the knowledge about the stimulus parameters can help to predict certain response features. In other words: it can restrict possible outputs *r*. This reduction in uncertainty is quantified by mutual information, which is measured on a log scale and is given in bits (1 bit of information reduces the number of possible outcomes by 50%) (A. Fairhall at al., 2012). Bit itself is not a dimensional unit, but rather a reminder of having used a certain system of quantification.

Assuming that there are X equally possible outcomes, the response would contain $\log_2(X)$ bits of information about the stimulus. As an example - if there are ten possible outcomes

restricted to digits between 0 and 9, and I would give an information that the target outcome is an even number, I would decrease the uncertainty by half and give one bit of information. Considering a simple example in the nervous system: if a neuron always produces a spiking response to a stimulus A, but never to stimulus B, and both of those stimuli are equally likely, then the evoked spike train would carry 1 bit of information about the stimulus ($\log_2(2) = 1$).

As mentioned before, neuronal representations of stimuli undergo trial-to-trial variability, which is also taken into account in Shannon's information theory and is quantified by response entropy (Eq. 2.3).

$$H(R) = \sum_r P(r) \log_2 P(r)$$

Equation 2.3. **Overall entropy of neural spiking responses**. Here the factor $h=log_2P(r)$, that quantifies the unpredictability associated with a given response, is multiplied by the probability of that response. In case of a neuron that always responds with the same firing rate, the entropy would equal to zero. Entropy corresponds to a "feel of surprise" when observing a neural response evoked by a specific stimulus.

Mathematically, mutual information *I(S;R)* quantifies to what degree the response encodes the stimulus, via measuring how much *P(r/s)* differs from *P(r)* (Eq. 2.4):

$$I(S;R) = \sum_{r,s} P(r|s) P(s) \log_2 \frac{P(r|s)}{P(r)}$$

Equation 2.4. **Mutual information between two variables**. This parameter measures the amount of information about one of them, knowing the other one; it relies on mutual dependence between the variables: the higher it is, the more information each variable carries about another one.

As we are considering the relation between the variables over certain time, therefore the value of mutual information would also depend either on the time window (for spike count) or on the bin size (for spike timing).

Overall mutual information used in Shannon's information theory can be used as a measure of amount of information in the neuronal response about the stimulus, for various encoding paradigms (spike timing, spike rate etc.) and many areas of the brain.

2.6.2. Actual analysis

Mutual information measures the average decrease in statistical uncertainty about the interval duration that an observer would obtain by measuring neuronal spiking (Cover &

Thomas, 2005; Shannon, 1948). For an ensemble of interval values $\{interv\}$, mutual information is defined as the difference between the overall entropy (indeterminacy) of the interval distribution and the average entropy of the interval distribution if the neuronal response is known (Eq. 2.5, compare with an Eq. 2.3 for general entropy):

$$I(\{interv\}, \{resp\}) = S[P(interv)] - \sum_{\{resp\}} S[P(interv|resp)]$$

$$= -\sum_{\{interv\}} P(interv) \log_2 P(interv)$$

$$+ \sum_{\{resp\}} P(resp) \sum_{\{interv\}} P(interv|resp) log2P(interv|resp)$$

Equation 2.5. **Quantification of mutual information in the actual data analysis**. Mutual information measures the level of correlation between at least two variables (such as input and output or stimulation and neuronal response) without the necessity of decoding one of them. In our calculations, "*interv*" refers to values of either the latest interval (the one immediately preceding the latest deflection) or the second-latest.

Adapted from (Pitas et al., 2016)

The distributions were determined as follows. After each whisker deflection, neuronal responses were monitored over a 40 ms window, chosen empirically based on the duration of the primary PSTH peak for responsive neurons. The response to each deflection was classified as either spiking or non-spiking: within the 40 ms window, the number of occasions where neurons spiked more than once was negligible, and creating extra response categories (two spikes, three spikes, etc.) did not qualitatively affect results. We then computed the distributions of latest intervals (those immediately before the latest deflection) or second-latest intervals, conditional on whether the neuron spiked or did not spike after the deflection. These values entered into the second (conditional entropy) term in the equation (Eq. 2.5). This term was subtracted from the entropy calculated from the overall (nonconditional) distribution of stimulus intervals.

Interval values were binned into nb = 4 equipopulated categories, but all qualitative results, particularly the decreased information about second-latest compared to latest intervals, were robust to varying nb within a reasonable range (2-8). Panzeri-Treves bias correction was applied to correct for possible undersampling (Panzeri et al., 2007; Panzeri & Treves, 1996). To test for any qualitative effects of bias, we also carried out analyses on decimated stimulus ensembles, finding no effects of bias with ensemble sizes down to

50% of the full one. All analyses were computed using Matlab; information estimated used the Information Breakdown Toolbox (ibtb.org) (Magri et al., 2009).

Based on information analysis, a neuron was classified as responsive to the stimulus if the mutual information between true spiking response and the stimulus was substantially greater than resulted from shuffling the correspondence between the latest interval and the spiking response. Shuffling was repeated 100 times and the resulting information averaged; for all neurons included in the data set information in the true stimulus-response relationship were > 2 orders of magnitude greater than this shuffled average. This approach to scoring responsiveness ensured that only neurons representing stimulus intervals were taken into account, while allowing visualization of the full variability in information values across responsive neurons. In addition, a neuron's spiking response had to convey least 0.05 bits about the value of the latest stimulus interval, consistent with the intuition that cells conveying very small information values are unlikely to participate in stimulus encoding. There were no neurons failing these criteria for inclusion but carrying significant information about earlier intervals. Out of a total of n = 136 cell-attached recordings, 84 satisfied these criteria and were included in the final dataset.

2.7. Behavioral training on sequence detection and discrimination

A significant part of my work as a doctoral student consisted of participation in a project on detection and discrimination of patterns (sequences) of whisker deflections in awake behaving animals (main researcher: Dr. Michael Bale). This new research line required designing a behavioral protocol to train mice at detecting and discriminating sequences of whisker vibrations in a Go / No-Go paradigm from scratch:

→ Setting up both hardware and software to register animal's behavior and control the task;

→ Adapting and testing the behavioral apparatus: the headbar, headbar holder, the tube / treadmill for loose body restraint, as well as the lickport for water delivery and lick detection;

→ Optimizing the implantation procedure.

2.7.1. Experimental subjects, housing and preparation

Mice (C57BL/6J, male and female) were housed singly under the same conditions as the animals assigned for awake electrophysiological experiments described above (see 2.3.1). They also underwent the same procedures of head-post implantation and handling (see 2.3.2.a, 2.3.2.b).

2.7.2. Behavioral training

a. Experimental set-up

The trial-based experiment, piezoelectric actuator and water delivery were controlled by a system comprising Matlab (Mathworks) and ActiveX (TDT) software controlling a custom-made combination of components (Bale et al., 2015). The system allowed manipulation of the task parameters, such as: relative frequency of stimuli, duration of the water valve release (reward), duration of time-out (punishment) etc. (Figure 2.12).
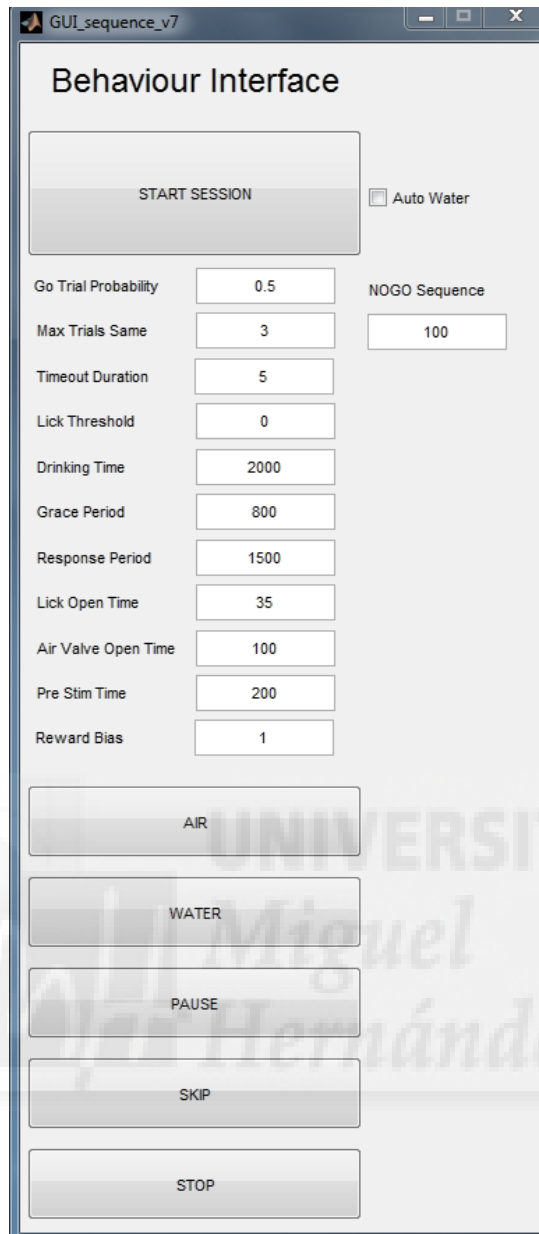
Figure 2.12. **Behavior interface allowing manipulation of the parameters of the task**. Thanks to the courtesy of Michael Bale (Sussex University, School of Life Sciences).

