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The emergence and plasticity of synapses at the axon initial segment

Pan Vazquez, Alejandro

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The emergence and plasticity of synapses at the axon initial segment

Alejandro Pan Vazquez

Thesis submitted for the degree of Doctor in Philosophy



King's College London, University of London

September 2018

Todo ser humano, si se lo propone, puede ser escultor de su propio cerebro.

Santiago Ramón y Cajal, 1897

Abstract

The axon initial segment (AIS) is a structure at the start of the axon with a high sodium and potassium channel density, defining the site of action potential generation. It is also the target of inhibitory synapses formed by a specific GABAergic interneuron, the Chandelier cell. Due to this strategic synaptic placement, Chandelier cells are thought to powerfully control network activity. Despite this, we know surprisingly little about this cell type. By visualising Chandelier cell interneurons and their boutons in somatosensory cortex at different developmental stages, we uncovered a critical temporal window of synapse formation at the AIS (P14-P16), which corresponds with the electrophysiological and morphological maturation of the Chandelier cell. Using a targeted chemogenetic approach, we showed that modulating the activity of either pyramidal cells or Chandelier cells themselves, resulted in a decrease in axo-axonic synapse number along the AIS. In parallel, we also observed a decrease in the length of the AIS and, as a consequence, a reduction in intrinsic excitability. Surprisingly, the same manipulation of neuronal activity in mature mice (P40-P46) showed the opposite effect, an increase in axo-axonic synapse number. Since axo-axonic synapses change polarity from depolarising to hyperpolarising late in development, this form of plasticity likely constitutes a homeostatic mechanism that serves to keep network activity stable. To confirm this, I studied the functional impact of this synaptic plasticity using whole-cell electrophysiology as well as voltage imaging. In conclusion, my work on the activity-dependent development of Chandelier cells has uncovered a bi-directional plasticity of axo-axonic synapses in juvenile and adult mice. I propose that the AIS and its synapses are highly plastic and form an important hub for stabilising neuronal activity.

Acknowledgements

First and foremost, I would like to thank my doctoral advisor Prof. Juan Burrone and Dr. Winnie Wefelmeyer; working with them has been a real pleasure. Without their support, this work would have never reached a successful end. I would also like to thank Prof. Beatriz Rico, my second supervisor, who advised me on key aspects of my research. I also would like to thank the rest of Burrone laboratory, current and past members, who taught me all I know and put up with me for so long. Among them, I would like to specially thank Dr. Federico Grillo who took me on as a rotation student and has been a great mentor and fantastic friend since then.

I also want to thank very especially my wife Rita Tavares. Thanks for supporting and accompanying me through the happy and less happy moments behind this work. Without her, this work would have never been finished. Many of the experiments here described, required a level of compromise and dedication that would have been impossible to fulfill without her unconditional support.

I would also like to thank the rest of my family for always supporting me in my academic endeavour. I especially need to thank my parents, Susana and Alejandro. Their support has always been unconditional and they have taken great sacrifices just to see me pursue what I wanted. This thesis is in many ways the culmination of the education and moral values they taught me since I was little. I hope the quality of this work is enough to honour it.

I would like to thank my friends, in the department and outside. They made this journey easier and more fun. I would like to specially thank Victoria, Sarah, Tristan, Tom Shallcross, Tom Sainsbury, Emily, Ankur and Susana with whom I shared hundreds of hours of work and enjoyment.

Finally, I would like to thank the Centre for Developmental Neurobiology; The Institute of Psychiatry, Psychology and Neuroscience; King's College London and the Medical Research Council for giving me the opportunity to pursue a PhD at their institution. I will carry the knowledge and skills I learned here for the rest of my career.

Statement

I declare that the work in this thesis was performed by myself except for the data in Figure 3.10 that was collected and analysed by Dr. Winnie Wefelmeyer.

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List of abbreviations

ACE-mNeon: ACE2N-mNeon-4AA

ACh: Acetylcholine

AIS: Axon Initial Segment

AP: Action potential

APV: 2-amino-5-phosphonopentanoic acid

Ca²⁺: Calcium

CaV: Calcium voltage gated channel

ChC: Chandelier cell

Cl⁻: Chloride

CGE: Cudate ganglionic eminence

CNO: Clozapine-N-Oxidase

DREADD: Designer Receptor Exclusively Activated by Designer Drugs

E_{Cl}: Chloride reversal potential

E_{GABA}: GABA reversal potential

E_{HCO₃⁻}: Bicarbonate reversal potential

EGTA: Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

EPSC: Excitatory Postsynaptic Current

FRET: Fluorescence resonance energy transfer

GABA: Gamma-Aminobutyric Acid

GABA_AR: GABA_A receptors

GABA_BR: GABA_B receptors

GEVI: Genetically encoded voltage indicator

GPCR: G protein coupled receptor

HCO₃⁻: Bicarbonate

IPSC: Inhibitory Postsynaptic Current

K⁺: Potassium

L: Layer

M: Molar

Mg²⁺: Magnesium

MGE: Medial Ganglionic Eminence

mM: millimolar

LIST OF ABBREVIATIONS

ms: millisecond

mV: millivolt

mW: milliwatt

Na⁺: Sodium

NaV: Sodium voltage gated channel

NBQX: 6-Nitro-2,3-dioxo-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide

Nkx2.1/*tdTomato*: Nkx2.1^{+/-}CreERT2::*Ai9*^{+/+}

Nkx2.1/*Ai32*: Nkx2.1^{+/-}CreERT2::*Ai32*

NRG1: Neuroregulin 1

ODP: Ocular dominance plasticity

POA: Preoptic area

PPR: Pair Pulse Ratio

PSP: Postsynaptic potential

PV: Parvalbumin

STT: Somatostatin

V: Volts

Chapter 1 : Introduction

1.1. Cortex and neuronal diversity

Among the different anatomical structures of the brain, the neocortex is perhaps the most characteristic example of the evolution of the mammalian nervous system. The unparalleled expansion of the pallium in mammals paved the way for this unique structure, which is believed by many to be the causal origin of the formidable cognitive skills of mammals (DeFelipe, 2011). By the 19th century, studies had already shown that the cortex could be divided into areas with specialised functions, from sensory to cognitive processing. Each of these areas is made up of thousands of neurons which tend to be vertically interconnected in what is often called a cortical column, as coined by Vernon Mountcastle over 60 years ago (Mountcastle, 1957). The cortex also shows a clear organisation along the cortical axis (i.e pial surface to white matter), where layers of cells with distinct identities and connectivity patterns can be stacked on top of each other making a total of 6 layers (DeFelipe, 2011).

However, it is not until we look at the cellular level that the cortex presents its extreme complexity. In what looks like an “impregnable jungle”, as originally described by Santiago Ramon y Cajal, cortical microcircuits present a large number of distinct cell types, which are tightly packed in space and form complex connectivity patterns. Early histological work from Cajal at the beginning of the 20th century already showed an extraordinary heterogeneity in the shape and specific location of cortical neurons

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(Ramón y Cajal, 1909) (Figure 1.1). Although we still do not know the full classification of neuronal types and new, methods in transcriptomics continue to increase our knowledge about neuronal heterogeneity (Johnson et al., 2015; Zeisel et al., 2015). However, despite the diversity, we can divide cortical neurons into two broad groups based on their function and on the neurotransmitter used for synaptic transmission: glutamatergic excitatory pyramidal cells and GABAergic inhibitory interneurons. From a computational perspective, pyramidal cells propagate information within and among microcircuits while interneurons shape overall cortical dynamics and gate information flow.

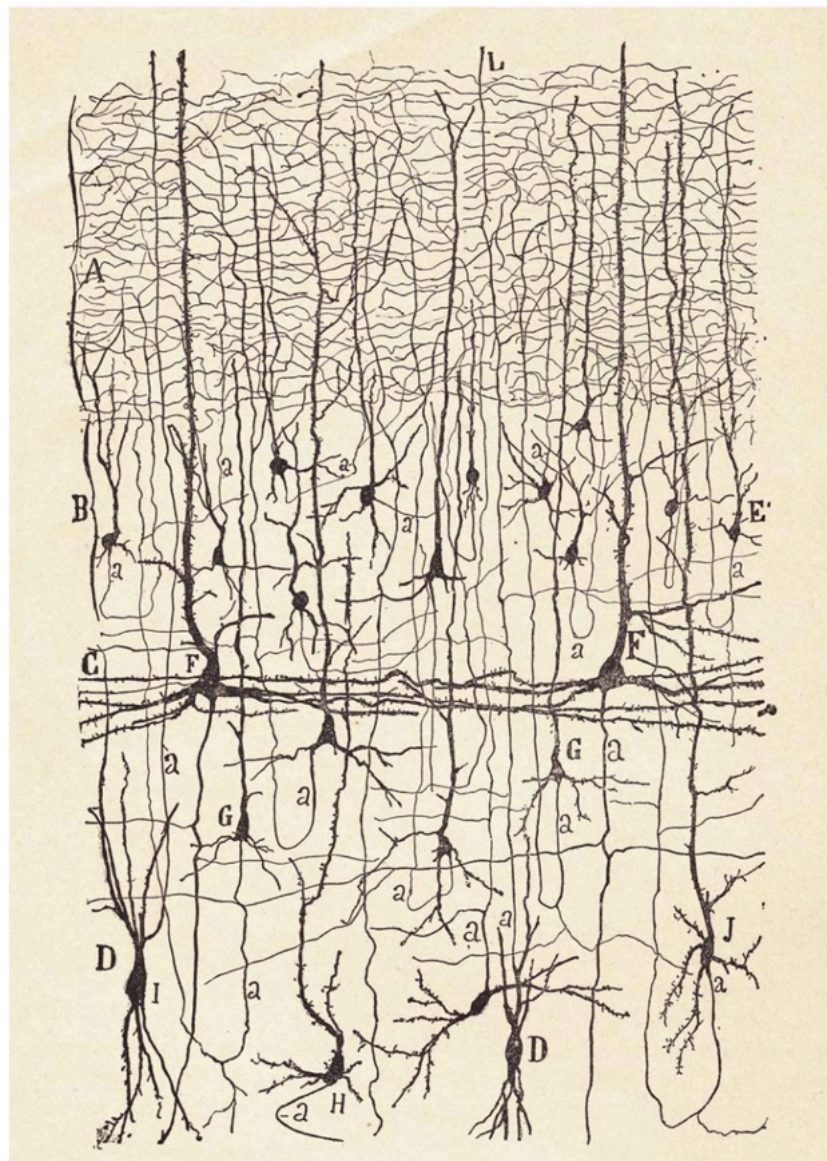


Figure 1.1. Drawing of Golgi stained cells in a 27 days old neonatal human cortex. (A) stellate cells layer; (B) granule cells layer, (C) giant cells layer, (D) polymorphic cells layer. Copyright Herederos de Santiago Ramon y Cajal (Ramón y Cajal, 1889).

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1.2. The inhibitory nervous system

Even though interneurons only represent 10-15% of the total neuronal population in the cortex (Meyer et al., 2011), they present unmatched diversity in their function, electrophysiological and morphological properties (Tremblay et al., 2016). In fact, based on our current understanding of neuronal heterogeneity, most of the neuronal diversity in cortical neurons could be attributed to interneurons alone.

The importance of interneuron diversity for brain function is perhaps better exemplified by their evolutionary age, 320 million years old. Specifically, it appears that the need for diverse GABAergic cell types with specialised functions is essential for complex nervous systems. In fact, recent work has shown that all amniotes contain the major interneuron families we find in humans, highlighting the evolutionary pressure for diversity (Tosches et al., 2018). Diversity across species does not stop there. Within mammals, several cell types are thought to be unique (e.g. rosehip neurons (Boldog et al., 2018)) or more prevalent (e.g. double-bouquet cell (Yáñez et al., 2005) in humans. We now know that GABAergic interneurons play a crucial role in regulating essential cortical functions such as activity-dependent plasticity (Kullmann et al., 2012; Donato et al., 2013) and the regulation of cortical dynamics (Buzsáki and Watson, 2012). Their malfunction is also thought to be behind several neurological disorders such as schizophrenia (Nakazawa et al., 2012), autism and epilepsy (Pavlov et al., 2013).

Interneurons can be classified according to their developmental origin, their molecular markers, morphology and function. Most of these characteristics can be associated with different molecular markers (Markram et al., 2004). In fact, the expression pattern of three of these markers divides the families that account for nearly the entirety of the neocortical interneuron population (Rudy et al., 2011). These are the Parvalbumin (PV), Somatostatin (SST) and 5HT3aR interneurons. These three genes show minimal overlap between populations (Zeisel et al., 2015; Tasic et al., 2016) and divide populations with shared functional properties. SST+ interneurons account for 30% of the total interneuron population. Their main interneuron type is the Martinotti cell, which synapses with the dendritic compartment of pyramidal neurons across several layers. 5HT3aR+ neurons are a varied group of interneurons (30% of total in neocortex), with a very particular synaptic targeting. They include VIP interneurons,

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which target other interneurons producing disinhibition, as well as other types of interneurons such as neurogliaform interneurons (NGF), which do not make synapses *per se*, as they release GABA via volume transmission. Lastly, parvalbumin (PV) interneurons are the most prevalent type of cortical interneurons, making 40% of the interneuron population. They consist of basket cells, which target the somatic compartment of cells in their vicinity, and chandelier cells, which form axo-axonic synapses onto the axon initial segment (AIS) of nearby cells (Tremblay et al., 2016).

The functional properties of the members of each of these families are unique. However, as in the case of their molecular markers, their functions are also distinctive and complementary. For example, SSTs are thought to mediate surround suppression in visual cortex by integrating activity from different areas of V1 and suppressing local networks (Adesnik et al., 2012; Nienborg et al., 2013). In turn, 5HT3aR interneurons, and in particular VIP interneurons, are thought to regulate cortical function during different behavioural states by disinhibition of SSTs (Lee et al., 2010). Finally, PV basket cells, the most common interneuron type in the cortex, are thought to be involved in gain control. For example, in the visual cortex they are known to decrease visual responses without affecting tuning (Atallah et al., 2012; Nienborg et al., 2013). These few descriptions are, however, just a glimpse of what we know about interneuron function. In the last two decades our knowledge on the function of interneurons, not just at the circuit level, but also in behaviour has improved substantially (Kepecs and Fishell, 2014). Despite this, several interneuron types have remained understudied.

In this thesis, I will focus on chandelier cells in the neocortex, a member of the PV family of interneurons. Due to the lack of targeted genetic mouse lines until recently (Taniguchi et al., 2011), we know surprisingly little about this cell type. However, based on their distribution, connectivity pattern and subcellular targeting of pyramidal cells, it is likely that these interneurons may play a pivotal in cortical function. Below I provide a detailed description of our current knowledge of chandelier cells in the mammalian brain.

1.3. Discovery of chandelier cells

Chandelier cells (ChCs), were one of the last types of interneurons to be identified. In fact, they were one of the few types of interneuron that Cajal did not discover. The first description of a ChC was given by the Hungarian anatomist János Szentágothai in the early 1970s (Szentágothai and Arbib, 1974). By using the Golgi staining method, the same method that Cajal used, they described an interneuron type whose axonal arbour formed rows of boutons that resembled candlesticks in a chandelier. Later, Jones described a similar looking cell, which he called interneuron type 4 (Jones, 1975). Szentágothai believed that chandelier cells formed their candlesticks with the apical dendrite of pyramidal cells and not with the AIS (Szentágothai, 1975). It was his graduate student Péter Somogyi who, by combining Golgi staining with electron-microscopy, found that in fact chandelier cells formed synapses directly onto the AIS (Somogyi, 1977). It is this axo-axonic contact that makes ChC unique and gives them their second name, axo-axonic cells. The story behind the co-existence of these two terms, chandelier cell and axo-axonic cell, dates back to their initial description by different people in the same lab, Somogyi and Szentágothai. Although initial descriptions based on the subcellular target of chandelier cells and axo-axonic cells suggested that these cells were distinct cell types. We now believe they were both referring to the same cell and the nomenclature is currently used interchangeably in the field. In this thesis, I will use 'chandelier cell' when referring to the cell type and 'axo-axonic' when referring to the synapses formed by these cells onto pyramidal neurons.

1.4. Chandelier cell morphology and connectivity

ChCs can predominantly be found in the neocortex (Schmidt et al., 1993; Taniguchi et al., 2013) and hippocampus (Soriano and Frotscher, 1989; Schmidt et al., 1993; Martínez et al., 1996), but can also be found in non-layered structures such as the amygdala (Kemppainen and Pitkänen, 2000). Within the neocortex, ChCs concentrate at the border between Layer 1 (L1) and Layer 2/3 (L2/3), as well as in Layer 5 (L5) and 6 (L6). In terms of their inter-areal distribution within the cortex, ChCs are thought to be more prevalent in frontal cortex than in sensory cortices (Taniguchi et al., 2013). They also appear to be present in all mammals. So far, they have been identified in

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rodents (Somogyi, 1977; Taniguchi et al., 2013), primates (Lund et al., 1981; DeFelipe et al., 1989) and humans (Kisvárdy et al., 1986; Szabadics et al., 2006).

ChCs share the main aspects of their morphology across all brain areas. They are bipolar cells with short aspiny dendrites, which branch close to the cell body and taper towards their distal end. In general, these dendrites are relatively simple in structure, with few branches and they are significantly shorter than those found in basket cells, the other type of PV cells (Papp et al., 2013). A main feature of ChCs is their large axonal arbours, with extensive preterminal branching, which allows them to make contacts with hundreds of pyramidal cells in their periphery (Somogyi, 1977; Peters et al., 1982). These contacts resembling candlesticks in a chandelier are commonly referred to as cartridges (Peters et al., 1982; Somogyi et al., 1982) (Figure 1.2). Cartridges are made directly onto the AIS, where they form rows of 2-12 boutons that extend over 10-50 μm in length. Interestingly, cartridges are thought to extend beyond the AIS to a limited extent, although these boutons account for less than 10% of the total number of axo-axonic boutons (Soriano et al., 1990; Buhl et al., 1994; Kawaguchi and Kubota, 1998).

Deviations from this basic cellular architecture can be found across different areas of neocortex depending on the layer in which they are positioned. ChCs in deeper layers have dendrites that project towards L 4 and an axonal arbour that projects within L 5. L 5 ChCs have a shorter dendritic and axonal arbour, which stay within the same layer. Finally, ChCs in L 2/3 (Figure 1.2) show a bipolar morphology with the main dendritic arbour extending vertically toward layer 1 with some horizontal projections staying in L 2. Their axonal arbour projects ventrally, invading layer 2/3 (Taniguchi et al., 2013).

In terms of the overall output, ChCs in the neocortex are thought to innervate mainly pyramidal cells. An individual ChC can make hundreds of cartridges, contacting 250-1200 pyramidal cells in their vicinity (Howard et al., 2005). This comprises nearly 50% of total AISs in their local area (90 μm radius), according to one report in somatosensory cortex (Inan et al., 2013). Conversely, an individual AIS is thought to be innervated by 3-6 cartridges each belonging to different chandeliers (Tamás and Szabadics, 2004; Inan et al., 2013). In the particular case of L 2/3, the only area where an accurate description of the identity of postsynaptic pyramidal cells contacted by ChCs has been made (Lu et al., 2017), ChCs are thought to connect mostly to pyramidal cells which are connected

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to deeper nuclei such as the basolateral amygdala (>85%), in comparison to pyramidal cells connecting calossally to the contralateral hemispheres.

In terms of their inputs, neocortical ChCs are thought to receive both excitatory and inhibitory inputs. Within prefrontal cortex, L 2/3 ChCs are thought to receive nearly 50% of their inputs from pyramids in L 2/3 and 30% from deeper layers (L5-6). They also receive GABAergic inputs amounting to nearly 10% of the total, from L1, L2 and L3. However, despite their large innervation of L 2/3 pyramids, reciprocal connectivity is thought to be small (circa 3%). Long range inputs are also present from thalamic nuclei and cholinergic input from the basal forebrain as shown in a monosynaptic rabies tracing study using ChCs as starter cells (Lu et al., 2017). Finally, ChCs are electrically coupled through gap junctions to PV basket cells (Woodruff et al., 2011), as well as PV negative neurons such as neurogliaform interneurons (Simon et al., 2005). This makes ChCs likely to fire synchronously with other interneurons.

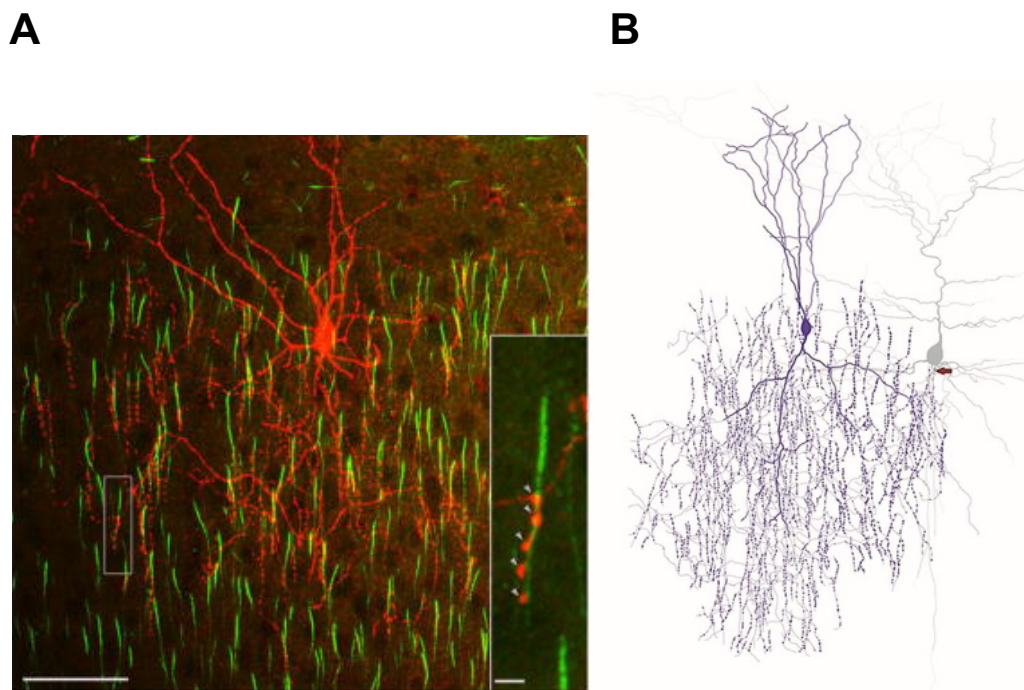


Figure 1.2 Neocortical L2/3 chandelier cells. (A) Immunostaining of ChC (red) innervating local AISs (green) (Taniguchi et al., 2013). Scale bar: 50 μm , Inset scale bar: 5 μm **(B)** Reconstruction of a ChC (magenta) and pyramidal cell (gray). Red arrow indicates ChC cartridge at the pyramidal cell AIS (Wang et al., 2016).

1.5. Chandelier cell function

What is the function of chandelier cells? Due to their synaptic targeting of the AIS, ChCs have been thought to be master regulators of cortical activity levels (Inan and Anderson, 2014). However, the unique properties of the axon have made ChC function a matter of heated debate during the past decade. I will address ChC function in this chapter at four different levels: 1) electrophysiological properties, 2) functional role in behaviour 3) brain dynamics and 4) synaptic properties.

1.5.1 Electrophysiological properties

ChCs are thought to be mostly PV⁺ interneurons and as such show fast spiking properties (Povysheva et al., 2013; Taniguchi et al., 2013). The fast spiking properties of the PV interneuron family are thought to come from the high expression of Kv3 potassium channels at their dendrites and soma, which deactivate quickly (Martina et al., 1998; Rudy and McBain, 2001). This is combined with high expression of Na⁺ channel at the AIS, which allow sustained fast activation (Hu and Jonas, 2014). However, major differences can be found between members of the PV family. ChCs are thought to fire at a higher rate and show more adaptation (Ganter et al., 2004; González-Burgos et al., 2005; Povysheva et al., 2013). Similarly, neocortical ChCs are thought to present lower spontaneous firing (Zhu et al., 2004), despite having a lower firing threshold than basket cells (Povysheva et al., 2013).

It is important to note that some ChCs might not be fast spiking, as has been reported before (Lewis and Lund, 1990). This is especially the case for PV negative interneurons. PV expression in interneurons is highly correlated with the expression of the K⁺ channels that generate fast-spiking (Toledo-Rodriguez et al., 2004). The population of PV negative ChCs was thought to be small until recently. However, in Taniguchi et al., 2013, it is reported that this can vary to a large degree between regions. For instance, in barrel cortex 50% of ChCs are thought to be PV negative, while in prefrontal cortex this number goes up to 85%. Nonetheless, PV negative ChCs are likely to also be fast-spiking. In fact, a recent study looking at ChC properties in prefrontal cortex observed that despite the low percentage of PV⁺ ChCs, on average ChCs still presented fast spiking properties (Miyamae et al., 2017).

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1.5.2 Role of chandelier cells in perception and behaviour

So far, no causal role for cortical ChCs in behaviour or perception has been found, as for much of cortex itself. However, *in vivo* recordings have shown differential firing of ChCs in L2/3 during particular behaviours. It has been reported that contrary to basket cells, ChCs do not respond directly to whisker stimulation in barrel cortex. However, they respond to high levels of cortical activity resulting from this stimulation (Zhu et al., 2004). This has suggested that the role of ChCs might be related to the control of overall levels of activity but not necessarily sensory processing.

Nevertheless, whether ChCs are simple fuses in cortical networks cannot be confirmed. In recent years, it has been shown that cortical interneurons have a more important role than previously thought in sensory processing. PV basket cells have been shown to be involved in spatial summation and the sharpening of cellular tuning in pyramidal cells of the visual cortex (Atallah et al., 2012). Complementary, SST+ interneurons are thought to compute surround suppression (Veit et al., 2017). The role that ChCs may have in these processes is unknown and will hopefully be investigated in the future.

1.5.3 Role of chandelier cells in network dynamics

Causal roles for neuronal types in cortex are often difficult to interpret due to the complexity of the information processed and computations performed. However, interneurons have been extensively studied at an intermediate level between behaviour and single cell computation. The mammalian brain has network wide synchronous events that show oscillatory behaviour at different frequencies. Each of these oscillations correlates with specific cognitive functions, such as attention or memory.

Interneurons and in particular fast-spiking interneurons due to their extensive innervation and synchronous activity are thought to play a major role in coordinating these oscillations (Buzsáki and Wang, 2012). A clear example of this are PV interneurons. PV firing is thought to enhance and entrain gamma oscillations (Cardin et al., 2009; Sohal et al., 2009), a network activity pattern commonly associated with high cognitive processes such as memory, attention and perception (Ray and Maunsell,

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2015). Despite the large body of work on PV function in network oscillations, we currently have a very scarce understanding of the role that ChCs may play. Some limited characterization has been performed at the level of the hippocampus and prefrontal cortex. Work from Klausberger and colleagues showed that hippocampal ChCs did not synchronize with gamma oscillations and were instead involved in the transition from theta rhythm to sharp wave ripples (Klausberger et al., 2003), two common activity patterns associated with movement and memory formation (Carr et al., 2011; Jadhav et al., 2012). More recently, in prefrontal cortex, it was shown that the two populations of fast spiking interneurons behaved differently during cortical gamma oscillations, in what could be a differential role for basket cells and ChCs (Puig et al., 2008). This has been confirmed by Massi and colleagues in 2012, who showed that prefrontal ChCs fired in the transition from slow oscillations to theta oscillations (Massi et al., 2012), while basket cells were involved in the entrainment of gamma oscillations.

1.6 Axo-axonic synapse function

1.6.1 Anatomy of the axo-axonic synapse

The most characterising feature of ChCs are their unique axo-axonic synapses. Paradoxically, their function is perhaps the most researched and yet unresolved matter in ChC physiology. Axo-axonic synapses form symmetric GABAergic synapses at the AIS (Steinecke et al., 2017). As mentioned before, the AIS is the site of action potential initiation (Palmer and Stuart, 2006). This privileged targeting confers on axo-axonic synapses the potential for total control over neuronal firing. At the postsynaptic end, GABA_A receptors (GABA_AR) accumulate at synaptic contacts (Nusser et al., 1996; Nyíri et al., 2001) on the AIS, as can be identified by both GABA_AR or gephyrin immunostaining (Wefelmeyer et al., 2015) (a postsynaptic protein which docks GABA_AR at the membrane (Essrich et al., 1998)). On the presynaptic side, GABA_B receptors (GABA_BR) modulate GABA release from axo-axonic boutons (Buhl et al., 1994). Upon GABA release from ChCs, GABA_AR receptors at the AIS are activated, allowing the flow of chloride (Cl⁻) (Rivera et al., 1999). Cl⁻ anions are negatively charged, thus their movement across the membrane will consequently affect the neuron's resting membrane potential (V_m).

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1.6.2 Hyperpolarising and depolarising GABA

What effect does GABA_AR activation have on neuronal computation? This is thought to depend on two factors: 1) the direction of Cl⁻ movement across the membrane which consequently affects the neuron's membrane potential, and 2) the reduction in input resistance generated by channel opening which has a shunting effect on voltage propagation. An ion will move across the membrane until electrical and chemical forces are in balance, resulting in no net flow of the ion. The membrane potential at which this happens is called the equilibrium potential (E_{ion}). Upon GABA_AR opening Cl⁻ and HCO₃⁻ will move in or out of the cell towards E_{Cl} and $E_{HCO_3^-}$ respectively (Kaila, 1994). However, GABA_AR are $\approx 2\times$ more permeable to Cl⁻ than HCO₃⁻ (Fatima-Shad and Barry, 1993). Therefore, in neurons with a high Cl⁻ intracellular concentration E_{Cl} will determine the equilibrium potential of GABA (E_{GABA}). Because Cl⁻ is negatively charged, if there is a net inflow of chloride, the cell will be hyperpolarised and the E_{GABA} will be said to be hyperpolarising. Conversely, a net outflow would result in a depolarising E_{GABA} .

We can see from this explanation that the role of GABA (i.e E_{GABA}) is tightly regulated by E_{Cl} . In turn, E_{Cl} is thought to depend on the expression of Cl⁻ transporters, which pump Cl⁻ in and out of the cell, and the intracellular concentration of impermeant anions (Glykys et al., 2014). Interestingly, the expression of Cl⁻ transporters can vary in response to different factors leading to changes in E_{GABA} . The most common example of this is the developmental GABA switch. In mature pyramidal cells, it is thought that the concentration of chloride inside the cell is lower than outside, which results in a hyperpolarising E_{GABA} . Conversely, it is widely believed that immature cells in the perinatal brain have a depolarising E_{GABA} (Ben-Ari et al., 2007), which transitions to its hyperpolarising state during the first postnatal weeks.

The molecular mechanism behind this switch is thought to be related to the differential expression of the chloride transporters NKCC1 and KCC2 throughout development (Kaila et al., 2014). NKCC1 pumps chloride, sodium and potassium into the cell and KCC2 extrudes chloride and potassium. In the perinatal brain, it is thought that the expression of NKCC1 is higher than KCC2 (Figure 1.3.A). This increases intracellular Cl⁻, producing a depolarising E_{GABA} . However, after early development (P7-P10 in the case of rodents), NKCC1 expression decreases, moving E_{GABA} to a hyperpolarising state (Rivera et al., 1999; Yamada et al., 2004; Romo-Parra et al., 2008) (Figure 1.3.B).

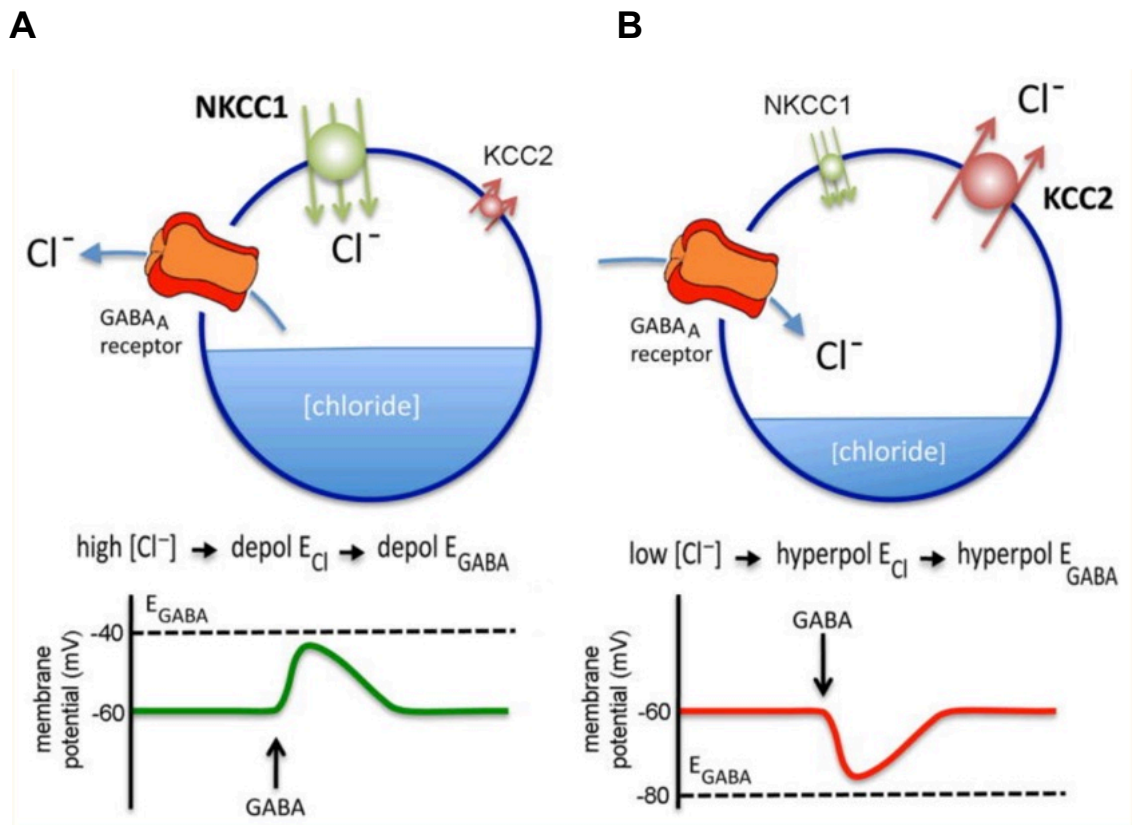


Figure 1.3 Schematic diagram describing the relationship between Cl⁻ transporters, Cl⁻ concentration and GABA_A function. (A) In immature neurons NKCC1 (which pumps Cl⁻ into the cell) is expressed at a higher concentration than KCC2 (which pumps Cl⁻ out of the cell). This leads to a higher intracellular concentration of Cl⁻ and a depolarising E_{Cl} in relation to V_m (-60 mV). This generates a depolarisation upon GABA_AR opening, as negatively charged Cl⁻ ions leave the neuron (green line). **(B)** In mature neurons, Cl⁻ transporter expression is reversed, leading to a decrease in intracellular Cl⁻ concentration compared to the extracellular space. This generates a hyperpolarising E_{Cl} in comparison to V_m, leading to hyperpolarisation upon GABA_AR opening (red trace) (Herbison and Moenter, 2011).

1.6.3 Axo-axonic synapses can be depolarising

Mature axo-axonic synapses were thought to be inhibitory like most adult GABAergic synapses. However, a seminal paper by Gábor Tamas's group in 2006, showed that the firing of ChCs led to GABA based depolarisations in pyramidal cells when recording from human acute slices (Szabadics et al., 2006). This observation was then repeated by several labs using acute slices from rodents (Khirug et al., 2008; Molnár et al., 2008;

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Woodruff et al., 2009), suggesting that E_{GABA} and E_{Cl} might be depolarising specifically at the AIS. However, this proposition has been controversial. A later study using extracellular recordings reported a hyperpolarising role of GABAergic synapses at the axon, contradicting the previous studies (Glickfeld et al., 2009). Likewise, correlates of interneuron function have suggested an inhibitory role of ChCs *in vivo*. Two studies showed that in response to chandelier cell activation *in vivo*, there was an associated reduction in the firing rate of nearby cells in both cortex and hippocampus (Viney et al., 2013; Lu et al., 2017). Similarly, Klausberger et al. 2003 showed that ChC firing happens during the hyperpolarising phase of the theta cycle.

The origin of these incongruities has been discussed extensively in the past decade. Glickfield et al. 2009 attributes the depolarising E_{GABA} observed in Szabadics et al., 2006 to an artefact in their methodology. E_{GABA} is often difficult to measure by standard intracellular recording techniques such as whole-cell patch clamp. This is because intracellular chloride gets dialysed by the internal solution of the recording pipette when accessing the cell. To solve this, Szabadics and colleagues used perforated patching. This is a technique where the antibiotic gramicidin is added to the recording patch pipette to generate nanoscale pores in the cellular membrane. These pores allow electrical access but block the flow of anions, leaving the intracellular chloride concentration intact. However as pointed out by Glickfield et al. 2009, gramicidin patches could still affect the concentration of cations such as sodium and potassium, which in turn could corrupt the recordings. This possibility is however unlikely, as shown by Woodruff et al. 2009. They recorded ChC-evoked potentials in the cell-attached configuration, where the intracellular milieu of the neuron is undisturbed, and still observed a depolarising role of ChCs.

Recently, a seminal paper from the Bender lab might have brought a final explanation to this discrepancy (Rinetti-Vargas et al., 2017). Their study shows that these differences can be mostly explained by the age of the specimens used (Rinetti-Vargas et al., 2017). As mentioned before, the switch in GABA polarity from a depolarising to a hyperpolarising role is thought to occur in rodents by the second postnatal week. However, it was not known if this was the same for all the compartments of the neuron. In Rinetti-Vargas et al., 2017 they show that the GABA switch appears to be delayed in the AIS in comparison to the somatodendritic compartment, with P21 mice still showing a depolarising E_{GABA} at the AIS (Figure 1.4). When putting previous studies into this

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developmental context, we can observe that studies using periadolescent rodents (P15-P21) showed a depolarising role of ChCs (Szabadics et al., 2006; Khirug et al., 2008; Woodruff et al., 2009, 2011), while studies using adult rodents reported a hyperpolarising role (Glickfeld et al., 2009; Viney et al., 2013; Wang et al., 2014).

It is important to note that this developmental change does not explain all the literature of axo-axonic physiology. The original Szabadics et al. 2006 study was done in tissue extracted from adult humans suffering from meningioma (52-58 years old), where according to this theory E_{GABA} should be hyperpolarising. Also, a recent study has shown that depolarising GABA at the soma could be detected *in vitro* but not *in vivo*, in anaesthetised neonatal mice (Valeeva et al., 2016). This is in accordance with a previous report showing that the cutting procedure for acute slices can result in the upregulation of NKCC1 and downregulation of KCC2, leading to an artificial depolarising E_{GABA} (Hasbargen et al., 2010). However, intact *in vitro* preparations, where no cutting is involved, have also shown depolarising GABA (Valeeva et al., 2013). Also, Valeeva et al., 2016 measured the role of GABA through secondary measures that could be affected by the fact that the mice were anaesthetised during the recordings. They recorded average sEPSC frequency in response to interneuron activation, a measure that could be abnormal in an anaesthetised state. In fact, *in vivo* recordings in other vertebrates such as tadpoles have confirmed the existence of excitatory GABA *in vivo* (Khakhlin and Aizenman, 2012). Ultimately, even if depolarising somatic GABA is an artifact of the slice preparation, this has only been tested in the soma. The relative difference in E_{GABA} between AIS and soma described in Rinetti-Vargas et al., 2017, could still apply *in vivo*.

Nonetheless, these studies have highlighted the need for studies to assess the developmental change in axo-axonic polarity in awake mice *in vivo*.

1.6.4 Can axo-axonic depolarisation lead to excitation?

A depolarising role of axo-axonic synapses does not necessarily mean that they are also excitatory and able to generate action potentials. So far, no study has shown direct evidence for an excitatory role of ChCs. However, secondary evidence has been found *in vitro*. In Rinetti-Vargas et al., 2017, E_{GABA} at the AIS was found to be above threshold in several occasions in acute slices from periadolescent mice. Similarly, in several studies

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polysynaptic EPSPs could be recorded after ChC activation in acute slices. This suggests that a population of neurons that has not been recorded from was firing in response to ChC stimulation (Szabadics et al., 2006; Glickfeld et al., 2009; Boldog et al., 2018).

