Establishing a combined optogenetic-electrophysiological method to characterize the activity of granule cells in the dentate gyrus of freely moving mice

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NANYANG
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**ESTABLISHING A COMBINED OPTOGENETIC-ELECTROPHYSIOLOGICAL
METHOD TO CHARACTERIZE THE ACTIVITY OF GRANULE CELLS IN THE
DENTATE GYRUS OF FREELY MOVING MICE**

VANJA CNOPS

SCHOOL OF BIOLOGICAL SCIENCES

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Abbreviations

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BrdU	5-bromo-2'-deoxyuridine
CA	<i>cornu ammonis</i>
ChR	Channelrhodopsin
DG	dentate gyrus
EC	entorhinal cortex
EYFP	enhanced yellow fluorescent protein
GABA	gamma-amino-butyric acid
GFP	green fluorescent protein
IEG	immediate early gene
LFP	local field potential
NMDA	N-methyl-D-aspartate
POMC	pro-opiomelanocortin- α
PSTH	peristimulus time histogram
SWR	sharp wave ripple

Abstract

Efforts to understand the role of the dentate gyrus have been hampered by the limitation of extracellular recordings to identify cell types. By combining extracellular recording with optogenetics, it has become possible to identify and characterize the activity of specific cell types. Here, we assessed the feasibility of this technique to identify granule cells of the dentate gyrus in freely behaving mice. We were able to detect units that responded to light stimulation with short latency and high reliability. Although the short latency suggested that units were directly activated by light stimulation, we could not exclude the possibility that they were indirectly activated postsynaptic neurons. We also detected voltage fluctuations after the light stimulus, which were presumably caused by photovoltaic artefacts and synchronous activation of multiple cells. We believe that optogenetic identification of granule cells could be a valuable technique provided that the uncertainty about the response latency is resolved.

1. Introduction

1.1. Hippocampal neuroanatomy

The hippocampus is a part of the allocortex, a phylogenetically old region of the brain which together with the neocortex forms the cerebral cortex (Andersen *et al.*, 2007). The main difference between the allocortex and neocortex is the number of cell layers. While the neocortex is characterized by six layers, the parts of the allocortex, like the hippocampus, contain only three to five layers. Another noteworthy feature of the hippocampus is that all principal cells are located in a single layer (Andersen *et al.*, 2007).

The terminology used to describe which regions of the brain belong to the hippocampus varies among authors (Andersen *et al.*, 2007). The *cornu ammonis* areas (CA1, CA2 and CA3) are sometimes called 'hippocampus proper', while the term 'hippocampal formation' refers to the CA areas together with the dentate gyrus, entorhinal cortex, subiculum, presubiculum and parasubiculum. In this thesis, the term "hippocampus" will refer to the CA regions CA1, CA2 and CA3 together with the dentate gyrus (DG).

The hippocampus is a bilateral structure; there is one hippocampus in each hemisphere. The three-dimensional structure of the rodent hippocampus resembles two bananas, connected at the dorsal side via the hippocampal commissure and spreading apart laterally and ventrally into the temporal cortex (Figure 1.1A; (Amaral *et al.*, 2007)). The anatomy of the hippocampus is largely conserved across mammals (Andersen *et al.*, 2007). Non-mammalian species like birds and reptiles have structures that are functionally homologous to the hippocampus, however it is still debated whether these structures have a common evolutionary origin (Andersen *et al.*, 2007). In humans the hippocampus is located in the medial temporal lobe. Early anatomists saw a seahorse in the shape of the human hippocampus, and named it after the Latin word for seahorse.

Histological sections through the rodent hippocampus are shown in Figures 1.1B and 1.1C. Nissl-stained coronal sections clearly show the characteristic structure of the hippocampus with two densely stained, interlocking cell layers. The V-shaped cell layer is the granule cell layer of the DG. It contains the cell bodies of granule cells, the principal cells of the DG. The other cell layer contains the cell bodies of pyramidal cells of CA1, CA2 and CA3 and is called pyramidal cell layer. According to early anatomists, the C-shaped structure of the hippocampus resembles the horns of a ram. They named it *cornu ammonis* (ammon's horn) after the Egyptian god Ammon, a god with a ram's head.

1.1.1. Basic hippocampal circuitry: the trisynaptic pathway

One of the remarkable features of the hippocampal circuit is that many of its connections are unidirectional (Amaral *et al.*, 2007). The entorhinal cortex (EC) projects to the DG, but there is no direct return projection from the DG to the EC. Likewise, CA3 does not directly project to the granule cells of the DG and CA1 has no projections to CA3.

The main input to the hippocampus is provided by the EC. Granule cells in the DG receive excitatory input from cells in layer II of the EC, via the so-called perforant pathway. Granule cells send their axons (called "mossy fibers") to CA3. The principal cells of CA3 are pyramidal cells, which project axons (Schaffer collaterals) to CA1. This simple, unidirectional series of connections (EC→DG→CA3→CA1) was first described by Ramon y Cajal in 1911 and was called "trisynaptic pathway" by Andersen (Andersen *et al.*, 1971). Because of this unidirectional connections, the hippocampus was seen as a simple model of

neural network organization. Computational models of the function of the DG (presented in section 1.2.3) were based on the strong, unidirectional connections between the DG and CA3.

It is now clear that the hippocampus contains more cell types and connections, which will be described in more detail in the following sections. Although many connections are left out in this “trisynaptic” view on the hippocampal circuitry, the unidirectional connections of the hippocampus are an interesting feature that makes the hippocampus different from most other brain regions (Figure 1.2).

1.1.2. The dentate gyrus

The DG contains three layers. From superficial to deep these are: the molecular layer, the granule cell layer and the polymorphic cell layer or hilus (Figure 1.1 C). The three layers of the DG are folded with the hilus on the inside. The fold is called crest and divides the DG in two blades: the upper (suprapyramidal) and lower (infrapyramidal) blade. Granule cells are the principal cells of the DG; they are the only cells whose axons leave the DG and project to the next area in the hippocampal circuit, the CA3.

Figure 1.1. Hippocampal neuroanatomy. (A) Location and three-dimensional organization of the hippocampal formation in the rat brain. Figure adapted with permission from (Amaral and Witter, 1989) (B) Nissl-stained coronal section of mouse brain (Lein *et al.*, 2007). (C) Nissl-stained coronal section of mouse brain with indication of layers of CA1 and DG. The hippocampal fissure separates the CA1 and DG.

1.1.3.1. Granule cells

The granule cell layer (*stratum granulosum*) contains the cell bodies of the granule cells. Granule cells have an elliptical cell body with a width of approximately 10 μm and a height of 18 μm (Claiborne *et al.*, 1990). The granule cell layer is a densely packed layer that is only 4-6 neurons or $\sim 60 \mu\text{m}$ thick in rats. The total number of granule cells in one hemisphere of the rat DG is about 1.2 million (Amaral *et al.*, 1990). Granule cells contain an extensive apical dendritic tree, which is located in the molecular layer. The dendrites form a cone-shaped tree in which the dendrites extend toward the hippocampal fissure. The molecular layer has an average thickness of 250 μm in rats and most dendrites terminate near the hippocampal fissure (Claiborne *et al.*, 1990). Besides the dendrites of granule cells, the molecular layer also contains different types of interneurons and axons coming from the EC and other sources. The molecular layer is divided in three parts, according to the differential origin of the incoming axons (Van Groen *et al.*, 2002). In the proximal third, closest to the granule cell body, granule cell dendrites receive axonal inputs from mossy cells. The middle and distal third of the molecular layer receive inputs from the medial and lateral EC, respectively.

1.1.3.1.1 Granule cell inputs

The EC is a part of the hippocampal formation that is located in the medial temporal lobe (Andersen *et al.*, 2007). It forms the main input and output structure of the hippocampus and is therefore often referred to as the gateway between hippocampus and neocortex. The entorhinal cortex receives processed sensory information from several neocortical sources. Cells in the superficial layers of the EC (layers II and III) provide the main input to the hippocampus, while the deep layers (IV-VI) make a minor contribution (van Groen *et al.*, 2003; Witter, 1993). The projection from the entorhinal cortex to the hippocampus is referred to as the perforant pathway. This pathway was first described by Cajal in 1911. It was called perforant pathway because its fibers run through the subiculum and hippocampal fissure, “perforating” it.

The EC is divided into a medial and a lateral region, which have distinct connectivity and convey different input information (Igarashi, 2016). The medial EC provides spatial information and projects to the middle third of the granule cell dendritic tree. The lateral EC provides non-spatial information (like odor and object information) and innervates the outer third of the granule cell dendrites (Deshmukh and Knierim, 2011; Hargreaves *et al.*, 2005; Young *et al.*, 1997).

The DG receives its major input from neurons in layer II of the EC. The fibers of layer II cells form mainly asymmetric, excitatory synapses on the dendritic spines of dentate granule cells. A small proportion of the fibers terminate on interneurons. In mice, at least in one of the strains (C57BL/6J), layer II cells project only to the DG and not to other hippocampal areas (van Groen *et al.*, 2003; Witter, 2007). Neurons in layer II of rats and monkeys on the other hand, also project to CA3 and subiculum (Witter and Amaral, 1991). Cells in layer III of the EC also project to CA3, and this projection is more prominent

in mice than in rats (Van Groen *et al.*, 2002). In conclusion, neurons in layer II of the EC provide the major, if not exclusive, input to the DG.

The perforant pathway is a divergent structure (Amaral and Witter, 1989). There are about five times more granule cells in the DG than projection neurons in layer II of the EC (200,000 EC layer II cells and 1 million granule cells in rat)(Amaral *et al.*, 1990). It was estimated that one perforant path fiber makes 15,000 to 19,000 synaptic connections with granule cells. One granule cell, on the other hand, receives more than 3,000 synaptic inputs from the EC (Patton and McNaughton, 1995).

Granule cells in the dentate gyrus also receive neuromodulatory inputs from dispersed brain regions like the locus coeruleus (norepinephrine), ventral tegmental area (dopamine) and septal nuclei (acetylcholine) (Andersen *et al.*, 2007). Finally, there are several types of interneurons which provide inhibitory feedback to the granule cells in the DG (see section 1.1.3.4).

1.1.3.1.2 Granule cell outputs

The axons of granule cells are called mossy fibers. Each granule cell gives rise to one mossy fiber (Amaral *et al.*, 2007). On their way to the CA3, mossy fibers run through the hilus, where they issue collaterals that synapse onto mossy cells and interneurons (Claiborne *et al.*, 1986). A mossy fiber is about 0.2–0.5 μm in diameter and gives rise to about seven smaller collaterals (Amaral *et al.*, 2007). Mossy fibers are unmyelinated axons (Amaral *et al.*, 2007). On their way to the CA3, mossy fibers run in a tight bundle just above the CA3 pyramidal layer (Blackstad *et al.*, 1970). This zone is visible without staining as a translucent area in brain sections and was therefore called “stratum lucidum” (Claiborne *et al.*, 1986).

Granule cell axons have an interesting characteristic that is not seen elsewhere in the mammalian nervous system: they form anatomically specialized synapses depending on the targets they innervate (CA3 pyramidal cells, mossy cells or interneurons)(Andersen *et al.*, 2007). Mossy fibers contain very large, complex boutons as well as small synaptic varicosities (Amaral *et al.*, 2007). The small synaptic varicosities (0.5-2 μm in diameter) terminate on inhibitory interneurons in the hilus. The large mossy fiber boutons (4-10 μm) are complex terminals, containing multiple release sites densely packed with synaptic vesicles. They terminate on mossy cells in the hilus and pyramidal cells in the CA3. While the number of small terminals innervating interneurons is around 200, there are only 20 to 30 large mossy fiber terminals along a single granule cell axon (Acsády *et al.*, 1998). This suggests that granule cells preferentially innervate inhibitory interneurons. However, because granule cell synapses onto mossy cells and pyramidal cells are much more intricate, they are more potent to activate their postsynaptic targets. Ramon y Cajal was the first who described the terminals of granule cell axons and called them mossy fibers due to the “mossy” appearance of the large boutons and synaptic varicosities.

Large mossy fiber boutons innervate equally remarkable structures on the postsynaptic target cells. The spines on CA3 pyramidal cells and mossy cells are unusually large and are engulfed by the giant mossy fiber boutons. These spines were called “thorny excrescences” because of their resemblance to the thorny protrusions on plants.

1.1.3.2 Adult-born granule cells

The DG is one of only a few regions in the mammalian brain where new neurons are continuously generated throughout life (Altman, 1962; Drew *et al.*, 2013). Adult neurogenesis is a multi-step process that starts with proliferation and fate specification of adult neural stem cells (Duan *et al.*, 2008). During maturation, newborn neurons undergo morphogenesis, migration, axonal and dendritic development. A large proportion of the newborn neurons die during the maturation process, but the surviving neurons become functionally integrated in the existing circuit (van Praag *et al.*, 2002). They acquire electrophysiological characteristics of mature dentate granule cells and eventually become indistinguishable from developmentally born granule cells. It was estimated that a substantial population (up to 10% in mice) of granule cells are adult-born neurons (Imayoshi *et al.*, 2008).

1.1.3.2.1 Unique physiological properties of immature granule cells

Immature, adult-born granule cells differ from mature granule cells in a number of ways. The main difference is the increased excitability and plasticity of immature neurons. It was shown that adult-born neurons exhibit excitatory responses to GABA, the major inhibitory neurotransmitter in the adult brain, up to approximately 24 days after mitosis (Ge *et al.*, 2005). This excitatory response is a result of high chloride concentrations in immature neurons. Both *in vitro* and *in vivo* studies have shown that immature neurons are more excitable than mature granule cells (Danielson *et al.*, 2016; Marín-Burgin and Schinder, 2012). Even though young neurons receive fewer and weaker inputs, they are equally or even more likely to be active as mature neurons (Danielson *et al.*, 2016; Mongiat *et al.*, 2009). Adult-generated granule cells also exhibit a period of heightened plasticity 4–6 weeks after mitosis. In this period, plasticity can be induced more easily in young neurons than in mature neurons (Ge *et al.*, 2007). The unique properties adult born neurons display during their development is likely to be tightly linked with their role in the network.

1.1.3.3 Mossy cells

Mossy cells are large, excitatory cells of the hilus. They receive inputs from granule cells and project to the proximal dendrites of ipsilateral as well as contralateral granule cells. This axonal projection is called the commissural/associational pathway. There are approximately 10,000 mossy cells in the DG of rats (Amaral *et al.*, 1990). Their large cell bodies (25–35 µm in diameter) are scattered in the hilus and are triangular or multipolar in shape. The dendrites of mossy cells form an extensive dendritic arbor that stays largely confined to the hilus. Occasionally, a dendrite crosses the granule cell layer and enters the molecular layer. This distribution indicates that mossy cells receive inputs from a wide range of granule cells and possibly also directly from the EC.

The somata and proximal dendrites of mossy cells are covered by numerous thorny excrescences. These large, complex spines are the termination sites of granule cell mossy fibers. The mossy fiber-thorny excrescence synapse is considered one of the most powerful excitatory synapses (Scharfman *et al.*, 1990). Mossy cells also receive excitatory input from CA3 pyramidal cells that send axon collaterals to the hilus (Scharfman, 1994).

Mossy cell axons project to the inner molecular layer of the ipsilateral and contralateral dentate gyrus. The contralateral projection targets granule cells at a similar septotemporal location as the cell bodies of origin. The ipsilateral projection, on the other hand, can spread very far in septotemporal direction. Mossy cells in the temporal part of the hippocampus in particular, have highly divergent axons that project to distant septal locations (Buckmaster *et al.*, 1996). Mossy cells also project to local inhibitory interneurons in the hilus and inner molecular layer. Because of their local connections with inhibitory interneurons and more distal connections with granule cells, it is thought that mossy cells are responsible for inhibition of local granule cells and excitation of distal granule cells.

1.1.3.4 Interneurons

Interneurons of the DG are a diverse population of cells, of which the majority play an inhibitory role in the circuit (Houser, 2007). Different types of interneurons can be distinguished based on morphological, neurochemical and electrophysiological characteristics. All DG interneurons use γ -aminobutyric acid (GABA) as their primary neurotransmitter (Houser, 2007). Interneurons are distributed in all layers of the DG and appear most concentrated in the hilus and at the interface of hilus and granule cell layer (Houser, 2007). Morphologically, interneurons of the DG can be divided into 2 broad classes based on the distribution of the axonal plexus. One group of interneurons terminates primarily at the perisomatic region and another group at the dendritic region of granule cells. The perisomatic-targeting interneurons can be further subdivided into cells targeting the soma and proximal dendrites (basket cells) and cells targeting axon initial segment (axoaxonic cells).

The most studied interneuron in the DG is the pyramidal basket cell (Amaral *et al.*, 2007). Pyramidal basket cells can be recognized by their large pyramidal-shaped cell bodies (25–35 μm in diameter), located predominantly at the border of the granule cell layer and hilus (Ribak and Seress, 1983). Basket cells receive inputs from granule cells, mossy cells and cells in the entorhinal cortex, and send axons to granule cells and interneurons of the DG. The 'basket' portion of the name refers to the dense pericellular plexuses their axons form around the cell bodies of granule cells. The axons of basket cells can cover large distances inside the granule cell layer, which allows a single basket cell to influence up to 10,000 (1%) of the granule cells.

Another major type of perisomatic-targeting interneurons has axons that make contacts with the initial segments of axons from granule cells (Somogyi, 1977). These are called axoaxonic or chandelier cells. The axon terminals of both basket cells and axoaxonic cells terminate close to the cell body of granule cells, which means that they have a strong influence over the output of granule cells (Houser, 2007).

Although they make up only about 10% of the neurons in the hippocampus, via strong connections with multiple granule cells, they exert a powerful control over the hippocampal circuit.

Apart from perisomatic-targeting interneurons, there is a variety of neurons targeting granule cell dendrites. These interneurons can be subdivided in several classes based on the location of their cell bodies and the distribution of their axons in the molecular layer. Interneurons with cell bodies in the hilus receive substantial input from dentate granule cells and can provide feedback inhibition. Interneurons with cell bodies in the molecular layer, on the other hand, receive input from the perforant path and provide feedforward inhibition.

In summary, interneurons in the dentate gyrus form a complex group of neurons that exert a powerful influence of granule cell activity by providing feedforward and feedback inhibition.

1.2. The function of the dentate gyrus

1.2.1. The role of the hippocampus in memory

Remembering specific events that happened in a particular context and at a particular time and place is a crucial cognitive ability that has been linked to the hippocampus. Appreciation for the role of the hippocampus in memory arose in the 1950s when the now famous patient H.M. had his hippocampus removed in an attempt to cure epilepsy (SCOVILLE and MILNER, 1957). This resulted in a severe anterograde amnesia (inability to form new memories). In the subsequent decades a range of lesion, electrophysiological, and optogenetic studies have associated the hippocampus with spatial, contextual and episodic memory processing (Goshen, 2014; Knierim, 2015).

The discovery of place cells in the hippocampus by O'Keefe and Dostrovsky in 1971 has led to the idea that the primary role of the hippocampus is to store spatial information (O'Keefe and Dostrovsky, 1971; O'Keefe and Nadel, 1978). Electrophysiological studies of place cell activity will be explained in the following sections. Here we briefly describe the most important studies that have implicated the hippocampus in spatial and non-spatial components of learning and memory.

Lesion studies in rodents have provided concrete evidence that the hippocampus is involved in spatial memory (Morris *et al.*, 1982). The most commonly used test for spatial memory deficits is the Morris water maze (Xavier and Costa, 2009). In this task, rodents are placed in a pool of opaque water and they learn the location of a hidden escape platform. Rats with hippocampal lesions were severely impaired in learning this task (Morris *et al.*, 1982). Other tasks that are used to test for hippocampal spatial deficits are the radial maze and contextual fear conditioning. Lesion studies targeting the dentate gyrus specifically have revealed that dentate gyrus granule cells are critically involved in spatial learning and memory (Xavier and Costa, 2009).

More recently, the incorporation of optogenetics into memory research has markedly increased our understanding of the hippocampal networks underlying learning and memory. In an elegant study by Liu *et al.*, optogenetics was used to investigate if a particular memory is encoded by a specific set of neurons in the hippocampus, so-called "engram cells" (Liu *et al.*, 2012). They labelled a set of granule cells in the DG that were activated during the encoding phase of a contextual fear conditioning task and showed that optogenetic reactivation of this set of cells was sufficient to induce freezing behavior, in an unrelated context. These findings provided the first proof that specific memories can be encoded by sparse populations of granule cells in the dentate gyrus.

1.2.2. The role of the hippocampus in spatial memory

Animals and human beings have a natural ability to remember places where they have been, orientate themselves and navigate effectively from one place to another. Squirrels for example are able to remember the place where they had hidden food in autumn and find the exact place months later, even when the forest is covered in a thick layer of snow (Jacobs+ and Limant, 1991). Certain birds can remember hundreds of locations where they had hidden seeds and nuts (Sherry, 1984). Where does this ability come from? Where is this information stored in the brain and how is it processed and updated with new information?

Several decades of research have revealed that the hippocampal formation has a specialized circuit for storing spatial information. Lesions of the hippocampus were shown to significantly impair spatial memory and learning (Morris *et al.*, 1982; Sutherland *et al.*, 1983). Certain cells in the hippocampus are thought to provide an animal with detailed information about its current location, direction and speed. These cells are thought to provide the basis for the so-called “cognitive map” of the brain, an internal representation of the environment (O’Keefe and Nadel, 1978). Just like a GPS tracks your location and calculates the best way to your destination, the cognitive map tells you where you are and how to reach your destination.

There are a few different types of spatially modulated cells in the hippocampal formation. In the following sections, I will present the five major types together with their characteristic spatial firing patterns.

1.2.2.1. Place cells

Place cells are cells that fire when an animal is at a specific place (Figure 1.3A). Place cells were discovered in 1971, when O’Keefe and Dostrovsky recorded unit activity in the hippocampus of freely moving rats (O’Keefe and Dostrovsky, 1971). They reported that the activity of certain cells in the hippocampus was closely related to the rat’s location. The cells fired at a high rate when the rat was at a particular part of the testing environment and were virtually silent when it was elsewhere. The location where a place cell was most active, was called the cell’s place field.

Subsequent experiments were carried out to determine which factors in the environment influence where a place cell fires (O’Keefe and Conway, 1978). Environmental manipulations like rotating or removing visual cues led to rotation or disintegration of place fields. This shows that visual cues exert a powerful influence on place cell firing. Other sensory information like olfactory and auditory cues also influence place cell activity (Shapiro *et al.*, 1997). In addition to these external sensory cues, place cells are also influenced by internal self-motion information from vestibular and proprioceptive systems (Hill and Best, 1981). It is a combination of this external and internal information that determines place cell firing.

Early extracellular unit recordings have demonstrated that place fields can remain stable for several months if the environment does not change (Thompson and Best, 1990). More recently, calcium imaging studies, which allow monitoring of ensemble activity of thousands of neurons over weeks, have shown that the ensemble activity of CA1 pyramidal cells is dynamic, yet a small subset of the population (15-25%) retained stable place fields, which allowed unambiguous representation of the environment (Ziv *et al.*, 2013).

If there is a change in the sensory input, place cells can change their firing fields (Robert U Muller and Kubie, 1987; Wilson and McNaughton, 1993). Place fields of a certain neuron may change in size or position, appear or disappear. These effects were called ‘remapping’. There are 2 main types of remapping: rate remapping and global remapping (S. Leutgeb *et al.*, 2005). In rate remapping, the locations of the place fields remain the same, but the firing rate changes (S. Leutgeb *et al.*, 2005). Rate remapping usually happens when there is a small change in the environment. If the change in the environment is big, or if the animal is moved to a different environment, place cells undergo global remapping (Robert U Muller and Kubie, 1987). In global remapping, both the location of the place fields and firing rate changes (S. Leutgeb *et al.*, 2005).

Figure 1.3. Five types of spatial cells. (A) Place cell. (B) Head direction cell. (C) Grid cell. (D) Border cell. For A, C and D a firing rate map is shown on the left and for B a directional firing polar plot, with peak firing rate indicated in hertz. On the right the path taken over whole trial is depicted (black line), with the locations where spikes were recorded in green. (E) Traces showing z-scores for firing rate (green) and speed (grey) for an entorhinal speed cell during 2 min of free foraging. Maximum values of firing rate and speed are indicated (left and right, respectively). Figures adapted from (Hartley *et al.*, 2013) and (Kropff *et al.*, 2015), with permission.

1.2.2.2. Other spatial cells in the hippocampal formation

While place cells fire when an animal is at a specific location, independent of its orientation, **head direction cells** preferentially fire when the animal is facing a certain direction ((Taube *et al.*, 1990); Figure 1.3B). Head direction cells are found in the EC, pre- and postsubiculum and also outside the hippocampal formation (Sargolini *et al.*, 2006; Taube, 2007; Taube *et al.*, 1990). Head direction cells are highly active when the animal's head is oriented in the cell's preferred direction and fire at low rates when the animal is facing any other direction. All head direction cells together act like a compass, representing the full range of orientations. The firing of head direction cells relies on distal cues in the environment (Taube *et al.*, 1990). If distal cues are rotated then the preferred firing direction of all head direction cells rotates, and this rotation is coherent with the rotation of the positions of place fields in the environment (Knierim *et al.*, 1995). Furthermore, it was shown that lesions of the head direction cell network do not abolish hippocampal place field formation, but result in instability of place fields (Calton JL1 *et al.*, 2003).

