A rewarding view on the mouse visual cortex: Effects of associative learning and cortical state on early visual processing in the brain

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1 | Introduction
Chapter 1. Introduction

When we see something we crave, like a cold beer on a warm summer night, our pupils dilate and saliva runs in our mouths when we anticipate the pleasure of the first cool sips. We, humans, are masters in communicating our subjective experience, but is our objective perception of the world outside affected by our internal state, like the anticipation of reward? In this PhD thesis I will address this question by discussing two experiments on how the learning of a stimulus-reward association affects the representation of reward-predicting visual information in the brain, and specifically in the visual cortex. In addition, results of a third experiment comparing awake and anesthetized visual processing in the cortex are included to study the influence of changes in internal state on visual cortex activity, as well as to validate and support generalization of the first two experiments. On the next few pages I will first introduce the main subject of this thesis, the mouse visual cortex, followed by a brief explanation of the most prevalent experimental method in my research, two-photon calcium imaging.

1.1 A comparative account of research on the mammalian visual cortex

The visual pathway is one of the most extensively studied systems of the brain. We know a great deal about the anatomical connectivity and about the response properties of various subtypes of cells located in each of the visual cortical- and subcortical areas (Werner et al., 2014). In addition much has already been learned about how cells in visual cortex change their connectivity and function in response to changes in the environment (see Espinosa and Stryker, 2012; Gilbert and Li, 2012). Even a summary of the current state of the field could exceed the volume of a book, therefore this introduction is designed to only bring the reader up to speed on the most relevant details of the brain’s visual system, highlighting differences and similarities between primate and rodent vision and with the ultimate aim to specifically introduce the experimental work that will follow in the subsequent chapters.

1.1.1 Organization of the subcortical visual system

The eye, the retina and the functional specialization ganglion cells

Light, coming from the world around us, is projected as an upside-down image on the two-dimensional curved surface of the retina within the eye. Visual processing starts with the detection of visible-light photons by a densely packed layer of rod- and cone photoreceptors in the outer retina. These photoreceptor cells excite and
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inhibit bipolar cells, directly, and also indirectly through horizontal cells that receive inputs from multiple neighboring photoreceptors. The output of the retina is carried by projections from retinal ganglion cells that receive inputs from the bipolar cells and from amacrine cells that additionally provide modulatory inhibition (Creutzfeldt and Sakmann, 1969; Kandel et al., 2000).

Within the retina, different types of retinal ganglion cells extract different features of an image resembling a set of parallel processing pathways. The classical retinal ganglion cell responds to an increase or decrease in luminance within a small patch of the visual field and additionally to the opposite change in luminance in the immediate surround of the center patch (Kuffler, 1953; Hartline, 1969), which is referred to as a center-surround ‘receptive field’. Retinal ganglion cells, however, can also have receptive fields that selectively respond to edges, overall luminance increases or decreases, absence of features (uniformity in luminance), direction of movement or overall movement, as has been observed in multiple mammalian species (Barlow et al., 1964; Spinelli, 1966; Creutzfeldt and Sakmann, 1969).

The retina is not uniformly organized. For instance, in humans and primates, the central region of the retina, the fovea, contains a much larger density of cone photoreceptors and is dominated by P (parvi)-class retinal ganglion cells, having small receptive fields and specialized in high acuity vision (Kandel et al., 2000). In rodents there is no such region that resembles a fovea (Lashley, 1934; Wagor et al., 1980), but also in mice the distribution of specific types of retinal ganglion cells is not uniform. Alpha-like retinal ganglion cells are more prevalent in the temporal part of the retina and may facilitate enhanced processing of the frontal visual field (Bleckert et al., 2014). In the ventral retina, that processes the upper visual field, highly specialized W3 retinal ganglion cells are much more prevalent compared to the dorsal retina (Zhang et al., 2012b). The receptive fields of these cells are sensitive to small moving objects against a uniform background and were proposed to specifically facilitate avian predator detection (Yilmaz and Meister, 2013). In summary, the joined output of the retinal ganglion cells can be considered to be a version of the original image, decomposed into a number of parallel channels each specialized in detecting different, relevant visual features (Masland, 2012; Werner et al., 2014).

**Paths of visual information into the brain**

Retinal ganglion cells project through the optical nerve, a vast bundle of axons, to the brain. At the optic chiasm, part of the projection fibers cross sides, in such a way that each brain hemisphere receives information from one visual hemifield rather than from one eye. Visual information in the left hemisphere originates from the right visual field and vice versa. Projections from retinal ganglion cells terminate mainly in
two structures of the central nervous system, the superior colliculus and the lateral geniculate nucleus (Figure 1.1; Werner et al., 2014).

The superior colliculus (SC), or optic tectum, is a phylogenetically old brain structure, located in the mesencephalic region of the brainstem. In brains of amphibians and fish, this is the main center where visual information is processed, as these animals do not possess much of a visual cortex (Kardong, 2006). Rodents do have a visual cortex, but the majority of retinal ganglion cell axons still terminate in the colliculus (Hofbauer and Dräger, 1985). The exact role of the colliculus in rodent vision is somewhat unclear, but the circuit is certainly involved in vision (Wang et al., 2010; Ahmadlou and Heimel, 2015; Feinberg and Meister, 2015) and potentially specialized in the detection of specific visual patterns (Shang et al., 2015; Wei et al., 2015). Moreover, there is considerable integration of sensory signals of multiple modalities (Ghose et al., 2014) and the colliculus can initiate eye-movements for the tracking of moving stimuli (Douglas et al., 2005). In fact, the role of the colliculus in
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rodents may be rather similar to its function in primates, where collicular neurons are involved in saccades, orienting responses, attention and visual target selection (Krauzlis et al., 2013; Inoue et al., 2015).

The lateral geniculate nucleus (LGN) is the most posterior-lateral nucleus of the thalamus. In higher mammals it is the dominant projection target of retinal ganglion cells and also in rodents it receives a large fraction of axons from the eye. The LGN is considered to be a relay station where visual information from the retina is passed on to the cortex. In mice, about half of the LGN cells have classical center-surround receptive fields while the remaining half responds to other features (Piscopo et al., 2013), like direction of movement (Marshel et al., 2012). These receptive fields may be directly inherited from the retina (Chen and Regehr, 2000) or produced by convergent recombination of information from afferent fibers (Hammer et al., 2015). A recent study, however, indicates that the mouse LGN contains, cells responding to self-locomotion (Erisken et al., 2014). Moreover, cells in the monkey LGN are modulated by perceptual decisions (Jiang et al., 2015) and respond differently when they are in the foreground or background of a scene (Jones et al., 2015). The concept of the LGN being merely a relay station is slowly breaking down, but whether modulations in the LGN reflect local visual processing or effects of top-down feedback remains to be seen.

1.1.2 Organization of the cortical visual system

Six layered cortex.

Most regions of the neocortex, including the primary visual cortex, consist of a six-layered circuit. In all but the uppermost cortical layer the majority of neurons is excitatory while an estimated 20% is inhibitory. Most excitatory cells are pyramidal neurons having a typical morphology with basal, within-layer dendrites around their cell body and a large apical dendrite reaching up to layer 1 where it branches out, except for part of the layer 6 pyramidal cells of which the apical dendrite only reaches up to layer 4. In layer 1 there are very few neurons altogether, it consists mostly of dendritic processes, axon terminals, and glial cells. The neurons that do reside in layer 1 are almost all of the inhibitory type. For a more comprehensive and in-depth discussion on basic cortical circuits, see Douglas and Martin (2004).