Could ChCs generate action potentials *in vivo*? This possibility has not been tested so far. However, one study has tried to simulate an *in vivo* like state in the slice. In Woodruff et al. 2011, they performed paired patching of pyramidal cells and depolarising ChCs. They observed that ChCs can fire more easily than pyramidal cells and that stimulation of the L1 afferents above ChC threshold but below pyramidal cell threshold, leads to a depolarising E_{GABA} . Finally, by introducing white noise to reproduce an 'awake like' condition in the slice, E_{GABA} at the AIS appeared to be hyperpolarising, as the V_m of the pyramidal cells increased (Woodruff et al., 2011). This experiment suggests that even if ChCs might be depolarising, in an awake state with high network activity ChCs would still be hyperpolarising. Only in conditions where cortical activity is minimal could ChCs have a facilitating role. This study was published before we knew about the delayed GABA switch at the AIS (Rinetti-Vargas et al., 2017). Therefore, it is entirely possible that in younger brains E_{GABA} at the AIS is above threshold, or depolarising enough to produce an action potential with minimal excitatory drive. In fact, modelling studies have shown that subthreshold depolarising GABA could be sufficient to generate action potentials (Gulledge and Stuart, 2003).

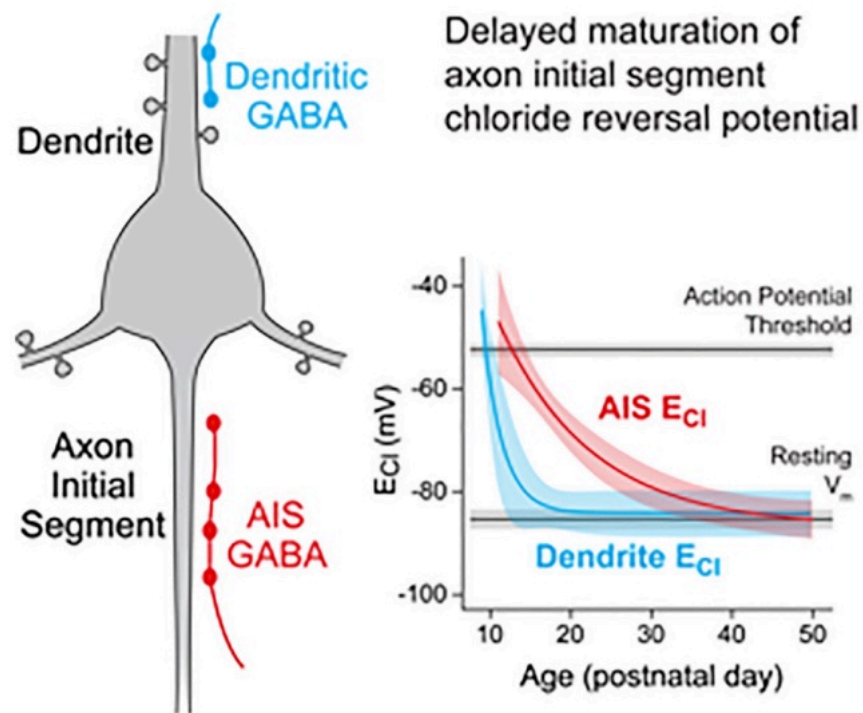


Figure 1.4. Development of E_{Cl} in different neuronal compartments after birth.

E_{Cl} at the AIS switches from a depolarising to a hyperpolarising role at a later age than in dendrites. (Rinetti-Vargas et al., 2017)

1.7. The axon initial segment

When reviewing axo-axonic synapses, it is important to not only examine the function of the axo-axonic bouton but also review the space they contact. The AIS is the site of action potential generation (Palmer and Stuart, 2006). This is due to the large number of low-threshold, voltage-gated Na^+ and K^+ channels it contains. This gives it a lower threshold than the soma. The AIS is situated at the proximal end of the axon; its exact length can vary and normally covers the first 20-50 μm of the axon (Gutzmann et al., 2014). Its morphological definition can vary depending on the method used for detection (e.g EM vs immunohistochemistry). In this thesis, I define the AIS as the axonal structure expressing the highest concentration of the anchor protein AnkerynG (a common hallmark in the field (Gutzmann et al., 2014)). In recent years, the AIS has been shown to be a highly plastic structure, involved in the electrophysiological homeostasis

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of the neuron. It can move and change its length, affecting the overall excitability of the cell (Grubb and Burrone, 2010; Kuba et al., 2010; Wefelmeyer et al., 2015) (Figure 1.5).

In vitro studies in neuronal cultures (Grubb and Burrone, 2010) and organotypic slices (Wefelmeyer et al., 2015) showed that high levels of activity in hippocampal pyramidal cells can move the AIS distally, causing a decrease in the intrinsic excitability of the neuron. Complementary, Kuba et al. 2010 showed in the chick auditory nucleus that sensory deprivation leads to an increase in AIS length and not position, which was associated with a homeostatic increase in excitability.

What are the molecular mechanisms underlying AIS plasticity? For changes in AIS position, it is thought that the activation of L-type Cav1 voltage-gated calcium channels is causal for this phenomenon to happen (Evans et al., 2013). However, the decrease in excitability associated with AIS movement is not associated with changes in ion channel composition. Interestingly, it is the relationship with axo-axonic synapses that makes this change homeostatic. Mathematical modelling of AIS plasticity shows that movement of the AIS alone in the distal direction should lead to an increase in excitability (Baranauskas et al., 2013). It is the shunting effect of the axo-axonic synapses that remain in position, at the proximal end of the axon (where the AIS used to be), that generate a homeostatic decrease in excitability (Wefelmeyer et al., 2015). Nonetheless, it is important to note that in *in vitro* hippocampal cultures, where axo-axonic innervation is very little or absent, increased activity has also been shown to trigger a distal movement of the AIS (Grubb and Burrone, 2010). This movement was also correlated with decreased excitability, suggesting that AIS movement on its own can generate this homeostatic change. This is in contrast to current AIS computational models (Baranauskas et al., 2013), suggesting that there must be an unknown component mediating the observed plasticity.

On the other hand, plastic increases in AIS length are associated with changes in ion channel expression. In response to sensory deprivation, neurons in the auditory nucleus are thought to replace Kv1 channels with Kv7s. This leads to a reduction in the shunting conductance generated upon action potential firing, which ultimately increases the neuron's excitability (Kuba et al., 2015).

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The length of the AIS of pyramidal cells has also been shown to change during early postnatal development in the cortex, when both excitatory and inhibitory afferents are being developed. The functional consequences of these changes are not known. However, it could be speculated that excitability changes related to AIS restructuring could be involved in the refinement of cortical circuits in the early postnatal brain (Cruz et al., 2009; Gutzmann et al., 2014; Kuba et al., 2014).

It is still an outstanding question how these different forms of AIS plasticity interact with each other and how specific they are to specific neuronal types. Activity dependent AIS movement has so far been shown to exist in hippocampus and the olfactory bulb. On the other hand, activity-dependent changes in AIS length have been observed in the chick auditory nucleus. Which cell types use each type of AIS plasticity? Are these two plastic mechanisms mutually exclusive? Are changes in length affected by or affect axo-axonic function? Answering these questions will be key for understanding not only AIS physiology but also the function of ChCs.

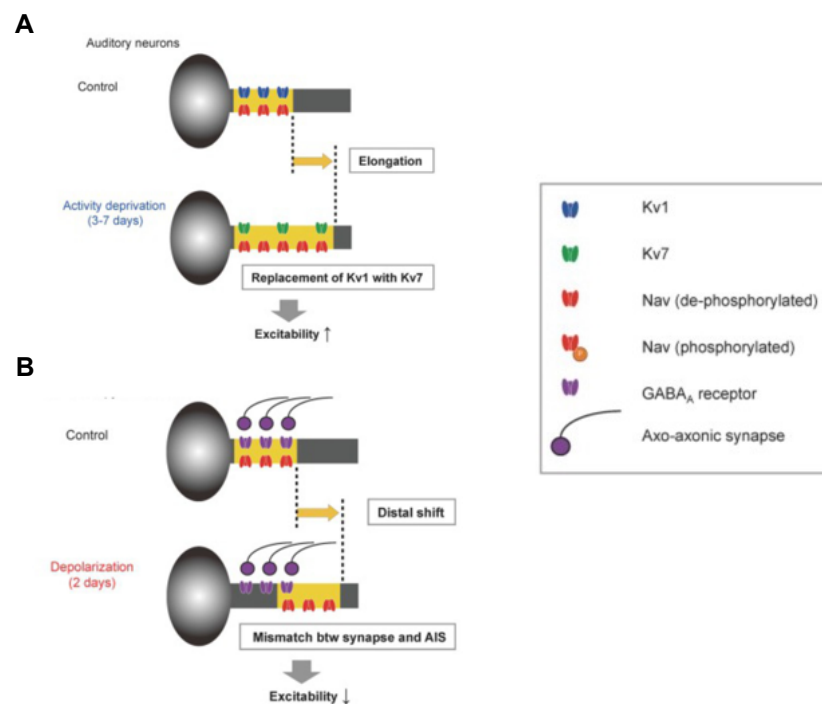


Figure 1.5. Structural plasticity of the AIS. (A) Chick auditory nucleus neurons show elongation of the AIS in response to sensory deprivation. This increases excitability by replacing Kv1 channels with Kv7 channels (Kuba et al., 2015). **(B)** Hippocampal CA1 pyramidal cells present a distal movement of the AIS after 2 days of chronic depolarisation, resulting in decreased excitability. It also creates a mismatch in axo-axonic synapse position, increasing the effect on excitability (Wefelmeyer et al., 2015). (Adapted from (Yamada and Kuba, 2016)).

1.8. Chandelier cell development

As highlighted by the different polarity of axo-axonic synapses at different ages, it is essential to understand ChC development to understand its function. Moreover, genetic and environmental factors that come into play during the development of neurons are often determinant on their final function and can be the cause behind many common neurological diseases. In this section I will cover the limited information we have on ChC development and comment on which external factors might affect this process.

1.8.1 Embryonic development and migration

Until recently, due to the lack of genetic mouse lines for the specific targeting of ChCs, it was difficult to study their developmental origin. The development of the *Nkx2.1^{+/-}CreER2* mouse line (Taniguchi et al., 2011) permitted the preferential labelling of ChCs in hippocampus and cortex. *Nkx2.1* is a homeodomain protein in charge of controlling the temporal competence of interneuron progenitors (Sussel et al., 1999; Butt et al., 2008). When inducing Cre at late embryonic stages (>E15.5) or postnatally in *Nkx2.1+* interneuron progenitors, ChCs and a minority of basket cells are labelled in a sparse and temporally controlled manner, allowing fate mapping (Taniguchi et al., 2013).

Interneurons follow a different migratory and developmental timeline than excitatory neurons in the cortex. Currently it is believed that most interneurons come from three structures in the subpallium: the medial ganglionic eminence (MGE), the caudal ganglionic eminence (CGE) and the preoptic area (POA) (Gelman et al., 2012). *Nkx2.1+* progenitors are present in the MGE, the ganglionic eminence that produces the majority of interneurons (50-60%). The MGE is thought to disappear at E15.5 and transition into a transient structure where *Nkx2.1+* interneuron progenitors are still present, called the ventricular germinal zone (VGZ). In Taniguchi et al. 2013 it is shown that it is in this latter structure where *Nkx2.1* progenitors differentiate mostly into ChCs (70-90%). Interestingly, knocking out *Nkx2.1* from VGZ progenitors leads to the disappearance of ChCs, confirming the VGZ origin of these cells.

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In the same study, Taniguchi and colleagues characterised the development and migration of ChCs during late embryonic development and early postnatal age. They found that immature ChCs migrate from the VGZ along the ventricle, reaching cortex by E18.5/P0. At this point two migratory streams are created. These two streams travel medially and laterally until they reach the cortical ventricular zone. Immature ChCs then cross the cortical plate, which is already formed by this age, reaching L1 by P3. After this stage, from P3 to P7, ChCs migrate to deeper layers to populate the L2, L2 border and L5 (Figure 1.6. A-D).

This unique developmental and migratory pattern makes ChCs the last set of interneurons to be born. They are born after the formation of all the cortical layers they innervate, and therefore do not follow the inside out developmental pattern of pyramidal cell development in cortex. This is unique in interneuron development and particularly different to other MGE interneurons, which are thought to follow pyramidal cell development (Miller, 1985).

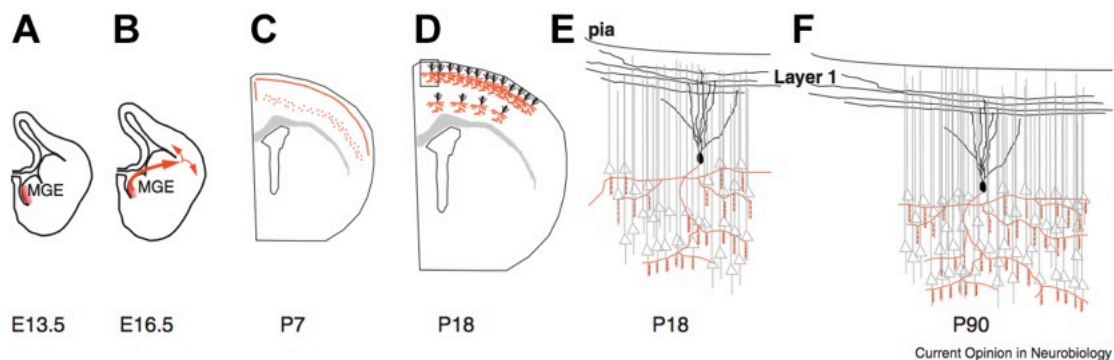


Figure 1.6. ChC development in the *Nkx2.1*^{+/-}CreER/tdtomato line. (A) ChCs originate from the ventral MGE, which later becomes the VGZ. ChCs are born as early as E13.5, but their birth rate peaks (B) at E16.5. From the VGZ, ChCs migrate to the cortical ventricular zone. (C) Postnatally, ChCs cross the cortical plate and form a plexus between L1 and 2, from which they migrate towards deeper layers. (D) After cell migration has finished, ChCs concentrate at the L1/L2 border and L5. (E-F) By P18, ChCs present a mature morphology. From this point, only minor increases in lateral span of the axonal arbour and total number of cartridges can be observed. Adapted from (Inan and Anderson, 2014).

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1.8.1 Maturation of chandelier cell morphology

After migration is complete by P7, ChC cartridges start to develop. These are thought to be visible by P14 and stable with mature characteristics by P18. After this point, it is thought that ChCs only undergo small changes in overall morphology, with small increases in the overall lateralization of their axonal arbour (Figure 1.6 E-F) (Inan and Anderson, 2014). Interestingly, in the case of monkeys it has been reported that cartridge density in cortex increases early after birth (3 months) but declines with age (3 years old) (Cruz et al., 2003; Fish et al., 2013); whether this is also the case in rodents is not known. Remarkably, it has been recently reported in a study in mice that the axonal arbour of ChCs present a significant decrease in the total number of varicosities between P16 and P21, in accordance with previous observations in monkeys and suggesting the presence of synaptic pruning in ChCs (Steinecke et al., 2017). Another study looking at long term changes in ChC morphology in mice detected an increase in the lateralisation of the axonal arbour between P18 and P90 (Inan et al., 2013).

1.8.2 Development of electrophysiological properties

As important to ChC development as the development of their morphology and connectivity, is the development of their fast-spiking properties. At this point, the body of literature is very small. A recent study has shown that ChCs present repetitive firing as early as P12, however they have a lower excitability, and lower fast-spiking capabilities than mature ChCs at P30 (Miyamae et al., 2017). Similarly, a previous study showed that by P23 ChCs presented mature spiking properties (Woodruff et al., 2009).

1.8.3 Activity dependent development of interneurons

The development of neural circuits is not only affected by their genetic composition but also by activity levels. We have known for a long time that activity levels driven both intrinsically and by sensory experience, shape the way neurons and circuits develop. Whether this is also the case for chandelier cells is not known. However, examples from other cell types in the literature suggest that this is likely.

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Early studies in visual cortex already showed that sensory experience, and consequently sensory driven neuronal activity, had profound effects on cellular connectivity. In the 1970s, Hubel and Wiesel showed that temporary monocular deprivation in kittens left neurons in the lateral geniculate nucleus permanently unresponsive to stimuli in the eye that was deprived. This only happened if sensory deprivation occurred during the neonatal stage that we now define as the ocular dominance critical period (Hubel and Wiesel, 1970; Hubel et al., 1977). Since then, numerous studies have shown that sensory driven and spontaneous activity in several brain structures, including the mammalian cortex, induce activity-dependent developmental plasticity (Spitzer, 2006). Most of the published work has focused on excitatory pyramidal cells (Cancedda et al., 2007; Wang et al., 2007). However, we now know that much of this plasticity, including the original Hubel and Wiesel ocular dominance plasticity, is driven to a large extent by inhibitory neurons of the PV family (Hensch, 2005; Kuhlman et al., 2013).

More recently, the wiring of interneurons has also been shown to be sensitive to activity levels in the brain during development. Two consecutive papers showed that thalamocortical activity drove the development of neurogliaform interneuron connectivity (De Marco García et al., 2011, 2015). Similarly, it has been shown that Reelin+ 5HT3R interneurons in barrel cortex require sensory stimulation during the first postnatal week in order to form functional excitatory-inhibitory networks (Che et al., 2018). Likewise, Marques-Smith et al. in 2016 showed that activity in SST cortical interneurons was necessary for triggering the formation of neonatal specific inhibitory circuitry (Marques-Smith et al., 2016).

At a synaptic level, activity and more particularly release of neurotransmitter are also known to be important for the maturation of GABAergic synapses. For example, release of GABA from GABAergic boutons is necessary and sufficient for the formation of GABAergic synapses onto pyramidal cell dendrites in the cortex during early periods of development (Oh et al., 2016). Likewise, it has been repeatedly shown that in the absence of GABAergic neurotransmission, cortical GABAergic synapses fail to mature (Chattopadhyaya et al., 2007; Lin et al., 2018).

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Finally, at a cellular level, it has recently been shown that the total number of interneurons in the cortex is also regulated in an activity dependent manner (Denaxa et al., 2018; Wong et al., 2018). In these studies, it was demonstrated that in response to high network activity there is a significant increase in interneuron survival. During early postnatal development (1st and 2nd postnatal week), a great number of interneurons undergo apoptosis. However, the extent of this cell death appears to depend on the level of activity of the network, which homeostatically promotes the survival of inhibitory interneurons.

Whether any of the activity dependent processes described above happen in ChCs is currently not known. However, due to their high innervation of entire circuits, it is likely that activity dependent remodelling is necessary to ensure optimal functioning.

1.9. Chandelier cells and disease

Understanding GABAergic function is not only critical for understanding cortical computation but also has major clinical implications. In recent years, it has become evident that many neurological disorders such as schizophrenia, autism and epilepsy (DeFelipe, 1999; Marín, 2012) are associated with deficits at the level of interneuron function. ChCs have been particularly associated with the pathophysiology of epilepsy and schizophrenia.

1.9.1 Schizophrenia

Schizophrenia is a type of psychosis with a complex pathophysiology and underlying genetic and environmental causes (Os et al., 2010). Among the many neural circuits thought to be disturbed in this psychiatric disorder, there have been several indications that the GABAergic system and particularly ChCs might be malfunctioning. Evidence for the involvement of ChCs in schizophrenia has come from post-mortem anatomical studies and identified common genes and environmental factors affecting ChC and schizophrenia development.

At a more general level, the schizophrenic brain is thought to present deficits in the expression of the GABA transporter GAT-1 and the synthetic enzyme in charge of the

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production of GABA, GAD67, suggesting a deficit in overall GABAergic function (Lewis et al., 2005). This appears to also affect ChCs, as post-mortem tissue from schizophrenic patients shows a decrease of 40% in the number of ChC cartridges positive for the GABA transporter GAT-1 (Woo et al., 1998). Similarly, at the AIS of cortical pyramidal cells from schizophrenic patients there is a higher concentration of GABA_AR, in what could be a compensatory effect for decreased axo-axonic innervation (Volk et al., 2002).

Schizophrenia often arises during adolescence (Woo, 2014). It is before and during this period that environmental risk factors are thought to trigger this disorder (Os et al., 2010). Similarly, both in rodent and primates ChCs are thought to be the last set of interneurons to integrate into brain circuits, and their development is thought to be mainly postnatal during the periadolescent period (Cruz et al., 2009; Fish et al., 2013; Taniguchi et al., 2013). Interneurons are specially plastic during their development (Wong and Ghosh, 2002; De Marco García et al., 2011, 2015; Denaxa et al., 2018) and it is therefore likely that strong environmental factors such as the ones thought to trigger schizophrenia might also lead to aberrant ChC function. In fact, schizophrenia is thought to arise at least in part from aberrant development of the GABAergic system (Schmidt and Mirnics, 2015). In favour of this hypothesis, it is thought that one of the highest genetic factors related with schizophrenia is also known to specifically affect ChC development. This is the NRG1/ErbB4 pathway (Stefansson et al., 2002). Disrupting the signalling of this pathway specifically in ChCs is thought to lead to a decrease in the number of boutons in a synaptic cartridge (Fazzari et al., 2010) (Figure 1.7). Similarly, in a different study, ErbB4 knockout in hippocampal fast-spiking PV cells led to a decrease in axo-axonic boutons and the presence of behavioural phenotypes (deficits in social behaviour and increase in locomotion) associated with schizophrenia (Del Pino et al., 2013).

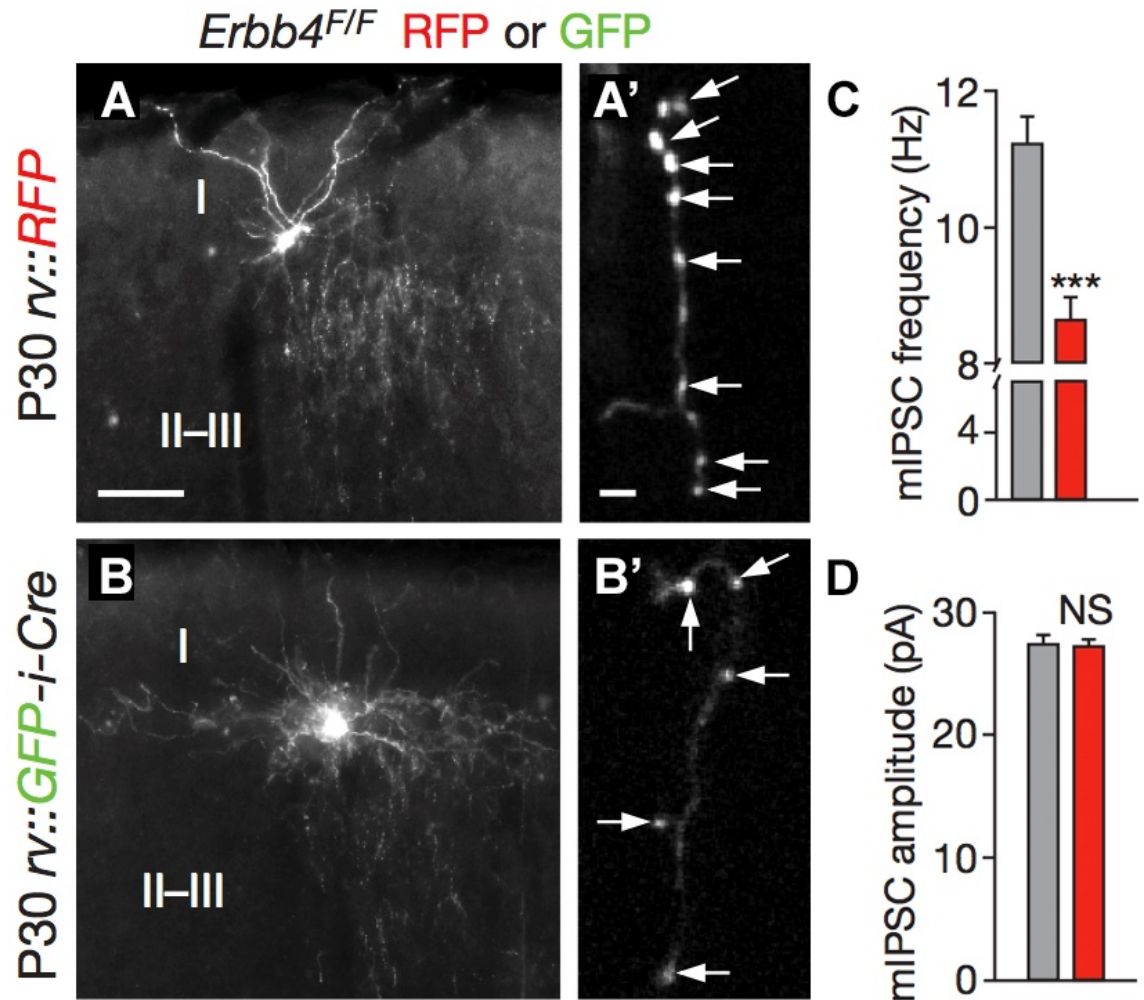


Figure 1.7. ErbB4 knockout in L2/3 ChCs of P30 mice. (A) Example image of wildtype (A) ChC and its (A') cartridge (B) ErbB4^{-/-} (B) ChC and (B') cartridge. Frequency (C) and amplitude (D) of mIPSCs in wild type (gray) and ErbB4^{-/-} ChCs (red). *** P<0.0001. Scale bar A,B = 100 μ m, A', B' = 2 μ m. Adapted from (Fazzari et al., 2010).

1.9.2 Epilepsy

Inhibition imbalances have long been associated with epilepsy (Fritschy, 2008). ChCs are also thought to be involved in the development of this neurological disorder. Early studies in monkey (Ribak, 1985) and rat models (Dinocourt et al., 2003) of epilepsy showed a decrease in axo-axonic innervation in the cortex and hippocampus. For example, kindled rats, a common epilepsy model, have been shown to have a lower number of axo-axonic synapses (Sayin et al., 2003). Similarly, human tissue from patients suffering temporal lobe epilepsy also showed a similar decrease in the number

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of cartridges (Marco et al., 1996; Marco and DeFelipe, 1997). However, a later study (Wittner et al., 2005) did not find a change in axo-axonic coverage and found that deficits in axo-axonic innervation were related to an actual decrease in pyramidal cell numbers.

Despite the ChC-related deficits reported, the role that ChCs may play in the pathophysiology of epilepsy is not known. Are they a causal feature of the runaway excitation we observe in epileptic seizures? Or are any changes in their morphology a secondary event to the presence of seizures? As mentioned before, whether ChCs are excitatory or inhibitory is still a matter of major debate. Understanding this function first will hopefully illuminate the functional consequences of a potential decrease in axo-axonic innervation in epilepsy. ChCs fire during periods of large excitation in cortex (Zhu et al., 2004). If ChCs are inhibitory, it could be hypothesized that ChCs are recruited to short-circuit epileptic activity (Paz and Huguenard, 2015) and that their malformation in epilepsy is causative of seizures. Alternatively, if ChCs are excitatory, the decrease in axo-axonic innervation might be a homeostatic adaption secondary to seizure appearance.

Understanding the role and plasticity of ChCs might not only give us an insight into the origins of seizure formation but also propose targets for future treatments. In recent years, research on closed-loop optogenetic systems for manipulating neuronal activity online have been proposed as a potential treatment for epilepsy. These approaches consist of the online detection of seizure onset and consequent optogenetic inhibition of pyramidal cells (Paz et al., 2013) or activation of PV interneurons (Krook-Magnuson et al., 2013) to limit propagation. In the case of closed-loop systems targeting PV interneurons (e.g. (Krook-Magnuson et al., 2013)), knowing whether PV basket cells and PV ChCs have the same function would improve the effectiveness of these methods.

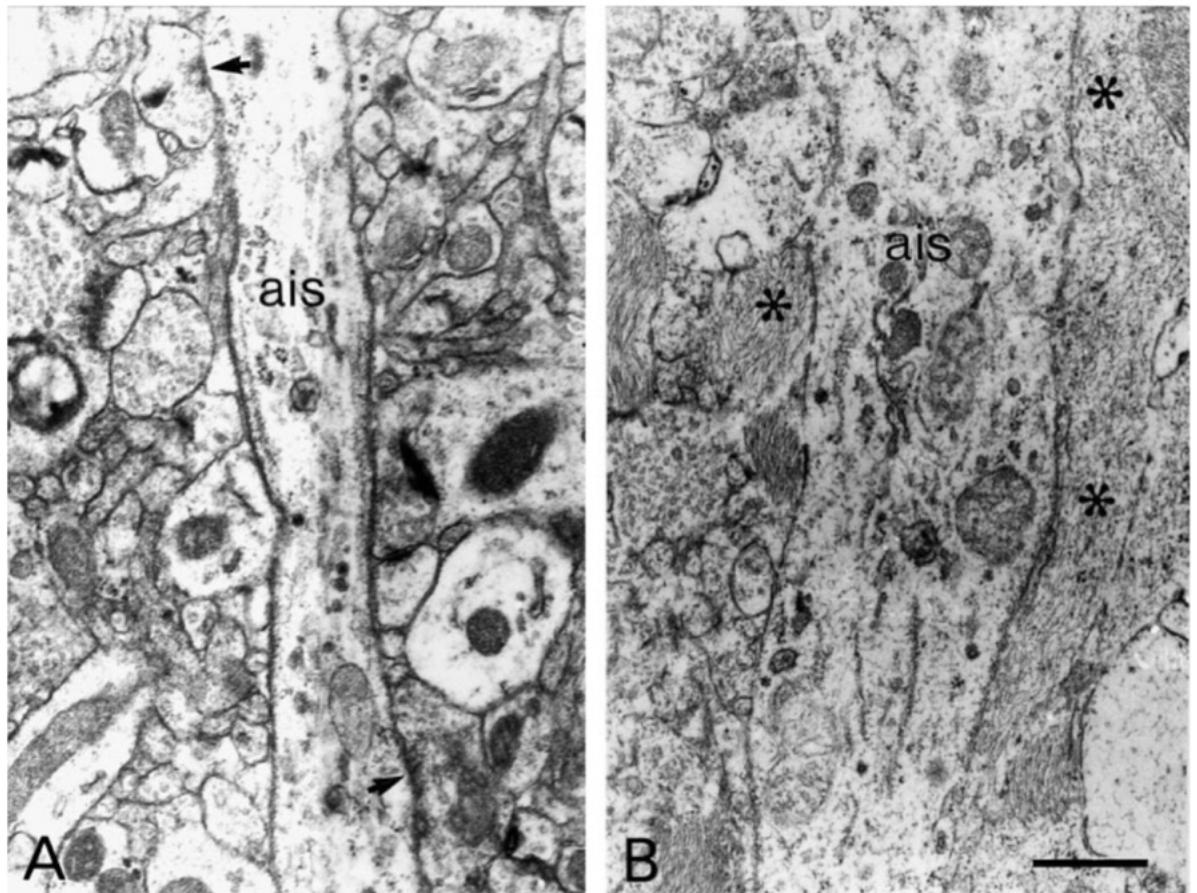


Figure 1.8 AIS and axo-axonic synapses in human epileptic cortex. Electron micrographs of a pyramidal cell AIS in **(A)** normal human temporal cortex with axo-axonic synapses and from a **(B)** human peritumoral neocortical region where no axo-axonic synapses can be found. Arrows indicate axo-axonic synapses, asterisks indicate glial processes. Scale bar: 0.5 μm (Adapted from: (DeFelipe, 1999)) .

1.10. Aims

In this chapter I have reviewed the current state of knowledge on the form, function and development of chandelier cells in the neocortex. Despite the recent progress on our understanding of these cells, questions remain regarding how these cells integrate into cortical microcircuits, how they adapt to them and what is their function. In the next three chapters I will try to answer some of these questions. More specifically:

1. In Chapter 3, I will investigate how ChCs develop their axonal arbour in the neocortex and how axo-axonic cartridges develop and mature structurally and functionally.

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2. In Chapter 4, I will investigate whether ChC development is affected by activity levels and whether they are plastic after maturation.
3. In Chapter 5, I will investigate what is the function/polarity of axo-axonic synapses during the periods of plasticity detected in Chapter 4.

Chapter 2 : Materials and Methods

2.1 Animals:

2.1.1 Transgenic lines:

Male and female mice were used for all experiments (See Appendix 2. For list of animals used in this thesis). They were housed in individually ventilated cages and provided with *ad libitum* food and water. Delivery of viral vectors was done in the Nkx2.1^{+/-}-CreER line (JAX014552). For morphology and electrophysiological experiments the Nkx2.1^{+/-}-CreER line was crossed with the Ai9 (JAX007909) or Ai32 line (JAX012569). Both homozygous and heterozygous mice were used. For morphological analysis Nkx2.1^{+/-}-CreER mice were crossed with the Ai9 (JAX007909) line. For measuring the intrinsic excitability of chandelier cells, Nkx2.1^{+/-}-CreER mice were crossed with both the Ai9 and the Ai32 line (JAX012569). For measuring the electrophysiological properties of the axo-axonic synapses Nkx2.1^{+/-}-CreER/Ai32 mice were used. For measuring sIPSCs frequency Nkx2.1^{+/-}-CreER/Ai32 were used. Finally, for recording the intrinsic excitability of pyramidal cells after DREADDs treatment, Ai9 mice were used. All procedures were carried out under project license PPL70/8573 in accordance with the UK Animal Scientific Procedures Act.

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2.1.2 Genotyping:

DNA extraction:

Ear samples were collected from animals age P10 or older. DNA was extracted by a 1h incubation in 100 µl of 50 mM NaOH at 95°C and shaking. The reaction was neutralised with 10 µl of Tris buffer (1 M). Samples were centrifuged at 14,000G to remove cell debris from suspension.

PCR:

The following mastermix was prepared for each PCR reaction:

PCR reaction (1x):

12.5µl Biomix Red (Bioline) / Biomix (Bioline) (for tdTomato reactions only)

10.3µl H₂O

1µl each primer

1µl DNA

Primers:

Nkx 2.1:

Wildtype reverse: 5' - CCTGGCCCTGTCTGTACG

Mutant reverse: 5'-ATGTTTAGCTGGCCCAAATG

Common: 5' - GCCTCCACTCAAGCCAATTA

tdTomato:

Wildtype Forward: 5' – AAGGGAGCTGCAGTGGAGTA

Wildtype Reverse: 5' – CCGAAAATCTGTGGGAAGTC

Mutant Forward: 5' – CTGTTCCTGTACGGCATGG

Mutant Reverse: 5' – GGCATTAAAGCAGCGTATCC

Ai32:

Wildtype Forward: 5' - AAGGGAGCTGCAGTGGAGT

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Wildtype Reverse: 5' - CCGAAATCTGTGGGAAGTC

Mutant Forward: 5' - ACATGGTCCTGCTGGAGTTC

Mutant Reverse: 5' - GGCATTAAAGCAGCGTATCC

All PCR reactions were set in 100µl PCR tubes (Eppendorf) placed in a Lifepro Thermocycler (BIOER) and went through the following protocol:

Step 1

95.0 °C – 120 sec

Step 2 (10x cycle)

95.0 °C – 20 sec

65.0 °C – 15 sec

72.0 °C – 10 sec

Step 3 (28x cycle)

95.0 °C – 20 sec

60.0 °C – 15 sec

72.0 °C – 10 sec

Step 4

72.0 °C – 120 sec

The PCR product was then run in a 2.5% agarose gel for 50min at 100V. The following bands were expected for each genotype:

Nkx2.1-CreER wildtype: 321 bp

Nkx2.1-CreER mutant: 475 bp

tdTomato wildtype: 297 bp

tdTomato mutant: 196 bp

Ai32 wildtype: 297 bp

Ai32 mutant: 212 bp

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2.2 Preparation and storage of injectables:

2.2.1 Clozapine-N-Oxidase (CNO):

For DREADDS experiments CNO (Enzo Life Sciences) was dissolved in 0.5% dimethyl sulfoxide (DMSO) and 0.9% saline to a final concentration of 0.5 mM. Once in solution CNO was aliquoted and stored at -20°C until the day of use. Animals were injected intraperitoneally twice daily with saline (0.9%) with DMSO (0.5%) or CNO in DMSO (0.5%) at 1mg/kg. The volume injected of saline was calculated based on the CNO equivalent for the given weight of the animal (on average 30 µl). On the final day of the experiment animals were only injected once, 2h before perfusion.

2.2.2 Tamoxifen:

For the optimal labelling of ChCs, Tamoxifen dependent Cre recombination was induced at P2. For experiments using cre-dependent viral vectors, tamoxifen was injected a P3. Tamoxifen (Sigma) was dissolved in corn oil (30 mg/mL) and injected intraperitoneally at a dose of 100µg/g.

2.3 Surgical procedures:

2.3.1 In-utero electroporation:

In utero electroporation was performed in E15.5 pregnant mice (Walker et al., 2017). Females were anaesthetised with isofluorine 2.5% before the abdomen was opened and the uterine horns exposed. DNA was delivered to the embryos via injection into the lateral ventricles with a glass micropipette. A total of 0.5-1µL of DNA solution was injected in each embryo. The DNA solution contained Fast Green (0.3% (wt/vol)) and DNA in TE buffer at (2000ng/ml concentration). Five square electric pulses (30 V, 50 ms) were passed at 1s intervals using a squarewave electroporator (CUY21EDIT; NEPA GENE).

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2.3.2 Intracranial neonatal injections:

Mice were anaesthetised with 2.5% isoflurane. Injections were targeted to layers 2/3 of the somatosensory cortex. The following coordinates were used: Anterior-Posterior-axis: 2.7 mm, Dorso-Ventral: -0.3 mm, Lateral-axis: -2.3 mm from Bregma. A total volume of 250 nl was infused at a flow rate 50 nl/min. For optimal labelling of chandelier cells, viral injections were conducted at P3. All injections were performed with a Nanoliter injector (WPI) controlled with a SMARTouch controller (WPI).

2.3.3 Cranial windows:

Cranial windows were implanted covering somatosensory cortex as in previous studies (Wong et al., 2018). P11-P12 mice were anaesthetised with 2% isoflurane in a nose-clamp and body temperature maintained at 37 °C with a heating pad. The local anaesthetic Bupivacaine was injected sub-cutaneously (10µl of 0.5 µg/ml) and the anti-inflammatory dexamethasone was applied intramuscularly (10 µl of 38 µg/ml solution) at the start of the procedure. The scalp was cleaned with betadine and cut open to expose the cranium. Lidocaine Hydrochloride (1% w/v) was then applied briefly to the cranial surface before the periosteal tissue was carefully removed with a scalpel. The cranial surface was then cleaned with Ringer's solution and a few drops of betadine. A custom-made metal head-post (Luigs and Neumann) was glued to the right hemisphere with cyanoacrylate glue (Henkel). Dental cement (Paladur) was applied around the head-post and the cranium to reinforce the implant. A 3 mm craniotomy was opened over the somatosensory cortex with a dental drill until the dura was exposed. Care was taken not to cross the bone sutures. Cortex buffer (NaCl [123.35 mM], KCl [5 mM], Glucose [10 mM], HEPES [10 mM], CaCl₂ [2 mM], MgSO₄ [2 mM]) was applied to the exposed surface and a 3mm glass coverslip (Harvard Apparatus) placed directly over the craniotomy, attached at the edges with cyanoacrylate glue and strengthened with dental cement. At the end of the procedure, mice were injected with saline and left to recover for 4h - 1 day before imaging.

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2.4 Molecular biology:

2.4.1 DNA constructs:

The following constructs were used in this study: CAG:hM3Dq+:GFP, CAG:hM3Dq+:RFP and CamKII:ACE2N-mNeon-4AA. All constructs were kept in TE buffer and stored at -20°C. Working aliquots were kept at 4°C.

2.4.2 Viral vectors:

The adeno-associated-viruses (AAV) AAV8-hsyn-hM3D(Gq)-mCherry (UNC Vector repository) and AAV9-CAG-Flex-EGFP-WPRE-bGH (UPenn Vector core) were used to drive the expression of GFP and hMD3q(Gq DREADDs) respectively in ChCs, upon tamoxifen mediated *Cre* recombination in the *Nkx2.1^{+/-}CreER2* mouse. Viral vectors were kept at -80°C prior to use.

2.5 Immunohistochemistry:

Immunohistochemistry was used for the detection of synaptic markers, *cfos* expression as well as for the imaging of all genetically expressed proteins (tdTomato, GFP and mCherry). Mice used for immunohistochemical analysis were transcardially perfused with 10ml of ice-cold saline solution followed by 20ml of 1% (wt/vol) PFA in HEPES (pH 7.3). The brains were carefully removed and put in 4% (wt/vol) PFA solution overnight at 4 °C. For optimal slicing, brains were embedded in blocks of 6% agarose (Sigma) and sectioned coronally (100 µm thick) with a vibratome (Leica). Slices were kept in in PBS supplemented with 0.05% Sodium Azide to improve preservation. Slices were incubated at RT for 2h in PBS-Triton (0.25%) supplemented with 10% Goat Serum (GS) (Sigma). This was followed by an overnight incubation in a primary antibody solution in PBS-Triton (0.25%)-GS (10%) (Table 1 for full list). After three 20 minute PBS-Triton (0.25%) washes, all slices were incubated for 2 hours at room temperature in secondary antibody solution in PBS-Triton (0.25%)-GS (10%) (Table 1 for full list). The secondary antibody solution was washed off as before and slices were mounted with Vectorshield mounting media with DAPI (VectorLabs) and kept at 4°C.