The behavioral apparatus was analogous to the one used in awake electrophysiology experiments (see 2.5.1): a mouse was head-fixed while its body remained comfortably within an acrylic tube. All whiskers, but one, were trimmed and the remaining C2 whisker was placed in a single tube attached to the piezoelectric actuator. The water delivery system was analogous to the one used in awake experiments, however in the present project valve release depended on the animal's performance. It was triggered only once the animal made a correct choice and, as indication, touched the lickport with its tongue on a Go trial. The mechanism of water delivery was based on the model proposed by Hayar et al (Hayar et al., 2006) allowing detection of licks in a highly temporally precise

way. It can be achieved by connecting a metal lickport to the input of a standard analog/digital (A/D) converter. The animal on the other hand should be connected to ground (e.g. via aluminum foil padding the inner walls of the tube). Each lick (tongue to metal lickport/water touch) would produce then a positive voltage step, which would unblock the valve for a set amount of time and release a drop of water (Figure 2.13).
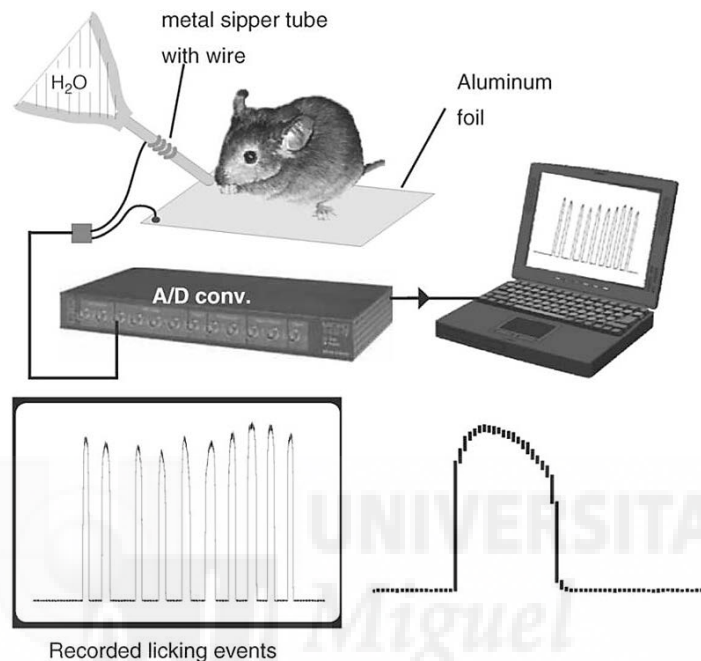
Figure 2.13. **Setup used for registering lick events with high temporal precision**. The upper panel shows the circuit: analog/ digital converter would be connected by one wire to the metal lickport and another wire to the aluminum foil padding the bottom of the acrylic tube. Once the animal touched the lickport with its tongue or paws (the latter avoided in our experiments by the position of the head fixation and acrylic tube assembly), it would close the circuit for the duration of the event and would produce a rise in voltage registered as lick event.
Adapted from (Hayar et al., 2006)

b. Design of the task

The task itself consists of various number of trials (typically mice performed 250-300 trials in a daily session lasting 30-40 min.) Each trial comprises several phases: stimulus onset and sequence playing (800 ms), response window (1.5 s) and inter-trial interval (4 s) (Figure 2.14).

Figure 2.14. **Content of a single trial in the behavioral task**. Single trial consists of sequence playing of 800 ms and a 1.5 s response window when animal's behavior is registered and classified as lick or no-lick. It is followed by 4 s inter-trial interval preceding following trial.

In each trial the mouse was presented with one of two possible stimuli: either Go or No-Go stimulus. If it replied by licking to a Go stimulus (Hit), it was rewarded with a drop of water. Withholding from licking on No-Go trials (Correct Rejections) didn't carry any consequence – the next trial would start within 4 s after the response period. Both types of behavior were regarded as correct responses. The animal could also withhold from licking on Go trial (Miss), which would not carry any negative consequence, but the animal would not receive reward, or lick on a No-Go trial (False Alarm), which would be punished with a time-out, forcing the animal to wait longer for the following trial (an additional 2-5 s) (Figure 2.15).
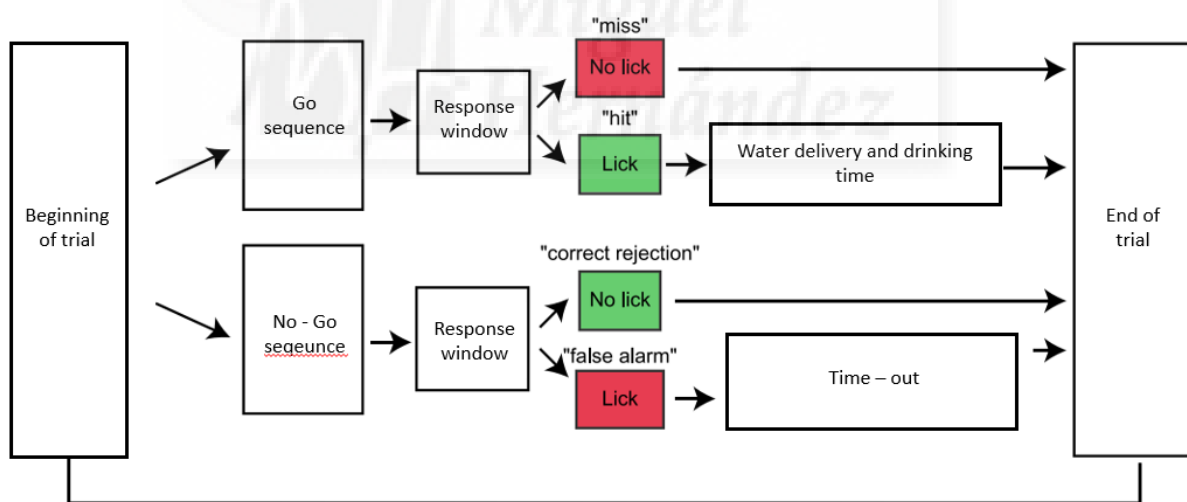
Figure 2.15. **Schematic drawing of the design of the behavioral task.** In every trial one, out of two sequences was played (either Go or No-Go). Animals' behavior (lick or no-lick) determined delivery of reward in case of Hit trials, punishment in case of False Alarm trials or no action as a consequence of Miss or Correct Rejection trails. This task design reinforced correct behavior.
Adapted from (O'Connor et al., 2010)

The stimuli themselves consisted of a sequence of 8 "syllables", each of them lasting 100 ms. Every syllable was pseudo-random white noise vibration. Those vibrations were

modulated with an amplitude envelope which implemented the sequence. Go and a No-Go sequence consisted of the same syllables; the only difference was their order (see Figure 2.16).

c. Protocol for behavioral training

As Go and No-Go sequences consist of the same, but scrambled, syllables, their discrimination and producing a correct response could be extremely difficult for naïve animals. Most likely it would result in withholding from licking throughout the entire session. Therefore we prepared a training protocol aimed at enabling the animals to acquire the ability to discriminate between Go and No-Go sequences in a gradual manner.

The animals went through the training once they were accustomed to head-fixation and to receiving water as a reward. It consisted of several stages: Go sequence would be the same at every stage but the No-Go one would differ and become closer to the Go sequence at each stage, making it harder to perform correct sequence discrimination (Figure 2.16). Proceeding to the higher stage would take place only if the animal would learn successfully the current one and achieve at least 75% of correct trials:

→ At first we trained animals to associate water reward with whisker stimulation; here Go stimulus would be opposed to no stimulation at No-Go trials; this way animals learn to drink in response to the detected Go sequence and withhold from licking on No-Go trials; this stage is called detection;

→ Once the goal was achieved (high hit rate and low false alarm rate), we introduced a simple No-Go sequence - a square wave, which was significantly different than Go sequence, and therefore, easy to discriminate. From now on the mouse had to learn to discriminate between both sequences;

→ In order to avoid associating reward delivery with any noisy stimulus, we proceeded to the next step. As mentioned before both stimuli consisted of 8 syllables, however in this step we haven't presented the "full No-Go" stimulus yet, but a "half No-Go", which consisted of 4, out of 8, target syllables. This design let us introduce elements of a final No-Go stimulus and at the same time provided sufficient difference between the Go and No-Go stimuli necessary for producing desired behavior and avoiding animal's frustration;

→ Once the animal reached over 75% of correct trials, a full No-Go stimulus was introduced.
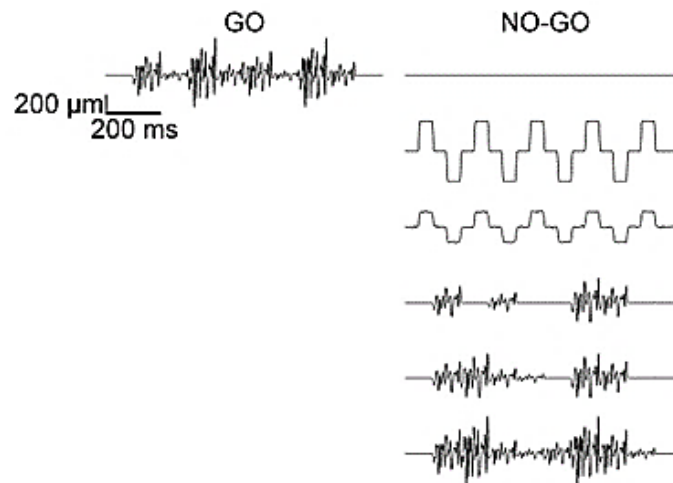
Figure 2.16. **Waveform of Go sequence and No-Go sequences played in subsequent stages of the training**. Square wave was played in the initial stage of the training, while half No-Go sequence required more expertise. Full No-Go consisted of exactly the same 8 syllables as Go sequence, however in scrambled manner – the only decision cue was the order of the syllables. Adapted from (Bale et al., 2015)

Some significant obstacles can occur during behavioral training:

→ High miss rate might indicate that either the animal is not thirsty enough to mark its decision by licking (lack of motivation), the lickport is misplaced (too far or too low preventing the animal from licking) or that the whisker slipped out from the tube attached to the piezoelectric wafer, preventing the animal from sensing and therefore discriminating the sequence and marking its decision;

→ Compulsive licking on both Go and No-Go trials might mean that either the animal is too thirsty or it did not acquire the knowledge on how to discriminate between both sequences; the required action would include increment of the No-Go trial probability and moving the lickport further away from the snout; time-out duration can also be increased (for instance from 2s to 5s) to discourage the animal from licking on No-Go trials;

We considered that animals achieved full learning if they reached 75% of correct responses (hits and correct rejections) in the 50 best trials of a session.

