Functional MRI of Synaptic Plasticity

Jesús Pacheco-Torres*, Andrea Moreno*, Begoña Fernández*, Laura Pérez-Cervera*, José María Caramés*, Luis Miguel Fernández-Mollá#, Elena Pérez-Montoyo*, Miriam Martínez-Sánchez*, David Moratal#, Santiago Canals*,

#Centre for Biomaterials and Tissue Engineering, Universitat Politècnica de València, Camí de Vera s/n, 46022, Valencia, Spain
**Instituto de Neurociencias, Consejo Superior de Investigaciones Científicas – Universidad Miguel Hernández, Santiago Ramón y Cajal s/n, 03550 Sant Joan d'Alacant, Alicante, Spain.*

Preprint for book chapter in Handbook of In Vivo Neural Plasticity Techniques

1. Introduction

1.1 Requirements for Brain-Wide Imaging

Brains are modular structures, implementing highly distributed and efficient coding (Bassett et al., 2010; Dehaene and Naccache, 2001; Goldman-Rakic, 1988; Rumelhart et al., 1986). Integration occurs at all levels in the hierarchy of the central nervous system. For instance, in an episode of our everyday life experience, highly processed multisensory information is combined with emotions, motivation, and previous experience to direct our behavior, first, and update our memory data- base, subsequently, with the outcomes of the selected actions as the basis of learning (Driver and Noesselt, 2008; Lavenex and Amaral, 2000; Shams and Seitz, 2008; Tanabe et al., 2005). Given the central role of memory in cognition, unveiling the mechanisms supporting integration and storage of information is a major challenge in neuroscience. Research over the years suggests that these processes depend on interactions at molecular, cellular, and systems-level scales, distributed in the brain and across milliseconds, minuets, days, or even years. Obtaining coherent information from organizational levels spanning so many orders of magnitude in spatial and temporal domains is a challenge that requires the integration of different experimental techniques.

With brain computations dispersed across neuronal networks, whole brain measures of neuronal activity are fundamental to delineate the scaffold of integrative brain units. Furthermore, due to the variable nature of the strength of neuronal connections, the almost fixed structural network of the brain can generate a multiplicity of functional configurations. As a consequence, a single brain region (a node in the network) may participate in several integrative units. Therefore, brain networks need to be investigated dynamically and in well-defined behavioral/cognitive states or well-controlled experimental conditions. In the case of the hippocampus, for instance, its role in the acquisition of declarative memories (Scoville and Milner, 1957; Squire, 1986, 1992) needs to be investigated for both computations supporting encoding of new events as well as supporting retrieval of stored information, not forgetting its role in spatial navigation (Fanselow and Dong, 2010; Moser and Moser, 1998). Overall, the broad function supported by a brain region is given by its characteristic and

anatomically defined inputs and outputs, and their dynamic properties, these ones being determined by context-dependent processes such as short- and long- term synaptic plasticity. The combination of both static and dynamic network descriptions is what we refer here as functional connectivity.

Experimental techniques useful to investigate brain activity in this context should ideally satisfy the following criteria:

- Applicable in vivo to study the intact brain.
- Provide readout of neuronal activity as accurate as possible.
- Provide adequate temporal and spatial resolution to monitor global processes.
- Applicable in human and nonhuman animal models to facilitate translation.

1.2 Functional Magnetic Resonance Imaging With Blood Oxygen Level Dependent Contrast

Over the past few decades, multiple noninvasive in vivo imaging methodologies have been developed to study brain function, although none of them have been able to fulfill all of the abovementioned criteria.

Functional magnetic resonance imaging (fMRI) utilizes MRI technology to indirectly measure neuronal activity. The most common form of fMRI uses the blood oxygen level dependent (BOLD) contrast (Ogawa et al., 1990). It relies on the brain's ability to locally modulate blood flow and volume to satisfy the increased energetic needs during neuronal activation. The relationship between increased local neural activity and changes in cerebral blood flow (CBF) is known as neurovascular coupling (or functional hyperemia). The correlation be- tween BOLD signals and concomitantly recorded electro- physiological measures is well established (Logothetis, 2008; Moreno et al., 2013), although the exact molecular mechanism remains unclear (Jego et al., 2014) and its homogeneity throughout brain regions debated (Moreno et al., 2013) (see below). Functional MRI allows imaging brain activations with a spatial resolution as low as a few hundreds of microns (limited by the low intrinsic sensitivity of MRI, the small expected changes in signal intensity, and the point spread function; for a review see

(Greve, 2011) and with a temporal resolution of seconds (constrained by the natural slow dynamics of the hemodynamic response).

It is important to stress that BOLD signals do not measure neuronal activity but changes in neuronal activity (see Greve, 2011, for a deeper discussion on the fundamentals of BOLD). Nevertheless, these changes in neuronal activity can occur spontaneously while the subject is not involved in any specific task. Under those conditions, the changes are of small amplitude and are referred to as resting-state activity. Alternatively, the change in activity can be boosted by introducing a specific paradigm that defines a high activity state (activation) and a low activity state (baseline) that are subsequently contrasted statistically. Common paradigms include the presentation of sensory stimuli, the resolution of a cognitive task, the administration of chemical substances, or the direct stimulation of the brain's parenchyma. In animal models, the availability of a larger repertoire of neuronal stimulation strategies allows the precise control of the stimulation parameters and neuronal populations being targeted. These strategies include intracranial direct electrical microstimulation and optogenetic/pharmacogenetic stimulation/inhibition of specific neuronal populations.

1.3 Direct Brain Stimulation Based Functional Magnetic Resonance Imaging

Direct manipulations of network nodes or links, that is, activation/inhibition of neuronal populations or tracts, overcome some limitations of sensory and cognitive stimulation. For instance, primary sensory regions are relatively easy to activate and can recruit the relevant structures in the processing pathway; however, for other structures (i.e., the nucleus accumbens (NAc), hypothalamic nuclei, etc.) there are no obvious (or easy to implement) exogenous activation protocols. Also, poly- synaptic activations in intricate brain networks, initiated by sensory stimuli or cognitive tasks, make it harder to investigate direct influences or causality. Direct activation of a specific pathway or a neuronal population eases such studies, allowing studies in which the consequences of precisely timed and accurately localized manipulation of neuronal activity, as those required in

synaptic plasticity studies, can be investigated in the context of broader network dynamics.

There are obviously very important advantages of using animal models in which the combination of fMRI with electrophysiological recordings or stimulation techniques are possible. However, the use of animals (especially small animals such as rats and mice) imposes certain limitations that need to be considered carefully, the use of anesthesia being the most important one. Also, spatial resolution issues, due to the smaller size of rodent's brains compared with primates, although partially countervailed by the implementation of systems with ultrahigh magnetic fields and stronger radio- frequency gradients, need to be considered carefully, especially for imaging subcortical regions.

Having these limitations in mind, the possibilities offered by electric-stimulation fMRI are still very attractive for systems neuroscience (Canals et al., 2009; Canals et al., 2008; Logothetis et al., 2010; Tehovnik et al., 2006; Tolias et al., 2005). The functional strength of defined neuroanatomical connections can be investigated in longitudinal experiments, and the consequences of diverse manipulations on functional connectivity can be systematically assessed. In the context of research dealing with neural plasticity, learning, and memory, this technique opens the possibility, for instance, to explore the consequences of synaptic plasticity induced in specific neuronal circuits on the global functional connectivity, aided by the brain-wide coverage of MRI.

1.3.1 Imaging the Consequences of Local Synaptic Plasticity on Brain-Wide Networks

The brain is said to be plastic, but, what does this mean? Long ago it was accepted that the brain does not grow in size with the acquisition of new information; instead, neurons rearrange themselves by the process of changing functional connections to add new information to already stored old information, and thereby build the knowledge specific to each individual (Cajal, 1894).

In 1973, Tim Bliss and Terje Lømo reported the first demonstration of a use-dependent strengthening of synaptic connections (Bliss and Lomo,

1973), a phenomenon known as long-term potentiation (LTP). They showed that neurons in the hippocampal formation can in fact undergo plastic changes in their synaptic inputs when stimulated repeatedly above a certain frequency threshold. This finding and others that followed were considered the experimental demonstration of Hebb's postulate on synaptic strength and learning (Hebb, 1949), and since then, LTP has been widely accepted as the prevalent model of an experience-dependent modification of brain circuits (Bliss and Collingridge, 1993, 2013). The link between memory and a change in synaptic efficacy was shown experimentally a few years later (Klein and Kandel, 1978).

Memory formation in the hippocampus, however, does not only need synaptic plasticity (cellular-level memory formation), but long-range functional interaction of the hippocampus with other brain regions is required (systems-level memory formation) (Buzsaki, 2006; Girardeau et al., 2009; McClelland et al., 1995; McNaughton and Morris, 1987; Morris, 2006; Squire, 1992). Humans with brain damage affecting the medial temporal lobe (Rempel-Clower et al., 1996; Scoville and Milner, 1957) and comparable lesion studies in animals (Kim and Fanselow, 1992; Olton and Papas, 1979; Zola- Morgan and Squire, 1990) very early indicated that memory storage and retrieval actually engage a large distributed network of reciprocal connections. However, while the evidence supporting the link between synaptic plasticity in the hippocampus and memory encoding was robust, little was known about how experimentally measured regional synaptic modifications alter the activity of more global, widespread networks supporting memory formation at the systems level.

In an attempt to link the cellular and system levels of memory formation, that is, to relate synaptic plasticity to widespread network dynamics, we simultaneously combined fMRI and in vivo electrophysiology in rats (Canals et al., 2008) and investigated, first, the global functional consequences of LTP (Canals et al., 2009) and then, the effects of short-term plasticity. Here we briefly review the obtained results that suggest a functional reorganization of long-range hippocampal circuits controlled by synaptic plasticity.

1.3.2 Long-Term Potentiation

When we stimulated hippocampal afferents of rats with an LTP-inducing protocol, inside a 7T MRI system, we discovered that the effects of synaptic potentiation were spreading into brain regions far apart from the induction site, the place where they had been typically investigated. Besides the potentiation of BOLD signals in the stimulated hippocampus, and an unanticipated increase in contralateral activation too, we found that stimulation of the perforant pathway after LTP induction activated a number of extrahippocampal structures as the prefrontal cortex (PFC), NAc, and perirhinal cortex (PrhC). Interestingly, the newly recruited structures by hippocampal activation after LTP had been repeatedly involved in memory processing over the years (Amaral and Lavenex, 2007; Brewer et al., 1998; Eichenbaum and Cohen, 2004; Fuster, 2001; Morris et al., 2003; Scoville and Milner, 1957; Siapas et al., 2005; Squire et al., 2004; Tse et al., 2011).