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Primary Antibody	Concentration	Supplier	Secondary Antibody	Concentration	Supplier
Anti-AnkyrinG-Mouse2b	1:1000	Millipore	Anti-Mouse2b-Alexa Fluor-488 or 647	1:500	Life Technologies
Anti-DsRed-Rabbit	1:500	Takara	Anti-Rabbit-Alexa Fluor-568	1:500	Life Technologies
Anti-GFP-Chicken	1:200	Abcam	Anti-Chicken-Alexa Fluor-488	1:500	Life Technologies
Anti-VGAT-mouseY3	1:500	Synaptic Systems	Anti-mouseY3-Alexa Fluor 647	1:500	Life Technologies
Anti-cFos-mouseY1	1:500	Santa Cruz	Anti-mouseY1-Alexa Fluor 647	1:500	Life Technologies
Anti-NeuN-mouseY1	1:500	Millipore	Anti—Alexa Fluor 568	1:500	Life Technologies

Table 1. List of primary and secondary antibodies.

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2.6. Fluorescence imaging:

2.6.1. Confocal Imaging:

A Nikon A1R inverted confocal microscope with a 40x water immersion objective (NA 1.1) was used for the acquisition of images from immunostained slices at 1024 x 1024 pixels. For synaptic measurements, a 3x zoom was used. Stacks had a z-step of 1 μ m. The following lasers were used: 408 nm, 488, 561 nm and 638 nm in combination with 450/50 nm, 525/50 nm, 595/50 nm and 700/75 nm filters, respectively. All images were acquired with NIS Elements software (Nikon).

2.6.2 2-photon *in vitro* imaging:

During electrophysiological recordings from chandelier cells, cell identity was confirmed by 2-photon imaging. A Chameleon femtosecond pulsed laser (Coherent) was used at a wavelength of 920nm and 1080nm depending if the reporter was positive for GFP-ChR2 or tdtomato respectively. Images were acquired at 1024x1024 pixels. Cell identity was confirmed by position (L1-L2/3 border) and the presence of axo-axonic cartridges.

2.6.3 2-photon *in vivo* imaging:

Mice were anaesthetised with Ketamine/Xylazine (100 mg/kg ketamine and 10 mg/kg xylazine). Imaging sessions lasted 2-3 hours. The same field of view was imaged over consecutive days. tdTomato was excited with a Ti-Sapphire laser (Coherent Chameleon) at 930 nm wavelength. Emitted light was collected by a GaAsP detector through a 1.0 NA, 20 $\times$ objective (Olympus). The excitation power was between 40 and 50 mW.

2.7 Electrophysiology:

2.7.1 Internal solutions:

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For intrinsic excitability measurements of chandelier and pyramidal cells the internal solution in the recording pipette contained: K-gluconate (130 mM), KCl (15 mM), 10 Na₂Phosphocreatine (10 mM), HEPES (10 mM), MgATP (4 mM), GTP (0.3 mM) and EGTA (0.3 mM), pH was adjusted to 7.3 with KOH and to 320 mOsm.

For recording IPSCs a high chloride intracellular solution was used. For chapter 3 experiments this was composed of: KCl (150 mM), 10 MgCl₂ (4.6 mM), CaCl₂ (0.1 mM), HEPES (10 mM), NaATP (4mM), NaGTP (0.4 mM) and EGTA (1 mM), pH was adjusted to 7.3 with KOH and to 320 mOsm. Because of the prevalent presence of action currents in >P18 and older animals, QX-314 (Tocris) (5mM) was included in the solution for IPSC recordings in the DREADDs experiments in Chapter 4.

2.7.2 Preparation of acute slices:

Nkx2.1-CreER-Ai9 mice (JAX) were anaesthetised with Isoflurane and their brains were rapidly removed in cold sucrose dissection media (Sucrose 240 mM, KCl 5mM, Na₂HPO₄ 1.25 mM, MgSO₄ 2 mM, CaCl₂ 1 mM, NaHCO₃ 26 mM and D-glucose 10mM). 300 um thick coronal slices were made with a V1000S vibrating blade microtome (Leica) in sucrose dissection media constantly carbonated (95% O₂ 5% CO₂). Slices were transferred to an immersion storage chamber in artificial cerebrospinal fluid (ACSF) with the following composition: NaCl (124 mM), KCl (5 mM), Na₂HPO₃ (1.25 mM), MgSO₄ (2 mM), CaCl₂ (2 mM), NaHCO₃ (26 mM), D-glucose (20 mM). Slices were left to recover for 1 hour at room temperature before recording.

2.7.3 Patch-clamp recordings:

Targeted whole-cell patch clamp recordings were made at the soma of ChCs or pyramidal cells at layer 2/3 of cortex under voltage and current clamp configurations. Acute slices were transferred to a recording chamber equipped with a BX51 (Olympus) microscope with Dodt gradient contrast optics. For targeted patching, tdTomato+ ChCs were illuminated with a 2-pE collimator (CoolLED) which resolved the soma and arbour of the cell. Slices in the recording chamber were constantly superfused with ACSF and constantly carbonated (95% O₂ 5% CO₂). For all recordings, the AMPA receptor blocker NBQX (10 µM), and the NMDA receptor blocker APV (25 µM) were present in the bath.

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Patching electrodes were made from thick-walled borosilicate glass (O.D: 1.5mm, I.D: 0.86 μ m, Sutter Instruments) and polished with a micro forge MF-900 (Narishige) obtaining a resistance between 3-4 $M\Omega$. Recordings were obtained with a Multiclamp 700B amplifier (Molecular Devices) and digitised with the Digidata 1440A digitizer (Molecular Devices). Data was acquired with the software Clampex (Molecular Devices) and Axon Multiclamp Commander Software (Molecular Devices). Signals were filtered at 10kHz and sampled at 50kHz. Pipette offsets were nulled before seal formation and pipette capacitance was compensated in the cell-attached configuration once a giga-seal was obtained. All measurements in voltage-clamp were made after series resistance compensation for 7 $M\Omega$ (Compensation table, *Appendix 1.*). All recordings were done at room temperature. Membrane resting potentials were checked in $I = 0$ mode and passive features were recorded on-line. For intrinsic excitability measurements, 10ms and 500ms current injections of increasing amplitude (10 pA steps) were injected in current clamp configuration. For measuring IPSCs, cells were held in voltage-clamp at -70mV, while ChC were stimulated with light with 2ms pulses from a 488nm LED (intensity at the level of the specimen: 5.8 mW/mm²). A 10 s interval was left between trials to allow for the full replenishment of the vesicular pool. For measuring spontaneous IPSCs, cells were hold at -70mV and left in a gap-free protocol for 3 min., Recordings were excluded if access resistance surpassed 30 $M\Omega$ and/or its leak current was below -300pA.

2.8 Voltage imaging:

2.8.1 Imaging:

Acute slices were prepared as explained in the previous section from mice electroporated with the voltage indicator ACE-mNeon in prefrontal and somatosensory cortex. Slices were transferred to a recording chamber equipped with a BX51 (Olympus) microscope with Dodt gradient contrast optics, 488nm & 568nm LEDs (CoolLED) and an Evolve 512 EMCCD camera (Photometrics). The slices were continuously superfused with carbonated ACSF containing NBQX (10 μ M) (Tocris), and APV (25 μ M) (Tocris). In the iontophoresis experiments the GABA_BR inhibitor CGP55845(10 μ M) (Tocris) was also always present in the bath. Cells were illuminated with a 488 nm LED at 29mW/mm² intensity while imaging at *circa* 120Hz with a EMCCD camera through a

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40x objective. To image at this acquisition rate, 4x4 binning was used, giving a final resolution of 128x128pixels. Each recording consists of 40-50 repeats with the same stimulus. To obtain a baseline of camera noise, acquisition started 50ms prior to LED illumination and finished 100ms after. LED illumination, camera acquisition and stimulation were synchronised with a Digidata 1440A digitizer (Molecular Devices) through the software Clampex (Molecular Devices).

2.8.2 Imaging input-voltage functions:

ACE-mNeon+ cells were patched in whole cell configuration as described in section 2.7.3. An input-voltage protocol was used in which the patched cell was passed through increasing voltage steps of 5mV from -90mV to -45mV lasting 50ms each.

2.8.4 Iontophoresis:

After selecting a ACE-mNeon+ cell an iontophoretic pipette was placed juxtaposed to the cell's AIS or soma. Iontophoresis pipettes were made from thick-walled borosilicate glass (O.D: 1.5mm, I.D: 0.86 μ m, Sutter Instruments) obtaining a resistance of 60-100M Ω . The pipettes were filled with a GABA solution containing: GABA (200mM, pH 5.0), Alexa Fluor 568 (10 μ M) and CGP55845(10 μ M) (Tocris). Alexa 568 was used to visualise GABA application. CGP55845 was also included in the iontophoretic solution to assure GABA $_B$ R inhibition from potential displacement of the blocker when GABA was ejected. A retention current of -30 nA was used when the pipette was in the bath to avoid spillage. A single pulse of 20ms and 150nA was used to eject the GABA solution from the pipette.

2.8.5 Stimulation of chandelier cells for imaging evoked potentials:

Chandelier cells with axonal arbors overlapping ACE-mNeon+ cells were patched in whole cell configuration as described in section 2.7.3. A train of 5 action potentials was generated by giving a 100Hz train of 1nA injections lasting 1ms each.

2.9. Analysis:

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2.9.1 Morphological analysis of AIS and axo-axonic boutons:

For morphological analysis, AISs and boutons were traced using the ROI manager and Simple Neurite Tracer (ImageJ). Custom MATLAB code was used to analyse the position of boutons along the AIS and their number. The fluorescent signal for the AIS fades on its ends and has its brightest points at the centre. To normalize AIS length measurements, custom MATLAB code was designed where the points delimiting the region where intensity dropped to a 20% of maximum fluorescence, were assigned as the start and end points. All analyses were performed blinded to the identity (i.e experimental condition) of the animals. Due to the sparsity of chandelier cells marked in somatosensory cortex, individual cartridges could be related to specific chandelier cells. All chandelier cell varicosities overlapping the AIS were included in the analysis of bouton number and density. For the analysis of contacts made by an individual chandelier, contact was defined as the presence of at least one ChC varicosity overlapping the AIS. For the measurement of cartridge size, a cartridge was defined as a collection of 2 boutons or more that project in the same axes as the AIS they synapse with. This measurement was based purely on axo-axonic cartridge morphology without taking into consideration if the boutons in the cartridge were contacting an axon.

2.9.2 Morphological analysis of basket cell boutons:

The number of synapses in an individual soma was analysed in a single plane as in (Del Pino et al., 2013). Somas were stained with NeuN and GABAergic synapses with VGAT. Thresholding for VGAT and NeuN immunostaining was adjusted in each individual image to ensure optimal segmentation. Segmentation and synapse counting was done automatically. Synapses were segmented with a watershed algorithm. Synapses were included if they were within a 1 μm radius of the segmented soma. Density of synapses was calculated by dividing synapse number over the perimeter of the soma. All analysis was done blinded to the identity of the animals.

2.9.3 Analysis of c-fos immunofluorescence:

To analyse the effect of DREADDs and its ligand on activity, mice were injected with CNO or saline 2h before perfusion and stained for c-Fos, a gene with activity dependent

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expression. A fluorescence threshold of 1.2x background fluorescence was set to classify cells as c-fos+. For measuring the effects of CNO on hM3Dq+ cells in hM3Dq-networks, cells were blindly selected before measuring their c-fos immunofluorescence profile.

2.9.4 Electrophysiological analysis:

Input-output curves were made with custom MATLAB scripts. Two types were used: 1) output in action potential (AP) frequency as a function of the current injected (pA) 2) output in AP frequency as function of current density (pA/Pf). This is the same as 1) but normalised for the capacitance of cell which correlates with its size. For the measurements of the voltage threshold, voltage threshold was determined by time of the first peak of the jerk (third derivative of voltage with respect to time). For the measurement of IPSC amplitudes, all analysis was done with the IGOR (Wavemetrics) package Neuromatic (Rothman and Silver, 2018). IPSCs were averaged over 15 sweeps and the maximum amplitude recorded. For the measurement of spontaneous IPSCs, template matching to a second exponential curve was used to automatically record the number of events. The parameters for template matching were as follows: rise time, 1ms, decay time, 20ms, zero-baseline before waveform, 1ms, total template waveform time, 40ms. For the measurement of release failures, only cells with at least two successful evoked postsynaptic potentials were included. Failures were defined as the absence of postsynaptic potentials in response to light stimulation on the presynaptic ChC.

2.9.5 Analysis of voltage imaging recordings:

For analysing voltage imaging recordings from evoked ChC activity, an ROI was selected covering the soma of the pyramidal cell of interest. From this ROI, the fluorescence intensity profile throughout time for each repeat (stimulation protocol repeat) was obtained and parsed through a custom-made bleach-correction algorithm (MATLAB). In this algorithm, the intensity profile for each repeat was fitted to a double exponential curve. From the resulting trace $\Delta F/F$ was calculated. F was determined as the average bleach-corrected baseline fluorescence of the last 10 frames prior to the evoked event. The resulting $\Delta F/F$ traces for each repeat were then averaged. For analysing voltage imaging recordings from the iontophoresis experiments, a slight variation to this

CHAPTER 2: MATERIALS AND METHODS

algorithm was introduced. In these experiments the bleach correction algorithm was trained in non-stimulation repeats in the same cells, where no GABA was released. More information on the analysis of voltage imaging recordings can be found in section 5.2 of this thesis (Chapter 5).

2.10 Statistics:

Statistical analysis was performed with Prism 6 (GraphPad), MATLAB and custom python scrips using the scipy.stats package. Before hypothesis testing, D'Agostino normality test was used in all data sets to determine if the data followed a normal distribution. Depending on the outcome, the parametric One-Way-ANOVA or non-parametric Kruskal-Wallis test was used when comparing over more than 2 groups. This was followed by a multiple comparison post-hoc analysis with a multiple comparisons correction. For comparison between two groups, a Student t-test or the non-parametric Mann-Wittney test was used depending on normality. Correlations were tested using Pearson's or Spearman's method depending on normality. For comparisons of frequency a χ^2 test was used. Values are presented as mean $\pm$ s.e.m. Unless otherwise stated confidence intervals and statistical tests were always based on the n number of neurons and not animals. Statistical significance was established at $P < 0.05$.

Chapter 3 : Development of chandelier cells in cortical networks

3.1 Introduction

As mentioned in the introduction of this thesis, ChCs probably remain one of the most enigmatic types of interneurons, both in terms of their development and their function. Since they were first described (Szentágothai and Arbib, 1974; Jones, 1975), our knowledge of their function and morphological features has been limited.

In this chapter I will focus on the development of the ChCs in layer 2/3 of somatosensory cortex. ChCs can be found predominantly in layers 2/3 and 5/6 of the neocortex (Taniguchi et al., 2013) and in the hippocampus (Soriano and Frotscher, 1989; Schmidt et al., 1993; Martínez et al., 1996). I chose to investigate ChCs in the somatosensory cortex since it is a well characterised region of the brain with well-defined sensory inputs (i.e whiskers) and behavioural outputs (e.g texture discrimination (Grillo et al., 2013)), in which any potential findings can be interpreted in a broader context of cortical function.

ChCs present a very defined arborisation pattern in cortex. They have short dendrites and a large axonal arbour which synapses onto the AIS (Somogyi, 1977; Fairén and

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Valverde, 1980) of hundreds of pyramidal cells in their periphery (Inan et al., 2013; Blazquez-Llorca et al., 2015). Importantly, all this information was obtained exclusively from mature ChCs. The lack of a ChC specific mouse line has limited studies of ChC morphology to mature brains, where their identification was based purely on morphological characteristics. Therefore, an accurate characterisation of how the chandelier arbour and its synapses mature is still lacking. This is critical information as their privileged synaptic targeting at the AIS, confers them with total control over microcircuit function during important plastic periods of the neonatal cortex (Hensch, 2005).

The Nkx2.1CreERT2 mouse line (Taniguchi et al., 2011, 2013) has provided the first insights into the development of ChCs more generally, and the arborisation of its axonal arbour more specifically. We now know that after migration has finished at P9, the ChC axonal arbour experiences important maturation changes. Cartridges are reported to be present at the AIS by P14 and to be stable in length and number of boutons by P18, in prefrontal cortex (Taniguchi et al., 2013; Steinecke et al., 2017). After this point, the lateralisation of the axonal arbour and the number of cartridges per ChC can also change but at a slower pace (Inan et al., 2013). However, we do not know if this timeline is similar across all cortical areas or whether the functional properties of axo-axonic synapses also change in line with their morphology.

Much like their morphological development, the maturation of the physiological properties of ChCs has not been explored in detail. Mature ChCs are fast spiking interneurons that hardly adapt to a constant current pulse, a feature they share with basket cells, but which distinguishes them from other interneurons in the cortex. A recent study has shown that ChCs develop their fast-spiking properties between P12 and P30 (Miyamae et al., 2017). However, a more detailed timeline of this process is lacking.

In this chapter I will characterise the following aspects of ChC development:

- 1) The morphological emergence of axo-axonic synapses in the cortex.
- 2) The functional emergence of axo-axonic synapses in the cortex.
- 3) The maturation of the passive and firing properties of ChCs.

3.2 Results

3.2.1 Imaging ChCs and their axo-axonic synapses

In order to label ChCs, I used the *Nkx2.1CreER* line. This line labels both ChCs (80%) and a small percentage of basket cells (20%). For the optimal labelling of ChCs, the original paper, recommended injections of Tamoxifen should be performed at E17.5 (Taniguchi et al., 2011). However, I found that this affected mouse labour, generally leading to early births that resulted in a poor survival rate of the litter. By injecting Tamoxifen postnatally at P2, I obtained optimal labelling of ChCs that was also sparse enough to allow the proper characterisation of ChC axon morphology (Figure 3.1.A). Similarly, sparse labelling permitted the exclusion of the minority basket cells labelled based on their characteristic morphology. To visualise ChCs, I crossed the *Nkx2.1 CreER* line with an *Ai9* reporter line, which expresses tdTomato in a *Cre* dependent manner. I will refer to this line as the *Nkx2.1/tdTomato* from here onwards.

As mentioned in the previous section I focused on layer 2/3 (L2/3) of the somatosensory cortex (Figure 3.1.B). I first quantified axonal arbour development immunohistochemically from P9 to P22, by sampling at regular time intervals and also included a late developmental timepoint at P42. Labelled ChCs in this area showed a clear polarity between their dendritic and axonal arbour by P12. ChC somas sat at the L1/L2/3 border with their dendrites projecting towards Layer 1 and their axonal arbours expanding ventrally into L2/3. A purely qualitative look at these cells, showed a progressive expansion of the axonal arbour from P12 until P18. This was not case for the dendritic arbour which remained constant from P12 onwards (Figure 3.1.C).

To quantify ChC cartridge development, I also had to label the AISs of pyramidal cells, as these represent the postsynaptic structures innervated by ChCs. To achieve this, I co-stained for AnkerynG (AnkG), a key structural protein of the AIS, as in a previous study (Wefelmeyer et al., 2015). As we can see in Figure 3.2 axo-axonic synapses overlapping with the AIS formed clear cartridges surrounding most AISs in the ChC's vicinity (Figure 3.2). However, AIS staining can be very variable with a high level of background in some

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cases. Moreover, AnkG immunofluorescence diminishes towards its ends, making the precise detection of the start and end positions of the AIS problematic. To partially solve this, I used an algorithm which normalises the fluorescence along a semi-automated trace of the AIS (Figure 3.3.A-B) to its maximum fluorescence (Figure 3.3.C), in 3 dimensions. The algorithm then determines the start and end positions of the AIS, as the points in which fluorescence drops to 20% of the maximum (Figure 3.3.C). (For more details see Chapter 2: Material and Methods). This approach allowed me to analyse AIS size in unbiased and systematic manner. The main limitation of this method is that due to the thresholding step, the actual length of the AIS might be different from the measure obtained by the algorithm. Similarly, due to the 20% fluorescence cut-off points, non-continuous AISs cannot be analysed.

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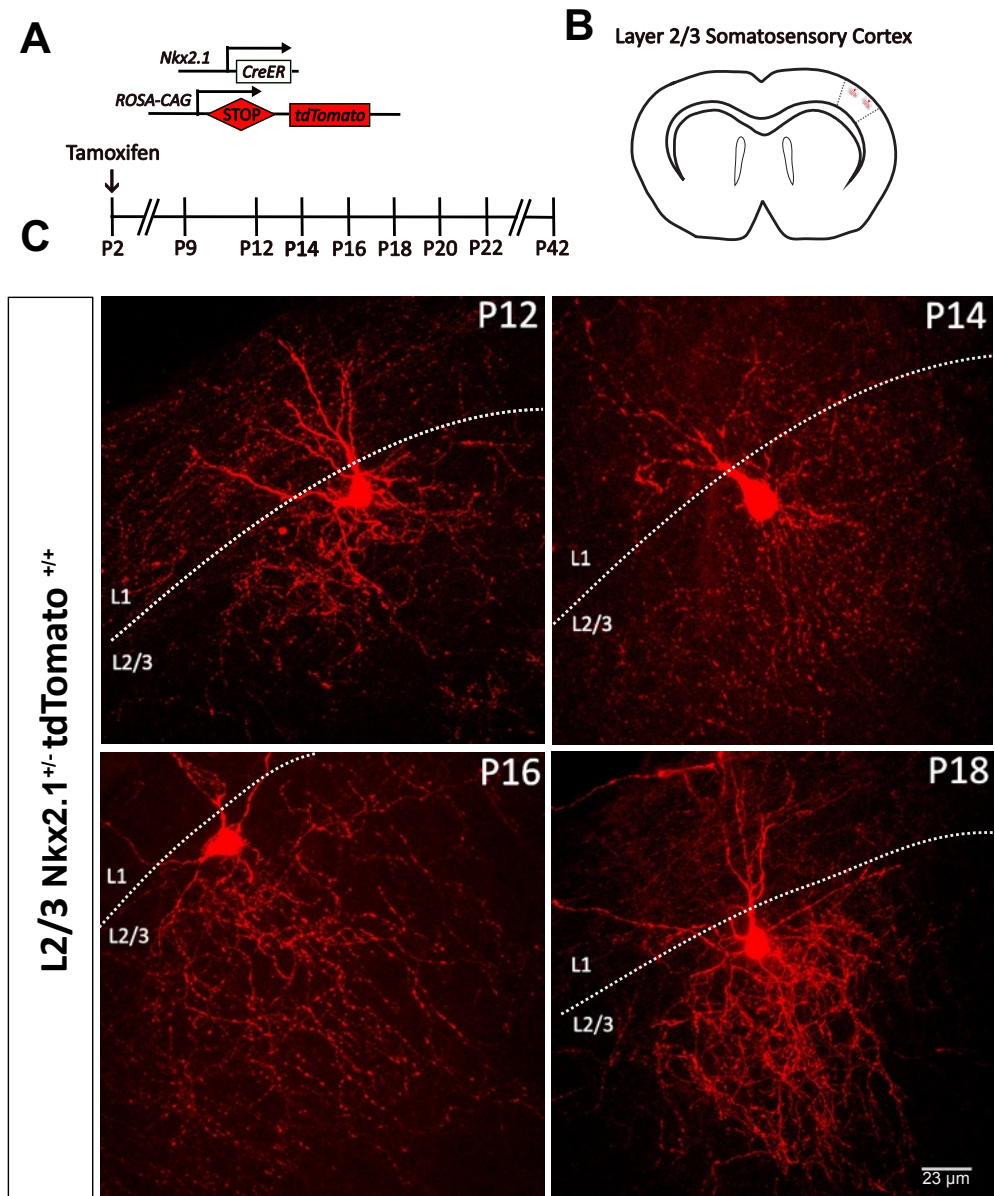


Figure 3.1 Developing ChCs in the *Nkx2.1^{+/-} Ai9(tdtomato)^{+/+}* mouse. **(A)** (Top) Genetic strategy, tamoxifen induced *Cre* recombination which activates *tdTomato* expression. (Bottom) Timeline for the induction and assessment of ChC development. **(B)** All measurements were done in layer 2/3 (L2/3) of somatosensory cortex. **(C)** Representative images of L2/3 ChCs in somatosensory cortex from P12 to P18. Dashed line indicates the L1,L2/3 border estimated from the characteristic decrease of cell body density in L1 .

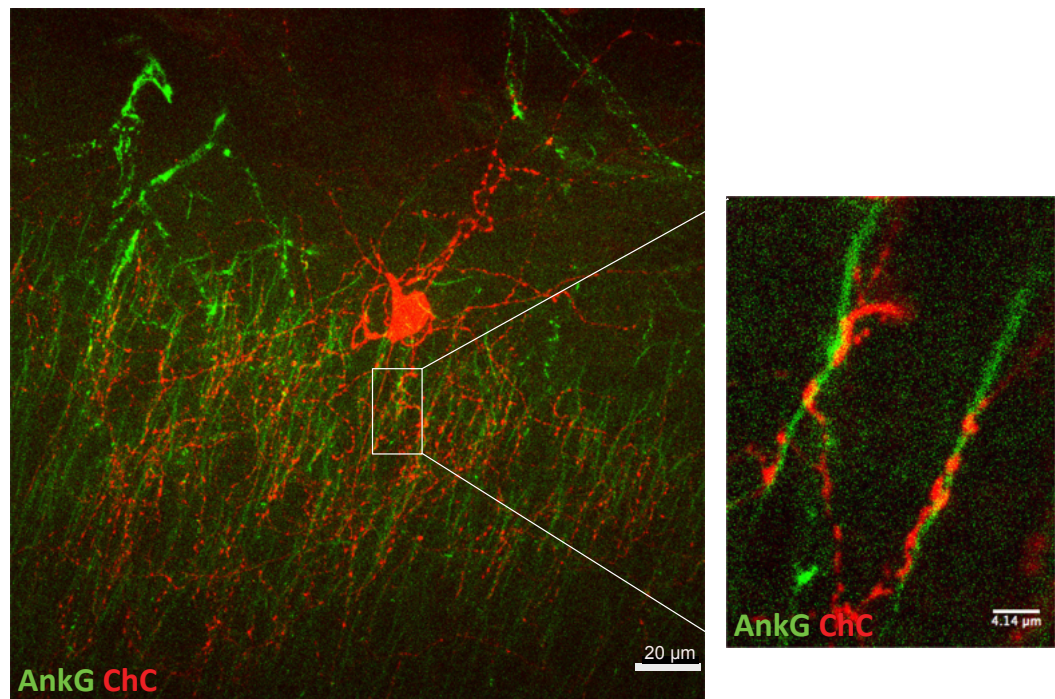


Figure 3.2 Mature ChC and cartridges. To the left, colabelling of ChC (tdtomato, red) and AISs (AnkerynG (AngK), green) at P18 in a Nkx2.1/tdtomato mouse. To the right, zoomed imaged of chandelier cell cartridge (red) and AIS (green).

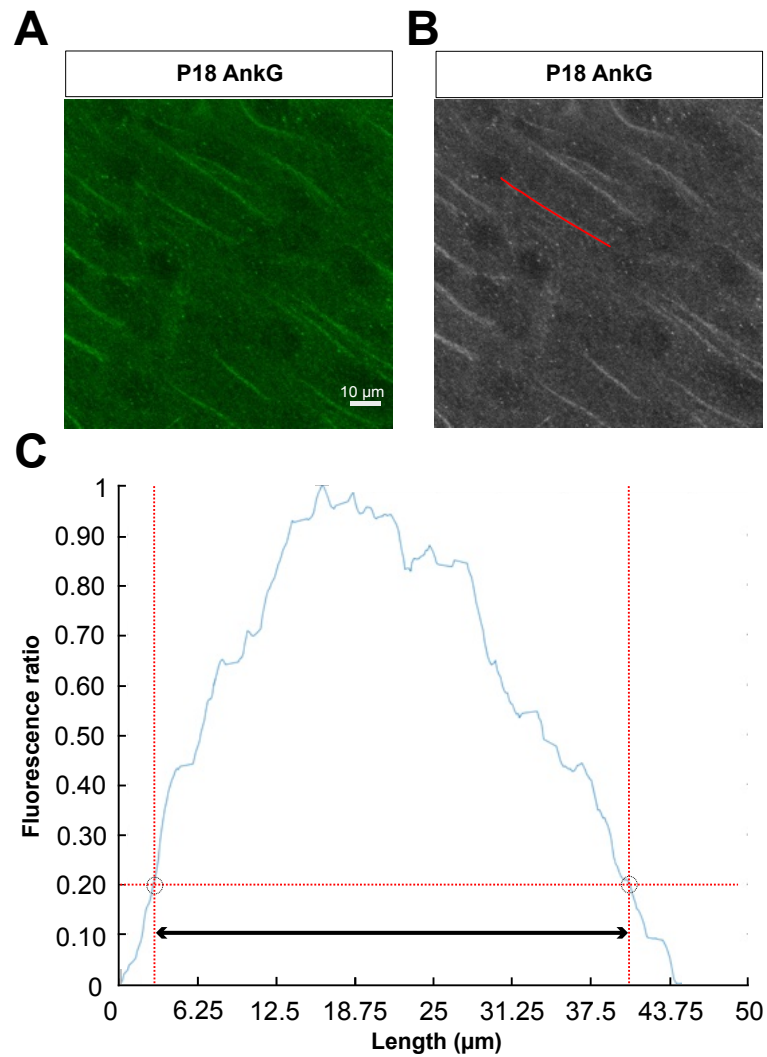


Figure 3.3 Fluorescence normalisation for the measurement of AIS length. (A) AnkG immunostaining for labelling of AIS in P18 Nkx2.1/tdtomato mouse. (B) Grayscale image of (A) with AIS traced (red line) (C) Fluorescence intensity profile of AIS labelled in (B). Blue, fluorescence values normalised to maximum fluorescence in the traced area. Dashed circles indicate the start and end of AIS. This was defined as the 20% (dashed red line) drop off points from maximum fluorescence. Black arrow indicates the resolved length of the AIS.

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3.2.2 P14-P16 constitutes a critical window for the development for axo-axonic synapses.

Using the tools described above, I quantified the development of axo-axonic synapses. Importantly, a bouton was defined as a tdTomato+ varicosity that overlaps an AIS. These varicosities are positive for the GABA transporter VGAT in >90% of cases (Steinecke et al., 2017), confirming their identity as presynaptic GABAergic boutons (Figure 3.4.A).

At P9, ChCs form a small number of boutons on AISs (Number: 2.564 ± 0.262 boutons, Density: 0.073 ± 0.007 boutons/ μm). This number stayed constant at P12 (Number: 3.844 ± 0.498 boutons, Density: 0.096 ± 0.013 boutons/ μm) and P14 (Number: 3.80 ± 0.451 boutons, Density: 0.073 ± 0.009 boutons/ μm). After this point, there was a significant increase in the density (0.167 ± 0.013 boutons/ μm) and number (7.269 ± 0.604 boutons, $P < 0.01$) of axo-axonic synapses in a period of just two days (P14-P16). These measures did not rise any further, but remained mostly constant until P42, suggesting that the P14-P16 period might constitute a critical window for axo-axonic development (Figure 3.4.B-C).

Interestingly, after P16 there appears to be a small, gradual decrease in synapse density and number, that was not significant when compared to P42 (Number: 4.986 ± 0.321 bouton, $p = 0.06$, Test: Kruskal-Wallis with posthoc Dunn's multiple comparisons). This finding, although only suggestive at this stage, may indicate the presence of axo-axonic synapse pruning. In comparison, the length of the AIS over the same developmental period did not change, remaining constant at around 20-30 μm (Figure 3.4.D). The emergence of axo-axonic synapses, therefore, appears to be independent of the postsynaptic structure it innervates.

The AIS has previously been shown to be organised into smaller spatial domains, broadly defined by their distance to the soma and the types of channels they preferentially express. Functionally, the distal AIS is thought to be the site of action potential (AP) initiation, in part due to the optimal electrical conditions in this region of the axon, but also as a result of the expression of specific channel subunits (e.g – Nav1.6). I therefore also mapped the spatial distribution of axo-axonic synapses along the AIS

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before, during and after the critical window of axo-axonic development (P12-P18). I first divided each AIS into three sections of equal length at different distances from the soma: Proximal, Medial and Distal (Figure 3.5.A). I then classified each bouton to one of these regions depending on its location. At P12, each domain was roughly equally innervated by axo-axonic synapses, with a small bias towards the medial domain ($24.6 \pm 4.1\%$, $38.9 \pm 4.1\%$, $36.4 \pm 4.7\%$ of boutons for proximal, medial and distal domains respectively). After this point, there was a gradual shift with age in synapse distribution towards distal synapses at the expense of proximal synapses. By P18 $45.9 \pm 7.7\%$ of boutons were in the distal third of the AIS and only $14.5 \pm 8\%$ were in the proximal third. This suggests that axo-axonic cartridges cluster their boutons at the distal end of the AIS as they mature (Figure 3.5.B).

Finally, I quantified the number of AISs contacted by an individual ChC over time. For this, I took a blind sample of 30 AISs in a $90\ \mu\text{m}$ radius cylinder around the soma of an individual ChC and counted the percentage of AISs that had at least one tdtomato+ varicosity, as a measure of connection probability (Figure 3.6.A). From P9 to P14, the percentage of AISs contacted was stable (P14: $36.7 \pm 6.1\%$). This value changed rapidly and significantly to $62.7 \pm 2.5\%$ at P16 ($p=0.0439$, One-way ANOVA with Sidak's posthoc multiple comparison) and remained constant thereafter, supporting the hypothesis that P14-P16 constitutes the critical developmental window for axo-axonic synapse development (Figure 3.6.B).

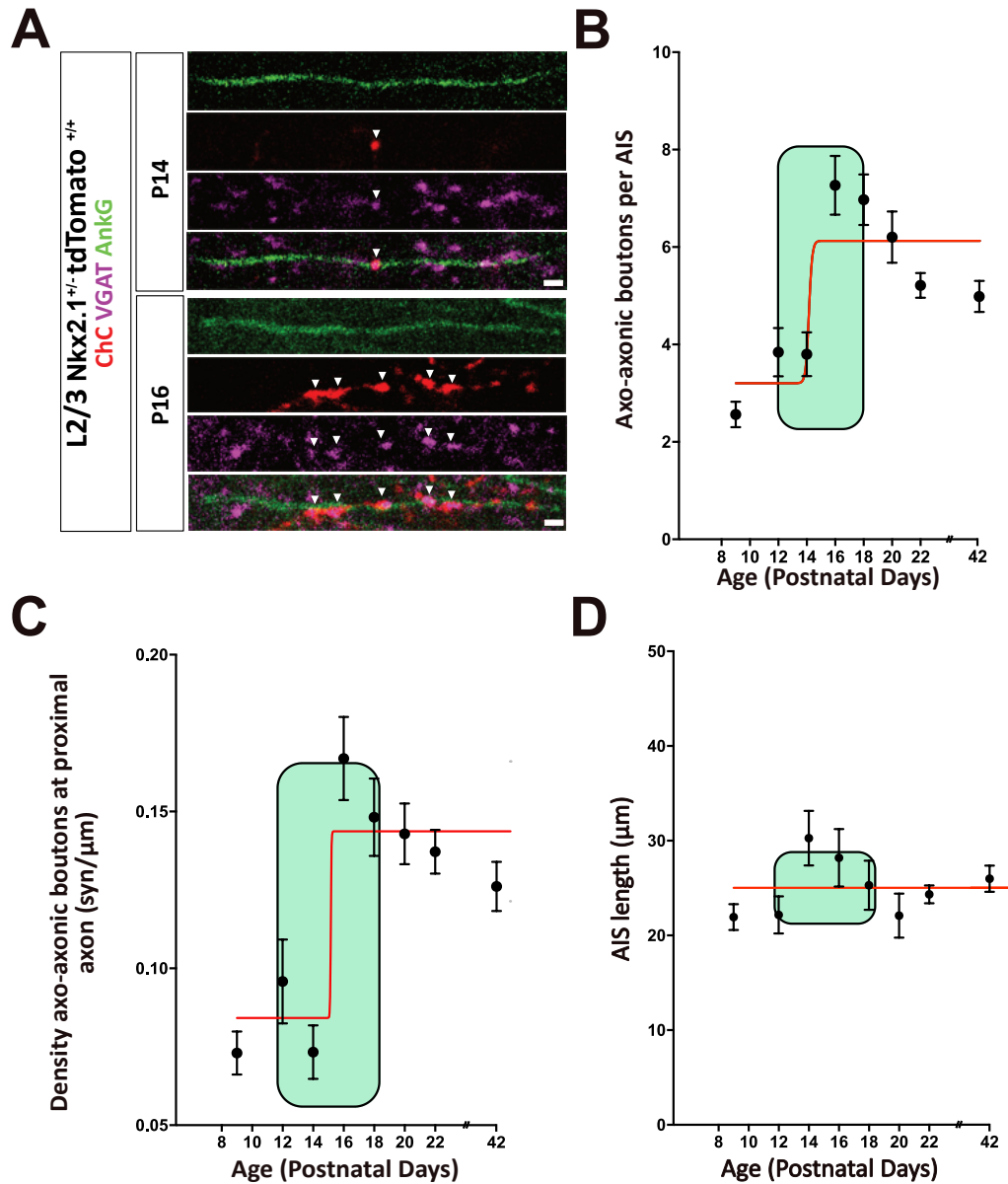


Figure 3.4 Axo-axonic synapse distribution throughout postnatal development. (A) Representative images of axo-axonic synapses (red) and AIS (green, AnkG) from at P14 and P16 in the Nkx2.1/tdtomato mouse. Axo-axonic bouton identity was confirmed by colabelling with VGAT (magenta) Average number. Scale bar: 2 μ m (B) and density (C) of axo-axonic boutons along an AIS, and average AIS length (D) as a function of postnatal age. Red line represents a sigmoidal fit of the data. Green area indicates the time period P12-P18 when most of morphological development of ChCs occurred. Values shown as mean $\pm$ s.e.m. n = 26-81 cartridges/AIS, n=2-4 mice per timepoint.

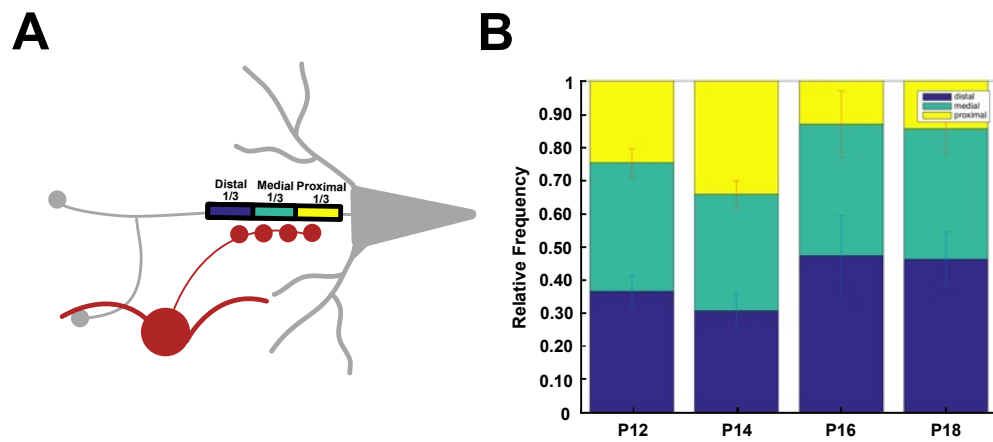


Figure 3.5 Clustering of axo-axonic synapses along the AIS throughout postnatal development. (A) Schematic diagram indicating axo-axonic boutons (red) synapsing with the proximal (yellow), medial (green) and distal (blue) third of the AIS. **(B)** Proportion of boutons along the AIS during the P12-P18 period in the Nkx2.1/tdtomato mouse. Results displayed as cumulative mean $\pm$ s.e.m for an individual cartridge. $n = 26-81$ cartridges/AIS, $n=2-4$ mice per timepoint.

