The connectivity logic of cannabinoid type-1 expressing interneurones in the mouse visual cortex

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Composition du Jury

Dr. Giovanni Marsicano:	Rapporteur
Dr. David Robbe:	Rapporteur
Dr. Maria-Cecilia Angulo:	Examinatrice
Dr. Pablo Méndez:	Examinateur
Dr. Rebecca Piskorowski:	Examinatrice
Dr. Laurent Venance:	Examinateur
Dr Alberto Bacci	Directeur de thèse

If the human brain were so simple

That we could understand it,

We would be so simple

That we couldn't.

Emerson M. Pugh

ACKNOWLEDGEMENTS

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Doing a phd is bittersweet experience, oscillating from moments of pure joy to unfiltered dismay. I am extremely lucky to have been surrounded by you for the last few years, and will sorely miss you when I leave.

ABBREVIATIONS

2-AG	2-Arachidonoylglycerol
5-HT3AR	serotonin receptor with subunit 3A
A1	primary auditory cortex
AC	adenylyl cyclase
AEA	anandamide / N-arachidonoylethanolamine
AM251	1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl- N-(1-piperidyl)pyrazole-3-carboxamide
BAC	backpropagation-activated Ca2+
BC	basket cell
CaN	calcineurin
CB1	cannabinoid receptor type 1
CC	corticocortical
CCK	cholecystokinin
CT	corticothalamic
CV	coefficient of variation
DGL	diacylglycerol lipase
dLGN	dorsal LGN
DNQX	(6,7-dinitroquinoxaline-2,3-dione)
DSE	depolarisation-induced suppression of excitation
DSI	depolarisation-induced suppression of inhibition
eCB	endocannabinoid
EEG	electroencephalogram
eIPSC	evoked (extracellular stimulation) IPSC
FAAH	fatty acid amide hydrolase
FBI	feedback inhibition
FFI	feedforward inhibition
fMRI	functional magnetic resonance imaging
FWHM	full width at half
GPCR	G-protein coupled receptor
IHF	immunohistofluorescence
iLTD	inhibitory LTD

iLTP	inhibitory LTP
IN	interneurone
ING	interneuron gamma
IP3	phosphatidylinositol-4,5-bisphosphate 3-kinase
IPSC	inhibitory postsynaptic current
ITN	intratelencephalic neurones
JNK	c-Jun N-terminal kinases
L	Layer
LGN	lateral geniculate nucleus
LTD	long-term depression
LTP	long-term potentiation
MAG	monoacylglycerol
mGluR	metabotropic glutamate receptor
MT	middle temporal visual area
mTOR	mammalian target of rapamycin
PC	principal cell
PING	pyramidal-interneuron gamma **or** petilla interneuron nomenclature group
PKA	protein kinase A
PLC	phospholipase C
PN	pyramidal /principal neurone
PC	pyramidal / principal cell (interchangable with PN in L2/3)
PPR	paired-pulse ratio
PV	parvalbumin
RELN	reelin
S1	primary somatosensory cortex
SEM	standard error of the mean
SPN	subcerebral projection neurones
SST	somatostatin
uIPSC	paired recording
V1	primary visual cortex
V2	secondary visual cortex
VGCC	voltage-gated calcium channel
VIP	vasointerstinal peptide
WIN55,212-2	11R)-2-methyl-11-[(morpholin-4-yl)methyl]-3-(naphthalene-1-carbonyl)-9-oxa-1-azatricyclo[6.3.1.$0^{4,12}$]dodeca-2,4(12),5,7-tetraene

INTRODUCTION

I- History of visual neuroscience

Understanding neural structures, pathways, and the mechanisms underlying their function can help us understand perception, thinking, and living. Sensory systems are well suited for investigating the role of experience in regulating the development and plasticity of neuronal circuits, as they include sensory organs as well as the structures that allow the inner representation of the external world. But how does the brain translate patterns of photons into what we experience as vision? The visual system comprises all the photoreceptors and neurones that underlie this process, and its study has been popular throughout history. Documents attempting to explain *how we see* go back millennia. Perhaps it is because humans rely on vision as a primary sense to evaluate the surrounding environment and direct their behaviour, or because it is so obvious that the eyes play a role in vision. The ancient Greeks believed a hollow nerve transported *pneuma* or fluids from the eye, meeting at the chiasma to form monocular vision. Ibn al-Haytham (c. 965-1040 AD), considered the founder of physiological optics, described the connection between the eyes and the head, starting to lay down the theory of how light bouncing off objects could be coherently collected by the eyes and relayed to the brain to create binocular vision. His drawings of the visual system (Fig. 1) are today considered some of the earliest surviving diagrams of the brain, affirming the deep connection between vision and neuroscience in general. More recently, Santiago Ramón y Cajal (1852-1934) used neuronal staining to describe the connection between the retina, the thalamus and the cortex, establishing a modern vision of the visual system by including the direction of information flow and projections from the cortex back into deep structures as well as to other cortices (Fig. 2)

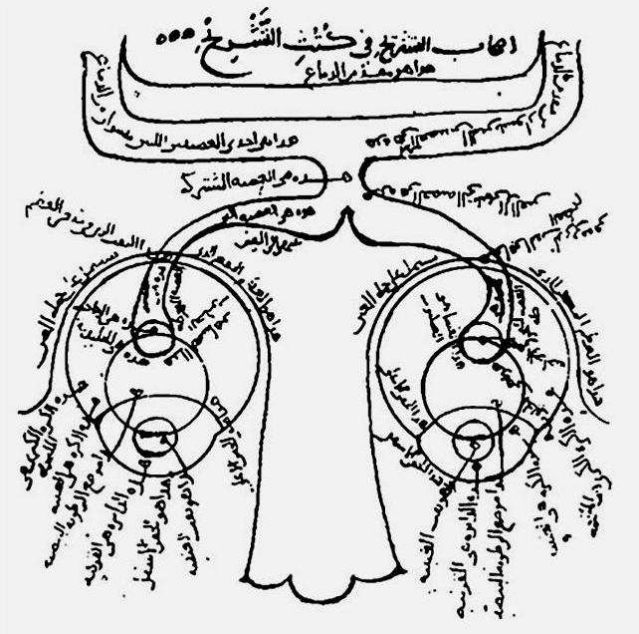

Figure. 1 The visual system according to Ibn al-Haytham (c. 965-1040 AD), perhaps the most ancient representation of the brain.

Neuroscience is by no means exclusive to anatomists and physiologists: psychologists have also played a crucial role in understanding and defining the experience of perception. The use of controlled stimulation and behavioural readouts (experimental design strategies that are still in use today) enriched visual neuroscience, for instance by using optical illusions to draw conclusions about how the brain might perceive. A dominant current of psychology in the early XXth century was gestalt psychology, which postulates that perception as a whole is different than the sum of percepts at a given time (Koffka, 2013). For instance, a specific sequence of displayed images can give the illusion of movement. This is known as the phi phenomenon (Dimmick, 1920), and demonstrates that perception is not simply the addition of the different images: the brain computes something else that gives the illusion of movement. Even simple thought experiments can lead to deep conclusions about perception. We can modulate what becomes salient in the visual field by focusing on a specific colour, shape, or any other feature, but some illusions cannot be unseen even if we know they are illusions (e.g. Fig. 3). This means that the amount of control we hold over our perception is somehow limited. The question is then, why can we control some parts of perception and others not? Is there something in the structure of neuronal networks that can explain this?

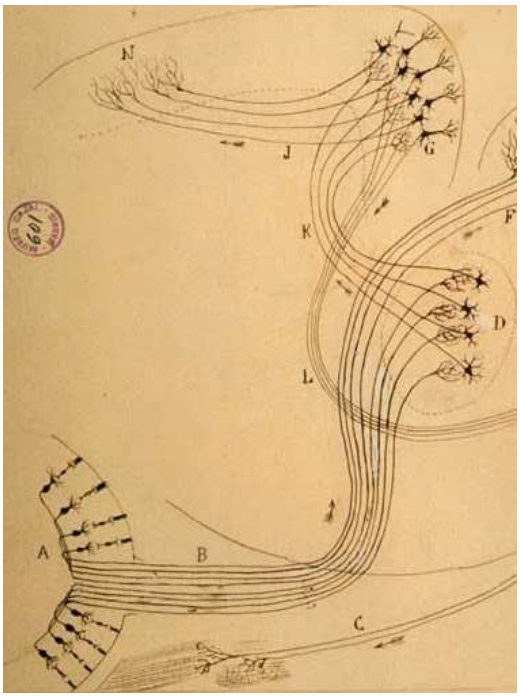

Figure 2. A drawing by Ramón y Cajal showing the connectivity between retina (A), LGN (D) and cortex (G).

Linking the observations of cognitive scientists, anatomists and physiologists, with the aim of providing a coherent picture that can illustrate how we experience the external world, remains the greatest challenge of neuroscience. In *The Organisation of Behavior* (1949), Hebb postulates that if neurone A contacts neurone B and takes part in its firing, repeated firing of neurone A will eventually lead to a strengthening of the connection of A onto B *via* physiological mechanisms. Although Hebb mainly focused on learning and memory, his postulate has direct implications for the entirety of neuroscience. Particularly, it gives a simple mechanism by which cell assemblies (or engrams) can be formed and maintained,

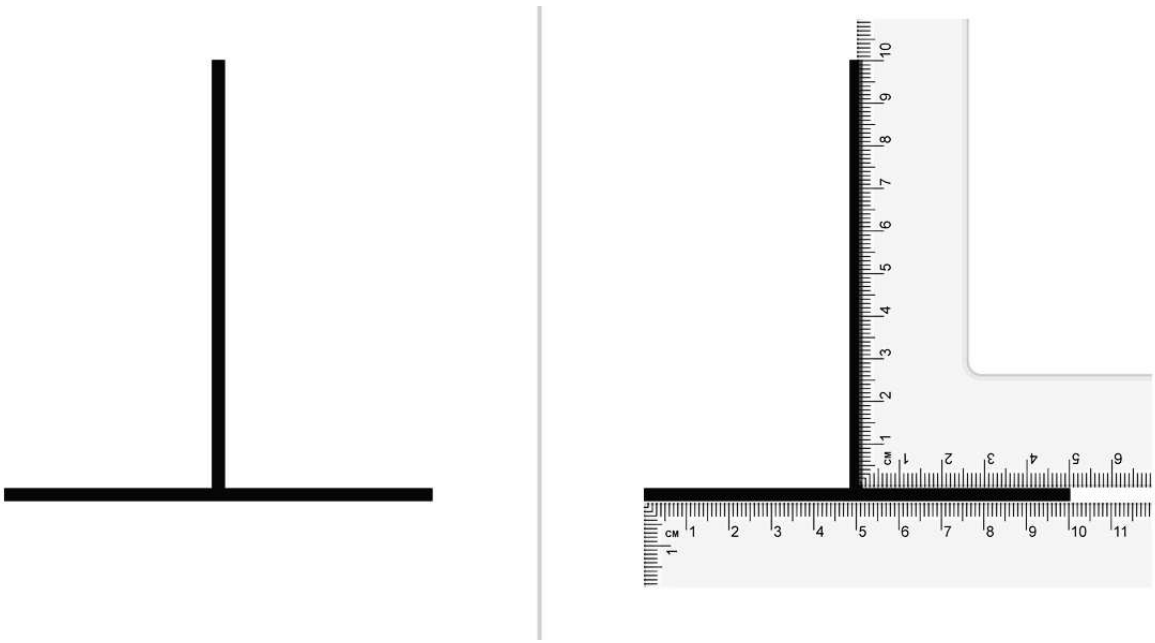

Figure 3. (left) The vertical-horizontal illusion gives the impression that the vertical bar is longer than the horizontal. (Right) This erroneous sensory experience cannot be corrected by adding digits, showing some aspects of perception cannot be modulated

switching from a passive to an active view of how the brain may process information. But technical limitations prevented direct testing of Hebb's postulate: although EEG had existed for decades, at the time there were no tools to record from small clusters of neurones, even less of single units. Indeed, the development of amplifiers and electrophysiological techniques in the second part of the twentieth century led to some of the greatest leaps in visual neuroscience. Notably, the work of David Hubel and Torsten Wiesel (1959) showed how different neurones encode features of vision, responding to specific stimuli such as orientated gratings or variations in contrast. By demonstrating responses to such specific features at the cellular level, their work gave experimental evidence for the type of code used by neural networks, and how it may be processed through the combination of sensory features to form a relevant visual field. Since then, there has been a drive to understand exactly how populations of neurones create such patterns of firing, and the development of the patch clamp technique by Erwin Neher, Bert Sakmann, and colleagues (1976; 1981) was essential in elucidating sub-cellular mechanisms of neuronal function and recording individual neurones. But there is still the problem that different brain areas may contribute to the processing of information, just as they may contribute to store a memory or a learned

behaviour, leading to elegant reductionist approaches (e.g. Kandel & Schwartz, 1982). For more complex neural networks, lesion work and experiments involving unnatural stimuli such as dark-rearing have been - and still are - extremely useful. Donald Hebb (1937) himself used dark-rearing in rats to understand whether vision was innate or the result of interplay between sensory experience and neurodevelopment during his thesis. The drawback of such methods is that the perturbation to the circuit is often permanent and does not reflect the timescale of neurological activity.

Recent development of optogenetic and cellular imaging tools have been a boon for neuroscientists trying to determine the contribution of specific cells and networks in cognitive processes (Deisseroth, 2015). It is easy to understate the revolution that it is today possible to identify, inhibit or stimulate specific sub-populations of neurones with millisecond precision, influencing behaviour in real time. However optogenetic experiments have the danger of demonstrating what some specific neurons in the brain *can* do rather than what they actually do, merely correlating with how the circuit works at the synaptic level, even when paired with electrophysiological recordings. Similarly, when calcium imaging is used to record the activity of hundreds of neurones simultaneously, it is difficult to infer whether any sequences or correlations are computationally meaningful due to the sheer amount of data such experiments produce. Moreover, Ca^{2+} signals are inherently slow, and do not resolve the precise temporal dynamics of synaptic integration and output spike trains. Therefore, using Ca^{2+} signaling as a proxy of neuronal activity is appropriate only for events relying on a rate code, but does not help revealing how neurons encode information within few milliseconds (i.e. using a temporal code). Nonetheless, these tools are today fuelling a revolution in the field, so much so that the mouse model that they often rely on is increasingly used in visual science, even though mice are not particularly visual animals (Baker, 2013). There is also evidence that cortical microcircuits are partially stereotyped, with patterns that transcend functional area and even species (Douglas & Martin, 2004; Harris & Shepherd, 2015), making the use of the mouse as an appropriate model to study sensory systems at a multiscale level: genetic, cellular, synaptic, circuit, systems and behavioral.

Indeed information flow within and across cortical networks strongly depends on how excitatory (E) and inhibitory (I) synaptic transmissions are integrated by individual neurons

(E/I ratio) (Isaacson and Scanziani, 2011). This integration is the mechanism by which neurones transform information, and, while excitation creates a signal that can be recorded, inhibition can mean the absence of a signal; isolating inhibitory synaptic inputs *in vivo* has remained an elusive achievement. *Ex vivo* preparations therefore remain extremely useful to quantify and study inhibitory synapses, their properties and plasticity. These preparations allow the pharmacological isolation of inhibitory conductances, but also facilitate the targeting of interneurones that are often incredibly sparse. Meanwhile, It has become clearer that inhibitory interneurones, through their far-ranging diversity, play a fundamental role in sculpting virtually all forms of cortical activity (Cardin *et al*., 2009; Sohal *et al*., 2009; Atallah *et al*., 2012; Buzsaki & Wang, 2012; Lee *et al*., 2012; Wilson *et al*., 2012). In the visual cortex, a number of studies performing intracellular measurements have attempted to infer changes in inhibition in response to visually evoked stimuli (Borg-Graham *et al*., 1996; Cardin *et al*., 2010; Mariño *et al*., 2005; Priebe & Ferster, 2008; Haider & McCormick, 2009; Haider *et al*., 2013), supporting the hypothesis that inhibition has a role in the spatial organisation of simple receptive fields.

During this introduction, I will first give an rapid overview of the visual system and describe receptive fields in more detail. I will then justify using the mouse in visual sciences, despite the fact that they are not particularly visual animals. Subsequently the role of the cortex as an associative machine will be described, with a focus on visual cortices and the comparison between primary and secondary visual areas. The basic rules of cortical architecture will be defined, both for excitatory and inhibitory circuits, and I will then focus on the diversity of interneurons, and particularly on basket cells and their connection with network oscillations. Finally, I will give a quick overview of the cannabinoid system and outline the aims of this study.

II- Overview of the visual system

Before the cortex: the retina and the lateral geniculate nucleus (LGN)

The retina is the first place where light encounters the central nervous system. The conversion from photon to synaptic potentials is performed by rods and cones, two cell types of the retina. Rods are sensitive to very low levels of light, while cones respond to high levels of light and can sense colour, mediating most of vision. Both express different members of the opsin family of light-sensitive transmembrane proteins, which are stacked on top of each other in a series of membranous disks to maximise the detection of incoming photons. When opsins are activated, they generate a G-protein mediated secondary messenger cascade, opening sodium channels and depolarising the cell. This initiates the long chain of synapses that will distribute and modify information throughout the visual system (Ehinger, 1982). Processing of visual information also begins there: cones express photopsins which are sensitive to different wavelengths, transducing complex information about colour through interactions between different opsins and downstream signal modulation (Buchsbaum *et al.,* 1983; Dacey, 2003). While not the topic of this thesis, it is relevant to note that in recent years it has become apparent that much processing happens in the retina, which contains heavily interconnected and plastic networks (Roska & Meister, 2014).

Retinal ganglion cells relay retinal information to the dorsal lateral geniculate nucleus (dLGN) of the thalamus: their axons form the optic nerve. The LGN is the major relay to the neocortex, but also processes information in all mammals. For instance, in rodents the LGN contains orientation-selective cells that remain unaffected during cortical silencing, suggesting they are locally generated and not a result of cortical feedback (Scholl *et al.,* 2013). In the rhesus monkey, LGN cells encode both spatial and chromatic qualities of visual stimuli (Hubel & Wiesel, 1966). The main driving input to the LGN is the retina, but it also receives afferents from a plethora of brain regions, from the cerebellum (Graybiel, 1974), to the brain stem (Bobillier, 1976). The LGN initiates a cascade of thalamo-cortical loops by mostly impinging upon neurones of Layer IV of the primary visual area (V1). Sensory information is relayed to other cortical layers in a stereotyped sequence (to Layer 2/3, then deep Layer 5) before being sent out of V1. These loops allow information to travel across different cortical areas both *via* direct connections and *via* cortico-thalamic pathways

involving either different thalamic nuclei (such as the pulvinar) or, again, the LGN. It is important to note that the information that sensory cortices transmit is already heavily processed, coding for multiple sensory features and timing. This implies that the cortex does not need to perform all these computations, but it must be able to maintain the coherence of the information it receives.

Receptive fields and information hierarchy

In a seminal publication, Hubel and Wiesel (1959) described groups of neurones in the primary visual cortex (V1) of the cat that would modulate their firing to specific changes in orientation of grids, bringing the concept of the receptive field at the centre of visual neuroscience. A receptive field is the region in sensory space where a stimulus causes a modification in the firing of a single cell. Hubel and Wiesel (1963) described simple cells, which are so-called because their receptive fields possess distinct excitatory and inhibitory regions with specific characteristics. Shining a spot of light in the excitatory region of the visual field (e.g. the top half, as in Fig 4. G) increased firing, while shining light in the inhibitory region (e.g. the bottom half in Fig 4. G) decreased firing. These regions were shown to be antagonistic: no change in firing was observed if a diffuse spot was shown in

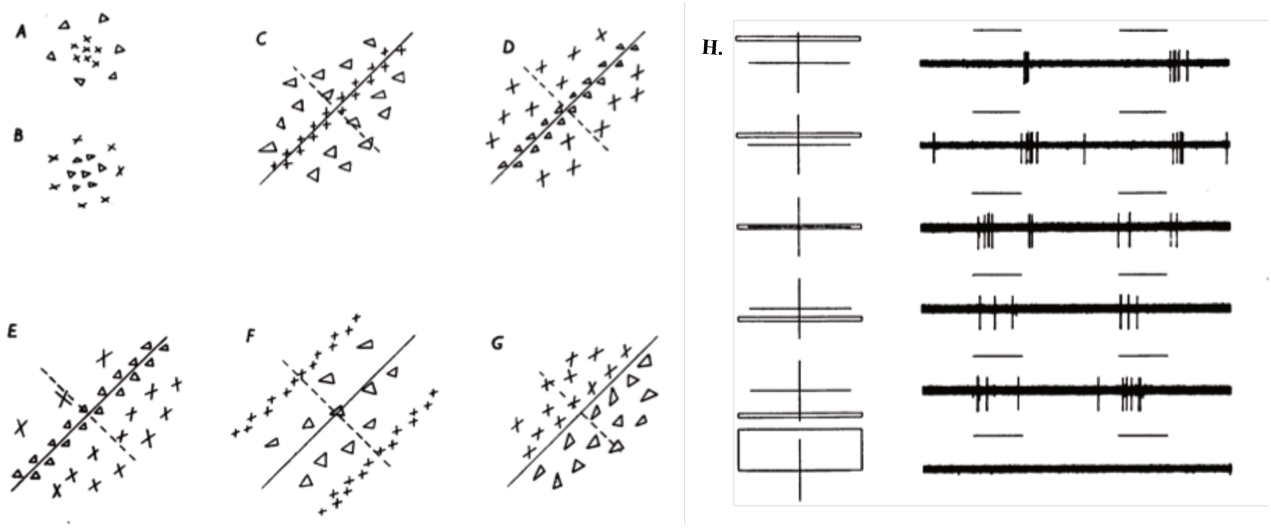

Figure 4. (A-G) Examples of simple cell receptive fields. Triangles represent inhibition of firing, and crosses excitation. (H) Example of a complex cell receptive field (stimulus to the left, response to the right). Adapted from Hubel & Wiesel (1962)

both excitatory and inhibitory regions. Increasing the size of the spot also increased the strength of the modulation in either region, giving evidence for summation. All these

characteristics mean that the behaviour of a simple cell can be predicted once a map of neuronal response from illuminating a spot across the visual field has been been established (e.g. Fig 4. A-G). In contrast, the firing of complex cells cannot be accurately predicted from such a map. Indeed, some cells didn't even respond to a stationary spot (making the map impossible), but responded to a luminous slit (e.g. Fig 4, H). Increasing the width of the slit to cover several excitatory regions did not summate the responses as expected from simple cells: on the contrary it inhibited the firing of the cell (Fig 4, H).

These findings led to the hypothesis that simple cells receive thalamic inputs while complex cells receive inputs from several simple cells as well as the thalamus, combining various features of the stimulus and only responding to precise combinations. Gilbert (1977) showed that layer IV contains more simple cells than superficial layers, consistent with LGN driven inputs onto layer IV, suggesting that local circuitry plays a role in the transformation from simple to complex receptive fields. An essential finding for all of neuroscience, this provides a concrete example of a mechanism by which networks can combine several features of a stimulus into a single signal. A direct implication is that there is a hierarchy in signal processing, with some features of the stimulus being passed on to other brain areas, and others being lost. As we go upstream, it is tempting to imagine single neurons encoding incredibly complex patterns of stimuli because they receive heavily processed inputs. However, it is dangerous to systematically imply causation from correlation - humorously illustrated by the finding there may be a Jennifer Aniston neurone (Quiroga *et al.,* 2005). It is nonetheless certain that neural networks use receptive fields to encode different characteristics of stimuli and combine them with very specific rules.

The rodent visual cortex

Mice have been used as a model organism in neuroscience research for decades. They breed well, are small in size, and are relatively easy to genetically manipulate. Because they are nocturnal animals, they were long considered to have a "simple" visual system. Undoubtedly, they generally rely on olfactory, tactile and auditory cues to guide their behaviour (Holy & Guo 2005; Jadhav & Feldman 2010; Stowers *et al*. 2013), and as far as humans are concerned mice would probably be considered legally blind (Baker, 2013). However, starved mice become diurnal and use dichromatic vision to navigate their environment (Jacobs *et al.,* 2004; Baden *et al*., 2013). Although their visual acuity is

considerably lower than that of higher mammals (0.5 cycles/degree, compared to about 6 cycles/degree in the cat and up to 80 cycles/degree in certain primate species (Gianfranceschi *et al*. 1999; Fig. 5), mice sometimes preferentially rely on visual information (Etienne *et al.,* 1996) and can be trained rapidly for visual tasks (Prusky & Douglas 2003). In fact, in studies using the Morris water maze (Morris, 1984), mice use visual cues to find their way. Such studies demonstrate that mice process multiple complex visual cues and associate them with relevant behaviour. Moreover, experiments on visual object recognition have shown that rats, and presumably mice, can use invariant shape information to identify landmarks from a variety of different viewing angles (Alemi-Neissi *et al*., 2013). Mice are able to distinguish features such as gratings (Hofer *et al*., 2011; Ko *et al*., 2011, Lee *et al.,* 2016) and experiments using increasingly complex cues are successfully being developed (Douglas *et al.,* 2005; Reinhold *et al.,* 2017). The anatomical organisation of the mouse visual system is far from simple, with the visual cortex receiving inputs from a multitude of areas (Wang & Burkhalter, 2007).

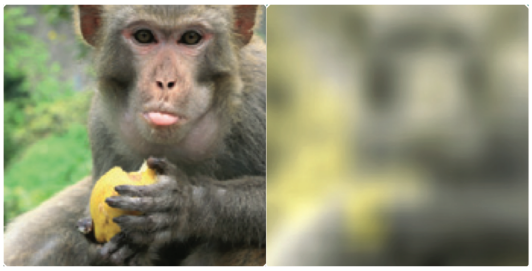

Figure 5. Simulated vision of a primate (left) and a mouse (right). Adapted from Baker (2013)

These computations are performed in interconnected cortical and subcortical networks, bringing up essential questions. What is the structure of these networks? How does the connectivity of neurones within a network reflect the computations that it performs? How do different areas communicate with each other in an orderly fashion? The ubiquity of mice in research has led to the development of a plethora of cutting-edge tools that can be used to answer these questions, making the mouse model particularly useful. For instance, significant progress was made using two-photon imaging of calcium transients in upper layer neurons of multiple areas in the mouse visual cortex, particularly V1 (Andermann *et al.,* 2011; Marshel *et al*., 2011; Roth *et al*., 2012). These data showed that tuning to high spatial frequencies in the visual cortex was actually more common than originally thought, with some regions displaying more selectivity for high temporal frequencies and the direction of motion. There is also evidence for the existence of highly connected neurons that form structured local subnetworks in the visual cortex of rodents (Song *et al*., 2005; Yoshimura & Callaway, 2005; Yoshimura *et al*., 2005). Similarly to cats and primates, some subnetworks respond to orientation selectivity (Hofer *et al*., 2011; Ko *et al*., 2011, Lee *et al.,* 2016). Also,

individual neurones can be tuned to correlations between spatial frequency and orientation (Ayzenshtat, 2016).

Over the last decades studying the mouse visual system gave promising insights on visual processing (Niell & Stryker, 2008, 2010; Liu *et al*., 2010; Huberman & Niell, 2011; Niell, 2011) cortical structure and function (Sohya *et al*., 2007; Cardin *et al*., 2009; Sohal *et al*., 2009; Marshel *et al*., 2011; Adesnik *et al*., 2012; Olsen *et al*., 2012), and visually-guided behavior (Dombeck *et al*., 2007; Andermann *et al*., 2010; Busse *et al*., 2011; Lee *et al*., 2012; Carandini & Churchland, 2013; Saleem *et al*., 2013).

The cortex as an associative machine

The responses of V1 neurons to visual stimuli are also modulated by contextual factors such as brain state (alertness, sleepiness; Stoelzel *et al.,* 2009; Sellers *et al.,* 2015), attention (Luck *et al.*, 1997; Hillyard *et al.,* 1998; McAdams & Reid, 2005; Thiele *et al.*, 2009; Harris & Thiele, 2011; Briggs *et al.,* 2013; Sanayei *et al.,* 2015), anticipation of reward (Stănişor *et al.*, 2013), etc. The overall spiking rate and feature selectivity of V1 neurons is greatly enhanced when the animal is running, suggesting that V1 in mice is both multi-sensory (e.g., receives direct motor inputs) and strongly modulated by brain state (e.g., alertness) (Niell & Stryker, 2010). In primates, spiking activity in V1 neurones is only modestly altered by cognitive influences like attention and reward, compared to the influence of attention on the spiking activity of extrastriate neurons in V4 and V5 (or middle temporal visual area, MT) (Hillyard *et al.*, 1998; Luck *et al*., 1997; McAdams & Maunsell, 1999, 2000). However, it is important to understand how cognitive factors influence the way in which visual information is encoded by early visual structures, as contextual modulation of sensory inputs is likely to be an evolutionarily conserved process.

Directly upstream of V1 is the secondary visual cortex V2, which is often called an associative cortex (Kennedy & Dehay, 1986; Felleman & Van Essen, 1991). The label “associative” can refer to the association of various features of stimuli, but also to the combinations of sensory modalities which are not processed independently. What we see is influenced by how we move, what we feel, and what we hear. A way to demonstrate this is by using illusions in which one sensory modality can influence the other. If you see an edited

video of someone saying ‘ga’ while mouthing ‘ba’, you would believe that person is saying ‘da’ (McGurk & MacDonald, 1976). This means that vision and hearing are combined to influence what we perceive. The cortex is ideally structured to allow these kinds of associations: it is heavily interconnected, both directly and *via* the thalamus. The Allen institute (e.g. Kuan *et al.,* 2015) and others (Zingg *et al.,* 2014) have published several connectivity maps of the mouse brain using tracer injections, quantifying the extent to which brain areas are wired together. But it is not sufficient to point out the ability of a brain area to associate information; after all, the complex receptive fields observed in V1 also require association, and the contextual factors influencing the firing mentioned above are also testimony to associativity. Still, some areas are better at combining information than others, and these associations must be plastic: if attention can modulate what we see it means synaptic connections in the network can change. We can distinguish the meanings of “by”, “buy” and “bye” because of the context in which they are used even if they sound exactly the same, again suggesting some processing of stimuli is non-deterministic. Primary areas combine features of specific modalities by processing information following a well-defined hierarchy (Yeo *et al.,* 2011): it is possible to predict stimulus-triggered patterns of connectivity. In contrast, “more-associative“ cortices are much less hierarchical, with connectivity patterns often switching boundaries in a widely distributed network. How can this association be performed at the cellular level?