3. Work distribution

This thesis describes work I performed within an overall project that included various parts developed by several lab members: Dr. Ana Lía Albarracín prepared the set up for electrophysiological recordings and whisker stimulation delivery, performed a minor number of the anesthetized experiments (6/35 mice) and introduced me to patch-clamp technique *in vivo*, neurosurgery and mouse training. Dr. Manuel Molano Mazón and Prof. Miguel Maravall performed data analysis and prepared stimulus waveforms. I performed the majority of electrophysiological patch-clamp recordings of single cells in barrel cortex of anesthetized mice (29/35 mice) as well as visualization of neurons marked with biocytin. In order to obtain neuronal recordings in awake animals I established and carried out the habituation and water deprivation protocol, modified a previously existing setup as well as stimulus delivery and added water delivery device to the set up.

In the behavioral project Dr. Michael Bale led the setting up of the equipment and automatization of the training, whereas I participated in the project by improving the training. Both I and Dr. Bale, as well as Dr. Albarracín had an equal contribution in choosing a proper design of the behavioral tube and head-post implant.

4. Results

4.1. Encoding of stimulation parameters

I performed cell-attached patch clamp recordings in barrel cortex of anesthetized and awake adult mice. During electrophysiological recordings, stimulation consisting of sequences of tactile pulses arriving at variable time intervals (Figure 3.1) was applied to the whiskers. Deflections of the piezoelectric wafer, that was the source of stimulation, were of equal size: peak-to-peak amplitude was 400 μm and maximum speed of each deflection reached 400 mm/s and median speed approximately 40 mm/s. The maximum angular velocity equaled to 7900 °/s and the median to 900 °/s. We focused on the variable inter-deflection interval (Figure 3.1) in order to estimate how well the sequences of this parameter are encoded in the neuronal response or, in other words, to what degree a neuronal response depends on its duration. We also wondered if this dependency, and further away also temporal integration, is conditioned by a location of a neuron within the circuit or its mean firing rate.

This approach allowed us to investigate the sensitivity of individual sensory neurons in the barrel cortex to a given temporal pattern of the stimuli. To be more exact we quantified how much individual neurons' spiking response after each deflection of whiskers would correlate with the preceding stimulus pattern: with the latest, pen-ultimate, antepenultimate interval etc. As both linear and non-linear relationships between the neuronal response and the stimulus were possible, in order not to exclude any of them, we used mutual information as the measure for correlation (Materials and Methods). Quantification of the amount of the information contained in the neuronal response about each of those intervals would give us an idea if single neurons in the barrel cortex are sensitive to the history of stimulation and, if so, to what extent it would condition the response. Only those neurons (n = 84 for anesthetized experiments and n = 8 for awake experiments) that carried significant amount of information about the latest interval just before the whisker deflection were included in the dataset (Figure 4.1).

Figure 4.1. **Information carried about the latest and second-latest interval: example of stimulation pattern and a corresponding neuronal response**. Top: whisker deflections applied by a piezoelectric wafer at pseudorandom intervals (40-500 ms); inset shows a waveform of a single deflection. All deflections were identical. Bottom: an example of cell-attached recording of a neuronal response to the stimulation visualized above; neuronal responses (both spiking and non-spiking) were analyzed after each deflection. This particular neuron spiked in response to longer but not shorter intervals, thus it encodes information about the interval duration. Scale bars: 100 ms, 2 mV.

Specifically, to confirm that the recorded response was correlated with the stimulation, and had not occurred by chance, for each neuron in the data set, we shuffled the correspondence between the latest interval and the spiking response. After processing the data in this way we expected a significant decrease in carried information to apply for neurons that encoded the stimulus. Indeed we observed that information diminished by at least two orders of magnitude, which confirms that the recorded response reflected the applied stimulation.

4.2. Temporal integration in somatosensory individual neurons

4.2.1. The amount of information over time

In order to determine whether neurons explicitly represent information extended over time, the information conveyed about both the latest interval and several previous intervals was computed. Compared to the latest interval, the information about earlier ones was significantly lower (Figure 4.2). To be exact, the information carried by neuronal response about the second-latest interval constituted 6.3% of that about the latest interval on average (population mean; $p < 10^{-22}$, $n = 84$, Wilcoxon rank-sum test), however appreciable variability around this value was observed (interquartile distance 6.5%). Information about third-latest and earlier intervals was even smaller (data not shown).
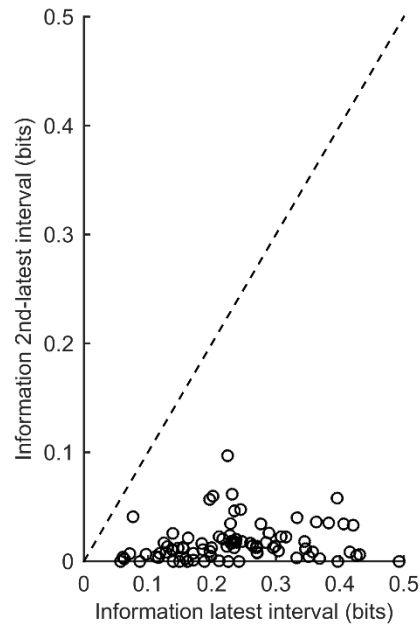
Figure 4.2. **Information carried about the latest and second-latest interval by single neurons in barrel cortex**. Each circular symbol represents a single neuron (n = 84). Information about the latest interval always exceeds the one about the second latest interval.

This result suggests that neurons within barrel cortex are heterogeneous in terms of the amount of information about the latest interval they carry. In other words, some of them are more informative about the interstimulus interval than others. We wondered if those neurons that contain the highest amount of information about the latest interval are also more informative about the preceding ones. Indeed, across the whole data set, the general tendency suggested that the higher the amount of information about the latest interval, the more informative were the neurons about the penultimate one (see Figure 3.2) (r = 0.25, p = 0.021, n = 84, Spearman correlation).

This tendency, however, had substantial variability: part of neurons stood out from this main group by providing high level of information about the latest interval but conveying little information about the earlier ones (Figure 4.3). This observation suggests that there is present not only a significant variability across recorded neurons in overall 'informativeness', i.e. in the amount of information carried about a certain parameter, but also some variation in the amount of temporal integration in those that are highly informative about the latest interval.
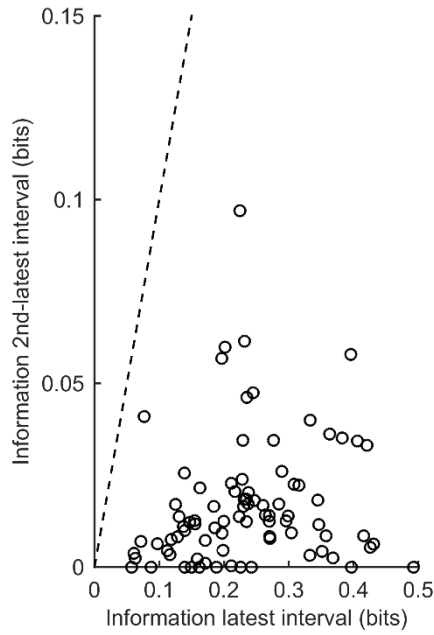
Figure 4.3. **Information carried about the latest and second-latest interval in magnification** (magnification of Figure 4.2): shows the variability in the ability to encode the information about the two variables. The majority of neurons that encode more information about the latest interval also encode more information about the penultimate one. Nevertheless there are neurons that are exception to this rule and even though they are sensitive to the latest interval, their spiking activity does not depend on the penultimate one.

4.2.2. Information ratio

To specifically visualize the amount of temporal integration (in other words: the ability to integrate over time) we quantified the so-called "integration ratio" – a variable equal to the ratio of information about the second-latest interval to information about the latest one (Eq. 4.1).

$$integration\ ratio\ = \frac{info\ _{second\ latest}}{info\ _{latest}}$$

Equation 4.1. **Integration ratio.** This parameter gives an idea about the ability of a neuron to integrate over time and therefore to encode sequences of pulses.