The flow of visual information within the primary visual cortex

The cortical projections of mammalian LGN relay cells terminate mainly in layer 4, although a smaller fraction is found in layer 2/3 (e.g. color vision in higher mammals) and layer 1 (Hubel and Wiesel, 1972; Lund, 1988; Cruz-Martín et al., 2014).
In the mouse, the overall flow of visual information through these six layers is quite stereotyped and similar to that in other mammals. Layer 4 cells receive input from the LGN and project mainly onto basal dendrites of cells in layer 2/3 (Thomson et al., 2002). Cells in layer 2/3 may be considered to perform local processing in the primary visual cortex although they can also receive LGN inputs. These cells do not only project onto dendrites of layer 5 (output) cells (Thomson et al., 2002), but also to a selection of neighboring layer 2/3 cells that respond to similar visual features (e.g. Gilbert and Wiesel, 1989; Ko et al., 2011) and to more distant layer 2/3 cells in higher visual cortical areas (e.g. Livingstone and Hubel, 1983; Glickfeld et al., 2013). Pyramidal cells that are located in layer 5 receive input from layer 2/3 (Thomson et al., 2002) and project to subcortical regions (e.g. SC), layer 6 and via the corpus callosum to the contralateral hemisphere (Swadlow, 1983; Kasper et al., 1994). A subset of layer 6 cells receives local inputs from upper cortical layers, while others receive inputs from higher visual areas and thalamic nuclei (Vélez-Fort et al., 2014). Layer 6 cells can project to thalamic structures (Claps and Casagrande, 1990), for instance to provide cortical feedback into the LGN (Olsen et al., 2012).

In humans and monkeys, projections from the retina through the P, M and K pathways—separated channels of visual information originating from different subsets of retinal ganglion cells—remain segregated in V1 and terminate in different sublayers of layer 4 and in spatially segregated blob and interblob subregions of layer 2/3 (Lachica et al., 1992). No such organization has been observed in mouse V1, but there is some evidence for the existence of similar parallel input channels, as in the monkey (Gao et al., 2010). One recently found exception is the projection from direction selective retinal ganglion cells, innervating the dorsal LGN and subsequently preferentially projecting to layer 1 of the visual cortex (Cruz-Martín et al., 2014). Nonetheless, most inputs to the visual cortex of the mouse are not considered to be spatially organized.

**Retinotopic organization of V1**

There is, however, at least one exception of a type of organization in the visual cortex that is conserved across all mammalian species (Kaas, 1980). Projections, originating from the retina and relayed by the LGN, innervate the primary visual cortex in a manner that is spatially organized with respect to visual field location (e.g. Nauta and Bucher, 1954). As in the retina, cells in the primary visual cortex selectively respond to a restricted region of the visual field (Hubel and Wiesel, 1959, 1968; Dräger, 1975; Wiesenfeld and Kornel, 1975). Because of the organization of the inputs, neighboring cells in V1 respond to visual stimulation in neighboring regions of the visual field in such a way that the 2-dimensional structure of an image projected onto the retina
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Figure 1.2: Retinotopic organization and orientation tuning in mouse V1. A An example of the retinotopic organization of V1 in the mouse (left panel; data acquired by P. M. Goltstein in the department of Tobias Bonhoeffer, Max Planck Institute of Neurobiology, 2015) The right panel shows the layout of a setup for mapping e.g. retinotopic organization using imaging of intrinsic signals in the brain (as used in the experiments of chapter 3). B An example of an orientation tuned neuron (data from the experiment of chapter 2). Left: Potential organization of center-surround receptive field inputs of an orientation tuned neuron. Middle: Calcium responses of a single neuron to moving gratings in 16 directions, reordered according to movement direction. Gray traces represent individual trials, the black trace is an average of 5 trials. Right: The average calcium response in this polar plot shows the amplitude of the response of the neuron in B, plotted in the actual direction of the moving grating.

is approximately maintained on the cortical surface (Figure 1.2A; Wagor et al., 1980; Tootell et al., 1988; Engel et al., 1997; Schuett et al., 2002).

The distance between two points in the retinal image does not map to a constant distance between two points on the surface of V1, but depends on the size of the receptive fields of V1 cells which increases with increasing eccentricity (Hubel and Wiesel, 1974b). Therefore, the central part of the visual field in humans and monkeys, especially the region of the fovea, has a larger representation on the cortex compared
to the peripheral visual field (Van Essen et al., 1984). The relation between the size of an object in the visual field and the size of its cortical representation is referred to as the cortical magnification factor (Cowey and Rolls, 1974). In rodents, this magnification factor is much more constant with increasing eccentricity, but their frontal visual field still has a slightly larger representation compared to the lateral visual field (Bleckert et al., 2014; Garrett et al., 2014).

**Orientation tuning**

Besides being responsive to only a specific location in the visual field, the receptive fields of neurons in the visual cortex can be tuned to respond preferentially to specific visual features. In 1959, Hubel and Wiesel published a study describing results from experiments in which they had recorded action potential responses of cells in the primary visual cortex (area 17) of anesthetized cats in response to various visual stimuli. The receptive fields of these cells typically consisted of multiple spatially restricted subregions that provided either excitatory or inhibitory drive to the neurons. In a number of cells, these ‘on’ and ‘off’ subfields were arranged in such a way that the cells responded maximally to a narrow rectangular shape that was aligned parallel to the boundary of the ‘on’ and ‘off’ subfields, while they would fail to respond at all when the same bar was projected perpendicularly to the arrangement of the subfields (Figure 1.2B). Because a cell with such a receptive field appears tuned to the orientation of an edge or a bar, these cells were defined to be ‘orientation-tuned’. By systematically varying the orientation of a bar, one can illustrate the orientation dependency of a neuron’s response and plot it as a tuning curve (Figure 1.2B). Defining features of orientation tuning curves are the orientation angle of the maximum response and the width of the response peak (named ‘preferred orientation’ and ‘bandwidth’; e.g. Swindale, 1998; Ringach et al., 2002).

Hubel and Wiesel (1962) classified a cell as simple or complex by how well the precise arrangement of ‘on’ and ‘off’ subregions of its receptive field predicted the response to visual stimuli. For instance, when a pattern of repeating bars (a grating) smoothly moves over the receptive field of a simple cell, the edge of each bar in the grating aligns in turn to the ‘on’-’off’ subfield boundary, intermitted by periods during which the edges do not align. The response of the simple cell to such a stimulus will reflect the repeated alignment and misalignment by a linear (increase/decrease) modulation of the response of the cell. Responses of complex cells were different. Hubel and Wiesel were unable to obtain subfields for these cells, while the cells would still respond to, for instance, the orientation or direction of a moving bar or grating; and did so without being modulated by the exact location of the edge of the bars in their receptive fields. The amount of this modulation (F1/F0 modulation) can be used
to classify cells into simple and complex, but some recent work indicates the existence of a continuum instead of two discrete groups (Mechler and Ringach, 2002). Hubel and Wiesel proposed that simple and complex cells reflected two successive stages in the computation of vision, which is supported by the observation of monosynaptic directional connectivity from simple to complex cells in cat V1 (Alonso and Martinez, 1998).

The function of inhibitory neurons in V1

Approximately 20%–30% of the cells in the neocortex are inhibitory; their axon terminals release the neurotransmitter GABA, which usually hyperpolarizes the postsynaptic cell and reduces its action potential activity (Kandel et al., 2000; Markram et al., 2004). Cortical inhibitory interneurons can be classified by which types of protein they express, by which neuromodulators they co-release and/or by the location on the postsynaptic neuron where they target their inhibition (Markram et al., 2004; Gonchar et al., 2007; Gentet, 2012). There are many types of interneurons, but three classes of inhibitory neurons have gained a lot of attention in the mouse visual cortex, partly because of the availability of genetic methods to selectively study their function (Taniguchi et al., 2011; Madisen et al., 2010).