Large L5 PNs are prominent computational units of the neocortex, projecting outside the neocortex and receiving a multitude of inputs. Their role as integrators is perhaps well illustrated by their long apical dendrites, which extend all the way to L1. These cells can therefore receive feedforward sensory information as well as feedback at the same time (Fig. 6). The feedback can contain information about context, meaning that L5 PNs can act as coincidence detectors: a spike initiated at the soma can lower the threshold for spike generation at apical dendrites, a mechanism known as backpropagation-activated Ca^{2+} (BAC) spike firing (Larkum *et al.,* 1999). This means that when there are coincident inputs at basal and apical dendrites the PN will start to fire in bursts. This greatly reduces the burden of complexity on networks compared to the ‘integrate and fire’ model, meaning that using their intrinsic properties, L5 PNs can process two streams of information separately and then combine them (Larkum, 2013; Fig. 6). In addition, it illustrates an elegant mechanisms by

which cell assemblies, representing different levels of information, can be bound together instantaneously.

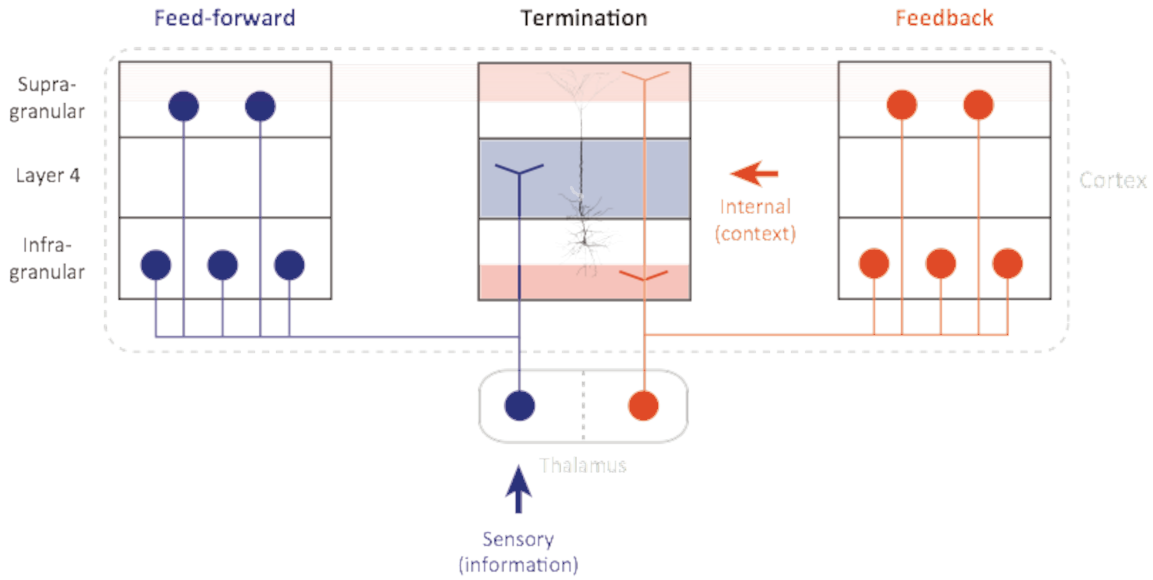

Figure 6. The feedforward stream driven by sensory organs (blue) and the feedback stream driven by other cortical areas and higher order thalamus (red) can be integrated by individual L5 PNs (from Larkum, 2013)

There is considerable evidence that the development of cortical circuit is not just encoded in genetic programs, but strongly influenced by network activity, resulting from the integration of external input with an internal model built on the memory of past events (Jabaudon, 2017). Rakic and colleagues (1991) provided the first evidence that modifying the thalamic inputs of a cortex could change its developmental fate: enucleation of the retina during mid-gestation caused a stark reduction in the number of cells in the LGN and transformed what should have been V1 into an abhorrent cortical structure, which the authors dubbed 'Area X'. This area shared more commonalities with V2, suggesting there may be adaptive mechanisms in play. Similarly, the onset of congenital blindness in humans has been found to impact the thickness (Li *et al.,* 2017) and functional connectivity (Wang *et al.,* 2015) of V1. In the congenital blind, 'V1' is involved in auditory and tactile motion stimuli processing (Sadato *et al.,* 2002; Poirier *et al.,* 2006; Klinge *et al.,* 2010). Higher cognitive tasks such as syntactic processing and episodic memory retrieval have been found to activates the 'V1' of the congenitally blind, which is not the case for regularly sighted individuals (Amedi *et al.*, 2003; Raz *et al.,* 2005; Lane *et al.,* 2015). All these data point towards activity-driven shaping of cortical circuits, though the mechanisms by which such shaping occurs remains poorly

characterised (Jabaudon, 2017). Furthermore, it points towards different activity patters emerging from sensory organs versus other cortical areas. What is the nature of such 'internal' signals?

Perception and predictive coding

During the early evolution of hominids, energy was a major factor constraining brain size (Leonard *et al.,* 2003). In fact, metabolic constraints are thought to cause a tradeoff between large bodies and large brains (Fonseca-Azevedo *et al.,* 2012). There was therefore considerable pressure for the brain to evolve to produce behaviours that are appropriate, fast, and energy efficient (Friston & Kiebel, 2009; Friston, 2010). Although we have the subjective impression of a richly detailed visual world, empirical evidence suggests that we actually see less than we think (Cohen *et al.,* 2016). For instance, change blindness occurs when objects are added in a picture or scene after a disruption (such as a flash, or a rapid concealment of the image) without being noticed by the onlooker (Simons & Levin, 1997; O'regan *et al.,* 1999). Similarly, attentional blindness occurs when someone is asked to pay particular attention to a particular feature and fails to see major events in a scene (Mack & Rock, 1998). If a group of people are asked to count the number of passes in a basketball game, they may fail to notice a man in a gorilla costume roaming the field (Simons & Chabris, 1999). Furthermore, the optic tract creates a blind spot on the retina that is 'filled in' by the brain using surrounding information, making it unnoticeable to us (Komatsu *et al.,* 2002 - test with Fig. 7). Then, there is cortical blindness whose sufferers cannot see but are still able to respond to visual stimuli (Goldenberg *et al.,* 1995). All these findings show that there is both less and more in perception than the eye meets.

Figure 7. Demonstration of the blind spot. Close your right eye and focus on the cross. As you move closer to the paper, the dot on the left will disappear and be replaced by the surrounding colour (white, in this case).

These findings have led to the theory of predictive coding: rather than processing every bit of information, what if the brain held an internal model of the external environment and

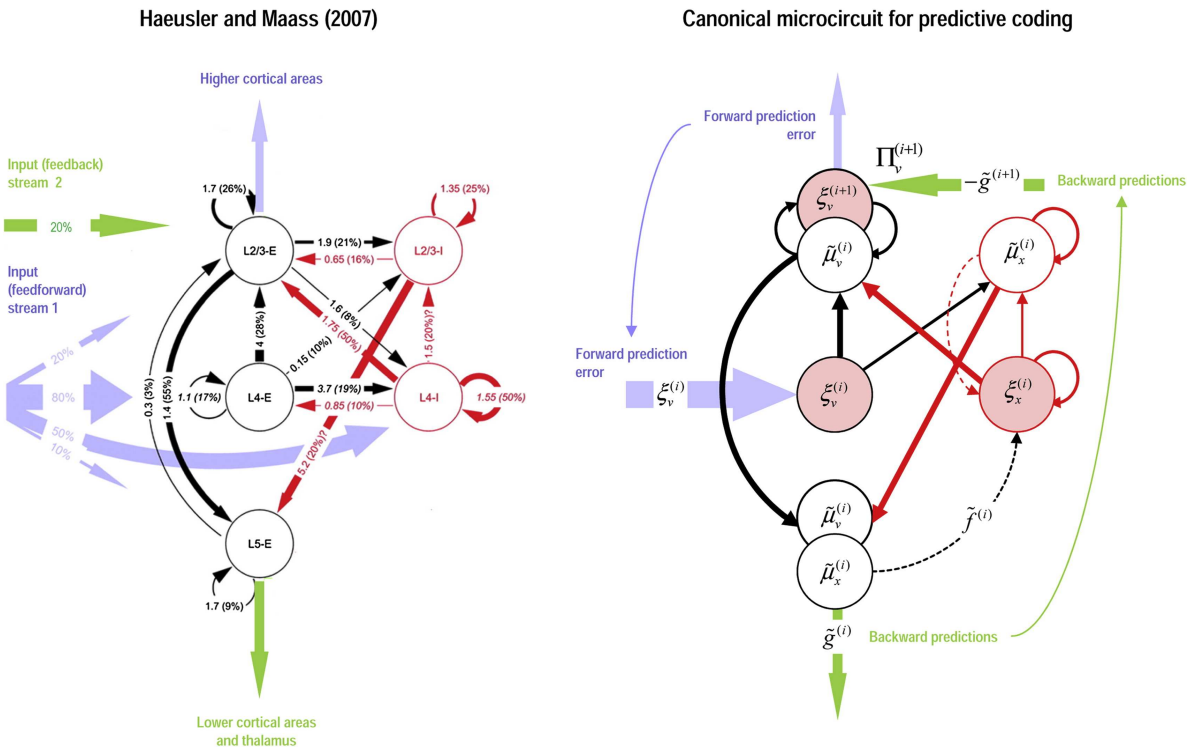

Figure 8. Striking similarities between observed cortical connectivity and an ideal predictive coding circuit. Left: the canonical excitatory microcircuit based on Haeusler and Maass (2007) with mean EPSP amplitude (in mV) and connection probabilities in parentheses. Right: the proposed cortical microcircuit for predictive coding. Prediction error populations are highlighted in pink, excitatory connections in black and inhibition in red. Connections that are not present in the microcircuit on the left are represented by dotted lines. Black circles represent PCs while red circles represent INs. From Bastos (2012)

constantly compared it with the inputs received by the senses using a form of Bayesian inference? Such a model would allow much faster responses to a changing environment as only the residual error would need to be computed, and can "flag" a relevant change in the environment. Following this change, the model can be updated as necessary. The theory that the brain generates models to make inferences about the sensorium has been around for over 150 years and has maintained traction ever since (Helmholtz, 1860; Gregory, 1968; Dayan *et al.,* 1995; Bastos, 2012). Interestingly, the requirements for computing predictive coding at the circuit level strikingly correspond to the connectivity of the canonical cortical microcircuit (Fig. 8**;** Bastos *et al.,* 2012). The differences in neuronal activity between cortical layers have become more apparent in recent years (de Kock *et al.,* 2007; Maier *et al.,* 2010). Superficial layers of the cortex tend to exhibit neuronal synchronisation and spike-field coherence in the gamma frequency, while the slower alpha and beta rhythms dominate in

the deeper layers (Buffalo *et al.,* 2010; Maier *et al.,* 2010). This implies that feedforward connections use higher frequencies than feedback connections (Bosman *et al.,* 2012), and that the canonical cortical circuit must not only synthesise spectrally distinct inputs, but also segregate its outputs (Bastos *et al.,* 2012). What is the cellular basis of cortical processing?

Rules of cortical architecture

The difficulty of finding a common blueprint throughout the cortex stems from the diversity of neuronal cell types and their respective connectivity. The brain is by far the organ with the largest amount of cell types, each expressing a panoply of different proteins (Sharma *et al.,* 2015); it is unsurprising that the component parts of neural networks reflect the variety and complexity of the behaviours they underly. In mammals, different sensory modalities such as vision, hearing, taste, smell and touch, are each represented in different cortical areas (O'Leary et al., 2007). The processing of all sensory information within cortical neuronal networks involves the integration of incoming information with past experiences and context. Cortical integration involves feedback and feedforward onto the same circuitry, regardless of the modality. All sensory cortices must detect, extract, and amplify features of stimuli in a chaotic sensory environment, although the precise character of these features will vary between senses. These common principles mean that there are generalisations that can be made across sensory cortices, and studying features of primary visual cortical microcircuits can also mean finding mechanisms that can be transposed to primary auditory or somatosensory cortex.

Early anatomists like Ramón y Cajal, and then physiologists dedicated much of their scientific lives to finding recurring modules in the cortex. Pioneers such as Lorente de Nó, Mountcastle (1955, 1957) and Powell (1959) found that when an electrode was perpendicularly inserted in the cortex of various mammals, radially organised groups of neurones had a tendency to have similar receptive fields, or at least responded to a single sensory modality. On the other hand, if the electrode penetrated the cortex at an angle, the likelihood to record from neurones sensitive to different modalities increased (Powell & Mountcastle, 1959). These groups of neurones extended radially from white matter to pia, so they were dubbed cortical columns. Theories started appearing that the cortex, which forms about 85% of our brain, evolved by multiplying a common blueprint and modifying it to suit a variety of functions, the so-called “swiss army knife model” (Barkow *et al.,* 1995). There is

some evidence for that: the cortex is often immediately recognisable, while evidently distinguishable from other laminated structures such as the hippocampus. Most cortical areas develop in a similar way, with pyramidal cells migrating from the sub-ventricular zone, usually forming six layers from pia to white matter. Similarly, major classes of inhibitory interneurons are represented throughout the cortex, but in different proportions (Whissel *et al.,* 2015), once again suggesting that the brain may have a 'toolkit' with diverse combinations to perform various tasks. It may therefore be more useful to think of cortical modules that are not solid structures, but as repeated, locally intertwined units, which form parts of a widely distributed network (Rockland, 2010).

The flow of information in cortical networks is also well conserved (Fig. 9; Douglas & Martin, 2004; Gong *et al.,* 2003; Harris & Mrsic-Flogel, 2013; Allene *et al.,* 2015). Cortical layers are defined according to the density of principal neurones (PNs) as well as their axonal projections. Layer (L) 4 receives dominant input from sensory relay (specific) nuclei of the thalamus, although to a lesser extent that is also the case for L5 and L6 (Constantinople & Bruno, 2013; Hooks *et al.,* 2013) and other cortical layers (Petreanu *et al.,* 2009; Smith *et al.,* 2012). Inputs from associative (non-specific) thalamic nuclei, on the other hand, mainly target L1 and L5b (Petreanu *et al.,* 2009). Similarly, contextual inputs from higher-order cortex avoid L4 and most densely target L1, L5 and L6 (Thomson & Lamy, 2007). Sensory

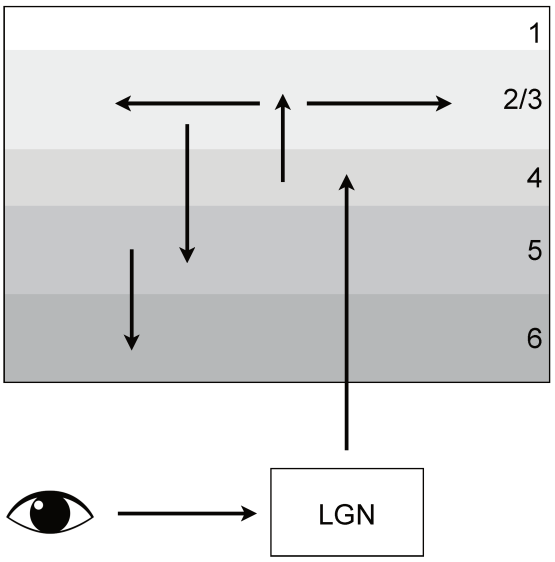

Figure 9. (extremely) Simplified stream of information from the eye to the cortex, and across the cortical layers. This pattern can be generalised to other sensory modalities such as hearing and touch. Black arrows represent excitatory connections.

processing is therefore mainly initiated in L4, the granular layer (Welker, 1976). Excitatory cells of L4 comprise pyramidal and spiny stellate cells, who have distinct morphologies but have largely similar intrinsic properties and coding strategies (Brecht & Sakmann, 2002). The architecture of L4 varies between species and modalities, which may reflect its developmental shaping by thalamic innervation and activity (Callaway & Borrell, 2011; Narboux-Nême *et al.,* 2012). Indeed, interactions between genetics and environment (including network activity) play an essential part in the development of microcortical circuits (Jabaudon, 2017). It is particularly interesting to note that reducing the size of LGN, for instance through enucleation of the retina during foetal development causes major changes in the size of L4 in primates (Rakic *et al.,* 1991). Also, rerouting auditory thalamus fibres to the visual cortex of the ferret transforms causes L4 neurones to adopt 'auditory' properties (Sur *et al.,* 1988; Pallas *et al.,* 1990).

From L4, information is then distributed throughout the cortex, albeit dominantly to layer 2/3 (Lefort *et al.,* 2009). Importantly, optogenetic stimulation of L2/3 does not significantly affect L4 cells, meaning the direction of information is almost unidirectional (Adesnik & Scanziani, 2010). While L2/3 excitatory cells are often considered homogeneous, their different subtypes are likely still to be fully characterised (Molyneaux *et al.,* 2009), with a variety of activity patterns being recorded *in vivo* (Chen *et al.,* 2013), and differential sensitivity of somatodendritic activation of cannabinoid receptor type 1 (CB1) coupled to specific dendritic arborisations (Marinelli *et al*., 2009). Following years of speculation, there is increasing evidence that pyramidal neurones in L2/3 coding for similar sensory features tend to be synaptically connected (Ko *et al.,* 2011; Lien & Scanziani, 2013; Wertz *et al.,* 2015). More specifically Lee and colleagues (2016) showed that pyramidal neurones responding to similar orientations tend to wire together, despite the fact that axons and dendrites of all orientation selectivities pass within 5 μm of each other with roughly equal probability. Such small distances mean that that the growth and pruning of dendritic spines can be the mechanism by which this connectivity can emerge. Excitatory neurones of L2/3 send long-range projections to contralateral cortices and local axons to L5, the main output layer of the cortex alongside L6 (Harris & Mrsic-Flogel, 2013). Intratelencephalic neurones (ITNs) tend to colonise upper L5, projecting upwards to L2/3 as well as the ipsi- and contralateral cortex, and striatum (Fig. 10). Subcerebral projection neurones (SPNs) are periodically expressed in L5 with a typical wavelength of 30 μm, and have extensive dendritic tufts in L1 (Fig. 10;

Maruoka *et al.,* 2011). In contrast to L4 cells, SPNs receive inputs from a diverse set of cortical cells and project very little axons locally (Kampa, 2006). SPNs can innervate sub-cerebral motor centres such as the basal ganglia and the ventral spinal cord, directly driving

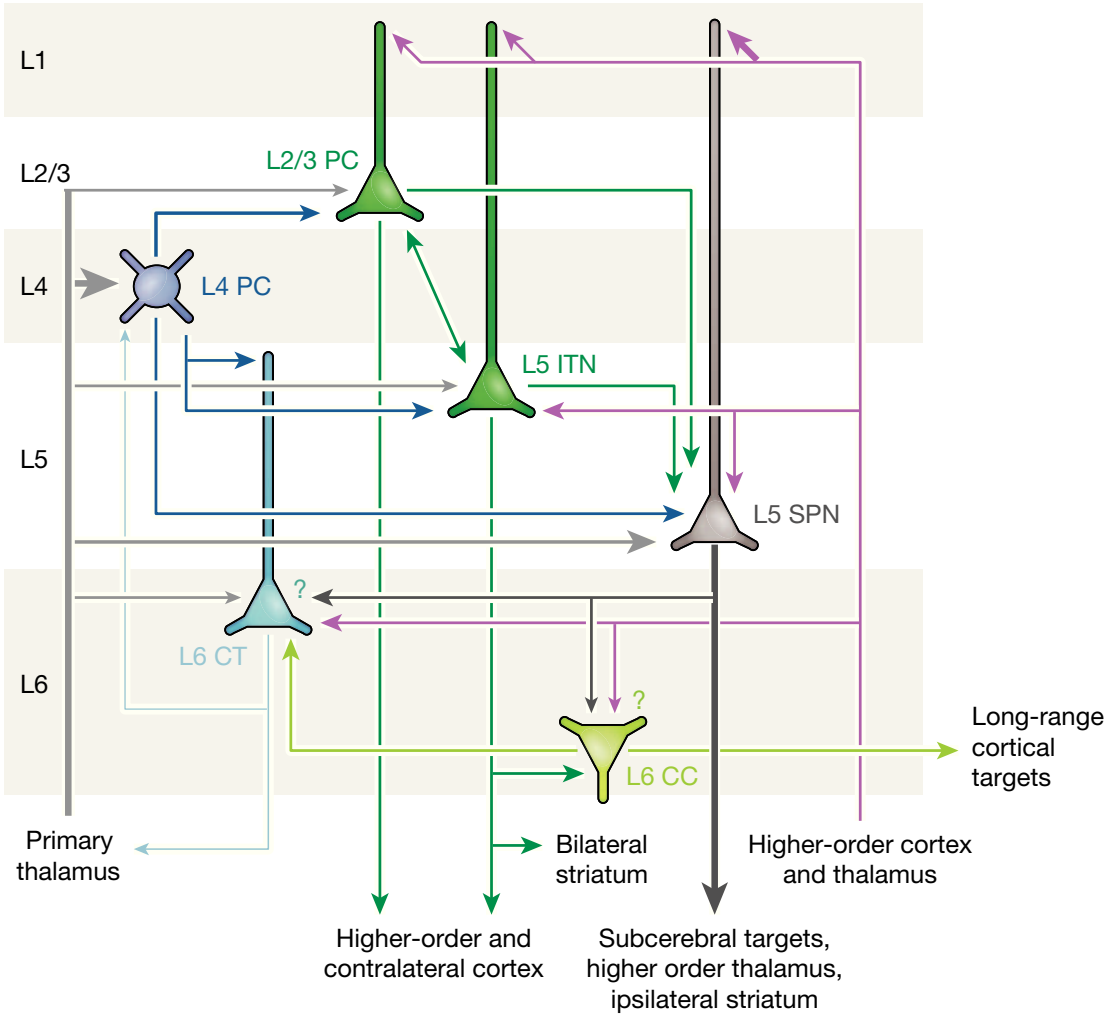

Figure 10. Canonical cortical excitatory connectivity. The strength of a pathway is represented by line thickness, with represents the strength of a pathway; question marks indicate connections that appear likely but have not yet been directly demonstrated. PC = principal cell, ITN = intratelencephalic neurones, SPN = Subcerebral projection neurones, CT = corticothalamic cells, CC = corticocortical cells. From Harris & Mrsic-Flogel (2013).

motor action (Matyas *et al.,* 2010). Alongside the projection of collaterals to higher-order thalamus and ipsilateral striatum (Kawaguchi, 2017), the connectivity pattern of SPNs points towards a neuronal class that integrates local information and distributes it to distant targets. In L6, at least two subclasses of PNs can be distinguished through both projection targets and molecular markers (Watakabe, 2012): corticothalamic (CT) cells project weakly to the

reticular and primary sensory thalamic nuclei while corticocoritcal (CC) cells have long-range small dendritic trees and horizontal axons directed to other cortical areas (Fig. 10). CT cells also strongly target interneurons in L4, consistent with the cortical silencing resulting from optogenetic activation of L6, and suggesting that layer may play a role in gain control of sensory processing (Olsen *et al.,* 2012).

The constant, bidirectional communication between different areas means that the brain can take advantage of the staggering diversity of neuronal cell types to make function-specific circuits. Rather than a morphologically defined area being useful for a single task (e.g. the cerebellum is for movement), a picture has emerged in which the processing advantages of different brain regions can be harnessed for different tasks. For instance, the cerebellum has an ideal structure for feedforward processing (Pisotta & Molinari, 2014; Mosconi *et al.,* 2015). This is both useful for executing a complex sequence of movement, as well as for decision-making, which also involves complex, probabilistic sequences (Blackwood *et al.,* 2004; Eccles, 2013). Using a common blueprint of rules, loosely coupled modules can be harnessed in parallel to perform different tasks at the same time, and to do so the cortex needs to be particularly efficient at associating information coherently.

III- Inhibitory circuits

The diversity of cortical interneurones

Inhibitory cells are fundamental elements of the cortical circuit and are believed to sculpt virtually all forms of cortical activity (Isaacson & Scanziani, 2011). Ramon y Cajal is thought to have said that the "functional superiority of the human brain is intimately bound up with the prodigious abundance and the unusual wealth of forms of the so-called neurones with short axons", noting that brains which had recently evolved contained more of such cells (DeFelipe *et al*., 2013). We now know that these cells are GABAergic interneurones (INs). INs exhibit a variety of somatic, dendritic and axonal morphologies (Markram *et al.,* 2004; *Jiang et al.,* 2015). Alongside the expression of a diverse set of ion channels and transporters, these features influence passive and active properties to form a wide array of heterogeneous firing signatures (Kawaguchi *et al.,* 1993; Cauli *et al.,* 1997; Gupta *et al.,* 2000; Kawaguchi & Kondo, 2002; Klausberger and Somogyi, 2008). In addition, intracellular signalling cascades and receptor expression means that INs respond differently to neuromodulators such as serotonin or endocannabinoids (eCBs), which are known to profoundly alter the manner in which information is computed by cortical networks (Morales & Bäckman, 2002; Aznar *et al.,* 2003). In recent decades it has become increasingly clear that interneurons heavily influence the integration of information (DeFelipe *et al.*, 2013; Kepecs & Fishell, 2014), and disturbing the balance between excitation and inhibition can lead to pathologies such as epilepsy or psychiatric diseases (Yizhar *et al.,* 2011; Marín, 2012; Mathalon & Sohal, 2015; Soltész, 2015). This diversity has led a drive to classify INs, and the method to do so has initiated a discussion that remains ongoing. The Petilla interneurone nomenclature group (PING) built a platform for the discussion, basing their system on three criteria: morphological, physiological and molecular (PING, 2008; DeFelipe *et al.,* 2013). The purpose of the nomenclature is to use the same adjectives for the different characteristics of interneurons (e.g. 'ascending' when a neurite extends from deep to superficial layers of the cortex), rather than attempting to categorise discrete, functionally relevant sub-types.

Since the Petilla meeting, the expression of certain molecular markers has emerged as a good starting point for further classification to identify the major classes of interneurones: the neuropeptides somatostatin (SST) and vasoactive intestinal peptide (VIP), the calcium-

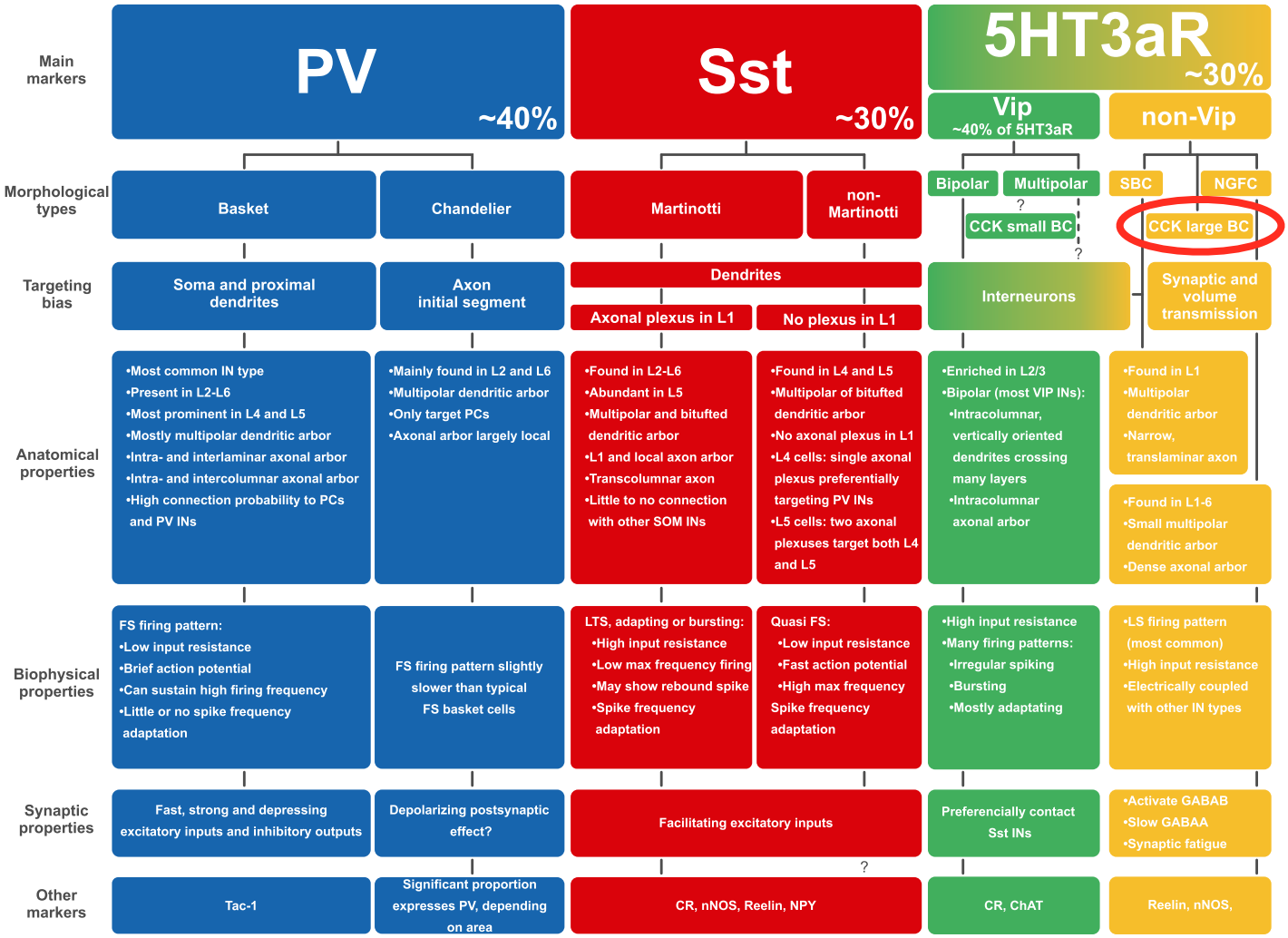

Figure 11. Classification and diversity of neocortical GABAergic interneurones. The IN under scrutiny in this thesis is circled in red (from Tremblay *et al.,* 2016).

binding protein parvalbumin (PV) and the signalling protein reelin (RELN) but not SST (Wamsley & Fishell, 2017). The non-SST/RELN expressing INs also typically express the serotonin receptor 5HT3aR, but not VIP, which have led to alternative classifications (Fig. 11; Tremblay *et al.,* 2016). By themselves or in combination these markers account for nearly all neocortical INs, although that should not imply that small number of remaining INs do not play an essential function. Also, many other markers have been used over the years, including (but certainly not limited to) calretinin, calbindin, neuropeptide Y, and - of particular interest to this thesis - cholecystokinin (CCK), a sub-type of RELN+ INs which often express the cannabinoid receptor type 1. Evidently, gene expression and the properties of a cell are not independent: for instance specific sub-types of voltage-gated ion channels determine the properties of an action potential (Hu *et al.*, 2014). It should be noted that the main classes of

INs described above have functional properties that are either prominent or exclusive to their class (Zeisel *et al.,* 2015; Tasic *et al.,* 2016). Some groups have concentrated on bridging the gap between the properties of a cell and gene expression, using a combination of whole-cell patch-clamp recordings and single-cell transcriptomics, a method known as “patch-seq” (Cauli *et al.,* 1997; Cadwell *et al.,* 2015).