Another type of spatial cells was found to fire at multiple locations that form a regular triangular pattern (Figure 1.3C; (Hafting *et al.*, 2005). These cells were called **grid cells**. They were discovered in the medial entorhinal cortex (MEC) in 2005 and have since also been found in pre- and parasubiculum (Boccaro *et al.*, 2010). Grid cells are thought to play a role in path integration, the use of self-motion cues to estimate a location (McNaughton *et al.*, 2006). Because the MEC forms a major input to the hippocampus, it is believed that grid cells provide a basis for place cell firing (McNaughton *et al.*, 2006). Theoretical and computational studies have suggested that place cells could be formed by linear summation of grid cell firing (Barry *et al.*, 2006; Solstad *et al.*, 2006) and recent experiments have confirmed that place cells in the hippocampus receive functional inputs from grid cells, as well as from head direction cells and border cells (Zhang *et al.*, 2014). However, there is accumulating evidence that the simple place to grid cell hypothesis might not be accurate (Bush *et al.*, 2014). Studies investigating spatial cells in rat pups have shown that place cells are present before grid cells in early postnatal development (Langston *et al.*, 2010; Wills *et al.*, 2010). Lesions of the medial entorhinal cortex in adult rats showed that place cells still exist after elimination of grid cells, albeit with decreased precision and stability (Hales *et al.*, 2014). Based on these observations, it was suggested that grid cells and place cells are not successive stages in spatial processing, but they are complementary and interact to form a reliable representation of space (Bush *et al.*, 2014).

Border cells are a fourth class of spatial cells. They fire at a specific distance and direction from a border in the environment (Figure 1.3D; (Lever *et al.*, 2009; Savelli *et al.*, 2008; Solstad *et al.*, 2008)). Border cells were identified in subiculum, parasubiculum, and medial entorhinal cortex. The most recently discovered type of spatial cell is the **speed cell** (Figure 1.3E; (Kropff *et al.*, 2015)). Speed cells were found in the MEC and hippocampus. Their firing rate shows a linear relationship to the speed the animal is moving.

Together, place cells, grid cells, head direction cells, border cells and speed cells are thought to generate a code that animals use to know their location in space. Spatial cells are mainly located in the hippocampal formation, where they form a network of functionally connected neurons. The firing of one type of spatial cells is often dependent on the correct functioning of other cells. How exactly spatial information flows through the hippocampal subfields and which forms of computation each subfield performs remain to be elucidated.

1.2.2.3. Place cells in the dentate gyrus

The first detailed description of place cells in the DG was provided by Jung and McNaughton in 1993. Based on Ranck's classification criteria from pyramidal cells in CA1 and CA3, cells recorded in the DG were classified into high-rate units and low-rate units. They found that the majority of cells recorded in the granule cell layer of the DG (88%) had mean rates below 0.5 Hz. The remaining cells fired at high rates (>2 Hz) and exhibited characteristics typical for interneurons. Because units with high firing rates are easier to detect than units with low firing rates, earlier reports had identified high firing rate units as granule cells (Rose *et al.*, 1983). Based on recordings in anesthetized rats, Mizumori proposed in 1989 that granule cells fired at very low rates and high firing rate cells were interneurons (Mizumori *et al.*, 1989). In addition, it was shown that cells in the DG could be classified based on a firing suppression ratio during paired-pulse stimulation of the perforant path. Putative granule cells showed strong paired-pulse suppression and putative interneurons showed little or no firing suppression. Jung and McNaughton recorded single unit activity in the dentate gyrus of freely moving rats and used Mizumori's criteria to classify units into granule cells and interneurons (Jung and McNaughton, 1993). The mean firing rate of the granule cells identified in this way was well below 1 Hz (mean, 0.15 ± 0.13 Hz; range, 0.01-0.48 Hz). They found that the granule cells showed spatially selective firing when the rat performed a spatial memory task. The place fields of granule cells were smaller than those of pyramidal cells and granule cells tended to have more subfields. The spike width of granule cells was broader than interneurons, but smaller than CA3 pyramidal cells. Granule cells fired single spikes as well as brief bursts. Bursts were composed of similarly sized spikes or spikes of decreasing size and were most often observed when the rat was resting.

More recently, Neunuebel and Knierim have demonstrated that the DG contains at least 2 different classes of spatial cells, with distinct physiological characteristics (Neunuebel and Knierim, 2012). They recorded unit activity in the DG while rats were sleeping or exploring an open arena. Putative excitatory neurons were divided in 2 groups: units with single place fields and units that had multiple place fields in the recording arena. They found that units with single fields tended to be recorded together with several other units that were mostly active during sleep and silent during behaviour. This suggests that the single-field cells were localized in a densely packed cell layer with only a small fraction cells active during behaviour. Since previous studies had shown that only a small fraction of cells in the granule cell layer is active in a given environment, it was likely that single-field cells represented granule cells (Chawla *et al.*, 2005). Because cells with multiple place fields were mostly recorded on tetrodes in or near the hilus, they suggested that multiple-field cells could be mossy cells or immature granule cells.

They further examined the firing characteristics of single- and multiple-field cells. Cells with single fields had significantly larger place fields than multiple-field cells, but the total area was comparable. The mean firing rate recorded during sleep was higher for multiple-field cells than for single-field cells (0.36 Hz vs. 0.05 Hz). During behaviour, there was no difference in mean firing rate. Cells with multiple fields were also more bursty. Furthermore, they detected differences in the spike waveform. The spike slope of the single-field cells was significantly steeper than the slope of multiple-field cells. In addition, single-field cells showed larger differences in spike amplitude between the 4 channels of the tetrode. The spike amplitude was often much larger on one of the channels, whereas the spike amplitude of multiple-field cells was similar for the 4 tetrode channels. This means that for single-field cells, one of the tetrode wires was very close to the cell. The small differences in spike amplitude of multiple-field cells indicate that the cells were located further away from the tetrode. Since it has been shown that mossy cells can be recorded over large distances ($>100\text{ }\mu\text{m}$), this observation adds support for the idea that multiple-field cells are mossy cells (Henze and Buzsáki, 2007).

Taken together, this study demonstrated that single- and multiple-field cells constitute distinct cell types. Based on the evidence explained above, it was proposed that mature granule cells fire in single locations, whereas cells with multiple fields may correspond to mossy cells or newborn granule cells.

1.2.3. The role of the dentate gyrus in pattern separation

1.2.3.1. Pattern separation is a computational concept

The process whereby distinct, but similar neuronal patterns of input information are transformed into more distinct neuronal representations is called pattern separation (Santoro *et al.*, 2013). The DG is believed to perform pattern separation by making small differences in input information from the EC more different and delivering well-separated patterns of output information to the CA3 cells (Marr, 1971).

The idea that the DG is involved in pattern separation was developed based on theoretical and computational models of the function of the hippocampus. There are several reasons why the DG seems to be well-suited to support a pattern separation function. Firstly, the number of granule cells is about five times larger than the number of EC neurons projecting to the DG (1,000,000 granule cells versus 200,000 EC cells in rats; Amaral 1990). In theory, a high number of granule cells could allow recruitment of distinct granule cell populations in response to different experiences. Secondly, the DG has a sparse coding scheme. While the number of granule cells is high, only a very small subset (< 5%) is active at any one time (Chawla *et al.*, 2005; Alme *et al.*, 2010). Immediate early gene (IEG) studies and *in vivo* recordings have shown that only a small fraction of DG granule cells is active at any given moment during behaviour (Alme *et al.*, 2010; Chawla *et al.*, 2005; Deng *et al.*, 2013; Jung and McNaughton, 1993; Neunuebel and Knierim, 2012). Sparse coding is beneficial for pattern separation, because the number of patterns that can be stored is inversely proportional to the number of active cells. The high number of granule cells combined with sparse coding could theoretically allow distinct sets of DG granule cells to be activated, even when input patterns are very similar. Then the chance that CA3 would receive input from the same set of granule cells would be very low. Besides, *in vitro* experiments have shown that single granule have the potential to activate CA3 pyramidal cells (Henze *et al.*, 2002). Although single spikes, evoked in granule cells by intracellular current injection, were generally not sufficient to activate postsynaptic CA3 target neurons, spike trains in granule cells reliably discharged CA3 pyramidal cells and interneurons (Henze *et al.*, 2002). The potential to activate postsynaptic cells, together with the high number of granule cells with sparse activity, suggest that granule cells could play a powerful role in separating similar patterns of input information to provide CA3 with a well-separated pattern of firing.

1.2.3.2. Experimental evidence for the pattern separation function of the DG

Experimental evidence for the proposed roles of DG and CA3 was provided by IEG experiments. IEGs are rapidly and transiently transcribed when a neuron is activated, and can be used as a method to assess population activity of neuronal cells. Several IEG studies have shown that only a very small fraction of granule cells (1-5%) is activated when an animal explores a given environment (Alme *et al.*, 2010; Chawla *et al.*, 2005; Deng *et al.*, 2013). Some IEG studies have also provided support for the pattern separation hypothesis by demonstrating that distinct populations of granule cells were recruited in response to small changes in environmental inputs (Chawla *et al.*, 2005; Deng *et al.*, 2013) or task demands (Savva *et al.*, 2011). When rats explored 2 different environments (a square platform with

walls and a rectangular, raised platform without walls in a different room with different lighting), a higher proportion of granule cells expressed the IEG Arc compared to when they explore only one environment or stay in their home cage (Chawla *et al.*, 2005). When rats performed 2 different tasks that required different behavioural strategies, more granule cells expressed the IEG *zif268* than when they repeated the same task twice (Satvat *et al.*, 2011). These studies suggest that the DG encodes different behavioral experiences by recruiting different populations of granule cells.

While IEG studies provided support for the pattern separation hypothesis, electrophysiological recordings have sparked controversy in the field. Single unit recordings have shown that units recorded in the DG are activated in many environments (Alme *et al.*, 2010; Leutgeb *et al.*, 2007). This is contrary to the idea that different granule cells are recruited to encode different experiences. Leutgeb and colleagues showed that the same population of granule cells (~1% of the total granule cell population) was activated repeatedly in different environments. Their data indicated that granule cells fire at different rates to distinguish different environments. As rats explored different environments, some neurons increased firing rate, while others decreased activity. The experimental design was slightly different from the IEG studies. While only 2 different boxes were used in the IEG experiments, Leutgeb also used a gradually morphing box to assess the effect of minimal changes in the environment. Units were classified as granule cells based on the location of the recording tetrode, spike waveform and firing rate. Recordings were made from the dorsal part of the DG granule cell layer. Unit activity was recorded from 67 putative granule cells. The experiment showed that changes in environmental information were represented by changes in patterns of activity within the same population of granule cells, not by recruiting different populations of cells. This is one possible way the DG could de-correlate incoming information, but it is not consistent with the pattern separation prediction made by computational models. Thus, the original computational models should be revised to take the option of rate coding into account.

The differences between the findings of electrophysiological and IEG studies can be explained by the strengths and limitations of the techniques. IEG studies have the advantage that many cells can be visualized and identified at the same time, which allows assessment of population coding. Using single-unit electrophysiological methods it is not possible to identify the recorded cell type, and only a small number of units can be recorded at the same time. Hence, unit recording has limited power to test population coding. However, the high temporal resolution (recording of individual spikes) allows assessment of rate coding, which is not possible using IEG methods.

The observation that the same, small population of granule cells is repeatedly activated in different environments has led to the idea that the population of neurons in the DG is not homogenous (Neunuebel and Knierim, 2012). Indeed, it seems likely that the DG contains different neuronal populations with distinct activation threshold. Neurons with low threshold are activated more easily and will fire regardless of the environment that is experienced. Neurons with high threshold on the other hand, may never be active in any environment. This theory was proposed by Alme and colleagues (Alme *et al.*, 2010). They further advocated that the active population is composed of newborn granule cells, while the inactive cells are “retired”, mature granule cells. Although the increased excitability and

plasticity of adult-born neurons likely gives them a special role in the circuit, more direct experiments are necessary to demonstrate the role of mature and newborn cells in the DG.

To summarize, computational models and some IEG experiments have suggested that the DG recruits distinct populations of granule cells to encode distinct events. *In vivo* electrophysiological recordings, on the other hand, have indicated that the DG recruits overlapping populations of granule cells to encode different events, and distinguishes between events using changes in firing rate. This difference may be explained by differences in the techniques used to study the phenomenon or by the fact that different populations of cells use different mechanism to perform pattern separation.

1.2.3.3. Pattern separation and pattern completion in other hippocampal areas

The CA3 is thought to play a role in pattern completion (Marr, 1971). Pattern completion is a process whereby incomplete neuronal representations are completed based on previously stored representations (Treves and Rolls, 1994). CA3 pyramidal cells have recurrent collaterals, axons that synapse onto dendrites of pyramidal cells within the same region. In fact, the main input of CA3 pyramidal cells comes from other pyramidal cells in the CA3 (Amaral *et al.*, 1990). Less than one-third of their inputs come from other cell populations (Amaral *et al.*, 1990). Recurrent collaterals make it possible to perform auto-association (McNaughton and Morris, 1987). This is the strengthening of connections among a set of pyramidal cells, which allows reactivation of this set of cells from an incomplete input pattern. This way, the CA3 can restore partial or noisy input patterns to obtain a complete, original pattern of activation.

The roles of the DG, CA3 and CA1 in pattern separation and pattern completion were tested in a series of local–global cue mismatch (double-rotation) experiments (Knierim and Neunuebel, 2016). In these experiments, rats explored a circular track while place cell activity was recorded in different hippocampal subregions. The local cues on the track and global cues on a curtain around the track were rotated in opposite directions and the changes in place cell activity were measured. It was shown that the changes in the DG were larger than the changes in the medial EC, supporting the theory of pattern separation in the DG (Knierim and Neunuebel, 2016). The changes in the CA3 on the other hand, were smaller than in the DG, providing support for a pattern completion role for the CA3 (Neunuebel and Knierim, 2014). It was also shown that the representations of the mismatch environments were more different in CA1 than in CA3 (Lee *et al.*, 2004). Some authors have proposed that CA1 performs a linear summation of CA3 and EC inputs (Knierim and Neunuebel, 2016). It was also proposed that its function is to compare the inputs from CA3 and EC (Hasselmo, 2005). How exactly the CA1 would perform these computations is not clear.

1.2.3.4. Potential behavioural correlate of pattern separation

Although pattern separation originally referred to a computational process, some researchers have started using it to describe behavioural phenomena (Santoro *et al.*, 2013). For example, certain behavioural tasks require an animal to distinguish similar stimuli like similar environments or nearby locations. Influenced by the pattern separation theory, behavioural scientists generated the idea that the DG could play a role in the discrimination of similar stimuli (Bakker *et al.*, 2008; Clelland *et al.*, 2009; McHugh *et al.*, 2007; Nakashiba *et al.*, 2012). The ability to discriminate similar stimuli is sometimes referred to as “behavioural pattern separation”. However, it should be kept in mind that pattern separation as computational and behavioural concept are fundamentally different. In this thesis, we use the term pattern separation to refer to the computational process whereby the output firing patterns of a network are less similar than the input firing patterns. The behavioural process of distinguishing similar information will be referred to as “behavioural discrimination”.

In the following paragraphs, I will present a number of behavioural experiments that have tested the idea that the DG is involved in behavioural discrimination. Although pattern separation may lay at the basis of behavioural discrimination, these studies do not prove that there is a link between the 2 processes.

Inspired by the pattern separation hypothesis, behavioural scientists have developed experiments to test the idea that the DG is involved in discriminating similar contexts. McHugh and colleagues demonstrated a role for dentate granule cells in discriminating similar contextual information (McHugh *et al.*, 2007). They generated a mouse line in which NMDA receptors were abolished specifically in DG granule cells. Using a contextual fear conditioning task, they showed that the NMDA receptor knock out mice were able to distinguish 2 environments that had substantially different sensory cues (a metal grid floor and white ambient lighting versus a smooth plastic floor and red lighting). However, knock out mice were impaired when the 2 environments were less distinct (identical metal grid floor, but unique odours, roofs, and lighting). This shows that dentate granule cells are involved in distinguishing similar environmental information.

Several studies have proposed a specific role for neurogenesis in context discrimination. Mice with reduced neurogenesis were impaired in a touch screen-based location discrimination task if stimuli were close together, but not if they were far apart (Clelland *et al.*, 2009). Similarly, in a radial arm maze task, they showed deficits only when they had to distinguish between adjacent arms, not when the arms were separated (Clelland *et al.*, 2009). Depleting adult-born neurons also caused deficits in discriminating similar contexts in a fear conditioning task (Tronel *et al.*, 2012). Increasing neurogenesis, on the other hand, improved the performance in touch screen location discrimination and contextual fear conditioning (Creer *et al.*, 2010; Sahay *et al.*, 2011).

Taken together, these data provide strong evidence that the DG plays an important role in behavioural discrimination.

1.2.4. The role of adult-born granule cells in the DG

As explained in section 1.1.3, immature granule cells undergo a period of increased excitability and plasticity. These unique properties suggest that adult-born neurons play a special role in information processing in the hippocampus. Behavioural studies have demonstrated a role for adult-born granule cells in a particular set of hippocampal-dependent tasks (Cameron and Glover, 2014). However, the exact contribution of adult born neurons is still unclear.

Several studies have investigated the influence of newborn neurons on spatial learning, yet the exact role of neurogenesis is still disputed (Arruda-Carvalho *et al.*, n.d.; Snyder *et al.*, 2005; Wojtowicz *et al.*, 2008). Neurogenesis does seem to play an important role when animals are challenged to discriminate very similar locations or contexts (Shors *et al.*, 2002). As explained in section 1.2.1.1, behavioural studies have consistently shown an effect of neurogenesis on behavioural discrimination (Becker, 2016; Clelland *et al.*, 2009; Creer *et al.*, 2010; Nakashiba *et al.*, 2012; Sahay *et al.*, 2011; Tronel *et al.*, 2012).

Some theories were developed to explain the mechanism by which young granule cells influence circuit function (Piatti *et al.*, 2013). According to one theory, young granule cells modulate the activity of mature granule cells, mainly through interactions with local interneurons. This is in line with recent findings by Drew and colleagues, who showed that adult-born granule cells robustly recruit local interneurons, which synaptically inhibit mature granule cells (Drew *et al.*, 2015). Another theory suggests that young granule cells themselves provide the DG's main output (Piatti *et al.*, 2013). One theory even suggested that mature granule cells become completely inactive or 'retire' after a short period of activity (Alme *et al.*, 2010). However this theory seems rather unlikely since recent studies have shown activity in both mature and newborn granule cells (Danielson *et al.*, 2016; Pilz *et al.*, n.d.). A more plausible hypothesis suggests that mature granule cells use sparse coding to represent very specific experiences, while immature neurons may respond to a broader set of events to provide a relatively low-information representation of many experiences (Aimone *et al.*, 2011). The combination of mature and immature granule cells would then provide a complete representation of many events with high information content.

1.3. Thesis objectives

1.3.1. Limitation of extracellular unit recording

Extracellular unit recording is a powerful technique to characterize the activity of individual cells in a behaving animal. The high temporal resolution of electrophysiological recordings allows for monitoring of individual spikes. Small diameter recording wires (<50 μm for tetrodes) can be implanted in the brain with minimal brain damage and minimal discomfort for the animal. This permits recording of neuronal activity while the animal is awake and engaged in tasks or normal behaviour. Long term recordings are possible without degeneration of the signal, which permits monitoring of individual units over many weeks or even months (Kentros *et al.*, 2004).

The first extracellular unit recordings were performed in 1939 and the technique is still widely used today (Renshaw *et al.*, 1940). There is no doubt that this technique has made a considerable contribution to our understanding of how the brain works. However, extracellular unit recording has one major shortcoming. It is not possible to determine the anatomical identity of the recorded cells.

The standard electrophysiological recording procedure involves slowly moving the tetrodes deeper into the brain until the target area is reached. While tetrodes are lowered, their depth is tracked and the location in the brain is estimated based on characteristic changes in LFP and spike activity. When recordings are finished, histological analysis of brain sections is performed to validate that recordings were made from the desired brain region. Although histological analysis may reveal the final location of recording electrodes, it does not provide information about the recorded cell type. A tetrode located in the brain is surrounded by many cells and often multiple cell types. In some brain areas, cell types can be distinguished based on spike waveform or firing rate. However, in areas like the DG, where several cell types are located closely together, it is currently impossible to verify the cell type based on electrophysiological data alone.

Previous studies have assumed that recordings were made from granule cells because recording sites were histologically located in the granule cell layer and granule cells are the most abundant cell type in the DG (Jung and McNaughton, 1993; Leutgeb *et al.*, 2007). However, if the majority of granule cells are silent, this argument becomes feeble. The DG does not only contain granule cells, but also mossy cells and several types of interneurons. In addition, neurogenesis continuously produces new neurons, so the granule cell population is composed of mature and newborn granule cells at different stages of maturation.

Histological analysis of recording sites is sometimes hard to interpret. In many cases it is not clear in which layer of the DG a tetrode was located. Published studies have shown recording sites near the interface between the granule cell layer and hilus. A tetrode at this location could possibly record activity from newborn and mature granule cells, as well as interneurons and mossy cells at the same time. Furthermore, it was shown that mossy cells can be recorded on tetrodes located 300 μm from each other (Henze and Buzsáki, 2007). Thus even when a tetrode is located in the granule cell layer, it would be uncertain if a recorded unit is a granule cell or mossy cell. Moreover, in some experiments the animal was terminated many days after the recordings were made. It is known that tetrodes can drift inside the

brain tissue over time, even if they were lowered very slowly to the target area (Neunuebel and Knierim, 2012). Therefore the tetrode location on the day of recording may be different from the histologically identified location. Taken together, histological analysis is inadequate to positively identify cell types in the DG.

Because histological analysis of recording sites is insufficient to identify cell types, attempts have been made to classify cells in the DG based on other criteria. Using primarily physiological criteria, Neunuebel and Knierim have demonstrated that the DG contains at least 2 different classes of cells with distinct physiological characteristics (Neunuebel and Knierim, 2012). Based on several lines of evidence, they proposed that cells that fire in single fields in a given environment are mature granule cells and cells with multiple place fields are mossy cells or immature granule cells. Despite the weight of the evidence they provided, the authors acknowledge that conclusive evidence of the firing characteristics of individual cell types requires different techniques, like juxtacellular recording or genetic labelling techniques. Nevertheless, this study greatly advanced our awareness of the variety of firing patterns in the DG. The demonstration that different firing patterns correspond to different cell types has important implications for functional theories of the DG (e.g., sparse coding and pattern separation). It also increases our understanding of how spatial information is processed in the DG. Although the combined evidence of this and previous studies is strong, it is important that the observed firing patterns are validated using techniques that conclusively identify the recorded cell type.

1.3.2. Optogenetic identification of granule cells

Extracellular unit recording has proven to be an invaluable technique to study the role of individual neurons in behaving animals. Notwithstanding the fact that a great amount of knowledge has been acquired using this technique, it does not offer precise anatomical information. Hence identification of the cell or cell type that generated the recorded activity has remained problematic.

The DG contains multiple excitatory cell types with distinct roles in the hippocampal circuit. Due to the limitation of extracellular recording to identify cell types, the role granule cells play in hippocampal information processing has remained controversial. Therefore, it is important to identify granule cells and define their role in the circuit.

Recent technical advances have opened up the possibility to identify and characterize the activity of individual cell types in awake, behaving animals. By combining extracellular recording with optogenetics, it has now become possible to identify neuronal cell types during *in vivo* electrophysiological recording (Lima *et al.*, 2009). The method is based on expressing light-activated channels like channelrhodopsin-2 (ChR2) in specific neuronal cell types. Illumination of ChR2-expressing neurons with brief flashes of blue light triggers short latency reliable action potentials. These action potentials can be detected by extracellular single-unit recording. With this method, ChR2-tagged neurons can thus be identified electrophysiologically and their physiological activity can be recorded during behavior.

It should be noted that manipulating the activity of neurons by optogenetics may result in a behavioural response. Indeed, Schoenenberger and colleagues showed that optogenetic silencing of place cells inside their place field can cause changes in spatial firing properties (remapping and lasting changes in firing rate)(Schoenenberger *et al.*, 2016). Diamantaki and colleagues have shown that optogenetic activation can induce place cell activity in previously silent granule cells (Diamantaki *et al.*, 2016). This ‘recruitment’ of silent granule cells into the coding population was most efficient if the granule cells were activated by theta-rhythmic trains of action potentials and under novelty. Together, these experiments show that optogenetic manipulation may result in changes in place cell activity.

Optogenetics is a relatively new technique that makes use of light to control the activity of neurons or other cell types (Boyden *et al.*, 2005; Nagel *et al.*, 2003). Neurons expressing light-sensitive proteins like ChR2 can be activated by light with millisecond precision. ChR2 is a light-gated nonspecific cation-permeable channel derived from algae. Blue light (~480 nm) induces a conformational change in the channel, opening of the pore and influx of positive ions, which depolarize the neuron and lead to generation of action potentials. Variants of ChR2 have been developed with altered kinetics, photocurrents, wavelength-, or ion selectivity. The H134R mutant of ChR2 for example, generates larger photocurrents than wild-type ChR2, but shows slower channel kinetics (Nagel *et al.*, 2005). In a similar way, light-driven pumps are used to silence neurons. Proteins like halorhodopsin and archaerhodopsin pump chloride ions into the cell or protons out of the cell, thereby hyperpolarizing and thus inactivating the cell.