Enhanced coupling of the hippocampus with the PFC, PrhC, and NAc was found immediately after LTP induction, paralleling the timing of synaptic strength potentiation in the dentate gyrus, and both synaptic potentiation and network reorganization were blocked by the N-methyl-D-aspartate receptor (NMDAR) antagonist, MK801, suggesting that the same NMDAR-dependent mechanism attributed to synaptic plasticity and memory encoding in the hippocampus may also explain the kind of network interactions required for systems consolidation. While systems-level interactions had been considered necessary for the consolidation of long-term memories, what could be its function in the initial stages of memory formation during encoding?

We have speculated (Alvarez-Salvado et al., 2014) that early network interactions triggered by synaptic plasticity may optimize the coordination of two memory buffers for systems consolidation, one in the hippocampus and another in the PFC. In experiments using a hippocampal-dependent pair-associate task for rats, it was demonstrated that systems consolidation can occur very rapidly when information is assimilated in a previously stored "schema" in the neocortex (Tse et al., 2007). In these cases, encoding is hippocampus-dependent but quickly consolidates in the neocortex, reflecting an influence of prior knowledge on the rate of consolidation

(Osada et al., 2008). Coordination between the hippo- campus and the prelimbic regions of the medial PFC were required not only for retrieval but also during memory encoding (Tse et al., 2011). In this view, parallel encoding in the cortex and hippocampus would establish the relevant associative links (orchestrated by the hippocampus) between objects and events initially disconnected but already represented in the cortex. Additional support of hippocampal-PFC interaction during encoding comes from early human fMRI studies showing that activations in the right PFC and bilateral parahippocampal cortex during memory encoding are predictive of how well a visual experience will be remembered (Brewer et al., 1998).

1.3.3 Short-Term Plasticity

More recently, we have investigated the role of short-term plasticity in the activity transfer from the hippocampus to the neocortex (Moreno et al., 2016). In that work we used stimulation protocols with frequencies ranging from 5 to 40 Hz, known to induce different forms of short-term plasticity in the Schaffer collateral input of CA1 in rats (Alger and Nicoll, 1982; Davies and Collingridge, 1993; Pouille and Scanziani, 2004). The key finding was that while BOLD signal recorded in hippocampus responded to all stimulation frequencies, it only responded to specific frequencies in extrahippocampal structures. Activity propagation across dorsal and ventral structures of the hippocampal formation, as well as the septum, increased with frequency, reaching a plateau at 20e40 Hz. Strikingly, at 10 and 20 Hz, activity spread extensively beyond the hippocampus into neocortical and subcortical regions. The brain regions receiving this hippocampal activity largely involved structures in the PFC, such as the orbitofrontal, prelimbic, infralimbic, and cingulate cortices, as well as the dorsal and ventral striatum (NAc).

However, the extrahippocampal spread of activity abruptly declined at 40 Hz, remaining circumscribed within the hippocampal formation with maximal BOLD amplitude.

These results were indicating that the output of CA3 implements a frequency-dependent gating mechanism that promotes long-range interactions at 10e20 Hz. Therefore, in addition to the well-known

functional roles of the dynamic CA3eCA1 interaction such as the modulation of synaptic plasticity (Davies et al., 1991; Manabe et al., 1993; Mott and Lewis, 1991; Perkel and Nicoll, 1993) and temporal fidelity of pyramidal cell firing (Pouille and Scanziani, 2001; Schaefer et al., 2006), our results were suggesting a new role of this local hippocampal circuit in routing activity propagation ac- cording to input frequency.

Overall, these fMRI studies draw attention, by virtue of observation of widespread neuronal activations, to important network implications of short- and long- term synaptic plasticity. Fig.1 shows example data obtained by short-term plasticity and LTP plasticity in- duction as recorded by fMRI.

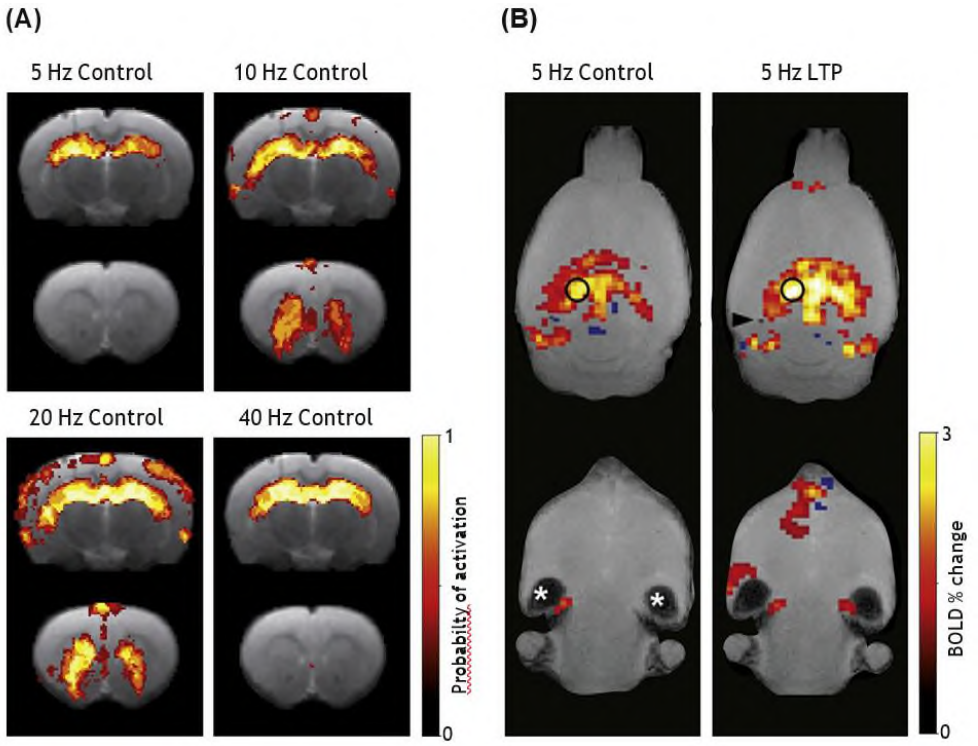

Figure 1. Short- and long-term potentiation measured in functional magnetic resonance imaging (fMRI). (A) Brain-wide short-term plasticity observed when stimulating the CA3 region of the hippocampus at different frequencies. The figure shows four stimulation frequencies (5, 10, 20 and 40 Hz) applied to the rat's hippocampus (showing probability activation < .001). (B) Long-term plasticity effects observed when stimulating at 5 Hz the perforant pathway (major hippocampal input coming from the entorhinal cortex) before and after long-term potentiation (LTP) induction in the same region. A stable (>3 h) potentiation is induced, whiche leads to a broard spread of activity observed after LTP induction and evoked by the same stimulus (% of BOLD signal change p < .01). *Asterisks* indicate the auditory channel artefacts; *circles* indicate the dorsal hippocampus; *arrow* indicates the stimulation electrode location. (A) Adapted from Moreno et al. (2016). (B) Adapted from Canals et al. (2008).

1.3.4 Considerations on the Origin of Functional Magnetic Resonance Imaging Signals

fMRI signals are mainly produced by the increase in CBF induced by vasoactive compounds released during neuronal activation, although brain blood volume does also contribute (for a review see Logothetis and Wandell, 2004). An important matter for neuroimaging is therefore to understand which aspects of neuronal work are reflected in increased CBF. Experiments simultaneously combining fMRI and electrophysiological recordings in the primary visual cortex of anesthetized monkeys showed that the imaging signal evoked by visual stimulation maximally correlates with the local field potential (LFP), an aggregate measure of synaptic and active dendritic currents (Logothetis et al., 2001). Although the correlation of the BOLD signal was only slightly higher toward LFP compared with spiking activity (multiunit and single unit activity), the LFP signal was the only predictor of the hemodynamic response when long stimulation protocols that habituate spiking activity were used. Consistent with these findings were studies in the rat cerebellar cortex, which convincingly showed that local CBF can indeed be dissociated from spiking activity while strongly correlated with LFPs (Mathiesen et al., 1998, 2000; Thomsen et al., 2004).

Based on the abovementioned results, it is believed that neuroimaging signals reflect the local processing of incoming neuronal activity to a particular area, rather than the output message being sent in outgoing efferent neuronal activity. The demonstration that local synaptic plasticity modulates the amplitude of the BOLD signal in the LTP experiment (Canals et al., 2009) reinforces this view. Of note, in the hippocampus, the axial organization of the cellular elements, with a rather precise alignment of dendritic shafts and somas, minimizes the cancellation of current sources from the LFP generators and facilitates the neurophysiological interpretation of the electrically evoked field potentials, such as synaptic currents reflected in the excitatory postsynaptic potential (EPSP) and spiking activity in the population spike. Using this preparation, we were able to unequivocally show that the EPSP slope was a precise predictor of BOLD signal amplitude, better than either the population spike or the electrical current used for stimulation (Canals et al., 2008, 2009). This result was confirmed in

experiments combining electrophysiological recordings with hippocampal CBF measurements based on laser Doppler flowmetry (Hamadate et al., 2011). Overall, fMRI with BOLD contrast offers quantitative and reliable measures of local synaptic processing.

2. Materials and Methods

2.1 Anesthesia

As previously introduced, most fMRI experiments in rodents are performed in anesthetized animals. Different anesthetics have been introduced for fMRI studies in rodents, each of which presents its particular advantages and drawbacks (for a review see Masamoto and Kanno, 2012), and all of them having an impact on the neurovascular coupling. At this point, it is important to emphasize that, in our experience, a successful fMRI experiment in rodents mostly relies on maintaining the animal's physiology at adequate and steady-state values. Body temperature (37+/- 0.5◦C), oxygen saturation (>95%), CO2 levels (35e50 mmHg), and blood pressure (130e140 mmHg) need to be fine-tuned. Precise monitoring of some of these values requires invasive interventions (i.e., blood pressure and accurate CO2 measurements require femoral artery cannulation and tracheotomy, respectively) or direct blood sampling, which is difficult to implement in longitudinal studies. However, important and accessible parameters such as blood oxygen saturation, heart and breathing rate, temperature, and even a trend of CO2 levels with subcutaneous measurements can be implemented, routinely providing robust and reproducible fMRI measurements. In all cases, pilot experiments with full monitoring of the animal's physiology are strongly recommended in setting up new anesthetic protocols.