Around 70% of cortical interneurones express either PV or SST, and as a consequence their contribution in cortical circuits and physiological properties are the most well-characterised (Wamsley & Fishell, 2017). The fast-spiking PV class contains basket cells, which form inhibitory synapses around the soma of other cells, and axo-axonic (or chandelier) cells that target the axon initial initial segment (Tanaguchi *et al.*, 2012). SST INs all target dendrites: Martinotti cells have ramifying axons in L1, forming synapses with the distal dendrites of PNs in L1 (Kawaguchi & Kubota, 1997), while the ‘non-Martinotti’ preferentially innervate L4 and L2/3 (Ma *et al.,* 2006; Xu *et al.,* 2010). There are however almost always exceptions to any rule surrounding INs: dendrite-targeting PV+ INs have also been reported in the mouse visual cortex (Buchanan et al., 2012). Similarly, SST+ INs have been observed to form axo-axonic synapses in the rat and monkey visual cortex (Gonchar *et al.,* 2002).

As a general rule, there is therefore a division of labor between dendritic and perisomatic inhibition (Fig. 12) . Perisomatic inhibitory synapses have been suggested to be strategically situated to control the timing and frequency of action potential generation, particularly during rhythmic activity (Cobb et al. 1995; Miles et al. 1996; Pouille & Scanziani, 2001; Klausberger & Somogyi, 2008; Royer *et al.,* 2012). On the other hand, dendritic inhibition could modify dendritic electrogenesis and integration (Miles *et al.*, 1996). Dendritic inhibition can also have major impacts on the output of a cell by efficiently suppressing non-linear dendritic processes such as calcium spikes, plateau potentials and NMDA spikes (Palmer, 2014). Indeed, a single IN can effectively inhibit the Ca^{2+} spike of a PN, preventing the bursting behaviour of layer 5 PNs (Larkum, 1999). Ca^{2+} spikes are required for some forms of synaptic plasticity and can therefore have profound impacts on circuit function (Kampa *et al.,* 2006; Golding *et al.,* 2002; Bar-Ilan *et al.*, 2013). It has been put forward that proximal inhibition causes divisive changes in gain while distal inhibition is subtractive (Koch *et al.,*

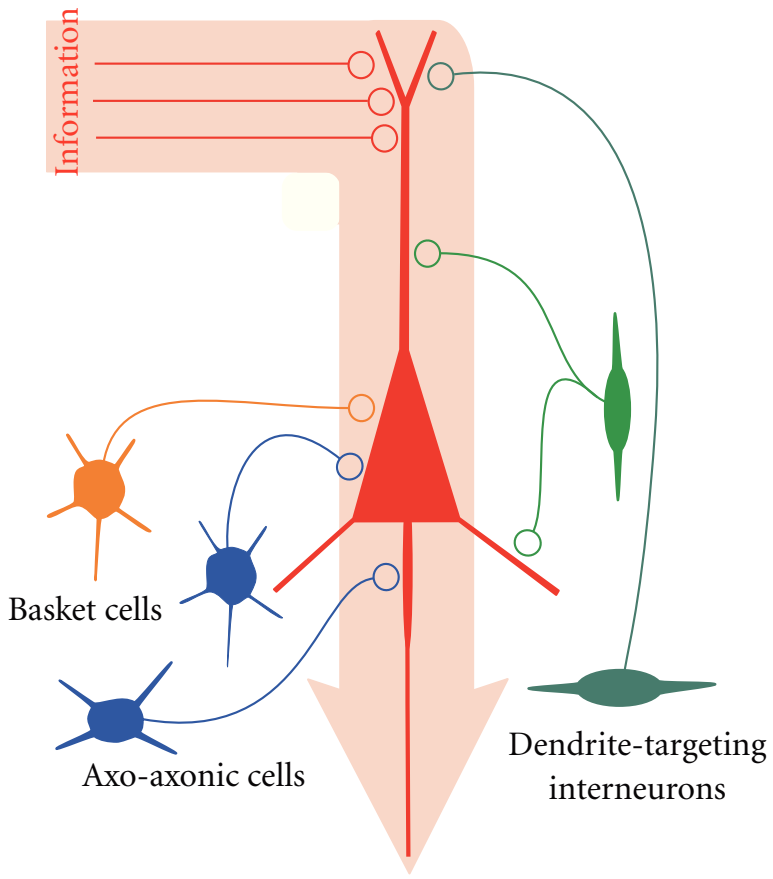

Figure 12. Different axonal targets of interneurones leading to a division of labor (from Méndez & Bacci, 2011)

1983; Atallah *et al.,* 2012; Pouille *et al.,* 2013). In fact, SST+ and PV+ INs have been found to perform both divisive and subtractive inhibition (Wilson *et al.,* 2012; Lee *et al.,* 2012; Atallah *et al.,* 2012; El-Boustani & Sur, 2014). Divisive inhibition is typically described as a form of gain control useful for scaling the response of neurones while maintaining their selectivity; attention can modulate motion processing in macaques through changes in gain, for instance (Treue & Trujillo, 1999). In contrast, subtractive inhibition is believed to sharpen the tuning of other cells (Ben-Yishai *et al.,* 1995). However, this view that proximal and distal inhibition are specialised for division and subtraction has also been disputed because the effects of inhibition depend on a plethora of factors, from dendritic morphology to the timing and site of inhibitory and excitatory synapses (Mitchell & Silver, 2003; Gidon & Segev, 2012).

Indeed, recent advances have allowed the precise control of SST or PV and have produced contradicting results regarding the specific roles of these cells during visual cortical

processing (Atallah *et al*., 2012; Lee *et al*., 2012; Wilson *et al*., 2012; El-Boustani & Sur, 2014). Activation of SST INs has been found to sharpen visual orientation tuning (Wilson *et al*., 2012), while others found the activation had no effect on tuning (Lee *et al.,* 2012). Similarly, PV IN activation has been found to cause changes in gain, having a minor effect on orientation tuning (Atallah et al., 2012; Wilson et al., 2012; El-Boustani & Sur, 2014), contradicting the sharpening observed by Lee *et al.* (2012). These inconsistencies are thought to largely stem from the differences in stimulation protocols used in these studies (Lee *et al.,* 2014), and provide good evidence that INs can perform subtractive or divisive inhibition depending on circuit dynamics rather than fixed properties. Furthermore, stimulating PV INs was shown to decrease the reliability of PNs during the processing of ambiguous visual stimuli, while activating SST INs increased reliability (Rikhye *et al.,* 2017). This effect was shown to be mediated by direct synaptic connections from SST onto PV INs (Rikhye *et al.,* 2017). Understanding the connectivity patterns of different IN classes is therefore of utmost importance to understand their functions in cortical circuits.

Cortical inhibitory connectivity rules

In recent decades, great progress has been made in elucidating the connectivity rules of INs, which have often been described as applying a vast “blanket of inhibition” locally, with axons contacting neighbouring excitatory cells with a high probability and contrasting with the specificity in connectivity pattern of principal cells (Fino & Yuste, 2011; Karnani *et al.,* 2014). This promiscuous connectivity with neighbouring PNs has been described both for SST (Fino & Yuste, 2011) and PV (Packer & Yuste, 2011). In the rat cortex it has been estimated that a PV BCs can form contacts with thousands of neurones (Karube *et al.,* 2004). Such omnipresent inhibition even raised the question of how excitatory activity can propagate at all (Karnani *et al.,* 2016). Furthermore, data have shown that non-pyramidal cells in the primary visual cortex do not show orientation tuning to the same extent as their PC counterparts (Sohya *et al.,* 2007; Kerlin *et al.,* 2010). Would it be accurate to describe this blanket of inhibition as simply preventing runaway excitation? Mounting evidence suggests that this is not the case.

In fact, there are patterns in both IN-PC and IN-IN connectivity. For instance, VIP INs have been implicated in forming transient ‘holes’ in the blanket by prefrentially inhibiting SST cells

(Pfeffer et al., 2013; Karnani *et al*., 2016), which could hypothetically lead to the apparition or extinction of cell assemblies during cognitive processes. In turn, it has been found that optogenetically inhibiting SST INs increased activity in L2/3, while decreasing activity in L4 (Xu *et al.,* 2013). These data suggest that SST cells have layer- and cell-specific connectivity: they preferentially innervate PNs in L2/3 and PV+ cells in L4 while avoiding other SST INs (Pfeffer et al., 2013). On the contrary, although PV BCs innervate hundreds of PNs, they have an affinity towards themselves, both *via* synapses and autapses, especially when the strength of connectivity is taken into account (Bacci *et al.,* 2003; Manseau *et al.,* 2010; Deleuze *et al.,* 2014). PV INs have also been found to inhibit type A L5 PCs that project sub-cortically more strongly than thin-tufted, callosally projecting PCs, while no such preference was found for SST INs (Lee *et al.,* 2014). However, another study found that Martinotti cells (a subtype of SST cells) also have a preference towards type A PCs (Hilscher *et al.,* 2017). On a sub-cellular scale, here is also evidence that SST INs can target specific dendritic spines, pointing towards very fine control of dendritic electrical and biochemical signalling (Chiu *et al*., 2013). Alongside the targets of INs, much work has also been put in studying their recruitment. VIP INs in V1 are mostly recruited by fibres coming from the cingulate cortex (Zhang *et al.,* 2014), while projections from primary motor cortex target those in S1 (Gentet, 2010; Lee *et al.,* 2013). This implies that higher-order cortico-cortical inputs can help modulate the output of primary cortical areas via VIP IN-mediated inhibition of SST cells.

Such studies have also led to the description of a canonical inhibitory connectivity pattern between three major types of INs in the primary visual cortex: (i) PV strongly inhibit one another yet show little contact with other INs (ii) SST avoid inhibiting one another but provide strong inhibition onto other INs and (iii) VIP cells preferentially inhibit SST cells (Pfeffer *et al.,* 2013). Interestingly this pattern seems to be repeated across different layers of the cortex, leading to the interesting conclusion that both the input and output of the visual cortex are regulated by similar inhibitory circuits (Pfeffer *et al.,* 2013).

Recurrent inhibitory circuit motifs

More generally, there are three recurrent circuit motifs that likely involve all types of INs (Fig. 10). Firstly, there is feedforward inhibition (FFI), which occurs when long-range excitatory connections simultaneously innervates a PN and an IN, and that the IN in turns innervates the PN. FFI is well illustrated by the recruitment of PV+ cells by sensory thalamic afferents in L4. Although many studies have been performed on the primary somatosensory cortex (S1) of rodents (e.g. Simons & Carvell, 1989; Gabernet *et al*., 2005), the motif appears to be reiterated in other areas such as V1 (Kloc & Maffei, 2014) and primary auditory cortex (A1) (Schiff & Reyes, 2012), as well as other species (Toyama *et* al., 1974). L4 thalamic inputs have been shown be both faster and stronger than those onto neighbouring PCs (Cruikshank *et al*., 2007; Gabernet *et al*., 2005; Inoue & Imoto, 2006). This efficient recruitment of PV+ INs leads to strong perisomatic inhibition, effectively suppressing PC output. However there is a monosynaptic delay in this inhibition, meaning the PC has a limited 'window of opportunity' to integrate and divulge sensory inputs (Pinto *et al.*, 2000). FFI has also been involved in the formation of receptive fields (Alonso & Swadlow, 2005), and as a mediator of coincidence detection by favouring synchronised over asynchronous thalamic inputs (Pinto *et al.*, 2003; Wilent & Contreras, 2005). The implications of PV-mediated FFI in cortical computing will be further discussed in a subsequent section.

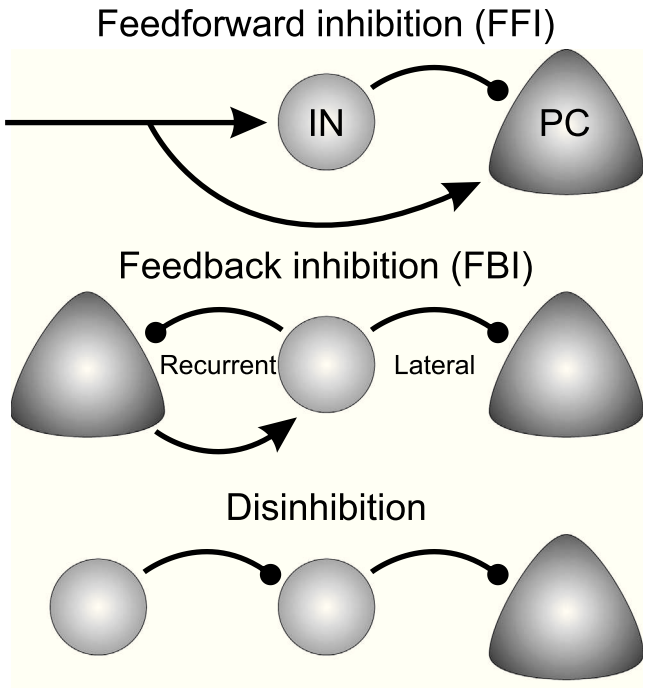

Figure 13. Archetypical circuit motifs involving INs. Arrows represent excitation while dots represent inhibition. From Tremblay *et al.,* 2016

Secondly, feedback inhibition (FBI) occurs when the source of excitation is local, with either recurrent inhibition onto the PN that provided the excitation, or so-called lateral inhibition onto PNs that did not participate in the recruitment of the IN. While FFI tracks the inputs onto a network, FBI depends on local circuitry, thereby tracking the outputs of the network. As mentioned earlier, FBI mediated lateral suppression has been implicated in cognitive processes such as size coding in the visual cortex (Adesnik *et al.*, 2012; Adesnik, 2017). Furthermore, FBI has been put forward as a mechanism by which cell assemblies can be selected and compete with each other (Roux & Buzsáki, 2015). Local connectivity patterns and dynamics can have interesting functional consequences: SST+ cells are recruited initially less efficiently by local excitatory inputs than PV+ INs, which means that initially FBI will be PV-mediated. When local circuitry produces high frequency input, however, PV+ IN recruitment weakens while SST IN strengthens (Pouille & Scanziani, 2004; Silberberg & Markram, 2007), giving rise to a frequency-dependent selection of the source of FBI. Consistent with this, several groups have found delayed inhibition exclusive to high frequency stimulation (Berger *et al*., 2010; Kapfer *et al*., 2007; Silberberg and Markram, 2007). This means that SST+ cells can act as burst detectors (Gentet *et al.,* 2012). In the case of size coding in the visual cortex, increased stimulus size increases inputs, recruiting SST cells that will then prevent activity in nearby circuits (Adesnik, 2012).

Lastly, there is disinhibition, which depending on the specific target can have opposite net effects in the circuit. I have mentioned that there are patterns in the way INs target each other (Freund *et al.,* 1983; Somogyi *et al.,* 1983; Gupta *et al.,* 2000). However, this does not necessarily imply disinhibition; the strength of connectivity is also relevant, and simply seeing IN-IN connections does not necessarily imply disinhibition. For instance, PV+ INs strongly innervate PCs, and it is not clear whether there is any situation in which PV+ INs perform disinhibition themselves (Tremblay *et al.,* 2016). Again, VIP cells are specialised in disinhibiting PCs from SST+ IN inputs in L2/3 of a variety of cortices (Lee *et al*., 2013; Pfeffer *et al*., 2013; Pfeffer, 2014). This indicates that some cell classes might have the tendency to performing certain circuit motifs. However, a single cell can also be part of several circuit motifs: for instance, SST+ INs can participate in both FBI and disinhibition. Still, network effects make it difficult to assess the computational effect of these mono- and disynaptic circuits, and it is therefore crucial to have quantitative information on inhibitory synapses.

PV and CCK basket cells exhibit radically different properties

Indeed, to better understand the function of INs in neuronal circuits, it is necessary to complement connectivity patterns and circuit motifs with physiological data. For the purpose of this thesis, I will focus on the physiology of BCs, and specifically the CCK+ INs. While the PV marker is non-overlapping with other neurones, clearly defining a major class of IN, the

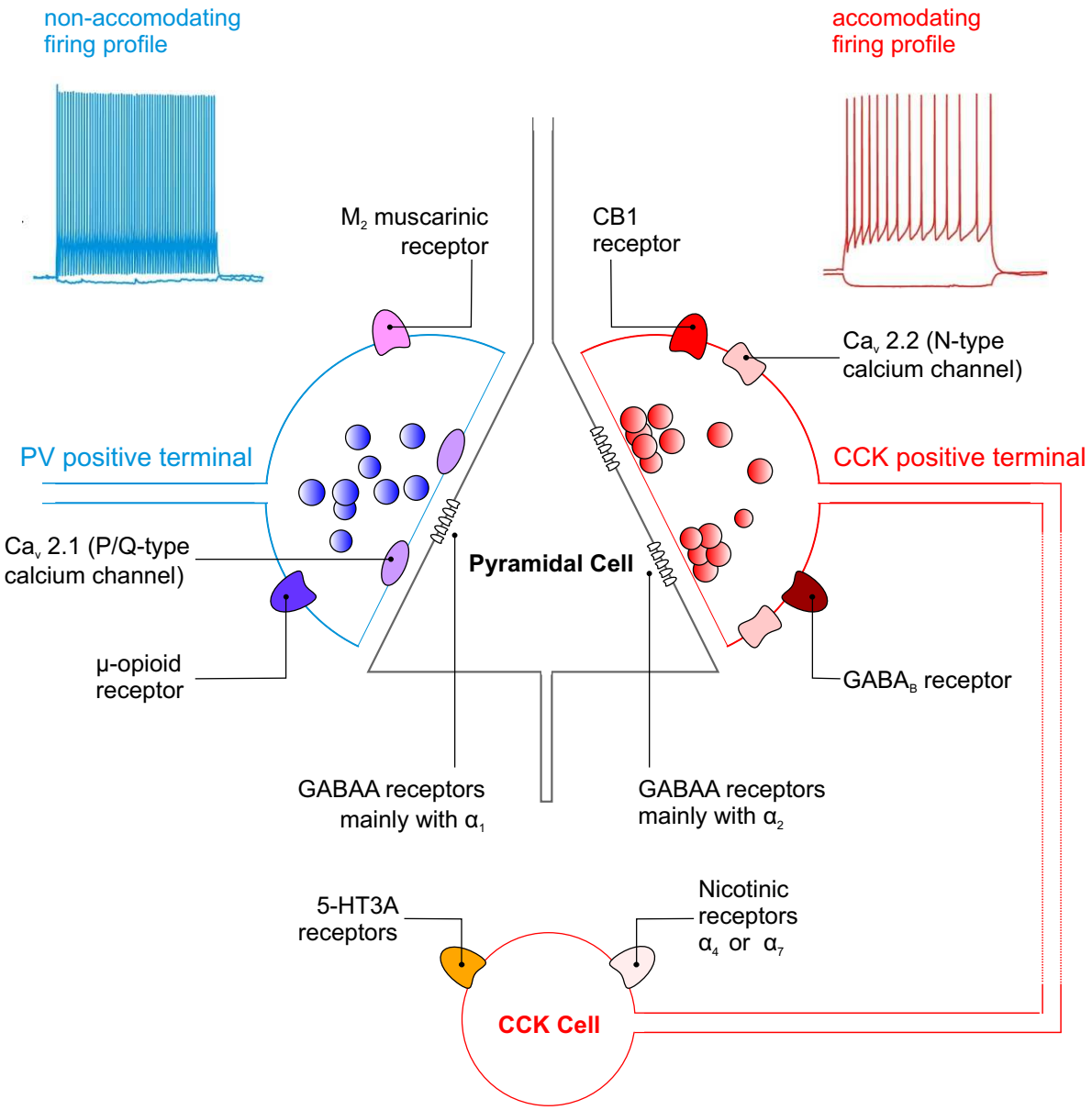

Figure 13. Schematic of two major types of basket cells. Although both PV+ and CCK+ BCs target the soma of other cells, they express distinct receptors and ion channels resulting in non-overlapping properties. Adapted from Freund and Katona (2007)

CCK neuropeptide is present in many different cell types, including excitatory neurones (Gallopin *et al.,* 2005). In INs, CCK can be co-expressed with or without VIP, with the two classes having specific properties. Approximately 10-30% of VIP INs co-express CCK, and likely correspond to the so-called “small basket cell” class, referring to the size of their soma and their limited dendritic and axonal arborisations (Freund *et al*., 1986; Kawaguchi & Kubota, 1996; Kubota, 2014; Wang *et al*., 2002). Largely found in L2, they likely exhibit a distinct connectivity pattern as compared to the SST-targeting VIP cell majority, which tend to co-express calretinin (Caputi *et al*., 2008; Jiang *et al*., 2015). Although it is certain that these VIP/CCK+ INs target the soma of both PCs and other INs (Dávid *et al*., 2007; Freund *et al*., 1986; Peters, 1990; Kawaguchi & Kubota, 1996; Staiger *et al*., 2004 Hioki *et al.*, 2013), it is less clear whether they are the only source of these VIP+ boutons. In contrast, non-VIP CCK+ INs tend to have a larger soma and more extensive dendritic and axonal arborisations (hence the “large basket cell” label) (Kubota & Kawaguchi, 1997; Kawaguchi & Kubota, 1998; Wang *et al*., 2002; Galarreta *et al*., 2004; Karube *et al*., 2004; Kubota, 2014). Non-VIP CCK BCs in the hippocampus and isocortex have been shown to express the vesicular glutamate transporter vglut3, meaning that they can also release glutamate, albeit at very low concentration because of enzymatic degradation (Somogyi *et al.,* 2004).

Both CCK+ and PV+ BCs project axons onto the soma of excitatory and inhibitory cells (Fig. 13; Hestrin & Armstrong, 1996 ; Kawaguchi & Kubota, 1998; Karube et al., 2004), although in the hippocampus some CCK INs target dendrites (Cope et al., 2002; Klausberger, 2009). There is also evidence for CCK having homotypic electrical connections (Blatow et al., 2003; Hestrin & Galarreta, 2004). Despite these similarities, CCK+ and PV+ INs also have radically different properties, suggesting distinct, complementary roles in microcircuits (Freund, 2003; Klausberger *et al.,* 2005; Freund & Katona, 2007; Armstrong & Soltész, 2012). Unlike PV BCs, CCK+ BCs cannot sustain high frequency firing and have accommodating spike discharge patterns as well as receiving multiple sub-cortical inputs (Freund, 2003; Freund & Katona, 2007). These differences mean that while PV BCs are easily recruited and can sustain their inhibition during high frequency patterns of activity, CCK BCs are better at integrating streams of information coming from different sources (Glickfeld & Scanziani, 2006). Furthermore, unlike the clock-like PV+ cells, CCK+ INs are not reliable; on the contrary, they tend to exhibit asynchronous synaptic release and high failure rates (Hefft & Jonas, 2005; Daw *et al.,* 2009). Asynchronous release is characterised by synaptic events

which are not tightly coupled to the presynaptic arrival of an action potential (e.g. Fig. 14), resulting in a long-lasting inhibition of the post-synaptic cell which can last for hundreds of milliseconds. Particularly, it tends to occur when the cell is stimulated at frequencies ranging from 50 Hz to 100 Hz, with more action potentials causing more asynchronous release (Hefft & Jonas, 2005; Daw et al., 2009). Interestingly, stimulations at rates known to occur *in vivo* has been shown to produce large asynchronous IPSCs independently of the target cell (Daw *et al.,* 2009). The underlying mechanism is though to involve the loose coupling of N-type calcium channels with different calcium sensors and modulation distinct from that of synchronous events (Soltész, 2011). The vesicle-fusion modulation protein Synapsin II has been shown to play contradicting roles in CCK vs PV+ IN synapses: deletion of Synapsin II removed the asynchronous component of CCK+ inputs and the synchronous component of PV+ inputs (Feliciano *et al.,* 2017). In PV+ INs, high frequency stimulation has been shown to trigger asychronous release from autapses and synapses (Manseau *et al.,* 2010) and artificially modulating pre-synaptic calcium concentrations suggested that the synchronous

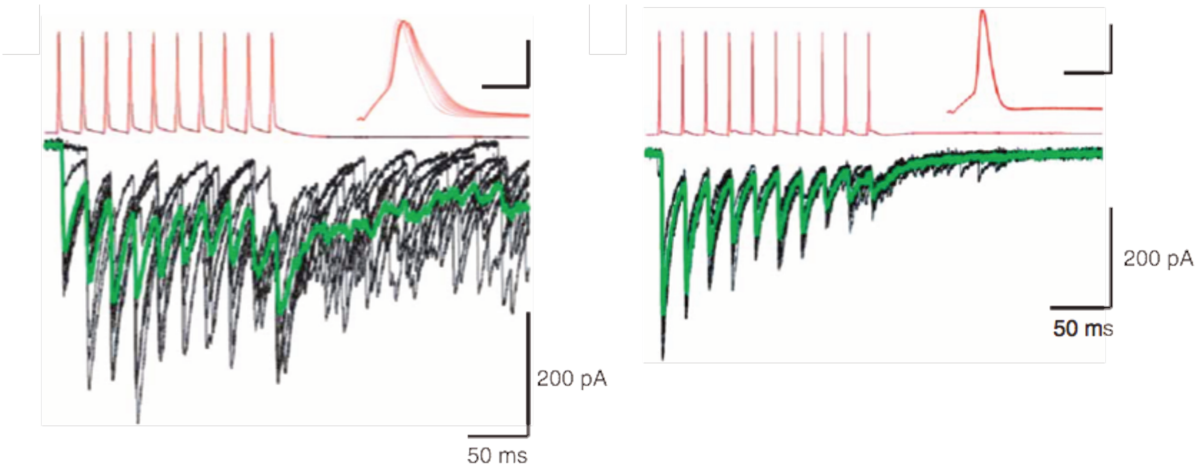

Figure 14. (Left) Jittery, slow action potential (AP)(red trace) and asynchronous release from CCK INs and (Right) Precise, rapid AP and synchronous release from PV INs. Presynaptic spikes in red, individual trials in black and average in green. Individual AP scale bar: 2 ms and 50 mV From Hefft & Jonas (2005)

and asynchronous component rely on distinct calcium sensors. This means that the output of the cell can be modulated in a complex manner, and again, this is likely to have functional significance: in the case of PV+ INs asynchronous release significant reduces the firing reliability of the cell itself, which then also affects the overall spiking precision and reliability of pyramidal cells (Manseau *et al.,* 2010). In CCK+ INs it has been suggested that the release of the CCK peptide requires asynchronous release (Verhage *et al.*, 1991).

Neuromodulators can influence presynaptic calcium concentrations, meaning the output of the cell can be influenced by a vast array of factors, from mood to specific activity patterns.

CCK INs as targets for neuromodulators

Indeed, large BCs are also a particularly interesting class because they post-synaptically express both the serotonin receptor sub-unit 5HT3a (Morales & Bäckman, 2002), the α7 and α4 sub-unit of the nicotinic receptor (Porter *et al.,* 1999), as well as CB1 presynaptically (Fig. 13; Katona *et al.,* 1999; Marsicano & Lutz, 1999). This means large BCs are the target of numerous neuromodulators and have therefore been seen as good candidates to transmit information about the autonomic state or mood of an animal. Although the highly plastic and unreliable CCK+ INs are often contrasted to the clock-like, rigid PV BCs (Freund, 2003; Freund & Katona, 2007), it is nonetheless caricatural to see PV+ BCs as monotonous metronomes: they have been shown to exhibit plasticity both structurally (Hensch, 2005) and synaptically (Nissen *et al.,* 2010; Lourenço *et al.,* 2014), though directly correlating synaptic plasticity with behaviourally-relevant processes *in vivo* has remained technically difficult. Previous work from our lab has shown PV and CCK BCs are sensitive to cell-autonomous forms of plasticity that involve the retrograde messengers nitrous oxide and endocannabinoids, which are released when PCs undergo repeated high frequency firing (Marinelli *et al.,* 2009; Lourenço *et al*., 2014), and have been found to simultaneously potentiate and depress PV and CCK+ IN inputs, respectively, leading to yet another mechanism by which different IN classes can be recruited in an activity-dependent manner.