To target the expression of ChR2 to genetically-defined neuronal populations, a variety of methods can be used (Cardin *et al.*, 2010). The use of viral vectors and transgenic mice are the most common strategies. *In utero* electroporation is also effective, but more invasive and less practical (Huber *et al.*, 2008). Viral-mediated gene delivery is a powerful strategy to achieve high levels of ChR2 expression in neurons. To target the expression of ChR2 to the desired cell type, cell type-specific promoters may be used. Targeted expression can also be achieved using the Cre-loxP system. In this approach, a Cre-dependent viral vector is injected in a Cre-expressing mouse driver line. Cell type specificity is obtained by the unique Cre expression pattern of the transgenic mouse line. Viral vectors with a strong general promoter and Cre-dependent expression of ChR2 are injected in the mouse brain. Cre-dependent expression of ChR2 can be achieved by inserting a loxP-flanked (‘floxed’) STOP sequence upstream of the ChR2 coding sequence or by using a double-floxed inversed open reading frame of ChR2 (Atasoy *et al.*, 2008; Cardin *et al.*, 2010; Kuhlman and Huang, 2008). In cells expressing Cre recombinase, the ChR2 sequence will be inverted or the STOP sequence will be excised, resulting in expression of ChR2.

One disadvantage of the use of viral vectors is variability in the number of transduced cells between animals. This limitation can be overcome by the use of transgenic animals. Recently generated transgenic mouse lines with Cre-dependent opsin-expression have greatly facilitated the expression of opsins in a wide range of cell types (Madisen *et al.*, 2012). These Cre-dependent reporter lines can be crossed with any of the available Cre-driver lines, which show cell type-specific expression of Cre recombinase. Combining a cell type-specific Cre-driver line with a Cre-dependent opsin line results in expression of the opsin in the desired cell type. This powerful approach has made it possible to control the activity of a wide range of neuronal cell types.

When targeted expression of ChR2 in the desired population of neurons is achieved, labelled neurons can be activated by light and detected by extracellular recording methods. It has been shown that illumination of ChR2-expressing neurons with brief flashes of blue light induces short latency reliable action potentials (Lima *et al.*, 2009). This study further claimed that the spikes induced by light stimulation were indistinguishable from spontaneous spikes, suggesting that triggering spikes with ChR2 was not perturbing waveform shape (Lima *et al.*, 2009). This is important because spike sorting strategies are based on similarity in waveform. In conclusion, by combining extracellular unit recording with optogenetics, ChR2-labeled neurons can be reliably identified *in vivo* and their activity can be characterized.

1.3.3. Thesis objectives

The objective of this PhD thesis is to demonstrate the feasibility of optogenetic identification of granule cells in the DG. To establish the technique, transgenic mice expressing ChR2 in dentate granule cells will be used. Illumination of the DG induces activity in ChR2-expressing cells, which can be monitored using tetrode-based extracellular unit recording. Our goal is to evaluate the feasibility of this technique, expose its limitations and difficulties, and to solve potential problems. Developing this technique is essential to positively identify granule cells in extracellular recordings and characterize their electrophysiological activity *in vivo*. Optogenetic identification of granule cells *in vivo* would allow us to determine the firing properties of granule cells during behaviour. Understanding the behavioural correlates of granule cell activity, in particular the spatial firing patterns of granule cells, would provide important insight into how spatial information is represented in the DG, which will contribute to our understanding of spatial information processing in the hippocampus. Furthermore, information about the physiological activity of granule cells is fundamental for developing computational models and functional theories about the role of the hippocampus.

2. Materials and Methods

2.1. Materials list

Chemicals	
Plating Solution	5% platinum solution (5% chloroplatinic acid, HCl, Lead-II-Acetate)
Dental cement	Meliodent Rapid Repair, Heraeus Kulzer, NY, USA
5-Bromo-2'-Deoxyuridine (BrdU)	Cat#B5002, Sigma, USA
Formaldehyde 36%	VWR International, Radnor, PA, USA
Paraformaldehyde	Cat # 16005-1KG-R, Sigma
Tissue cryoprotectant solution	25% Glycerin, 30% Ethylene Glycol in 0.1M phosphate buffer (PB)
Cresyl Violet Acetate	C5042-10g, Sigma
Materials for construction of Versadrive8 Optical	
Versa Drive 8 Optical Kit	Neuralynx, Inc., Bozeman, MT, USA
Tetrode wire	17 μ m polyimide-coated Pt-Ir (90/10%) wires. California Fine Wire Company, Grove Beach, CA, USA
Mono-Fiber Optic Cannula	MFC_200/245-0.37_15.0mm_ZF1.25_FLT. Doric Lenses Inc, Quebec, Canada
Polyimide Tubin	0.20 mm (Inside diameter). MicroLumen, Oldsmar, FL USA
Anchor Screws	Stainless Steel, 00-90 x 0.0625" Binding Head
Equipment	
Digital Lynx 4SX 64ch Package	Neuralynx, Inc., Bozeman, MT, USA
HS-18 MM Double recording cable	Neuralynx, Inc., Bozeman, MT, USA
473 nm Blue DPSS Laser System	Laserglow Technologies, Ontario, Canada
3mm Laser Shutter	LS3T2 Vincent Associates, Rochester, NY, USA
Optic Fiber Patch Cord	Mono-fiber Optic Glass cord, NA=0.22, ZF 1.25 connector
Arduino board	Arduino UNO

2.2. Methods

2.2.1. Animals

All experimental and surgical procedures were approved by the Biological Resource Centre (BRC) Institutional Animal Care and Use Committee (IACUC) of Biopolis (Singapore).

To tag genetically defined DG neurons with ChR2, we crossed a Cre driver line with a ChR2 reporter line (Tg(POMC-cre)¹Lowl × Ai32). Both mouse lines were obtained from The Jackson Laboratory. In the POMC-Cre driver line, the mouse pro-opiomelanocortin-alpha (POMC) promoter drives expression of Cre primarily in the granule cells of the dentate gyrus. Ai32 (RCL-ChR2(H134R)/EYFP) mice express an improved Channelrhodopsin-2 (ChR2(H134R)) that is fused to enhanced yellow fluorescent protein (EYFP) after exposure to Cre recombinase. Without Cre recombinase, the expression of the fusion protein is blocked by an upstream STOP cassette that is flanked by two LoxP sites. Expression of Cre removes the STOP cassette, allowing expression of ChR2-EYFP.

Breeding was done with one heterozygous (POMC-Cre^{+/-}) and one homozygous Ai32 mouse. Mice were genotyped and the Cre-positive offspring (abbreviated here as POMC-ChR2Y) were used for all experiments, unless stated otherwise. Cre-negative mice were used as control mice to test the effect of light stimulation in tissue without ChR2 expression.

The total number of mice used for the *in vivo* experiments was 22. Each of these mice was implanted with a micro-drive containing 8 tetrodes and 1 optic fibre (except for one micro-drive which contained only 7 tetrodes and 1 optic fibre). Histological analysis of tetrode tracks showed that 8 Pomc-ChR2Y mice had at least one tetrode that ended in or had passed through the DG. In the remaining 14 mice, either none of the tetrodes reached the DG or the quality of the brain sections was not good enough to identify tetrode tracks. The total number of tetrodes that ended in the granule cell layer of the DG was 20 and the number of tetrodes that had passed through the granule cell layer was 8.

Mice were housed in transparent cages in a temperature and humidity controlled environment (20-22°C; 50-60%) on a 12 h light/dark cycle. Mice were habituated to being picked up, handled and weighed before the start of experimental procedures. The amount of food was restricted to provide motivation for the behavioural task. Food restriction was adjusted so that mice showed good performance in the task while keeping at least 85% of *ad libitum* body weight. Water was available at any time in the home cage.

2.2.2. Construction of VersaDrive

The VersaDrive 8 Optical was used for optogenetic stimulation and electrophysiological recording in freely moving mice. The drive housed 8 independently movable tetrodes within a circular region 1.2 mm in diameter (0.5 mm spacing between adjacent electrodes) and an optical fibre in the centre. Tetrodes were made by twisting four strands of 17 µm polyimide-insulated platinum/iridium wires. The twisted wires were heated for 2 min at 200 °C to fuse the insulation without short-circuiting the electrodes. The

electrode tips were cut with sharp serrated scissors and gold plated to reduce the impedance to 200–300 k Ω measured at 1 kHz. Tetrodes and optic fibre could be moved independently by turning small screws. One full rotation of the screw moved the tetrode or optic fibre by 250 μ m. The weight of the fully-assembled drive was approximately 3.2 g and did not restrict the free movement of the mouse. During recording, when the recording cables were connected to the drive, the weight was partly lifted by a pulley and counterweight, which allowed the mouse to explore freely.

2.2.3. Spatial exploration

Spatial exploration consisted of 3 sessions of approximately 10 min during which the mouse was foraging for small crumbs of chocolate cereal inside an open box (60 cm x 60 cm; 50 cm high) with black floor and black walls (Figure 2.1). A white A4 paper was placed on one of the walls inside the enclosure to serve as a visual cue.

Figure 2.1. Spatial exploration setup.

2.2.4. Surgery and tetrode lowering

Mice were anaesthetized with isoflurane (induction 4% isoflurane in 2 l/min O₂, maintenance 2% isoflurane in 1 l/min O₂). Before surgery, the analgesic buprenorphine (0.6 mg/kg body weight) and the local anaesthetic lignocaine (max 1.33 mg/kg) were injected subcutaneously. The fur on top of the head was shaven and the head was mounted in a stereotaxic frame. The skin at the surgical site was cleaned with iodine and 70% ethanol. The skull was exposed by a midline incision of approximately 2 cm and the underlying connective tissue was cleared from the surface of the skull. Six small holes were drilled into the skull to accommodate surgical anchor screws, 2 of which serve as ground (Figure 2.2). A craniotomy (2 mm in diameter) was made 1.9 mm posterior to the bregma and 1.4 mm from midline (Figure 2.2). The drive was moved down using the stereotactic apparatus until the tip of the tetrodes came into contact with the cortex. At this point (depth=0), the tetrodes were lowered 400-1000 µm into the cortex. The space between the skull and the tetrodes was covered with surgical sponge to avoid contact between dental cement and tetrodes. Dental cement was applied to secure the drive to the skull. The analgesic Meloxicam (5 mg/ml) and antibiotic Enrofloxacin (50 mg/mL) were added to the drinking water for 5 days post-surgery.

After surgery, tetrodes were gradually lowered to the DG. The position of the tetrodes in the brain was estimated by monitoring characteristic changes in local field potential (LFP) and unit activity.

Figure 2.2. Top view of mouse skull showing approximate position of structural screws, ground screws and the opening in the skull where the electrodes are inserted. Adapted with permission from www.discoverlife.org

2.2.5. Data acquisition

Electrophysiological recordings were made using the data acquisition system DigitalLynx SX (Neuralynx). Unit activity was amplified by a factor 3000-5000 and band-pass filtered at 600-6000 Hz. Spikes with amplitude above a certain threshold (defined by the experimenter, for each tetrode individually) were time-stamped and digitized at 32 kHz. These data were stored together with video tracking data on a computer running Cheetah 5.7.0 software (Neuralynx). Local field potential (electric potential in the extracellular space around neurons; LFP) data were recorded from each individual electrode of all tetrodes.

2.2.6. Video tracking

Two light emitting diodes (1 red and 1 green LED) attached to the headstage (HS-18 mm, Neuralynx), allowed tracking of the position of the mouse via a video camera on the ceiling. The LED signal was sampled at 60 Hz with a resolution of 0.2 cm per pixel (640 × 480 pixels).

2.2.7. Experimental procedures

Mice were food-deprived and habituated to exploration before surgery. When performance was good, mice were given free access to food for at least one day and the recording drive was implanted. Tetrodes were lowered at least once every two days from the day of surgery until they reached the dentate gyrus. When the mouse had recovered from surgery, food restriction and spatial exploration were restarted. When LFP showed that tetrodes were approaching the DG (increasing gamma oscillations), light stimulation sessions were started and tetrodes were moved in small steps until light-responsive units were found. When at least one light responsive unit was found, recording sessions composed of three spatial exploration sessions and three laser stimulation sessions were started (see below). In another group of mice, light stimulation was not started before at least one place cell was recorded in the dentate gyrus. A general overview of the experimental procedures is shown in Figure 2.3.

2.2.8. Recording procedure

Before recording, the mouse was resting on a towel inside a pot, the recording cables and optic fibre were connected, and the LFP and spike signals were monitored. The standard recording procedure was composed of three exploration sessions in the box and three optogenetic stimulation sessions, of which one was a control session with the laser light turned off and the shutter on. This control session was done to check if a neuronal response was caused by the laser light stimulus or by confounding factors like the sound of the shutter.

Figure 2.3. Experimental procedures. Mice were pre-trained for ~1 week before the Versadrive was surgically implanted. (A) In a first set of experiments, we tried to identify granule cells by optogenetic stimulation. Then we characterized place cell activity of the identified granule cells. (B) In a second set of experiments, we tried to identify place cells in the DG first and subsequently checked if the place cells were light-responsive granule cells.

2.2.9. Light stimulation

During light stimulation sessions, the mouse was resting on a towel inside a pot on a pedestal. Before every recording session, laser light intensity was measured at the tip of the optic fibre patch cord and adjusted to 15 mW. The laser was left on constantly and the beam was modulated with an external mechanical shutter. An Arduino board was used to control the shutter and the Arduino output signal was also recorded by the Neuralynx system. Light stimulation consisted of 3-ms pulses with a 2.5 s interval between each pulse (0.4 Hz; ~250 pulses/10 min stimulation session).

In order to measure the time it takes for the shutter to open and close, a light-to-voltage sensor was used to capture the laser light after it passed the shutter and convert it to a voltage signal. This signal and the signal from the Arduino were sent to an oscilloscope and the time difference between the two signals was measured. The difference between the signal from Arduino and the opening of the shutter was 2.778 ± 0.007 ms. The duration of the light stimulus was also measured with the light-to-voltage sensor (3.745 ± 0.021 ms). The Arduino board delivered 3-ms pulses to the shutter, but because the delay to close the shutter was longer than the delay to open the shutter, the actual duration of the laser light pulse was 3.7 ms.

2.2.10. Data analysis

2.2.10.1. Spike sorting

Spikes were sorted off-line using the cluster-cutting software MClust 3.5 (A.D. Redish), written in Matlab. Clustering was performed manually in two-dimensional projections of the spike parameters peak amplitude, energy, peak-valley difference and peak 6 to 11. Each spike was stored as 32 data points, sampled over 1 ms. 'Peak 6 to 11' is the peak amplitude from the 6th to the 11th data point. A group of spikes was considered to be generated from a single unit if the spikes formed a discrete, isolated cluster in the scatterplots, if the waveforms were similar and if there were no spikes with interspike intervals of less than 1 ms.

2.2.10.2. Firing rate map construction and analysis

For well isolated units, trajectories with spike locations and firing rate maps were constructed using MATLAB. Briefly, the total number of spikes recorded in a given location bin (5 cm × 5 cm) was summed and smoothed with a Gaussian centred on each bin (smoothing factor for Gaussian kernel was 5 cm) and then divided by the amount of time that the animal spent in that bin. The peak firing rate was defined as the firing rate in the bin with the highest firing rate. A place fields was defined as a group of at least 8 contiguous bins with an average firing rate exceeding 20% of the peak firing rate, and peak rate above 2 Hz.

2.2.10.3. Light response analysis

To visualize the light response of single units, spike raster plots and peristimulus time histograms were constructed by aligning all trials of a session at the light onset. Spike raster plots show individual trials in horizontal rows and spikes as black dots. Peristimulus time histograms show the sum of the spikes occurring per bin. Laser light was on from 0 to 3.7 ms.

In order to determine if the light stimulus caused a significant increase in firing, the number of spikes emitted after the light stimulus was compared to the number of spikes expected to occur by chance. The expected number of spikes was determined by shuffling the timestamps of the spikes within a 200-ms interval around the light stimulus (-100 ms to +100 ms). Spike timestamps were shuffled 10,000 times and then spikes were counted for bins of 1 ms in a 100-ms time window after the stimulus. The 3-ms block with the maximum number of spikes was identified for the real data as well as for the shuffled data. The cell was defined as light responsive if the number of spikes in the most active 3-ms block in the real data exceeded the 99.9th-percentile value for the proportion of spikes in the most-active 3-ms block in the shuffled distribution.

2.2.10.4. Changing spike detection threshold of previously recorded data

In order to change the spike detection threshold of previously recorded data, continuously sampled channel (CSC) files were filtered between 600 Hz and 6000 Hz using the program NlxCSCFiltering.exe (Neuralynx). The program CscSpikeExtractor.exe was used to determine a new threshold and extracted spikes from the filtered CSC files.

2.2.11. Tetrode tracking

Mice were perfused transcardially using 0.9% saline followed by 4% formaldehyde in phosphate buffered saline (PBS) while the tetrodes remained in the brain. The head of the mouse was decapitated and kept for two to three days in 4% formaldehyde (or 4% PFA) solution at room temperature. The tetrodes and optic fibre were turned up and the brain was carefully removed from the skull and stored in 4% formaldehyde solution (or 4% PFA) in PBS for three to five days before sectioning.

For tetrode tracking, frozen sagittal sections (35 μm) were cut using a cryostat and mounted on gel-coated slides. The slides were allowed to dry for minimum 24 h, before staining with cresyl violet to reconstruct the tetrode tracks.

3. Results

3.1. ChR2Y is expressed in 10% of granule cells

ChR2 expression in granule cells of the DG was obtained by crossing a Cre driver line with a ChR2 reporter line (POMC-Cre × Ai32)(Balthasar *et al.*, 2004; Madisen *et al.*, 2012). The Cre-positive offspring (abbreviated here as POMC-ChR2Y) show specific and high expression of ChR2 in 10% of granule cells in the DG and were used for optogenetic identification experiments.

Ai32 mice (Rosa-CAG-LSL-ChR2(H134R)-EYFP-WPRE) express an improved Channelrhodopsin-2 ChR2(H134R) fused to enhanced yellow fluorescent protein (EYFP) following exposure to Cre recombinase (Madisen *et al.*, 2012). In absence of Cre, expression of the fusion protein (abbreviated here as ChR2Y) is inhibited by an upstream STOP cassette which is flanked by two LoxP sites. In Cre-positive cells, the STOP sequence between the LoxP sites will be excised by Cre-recombinase, allowing expression of ChR2Y (Madisen *et al.*, 2012).

In the POMC-Cre driver line, Cre is expressed under control of the mouse pro-opiomelanocortin-alpha (POMC) promoter. POMC is normally not expressed in the hippocampus, but the POMC promoter region carries sequences that consistently direct gene expression to the DG (Overstreet *et al.*, 2004). The factors responsible for POMC promoter dependent expression in dentate granule cells are not completely understood. In POMC-EGFP transgenic mice, EGFP was expressed selectively and transiently in newly born granule cells of the DG (Overstreet *et al.*, 2004). BrdU-labeling experiments in adult POMC-EGFP mice showed that EGFP expression was peaking in granule cells 2 weeks after mitosis and turning off by 1 month. Most, if not all, postnatally generated granule cells transiently expressed EGFP.

In POMC-ChR2Y mice, ChR2Y was expressed throughout the granule cell layer, and not only in the inner third, where the majority of postnatally generated granule cells are located (Figure 3.1B; (Jessberger and Kempermann, 2003)). Therefore, we believe that not only postnatally, but also developmentally generated granule cells underwent Cre-mediated recombination.

We presume that the reason why only 10% of granule cells express ChR2Y in this mouse line is because not all granule cells underwent Cre-mediated recombination. Even though all granule cells might transiently express Cre, some cells may undergo recombination, while others don't.

It has been shown by our lab that ChR2Y is specifically expressed in granule cells of the DG in POMC-ChR2Y mice (Figure 3.1A; (Blankvoort, 2015)). Immunostaining for YFP and the granule cell marker Prox1 showed that approximately 10% of granule cells showed ChR2-YFP expression (Blankvoort, 2015). ChR2Y is targeted to the cell membrane and is expressed in the cell body as well as in the axon and dendrites (Figure 3.1 A,B).

Further, it was shown that light stimulation induced the expression of the immediate early gene c-FOS in granule cells in the DG (Blankvoort, 2015). 473-nm, 10-ms light pulses were delivered for 3 min at 10 Hz through an implanted optic fibre. Light stimulation increased the expression of c-FOS in DG granule cells of POMC-ChR2Y mice compared with non-stimulated mice. This means that the expression level of

ChR2Y was sufficiently high and optogenetic stimulation effectively induced activity in granule cells of the DG. c-FOS expression was not only increased in ChR2-positive granule cells, but also in ChR2-negative cells. This could be explained by excitatory connections between granule cells and mossy cells: mossy cells in the hilus receive powerful input from a small number of granule cells and provide excitatory output to a large number of granule cells (Scharfman, 2016).

Figure 3.1. ChR2Y expression pattern in POMC-ChR2Y mice. (A) ChR2Y expression in the brain of a POMC-ChR2Y mouse. Green, anti-GFP immunostaining. Scale bar: 1mm. Image courtesy of Dr. Stefan Blankvoort. (B) ChR2Y is expressed in ~10% of DG granule cells. Green, anti-GFP immunostaining. Magenta, anti-NeuN immunostaining. Scale bar: 250 μ m

3.2. Tetrode lowering based on hippocampal network patterns

After implantation of the drive, tetrodes were gradually lowered to the granule cell layer of the DG. Tetrodes were lowered at least once every 2 days in order to prevent them from getting stuck in the brain. The depth of the tetrodes was measured by the number of turns of the tetrode lowering screws. One full rotation of the screws moved the tetrodes by 250 μm . The location of the tetrodes was estimated based on their depth and characteristic changes in local field potential (LFP) in the hippocampus. The characteristic changes in LFP will be presented in this section.

During surgery, tetrodes were implanted at 500 μm to 1000 μm below the brain surface. The depth of the CA1 pyramidal cell layer is about 1400-1700 μm . The LFP of CA1 is characterized by theta oscillations during exploration and sharp wave-ripple complexes during rest (Figures 3.2 and 3.3; (Buzsáki *et al.*, 2003)). Theta oscillations or theta waves are slow oscillations (6-9 Hz) that are characteristic for the hippocampus. They occur during locomotor activities like exploring and during REM sleep (Jouvet, 1969; Vanderwolf, 1969). The amplitude of theta oscillations increased gradually from the neocortex to the CA1 pyramidal cell layer, reversed below the CA1 pyramidal cell layer and was largest at the hippocampal fissure (Figure 3.2). When the tetrode moved from the CA1 to the DG, gamma oscillations gradually increased (Buzsáki *et al.*, 2003). Gamma oscillations are 40-100 Hz oscillations that are most prominent in the DG of mice.

During immobility, sharp waves were observed in the CA1 (Figure 3.3). When the tetrode was at the dorsal side of the CA1 pyramidal cell layer, sharp waves were upward deviations from baseline (Figure 3.3A). When the tetrode was on the ventral side of the pyramidal cell layer, sharp waves were downward deviations (Figure 3.3B). Together with sharp waves, ultra-fast oscillations occur in CA1. These 140-200 Hz oscillations are called “ripples”. Many CA1 neurons fired during these “sharp wave-ripples” (SWR), resulting in many clusters in the spike window (Figure 3.3C).

When the tetrode reached the granule cell layer of the DG, gamma amplitude was very prominent and units started firing sparsely (Figure 3.2D). Previous studies reporting unit activity in the DG of rats monitored long periods of sleep in order to determine if the tetrode was in a layer of sparsely firing granule cells (Neunuebel and Knierim, 2012). However, mice hardly sit quietly and therefore we may have moved several tetrodes through the granule cell layer without noticing any activity.

A CA1 stratum radiatum

B Hippocampal fissure

C DG molecular layer

D DG granule cell layer

E DG hilus

Figure 3.2. Theta oscillations at estimated hippocampal areas. In each plot, 1 second of LFP recordings at different depths in the hippocampus is shown for the 4 channels of a tetrode. (A) Low amplitude theta oscillation at the *stratum radiatum*, just below the pyramidal cell layer of CA1. (B) Large amplitude theta when the tetrode approached the HF. The 7-8 Hz theta oscillation is indicated on channel 4. (C,D,E) Gamma oscillations recorded when the tetrode moved from the molecular layer to the hilus of the DG. The gamma oscillation (60-70 Hz) is indicated for $\sim 160 \mu\text{s}$ of recording from channel 4 of the tetrode in the granule cell layer (D).

Figure 3.3. LFP characteristics and unit activity of the CA1 pyramidal cell layer. (A,B) One second of LFP recordings in the CA1 pyramidal cell layer. The upper 4 traces are high-pass filtered (600-6000 Hz) to show unit activity. The 5th trace is filtered between 150 Hz and 250 Hz to show ripples. The lower trace shows all frequencies from 0.1 Hz to 9000 Hz (A) Upward SWR (red arrow) are seen when the tetrode is on the dorsal side of the CA1 pyramidal cell layer. (B) Downward SWR (red arrow) occur on the ventral side of the CA1 pyramidal cell layer. (C) Spike window showing several units recorded in CA1. The input range is indicated in white in μV and the spike detection threshold in green.

Figure 3.4. LFP characteristics and unit activity of the DG granule cell layer. (A) LFP traces (as in Figure 3.2A) and (B) unit activity, presumably recorded in the granule cell layer of the DG.