The final election of an anesthetic method will depend on multiple factors such as the species utilized [i.e., rats (Hendrich et al., 2001) *vs* mice (Schroeter et al., 2014)], the duration of the experiment (Sonnay et al., 2015), whether it is an acute or longitudinal experiment, and even the type of stimuli used (Paasonen et al., 2016). As a general rule, injectable anesthetics provide a stable imaging condition for up to 2 h, whereas inhaled anesthesia allows longer

imaging sessions. An exception to this rule is urethane, which provides a stable and long-lasting (more than 8 h) anesthetized state with a single intraperitoneal injection and minimal cardiovascular effect (Maggi and Meli, 1986). Importantly, urethane also preserves most of the characteristic electrophysiological rhythms recorded in the hippocampus and other neocortical regions (Moreno et al., 2016). However, urethane is restricted to terminal experiments due to its hepatotoxic and carcinogenic effects, for which it is compulsory to euthanize the animal at the end of the experiment. For chronic rat experiments and when working with mice, dexmedetomidine is the usual election (Jego et al., 2014). It allows animal recovery but provides, in our hands, shorter periods of stable anesthesia (in the range of 1.5e2 h). An alternative administration regime for dexmedetomidine has been proposed to extend this period (Pawela et al., 2009). We have found significant differences between different rats and mice strains.

2.2 Electric Stimulation

2.2.1 Different Types of Electrodes Available

Traditionally, electrophysiological stimulation in vivo is made via intracerebral implantation of stimulating electrodes made with some kind of metal (e.g., platinum, iridium, gold, stainless steel, tungsten, etc.). A variety of metals or their alloys are used successfully to stimulate and record LFP signals. But one of the difficulties faced when trying to combine electrophysiological techniques with fMRI is that of the susceptibility of the magnetic field to metals. This is straightforward for a ferromagnetic material such as steel, but even when the material is nonferromagnetic (that is, not susceptible to conserve magnetism), the presence of a metallic electrode creates an inhomogeneity of the magnetic field as applied to the brain, which can disrupt any detectable signal. Structural scans [rapid acquisition relaxation enhanced (RARE) sequences] are less sensitive to these slight changes in the field homogeneity (due to their slower nature), but functional imaging [echo planar imaging (EPI) sequences] is especially affected (see (Chao et al., 2014) for an example of image distortion using tungsten electrodes). Fig. 2, panel C, shows an example of this effect using a PteIr electrode implanted in the

perforant path of the hippocampus. The area around the electrode is severely distorted, making it impossible to extract any information from this region. When the electrode is located in a region that is relatively far away from the region of interest, the problem is manageable, as the signal loss does not affect the region of interest. But if the electrode is in a very central location in the brain (as is the case of the dorsal hippocampus), or more than one electrode is inserted, the distortion problem has to be faced.

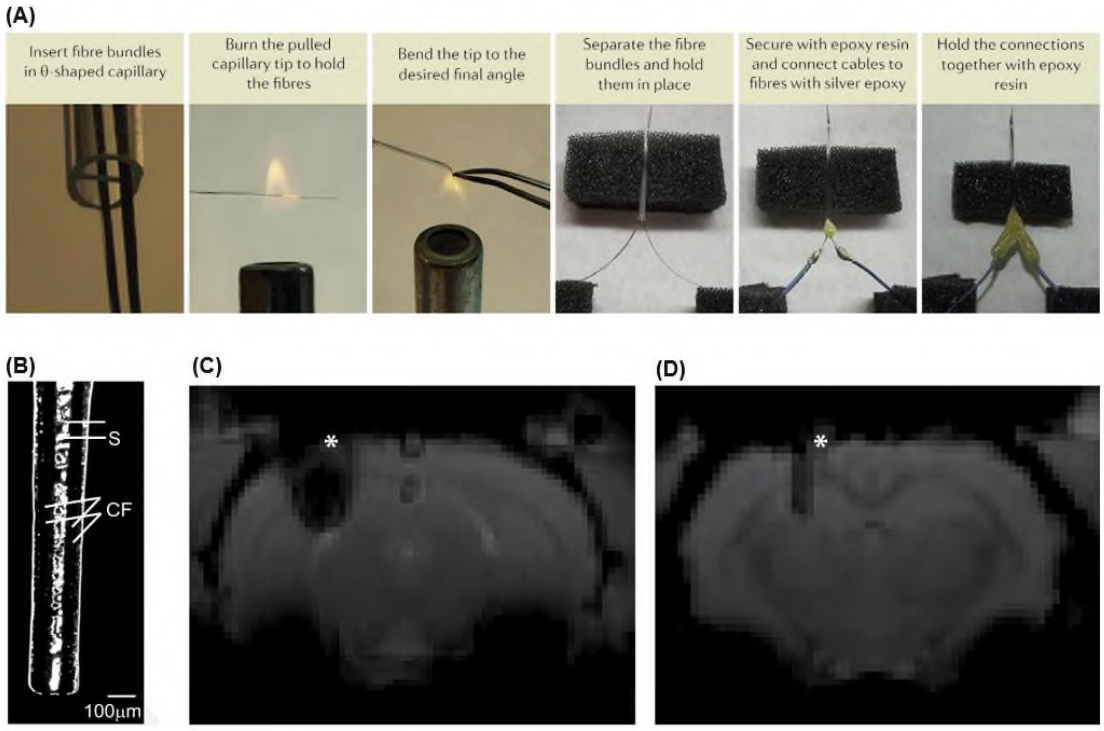

Figure 2. Carbon fiber electrodes construction. (A) Step-by-step illustrated guide for building custom-made carbon fiber bipolar elec- trodes. (B) Photomicrograph of the tip of a bipolar carbon fiber electrode (S, septum of the theta-shaped capillary; CF, carbon fiber bundles). (C) Functional echo planar imaging (EPI) with a conventional platinumeiridium electrode implanted in the perforant pathway of a rat's brain. Note the signal loss (asterisk) around the electrode tip. (D) Functional EPI with a CF bipolar electrode built as described here, implanted in the CA3 hippocampal region. The signal loss around the electrode tip (asterisk) is comparably smaller as the one in (C).

On the exploration of new nonmetallic materials that could be used to stimulate the brain in vivo, we developed custom-made glass-coated carbon fiber electrodes. The electrical properties of carbon fibers are highly robust (McCreery, 2008). Several laboratories had already published methods in which bundles of individual carbon fibers can be grouped together and attached to an

external wire connector to complete the stimulating circuit. Very reliable stimulation with these electrodes has been described (Duffy et al., 2015; Dunn et al., 2009; Guitchounts et al., 2013; Vitale et al., 2015). We modified these methods to create a custom bipolar stimulation electrode that could be placed in nuclear magnetic resonance environments and minimally affect the BOLD signal. The procedure for creating these electrodes is detailed in the following section.

2.2.2 Carbon Fiber Electrode Construction

To prepare carbon fiber electrodes we use bundles of fibers inserted into a theta-shaped glass capillary previously pulled to form 7-mm-long pipettes with w200 mm tip diameter and adjusted to produce an electrical impedance of 40e65 kU. Small electrode tips will cause less tissue damage during implantation, but higher electric impedance would require higher voltages and there- fore the possibility to overheat and damage the tissue. Thus, there is a compromise between these two parameters. In our experience, an electrode tip of w200 mm renders good stimulation while minimizing tissue dam- age. A regular wire with a pin connector is attached to the pipette, connected to the carbon fibers using silver conductive epoxy resin (RS Components, UK), and isolated with clear epoxy resin (Moreno et al., 2016).

Depending on the configuration used in the MRI, the glass electrode can be bent to accommodate the receiver coil, minimizing its distance to the brain, and maximizing the signal-to-noise ratio (SNR). In our preparation, with stimulation electrodes implanted either in the perforant pathway or the area CA3 of the dorsal hippocampus, the tip of the electrode was bent using a Bunsen burner and some forceps to form a 90 degree angle so it could go inside the brain, leaving the main body of the pipette outside, parallel to the head of the rat, minimizing the implant's height, and allowing a closer proximity between the MRI array coil and the head of the animal.

2.3 Parameters for Inducing/Triggering Neural Plasticity/Neural Activity

2.3.1 Stimulation Protocols

In previous work, where we applied electric stimulation fMRI to study the frequency response of the perforant pathway in rats, the major neocortical input to the hippocampus (Canals et al., 2008), we showed the existence of an activity threshold to elicit a detectable fMRI response. More specifically, we showed that (1) a certain level of activity, in an approximately constant population of neurons, must be reached to start a detect- able BOLD signal, (2) the activity threshold for BOLD elicitation can be reached by applying trains of pulses at relatively low frequencies (4e5 Hz for the perforant path), (3) once the threshold is crossed, the BOLD signal (magnitude and extension of the activation) is linearly correlated with the stimulating current, (4) at current intensities evoking a half-maximal neuronal spiking response, the activity spreads polysynaptically with increasing stimulation frequencies up to 20 Hz. Thus, stimulation protocols often consisted in 6e10 trains of electrical pulses (100 ms biphasic pulses) repeated every 30e60 s (total duration of the trial 180e600 s) and trials repeated 3e5 times per condition. The duration of the stimulation train can be adjusted to the specific needs, but durations between 2 and 6 s at frequencies ranging from 4 to 20 Hz produce BOLD responses of excellent amplitude (larger than 4% change) in a variety of preparations (Alvarez-Salvado et al., 2014; Canals et al., 2008; Godino et al., 2013; Hadar et al., 2016; Moreno et al., 2016). Off periods between stimulation trains sufficiently long as to allow a full recovery of the hemodynamic response (25e30 s in rats and mice) increase the amplitude of the response and the statistical power of the anal- ysis. A good synchronization between image acquisition and timing of stimulus presentation is necessary and easily achievable using transistor-transistor logic (TTL) signals either generated by the imaging console to control the pulse generator or vice versa, generated by the stimulation device to trigger image acquisition. Duration of the stimulation train, pulse shape, amplitude and duration, frequency, and any other stimulus parameter can be systematically varied for specific purposes (Tehovnik et al., 2006), such as investigating short-term plasticity (Moreno et al., 2016) (Fig. 3).