Parvalbumin-expressing (PV+) interneurons provide strong local inhibition, helping to keep overall activity levels in the cortex within bounds (Hofer et al., 2011). Inhibition shapes feature selectivity in the retina (Barlow and Levick, 1965; Briggman et al., 2011) and a similar role has been theorized for visual cortex inhibitory interneurons (Ben-Yishai et al., 1995; Ursino and La Cara, 2004) with the PV+ interneuron being a prime candidate effector. More recently, Priebe and Ferster (2008) argued that the precise feature selectivity of orientation-tuned visual cortical neurons does not necessarily depend on inhibition. Moreover, studies in mouse visual cortex have shown that PV+ cells are generally poorly orientation selective (Liu et al., 2009; Kerlin et al., 2010; Runyan et al., 2010). A few other studies demonstrate a (moderate) role for PV+ interneurons in shaping tuning width of orientation-tuned neurons in mouse V1, namely by activating the inhibitory cells using optogenetic stimulation (Atallah et al., 2012; Lee et al., 2012; Wilson et al., 2012), but the increase in selectivity of tuning curves is rather small and may be attributed to changes in overall response gain (Atallah et al., 2014; El-Boustani et al., 2014; Lee et al., 2014b).

Somatostatin expressing (SOM+) interneurons mainly provide inhibition onto apical dendrites of pyramidal cells that branch out in layer 1, as well as onto other types of inhibitory neurons. Some SOM+ cells are tuned to visual features like orientation, but others are not (Kerlin et al., 2010; Ma et al., 2010). In mouse somatosensory cortex, SOM+ cells control the gain of inputs to layer 1 and thereby modulate local
intra-cortical processing via lateral connectivity of layer 2/3 cells (Gentet, 2012). In
the mouse visual cortex, SOM+ interneurons are involved in setting a spatial limit on
visual processing by providing surround suppression (Adesnik et al., 2012; Haider et
al., 2012). Inhibition by SOM+ neurons could be involved in circuitry of end-stopped
cells, also referred to as hypercomplex cells. These cells respond like complex cells,
but are additionally selective for the length of a stimulus (Hubel and Wiesel, 1965).
Surround suppression by SOM+ neurons may provide the inhibition that has been
suggested to shape this feature (Orban et al., 1979; Dobbins et al., 1987).

Vasoactive intestinal protein expressing (VIP+) interneurons are a bit of a special
case because they only appear to provide inhibition onto other interneurons, namely
of the SOM+ and PV+ type (Pfeffer et al., 2013). In mouse visual cortex, VIP+ neurons
are broadly tuned to orientation of moving stimuli (Kerlin et al., 2010; Mesik et al.,
2015) and a recent study found that VIP+ neurons are a crucial link in the facilitation
of local visual responses by long-range inputs from the anterior cingulate cortex (Zhang
et al., 2014). These properties are in line with a general role for VIP+ interneurons
in state-dependent gain control of visual responses by higher cortical regions or
subcortical nuclei (Alitto and Dan, 2012; Pi et al., 2013; Fu et al., 2014).

Finally, in the visual cortex of many mammals, including the mouse, the local
field potential (LFP) shows prominent oscillations in e.g. the gamma frequency range
(Gray et al., 1989; Engel and Singer, 2001; Nase et al., 2003). Cortical LFP oscilla-
tions reflect the synchronized activity of subsets of neurons, which is thought to be
achieved through timed inhibition of, for instance, parvalbumin positive basket cells
(Creutzfeldt et al., 1966; Hasenstaub et al., 2005; Mann and Paulsen, 2007; Bartos
et al., 2007; Cardin et al., 2009; Suffczynski et al., 2014; Kuki et al., 2015). The orga-
nization of the spatiotemporal structure of activity patterns in multiple frequency
bands may facilitate communication within and between cortical and subcortical
structures (Buzsáki and Chrobak, 1995; Fujisawa and Buzsáki, 2011) and has been
proposed to mediate effects of e.g. long-range attentional modulation and behavioral
inhibition (Fries et al., 2001; Gregoriou et al., 2009; Pritchett et al., 2015). Moreover,
the presence of LFP oscillations in certain frequency bands is indicative of the cortical
brain state (Steriade et al., 1991; Contreras and Steriade, 1997; Poulet and Petersen,
2008). In summary, even if interneurons are not crucial for shaping feature selectivity,
inhibitory actions and interactions in the visual cortex vastly increase the complexity
and flexibility of local circuit dynamics.

Columns, pinwheels and mesoscale functional organization

When Hubel and Wiesel made their observations of orientation-tuned neurons in the
cat brain, they noticed a tendency of neighboring cells to have the same preferred
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orientation (Hubel and Wiesel, 1962). They proposed that the visual cortex consists of functional columns, spanning multiple cortical layers, in which simple and complex cells interact to give rise to a columnar orientation preference. In a later study, they discovered that the orientation preference progressed continuously between two different locations in the visual cortex and confirmed the two-dimensionality of the columnar organization (Hubel and Wiesel, 1974a; Hubel et al., 1977). The full extent of the organization became visible with the introduction of functional optical imaging techniques in neuroscience, revealing a two-dimensional map of orientation preference slabs (Blasdel and Salama, 1986). With electrophysiological and imaging techniques improving, high quality maps were made, describing in great detail the continuous organization of iso-orientation patches and their organization around centers, coined pinwheels or singularities (Swindale et al., 1987; Bonhoeffer and Grinvald, 1991; Ohki et al., 2006). Besides for orientation, the visual cortex of higher mammals is also functionally organized to represent movement direction (Payne et al., 1981; Weliky et al., 1996), ocular dominance (LeVay et al., 1975) and spatial frequency (Tootell et al., 1981; Bonhoeffer et al., 1995). These maps are laid out across the cortical surface such that they provide orthogonal coverage of all of the mapped features (Hübener et al., 1997; Swindale et al., 2000).

In some smaller mammalian species like the rat or mouse, spatial organization of orientation, ocular dominance or spatial frequency preference is absent (Ohki et al., 2005; Mrsic-Flogel et al., 2007; Bonin et al., 2011), although there is an indication of ocular dominance patches in rat V1 (Laing et al., 2014). One reason for the absence of orientation columns in small mammals could be a disadvantage of covering too large subregions of the visual field by single orientation columns. If one would imagine mouse V1 having orientation columns of only a few hundred micrometers in diameter, it could lead to a ‘clumsy’ arrangement with less than a dozen columns in total and therefore having large parts of the visual field being processed by cells that are all tuned to the same orientation (Kaschube, 2014). This situation, however, is not unlike the recently discovered ‘orientation columns’ in the mouse superior colliculus (Ahmadlou and Heimel, 2015; Feinberg and Meister, 2015). Animals that do have orientation columns share many similarities in how these columns are organized, which could point to an underlying functional advantage leading to superior visual processing (Kaschube et al., 2010). However, the discovery of the gray squirrel—a highly visual mammal—entirely lacking orientation columns argues against a critical advantage (Van Hooser et al., 2005; Kaschube et al., 2010).
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Organization of higher visual areas

In two papers published in 1991 and 1992, Felleman and Van Essen reported a set of experiments tracing the anatomical circuit of 25 visual areas and an additional 7 visual-association areas of the neocortex. Using a brute-force tracing approach, they revealed an extended network of feed-forward and feedback projections suggesting that visual processing in the macaque neocortex consists of at least 10 hierarchical levels with a high level of interconnectivity (Figure 1.3A). Later, a similar but more simplified network was uncovered in the neocortex of the rat (Burwell and Amaral, 1998), revealing striking similarities in overall hierarchical cortical organization of the rodent and primate visual system (Figure 1.3B).