We plotted the integration ratio against the amount of information about the latest interval (Figure 4.4), which demonstrated that neurons which are the most informative about the latest interval are not the ones that integrated the most. On the other hand, a low information amount about the latest interval (i.e. a small denominator) was not a cause of low integration ratio. Rather those neurons which conveyed intermediate levels of

information about the latest interval were observed to be strong integrators. These neurons were able to achieve integration ratios of approximately 20% and above.
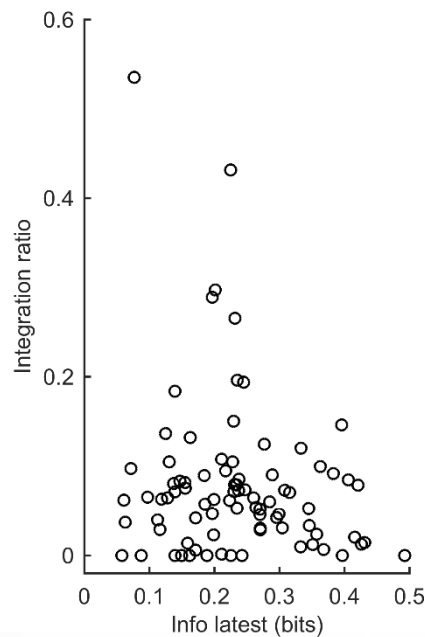
Figure 4.4. **Integration ratio**: integration ratio versus the information conveyed by single neurons about the latest interval. The strongest integrators were the neurons which encoded intermediate information about the latest interval.

4.3. Controlling the duration of the latest interval

The results presented above were obtained upon applying the full range of intervals, which varied between 40 and 500 ms (see paragraph 2.5.2.b). This implies that the dataset used to estimate information values included cases where the duration of the latest interval was as short as 40 ms or as long as 500 ms. Therefore in case of the longest applied intervals, the penultimate ones would end up 500 ms before the neuronal response under consideration, which, for neurons of small integration capability, would mean that they would not encode the information about the penultimate interval.

If we consider a hypothetical neuron which conveys information over up to, e.g., 300 ms, the data set would include both second-latest interval values that the neuron might carry information about (because they ended less than 300 ms before the response) and ones about which it would carry none (because they ended up too far in the past). Thus if the latest interval lasted, for instance, 100 ms, then such a hypothetical neuron would discriminate penultimate interval values up to 200 ms. Nevertheless, if the latest interval

lasted 400 ms, this neuron's response would not convey any information about the preceding interval (Figure 4.5).
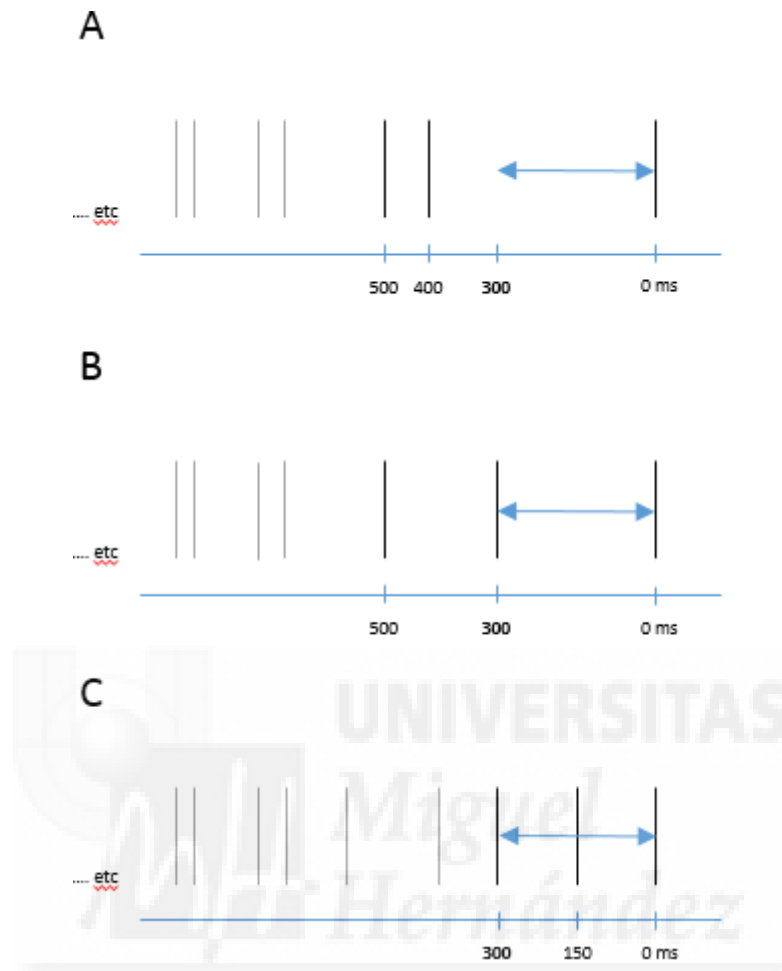
Figure 4.5. **Temporal integration capability**. An example of a neuron which effective integration time equals to 300 ms. The blue horizontal line depicts the timeline, where the times of 3 last pulses are marked, the black vertical lines depict pulses applied to the vibrissae and the blue arrow shows the effective integration time of this particular neuron. Anything beyond the integration capability time, will not influence its response. (A) If the latest interval is longer than 300 ms, then, as the neuron's response is influenced only by events within 300 ms prior to the last pulse, the response will not carry information about earlier intervals (B) The latest interval can be equal to 300 ms, therefore it will influence the response of the neuron, but the duration of the penultimate interval will still not be encoded. (C) The duration of the latest interval can be equal to 150 ms and the neuron will encode it. Its response could also contain some information about the second-latest interval.

If we follow this reasoning: using the previous stimulus set which contained intervals of any possible duration between 40 and 500 ms, could give us only a partial image of temporal integration in individual somatosensory neurons.

To refer to this possibility and extend the probability of discovering a reliable measure of temporal integration in single neurons, we analyzed the information about the second-latest interval in a slightly different manner. We repeated the previous step and analyzed the amount of information about the second latest interval but, this time, having control over the duration of the latest interval. For a maximum size of the latest interval the information about the second-latest interval was calculated, taking its full range of variations into account. Subsequently it was divided by information about the latest interval to obtain the information ratio. In other words: we calculated the amount of information about two latest intervals if the latest one lasted correspondingly: up to 500 ms, 400 ms, 350 ms, 300 ms, 200 ms, 150 ms etc. The calculated information ratio was then specific for a given maximum duration of the latest interval.

For this analysis we used a separate set of recordings based on a specifically designed and amplified stimulus set (see paragraph 2.5.2.b). We first checked that results on information about the latest interval and on amount of temporal integration did not differ for this stimulus set as compared to those for recordings acquired with different stimulus ensembles ($p = 0.44$ and $p = 0.31$ respectively, $n = 24$ and $n = 20$ for original and enlarged stimulus set, Kruskal-Wallis test).

Analysis of these data displayed little increase in temporal integration: plotting the information ratio as a function of the maximum duration of the latest interval revealed that over the range 75-500 ms integration remained relatively flat and, for most neurons, weak (Figure 4.6). The integration ratio value for a maximum allowed latest interval of 500 ms was not significantly different than values for shorter maximum allowed intervals (Figure 4.6; $P = 0.29$, $n = 20$, generalized linear model with Bonferroni correction). Interestingly, neurons tended to convey the strongest integrated information about the penultimate interval when the latest interval was constrained to end 200-300 ms before the response, not when it was constrained to end more recently (Figure 4.6; $P<10^{-7}$, $n = 20$, generalized linear model with Bonferroni correction).
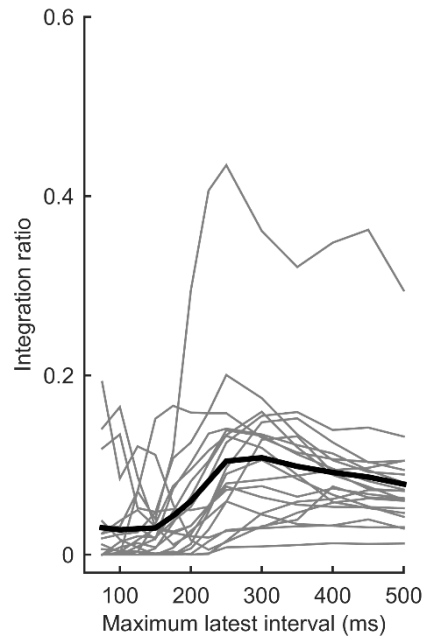
Figure 4.6. **Integration ratio as a function of cutoff**: Effect on integration of limiting the allowed duration of the latest interval. Information about the second-latest interval was computed while restricting the maximum allowed duration of the latest interval (n=20 neurons). This grey lines: individual neurons; thick black line: population mean.

This observation can be justified by a general tendency of significant number of neurons to not respond to latest intervals shorter than 100 ms (Chung et al., 2002). This effective refractory period voided any ongoing integration (memory) of the information about the earlier interval values when the latest one was very short.

In summary, for the neurons with capability of integrating over time, this phenomenon extends over several hundred milliseconds.

4.4. Correlation between temporal integration and location in the circuit or responsiveness of a neuron.

4.4.1. Temporal integration vs. responsiveness

The results we obtained strongly suggest that the majority of neurons in barrel cortex represent information principally about the latest stimulus values, however some neurons display some ability to integrate over hundreds of milliseconds. We wondered if the variability in the capacity of neurons to integrate over time can be related to neurons' responsiveness, i.e. the amount of spikes fired per time window (firing rate). It has been already proven that the amount of transmitted information per spike is maintained on similar level throughout the majority of neurons of a given category. Therefore those

neurons that have higher firing rate convey more information (Borst & Haag, 2001). This finding of Borst and Haag in invertebrates has been reproduced in vertebrates as well (Tripathy et al., 2013), including in the primary auditory cortex (Klampfl et al., 2012).