3.3. Histological verification of tetrode tracks

When electrophysiological recordings were finished, mice were perfused transcardially with saline and 4% formaldehyde to prepare brain sections for verification of tetrode tracks. Brain sections were stained with cresyl violet and tetrode tracks were reconstructed based on epifluorescence images.

Histological analysis of tetrode tracks confirmed that 28 out of 175 tetrodes in 22 mice ended in or had passed through the DG granule cell layer (Figure 3.5). On 2 of these, we identified putative granule cells. The first one was located in the granule cell layer of the lower blade of the DG (mouse 133 tetrode 1; Figure 3.5, red arrow) and the second one in the molecular layer of the lower blade (mouse 218 tetrode 4; Figure 3.5, red arrow). In both cases, we continued recording after granule cells were identified in an attempt to identify more light-responsive granule cells. Therefore, the mice were perfused several days after the identification of granule cells. In the days between identification of granule cells and perfusion, the tetrodes were not manually lowered, but may have drifted in the brain tissue.

Of the tetrodes that did not reach the DG, many got stuck in the brain tissue and could not be lowered anymore after a certain depth. Others were bent in or above the brain and missed the DG. Some tetrodes passed through the DG granule cell layer without we noticed it, possibly because the granule cells were very silent.

Figure 3.5. Histological verification of tetrode tracks. (A) Cresyl violet stained brain sections of all tetrodes that ended in the DG. The position of the tetrode is indicated with an arrow. (B) Cresyl violet stained brain sections of all tetrodes that had passed through the DG. Both the brain sections where the tetrode is in the DG granule cell layer and where the tetrode is at its deepest position are shown. The tetrode tracks of the tetrodes on which putatively light responsive granule cells were detected are indicated with red arrows.

3.4. Optogenetic identification of granule cells

3.4.1. Light responsive unit example 1

An example of a light-responsive unit is presented in the following sections. The unit was recorded on a tetrode in the granule cell layer of the DG of a ChR2Y-positive mouse. In order to conclude that the unit is a granule cell, the light response was investigated extensively. The electrophysiological characteristics when the mouse was in rest and location specific firing during spatial exploration will also be described.

The light-responsive unit was identified in POMC-ChR2Y mouse #133, on tetrode 1 on 11th November, 2014. The tetrode was lowered to its final depth of approximately 2000 μm on 7th November. At this depth, the LFP showed high amplitude gamma oscillations, characteristic for the DG. Place cells were also identified on 10th November, but these did not show any light-evoked activity. The light-responsive unit described below was recorded on 11th November. Tetrodes were not lowered after this day, and the mouse was terminated on 17th November.

3.4.1.1. Recording sessions

Seven sessions were recorded: 1 rest session in which the mouse was resting on a towel inside a pot on a pedestal, 3 spatial exploration sessions in which the mouse was foraging for food in an open arena, 2 light stimulation sessions with 3.7 ms laser pulses at 0.4 Hz, and 1 control session with the laser light off (Figure 3.6). The laser light intensity at the tip of the optic fibre patch chord was 16.2 mW. The duration of each session was 10 minutes. Light stimulation was given while the mouse was resting on the pedestal. The pedestal setup was used to limit the movement of the mouse. The mouse was thoroughly habituated to the pedestal and no signs of stress were observed.

When a single neuron fires an action potential, it produces a characteristic pattern of voltage changes that can be detected by extracellular electrodes. The amplitude and waveform of the detected spikes is dependent on the relative position of the neuron and electrode. In comparison with single electrodes, tetrodes have the advantage that the voltage changes caused by firing of a given neuron are measured on 4 different channels. For this reason, tetrodes provide more information per spike and thus allow isolation of more neurons with higher reliability (Gray *et al.*, 1995).

Figure 3.6 shows spike scatter plots and spike waveforms of a single unit recorded during the 7 recording sessions. Spike scatter plots show features of the spikes (for example, peak amplitude or energy). Each dot represents one spike. The scatter plots in Figure 3.6 show the peak amplitude of all spikes, as recorded on two channels of the tetrode (peak amplitude recorded on channel 1 vs. peak amplitude recorded on channel 3). Because spikes originating from the same cell have similar waveforms, they form clusters in the scatter plots. This is one of the fundamental principles of spike sorting. Spikes were manually sorted based on projections of spike peak amplitude, energy and peak-valley difference. The red spikes in the scatter plots in Figure 3.6 were clustered together in all projections, and were therefore assumed to originate from a single cell. Spikes in black were excluded, because their waveform was different. For each recording session, the waveforms of the spikes of the red cluster are shown on the right in Figure 3.6.

Figure 3.6. Cluster stability. Cluster stability was assessed by visually comparing spike scatter plots and waveforms. (Left) spike scatter plots showing the peak amplitude recorded on channel 1 vs. channel 3. Red dots belong to the cluster, black dots were excluded. Peak amplitude is shown in AD values. AD value 20,000 corresponds to 427 μV . (Top right) waveforms of spikes of the red cluster, as recorded on tetrode channel 3. Different colours were used in this plot to visualize individual spikes, but all spikes belong to the red cluster. The duration of the spike record is 1 ms. Amplitude is shown in AD values. (Bottom right) Average waveform of the spikes of the red cluster, as recorded on the 4 channels of the tetrode.

3.4.1.2. Light response

The light response of the isolated red cluster in Figure 3.6 is shown in Figure 3.7. Both light stimulation sessions consisted of 240 light stimuli. Light stimulation was controlled by a mechanical shutter. The duration of the light pulses was 3.7 ms and the interval between two pulses was 2.5 s. Two 10-min light stimulation sessions were recorded and one control session with the shutter working, but the laser light turned off. The intensity of the 473-nm laser light was 16.2 mW measured at the tip of the optic fibre patch cord.

To visualize the response of a single unit after light stimulation, peri-stimulus time histograms and spike raster plots were constructed with all trials in a session aligned at the onset of the light pulse (Figures 3.7A-D). The spike raster plot at the bottom of the figure displays each spike as a black dot. The 240 trials of the session are shown in horizontal rows. The period of light illumination is indicated by a grey bar in the raster plot. In the non-grayed area the laser light is off. Shown on top is a histogram of the sum of the spikes. Figures 3.7A and 3.7B show spikes in the interval from 100 ms before to 100 ms after the onset of the light pulse. The baseline firing rate, as seen in the 100 ms before the light pulse, is very low (~1 Hz; see below). Within 10 ms after the light pulse, there is a great increase in firing.

3.4.1.2.1. Light stimulation caused a significant increase in firing

Although it was obvious in this example that light stimulation caused an increase in firing, we used a statistical test to confirm that the firing rate was significantly changed (based on (Zhang *et al.*, 2013)). This test compared the number of spikes emitted after the light stimulus with the number of spikes expected to occur by chance. The expected number of spikes was determined by shuffling the timestamps of the spikes within a 200-ms interval around the light stimulus (-100 ms to +100 ms). Spike timestamps were shuffled 10,000 times and then spikes were counted for bins of 1 ms in a 100-ms time window after the stimulus. The 3-ms block with the maximum number of spikes was identified for the real data as well as for the shuffled data. The results of the shuffling procedure are shown in Figure 3.8. A unit was identified as light responsive if the number of spikes in the most active 3-ms block in the real data exceeded the 99.9th-percentile value for the proportion of spikes in the most-active 3-ms block in the shuffled distribution. Based on this criterion, the isolated unit showed indeed a significant increase in firing upon illumination, for both light stimulation sessions. In laser stimulation session 1 the maximum number of spikes in 3 consecutive bins was 343, while the 99.9th-percentile value of the shuffled distribution was 21 spikes. For laser stimulation session 2, these numbers were 383 and 23. The 3 bins with the highest number of spikes were the bins from 5 ms to 8 ms for both sessions. In the control session, when the shutter was working, but the laser light was turned off, there was no significant increase in firing (Figure 3.11A).

3.4.1.2.2. Baseline firing rate

Apart from the spikes evoked by the light stimulus, the unit also fired spontaneous spikes. A baseline firing rate was calculated based on the firing activity of the unit independently of the light stimuli. Visual inspection of the peri-stimulus time histograms showed that the light stimulus induced firing within 10 ms after light onset, after which there was a period of silence for at least 50 ms. Assuming that the light pulse did not have any influence after more than 1 second, we calculated a baseline firing rate based on the interval from 1 second to 2.5 seconds after the light stimulus (the interval between 2 stimuli was 2.5 seconds). The average firing rate in the baseline interval was 1.02 Hz. During the control session when the laser light was turned off, but the shutter was working, the average firing rate was 1.15 Hz. We also recorded a 10-min session when the mouse was resting on the pedestal before the start of laser stimulation and exploration sessions. The average firing rate in this session was 0.65 Hz. Differences in firing could be caused by differences in arousal state of the mouse, which could be influenced by the noise of the shutter or the prospect of food.

3.4.1.2.3. Light response characteristics

A closer look at the response in individual trials shows that the unit responded to light stimulation by firing one, two or sometimes three spikes (Figures 3.7C,D). This resulted in 2 large and 1 small peak in the PSTH's in Figures 3.7C,D. Out of the 240 light pulses per session, 24 in the first session and 17 in the second session did not evoke any response. The average response reliability (percentage of stimuli that evoked a response) was 91.5%.

It is known that brief pulses of light can induce more than one spike in ChR2-expressing cells (Boyden *et al.*, 2005). The extra spikes are assumed to be caused by the slow inactivation kinetics of ChR2 and ChR2(H134R) (Gunaydin *et al.*, 2010). In order to characterize the light response in more detail, we separately investigated the spikes from the 3 peaks. In both light stimulation sessions, the second peak had more spikes than the first one (Table 3.1). All spikes in the first peak fell within a time interval from 4.7 ms to 6.2 ms after the onset of the light pulse. Spikes in the second peak occurred between 6.3 ms and 8 ms after light onset and spikes in the third peak between 8.1 ms and 9.6 ms. The average latency of the spikes in the first peak was 5.5 ms and the first presumably light-evoked spike occurred 4.7 ms after the onset of the laser stimulus. The average latency of the spikes in the second peak was 7.1 ms and for the third peak, the average latency was 8.9 ms (Table 3.1).

Figure 3.7. Increase in neuronal activity after light stimulation. Peri-stimulus time histograms (PSTHs; top) and spike raster plots (bottom) showing spikes from the red cluster in Figure 3.6 during laser stimulation session 1 (A and C) and session 2 (B and D). (A,B) PSTH and raster plot showing the time interval from 100 ms before to 150 ms after the stimulus. Bin size in the histograms is 0.1 ms. In the raster plots, the light stimulus is represented by a cyan bar and spikes are shown as black dots. Light was on from 0 to 3.7 ms. Each row in the raster plot is derived from one trial (trial duration, 2.5 ms). Note the low baseline firing before the light stimulus and absence of firing between 10 ms and ~75 ms after the light pulse. (C,D) The time interval from light onset to 15 ms after onset is shown. Bin size is 0.1 ms.

Table 3.1. Light response characteristics. Both light stimulation sessions consisted of 240 light pulses. Peak 1, 2 and 3 refer to the 3 peaks in the histograms in Figure 3.7C,D.

Light evoked spikes	Laser ON (1)	Laser ON (2)		Spike latency
Number of spikes in peak 1	150	175	Peak 1	4.7-6.2 ms
Number of spikes in peak 2	189	196	Peak 2	6.2-8.0 ms
Number of spikes in peak 3	65	55	Peak 3	8.0-10.0 ms
Total number of spikes	404	426		
Latency of spikes in peak 1 (ms)	5.44±0.28	5.48±0.27		
Latency of spikes in peak 2 (ms)	7.10±0.32	7.14±0.33		
Latency of spikes in peak 3 (ms)	8.91±0.36	8.86±0.33		
Latency of first spike (ms)	4.72	4.82

Figure 3.8. Results of shuffling procedure. Histograms of the number of spikes in the 3-ms block with the highest number of spikes for the 10,000 times shuffled data, with the 99.9 percentile indicated by a red line. For the real data, the number of spikes in the 3-ms block with the highest number of spikes was 343 in laser stimulation session 1 and 383 in session 2.

3.4.1.2.4. Spike waveform

In order to confirm that light-evoked and spontaneous spikes were generated by the same neuron, we compared the waveform of light-evoked spikes with the waveform of the spontaneous spikes (Figure 3.9). Figure 3.9A shows the waveforms of all spikes that occurred more than 50 ms after the light stimulus, with the average waveform in a different colour. We assume that these are spontaneous spikes and their waveform was not influenced by laser stimulation. Figure 3.9B shows all waveforms of the spikes from the first peak in figure 3.7C. Light-induced spikes showed more variability in waveform, which was most obvious in the part of the spike record after the trough of the spike. However, the average waveforms of spontaneous spikes and light-induced spikes from the first peak were very similar. There were no detectable changes in peak and trough amplitudes between both groups of spikes.

Next, we studied the waveforms of the light-induced spikes for each peak of figure 3.7D separately. Figure 3.9B, C and D show the spikes of peak 1, 2 and 3, respectively. It is clear that the peak amplitude of the spikes in the second and especially the third peak was smaller than in the first peak. Although some spike amplitudes were similar in the first and second peak, there was a clear decrease in average spike amplitude.

3.4.1.2.5. Variability in spike waveforms

Visual inspection of the spike waveforms of the red cluster in Figure 3.6 suggested that spike waveforms of light-induced spikes showed more variability than spontaneous spikes. We investigated how this variability was represented the spike scatter plots. More variability in spike waveform results in more variability in spike features like amplitude and energy. Therefore, it is expected that light-evoked spikes are less densely clustered together than spontaneous spikes. To visualize this, we plotted all spikes from the single, isolated unit from Figure 3.5 in scatter plots showing the peak amplitude of the spikes on each pair of channels (Figure 3.10). Spontaneous spikes (defined here as spikes more than 10 ms after the light stimulus) were plotted in black and light induced spikes (spikes within 3 to 10 ms after the onset of the stimulus) in cyan. As expected, most of the spontaneous spikes (black) formed a dense cluster, with few spikes scattered around the cluster. The light-evoked (cyan) spikes mostly overlapped with the spontaneous spikes, but some cyan spikes were also scattered around the black cluster.

Figure 3.9. Waveform comparison of spontaneous and light evoked spikes (data from laser stimulation session 2). The 4 plots in each column show the spike waveforms as recorded on the 4 channels of a tetrode. Waveforms of (A) spontaneous spikes, defined as spikes recorded more than 50 ms after light onset, (B) spikes in the first peak of the histogram in Figure 3.7D. (C) spikes in the second peak, (D) spikes in the third peak. The average of the waveforms is shown in a different colour. Amplitude is shown in AD values. AD value 20,000 corresponds to 427 μV . The duration of the spike record is 1ms. Note the difference in scale in the plots.

Figure 3.10. Scatter plots showing light-evoked and spontaneous spikes of a single light-responsive unit (the red cluster in Figure 3.6). Spikes in black are spontaneous spikes (>10 ms after light onset) and spikes in cyan are light-evoked (3-10 ms after light onset). Plotted here is the peak amplitude of the spikes as recorded on each pair of channels (peak1 is the peak amplitude of the spikes as recorded on channel 1, and so on). (A) Laser stimulation session 1. (B) Laser stimulation session 2.

3.4.1.2.6. Variability in spike waveform of light-induced spikes

Since spike sorting is based on similarity of waveforms, one may argue that the spikes scattered around the dense black cluster could have originated from a different neuron. In the following sections I will explain why this is very unlikely.

First of all, the interspike interval (ISI) histograms in Figure 3.11C,D show that very few (less than 5) spikes had an ISI under 1 ms. If the spikes had originated from more than one light-responsive unit, the units would fire at similar latencies after each light pulse, and many spike pairs with an ISI of < 1 ms would be expected.

Secondly, when the cluster was cut in a way that all spikes scattered around the dense cluster core were eliminated, thus only spikes with very similar waveforms were included in the cluster, the unit still showed a highly significant light-response (Figure 3.12). In both light stimulation sessions, more than 50 light-evoked spikes were observed in the 3-10 ms interval after light stimulation (Figure 3.12E,F). Since all the spikes in this cluster were very similar, they most likely originated from one single unit.

Figure 3.11. Unit activity and ISI histograms for control and laser stimulation sessions (data from red cluster in Figure 3.6). (A) PSTH and raster plot for control session (laser was off, but shutter was working). The time period where the light stimulus would have happened if the laser was on is represented by a grey bar in the raster plot. (B,C,D) ISI histogram for control session 2 and laser stimulation session 1 and 2, respectively. In laser stimulation session 1, the first bin (0-1 ms) has no spikes and in session 2 the first bin has 3 spikes.

Figure 3.12. Unit with only very similar spikes also contains light-evoked spikes. (A,B) For both laser stimulation sessions, a scatter plot of spikes showing the peak amplitude recorded on channel 1 vs. channel 3. The blue cluster was cut so that it does not include spikes scattered around the dense cluster core. Axes are the same as in Figure 3.6. (C,D) Waveform of all spikes of the blue cluster, as recorded on channel 2 of the tetrode. Note that all spikes of the blue cluster have very similar waveforms. (E,F) PSTHs and spike raster plots showing spikes from the blue cluster in the time interval from 10 ms before to 60 ms after the laser stimulus. Bin size in the histograms is 1 ms. (G,H) Scatter plots showing light-evoked (3-10 ms after light onset; cyan) and spontaneous (>10 ms after light onset; black) spikes of the blue cluster.

Thirdly, the amplitude of the spikes in this cluster was very large (300-600 μV), which suggested that the tetrode was very close to the neuron. If spikes are large, they are usually clearly separated from noise and other clusters. Because the spike detection threshold was high ($\sim 100 \mu\text{V}$), no other isolated clusters and hardly any noise was recorded (see scatter plots in Figure 3.6). If there would be spikes from two units in this cluster, one light responsive and one not light responsive, then there would be a systematic difference between light-induced and spontaneous spikes. In Figure 3.9, no such systematic difference could be detected; some light evoked spikes have larger peaks, some have smaller peaks. Therefore, we conclude that the variation in the spike waveform of light-induced spikes is random and the spikes scattered around the dense cluster core are most likely generated by the same cell.

3.4.1.2.7. Scattered spikes

As can be seen in Figure 3.6, many spikes were scattered around the red cluster in the light stimulation sessions. Some spikes were excluded from the red cluster because the waveform was considered too different. Since we showed above that the waveform of light-induced spikes may show some variation, we wondered if the excluded spikes should have been included in the red cluster. To learn more about the spikes scattered around the cluster, we re-analyzed the data with most or all of the scattered spikes included (green cluster in Figure 3.13A,B). The waveform of these spikes showed much more variability (Figure 3.13C,D). In addition, the peak of the waveform was not aligned for several spikes. However, the fact that the ISI histograms had very few spikes in the 0-1 ms interval, suggests that all spikes of the green cluster were derived from a single cell. The light response of the green cluster was larger than the response of the red cluster (Figure 3.13G,H). Only 3 trials in the first session and 6 trials in the second session did not show any light-evoked response. The average response reliability was 98%. The arguments explained here support the idea that all spikes in the green cluster were generated by the same neuron. However, because the peak of the spikes was not aligned for all spikes, the timestamps of the non-aligned spikes may be not recorded accurately and the data were not used for further analysis.

Figure 3.13. Unit including scattered spikes. (A,B) Scatter plots of spikes showing the peak amplitude recorded on channel 1 vs. channel 3. The green cluster was cut so that it contains many spikes scattered around the dense cluster core. Axes as in figure 1. (C,D) Waveform of all spikes of the green cluster, as recorded on channel 2 of the tetrode. (E,F) ISI histograms and (G,H) PSTHs and spike raster plots for spikes of the green cluster.

3.4.1.3. Location specific firing

The previous sections demonstrated the light response of the red cluster from Figure 3.6. In the following section, location specific firing of the unit will be presented. Visual inspection of spike waveforms and spike scatter plots indicated that the same unit was isolated in light stimulation and exploration sessions (Figure 3.6).

To show location specific firing, the mouse was trained to explore an open arena (60 cm x 60 cm) for 3 sessions of 10 min each. The laser light was off during all spatial exploration sessions. Unit activity was recorded while the location of the mouse was tracked with a camera above the centre of the arena. Trajectories with spike locations and firing rate maps were constructed using MATLAB (J. K. Leutgeb *et al.*, 2005). Briefly, the total number of spikes recorded in a given location bin (5 cm x 5 cm) was summed and smoothed with a Gaussian centered on each bin and then divided by the amount of time that the animal spent in that bin. A place field was defined as a group of at least 8 contiguous bins with an average firing rate exceeding 20% of the peak firing rate, with the peak rate above 2 Hz. Peak firing rate was defined as the highest firing rate in any of the bins (number of spikes divided by time spent in the bin). The spike maps and firing rate maps in Figure 3.14 reveal that the unit shows location specific firing. Quantitative analysis shows that there were 3 place fields in the first and third exploration session, but only one place field in the second session. The location of the place fields remained roughly the same, but the firing rate changed, perhaps because the mouse did not cover all areas in the three sessions (Table 3.2).

The ISI histogram in Figure 3.14H shows an increase in firing at the ~120-ms interval. This shows that firing is theta modulated. Another interesting observation is that the ISI peaks seen at 1-2 ms and 3-4 ms intervals during light stimulation (Figures 3.11C,D) are completely absent in during spatial exploration (Figure 3.14G). This means that bursting with ISI < 2 ms, as seen in figure 3.11C,D, only happens after light stimulation and not in physiological conditions. If we define burst firing as consecutive spikes fired within an ISI of less than 10 ms, 16% of spikes were fired in bursts during spatial exploration. In the rest session, 15% of spikes were fired in bursts.

Figure 3.14. Location specific firing (data from red cluster in Figure 3.6). (A-C) Trajectories with spike locations and (D-F) corresponding colour-coded firing rate maps. Each rate maps is scaled to the session's peak firing rate, which is indicated above the map. Red is peak firing, blue is no firing. White indicates bins which the mouse did not explore. The laser light was off during all exploration sessions. (G,H) ISI histograms of spatial exploration session 1.

Table 3.2. Firing characteristics during exploration and rest

Firing characteristics	Exploration	Exploration	Exploration	Average	Rest	Laser Off
	Session 1	Session 3	Session 5		Session 0	Session 2
Average firing rate (Hz)	0.84	0.81	1.54	1.06	0.65	1.15
Peak rate (Hz)	4.29	5.90	9.62	6.60		
Bursting	0.16	0.14	0.19	0.16	0.15	0.23
Sparseness	0.37	0.29	0.27	0.31		
Information	0.97	0.69	1.25	0.97		
Selectivity	5.98	8.45	7.03	7.15		

3.4.2. Light responsive unit example 2

In the previous section, light-evoked firing of a single, well-isolated unit was described. The day after this unit was detected, we did the same light stimulation and spatial exploration experiment. Again we detected a light-responsive place cell, on the same tetrode as the previous day. The waveform and light response characteristics were similar on both days, but the location of the place field was different. The tetrode was not lowered between the 2 recording days, but may have drifted in the brain tissue. Therefore, we cannot ascertain that the unit is the same one as recorded on the day before or a different light-responsive place cell. The light response and place cell properties of the unit will be presented below.

3.4.2.1. Recording sessions

As on the previous day, we recorded 3 spatial exploration sessions, 2 light stimulation sessions (3.7 ms stimuli at 0.4 Hz) and 1 control session with the laser light turned off. The light intensity was 16.2 mW, measured at the tip of the optic fibre patch chord. Additionally, one 10-min light stimulation session was recorded with light intensity at 4.4 mW. For each recording session, a spike scatter plot and waveform plot are shown in Figure 3.15.

3.4.2.2. Light response

Light-induced activity of the isolated single unit is shown in Figure 3.16. Light stimulation at 16.2 mW caused a remarkably similar light-response as recorded on the previous day (Pearson correlation for number of spikes per 1-ms bin in the first 15 ms after light stimulation, $r > 0.95$, $p < 0.001$). PSTH's showed a highly significant increase in firing in the time interval from 4 ms to 11 ms after light stimulation onset. There was no increase in firing in the control session, when the shutter was working, but the laser light was turned off (Figure 3.16C). The first presumably light-evoked spike occurred in both light stimulation sessions within 5 ms after the light onset. Both sessions consisted of approximately 250 trials (Table 3.3). The average response reliability was 93.5%.

As in the previous example, we divided the response into 3 peaks based on visual inspection of the histograms and separately investigated the spikes in the 3 peaks (Table 3.3). The average response latency of the spikes in the 3 peaks was slightly longer (~0.40 ms) than on the day before (compare Table 3.1 and 3.3). The variability in response latency was slightly higher on the second day (higher s.e.m.). The number of spikes in the first and second peak was higher on the second day and the number of spikes in the third peak was lower. The average response reliability was similar on the 2 days. . As on the first day, the number of spikes in the second peak was higher than the number of spikes in the first peak.

Figure 3.15. Clusters were stable over the 7 recording sessions. For each recording session, a scatter plot of spikes showing the peak amplitude recorded on channel 3 vs. channel 4 (left) and the waveform of the spikes of the red cluster, as recorded on channel 2 (right). Amplitude is shown in AD values. AD value 20,000 corresponds to 427 μ V. The duration of the spike record (right) is 1ms.

Table 3.3. Light response characteristics. Peak 1, 2 and 3 refer to the 3 peaks in Figure 3.16A,B.