The CCK peptide itself also has opposite effects on PV+ and CCK+ inputs: exploiting the difference in expression of pre-synaptic calcium channels, Foldy *et al.* (2007) pharmacologically isolated CCK+ IN and PV+ IN inhibitory currents onto PCs and found that applying CCK stimulated PV+ inputs while simultaneously suppressing CCK+ inputs. This finding echoes the “push/pull” effect of cell autonomous plasticity, suggesting CCK can also act as a selective switch in inhibitory networks, changing the source of the perisomatic inhibition a PC receives (Lee & Soltész, 2011). In fact, it has been shown that CCK is important in a panoply of behaviours, such as learning and memory (Hadjiivanova *et al.,* 2003), feeding (Moran & Kinzig, 2004) and nociception (Faris *et al.,* 1984; Baber *et al.,* 1989). In addition several diseases of the CNS have been linked to CCK, such as anxiety and panic attacks (Ravard & Dourish, 1990), deficits in fear extinction (Myers & Davis,

2007), and schizophrenia (Ferrier *et al.,* 1983; Hashimoto *et al.,* 2008). Even though the peptide is expressed by both PCs and INs (Gallopin *et al.,* 2005), it has been difficult to quantify to which extent immunohistochemically, due to a high number of isoforms and differences in post-translational modifications (Toledo−Rodriguez *et al.,* 2005).

CCK INs and behaviour

There is increasing evidence that CCK+ BCs are involved in a diversity of behaviours based on specific circuits. For instance, long-range cortico-thalamic inhibitory connections have been shown to efficiently suppress CCK+ BCs in CA1, resulting in the disinhibition of PV+ cells which normally receive feedforward inhibition from CCK+ cells, a pathway which has been implicated in fear memory formation (Basu *et al*., 2016). Furthermore, specifically disrupting CCK+ IN wiring has been shown to disrupt the generation of place cells and novel object recognition (del Pino *et al.,* 2017). There is also unpublished evidence that enriching the environment of mice increases the number of CCK+ IN synaptic contacts in the hippocampus (Hartzell *et al.,* personal communication). Together, this suggests that CCK+ INs may have a role in encoding details in the environment, and potentially combine them with different behavioural states.

However, much of the work described above has been performed in the hippocampus, which not only benefits from a simpler laminar structure as well as the robust targeting of superficial and deep stratum pyramidale PCs by CCK and PV+ INs, respectively (Valero *et al.,* 2015). The rules surrounding the wiring and properties of CCK+ INs in the neocortex remain poorly characterised. For instance there is evidence that PV and CCK BCs are present in different proportions according to cortical area (Whissell *et al.,* 2015), and it is interesting to note that CCK+ INs are relatively more abundant in secondary sensory areas (including V2) of the mouse cortex, than in corresponding primary areas. One of the best markers for CCK+ INs has remained CB1, which plays interesting roles in the modulation and plasticity of synaptic release (described in a further section). Interestingly, there also appears to be a laminar-specific patterns of CB1+ and PV+ inhibitiory boutons in the cortex (Fig. 15), although for CCK+ cells the characterisation of synaptic properties lags behind that of PV cells. The paucity of such information is a direct consequence of technical and biological limitations which have until recently prevented the precise targeting of CCK+ INs

in the cortex, and now that tools are available it is worthwhile to investigate this cell type in cortical circuits.

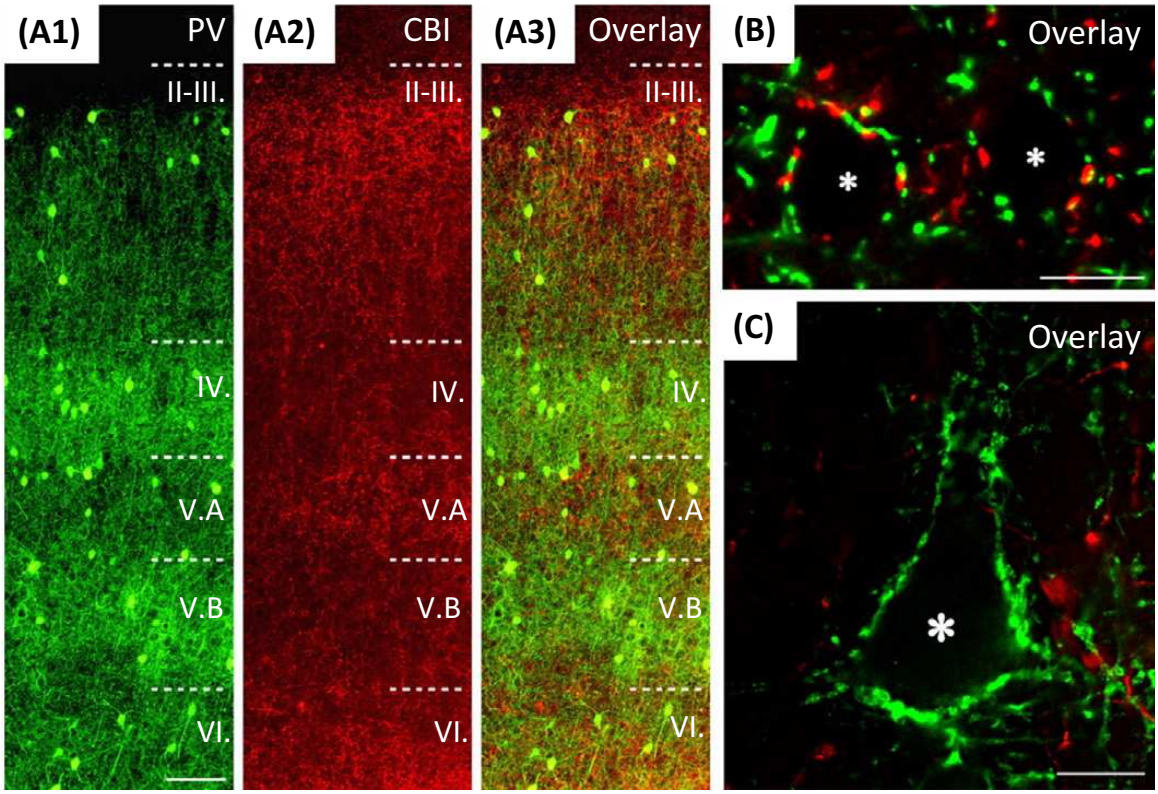

Figure 15. Complementary expression of markers for major basket cells in the cortex. Patterns of PV (A1) and CB1 (A2) show layer-specific distribution of perisomatic IN afferent projections on to PCs. There is concomitant expression in some layers (A3, B), while in others PV are almost exclusively present (A3, C). From Bodor (2005), adapted by Allene *et al.* (2015)

Basket cells and network oscillations

There are so many electrical and biochemical interactions in the brain that it is amazing to contemplate how neural networks manage to deal with noise and error, both at the molecular and cognitive level. Much of the cortex is bombarded with both bottom-up and top-down inputs simultaneously. Even simple actions require the orderly firing of millions of synapses in the right order. How is this cacophony organised? Although there has been considerable debate around the issue, network oscillations appear as good candidates for introducing a conductor in this unruly orchestra. Evidence that the brain produces rhythmic electromagnetic signals goes back centuries (Berger, 1929), and studies that correlate them with behavioural states such as sleep (Feinberg *et al.,* 1967; Borbély *et al.,* 1983; Steriade *et al*., 1993; Carskadon & Dement, 2005; Riedner *et al*., 2007) and cognitive processing

(Rowland *et al.,* 1985; Klimesch, 1996) are too numerous to count. Detractors argue that oscillations might be the result rather than a substrate for cortical computations, analogical to the BOLD signal recorded with fMRI (Merker, 2013). No matter how tempting to accept that oscillations have a computational role in the cortex, it remains difficult to prove precisely because the hypothesis relies on a close interaction between oscillations and processing, making it difficult to separate them. Still, there are both conceptual arguments and data which suggest that oscillations are not just by-products.

Indeed, disruption of oscillations has been shown to impede cognitive processes and has been involved in psychiatric diseases (Hajós *et al.,* 2008; Raver *et al.,* 2013). Although the tools remain exceedingly rough, enhancing oscillations, for example by using transcranial magnetic stimulation, has also been shown to improve a variety of cognitive tasks, although many of these results are controversial (Luber & Lisanby, 2014). Having a clock coordinating circuits also solves many computational problems that arise from the substrate being biological, and it is unrealistic to expect synapses to behave like computers. Without having a clock that can synchronise populations of neurones, noise becomes problematic: any delay could be amplified, and the temporally precise coordination of activity that mediates a lot of plasticity and feature coding would be disturbed (e.g. Kampa *et al.,* 2006; Basu *et al.,* 2013). Brain oscillations can provide a framework for neural syntax, separating streams of messages into distinct packets and facilitating the communication between distant areas (Varela *et al.,* 2001; Gregoriou *et a*l., 2009; Buszáki, 2012). Buszáki (2006) gives a great overview of the links between oscillations and the brain. A particularly interesting observation is that the first neural networks most likely evolved to rhythmically control the muscles of sea-living filter feeder by producing an oscillatory output. Movement therefore preceded sensory systems, challenging the view that stimulus is interpreted and then gives rise to adaptive behaviour; rather, movement modulates the sensorium, and the state of the network will influence the interpretation of the stimuli it receives.

What is the cellular basis for oscillations? Oscillations are typically classified relative to their frequency, spanning between 0.05 Hz and 500 Hz (Buszáki, 2012). These rhythms can also be present in combination, with the slower oscillations typically modulating the power of the faster one (e.g. Bragin *et al.,* 1995; Buszáki & Wang, 2012). It is beyond the purpose of this thesis to describe all the different oscillations, their mechanism and computational role. We

will therefore focus on the gamma (30 Hz to 80 Hz) and theta (4 Hz to 10 Hz) band, the generation of which has been suggested to involve basket cells (Klausberger *et al.,* 2003; Bartos *et al.,* 2007; Freund & Katona, 2007; Tukker *et al.,* 2007 Klausberger & Somogyi, 2008). Indeed, because BCs modulate the timing of the output of their targets, they are ideal candidates to play a part in controlling oscillations. PV+ INs have many features that make them able to play a role in fast oscillations. They can sustain high rates of firing thanks to specific channel expression (e.g. Kv3 voltage-gated potassium channel; Chow *et al.,* 1999; Rudy *et al*., 1999), low input resistance and short membrane time-constants (Nörenberg *et al.,* 2010), as well as a high-density of voltage-gated sodium channels for fast action potential propagation (Hu *et al*., 2014). Oscillations can then be generated through autaptic inhibition (Hermann & Klaus, 2004) and recurrent homotypic synaptic and electrical connections (Galarreta & Hestrin, 2001; Hu & Agmon, 2015), although the role of electrical connections in gamma generation has been put into question (Neske & Connors, 2016). An oft proposed model of gamma generation relies on recurrent connections between PC and PV+ INs; the so-called pyramidal IN gamma (PING), which is to be contrasted to IN gamma (ING) (Tiesinga & Sejnowski, 2009). Both models rely on the simple principle of alternating IN recruitment (thereby increasing inhibition) with IN inhibition (thereby decreasing inhibition and increasing PC spiking), with recurrent connections providing the loop required for oscillations to take place. Indeed, Sohal *et al*. (2009) used a panel of optogenetic tools to show that inhibiting PV+ INs suppressed gamma oscillations *in vivo*. Meanwhile, Cardin *et al.* (2009) found evidence that rhythmically driving PCs tends to increase the power of lower frequencies, while doing the same for PV+ INs generated gamma. This latter result is not incompatible with the PING hypothesis; it is still compatible with a view that PV+ INs are more efficient at gamma generation than their PC counterparts. While it is certain that PV are crucial for these rhythms, SST INs have also recently been shown to play a role in gamma band synchronisation (Veit *et al.,* 2017).

The implication of CCK BCs in the driving of oscillations has gained more and more traction. Disrupting CCK BC wiring in the hippocampus has been shown to disrupt both theta band oscillations and the generation of place cells (del Pino *et al.,* 2016). Furthermore, analysis of the relationship between IN firing and theta waves in the hippocampus has shown that CCK+ IN firing specifically correlates most strongly with theta (Li *et al*., 2017). Although it comes with the major caveat that CB1 is not exclusively expressed at CCK synapses, there

is ample evidence that exogenous cannabinoids affect oscillations (e.g. Hájos *et al.,* 2000; Robbe *et al.,* 2006). A study by Kucewicz *et al.,* (2011) found that in awake rats synthetic cannabinoids decreased the power of the local field potential between 0.1–30 Hz in CA1 and between 30–100 Hz in mPFC. Interestingly, this study also found that a reduction in accuracy in the performance of a spatial memory task was correlated with a decrease in theta-band coherence between the two areas during the decision-making phase of the task. Furthering this idea, exogenous cannabinoids have also been found to disturb the spike-timing coordination of hippocampal place cells without affecting place fields (Robbe & Buzsáki, 2009), and to cause memory impairments which correlate with the desynchronisation of cell assemblies without any change in firing rate (Robbe *et al.,* 2006). From these data a view has emerged in which CCK+ cells fine-tune the timing of PCs rather than imposing a strict tempo on their targets.

There is also evidence to suggest that oscillations are also affected by cross-talk between different BCs classes, at least in the hippocampus. Application of the cholinergic agonist carbachol is known to produce IPSCs at theta band frequencies (Nagode *et al.,* 2011), and silencing of PV BCs does not inhibit these suggesting they originate from CCK BCs (Nagode *et al.,* 2014). However, μ-opioid receptor agonists also suppressed these theta-oscillations, even though the μ-opioid receptor is known to be expressed in PV, but not CCK BCs. Similarly, serotonergic drugs can modulate gamma oscillations (Huang *et al.,* 2011) even if 5-HT3aRs are expressed in CCK, but not PV BCs (Freund & Katona, 2007). Indeed, it is known that in the hippocampus there are synapses between PV and CCK+ INs (Karson *et al.* 2009), and that activation of 5HT3aRs can lead to the impairment of gamma *via* a CCK-mediated desynchronisation of PV-BC firing (Huang *et al.,* 2011). It is therefore increasingly clear that PV and CCK BCs not only contribute to oscillations but can also influence each other, as well as being the target of various neuromodulators. In normal circuits oscillations have been correlated assembly selection and competition (Roux & Buzsáki, 2015), oscillatory coupling (Buzsáki & Wang, 2012), and grid field formation (Couey *et al*., 2013). Conversely, abnormal oscillations have been documented in a wide-range of psychiatric diseases, making it is worthwhile to understand these two BC types in detail (Mathalon & Sohal, 2015). CCK INs express high levels of CB1. In the next section, I will review the endocannabinoid system.

III- The endocannabinoid system

Cannabis as a gateway drug to endocannabinoid research

While cannabis has been consumed by humans for millennia, the physiological understanding of its action lagged behind that of other drugs for the majority of the 20th century. Research was long hampered by a number of factors ranging from prohibition to the chemical nature of cannabinoid compounds, the number of scientific papers on the topic has steadily increased since the mid 1990s to become a major research field in the neurosciences and beyond. Looking at the behavioural and physiological effect of cannabis can give clues to the role of the underlying neural networks in a healthy, sober mind. Writings detailing the medical and recreational use of cannabis are peppered through history, going as far back as the Assyrians (ca. 2000 BC). The plant was then thought to be a cure for a variety of ailments, from depression to banishing 'ghosts' or 'witchcraft', probable early interpretations of psychiatric disorders. Interestingly, the dose-dependent triggering of psychosis by the plant was recorded in Ben Ts'ao's pharmacopoeia: "when taken in excess it could cause seeing devils". When the drug was spread to Europe in colonial times, the psychiatrist Jacques-Joseph Moreau (1845) thought that hashish could serve as a powerful tool to explore pathogenicity in the brain. Following self-experimentation, Moreau describes a perceived separation in psyche: one that results from interacting with the external world, and another, that feeds itself from the first, but remains internal and distinct nonetheless, echoing the bottom-up and top-down interactions that have been discussed above. However, cannabinoid research stagnated for many decades with reports of oral administration of cannabis extracts leading to unpredictable and variable responses. It is now known that Δ-9-tetrahydrocannabinol (THC; Fig. 16), the main psychoactive compound of cannabis, has paradoxical dose-dependent effects and also interacts with other phytocannabinoids (Grisham & Ferraro, 1972; Agurell *et al.,* 1986; Katsidoni *et al.*, 2013). THC was discovered in the 1960s (Gaoni & Mechoulam, 1964), and synthesised three years later (Mechoulam *et al.*, 1967). Only then it became possible to precisely characterise the effects of cannabis by systemic administration of various doses of THC in animals, proving that this compound mediates sedative, hyperphagic and antinociceptive effects (Carlini *et al*., 1974; Kiplinger & Manno, 1971). Studies using chromatography showed that many phytocannabinoids are lipophilic, thereby leading to the hypothesis that their mechanism of

action was membrane-dependent and non-specific. However, the high stereospecificity of synthetic cannabinoids, the dose-dependent modulation of cyclic AMP (Howlett, 1990), and the displacement of tritium-labeled cannabinoids by GTP and other cannabinoids allowed the identification and cloning of a pharmacologically distinct receptor (Devane *et al.,* 1988; Matsuda *et al.,* 1990). Moreover, the localization of the potential cannabinoid receptor in the central nervous system (Matsuda *et al.*, 1990) was consistent with the cognitive and psychotropic effects of cannabinoids (Herkenham *et al.*, 1990). Later on, a second receptor (CB2) was identified, and is mainly found in peripheral nervous and immune system (Munro *et al.*, 1993) and at lower levels in microglial brain cells (Nunez *et al.*, 2004). The presence of CB2 in neurons is still under scrutiny, although several reports address its role in neural transmission and excitability (Morgan *et al.*, 2009; den Boon *et al.*, 2012; Stempel *et al.*, 2016). Given the lipophilic nature of exogenous cannabinoids, it was assumed that hypothetical endogenous cannabinoid molecules would also be lipids. Indeed, anandamide (AEA; Fig. 16), composed of arachidonic acid and ethanolamide, was the first discovered eCB (Devane *et al.*, 1992) followed by 2-arachidonoylglycerol (2-AG; Fig. 16; Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). Later on, their specific synthesis and degradation pathways were uncovered (Marzo *et al.*, 1994). The identification of endogenous receptors (CB1 and CB2) and ligands (eCBs) promoted the concept that an “endocannabinoid system” (ECS) exists in the body, participating in the regulation of physiological processes (Piomelli, 2003).

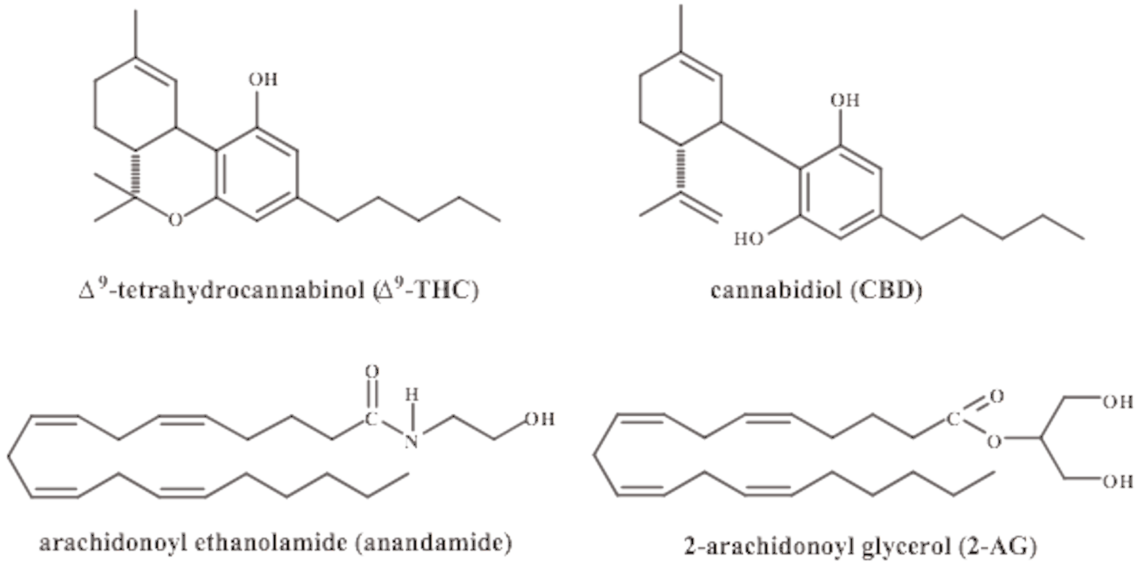

Figure 16. Structure of two major phytocannabinoids (top) and endocannabinoids (bottom)

But where exactly is the CB1 receptor expressed? CB1 is widely distributed throughout the CNS and within cortical regions (Matsuda *et al*., 1990; Tsoua *et al.,* 1998), with CB1 mRNA mainly found in GABAergic interneurons co-expressing glutamic acid decarboxylase (GAD) and cholecystokinin (CCK), while they are virtually absent in PV+ INs (Marsicano & Lutz, 1999; Katona *et al.,* 1999). This is of particular importance for this thesis, because we use the CB1 as a marker for our CCK+ IN. CB1 is primarily expressed presynaptically on both central and peripheral system neurones, although electrophysiological data strongly suggests that somatodendritic CB1 receptors participate in the autocrine regulation of neuronal excitability (Bacci et al., 2004; Marinelli et al., 2009). Moreover, there is also evidence that CB1 is present in mitochondria and astrocytes, where it has physiologically relevant roles (Han *et al*., 2012; Bénard *et al*., 2012; Da Cruz *et al*., 2016; Hebert-Chatelain *et al.,* 2016). Although the density of expression is particularly high in GABAergic synapses, there is also evidence for CB1 modulation in a variety of glutamatergic synapses (Gerdeman & Lovinger, 2001; Hájos *et al.,* 2001; Melis *et al*., 2004; Takahashi & Castillo, 2006). Similarly, not all CB1 expressing INs are CCK positive (Yu *et al.,* 2015). Caution should always be applied when using mRNA or even protein expression as proxies for functional importance. For instance, knocking-out CB1 exclusively in glutamatergic cell-types (which normally express it at low levels) revealed major functions such as resistance to excitotoxic insults in the hippocampus (Monory *et al*., 2006), fear coping, stress and anxiety (Dubreucq *et al*., 2012; Metna-Laurent *et al*., 2012) and food intake (Bellocchio *et al*., 2010). Also, the receptor cannot be considered in isolation: intracellular signalling cascades can also contribute to the dynamics of endocannabinoid signalling (Mechoulam & Parker, 2013). Indeed, the CB1 is a G-protein coupled receptor (GPCR), associated with a variety of down-stream signalling pathways. Cannabinoids inhibit the cAMP-dependent protein kinase (PKA) pathway via CB1 activation coupled to Gi/o proteins (Howlett *et al*., 1986; Di Marzo et al., 1998; Piomelli, 2003; Howlett, 2005) while they stimulate other pathways including (among others): the mitogen-activated protein kinases / extracellular signal-regulated kinase (MAPK/ERK), c-Jun N-terminal kinases (JNK), mammalian target of rapamycin (mTOR) and phosphatidylinositol 3-kinases (PI3K) pathways both in vitro and in vivo (Bouaboula *et al*., 1995; Valjent *et al*., 2001; Puighermanal *et al*., 2009; Andre & Gonthier, 2010). Interestingly, some of these pathways have been implicated in the maintenance of long-term synaptic plasticity (Chevaleyre *et al.,* 2007; Heifets & Castillo, 2009). Gi/o protein-mediated CB1 signalling has also been found to modulate calcium and potassium channels (Twitchell *et al.,*

1997; Guo & Ikeda, 2004). This can have profound effects on how CB1 modifies the synaptic release probability on shorter time-scales; the most well defined result of presynaptic CB1 activation is the inhibition of neurotransmitter release.

The biochemistry behind ligand synthesis and degradation can also play a modulating cannabinoid signalling. The two major endogenous ligands AEA and 2-AG have been extensively studied, although their relative contribution to different processes remains debated (Castillo *et al.,* 2012). Similarly to phytocannabinoids, these are lipid compounds, and therefore unlike other neurotransmitters they are not released in vesicles. Rather, they are synthesised when needed through mechanisms that can be influenced by the state of the postsynaptic cell. The synthesis of 2-AG requires several enzymes, such as phospholipase Cβ (PLCβ) and diacylglycerol lipases (DGL), which can both be modulated from a variety of pathways. For instance, PLCβ can be upregulated both through activation of post-synaptic group I metabotropic receptors (I mGluR) (Maejima *et al.,* 2001; Varma *et al.,* 2001) and calcium influx *via* voltage gated calcium channels (VGCC) (Hashimotodani *et al*., 2005) leading to the hypothesis that PLCβ can act as coincidence detectors of pre-synaptic and post-synaptic activity. DGLα, which synthesise 2-AG, is specifically present in post-synaptic terminals (Fig. 17; Katona *et al.,* 2006; Yoshida *et al.,* 2006; Lafourcade *et al.,* 2007) and has been shown to be required for both short and long-term depression of synaptic release (Tanimura *et al.,* 2010; Yoshino *et al.* 2011). On the other hand, anandamide synthesis is less well characterised, and although links to calcium influx and post-synaptic depolarisation have been established, calcium independent synthesis has also been reported (Varga *et al.,* 2014). Indeed, several pathways can lead to anandamide synthesis (Simon & Cravatt, 2008; Di Marzo, 2015), leading to many potential targets for fine-tuning. A particularly interesting feature of endocannabinoids is that they often retrograde across the synapse to activate pre-synaptic receptors, which means that the activity of a neurone will modulate the inputs it receives. However the mechanism by which this occurs remains obscure for 2-AG. The transport of anandamide could be facilitated by a lipophilic cargo protein (Beltramo *et al.,* 1997). Degradation of anandamide is mainly performed inside the post-synaptic cell by fatty acid amide hydrolase (FAAH)(Gulyas et al., 2004). In contrast, the enzyme responsible for 2-AG degradation (MAG lipase) is present in the pre-synapse (Gulyas *et al*., 2004), albeit irregularly (Tanimura *et al.,* 2012). The presence, quantity and distribution of degrading enzymes gives yet another point of control

of CB1-mediated modulation: data have shown that MAG lipase does indeed influence the magnitude of 2-AG-dependent plasticity (Pan *et al.,* 2011).

eCB-mediated modulation of synaptic release

There is therefore a tremendous amount of control that can be exerted on and by the endocannabinoid system. In our case, it is particularly interesting to look at how the CB1-receptor affects the platicity between neurones, and what impact this may have in information processing, with a particular focus on inhibitory transmission. Although the process was observed earlier (Pitler & Alger, 1992; Vincent *et al.,* 1992) the first CB1-dependent retrograde plasticity described in the literature was depolarisation induced suppression of inhibition (DSI; Fig. 17; Ohno-Shosaku *et al*., 2001; Wilson & Nicoll, 2001) and its excitatory counterpart DSE (Kreitzer & Regehr, 2001). In these papers, the authors show that depolarising the postsynaptic cell results in the suppression of neurotransmitter release from its inputs for around a minute and block the process with CB1 antagonists, and behaviourally relevant spike trains have also been shown to cause DSI (Hampson *et al.,* 2003). DSI/E relies on the inhibition of Ca2+ influx through VGCCs, which can be directly modulated by the G-proteins associated to CB1 (Fig. 17; Wilson *et al.,* 2001; Kreitzer &

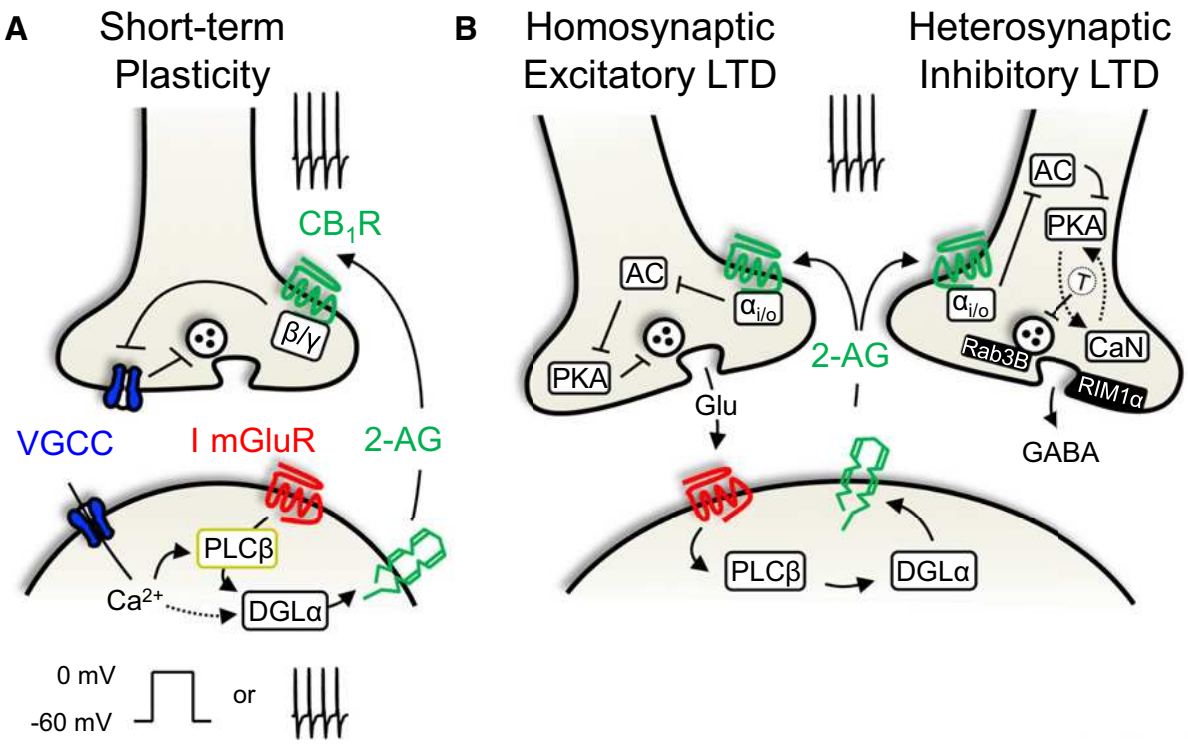

Figure 17. Molecular mechanisms underlying phasic CB1-mediated plasticity (**A**) Short-term depression involves post-synaptic calcium influx and eCB synthesis (**B**) eCB LTD and iLTD respectively involve homosynaptic and heterosynatic processes. From Castillo (2012)

Regehr, 2001; Brown *et al.,* 2003). Endocannabinoids also mediate long term depression (eCB-LTD) of excitatory (Gerdeman *et al.,* 2002; Robbe *et al.,* 2002) and inhibitory (Marsicano *et al.,* 2002; Chevaleyre & Castillo, 2003) inputs. A sustained release of eCB is required for the induction of eCB-LTD, and usually involves downregulation of the cAMP/PKA signalling pathway (Chevaleyre *et al.,* 2007) and P/Q type calcium channels (Mate *et al.,* 2008). In the case of inhibitory LTD (iLTD), the active zone proteins RIM1α and Rab3b are needed, hinting towards changes in the vesicular release machinery (Fig. 17; Chevaleyre *et al*., 2007; Tsetsenis *et al*., 2011).