In order to visualize whether this tendency was also present in our data we plotted the information conveyed by each neuron about the latest interval against its mean firing rate (Figure 4.7).
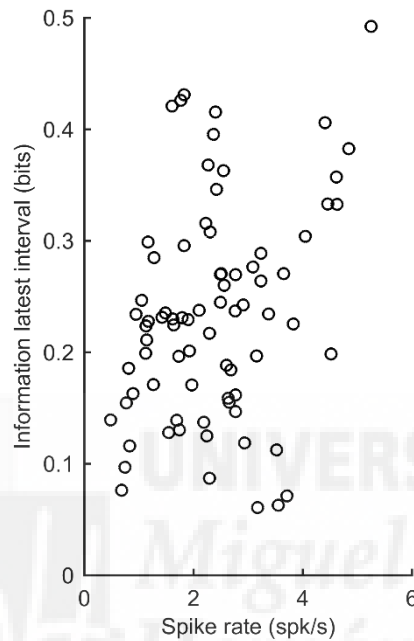
Figure 4.7. **Influence of firing rate on information encoding: "Informativeness" as a function of neuron's firing rate:** information about the latest interval vs. average spike rate over the duration of the entire stimulus. Here more active neurons (n = 77) convey more information.

As expected from previously established results (Borst & Haag, 2001; Klampfl et al., 2012; Tripathy et al., 2013), those neurons that fired more, also tended to convey more information about the latest interval (r = 0.25, p = 0.03, n = 77, Spearman correlation). Subsequently, to reveal the capacity of neurons to integrate information over longer timescales, we plotted integration ratio as a function of firing rate (Figure 4.8). We found that neurons that fired more were weaker integrators (r = - 0.31, p = 0.0053, n = 77, Spearman correlation).
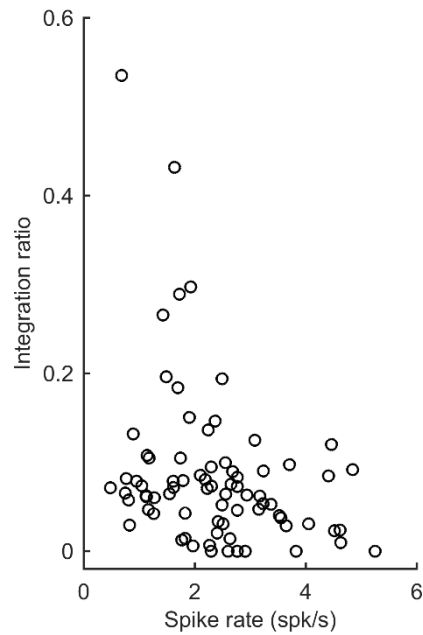
Figure 4.8. **Influence of firing rate on information encoding: integration ratio as a function of firing rate.** The analysis of the ability to integrate over time as a function of the neuron's responsiveness shows that neurons that fire more spikes (and so are more informative) integrate less over time.

4.4.2. Temporal integration vs. location in the circuit

Laminar location of a cortical neuron is an indicator of its position within the cortical circuit. Neurons in different layers represent different stages of cortical processing. The depth at which a neuron is located, i.e. the depth of an experiment's recording site indicates the neuron's layer (Lefort et al., 2009). Neurons in layer 4 receive a direct and strong thalamic input and, at the same time, no input from other cortical layers or other brain regions external to barrel cortex. They reliably encode information arriving from the thalamus. However neurons in layers 2/3 receive inputs not only from layer 4, but also from other cortical areas.

Given that neurons in barrel cortex have variable informativeness and temporal integration we wondered whether the position of recorded neurons in the circuit could be correlated to their ability to convey information and integrate over time. We found out that neurons across all depths of the cortex were capable of encoding appreciable amount of information about the latest interval, however there was no laminar specificity observed (Figure 4.9). The integration ratio varied from neuron to neuron, even between those located at proximal positions (Figure 4.10). This is consistent with the notion that the ability for temporal integration is heterogeneous even across proximate neurons within barrel cortex circuits. According to recent findings heterogeneity in neural

responses occurs at all stages of tactile processing in rodents and reflects in behavior (O'Connor et al., 2010). Even those neurons which are in the same circuit might be tuned to different stimulus features (Estebanez et al., 2012) as well as might have diverse activity levels (so differences in activity and responsiveness across the whole tactile pathway) (Barth & Poulet, 2012; O'Connor et al., 2010), which can also be observed in other sensory modalities (Tripathy et al., 2013).
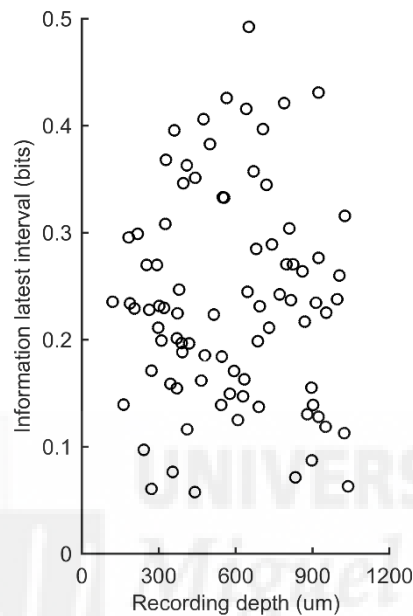
Figure 4.9. **Influence of location in the circuit on information encoding: "Informativeness" as a function of neuron's recording depth.** information about the latest interval versus cortical depth of the recorded neurons (n = 84) showed that across all cortical layers neurons encode significant amount of information and that there is a substantial variability in the amount of conveyed information even between proximal neurons.

Across the whole population there was a small subset of neurons that integrated more strongly and those had supposed location in the upper layers of the cortex (302-468 µm, reading error estimated as around 35 µm, see paragraph 2.5.3.c) (Figure 3.10). Conversely, the most active neurons, those with high spiking activity which integrated weakly (Figure 4.8), were found at depths consistent with locations in the deeper layers (Figure 4.10, magenta symbols) (Lefort et al., 2009).
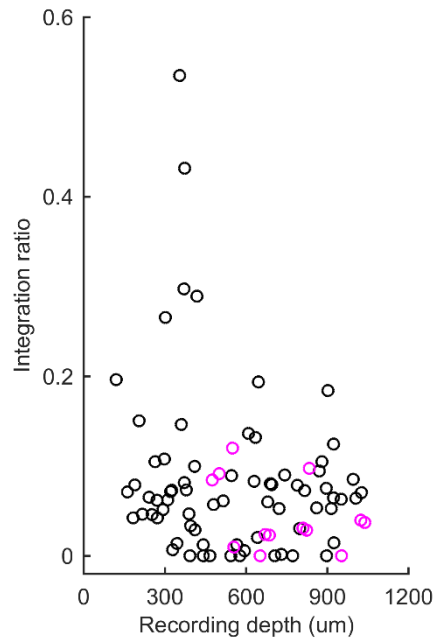
Figure 4.10. **Influence of location in the circuit on information encoding: integration ratio as a function of neuron's recording depth.** Integration ratio versus cortical depth of recorded neurons showed that across all cortical layers neurons displayed integration ratio well below one, however there is a significant variability observed: even neighboring neurons might have different ability to integrate over time. A subset of neurons in the upper layers achieved values beyond 0.2. Symbols in magenta are the most active neurons from figures 3.7 and 3.8 (mean firing rate > 3.4 spike/s), which were located in the deeper layers and had low integration ratio.

4.5. Experiments in awake animals confirm weak temporal integration in individual neurons in barrel cortex

The experiments carried out in anesthetized animals gave an insight into how stimuli, which arrived further in the past, influence ongoing sensory coding in the barrel cortex. It is important, however, to keep in mind that anesthetic agents, especially ketamine – an NMDA receptor antagonist, impair normal functioning of neuronal circuits and therefore affect processing of information.

Active (desynchronized) state, which takes place in a vigilant animal or during REM sleep, is characterized by neuronal activity of low amplitude but high frequency oscillations, whereas inactivated (synchronized) state consists of high amplitude slow and low frequency waves. It is observed in animals under the influence of anesthesia, non-REM sleep or drowsiness (Cohen & Castro-Alamancos, 2010). There is an essential difference in terms of overall spontaneous activity between both behavioral states and the mechanisms that regulate them. For example sensory adaptation is more pronounced in

inactivated (synchronized) state and decreases when animal is attentive and vigilant. Sensory suppression of thalamocortical synapses occurs robustly in the activated state diminishing adaptation (Castro-Alamancos, 2004a).

The pronounced differences in the dynamics of the nervous system between the activated and inactivated states were the reason to check if the result of limited temporal integration in anesthetized animals still applies to awake ones.