The functional organization of the mouse visual cortex had so far remained rather unexplored, apart from a handful of studies on V1 receptive field organization (Dräger, 2012).
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1975; Wagor et al., 1980; Schuett et al., 2002; Kalatsky and Stryker, 2003) and studies using the primary visual cortex as a model for studying plasticity (see below; e.g. Gordon and Stryker, 1996; Hensch and Stryker, 1996; Hübener, 2003). In 2007, Wang and Burkhalter showed that, also in the mouse, the visual cortex projects to as many as 15 higher cortical areas, nine of which having a retinotopic organization. Moreover, in subsequent studies they showed that the overall connectivity of the murine visual system follows a similar, though simpler, pattern as found in visual areas in primates and humans (Figure 1.3B and 1.3C; Gao et al., 2010; Wang et al., 2011, 2012).

As Felleman and Van Essen (1991) proposed, the existence of these densely connected cortical networks is highly suggestive of a hierarchical system of visual processing. Comparison of electrophysiological recordings from cells in the secondary visual cortex (V2) of the monkey and fMRI recordings from V2 in humans, with recordings from V1, revealed that cells in V2 are more responsive to pictures that are from natural scenes or have the same higher order statistical dependencies as natural scenes (Freeman et al., 2013). In recordings from areas further upstream from V1, into the temporal regions of the primate brain, feature selectivity becomes increasingly more complicated, with cells being selective for composites of forms, like corners or simple shapes (Tanaka et al., 1991; Logothetis et al., 1995). Other regions, more dorsally located in the neocortex, are specialized in computation of motion direction (e.g. area MT; Albright, 1984; Rodman and Albright, 1989) or color (e.g. V3, V4; Schein et al., 1982; Burkhalter and Van Essen, 1986). The exact organization of cortical areas into processing levels and/or hierarchies is under scrutiny (Van Essen et al., 1992; Hilgetag and Kaiser, 2004; Vezoli et al., 2004), but there is considerable theoretical and experimental agreement on the overall flow of information through visual cortical areas.

In 2005, Quiroga et al. published a most striking experiment. Before a surgery was performed on human epileptic patients, the source of the seizures was located with temporarily implanted microelectrodes. Quiroga et al. (2005) used these electrodes to additionally record from nearby cells in different regions of the medial temporal lobe (MTL), which is part of the ventral visual stream and mostly associated with memory, but also with perceptual representations (Tulving and Schacter, 1990; Bussey and Saksida, 2007). They observed a subset of neurons in the MTL that were highly selective for specific landmarks, objects or individuals, even if the pictures of these objects or individuals were visually very different from each other. In some cases, a neuron would even respond to the written name of a person. The observation of invariant tuning for objects and individuals resembles more our subjective perception, which includes a conceptual representation of a person’s or object’s identity, rather than low-level image features. One may consider the outputs of these cells to be part of object centered representations (Marr, 1982), which can be considered an endgoal...
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of the visual computation in the brain.

The visual features that neurons in the higher visual areas of the mouse brain specialize in are still mostly unknown. There is some consensus that the posterior medial visual area (area PM) is more responsive to slowly moving stimuli of high spatial frequency (finer details), while neurons in the anterior lateral region (area AL) are more responsive to fast moving stimuli with low spatial frequencies (Andermann et al., 2011; Marshel et al., 2011). These higher visual areas receive selective inputs from cells in the primary visual cortex that prefer matching visual features (Glickfeld et al., 2013). Although the cells that project to different visual processing streams in mouse V1 are not spatially segregated (Gao et al., 2010) like in the monkey, the overall principles governing the organization of the mouse visual cortex make it a suitable model for studying visual learning (Glickfeld et al., 2014).

1.1.3 Plasticity in primary visual cortex

Neural plasticity is the ability of a neural circuit to adapt its function and/or connectivity to changes in the internal or external environment. This adaptive ability has many faces, from the slow recovery from a brain trauma to rapid changes in the circuits mediating learning and memory. Although plasticity induced changes in the brain are certainly not all identical, to some extent they do reflect the same underlying processes of synapse rearrangement and synaptic plasticity. In the following sections, I will introduce a few different plasticity paradigms that have been observed to affect connectivity and cellular responses in sensory areas of the neocortex.

On synaptic plasticity, LTP and memory

At the synapse, the presynaptic neuron releases a neurotransmitter in the small space between its axon terminal and the dendrite of the postsynaptic neuron. The strength of the transmitted signal depends, amongst others, on the amount of neurotransmitter released, and on the amount of postsynaptic receptors. When the pre- and postsynaptic neurons are repeatedly co-activated, the synapse is likely to strengthen and remain stronger for a long time (up to hours, weeks or more), a process called long-term potentiation (LTP; Bliss and Lomo, 1973). The opposite process, the weakening of a synapse, can occur when the two neurons are weakly active together (Schuman and Madison, 1994) and is referred to as long-term depression (LTD Bear and Malenka, 1994);

The relation between LTP and memory was first demonstrated in experiments that interfered with the function of NMDA receptor channels in the hippocampus, showing that this did not only impair LTP, but also hippocampus-dependent spatial
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learning (Morris et al., 1986; Davis et al., 1992; Tsien et al., 1996). A subsequent study showed that electrophysiological LTP emerges in the amygdala after (behavioral) fear-learning, only potentiating responses to the conditioned stimulus (Rogan et al., 1997). Even stronger evidence of LTP being the mechanism underlying memory formation has been provided by Whitlock et al. (2006), showing that when LTP is electrically induced in the CA1 area of the hippocampus, it becomes more difficult to additionally store a new memory that depends on this circuit. Next, they showed the reverse, when a new memory was stored in the brain it became more difficult to elicit LTP using electrical stimulation in CA1. Together, these studies provide strong support for a causal relation to exist between synaptic plasticity, long-term potentiation and learning and memory.

Experience-dependent plasticity during development and adulthood

Neurons in sensory regions of the brain are not entirely hardwired to process or represent always precisely one specific unit or feature of sensory information. Experience (sensory input) is required for cells in the sensory cortices to develop or refine their wiring and response properties (Stryker and Harris, 1986; Katz and Shatz, 1996; Rochefort et al., 2009). This instructive effect was directly demonstrated in the visual cortex of the ferret, where orientation-tuned neurons do not discriminate motion directions at the time of eye-opening, but acquire direction selectivity in the first days after eye-opening as a consequence of visual motion experience (Li et al., 2008). A potential advantage of using visual experience to shape the development of a visual cortical circuit is to fine-tune the circuit to actual environmental demands. Such an effect was observed in the visual cortex of cats that were raised seeing only horizontal or vertical bars, where the majority of cells preferentially responded to the overexposed stimulus, biasing the cortical representation of orientations (Hirsch and Spinelli, 1970; Hirsch et al., 1983). A similar effect was recently observed in mice (Kreile et al., 2011). Both studies illustrate how, in the case of development under conditions that do not resemble what will be experienced in adulthood, adjustment to the environment could become maladaptive.