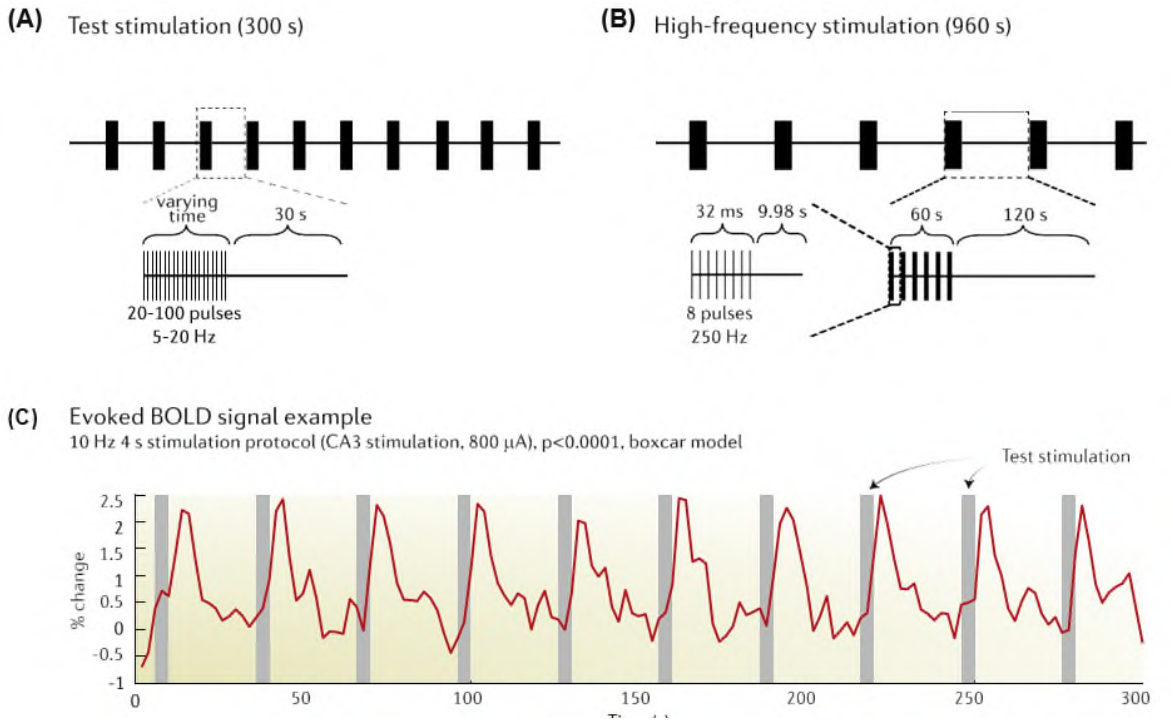

Figure 3. Example stimulation protocols used to elicit blood oxygen level dependent (BOLD) signals and induce different forms of plasticity in a functional magnetic resonance imaging (fMRI) experiment. (A) Test train stimulation is used to elicit enough neuronal activation to generate a BOLD response, without producing long-term changes in synaptic strength. The stimulation consists of several trains of electric pulses separated by longer time intervals to allow the BOLD signal to recover to baseline levels. The duration, number, and frequency of the electric pulses in a train are adjusted to the experimental requirements, but single-pulse stimulation before and after a train test stimulation must induce an evoked potential of constant characteristics (i.e., Canals, et al., 2009), as a proof for long-term preservation of synaptic responses. The frequency of stimulation in a train can also be systematically changed to investigate the effect of short-term plasticity on activity propagation (i.e., Moreno,et al., 2016). (B) High-frequency stimulation protocol used to evoke long-term potentiation in the perforant pathway of the hippocampus during an fMRI experiment (C). Example of an evoked BOLD signal observed with 10Hz stimulation in the CA3 region of hippocampus (the BOLD signal is correlated with the stimulation protocol using a boxcar model, $P < .0001$). Stimulation periods are marked by the gray bars. Note the consistent response throughout all train stimulations.

2.4 Acute Intracerebral Electrode Implant for Its Use in Functional Magnetic Resonance Imaging Experiments

Most of BOLD-based fMRI data are acquired using EPI images, which are very sensitive to T2* changes. Practically speaking, a number of factors can confabulate causing a deterioration of the quality of the functional images. When working with surgically manipulated animals, especially in acute preparations, extreme care has to be taken to minimize their impact.

Because of the presence of strong magnetic fields, surgery is performed in

an area separated from the magnet room. Thus, it will be necessary to fix the stimulating and/or recording electrode to the animal's skull so that the animal can be safely transferred to the magnet room at the end of the surgery. To ease this transfer, we have developed a customized head fixation system (shown in Fig. 5). Furthermore, due to the common use of surface coils in fMRI experiments, both the electrode positioning and its fixations must be done in such a way that allow maximal proximity between the MRI coil and the brain of the animal. Special care must be taken with bleeding during surgery because any trace of blood will have a deep impact in the image quality, making it very difficult to obtain a reliable BOLD signal.

In an anesthetized rodent (using, e.g., urethane for acute experiments, 1.2e1.4 g/kg for rats, and 1.4e1.6 g/kg for mice), surgery starts by placing the animal in a stereotaxic frame, the head is shaved, and a local anesthetic is injected subcutaneously in the incision points [0.2 mL of 0.5% bupivacaine (Braun Medical, USA)]. In our experience, when working with mice, the complete removal of the skin over the skull significantly increases the quality of the fMRI images. To do that, the skin is cut and borders cauterized (with extreme care taken to avoid overheating the skull). Hydrogen peroxide (3%) can be applied to the bone and skin to remove any blood traces before trephines are made. To prevent eye damage, employ ophthalmic gel on each eye; the gel needs to be re-applied during the surgery to ensure that eyes are covered at all times. The comfort of the animal during the complete experiment is an important factor determining the success of the fMRI session.

Trephine holes are made by hand with a manual drill (2 mm diameter) to prevent heating caused by an electric driller in the target coordinates, and the dura is perforated with a needle at the incision points to allow the penetration of the electrodes. It is important not to remove more of the dura than needed for a precise insertion of the electrode tip: the intact meninges will prevent brain tissue from swelling. In addition, constant moisture must be provided to the exposed brain surface to prevent dryness and inflammation (e.g., by irrigating the surface with 9% saline solution). Electrodes are then lowered in the tissue slowly, avoiding excessive bleeding, and located by coordinates close to its final position. In the case that recording electrodes are inserted, the final positioning is done by electrophysiological vali- dation, that is, optimizing the

amplitude of the evoked field potential by fine adjustments in the position of the stimulating and recording electrodes. During the whole surgery, the animals need to be maintained with constant temperature (37.0 0.5◦C) with a heating blanket. Vital constants (pulse and breath distention, heart and breath rate, and oxygen saturation) are monitored using a paw-clip pulse oximeter (MouseOx Plus, Starr Life Sciences, USA).

Once the electrodes are placed in their final positions, they must be secured to the skull with dental acrylic. To do this, first dry-clean the bone surface (adding a small drop of tissue adhesive can help to dry up and create a surface to which the acrylic can adhere better) and then pour a thin layer of dental acrylic that covers the exposed brain, electrode tips, and bone (Super-Bond, Sun Medical, Japan). When this initial layer is completely dry, complete the implant by adding more dental acrylic or bone cement (e.g. PALACOS, Heraeus Medical GmbH, Germany) until the electrodes are covered to ensure a robust hold. When the implant is secured and dry, the animal can be transferred and fixed in the MRI bed, where temperature and other physiological constants will be again carefully maintained.

2.5 Functional Magnetic Resonance Imaging Acquisition

Physiological monitoring inside the magnet is routinely performed using an MRI-compatible thermometer (Multi-Sens signal conditioner, Opsens, Quebec, Canada) and pulse oximeter (MouseOx, Starr Life Sciences, Oakmont, USA). Additionally, breathing rate can be monitoring alone using a simple custom-designed piezoelectric device (sensitive to pressure) positioned in the chest of the animal. Stable physiological parameters are fundamental to provide a stable baseline of im- aging signals against which experimental conditions can be efficiently contrasted, such as, for instance, conventional investigations on the effects of short- and long- term synaptic plasticity. In addition, the recorded physiological parameters can be used to feed the analysis of BOLD signals (used as nuisance factors) which might be especially important in resting-state experiments. It is crucial to keep the animal's temperature in the physiological range and to maintain it stable (37 +/- 0.5◦C) to preserve vascular reactivity in response to neuronal activation. We use a water blanket connected to a water

bath (Thermo Scientific SAHARA Heated Bath Circulators S5P) controlled by a temperature regulatory system (Thermo Scientific STANDARD Series Thermostats SC150). A custom-made system is used to loop the animal's temperature into the temperature controller to keep a precise control of body temperature (see Fig. 4).

We use a horizontal 7 T scanner with a 30 cm diameter bore (Biospec 70/30v, Bruker Medical, Ettlingen, Ger- many). The system has a 675 mT/m actively shielded gradient coil (Bruker, BGA 12-S) of 11.4 cm inner diameter. Data are acquired and preprocessed with a HewlettePackard console running ParaVision 5.1 soft- ware (Bruker Medical GmbH, Ettlingen, Germany) operating on a Linux platform. We employ a 1H rat brain receive-only phase array coil with integrated combiner and preamplifier, no tune/no match, in combination with the actively detuned transmit-only resonator (Bruker BioSpin MRI GmbH, Germany).

Typical imaging sequences and parameters in our system are as follows:

Gradient echo (GE)eEPI sequence: field of view (FOV), 25 × 25 mm; slice thickness, 1 mm; 15 slices; matrix, 96 × 96; segments, 1; flip angle, 60 degrees; echo time (TE), 15 ms; repetition time (TR), 2000 ms and four dummy scans. Although we typically use slice thickness (coronal orientation) of 1 mm for rats and 0.8 mm for mice, depending on the SNR and the expected level of activation, it can be reduced.