The 'awake part' of the project had an analogous structure to the anesthetized one presented above: experiments were carried out on animals trained to remain quietly under head-fixation during delivery of whisker stimuli identical to those applied to anesthetized mice (see paragraph 2.7). We focused on the overall amount of information conveyed about the latest interval as compared to the second-latest interval. Again single neurons in barrel cortex conveyed much less information about the penultimate interval comparing to the latest one (Figure 4.11). We note that information about both the latest and the second-latest interval was around 10-fold lower than in anesthetized experiments. There are a few possible reasons for this: first of all whiskers might have been attached to the stimulator too loosely and/or driven too weakly. Nevertheless this alternative seems unlikely as anesthetized trials carried out with the same attachment as in the awake experiments resulted in no decrease in temporal integration for the data obtained. Alternatively, awake animals might have paid attention to other cues, or have initiated whisker movement actively, and so the recorded responses might not result purely from the tactile stimulation we delivered. In any case, temporal integration was as weak in awake as in anesthetized animals and therefore the observed tendency was maintained.
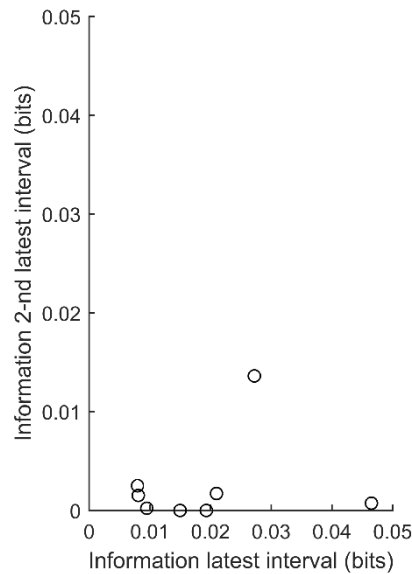
Figure 4.11. **Information carried about the latest and second-latest interval by single neurons in barrel cortex**: each circular symbol represents a probability of spiking after the whisker deflection about and penultimate interval by a single neuron (n = 8). The information level about both latest and second latest interval dropped by around a factor of 10 compared to the experiments carried out in anesthetized animals, however the general tendency is maintained: neurons that encoded some information about the latest interval "did not care" about the second latest interval.

4.6. Behavioral training

Behavioral training aims at preparing implanted and water-deprived animals for participation in Go/No-Go task, where the decision based on sensory percept is expressed by licking or withholding from licking. At the final stage of the training, the animal should be able to distinguish between two pseudo-random white noise vibrations consisting of the same eight syllables, but placed in different order (see paragraphs 2.7.2.b and 2.7.2.c).

We have established a protocol for efficient behavioral training. The training proceeds through several stages of increasing difficulty. The initial, and the easiest, stage includes a No-Go part, which, in fact, is lack of whisker stimulation, opposed to a Go stimulus that remained unchanged throughout all the stages of training. The final, most difficult, stage contained a No-Go stimulus which differed from the Go one only in its temporal patterning. The requirement for passing to the next stage of the training was obtaining at least 75% of correct behavior in 50 best trials (Figure 4.12A). The most successful animals (four mice) reached the final Go/No-Go stage and performed at an average level of 65% correct (Figure 4.12B).
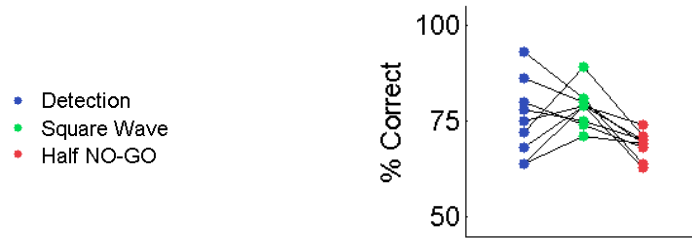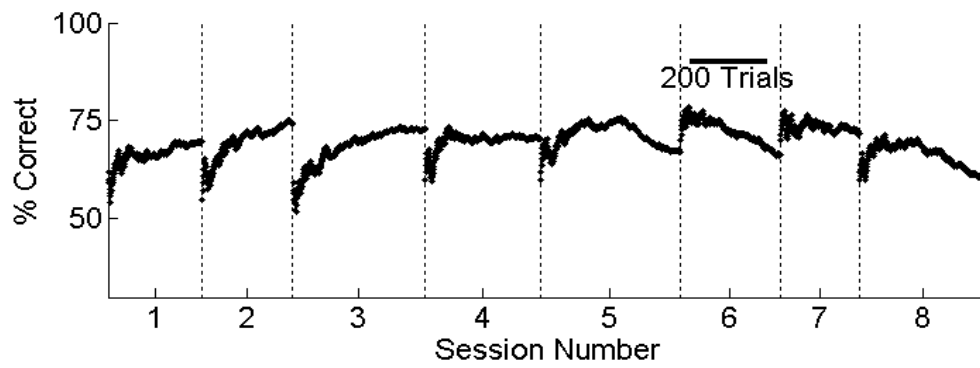
Figure 4.12. **Performance across subsequent sessions and stages of the training.** (A) Percentage of correct performance for eight mice; performance in detection and discrimination between Go sequence and square wave appeared to be easier than discrimination between Go and half No-Go sequence. (B) Performance of the best performing mouse in the discrimination between Go and full No-Go sequence (complete task): for four mice who reached the final stage the best performance averaged to 65% correct, whereas the best performance reached up to 74% correct.

5. Discussion

5.1. Motivation of the study

In order to survive living organisms have to produce an appropriate reaction in response to present and past environmental stimuli. For this to happen the physical parameters of the stimulation must be translated into neuronal language – patterns of action potentials. Whilst such encoded information is precisely transferred from the receptors up the processing pathway (Montemurro et al., 2007b; Salinas et al., 2000; Yang & Zador, 2012), it is in the cerebral cortex where complex higher order processes take place and where a given piece of information is placed in context. We assume that one of the features of the neocortex, crucial for encoding complex spatial and temporal characteristics of the world, is sequence sensitivity and sequence recognition. Necessary for sequence recognition is the ability to integrate the information over time. Neurons capable of integrating over time would be able to combine information about the present and the past, and so their response would not be evoked purely by the instantaneous value of the stimulus, but by the history of stimulation over a certain time course.

Indeed, already a few decades ago it was observed that single neurons can be sensitive to temporal patterns of stimuli (Segundo et al., 1963), which means that even subtle differences in stimulus temporal distribution can be reflected in the neuronal response. Certain mechanisms underlying sequence sensitivity at the level of single neurons have been discovered too in studies proposing the effect of dendritic computations in this process (Branco et al., 2010).

The goal of the present study was to investigate if sequence sensitivity and temporal integration are present *in vivo* and whether they play an important role in a system where discrimination of temporal patterns is crucial. We aimed to answer this question by studying single neurons in the barrel cortex of mice.

5.2. Integration of ongoing stimuli in the barrel cortex

What we found out is that neurons in the vibrissal somatosensory cortex carry significantly more information about the last interval than about the penultimate or the earlier ones. We assumed that those neurons that fired more action potentials in a determined period of time, and so were more responsive, should also be more informative about certain aspect of the stimulation. This turned out to be true for information about

the latest interval. However, we also discovered that neurons that stand out in terms of their responsiveness to the present vibrissae stimulation integrate less over time – in other words, they encode less information about the past stimulation. We found that neurons integrating most strongly were a subset located in the upper (supragranular layers); apart from this subset, neurons across cortical depths tended to integrate little (with less than 20% of information conveyed about the duration of the second-latest than about the latest stimulus interval). However the subset of stronger integrating neurons in the upper layers showed ability to convey information about stimulus parameters on the timescale of several hundreds of milliseconds.

Neuronal responses convey information encoded in various ways (Maravall & Diamond, 2014a; Panzeri et al., 2002; Panzeri et al., 2010; Zuo et al., 2015). Research has tended to differentiate between two main mechanisms for coding: spike timing which can signal rapid fluctuations in a precise way and makes an especially important contribution in subcortical tactile circuits (as the number of possible spike patterns increases exponentially with spike timing resolution) (Arabzadeh et al., 2005; Bale et al., 2015; Bale et al., 2013; Montemurro et al., 2007b) and spike rate, which tends to reflect more slowly varying features of the stimulus (Lundstrom et al., 2010). Although spike timing is not quite as precise in the cortex as subcortically, its importance – in the form of latency codes – has been supported by several studies (Panzeri et al., 2002; Zuo et al., 2015). We thus wondered if the total amount of information would increase if we considered spike timing as a code, and whether the results for integration would be different. For that purpose first we analyzed if the responses occurred at short or at long latencies and subsequently we measured the information that variations in latency conveyed about the latest and second-latest stimulus interval. As a result we obtained some extra information about the latest interval but not any more about the second-latest one: this left unaffected the qualitative conclusion that information conveyed about second-latest interval is considerably less than about latest interval.

The observation we made, that neurons integrating over hundreds of milliseconds (which stands for several intervals) are found only in supragranular layers is consistent with the current knowledge about the connectivity of barrel cortex (Feldmeyer et al., 2013; Lefort et al., 2009). Layer 4 and 6 receiving direct thalamocortical input exhibit the shortest latencies between the whisker stimulus onset and the most precise response (Hires et al., 2015). Moreover thalamic projections arriving to layer 4 innervate fast spiking (FS) cells

that project to spiny stellate cells creating an inhibitory feed-forward mechanism and providing relatively short window for synaptic integration in layer 4 excitatory neurons (Brecht & Sakmann, 2002; Bruno & Sakmann, 2006; Pinto et al., 2003; Tan et al., 2008; Gabernet et al., 2005). Layer 5 thick tufted neurons, anatomically located in the end of the processing pathway, receive direct thalamic projections (Constantinople & Bruno, 2013) and innervate layer 4 and 6, enabling reception of reliably encoded thalamic information and skipping transformation process in supragranular layers (Meyer et al., 2010). Supragranular (superficial) layers, on the other hand, are not direct recipients of thalamic input.