Cells in the primary visual cortex respond with different strengths to inputs from each of the eyes (Hubel and Wiesel, 1962; Mrsic-Flogel et al., 2007). If the input from one eye is blocked during development, cells in the visual cortex become overall more responsive to the other (open) eye (Wiesel and Hubel, 1963; Dräger, 1978). This effect is strongest during a specific period in early life, the critical period for ocular dominance plasticity (Wiesel and Hubel, 1963; Hubel and Wiesel, 1970; Hensch, 2005; Leveit and Hübener, 2012), when the incoming visual features from the two eyes are matched for binocular vision (Wang et al., 2010) and GABAergic
inhibition in the visual cortex matures (Hensch et al., 1998). Besides being a canonical model for developmental and experience-dependent cortical plasticity (Hübener and Bonhoeffer, 2014), ocular dominance (OD) plasticity is specifically used to study circuit background and treatment of the condition of amblyopia (Sengpiel, 2014).

Even when the brain is fully matured, primary sensory neurons in the neocortex can still ‘find’ different presynaptic partners, for instance when their original inputs are removed as in the case of a peripheral lesion or amputation (Kaas et al., 1983). A classical experimental paradigm for studying such plasticity is the retinal lesion (Sammons and Keck, 2015). Here, a small patch of the retina is destroyed so that the corresponding part of the cortical retinotopic representation (the lesion projection zone, LPZ) will receive no more visual drive, even though thalamic relay neurons are still intact (Kaas et al., 1990). Immediately after a retinal lesion, the cortical region that used to respond to stimuli in the lesioned visual field is unresponsive to visual stimuli. After some period of time, however, the cortex starts responding again, but now to visual stimuli in neighboring regions of visual space (Kaas et al., 1990; Darian-Smith and Gilbert, 1995; Keck et al., 2008). Furthermore, by following the morphological structure of neurons in the visual cortex using in vivo two-photon microscopy, Keck et al. (2008, 2011) showed that the recovery of visual responses was associated with a large-scale rearrangement of excitatory and inhibitory synaptic connectivity. While in cats and monkeys OD plasticity is only observed in juvenile subjects, mice can still undergo OD plasticity far into adulthood. The mechanisms, however, differ to some extent from critical-period OD plasticity (Hofer et al., 2006b) and may require conditions of enriched environment (Greifzu et al., 2014), exercise (Kalogeraki et al., 2014) or additional coherent visual stimulation (Matthies et al., 2013).

**A role for V1 in perceptual refinement and novelty signaling**

Plasticity in the primary visual cortex does not only occur as a consequence of rather strong interference with V1 inputs, but has also been proposed to underlie learning to discriminate fine visual details (Perceptual learning; Ramachandran and Braddick, 1973). In an effort to test where in the visual pathway perceptual learning occurs, Karni and Sagi (1991) trained human subjects extensively to discriminate a texture from a distracting background at a specific region in their visual field and with one eye only. The learning-related improvement turned out to be lost when the retinotopic location of the task was altered, or when the subjects were instructed to perform the task with the untrained eye. This retinotopic selectivity, and even more so this ocular specificity, were taken as an indication that plasticity underlying perceptual learning occurs in the very first stages of cortical visual processing, possibly in V1 (see also Gilbert et al., 2009). Similar experiments have been done using monkeys while
performing electrophysiological recordings. Indeed, extensive training produced very specific changes in orientation tuning of V1 neurons which were found to result in enlarged differences in response amplitude between the two discriminated orientations (Schoups et al., 2001). However, perceptual learning does not always result in changes in V1 tuning curves (Ghose et al., 2002) and can also alter responses at intermediate levels of visual processing (Yang and Maunsell, 2004; Raiguel et al., 2006).

Besides being involved in learning to discriminate visual stimuli, the visual cortex may be required for remembering the novelty, as opposed to familiarity, of stimuli. Frenkel et al. (2006) showed that daily exposure to a grating with a specific orientation leads to a progressive increase of evoked responses in the primary visual cortex of the mouse (stimulus-selective response potentiation, SRP), that is dependent on LTP of the thalamocortical synapse in V1 layer 4 (Cooke and Bear, 2010). The emergence of this increase in response amplitude was suggested to be indicative of how familiar a mouse is with a grating having the specific presented orientation (Cooke et al., 2015). Evidence for this proposal was provided as follows: Animals naturally explore novel stimuli and therefore also approach and investigate oriented gratings. Cooke et al. described that in a time course parallel to that of the built-up of SRP, the animals showed less and less interest in the stimulus used for SRP. The surprising finding was, however, that local blockade of V1 plasticity did not only prevent SRP, but also prevented the mice from becoming familiar with the stimulus used for SRP; the mice explored the oriented grating on each day of the experiment as if they saw it for the first time. Although a direct causal relation between V1 plasticity and the memory for novelty of a stimulus may require further evidence, this series of studies strongly suggests that cells in the primary visual cortex at least facilitate the detection and memory of novel versus familiar stimuli.

**Reinforcement learning and V1 plasticity**

The concept of reinforcement learning is, through trial and error, finding the most appropriate action in response to a specific stimulus, location, or situation, without receiving specific instructions other than positive or negative scalar feedback on the outcome of each action (Sutton and Barto, 1998). Classical and operant conditioning are used to investigate effects of reinforcement learning in animals and humans. In classical conditioning, a stimulus is paired with an unconditioned stimulus resulting in a conditioned association between stimulus and reward (Pavlov, 1927). Operant conditioning requires the animal to learn, through trial and error, that an operant response like pressing a lever or breaking a photobeam results in a reinforcement (Skinner, 1938). Although learning and memory are mostly associated with higher
cortical areas and the hippocampal formation in the medial temporal lobe (Gelbard-Sagiv et al., 2008), the frontal cortex (Asaad et al., 1998; Lee and Solivan, 2008), the striatum (Lansink et al., 2009) and the cerebellum (Thompson, 1986), conditioning does affect the perception of visual stimuli (Begleiter et al., 1967).

Visual cortex cells possess the ability to change response patterns as a consequence of a behaviorally relevant outcome, which was first shown by operant conditioning of the response amplitude of a neuron in the cat visual cortex (Shinkman et al., 1974). When the activity of this neuron exceeded a threshold in a specific period after a visual stimulus had been shown, reinforcement was given by electrical stimulation of a hypothalamic region (presumably including the medial forebrain bundle). Repeated stimulus-response-reward pairing led to larger responses of the cell, specifically in the reward related post-stimulus period, indicating that reinforcement information was likely fed back into the visual cortex. A much more recent study showed in a more naturalistic, though head-restrained, setting that cellular responses of V1 cells to rewarded stimuli increased in response amplitude and selectivity during the period of a conditioning paradigm (Poort et al., 2015). Such effects may help perception; Seitz et al. (2009) showed that pairing presentations of oriented bars, embedded in a noisy image such that they could not be directly perceived by the human subjects, with a reward led to an eye-specific and retinotopically selective improvement in the detection threshold for bars of this orientation.

If stimulus-reward pairing enhances low-level visual perception and V1 cells can change their response properties as a result of operant conditioning, an outcome signal must be fed back into the local circuit (Sutton and Barto, 1998; Gavornik et al., 2009). Possible candidate circuits transmitting such signals contain cells that release slowly acting neuromodulators like dopamine and acetylcholine (Schultz, 1986; Hasselmo, 1995). Dopaminergic cells respond to unexpected reward and appear to code for the difference between the expected outcome and the actual outcome of a stimulus or action (Schultz et al., 1997). Moreover, dopamine release in the extracellular space of the striatum can provide spatially and temporally specific modulation of synapses connecting two other cells (Yagishita et al., 2014), but it has not yet been resolved whether dopamine provides a similar modulation of synaptic connectivity in V1. Although there are some indications that dopamine neurons project to the visual cortex in the rat (Febvret et al., 1991) and occipital cortex dopamine levels increase in response to visual stimulation (Müller and Huston, 2007), these studies used techniques that may have misidentified the neurotransmitters and fiber connections. There are also a number of contesting theories on the function of dopamine signals in the brain, suggesting a role in novelty signaling and attentional switching (e.g. Pennartz, 1995; Redgrave et al., 1999).