Light evoked spikes	Laser ON (1) (246 trials)	Laser ON (2) (252 trials)		Peak	Spike latency
Latency of spikes in peak 1 (ms)	5.83±0.32	5.83±0.36		Peak 1	4.90-6.65 ms
Latency of spikes in peak 2 (ms)	7.53±0.36	7.55±0.42		Peak 2	6.65-8.75 ms
Latency of spikes in peak 3 (ms)	9.47±0.17	9.35±0.33		Peak 3	8.75-10 ms
Number of spikes in peak 1	205	201			
Number of spikes in peak 2	217	216			
Number of spikes in peak 3	29	30			
Total number of spikes	451	447			
Response reliability	93%	94%

Figure 3.16. Light-evoked firing. PSTHs (top) and spike raster plots (bottom) showing spikes from the isolated unit (red cluster in Figure 3.15) during laser stimulation session 1 (A), session 2 (B), control session with laser light off (C) and laser stimulation session with light intensity at 4.4 mW (D). In the laser stimulation sessions (A,B,D), the time interval from light onset to 15 ms after onset is shown and bin size is 0.1 ms. In the control session (C), the time interval from 10 ms before to 60 ms after light onset is shown and bin size is 1 ms.

3.4.2.2.1. Spike waveform

The similarity of the scatter plots and spike waveforms shown in Figure 3.15 provide strong evidence that the cluster was stable in all sessions and the spikes were generated by the same neuron. In addition, to assure that light-evoked and spontaneous spikes were generated by the same cell, we compared the waveform of light-evoked and spontaneous spikes recorded in the same session. Figure 3.17 shows the waveform of all spikes recorded in laser stimulation session 1. Spontaneous spikes (defined here as all spikes that happened more than 50 ms after light stimulation) were very similar as spikes of the first peak of the light response (Figure 3.17A,B). Although light-evoked spikes showed more variability in waveform, the average waveform was similar as the average waveform of the spontaneous spikes. The spikes of the second peak were slightly smaller in amplitude and the spikes of the third peak were even smaller. In order to confirm that there was no systematic difference between light-evoked and spontaneous spikes, we examined scatter plots in which spontaneous spikes were plotted in black and light-evoked spikes in cyan (Figure 3.18).

Figure 3.17. Waveform comparison of spontaneous and light evoked spikes (data from laser stimulation session 1). Waveform of (A) spontaneous spikes, happening more than 50 ms after light onset, (B) spikes in the first peak of the histogram in Figure 3.16A, (C) spikes in the second peak, (D) spikes in the third peak. The average of the waveforms is shown in a different color. Amplitude is shown in AD values. AD value 20,000 corresponds to 427 μV . The duration of the spike record is 1ms (shown here as 32 data points). Note the difference in scale in the plots.

Figure 3.18. Scatter plots showing light-evoked and spontaneous spikes of a single light-responsive unit (red cluster in figure 3.15). Spikes in black are spontaneous spikes (>10 ms after light onset) and spikes in cyan are light-evoked. The peak amplitude of the spikes as recorded on each pair of channels is plotted. (A). All light-evoked spikes in the 4-10 ms interval after light onset are shown in cyan. (B,C,D) Spikes of the first peak (B), second peak (C) and third peak (D) in cyan.

3.4.2.2.2. Response to low light intensity stimulation

In order to assess the influence of light intensity on the light response, we did one light stimulation session with a lower light intensity (4.4 mW, measured at the tip of the optic fibre patch chord; Figure 3.16D). Light stimulation at 4.4 mW caused a highly significant light-response, but the reliability was lower and the response latency was longer than for 16.2 mW light stimulation. While the response reliability for light stimulation at 16.2 mW was more than 90%, reliability for 4.4 mW stimuli was only 65%. In contrast to the 3-peak response induced by 16.2 mW light, 4.4 mW light pulses resulted in only one broad peak (Figure 3.16). The first presumably light-evoked spike occurred 6.42 ms after light onset, ~ 1.5 ms later than for 16.2 mW stimuli. While the average latency of spikes in the first peak of the 16.2 mW response was 5.83 ± 0.34 ms, the average latency for 4.4 mW pulses was 8.43 ± 0.87 ms. In 9 out of a total of 246 4.4-mW light stimulation trials, 2 spikes were detected after the light pulse. To conclude, decreasing the light intensity resulted in a lower response reliability and longer response latency.

Figure 3.19. Spontaneous and light-evoked spikes comparison for 4.4 mW light stimulation. Waveform of all light-evoked spikes (A) and spontaneous spikes (B), as recorded on the 4 channels of the tetrode. (C) Scatter plots of peak amplitude of all spontaneous (black) and light-evoked (cyan) spikes.

3.4.2.3. Location specific firing

To show location specific firing, the mouse performed 3 10-min spatial exploration sessions in the same square arena as on the previous day. The isolated unit showed location specific firing that was different from the day before (compare Figure 3.14 and 3.20). The location of the place fields was different and firing was less specific on the second day of recording. Visual inspection of the firing rate maps recorded on the second day made clear that the location of the place fields varied between the 3 sessions (Figure 3.20). Based on the same criteria for quantitative place field analysis as used on the first day, session 1 and 3 had 2 place fields and session 2 had only 1 place field. Sparseness was higher on the second day and information and selectivity were lower (Table 3.4). The average firing rate, peak firing rate and burstiness were also higher on this day. ISI histograms show that the unit was theta modulated, like the unit recorded on the previous day.

Figure 3.20. Location specific firing. (A-C) Trajectories with spike locations. (D-F) Corresponding colour-coded firing rate maps. Each rate maps is scaled to the session's peak firing rate, which is indicated above the map. Red is peak firing, blue is no firing. White indicates bins which the mouse did not explore. (G,H) ISI histograms of spatial exploration session 1.

Table 3.4. Firing characteristics during exploration

Firing characteristics	Exploration (1)	Exploration (2)	Exploration (3)	Average
Average firing rate (Hz)	2.19	2.74	2.99	2.64
Peak firing rate (Hz)	11.22	15.81	12.73	13.25
Bursting	0.24	0.25	0.27	0.25
Sparseness	0.47	0.47	0.50	0.48
Information	0.65	0.69	0.63	0.66
Selectivity	5.28	5.75	4.27	5.10

3.4.3. Light-responsive place cell example 3: place cell activity recorded before light stimulation

The previous sections demonstrated light-evoked firing of 2 units recorded on 2 consecutive days on the same tetrode. Another light responsive unit was detected in another POMC-ChR2Y mouse. The light response and location specific firing of this unit will be described in the sections below. Histological analysis of tetrode recording sites showed that the tetrode on which the light responsive unit was recorded had crossed the granule cell layers of the upper and lower blade of the DG, and was located in the molecular layer of the lower blade of the DG at the end of the experiment. The light responsive place cell was recorded on the 3rd of January 2015 on tetrode 4 in mouse #218. The mouse was perfused 16 days later. The depth of the tetrode was 1937.5 μm , measured by monitoring rotations of the tetrode lowering screw. The tetrode was not lowered after recording the light responsive unit, but spike scatter plots changed during subsequent recording days, suggesting that the tetrode was drifting inside the brain tissue. Therefore, the light responsive unit described here may have been recorded in the molecular layer, granule cell layer or hilus of the DG.

3.4.3.1. Recording sessions: In the previous examples, light stimulation sessions were recorded for several days before a light responsive unit was detected. It is conceivable that extensive light stimulation and thus activation of ChR2-expressing cells in the DG might induce or alter place cells activity in those cells (Diamantaki *et al.*, 2016). In order to test this we did the experiment in a different way. In the experiment described below, our aim was to detect a place cell in the DG first, before light stimulation sessions were started. Once a place cell was recorded on a tetrode that was believed to be in the DG, light stimulation was started. After light stimulation spatial exploration was recorded again to check if the place fields had changed. Spike scatter plots and waveforms of the light-responsive unit are shown in Figure 3.23. Three sessions of spatial exploration were recorded to assess place field stability. One light stimulation session is shown and one control session with the laser light off. In addition, a rest session was recorded to characterize the activity of the unit when the mouse was in rest.

3.4.3.2. Light response

The isolated unit in Figure 3.21 showed a significant increase in firing in response to light stimulation. Light stimulation consisted of 245 brief light stimuli (pulse duration, 3.7 ms; frequency, 0.4 Hz). The intensity of the laser light was 15 mW at the tip of the optic fibre patch cord. Before implantation, light transmission through the optic fibre inside the drive reduced light intensity by 29%. Therefore, the light intensity at the tip of the optic fibre inside the brain would have been 10.65 mW. PSTHs and raster plots were constructed as described previously. Figure 3.22A shows a great increase in firing after the light stimulus. Figure 3.22A,B show that the baseline firing rate is very low. However, after light stimulation (cyan bar) there is a great increase in firing. Figure 3.22C shows the light response in more detail. There is one big peak at 5-7 ms after the onset of the light pulse, and more spikes up to 10 ms after light onset. The total number of spikes between 4 and 10 ms after light onset was 231. Out of the 245 trials, 51 did not induce any response. The response reliability was 79%. The average latency of all light-induced spikes (defined as spikes in the interval 4-10 ms after light onset) was 6.284 ms. The earliest putatively light-induced spike had a latency of 4.7 ms after light onset.

Figure 3.21. Recording stability. For each recording session, a spike scatter plot with spikes assigned to the isolated unit in red (left) and waveform of all spikes in the unit (right). The scatter plots show the peak amplitude of the spikes recorded on tetrode channel 1 vs. channel 4. The waveform (right) was recorded on channel 3. Amplitude is shown in AD values in both plots. AD value 20,000 corresponds to 427 μ V. The duration of the spike record (right) is 1ms.

Figure 3.22. Increase in firing after light stimulation. PSTH's and spike raster plots showing spikes from the red cluster in figure 1 recorded during the laser stimulation session. (A,B) Time interval from 100 ms before to 150 ms after light stimulation. Bin size, 1 ms. (C) Time interval from light onset to 15 ms after light onset. Bin size is 0.1 ms.

In order to **confirm that all spikes were generated by the same cell**, we inspected spike waveforms and scatter plots for all combinations of the spike features peak amplitude, energy and “peak-valley difference”, for the 4 tetrode channels. All waveforms and plots appeared very similar across recording sessions. Only the session with light stimulation showed slightly more variation in spike waveforms and spikes were less densely clustered in the scatter plots. To confirm that the spikes in this session were indeed from the same cell, the spikes were inspected in more detail. First, we plotted the spikes induced by light stimulation, and compared these with spontaneous spikes recorded in the same session (Figure 3.23). The spikes induced by light stimulation were more variable than the spontaneous spikes, but the average spike waveform was similar. The peak amplitude of the average light-induced waveform was smaller than the peak amplitude of the average spontaneous waveform. This was most obvious on channel 3, the channel with the highest amplitude spikes. The difference in amplitude can be explained by the fact that the light-induced waveforms contain spikes that came second in the light-induced spike

burst. As shown before, the second spike was usually smaller than the first one. Burst firing also happened spontaneously, but then the time between 2 consecutive spikes was longer and the decrease in amplitude was smaller. In addition, some light-induced spikes were not well aligned, which also led to a decreased peak amplitude. If the spikes were generated by 2 different cells, one light-responsive and one not light responsive cell, we would expect a systematic difference between these 2 groups of spikes. Figure 3.23 shows that the variability in light-induced spikes was random. No systematic difference in spike waveform could be detected, apart from the difference in peak amplitude. According to spike sorting principles this indicates that spikes were generated by a single cell. We also plotted light-induced and spontaneous spikes in scatter plots showing the peak amplitude of the spikes for each recording channel (Figure 3.23C). Also in these plots, there was no systematic difference in the 2 groups of spikes. Furthermore, ISI histograms showed that no spikes were recorded with an ISI of less than 1 ms (Figure 3.24A). If the spikes in this cluster were generated by 2 different light-responsive cells, we would expect many spikes responding to the light stimulus with a similar latency. In this case the chance of 2 spikes occurring within 1 ms from each other would be quite high (if both cells had a high response reliability). The fact that there were no spikes at all in the 0-1 ms interval, together with the fact that there was no systematic difference in spike waveform, strongly suggests that all spikes were generated by a single cell.

Figure 3.23. No systematic difference between spontaneous and light-induced spikes. (A) Waveforms of all light-induced spikes (defined as spikes recorded between 4 ms and 10 ms after light pulse onset), plotted for each of the 4 recording channels. (B) Waveform of all spontaneous spikes recorded in the same light stimulation session (spikes recorded more than 12 ms after light pulse onset). (C) Scatter plots displaying peak amplitude recorded on each combination of channels for spontaneous spikes (black) and light-induced spikes (cyan).

In the ISI histogram of the light stimulation session, shown in Figure 3.24A, a high number of spikes are present in 2-3 ms ISI. Most of these spike pairs were detected after light stimulation. In 37 out of 245 light stimulation trials, 2 spikes were detected after the light pulse. The delay between 2 light-induced spikes varied from 1.9 ms to 2.9 ms (average, 2.346 ms; SEM, 0.233 ms).

Figure 3.24. ISI histograms for spikes recorded during a light stimulation session (A) and a spatial exploration session (B). Note the absence of spikes in the interval 0-1 ms and the peak at 2-3 ms in the light stimulation session.

3.4.3.3. Location specific firing

As mentioned above, the aim of this experiment was to investigate if light stimulation caused a change in place cell activity. Three spatial exploration sessions were recorded, of which the first one before any light stimulation was given in the brain of this mouse. This spatial exploration session was followed by a control session, in which the laser light was off, and a light stimulation session with light intensity at 15 mW (Figure 3.21). Next, another spatial exploration session was recorded in the same arena, followed by again a light stimulation session and a spatial exploration session. At the end, a rest session was recorded, in which the mouse was resting on a towel inside a pot on a pedestal.

The location of the place fields was very similar in the 3 spatial exploration sessions (Figure 3.25). The firing rate was harder to compare, because there were some bins which the mouse did not explore. Other place cell characteristics like sparseness, information and selectivity were comparable between sessions, suggesting that light stimulation had little or no effect on the location specific firing of this unit (Table 3.5). ISI histograms were also similar before and after light stimulation. Representative examples of ISI histograms are shown in Figure 3.25G,H. All together, these results indicate that light stimulation had little or no effect on the activity of the light-responsive unit.

Table 3.5. Firing characteristics during exploration and rest

Firing characteristics	Exploration (1)	Exploration (2)	Exploration (3)	Average for exploration	Rest
Average firing rate (Hz)	1.06	1.77	1.32	1.38	1.18
Peak firing rate (Hz)	7.41	8.20	11.99	9.20	
Bursting	0.19	0.25	0.24	0.23	0.17
Sparseness	0.49	0.45	0.31	0.42	
Information	0.58	0.72	1.07	0.79	
Selectivity	7.62	4.70	9.45	7.26

Figure 3.25. Location specific firing was similar before and after light stimulation. (A-C) Trajectories with spike locations. (D-F) Corresponding colour-coded firing rate maps. Each rate maps is scaled to the session's peak firing rate, which is indicated above the map. Red is peak firing, blue is no firing. White indicates bins which the mouse did not explore. (G,H) ISI histograms of spatial exploration session 2.

3.5. Light-induced response latency

An increase in spikes after light stimulation can be caused in 2 ways. The spikes could be a direct response from a ChR2-expressing granule cell. Alternatively, spikes could be an indirect, synaptic response from a cell postsynaptic to a ChR2-expressing granule cell. If a granule cell fires a light-evoked action potential and thereby activates a postsynaptic cell, we could record “light-induced” spikes generated by the postsynaptic cell. Granule cells send axons to CA3 pyramidal cells and interneurons and issue collaterals that synapse onto mossy cells and interneurons of the hilus. Therefore, a tetrode in or near the DG could record “light-induced” spikes from granule cells, pyramidal cells, mossy cells or interneurons.

Because ChR2-expressing granule cells are directly activated by the light stimulus, while postsynaptic cells are activated only after granule cells fired, there should be a difference in response latency between granule cells and postsynaptic cells. In order to distinguish direct and indirect activation, we measured the response latency of ChR2-expressing granule cells *in vitro*. *In vitro* experiments have the advantage that Chr2Y-positive granule cells can be identified based on YFP expression. Therefore the response delay is undeniably a direct response. All *in vitro* patch clamp experiments were done by Dr. Congshu Liao. In addition, we sought to identify the minimal response latency for indirectly activated cells. If the response latency of a presumably light-activated granule cell measured *in vivo* is shorter than the minimal latency for an indirect response, we have strong evidence that the response recorded *in vivo* is a direct response from a granule cell.

3.5.1. *In vitro* response latency of ChR2-positive granule cells

First we measured the response latency of ChR2-positive cells by patch clamp experiments in brain slices. The same POMC-ChR2Y mouse line was used for *in vivo* and *in vitro* experiments. The light stimulation protocol *in vitro* consisted of 10-ms, 473-nm light stimuli at 0.4 Hz. ChR2-positive granule cells could be identified by EYFP expression. The response latency was determined by measuring the time between the onset of the light stimulus and the action potential threshold (Figure 3.26).

Figure 3.26. Measuring action potential latency. The action potential latency was measured from the start of the light stimulus (red trace on top) to the action potential threshold. The black trace at the bottom is the voltage trace measured in current clamp.

3.5.1.1. Increasing laser light intensity resulted in a decrease in response latency

In vitro patch clamp experiments were carried out to determine the response latency of light responsive granule cells. Response latency is not only dependent on the cell type, but also on the level of ChR2 expression, laser light intensity and optic properties of the tissue (Lima *et al.*, 2009). The exact light intensity *in vivo* is not known, because it is dependent on the distance between tetrode and optic fibre. In order to identify a range of possible response latencies, we varied the conditions of light stimulation *in vitro*. Two different neutral density (ND) filters were used and laser light intensity was increased in 20 steps while light-induced action potentials were recorded. The response delay was measured for 3 ChR2Y-expressing granule cells. Summary statistics are shown in Table 3.6.

Table 3.6. *In vitro* granule cell response delay statistics for 2 different ND filters and 20 light intensities. Action potentials of 3 granule cells were recorded. For granule cell 1, the response delay was measured 5 times at each light intensity with ND filter 1 and 6 times at each light intensity with ND filter 2. For granule cell 2, each light intensity was used once for both ND filters. For granule cell 3, each light intensity was used once for ND filter 2.

Light intensity (arbitrary units)	ND1 (n = 2 cells)				ND2 (n = 3 cells)			
	Average delay (ms)	SD	Min	Max	Average delay (ms)	SD	Min	Max
1	12.45	2.33	10.80	14.10	14.47	7.47	10.12	23.10
2	9.42	1.95	8.04	10.80	9.99	5.05	6.70	15.80
3	8.01	1.68	6.82	9.20	8.20	3.03	6.40	11.70
4	7.85	1.77	6.60	9.10	8.06	2.81	6.40	11.30
5	7.87	1.74	6.64	9.10	7.99	3.13	5.97	11.60
6	7.71	2.11	6.22	9.20	7.08	2.35	5.70	9.80
7	7.34	1.64	6.18	8.50	7.13	2.23	5.70	9.70
8	7.19	1.85	5.88	8.50	7.02	2.49	5.47	9.90
9	6.94	1.64	5.78	8.10	6.49	1.57	5.48	8.30
10	6.87	1.60	5.74	8.00	6.62	1.81	5.45	8.70
11	6.91	1.12	6.12	7.70	6.56	1.94	5.40	8.80
12	6.83	1.37	5.86	7.80	6.42	2.07	5.05	8.80
13	6.86	1.33	5.92	7.80	6.36	1.77	5.28	8.40
14	6.78	1.16	5.96	7.60	6.20	1.74	5.00	8.20
15	6.71	1.26	5.82	7.60	6.01	1.48	4.93	7.70
16	6.64	1.36	5.68	7.60	5.94	1.70	4.92	7.90
17	6.34	1.22	5.48	7.20	5.81	1.38	5.00	7.40
18	6.52	1.24	5.64	7.40	5.86	1.59	4.88	7.70
19	6.27	1.03	5.54	7.00	5.69	1.40	4.77	7.30
20	6.32	1.10	5.54	7.10	5.69	1.39	4.78	7.30

We found that increasing the light intensity resulted in a decrease in granule cell response latency (Figure 3.27). There was a significant relationship between the light intensity and response delay (Pearson correlation, $r = -0.760$ for ND1 and $r = -0.761$ for ND2, $p < 0.001$ for both ND1 and ND2). For high light intensities, the latency reached a plateau value. The minimal response latency varied between cells from 3.3 ms to 7.0 ms. At low light intensities, cells sometimes fired more than 10 ms after the light stimulus. Thus, these *in vitro* experiments show that the response latency of granule cells can vary from 3.3 ms to more than 10 ms.

Figure 3.27. The response latency of ChR2-expressing granule cells decreased with increasing light intensity. Response latencies for 3 different cells are shown in 3 different colours. Each trace represents a measurement of 20 light stimuli with increasing intensity. Each dot represents one measurement.

3.5.2. Response latency of postsynaptic cells

Since granule cells show a wide range of response latencies, dependent on light intensity and other conditions, we need another criterion to distinguish direct from indirect responses. If the response latency of postsynaptic cells would be very different, we could use this as criterion to distinguish directly and indirectly activated cells.

First, we tried to measure the response from a postsynaptic cell *in vitro*. We could not detect any action potential response in postsynaptic cells in the DG. Perhaps granule cells activated by light stimulation were not able to induce action potentials in postsynaptic cells. We did however record an excitatory postsynaptic potential (EPSP) in one cell in the hilus. The response latency measured from the onset of the light stimulus to the onset of the EPSP was 5.89 ± 2.07 ms. If this cell was activated by a ChR2-expressing granule cell, all cells responding faster than 5.89 ms could be considered directly activated. However, we cannot say for sure that this cell was directly activated by a granule cell. Although we believe that it is unlikely, the cell could have been 2 synapses away from a ChR2-expressing granule cell.

Since we were able to record only one cell, of which we don't know the identity, we also tried to identify the response latency of indirectly light-activated units *in vivo*.

3.5.2.1. *In vivo* indirect response latency

While trying to detect light-activated granule cells *in vivo*, we sometimes recorded an increase in firing after the light stimulus in putative interneurons. Putative interneurons were defined here as cells firing at > 2 Hz; Figure 3.28. Because interneurons do not express ChR2, this increase in firing was not a direct response to light stimulation, but an indirect, synaptic effect.

We found a significant increase in firing after the light stimulus in 35 interneurons. Interneurons were classified based on high firing rate (> 2 Hz) and the shuffling method was used to identify a significant increase in firing. The response of interneurons was rather variable between cells. Some interneurons fired spikes after a well-defined delay, others fired in a broad latency range, and some even fired at 2 distinct latencies (Figure 3.28).

We defined the response latency as the average latency of all spikes in the 3 consecutive 1-ms bins with the highest number of spikes. The average response latency was considerably longer for interneurons than for putative granule cells (2-tailed t-test, $p < 0.001$; Table 3.6). In comparison to the consistent response latency of the 3 putative granule cells, the response latency of interneurons showed a great variability. Although the average response latency of interneurons was longer, some interneurons showed a response that was faster than the response of the putative granule cells.

3.7. Comparison of the response latency of interneurons and putative granule cells.

	n	Response latency			
		Average	SEM	min	max
Interneurons	35	13.136	8.373	4.5931	46.2
Putative granule cells	3	6.324	0.167	6.133	6.441

To summarize, we showed that the response latency of the putative granule cells recorded *in vivo* falls within the range of response latencies recorded *in vitro*. In addition, the average response latency of interneurons recorded *in vivo* was longer than the average response latency of the putative granule cells. These data provide indications that the putative granule cells were directly activated by the light stimulus.

Figure 3.28. Interneuron response latency. Scatterplots (left) and PSTH's and rasterplots (right) for 5 examples of interneurons with a significant increase in firing after light stimulation. The timing of the light stimulus is indicated by a gray bar in the raster plots.

3.6. Scattered spikes and units with low light response reliability

The light responsive units presented in previous sections showed a highly significant response to light stimulation and high response reliability. 79% to 98% of light stimulation trials evoked a response. In addition, spike amplitudes were relatively large, units were well isolated and most of the “scattered spikes” in the light stimulation session were most likely generated by the same cell. In addition to these highly reliable light responsive units, we recorded 4 units that seemed to respond to light stimulation with a much lower reliability. The average response reliability of these units was 11.4% (range 2.5% - 30.4%). Although reliability was low, a small number of spikes after the light stimulus could be sufficient to qualify as a significant response, because the baseline firing rate of the units was often very low. As in the previous examples, these units were surrounded by scattered spikes, but here it was not clear if these were also generated by the same cells.

3.6.1. Light responsive units with low response reliability

An example of a light responsive unit with low light response reliability is shown in red in Figure 3.29. The unit is composed of the spikes shown in red in Figure 3.29A-C. Other spikes recorded in the same session are shown in black. The isolated red cluster was surrounded by many scattered spikes in the light stimulation session; (Figure 3.29A-C, middle row). The cluster was not well isolated in this session. Scattered spikes were present only in the light stimulation sessions, not in spatial exploration or control sessions. Because scattered spikes were dispersed around the red cluster in all projections, it was hard to sort spikes. If there was doubt if spikes should be included or excluded from the cluster, we chose to exclude them in order to provide more robust evidence if the unit were light responsive. The light response of the unit is shown in Figure 3.30A. The laser stimulation session consisted of 240 light stimulation pulses (3.7 ms pulses at 0.4 Hz, 15 mW). Only 6 spikes occurred within 10 ms after light onset (response reliability, 2.5%). Although the number of light-evoked spikes was very low, it was a statistically significant increase (Figure 3.30D). The average response latency was 7.50 ± 0.50 ms and the first spike occurred 6.62 ms after light onset. Both the average response latency and first spike occurred more than 1 ms later than in previous examples of units with high response reliability.