Excitatory eCB-LTD can require the combination of presynaptic firing (through mGluR-I) with CB1 activation, meaning it will only be expressed as specific synapses (Singla *et al.,* 2007; Heifets *et al.,* 2008). Likewise, heterosynaptic iLTD at specific inhibitory synapses has also been observed (Fig. 17). iLTD requires postsynaptic activation of glutamatergic receptor in order to induce eCB release which will leak onto neighbouring CB1+ GABAergic synapses (Chevaleyre & Castillo, 2003). In contrast, DSI provides a mechanism by which cell autonomous activity can cause disinhibition from specific (likely large CCK BC) inputs more widely onto somatodendritic compartments. By themselves, eCB-iLTD and DSI have an entirely different impact on the CB1+ inputs the target receives. Considering the properties of large BCs described earlier, it is tempting to see DSI as a potential mechanism of fine-tuning the output of PCs (and perhaps other INs) following specific patterns of activity. Indeed, there is evidence for CCK+ IN disinhibition increasing the likelihood of PC spiking (Chevaleyre & Castillo, 2003; Younts *et al.,* 2013). A single CB1+ IN can maintain inhibition on PNs which have not synthesised eCB while disinhibiting the ones that have, a process by CCK-INs can influence the competition between outputs. On top of that, both DSI and iLTD have been shown to facilitate the quintessential LTP of glutamatergic Schaffer collateral synapses onto the dendrites of CA1 PNs (Carlson et al., 2002; Chevaleyre & Castillo, 2004; Zhu & Lovinger, 2007). This form of metaplasticity also has interesting implications because of the distinct spatial and temporal profile of iLTD and DSI. It has also recently been shown that CCK+ IN circuits play a crucial role in providing feedforward inhibition in in CA1, and are efficiently recruited by inputs from both the cortex and the hippocampus (Basu *et al.,* 2013). This is particularly interesting because a type of synaptic learning rule known as input-timing dependent plasticity (ITDP) has been shown to cause disinhibition of CA1 PCs as well as excitatory LTP (Basu *et al.,* 2013). ITDP depends on coincident inputs in a short time window

(20ms), which (maybe coincidentally) corresponds to a gamma oscillation period (50 Hz). More recently the same team (Basu *et al.,* 2016) demonstrated that long-range inhibition from the entorhinal cortex could also disinhibit PCs from CCK+ INs. These circuit properties once again suggest that with the help of eCBs, CCK+ INs can contribute to information processing by timing and gating information flow, producing sparsely coded cell assemblies and increasing the contrasts between signals.

Tonic modulation by endocannabinoids

Although less well studied than its phasic counterpart, control of GABAergic release properties by cannabinoids can also occur tonically (Soltész *et al.,* 2015). Typically, tonic eCB signalling is revealed by applying a CB1 antagonist such as AM-251, and causes an increase in IPSC amplitude (Fig. 18). As AM-251 has inverse agonist properties (thereby decreasing receptor activity under a basal level), this effect could be due to a lowering of basal CB1 activity (Howlett *et al.,* 2011). However there is also data showing that tonic effects can be blocked by chelating post-synaptic Ca2+ (Neu *et al*., 2007) or changes in eCB concentration (Navia-Paldanius *et al.,* 2015). In some cases, tonic eCB signalling has been shown to effectively silence synapses, which can become 'awoken' by the application of the antagonist as well as high frequency presynaptic stimulation (Losonczy *et al*., 2004). While the mechanism remains poorly understood, there is also evidence that tonic eCB signalling can be specific to CCK BCs (Lee *et al.,* 2010). By providing powerful synaptic suppression, tonic CB1-signalling could also have a significant impacts on CCK BC function, radically influencing the processing properties of PNs (Isaacson & Scanziani, 2011). There is not yet any data showing tonic signalling *in vivo,* and it should also be noted that selectively affecting tonic inhibition is not yet pharmacologically feasible. Still, tonic eCB signalling has been linked with psychiatric diseases; for instance, mutations of neuroligin-3 have been associated with autism and are known to disrupt tonic, but not phasic CB1 signalling (Földy *et al*., 2013). A recent study using a variety of pharmacological tools found that several pathways seem to be involved in the regulation of tonic eCB signalling (Lee *et al.,* 2015). It would be of great interest to find out in more detail how such a system might be harnessed in different cell types and brain areas, and again there is a particular paucity of information in the neocortex.

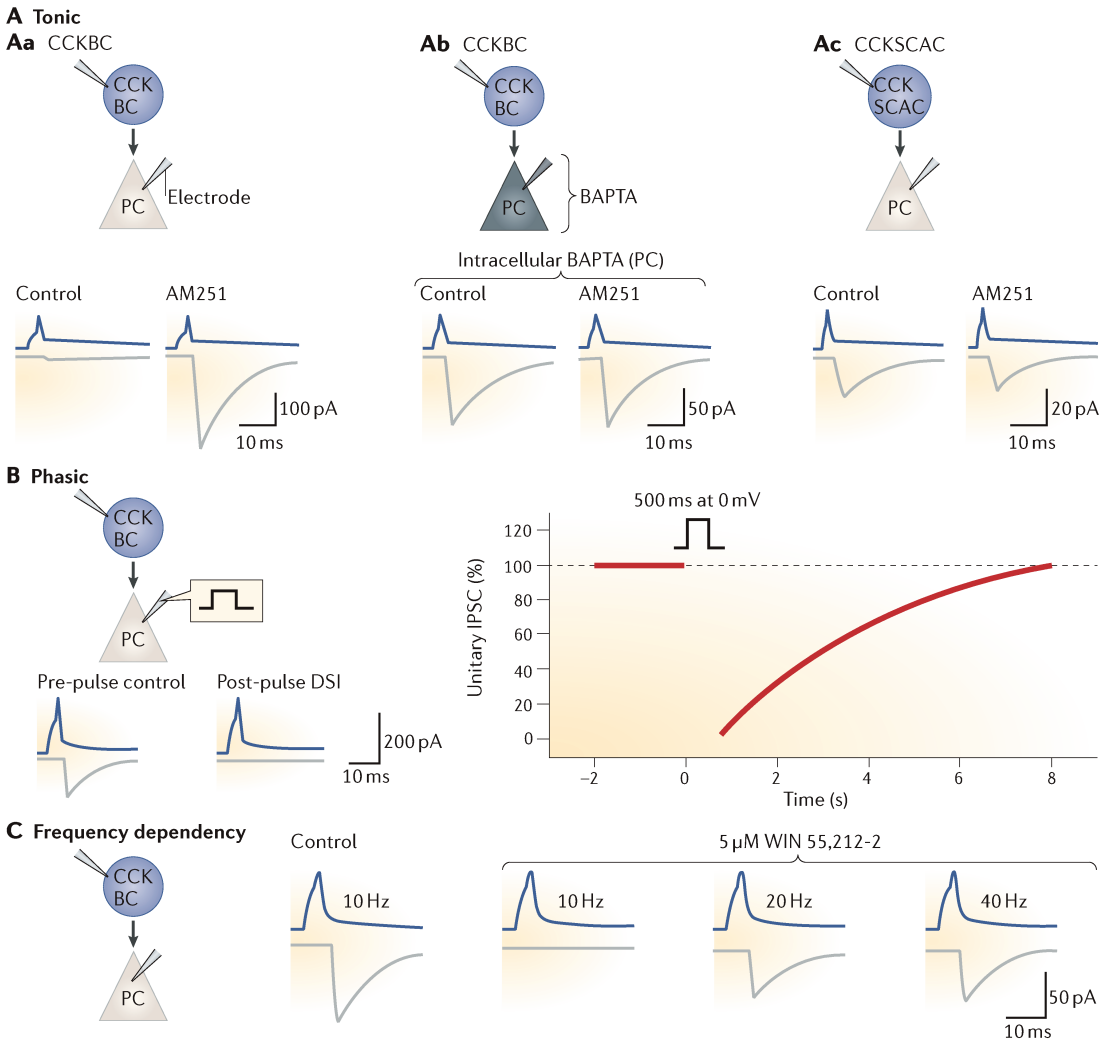

Figure 18. eCB-mediated processes (**Aa**) Tonic inhibition revealed by application of the CB1antagonist and inverse agonist AM-251 (**Ab**) AM-251 has no effect on IPSC amplitude when calcium is chelated post-synaptically by BAPTA (**Ac**) Tonic eCB appears not be present in dendrite-targeting Schaffer collateral-associated cells (CCKSCACs) (**B**) Phasic eCB signalling mediating DSI (**C**) The CB1 agonist WIN55,212-2 inhibits CCK IPSCs, which can then be "awoken" using high frequency trains of action potentials. From Soltész (2015)

Cortical phasic eCB-mediated modulation

Until recently, it has been incredibly difficult to target CB1+ INs, and because they are sparse, blindly targeting them is a laborious process. There are no fibres which can be reliably excited to segregate inputs from different IN types. Indeed, extracellular stimulation will mostly evoke responses from PV+ cells. Until the advent of intersectional strategies, the best way to identify CCK+ INs in the neocortex was by using CB1-dependent mechanisms as a marker. Studies in the cortex point towards similarities with the hippocampus: both DSI and LTDi have been observed in somatosensory areas (Galarreta *et al.,* 2008; Marinelli *et*

al., 2009), and CB1+ INs have high failure rates, asynchronous firing, gap junctions and variable short-term dynamics (Galarreta *et al.,* 2008). Furthermore, cannabinoids have been found to potentiate backpropagating action potential-induced dendritic calcium transients in L2/3, but not L5 of the somatosensory cortex, pointing towards a role of CB1-signalling in dendritic processing (Hsieh & Levine, 2012). Similarly, to the hippocampus, this could implicate CB1+ INs in increasing the excitability of cells that receive simultaneous inputs. Another study observed CB1-independent DSI in some IN-PN pairs in L2-4 (De-May & Ali, 2013), hinting there is cell-type specificity of CB1-dependent modulation. This is also supported by the finding that CB1+ GABAergic cells in the entorhinal cortex preferentially innervate PCs that project outside the hippocampus, and avoid nearby PCs that will give rise to hippocampal excitatory inputs (Varga *et al.,* 2010). The pattern of eCB system components is also laminar: in the barrel cortex there is higher receptor expression in L1-3 and L5b-6 than in L4-5a, which coincides with the expression of FAAH (Egertová, 2003; Bodor *et al.,* 2005). In V1, this pattern can be modified by sensory deprivation (Yoneda, 2013), indicating there may be activity-dependent mechanisms similar to those of the hippocampus (Hartzell *et al.,* personal communication), and hinting that increases in inputs can enhance CB1 expression. In addition, Jiang *et al.* (2010) showed that eCB-LTDi during the critical period is necessary for the maturation of GABAergic transmission in the visual cortex. There is also a laminar component to this increase of GABAergic transmission during development, with L4 maturing earlier than L2/3, and the latter being sensitive to dark rearing while the former is not (Jiang *et al.,* 2010). CB1 KO mice showed deficits in GABAergic transmission similar to those caused by sensory deprivation exclusively in L2/3, indicating CB1 can have layer-specific roles in shaping inhibition (Jiang *et al.,* 2010).

Furthermore there is also evidence of area-specific patterning of CB1: for instance it has been shown that there is more CB1 in V2 than in V1 (Yoneda *et al.,* 2013). This pattern of higher expression in associative cortices can be generalised to other sensory systems, and has also been corroborated in monkeys (Eggan & Lewis, 2006). It should also be noted that as described earlier, L4 is primarily driven by sensory thalamic inputs while the other layers receive signals from a multitude of brain areas, once again reinforcing the idea that the CB1 receptor tends to be present in circuits which are more associative. This is also true at a more synaptic level, at least for excitatory synapses: cannabinoids are known to play a role in gating spike-timing dependent plasticity of corticostriatal synapses (Fino *et al.,*2010; Cui *et*

al., 2013; Cui *et al.,* 2016). Taken together, these data support layer, area and cell-specificity in CB1 IN properties and modulation, but the precise contribution they make in the visual cortex remains poorly characterised.

Aim of the study

The aim of this study is to fill in a gap currently present in the literature surrounding the properties of CB1+ (likely large CCK+ BC) in the cortex. We have seen that the pattern of CB1 differs between cortical layer, but also between cortical areas (Yoneda *et al.,* 2013), with a general positive correlation between associativity and the density of CB1. We also know that there tends to be a higher ratio of CCK+ to PV+ INs in secondary rather than primary sensory areas (Whissell *et al.*, 2015) and that CCK BC somata are most numerous in L2/3. It is however unclear whether the differences in pattern of CB1 are due to variations in morphology or directly correlate with the number of CB1+ cells, the density of the receptor on axonal fibers and/or their morphology. And does this reflect different intrinsic properties? We chose V1 and V2 as representatives of primary and secondary sensory cortices.

The connections between cortical CB1+ INs and PCs remain poorly characterised, especially in the visual cortex. Do these synapses look like the ones in the somatosensory cortex, or is there a difference between areas? We aimed to characterise both intra-laminar (within a cortical layer) and extra-laminar connectivity: because of low expression of CB1 in L4 of primary sensory areas, we were particularly interested in knowing whether there are differences in projections from L2/3 to 4 in V2. Such differences in connectivity could reflect the specialisation of cortical circuits for specific computations.

Following this morphological and connectivity comparison between V1 and V2, we also wanted to see whether CB1-dependent mechanisms differed between the two areas. We chose to use DSI as a proxy for phasic CB1-modulation, but also inquired tonic effects using pharmacological tools. Thankfully, these aims were made easier to accomplish through the use of a transgenic mouse line in which CB1+ cells contain a fluorescent reporter (collaboration with Oliver Schlüter, University of Pittsburgh), allowing us to target large BCs easily for electrophysiological recordings

MATERIALS & METHOD

Animals

Experimental procedures followed French and European guidelines for animal experimentation. Experiments for paired recordings were performed on both female and male CB1-tdTomato mice, which were kindly provided by the lab of Oliver Schlüter (Winters *et al.*, 2012). This team generated a bicistronic gene for CB1 with tdT expressed after an internal ribosome entry site (IRES). Hence, the soluble fluorophore was expressed under the control of the CB1 promoter, without being attached to the receptor itself. The mice have been shown to express the same amount of CB1 protein than wild-type animals, with the same pattern of expression (Winters *et* al., 2012). We also crossed the CB1-tdTomato line with GAD67-GFP mice (graciously donated by the Lubetzki-Stankoff group at the ICM in Paris) to identify CB1-expressing INs for cell counting. In experiments for Fig. 2, C57BL/6 wild-type were purchased from Janvier laboratories. Mice were housed in an animal facility with a 12h light/dark cycle, with food and water available *ad libitum*.

Immunohistochemistry and cell counting

All animals were deeply anesthetized with ketamine-xylazine and intracardially perfused first with cold PBS (20ml) followed by 30-40 ml of cold 4% PFA (paraformaldehyde, diluted in PBS). Brains were removed and post-fixed in 4% PFA overnight at 4 degrees. For cryoprotection they were next put in 30% sucrose (diluted in PBS) overnight and frozen at -45°C in isopentane. The brains were cut with a microtome (Thermo Fisher), with nominal section thickness set to 20 µm. After rinsing with PBS, slices where incubated 2h at room temperature in 0.3% PBT (0.3% Triton X in PBS) and 10% BSA blocking solution. Primary antibodies diluted in 0.3% PBT were incubated overnight at 4 degrees. The following antibodies were used: anti-CB1 (Frontiers institute, goat 1:400), anti-GFP (Millipore, MAB 3580 mouse 1:500), anti-DsRed (Clontech, rabbit 1:500). and anti-PV (Sigma PARV-19, mouse 1:1000). The CB1 antibody was raised agains a 31 amino acid C-terminal sequence of the receptor, and has been shown to mainly stain GABAergic axonal fibres, resulting in an immunopositive mesh (Bellochio *et al.,* 2010). Anti-GFP and anti-DsRed (i.e. tdTomato) were used to enhance the signals of GFP and tdTomato allowing us to increase the signal to noise ratio, particularly for counting somata. Slices were then rinsed with PBS and incubated for

2h at room temperature with the secondary antibodies Alexa 488 anti-goat, Alexa 488 anti-mouse and Alexa 633 anti-rabbit, all obtained from Life Technologies and diluted 1:500 in PBT 0.3%. Slices were mounted with Fluoromount (Sigma) and stocked at 4°C. Whole brain slices were imaged using an epifluorescence slice scanner (Zeiss).

CB1 immunofluorescence pattern: V1 and V2 were determined using the Allen brain atlas, and only slices in which both areas were present were analysed in order to be able to quantitatively compare the fluorescence patterns. To obtain the pattern, the “straight” option in FIJI (NIH) with a line width of 400 µm was used to calculate the grey value intensity of CB1 immuno-staining from pia to white matter. For each slice, the maximum fluorescence intensity over a length of 10 µm of either V1 and V2 was used to normalise fluorescence in the rest of the analysed areas. Also, because of differences in cortical length between animals and slices, we determined cortical layer thickness by using a ratio obtained from measuring each layer in the Allen atlas with total cortical length set as 1.

C*ounting CB1 INs:* CB1-tdTomato/GAD67-GFP positive cells were counted in both V1 and V2; for each area a region of interest (ROI) was drawn. The ITCN (Image-based Tool for Counting Nuclei) plugin in ImageJ was modified (courtesy of Brahim Abbes) in order to be able to count the cells and their distance from the pia simultaneously. The pia was drawn on each image, and cells expressing both tdTomato and GFP were manually marked. Indeed, although the ITCN was designed to automatically count cells, the widespread expression of tdTomato in both soma and neurites made automatic detection too unreliable. The software then calculated the minimal distance from marked cells to the pia. Once these distances were established, we were able to bin cell counts in the different layers of the cortex. Layer (bin) size was determined using the online mouse reference atlas, determining the % of cortical thickness each layer represented. We then used these ratios to calculate actual layer thickness on our slices. Also, ROIs could differ widely in width, hence to compensate for this we divided the obtained laminar density by the width of the ROI.

Morphological reconstruction

Biocytin Fills: To reliably reconstruct the fine axonal branches of mature CB1 INs, dedicated experiments were performed following recent updates by Jiang *et al.* (2015) to the classical

avidin-biotin-peroxidase method (e.g. Jiang *et al.* 2013). Biocytin (Sigma) was added to the intracellular solution at a high concentration (0.5g/100ml), which required extensive sonication. To avoid excessive degradation of fragile molecules such as ATP, sonication was performed in an ice bath. The intracellular solution was then filtered twice to prevent the presence undissolved lumps of biocytin in the patch pipette. Extra care was applied in verifying that the micro manipulators and slice were stable for recordings of at least 1h. During that time, neurones were injected with large depolarizing currents in current clamp mode for fifteen times (100ms, 1-2nA, 1Hz); indeed biocytin is charged and these currents are thought to help expedite the molecule in the fine axonal structures of mature INs. At the end of recordings, the patch pipette was removed carefully with the aim of resealing the cell properly, equivalent to obtaining an inside out patch. The slice was then left in the recording chamber for a further 5-10 min to allow further diffusion. Slices were then fixed with 4% paraformaldehyde in phosphate buffer saline (PBS, Sigma) for at least 48 h. Following fixation, slices were incubated with the avidin-biotin complex (Vector Labs) and a high concentration of detergent (Triton-X100, 5%) for at least two days before staining with 3,3′-Diaminobenzidine (DAB, AbCam).

Cells were then reconstructed and cortical layers delimited using neurolucida 7 (MBF Bioscience) and the most up to date mouse atlas (Allen Institute). Because cortical layer thickness differs within and across areas, we normalised neurite lengths relative to layer thickness to obtain the most accurate measure of density in each layer using an arithmetic method comparable to the graphic method used by Bortone *et al. (*2012). To obtain heat maps, we imported reconstructions in Illustrator (Adobe) and aligned the soma horizontally, and pia and white matter vertically. From there, individual bitmaps were generated separating dendrites and axons. These were subsequently blurred in ImageJ (NIH) using a gaussian filter with a radius equivalent to 20 µm. The contrast of blurred images was then adjusted to obtain the highest possible pixel intensity, and were then overlapped and averaged. The resulting group average image was also adjusted to the highest pixel intensity, and a lookup table (ImageJ's "Fire", inverted) was applied to colour code the density of neurites across cortical layers.

In Vitro Slice Preparation

Coronal slices (350 µm thick) of visual cortex were obtained from 30-35 day-old mice. Males and females were used in equal quantities. The area of interest was identified using the Allen adult mouse brain reference atlas. Animals were deeply anaesthetised with a mix containing 120 mg/kg body weight of ketamine and 24 mg/kg body weight Xylazine in a vehicle solution containing 0.9% sodium chloride. To increase slice quality and durability, a transcardiac perfusion was performed using an ice cold "cutting" solution containing the following (in mM): 126 choline chloride, 16 glucose, 26 $NaHCO_2$, 2.5 KCl, 1.25 NaH_2PO_4, 7 $MgSO_4$, 0.5 $CaCl_2$, (equilibrated with 95% O_2 / 5% CO_2). Following decapitation, brains were quickly removed and sliced with a vibratome (Leica) while immersed in ice cold cutting solution. Slices were then incubated in oxygenated (95% O_2 / 5% CO_2) artificial cerebrospinal fluid (ASCF) containing the following (in mM): 126 NaCl, 20 glucose, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, $2CaCl_2$ (pH 7.4, 310-320mOsm/L), at 34°C for 20 min, and subsequently at room temperature before transferring to the recording chamber. The recording chamber was constantly perfused with warm (32°C), oxygenated ACSF at 2.5-3 mL/min. Throughout all experiments slices were kept in the same orientation during incubation, recording, and immunohistochemistry.

Electrophysiology

Synaptic currents were recorded in whole-cell voltage or current clamp mode in principal cells of either L2/3 or L4 of primary and secondary visual cortex. Excitatory cells of L2/3 were visually identified by their triangular somata and apical dendrites projecting towards the pia, while in L4 they were identified by their round somata and by verifying they did not exhibit fast-spiking properties. L5 pyramidal neurones were identified by their large somata and apical dendrites. Meanwhile, INs were targeted using CB1-tdTomato fluorescence elicited by a green (λ=530 nm) LED (Cairn research) coupled to the epifluorescence path of the microscope, alongside their characteristic large somata and bi- or multipolar dendritic morphology. To study the intrinsic excitability of INs, the AP waveform and the input/output firing properties of cells, electrodes were filled with an intracellular solution containing (in mM): 127 K-gluconate, 6 KCl,10 Hepes, 1 EGTA, 2 $MgCl_2$, 4 Mg-ATP, 0.3 Na-GTP, with pH

adjusted with KOH to 7.2 resulting in an osmolarity of 290-300 mOsm. Based on the Nernst equation, the estimated reversal potential for chloride (ECl) was approximately –69 mV. For these experiments the following drugs were also present in the superfusate (in μM): 10 DNQX (Tocris), 10 gabazine, and 50 D-APV (all from Tocris).

To record GABAergic uIPSCs from paired recordings, we used a “high chloride” intracellular solution containing (in mM): 65 Kgluconate, 70 KCl, 10 Hepes, 1 EGTA, 2 $MgCl_2$, 4 Mg-ATP, 0.3 Na-GTP; again, the pH was adjusted to 7.2 with KOH and resulted in an osmolarity of 290-300 mOsm. For this solution, the ECl was calculated to be at -16 mV based on the Nernst equation, which means that when clamping the cell at -70mV, activation of GABAA receptors resulted in inward currents. We confirmed that the currents were GABAergic by demonstrating they were unaffected by DNQX (10 μM) (Tocris Bioscience) and extinguished by gabazine (10 μM; data not shown). In most paired-recording experiments the ACSF was left drug-free as INs were reliably targeted. For extracellularly evoked IPSCs, a tungsten electrode wired to a battery powered stimulus isolation unit (A.M.P.I.) was used, and recordings were made in the presence of DNQX (10 μM). To avoid spiking of the recorded pyramidal cell during stimulation the voltage-gated sodium channel antagonist (QX-314, 5 mM) (Tocris) was added to the intracellular solution. Signals were amplified using a Multiclamp 700B patch-clamp amplifier (Axon Instruments), digitized with a Digidata 1440A (Axon Instruments), sampled at 50 kHz and filtered at 2 kHz or 10 kHz respectively for voltage and current clamp recordings.

Stimulation protocols: pClamp v. 10.3 (Axon instruments) was used to record the signal and generate stimulation protocols. All voltage-clamp protocols contained a 5 mV step used to monitor the series resistance (Rs), which was kept under 25 MΩ. Recordings in which the Rs had deviated by more than 25% were discarded. For paired recordings, a brief pulse was used to elicit a single action current, followed by a train of 5 pulses at 50 Hz. This pattern was repeated every 5s (0.2 Hz). For extracellular stimulation, a single pulse was elicited every 10s (0.1 Hz).

DSI: DSI was induced in voltage clamp by holding the post-synaptic cell at 0 mV for 5 s in the post-synaptic cell. Because we encountered high failure rates and no clear trend in use-

dependent plasticity (see Fig. 6 in results), we averaged the IPSC amplitudes of the entire train to obtain a single amplitude for each sweep, allowing us to have stable baselines and time-courses following depolarisation. Between three and five depolarisations were performed on each cell, separated by at least 2 min.

AM251 pharmacology: in experiments using the CB1 antagonist AM251 (3 μM)(Tocris and Adooq biosciences), the drug (diluted in DMSO, less than 0.1% in ACSF) was added directly to the resting chamber. Indeed, AM251 can take over 40 min to act fully (Báldi *et al.,* 2016), and the chamber must be extensively rinsed between recordings, making time-courses in paired recordings particularly difficult. Therefore, we took advantage of the first connected pair of a recording session to add the drug in the superfusate, obtaining a few time-course experiments.

WIN 55,212-2 pharmacology: the CB1 agonist WIN 55,212-2 was used at 3 μM in experiments in which IPSCs were elicited with an extracellular electrode. In a preliminary study on the effect of the drug on paired recordings we used several concentrations (1 μM, 500 nM and 150 nM). We also included bovine serum albumin (BSA; 0.2%) in the ACSF because the drug is highly lipophillic and BSA is thought to facilitate drug delivery. Between each recording, the tubing and chambers were rinsed for at least 10 min with hot water (50°C).

Data analysis

AP waveform and firing profile: Using an adapted script in MATLAB (Mathworks) courtesy of Jean Simmonet (Humboldt University of Berlin). The average trace (20 sweeps) of a single spike was differentiated and plotted against Vm. The threshold was defined as the first moment in which the slope of the action potential crosses a threshold typically set at 30 mV/ms, then determining the rise time and peak of the AP. The rest of the analysis was performed in Clampfit 10 (Molecular Devices). The adaptation coefficient was determined at max firing rate, by dividing the steady state spike instantaneous frequency (last two APs of depolarising step) by the initial instantaneous frequency (first two APs of depolarising step). Input resistance was measured in the late portion of the membrane potential relaxation from a step current injection of –100 pA, while the membrane time constant (tau) was obtained

from fitting a single exponential to the early portion of the step until relaxation. Using these parameters, the capacitance was obtained arithmetically (C=tau/R). Vrest was measured during stable current clamp recordings without current injection. The relative variability of the inter-spike interval was determined by computing the coefficient of variation of the times between two consecutive APs. The full width at half maximum (FWHM) was determined by determining the width of the AP at half of it's total amplitude (from threshold to peak).

uIPSC analysis: uIPSC amplitudes were obtained using a custom script in MATLAB. Failures were defined as any value inferior to twice the standard deviation of the noise (usually around 7 pA) and were included in all relevant analyses.

Asynchronous release: custom written software (Detecta, courtesy of J. R. Huguenard, Stanford University - available online) was used to detect the timing of asynchronous and spontaneous events. The software uses an algorithm to differentiate (dV/dt) the trace on a short time window, defining events as moments where dV/dt crosses a threshold for a specific amount of time. Subsequently, events were binned in 10 ms bins and averaged across sweeps to obtain an average instantaneous frequency.

Statistical Analysis

All statistical analysis were performed in Prism (GraphPad). The normality of data was systematically tested with a D'Agostino and Pearson omnibus normality test. When normal, two datasets were compared using independent t-tests. When more than two data sets were compared, one-way ANOVAs and two-way ANOVAs were used. In situations where data was not normal or samples were small, we used non-parametric tests (Mann Whitney t-test and Kruskal-Wallis test followed by Dunn's multiple comparison test for more than two groups, respectively) unless stated otherwise. Time courses were compared using non-linear fitting and comparison of the resulting fitting parameters. Differences were considered significant if $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$) and means are always presented with the SEM.