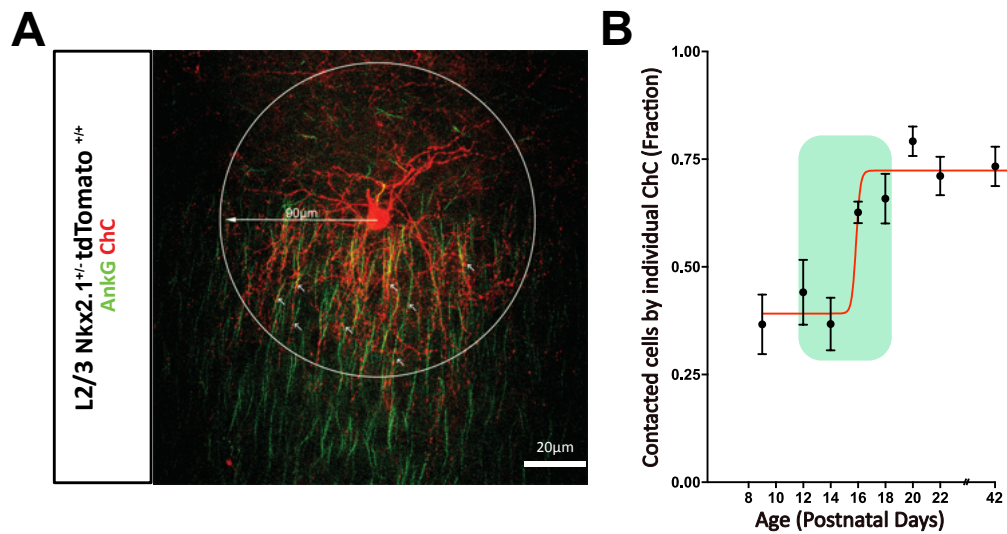


Figure 3.6 Connection probability of individual ChCs and AISs in their vicinity. **(A)** Representative images of ChC (red) and AIS (green, AnkG) in a P20 Nkx2.1/tdtomato mouse. White circle marks the area used to measure connection probability, a 90 μ m radius cylinder around soma of the ChC. Arrows indicate examples of contacted AISs. **(B)** Average connection probability of ChCs as a function of postnatal age. Red line represents a sigmoidal fit of the data. Green area indicates the time period P12-P18 when most of morphological development of ChCs occurred. Values shown as mean $\pm$ s.e.m. n = 4-6 ChCs, n=2-4 mice per timepoint.

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3.3.3 P14-P16 constitutes a critical window for the electrophysiological maturation of ChCs and axo-axonic synapses

Having described a critical window for the development of ChCs at a morphological level, I explored whether the electrophysiological properties of ChCs and their synapses also changed during this time.

In the previous section I described a critical window for the development of axo-axonic synapses. However, whether axo-axonic synapses are functional before and after this period, not only depends on the presence of synaptic machinery as shown in the previous section but also on the ability of ChCs to fire APs. I assessed this by measuring the intrinsic excitability of ChCs in immature (P12-P14) and mature cells (P18-onwards) (Figure 3.7.A). I found that both mature and immature ChCs were capable of firing action potentials (Figure 3.7.B-C). However immature ChCs were significantly less excitable than mature cells and had slower spiking properties ($P < 0.001$) when looking at their input-output relationship (Figure 3.7.D). Immature ChCs tended to have a higher rheobase (211.4 ± 60.41 pA), smaller maximum firing frequency (40 ± 8.859 Hz) (Figure 3.7.D) and higher latency to the first action potential (232.9 ± 41.93 ms) than mature ChCs (rheobase: 135 ± 19.62 pA, maximum firing rate: 61 ± 12.47 Hz, latency to first action potential: 118.9 ± 32.94 ms) (Figure 3.7.E-G).

I then proceeded to examine the synaptic properties of the axo-axonic synapse before and after the P14-P16 window. Based on the morphological data I hypothesised that inhibitory postsynaptic currents (IPSCs) from mature ChCs (P16-onwards) should have a significantly larger amplitude than those originating from immature ChCs (P12-P14). I crossed the Nkx2.1CreERT2 line with the Ai32 line which conditionally expresses ChR2 in ChCs (Nkx2.1Ai32 line). This allowed me to optically generate action potentials in ChCs and simultaneously record IPSCs in pyramidal neurons through whole-cell patch clamping.

To use this method, I first had to establish the amount of light needed to optically stimulate ChCs to induce action potentials reliably. Moreover, due to the differences found in intrinsic excitability at different ages (Figure 3.7), I suspected that different light stimulation protocols would be needed for mature (P18- onwards) and immature

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(P12-P13) ChCs. I therefore patched ChCs expressing ChR2 at both time periods, in both cell-attached and whole-cell configurations (Figure 3.8.A). I measured the relationship between the amount of light delivered and firing frequency by keeping the intensity of light constant (20% LED power) and changing the stimulus length. As expected, immature ChCs were less responsive to light pulses than mature cells, with some immature cells failing to generate action potentials in response to short light pulses (Min 1ms stim: 0.6 ± 0.245 AP, Max 20ms stim: 1.5 ± 0.447 AP) (Figure 3.8.B). In comparison, mature ChCs always generated action potentials in response to light. Short light stimulation of 1ms always generated at least 1 action potential (Min 1 ms stim: 1.5 ± 0.5 AP, Max 20ms stim: 3 ± 0 AP) (Figure 3.8.B-D). Based on this information I decided to use 2 ms as my stimulation standard, which would reliably elicit an AP in both immature and mature ChCs. Under these stimulation conditions mature ChCs would sometimes fire 2 APs, however these were always sufficiently apart so that their correspondent IPSCs were not perfectly summated. This allowed the measurement of the amplitude of the IPSC generated by the first AP.

I then measured IPSCs in pyramidal cells connected to immature (P12-P13) and mature ChCs (P18-P20) (Figure 3.9.A-B). I found that the average IPSC amplitude from mature ChCs was significantly higher (64.32 ± 13.81 pA, $P < 0.01$) than from immature ChCs (30.48 ± 3.55 pA) (Figure 3.9.C-D). Likewise, the maximum IPSC amplitude taken from 15 consecutive sweeps for each cell was significantly higher in mature (129.5 ± 33.89 pA) rather than immature ChCs (57.75 ± 8.867 pA, $P < 0.05$) (Figure 3.9.E). These results further supported the presence of the critical window of axo-axonic development that I characterised morphologically.

Finally, I measured the short-term plasticity properties of axo-axonic synapses. I stimulated ChCs twice at 20Hz and measured the pair-pulse-ratio (PPR) at each developmental period. PPR is the ratio of the amplitude of the second IPSC to the first IPSC. Both facilitating and depressing immature axo-axonic synapses could be found (PPR: 1.089 ± 0.064) while mature synapses were mostly depressing (0.9057 ± 0.091) (PPR < 1) (Figure 3.9.F).

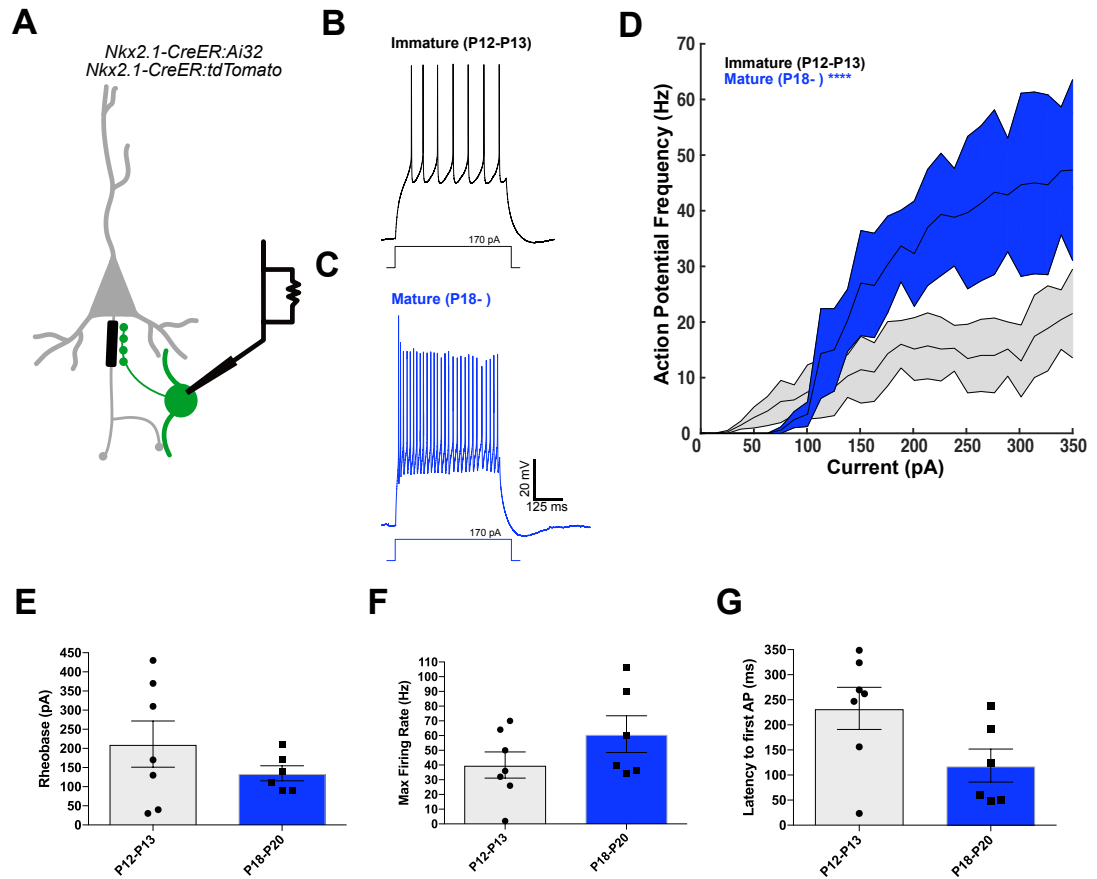


Figure 3.7 Intrinsic excitability of ChCs throughout development. (A) Chandelier cells were patched in whole cell configuration from *Nkx2.1^{+/+}-CreER* crossed with both the *Ai32* line and the *Ai9* line. (B-C) Representative traces from 500 ms current injections of 170 pA in immature (black) and mature (blue) ChCs (D) Input-Output curves showing action potential frequency as a function of the current injected into immature (black) and mature (blue) ChCs. Current injections consisted of increasing steps of 10 pA and lasted 500 ms. Centre line for each group represents mean firing rate. (E) Rheobase, (F) Max firing frequency and (G) Latency to the first action potential in immature and mature ChCs. Statistics: (E-G) Tested with Mann-Whitney test. (D) One-Way repeated measures ANOVA. Shaded area represents $\pm$ s.e.m. Immature: $n = 7$ ChCs, $n = 3$ mice; Mature: $n = 6$ ChCs, $n = 4$ mice.

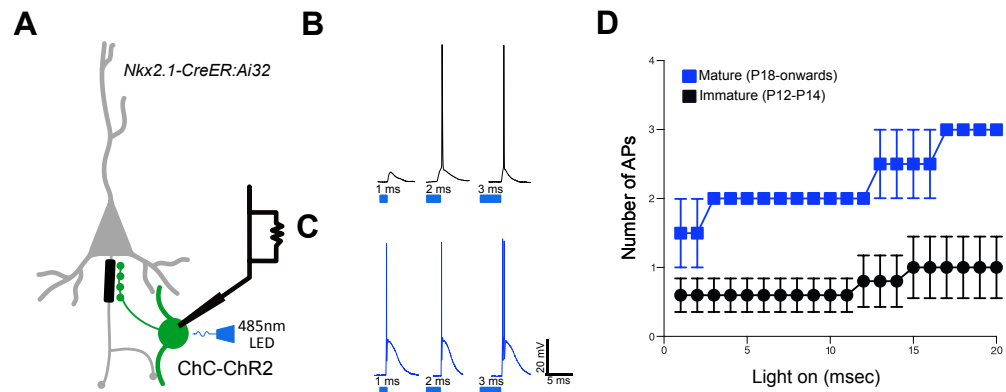


Figure 3.8 ChR2 characterisation in ChCs **(A)** Chandelier cells were patched in whole cell configuration from *Nkx2.1^{+/+}-CreER* mice crossed with both the *Ai32* line and stimulated with a 485nm LED **(B-C)** Representative action potentials generated with 1,2 and 3 ms light in immature (black) and mature (blue) ChCs **(D)** Input-Output curves showing action potential number as a function of light pulse length in immature (black) and mature (blue) ChCs. Values shown as mean $\pm$ s.e.m. Immature: $n = 5$ ChCs, $n = 3$ mice; Mature: $n = 2$ ChCs, $n = 2$ mice.

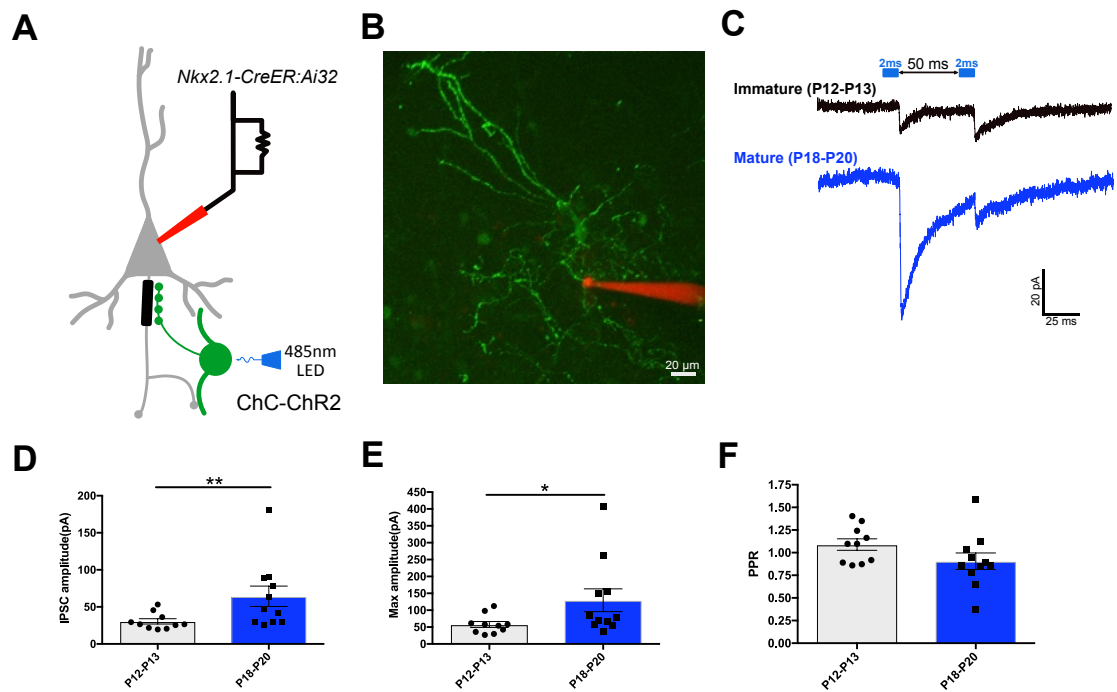


Figure 3.9 Electrophysiological maturation of the axo-axonic synapse (A) Pyramidal cells near ChCs were patched in whole cell configuration from Nkx2.1^{+/+} Ai32 mice. ChCs were stimulated with a 485nm LED (B) Representative 2-photon image of a patched pyramidal cell (red) connected to a ChC expressing ChR2-GFP (green) (C) Representative IPSCs from pyramidal cells, evoked by light stimulation (2ms, 50ms interval) of immature (black) and mature (blue) ChCs. (D) Average IPSC amplitude, (E) maximum IPSC amplitude and (F) Pair Pulse Ratio from pyramidal cells connected to immature (black) and mature (blue) ChCs. Statistics: **P<0.01, *P<0.05 (D-E) tested with Mann-Whitney test. (F) Student's t-test. Values shown as mean $\pm$ s.e.m. Immature: n = 10 neurons, n = 4 mice; Mature: n = 11 neurons, n = 4 mice.

3.3.4 *In vivo* tracking ChC development

The critical window of development described in the previous section comes from averaging the properties of multiple cells in a population. However, it is likely that some heterogeneity exists, such that individual cells will mature at different rates and to different extents. To assess the individual variability in ChC development, my colleague, Dr. Wefelmeyer, performed 2-photon- *in vivo* imaging to track individual ChCs from P12 to P22 (Figure 3.10.B). In agreement with my immunohistochemical analysis she found that from P12 onwards the majority of changes in ChCs took place in the axonal compartment, whilst dendritic trees remained stable throughout the imaged period (Figure 3.10.A). She also quantified the numbers of cartridges formed by an individual ChC over time, a measure that is comparable to my measure of ChC connection probability in Figure 3.6. Similarly, she found that the majority of changes happened during the P12 -P16 window (P12: 27.476 ± 15.66 cartridges, P16: 63.449 ± 10.049 cartridges, Change: $293.44 \pm 75.737\%$, Figure 3.10.C). However, she did not observe such a clear step change from P14 to P16 (P14: 47.093 ± 11.379 , P16: 63.449 ± 10.049 Change: $71.968 \pm 11.802\%$, Figure 3.10.C).

Remarkably, the one ChC that could be tracked from P14 to P24 showed a decrease (-24.8%) in the number of cartridges made from a maximum of 77.446 cartridges at P16 to 58.258 cartridges at P24. Again, this observation supports the results from the immunohistochemical analysis in Figure 3.4, suggesting that ChCs might undergo some form of synaptic pruning (Figure 3.10.C). This subtle, yet very interesting feature of ChC development will require further replication with more animals and more ChCs to be confirmed.

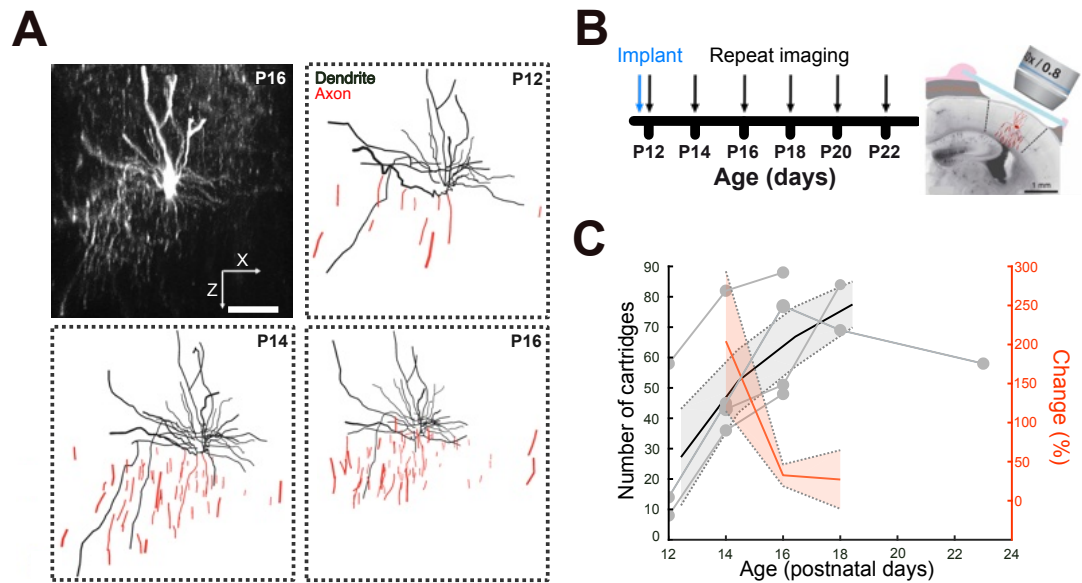


Figure 3.10 In vivo imaging of ChC development (A) Representative image of P16 ChC (Top left corner, grayscale). Reconstructions of dendritic (red) and axonal arbour (red) of the same ChC at P12, P14 and P16. Scale bar: 20 μ m **(B)** Timeline for the implantation of cranial windows and the repeated imaging of individual ChCs. **(C)** Number of cartridges made by individual chandelier cells (grey traces) throughout postnatal development. Black line shows average number of cartridges during the critical period of axo-axonic development (P12-P18). Red line shows the average percentage change on the number of cartridges present from P12 to P18. Shaded areas show s.e.m. $n = 4$ ChCs, $n = 3$ mice.

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3.3 Discussion

In this chapter I described a critical window for the development of the ChC axonal arbors between P14-P16. During this period, ChCs showed a significant increase in the connection probability and number of boutons made on nearby AISs. In a period of just two days these two parameters doubled and remained constant thereafter. These results were correlated with electrophysiological measures of synaptic development. Recordings from pyramidal neurons showed that IPSCs elicited by activation of ChCs from P16 onwards had approximately double the amplitude than IPSCs from the P12-P14 period. Similarly, the fast spiking properties of ChCs also significantly developed during this period. Immature ChCs (P12-P14) had a smaller maximum average firing frequency than mature ChCs (P16-onwards). Finally, I also present data from a collaborator corroborating this critical window of axo-axonic development by tracking ChCs *in vivo*. She found that over the period from P12 to P18, individual ChC axons undergo a rapid transition, generally within two days, from an immature state with few cartridges, to a highly complex arbour with multiple cartridges.

3.3.1 P14-P16 constitutes a critical developmental window for the developmental of the ChC axonal arbour

The morphological characterisation of ChC development showed that most of axonal arbour development occurred in the P14-P16 period. Previous studies on the migration of ChCs showed that these neurons reach their final location at P7-P9, relatively late when compared to other cortical interneurons. By the end of migration, the characteristic cartridges along the axon have not yet developed (Taniguchi et al., 2013). Here, I showed that cartridge development happens rapidly and within a very defined temporal window, from P14 to P16. After this time-point mature cartridges could be observed. This is in line with other studies that have shown mature ChCs and cartridges in the P16-P18 period (Inan et al., 2013; Taniguchi et al., 2013; Steinecke et al., 2017). We also observed that cartridge development stalls from P18 onwards, which is in line with the stability observed in a previous study reporting similar axo-axonic cartridges at P18, P30 and P90 (Inan et al., 2013). It should be noted that at P9, the earliest time point in this study, axo-axonic synapses could already be observed (on average 2 per AIS) even though no clear cartridges were apparent. Unfortunately, there is currently

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no data on earlier events that may shed light on how these initial, pioneer synapses are made. It is possible that upon arrival to the correct layer of the cortex, initial arbour extension produces a bout of synaptogenesis that innervates neighbouring pyramidal cells. However, whether these synapses are functional or not is not known, nor do we know if ChCs play any functional role in the brain at these very early developmental periods.

In this chapter I also present data from tracking the development of individual ChCs *in vivo*. From P12 to P16 most of the tracked ChCs transition from a poorly formed axonal arbour with few cartridges to a complex axonal arbour with a high number of cartridges. This partially agrees with the P14-P16 critical window I observed at a population-wide level in fixed tissue. *In vivo* imaging showed a rapid burst (generally 2 days) of synaptogenesis that took place at some point during the P12 to P16 period. However, most cells (3/4) experienced the majority of their axonal expansion from P12 to P14, earlier than for fixed tissue data. It is unclear why we observe this difference. One explanation might be due to the difference in sample sizes between *in vivo* (n=4 ChCs) and fixed tissue data (n=4-6 ChCs at each timepoint). Another possibility is that when analysing axonal development in fixed tissue, I am also including ChCs in immature timepoints (P9-P14) that will undergo apoptosis. Previous work has shown that ChC apoptosis is thought to occur during this period (Taniguchi et al., 2013) and our *in vivo* imaging also showed ChCs that disappeared along consecutive imaging timepoints. These ChCs were not used in our analysis, since they could not be tracked during the critical period of synaptogenesis. However, this could introduce a potential difference in our measures between the two conditions, since apoptotic cells would have been included in the fixed data set. No study has characterised the morphology (e.g blebbing) of apoptotic ChCs, however it is possible that their development might be abnormal or immature, and could have biased my early measures of axon development in the fixed data set to appear more immature than they really were.

In support of the critical window of synaptogenesis described here, a recent study was published while undertaking my experiments, which showed very similar findings (Steinecke et al., 2017). They also describe axo-axonic development during the P12 to P21 period and find a significant increase in the complexity of the ChC arbour between

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P12 and P16. Likewise, they also observed a significant change in the total number of axo-axonic varicosities during this period.

The P14-P16 ChC development makes ChCs the last set of MGE cortical interneurons to fully develop (Hu et al., 2017). This period is a particularly dynamic time in the somatosensory cortex, where important changes in activity occur and may help explain the context in which ChCs wire up. For example, active whisking is first noticeable in the rodents during this time period. This behavior might generate novel patterns of cortical activity (Landers and Philip Zeigler, 2006; Grant et al., 2009) that will likely impact ChC plasticity. More broadly, the appearance of this new behaviour may require the modulation of somatosensory networks by cortical interneurons that can actively control circuit function and are sensitive to ongoing activity levels. Therefore, it could be speculated that mature ChCs may be required to perform this task.

One possible role for the late development of ChCs could be related to the regulation of critical periods of developmental plasticity. Other interneuron types have been shown to be essential in the regulation of critical periods in the brain. For instance, PV interneurons regulate the closure of the ocular dominance critical period. For example, promoting PV interneurons to develop faster leads to an earlier closure of this critical period (Huang et al., 1999). Whether ChCs could be involved in a similar process in the somatosensory cortex is not known. However, it is interesting to note that ChC development takes place during a late form of critical period plasticity in layer 2/3 of somatosensory cortex. Thalamic inputs from the whiskers reach layer 4 of the cortex before P3, opening an early somatosensory critical period for layer 4 connectivity (Erzurumlu and Gaspar, 2012). However, a second critical period has been shown to be required for the adequate maturation of layer 2/3 somatosensory maps between P12 and P14, when sensory drive from active whisking is needed for appropriate network development (Stern et al., 2001). Therefore, it seems possible that ChCs might also play a role in the closure of this critical period or that alternatively require a fully mature L2/3 (post-critical period) in order to fully develop. In relation to the latter possibility, recent work imaging the activity of neonatal somatosensory circuits *in vivo*, has shown that from the first to the second postnatal week, activity in somatosensory cortex passes from synchronicity (Yang et al., 2013) to asynchronicity (Golshani et al., 2012, Mizuno

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et al., 2017). It is therefore tempting to speculate that it is this change in activity patterns that is permissive or even instructive to ChC development.

3.3.2 Mature ChC morphology matches previous data

Previous work has provided detailed characterisations of ChC morphology. Similar to our findings in this chapter, mature axo-axonic cartridges in somatosensory cortex are thought to have 2-12 synaptic boutons (Peters et al., 1982; Somogyi et al., 1982), with on average 3-5 boutons per cartridge (Inan et al., 2013). Similarly, when normalising to the length of the AIS, our density measurements are similar those presented in a previous study in ErbB4+ ChCs (Fazzari et al. 2010), but slightly larger than those measured by others (Inan et al., 2013). This discrepancy may arise from differences in the way axo-axonic synapses were analysed. In my experiments, boutons were counted manually and at a high magnification, while previous studies have performed this characterisations at a lower magnification that may have missed putative boutons. More likely, however, this difference arises from a different inclusion criteria. In Inan et al., 2013, only boutons along an individual cartridge that opposes an AIS were included, ignoring any other isolated boutons from the same axon that do not belong to the cartridge. The analysis performed here does not bias against these boutons – it includes all axo-axonic bouton that form onto an AIS, regardless of whether they originate from the same cartridge. In fact, I frequently observed that isolated boutons from other axonal branches of the same neuron contacted an already innervated AIS. As shown in Figure 3.4.A, these isolated boutons also express VGAT and are therefore likely to also release GABA. In my opinion, my inclusion criteria reflects more accurately the total number of GABAergic boutons that a given ChC makes onto an AIS and may help explain the lower numbers reported by Inan et al. 2013.

3.3.3 Axo-axonic synapses cluster at the distal AIS

Our data shows that during the third week of postnatal development, axo-axonic boutons cluster at the distal end of the AIS. This is in agreement with what has been described in mature chandelier cells (Inda et al., 2006; Taniguchi et al., 2013). What might be the role for this unique positioning?

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Within the AIS, the distal domain is thought to be the preferred site of action potential generation (Hu et al., 2009). This is due to the significantly higher proportion of low-threshold $\text{Na}_v1.6$ channels distally and a comparatively low proportion of the high-threshold $\text{Na}_v1.2$ channels, which are located primarily at the proximal AIS (Hu et al., 2009). It has been suggested that $\text{Na}_v1.6$ channels confer the distal AIS with the control of action potential generation, whereas $\text{Na}_v1.2$ channels in the proximal AIS gate the backpropagating action potential and set up the action potential threshold in the somatodendritic compartment (Hu et al., 2009). Moreover, the cable properties of the distal AIS favour AP initiation on their own, as this site maximises the ratio of sodium conductance to the electrical load of the somatodendritic compartment, increasing excitability (Baranauskas et al., 2013). This makes the distal AIS the perfect site for controlling AP initiation, a function commonly attributed to ChCs.

Another function that has been proposed for the distal positioning of axo-axonic synapses is that it gives ChCs effective control over the backpropagation of ectopic action potentials initiated in the axon (beyond the AIS) during fast network oscillations. The small diameter of the distal AIS limits the depolarising current flowing into the soma from the axon, making it again the ideal site for the control of antidromic AP backpropagation by ChCs (Dugladze et al., 2012).

Therefore, it appears that the distal clustering of axo-axonic synapses during the 3rd postnatal week described in this chapter, is well suited for supporting mature ChC function, whether it is controlling AP initiation or backpropagation.

3.3.4 Synaptic pruning in chandelier cells

The formation of redundant synapses followed by elimination based on computational needs, is a common strategy in development and refinement of neuronal circuitry. Interestingly, I observed a small non-significant decrease in the number of axo-axonic synapses between P18 and P42. This decrease was also observed when tracking individual ChCs *in vivo* from P18 to P24. Remarkably, similar to what I show here, a recent study showed significant decrease in the number of varicosities and total arbour complexity from P16 to P21 (Steinecke et al., 2017). Could this be an example of synaptic pruning? Synaptic pruning has been described in several neuronal types in CNS

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and PNS and thought to play an important role in the refining connections within a circuit (Riccomagno and Kolodkin, 2015). Similarly, dendritic GABAergic inputs in layer2/3 of neocortex are thought to undergo developmental synaptic pruning of inhibitory synapses in pyramidal cell dendrites (Versendaal et al., 2012). Could this also be happening during the wiring of ChC axons? At this moment, it is difficult to confirm whether synaptic pruning is part of ChC development. Future experiments examining the development of the ChC axonal arbour *in vivo*, in combination with GABAergic postsynaptic markers in pyramidal neurons, may provide an answer to this question. By simultaneously tracking the pre and postsynaptic compartments of axo-axonic synapses, the dynamics of synapse turnover could be visualised at different developmental times to assess the stability of synapses during periods of synaptic pruning.

3.3.5 ChC develop dendrites before their axonal arbour

In comparison to the axonal arbour, dendrites appear to be fully mature by P12. As is clearly seen in the *in vivo* example images of ChCs taken from P12-P16 (Figure 3.10), the dendritic arbour shows no major changes in development in comparison to the striking changes on axonal growth. This is in line with a previous study which showed that dendrites from L2/3 ChC showed a polar projection towards the pial surface and were present and stable from P12 onwards (Miyamae et al., 2017). The presence of dendrites suggest that ChCs might be receiving inputs before their axonal arbour fully develops (< P16) and these inputs would likely drive activity in ChCs (Fariñas and DeFelipe, 1991; Lu et al., 2017). Whether this ongoing network activity plays a role in ChC development is not known, however other interneurons have been shown to be sensitive to changes in activity at early stages of development (De Marco García et al., 2011, 2015). In the next chapter I will explore the possibility that activity plays a role in the development of ChCs in the cortex.

3.3.3 Electrophysiological maturation of chandelier cells

The characterisation of ChC's intrinsic excitability showed that their fast spiking properties develop during the third postnatal week, in agreement with a recent study

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that reports higher firing frequencies for P30 ChCs, when compared to P12 (Miyamae et al., 2017). My results provide a narrower window over which this development in intrinsic excitability occurs. This is in agreement with previous studies showing that in rodents, ChCs have mature fast spiking properties by P23 (Woodruff et al., 2009). Moreover, the firing frequency and rheobase values presented here for mature ChCs (P18-onwards), are similar to those observed in previous studies using much older animals (>P50) (Povysheva et al., 2013). It is important to note that in my experiments I classified all recordings in two age groups, immature (P12-P13) and mature (P18 onwards). This coarse distinction will need to be probed in more detail to establish if the rapid changes in axonal morphology during this period are also mirrored by equally rapid changes in intrinsic excitability.

I then looked at the synaptic properties of axo-axonic synapse. IPSC amplitude depends on three factors: the quantal amplitude (a mix of the number of postsynaptic receptors and the amount of neurotransmitter released), the number of synapses and the release probability of presynaptic boutons. I hypothesised that the increase in axo-axonic synapses I observed morphologically from P14 to P16 should translate into larger IPSC amplitudes, assuming all other factors remain constant. Indeed, ChCs doubled their IPSC amplitude in the space of just two days (from P14 to P16) in agreement with the increase in bouton number measured morphologically. Similarly, the maximum IPSC amplitude also increased significantly during this period. Although other factors could also have played a role in driving this developmental increase in amplitude (e.g – changes in the number of postsynaptic GABA receptors), the close agreement with our morphological findings suggest that bouton number likely plays an important role in increasing GABAergic drive during development.

Finally, I also measured the short-term plasticity properties of the axo-axonic synapse at different ages. In general, short-term plasticity is thought to correlate with release probability, such that low release probability synapses facilitate (high PPR), while high release probability synapses depress (low PPR). I found that axo-axonic synapses did not always show synaptic depression, as is commonly associated with interneurons of the PV family. Although there are many possible mechanisms that are thought to control short-term plasticity at the synapse, it has been suggested that the parvalbumin protein itself (a mobile calcium buffer) might be essential for synaptic depression in these

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interneurons (Caillard et al., 2000). It is therefore possible that the subset of facilitating axo-axonic synapses measured here are unique to ChCs that do not express PV. Interestingly, I detected a small non-significant decrease in PPR between immature (P12-P13) and mature cells (P18-P20). In addition, PV expression starts at the end of the second postnatal week (Sun et al., 2016). Thus, it is possible that immature ChCs form slightly more facilitating synapses due to a lack of PV expression.

3.3.2 Concluding Remarks

In this chapter I have described a critical window of ChC axonal arbour development between P14 and P16. This critical window was demonstrated at a morphological and electrophysiological level. *In vivo* imaging of ChC development showed variability in the timing of the critical window, which started earlier in many occasions. Interestingly ChC dendrites, which are thought to receive intracortical excitatory inputs, were fully established before the ChC axon developed. This may provide an indication that ChC activity may play a role in guiding ChC development. In the next chapter I will explore this question by manipulating activity levels in ChCs and their local environment during the critical window of axonal development I identified in this chapter.

Chapter 4 : Plasticity of axo-axonic synapses

4.1 Introduction

In the previous chapter I described a critical window for axo-axonic synapse formation at the AIS in somatosensory cortex, during the periadolescent period, extending from P14 to P16. During that period, the cortex is known to experience significant changes in overall network activity that could affect neuronal development (Stern et al., 2001).

Both neuronal connectivity and development are known to be affected by activity levels of the cell itself and their local microcircuits. This is especially true during early neonatal development, where circuitry is fine-tuned in response to environmental cues (De Marco García et al., 2011, 2011; Dehorter et al., 2015). Moreover, activity-dependent processes are not only guiding neuronal circuits toward its mature state but, in many cases, are serving functions unique to the developing brain. This is the case for many mechanisms of homeostatic plasticity. The fast and drastic changes in connectivity in the early postnatal brain can lead to a destabilization of neuronal activity. In fact, the neonatal period is the stage in life where epileptic seizures are more likely to occur (Glass, 2014). As a result, homeostatic mechanisms are thought to be in place to safeguard neurons from abnormal activity levels during this highly dynamic period. The neuron can achieve this by negative feedback mechanisms at the level of its

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intrinsic (e.g ion channel expression) and synaptic properties (strength of its excitatory or inhibitory inputs). Homeostatic mechanisms can be present in both excitatory and inhibitory cells. In the case of inhibitory GABAergic cells for example, sensory deprivation in somatosensory cortex is known to lead to a homeostatic reduction in GABAergic innervation in the neonatal brain (Jiao et al., 2006; Flores and Méndez, 2014). Other homeostatic plasticity processes are not exclusive to the developing brain but change with age. For example, young cortical and hippocampal neurons show homeostatic responses in the strength but not frequency of their excitatory input, in contrast to mature neurons that present homeostatic changes in both (Wierenga et al., 2006; Echegoyen et al., 2007). Finally, it is important to note that interneuron plasticity more generally, is not only confined to the developing brain. Interneurons are highly plastic in the adult brain, a feature that is thought to be important for supporting essential brain functions such as the consolidation of long term memory (Donato et al., 2013; Caroni, 2015; Karunakaran et al., 2016).

How neuronal activity might affect ChC development is currently unknown. ChCs have been proposed as major regulators of cortical activity due to their extensive axonal arbour and privileged site of synaptic innervation. The period when their arbour develops (P14-16) coincides with the time that major somatosensory behaviour arise, such as active whisking (Landers and Philip Zeigler, 2006). Therefore, it is likely that major changes in neuronal activity patterns as well major circuit reorganization will be taken place around this time. In fact, it is thought that the critical period for the formation of intracortical connections at layer 2/3 of somatosensory cortex occurs just before this period (P10-P14) (Lendvai et al., 2000; Stern et al., 2001; Maravall et al., 2004). It is therefore possible that chandelier cells would also show activity-dependent plasticity of some sort to adapt to this changing environment. Moreover, ChCs innervate the AIS, which is a structure known to be highly plastic, especially during early development. It can change its length and position, modulating intrinsic excitability (Grubb and Burrone, 2010; Kuba et al., 2010; Wefelmeyer et al., 2015). However, we do not know whether ChCs are also plastic, nor whether they contribute to the homeostatic changes observed at the AIS to control neuronal output.

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In this chapter I will use chemogenetic methods (i.e DREADDs) to investigate the role that activity plays in the plasticity of axo-axonic synapses and of the AIS. I will specifically examine the following questions:

- 1) Can I use chemogenetic methods to increase activity in periadolescent mice?
- 2) What is the effect of high network activity on chandelier cell development and plasticity?
- 3) Does high network activity lead to AIS plasticity *in vivo*?
- 4) Are early forms of plasticity permanent or age specific?

4.2 Results

4.2.1 Using DREADDs to increase network activity chronically in the periadolescent cortex.

Two of the most popular strategies to manipulate the activity of specific neuronal populations are optogenetic and chemogenetic techniques. Optogenetics uses light-activated channels to drive the activity of a neuronal population of interest. This offers exceptional cellular specificity and temporal control (Fenno et al., 2011). However, it requires a light source that can directly illuminate the targeted neurons, which can limit the extent of its use *in vivo*, as it often requires the implantation of illumination devices through invasive surgeries. On the other hand, chemogenetic methods use a designer receptor expressed in the neuronal population of interest that can then be activated by a synthetic drug. The drugs can be injected into the animal allowing for easy *in vivo* activity manipulation, sacrificing temporal control over the activity induced. These methods are commonly referred to by their acronym DREADDs (designer receptor exclusively activated by designer drugs). The most common type of DREADDs available are the excitatory version hM3Dq and the inhibitory version hM4Di (Urban and Roth, 2015). hM3Dq is a Gq-PCR derived from a muscarinic receptor that has been mutated so that it no longer responds to the native neurotransmitter acetyl-choline (ACh), but does respond to the designer drug, clozapine-n-oxidase (CNO). Activation of this receptor by CNO increases neuronal activity by releasing calcium from internal stores (Figure 4.1.A). hM4Di is a Gq-PCR mutated from an M4 muscarinic receptor. When activated by CNO it will activate Gi signalling which, in turn will lead to the opening of potassium channels hyperpolarising the neuron's membrane potential (Armbruster et al., 2007).

In theory, CNO was thought to be a fully inert designer drug that would not activate any endogenous receptors in the brain. However, a seminal paper (Gomez et al., 2017) showed that CNO is in fact metabolised into the psychoactive compound clozapine, which in turn activates hM3Dq receptors. However, clozapine unlike CNO can have off-target effects as it is a strong antagonist of 5-HT/2A receptors, which can also act on cholinergic and dopaminergic receptors (Seeman, 2013). The off-target effects of CNO require the use of proper controls for the drug itself. It is important to note that even

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though CNO might lead to the non-targeted activation of other receptors, aside from hM3Dq, its high affinity for hM3Dq allows its use at low concentrations, which in turn should minimise off-target interactions.

In this study, I decided to use a chemogenetic approach (hM3Dq excitatory DREADDs) to modulate the activity of cortical networks. Alternative methods, such as optogenetic approaches were considered more invasive and generally less suitable for neonatal and periadolescent *in vivo* approaches in mice. Mice pups require constant care from their mother, which may malnourish the pup in the presence of a foreign object such as a head implant for an illumination device. Similarly, mice pups cannot go through the intense surgeries required for the implantation of these devices as they cannot survive long periods of time without feeding.