A stronger candidate circuit for relaying outcome-related signals into sensory
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areas of the brain is the nucleus basalis/substantia innominata region, which releases the neurotransmitter acetylcholine that can also act as a neuromodulator affecting visual cortex plasticity (Bear and Singer, 1986; Kilgard and Merzenich, 1998). A subset of neurons in the visual cortex of the rat strongly responds to upcoming reward in the absence of simultaneous visual stimulation (Shuler and Bear, 2006). These reward anticipating response patterns critically depended on cholinergic modulation (Chubykin et al., 2013) and may function as a feedback signal in reinforcement learning (Gavornik et al., 2009). Acetylcholine release has also been implicated in attention (Hagan and Morris, 1988; Muir et al., 1994; Han et al., 2014), but it is difficult (and potentially irrelevant) to fully disambiguate the effects of reward and attention (Stǎnişor et al., 2013). Conditioning related modifications in V1 may also depend on glutamatergic inputs relaying a reinforcement signal from frontal cortical areas like cingulate and medial prefrontal cortex (Pennartz et al., 2000; Zhang et al., 2014), potentially mediated though local V1 inhibitory networks (Fu et al., 2014; Petro et al., 2014). In summary, visual cortex plasticity is not only observed following changes in statistical input structure, but also drives adjustment of local representations or computations suiting the behavioral needs of the animal.

1.1.4 Is the visual cortex a bottom-up feature detector?

The organization of the visual system has so far been presented such that one gets the impression of information being processed in an organized system with hierarchical levels, each processing step recombining inputs from the previous step in order to represent more complicated visual features and integrate information across larger fields of view (Van Essen et al., 1992). Indeed, neurons in the visual stream seem to respond to visual stimuli just as they would be expected at their respective level (Hubel and Wiesel, 1962; Schein et al., 1982; Burkhalter and Van Essen, 1986; Tanaka et al., 1991; Logothetis et al., 1995; Freeman et al., 2013). Many of these observations, however, were made in anesthetized animals or under very controlled experimental and behavioral conditions. In awake, free viewing monkeys it turned out to be much more difficult to predict what the animal was viewing from activity in the primary visual cortex (Livingstone et al., 1996), which is surprising given the reliability of responses of orientation-tuned cells in V1 of anesthetized monkeys (Hubel and Wiesel, 1968). This problem of unreliability may be attributed to uncontrollable experimental conditions and can be reduced by restricting head movements and enforcing gaze fixation (Wurtz, 1969). On the other hand, the unreliability of responses of cells in the visual cortex could indicate a caveat in our understanding of their computation.

A growing body of literature indicates that responses of cells in the primary visual cortex are modulated by context, behavioral state and attention (Wörgötter and Eysel,
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2000; Maimon, 2011). These modulations may originate from a variety of structures, including strong inputs relaying feedback information from higher visual and frontal cortical areas and projections from the superior colliculus into V1 (Allman et al., 1985; Lamme et al., 1998a; Polack and Contreras, 2012; Muckli and Petro, 2013; Sellers et al., 2015) and from subcortical structures potentially mediated by neuromodulator release (Pinto et al., 2013; Lee et al., 2014a). Monkey visual cortex cells are indeed strongly modulated by whether their receptive field covers a foreground object or an object in the background (Lamme et al., 1998b; Spillmann et al., 2015) and by whether the field is part of an object that is in the focus of attention or predictive of reward (Chen et al., 2008; Stănişor et al., 2013). Human visual cortex shows similar modulations by attention and reward (Poghosyan and Ioannides, 2008; Serences and Saproo, 2010). Moreover, specific timed disruption of the feedback activity in V1 blocks recognition of visual stimuli (Camprodon et al., 2010) and prevents visual awareness under specific circumstances (Ro et al., 2003).

Many of the state dependent or contextual modulations of V1 activity in monkeys and mice are affected, or even lost, under anesthesia (e.g. Lamme et al., 1998b; Pack et al., 2001; Movshon et al., 2003; Pack et al., 2003; Haider et al., 2012; Adesnik et al., 2012; Vaiceliunaite et al., 2013). Anesthesia can be used as a manipulation to study state-dependent brain changes in the context of loss of consciousness (Collins et al., 2007; Alkire et al., 2008; Lee et al., 2009b,a; Schröter et al., 2012; Untergehrer et al., 2014) and has been reported to change the spatiotemporal structure of spontaneously emerging (ongoing) and evoked activity patterns in mouse visual cortex (Greenberg et al., 2008; Golshani et al., 2009; Pisauro et al., 2013). Attention- and state-dependent influences have a widespread effect on the primary visual cortex activity and the currently accepted view of V1 acknowledges bottom-up and context-dependent (through lateral interactions) feature detection as well as an extended palette of top-down modulations (Petro et al., 2014).

When Niell and Stryker (2010) were recording neuronal activity in the visual cortex of awake mobile mice, they found that cells responded much more vigorously to a visual stimulus when the mouse was running compared to when it was immobile. This observation kicked off a series of publications specifically studying state-dependent modulations in the mouse visual cortex. Polack et al. (2013) demonstrated that the running-associated gain increase was dependent on local noradrenaline, but not acetylcholine. Combined results from three other studies, however, sketch a different circuit. Basal forebrain acetylcholine cells that project to V1 were found to be driven by neurons in the mesencephalic locomotor region (MLR), that can also induce running behavior (Lee et al., 2014). Subsequently, these cholinergic projections were shown to activate a specific circuit of VIP+ interneurons in V1 that disinhibit layer 2/3 pyramidal cells during locomotion (Pinto et al., 2013; Fu et al., 2014). Mouse visual
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cortex cells are also modulated by pupil dilations (Reimer et al., 2014) and locomotion independent arousal (Vinck et al., 2015) and a subset directly encodes the running speed of the animal in their firing patterns (Saleem et al., 2013).

The abundance of non-visual signals reflecting internal state and motor output may indicate that the visual cortex follows a coding strategy of signaling unexpected changes in the visual environment. This strategy of predictive coding can be considered more efficient compared to continuously transmitting full images to higher cortical areas, because the strong spatial and temporal correlations in natural images would make such full-image transmission redundant in information (Srinivasan et al., 1982; Mumford, 1992; Bastos et al., 2012). The possibility of V1 encoding the image using this principle has been explored in a modeling framework, which explained extraclassical receptive field properties like end-stopping and contextual modulations (Rao and Ballard, 1999). A recent study in mouse visual cortex discovered neurons in layer 2/3 that responded only when visual and running speed did not match, indicating that V1 indeed contains circuitry to compare expected and detected visual signals (Keller et al., 2012). The question of how vision is computed in the brain remains elusive, but it is certain that the answer will extend beyond the concept of a feed-forward hierarchical feature detector.