Alternatively a spin echo (SE) sequence with similar parameters can be used. There is extensive literature reviewing the impact of the employed sequence methodology in the obtained fMRI results (Greve, 2011). Briefly, SE is more specific to microvasculature changes but less sensitive, whereas GE is more influenced by changes in macrovasculature but overall more sensitive. An anatomical image with the same geometry than the EPI images is acquired with higher (at least double) in- plane resolution. This will help to identify anatomical landmarks and localize brain activations. For T2-weighted anatomical images, we use a RARE sequence with the following acquisition parameters: FOV, 25 × 25 mm; slice thickness, 1 mm; 15 slices; matrix, 192 × 192; RARE factor, 8; effective TE (TEeff), 56 ms; TR, 2000 ms.

2.5.1 Time line of an Electrical StimulationeFunctional Magnetic Resonance Imaging Experiment

In this section, an example of the temporal sequence of events taking place during an electrical stimulation fMRI experiment is described for reference.

To prevent temperature drop, the magnet's temperature control system must be preheated before the animal is fixed. After placing the animal in the MRI bed, reflexes are checked to ensure that a correct level of anesthesia has been maintained and comfort is assured. Once the animal is positioned, all monitoring and stimulating de- vices should be connected (temperature probe, pulse oximeter, and electrode connections) and checked to be working prior to introducing the animal inside the mag- net's bore and starting the experiment. The coil is fixed to the MRI bed over the head of the animal, as close as possible to the skull, avoiding excessive pressure be- tween the coil and the electrode/s. The animal is then placed inside the magnet aligning the approximate center of the brain with the magnet isocenter.

T2-weighted anatomical images are acquired in the three orthogonal planes for anatomical reference. Even when the animal positioning is accurate, there can be small inter-animal differences when defining an exact position. To facilitate grouped analyses, it is interesting to minimize this variability. Thus, we recommend using anatomical landmarks to position EPI slices always in the same anatomic location. A possible strategy is as follows:

1. Locate the plane that cuts the base of the cerebellum and the anterior commissure.

2. Locate the midline plane that separates the brain in left and right hemispheres.

3. Use the above anatomical planes to define the angle and positioning of the slices for functional imaging. In our case, 15 slices are positioned perpendicular to the planes with the sixth more anterior slice containing the anterior commissure.

Using a shimming procedure, the brain's field homogeneity is adjusted. In our case, we use the Mapshim macro implemented by Bruker. EPI images'

geometry is then adjusted according to the landmarks mentioned earlier and saturation slices are located covering the eye- balls. A set of EPI images is then acquired to check proper image acquisition (no folding, ghosts, etc.) before the start of the experiment.

2.6 Data Analysis

The development of either commercial or open- source software tools for fMRI analysis has greatly facilitated the applicability of fMRI and has contributed to its massive widespread. Nevertheless, due to the complex mathematical work behind the generation of brain activation maps, it is important to know and apply proper and robust statistical methods (Eklund et al., 2016). A detailed description of all possibilities for the analysis of fMRI data sets is out of the scope of this chapter. For a deep discussion about fMRI analysis, see Ashby, 2011; Poldrack et al., 2011. A typical fMRI analysis in our electric stimulation fMRI experiments involves several preprocessing steps, including brain realignment and coregistration with a neuroanatomical template (rat or mouse), intensity normalization, linear detrending, temporal and spatial filtering (3 × 3 full width at half maximum Gaussian kernel of 1.5 sigma), followed by a general linear model analysis or cross- correlation analysis with a simple boxcar model shifted forward in time (typically by the employed TR) or a boxcar convolved with the hemodynamic response function (for details see Pallares et al., 2015). Preprocessing and analysis of the images are implemented in MATLAB® (The Mathworks, Inc., Natick, MA, USA) using custom software, which includes Statistical Parametric Mapping package (SPM8, http://fil.ion.ucl.ac.uk/spm) and FSL Software (FMRIB, http://fsl.fmrib.ox.ac.uk/fsl).

3. Tips and Troubleshooting

Many of the most common problems and how to solve/overcome them have been mentioned earlier in the chapter. In this section, we would like to focus on three aspects that have not been treated in detail in previous sections.

3.1 Echo Planar Imaging Distortions

EPI is highly sensible to abrupt changes in magnetic susceptibility originating artefacts in the border where the variation occurs. This phenomenon is particularly important in the regions where the animal's skull has been exposed for electrode implantation. In our experience, these artefacts can be reduced by covering the exposed region with a layer of semisolid agarose (0.5% solution) applied with a syringe and filling the space between the MRI coil and the animals' head. Agarose can be dissolved in deuterium oxide (heavy water or 2H2O) to avoid signal coming from the applied solution.

Other brain areas affected by susceptibility artefacts are those surrounding the auditory canal, which is air filled and so causes signal loss from the posterior ventral part of the brain (including the medial temporal lobe, amygdala, etc.). A strategy to overcome this problem, in addition to using EPI sequences less susceptible but also less sensitive, is to fill the canal with the above-mentioned agarose solution. The tympanic membrane can be perforated with a blunt needle and the auditory canal filled in with 0.5% agarose. At this concentration, a small amount of agarose (w200 mL per ear) completely fills up the auditory canal, removing the air that disrupts fMRI signals. Animals must be deeply anesthetized, and all the experiments must be terminal (acute) to perform this procedure.

3.2 Temperature Control

As discussed earlier, changes in the temperature of the subject could result in important changes in the fMRI signal. Therefore, it is necessary to monitor and keep constant the temperature of the animal during the whole MRI session. This is of critical importance in experiments lasting several hours as those typically done in long-term synaptic plasticity investigations.

In this section, we describe a custom-made and inexpensive closed-loop regulation system that provides precise control of body temperature by feeding the rectal temperature of the animal recorded by a commercial MRI-compatible thermometer (OTP-M fiber optic high accuracy temperature sensor, Opsens Inc., Quebec, Canada) into the thermostat of a standard

water bath connected to the animal's MRI bed (SAHARA Heated Bath Circulators S5P, Thermo Fisher Scientific, Waltham, MA) (see Fig. 4). This automatic temperature control system is based on the Arduino microcontroller (Arduino MEGA 2560, Arduino SRL, Italy). To maintain the physiological temperature of the animal stable automatically, a PID (proportional-integral-derivative) controller has been developed. The microcontroller obtains, through serial communication (using an RS232 Shield V2, LinkSprite Technologies, Inc., Longmont, CO), the temperature of the animal from the signal conditioner, and it generates a control action that is transmitted to the thermostat (using a USB Host Shield V2.0 for Arduino) to control the temperature of the fluid pumped to the bed. This control system allows automatic temperature control, maintaining an almost constant temperature of the animal in the scanner. Interaction with the automatic temperature control system is possible through a keypad and a display (LCD Keypad Shield for Arduino), being able to set the desired temperature for the animal. Circuit details and routines programmed in the Arduino can be downloaded from http://dmoratal.webs.upv.es/research.html.

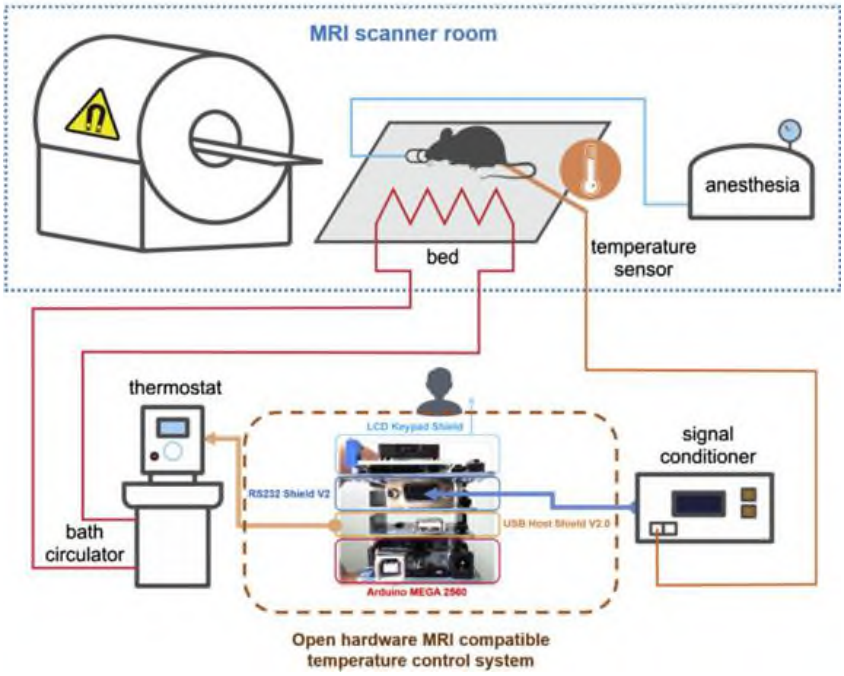

Figure 4. Custom-made temperature control system. This figure depicts the whole process involved in the automatic regulation of the temperature of the animal being scanned: a temperature sensor connected to a signal conditioner measures the body temperature of the animal under study. An Arduino-based microcontroller obtains, through serial communication (RS232 Shield V2), the temperature of the animal from the signal conditioner, and it generates a control action that is transmitted to the thermostat (USB Host Shield V2.0) to control the temperature of the fluid pumped to the bed through a bath circulator. A user can interact with the automatic temperature control system to establish the desired temperature for the animal through a keypad and a display (LCD Keypad Shield). MRI, magnetic resonance imaging.

3.3 Rodent Magnetic Resonance Imaging Compatible Bed

MR manufacturers offer a reduced variety of animal holders, restrainers, and MRI-compatible beds. Further- more, the commercially available MRI accessories often suppose abusive expenditure, especially when customization is required. Supported with the growing availability of 3-D printers at most institutions, here we share a solution we developed to speed up the process of precise and reproducible fixation of the animal in the MRI system, as well as the transfer of the animal from the stereotaxic devise to the MRI bed. In the following link we provide a customizable free template of an MRI-compatible rodent bed (with several attachments) that can be easily fed to a 3D-printer software and produced individually at a reduced cost. The template and a description of its use can be freely downloaded from http://dmoratal.webs.upv.es/ research.html.