Fixed tissue analysis of ChC morphology showed that the P14-16 window was the time that, on average, most axo-axonic synapses were formed. However, the *in vivo* tracking of the development of individual ChCs showed that the rapid development of axo-axonic synapses varied across cells and could fall either side of this narrow window. To account for this heterogeneity, I decided to increase activity over a broader time-window, from P12-P18 (Figure 4.1.B), that would encompass the critical period of synaptogenesis for all ChCs.

Mice were injected twice a day to ensure a continued increase in activity (Figure 4.1.B). However, no study has accurately measured the pharmacokinetics of CNO during the neonatal period, only in adult mice where a single CNO injection was shown to last 6-8h (Alexander et al., 2009; Urban and Roth, 2015). These values are likely to be affected by the concentration of the drug, the age of the animal and its genetic strain. Here, I used a concentration (1mg/kg) that was the lowest concentration used by previous studies to successfully increase neuronal activity (Sciolino et al., 2016; You et al., 2016; Alcacer et al., 2017; Ben-Shaanan et al., 2018) and that is also known to minimise detectable off-target behavioural effects (MacLaren et al., 2016). I injected the drug twice a day by peritoneal injection as previously described (Wong et al., 2018), with an interval of 10-12h between each injection. On the day of perfusion (P18) mice received a single dose in the morning, 2h before perfusion (for more details see Chapter 2: Material and Methods).

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I delivered hM3Dq specifically to Layer2/3 of somatosensory cortex via *in utero* electroporation at embryonic day E15.5. This resulted in DREADD expression in large areas of somatosensory cortex, where 10-20% of pyramidal cells in layer 2/3 expressed the receptor (Figure 4.1.C). I then divided the animals into four groups: 2 treatment group and 2 control groups (Figure 4.1.E). The two treatment groups were obtained from mice electroporated with hM3Dq and treated with CNO, and included pyramidal cells that expressed DREADD (hM3Dq+) and neighbouring pyramidal cells that did not express DREADD but were part of the network (hM3Dq-network). The latter group would not be stimulated by CNO directly, but may be affected by the overall activity of the network. I will refer to these groups as 'hM3Dq+' and 'hM3Dq-network' respectively, for the rest of the thesis (Figure 4.1.D). I also used two control sets. One consisted of animals where hM3Dq was electroporated but a vehicle saline was injected instead of CNO (Saline Control). This group controlled for any effects that the expression of the hM3Dq receptor might have, which is unrelated to its activation by CNO. The second control set consisted of mice that had not being electroporated and therefore did not express the DREADD receptor, but where injected with CNO in the same regime as the other groups (CNO control/Drug control). This group controlled for any off-target effects of CNO.

4.2.2 Acute activation of hM3Dq+ increases activity in a cell autonomous manner at P12

To test if hM3Dq could be used to increase activity in neonatal mice pups at the beginning of the treatment, CNO/Saline was injected across groups at P12, two hours before perfusion. Animals were then stained for c-Fos, a gene whose expression is highly correlated with activity levels in the neuron (Bullitt, 1990), and which reaches maximum expression 1-2 hours after activity is increased (Barros et al., 2015)(Figure 4.2.A). I found that neurons expressing hM3Dq (hM3Dq+) from CNO treated mice were positive for c-Fos at a significantly higher percentage ($80\% \pm 16\%$) than hM3Dq-network neurons ($5.1\% \pm 2.1\%$, $P < 0.001$) from the same pup or hM3Dq+ ($5.5 \pm 0.5\%$, $P < 0.001$) and hM3Dq-network ($7.1 \pm 0.6\%$, $P < 0.001$) cells from saline control mice (Figure 4.2.B). It should be noted that for this experiment, the CNO control group was not included. However, due to lack of c-Fos expression in the 'CNO+ hM3Dq-network'

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group ($5.1\% \pm 2.1\%$, $P < 0.001$), I could confidently infer that acute CNO treatment (1mg/kg) on its own did not affect activity levels in cells lacking hM3Dq. On the contrary, acute CNO treatment in hM3Dq+ cells led to a cell-autonomous significant increase in network activity.

I then tested if there was linear relation between receptor expression and levels of activity. For this, I tested the correlation of c-Fos expression as a function of hM3Dq expression in both CNO and saline injected mice (Figure 4.2.C.). As expected, saline injected mice showed no significant correlation between the two, but did show an overall lower expression of c-Fos than CNO treated mice (Saline: 93.4 ± 0.9 , CNO: 133.4 ± 2.23). Interestingly, for CNO treated neurons there was a significant linear correlation between hM3Dq expression and c-Fos expression ($P < 0.05$). This suggest a potential relation between the expression of the DREADD receptor and overall activity levels within the cell after CNO injection.

4.2.3 Chronic activation of hM3Dq+ (P12-P18) increases activity in a non-cell autonomous manner.

I then tested what were the effects of chronic activation of hM3Dq+ cells after 6 days of treatment (P12-P18). As before, mice were injected 2h before the last CNO injection and c-Fos expression levels were analysed.

In contrast to the results obtained from the acute treatment at P12, I found that chronic treatment for 6 days led to a significant increase in the percentage of c-Fos+ cells in both the hM3Dq+ ($56 \pm 2.51\%$, $P < 0.01$) and hM3Dq-network cells ($50.3 \pm 4.1\%$, $P < 0.01$) when compared to the CNO control ($21 \pm 10\%$) and saline control groups (hM3Dq+: $12 \pm 2.96\%$, hM3Dq-network: $21.7 \pm 2.96\%$) (Figure 4.3.A-B). Together, this data suggests that chronic hM3Dq+ activation generates a network-wide increase in activity in the cortex. Interestingly, the percentage of c-Fos+ hM3Dq+ cells at P18 was smaller than at P12 ($80 \pm 16\%$), which may reflect some small levels of adaptation to the stimulus in a subset of neurons during the 6 days of CNO delivery. Nonetheless, this should not necessarily be taken to reflect a lower amount of overall cortical activity. In fact, due to the non-cell autonomous increase in activity at P18, a much larger number of cells are active at this time point than at P12. Finally, no significant differences were found in the

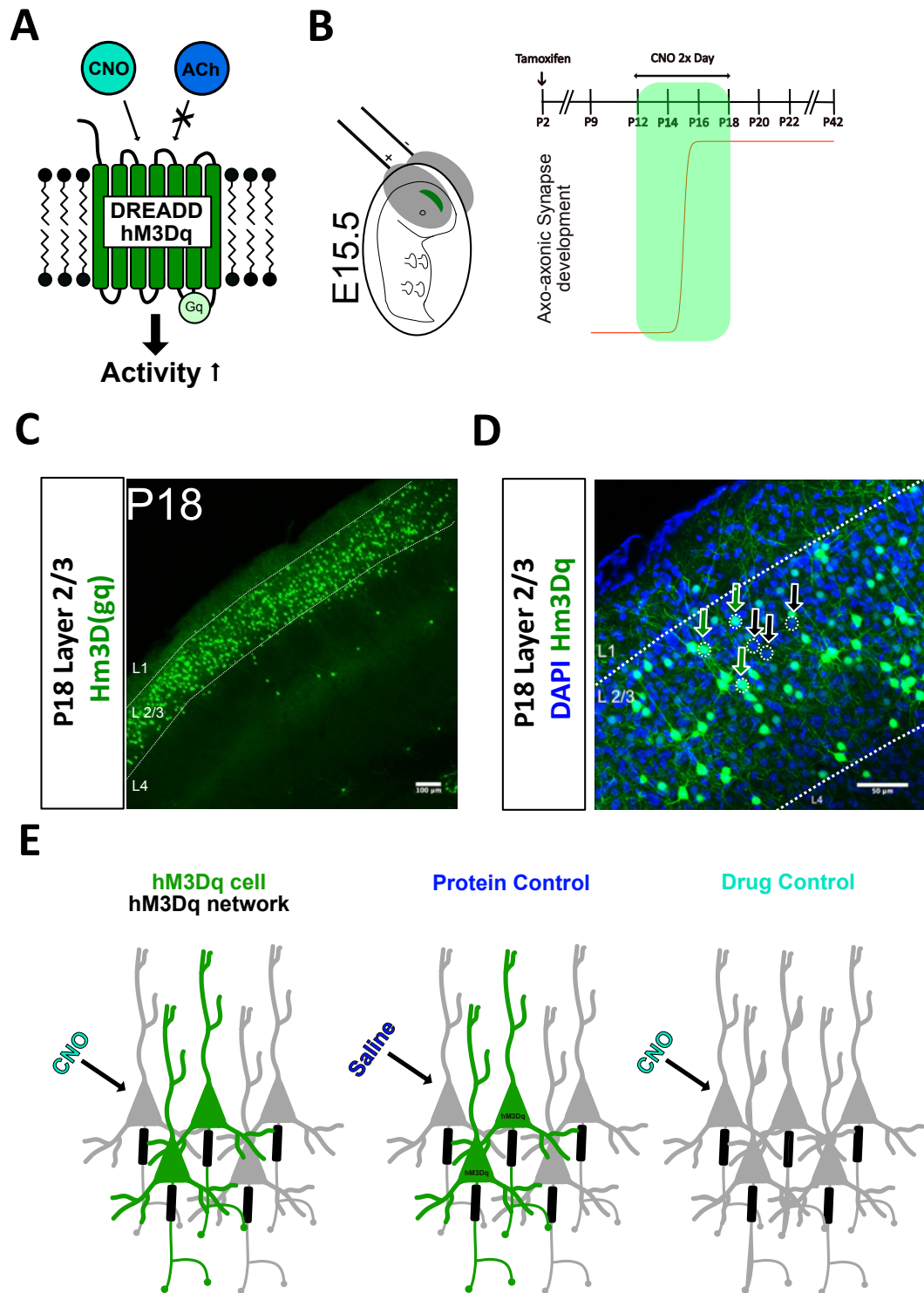
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percentage of c-Fos+ cells between the saline and CNO control groups ($P>0.05$), showing that at the concentration used, CNO has no off-target effects on neuronal activity.

It is important to note that the n number for these experiments (Fig.4.2 & 3), was low (n=2-3 mice per condition). However, due to the strength of the effect observed I concluded that chronic activation of hM3Dq+ can increase activity in a non-cell autonomous manner during the P12-P18 period.

Figure 4.1 Manipulating cortical activity with DREADDs. (A) Structure and function of the excitatory DREADD hM3Dq **(B)** Delivery and timeline of activation of hM3Dq compared to axo-axonic development. Mice were electroporated *in utero* at E15.5 with hM3Dq. Mice were then injected with either CNO or Saline 2x a day (morning and evening) from P12 to P18. At P18 mice were perfused 2h after the morning injection and axo-axonic and AIS plasticity was assessed. **(C)** Representative image of L2/3 electroporation of somatosensory cortex with hM3Dq (green) in a P18 mouse **(D)** Representative image of the mixed population of pyramidal cells in an electroporated brain. Cell nuclei marked with DAPI (blue). hM3Dq+ cells (green). Green arrow points at hM3Dq+ cells. Black arrow points at hM3Dq- cells (hM3Dq-network) in hM3Dq electroporated network. **(E)** Summary diagram showing the 4 populations that were assessed: The effect of hM3Dq activation with CNO at cell autonomous (hM3Dq+) and network (hM3Dq-network) level, the control for the effect of hM3Dq expression (Saline/Protein control) and the effect of CNO application (CNO/Drug control). *(Figure on the next page)*

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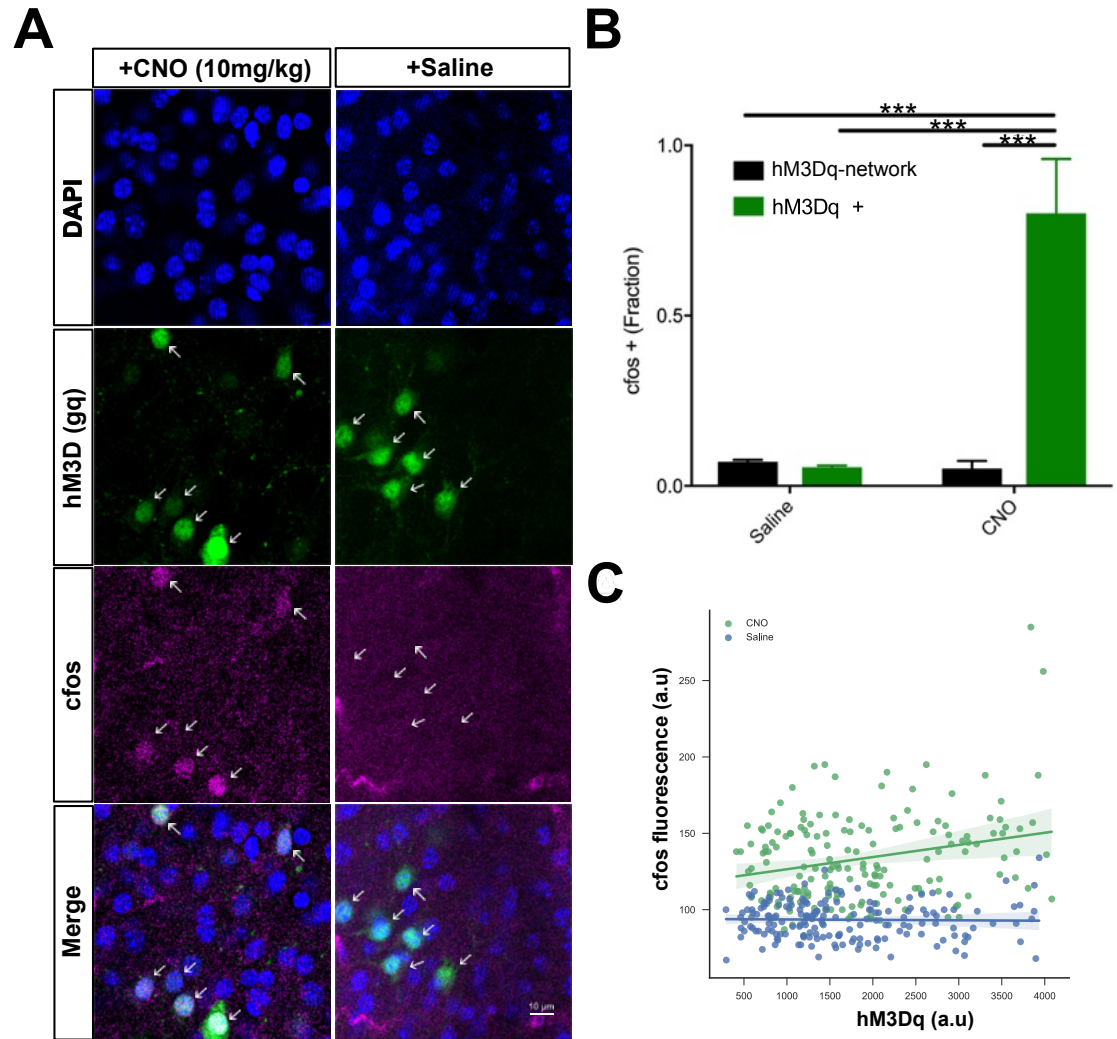


Figure 4.2 c-Fos expression increases in hM3Dq+ cells at P12 mice following CNO injection. **(A)** Representative images of c-Fos expression in L2/3 cells in somatosensory cortex following a CNO or Saline injection in P12 mice. Nuclei (Blue), hM3Dq+ cells (green), c-Fos (magenta) **(B)** Fraction of c-Fos positive cells in saline and CNO injected animals. hM3Dq+ and hM3Dq-network cells in green and black respectively. Values expressed as mean $\pm$ s.e.m. *** $P < 0.001$ tested Chi-Square by merging data from each condition prior to plotting. **(C)** c-Fos fluorescence as a function of hM3Dq fluorescence. CNO and saline injected in green and blue respectively. Linear regression, shade (95% confidence interval). Spearman's correlation ($P < 0.05$, Spearman $R = 0.152$). CNO (hM3Dq+ $n = 57$ cells, hM3Dq-network $n = 543$ cells, $n = 2$ mice), Saline (hM3Dq+ $n = 51$ cells, hM3Dq-network = 549 cells, $n = 2$ mice).

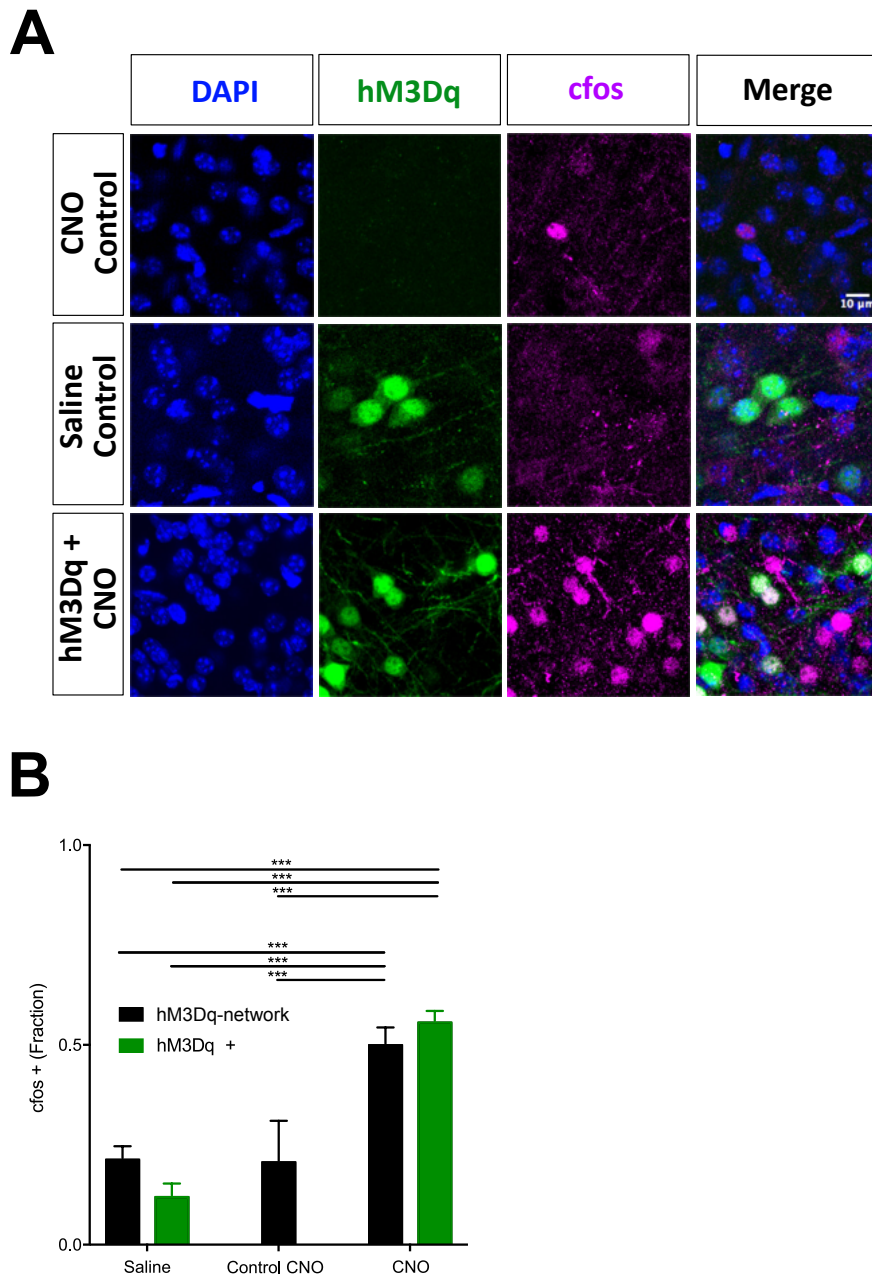


Figure 4.3 c-Fos expression increase in hM3Dq+ and hM3Dq-network cells from P18 mice following prolonged activation with CNO. (A) Representative images of c-Fos expression in L 2/3 cells in somatosensory cortex following continuous injections of CNO/saline for 6 days (P12-P18, 2x day). Nuclei (Blue), hM3Dq+ cells (green), c-Fos (magenta). **(B)** Fraction of c-Fos positive cells in saline and CNO injected animals with (CNO/Saline) and without hM3Dq+ cells (CNO control). hM3Dq+ and -hM3Dq-network cells in green and black respectively. Values expressed as mean $\pm$ s.e.m. *** $P < 0.001$ tested with Chi-Square by merging data from each condition prior to plotting. CNO (hM3Dq+ $n = 300$ cells, hM3Dq-network $n = 300$ cells, $n = 3$ mice), Saline (hM3Dq+ $n = 300$ cells, hM3Dq-network $n = 300$ cells, $n = 3$ mice), CNO control ($n = 200$ cells, $n = 2$ mice).

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4.2.4 High network activity decreases the density and number of axo-axonic synapses

Once I established that DREADDs could be used to increase network activity chronically, I proceeded to test the effect of high network activity on axo-axonic synapse development (Figure 4.4.A). After increasing activity from P12 to P18 in *Nkx2.1*/tdTomato mice, I performed an immunohistochemical analysis where I traced ChC axonal varicosities that formed onto the AIS of pyramidal neurons, as a measure of axo-axonic synapses. I found that there was a significant decrease in both the density (Figure 4.4.B) and number of axo-axonic synapses (Figure 4.4.C) at the AIS of both hM3Dq+ (Density: 0.106 ± 0.013 syn/ μ m, $P < 0.01$ to CNO control, $P < 0.01$ to Saline Control; Number: 3.91 ± 0.434 , $P < 0.01$ to CNO control, $P < 0.01$ to Saline Control) and hM3Dq-network cells (Density: 0.095 ± 0.008 syn/ μ m, $P < 0.001$ to CNO control, $P < 0.01$ to Saline Control; Number: 3.71 ± 0.29 , $P < 0.001$ to CNO control, $P < 0.01$ to Saline Control) in mice injected with CNO in comparison to both the saline (Density: 0.162 ± 0.0165 syn/ μ m; Number: 6.75 ± 0.833) and CNO controls (Density: 0.1501 ± 0.006 syn/ μ m; Number: 6.182 ± 0.288). One particularly interesting finding from this data set is that the decrease (approx. 1/3) in axo-axonic innervation was network wide, with both hM3Dq+ and hM3Dq-network cells showing a similar number/density of synapses. There were no significant differences between control groups.

4.2.5 High network activity decreases AIS length

I then tested whether AIS size also changed in response to increased activity (Figure 4.5.A) using the same method described in Chapter 3. I found that activity appeared to change the average length of the AIS across groups ($P < 0.05$, ANOVA). However, this difference was relatively small, with the posthoc multiple comparison test failing to reach significance (Figure 4.5.B). This change appeared to be specific to hM3Dq+ cells (26.6 ± 1.23 μ m) which showed shorter AISs than hM3Dq-network cells (30.92 ± 1.49 μ m), as well as both the saline (32.04 ± 1.64 μ m) and CNO control (30.76 ± 1.49 μ m) groups. Interestingly, when looking at mice at an individual level, some striking plastic changes could be seen within a single brain. In fact, in one single brain, AISs were almost completely absent in the electroporated hemisphere but were of a normal size in the contralateral hemisphere (Figure 4.5.C). This network wide decrease cannot be

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explained by a failure of AIS immunolabelling as tissue in the same sample, but in other brain regions, contained normal AISs.

The variability in AIS plasticity, especially in hM3Dq-networks cells, could be related to differences in network-wide activity levels between different animals. One likely difference between mice may arise from the variability in transfection efficiency achieved with *in utero* electroporations, resulting in a heterogeneity in the density of pyramidal cells that express hM3Dq. This, in turn, could influence the overall levels of network activity. Therefore, I tested whether AIS length in hM3Dq-network cells was related to the total density of the hM3Dq cells in the brain (Figure 4.5.D). I found an apparent correlation between the size of the AIS in hM3Dq-network cells and the density of hM3Dq cells, although it did not reach statistical significance ($R^2 = 0.86$, $P = 0.07$). The lack of significance is likely due to the low number of points (only 4 brains were used), however the high R^2 does suggest a correlation between the two variables. Perhaps not surprisingly, this correlation was absent in hM3Dq+ cells.

Based on the information above, I concluded that the AIS shortened in response to heightened activity in a cell autonomous manner. In networks with low electroporation densities, only hM3Dq+ neurons showed heightened activity and thus AIS plasticity. On the contrary, under high electroporation densities, the activation of hM3Dq+ neurons probably lead to a network wide increase in activity, which in turn affected the AIS length of all neurons involved.

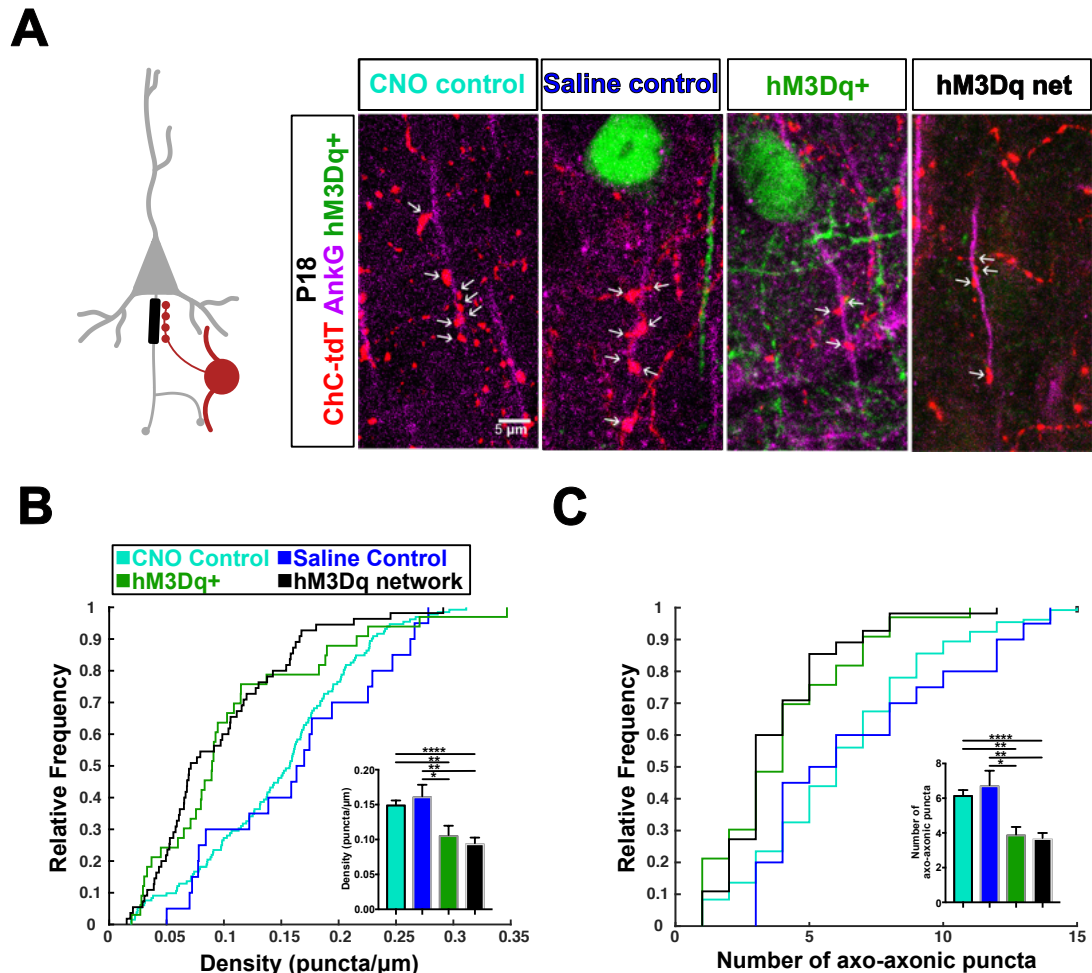
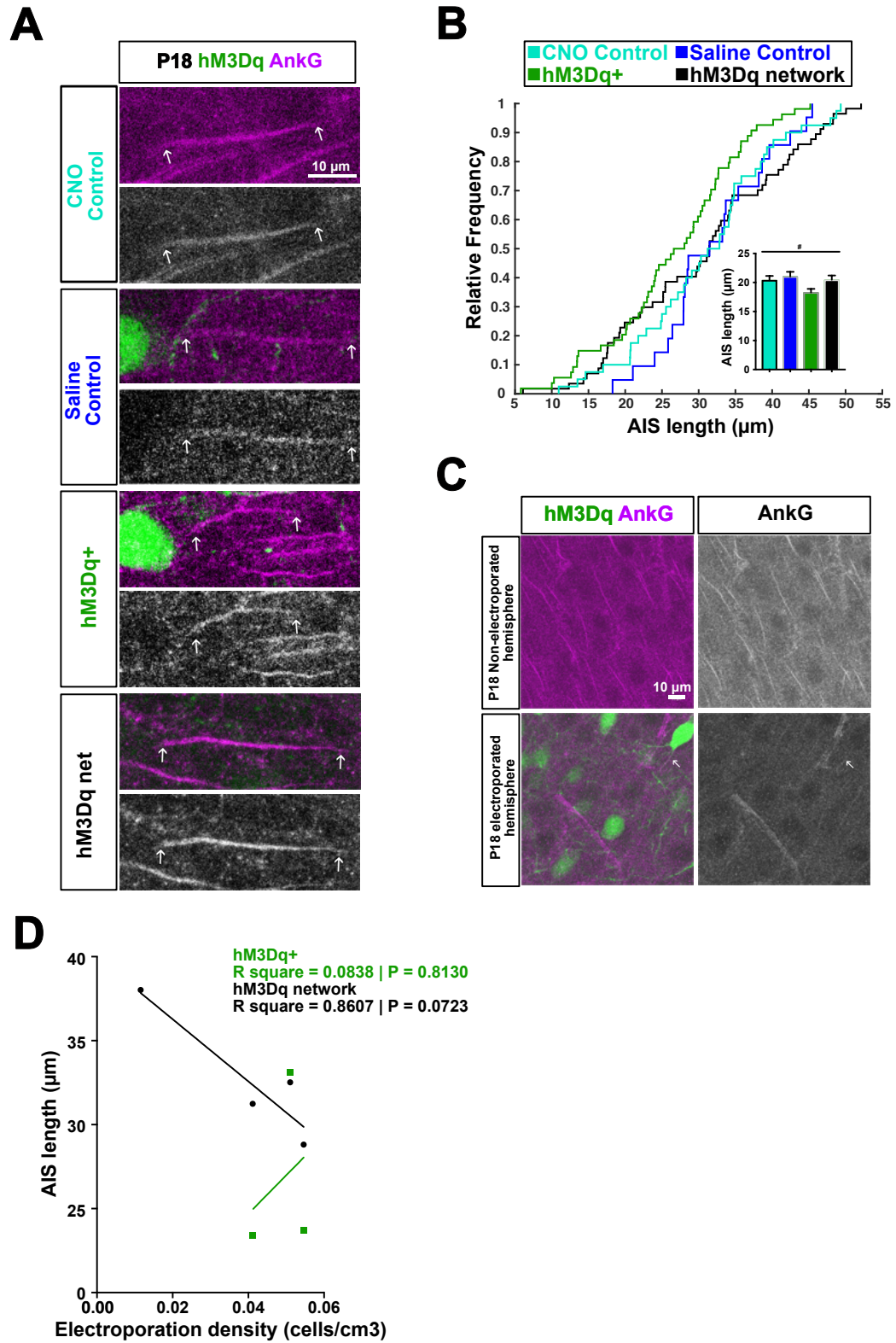


Figure 4.4 High network activity decreases the number of axo-axonic synapses at the AIS. (A) Representative images of AIS and axo-axonic synapses from layer2/3 somatosensory cortex from P18 mice after repeated treatment with CNO/saline (P12-P18), across groups. AIS (magenta), ChC axo-axonic synapses (red) and hM3Dq+ (green). (B-C) Cumulative distribution plot and bar chart showing the density (B) and number (C) of axo-axonic synapses along the AIS in each different group. Values expressed as mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ tested with one-way Kruskal-Wallis test with Dunn's multiple comparison test. CNO control: $n = 132$ neurons, $n = 3$ mice; Saline control: $n = 20$ neurons, $n = 4$ mice; hM3Dq: $n = 33$ neurons, $n = 4$ mice; hM3Dq-network: $n = 55$ neurons, $n = 4$ mice.

Figure 4.5 High network activity decreases AIS length. (A) Representative images of AISs from L2/3 somatosensory cortex from P18 mice after repeated treatment with CNO/saline (P12-P18), across groups. For each group, top image: AIS (magenta) and hM3Dq+ (green), bottom image: grayscale image of AIS. White arrows delimit AIS. **(B)** Cumulative distribution plot and bar chart showing the length of the AIS in each different group. Values expressed as mean $\pm$ s.e.m., # denotes $P < 0.05$ for one-way ANOVA without reaching significance in posthoc comparisons. **(C)** Representative image of extreme case of AIS plasticity at L2/3 of somatosensory cortex in a hM3Dq+ network after repeated treatment with CNO (P12-P18). Electroporated hemisphere (bottom) compared to non-electroporated hemisphere (top). For each hemisphere, left panel: AIS (magenta) and hM3Dq+ (green), right panel: grayscale image of AISs. **(D)** AIS length as a function of hM3Dq electroporation density. Green: hM3Dq+ cells, Black: hM3Dq-network cells. Lines represent linear regression. P values from Pearson's correlation. CNO control: $n = 57$ neurons, $n = 3$ mice; Saline control: $n = 21$ neurons, $n = 4$ mice; hM3Dq $n = 54$ neurons, $n = 4$ mice; hM3Dq-network = 40 neurons, $n = 4$ mice.

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4.2.6 High network activity decreases the number of boutons per cartridge

The shortening of the AIS after increasing network activity left the possibility that the decrease in the number axo-axonic synapses measured at the AIS in Figure 4.4 reflected: a) an actual decrease in axo-axonic innervation that mirrors the shortening of the AIS, or b) a decrease in AIS size but no change in the total number of boutons, leaving synapses beyond the AIS unaccounted for (Figure 4.6.A).

To distinguish between the two, I designed a measure for counting the number of varicosities (putative boutons) in a cartridge (for details see Chapter 2.9.1) independently of AIS length. Cartridges are the rows of boutons that compose the axonal arbour of ChCs and will give a measure of bouton number that is not normalised by the length of the AIS. The results of this analysis showed that high network activity from P12-P18 lead to a network wide decrease in cartridge size. hM3Dq+ (5.542 ± 0.43 , $P < 0.01$ to CNO control, $P < 0.05$ to Saline Control) and hM3Dq-network cells (5.9 ± 0.38 , $P < 0.001$ to CNO control, $P < 0.05$ to Saline Control) had cartridges with significantly less varicosities than the saline (8.625 ± 0.892) and CNO control groups (8.433 ± 0.423) (Figure 4.6.C). There were no significant differences between control groups. Thus, I concluded that the plasticity observed in response to network activity, was a decrease in the number of axo-axonic boutons in a cartridge.

4.2.7 High network activity decreases the number of AIS contacted by an individual chandelier cell.

After confirming a decrease in axo-axonic innervation in response to high network activity, I tested whether there was an associated decrease in the number of AISs contacted by an individual ChC at P18, across groups. For this, I quantified the percentage of AISs contacted within a 90 μm radius sphere centred at the ChC soma and within the toroid formed by the area between a 90-180 μm from the soma (Figure 4.7.A). A sample of 30 AISs was taken from each area, blinded to whether they were receiving a ChC contact, and the number AISs containing at least 1 bouton were counted. I found that ChCs in hM3Dq-networks treated with CNO made significantly less contacts in both areas (90 μm : 72.6%, $P < 0.001$ to saline control, $P < 0.001$ to CNO control; 90-180 μm : 47.8%, $P < 0.001$ to saline control, $P < 0.001$ to CNO control) than in the saline control

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(90um: 90.7%, 90-180um: 72.3%) and CNO control (90um: 89.3%, 90-180um: 64.8%) groups (Figure 4.7.B). Again, there were no significant differences between control groups. Thus, I concluded that ChCs in response to high network activity, not only reduce the number of synapses in each cartridge, but also innervate less cells in their local network.

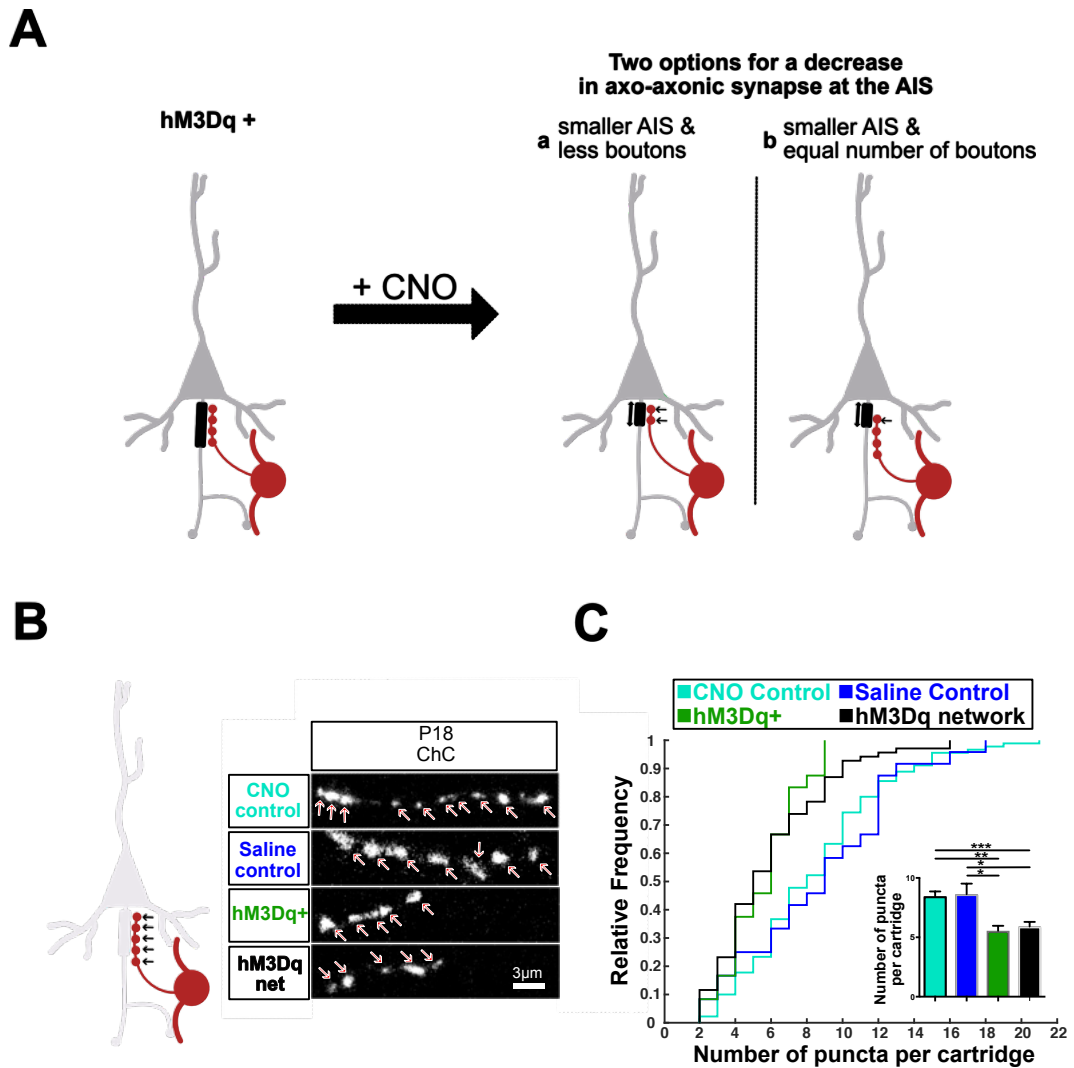


Figure 4.6 High network activity decreases cartridge size. (A) Diagram explaining the need for a purely presynaptic measure of bouton number to confirm the presence of structural plasticity at axo-axonic synapses. Due to the decrease in AIS size in response to high levels of activity, a decrease in axo-axonic synapses at the AIS could be due to a) a decrease in the total number of boutons in a cartridge, b) a decrease in AIS size with an equal number of boutons that synapse beyond the AIS. **(B)** Representative images of cartridges (grayscale) from layer2/3 somatosensory cortex from P18 mice after repeated treatment with CNO/saline (P12-P18), across groups. Arrows point at individual axo-axonic boutons in a cartridge. **(C)** Cumulative distribution plot and bar chart showing the number of boutons per cartridge in each different group. Values expressed as mean $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ tested with Kruskal-Wallis test with Dunn's multiple comparison test. CNO control: $n = 57$ neurons, $n = 3$ mice; Saline control: $n = 21$ neurons, $n = 4$ mice; hM3Dq: $n = 54$ neurons, $n = 4$ mice, hM3Dq-network: $n = 40$ neurons, $n = 4$ mice.