Since the light response of this unit was very different from previous examples, we investigated the light-induced spikes in more detail. First, we plotted all spikes of the red cluster in scatter plots displaying the peak amplitude of the spikes on each pair of tetrode channels (Figure 3.31C). Light-induced spikes were plotted in cyan and spontaneous spikes (spikes from the same session, occurring more than 10 ms after light pulse onset) in black. In all scatter plots, the light-induced spikes were overlapping with the cluster of spontaneous spikes. That is, they were not scattered outside the cluster formed by the spontaneous spikes. This means that the peak amplitude and the ratio of peak amplitudes on each pair of channels were comparable for spontaneous and light-induced spikes. Spike sorting is based on the idea that spikes originating from the same cell have similar waveforms. The observed consistency in peak amplitudes therefore indicates that the light-evoked and spontaneous spikes were generated by the same cell. In addition, we compared spike waveforms of the 6 light-evoked spikes with 100 randomly chosen spontaneous spikes (figure 3.31D). We did not find any obvious or systematic difference between spontaneous and light-induced spike waveforms.

Figure 3.29. Cluster isolation quality. (A-C) Scatterplots show all spikes recorded during one spatial exploration session (left), one laser stimulation session (middle) and one control session with the laser light off (right). Scatter plots display (A) spike peak amplitude on tetrode channel 2 vs. channel 4, (B) peak amplitude on channel 1 vs. energy on channel 2 and (C) the difference in voltage between the peak and valley of the spike, “peak-valley difference” on channel 2 vs. channel 3. Scattered spikes are encircled in green. (D) Spike waveforms for all spikes in the red cluster. Voltage is displayed in AD values. AD value 20.000 corresponds to 305 μV .

Figure 3.30. Light-evoked spikes. (A) PSTH and raster plot showing spikes from 10 ms before to 60 ms after light stimulation onset. (B) Scatterplots showing peak amplitude of all spikes in the red cluster of Figure 3.29. Light-induced spikes (3-10 ms after light onset) in cyan and spontaneous spikes (>10 ms after light onset) in black. (C) Waveform of light-induced spikes (cyan, left) and 100 spontaneous spikes (black, right). (D) Histogram of the number of spikes in the 3-ms block with the highest number of spikes for the 10,000 times shuffled data, with the 99.9 percentile indicated by a red line.

Another example of a unit with a significant light response, but low response reliability is shown in Figure 3.31. As in the previous example, scattered spikes were present around the cluster in the light stimulation session, which made it hard to isolate the cluster. With 16 spikes occurring within 10 ms after light stimulus onset, the unit showed a significant response to light stimulation (Figure 3.31B). Response reliability was 5.5%. The average response latency was 7.31 ± 0.68 ms and the earliest spike occurred 5.72 ms after light onset. Waveform and scatter plots showed no systematic difference in peak amplitude between light-evoked and spontaneous spikes (Figure 3.31C,D). Individual waveforms of light-induced spikes show more variability than waveforms of spontaneous spikes, yet there was no prominent difference in average waveforms. This similarity in waveform indicates that spontaneous and light-evoked spikes were generated by the same, light-responsive cell.

Figure 3.31. Light responsive unit with low response reliability. (A) Scatter plot displaying energy of the spikes recorded on tetrode channel 1 vs. channel 2. (B) PSTH and raster plot. (C) Scatterplot displaying peak amplitude on each pair of channels light-induced spikes in cyan and spontaneous spikes in black. (D) Spike waveforms. Light-induced spikes in cyan and spontaneous spikes in black.

3.6.2. A spike sorting problem caused by scattered spikes

In many laser stimulation sessions, clusters were surrounded by scattered spikes. Scattered spikes can create difficulties during spike sorting if they overlap with clusters. Often it was not straightforward to isolate single units in light stimulation sessions, even when units were clearly isolated in sessions without laser stimulation.

An example of a laser stimulation session where spike sorting was problematic is shown in Figure 3.32. The red cluster was well-isolated in the spatial exploration and control sessions, when the laser light was off. Nonetheless, it was surrounded by many scattered spikes in the laser stimulation session. A first attempt to sort spikes resulted in the red cluster shown in Figure 3.32A. The unit showed a significant increase in firing after light stimulation (Figure 3.32B,C). The waveforms of light-induced spikes were more variable than those of spontaneous spikes (Figure 3.32E). When we plotted light-induced and spontaneous spikes together in scatter plots, it was apparent that many of the light induced spikes were located outside the cluster formed by the spontaneous spikes (Figure 3.32D). For many of the light induced spikes, the peak amplitude was larger on tetrode channels 1 and 2, and smaller on tetrode channel 3. This systematic difference in peak amplitudes could signify that light-induced and spontaneous spikes were generated by different cells.

3.6.3. A false positive result caused by scattered spikes

Spike analysis suggested that the cluster isolated above may contain spikes that did not belong to the cluster. The cluster was overlapped by many scattered spikes, which may have been generated by different cells. In order to isolate a cluster so that it contained only spikes generated by one single cell, we sorted spikes again, so that the new cluster only contained very similar spikes. The spikes of this newly isolated unit, shown in blue in Figure 3.33A, were tightly clustered in all spike sorting projections. Spikes that were included in the first spike sorting attempt are shown in red. The PSTH and scatterplot for this unit show that 4 spikes were fired within 10 ms after light stimulation (Figure 3.33B). This was not a statistically significant response (percentile 99.7; Figure 3.32C). The scatterplot in Figure 3.33D shows that the 4 spikes were located on the outer edge of the cluster of spontaneous spikes. Although we tried to include only spikes with very similar waveforms, the 4 spikes still showed some waveform variability (Figure 3.33E).

To summarize, we believe that the isolated red cluster in the first spike sorting attempt was not light-responsive, but contained some of the scattered spikes. Scattered spikes seemed to be randomly distributed over the spike sorting projections, which made spike sorting tricky and may have caused a false positive result. Because we were not sure if the light-induced “scattered” spikes were generated by the same unit or not, we sorted the spikes again, trying to exclude scattered spikes. This new cluster, which included only spikes with very similar waveforms, did not show a significant light response.

Figure 3.32. Cluster including some scattered spikes shows a significant response to light stimulation. (A) Scatter plots showing all spikes recorded during a spatial exploration session (left), a control session without light stimulation (middle) and a light stimulation session (right). Spikes in red are sorted into a cluster. Scattered spikes are encircled in green. (B) PSTH and raster plot for spikes of the red cluster. (C) Histogram of the number of spikes in the 3-ms block with the highest number of spikes for the 10,000 times shuffled data, with the 99.9 percentile indicated by a red line. (D) Scatter plots for spikes of the blue cluster with spontaneous spikes in black and light-evoked spikes in cyan. (E) Waveforms of light-evoked spikes (left, cyan) and 100 spontaneous spikes (right, black).

Figure 3.33. Cluster excluding scattered spikes shows no significant light response. (A) Scatterplot showing all spikes recorded during a light stimulation session. Spikes in blue are included in the cluster. Spikes in red were included in figure 3.32, but are excluded from the blue cluster. (B) PSTH and raster plot for spikes of the blue cluster. (C) Histogram of the number of spikes in the 3-ms block with the highest number of spikes for the 10,000 times shuffled data, with the 99.9 percentile indicated by a red line. (D) Scatter plots for spikes of the blue cluster with spontaneous spikes in black and light-evoked spikes in cyan. (E) Waveforms of light-evoked spikes (left) and 100 spontaneous spikes (right).

Spike sorting when clusters are surrounded by scattered spikes is problematic for 2 reasons. First of all, the spikes scattered around the clusters in the light stimulation session lead to poor isolation quality. Usually, poorly isolated clusters are not included in analysis, because it is not clear if all spikes were generated by the same cell. Secondly, spikes scattered around the cluster may or may not be generated by the same cell. Spike sorting is based on similarity in waveform. Spikes with similar waveforms are supposed to be generated by the same cell. However, spikes generated after light stimulation might be slightly different. Small differences in waveform can be caused by opening of ChR2 channels. Another possible explanation for variation in waveform is that after light stimulation, several ChR2-positive cells fire together, which may cause changes in baseline voltage (see further).

3.6.4. Scattered spikes are evoked by light stimulation

In previous sections, we demonstrated a spike sorting problem caused by scattered spikes. Scattered spikes were only present in light stimulation sessions, not in exploration or control sessions, when laser light was turned off. In the following sections, we will explore what caused scattered spikes in light stimulation sessions, and how they can be prevented.

Since scattered spikes were only present in light stimulation sessions, we expected that they were caused by the light stimuli. To check this, we plotted all spikes recorded during a light stimulation session, with spikes that were fired within 10 ms from light stimulation onset in cyan (Figure 3.34). We demonstrate this for the laser stimulation session presented in Figure 3.29 (Figure 3.29A, laser ON). This figure makes it clear that the scattered spikes were evoked by light stimulation. The red cluster in Figure 3.29 is circled in red in Figure 3.34. In all plots, some light-evoked spikes were overlapping with the red cluster. Yet again, it cannot be deduced from this figure if they were a part of the red cluster or of the cloud of scattered cyan spikes.

Figure 3.34. Scatter plots of all spikes recorded during a light stimulation session. Light-induced spikes (spikes within 10 ms from light onset) in cyan and other spikes in black. The red cluster from Figure 3.29 is circled in red.

3.7. Photovoltaic artefact

3.7.1. A cluster that was only present in light stimulation sessions

Most of the scattered spikes were dispersed over the plots in all projections and did not seem to form clusters. However, in some light stimulation sessions, we noticed that a certain group of light-induced spikes were clustered. Figure 3.35 shows a laser stimulation session with light-evoked spikes (spikes within 10 ms from light onset) in cyan. Also in this example, light-evoked spikes were scattered over the plots. However, light-evoked spikes seemed to form 1 clear cluster. This cluster was not present in the spatial exploration or control sessions. One possibility is that this cluster was formed by light-evoked spikes of a granule cells that was quiet during spatial exploration and rest sessions. Another possibility is that this cluster was composed of artifacts caused by light stimulation, which look like spikes. This second possibility will be explored in the following section.

Figure 3.35. Scatter plots of all spikes recorded during a light stimulation session and spatial exploration. Light-induced spikes (spikes within 10 ms from light onset) in cyan and other spikes in black. Light-evoked spikes that formed a cluster in the light stimulation session are circled in red. The red circle in the spatial exploration plots indicates the absence of the cluster.

3.7.2. Short latency response with abnormal waveform

In some light stimulation sessions, a cluster appeared that was not present in exploration or control sessions. In the example shown in Figure 3.36 (same data as in section 6.3.2), 3 clusters were isolated in the spatial exploration session. All 3 showed location specific firing, but no light-induced activity (Figure 3.36B). In the laser stimulation sessions, a cluster appeared that was not present in the other sessions. (purple cluster in Figure 3.36A). The unit was overlapping with some scattered spikes, but could be easily isolated because the spikes were relatively tightly clustered. Waveform analysis showed that the amplitude of the spikes was roughly the same on all 4 tetrode channels (Figure 3.36C). This is not often seen in unit recording, but it could happen if a cell is located at a similar distance from all 4 tetrode wires. The waveform was also wider than the waveform of regular spikes (compare red and purple spikes in Figure 3.36C). The spikes seemed to have a slower depolarization and repolarization phase. The atypical waveform hinted that these were not normal spikes. Analysis of the light response showed that the unit fired 1 spike after nearly every light pulse (Figure 3.36D). The response was very time-specific; all spikes happened between 2.5 and 3 ms after light stimulation onset. No spikes were detected outside of this time interval. The average response latency was 2.82 ± 0.08 ms. This was faster than any granule cell response recorded *in vitro*. Given the abnormal waveform and conspicuously fast response, it seemed implausible that these “spikes” were generated by granule cells.

Figure 3.36. Short latency response with abnormal waveform. Scatterplots of spikes recorded during (left) spatial exploration, (middle) laser stimulation and (right) a control session with laser light off. Peak amplitude on channel 1 vs. channel 4 is displayed in AD values. AD value 10.000 corresponds to 122 μ V. (B) Location specific firing of the red, blue and green units recorded during spatial exploration. (C) Waveform of all spikes in the red and purple cluster, as recorded on the 4 channels of the tetrode. (D) PSTH and raster plot for spikes of the purple cluster.

Another example of a short-latency response with abnormal waveform is shown in Figure 3.37. In this case, 2 new clusters appeared in the light stimulation session, which were not present in spatial exploration and control sessions (Figure 3.37A). The spikes had a very high amplitude, so that the peak was sometimes outside of the detection range. Still, the spike amplitude seemed similar on all 4 tetrode channels, as in the previous example, and the spike width was wider than usual (Figure 3.37B). All spikes happened between 2.00 ms and 2.33 ms for the blue cluster and between 3.29 ms and 3.74 ms for the green cluster. There were no spontaneous spikes outside of this interval. The average latency was 2.20 ± 0.05 ms for the blue cluster and 3.50 ± 0.08 ms for the green cluster. For every single stimulus, there was a response in both clusters.

This short latency response with abnormal waveform was observed 32 times in 76 recording sessions and on 12 different tetrodes. The response latency was 2.77 ± 0.450 ms (range, 1.96 – 3.53 ms).

Figure 3.37. Short latency responses with abnormal waveform. (A) Scatterplot displaying spikes recorded during a light stimulation session. (B) Waveform of all spikes of the blue and green cluster. (C, D) PSTH and raster plot for the blue and green cluster, respectively.

3.7.3. Photovoltaic artefact

The abnormal waveform and extremely short response latency make it hard to believe that the response presented here was generated by granule cells. Since the response is only present after light stimulation, it is likely to result from a photovoltaic artefact. Photovoltaic artefacts can be caused by interaction between the light and the electrodes. It is known that illumination of electrodes placed in an electrolyte causes electrons to be emitted from the electrode. This effect, called the Becquerel effect or photovoltaic effect, has been shown to cause artifacts in optogenetic experiments (Mikulovic *et al.*, 2016; Roux *et al.*, 2014). Since the photovoltaic effect is independent of ChR2 expression, it should also be present in ChR2-negative mice. To test this, we did the same light stimulation experiment in a Cre-negative mouse, which should not have any ChR2 expression.

On one of the tetrodes placed in the brain of a ChR2-negative mouse, a small response was recorded that could represent the photovoltaic effect. The tetrode was located in the *stratum radiatum* of area CA1 and the optic fibre was in the *stratum oriens* of CA1. The estimated distance between the optic fibre and tetrode was 500 μm , comparable to the distance between optic fibre and tetrodes in ChR2-positive mice. The photovoltaic effect was hard to identify in the recorded local field potential (LFP) trace by observing individual trials. Therefore, we aligned the LFP at the trial onset and displayed the averaged LFP (Figure 3.38A). The average LFP showed a small increase in voltage at approximately 2 ms after light onset. This response was observed at all 4 channels of the tetrode.

However, the effect was well within standard deviation and would not be a significant deviation from the baseline value (amplitude $\sim 10 \mu\text{V}$). It was only visible on 1 of the tetrodes (the other tetrodes were possibly located not deep enough inside the brain to observe the effect). We did the same analysis for the LFP of the 2 examples shown in previous sections (Figure 3.36 and 3.37). In these examples, the presumed photovoltaic effect is clearly visible (arrows in Figures 3.38B, C). The amplitude is also 10 times larger (100 μV - 300 μV).

The photovoltaic effect is dependent on the relative position and orientation of the optic fibre and tetrode, so the difference between the ChR2-positive and ChR2-negative mice could be explained by a different position or orientation of the optic fibre and tetrodes. Since we only have results from 1 ChR2-negative mouse, we cannot confirm that the response was caused by the photovoltaic effect. More experiments are required to determine the origin of the short-latency response with abnormal waveform.

Figure 3.38. Average filtered LFP aligned at the light stimulus onset. The LFP was filtered between 600 Hz and 6.000 Hz, aligned at light stimulus onset and averaged over all trials. The light stimulus on and off times are indicated by blue lines. The grey area indicates the standard error. (A) LFP is shown for the 4 channels of a tetrode in the brain of a ChR2-negative mouse. One of the channels is shown larger on the left and the other 3 in small on the right. The presumed photovoltaic effect is indicated by a red arrow. (B) Average LFP of one channel of the example shown in Figure 3.36. (C) Average LFP of one channel of the example shown in Figure 3.37. The average LFP was very similar on all channels of the tetrode.

3.8. Summary of all units

Table 3.8 provides an overview of all well isolated units ($n = 191$). Units were defined as well isolated if the L-ratio was smaller than 0.05 (Schmitzer-Torbert *et al.*, 2005). The units were recorded on 19 tetrodes in 6 mice during 71 recording sessions. A ‘recording session’ consisted of 3 spatial exploration sessions (~10 min each), 2 light stimulation sessions (3.7 ms laser pulses, 0.4 Hz, ~10 min, ~250 stimuli), and 1 control session with the laser light off (~10 min).

A unit was classified as a place cell if it had at least one place field. A place fields was defined as a group of at least 8 contiguous bins with an average firing rate exceeding 20% of the peak firing rate, and peak firing rate above 2 Hz. Units were classified as putative interneurons if the average firing rate was above 2 Hz. Units were light responsive if they showed a significant increase in firing rate within 25 ms after the light stimulus and the waveforms of light-evoked and spontaneous spikes were visually similar. Response latency was calculated as the average latency of all spikes in the 3 consecutive 1-ms bins with the highest number of spikes. The response reliability was calculated as the percentage of light stimuli that evoked at least one spike. Multiple spikes per stimulus were only observed in the units with high response reliability. Units were considered photovoltaic artefacts if the spike amplitude was similar on all 4 tetrode channels and the response delay was less than 3.6 ms.

Table 3.8. Overview of light response characteristics and firing rate of all well-isolated units.

		Laser stimulation		Spatial exploration (laser OFF)
		response latency (ms)	response reliability (%)	firing rate during exploration (Hz)
place cell	74	-	-	0.80 ± 0.62
no light response				
putative granule cell	3	6.324 ± 0.167	90.2 ± 9.9	1.63 ± 0.90
light response with high response reliability				
putative granule cell	4	6.595 ± 0.954	11.4 ± 12.8	1.16 ± 0.99
light response with low response reliability				
putative interneuron	19	-	-	14.41 ± 12.95
no increase in firing after light stimulus				
putative interneuron	35	13.136 ± 8.373	-	10.80 ± 8.68
increase in firing after light stimulus				
photovoltaic artefact	31	2.767 ± 0.450	-	-
other units	25	-	-	-
Total	191			

4. Discussion

One of the most fundamental goals in neuroscience is to understand the function of individual neurons in neural networks and their role in behaviour. Extracellular unit recording is the most commonly used method to monitor the activity of single neurons in behaving animals. The major drawback of the technique is the inability to determine the anatomical identity of the cells that generated the activity. This is problematic especially in brain areas containing multiple excitatory cell types, like the DG. Attempts have been made to classify the cells of the DG based on physiological criteria, but these were insufficient to conclusively determine the anatomical identity (Neunuebel and Knierim, 2012). In order to fully understand the function of the DG, the development of techniques with the potential to identify and record activity of specific cell types is crucial.

Recently, the introduction of optogenetics has made it possible to identify neuronal populations during *in vivo* extracellular recording (Lima *et al.*, 2009). By combining extracellular unit recording with optogenetics, specific neuronal cell types can be stimulated and their response can be recorded at the same time. The technique involves expression of light-sensitive proteins like ChR2 in specific neuronal populations. Illumination of ChR2-expressing neurons with brief flashes of blue light evokes short latency action potentials, which can be monitored using tetrode-based unit recordings. The technique has been applied to identify inhibitory and excitatory neurons in a few different brain regions (Arenkiel *et al.*, 2007; Cardin *et al.*, 2010; Chaves-Coira *et al.*, 2016; Cohen *et al.*, 2012, 2015; Han, Qian, Bernstein, H. Zhou, *et al.*, 2009; Kvitsiani *et al.*, 2013; Lima *et al.*, 2009; Royer *et al.*, 2010; Wu *et al.*, 2013; Xu *et al.*, 2015; Zhang *et al.*, 2013). While these studies have demonstrated that optogenetic identification of neuronal cell types is feasible, they also made clear that combining optical stimulation and electrophysiological recordings poses several challenges.

The objective of this PhD project was to establish and evaluate the combined optogenetic-electrophysiological technique for identification of granule cells in the DG of freely moving mice. We stimulated ChR2 expressing granule cells in the DG and were able to detect units that responded to light stimulation with short latency and high reliability. However, we could not provide conclusive evidence that the units were granule cells. Careful analysis of the light response revealed that the optogenetic identification technique has limitations that have to be overcome to identify granule cells. Here, we will evaluate the use of the optogenetic identification technique, discuss its strengths and weaknesses and propose ways to solve the limitations we identified.

4.1. Granule cell specific expression of ChR2

Cell type specific expression was obtained by crossing a granule cell specific Cre driver line with a Cre-dependent ChR2 reporter line (POMC-Cre × Ai32). The Ai32 ChR2 reporter line was specifically designed to facilitate the expression of ChR2 in specific, genetically defined cell types (Madisen *et al.*, 2012). By crossing this line with any one of the hundreds of available Cre driver lines, reliable and accurate expression of ChR2 can be obtained in a wide range of cell types. Crossing the Ai32 mice with POMC-Cre

mice resulted in specific expression of ChR2 in 10% of granule cells (Figure 3.1). While many other studies have used viral vectors to obtain high levels of ChR2 expression in the desired cell type, we opted for a fully transgenic approach. This way, we bypassed the need to produce viral vectors and obtained highly reliable and reproducible expression of ChR2.

Since each ChR2 channel produces only a small current, high levels of ChR2 are required to effectively evoke action potentials. In order to test if the expression level of ChR2 was sufficient to evoke action potentials in ChR2Y-positive granule cells in POMC-ChR2Y mice, Dr. Stefan Blankvoort, a previous PhD student of the lab, used immunostaining to demonstrate that light stimulation increased the expression of the IEG c-FOS in ChR2-positive granule cells compared to non-stimulated mice (Blankvoort, 2015). This experiment proved that light can effectively induce activity in ChR2-expressing granule cells in POMC-ChR2Y mice. However, it should be noted that the light stimulation protocol in the c-FOS experiment was different from the light stimulation protocol used in the optogenetic identification experiments (10-ms pulses at 10 Hz for 3 min vs. 3.7-ms pulses at 0.4 Hz for 10 min). This is an important difference, because longer pulses and higher frequency increase the chance that a neuron is activated. However, our *in vitro* experiments showed that some granule cells fired 3.3 ms after the onset of the light stimulus. In addition, multi-unit analysis of the *in vivo* data showed a great response after the light stimulus, which suggests that 3.7 ms pulses were sufficient to evoke a light response.

We chose to use a mouse line in which Cre expression was restricted to 10% of granule cells despite the fact that mouse lines with Cre expression in nearly all granule cells are available (for example Dock10 Cre mice; (Kohara *et al.*, 2013)). We reasoned that if all granule cells would express ChR2, light stimulation would cause firing of many or all granule cells at the same time. Besides the fact that this is a very non-physiological state, it may also cause problems for spike detection and spike sorting. The granule cell layer is a very dense cell layer (900.000 granule cells /mm³ in the mouse dorsal DG; (Jinno and Kosaka, 2009)). A tetrode in this layer would be surrounded by a high number of granule cells. Based on large-scale recordings in the CA1 region, it was estimated that a tetrode can record spikes fired by neurons within a cylinder with a radius of 50 µm (Buzsáki, 2004). If the same holds true for the DG, a cylinder of this size would contain approximately 350 granule cells. If all these cells would fire within 10 ms after light stimulation, it would be impossible to isolate the spikes from individual cells. Hence, a low percentage of granule cells expressing ChR2 would be beneficial for spike detection and spike sorting.

With 10% of granule cells expressing ChR2, the number of ChR2-expressing granule cells that theoretically can be detected by a single tetrode in the granule cell layer would be 35. Yet, in practice, only a small fraction of the neurons can reliably be detected with currently available tetrodes and spike sorting methods. The remaining neurons may be damaged by the blunt end of the tetrodes, or may be too small in amplitude. Thus there is a large gap between the numbers of theoretically recordable and routinely recorded neurons (Buzsáki, 2004).

If a tetrode in the granule cell layer can record up to 35 ChR2-expressing granule cells, why did we identify only 3 reliably light-responsive cells?

First of all, many of the tetrodes did not reach the granule cell layer. Histological analysis of tetrode tracks showed that only 28 out of 175 tetrodes (16%) had reached the granule cell layer. Tetrodes are very thin wires that can easily get stuck inside the brain or bend and move in the wrong direction. For the tetrodes that did reach the DG, we may have lowered them through the thin layer of sparsely firing granule cells without noticing. In theory, we could have used light stimulation to search for the granule cell layer. If we monitored unit activity while stimulating with light and slowly lowered the tetrodes to the granule cell layer, it should be easy to detect the light-responsive units of the granule cell layer. However, light-evoked activity was not only detected in the granule cell layer, but also in the surrounding regions. For example, mossy cells in the hilus or CA3 pyramidal cells could be indirectly activated by input from ChR2-expressing granule cells. The difference in response latency between directly and indirectly activated cells was impossible to detect during recording. In addition, the photovoltaic effect could be detected in any brain region where the light illuminated the tetrode tip. This effect was hard to distinguish from spikes, especially during recording. For these reasons, we could not depend on light-evoked activity during recording to determine if the tetrode was in the granule cell layer.