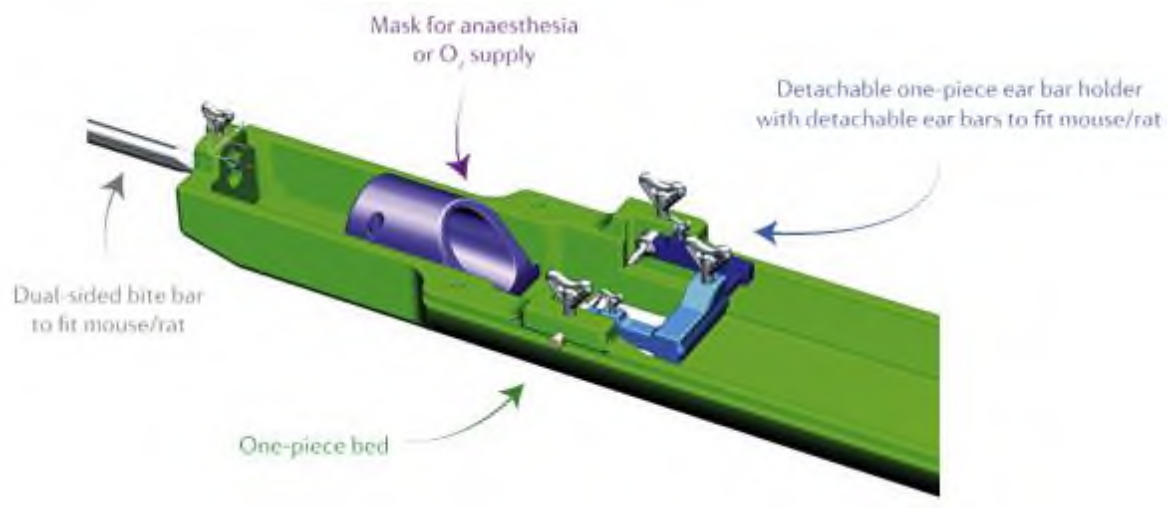

Figure 5. Free downloadable small rodent magnetic resonance imaging (MRI) bed. 3-D rendered image of the different parts of the proposed MRI rodent bend. The multifunctional (mice and rats) apparatus provides a customizable body, ear bar, bite bar and anesthesia mask. The main feature of the system is the detachable ear bar holder that allows precise and simple location of the ear bars in the animal, first, and in the MRIbed then, assuring reproducible imaging sessions in longitudinal experiments. Furthermore, it facilitates the transfer of the animal from a stereotaxic devise, for the surgical implantation of electrodes, to the MRI bed without the need of repositioning the ear bars.

4. Future perspectives

In this chapter we have described, stepwise, how to perform a BOLD fMRI experiment with intracerebral electric microstimulation. This protocol has allowed us to (1) perform precise and controlled activations of selected brain regions, (2) reproducibly acquire data within and between animals in rats and mice, (3) inves- tigate the frequency dependence of activity propagation in brain networks, and (4) provide several insights into the synaptic plasticity control of long-range connectivity. In this last section about future perspectives, we will discuss the need of awake animal fMRI studies and the available procedures to date.

4.1 Functional Magnetic Resonance Imaging in the Awake Animal

The need for the absolute immobility of the subject during image acquisition has favored the use of anesthetized animals in most studies. Besides the obvious impact of anesthesia on neuronal activation, additional factors need to be considered in fMRI studies because anesthetics directly impact on the hemodynamic response and the neurovascular coupling (Masamoto and

Kanno, 2012). Therefore, an important step forward in rodent brain imaging would be the implementation of protocols that allow to routinely acquire fMRI images in the awake, conscious animal, thus avoiding the anesthetics' effects.

Some alternatives to avoid anesthetics exist, e.g., habituation training protocols which allow to do fMRI experiments in nonanesthetized rodents. These proto- cols have been traditionally implemented in humans to reduce the stress and anxiety evoked by the MRI environment (using preexposure to the stressful conditions as closed and reduced space, high noise levels, etc.). In 1998, Lahti and colleagues proposed the first successful protocol to image awake rodents in MRI (Lahti et al., 1998, 1999) without any prior exposure; some adverse effects (somatosensory cortex activation presumably caused by the acute stress) were reported. The first habituation protocol though was introduced by King and colleagues (King et al., 2005) and included a progressive habituation (starting with lower exposure times and building up until reaching the final needed scanning time) in a mock scanner, also using local anesthetics to minimize the pain in precise points (where the restrainer is fixed). Since then, several studies have perfected the habituation technique (Dunn et al., 2011, 2009; Ferris et al., 2006; Liang et al., 2012, 2015; Upadhyay et al., 2011). All available protocols start with the animal sedated (low isoflurane dose), and then sedation is progressively removed, and the animal recovers consciousness immobile inside the magnet.

Although these procedures are very appealing and pave the way to advancements in rodent fMRI, the currently available protocols involve an initial period of severe and intense stress during habituation to the MRI environment, which could interfere with the particular scientific question at hand. In fact, there is extensive literature investigating the effect of strong acute stress induced, for instance, by animal restraining, which include potential behavioral alterations such as learned helplessness, anxiety, molecular, neurophysiological, and neuroanatomical alterations (Bardin et al., 2009; Vyas and Chattarji, 2004; Wood et al., 2008). Therefore, when using this strategy to train animals in awake fMRI experiments, it is advisable to use mild protocols to reduce stress to a minimum, and always control for potential collateral alterations introduced by training it- self. Other strategies involve the use of chronic MRI-compatible head implants to restrain the animals. These implants allow for the habituation of the

animals without the need of initial periods of anesthesia by gradually habituating the animal to be restrained by the previously implanted headcap (Chang et al., 2016; Harris et al., 2015; Kenkel et al., 2016; Khubchandani et al., 2003). Although promising, the available evidence does not allow yet discarding stress interference in these protocols.

While improvement in training protocols for awake fMRI experiments is still necessary, we believe that this preparation, in combination with direct brain stimulation protocols, provide a powerful framework to investigate the consequences of local alterations in synaptic plasticity on global network dynamics.

Funding

This work was supported in part by the Spanish Ministerio de Econom´ıa y Competitividad (MINECO) and FEDER funds under grants BFU2015-64380-C2-2-R (D.M.) and BFU2015-64380-C2-1-R and EU Horizon 2020 Program 668863-SyBil-AA grant (S.C.). S.C. acknowledges financial support from the Spanish State Research Agency, through the "Severo Ochoa" Programme for Centres of Excellence in R&D (ref. SEV- 2013-0317). D.R.Q. was supported by grant "Ayudas para la formación de personal investigador (FPI)" from the Vicerrectorado de Investigación, Innovación y Transferencia of the Universitat Politècnica de València.

References

Alger, B.E., Nicoll, R.A., 1982. Feed-forward dendritic inhibition in rat hippocampal pyramidal cells studied in vitro. J. Physiol. 328, 105e123.

Alvarez-Salvado, E., Pallares, V., Moreno, A., Canals, S., 2014. Functional MRI of long-term potentiation: imaging network plasticity. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 369, 20130152. https://doi.org/10.1098/rstb.2013.0152.

Amaral, D., Lavenex, P., 2007. Hippocampal Neuroanatomy. In: The hippocampus Book, vol. 1, pp. 37e114.

Ashby, F.G., 2011. Statistical Analysis of FMRI Data. MIT Press. Bardin, L., Malfetes, N., Newman-Tancredi, A., Depoortere, R., 2009. Chronic restraint stress induces mechanical and cold allodynia, and enhances inflammatory pain in rat: relevance to humanstress-associated painful pathologies. Behav. Brain Res. 205, 360e366. https://doi.org/10.1016/j.bbr.2009.07.005.

Bassett, D.S., Greenfield, D.L., Meyer-Lindenberg, A., Weinberger, D.R., Moore, S.W., Bullmore, E.T., 2010. Efficient physical embedding of topologically complex information processing networks in brains and computer circuits. PLoS Comput. Biol. 6, e1000748. https:// doi.org/10.1371/journal.pcbi.1000748.

Bliss, T.V., Collingridge, G.L., 1993. A synaptic model of memory: long- term potentiation in the hippocampus. Nature 361, 31e39. https:// doi.org/10.1038/361031a0.

Bliss, T.V., Collingridge, G.L., 2013. Expression of NMDA receptor- dependent LTP in the hippocampus: bridging the divide. Mol.Brain 6, 5. https://doi.org/10.1186/1756-6606-6-5.

Bliss, T.V., Lomo, T., 1973. Long-lasting potentiation of synaptic trans- mission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J. Physiol. 232, 331e356.

Brewer, J.B., Zhao, Z., Desmond, J.E., Glover, G.H., Gabrieli, J.D., 1998.Making memories: brain activity that predicts how well visualexperience will be remembered. Science 281, 1185e1187.

Buzsaki, G., 2006. Rhythms of the Brain. Oxford University Press.

Cajal, S.R.Y., 1894. The Croonian Lecture: La Fine Structure des Centres Nerveux. Proc. Roy. Soc. Lond. 55, 444e468. https://doi.org/ 10.1098/rspl.1894.0063.

Canals, S., Beyerlein, M., Merkle, H., Logothetis, N.K., 2009. FunctionalMRI evidence for LTP-induced neural network reorganization. Curr. Biol. 19, 398e403. https://doi.org/10.1016/j.cub.2009.01.037.

Canals, S., Beyerlein, M., Murayama, Y., Logothetis, N.K., 2008. Electric stimulation fMRI of the perforant pathway to the rat hippocampus. Magn. Reson. Imaging 26, 978e986. https://doi.org/10.1016/ j.mri.2008.02.018.

Chang, P.C., Procissi, D., Bao, Q., Centeno, M.V., Baria, A., Apkarian, A.V., 2016. Novel method for functional brain imaging in awake minimally restrained rats. J. Neurophysiol. 116, 61e80. https://doi.org/10.1152/jn.01078.2015.

Chao, T.H., Chen, J.H., Yen, C.T., 2014. Repeated BOLD-fMRI imaging of deep brain stimulation responses in rats. PLoS One 9, e97305. https://doi.org/10.1371/journal.pone.0097305.

Davies, C.H., Collingridge, G.L., 1993. The physiological regulation of synaptic inhibition by GABAB autoreceptors in rat hippocampus. J. Physiol. 472, 245e265.