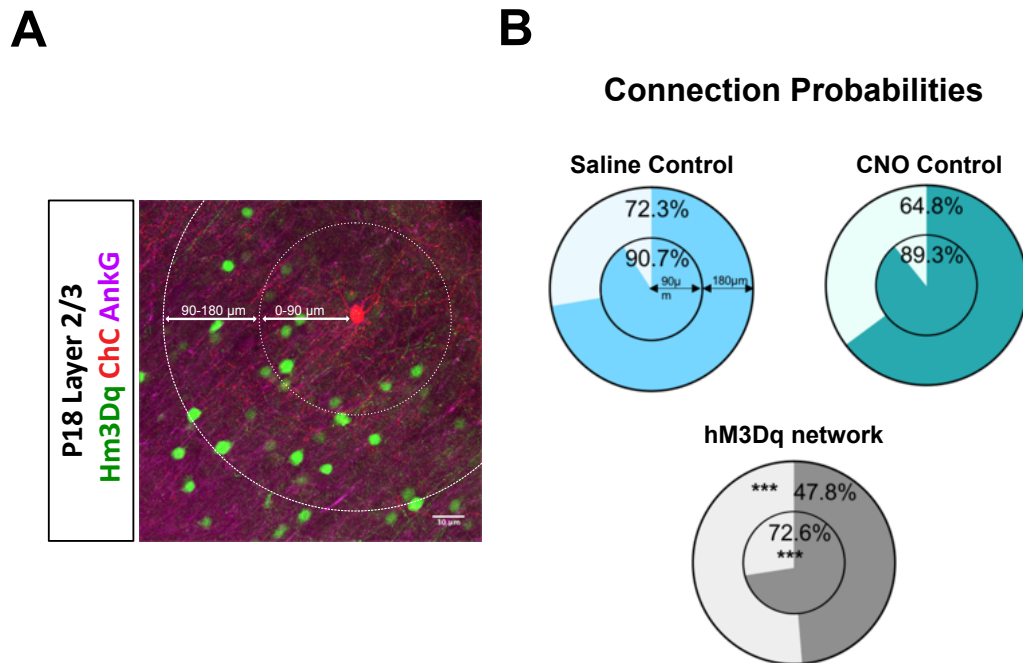


Figure 4.7 High network activity decreases ChC connection probability. (A) Representative image of ChC in L2/3 somatosensory cortex in a hM3Dq+ network at P18 after repeated treatment with CNO/saline (P12-P18). AIS (magenta), axo-axonic synapses (red) and hM3Dq+ (green). Dashed lines mark the areas used to measure connection probability. Inner circle: 90 μm radius cylinder around soma. Outer circle: 90-180 μm radius toroid around the soma. **(B)** Pie charts showing connection probability in an 90 μm (inner circle) and 90-180 μm radius (outer circle) across groups. Values expressed as %. *** $P < 0.001$ tested with χ^2 test. CNO control: $n = 270$ AIS, $n = 9$ ChCs, $n = 3$ mice; Saline control: $n = 300$ AIS, $n = 10$ ChCs, $n = 4$ mice; hM3Dq-network: $n = 390$ AIS, $n = 13$ ChCs, $n = 4$ mice.

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4.2.8 Electrophysiological signature of axo-axonic plasticity

After analysing plasticity at a morphological level, I tested if we could validate these results through electrophysiological methods. The decrease in the number axo-axonic synapses and pyramidal cell contacts in response to high network activity generates two predictions:

- 1) A decrease in axo-axonic innervation should carry a decrease in IPSC amplitude measured in pyramidal neurons.
- 2) A decrease in the number of contacts made by an individual ChC should lead to a decrease in connection probability between ChCs and pyramidal neurons in their vicinity.

To test for this I used the Nkx2.1/Ai32 line, electroporated the mice with hM3Dq, delivered the same CNO treatment protocol (P12-P18) as in the previous section (Figure 4.1) and prepared acute slices for patch-clamp recordings. Due to the added difficulty of these experiments and the similarity in results among control groups shown in the previous chapter, only the CNO control group was included in this experimental set. Mice aged between P17-P19 were included in both groups to maximise the number of recordings from a litter. In P19 mice CNO treatment was prolonged by 1 day. In the case of P17 mice CNO treatment was shortened by 1 day.

Whole cell patch-clamp recordings showed that the average amplitude of IPSCs in connected neurons was smaller in hM3Dq-network cells (51.6 ± 11.41 pA, $P < 0.05$) than in recordings from CNO controls (95.7 ± 17.31 pA) (Figure 4.8.A-B), confirming the first prediction. The maximum amplitude across 15 sweeps in connected neurons was also higher, but not significant, in the CNO control compared to the hM3Dq-network group (CNO control: 161.1 ± 30.8 pA, hM3Dq-network: 113.6 ± 25 pA, $P > 0.05$) (Figure 4.8.E). This measure was used as an extra correlate for innervation by ChCs. Finally, I also measured the failure rate of IPSCs in connected neurons during 15 sweeps of ChC stimulation. I surmised that a lower number of boutons innervating an individual axon could lead to an increase in release failures. Indeed, ChC inputs onto hM3Dq-network neurons ($32.8 \pm 8.4\%$, $P < 0.001$), presented a significantly higher failure rate than neurons from the CNO control group ($14.8 \pm 4.4\%$) (Figure 4.8.D).

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Finally, I also found that the connection probability of ChCs was significantly lower in hM3Dq-network (24.6%, $P < 0.001$) than in the CNO control (52.8%) (Figure 4.8.C), confirming the second prediction.

Overall, both the decrease in ChC evoked IPSCs amplitude and connection probability in hM3Dq-networks, further supported the decrease in axo-axonic innervation in response to high activity that I observed morphologically.

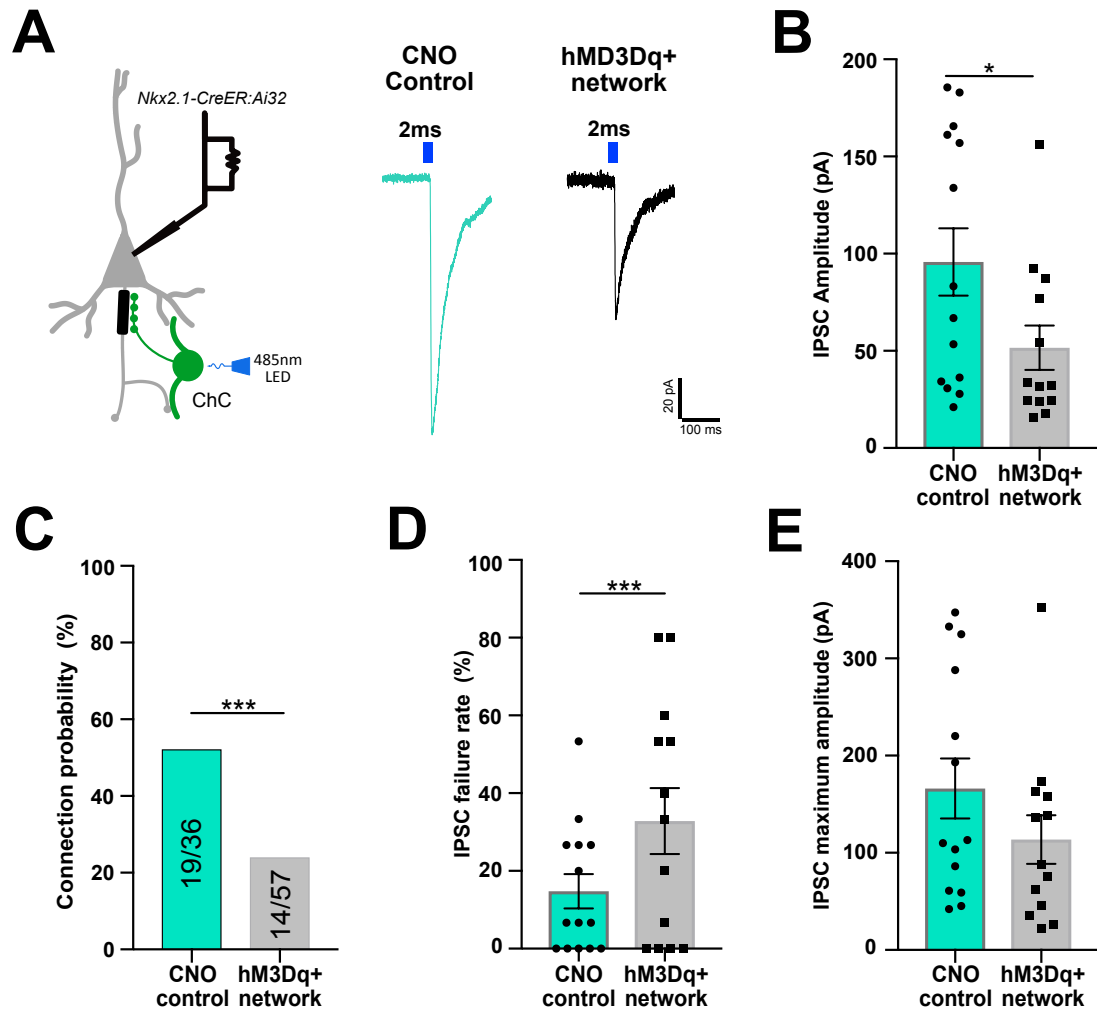


Figure 4.8 Electrophysiological signature of axo-axonic plasticity. (A) Representative IPSCs from chandelier cells in the CNO control and hM3Dq-network group. (B) Average IPSCs amplitudes for each neuron. (C) Connection probability (number of connected cells/tested pairs). (D) IPSC failure rate (number of IPSCs failures/15 trials in connected pairs). (E) Maximum IPSC amplitude in 15 trials for each cell. Values expressed as mean $\pm$ s.e.m. Each circle and square represent individual cells. (A, E) $*P < 0.05$ tested with Mann-Whitney test. (C-D) $***P < 0.001$ tested with χ^2 test. Significance of IPSC failure rate was measured by merging all sweep across all neurons in each condition. For IPSC analysis: CNO control: $n = 14$ neurons, $n = 4$ mice; hM3Dq-network = 13 neurons, $n = 4$ mice. For connection probability: CNO control: $n = 36$ neurons, $n = 5$ mice; hM3Dq-network: $n = 57$ neurons, $n = 9$ mice.

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4.2.9 Electrophysiological signature of AIS plasticity

Previous studies showed that structural changes of the AIS are correlated with significant changes in the intrinsic excitability properties of neurons. Therefore, based on the AIS shortening observed after six days of high activity in hM3Dq+ cells (Figure 4.5), I measured the intrinsic excitability of layer2/3 in these cells after CNO treatment. Interestingly I found that hM3Dq+ cells (Figure 4.9.A) were significantly less excitable as measured by their input-output curve (Figure 4.9.C) than neurons in the CNO control group (Figure 4.9.B).

These changes were not related with any differences in the passive properties of the neurons. Both the input resistance (hM3Dq+: 207.5 ± 37.35 m Ω , CNO control: 275.7 ± 65.6 m Ω) and capacitance (hM3Dq+: 61.51 ± 7.77 pF, CNO control: 51.13 ± 2.87 pF) of neurons were not significantly different between conditions (Figure 4.9.ext.A-B). Other measures of excitability such as voltage threshold (hM3Dq+: -42.75 ± 1.82 mV, CNO control: -42.7 ± 1.26 mV) and rheobase (hM3Dq+: 111.3 ± 17.8 pA, CNO control: 98.57 ± 15.19 pA) showed no significant differences between both conditions (Figure 4.9.ext.C-D).

Finally, I also analysed the time difference between the first two peaks of the second derivative of voltage with respect to time (Figure 4.9.ext.E). This can be used as a proxy for measuring AIS movement, a type of AIS plasticity previously described in hippocampal neurons (Grubb and Burrone, 2010). During action potential initiation, an action potential is first generated at the AIS, which propagates to the soma where it initiates for a second time. This generates two peaks when we look at the second derivative of voltage with respect to time, which correspond to the action potential initiation event in each neuronal compartment. Thus, the time difference between these two peaks can be used as a measure of AIS distance from the soma. If the AIS has moved away from the soma the propagation of the action potential into the soma will take longer, which will in turn increase the time difference between the peaks (Wefelmeyer et al., 2015). This measure showed no difference between hM3Dq+ (0.625 ± 0.067) and CNO control cells (0.62 ± 0.02) (Figure 4.9.ext.E), suggesting cortical neurons only undergo a shortening of the AIS, without any noticeable change in position along the axon. Future studies should validate this result morphologically.

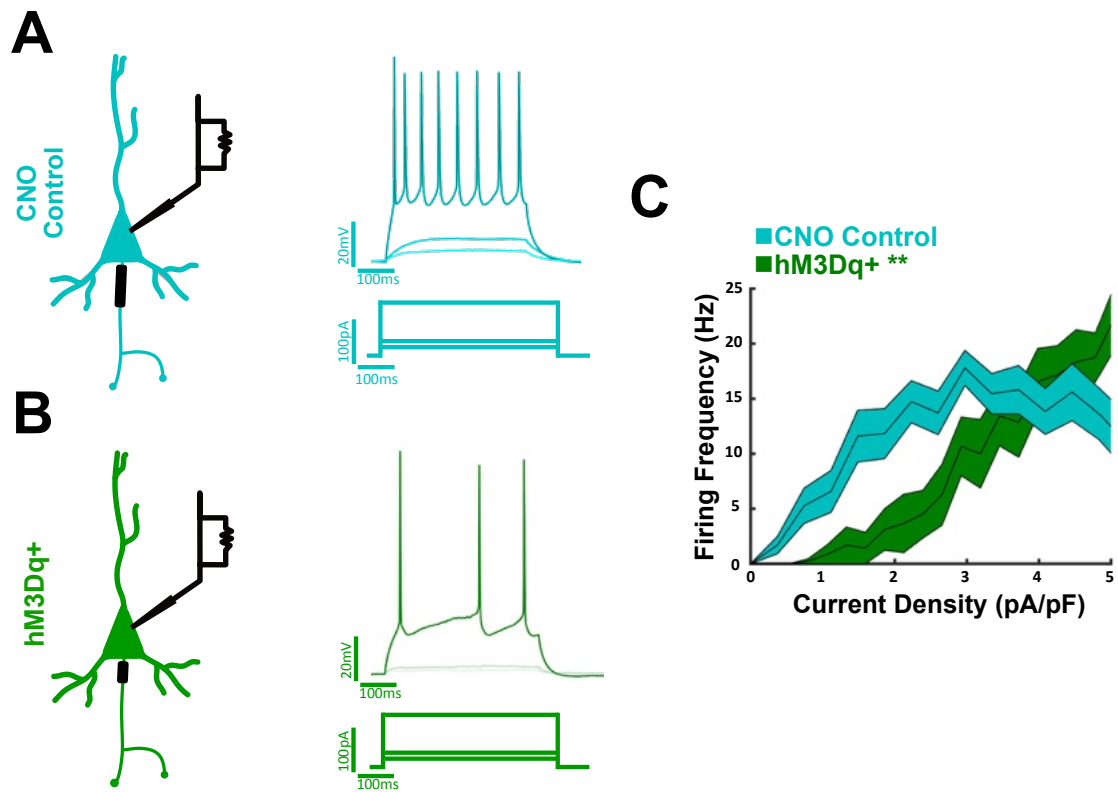


Figure 4.9 Electrophysiological signature of AIS plasticity. (A-B) Representative firing profile of pyramidal cells in the CNO control and hM3Dq-network group after injecting increasing 10 pA steps of 500ms. 1st step, 10 pA, 2nd step, 20 pA, 3rd step, 120 pA. **(C)** Input-output curve for pyramidal cells in each group. Centre line for each group represents mean firing rate for each current density bin. Shaded area between outer lines represents $\pm$ s.e.m. ** $P < 0.05$ Repeated measures ANOVA. CNO control: $n = 7$ neurons, $n = 2$ mice; hM3Dq+: $n = 8$ neurons, $n = 2$ mice.

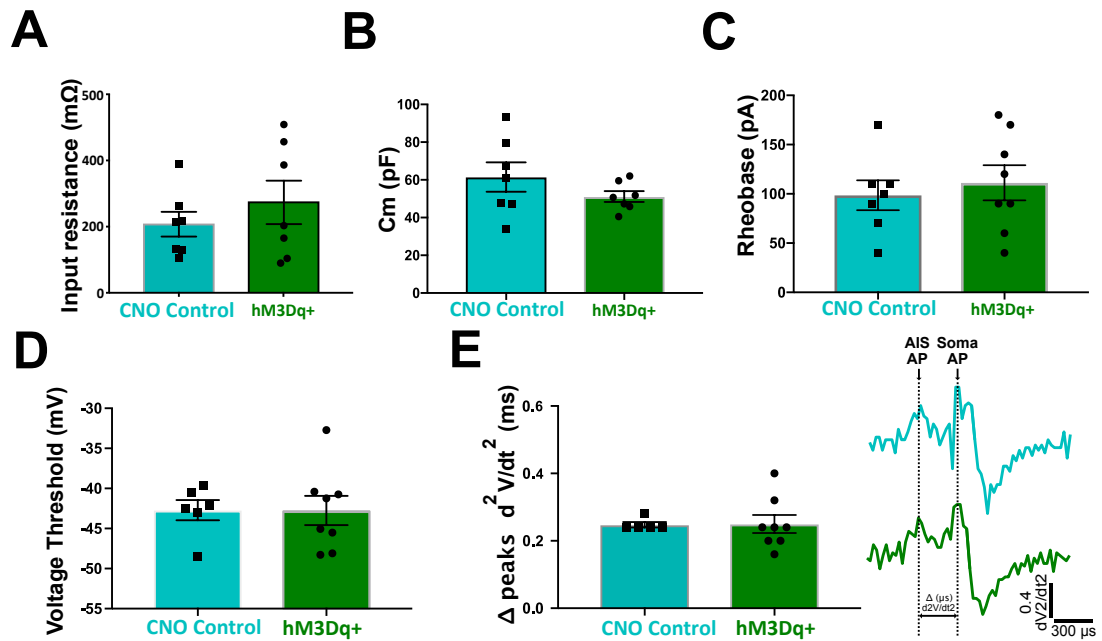


Figure 4.9 Electrophysiological signature of AIS plasticity (extended). Passives and intrinsic excitability measure across groups. **(A)** Input resistance, **(B)** capacitance, **(C)** rheobase, **(D)** voltage threshold, **(E)** Left, time difference between peaks of the second derivative of voltage in respect to time. Right, example single trial sweeps of the second derivative of voltage in respect to time across condition. Vertical arrows indicate peaks corresponding to the time of action potential initiation at the AIS and soma. Values expressed as mean ± s.e.m. Significant difference tested with Mann-Whitney test. CNO control: n = 5-7 neurons, n = 2 mice; hM3Dq+: n = 8 neurons, n = 2 mice.

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4.2.10 High network activity reduced the number of axo-somatic basket cell synapses

After confirming a reduction in axo-axonic synapses in response to heightened network activity, I decided to test if synapses from other types of interneurons also changed. I focused on basket cell synapses. Like ChCs, basket cells are fast-spiking, PV+ interneurons that are thought to control the overall levels of activity in the neocortex (Kepecs and Fishell, 2014). Unlike ChCs, they synapse directly onto the soma of pyramidal neurons, forming a basket-like structure that engulfs the cell body (Hu et al., 2014).

I first counted the number of GABAergic synapses at the soma of L2/3 cells (Figure 4.10.A), following the same treatment protocol used in the previous section (Figure 4.1). To quantify basket cell synapses I measured VGAT+ puncta (a marker for GABA transporters found in all interneurons) in direct contact with somas that were stained with the neuronal somatic marker NeuN. I used a previously published automated single plane analysis to detect and count synapses that overlapped with the somatic stain (del Pino et al., 2013) (Figure 4.10.B). The results showed that in response to heightened network activity from P12 to P18, there was a significant increase in the density of synapses contacting the soma of hM3Dq+ (0.4153 ± 0.014 syn/ μm , $P < 0.001$ to saline control, $P < 0.001$ to CNO control) and hM3Dq-network cells (0.411 ± 0.012 syn/ μm , $P < 0.05$ to saline control, $P < 0.05$ to CNO control) in comparison to both saline control (0.353 ± 0.012 syn/ μm) and CNO control groups (0.355 ± 0.013 syn/ μm) (Figure 4.10.C).

4.2.11 High network activity increased the frequency of spontaneous IPSCs

I then recorded spontaneous inhibitory events as an electrophysiological correlate of basket cell synapse number (Figure 4.11.A). When patch-clamping cortical neurons at the soma, most spontaneous events recorded in the presence of glutamatergic blockers are thought to originate from basket cell synapses. This is due to the large number of them and their proximity to the recording electrode. Experiments measuring spontaneous IPSCs were carried out in acute slices from P17-P19 mice after CNO

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treatment. Again, as for the electrophysiological analysis of axo-axonic plasticity, mice P17-P19 were used and only one control group was used (CNO control).

I used two measures of spontaneous IPSC (sIPSCs) frequency by looking at the average sIPSC frequency in an individual neuron. Interestingly, the results showed that in response to high network activity, hM3Dq+ cells had a higher sIPSCs frequency (1.575 ± 0.39 Hz, $P < 0.05$) than CNO control neurons (0.428 ± 0.07 Hz). No significant difference was found in sIPSC frequency between hM3Dq+ and hM3Dq-network cells (0.78 ± 0.16 Hz). Likewise, no significant difference was found in sIPSC frequency between the hM3Dq-network and the CNO control group (Figure 4.11.C). The average amplitude of individual sIPSCs was similar across all groups (CNO control: 50.32 ± 2.71 ; hM3Dq-network: 46.13 ± 2.33 ; hM3Dq+: 48.78 ± 2.15) (Figure 4.11.D). This was expected, since as shown in the previous section (3.2.10) in response to high network activity there was a significant increase in the number of axo-somatic boutons, which may lead to an increase sIPSC frequency.

Based on the above information I concluded that increased neuronal activity led to a cell autonomous increase in axo-somatic innervation which followed a homeostatic direction.

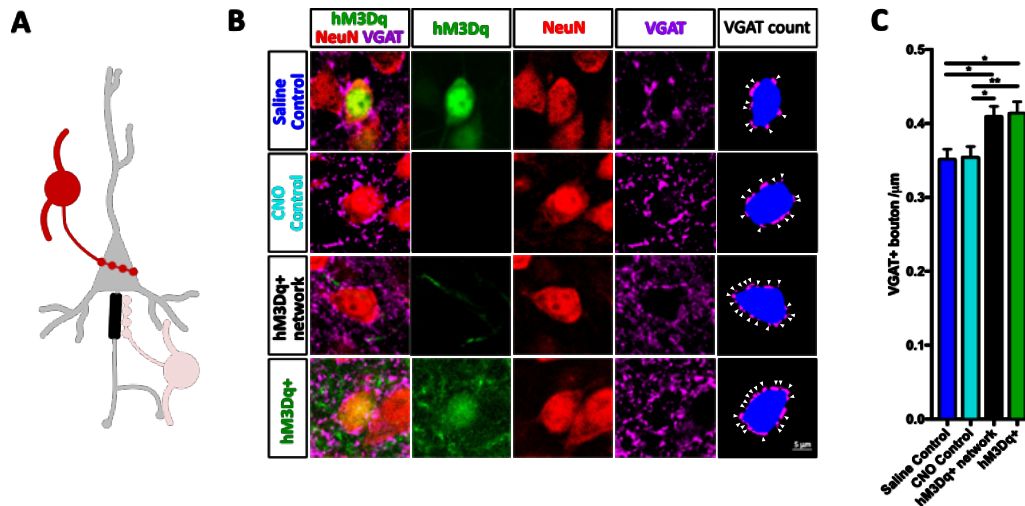


Figure 4.10 High network activity decreases the number of basket cell synapses at the soma. (A-B) Representative images of somas and basket cell synapses from L2/3 somatosensory cortex from P18 mice, after repeated treatment with CNO/saline (P12-P18), across groups. Soma (red), VGAT puncta (magenta) and hM3Dq+ (green). Cells were thresholded and automatically segmented as shown in 'VGAT count': segmented VGAT puncta (magenta), segmented soma (blue) **(C)** Bar chart showing the density of basket cell synapses across groups. Values expressed as mean $\pm$ s.e.m. * $P < 0.05$ ** $P < 0.01$ tested with one-way ANOVA with Sidak's posthoc comparison test. CNO control: $n = 30$ neurons, $n = 3$ mice; Saline control: $n = 20$ neurons, $n = 3$ mice; hM3Dq: $n = 30$ neurons, $n = 3$ mice, hM3Dq-network: $n = 30$ neurons, $n = 3$ mice.

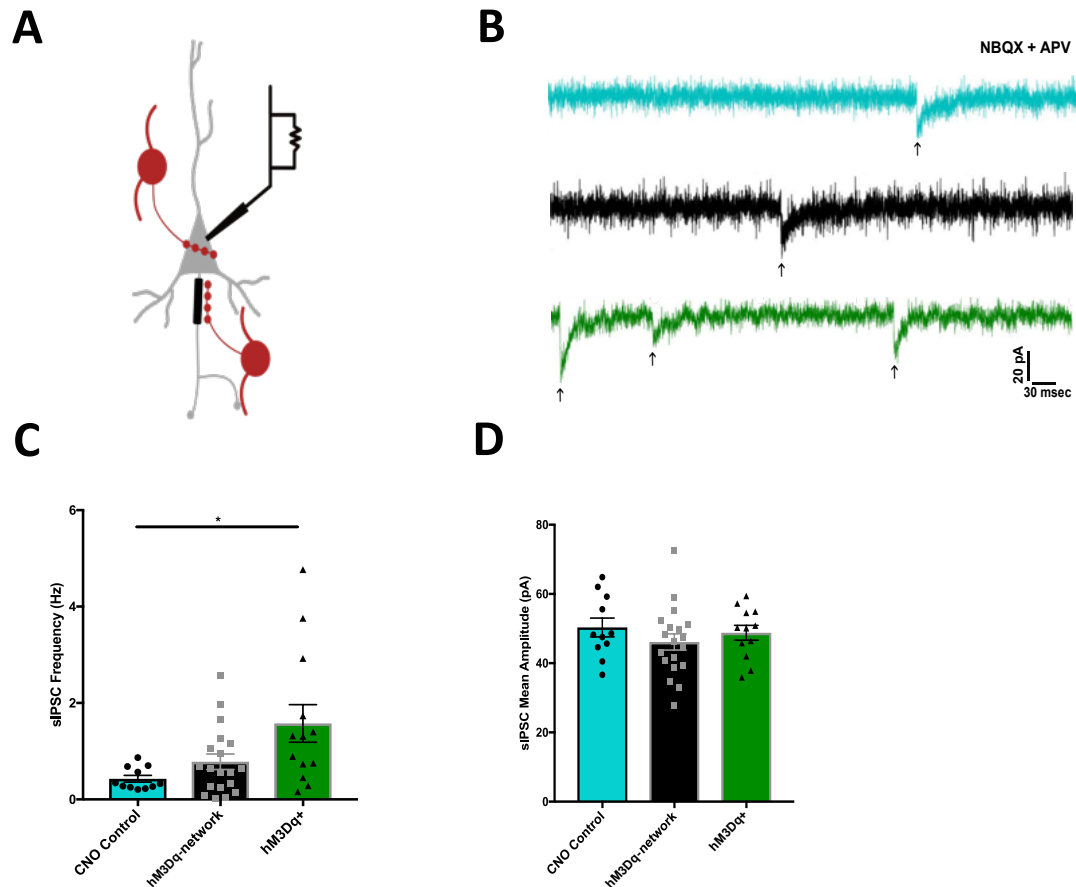


Figure 4.11 Electrophysiological signature of basket cell plasticity. **(A)** Pyramidal cells were patched in whole cell configuration after repeated treatment with CNO (P12-P18). Spontaneous events were recorded in the presence of glutamatergic blockers (NBQX and APV). **(B)** Representative traces of spontaneous IPSCs (sIPSCs) across groups. **(C)** Average sIPSC frequency and **(D)** amplitude for each cell. Each square circle and triangle represent and individual cell. Values expressed as mean $\pm$ s.e.m. * $P < 0.05$ tested with Kruskal-Wallis test followed by Dunn's multiple comparisons test. CNO control: $n = 11$ neurons, $n = 3$ mice; hM3Dq-network: $n = 19$ neurons, $n = 5$ mice, hM3Dq+: $n = 13$ neurons, $n = 3$ mice.

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4.2.12 Axo-axonic plasticity is reversible

I next tested whether the plasticity of axo-axonic synapses during development was reversible (Figure 4.12). For this I followed the CNO treatment protocol outlined in Figure 4.1 (P12-P18) with an added recovery period of 5 days where no CNO was injected (Figure 4.12.A). Mice across all groups were perfused at P22.

Morphological analysis (Figure 4.12.B) showed that after 5 days of recovery both the density and number of axo-axonic synapses were significantly increased in the hM3Dq-network condition (Density: 0.1428 ± 0.006 syn / μm , Number: 5.97 ± 0.267 , $P < 0.001$) in comparison to its P18 counterpart (Density: 0.095 ± 0.008 syn / μm , Number: 3.709 ± 0.29) (Figure 4.12.C-D). The recovery appeared to be nearly complete, nonetheless subtle differences could still be found. The density of synapses at P22 in hM3Dq+ (0.129 ± 0.012 syn / μm) and hM3Dq-network conditions (0.143 ± 0.006 syn / μm) did not fully recover back to control levels (0.164 ± 0.009 syn / μm) (Figure 4.12.C). This difference was subtle as suggested by the post-hoc multiple comparison tests, which failed to locate specific paired differences (differences across groups were still significant as indicated by $P < 0.05$, Kruskal-Wallis). The recovery was complete for the number of axo-axonic synapses, where average values were comparable across conditions (P22 CNO control: 6.1 ± 0.336 , P22 hM3Dq+: 4.878 ± 0.435 , P22 hM3Dq+ network: 5.97 ± 0.267) (Figure 4.12.D).

I then tested if the apparent shortening of the AIS in response to high network activity was also reversible (Figure 4.13). Morphological analysis (Figure 4.13.A) showed that there was a significant increase in AIS length in the hM3Dq+ condition (only condition in which we observed a potential change at P18) after 5 days of recovery (P22: 35.5 ± 2.17 μm , P18: 26.6 ± 1.23 μm , $P < 0.001$) (Figure 4.13.B). Unexpectedly, there was not only a full recovery of AIS size, but a significant increase in AIS size of both P22 hM3Dq+ (35.5 ± 2.17 μm , $P < 0.05$) and P22 hM3Dq-network (35.5 ± 0.757 μm , $P < 0.001$) neurons in comparison to the P22 CNO control (30.6 ± 1.364 μm), suggesting some kind of rebound effect.

Finally, I tested if the decrease in connection probability observed after high network activity had also recovered (Figure 4.7). As expected from the partial recovery of density and number of axo-axonic synapses, connection probability of chandelier cells fully

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recovered (Figure 4.14.A). Connection probability within a 90 μm radius of the soma significantly increased from 72.6% at P18 to 88.3% following the recovery period at P22 ($P < 0.001$) for hM3Dq-network ChCs. The latter was not significantly different to the P22 CNO control (Figure 4.14.B).

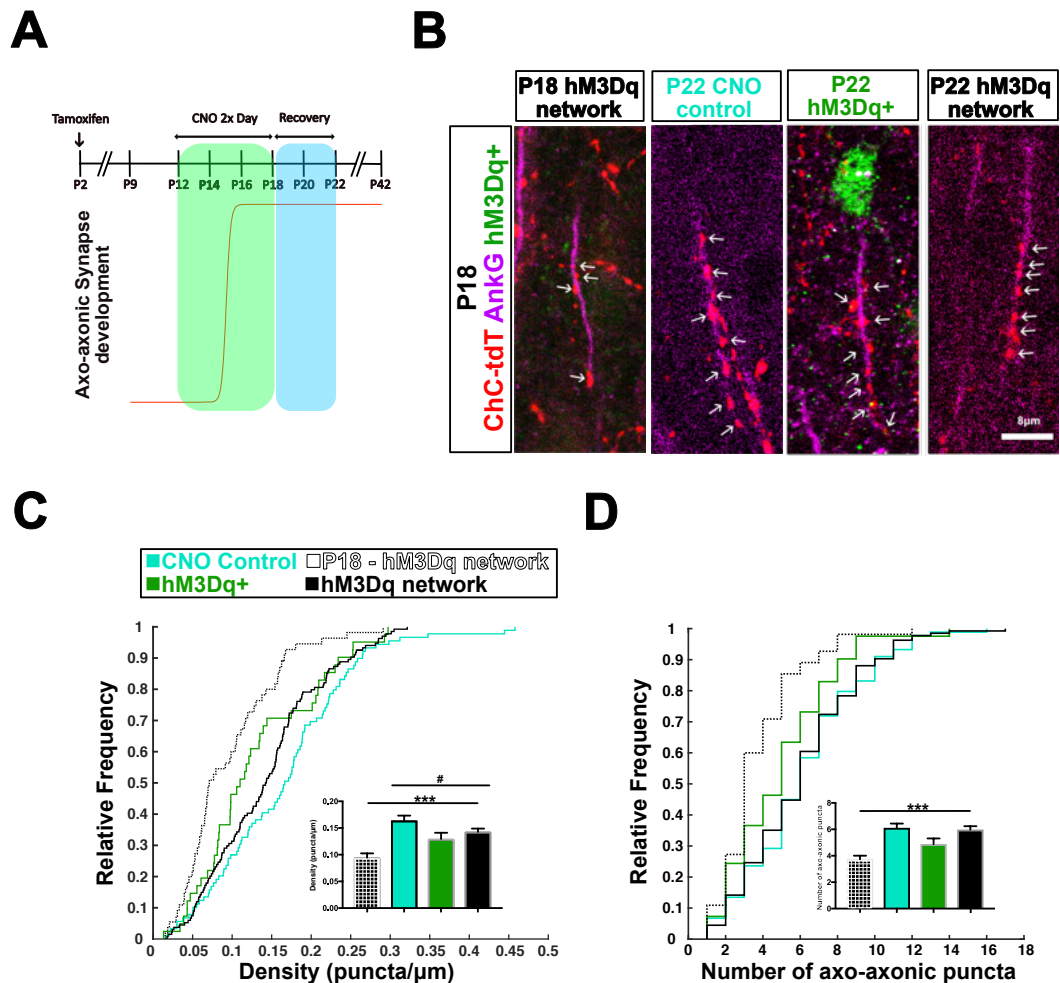


Figure 4.12 Axo-axonic synaptic plasticity is reversible. (A) After repeated treatment with CNO/saline (P12-P18, green), mice were not injected for a period of 5 days until P22 (cyan), when axo-axonic synapse density and number at the AIS was quantified. (B) Representative images of AIS and axo-axonic synapses from L2/3 somatosensory cortex from P22 mice across groups after recovery period. AIS (magenta), axo-axonic synapses (red) and hM3Dq+ (green). (C-D) Cumulative distribution plot and bar chart showing the density (C) and number (D) of axo-axonic synapses along the AIS in each different group (empty letters represent dashed line). Values expressed as mean $\pm$ s.e.m. P22 conditions tested with Kruskal-Wallis test followed by Dunn's multiple comparisons test. # denotes $P < 0.05$ for Kruskal Wallis test without reaching significance in Dunn's posthoc comparisons. *** $P < 0.01$ P18 hM3Dq-network – P22 hM3Dq-network comparison tested with Mann-Whitney test. CNO control: $n = 89$ neurons, $n = 2$ mice; hM3Dq: $n = 41$ neurons, $n = 3$ mice, hM3Dq-network: $n = 134$ neurons, $n = 3$ mice.

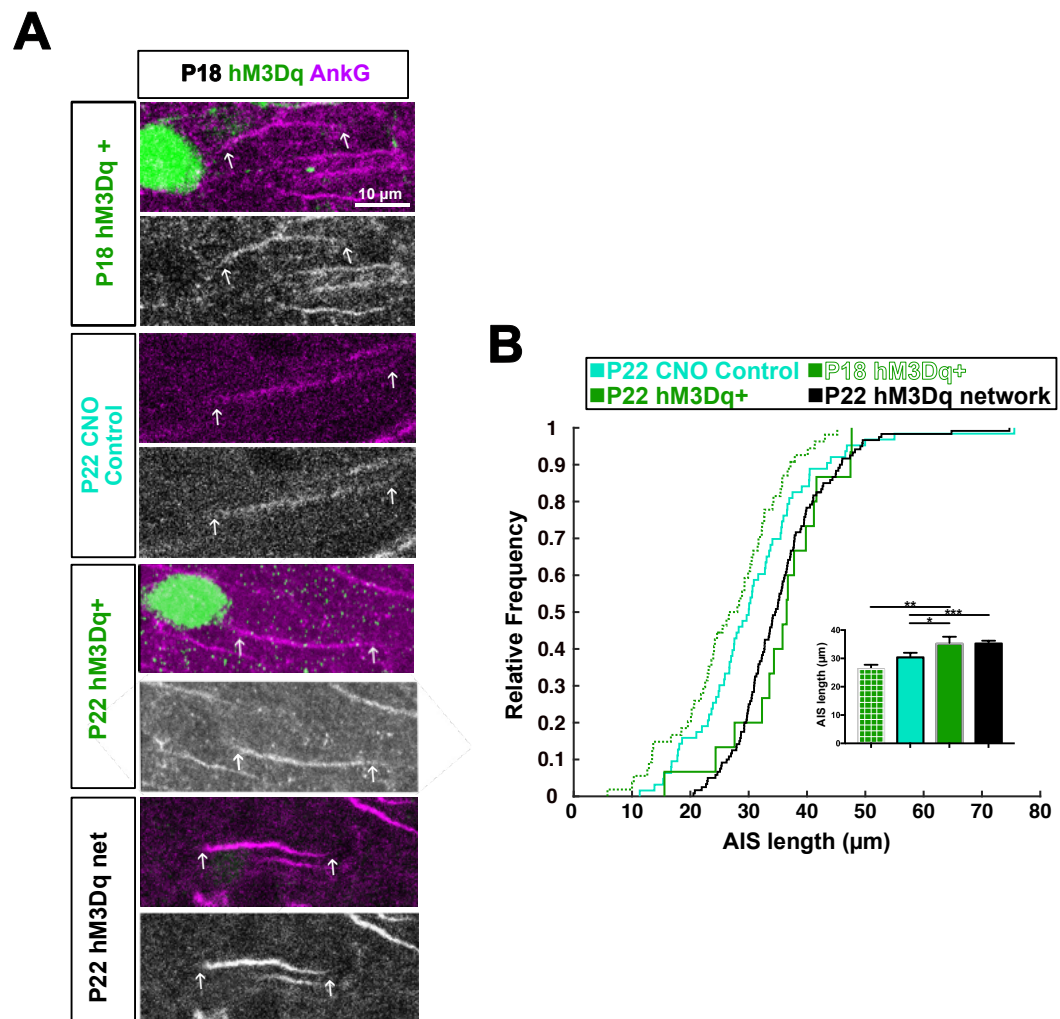


Figure 4.13 AIS plasticity is reversible. (A) Representative images of AISs from L2/3 somatosensory cortex from P22 mice across groups, after repeated treatment with CNO/saline (P12-P18) and a 5 days recovery period. For each group, top image: AIS (magenta) and hM3Dq+ (green), bottom image: grayscale image of AIS. White arrows delimit AIS. **(B)** Cumulative distribution plot and bar chart showing the length of the AIS in each different group. Values expressed as mean $\pm$ s.e.m (empty letters represent dashed line). P22 conditions tested with Kruskal-Wallis test followed by Dunn's multiple comparisons test. * $P < 0.05$, *** $P < 0.001$. ** $P < 0.01$ P18 hM3Dq+ – P22 hM3Dq+ comparison tested with unpaired student t-test. CNO control: $n = 63$ neurons, $n = 2$ mice; hM3Dq: $n = 15$ neurons, $n = 3$ mice, hM3Dq-network: $n = 120$ neurons, $n = 3$ mice.