RESULTS

CB1 is highly expressed in secondary visual cortex

CB1 are known to modulate neurotransmitter release in virtually all brain areas (Mechoulam & Parker, 2013). Morphological, biophysical and synaptic properties of CB1 IN-PC

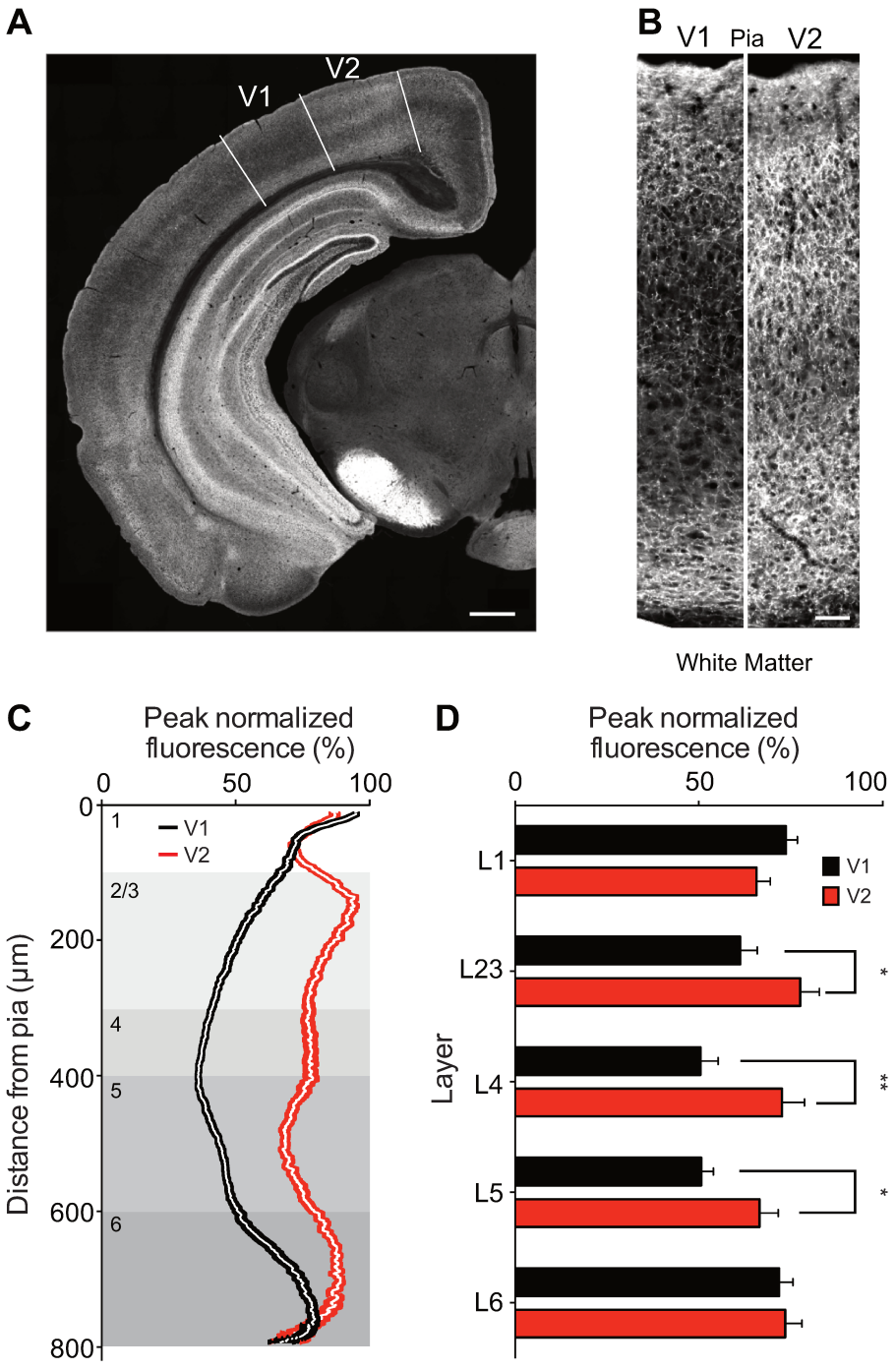

Figure 1. CB1 expression is higher in L2/3, 4 and 5 of V2 than in V1. (**A**) CB1 immunohistofluorescence (IHF), coronal slice (20 μm thick) with V1 and V2 delimited. Scale: 500 μm (**B**) Side to side comparison of CB1 IHF between V1 and V2, with cutouts obtained from the same slice, using the same acquisition settings. Scale: 50 μm. (**C**) CB1 fluorescence pattern from pia to white matter in V1 and V2, normalised to the peak of fluorescence in either region on the same slice (n=9 slices on 3 animals). Layers are delimited by white/grey boxes, with numbers in top left corners. (**D**) Data from (**C**) binned into cortical layer. Error bars are SEM. *p<0.05 and **p<0.01; n=9.

connections have been well characterised in the hippocampus, but the situation is less clear in the cortex. It has been demonstrated that the expression of CB1 is higher in V2 than in V1 (Yoneda *et al.,* 2013). However, the exact expression across layers in V1 and V2 has not yet been examined. We therefore sought to describe this pattern in more detail using CB1 immunofluorescence in wild-type mice (Fig. 1A and 1B). We observed a fibrous mesh of CB1, which has also been previously described (Bellochio *et al.,* 2010). In V1 the intensity of peak-normalised fluorescence gradually decreased from L1 to L4 (respectively 95±1 % and 35±1 %, n=9 for all), and from there steadily increased until the bottom of L6 (80±2 %; Fig. 1 C, black). In contrast, in V2 the peak-normalised fluorescence from pia to white matter was always comprised between a maximum in L2/3 and a minimum in L5 (respectively 95±1 % and 70±1 %; Fig. 1 C, red line). To compare the laminar expression between the two areas, we binned this data into distances corresponding to cortical layers (Fig. 1D). We found that the level of expression of CB1 was higher in L2/3, 4 and 5 of V2 than in corresponding layers of V1 (respectively $p<0.05$, $P<0.01$ and $p<0.05$; two-way ANOVA with correction for multiple comparisons). While there was no significant difference in CB1 expression between layers of V2, in V1 there was less receptor in L4 and 5 (respectively 51±5 % and 51±3 %) than in L1 (74±3; $p<0.01$, $p<0.01$, respectively) and L6 (72±4 %; $p<0.05$ and $p<0.05$, respectively; Fig. 1D). There is therefore a starkly contrasting pattern of CB1 expression in primary and secondary visual areas, with differences largely attributed to a relative scarcity of receptor in L2/3-5 of V1.

A CB1 agonist affects eIPSCs in V2, but not V1

To test whether CB1-dependent modulation of inhibitory responses were more strongly modulated in V2 than V1, we recorded from large layer 5 PNs and extracellularly evoked monosynaptic inhibitory postsynaptic currents (IPSCs) by blocking AMPA-kainate mediated neurotransmission (DNQX, 10 µM), and monitored the change in amplitude triggered by the CB1 agonist WIN 55,212-2 (3 µM; Fig. 2A). We found that although in V2 the drug caused a significant decrease in amplitude, there was no effect in V1 (Fig. 2B-C). The difference can therefore likely be explained by the higher amount of CB1+ fibres in L5 of V2 than V1. Extracellular stimulation indiscriminately activates axonal fibres from many interneurone subclasses, including those that do not express CB1Rs on their terminals. In the preliminary phase of this study, we could not discriminate and thus target INs expressing CB1. We then established a collaboration with Dr. Oliver Schlüter (Univ. Pittsburgh, USA) and used CB1-

tdTomato mice, in which neurons expressing CB1Rs could be visualized. Indeed, the small magnitude of the decrease in V2 (ca. 30 %) is to be contrasted with the full extinguishing of response caused by the agonist on paired CB1 IN -> PC recordings, even at nanomolar concentrations (albeit in a different layer) (Fig. 2D). This finding emphasizes the use of this mouse line as fundamental for understanding the connectivity properties of CB1-INs and thus for the prosecution of our study. Nonetheless, these data provide evidence for significant sensitivity of evoked IPSCs to CB1 activation in deep cortical layers of different visual areas.

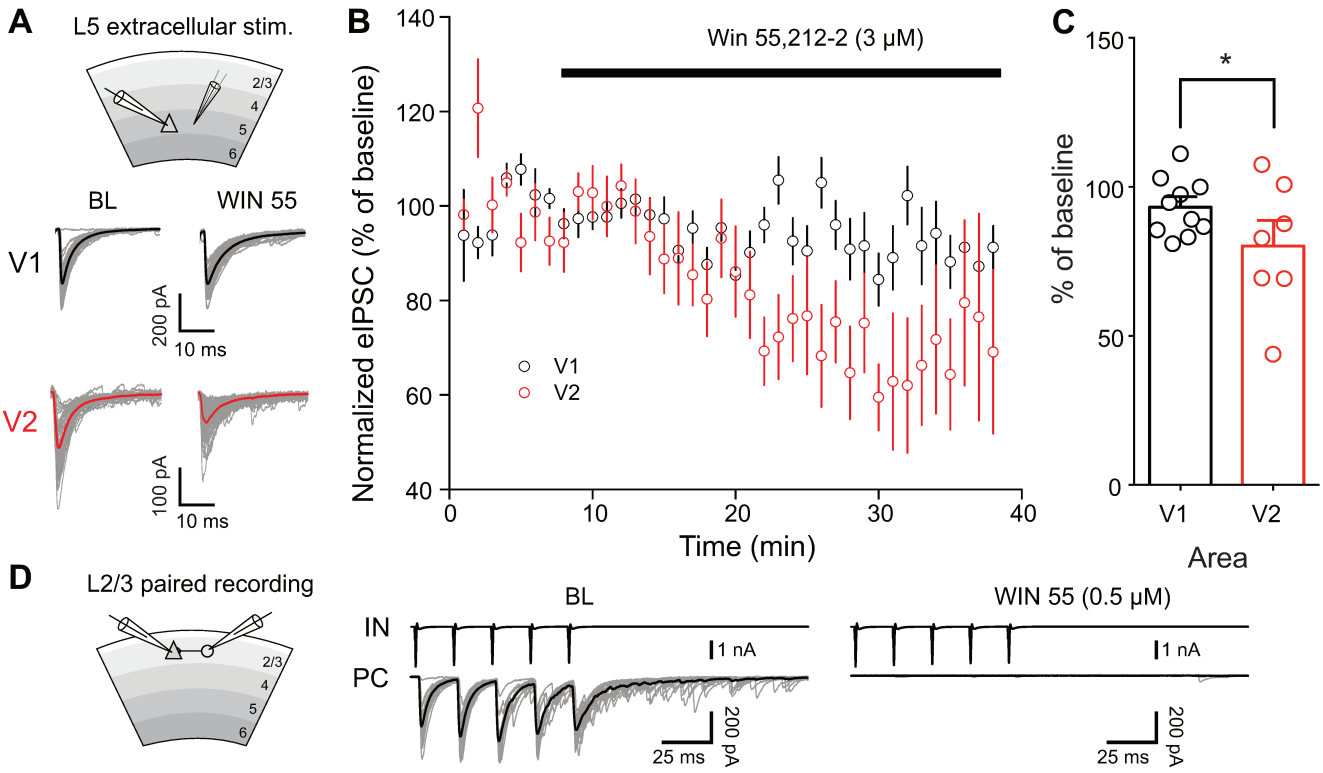

Figure 2. eIPSC recorded in L5 pyramidal neurones are affected by the CB1 agonist WIN 55,212-2 in V2, but not V1. (**A**) Experimental setup and representative traces in V1 (top, black) and V2 (bottom, red) during baseline (left) and 15 min after application of WIN55,212-2 (**B**) Time course (binned into single minutes) following WIN55,212-2 application (black bar above the graph). (**C**) WIN55,212-2 had more effect in V2 than in V1 ($p<0.05$, V1: n=10, V2 n=7; unpaired t-test). Values were obtained by averaging values for 5 min, 15 min after WIN55,212-2 wash-in. (**D**) uIPSCs from CB1 INs recorded in L2/3 are fully extinguished by sub-micromolar concentrations of WIN55,212-2

Distribution of CB1 INs in V1 and V2

CB1 is most prominently expressed on inhibitory axonal fibres. Hence, the difference in CB1 expression pattern between V1 and V2 shown in Figure 1 could be explained by: *i)* different numbers of CB1 INs between the two areas, *ii)* distinct fibre density originating from the same interneurons, *iii)* different level of expression of CB1 in each given axon. However, CB1 is not expressed exclusively in GABAergic interneurons. Therefore, in order to count CB1 INs selectively, we crossed the CB1-tdTomato with GAD67-GFP mice. The fluorescent reporters were under the control of the endogenous promoters of the CB1 and GAD67 genes. We thus counted cells which expressed both tdTomato and GFP reporters in these CB1-tdTomato::GAD67-GFP mice to measure the density of CB1 INs in each layer of the two areas (Fig. 3A). We found that virtually all the CB1+ cells in upper cortical layers (including L2/3) co-expressed GAD67 (Fig. 3A,1; orange arrows). However, in deeper layers (most notably L6), we found evidence of cells expressing CB1, but not GAD67, meaning they are likely putative excitatory cells expressing CB1 (Fig. 3A,2; red arrows). Importantly, CB1+ cells did not co-localise with PV, the other major type of BC (Fig. 3B). In both areas, the density of CB1 INs in the CB1-tdTomato mice followed the same laminar pattern, and there was no difference in density between the two areas (Fig. 3C; all $p>0.05$, t-tests with correction for multiple comparisons). The distribution of soma in both areas (Fig. 3) recapitulate well the immunofluorescence pattern observed in V1 (Fig 1B and C), with high density in L2/3 (V1: 26±2 cells/mm, V2: 30±2 cells/mm), and a minimum density in L4 (7±1 cells/mm in V1 and 5±1 cells/mm in V2; $p<0.001$ for both, Kurskall-Wallis ANOVA corrected for multiple comparisons). Unlike the immunohistofluorescence pattern, however, there are less CB1 INs in deeper than superficial layers, particularly L6 (V1:12±2 cells/mm and V2:13±2 cells/mm, $p<0.001$ and $n=15$ for both when compared to L2/3). This discrepancy could be due to the presence of non-IN cells expressing CB1 in these layers (Fig. 3A,2). Taken together, these observations make the case that the higher CB1 expression observed in the internal layers of V2 cannot be accounted for by differences in the number of CB1 INs, and therefore prompt the question of differential innervation patterns in the two visual areas.

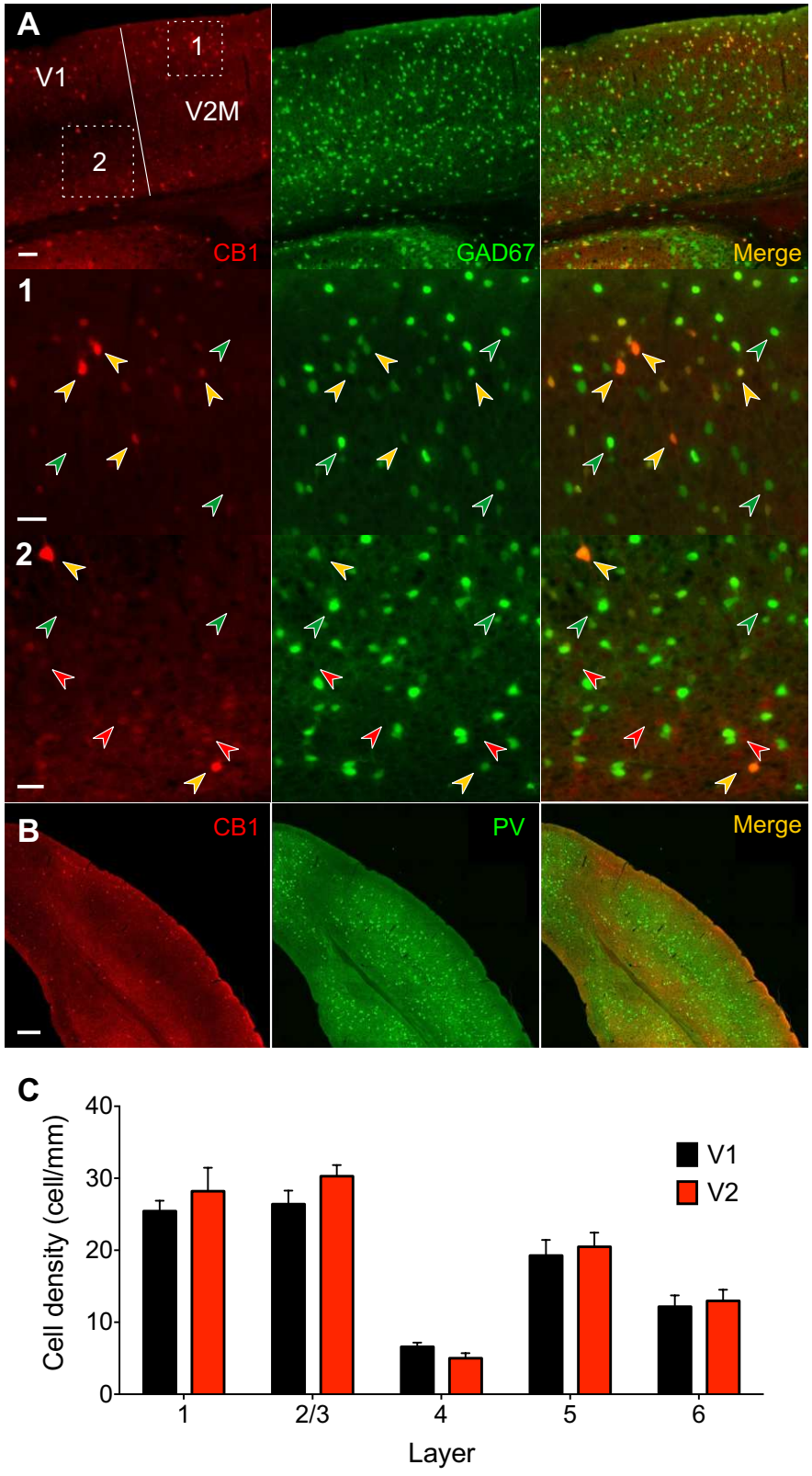

Figure 3. CB1 IN densities do not differ between V1 and V2. (**A**) Co-localisation of tdTomato (red, left panel) and GFP (green, middle panel) immunoreactivity in a CB1-tdTomato::GAD67-GFP mouse. Cell density was established by counting cells co-expressing the two reporters (superimposed epifluorescence image, right panel). Scale: 50 µm (**1**) Zoom from insert 1 in (A) CB1+ cells in upper cortical layers virtually always co-expressed GAD67. Scale: 20 µm (**2**) In deeper layers, some CB1+ cells did not express GAD67, and are putative CB1 expressing excitatory neurones. Scale: 20 µm (**B**) In the cortex, no CB1+ cell co-localised with parvalbumin, the marker of another major basket cell type. (**C**) Cortical laminar density of CB1 INs in V1 and V2. Mean±SEM, n=15 for each condition.

L4 of V2 is highly innervated by L2/3 CB1 IN axons

It is known that members of major IN classes can exhibit morphological diversity (Jiang *et al.,* 2015). We therefore sought to ascertain whether the difference in CB1 expression pattern was due to a variation in morphology of CB1 INs in V1 and V2. We took advantage of the CB1-tdTomato mouse to examine the morphology of CB1 INs, in which the brightest cells of L2/3 were certified CB1 INs (Fig. 3). We performed whole-cell patch clamp recordings from coronal acute brain slices during one hour or more, followed by post hoc morphological recovery of each neurone with an optimised DAB staining method that allowed us to reconstruct the fine axonal arborisations of mature cortical INs (for details refer to methods). We reconstructed 7 CB1 INs in each area (Fig. 4), which we scaled, aligned, and overlapped to create heat maps of axonal projections (Fig. 5A-B). Strikingly, in V2 the reconstructed axonal branchings (Fig. 4, red tracing) exhibited hotspots in L2/3 and L4 (Fig. 5B), while in V1 there were few projections to deeper cortical layers (Fig. 5A). We found no significant difference in relative axonal density per layer between L2/3 of V1 and V2 (respectively 260±54 and 324±29; $p=0.32$ and $n=15$ for both V1 and V2, unequal variance t-test corrected for multiple comparisons; Fig. 5C). On the other hand, we found higher relative axonal density in L4 and 5 of V2 (220±25 and 62±15) than in corresponding layers of V1 (47±20 and 10±6; $p<0.001$ and $p<0.01$), echoing the difference in pattern described in Fig. 1. Nonetheless, this pattern could also be affected if CB1 INs of V2 had different axonal lengths, and although there is a trend for longer axons in V2 than V1 (12±2 mm vs. 8±5 mm), it was not found to be significant ($p=0.06$; unpaired t-test; Fig. 5D). We then performed the same analyses for dendrites (Fig. 5E-H), first producing heat maps (Fig. 5E and F), and then comparing the relative density between the layers of V1 and V2 (Fig. 5F). We found that there was virtually no dendrites in L4 of V1, compared to a low relative density in L4 of V2, although this difference was not statistically significant (respectively 0±0 and 18±6; $p>0.05$, unequal variance t-test corrected for multiple comparisons; Fig. 5F). In other layers, relative dendritic density was comparable between V1 and V2 (all $p>0.05$). Putting together the data from Fig. 1, 3, 4 and 5, we can conclude that CB1 IN morphology contributed to differences in laminar expression of CB1 between V1 and V2.

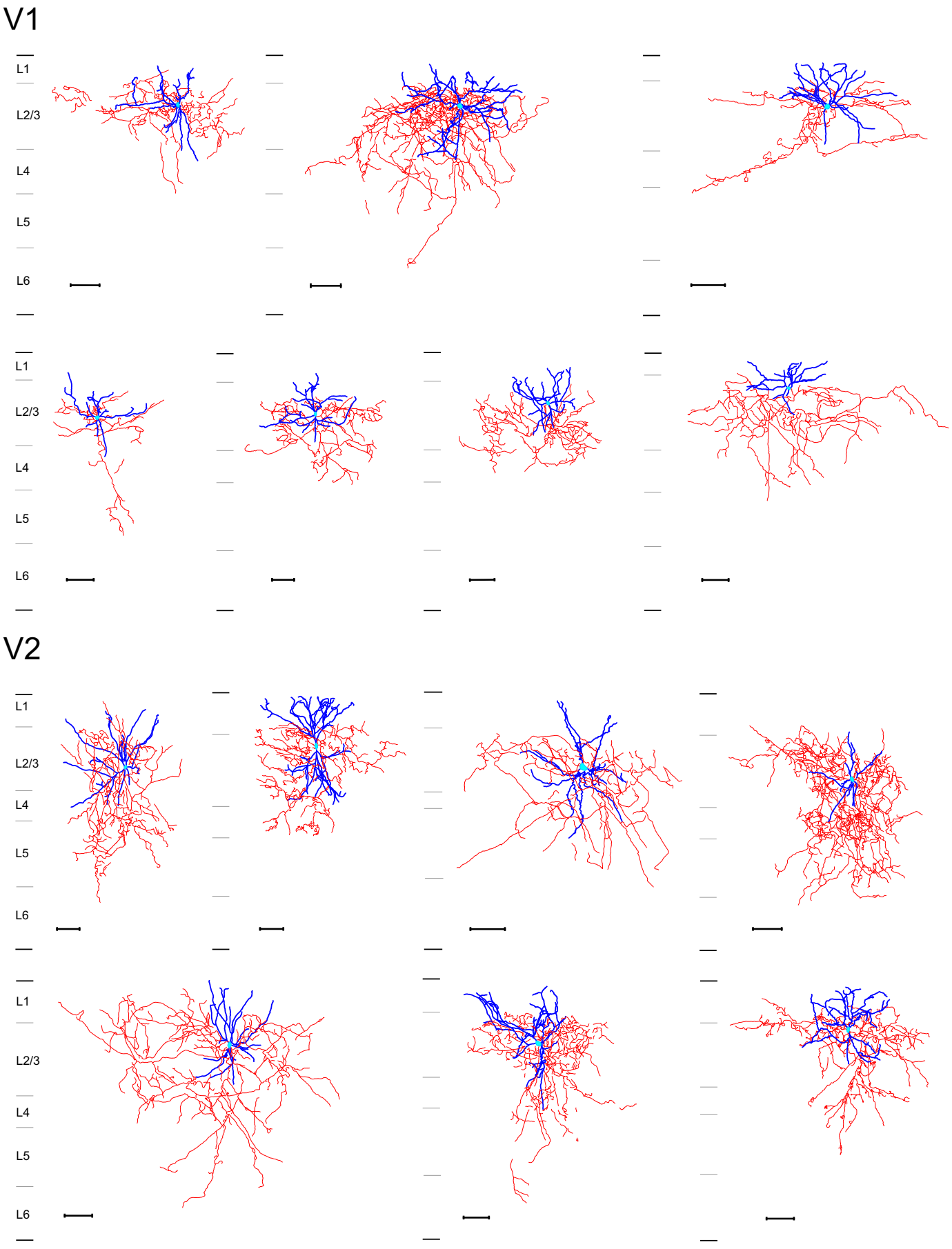

Figure 4. Individual reconstructions of CB1 INs in V1 and V2. Dendrites are in blue, axons in red, and soma in turquoise. Scale bars = 100 µm.

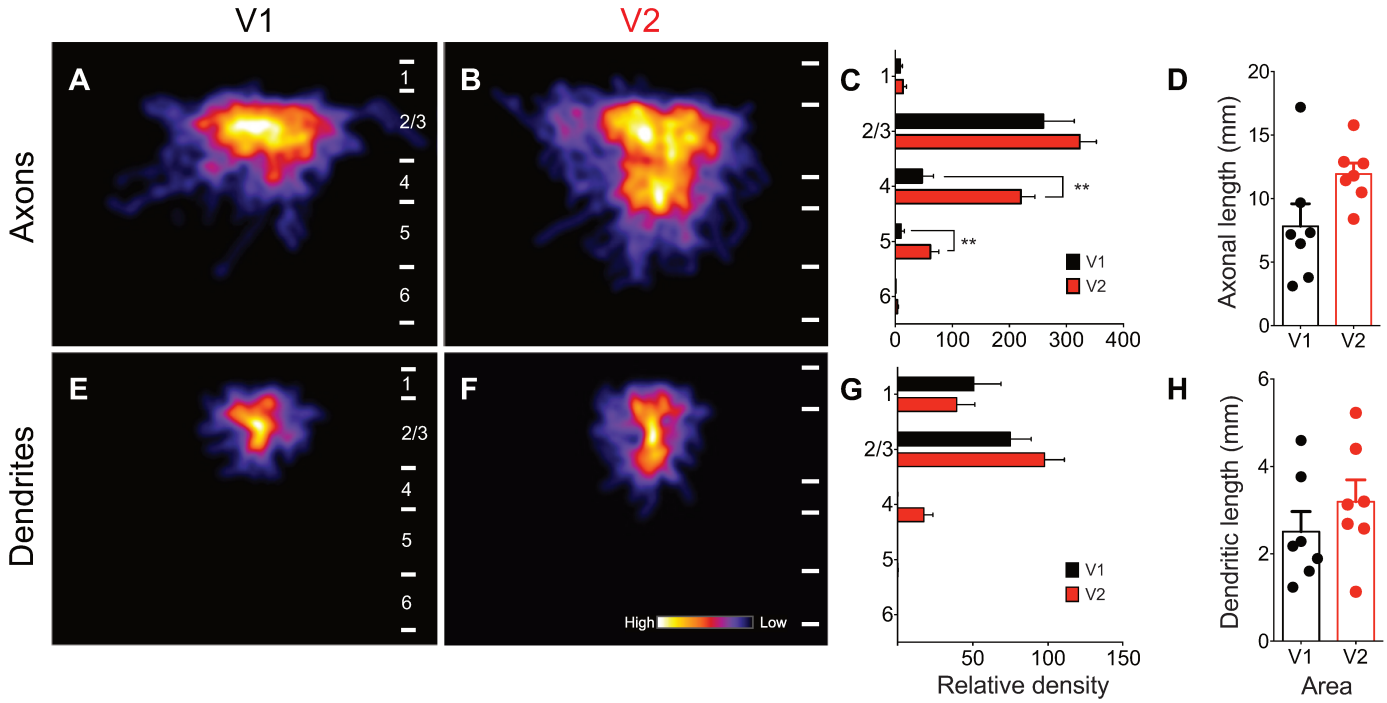

Figure 5. L2/3 CB1+ IN exhibit distinct morphological properties in V1 and V2. (**A**) Heat map of of CB1 IN axonal density in V1 (n=7). Numbers designate cortical layers. The colour scale can be found in (F). There are no scale bars because the image contains several superimposed images with different scale bars. (**B**) Corresponding axonal heat map in V2 (n=7). White lines indicate cortical layers, which are numbered on the neighbouring panel (**C**) Relative axonal density, normalised by layer thickness (see methods). Means±SEM. *p<0.05 and **p<0.01 (**D**) Total axonal length in the two areas (means±SEM). (**E-H**) Same as (A-D) but for dendrites.

CB1 INs of V1 and V2 share intrinsic firing dynamics

We reliably targeted CB1 INs (Fig. 3A) with different arborisations in V1 and V2 (Fig. 4 and 5), leading us to ask whether these constituted two subtypes, with possible differences in passive and active properties. We performed patch-clamp recordings of CB1 INs in current clamp. CB1 INs from V1 and V2 did not display differences in their passive properties (e.g. Vrest, tau membrane time constant, input resistance - see Tab. 1). Analysis of the shape of action potentials evoked by minimal current injection step (Fig. 6A) showed that the AP threshold, rise time, peak and full width at half maximum (FWHM) of CB1 INs of V1 and V2 were not significantly different (Fig. 6B-E; Tab. 1). In addition, we studied firing dynamics of CB1 INs by constructing input-output relationships, resulting from delivering constant current pulses (800 ms duration) of increasing amplitude, and measuring the resulting firing rate (frequency-current or *f-i* curves; Fig. 6F). The increase in spike frequency associated with increasing current step injection was the same in both areas (p=0.08, V1: n=17, V2: n=11; two-way ANOVA; Fig 5F). The coefficient of variation (CV) of the inter-spike interval (ISI), was not found to be different between V1 and V2 (Fig 6G, Tab. 1). Consistent with the

literature (Freund & Katona, 2007), we found evidence of spike adaptation in both V1 and V2 CB1 INs, and there was no difference between the two areas (Fig. 6H; Tab. 1). A comprehensive list of the parameters under scrutiny can be found in Table 1. All of them indicate that CB1+ INs in L2/3 of the mouse visual cortex have homogeneous intrinsic passive and active properties and characteristics of adapting INs (Glickfeld & Scanziani, 2006).