These observations support the increased responsiveness of infragranular layers recorded in the present research and their reduced ability to integration over time compared to supragranular layers. We propose that neurons at initial cortical stages of tactile processing behave as encoders of current stimulus parameters, while some traces of integration can be observed in later stages of processing in the barrel cortex.

5.3. Other sensory modalities

Recent work in other primary sensory cortices has found responses that correlate not just with current stimulus parameters but with the earlier ones as well. In the visual and auditory modalities, compared to the simpler three-synapse whisker tactile modality, there is a significantly greater number of synaptic processing stages between sensory transduction and primary cortex, potentially creating the scope for greater temporal integration. Nevertheless, timescales for integration (up to a few hundreds of milliseconds) appear comparable across different modalities.

In cat primary visual cortex, population responses to a sequence of visual stimuli (letters of alphabet) encode information about letters presented up to several hundred millisecond before the current one (Nikolić et al., 2009). In ferret primary auditory cortex, responses to presentation of a sequence of tones convey information not just about the current tone but also about the direction of the frequency step from previous to current tone, up to approximately one hundred milliseconds after the step had occurred (Klampfl et al., 2012).

5.4. Mechanism underlying temporal integration

5.4.1. Dendritic computations

Individual neurons contain certain mechanisms which allow them to integrate synaptic input and modulate their response according to the present and past input. It has been observed that dendrites do not only provide and enable a unidirectional flow of information but also carry on local computations forming independent processing units (Branco & Häusser, 2010). Dendritic spikes, potentials local to dendritic branches appearing once the synaptic input crosses the threshold, are regarded as the hallmark of those localized computations. The effect of those dendritic processes on integrated synaptic input is later either kept local, due to unfavorable impedance at the branchpoints, or transferred to the soma, where the global integration takes place in order to produce an adequate output pattern (Branco & Häusser, 2010).

For instance, directionally selective retinal ganglion cells display dendritic sodium spikes that, subsequently, trigger somatic spikes and amplify the cells' selectivity (Oesch et al., 2005). How sequence selectivity is accomplished by synaptic and dendritic integration mechanisms in central brain circuits in vivo remains difficult to investigate. Nevertheless a few studies have explored this. One of them claims that the stimulation of the hindlimb of awake and anesthetized rats, evokes dendritic calcium spikes in layer 5 pyramidal cells (Murayama & Larkum, 2009; Murayama et al., 2009). Moreover, as observed *in vitro*, both preceding dendritic action potentials and following somatic depolarizations have been observed in cortical pyramidal neurons upon activating input synapses in predetermined sequence. The mechanisms such as local impedance gradients and NMDA receptors let single dendritic branches display sensitivity to the patterns of synaptic input. Such basic computational elements as dendritic branches are capable of detecting synaptic sequences and discriminating input patterns before the global cellular integration takes place (Branco et al., 2010). Further research in this topic is needed to unravel the mechanisms of temporal integration in higher order areas.

5.4.2. Adaptation

Adaptation occurs mostly during quiescence states (anesthesia, sleep SEM and awake immobility) (Castro-Alamancos, 2004a) but also while performing in a well-learned tasks (Ollerenshaw et al., 2014). It leads to overall decrease of spike probability in response to

repetitive stimuli and enhancement of information flow in sensory systems. Behaviorally, adaptation means an increment of stimulus discriminability at the cost of detectability of the weakest stimuli (Ollerenshaw et al., 2014). A proposed mechanism for higher discrimination involves modulation of cortical window of opportunity for integration of thalamic input (Gabernet et al., 2005; Mease et al., 2014; Wang et al., 2010). The shorter the integration window, so the time during which EPSPs can be effectively summated (Lloyd, 1946), the more difficult it is to trigger a spike and the more coincident has to be the input. It therefore indicates how precisely a post-synaptic neuron can reflect the temporal structure of inputs.

Particularly in the whisker pathway, neurons display adaptation and context-dependent sensitivity modulation over timescales on the order of hundreds of milliseconds and even seconds (Lundstrom et al., 2010; Maravall & Diamond, 2014a; Maravall et al., 2007). Here fast-spiking (FS) neurons which, as mentioned earlier, create a feed-forward inhibition loop on excitatory neurons in the barrel cortex, decide about the integration window duration. In anesthetized animals and acute slices, where spontaneous activity is low, the feed-forward inhibition tends to be very high, narrowing the integration window. Increasing stimulus frequency both decreases the FS inhibition on excitatory neurons and broadens the integration window making the responses less specific. In awake animals ongoing spontaneous thalamic activity depresses the thalamic input onto the FS neurons, decreasing the range of feed-forward inhibition and maintaining wide integration window. However in behaving animals, additional factors, such as neuromodulators or network activity, can raise the threshold value of FS neurons, making them easier to activate, even if thalamic inputs are depressed, which results in short integration window of the tactile neurons and high sensory resolution (Gabernet et al., 2005).

Therefore, although the vibrissal thalamocortical circuit seems to have a great temporal precision at the expense of short integration window, it has been shown that the inhibitory circuit adapts as much as the excitatory one widening the window of opportunity for integration, depending, to great extent, on the behavioral state of the animal.

5.5. Comparison with previous studies

Although previous experimental designs did not specifically measure temporal integration in terms of information conveyed about stimulus sequences, our data can be compared with earlier estimates of tactile integration times.

Research on stimulus feature selectivity has observed that in subcortical stages (from TG to VPm) straightforward physical parameters of the stimulus features are reflected in the activity of neurons (Bale et al., 2013; Petersen et al., 2008), whereas at the cortical level complex high dimensional features are necessary to explain the activity of neurons (Estebanez et al., 2012). In general, the duration of stimulus features encoded by barrel cortex neurons is short and oscillates around tens of milliseconds (Estebanez et al., 2012; Maravall et al., 2007). This suggests that, as in our work, barrel cortex neurons essentially encode current and recent stimulation.

Our findings also appear to agree with a study where rats learned and carried out a vibrotactile detection task. There the animal's behavior was consistent with weak temporal integration of the responses of barrel cortex neurons. The minimum time required for temporal integration and necessary for a behavioral choice was estimated to be 25 ms (Stüttgen & Schwarz, 2010).

5.6. Behavioral state

5.6.1. Anesthetized vs. awake state

The main data set in the present study is based on experiments carried out in anesthetized mice. Anesthetics apart from changing network dynamics impair cross-communication between various brain regions, which are normally engaged in creating a fully-meaningful sensory percept and producing behavior in awake animals. For example, in mice trained on an object localization discrimination task, barrel cortex neurons have access to long lasting location signals related to past touches of specific whiskers, conveyed by axons that project from primary motor cortex to layer 1 of primary somatosensory cortex (Petreanu et al., 2012). Thus pyramidal neurons receiving layer 1 input could potentially respond selectively to sequences of touches with different whiskers, effectively integrating over time. On the other hand, anesthetized animals, are unable to control their sensorimotor system in order to collect valid task-relevant information. Similarly, any influence of higher order areas participating e.g. in integrating and storing the information about the stimulation as well as decision making is limited. The importance of associative areas was reviewed by Romo and de Lafuente (Romo & de Lafuente, 2013): they remarked that in monkeys performing a vibrotactile discrimination task, many parts of parietal and frontal lobes (MPC, DPC, VPC, PFC) are actively engaged in detection and

discrimination of sensory stimuli (which might require temporal integration) as well as in processing of information preceding and influencing decision making.

Motivated by these considerations we decided to perform a second set of experiments in awake naïve mice receiving passive whisker stimulation. We observed that temporal integration under those circumstances was no greater than under anesthesia. This fact indicates that higher temporal integration of tactile stimuli is likely to occur at higher stages of cortical processing, rather than in primary somatosensory cortex.

5.6.2. Activated vs. non-activated states

Primary somatosensory cortex is required for creating whisker sensation upon active and passive whisker stimulation (Lak et al., 2010; Miyashita & Feldman, 2013; Stüttgen & Schwarz, 2008). Passive stimulation tasks have the advantage of enabling the experimenter to control the distribution and parameters of applied stimuli and investigate the exact coding mechanisms. However, they raise the concern that non-active participation in the task, where full attention is not demanded, might bring the animal to quiescence, leading to a different brain state than the activated one found in motivated, attentive animals (Rigas & Castro-Alamancos, 2009).

Activation of cortical network implies enhanced excitatory input from the thalamus. The firing rate of inhibitory FS cells in layer 4 becomes increased as well, which overall avoids overexcitation of barrel cortex and provides balance between excitatory and inhibitory cortical activity (Hirata & Castro-Alamancos, 2011). This and other mechanisms could regulate the window of integration of cortical neurons differentially during activated and inactivated states. Moreover the study by Castro-Alamancos in urethane-anesthetized rats concludes that driving cortex to activated state suppresses low frequency firing rates (caused by an increment of activity of thalamocortical cells leading to the depression of thalamocortical synapses during the activated state) (Castro-Alamancos, 2004a, 2004b).

Activated states in some ways resemble the "up state" of cortical spontaneous activity found during the slow oscillations characteristic of anesthesia and slow-wave sleep (Destexhe et al., 2007; Hirata & Castro-Alamancos, 2011). We took steps to analyze the activity recorded during anesthetized up and down states separately, in order to investigate if barrel cortex cells' capability of integrating over time depended on the

current state of the cortical network. However, we found no such dependence (data not shown).