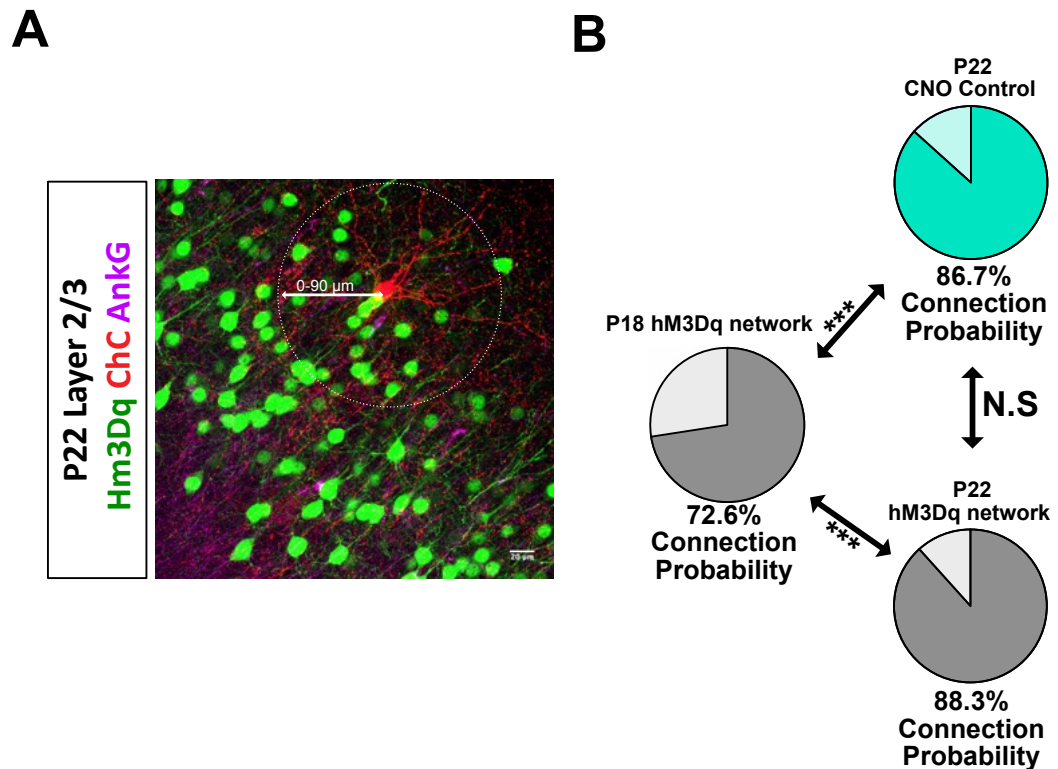


Figure 4.14 Single chandelier cell connectivity returns to baseline after 5 days of recovery. **(A)** Representative image of ChC in L2/3 somatosensory cortex in a hM3Dq+ network at P22 after repeated treatment with CNO/saline (P12-P18) and recovery period of 5 days. AIS (magenta), axo-axonic synapses (red) and hM3Dq+ (green). Dashed lines mark the area used to measure connection probability. Inner circle: 90 μm radius cylinder around soma **(B)** Pie charts showing connection probability in a 90 μm (inner circle) across groups. Values expressed as %. N.S (not significant), *** $P < 0.001$ tested with χ^2 test. CNO control: $n = 180$ AIS, $n = 6$ ChCs, $n = 2$ mice; hM3Dq-network: $n = 240$ AIS, $n = 8$ ChCs, $n = 3$ mice.

4.2.13 High network activity in adult somatosensory cortex leads to an increase in axo-axonic synapses but not ChC connection probability

Some forms of plasticity are thought to be unique to the developing brain (e.g. ocular dominance plasticity (Hubel and Wiesel, 1970)). Therefore, after confirming that axo-axonic plasticity was reversible, I wanted to check whether this was unique to the periadolescent brain or if it would happen during young adulthood. As before, I electroporated Nkx2.1/tdTomato embryos at E15.5 with hM3Dq, but applied CNO treatment (6 days, 2x a day) from P40 to P46. At P46, I perfused the animals for morphological analysis after the first CNO injection (Figure 4.15.A).

Morphological analysis (Figure 4.15.B) showed that in response to high network activity there were network-wide significant increases in the density and number of synapses across groups. In contrast to the periadolescent brain, hM3Dq-network neurons in young adults had significantly more axo-axonic synapses (7.467 ± 0.38 , $p < 0.001$ for CNO control and $p < 0.01$ for saline control) than both Saline (5.6 ± 0.464) and CNO control (4.7 ± 0.342). hM3Dq+ neurons (7.226 ± 0.48) showed the same tendency, with a significant increase in comparison to the CNO control ($p < 0.01$) but failing to reach significance when compared to the saline control ($p = 0.08$) (Figure 4.15.D). Measurements of synapses density displayed a similar pattern, showing overall significance across the conditions ($P < 0.05$) but failing to reach significance in the posthoc multiple comparisons. hM3Dq+ (0.172 ± 0.01 syn/ μm , $p = 0.08$ for saline control, $p = 0.1$ for CNO control) and hM3Dq-networks neurons (0.17 ± 0.08 syn/ μm , $p = 0.06$ for saline control, $p = 0.08$ for CNO control) tended to have a higher density of axo-axonic synapses than neurons from both the Saline (0.14 ± 0.01) and CNO controls (0.14 ± 0.07 syn/ μm) (Figure 4.15.C). There were no significant differences between the two controls for both density and number of synapses.

In the periadolescent brain I observed a potential decrease in AIS length in response to high network activity. When looking at AIS length in young adults there was a significant increase in AIS length in response to high levels of network activity (Figure 4.16.A). Both hM3Dq+ (35.63 ± 1.11 μm , $p < 0.01$) and hM3Dq-network cells (36.2 ± 1 μm , $p < 0.001$) presented a longer AIS than the CNO control (30.47 ± 0.84 μm). However, this was

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inconclusive as there was not a significant difference with the saline control (33.8 ± 0.78 μm , $P = 0.2$ for hM3Dq-network, $P = 0.4$ for hM3Dq+) (Figure 4.16.B). Again, there were no significant differences between both control groups.

Finally, in the periadolescent brain I also observed a decrease in the number of AISs contacted by an individual chandelier cell in hM3Dq+ networks after CNO treatment. When testing for this phenotype in adults (Figure 4.17.A), ChCs in hM3Dq-networks presented similar connection probabilities (93.3%) to both ChCs in the Saline (85.3%) and CNO control groups (90.7%) within 90 μm , suggesting that at this age activity dependent ChC plasticity is limited to the axo-axonic cartridge (Figure 4.17.B).

Overall this data suggested that there might be a switch in the direction of activity dependent plasticity in adult mice. At this age, increases in network activity lead to a significant increase in axo-axonic synapse number, the opposite to what was observed in periadolescent mice.

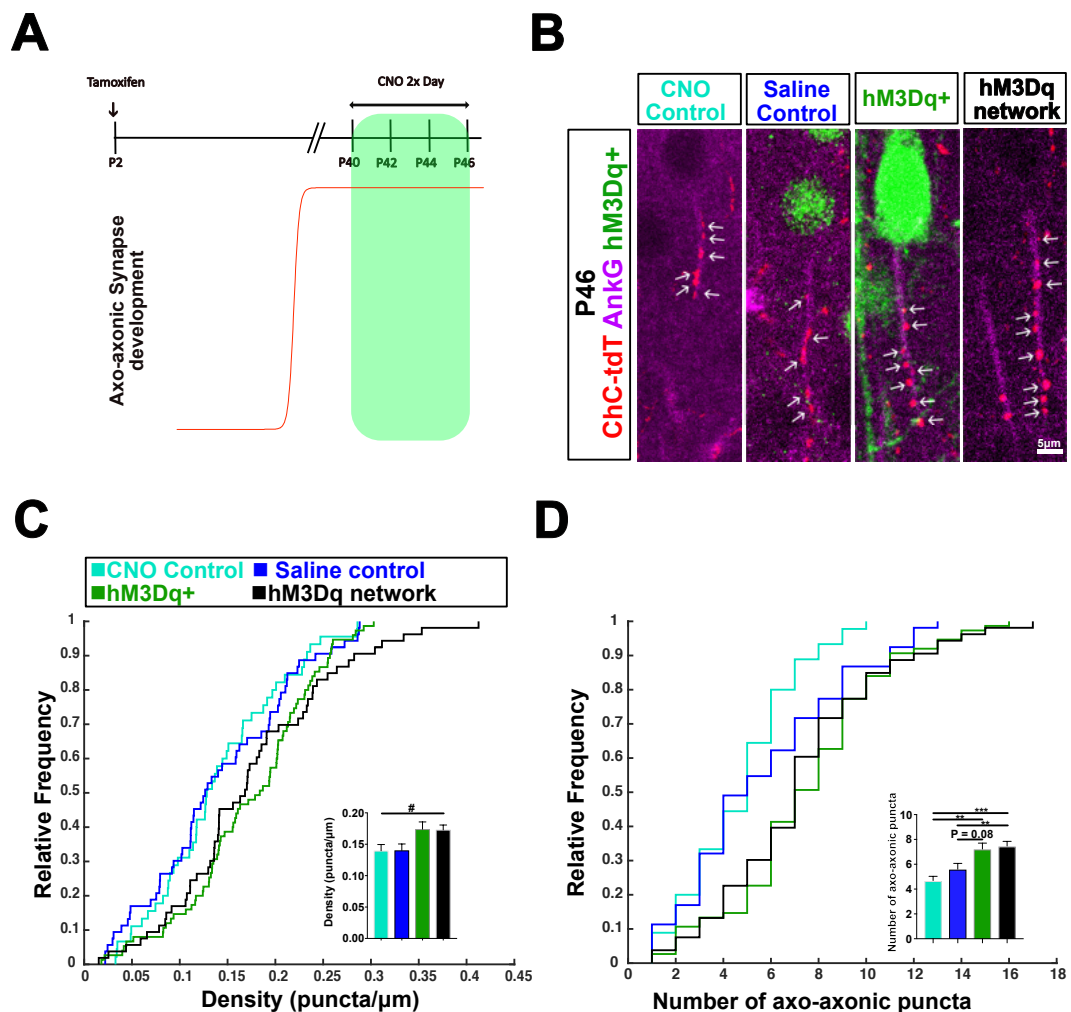


Figure 4.15 High network activity increases the number of axo-axonic synapses at the AIS in adult mice. (A) Repeated treatment with CNO/saline (P40-P46, green) twice a day. At P46 axo-axonic synapse density and number at the AIS was quantified. **(B)** Representative images of AIS and axo-axonic synapses from L2/3 somatosensory cortex from P46 mice, after repeated treatment with CNO/saline across groups (P40-P46). AIS (magenta), axo-axonic synapses (red) and hM3Dq+ (green). **(C-D)** Cumulative distribution plot and bar chart showing the density (C) and number (D) of axo-axonic synapses along the AIS in each different group. # denotes $P < 0.05$ for one-way ANOVA without reaching significance in posthoc comparisons. Values expressed as mean $\pm$ s.e.m., * $P < 0.05$ ** $P < 0.01$, *** $P < 0.001$ tested with Kruskal-Wallis test and Dunn's posthoc comparisons test. CNO control: $n = 41$ neurons, $n = 3$ mice; Saline control: $n = 44$ neurons, $n = 5$ mice, hM3Dq: $n = 43$ neurons, $n = 3$ mice, hM3Dq-network: $n = 72$ neurons, $n = 3$ mice.

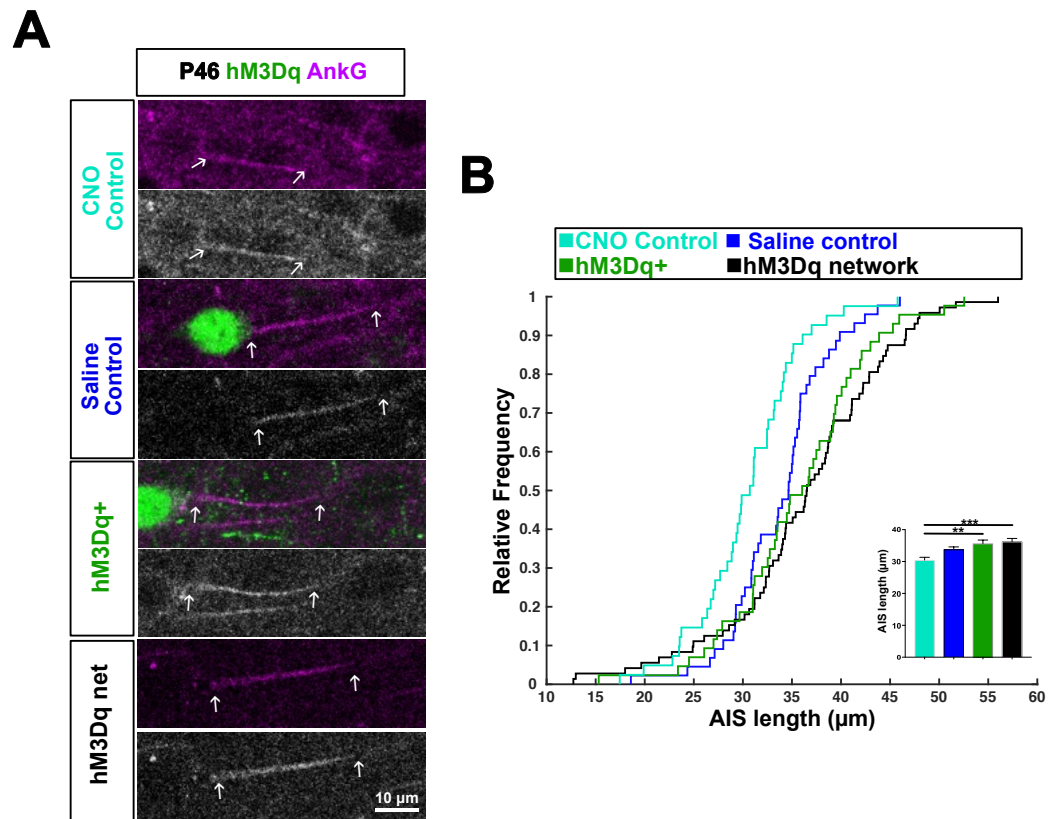


Figure 4.16 AIS length might be increased by high network activity in adult mice. **(A)** Representative images of AISs from L2/3 somatosensory cortex from P46 mice, after repeated treatment with CNO/saline (P40-P46), across groups. For each group, top image: AIS (magenta) and hM3Dq+ (green), bottom image: grayscale image of AIS. White arrows delimit AIS. **(B)** Cumulative distribution plot and bar chart showing the length of the AIS in each different group. Values expressed as mean $\pm$ s.e.m. ** $P < 0.01$, *** $P < 0.001$ tested with Kruskal-Wallis test and Dunn's posthoc comparisons test. CNO control: $n = 45$ neurons, $n = 3$ mice; Saline control: $n = 53$ neurons, $n = 5$ mice, hM3Dq: $n = 53$ neurons, $n = 3$ mice, hM3Dq-network: $n = 75$ neurons, $n = 3$ mice.

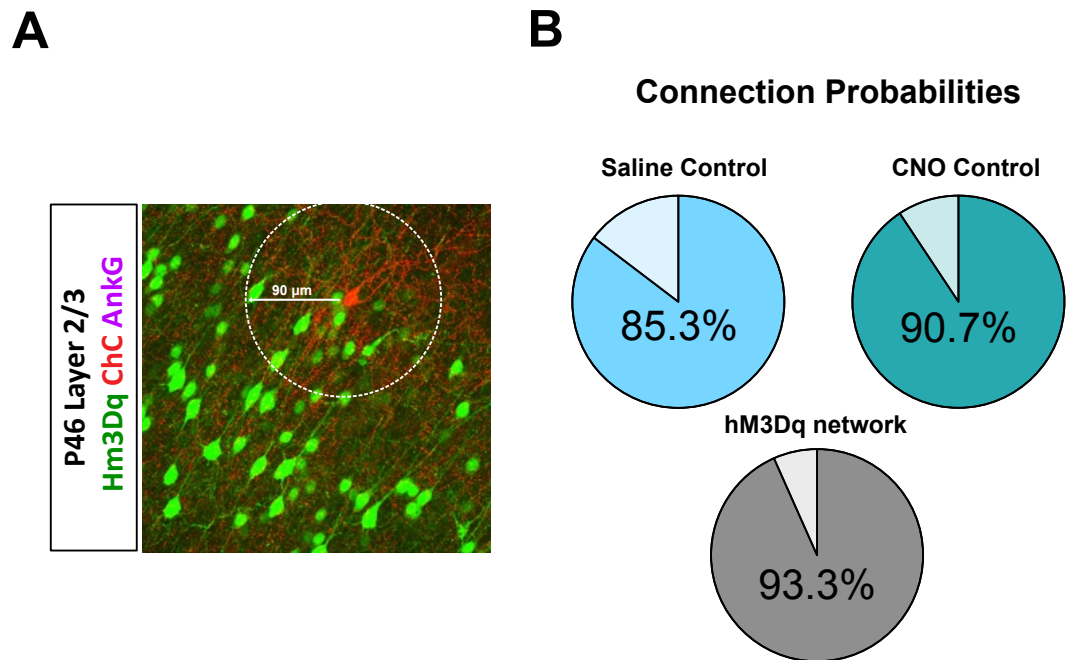


Figure 4.17 High network activity does not affect ChC connection probability in adult mice. **(A)** Representative image of ChC in L2/3 somatosensory cortex in a hM3Dq+ network at P46 after repeated treatment with CNO/saline (P40-P46). AIS (magenta), axo-axonic synapses (red) and hM3Dq+ (green). Dashed lines mark the areas used to measure connection probability. Inner circle: 90 μm radius cylinder around soma. **(B)** Pie charts describing connection probability in an 90 μm (dashed circle) across groups. Values expressed as %. Differences tested with χ^2 test. CNO control: n = 150 AIS, n = 5 ChCs, n = 3 mice; Saline control: n = 150 AIS, n = 5 ChCs, n = 4 mice; hM3Dq-network: n = 180 AIS, n = 6 ChCs, n = 3 mice.

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4.2.14 High activity in ChCs leads to a cell autonomous decrease in axo-axonic synapses and ChC connection probability

In the previous sections of this chapter I showed that cortical ChCs significantly decrease the number of axo-axonic synapses made in response to high activity in their local network. These changes happen in a network wide (non-cell autonomous) manner, irrespective of whether individual cells expressed hM3Dq. As suggested by the c-Fos results at P18 (Figure 4.3), the increase in the activity of hM3Dq+ cells might spread to the rest of the network. Increases in network activity are also likely to drive activity in ChCs through local intracortical connections (Lu et al., 2017). To establish if axo-axonic synapse plasticity only requires the activity of pyramidal cells or can also be driven by increasing the activity of ChCs in a cell-autonomous manner, we expressed DREADDs (hM3Dq) solely in ChCs.

To increase the activity of ChC I expressed hM3Dq in ChCs themselves in L2/3 of somatosensory cortex of Nkx2.1CreER^{+/−} mice. To maintain the cell-autonomous features of this manipulation I attempted to reduce the effect of increasing ChC activity on the network by sparsely expressing hM3Dq in ChCs. I used a viral approach that allowed infection of isolated ChCs (Figure 4.18.A) in an extremely low number; a maximum of 2 ChCs were infected per brain (see methods for more details). Since the hM3Dq-RFP expression did not distribute well along the entire cell (I found it localised mainly to the soma and dendrites) I co-infected with a Cre-dependent GFP virus that would reveal the whole morphology of the ChC (Figure 4.18.B). This also provided the added advantage of an internal control in the form of ChCs that were infected with GFP but did not express hM3Dq. Both cells would be exposed to CNO, but only the hM3Dq+ ChCs would be activated, generating an internal “CNO control” condition. As in the original network manipulation, I injected CNO in the mice infected with the hM3Dq/GFP virus twice a day from P12 to P18.

Increasing activity in ChC themselves led to similar results to those observed when increasing activity at a network level. Morphological analysis (Figure 4.19.A) showed that hM3Dq+ ChCs treated with CNO formed significantly less axo-axonic synapses (Density: 0.11 ± 0.011 syn/ μ m, $P < 0.01$; Number: 4.617 ± 0.401 , $P < 0.01$) than ChCs in

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the CNO control group (Density: 0.14 ± 0.009 syn/ μm ; Number: 6.267 ± 0.403) (Figure 4.19.B-C).

In contrast to increasing activity in pyramidal cells, increasing activity in ChCs did not lead to a significant shortening of the AIS. AIS contacted by hM3Dq+ ChCs (34.49 ± 7.732 μm) had similar lengths to the ones contacted by CNO control ChCs (37.02 ± 0.78 μm) (Figure 4.19.D-E).

Finally, I assessed whether the decrease in connection probability observed when increasing activity at a network level was also present when increasing activity in ChCs themselves (Figure 4.20.A). Interestingly, identical results were obtained. hM3Dq+ ChCs (74.00 ± 0.064 %, $P < 0.05$) presented a significantly lower connection probability within 90 μm of the soma than CNO control ChCs (90.00 ± 0.026 %, %). Additionally, the connection probability in hM3Dq+ ChCs was comparable to the one seen in hM3Dq-networks (74% and 72.6% respectively) (Figure 4.20.B).

Overall, these results show that increasing activity of ChC's themselves can lead to the same axo-axonic plasticity than the one generated by heightening network activity. Nonetheless, it should be noted that due to the difficulty of these experiments the N number is low. The data comes from 3-4 cells in 4 different animals. Further replications of this experiment are needed to confirm the observation presented here.

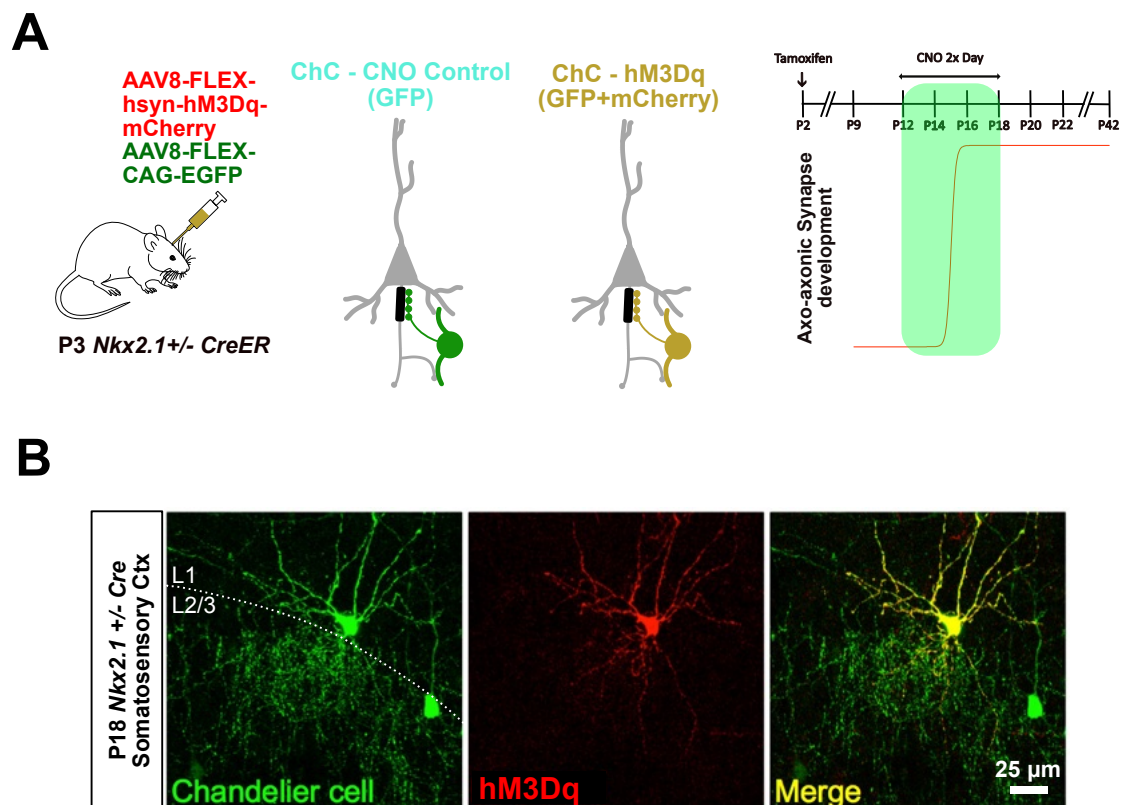
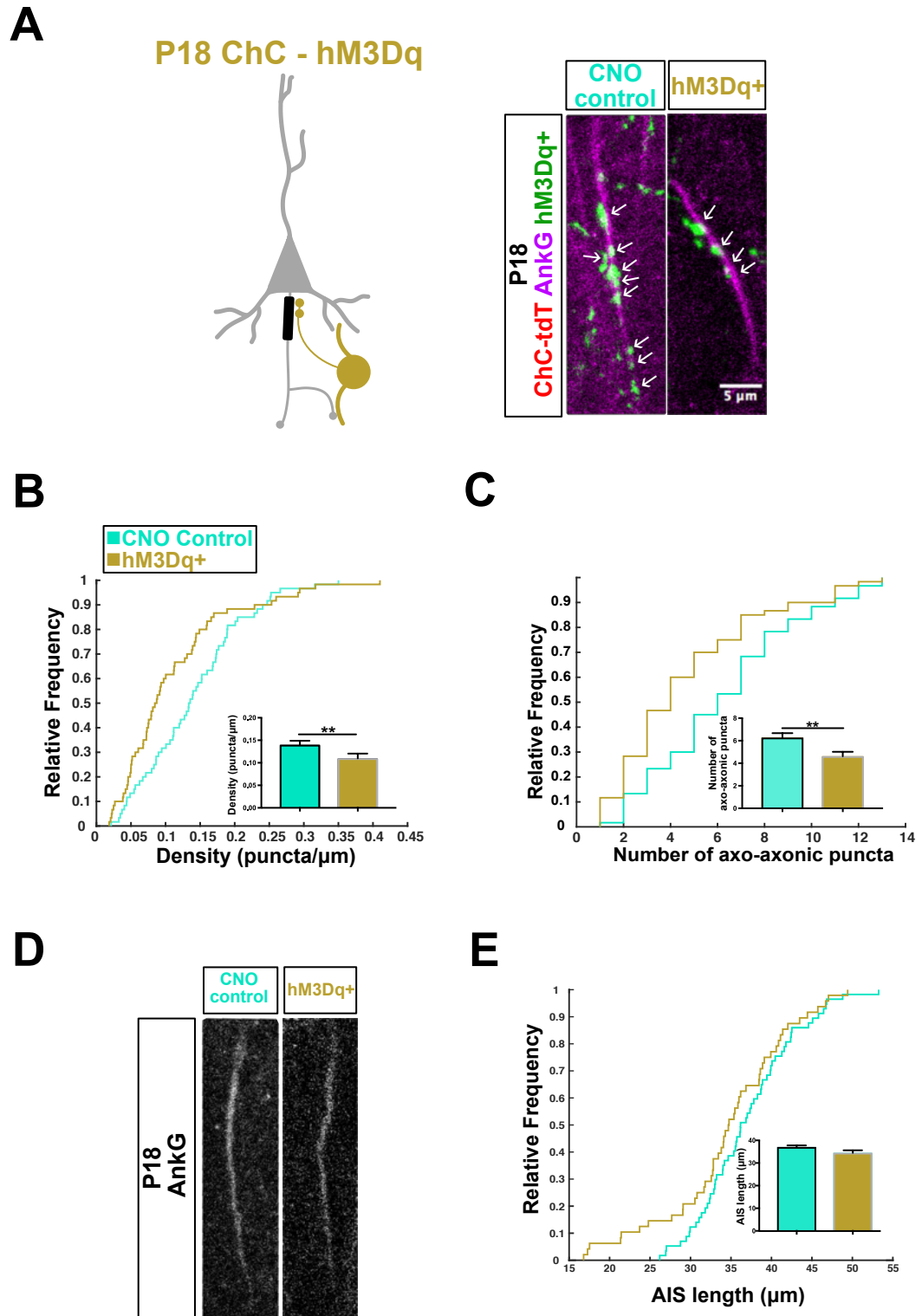


Figure 4.18 Delivery of hM3Dq to chandelier cells. (A) Viral vectors AAV8-FLEX-CAG-EGFP (GFP) and AAV8-FLEX-hsyn-hM3Dq-mCherry (hM3Dq) were injected transcranially in P3 *Nkx2.1*CreER^{+/-} mice. This resulted in two populations of chandelier cells in the same mouse: 1) GFP+ ChCs that were used as an intracranial control (ChC-CNO control) and 2) GFP+ hM3Dq+ ChCs (ChC-hM3Dq). Mice were injected with CNO (morning and evening) from P12 to P18 (the critical period of axo-axonic synapse development). At P18 mice were perfused 2h after the morning injection and axo-axonic and AIS plasticity was assessed across groups. (B) Representative picture of successfully infected ChC with both the viral vector containing GFP (green) and hM3Dq (red).

Figure 4.19 High ChC activity decreases the number of axo-axonic synapses at the AIS. **(A)** Representative images of AIS and axo-axonic synapses from P18 L2/3 somatosensory ChC infected with hM3Dq, after repeated treatment with CNO (P12-P18). AIS (magenta), axo-axonic synapses (green) and hM3Dq (red). Cumulative distribution plots and bar charts showing the density **(B)** and number **(C)** of axo-axonic synapses along the AIS for each different group. **(D)** Representative images of AISs (grayscale) from CNO control and hM3Dq+ cells. **(E)** Cumulative distribution plot and bar chart showing the length of the AIS in each different group. Values expressed as mean $\pm$ s.e.m. ** $P < 0.01$ tested with Mann-Whitney test. For axo-axonic analysis: CNO control: $n = 60$ AIS, $n = 3$ mice; hM3Dq-network = 60 AIS, $n = 3$ mice. For AIS analysis: CNO control: $n = 57$ AIS, $n = 3$ mice; hM3Dq-network = 48 AIS, $n = 3$ mice.

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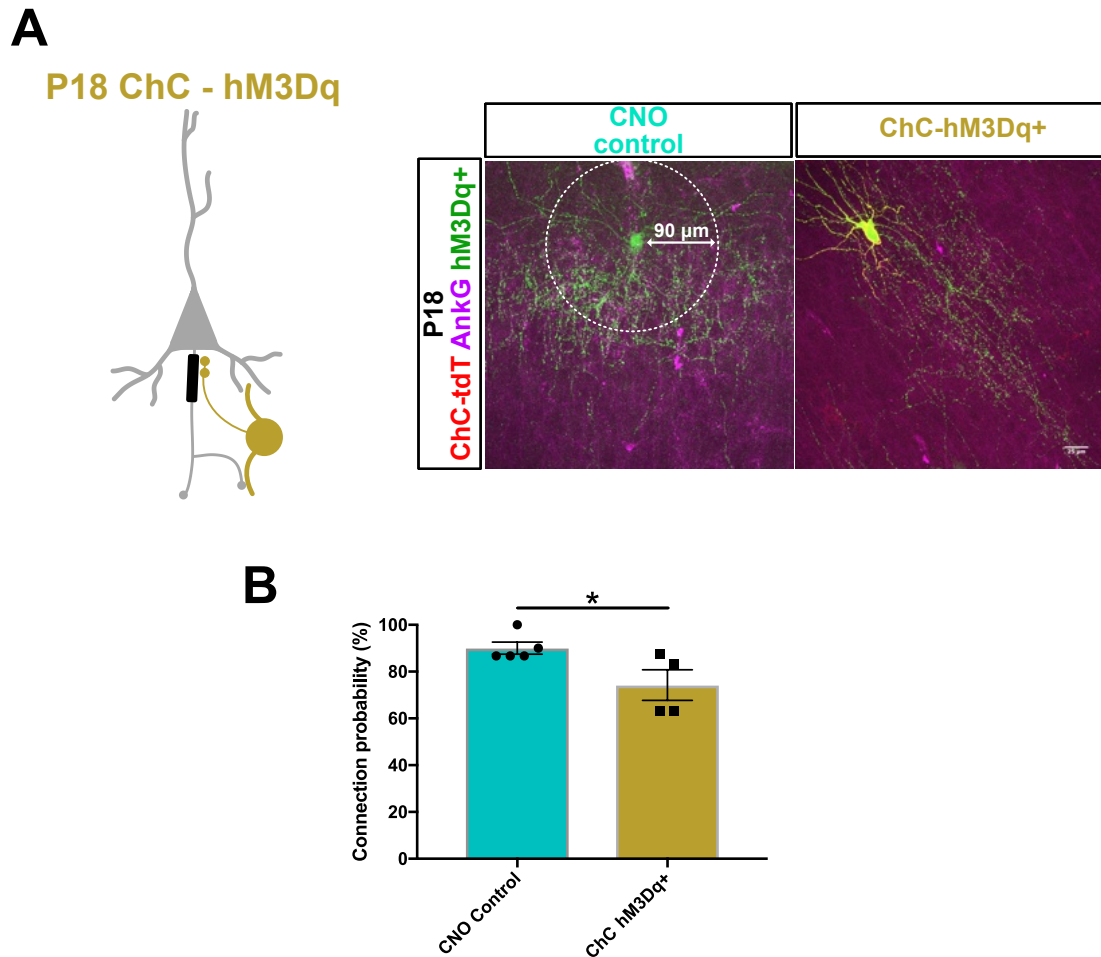


Figure 4.20 High ChC activity decreases ChC connection probability. (A) Representative image of P18 CNO-control and hM3Dq+ ChCs in L2/3 somatosensory cortex after repeated treatment with CNO (P12-P18). AIS (magenta), chandelier cell (green) and hM3Dq+ (red). Dashed lines mark the area used to measure connection probability. Inner circle: 90 µm radius cylinder around soma. **(B)** Bar plot showing connection probability in an 90 µm (inner circle) across groups. Values expressed as %. * $P < 0.05$ tested with Mann-Whitney test after arcsin transformation. CNO control: $n = 5$ ChCs, $n = 3$ mice; hM3Dq-network $n = 4$ ChCs, $n = 3$ mice.

4.2.15 Feedback model of chandelier cell plasticity

Based on the similarity of the results when increasing the activity of either pyramidal cell networks or ChCs themselves, I propose the following model of axo-axonic synapse plasticity in the periadolescent brain: increased activity in the cortical network is read-out by the ChC through increases in their activity levels, which in turn triggers a decrease in the number of axo-axonic synapses formed. Is this model biologically plausible? For this model to hold true, ChCs must be able to read-out activity from their local network through either direct or indirect connections (Figure 4.21).

Strong data on the afferent connections of ChCs is lacking and the only study looking at local connectivity of ChCs in a sensory cortex shows that there appears to be no direct pyramidal-to-ChC connections in layer 2/3 of visual cortex (Jiang et al., 2015). However, their entire data-set is based on only 6 paired recordings between ChCs and pyramidal neurons. As a result, more information is needed to understand how/if pyramidal cells are connected to neighbouring ChCs. I conducted a preliminary experiment where I performed pair recordings of ChCs and pyramidal cells in layer 2/3 networks from P18-P22 Nkx2.1/tdTomato mice. Unfortunately, due to time limitations, this experiment could not be completed. However, I was able to show, in one example out of 3 paired recordings, that the pyr-ChC connection does exist in Layer 2/3 of somatosensory cortex at P18, and that unless it was a serendipitous observation, this connection is likely to be more common than previously thought (Figure 4.21).

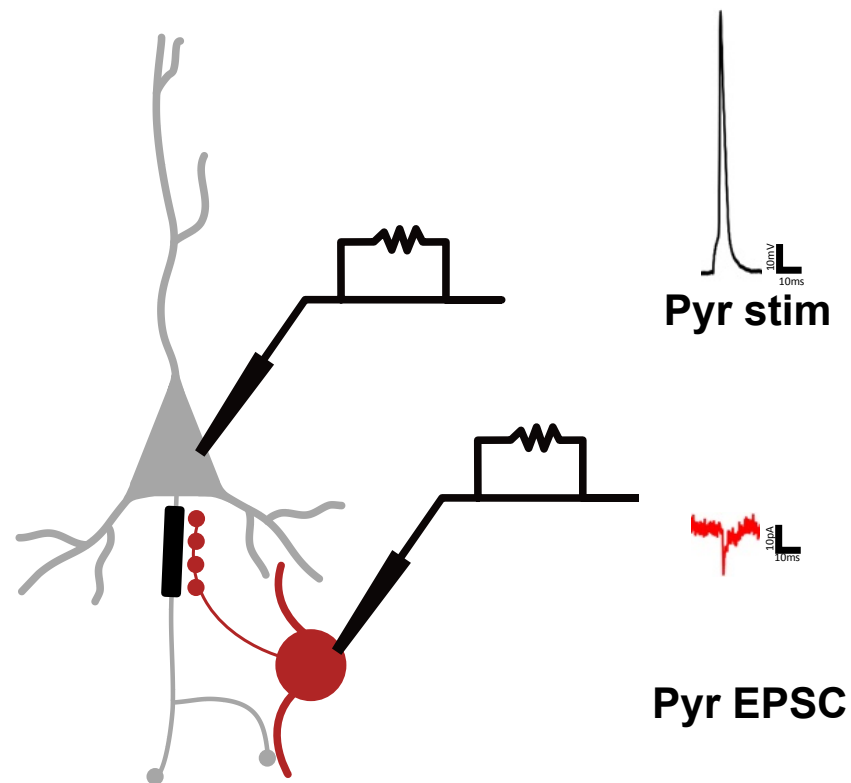


Figure 4.21 Pair whole-cell electrophysiological recording from a chandelier and pyramidal cell. Action potentials were generated in current clamp in a pyramidal cells in L2/3 (black) (Pyr stim) connected to a ChC. Evoked EPSCs were recorded in voltage clamp in ChCs.

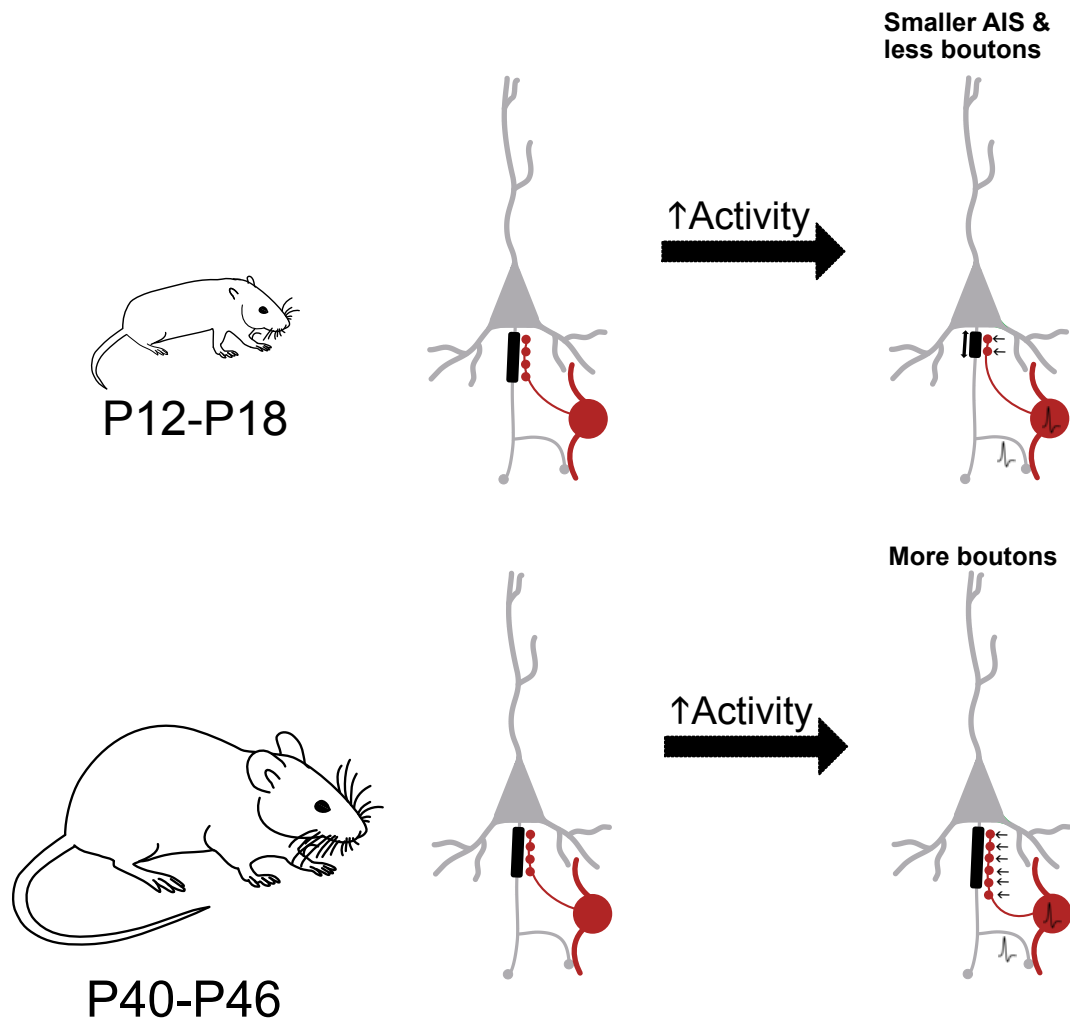


Figure 4.22 Summary diagram of axo-axonic and AIS plasticity in periadolescent and adult mice. In periadolescent mice high levels of network activity lead to a decrease in the number of axo-axonic synapses and AIS length. Similarly, increased ChC activity also leads to a decrease in the number of axo-axonic synapses. Inversely, in adult mice high levels of network activity lead to an increase in the number of axo-synapses. We speculate this might follow a feedback model where elevated levels of network activity lead to an increase in chandelier cell activity, which in turn leads to plastic changes in axo-axonic synapses.