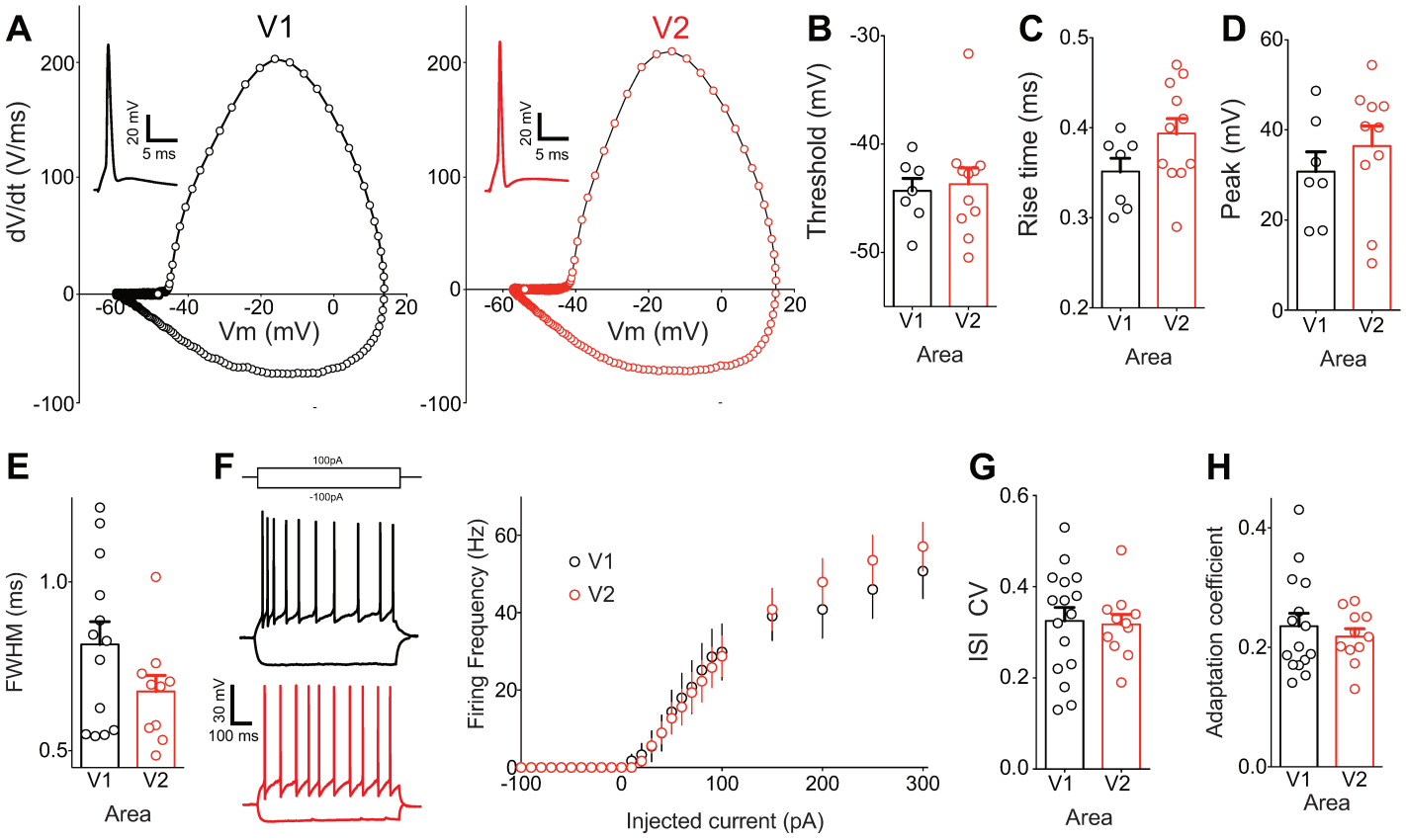

Figure 6. L2/3 CB1 INs exhibit similar active and passive single-cell properties in V1 and V2. (**A**) Phase plane plots of the derivative of a single AP (insert) evoked by minimal current injection step (1.5 ms) in V1 (black) and V2 (red). (**B**) Action potential (AP) threshold (**C**) AP rise time (**D**) AP peak (**E**) AP full width at half maximum (**F**) Injection of negative and positive current in V1 (black trace) and V2 (red trace), with the resulting frequency/injected current curve (right) (**G**) Coefficient of variation of the inter-spike interval of spikes elicited by depolarising pulses. (**H**) Adaptation coefficient corresponding to the ratio of the interval between the two first and two last spikes elicited by a depolarising pulse. Although Vrest was not different between V1 and V2 (see Tab. 1), to avoid biases arising from cell to cell variability in Vrest each intrinsic excitability property was measured at a Vhold of -65 mV. Plots from (B) to (H) display mean±SEM. Values, sample size and statistics are summed up in Tab. 1.

Table. 1 Comparison of intrinsic passive and active membrane properties CB1 INs in V1 and V2

	V1			V2			Stats	
	Mean	**±SEM**	**n**	**Mean**	**±SEM**	**n**	**p**	**Test**
Vrest (mV)	-65.00	1.88	10	-59.88	2.32	8	p=0.10	unpaired t-test
R_{in} (MΩ)	268	33	14	283	44	11	p=0.80	Mann-Whitney
Cm (pF)	84	9	14	89	11	11	p=0.75	unpaired t-test
tau (ms)	19.90	1.42	14	21.43	1.70	11	p=0.46	Mann-Whitney
AHP (mV)	5	1	15	6	1	11	p=0.34	unpaired t-test
Rheobase (pA)	739	71	14	671	45	11	p=0.07	unpaired t-test
Threshold (mV)	-44	1	7	-44	1	11	p=0.76	unpaired t-test
Rise Time (ms)	0.35	0.02	7	0.39	0.02	11	p=0.11	unpaired t-test
Peak (mV)	30.75	4.40	7	36.42	4.47	11	p=0.40	unpaired t-test
FWMH (ms)	0.82	0.07	13	0.68	0.05	10	p=0.13	unpaired t-test
ISI CV	0.33	0.03	16	0.32	0.02	11	p=0.85	unpaired t-test
Adapt Coef	0.24	0.02	15	0.22	0.01	11	p=0.53	unpaired t-test

Intra- and infra-layer connectivity patterns of CB1 INs in V1 and V2

The anatomical and morphological results that are illustrated in Figures 1, 3, 4 and 5 indicate that layer 2/3 CB1 INs of V2 project extensively in deeper cortical layers. Remarkably, this projection pattern is not evident in V1. We therefore sought to determine if the probability of connected pairs within and across layers was different in V1 and V2. We performed simultaneous whole-cell pair recordings between presynaptic CB1 INs in layer 2/3 and postsynaptic PNs either in layer 2/3 or layer 4, in V1 and V2 (Fig. 7A-B). In both areas, we reliably obtained connected pairs when the post-synaptic PN was also located in L2/3 (Fig. 7A; connected pairs: 43/124 vs 37/77 in V1 and V2, respectively). However, when the post-synaptic cell was located in L4, the likelihood of obtaining connected pairs was very low in

V1 while remaining high in V2 (5/70 and 27/74, respectively). These connectivity rates are largely consistent with the axonal morphologies of these cells (Fig 4, 5A-B) and the distribution of CB1 in these areas (Fig. 1). Also, because of the low sample size of L2/3-4 connections in V1, we do not discuss them further, although corresponding values can be found in Table 2**.** These results confirm that layer 2/3 CB1 INs possess remarkably different functional projection patterns in the two visual areas. In particular, in V2, CB1 INs connect with PNs in both superficial and deep layers with equal probability.

Synaptic properties of CB1 INs are different in V1 and V2

We then explored the synaptic properties of CB1 INs in connected pairs with postsynaptic PNs located in either L2/3 or L4 in V2, and in L2/3 of V1. Remarkably, we found that the amplitudes of unitary IPSCs (uIPSCs) were ~6 fold larger onto PNs in L2/3 of V1 than V2 ($p<0.001$; Kurskal-Wallis ANOVA with multiple comparisons; Fig 7C and Tab. 2). Similarly, failure rate and variability (measured as coefficient of variation, or CV) of uIPSCs was much lower in CB1 IN-PN connections in L2/3 of V1 than V2 ($p<0.01$ for both; Kurskal-Wallis ANOVA with multiple comparisons; Fig. 7D and Tab. 2). Importantly, failure rates in L2/3 of V1 were consistent with those described in other areas (Galaretta *et al.,* 2008). It is worthwhile to note that we never encountered a connected pair that did not fail to some extent in V2 L2/3, and in that area we also observed a cell with a silent synapse that was only revealed by a 50 Hz train of presynaptic spikes (Fig. 7D). Curiously, we did not observe any differences in the paired pulse ratio (PPR) ($p=0.43$, one way ANOVA; Fig. 7E; Tab. 2). This is atypical for high failure rate synapses, and will be discussed further below. Surprisingly, connected pairs between CB1 INs and postsynaptic PNs located in Layer 4 of V2 were strong and reliable, as opposed to connections formed by the same interneurons, but in L2/3 ($p<0.05$ Kurskal-Wallis ANOVA, Fig. C-E, Table 2). Indeed these infra-laminar connections (from L2/3 to Layer 4 in V2) showed similar amplitudes, failure rate and CV of CB1 IN-PN pairs in L2/3 of V1 ($p>0.05$ Kurskal-Wallis ANOVA, Fig. C-E, Table 2. In sum, connections in L2/3 of V2 were the weakest and most unreliable, when compared to the neighbouring area or layer. In a few cases in V2, we kept the same IN and changed the layer of the postsynaptic target to obtain sequential triple recordings (Fig. 7G). Although sample size is low, we found that in 2 out of 4 consecutive pairs, uIPSCs were stronger (almost seventy-fold, in one case) and more reliable in L4 than L2/3, suggesting target-specific presynaptic release properties (Fig. 7G).

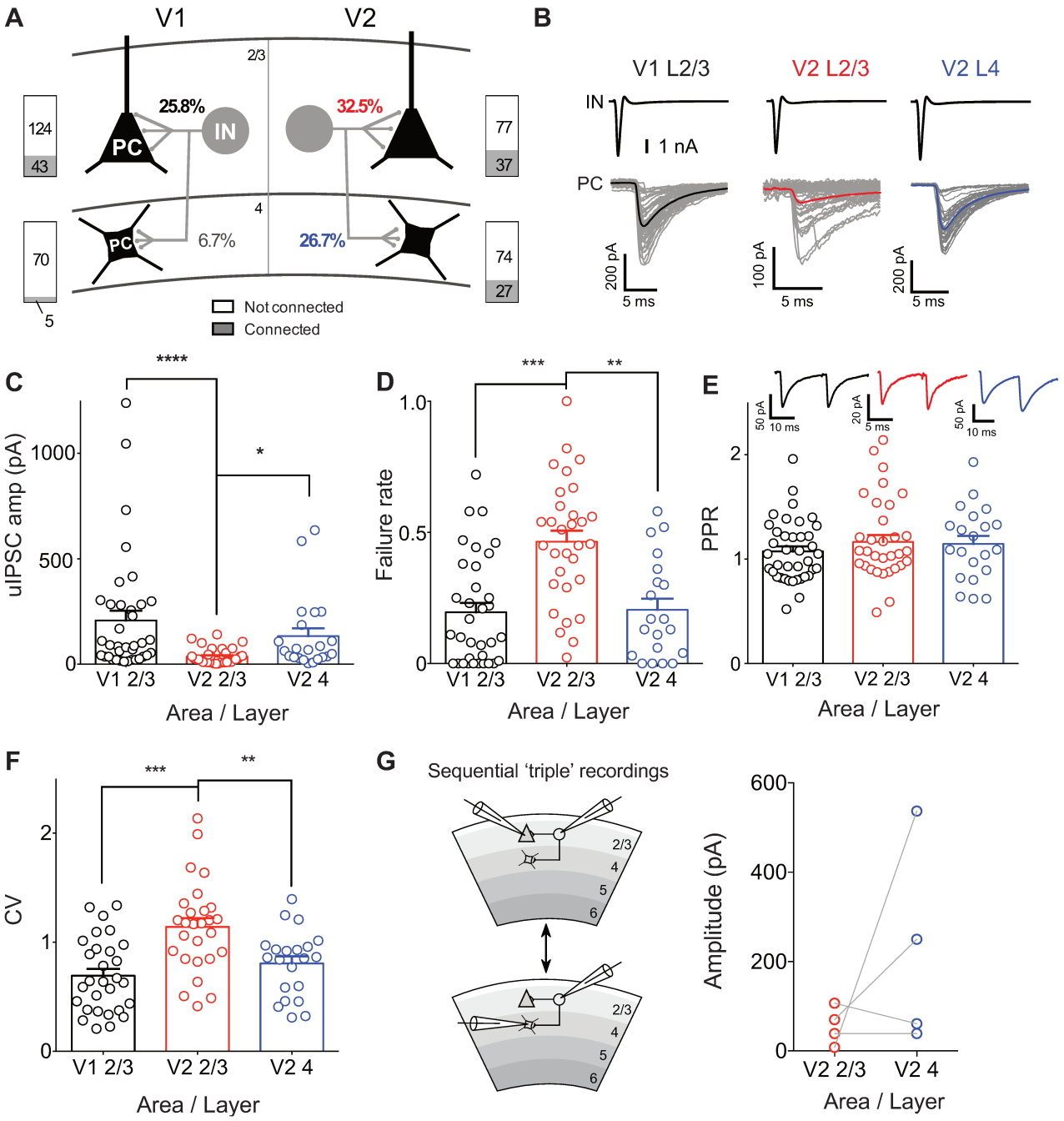

Figure 7. CB1 INs give rise to different uIPSCs in postsynaptic principal cells (PC) depending on the visual cortical area and layer of the target. Values, sample size and statistics are in the text or in Tab. 2. (**A**) Rates of IN → PC connectivity within L2/3 and to L4 in both V1 and V2. (**B**) Representative traces from evoked uIPSCs in the postsynaptic PC (presynaptic action current above, in black) in monosynaptically connected pairs in V1 L2/3 (avg. in black), V2 L2/3 (avg. in red) and V2 L4 (avg. in blue) principal cells. Individual sweeps (T=30) in light grey. (**C**) uIPSC amplitude population data. We found lower amplitudes in V2 L2/3 than in V2 L4 and V1 L4 (*p<0.05, ****p<0.0001)(**D**) Failure rate population data. V2 L2/3 connections were found to fail significantly more than in V2 L4 and V1 L2/3 (***p<0.0001, **p<0.01). (**E**) Average plot of the paired-pulse ratio (PPR). There was no difference in PPR between the three conditions. Representative average traces in V1 L2/3 (black), V2 L2/4 (red) and V2 L4 (blue). (**F**) Average plot of uIPSC amplitude coefficient of variation (CV). The CV was higher in V2 L2/3 than in V2 L4 and V1 L2/3 (***p<0.0001, **p<0.01). (**G**) Scheme of sequential triple recordings performed in V2 in which the presynaptic IN in L2/3 was kept while the post-synaptic target was changed (Right) Trend of larger amplitudes in V2 L4 than V2 L2/3, despite coming from the same pre-synaptic cell (n=4). Black circles represent V1 L2/3 connections. V2 L2/3 is in red, and V2 L4 is in blue. All bar graphs represent mean±SEM.

Overall, these data indicate that inputs from CB1+ INs in the visual cortex have area- and layer-specific properties. Given the marked difference in CV and failure rate, it is likely that the synapses from these interneurons possess visual-area, layer- and target-specific differences of presynaptic release of GABA.

Table. 2 Comparison of L2/3 CB1 IN → PC synaptic properties

	V1 L2/3			V1 L4			V2 L2/3			V2 L4		
	Mean	**±SEM**	**n**	**Mean**	**±SEM**	**n**	**Mean**	**±SEM**	**n**	**Mean**	**±SEM**	**n**
uIPSC amp. (pA)	207	46	37	30	13	5	35	6	31	133	36	21
Failure rate	0.19	0.03	37	0.28	0.03	5	0.46	0.04	31	0.20	0.18	21
PPR	1.07	0.04	37	1.32	0.32	4	1.17	0.07	31	1.14	0.07	21
CV	0.79	0.07	37	0.50	0.09	4	1.20	0.10	31	0.81	0.06	21

Asynchronous release of GABA is commonly present at CB1 IN→PC connections

Asynchronous release coming from CB1 or CCK INs has been well documented in the hippocampus (Hefft & Jonas, 2005). We found evidence of asynchronous release in individual IN→PC connections of all three areas, with a characteristic increase in instantaneous event frequency following repeated presynaptic stimulation (Fig. 8A-B). To compare the peak of asynchronous release, we binned the data into a 20 ms window following a train of 5 APs at 50 Hz (Fig. 8C-D). We then compared this value to the baseline frequency preceding the train, and found a significant increase in V1 L2/3 and V2 L4, but not V2 L2/3 (Fig 8D) (respectively $p<0.001$, $p<0.01$ and $p=0.2$, one sample t-tests). However, there was no difference in peak asynchronous release between the three conditions (Fig. 8C; $p=0.10$, Kurskal-Wallis ANOVA). It should be noted that due to technical constraints many cells had to be excluded from this analysis, and that the stimulation protocol that we used was not specifically designed to answer such a question - typically higher frequencies (100 Hz) and longer trains are used (e.g. Hefft & Jonas, 2005). Indeed, these patterns of stimulation did induce asynchronous release in all areas (not shown). Although it is certain

that asynchronous release is common in CB1 INs in the cortex, it remains unclear whether there are differences in asynchronous release between the three areas.

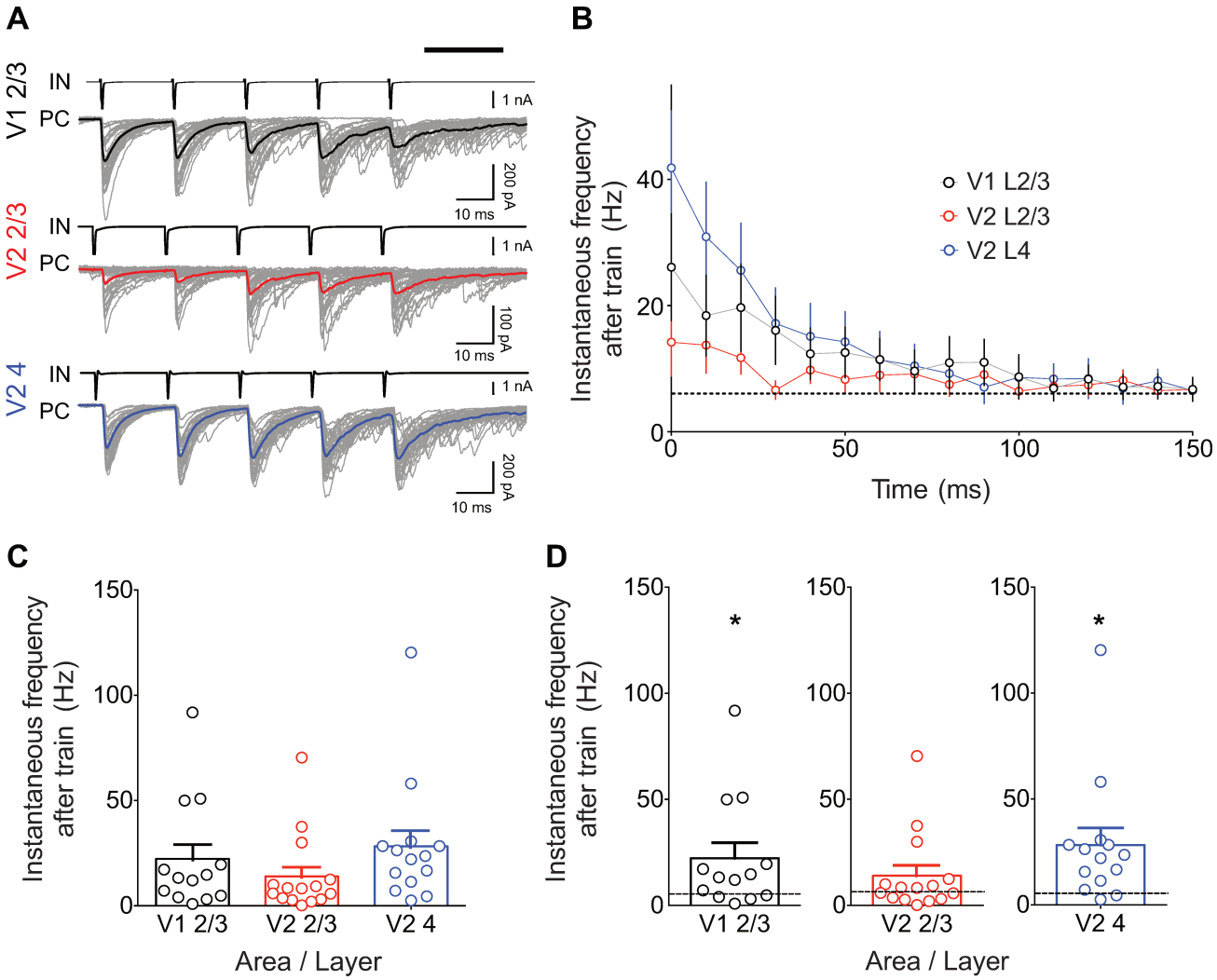

Figure 8. Asynchronous release is present in V1 L2/3 and V2 L4, but not V2 L2/3. (**A**) Representative traces in voltage clamp showing asynchronous release, observable by a marked increase in instantaneous frequency following a 5 pulse train at 50 Hz. Grey traces are individual trials and average traces are in corresponding colours: black for V1 L2/3, red for V2 L2/3 and blue for V2 L4. Presynaptic spikes of CB1 INs in voltage clamp above uIPSCs in black. The black bar on top of the panel corresponds to the window of analysis used in panel (C) and (D) (**B**) Population averages of the instantaneous frequency after the train. The dotted line represents the average instantaneous frequency during the baseline period. (**C**) There was no significant difference between peak asynchronous release (20 ms after the train) between V1 L2/3, V2 L2/3 and V2 L4 (**D**) Comparison of peak asynchronous release (20 ms after the train) with pre-train baseline frequency (dotted lines). V1 L2/3 and V2 L4 displayed significant asynchronous release, but this was not the case for V2 L2/3.

Plasticity of L2/3 CB1 IN→PC synapses exhibits specific properties in different cortical visual areas and layers

Following the observation of the differences in synaptic properties detailed above (Fig. 7 & 8), we wanted to ascertain whether there were any differences in the plasticity of these synapses. DSI is the classical electrophysiological paradigm to test presynaptic eCB modulation (Wilson & Nicoll, 2001). We reliably induced DSI by depolarising the post-synaptic cell for 5 s at 0 mV (Fig. 9A). Because of a prevalence of high failure rates (particularly in V2), using single event amplitudes resulted in occasional spurious relative amplitudes. We therefore chose to average the amplitudes of the five pulses of the 50 Hz train to minimise changes in amplitudes from sweep to sweep (Fig. 9A). This approach has been previously used to study highly failing GABAergic synapses of CCK/CB1 interneurons of the baso-lateral amygdala (Vogel *et al.,* 2016). To obtain a value of DSI comparable between our three conditions, we averaged the values of three sweeps following depolarisation (corresponding to 15 s). Although there was a trend of a stronger DSI amplitude in V1 L2/3 and V2 L4 than in the already unreliable and weak synapses in V2 L2/3, we did not find any significant difference between our three conditions ($p=0.11$, one way ANOVA; Fig. 9B). We then looked at the time-course of DSI, and found that the time it took for responses to recover to baseline amplitudes was shorter in V2 L2/3 than in V1 L2/3 and V2 L4 (Fig. 9D-E; comparison of exponential fit time constants (tau), $p<0.01$, V2 L2/3: tau= 3.6 s and n=17, V1 L2/3: tau= 10 s and n=24, V2 L4: tau= 8.3 s and n=19). It should also be noted that we were looking at changes in amplitude with relation to the baseline, and that baseline amplitudes were different between conditions (Fig. 7C), meaning DSI may effectively suppress larger quantities of charge in V1 L2/3 and V2 L4. Finally, we verified that the CB1 antagonist AM251 blocked DSI in all three synapses (Fig. 9F). This area-specific difference in plasticity kinetics reinforces the finding that V2 L2/3 synapses are functionally different from their counterparts in V1 L2/3 or V2 L4.

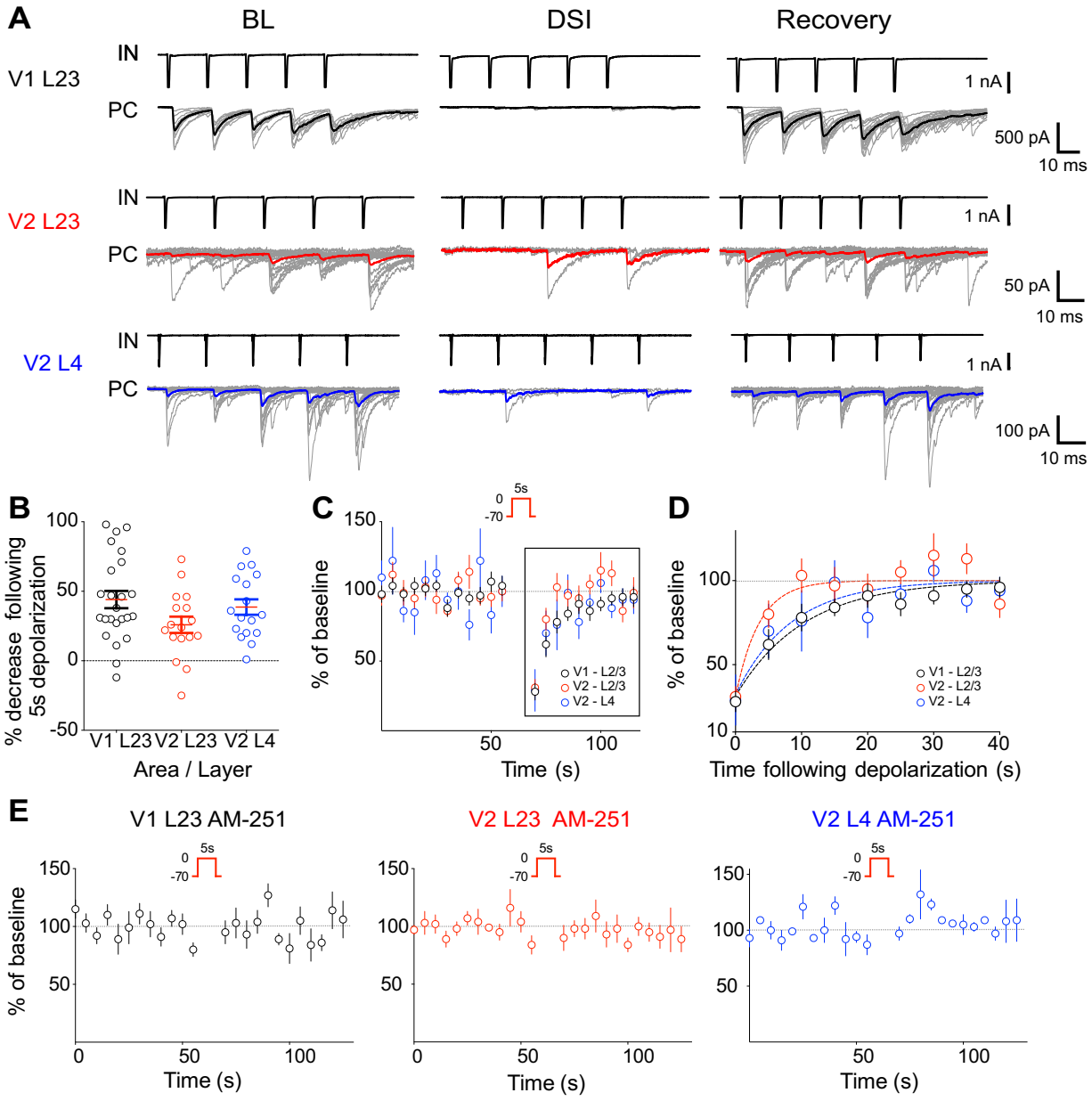

Figure 9. DSI in CB1 IN → PC pairs recovers faster in V2 L2/3 than in V1 L2/3 or V2 L4 (**A**) Representative traces in V1 L23 (top, black) V2 L23 (middle, red) and V2 L4 (bottom, blue) during the baseline (left), DSI (middle) and recovery from DSI (right). In all cases, presynaptic spikes above uIPSCs (black). Individual traces in grey and averages in the corresponding colour. Three sweeps were averaged for DSI and ten for baseline and recovery. To stabilise responses, we averaged the responses to a train to get a single sweep value (**B**) Plot of individual average uIPSC amplitude after DSI. There is no differences in the maximum DSI values between connections in V1 L2/3, V2 L2/3 or V2 L4. Values corresponding to three averaged sweeps directly after depolarisation (as in (A) DSI) as a percentage of decrease of the baseline prior to the depolarisation. Therefore positive values represent depression, while negative values represent potentiation. (**C**) Time course of average uIPSCs during baseline, DSI and recovery periods DSI in the three connections. The black dotted line represents the baseline value of 100 % (**D**) Focus on the recovery from DSI, with dotted coloured lines representing single exponential fits which were compared for analysis. V2 L2/3 recovered significantly faster than in V1 L2/3 or V2 L4 ($p<0.01$) (**E**) DSI is blocked by the CB1 receptor antagonist AM-251 (single sample t tests on max DSI with theoretical mean = BL; V1 L2/3: $p=0.53$ and $n=8$; V2 L2/3 $p=0.15$ and $n=8$; V2 L4: $p=0.72$ and $n=4$).