1.1.5 Do small rodents rely on vision to guide their behavior?

Mice are nocturnal animals and have a visual system with a relatively low visual acuity compared to humans – leading to the assumption that mice do not rely much on vision in their natural behavior (Huberman and Niell, 2011). However, by now it is clear that mice have a relatively well developed visual system including a primary visual cortex (Dräger, 1975; Schuett et al., 2002; Niell and Stryker, 2008), a minimum of 9 higher visual areas (Wang and Burkhalter, 2007) with differential visual processing (Andermann et al., 2011; Marshel et al., 2011; Glickfeld et al., 2013) and a connection pattern that resembles the dorsal and ventral visual stream (Gao et al., 2010; Wang et al., 2011, 2012). But whether mice use their visual system to guide their behavior is a question that goes beyond neural circuitry. First, we must ask whether mice can discriminate complex visual patterns at all, and whether they can associate them with actions and outcomes. The answer to this question is unequivocally ‘yes’. For instance, mice can discriminate pictures in nose-poke or touch-screen operant chambers where touching a specific picture results in delivery of reward (Bussey et al., 2001; Morton et al., 2006; Horner et al., 2013; Oomen et al., 2013). Alternatively, they can swim towards a visual motion coherence target that indicates the location of a hidden underwater platform (Douglas et al., 2006). Moreover, mice can use vision for navigation in the Morris water maze where only distal visual cues are indicative of spatial orientation.
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(Morris, 1981; Stackman et al., 2012).

The second question we have to ask is to what extent a mouse in its natural environment uses visual information. From the observation that mice can solve visual tasks in a laboratory setting does not necessarily follow the conclusion that the animals must use this sense for a similar purpose in their natural environment. The key to the answer lies in innate visual behaviors of mice that evolved potentially long ago, outside of the laboratory. As Yilmaz and Meister (2013) argue, we know only of a handful of these behaviors and most are reflex-like. Two such behaviors, the optokinetic reflex and the optomotor response, occur when a moving stimulus passes in front of the eyes and the mouse follows the direction of movement with its eyes (optokinetic reflex; Stahl, 2004) or head (optomotor response; Prusky et al., 2004). This kind of behavior likely supports image stabilization and indicates that accurate detection of visual detail is relevant to their behavior. Another type of non-learned behavior is the innate fear response to overhead looming stimuli (Yilmaz and Meister, 2013; Shang et al., 2015; Wei et al., 2015). Vision is usually the only reliable pre-warning for an aerial attack and it is hard to deny the relevance of detecting and discriminating predators. Last, even though the binocular zone spans only a relatively small part of the visual cortex, mice do have the neural circuitry for depth perception (Scholl et al., 2013) and avoid foraging on sections of a transparent piece of glass that has a deep gap underneath (Fox, 1965). Extrapolating these innate behaviors to learned behaviors like context-dependent object discrimination is probably too large of a step, but the visual behaviors of mice are certainly ecologically relevant and more complex than one might intuitively expect from a small nocturnal rodent.

1.2 How two-photon microscopy revolutionized neuroscience

The development of the two-photon microscope opened up the microcosmos of the living brain for public viewing. Two-photon microscopy generates multicolor images from a plane or volume in the brain with subsecond temporal precision and has a spatial resolution that is sufficient to reconstruct the morphology of a neuron. It is relatively non-invasive and has therefore the potential to be used for long-term repeated recordings of the same optically identified cells. In the next few paragraphs I will explain in more detail how two-photon microscopy in living mice works and how the data can and should be interpreted.
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Figure 1.4: Fluorescence and two-photon microscopy. A Schematic depiction of the principle of fluorescence imaging. The green triangle represents a neuron filled with a fluorescent molecule in the brain, surrounded by unlabeled cells (dotted triangles). The blue excitation light is absorbed by fluorescent molecules in the neuron, emitting green photons. A chromatic filter blocks the excitation light, selectively enhancing the contrast of the fluorescently labeled neuron. B Inset: Two-photon absorption can occur when two photons impinge on a single fluorescent molecule at the same time. Main panel: Optical sectioning using two-photon microscopy. Only the focal point of the converging laser beam (red) has sufficient density of photons to have a high chance of simultaneous absorption by a fluorescent molecule. Emitted photons come only from the location of the focal point of the laser and can therefore be all collected using a single highly sensitive detector (e.g. a photo multiplier tube).

Fluorescence

Under normal conditions, the mouse brain does not reveal much of its intricate circuits of cells and connections, but looks rather like a uniform slightly pink substance with a number of blood vessels running through and around it. Using the principle of fluorescence, however, one can make specific cells in the brain stand out from the background. A fluorescent molecule can absorb light of one wavelength (the excitation wavelength), which brings it to a higher energy state. Subsequently, the molecule falls back to a lower energy state and returns the energy difference by emitting a photon. In the process of changing energy states, however, the molecule loses a small amount of energy and the returned photon will therefore have a lower energy. Because the energy of a photon is proportional to its wavelength, the wavelength of the emitted photon will be longer (a more red shifted color). When one would illuminate a part of the brain that contains a cell that is filled with a fluorescent molecule, the fluorescent molecules would absorb some of the impinging photons (e.g. of blue color) and subsequently emit photons with a red-shifted wavelength (e.g. of green color). By looking through a chromatic filter, one now simply blocks the excitation light from reaching our eyes, and the one cell that contains the fluorescent molecules emitting
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green light will still be visible, appearing as if it was hovering lonely in the darkness of the brain (Figure 1.4A; see also e.g. Yuste and Konnerth, 2004).

There are multiple ways of getting fluorescent molecules into a neuron. First, one can fill a small glass pipette with a solution containing a fluorescent molecule and use patch clamping or electroporation to transfer the fluorescent molecules into the cell (Nevian and Helmchen, 2007; Kitamura et al., 2008). Some types of designed fluorescent molecule can freely travel through a cell’s membrane, but once inside the cell they become trapped because of local biochemical processes altering the structure of the molecule. Thus, when a small volume of these molecules is injected into the brain, they will spread out to nearby cells and automatically become trapped (multicell bolus loading; Stosiek et al., 2003). Currently, the most popular way of getting a neuron to become filled with a fluorescent molecule is to use genetic expression of a fluorescent protein (FP), for instance green fluorescent protein (GFP; Heim and Tsien, 1996). The GFP coding region in the DNA can be preceded by a promoter region that is only transcribed in certain cell types, which restricts expression of GFP and serves to identify specific cells and circuits in the brain (Gong et al., 2003). The snippets of genetic code that express these FP’s can be built into the DNA of the mouse, but also transduced into a local group of cells by injecting a virus into a specific brain region (Luo et al., 2008).

Two-photon microscopy

High-resolution images from fluorescent neurons in a brain can be made with a two-photon laser-scanning microscope (Denk et al., 1990; Tan et al., 1999). The principle of operation is the use of a focused infrared laser beam that illuminates only one very small point in the brain at a time. The light that is emitted from the brain can be filtered (as explained above) to reject the excitation wavelength of the laser, so that highly sensitive photo multiplier tubes (PMTs) can collect all remaining photons. By scanning the focal point of the laser in a grid like fashion across a single plane in the brain, fluorescence at each point in this plane is sampled sequentially. Using information on where the laser’s focal point was at each sampling point, one can now reconstruct the full image of fluorescence intensities. Conventional two-photon microscopes acquire images at about two frames per second, systems fitted with a resonant mirror scan frames faster (at 20-60 Hz), but the fastest systems use non-mechanical acousto-optic deflectors (AODs) to steer the beam. These can acquire full frame images up to a speed of 500 Hz (Grewe et al., 2010).