Davies, C.H., Starkey, S.J., Pozza, M.F., Collingridge, G.L., 1991. GABA autoreceptors regulate the induction of LTP. Nature 349, 609e611.https://doi.org/10.1038/349609a0.

Dehaene, S., Naccache, L., 2001. Towards a cognitive neuroscience of consciousness: basic evidence and a workspace framework. Cogni- tion 79, 1e37.

Driver, J., Noesselt, T., 2008. Multisensory interplay reveals crossmodal influences on 'sensory-specific' brain regions, neural responses, and judgments. Neuron 57, 11e23. https://doi.org/10.1016/ j.neuron.2007.12.013.

Duffy, B.A., Choy, M., Chuapoco, M.R., Madsen, M., Lee, J.H., 2015. MRI compatible optrodes for simultaneous LFP and optogenetic fMRI investigation of seizure-like afterdischarges. NeuroImage 123, 173e184. https://doi.org/10.1016/j.neuroimage.2015.07.038.

Dunn, J.F., Khan, M.N., Hou, H.G., Merlis, J., Abajian, M.A., Demidenko, E., Grinberg, O.Y., Swartz, H.M., 2011. Cerebral oxygenation in awake rats during acclimation and deacclimation to hypoxia: an in vivo electron paramagnetic resonance study. High Alt. Med. Biol. 12, 71e77. https://doi.org/10.1089/ ham.2010.1038.

Dunn, J.F., Tuor, U.I., Kmech, J., Young, N.A., Henderson, A.K., Jackson, J.C., Valentine, P.A., Teskey, G.C., 2009. Functional brain mapping at 9.4T using a new MRI-compatible electrode chronically implanted in rats. Magn. Reson. Med. 61, 222e228. https:// doi.org/10.1002/mrm.21803.

Eichenbaum, H., Cohen, N.J., 2004. From Conditioning to Conscious Recollection: Memory Systems of the Brain: Memory Systems of the Brain. Oxford University Press, USA.

Eklund, A., Nichols, T.E., Knutsson, H., 2016. Cluster failure: why fMRIinferences for spatial extent have inflated false-positive rates. Proc. Natl. Acad. Sci. U. S. A. 113, 7900e7905. https://doi.org/10.1073/pnas.1602413113.

Fanselow, M.S., Dong, H.W., 2010. Are the dorsal and ventral hippocampus functionally distinct structures? Neuron 65, 7e19. https://doi.org/10.1016/j.neuron.2009.11.031.

Ferris, C.F., Febo, M., Luo, F., Schmidt, K., Brevard, M., Harder, J.A., Kulkarni, P., Messenger, T., King, J.A., 2006. Functional magneticresonance imaging in conscious animals: a new tool in behavioural neuroscience research. J. Neuroendocrinol. 18, 307e318. https://doi.org/10.1111/j.1365-2826.2006.01424.x.

Fuster, J.M., 2001. The prefrontal cortex an update: time is of the essence. Neuron 30, 319e333.

Girardeau, G., Benchenane, K., Wiener, S.I., Buzsaki, G., Zugaro, M.B.,2009. Selective suppression of hippocampal ripples impairs spatialmemory. Nat. Neurosci. 12, 1222e1223. https://doi.org/10.1038/nn.2384.

Godino, M.D.C., Romera, V.G., Sanchez-Tomero, J.A., Pacheco, J.,Canals, S., Lerma, J., Vivancos, J., Moro, M.A., Torres, M., Lizasoain, I., Sanchez-Prieto, J., 2013. Amelioration of ischemic brain damage by peritoneal dialysis. J. Clin. Invest. 123, 4359e4363. https://doi.org/10.1172/JCI67284.

Goldman-Rakic, P.S., 1988. Topography of cognition: parallel distributed networks in primate association cortex. Annu. Rev. Neurosci.11, 137e156. https://doi.org/10.1146/annurev.ne.11.030188.001033.

Greve, J.M., 2011. The BOLD effect. Meth. Mol. Biol. 771, 153e169. https://doi.org/10.1007/978-1-61779-219-9_8.

Guitchounts, G., Markowitz, J.E., Liberti, W.A., Gardner, T.J., 2013.A carbon-fiber electrode array for long-term neural recording. J. Neural. Eng. 10, 046016. https://doi.org/10.1088/1741-2560/ 10/4/046016.

Hadar, R., Vengeliene, V., Barroeta Hlusicke, E., Canals, S., Noori, H.R.,Wieske, F., Rummel, J., Harnack, D., Heinz, A., Spanagel, R., Winter, C., 2016. Paradoxical augmented relapse in alcohol- dependent rats during deep-brain stimulation in the nucleus accumbens. Transl. Psychiatry 6, e840. https://doi.org/10.1038/ tp.2016.100.

Hamadate, N., Yamaguchi, T., Sugawara, A., Tsujimatsu, A., Izumi, T., Yoshida, T., Ohmura, Y., Yoshioka, M., 2011. Regulation of cerebralblood flow in the hippocampus by neuronal activation through theperforant path: relationship between hippocampal blood flow andneuronal plasticity. Brain Res. 1415, 1e7. https://doi.org/10.1016/ j.brainres.2011.08.008.

Harris, A.P., Lennen, R.J., Marshall, I., Jansen, M.A., Pernet, C.R.,Brydges, N.M., Duguid, I.C., Holmes, M.C., 2015. Imaging learned fear circuitry in awake mice using fMRI. Eur. J. Neurosci. 42, 2125e2134. https://doi.org/10.1111/ejn.12939.

Hebb, D.O., 1949. The Organization of Behavior: A Neuropsychological Theory. Wiley.

Hendrich, K.S., Kochanek, P.M., Melick, J.A., Schiding, J.K., Statler, K.D., Williams, D.S., Marion, D.W., Ho, C., 2001. Cerebral perfusion during anesthesia with fentanyl, isoflurane, or pentobarbital in normal rats studied by arterial spin-labeled MRI. Magn. Reson. Med. 46, 202e206.

Jego, P., Pacheco-Torres, J., Araque, A., Canals, S., 2014. Functional MRI in mice lacking IP3-dependent calcium signaling in astrocytes. J. Cerebr. Blood Flow Metab. 34, 1599e1603. https://doi.org/ 10.1038/jcbfm.2014.144.

Kenkel, W.M., Yee, J.R., Moore, K., Madularu, D., Kulkarni, P., Gamber, K., Nedelman, M., Ferris, C.F., 2016. Functional magneticresonance imaging in awake transgenic fragile X rats: evidence of dysregulation in reward processing in the mesolimbic/habenular neural circuit. Transl. Psychiatry 6, e763. https://doi.org/ 10.1038/tp.2016.15.

Khubchandani, M., Mallick, H.N., Jagannathan, N.R., Mohan Kumar, V., 2003. Stereotaxic assembly and procedures for simulta-neous electrophysiological and MRI study of conscious rat. Magn.Reson. Med. 49, 962e967. https://doi.org/10.1002/mrm.10441.

Kim, J.J., Fanselow, M.S., 1992. Modality-specific retrograde amnesia of fear. Science 256, 675e677.

King, J.A., Garelick, T.S., Brevard, M.E., Chen, W., Messenger, T.L., Duong, T.Q., Ferris, C.F., 2005. Procedure for minimizing stress for fMRI studies in conscious rats. J. Neurosci. Meth. 148, 154e160. https://doi.org/10.1016/j.jneumeth.2005.04.011.

Klein, M., Kandel, E.R., 1978. Presynaptic modulation of voltage- dependent Ca^{2p} current: mechanism for behavioral sensitization in *Aplysia californica*. Proc. Natl. Acad. Sci. U. S. A. 75, 3512e3516.

Lahti, K.M., Ferris, C.F., Li, F., Sotak, C.H., King, J.A., 1998. Imaging brain activity in conscious animals using functional MRI. J. Neurosci. Meth. 82, 75e83.

Lahti, K.M., Ferris, C.F., Li, F., Sotak, C.H., King, J.A., 1999. Comparisonof evoked cortical activity in conscious and propofol-anesthetizedrats using functional MRI. Magn. Reson. Med. 41, 412e416.

Lavenex, P., Amaral, D.G., 2000. Hippocampal-neocortical interaction:a hierarchy of associativity. Hippocampus 10, 420e430. https://doi.org/10.1002/1098-1063(2000)10:4<420::AID-HIPO8>3.0.CO; 2e5.

Liang, Z., King, J., Zhang, N., 2012. Anticorrelated resting-state func- tional connectivity in awake rat brain. NeuroImage 59, 1190e1199. https://doi.org/10.1016/j.neuroimage.2011.08.009.

Liang, Z., Liu, X., Zhang, N., 2015. Dynamic resting state functional connectivity in awake and anesthetized rodents. NeuroImage 104,89e99. https://doi.org/10.1016/j.neuroimage.2014.10.013.

Logothetis, N.K., 2008. What we can do and what we cannot dowith fMRI. Nature 453, 869e878. https://doi.org/10.1038/ nature06976.

Logothetis, N.K., Augath, M., Murayama, Y., Rauch, A., Sultan, F., Goense, J., Oeltermann, A., Merkle, H., 2010. The effects of electricalmicrostimulation on cortical signal propagation. Nat. Neurosci. 13,1283e1291. https://doi.org/10.1038/nn.2631.

Logothetis, N.K., Pauls, J., Augath, M., Trinath, T., Oeltermann, A., 2001. Neurophysiological investigation of the basis of the fMRI signal. Nature 412, 150e157. https://doi.org/10.1038/35084005.

Logothetis, N.K., Wandell, B.A., 2004. Interpreting the BOLD signal. Annu. Rev. Physiol. 66, 735e769. https://doi.org/10.1146/ annurev.physiol.66.082602.092845.

Maggi, C.A., Meli, A., 1986. Suitability of urethane anesthesia for physiopharmacological investigations in various systems. Part 2: cardio-vascular system. Experientia 42, 292e297.

Manabe, T., Wyllie, D.J., Perkel, D.J., Nicoll, R.A., 1993. Modulation ofsynaptic transmission and long-term potentiation: effects on paired pulse facilitation and EPSC variance in the CA1 region of the hippocampus. J. Neurophysiol. 70, 1451e1459.