V2 L2/3 CB1 IN→PC synapses are under the control of tonic eCB signalling

The observation that tonic CB1 signalling can inhibit neurotransmitter release in the hippocampus is widespread (Soltesz, 2015). We therefore asked whether tonic signalling could play a role in explaining why the inhibitory synapses in L2/3 exhibit high failure rates and low amplitudes. We performed paired recordings in slices pre-incubated with the CB1 antagonist AM-251 (3 µM) (Fig. 10A). For these experiments, we only compared V2 L2/3 with V1 L2/3. Remarkably, we found that when AM-251 was present in the superfusate, the normally weak and unreliable synapses in V2 started to exhibit characteristics similar to V1, while V1 was unaffected by the drug (Fig. 10B-C). Mean control amplitudes in V2 averaged 36±6 pA, while incubation with AM-251 yielded significantly higher amplitudes of 148±51 pA (respectively n=37 and n=15, p<0.001, Mann-Whitney unpaired t-test). In V1, AM-251 had no effect on amplitudes (Ctrl: 208±46 pA and n=37; AM-251: 201±49 pA and n=15; p=0.98,

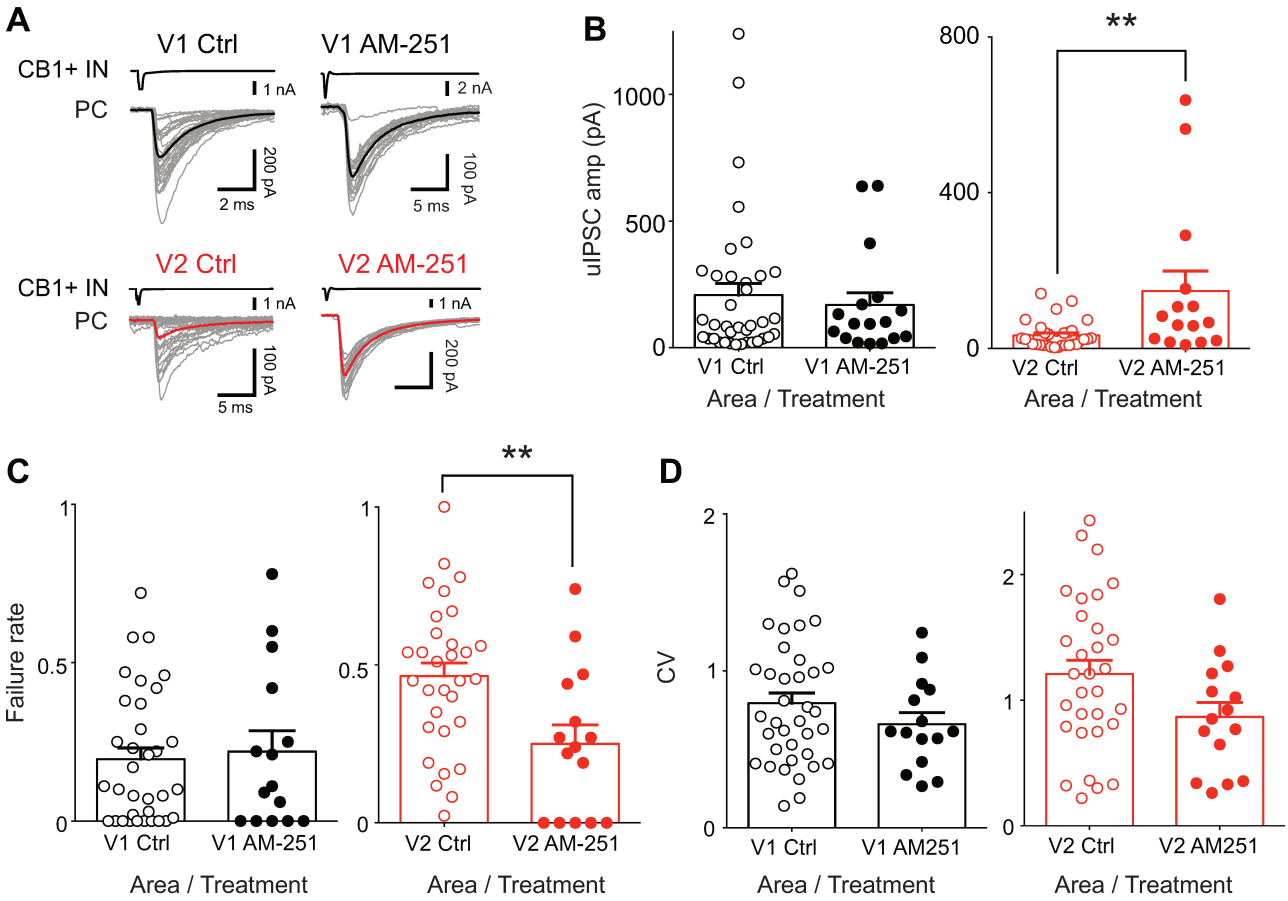

Figure 10. Tonic endocannabinoid signalling is visual area specific. (**A**) Representative traces of uIPSCs in the absence (Ctrl, left) and presence of 3 µM AM-251 (right) in V1 (top, black) and V2 (bottom, red). Presynaptic spikes above uIPSCs in black. Grey traces are individual sweeps. (**B**) Application of AM-251 increased uIPSC amplitude in V2, but not in V1. (**C**) Application of AM-251 decreased failure rate in V2, but not in V1. (**D**) The coefficient of variation of uIPSC amplitudes was not affected by AM-251 in either V1 or V2.

Mann-Whitney unpaired t-test). Strikingly, AM-251 significantly reduced the failure rate of synapses in V2 L2/3 (Fig. 10C) (Ctrl: 0.46±0.04 and n=31; AM-251: 0.25±0.06 and n=15; $p<0.01$, Mann-Whitney unpaired t-test). This was the first time that we witnessed synapses that never failed or had average amplitudes superior to 200 pA in V2 L2/3. In V1, failures remained unaffected by AM-251 (Ctrl: 0.20±0.03 and n=37; AM-251: 0.22±0.07 and n=15; $p=0.93$, Mann-Whitney unpaired t-test). However, although there was a clear trend, we did not find that AM-251 significantly affected the CV in V2 L2/3 (Fig. 10D)(Ctrl: 1.21±0.11 and n=31; AM-251: 0.87±0.12; $p=0.06$; unpaired t-test). In V1, AM-251 had no effect on the CV (Ctrl: 0.70±0.07 and n=37; AM-251: 0.66±0.3 and n=15). Taken together, these data suggest that the different synaptic properties of CB1 INs are highly likely due to the presence of tonic CB1 signalling in V2 L2/3.

PPR is an unreliable measure of presynaptic effects in CB1 IN→PC connections

Typically, paired-pulse facilitation is observed in high failure synapses and changes alongside pre-synaptic release probability. It is however known that CCK/CB1-INs can exhibit neither facilitation nor depression, despite high failure, and that AM-251 can increase amplitudes without affecting the PPR (Rovira-Esteban *et al.*, 2017). Indeed we found that AM-251 did not affect the PPR in either V1 or V2 (Fig. 11A) (respectively $p=0.08$ and $p=0.17$; unpaired t-tests). Even more surprisingly, and unlike other studies, we found that even in the extremely low release probability case of DSI, there was no clear trend towards an increase in PPR in either V1 or V2 (Fig. 11B) (respectively $p=0.93$ and $p=0.13$, Wilcoxon-signed rank test), although it should be noted that some cells were excluded because of persistent failure during DSI. For these synapses, PPR of two consecutive pulses does not seem to be a good parameter to infer changes in presynaptic release probability.

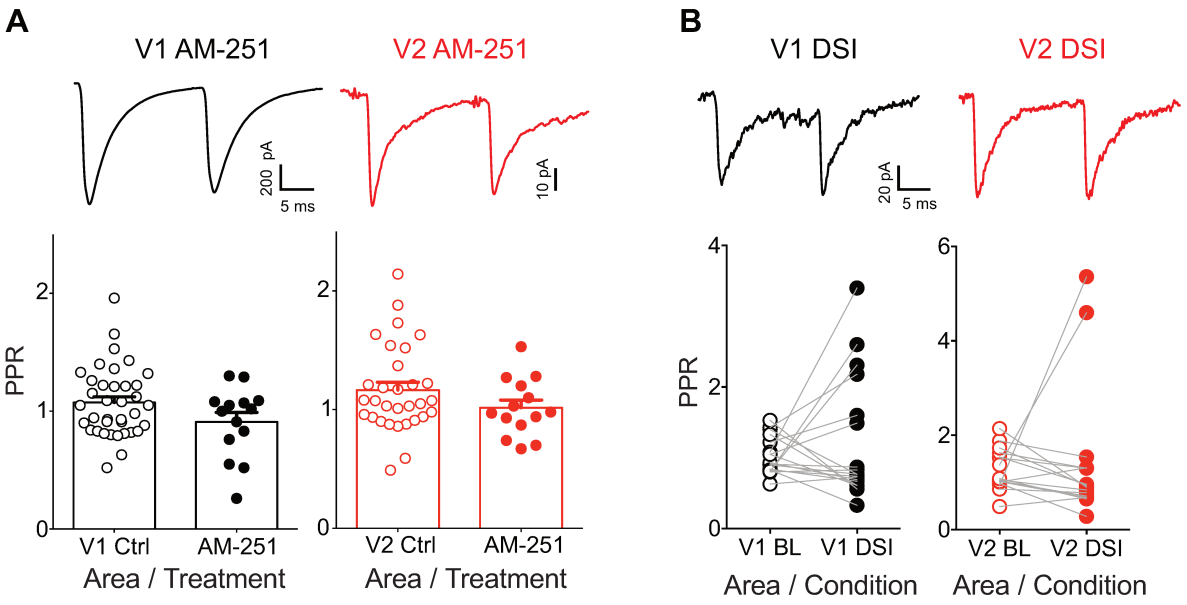

Figure 11. Changes in release probability are not reliably associated with changes in paired pulse ratio (PPR) in CB1 IN → PC synapses. (**A**) AM-251 has no effect on the PPR in either V1 or V2. (**B**) Even in the extremely low release state, there is no clear trend of increase in PPR.

DISCUSSION

Summary of findings

In this study, we examined the connectivity pattern of CB1/CCK INs in the mouse visual cortex. Indeed, this cell type is a prominent element of the cortical microcircuit, but its characteristics and functional properties are elusive as compared to other inhibitory cell types, such as SST and PV cells; some authors (Jiang *et al.,* 2015) have even put forward that CCK BCs are not present in V1. We found that CB1 expression was much higher in V2 across layer 2/3 to 5 as compared to V1. Moreover, we found that CB1-expressing INs (which also express CCK; Katona *et al.,* 1999; Marsicano & Lutz, 1999) possessed different anatomical and functional connectivity patterns: synaptic connections were strong and reliable in layer 2/3 of V1 and in layer 4 of V2, but they were weak and unreliable in layer 2/3 of V2. This resulted in different kinetics of CB1-dependent plasticity in different layers and visual areas. Therefore, it appears that layer 2/3 of V2 conferred a lower efficacy of synaptic release from CB1 INs. This was due to a tonically active CB1 signalling in this specific layer and cortical visual area (Fig. 1).

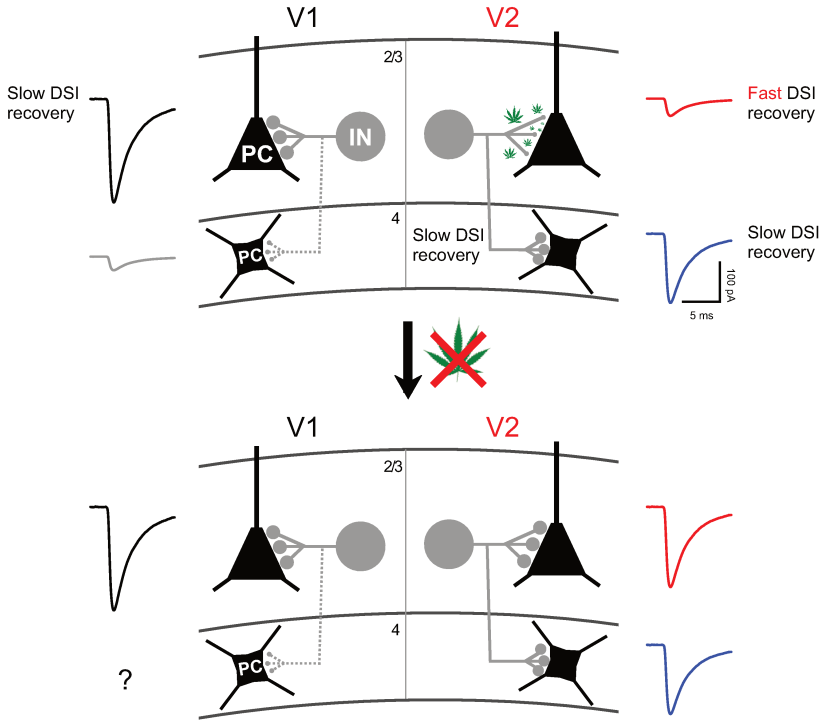

Figure 1. Summary of the main findings of this study. Scale bar is valid across the figure. Larger boutons represent larger amplitudes, and the crossed out cannabis leaf represents the blocking of CB1 by AM251.

Differential expression of CB1 in V1 and V2: are endocannabinoids associative?

Our anatomical data are in good agreement with previously reported differences of expression of CB1 in different cortical areas (Yoneda *et al.,* 2013). More CB1 in V2 could be due to *i)* differences in the number of CB1 INs; *ii)* differences in their projections; *iii)* increased expression of CB1 in each axonal terminal. Our data allowed us to exclude i) because we found similar cell counts across the cortical layers of V1 and V2. On the other hand, we found support for ii): we report high levels of axonal innervation originating in layer 2/3 and invading layer 4 only in V2. Regarding iii), our results cannot exclude higher expression of CB1 in V2, and future pharmacological studies are required to directly address this question. However, it is notable that comparable levels of CB1 in layer 2/3 and layer 4 of V2 yield completely different CB1-dependent modulation of synaptic transmission and plasticity. In the case of tonic CB1-mediated inhibition, modulation of GABA release has been shown to be independent of the quantity (assessed immunohistochemically) of CB1 on axon terminals (Lenkey *et al.,* 2015). What the actual function of higher CB1 expression in V2 is, as compared to V,1 remains to be understood. One possibility is that CB1-mediated plasticity phenomena are already saturating at the receptor levels shown in V1, with the larger amount of CB1 expression in V2 serving other cellular functions (Gaffuri *et al.,* 2012). Alternatively, it is possible that in V2, there is a higher level of expression in specific subcellular compartments, such as mitochondria (Hebert-Chatelain *et al.,* 2017).

Interestingly, we found that layer 5 PNs receive CB1-mediated modulation of monosynaptic inhibitory responses only in V2. This contrasts with previous data indicating that large layer 5 PNs of the somatosensory cortex are almost devoid of CB1-positive GABAergic boutons (Bodor, 2005) and do not express CB1-dependent plasticity (Bodor, 2005; Lourenço, 2014). The specific effects of CB1 agonists in V2 but not V1 suggest that deep-layer cortical PNs of primary sensory cortices are not modulated by eCBs. It has been proposed that CB1/CCK INs are better suited at integrating consecutive activation by independent afferents (Glickfeld & Scanziani, 2006). Strikingly, more associative cortices, which presumably receive more independent afferents, seem to require more CB1-dependent modulation. In line with this idea, more associative layers (such as L2/3) within traditionally non-associative cortices, such as V1, also express higher levels of CB1. Whissell and colleagues (2015) found that secondary sensory cortices tend to have a higher ratio of PV to CCK BCs than their primary counterparts, suggesting there may be a division of labour between these major BC cell

types, reflecting the specialised computational role of different brain areas. Accordingly, it is known that although they are ubiquitous, PV cells dominate L4 (Ascoli *et al.,* 2008; Tremblay *et al.,* 2016), while we find higher CCK/CB1 cell density in L2/3 and L5/6.

The V2-specific innervation pattern onto layer 4 is particularly interesting. The lack of infra-layer innervation in V1 could be due to a consistent inability of filling axons with biocytin, or bias in selecting CB1 INs in specific cortical areas. This is unlikely for at least two reasons. First, prominent L4 innervation is in agreement with the CB1 staining pattern. Second, the probability of finding infra-laminar connected pairs is dramatically different in the two visual areas; we could find very few connected pairs when the postsynaptic PNs was in layer 4 of V1 (5 pairs out of 74 double recordings). Moreover, even those few connections yielded small and unreliable uIPSCs, arguing against a strong modulation of layer 4 neurons by this IN class from layer 2/3. Although we have shown a relative scarcity of CB1 in L4 of V1, in absolute terms the staining was still prominent, and we cannot exclude inhibitory projections from other layers. Nevertheless, here we provide evidence of a different connectivity logic of CB1 INs from L2/3 in different visual areas. It will be interesting to study the functional role of this differential control of layer 4 of V1 and V2, and whether they modulate visually relevant behaviours. The specific infra-laminar projection of CB1 INs in V2 might reveal different circuit motifs in different cortical areas. Indeed, L4 activation by thalamo-cortical fibers is relayed to layer 2/3 where it could generate a feedback inhibitory loop operated by CB1 INs only in associative cortices. This area-specific routing of information could have important consequences in sensory perception.

CB1 INs of L2/3 of V1 and V2 have similar intrinsic properties, but form different synapses onto PNs

Differences in anatomical parameters, such as dendritic and axonal projections have traditionally been used to distinguish different interneurone subclasses (Markram *et al.*, 2004; Ascoli *et al.,* 2008). Accordingly, different cell types identified by their morphology, exhibit specific electrophysiological properties (Markram *et al.*, 2004; Ascoli *et al.,* 2008). Here we found that, despite the different axonal projections in the two visual areas, CB1 INs exhibited similar electrophysiological signatures. Moreover, synaptic properties share many similarities, such as high failure rate, variable short-term dynamics and asynchronous release, consistent with their counterparts in the hippocampus (Neu *et al.,* 2007), amygdala

(Rovira-Esteves *et al.,* 2017) and other cortical areas (Galarreta *et al.,* 2008). This argues against the existence of subtypes of cortical CB1 INs in visual areas, although a thorough molecular investigation using single cell transcriptomics is worth pursuing.

Importantly, we found that GABAergic synapses from CB1 INs onto layer 2/3 PNs are the weakest and most unreliable, and this was due to tonic CB1 activation. Functional synaptic efficacy is influenced by pre- and postsynaptic factors: the number, density and location of participating synaptic vesicles, calcium channels, receptors and fusion machinery proteins. Specific interactions of these molecular players at the nanometer (nanoscale) level are believed to define synaptic efficacy and plasticity. In particular, presynaptic factors, such as the distance between presynaptic voltage-gated Ca^{2+} channels and the sensor for synaptic vesicle fusion, sets the functional coupling strength that influences release probability, and hence synaptic efficacy. Indeed, this coupling strength has been shown to vary widely across synapses (Hefft & Jonas, 2005). In principle, the visual area- and layer-specific synaptic differences that we found could be explained by specific expression of any of these molecular synaptic components. Yet, this does not seem to be the case, as the application of the CB1 antagonist AM251 almost completely erased the differences between cortical areas and layers, if we assume equal release probability. Therefore, we do report functional synaptic diversity, but this was due to constant modulation of synaptic transmission by CB1 only in layer 2/3 of V2.

Despite an increase in uIPSC amplitude and a decrease in failure-rate, the lack of change in PPR in the presence of AM251 is surprising, but also consistent with other studies (Rovira-Esteves, 2017). Unlike the latter, however, we did find AM-251 decreased the failure-rate, and this result was consistent with a study by Neu and colleagues (2007). As we mentioned, presynaptic release, and by extension short-term plasticity dynamics, can depend on a plethora of presynaptic factors. These can often converge onto the same biochemical pathways, such as Ca^{2+}, and for CB1/CCK BC synapses PPR with two consecutive IPSCs may not serve as an appropriate measure of presynaptic effects. Also, using PPR to infer presynaptic release probability of GABAergic synapses has been questioned due to spurious facilitation caused by occasional synaptic failure (Kim & Alger, 2001). Yet, it is still surprising that we find no clear trend in PPR even in the extremely low-release probability case of DSI. However, many pairs had to be removed from this analysis precisely because of high failure,

and in future studies it may be more appropriate to elicit longer presynaptic spike trains, allowing normalization of the last evoked IPSC to the first response (e.g. $uIPSC_{10}/uIPSC_1$ - Barsy *et al.,* 2017).

Remarkably, we found that the quintessential CB1-dependent plasticity DSI was also different in V1 and V2. Strong GABAergic synapses in layer 2/3 of V1 and layer 4 of V2 showed a slower DSI recovery than those in layer 2/3 of V2. This could be due to the actual differential strength in the examined areas: indeed, recovering to tens of pA values might take less than recovering all the way back to hundred-pA levels. In this way, tonic CB1 signaling would act as a ceiling for plasticity. It will be interesting to test whether milder DSI-inducing and/or long-term plasticity protocols will differently affect connections in the areas under scrutiny. Importantly, despite the *relative* DSI amplitude was similar in V1 and V2, it is noteworthy to stress here that from the perspective of single PNs, DSI produced a massive reduction of PN perisomatic inhibition in L2/3 of V1 (synaptic currents went from several hundred-pA to zero). In contrast, L2/3 PNs of V2 sensed a reduction of perisomatic inhibition from CB1 INs, which was already weak (~6 fold less powerful) already before DSI stimuli. This differential absolute change of acute eCB modulation of inhibitory transmission from CB1 INs will likely produce distinct effects in the output spiking properties of single PNs in the two cortical areas.

Area- and layer- specific tonic inhibition

Importantly, the CB1-dependent tonic reduction of inhibition reported here is consistent with previous observations that CCK-positive INs elicit inhibitory postsynaptic potentials that are modulated by CB1 receptors (Losonczy et al., 2004; Földy et al., 2006; Ali, 2009). However, despite the fact that a number of groups reported tonic CB1 modulation, others have failed to observe this phenomena (Castillo et al., 2012). This has raised the possibility that tonic inhibition could arise from the health of the slice preparation and/or recording conditions. To our knowledge, we are the first to demonstrate in adult tissue that the same CB1 IN could be responsible for phasic and tonic modulation at difference synapses. Tonic CB1 activation could be due to different mechanisms, including a constitutively active receptor in the absence of a natural ligand (Leterrier *et al.,* 2004; Losonczy et al., 2004), or a persistently activated receptor by a tonic presence and/or synthesis of eCBs (Neu *et al,.* 2007). It has been suggested that this tonic endocannabinoid mobilisation from postsynaptic pyramidal

cell is regulated by mGluRs and muscarinic receptors (Földy et al., 2007). Distinguishing between these possibilities is very challenging and requires highly specific pharmacology and/or genetic approaches. Yet, we believe that this tonic modulation of CB1 is due to a constant presence of eCBs: indeed, the same axons exhibited quasi-opposite CB1-dependent synaptic properties in V2 depending on the postsynaptic target, thus likely excluding a target-specific CB1 endogenous state in the two layers. It will be interesting to determine the possible sources of eCBs: these could be postsynaptic neurons or non-neuronal elements in the neuropil (such as astrocytes). Alternatively, tonic CB1 activation could derive from a lack of enzymatic eCB degradation (Lenkey *et al.,* 2015).

Independently of the actual underlying molecular mechanism, it has been shown that tonic CB1 activity can be overrun by high frequency firing of the presynaptic INs (Chen et al., 2003, 2007; Földy et al., 2006) or could be facilitated by presynaptic activity (Zhu & Lovinger, 2007; Heifets *et al.*, 2008; Lourenço et al., 2010). Maturation of the visual cortex and stress could possible be factors altering CB1 signalling (Jiang *et al.,* 2010; Cusulin *et al.,* 2014). It is therefore tempting to speculate that this specific doping of GABA release from this interneurone subtype only in layer 2/3 of V2 might be used as a strategy to alter the input/ output transformations of postsynaptic PNs. Indeed, the dampening of GABA release from the CCK/CB1 BCs might favor the inhibitory control of PNs by other inhibitory cell subclasses. In particular, PV BCs are known to compete with CCK/CB1 INs in the perisomatic control of PNs (Hefft & Jonas, 2005; Freund & Katona, 2007). It will be important to investigate whether tonic CB1 activation exists *in vivo*, and, in that case, what behavioural states might awaken these synapses to regain control of layer 2/3 PNs.

The highly specific inhibition of PN output can profoundly affect the participation and orchestration of populations of PNs to behaviorally relevant network activity, such as coordinated oscillations, believed to underlie several cognitive functions, including sensory perceptions (Buzsáki, 2010). It will be therefore interesting to establish whether the anatomical and functional connectivity patterns that we describe here could shed new light on the role of V2 during sensory perception. Moreover revealing, the connectivity logic of different cortical circuits is important to advance our knowledge on the finely regulated ecosystem that underlies virtually all behaviours.

Future Directions

We have uncovered a visual area- and layer-specific connectivity logic of a prominent interneurone subtype, whose function is still unknown. This research opens a series of questions:

1. Is this connectivity logic also reflected in other sensory cortices?
2. How are cortical CB1/CCK INs recruited?
3. Do they target specific PNs? How do they compete with PV synapses?
4. Cell autonomous plasticity: what is it good for?
5. What is the mechanism underlying tonic inhibition in V2 L2/3?
6. Does tonic CB1-mediated inhibition in V2 occur *in vivo*, and what role does it have?
7. Do presynaptic calcium transients differ in L2/3 and 4 of V2, and how may they be modulated by CB1?
8. How does cortical maturation or experience affect the synapses formed by CB1/CCK neurones? Is there an activity-dependent mechanism
9. What is the spiking activity pattern of visual cortical CB1/CCK INs *in vivo*? How does this activity correlate with visual sensory features?
10. Do CCK/CB1 INs play a role in cortical network oscillations?
11. What is the specific role of V2 in mice? Are there any behaviours that reliably depend on it?

Answering these questions will help understanding how specialised cortical circuits contribute to the internal representation of the external world that we experience as perception. Considering the involvement of CB1 INs in various pathologies, characterising the role and molecular peculiarities of this cell type may lead to a better understanding of the development and persistence of neuropsychiatric disease.

ABSTRACT

During sensory processing, the correct subjective representation and interpretation of the external world is accomplished by a constant bi-directional communication between primary and secondary sensory cortices. Yet, the synaptic rules governing the processing of sensory information in these distinct cortical areas, and the specific microcircuit players involved, are still poorly characterized. After finding that the cannabinoid receptor (CB1) is more widely expressed in secondary than in primary sensory areas, we asked whether the neurons expressing CB1 had different properties in the two areas. CB1 is mainly expressed in large inhibitory basket cells that target the soma of other neurons, likely modulating the timing of their output. Using whole-cell patch clamping in acute brain slices from adult mice, we found that in layer 2/3 of the primary visual area of mice (V1), CB1+ interneurons exerted strong and reliable inhibition onto pyramidal cells. In contrast, in L2/3 of the secondary visual area (V2) CB1+ interneurons exerted small and unreliable inhibition onto excitatory cells. Interestingly, pharmacological blocking of CB1Rs in V2 led to a potentiation of inhibition, while in V1 this effect was not observed, suggesting that CB1 interneurons in V2 are ‘doped’ by tonically or constitutively activated receptors. Differences in the kinetics of depolarization-induced suppression of inhibition (DSI) also support this hypothesis. In addition, we found a different innervation pattern of CB1+ interneurons in the two visual areas: in V2, CB1+ interneurons projected their axons both within their cortical layer and to deeper layers, while in V1 these projections were principally intralaminar. Strikingly, infra laminar connections in V2 shared synaptic characteristics with those of L2/3 in V1. This study therefore suggests that different visual areas exhibit differential CB1-mediated modulation of perisomatic inhibition onto layer 2/3 and 4 principal neurons. Moreover, our results show differences in microcircuit connectivity patterns, suggesting area-specific differences in the integration of visual information.

RÉSUMÉ

La perception sensorielle dépend d'une interaction constante entre plusieurs zones corticales. Typiquement, pour chaque sens il y a une zone primaire qui reçoit directement des informations sensorielles du thalamus et une zone secondaire qui associe cette information avec des centres cognitifs plus élaborés (information descendante). Pourtant, les propriétés synaptiques et les microcircuits impliqués dans ces différents processus ne sont toujours pas élucidés. Nous savons que le récepteur cannabinoïde de type 1 (CB1) est exprimé a un plus grand niveau dans les cortex sensoriels secondaires. Ce récepteur est principalement exprimé par des cellules inhibitrices qui expriment aussi le peptide cholecystokinin (CCK), et font partie d'une classe cellulaire appelée cellules panier. Ces interneurones (IN) sont connus comme étant idéalement placés pour controller la sortie des cellules qu'elles visent, notamment les neurones pyramidaux (NP). En utilisant des enregistrements entre des paires d'IN CCK/CB1 et NP dans la couche 2/3 (C2/3) des centres visuels primaires (V1) et secondaires (V2) de souris adultes, nous avons demontré que ces neurones expriment une morphologie différente dans ces deux cortex. En effet, dans le V1 les IN CCK projettent quasiment exclusivement dans leur propre couche, tandis que dans le V2 ils projettent aussi dans la couche 4 (C4). Malgré cette difference morphologique, ces IN avaient les mêmes signatures électrophysiologiques, suggérant qu'il s'agisse bien d'un type cellulaire homogène. En revanche, les connections synaptiques avec les NP étaient nettement plus petites et non fiable dans la C2/3 de V2. Cette différence disparut avec l'application d'un antagoniste de CB1, suggérant que ce récepteur médie une inhibition tonique qui est spécifique à une zone et couche corticale. Cette étude montre ainsi qu'il existe des microcircuits inhibiteurs particuliers dans les aires primaires et secondaires visuelles.

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