Under normal conditions, a focused laser beam will also excite fluorescent molecules above and below the focus point. This is where two-photon excitation gives an edge (Denk et al., 1994). Two-photon excitation happens when two photons
1.2. How two-photon microscopy revolutionized neuroscience

collide onto a fluorescent molecule simultaneously. If the summed energy of the two photons corresponds roughly to the energy needed for single photon fluorescence excitation, the two photons can have the same effect as this single photon, and push the molecule to the same higher energy level as with one-photon excitation (Figure 1.4B, inset). Because photons and fluorescent molecules are small, there is only a limited chance that a single photon impinges on a molecule. In the focal point of the laser beam this chance is the highest, but the chance of exciting a fluorescent molecule with a single photon above or below the focal point is reduced linearly with the increasing diameter of the laser bundle. Using two-photon excitation, the chance of exciting a molecule above or below the focal point is reduced quadratically instead of linearly, because we now need the excitation to happen twice at the same time. This effect effectively reduces the amount of fluorescence excitation above and below the focal point to zero, guaranteeing that virtually all emitted photons come from the single focal point of the infrared laser beam (Figure 1.4B). This principle is called optical sectioning.

One specific additional advantage of two-photon microscopy for imaging in the living brain is that most commonly used fluorescent molecules require two-photon excitation using infrared wavelengths, which penetrate tissue better than visible wavelengths. Because of its superior optical sectioning and the ability of non-invasive imaging of live brain tissue, two-photon microscopy is now widely used in neuroscience (Svoboda and Yasuda, 2006).

Calcium imaging

Besides for studying morphological structure of neurons, two-photon imaging can be used to study activity patterns of cells in the brain by imaging fluorescent calcium indicators (Stosiek et al., 2003). Most calcium indicators consist of a single fluorescent molecule that can bind or trap one or more calcium ions. In addition, these molecules must have a different likelihood of fluorescence excitation in the ‘calcium-bound’ state compared to the ‘calcium-unbound’ state. Currently, calcium indicators come in two forms. Synthetic indicators such as Oregon Green BAPTA1 (OGB1) have the advantage that they are small, bright, resistant to photo-bleaching, fast in their calcium-binding kinetics and their fluorescence intensity reflects the intracellular calcium concentration rather linearly, but their drawback is that they can only be imaged for up to 12 hours (Stosiek et al., 2003). Genetically encoded calcium indicators like GCaMP, Twitch or Yellow-Cameleon consist of one or more fluorescent protein(s) fused to a calcium binding protein and have generally less ideal binding kinetics and linearity in their response to varying calcium concentrations (although there are more and more diverse variants available; see Rose et al., 2014). Their major advantage
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...is that neurons can express the indicator genetically over long periods of time and thereby allow tracking of neuronal activity over the course of many days, weeks or months (Horikawa et al., 2010; Lütcke et al., 2010; Chen et al., 2013; Thestrup et al., 2014).

Two-photon calcium imaging data acquired by using the OGB1 calcium indicator can be analyzed by averaging the fluorescence in manually outlined cell bodies in each image of a series and interpreting the fluorescence brightness of the cell as an indication of its activity (e.g. Ohki et al., 2005). There are a few complications that one has to be aware of. First, the fluorescence intensity compared to baseline ($\frac{\Delta F}{F}$) may not reflect action potential activity linearly. Action potentials result in increases in intracellular calcium concentration, but we image the fluorescence intensity of a calcium indicator. This is a two-step, indirect measurement introducing additional noise and the $\frac{\Delta F}{F}$ can increase non-linearly in relation to the cell’s action potential frequency (Vogelstein et al., 2009). Second, two-photon microscopes are not perfect and there will be optical aberrations, which blur the boundaries between intracellular and extracellular regions. Because the neuropil (axons, dendrites, glial processes) is often also loaded with the calcium indicator, signals from the neuropil will partially mix with cellular signals (Kerlin et al., 2010). This can to some extent be compensated for, as we have also done in the experimental chapters (Chapters 2 to 4), but it remains a potential confound. Last, the brain of a mouse will move during imaging. Although we use reliable methods compensating for within-plane movement (Guizar-Sicairos et al., 2008), movement can also be out of the imaged plane and this may result in apparent fluorescence increases or decreases. This can be especially critical when imaging brain activity in awake moving animals (Andermann et al., 2010), but even in anesthetized mice a slow drift of the focal plane may affect the brightness of individual cells.

Analysis of imaging data

Why use two-photon calcium imaging for studying response properties of cells in the visual cortex, if other techniques like electrophysiology can directly record action potential activity from cortical cells with a much higher temporal resolution? Two-photon microscopy offers at least two key advantages over electrophysiology. The most obvious one is that two-photon imaging has the ability to visually identify cell types and study their morphological structure using targeted labeling of single cells or by selective genetic expression of fluorescent proteins (Gong et al., 2003; Luo et al., 2008). In the experimental chapters, for instance, astrocytes were distinguished from neurons using an additional labeling with the red fluorescent molecule SR101 (Nimmerjahn et al., 2004). Moreover, two-photon imaging reveals information about...
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the precise functional and anatomical micro-organization of neurons in small cortical regions, which will turn out to be a relevant variable in all three studies.

A second advantage of calcium imaging is the relative unbiased sampling of cells in an imaging region. Using conventional two-photon microscopy, each pixel in a field of view is sampled in every image. Electrophysiological methods for recording from multiple cells in the brain (i.e., ensemble recordings) depend on the detection of electrical activity of these cells and are therefore unable to sample non-active cells. Two-photon imaging offers a more complete view of the local circuit. By imaging tissue that is densely labeled with a calcium indicator one can obtain simultaneous activity patterns of many cells (up to 350 in the experimental chapters of this thesis), which can be used to study how activity patterns of neurons functionally interact while they process information (Seung and Sompolinsky, 1993; Abbott and Dayan, 1999). These types of analyses of population coding will be a topic in Chapters 3 and 4. As with any scientific technique, two-photon imaging offers distinct advantages as well as drawbacks, which should both be considered in the design of an experiment.

1.3 Are primary visual representations affected by appetitive conditioning and changes in cortical state?

In the following chapters I will report on three separate sets of experiments that can be combined to address the question of how stimulus processing and ongoing activity in the mouse primary visual cortex adjusts as a consequence of associative learning and overall changes in cortical state. To answer this question I have divided the results in two sections. The first section contains chapter 2 and 3, which investigates the consequences of appetitive conditioning on stimulus representations in the visual cortex. Specifically, in chapter 2 we asked whether learning to expect an outcome, associated with a visual stimulus, results in a modulation of the neural representation of this stimulus, already at the earliest levels of visual processing in the cortex.

The experiments reported in chapter 3 followed up on results of the first experiment and examined in more detail how the brain improves its representation of visual information in the neural circuits of the primary visual cortex as a consequence of learning. The main difference with chapter 2 is that we now performed conditioning using stimuli that had spatially adjacent cortical representations, which allowed to investigate the effects of conditioning at multiple spatial scales and test whether reward association, stimulus discrimination and/or task-related exposure underlies the observed changes in single cell tuning curves and population coding.

In the final experimental chapter (Chapter 4) we tested and discussed to which degree neural circuits in awake, active brains have different visual processing abilities,
compared to the exact same circuits in anesthetized, passive brains. This chapter did not only test state-dependent differences in neuronal tuning to visual stimuli, but also explored how ongoing (spontaneously emerging) activity patterns changed in their spatial and temporal dynamics under anesthesia. I will conclude the PhD thesis with a discussion of how the results of these chapters fit in the context of the above-mentioned overarching question, by specifically addressing how learning related plasticity manifests itself in the primary visual cortex and whether an anesthesia induced state-change results in a reduction of the complexity of stimulus representations in the brain.