Masamoto, K., Kanno, I., 2012. Anesthesia and the quantitative evaluation of neurovascular coupling. J. Cerebr. Blood Flow Metab. 32, 1233e1247. https://doi.org/10.1038/jcbfm.2012.50.

Mathiesen, C., Caesar, K., Akgoren, N., Lauritzen, M., 1998. Modification of activity-dependent increases of cerebral blood

flow by excit- atory synaptic activity and spikes in rat cerebellar cortex. J. Physiol.512 (Pt 2), 555e566.
Mathiesen, C., Caesar, K., Lauritzen, M., 2000. Temporal coupling between neuronal activity and blood flow in rat cerebellar cortex as indicated by field potential analysis. J. Physiol. 523 (Pt 1), 235e246.
McClelland, J.L., McNaughton, B.L., O'Reilly, R.C., 1995. Why there arecomplementary learning systems in the hippocampus and neocortex: insights from the successes and failures of connectionist models of learning and memory. Psychol. Rev. 102, 419e457.
McCreery, R.L., 2008. Advanced carbon electrode materials for molecular electrochemistry. Chem. Rev. 108, 2646e2687. https://doi.org/ 10.1021/cr068076m.
McNaughton, N., Morris, R.G., 1987. Chlordiazepoxide, an anxiolytic benzodiazepine, impairs place navigation in rats. Behav. BrainRes. 24, 39e46.
Moreno, A., Jego, P., de la Cruz, F., Canals, S., 2013. Neurophysiological, metabolic and cellular compartments that drive neurovascular coupling and neuroimaging signals. Front. Neuroenergetics 5, 3. https://doi.org/10.3389/fnene.2013.00003.
Moreno, A., Morris, R.G., Canals, S., 2016. Frequency-dependent gating of hippocampal-neocortical interactions. Cerebr. Cortex 26,2105e2114. https://doi.org/10.1093/cercor/bhv033.
Morris, R.G., 2006. Elements of a neurobiological theory of hippocampal function: the role of synaptic plasticity, synaptic tagging and schemas. Eur. J. Neurosci. 23, 2829e2846. https://doi.org/ 10.1111/j.1460-9568.2006.04888.x.
Morris, R.G., Moser, E., Riedel, G., Martin, S., Sandin, J., Day, M.,O'Carroll, C., 2003. Elements of a neurobiological theory of the hippocampus: the role of activity-dependent synaptic plasticity in memory. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 358, 773e786.
Moser, M.B., Moser, E.I., 1998. Functional differentiation in thehippocampus. Hippocampus 8, 608e619. https://doi.org/10.1002/(SICI)1098-1063(1998)8:6<608::AID-HIPO3>3.0.CO;2-7.
Mott, D.D., Lewis, D.V., 1991. Facilitation of the induction of long-term potentiation by GABAB receptors. Science 252, 1718e1720.
Ogawa, S., Lee, T.M., Kay, A.R., Tank, D.W., 1990. Brain magnetic resonance imaging with contrast dependent on blood oxygenation. Proc. Natl. Acad. Sci. U. S. A. 87, 9868e9872.
Olton, D.S., Papas, B.C., 1979. Spatial memory and hippocampal function. Neuropsychologia 17, 669e682.
Osada, T., Adachi, Y., Kimura, H.M., Miyashita, Y., 2008. Towards understanding of the cortical network underlying associative memory. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 363, 2187e2199.
Paasonen, J., Salo, R.A., Shatillo, A., Forsberg, M.M., Narvainen, J.,Huttunen, J.K., Grohn, O., 2016. Comparison of seven different anesthesia protocols for nicotine pharmacologic magnetic resonance imaging in rat. Eur. Neuropsychopharmacol. 26, 518e531.https://doi.org/10.1016/j.euroneuro.2015.12.034.
Pallares, V., Moya, J., Samper-Belda, F.J., Canals, S., Moratal, D., 2015. Neurosurgery planning in rodents using a magnetic resonance im- aging assisted framework to target experimentally definednetworks. Comput. Meth. Progr. Biomed. 121, 66e76. https:// doi.org/10.1016/j.cmpb.2015.05.011.
Pawela, C.P., Biswal, B.B., Hudetz, A.G., Schulte, M.L., Li, R.,Jones, S.R., Cho, Y.R., Matloub, H.S., Hyde, J.S., 2009. A protocol for use of medetomidine anesthesia in rats for extended studies using task-induced BOLD contrast and resting-state functional connectivity. NeuroImage 46, 1137e1147. https://doi.org/ 10.1016/j.neuroimage.2009.03.004.
Perkel, D.J., Nicoll, R.A., 1993. Evidence for all-or-none regulation of neurotransmitter release: implications for long-term potentiation.J. Physiol. 471, 481e500.
Poldrack, R.A., Mumford, J.A., Nichols, T.E., 2011. Handbook of Functional MRI Data Analysis. Cambridge University Press.
Pouille, F., Scanziani, M., 2001. Enforcement of temporal fidelity in pyramidal cells by somatic feed-forward inhibition. Science 293,1159e1163. https://doi.org/10.1126/science.1060342.
Pouille, F., Scanziani, M., 2004. Routing of spike series by dynamic circuits in the hippocampus. Nature 429, 717e723. https://doi.org/ 10.1038/nature02615.
Rempel-Clower, N.L., Zola, S.M., Squire, L.R., Amaral, D.G., 1996.Three cases of enduring memory impairment after bilateral damage limited to the hippocampal formation. J. Neurosci. 16, 5233e5255.
Rumelhart, D.E., McClelland, J.L., Group, P.D.P.R., 1986. ParallelDistributed Processing, vols. 1 and 2. The MIT Press, Cambridge, MA.
Schaefer, A.T., Angelo, K., Spors, H., Margrie, T.W., 2006. Neuronal oscillations enhance stimulus discrimination by ensuring action po- tential precision. PLoS Biol. 4, e163. https://doi.org/10.1371/ journal.pbio.0040163.
Schroeter, A., Schlegel, F., Seuwen, A., Grandjean, J., Rudin, M., 2014.Specificity of stimulus-evoked fMRI responses in the mouse: the in-fluence of systemic physiological changes associated with innocuous stimulation under four different anesthetics. NeuroImage 94, 372e384. https://doi.org/10.1016/j.neuroimage.2014.01.046.
Scoville, W.B., Milner, B., 1957. Loss of recent memory after bilateral hippocampal lesions. J. Neurol. Neurosurg. Psychiatry 20, 11e21.
Shams, L., Seitz, A.R., 2008. Benefits of multisensory learning. Trends Cogn. Sci. 12, 411e417. https://doi.org/10.1016/j.tics.2008.07.006.

Siapas, A.G., Lubenov, E.V., Wilson, M.A., 2005. Prefrontal phase locking to hippocampal theta oscillations. Neuron 46, 141e151. https:// doi.org/10.1016/j.neuron.2005.02.028.
Sonnay, S., Just, N., Duarte, J.M., Gruetter, R., 2015. Imaging of prolonged BOLD response in the somatosensory cortex of the rat. NMR Biomed. 28, 414e421. https://doi.org/10.1002/nbm.3263.
Squire, L.R., 1986. Mechanisms of memory. Science 232, 1612e1619.
Squire, L.R., 1992. Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. Psychol. Rev. 99, 195e231.
Squire, L.R., Stark, C.E., Clark, R.E., 2004. The medial temporal lobe*. Annu. Rev. Neurosci. 27, 279e306.
Tanabe, H.C., Honda, M., Sadato, N., 2005. Functionally segregated neural substrates for arbitrary audiovisual paired-association learning. J. Neurosci. 25, 6409e6418. https://doi.org/10.1523/ JNEUROSCI.0636-05.2005.
Tehovnik, E.J., Tolias, A.S., Sultan, F., Slocum, W.M., Logothetis, N.K., 2006. Direct and indirect activation of cortical neurons by electricalmicrostimulation. J. Neurophysiol. 96, 512e521. https://doi.org/ 10.1152/jn.00126.2006.
Thomsen, K., Offenhauser, N., Lauritzen, M., 2004. Principal neuron spiking: neither necessary nor sufficient for cerebral blood flow inrat cerebellum. J. Physiol. 560, 181e189. https://doi.org/10.1113/jphysiol.2004.068072.
Tolias, A.S., Sultan, F., Augath, M., Oeltermann, A., Tehovnik, E.J., Schiller, P.H., Logothetis, N.K., 2005. Mapping cortical activity eli- cited with electrical microstimulation using FMRI in the macaque. Neuron 48, 901e911. https://doi.org/10.1016/ j.neuron.2005.11.034.
Tse, D., Langston, R.F., Kakeyama, M., Bethus, I., Spooner, P.A., Wood, E.R., Witter, M.P., Morris, R.G., 2007. Schemas and memoryconsolidation. Science 316, 76e82.
Tse, D., Takeuchi, T., Kakeyama, M., Kajii, Y., Okuno, H., Tohyama, C., Bito, H., Morris, R.G., 2011. Schema-dependent gene activation and memory encoding in neocortex. Science 333, 891e895.
Upadhyay, J., Baker, S.J., Chandran, P., Miller, L., Lee, Y., Marek, G.J., Sakoglu, U., Chin, C.L., Luo, F., Fox, G.B., Day, M., 2011. Default- mode-like network activation in awake rodents. PLoS One 6, e27839. https://doi.org/10.1371/journal.pone.0027839.
Vitale, F., Summerson, S.R., Aazhang, B., Kemere, C., Pasquali, M., 2015. Neural stimulation and recording with bidirectional, soft carbon nanotube fiber microelectrodes. ACS Nano 9, 4465e4474. https://doi.org/10.1021/acsnano.5b01060.
Vyas, A., Chattarji, S., 2004. Modulation of different states of anxiety- like behavior by chronic stress. Behav. Neurosci. 118, 1450e1454. https://doi.org/10.1037/0735-7044.118.6.1450.
Wood, G.E., Norris, E.H., Waters, E., Stoldt, J.T., McEwen, B.S., 2008. Chronic immobilization stress alters aspects of emotionality and associative learning in the rat. Behav. Neurosci. 122, 282e292. https://doi.org/10.1037/0735-7044.122.2.282.
Zola-Morgan, S.M., Squire, L.R., 1990. The primate hippocampal formation: evidence for a time-limited role in memory storage. Science 250, 288e290.