4.2. Response latency

One of the main challenges in optogenetic identification experiments is distinguishing directly light-activated cells from indirectly, synaptically activated units.

Granule cells of the DG innervate CA3 pyramidal cells, hilar mossy cells and interneurons of the hilus and CA3. They form specialized, powerful excitatory connections with their target neurons. It was shown that **a single granule cell is able to reliably activate target neurons in the hilus and CA3** (Henze *et al.*, 2002; Scharfman *et al.*, 1990). While a train of spikes is needed to activate target neurons in CA3, even a single action potential of a granule cell is sufficient to evoke a burst in mossy cells. Thus, optogenetic activation of granule cells most likely activates postsynaptic mossy cells and perhaps also CA3 pyramidal cells and interneurons.

Besides mossy cells and CA3 pyramidal cells, granule cells also make numerous excitatory synapses on inhibitory interneurons in the hilus and CA3 region (Acsády *et al.*, 1998). In fact, interneurons are the main postsynaptic target of dentate granule cells. Therefore, activation of granule cells could evoke feed-forward inhibition of CA3 pyramidal neurons and the net effect of optogenetic activation of granule cells could be inhibition of CA3 pyramidal cells.

A few different approaches have been used to distinguish direct from indirect activation in optogenetic identification experiments. The most convincing way to demonstrate that a cell is directly activated is by **blocking synaptic transmission** (Lima *et al.*, 2009; Zhang *et al.*, 2013). This can be achieved by applying synaptic blockers like the AMPA receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX). For deep brain regions like the DG, the synaptic blocker has to be infused into the brain. This carries a risk of losing the recorded unit due the injection of a small volume of fluid in the target area. In addition, blocking synaptic activity may

change the excitability of neurons, and thereby change the response latency of ChR2-expressing neurons. Apart from the risks of losing the recorded unit or changing the excitability, it would also be cumbersome to do this experiment for every putatively light-responsive unit.

Another way to distinguish direct and indirect activation is based on **high frequency stimulation** (Lima *et al.*, 2009). It was shown for cortical neurons that directly activated ChR2 expressing neurons reliably follow 5 Hz stimulation, whereas synaptically activated cells failed to follow 5 Hz stimulation. It is not known if this is also true for dentate granule cells. However, if it is true, high frequency stimulation could be a reliable, easy and fast approach to distinguish direct and indirect activation.

A more straightforward method to separate directly and indirectly activated cells is based on **response latency**. If light-activated neurons fire within a few ms after light onset with little variation in latency, and the latency for a postsynaptic cell to fire is relatively long, the difference in response latency could be used as a criterion to distinguish directly and indirectly activated neurons. The latency for a ChR2-expressing cell to fire after light stimulation is dependent on the cell type, light intensity and ChR2-expression level. Different cell types show different response latencies, as shown by a variety of published optogenetic identification studies (Cohen *et al.*, 2012; Kvitsiani *et al.*, 2013; Lima *et al.*, 2009; Zhang *et al.*, 2013). Inhibitory interneurons generally fire at shorter latencies than excitatory neurons. All directly activated neurons identified so far fired between 2 ms and 10 ms after the onset of light stimulation. Zhang *et al.* provided evidence that indirectly activated cells could be distinguished based on a difference in response latency (Zhang *et al.*, 2013). However, other studies have put forward that response latency cannot be used as criterion to separate direct and indirect firing (Henze *et al.*, 2002; Kvitsiani *et al.*, 2013; Lima *et al.*, 2009; Stark *et al.*, 2015). They argued that synaptic transmission from principal cells to interneurons may be rapid and strong. Indeed, the latency between a CA1 pyramidal cell action potential and interneurons spike was shown to be only 1.5 ms (Marshall *et al.*, 2002). Moreover, the response latency of a certain cell type can vary between cells because it is dependent on ChR2 expression levels and the amount of light that reaches the cell. In this regard, separation of directly and indirectly activated neurons by latency criteria can be highly unreliable.

In order to assess if response latency could be used to distinguish direct and indirect responses in our experiments, **we determined the response latency of granule cells and postsynaptic cells**. The response latency of optogenetically activated granule cells was measured *in vitro* in patch clamp experiments. The latency was found to be highly variable between cells and was also dependent on light intensity. Because of this high variability, we could not rely on this measure to identify directly activated cells. Instead, we tried to identify the minimal response latency for synaptically activated cells. If the response latency of a certain unit was shorter than the minimal response latency of indirectly activated cells, then this unit must be directly activated. Detecting indirectly activated mossy cells or interneurons by patch clamp experiments *in vitro* was more difficult than expected. Only one putatively indirectly activated cell could be detected. In our *in vivo* experiments, on the other hand, we detected several interneurons with a significant increase in firing after light stimulation. The response latency of the very first putatively postsynaptic spike of all interneurons was 5.9 ms. The three examples of light-responsive units (putative granule cells) all fired spikes with a latency shorter than 5.9 ms, suggesting that they were directly activated. In addition to the minimal response latency, the average response latency was also longer for

interneurons than for putative granule cells. Although these data suggest that the putative granule cells were directly activated, they do not provide conclusive evidence. Because the latency criterion is based on data from a limited number of interneurons, we cannot ascertain that this is the absolute minimum for all postsynaptic cells. It remains possible that other interneurons or mossy cells could fire at shorter latencies. In order to prove that the putative granule cells were indeed directly activated, other experiments like blocking of synaptic transmission are necessary.

Based on the available data, **we cannot confirm that the 3 examples of putative granule cells were directly light-activated.** They could also be mossy cells of the hilus, transsynaptically activated by light-responsive granule cells. The third example of light responsive unit could also be a CA3 pyramidal cell, because the tetrode had crossed the CA3 pyramidal cell layer. Interestingly, a study in anesthetized rats has shown that the delay between granule cell action potentials and CA3 pyramidal cell spikes is on average 2.7 ms (Marshall *et al.*, 2002). If we assume that this delay is similar in awake mice, and the minimal delay between the light stimulus and granule cell action potential is 3.3 ms (as shown in our *in vitro* experiments), then the minimal delay between the light stimulus and CA3 pyramidal cell spike would be 6 ms. Since the light-responsive unit fired several spikes with a delay under 6 ms, it is rather unlikely that the unit was a pyramidal cell. Moreover, the unit had multiple place fields in the exploration arena, whereas CA3 pyramidal cells usually show only one place field in a standard size arena (Leutgeb *et al.*, 2007). The data presented here indicate that the 3 light-responsive units are probably not CA3 pyramidal cells.

As mentioned above, granule cells make strong synaptic connections with hilar mossy cells. Using paired intracellular recordings in rat hippocampal slices, it was demonstrated that a single action potential in a granule cell can evoke a burst of action potentials in mossy cells (Scharfman *et al.*, 1990). Thus it is very likely that optogenetic activation of granule cells causes synaptically evoked activity in mossy cells.

There are about 100 granule cells for each mossy cell in the DG (Amaral *et al.*, 1990). Granule cells form sparse, but powerful connections with mossy cells. It was estimated that each granule cell innervates only one or two mossy cells and a typical mossy cell receives input from up to 100 granule cells (Scharfman, 2007). Given the strength of the connections, it can be predicted that mossy cell firing is closely related to the firing of the input cells. If the granule cells which provide input to a mossy cell show place cell activity, then the mossy cell probably shows related location specific firing. It has been demonstrated for CA1 that monosynaptic connections between pyramidal cells and neighbouring interneurons can induce place-related firing in the interneuron (Marshall *et al.*, 2002)(Marshall 2002). Based on these ideas, it is conceivable that mossy cells show place-related firing that is dependent on the place cell activity of granule cells.

Other indications suggesting that the light-responsive units could be granule cells include the relatively high firing rate and the high spike amplitude, both characteristics of mossy cells (Henze and Buzsáki, 2007; Scharfman, 1994).

Briefly summarized, in this section we assessed if the light response latency could be used to distinguish directly and indirectly activated units. Despite the difference in minimal and average response latency

between the putative granule cells and interneurons, a latency criterion was not sufficient to provide conclusive evidence for direct light activation. Therefore, the putative granule cells could also be synaptically activated mossy cells of the hilus. Besides this uncertainty, we also noticed that the light-responsive units showed a relatively high firing rate, large amplitude spikes and multiple place fields, which could be characteristics of mossy cells.

4.3. Light-induced artefact

Several studies in which optical stimulation and extracellular recording were combined have mentioned artefacts caused by the interaction between light and electrodes (Ayling *et al.*, 2009; Cardin *et al.*, 2010; Han, Qian, Bernstein, H.-H. Zhou, *et al.*, 2009; Kozai and Vazquez, 2015; Mikulovic *et al.*, 2016; Roux *et al.*, 2014; Zorzos *et al.*, 2009). The light artefacts are often referred to as "photoelectric effect", but this is not correct because the photoelectric effect requires very high energy photons like X-rays to eject electrons from a metal. The artefacts seen in optogenetic experiments are most likely caused by the photovoltaic effect. In this effect, light is absorbed by an electron, which causes the electron to move to a higher energy orbital. The electron is not ejected from the material, but an electric potential is produced by the separation of charges. This potential can cause artefacts in extracellular recordings.

Although light artefacts can confound LFP as well as spike recordings, only a few studies have given a clear description of the artefact (Cardin *et al.*, 2010; Han, Qian, Bernstein, H.-H. Zhou, *et al.*, 2009; Mikulovic *et al.*, 2016). Han *et al.* immersed electrodes in saline to characterize the effect of light on electrode recordings (Han *et al.*, 1993). They showed that the effect is proportional to the light intensity and dependent on the wavelength and orientation of the electrode and light beam. For a given position of electrode and light beam, artefacts remain the same in amplitude and duration throughout the delivery of repeated light pulses. The artefact is also very tightly time-locked to the light pulse, in contrast to spikes evoked by light stimulation of ChR2-expressing cells (Dugué *et al.*, 2012). In continuous voltage recordings (LFP), the effect appears as an exponential charging and discharging, with a duration of approximately twice the length of the light stimulus (Dugué *et al.*, 2012). In spike recordings, the photoelectric artefact can distort spike waveforms, especially when the light stimulus is short. If the voltage transient caused by the light stimulation is above the spike detection threshold, it will be detected as a spike. Additionally, our own data showed that the time between the onset of the light stimulus and the presumed light artefact was different for different tetrodes, suggesting that it is dependent on the positioning of the tetrode and light beam. Furthermore, the latency of the effect was clearly shorter than the latency of light-evoked spikes in our experiments. Because of this very short and highly consistent latency, the effect was easy to distinguish from real spikes.

One way to reduce the photovoltaic effect is by reducing the light intensity. The currently available opsins require rather high light intensities to be effective, but with the development of more sensitive light-activated ion channels, low light intensities will be sufficient to activate neurons. In addition, "light-proof" recording electrodes are being developed with materials or coatings that attenuate light artefacts (Zorzos *et al.*, 2009).

As the photovoltaic effect is independent of the expression of optogenetic proteins, it should be present in ChR2-negative mice. However, in our experiment we did not find a clear effect of light stimulation in a ChR2-negative mouse. Possibly, the positioning of optic fibre and tetrodes was not optimal to induce the effect. Many of the tetrodes in ChR2-positive mice also did not detect any photovoltaic artefact. Further experiments are needed to determine the exact positioning and orientation that causes the effect. A better understanding of the photovoltaic effect will help us to recognize, minimize or prevent it.

4.4 Scattered spikes and units with low response reliability

Besides a few units with very high response reliability, we also recorded numerous units with a response reliability of less than 10%. Most of these were significantly light-activated, because even a few spikes can be significant if the baseline firing rate is low. However, the observation that these units were surrounded by many light-evoked, scattered spikes made us **doubt if the units were really ChR2-expressing granule cells or if it was a false positive result.**

In extracellular unit recording, spikes are sorted into units based on similarity in waveform. Spikes generated by the same neuron form clusters in spike feature scatter plots. The majority of the low response reliability spikes formed well-isolated clusters in the spatial exploration and control sessions, but they were surrounded by scattered spikes in the light stimulation sessions. These scattered spikes were dispersed over the plot and could not be isolated in clusters. They caused difficulties in spike sorting, as there was no clear boundary between spikes of the cluster and scattered spikes. Furthermore, we showed that the scattered spikes were all evoked by light stimulation. Since this 'cloud' of scattered spikes was overlapping with the isolated units, the units might contain some scattered spikes that were not generated by the same cell.

In order to find out if the light-evoked and spontaneous spikes of a cluster were generated by the same cell, we first inspected the spike waveforms. An in-depth comparison of individual waveforms showed that for some units there were small differences between the spike waveforms of spontaneous and light-evoked spikes. For other units, we did not detect apparent differences. If the latter were ChR2-expressing granule cells, then why was the response reliability so different from the response reliability of the other light-responsive units?

There could be several reasons why a unit shows a low response reliability. Whether or not a cell fires an action potential in response to light stimulation is dependent on the amount of light that reaches the cell, the level of ChR2-expression and the excitability of the cell. Therefore, it is expected that different cells show different levels of response reliability. In published work, the response reliability of a certain cell type also varied from less than 10% up to 100% (Kvitsiani *et al.*, 2013).

Based on these data, we conclude that it is possible that units with low response reliability are ChR2-expressing granule cells, but this could also be a false positive result caused by scattered spikes.

Because **scattered spikes** formed a major issue in spike sorting, we tried to find out what they were and where they came from. By plotting all spikes that occurred within 10 ms from light stimulation in a different colour than the spontaneous spikes, we demonstrated that scattered spikes were evoked by light stimulation. We also verified that the waveforms of scattered spikes were similar to the waveform of regular spikes. Since there was no obvious difference, we assume that the scattered spikes were not light artefacts, but real spikes. Based on these data, we presume scattered spikes are light-evoked spikes generated by ChR2-positive granule cells or their postsynaptic targets. Interestingly, scattered spikes were distributed over the entire spike plot and did not form any clusters. A possible explanation would be that they were generated by many different ChR2-positive neurons, and the variability in spikes evoked by a single neuron was large. Normally, spikes generated by a single neuron are very similar. However, after light stimulation, we observed large voltage fluctuations, presumably caused by activity of light-responsive neurons. These voltage fluctuations could cause distortions and variability in spike waveform.

In a previous section, we estimated that a tetrode in the granule cell layer could detect spikes from approximately 35 ChR2-positive granule cells. If some or all these cells fire shortly after every light pulse, large fluctuations in voltage can be expected. Spikes located near the tetrode tip will cause large voltage fluctuations, while spikes located further away will evoke small deflections in voltage. The number of responding cells and their response latency will be different for every light stimulus. Therefore, the voltage fluctuations will also be different in each trial.

Based on these ideas, **it is plausible that there is large variability in spikes recorded shortly after light stimulation**, even if they were generated by the same cell. This could explain why scattered spikes do not form clusters.

It should be noted that there was another big difference between the 3 units with high response reliability and the units with low response reliability. The ‘high response’ units had spike amplitudes between 300 μ V and 700 μ V, while the spikes of ‘low response’ units had amplitudes below 200 μ V. If the amplitude of a spike is low, fluctuations in baseline voltage are expected to have a relatively large impact on the spike waveform. Perhaps the units with small spikes were ChR2-positive, but the spikes evoked by light stimulation were distorted by voltage fluctuations. Distorted spikes would fall outside the cluster of normal, spontaneous spikes and be excluded from the cluster during spike sorting. If many or all the light-evoked spikes were excluded from the cluster, then the light response would obviously be very low.

The waveform of units with large amplitude spikes, on the other hand, would be less influenced by fluctuations in voltage. Therefore the waveform of light-evoked spikes is expected to look similar to the waveform of spontaneous spikes. The light-evoked spikes would be included in the cluster and contribute to the light response. The slight variation in spike waveform caused by light stimulation can be seen in Figure 3.9: the waveforms of the spikes recorded in the light stimulation session show slightly more variability than the waveforms recorded in sessions without light stimulation.

Whether or not units with low light response reliability were light-responsive granule cells, the **poor isolation quality** caused by scattered spikes remains the main issue in these experiments. Usually, if the isolation quality of a unit is poor, the data are not included in analysis. Here we analysed the response of these poorly isolated units in detail because we were trying to establish the technique. If our goal was to characterize identified granule cells, then we would not include the data in the results.

It is possible to **avoid the problems caused by scattered spikes** by detecting high amplitude spikes. In the 3 examples of units with high amplitude spikes, there were hardly any scattered spikes surrounding the cluster. If a unit is close to the tetrode, spike amplitudes can be very large. In this case the spike detection threshold can be set at a relatively high value. Scattered spikes are presumably generated by units further away from the tetrode. If the spike detection threshold is higher than the amplitude of the scattered spikes, no scattered spikes will be recorded. This is easy in theory, but how can we practically increase the detection of light-responsive cells near the tetrode? One way is to increase the number of tetrodes, but this is limited by the weight of the drive. Another option is to increase the number of ChR2-positive cells. However, as explained before, if 2 or more cells fire synchronously, the spike waveforms will be overlapping, which will prevent the reliable isolation of single units. A solution to this “synchrony-induced spike superimposition” was proposed by Royer et al. (Royer *et al.*, 2010). They showed that low frequency sine wave stimulation activates different neurons at different times, so that spikes will be detected separately and units can be reliably isolated. So, if we use mice which express ChR2 in a higher proportion of granule cells, and we use sine wave light stimulation, we might be able to detect more reliably light-activated granule cells.

5. Conclusion and future directions

Based on the data we acquired, we have to conclude that combining extracellular recording with optogenetics is currently not an efficient technique to identify granule cells of the DG. Even though we were able to record the activity of a few light-responsive cells, uncertainty about the recorded cell type remains. None of the units could be conclusively identified as granule cell. Units that responded to light stimulation with high reliability could be either granule cells or postsynaptic cells, while units with low light response reliability were surrounded by scattered spikes and should be excluded based on poor isolation quality.

We identified 3 units that showed place cell activity and responded to light stimulation with short latency and high reliability, of which 2 were recorded on the same tetrode on consecutive days. The response latency of these units was shorter than any postsynaptic response of interneurons. Although this suggests that the units were directly activated by light, we could not exclude the possibility that they were indirectly activated postsynaptic cells. In vitro experiments showed that the response latency of granule cells was dependent on light intensity and also varied between cells. The difference in latency between directly and indirectly activated cells was expected to be small. Therefore, response latency was not a reliable criterion to distinguish directly and indirectly activated cells.

Light stimulation evoked up to 3 spikes in the cells with high response reliability. We and others have shown that stimulation with high light intensity can evoke multiple spikes in a single cell (Figure 3.7; (Han, Qian, Bernstein, H.-H. Zhou, *et al.*, 2009). An alternative explanation is that one or more light-activated granule cells evoked a burst of spikes in a postsynaptic mossy cell. We also noticed that the spike amplitude of the putatively identified granule cells was very large. Large spikes could have been the result of close proximity between granule cells and tetrode or the spikes could have been generated by mossy cells, which are known to produce large spike waveforms (Scharfman, 2007).

In conclusion, the units that responded to light stimulation with short latency and high reliability could be granule cells, but the possibility that they were postsynaptic cells cannot be excluded.

New developments in the field (e.g., more sensitive opsins, light-proof recording electrodes and better spike detection and spike sorting methods) could eventually make optogenetic identification of granule cells an efficient technique to study the function of granule cells in the DG. The method will remain an indirect approach because spikes evoked during behaviour have to be compared to spikes evoked by light stimulation. The need to compare spikes within a cluster and compare clusters between sessions is a weak point of the technique. However, improvements in recording and spike sorting techniques are expected to make extracellular unit recording more efficient and more reliable in the future.

Meanwhile, new techniques that allow direct identification of cell types are being developed. In vivo intracellular recording, for example, can be combined with fluorescence imaging to target specific cell types. This allows identification of the recorded cell type, but it is a technically very challenging and rather slow technique because only one unit can be recorded at the same time. Juxtacellular recording is another technique for extracellular single-unit recording which makes it possible to label the recorded

neuron for post hoc morphological reconstruction (Pinault, 1996). A method that may offer a higher yield is calcium imaging. This method makes use of genetically encoded calcium indicators to monitor neuronal activity. The technique has been used in the DG to record the activity of mature and newborn granule cells ('Danielson 2016 Neuron Suppl Distinct Contribution of Adult-Born Hippocampal Granule Cells to Context Encoding', n.d.). Although this technique is more invasive and the temporal resolution is lower than electrophysiological recordings, it is a very efficient method to monitor the activity of multiple genetically identified neurons simultaneously.

References

- Acsády L, Kamondi A, Sík A, Freund T, Buzsáki G. GABAergic cells are the major postsynaptic targets of mossy fibers in the rat hippocampus. *J. Neurosci.* 1998; 18: 3386–403.
- Aimone JB, Deng W, Gage FH. Resolving New Memories: A Critical Look at the Dentate Gyrus, Adult Neurogenesis, and Pattern Separation. *Neuron* 2011; 70: 589–596.
- Alme CB, Buzzetti R a., Marrone DF, Leutgeb JK, Chawla MK, Schaner MJ, et al. Hippocampal granule cells opt for early retirement. *Hippocampus* 2010; 20: 1109–1123.
- Altman J. Are New Neurons Formed in the Brains of Adult Mammals. *Science* (80-.). 1962; Volume: 1127–1128.
- Amaral DG, Ishizuka N, Claiborne B. Neurons, numbers and the hippocampal network. *Prog. Brain Res.* 1990; 83: 1–11.
- Amaral DG, Scharfman HE, Lavenex P. The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). *Prog. Brain Res.* 2007; 163
- Amaral DG, Witter MP. The three-dimensional organization of the hippocampal formation: A review of anatomical data. *Neuroscience* 1989; 31: 571–591.
- Andersen P, Bliss TVP, Skrede KK. Unit analysis of hippocampal population spikes. *Exp. Brain Res.* 1971
- Andersen P, Morris R, Amaral D, Bliss T, O'Keefe J. *The Hippocampus Book*. Oxford University Press; 2007.
- Arenkiel BR, Peca J, Davison IG, Feliciano C, Deisseroth K, Augustine G, et al. In Vivo Light-Induced Activation of Neural Circuitry in Transgenic Mice Expressing Channelrhodopsin-2. *Neuron* 2007; 54: 205–218.
- Arruda-Carvalho M, Sakaguchi M, Akers KG, Josselyn SA, Frankland PW. Behavioral/Systems/Cognitive Posttraining Ablation of Adult-Generated Neurons Degrades Previously Acquired Memories.
- Atasoy D, Aponte Y, Su HH, Sternson SM. A FLEX switch targets Channelrhodopsin-2 to multiple cell types for imaging and long-range circuit mapping. *J. Neurosci.* 2008; 28: 7025–30.
- Ayling OGS, Harrison TC, Boyd JD, Goroshkov A, Murphy TH. Automated light-based mapping of motor cortex by photoactivation of channelrhodopsin-2 transgenic mice. *Nat. Methods* 2009; 6: 219–224.
- Bakker A, Kirwan CB, Miller M, Stark CEL. Pattern separation in the human hippocampal CA3 and dentate gyrus. *Science* 2008; 319: 1640–1642.
- Balthasar N, Coppari R, McMinn J, Liu SM, Lee CE, Tang V, et al. Leptin receptor signaling in POMC neurons is required for normal body weight homeostasis. *Neuron* 2004; 42: 983–991.
- Barry C, Lever C, Hayman R, Hartley T, Burton S, O'Keefe J, et al. The boundary vector cell model of place cell firing and spatial memory. *Rev. Neurosci.* 2006; 17: 71–97.
- Becker S. Neurogenesis and pattern separation: time for a divorce. *Wiley Interdiscip. Rev. Cogn. Sci.* 2016

- Blackstad TW, Brink K, Hem J, June B. Distribution of hippocampal mossy fibers in the rat. An experimental study with silver impregnation methods. *J. Comp. Neurol.* 1970; 138: 433–449.
- Blankvoort SMA. Catering new neurons to environmental needs. 2015
- Boccara CN, Sargolini F, Thoresen VH, Solstad T, Witter MP, Moser EI, et al. Grid cells in pre- and parasubiculum. *Nat. Neurosci.* 2010; 13: 987–94.
- Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K. Millisecond-timescale, genetically targeted optical control of neural activity. *Nat. Neurosci.* 2005; 8: 1263–1268.
- Buckmaster PS, Wenzel HJ, Kunkel DD, Schwartzkroin PA. Axon arbors and synaptic connections of hippocampal mossy cells in the rat in vivo. *J. Comp. Neurol.* 1996; 366: 271–92.
- Bush D, Barry C, Burgess N. What do grid cells contribute to place cell firing? *Trends Neurosci.* 2014; 37: 136–45.
- Buzsáki G, Buhl DL, Harris KD, Csicsvari J, Czéh B, Morozov a. Hippocampal network patterns of activity in the mouse. *Neuroscience* 2003; 116: 201–211.
- Buzsáki G. Large-scale recording of neuronal ensembles. *Nat. Neurosci.* 2004; 7: 446–451.
- Calton JL1, Stackman R, Goodridge J, Archey WB, Dudchenko PA, Taube JS. Hippocampal place cell instability after lesions of the head direction cell network. *J. Neurosci.* 2003
- Cameron HA, Glover LR. Adult Neurogenesis: Beyond Learning and Memory. 2014; 32
- Cardin J a, Carlén M, Meletis K, Knoblich U, Zhang F, Deisseroth K, et al. Targeted optogenetic stimulation and recording of neurons in vivo using cell-type-specific expression of Channelrhodopsin-2. *Nat. Protoc.* 2010; 5: 247–254.
- Chaves-Coira I, Barros-Zulaica N, Rodrigo-Angulo M, Núñez Á. Modulation of Specific Sensory Cortical Areas by Segregated Basal Forebrain Cholinergic Neurons Demonstrated by Neuronal Tracing and Optogenetic Stimulation in Mice. *Front. Neural Circuits* 2016; 10: 28.
- Chawla MK, Guzowski JF, Ramirez-Amaya V, Lipa P, Hoffman KL, Marriott LK, et al. Sparse, environmentally selective expression of Arc RNA in the upper blade of the rodent fascia dentata by brief spatial experience. *Hippocampus* 2005; 15: 579–586.
- Claiborne BJ, Amaral DG, Cowan WM. A light and electron microscopic analysis of the mossy fibers of the rat dentate gyrus. *J. Comp. Neurol.* 1986; 246: 435–458.
- Claiborne BJ, Amaral DG, Cowan WM. Quantitative, three-dimensional analysis of granule cell dendrites in the rat dentate gyrus. *J. Comp. Neurol.* 1990; 302: 206–219.
- Clelland CD, Choi M, Romberg C, Clemenson GD, Fragniere A, Tyers P, et al. A functional role for adult hippocampal neurogenesis in spatial pattern separation. *Science* 2009; 325: 210–3.
- Cohen JY, Amoroso MW, Uchida N. Serotonergic neurons signal reward and punishment on multiple timescales. *Elife* 2015
- Cohen JY, Haesler S, Vong L, Lowell BB, Uchida N. Neuron-type-specific signals for reward and

punishment in the ventral tegmental area. *Nature* 2012; 482: 85–8.