5.6.3. Behavioral training

Future studies aiming at revealing the foundations of temporal integration in the tactile whisker system will need to focus on animals trained on discrimination of temporally patterned stimuli collected in either active or passive way (Miyashita & Feldman, 2013). Extending the research to awake trained animals will give us more knowledge on how temporal integration expands within vibrissae circuit in intact tissue (Fassihi et al., 2014; Maravall & Diamond, 2014b; Miyashita & Feldman, 2013). Apart from investigating the brain in its activated state, it will give the possibility of studying how temporally integrated information is stored and used for decision making.

We have made first steps in training animals on a Go/No-Go task involving discrimination of temporal patterns and have obtained preliminary behavioral data. Upon receiving whisker stimulation corresponding to the Go or No-Go sequence the subject had to make the decision whether to respond to it by licking water or withdrawing from licking respectively. This results in four possible types of outcomes: "hit" when the animal licks on Go trials and "correct rejection" when it withdraws from licking on No-Go trials; "miss" (no licking on Go trials) and "false alarm" (licking on No-Go trials). Correct behavior was reinforced by delivering a drop of water on "hit" trials and the wrong one was punished by so-called "time-out" (a 2 – 5 s delay in starting the next trial). Stimulation sequences consisted of noise vibrations of different amplitudes and training itself was divided into several stages. The Go sequence was always the same and consisted of 8 parts, called syllables. The No-Go stimulation consisted of exactly the same syllables but in a scrambled order. At first though we trained the animals to respond with licking to the Go sequence while also providing trials with no stimulation. Once the mouse learned the association between the water reward and the presence of the Go sequence we introduced unrewarded No-Go sequences of vibrations. This began with easy to discriminate sequences (such as square waves substantially different from the Go sequence) but became more complicated as scrambled syllables were gradually inserted.

Mean performance for discrimination between Go and No-Go sequences for four mice amounted to 65% (or D' = 1.18) and the best one to 74% and D' = 2.39. We concluded that mice are able to discriminate between sequences of variable amplitude and very likely

temporal integration plays here a major role (Bale et al., 2015). Our group also carried out an analogous vibrotactile discrimination task in humans. The vibration was applied to the fingertip and the participant was supposed to recognize a repeating (Go) sequence and press a button whenever it appeared. The result was analogous to the one obtained in the mouse project.

Humans have been shown to display similar learning in other modalities. A study by Agus et al (Agus et al., 2010) demonstrates that humans are able to detect repeated, semantically meaningless auditory noise sequences and differentiate them from other, unrepeated ones. This form of learning applies to visual cortex as well, although learning in this case takes more time than for auditory stimuli (Gold et al., 2014). Both studies support the idea that sequence learning is not restricted to only one modality but rather emerge as a general neuronal capability throughout cortical circuitries.

5.7. Future work

Overall the ability of neurons in the barrel cortex does not support strong temporal integration at the early stages of processing (Stüttgen & Schwarz, 2010), however present work states that almost a third of responsive neurons in supragranular layers carries information about past stimulus parameters over hundreds of ms. Therefore the supragranular layers might be the first stage of cortical processing that contains integrated information about patterns of stimuli. Moreover responses of the neurons in supragranular layers can summate over time in a manner that depends on their target (Yamashita et al., 2013). Neurons projecting to secondary somatosensory cortex may be able to integrate more than those projecting to primary motor cortex. This predicts that neurons in secondary somatosensory cortex may integrate more, suggesting that it would be important to study rodent secondary somatosensory cortex neurons in terms of sensitivity to temporally patterned stimuli.

A further important question for future work concerns the mechanisms that generate subsets of neurons with longer integration times. Neurons within a population could become sensitive to stimulus structure over particular timescales by having synaptic inputs with specific dynamical properties: synapses with distinct temporal filtering properties render their postsynaptic targets sensitive to particular features in the stimulus (Buonomano & Maass, 2009; Carlson, 2009; Díaz-Quesada et al., 2014). As mentioned before, certain layer 2/3 neurons favor temporal integration; in assessing the mechanisms

shaping temporal integration and sequence selectivity, it will be necessary to parse neurons by identity and projection pattern.

Awake experiments, that require training on a Go/No-Go discrimination task, can give an insight into the activity of neurons in primary and secondary somatosensory cortex in alert behaving animals, upon performing electrophysiological recordings or visualizing populations of neurons via two-photon microscopy. They can also bridge the delivered stimulation, neural activity and the behavior.

Conclusions

1. Using juxtacellular patch clamp recordings *in vivo*, I examined whether neurons in the barrel cortex integrate over time by comparing information about different intervals in a sequence.

2. Compared to the latest interval before a response, information carried about the earlier ones was significantly lower: on average, the information carried by neuronal responses about the second-latest interval equaled to 6.3% of that about the latest interval. Information about the third-latest interval and earlier ones was even smaller.

3. The strongest integrating neurons tended to convey intermediate levels of information about the latest interval. They achieved a ratio of information about the second-latest to latest interval reaching above 20%. These neurons were therefore capable of integrating over timescales up to several hundred milliseconds.

4. Neurons that fired more tended to convey more information about the latest interval but were weak integrators.

5. Neurons across all depths of the cortex were capable of encoding appreciable amount of information about the latest interval, with little laminar specificity. The integration ratio varied from neuron to neuron, even between those located at proximal positions. The subset of neurons that integrated more strongly was located at depths consistent with layer 3.

6. In awake naïve animals, single neurons in barrel cortex also conveyed much less information about the penultimate interval comparing to the latest one. Therefore temporal integration was no greater in awake animals.

7. We have developed a paradigm to train the mice to perform in a Go/No-Go tactile discrimination task. We observed that mice are able to learn to recognize and discriminate meaningless sequences, different only in their temporal pattern, and report the Go sequence.

Conclusiones

1. Mediante registros *patch-clamp in vivo*, investigué si las neuronas en la corteza en barriles son capaces de integrar la información táctil, comparando la información contenida en las respuestas neuronales sobre diferentes propiedades de una secuencia de estímulos.

2. La información acerca del último intervalo de la secuencia anterior a una respuesta era significativamente mayor que acerca de los anteriores: en promedio, la información sobre el penúltimo intervalo constituía 6.3% de la del último. La información acerca de los intervalos anteriores era todavía más pequeña.

3. Para las neuronas que integraban más fuertemente, el cociente de información sobre el penúltimo intervalo e información sobre el último intervalo alcanzó niveles de 20% o más. Estas neuronas eran capaces de integrar información a lo largo de duraciones hasta varios cientos de milisegundos.

4. Las neuronas más activas transmitían más información sobre el último intervalo, pero tendían a integrar débilmente.

5. Neuronas situadas en todas las capas de la corteza en barriles eran capaces de transmitir una cantidad significativa de información sobre el último intervalo, sin mostrar grandes diferencias entre capas. Los niveles de integración eran variables dentro de cada capa. Un subconjunto de neuronas situadas a profundidades consistentes con la capa 3 mostró la mayor capacidad de integración

6. En animales despiertos y no entrenados, la información transmitida acerca del penúltimo intervalo también era menor que acerca del último. La integración temporal en animales despiertos no era mayor que en animales anestesiados.

7. Hemos desarrollado un paradigma de entrenamiento de ratones en una tarea de discriminación de estímulos táctiles aplicados a las vibrisas (Go/No-Go). Hemos observado que los ratones son capaces de aprender a reconocer patrones de estimulación sin significado a priori y que se diferencian de otros únicamente por su organización temporal.

Abbreviations

TG	:	trigeminal ganglion
TN	:	trigeminal nuclei
SpV	:	nucleus principalis
SpVo	:	nucleus oralis
SpVi	:	nucleus interpolaris
SpVc	:	nucleus caudalis
VPm	:	ventroposterior medial nucleus
POm	:	posterios medial nucleus
TRN	:	reticular nucleus
CO	:	cytochrome oxidase
BC	:	barrel cortex
S1	:	primary somatosensory cortex
S2	:	secondary somatosensory cortex
M1	:	primary motor cortex
V1	:	primary visual cortex
A1	:	primary auditory cortex
PW	:	principal whisker
LD	:	ligh-dark cycle
SD	:	standard deviation
i.p.	:	intraperitoneal (injection)
s.c.	:	subcutaneous (injection)
NMDA	:	N-methyl-D-aspartate (receptor)
PB	:	phosphate buffer
REM	:	rapid-eye movement (sleep)
PFC	:	prefrontal cortex
MPC	:	medial premotor cortex
DPC	:	deep prepyriform cortex
VPC	:	ventromedial prefrontal cortex

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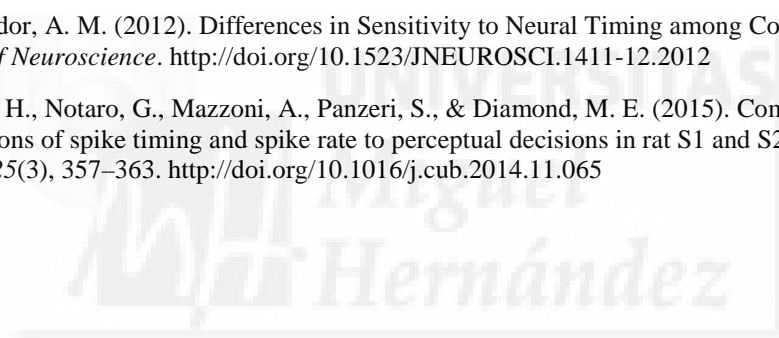Lee, L. J., & Erzurumlu, R. S. (2005). Altered parcellation of neocortical somatosensory maps in N-

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Annex