Creer DJ, Romberg C, Saksida LM, van Praag H, Bussey TJ. Running enhances spatial pattern separation in mice. *Proc. Natl. Acad. Sci.* 2010; 107: 2367–2372.

Danielson NB, Kaifosh P, Zaremba JD, Lovett-Barron M, Tsai J, Denny CA, et al. Distinct Contribution of Adult-Born Hippocampal Granule Cells to Context Encoding. *Neuron* 2016: 1–12.

Deng W, Mayford M, Gage FH. Selection of distinct populations of dentate granule cells in response to inputs as a mechanism for pattern separation in mice. *Elife* 2013; 2013: 2–5.

Deshmukh SS, Knierim JJ. Representation of non-spatial and spatial information in the lateral entorhinal cortex. *Front. Behav. Neurosci.* 2011; 5: 69.

Diamantaki M, Frey M, Preston-Ferrer P, Burgalossi A. Priming Spatial Activity by Single-Cell Stimulation in the Dentate Gyrus of Freely Moving Rats. *Curr. Biol.* 2016: 1–6.

Drew LJ, Fusi S, Hen R. Adult neurogenesis in the mammalian hippocampus: Why the dentate gyrus? *Learn. Mem.* 2013; 20: 710–729.

Drew LJ, Kheirbek MA, Luna VM, Denny CA, Cloyd MA, Wu M V, et al. Activation of local inhibitory circuits in the dentate gyrus by adult-born neurons. *Hippocampus* 2015; 26: 763–78.

Duan X, Kang E, Liu CY, Ming G-L, Song H. Development of neural stem cell in the adult brain. *Curr. Opin. Neurobiol.* 2008; 18: 108–15.

Dugué GP, Akemann W, Knöpfel T. A comprehensive concept of optogenetics. In: *Progress in brain research*. 2012. p. 1–28.

Ge S, Goh ELK, Sailor KA, Kitabatake Y, Ming G, Song H. GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature*, Publ. online 11 December 2005; | doi10.1038/10.1038/nature04404 2005; 439: 589.

Ge S, Yang C-H, Hsu K-S, Ming G-L, Song H. A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. *Neuron* 2007; 54: 559–66.

Goshen I. The optogenetic revolution in memory research. *Trends Neurosci.* 2014

Gray CM, Maldonado PE, Wilson M, McNaughton B. Tetrodes markedly improve the reliability and yield of multiple single-unit isolation from multi-unit recordings in cat striate cortex. *J. Neurosci. Methods* 1995; 63: 43–54.

Van Groen T, Kadish I, Wyss JM. Species differences in the projections from the entorhinal cortex to the hippocampus. *Brain Res. Bull.* 2002; 57: 553–556.

van Groen T, Miettinen P, Kadish I. The entorhinal cortex of the mouse: organization of the projection to the hippocampal formation. *Hippocampus* 2003; 13: 133–49.

Gunaydin L a, Yizhar O, Berndt A, Sohal VS, Deisseroth K, Hegemann P. Ultrafast optogenetic control. *Nat. Neurosci.* 2010; 13: 387–392.

Hafting T, Fyhn M, Molden S, Moser M-B, Moser EI. Microstructure of a spatial map in the entorhinal

cortex. *Nature* 2005; 436: 801–6.

Hales JB, Schlesiger MI, Leutgeb JK, Squire LR, Leutgeb S, Clark RE. Medial Entorhinal Cortex Lesions Only Partially Disrupt Hippocampal Place Cells and Hippocampus-Dependent Place Memory. 2014.

Han X, Qian X, Bernstein JG, Zhou H, Talei G, Stern P, et al. Supplemental Data Millisecond-Timescale Optical Control of Neural Dynamics in the Nonhuman Primate Brain. 2009; 61: 1–18.

Han X, Qian X, Bernstein JG, Zhou H-H, Franzesi GT, Stern P, et al. Millisecond-timescale optical control of neural dynamics in the nonhuman primate brain. *Neuron* 2009; 62: 191–8.

Han ZS, Buhl EH, Lörinczi Z, Somogyi P. A high degree of spatial selectivity in the axonal and dendritic domains of physiologically identified local-circuit neurons in the dentate gyrus of the rat hippocampus. *Eur. J. Neurosci.* 1993; 5: 395–410.

Hargreaves EL, Rao G, Lee I, Knierim JJ. Major dissociation between medial and lateral entorhinal input to dorsal hippocampus. *Science* 2005; 308: 1792–4.

Hartley T, Lever C, Burgess N, O’Keefe J. Space in the brain: how the hippocampal formation supports spatial cognition. *Philos. Trans. R. Soc. B Biol. Sci.* 2013; 369: 20120510.

Hasselmo ME. The Role of Hippocampal Regions CA3 and CA1 in Matching Entorhinal Input With Retrieval of Associations Between Objects and Context: Theoretical Comment on Lee et al. (2005). *Behav. Neurosci.* 2005; 119: 342–345.

Henze D a., Buzsáki G. Hilar mossy cells: functional identification and activity in vivo. *Prog. Brain Res.* 2007; 163

Henze DA, Wittner L, Buzsáki G. Single granule cells reliably discharge targets in the hippocampal CA3 network in vivo. *Nat. Neurosci.* 2002; 5: 790–795.

Hill AJ, Best PJ. Effects of deafness and blindness on the spatial correlates of hippocampal unit activity in the rat. *Exp. Neurol.* 1981; 74: 204–217.

Houser CR. Interneurons of the dentate gyrus: an overview of cell types, terminal fields and neurochemical identity. *Prog. Brain Res.* 2007; 163: 217–233.

Huber D, Petreanu L, Ghitani N, Ranade S, Hromádka T, Mainen Z, et al. Sparse optical microstimulation in barrel cortex drives learned behaviour in freely moving mice. *Nature* 2008; 451: 61–4.

Igarashi KM. The entorhinal map of space. *Brain Res.* 2016

Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, et al. Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. *Nat. Neurosci.* 2008; 11: 1153–61.

Jacobs+ LF, Limant ER. Grey squirrels remember the locations of buried nuts. *Anim. Behav* 1991; 41

Jessberger S, Kempermann G. Adult-born hippocampal neurons mature into activity-dependent responsiveness. *Eur. J. Neurosci.* 2003; 18: 2707–2712.

Jinno S, Kosaka T. Stereological estimation of numerical densities of glutamatergic principal neurons in

the mouse hippocampus. *Hippocampus* 2009; 20: NA–NA.

Jouvet M. Biogenic amines and the states of sleep. *Science* 1969; 163: 32–41.

Jung MW, McNaughton BL. Spatial selectivity of unit activity in the hippocampal granular layer. *Hippocampus* 1993; 3: 165–182.

Kentros CG, Agnihotri NT, Streater S, Hawkins RD, Kandel ER. Increased attention to spatial context increases both place field stability and spatial memory. *Neuron* 2004; 42: 283–295.

Knierim JJ, Kudrimoti HS, McNaughton BL. Place cells, head direction cells, and the learning of landmark stability. *J. Neurosci.* 1995; 15: 1648–59.

Knierim JJ, Neunuebel JP. Tracking the flow of hippocampal computation: Pattern separation, pattern completion, and attractor dynamics. *Neurobiol. Learn. Mem.* 2016; 129: 38–49.

Knierim JJ. From the GPS to HM: Place cells, grid cells, and memory. *Hippocampus* 2015; 25: 719–725.

Kohara K, Pignatelli M, Rivest AJ, Jung H-Y, Kitamura T, Suh J, et al. Cell type-specific genetic and optogenetic tools reveal hippocampal CA2 circuits. *Nat. Neurosci.* 2013; 17: 269–279.

Kozai TDY, Vazquez AL. Photoelectric artefact from optogenetics and imaging on microelectrodes and bioelectronics: New Challenges and Opportunities. *J. Mater. Chem. B. Mater. Biol. Med.* 2015; 3: 4965–4978.

Kropff E, Carmichael JE, Moser M-B, Moser EI. Speed cells in the medial entorhinal cortex. *Nature* 2015; 523: 419–424.

Kuhlman SJ, Huang ZJ. High-resolution labeling and functional manipulation of specific neuron types in mouse brain by Cre-activated viral gene expression. *PLoS One* 2008; 3: e2005.

Kvitsiani D, Ranade S, Hangya B, Taniguchi H, Huang JZ, Kepecs A. Distinct behavioural and network correlates of two interneuron types in prefrontal cortex. *Nature* 2013; 498: 363–6.

Langston RF, Ainge JA, Couey JJ, Canto CB, Bjerknes TL, Witter MP, et al. Development of the Spatial Representation System in the Rat. *Science* (80-.). 2010; 328: 1576–1580.

Lee I, Yoganarasimha D, Rao G, Knierim JJ. Comparison of population coherence of place cells in hippocampal subfields CA1 and CA3. *Nature* 2004; 430: 456–459.

Lein ES, Hawrylycz MJ, Ao N, Ayres M, Bensinger A, Bernard A, et al. Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 2007; 445: 168–176.

Leutgeb JK, Leutgeb S, Moser M-B, Moser EI. Pattern separation in the dentate gyrus and CA3 of the hippocampus. *Science* 2007; 315: 961–966.

Leutgeb JK, Leutgeb S, Treves A, Meyer R, Barnes CA, McNaughton BL, et al. Progressive transformation of hippocampal neuronal representations in ‘morphed’ environments. *Neuron* 2005

Leutgeb S, Leutgeb JK, Barnes C a, Moser EI, McNaughton BL, Moser M-B. Independent codes for spatial and episodic memory in hippocampal neuronal ensembles. *Science* 2005; 309: 619–623.

- Lever C, Burton S, Jeewajee A, O'Keefe J, Burgess N. Boundary Vector Cells in the Subiculum of the Hippocampal Formation. *J. Neurosci.* 2009; 29: 9771–9777.
- Lima SQ, Hromádka T, Znamenskiy P, Zador AM. PINP: A new method of tagging neuronal populations for identification during in vivo electrophysiological recording. *PLoS One* 2009; 4
- Liu X, Ramirez S, Pang PT, Puryear CB, Govindarajan A, Deisseroth K, et al. Optogenetic stimulation of a hippocampal engram activates fear memory recall. *Nature* 2012; 484: 381–385.
- Madisen L, Mao T, Koch H, Zhuo J, Berenyi A, Fujisawa S, et al. A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. *Nat. Neurosci.* 2012; 15: 793–802.
- Marín-Burgin A, Schinder AF. Requirement of adult-born neurons for hippocampus-dependent learning. *Behav. Brain Res.* 2012; 227: 391–399.
- Marr D. Simple memory: a theory for archicortex. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 1971; 262: 23–81.
- Marshall L, Henze D a, Hirase H, Leinekugel X, Dragoi G, Buzsáki G. Hippocampal pyramidal cell-interneuron spike transmission is frequency dependent and responsible for place modulation of interneuron discharge. *J. Neurosci.* 2002; 22: RC197.
- McHugh TJ, Jones MW, Quinn JJ, Balthasar N, Coppari R, Elmquist JK, et al. Dentate gyrus NMDA receptors mediate rapid pattern separation in the hippocampal network. *Science* (80-.). 2007; 317: 94–99.
- McNaughton BL, Battaglia FP, Jensen O, Moser EI, Moser M-B. Path integration and the neural basis of the 'cognitive map'. *Nat. Rev. Neurosci.* 2006; 7: 663–678.
- McNaughton BL, Morris RGM. Hippocampal synaptic enhancement and information storage within a distributed memory system. *Trends Neurosci.* 1987; 10: 408–415.
- Mikulovic S, Pupe S, Peixoto HM, Do Nascimento GC, Kullander K, Tort ABL, et al. On the photovoltaic effect in local field potential recordings. *Neurophotonics* 2016; 3: 015002.
- Mizumori SJY, Mcnaughton BL, Barnes CA. A Comparison of Supramammillary and Medial Septal Influences on Hippocampal Field Potentials and Single-Unit Activity. *JOURNAL OF Neurophysiol.* 1989; 61
- Mongiat LA, Espósito MS, Lombardi G, Schinder AF. Reliable Activation of Immature Neurons in the Adult Hippocampus. *PLoS One* 2009; 4: e5320.
- Morris RGM, Garrud P, Rawlins JNP, O'Keefe J. Place navigation impaired in rats with hippocampal lesions. *Nature* 1982; 297: 681–683.
- Muller RU, Kubie JL. The effects of changes in the environment on the spatial firing of hippocampal complex-spike cells. *J. Neurosci.* 1987; 7: 1951–1968.
- Muller RU, Kubie JL. The Effects of Changes in the Environment on the Spatial Firing of Hippocampal Complex-Spike Cells. *J. Neurosci.* 1987; 7: 1951–988.
- Nagel G, Brauner M, Liewald JF, Adeishvili N, Bamberg E, Gottschalk A. Report Light Activation of Channelrhodopsin-2 in Excitable Cells of *Caenorhabditis elegans* Triggers Rapid Behavioral Responses.

Curr. Biol. 2005; 15: 2279–2284.

Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, et al. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc. Natl. Acad. Sci. U. S. A.* 2003; 100: 13940–5.

Nakashiba T, Cushman JD, Pelkey KA, Renaudineau S, Buhl DL, McHugh TJ, et al. Young dentate granule cells mediate pattern separation, whereas old granule cells facilitate pattern completion. *Cell* 2012; 149: 188–201.

Neunuebel JP, Knierim JJ. Spatial Firing Correlates of Physiologically Distinct Cell Types of the Rat Dentate Gyrus. *J. Neurosci.* 2012; 32: 3848–3858.

Neunuebel JP, Knierim JJ. CA3 retrieves coherent representations from degraded input: direct evidence for CA3 pattern completion and dentate gyrus pattern separation. *Neuron* 2014; 81: 416–27.

O'Keefe J, Conway DH. Brain Hippocampal Place Units in the Freely Moving Rat : Why They Fire Where They Fire. *Exp. Brain Res.* 1978; 31: 573–590.

O'Keefe J, Dostrovsky J. The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. *Brain Res.* 1971; 34: 171–175.

O'Keefe J, Nadel L. *The Hippocampus as a Cognitive Map*. Oxford: Oxford University Press; 1978.

Overstreet LS, Hentges ST, Bumaschny VF, de Souza FSJ, Smart JL, Santangelo AM, et al. A transgenic marker for newly born granule cells in dentate gyrus. *J. Neurosci.* 2004; 24: 3251–3259.

Patton PE, McNaughton B. Connection matrix of the hippocampal formation: I. The dentate gyrus. *Hippocampus* 1995; 5: 245–286.

Piatti VC, Ewe L a., Leutgeb JK. Neurogenesis in the dentate gyrus: Carrying the message or dictating the tone. *Front. Neurosci.* 2013; 7: 1–11.

Pilz G-A, Carta S, Stäubli A, Ayaz A, Jessberger S, Fritjof Helmchen X. Functional Imaging of Dentate Granule Cells in the Adult Mouse Hippocampus.

Pinault D. A novel single-cell staining procedure performed in vivo under electrophysiological control: morpho-functional features of juxtacellularly labeled thalamic cells and other central neurons with biocytin or Neurobiotin. *J. Neurosci. Methods* 1996; 65: 113–136.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. Functional neurogenesis in the adult hippocampus. *Nature* 2002; 415: 1030–1034.

Renshaw B, Forbes A, Morison BR. ACTIVITY OF ISOCORTEX AND HIPPOCAMPUS: ELECTRICAL STUDIES WITH MICRO-ELECTRODES. *J. Neurophysiol.* 1940; 3: 74–105.

Ribak CE, Seress L. Five types of basket cell in the hippocampal dentate gyrus: a combined Golgi and electron microscopic study. *J. Neurocytol.* 1983; 12: 577–97.

Rose G, Diamond D, Lynch GS. Dentate granule cells in the rat hippocampal formation have the behavioral characteristics of theta neurons. *Brain Res.* 1983; 266: 29–37.

Roux L, Stark E, Sjulson L, Buzsáki G. In vivo optogenetic identification and manipulation of GABAergic

interneuron subtypes. *Curr. Opin. Neurobiol.* 2014

Royer S, Zemelman B V, Barbic M, Losonczy A, Buzsáki G, Magee JC. Multi-array silicon probes with integrated optical fibers: light-assisted perturbation and recording of local neural circuits in the behaving animal. *Eur. J. Neurosci.* 2010; 31: 2279–91.

Sahay A, Scobie KN, Hill AS, O’Carroll CM, Kheirbek MA, Burghardt NS, et al. Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature* 2011; 472: 466–70.

Santoro A, Frey JU, Yassa MA. Reassessing pattern separation in the dentate gyrus. 2013

Sargolini F, Fyhn M, Hafting T, McNaughton BL, Witter MP, Moser M-B, et al. Conjunctive representation of position, direction, and velocity in entorhinal cortex. *Science* 2006; 312: 758–62.

Satvat E, Schmidt B, Argraves M, Marrone DF, Markus EJ. Changes in task demands alter the pattern of zif268 expression in the dentate gyrus. *J. Neurosci.* 2011; 31: 7163–7167.

Savelli F, Yoganarasimha D, Knierim JJ. Influence of boundary removal on the spatial representations of the medial entorhinal cortex. *Hippocampus* 2008; 18: 1270–1282.

Scharfman HE, Kunkel DD, Schwartzkroin PA. Synaptic connections of dentate granule cells and hilar neurons: Results of paired intracellular recordings and intracellular horseradish peroxidase injections. *Neuroscience* 1990; 37: 693–707.

Scharfman HE. Evidence from simultaneous intracellular recordings in rat hippocampal slices that area CA3 pyramidal cells innervate dentate hilar mossy cells. *J. Neurophysiol.* 1994; 72: 2167–80.

Scharfman HE. THE DENTATE GYRUS : A COMPREHENSIVE GUIDE TO STRUCTURE, FUNCTION, AND CLINICAL IMPLICATION S. Oxford: Elsevier; 2007.

Scharfman HE. The enigmatic mossy cell of the dentate gyrus. *Nat. Publ. Gr.* 2016

Schmitzer-Torbert N, Jackson J, Henze D, Harris K, Redish AD. Quantitative measures of cluster quality for use in extracellular recordings. *Neuroscience* 2005

Schoenenberger P, O’Neill J, Csicsvari J. Activity-dependent plasticity of hippocampal place maps. *Nat. Commun.* 2016; 7: 11824.

SCOVILLE WB, MILNER B. Loss of recent memory after bilateral hippocampal lesions. *J. Neurol. Neurosurg. Psychiatry* 1957; 20: 11–21.

Shapiro ML, Tanila H, Eichenbaum H. Cues that hippocampal place cells encode: dynamic and hierarchical representation of local and distal stimuli. *Hippocampus* 1997; 7: 624–42.

Sherry D. Food storage by black-capped chickadees: Memory for the location and contents of caches. *Anim. Behav.* 1984; 32: 451–464.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E. Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus* 2002; 12: 578–84.

Snyder JS, Hong NS, McDonald RJ, Wojtowicz JM. A role for adult neurogenesis in spatial long-term memory. *Neuroscience* 2005; 130: 843–852.

- Solstad T, Boccara CN, Kropff E, Moser M-B, Moser EI. Representation of geometric borders in the entorhinal cortex. *Science* 2008; 322: 1865–8.
- Solstad T, Moser EI, Einevoll GT. From grid cells to place cells: A mathematical model. *Hippocampus* 2006; 16: 1026–1031.
- Somogyi P. A specific ‘axo-axonal’ interneuron in the visual cortex of the rat. *Brain Res.* 1977; 136: 345–50.
- Stark E, Bere A, Khodagholy D, Kipke DR, Yoon E, Wise KD. *Primer Tools for Probing Local Circuits : High-Density Silicon Probes Combined with Optogenetics.* 2015
- Sutherland RJ, Whishaw IQ, Kolb B. A behavioural analysis of spatial localization following electrolytic, kainate- or colchicine-induced damage to the hippocampal formation in the rat. *Behav. Brain Res.* 1983; 7: 133–153.
- Taube JS, Muller RU, Ranck JB. Head-direction cells recorded from the postsubiculum in freely moving rats. I. Description and quantitative analysis. *J. Neurosci.* 1990; 10: 420–435.
- Taube JS. The Head Direction Signal: Origins and Sensory-Motor Integration. *Annu. Rev. Neurosci.* 2007; 30: 181–207.
- Thompson LT, Best PJ. Long-term stability of the place-field activity of single units recorded from the dorsal hippocampus of freely behaving rats. *Brain Res.* 1990; 509: 299–308.
- Treves A, Rolls ET. Computational analysis of the role of the hippocampus in memory. *Hippocampus* 1994; 4: 374–391.
- Tronel S, Belnoue L, Grosjean N, Revest J-M, Piazza P-V, Koehl M, et al. Adult-born neurons are necessary for extended contextual discrimination. *Hippocampus* 2012; 22: 292–298.
- Vanderwolf CH. Hippocampal electrical activity and voluntary movement in the rat. *Electroencephalogr. Clin. Neurophysiol.* 1969; 26: 407–18.
- Wills TJ, Cacucci F, Burgess N, O’Keefe J. Development of the Hippocampal Cognitive Map in Preweanling Rats. *Science* (80-.). 2010; 328
- Wilson M a, McNaughton BL. Dynamics of the hippocampal ensemble code for space. *Science* 1993; 261: 1055–1058.
- Witter MP, Amaral DG. Entorhinal cortex of the monkey: V. Projections to the dentate gyrus, hippocampus, and subicular complex. *J. Comp. Neurol.* 1991; 307: 437–459.
- Witter MP. Organization of the entorhinal-hippocampal system: a review of current anatomical data. *Hippocampus* 1993; 3 Spec No: 33–44.
- Witter MP. The perforant path: projections from the entorhinal cortex to the dentate gyrus. *Prog. Brain Res.* 2007; 163: 43–61.
- Wojtowicz JM, Askew ML, Winocur G. The effects of running and of inhibiting adult neurogenesis on learning and memory in rats. *Eur. J. Neurosci.* 2008; 27: 1494–502.

Wu F, Stark E, Im M, Cho I-J, Yoon E-S, Buzsáki G, et al. An implantable neural probe with monolithically integrated dielectric waveguide and recording electrodes for optogenetics applications. *J. Neural Eng.* 2013; 10: 056012.

Xavier GF, Costa VCI. Dentate gyrus and spatial behaviour. *Prog. Neuro-Psychopharmacology Biol. Psychiatry* 2009; 33: 762–773.

Xu M, Chung S, Zhang S, Zhong P, Ma C, Chang W-C, et al. Basal forebrain circuit for sleep-wake control. *Nat. Neurosci.* 2015; 18: 1641–7.

Young BJ, Otto T, Fox GD, Eichenbaum H. Memory representation within the parahippocampal region. *J. Neurosci.* 1997; 17: 5183–95.

Zhang S, Ye J, Miao C, Tsao A, Cerniauskas I, Ledergerber D, et al. Optogenetic Dissection of Entorhinal-Hippocampal Functional Connectivity Optogenetic Dissection of. 2013; 340: 0–14.

Zhang S-J, Ye J, Couey JJ, Witter M, Moser EI, Moser M-B. Functional connectivity of the entorhinal-hippocampal space circuit. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 2014; 369: 20120516.

Ziv Y, Burns LD, Cocker ED, Hamel EO, Ghosh KK, Kitch LJ, et al. Long-term dynamics of CA1 hippocampal place codes. *Nat. Neurosci.* 2013; 16: 264–6.

Zorzos AN, Dietrich A, Talei Franzesi G, Chow B, Han X, Fonstad CG, et al. Light-proof neural recording electrodes. 2009.