4.3 Discussion

In this chapter I showed an activity-dependent structural plasticity of chandelier cells at the level of axo-axonic synapses (Figure 4.22). Enhancing neuronal activity with excitatory DREADDs (hM3Dq) in the periadolescent L2/3 somatosensory cortex (P12-P18) either at the network level or in ChCs themselves, led to a decrease in ChC output. Morphologically, I observed a decrease in connection probability between ChCs and pyramidal neurons, as well as an overall decrease in the total number of axo-axonic synapses formed onto the AIS of a pyramidal cell. These findings were also supported by electrophysiological recordings, which showed that IPSCs elicited by ChCs in pyramidal cells showed a smaller amplitude and a decrease in connection probability when pyramidal cell networks were activated chemogenetically. This plasticity was partially reversible within 5 days of recovery, where network activity was not enhanced. Interestingly, the same manipulation in adult mice (P40-P46) led to opposite results, showing a tendency towards an increase in the number of axo-axonic synapses in response to high network activity. Axo-axonic plasticity was accompanied by a homeostatic change in the length of AIS between P12 and P18. AIS length decreased in response to high activity levels which was correlated with a decrease in intrinsic excitability. Inconclusive results showed an increase in AIS size in response to high network activity in the adult.

4.3.1 Sparse activation of pyramidal cell activity causes network-wide changes in activity.

Acute CNO injections at P12 led to an acute increase in activity in neurons expressing hM3Dq. However chronic activation over six days (P12-P18) led to a network wide increase in activity. There are several possibilities for this difference. One possibility is that sparse activation of pyramidal cells gradually spreads to the network through intracortical connections. It is thought that in mature L2/3 neurons, connection probability between independent pyramidal cells is close to 20% (Avermann et al., 2012; Ko et al., 2014), which combined with the 10-20% expression of hM3Dq+ cells, may allow the spread of activity to the local network. In comparison, the immature network (P12) may have insufficient (or weak) intracortical connectivity for the spread

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of activity in this manner. One way to test for this would be to inject CNO in hM3Dq+ electroporated mice and then perform *in vivo* calcium imaging of the whole network.

Another possibility is that a chronic enhancement of neuronal activity is directly boosting intracortical connectivity. This would allow for an easier propagation of activity from hM3Dq+ cells firing in response to CNO. How could this be happening in our system? Following the Hebbian axiom ‘fire together, wire together’, it is possible that increasing activity in hM3Dq+ cells causes enhanced connectivity between these cells due to an increase in coincident firing. Similarly, increasing the firing frequency of hM3Dq+ neurons, would increase the probability of coincident firing with spontaneously firing hM3Dq-network cells, ultimately increasing network wide interconnectivity. In fact, generating coincident firing with optogenetic tools has been shown to generate interconnected cortical ensembles in cortex (Carrillo-Reid et al., 2016). Similarly, coincident firing of cortical pyramidal cells is known to underlie functional connectivity in both somatosensory (Kim et al., 2016) and visual cortex (Ko et al., 2011; Cossell et al., 2015). Likewise, decreasing evoked activity in visual cortex by dark rearing leads to decreased intracortical connectivity (Ko et al., 2014). One way to test this hypothesis would be to perform pair recordings of pyramidal cells in hM3Dq-networks before and after CNO treatment and measure their connection probability.

Although it is not possible to conclude whether intracortical connectivity has been affected by increased activity. I can conclude that activity certainly increased in a network wide manner. This was beneficial to this study as it allowed me to investigate the role of network activity in ChC wiring. It also provided an added control for the use of chemogenetic approaches. DREADDs, as all Gq-GPCRs, can activate several molecular cascades within the cell independently of changes in electrical activity (Roth, 2016). This can be a significant confound in studies where results from DREADDs are interpreted solely as consequence of changes in activity. However, the network-wide increase in activity observed for neighbouring pyramidal that do not express DREADDs, provided a condition in which activity had changed without expression of DREADD protein. I was therefore able to more confidently conclude that the forms of plasticity described were likely driven by activity and were not an artefact of DREADD activation.

4.3.2 Heightened neuronal activity leads to a decrease in axo-axonic innervation in periadolescent mice.

Increasing activity in L2/3 pyramidal cells from P12 to P18 led to a 1/3 decrease in axo-axonic innervation. These results were robust, showing similar effects with both morphological and electrophysiological methods. To my knowledge this is the first report of an activity-dependent plasticity of axo-axonic synapses. Nonetheless, questions remain as to the mechanism behind the decrease in axo-axonic innervation.

One possibility is that increased activity delays the development of ChCs. Axo-axonic innervation measures from the 'hM3Dq-network' condition were comparable with those described for immature ChCs (P12-P14) (See chapter 3; P18 hM3Dq-network: 0.095 ± 0.008 syn/ μ m; P12: 0.096 ± 0.013 syn/ μ m). Activity-dependent processes that control neuronal development have been previously described. For example, other types of interneurons have shown that activity can play a fundamental role in the development of several of morphological and functional features. For instance, studies from De Marco Garcia and colleagues (De Marco García et al., 2011, 2015) have shown that thalamic activity is necessary for the appropriate development of neurogliaform interneurons. In this case, decreased activity results in defective development of their dendritic and axonal arbours. Similarly, Dehorter et al., 2015 showed that neonatal activity is necessary to determine the fast-spiking properties of PV interneurons through the expression of specific potassium channels (Dehorter et al., 2015). It is therefore possible that the increased levels of activity generated by the activation of DREADDs, acts to halt any further development of ChC axons, so that their properties remain similar to those observed at P12. An alternative possibility, is that high network activity actively decreases innervation. Under this scenario, axo-axonic synapses form normally, but they subsequently retract in response to high network activity. My experiments could not distinguish between these possibilities. However, one way to tease the two options apart would be to track the development of individual ChCs *in vivo* (see Chapter 3) during increases in network activity. However, this proved very difficult as tracking the same cartridges over consecutive days is challenging, due to displacement of the imaged area under the window as both the brain and the cranium expand during early life. Although this approach was successfully carried out in the previous chapter, the added difficulty of targeting the electroporation to the small area

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under the imaging window (3 mm²), combined with the sparse labelling of ChCs in the *Nkx2.1^{+/−}CreER* line and ChC apoptosis over the third postnatal week, made this experiment very challenging.

In addition to the two options discussed above, there remains another possibility that does not depend on the dynamics ChC synapses, but on the identity of the target pyramidal cell. Recent work from the Huang lab has shown pyramidal cells projecting intracortically received weaker axo-axonic innervation than pyramidal cells projecting to the amygdala. From this study, it is difficult to conclude if the nature of the observed decrease in innervation was due to a decrease in the number of presynaptic axo-axonic boutons, as we see here, or due postsynaptic differences in GABA_AR receptor expression. However, if these observations are in fact due to differences in the number of axo-axonic synapses in each cell type. It seems plausible that the difference in activity levels between these two populations of pyramidal cells, is the signal that ChCs use to determine the axo-axonic innervation that they should receive. Hence, one could speculate that in my experiments the increase in pyramidal cell activity in hM3Dq-networks, mimics the cortical state generated by intracortically projecting pyramidal cells, which signals ChCs to reduce axo-axonic innervation. Future experiments, looking at the activity patterns of different populations of pyramidal cells targeted by ChCs will hopefully shed some light towards this aspect.

Increasing activity in ChCs in a cell-autonomous manner led to the same decrease in ChC output to the one observed by increasing network activity. This raises the possibility that both manipulations are activating the same plasticity programs. This is perhaps not too surprising if one considers ChCs as part of the local network that it also serves to modulate. Increases in network activity would then also drive activity in ChCs, in turn decreasing axo-axonic innervation. In this ‘feedback model’ of plasticity, network activity lies above ChC activity in the cascade of events that lead to a decreased output and relies on ChCs being able to read-out the ongoing activity levels of neighbouring pyramidal cells. Alternatively, it is also possible that both activity manipulations (network vs ChC) may converge on the same phenotype by using different mechanisms. In this situation, network and ChC activity can act as two independent variables that control ChC output. However, it has been suggested that specific types of interneuron plasticity such as homeostatic plasticity happen through network mechanisms and not

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in a cell autonomous manner, supporting a feedback model of ChC plasticity (Hartman et al., 2006). Moreover, as described here (Figure.4.21), the local connections between pyramidal and ChCs do appear to exist, providing some evidence that ChCs are imbedded in their local network and may indeed follow their activity levels. I believe that it is likely that ChCs operate via a “feedback model” that locally controls network activity. To further test this model a possible experiment would be to express inhibitory DREADDs in chandelier cells (e.g hM4Di (Urban and Roth, 2015) within hM3Dq+ electroporated networks. This would lead upon CNO injection to a simultaneous increase in network activity but a decrease in ChC excitability. If the feedback model is correct, ChC under this experimental condition might be unable to read-out the increase in network activity and would not show any plastic changes.

Finally, the changes observed in axo-axonic boutons were almost totally reversed 5 days after the end of the CNO treatment. This suggests that the plasticity of axo-axonic synapses observed here does not constitute the activity-dependent rules that dictate the wiring of ChCs, where changes in activity levels lead to permanent effects in overall connectivity. Such forms of plasticity are observed in classical ocular dominance plasticity (Hensch, 2005), as well as in the development of specific interneuron types such as neurogliaform interneurons (De Marco García et al., 2011, 2015). In this case, ChCs appear to tune their output to ongoing levels of activity of the network during development. There is a possibility that a more prolonged period of increased activity could have led to permanent changes, however this was not tested. The activity manipulations carried out here started at the end of the critical period of L2/3 somatosensory cortex (P10-P14) (Stern et al., 2001; Maravall et al., 2004). It is therefore possible that if CNO treatment started earlier they would have induced permanent changes in connectivity.

4.3.3 Heightened neuronal activity leads to an increase in axo-axonic innervation in adult mice.

Increasing network activity led to the opposite effect in young adults (P40-P46), where I observed an increase in synaptic density and number. These results should be taken with care, as the morphological changes were more subtle than plastic changes during the P12-P18 period. Also, due to time limitations I did not obtain electrophysiological

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corroboration of the synaptic phenotype observed morphologically. Nonetheless the possibility that the plasticity rules of chandelier cells and its synapses change with age is fascinating. The reasons for this difference are not known, but as I will discuss later in this thesis, they may be related to changes in the function of ChC synapses themselves.

Interestingly, I did not observe a change in ChC connection probability in the adult brain. The reasons for this are not known but it is possible that once axonal arbours are fully developed, structural plasticity is more limited, and can only occur at the level of ChC cartridges. More specifically, the lack of plasticity in adult ChC axonal arbours could be related to changes in the perineuonal nets (PNNs) of ChCs. During early adulthood, PNNs develop around neurons stabilising their structure and limiting morphological reorganisation. For example, the development of PNNs with age is thought to limit PV plasticity (Pizzorusso et al., 2002; Spatazza et al., 2013) and neurite outgrowth (Karetko and Skangiel-Kramska, 2009). In future experiments, it would be interesting to see if the disruption of PNNs in adult ChCs could reopen plasticity of the axonal arbour. Indeed, experiments where PNNs were digested in the adult visual cortex reinstated ocular dominance plasticity in a manner similar to developing mice (Pizzorusso et al.).

Finally, to further understand axo-axonic plasticity it would be interesting to know whether it is bidirectional. Future experiments should assess whether decreased levels of activity would lead to opposite effects. This could be done by using inhibitory DREADDs in the network (Urban and Roth, 2015) or by the over expression of the potassium rectifying channel, Kir.2.1 (Hartman et al., 2006; De Marco García et al., 2011 p.20).

4.3.4 Physiological role of axo-axonic plasticity

Several physiological functions could be attributed to the ChC plasticity described in this chapter. A tempting hypothesis is that it is part of a homeostatic form of plasticity. In this scenario, in response to high levels of network activity, ChCs would respond by changing their output to maintain cortical activity levels constant. The switch in polarity of axo-axonic synapses (Rinetti-Vargas et al., 2017) is in line with the switch in the direction of synaptic plasticity and is consistent with a homeostatic role for ChCs in the cortex. In other words, increases in network activity that result in the removal of

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excitatory synapses (as is the case for axo-axonic synapses early in development) or the addition of inhibitory synapses (as in mature axo-axonic synapses) are generally seen as homeostatic forms of plasticity, since they oppose ongoing activity levels. This argument relies on the assumption that ChCs are depolarising in the young brain and hyperpolarising in the adult. This possibility has recently gained ground with a study showing that axo-axonic synapses are depolarising before P21, point after which they switch to a traditional GABAergic hyperpolarising role (Rinetti-Vargas et al., 2017). Moreover, interneurons have been previously shown to be plastic in a network wide manner and in a homeostatic direction (Tien and Kerschensteiner, 2018). Examples of these features have been found in several brain areas. Looking at network vs cell-autonomous interneuron adaptation, a study showed that blocking activity in developing hippocampal neurons in a network-wide manner but not in individual pyramidal cells, led to a reduction in GABAergic innervation (Hartman et al., 2006). Homeostatic mechanisms in inhibitory synapses have also been described in several systems. For example, in visual cortex it has been shown that monocular deprivation leads to a decrease in the number of inhibitory dendritic synapses (van Versendaal et al., 2012; Villa et al., 2016), in what appears to be a homeostatic adaption for a decrease in sensory input. Similar observations have been described in somatosensory cortex. Somatosensory deprivation has been shown to reduce inhibitory input in layer 4 of the cortex (Jiao et al., 2006; Flores and Méndez, 2014). On the contrary, whisker stimulation has been shown to lead to an increase in inhibitory (Knott et al., 2002; Hayama et al., 2013). Generally, this suggests the presence of bidirectional homeostatic plasticity in somatosensory inhibitory systems. Importantly, inhibitory homeostatic plasticity is not exclusive to postsynaptic modifications (e.g GABA_AR expression) but can also be observed presynaptically as in the case of the ChC plasticity described here. In the case of somatosensory cortex, it has been shown that in response to whisker plucking, interneuron boutons disappear (Marik et al., 2010). Similar observations have been made in other brain areas such as visual cortex (Chen et al., 2011; Keck et al., 2011) in response to visual deprivation. In the next chapter I will explore this homeostatic model in more detail by investigating the function of the axo-axonic synapses in the P12-P18 plasticity period.

Interestingly, ChCs in rodent epilepsy models present a decrease in axo-axonic synapses (Dinocourt et al., 2003; Sayin et al., 2003). This could suggest that the

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plasticity observed here might be a pathological consequence of abnormally high levels of brain activity. We did not test for hallmarks of epilepsy. However, I can report that mice under CNO and saline conditions had identical weight and survival rates across groups. Moreover, in the mentioned studies they also report a seizure induced decrease in axo-somatic synapses (basket cell synapses), while we see an increase in periadolescent mice (Sayin et al., 2003). Furthermore, increasing activity directly in isolated ChCs, a manipulation which should have not generated seizures, led to identical effects. Additionally, all plastic changes observed in the P12-P18 period recovered after 5 days, suggesting this was not a terminal effect due to injury.

Finally, to understand the importance ChC plasticity in the wildtype brain, it would be interesting to know if these plastic changes have any effect in cortical function. Several behavioural tasks, such as texture discrimination, are thought to depend on somatosensory cortex (Heimendahl et al., 2007; Diamond et al., 2008; Morita et al., 2011; Garion et al., 2014). In future experiments, it would be interesting to know if the manipulations observed here lead to any detectable phenotype on cortical-driven somatosensory behaviours. Conversely, it would also be important to know whether sensory manipulations could also lead to similar plastic changes. This could be done by using whisker stimulation (Galvez et al., 2009) or trimming (Lendvai et al., 2000) to recreate the effects of enhanced and deprived network activity respectively.

4.3.5 Molecular mechanisms underlying axo-axonic plasticity

The molecular mechanism through which the axo-axonic plasticity observed occurs is unknown. A potential genetic target is ErBB4 or its ligand the cell-adhesion molecule NRG1 (Chung et al., 2016). ErBB4 is exclusively present in inhibitory interneurons of many types, including the PV family (Fazzari et al., 2010; del Pino et al., 2013). It is known that this pathway has critical consequences in ChC development. ErbB4 knockout is known to decrease ChC innervation of nearby AISs (Fazzari et al., 2010). Moreover, its signalling is known to be regulated in an activity-dependent manner (Eilam et al., 1998; Ozaki et al., 2004). In fact, the ErBB4/NGR1 pathways is thought to be the causal mechanism behind basket cell plasticity during ocular dominance plasticity (ODP) (Sun et al., 2016). The reduction in cortical activity due to sensory deprivation in ODP is known to decrease NRG1 signalling, which in turns leads to a

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decrease in the strength of the connection between pyramidal cells and PV interneurons. This when put together with the results in Fazzari et al., 2010, suggest that the ODP activity-dependent decrease in NRG1 signalling, might also lead to a decrease in axo-axonic and axo-somatic synapse density. This would explain the observations I made on basket cell plasticity and ChC plasticity in the adult. Nonetheless, at a first glance, this would also imply an increase in NRG1 signalling and axo-axonic synapses in response to high ChC activity, which is the contrary to what I observed. However, we now know that NRG1/ErbB4 appears to behave in a state dependent manner throughout development, varying its function depending on the age of the animal (Sun et al., 2016). It is therefore plausible this pathway might regulate axo-axonic plasticity in ChCs in somatosensory cortex in a compatible manner to the results presented here.

Another possibility is that changes in GABA neurotransmission underlie axo-axonic plasticity. Previous reports have shown that neurotransmission plays a critical role in the maturation of GABAergic synapses. If GABAergic release is impaired, GABAergic synapses fail to mature in developing interneurons (Huang, 2009; Lin et al., 2018). Similarly, GABA release alone is sufficient for the formation of somatostatin GABAergic synapses onto dendrites (Oh et al., 2016). In our experiments, increases in network activity should lead to an increase in total GABA release over time, however this did not translate into an increase in axo-axonic synapse formation. It is possible that the role of neurotransmission in synapse stabilisation saturates with minimal levels of GABA release, with increasing levels of neurotransmission not necessarily leading to the formation of more synapses. This would consequently leave space for other plastic mechanisms such as the one shown in this thesis.

4.3.6 Plasticity in basket cells

In this chapter I also showed that basket cells showed an opposite direction in the plasticity of their axons in comparison to ChCs, increasing the number of synapses onto a pyramidal neuron in response to high network activity. Basket cells are thought to be hyperpolarising at this age (Ben-Ari et al., 2007) and therefore this could also constitute a homeostatic plasticity mechanism. In fact, previous studies support a homeostatic role for basket cell activity-dependent plasticity. For example, it has been

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shown that neuronal activity blockade with tetrodotoxin (TTX) in neocortical *in vitro* cultures leads to a decrease in basket cell connectivity and IPSC amplitude. (Bartley et al., 2008). Likewise, in a different study, it was shown that in the dentate gyrus, an increase in excitatory drive leads to a homeostatic strengthening of basket cell axo-somatic synapses onto granule cells (Pieraut et al., 2014).

How are activity related changes leading to structural plasticity? A potential molecular pathway that may underlie the plasticity observed in basket cells also involves the NRG1/ERBB4 pathway (Sun et al., 2016) (see previous section 4.3.5). Another potential pathway involves the transcription factor Npas4, which is thought to regulate the formation of axo-somatic inhibitory synapses. Its expression has been shown to be regulated in an activity-dependent manner and could therefore underlie many of the plastic changes presented here (Bloodgood et al., 2013).

Finally, a main limitation of my assessment of basket cell plasticity should be noted. Since basket cell plasticity was not the focus of this study, I recorded spontaneous events received by pyramidal cells as a correlate of synapse number. However, even though most inhibitory events recorded at the soma of a pyramidal cell likely originate from basket cells (due to their high number (Rudy et al., 2011) and proximity to the recording pipette) the firing of somatostatin and other dendritic targeting interneurons will also affect this measure. Paired recordings of basket and pyramidal cells would have given a more accurate account of the change in the strength of basket cell axo-somatic synapses. Similarly, I cannot discard the possibility that a change in the firing frequency of interneurons and not an increase in synapses is underlying the significant increase in sIPSC frequency. The measure of miniature IPSC (mIPSC) by blocking all activity with TTX would have been a better correlate of perisomatic GABAergic innervation.

4.3.7 AIS Plasticity

In this chapter I also assessed if changes in network activity affected AIS length. In the past decade several studies have shown that the AIS is a plastic structure that can change in length and position, leading to changes in the intrinsic excitability of the

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neuron (Grubb and Burrone, 2010; Kuba et al., 2010; Wefelmeyer et al., 2015). In response to high network activity from P12-P18 we observed a discrete decrease in AIS length, in what could be a homeostatic mechanism. This change appeared to be cell-autonomous, depending entirely on the activity levels of each cell. A network wide effect could only be observed in brains with a high concentration of DREADDs responsive cells, in which it is likely that activity was increased in all neurons. This decrease in AIS size was correlated with a decrease in intrinsic excitability. Previous studies have corroborated the same observation. For example, a study in the pallium of the chicken, the avian homolog of the mammalian neocortex, showed the same kind of plasticity. In response to auditory sensory deprivation, the AIS increased in size and increase in excitability (Kuba et al., 2010). Similarly, heightened neuronal activity in dentate granule cells is known to decrease AIS length (Evans et al., 2015), leading to a decrease in excitability. Nevertheless, it is important to note that even though theory tell us that changes in AIS morphology should carry changes in intrinsic excitability, it is not possible to proof that the decrease in AIS length observed here is the casual link for the decrease in excitability observed. Many other homeostatic mechanism which did not assess may be working in parallel to decrease excitability (For review: (Turrigiano, 2011)).

Interestingly, after the recovery period of 5 days, AIS size not only recovered but significantly increased in length. The reasons behind this effect are unknown. One possibility is that during the CNO treatment period, other homeostatic mechanisms come into play to reduce network excitability. These mechanisms could have a more permanent or a slower response than AIS plasticity. This could lead to an abnormal decrease in baseline neuronal activity during the recovery period, which in turn leads to a homeostatic increase in AIS length. In fact, AIS length is thought to change in a timescale of hours (Dumitrescu et al., 2016), being much faster than other homeostatic mechanisms such as synaptic scaling (Wefelmeyer et al., 2016). *In vivo* recordings to measure network activity before and after CNO treatment could shed some light into this unexpected result.

When increasing activity in the adult I obtained inconclusive results on AIS length. There was a network wide increase in AIS length in hM3Dq activated networks in comparison to the CNO control but not the saline control. Based on these inconclusive

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results it is not possible to determine whether the AIS is plastic at this age. So far, all studies looking at AIS plasticity have been done in the developing brain, and it is therefore unknown whether this also happens in mature networks (Grubb and Burrone, 2010; Kuba et al., 2010; Evans et al., 2015). It is likely that this type of plasticity is much more restricted in the adult than during development, as in mature neurons the presence of a mature extracellular matrix around the AIS may limit the AIS' plastic capacity (John et al., 2006). Similarly, due to the lack evidence of AIS plasticity in the adult, it is not possible to conclude whether this would also be homeostatic. Nonetheless, anatomical evidence from adult rodent neuronal from a traumatic injury (Baalman et al., 2013) or in a genetic epilepsy model (Harty et al., 2013) show a shortening or distal movement of the AIS in what is thought to be a homeostatic mechanism. However, whether this homeostatic plasticity event is unique to injury or it generalises to networks in the healthy brain is currently unknown. Since this study was focused on the assessment of axo-axonic plasticity, I did not perform an electrophysiological assessment of intrinsic excitability in pyramidal cells following CNO treatment in the adult. This information would however be very useful to confirm whether the inconclusive changes in morphology observed here carried any functional effect.

4.3.8 Concluding remarks

In conclusion, in this chapter I showed for the first-time structural plasticity in axo-axonic synapses in the periadolescent and adult brain. In response to high levels of network activity or high activity in chandelier cells, the number of axo-axonic synapses decreased in the periadolescent brain but increased in the adult brain. This potentially constitutes a unique switch in structural plasticity. In parallel, I also described homeostatic changes in AIS length and basket cell innervation of pyramidal cells. Is axo-axonic plasticity also homeostatic? In the next chapter I will explore the function of the axo-axonic synapse during the periadolescent period to shed some light into this question.

Chapter 5 : Function of axo-axonic synapses in the periadolescent brain

5.1 Introduction

In the previous chapter I described a synaptic plasticity mechanism whereby high levels of cortical activity result in a decrease in the number of cortical axo-axonic synapses formed onto pyramidal neurons in the mouse periadolescent brain (P12-P18). This effect could have numerous consequences on pyramidal cell output depending on the function of GABA at the AIS during this period.

As mentioned in my introduction, the polarity of GABA, whether it is hyperpolarising or depolarising, depends on its reversal potential (E_{GABA}). This is set primarily by the equilibrium potential of Cl^- (E_{Cl}), since this is the main ion carried by GABA receptors across the membrane. E_{Cl} depends on the concentration of Cl^- inside ($[\text{Cl}^-]_{\text{in}}$) and outside ($[\text{Cl}^-]_{\text{out}}$) of the neuron. This is set by various mechanisms including the Cl^- transporters (Kaila et al., 2014) and local impermeant anions in the cell cytoplasm (Glykys et al., 2014). In adult mice E_{Cl} is thought to be hyperpolarising in most occasions. However, there has been a heated debate on whether E_{Cl} might be depolarising at the axon including the AIS, which would make axo-axonic synapses depolarising (Szabadics et al., 2006; Khirug et al., 2008; Glickfeld et al., 2009; Woodruff et al., 2011). More recently, by

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focusing on the developmental time-course of changes in E_{Cl} values along different subcellular compartments, Rinetti-Vargas et al. (2017) managed to bring together many of the discrepancies from previous studies. They showed that whereas GABAergic synapses on the somato-dendritic compartment switch polarity within the second postnatal week, those on the AIS remain depolarising well into the third postnatal week, providing a temporal window over which different interneurons could potentially drive pyramidal cell activity in opposite directions.

Based on these observations, the axo-axonic and basket cell plasticity described in Chapter 4 would follow a homeostatic direction. In the periadolescent brain an increase in network activity leads to a decrease in axo-axonic synaptic output, which carries a decrease in depolarising drive. Similarly, at the soma where GABA is already hyperpolarising, an increase in network activity leads to an increase in basket cell synaptic output and a consequent increase in inhibitory drive. However, a model such as this one has not yet been replicated in different labs or with alternative methods. Similarly, Rinetti-Vargas and colleagues (Rinetti-Vargas et al., 2017) measured E_{GABA} at the AIS through the local iontophoretic delivery of GABA, but did not measure the direct effect of ChC activation. This information is needed to better understand cortical function in the developing brain and to put plastic mechanisms such as the ones described above into a functional context. Moreover, most studies looking at GABA polarity tend to use perforated gramicidin patches to gain electrical access but avoid the dialysis of the cell's intracellular milieu (see Chapter 1 for more details). However, newly developed genetically-encoded-voltage-indicators (GEVIs) which can report subthreshold voltage changes (Hoppa et al., 2014; St-Pierre et al., 2014; Gong et al., 2015) could be used for answering similar questions less invasively and with higher throughput. Nonetheless, GEVIs have so far not been used towards these ends.

In this chapter I set out to examine the role of axo-axonic synapses in layer 2/3 of the periadolescent cortex with three objectives:

- 1) To test the use of GEVIs to investigate subthreshold GABA responses.
- 2) To determine the polarity of GABA at the AIS and soma by voltage imaging.
- 3) To establish the polarity of axo-axonic synaptic events at the AIS.

5.2 Results

5.2.1 Using GEVIs to measure subthreshold postsynaptic potentials (PSPs)

Investigating GABAergic polarity is challenging as the researcher must ensure the intracellular milieu of the neuron (mostly Cl^- ions) is preserved as this will determine E_{GABA} . This makes traditional electrophysiological techniques such as whole-cell patch clamp unusable. To circumvent this, studies often rely on the use perforated gramicidin patches, which allows electrophysiological access through small pores in the membrane that are impermeable to anions, but which will maintain Cl^- concentrations within the cell unaffected. Although extremely useful, the perforated-patch technique suffers from many drawbacks, including poor access resistance and the possibility that the intracellular milieu may have been compromised, even transiently, during the recording and therefore affecting intracellular Cl^- levels. In addition, perforated patches are generally low-throughput in comparison to other patch-clamp configurations.

To avoid these problems, I used an optical approach for the measurement of subthreshold GABAergic PSPs, by making use of genetically encoded voltage indicators (GEVIs). This approach allows for non-invasive, easily scalable measurements of voltage. I used the ACE2N-mNeon-4AA (ACE-mNeon) a GEVI that offers optimal signal-to-noise ratio and fast kinetics (Gong et al., 2015) (Figure 5.1.B-C). In fact, an in depth characterisation by Victoria Gonzalez Sabater in the lab (Gonzalez-Sabater, 2018, *in preparation*) has shown that this indicator is the brightest and has the best signal to noise of the currently available probes. ACE-mNeon combines the archeorhodopsin (ACE) with the fluorescent protein mNeon, to allow voltage-sensitive fluorescence resonance energy transfer (FRET) (Zou et al., 2014). In response to a membrane voltage depolarisation, protonation of a Schiff base which links retinal chromophore to the protein core in ACE results in a change in its absorbance that allows the non-radiative quenching of mNeon. As a result, depolarisations result in a decrease in mNeon fluorescence, whereas hyperpolarisations are seen as an increase fluorescence (Figure 5.1.C).

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It should be noted that although targeting of this GEVI to the plasma membrane is better than others, it is also expressed in some internal membranes. Therefore, not all baseline fluorescence will provide a signal that reflects changes in membrane voltage. This makes $\Delta F/F$ comparisons based on amplitude across cells invalid without further calibration, since the fraction of fluorescence responsive to voltage will vary from cell to cell and compartment to compartment. Nonetheless, this does not affect the experiments carried out here since we are solely interested in determining the polarity of GABA PSPs, not the amplitude.

I delivered ACE-mNeon to pyramidal cells in layer 2/3 of cortex via in utero electroporation at E15.5 as described in Chapter 4 in the *Nkx2.1/tdtomato* mice. I then prepared acute slices at P14-P19 and performed wide-field imaging of Ace-mNeon responses using an EMCCD camera and a 488nm LED illumination (Figure 5.1.A). Images were acquired at 100Hz, a sampling frequency that is sufficiently fast to catch the comparably slow GABAergic synaptic events measured by electrophysiology.

To correct for bleaching of the probe and filtering noise (Figure 5.2), first, the raw fluorescence traces (excluding the stimulation time-points) were fitted with a double exponential that accurately accounted for the bleaching of the probe (Figure 5.2.A). This generated a bleach-corrected trace with a stable baseline from which I measured ΔF (Figure 5.2.B). I then averaged the bleach-corrected traces obtained under the same stimulation conditions (Figure 5.2.C). Finally, the resulting trace was passed through a 1-D digital filter to reduce camera noise and $\Delta F/F$ was calculated. Resting F was calculated as the average fluorescent for 10 frames before the event (aprox. 82ms) (Figure 5.2.D)

In the first experiment, I tested the reliability of ACE-mNeon for measuring subthreshold responses in acute slices by patching ACE-mNeon expressing pyramidal neurons and comparing electrophysiological and fluorometric recordings.

I patched ACE-mNeon+ cells in voltage-clamp mode, holding the cell at -70 mV and performed an I-V protocol with incremental steps of 5 mV, from -90 mV to -45 mV (Figure 5.3.A). Voltage steps were 50 ms in duration, which resembles the duration of typical GABAergic PSPs. Simultaneously, I imaged ACE-mNeon at the soma of the

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patched cell and repeated each voltage step 3 times to obtain an averaged fluorescence trace. The IV responses shown, shows that even the smallest 5mV steps were easily distinguishable from above the noise, despite the small changes in fluorescence observed for each step ($\Delta F/F$ $0.2 \pm 0.02\%$, mean $\pm$ S.E.M) (Figure 5.3.B). I also observed that the voltage steps generated linear changes in fluorescence (Figure 5.3.C) ($R^2 = 0.9944$, $P = <0.0001$).

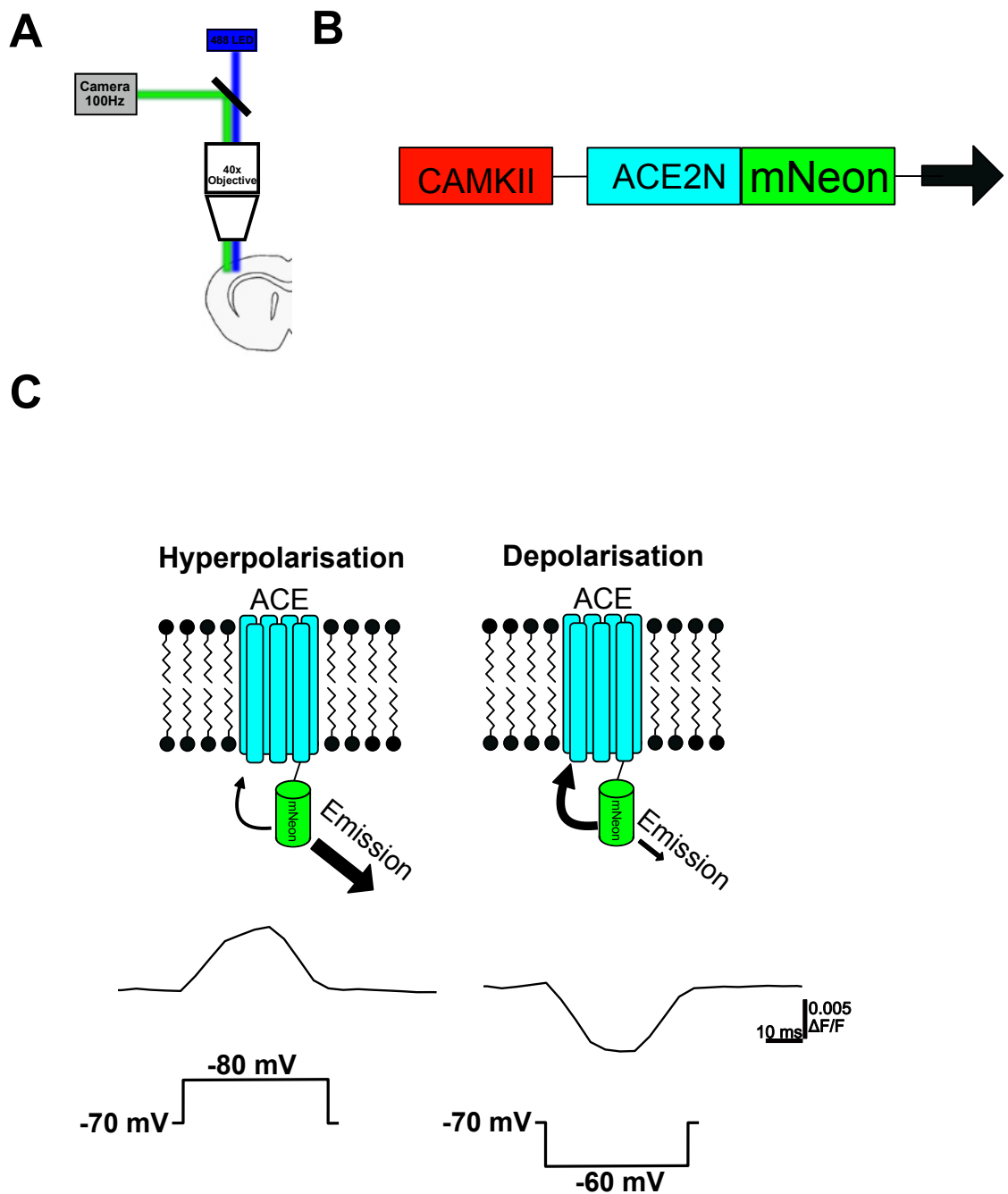


Figure 5.1 Voltage imaging using the genetically encoded voltage indicator ACE-mNeon. (A) The LED emits at 488nm it excites ACE2N-mNeon in the slice. Green photons are emitted which are then directed by a dichroic mirror into EMCCD camera acquiring at 100Hz (B) Plasmid used for ACE2N-mNeon with a neuron specific CAMKII promoter (C) ACE2N-mNeon is a FRET based voltage indicator where ACE works as an acceptor (blue) and its linked to a donor mNeon (green). Hyperpolarisations lead to the emission of electrons from mNeon while depolarisation leads to a transfer of electrons to ACE and a corresponding decrease in mNeon fluorescence.

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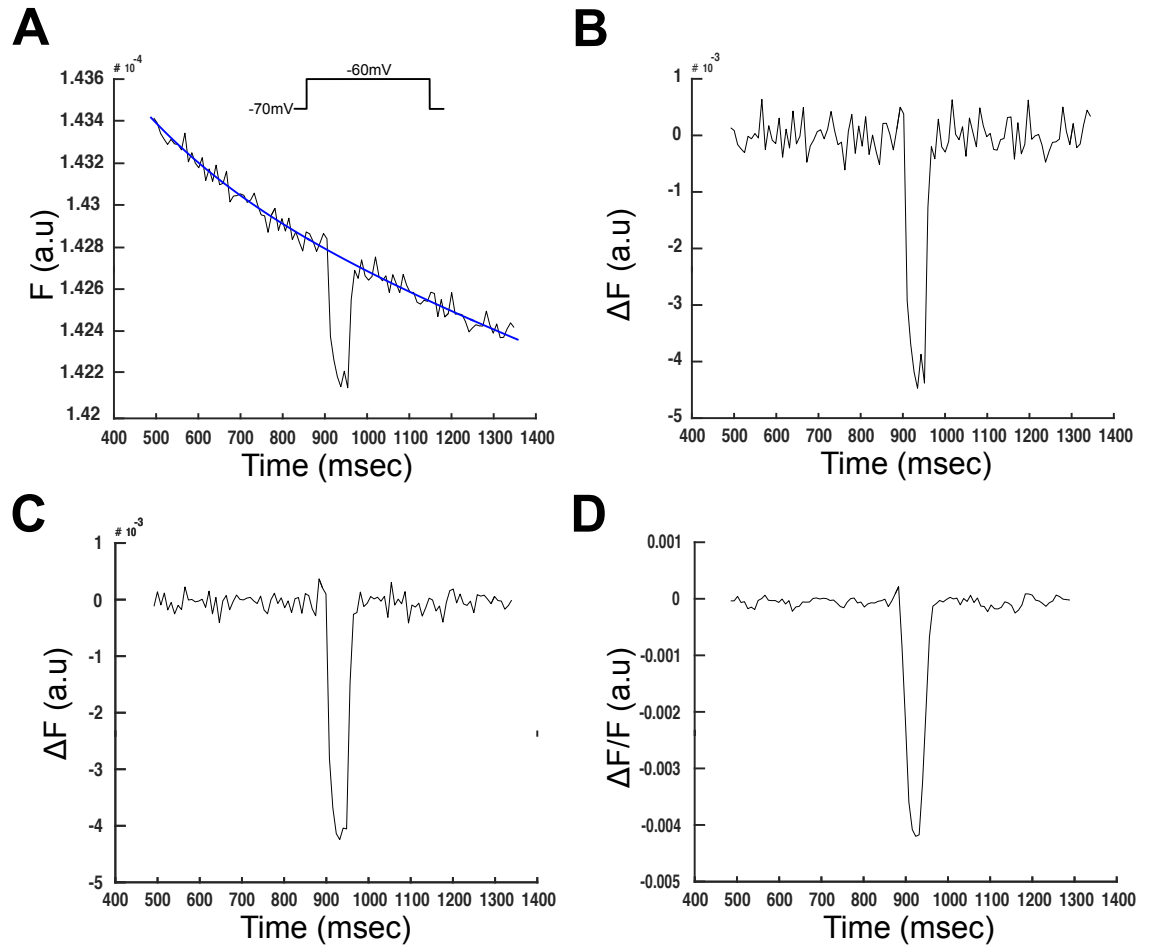


Figure 5.2 Post-hoc processing of voltage imaging traces. (A) Raw fluorescence trace for a voltage step of +10mV. Blue trace, double exponential fit. **(B)** ΔF Bleach corrected fluorescence trace. **(C)** $\Delta F/F$ averaged trace over 3 repeats. **(D)** $\Delta F/F$ filtered trace with a 1-D digital filter.

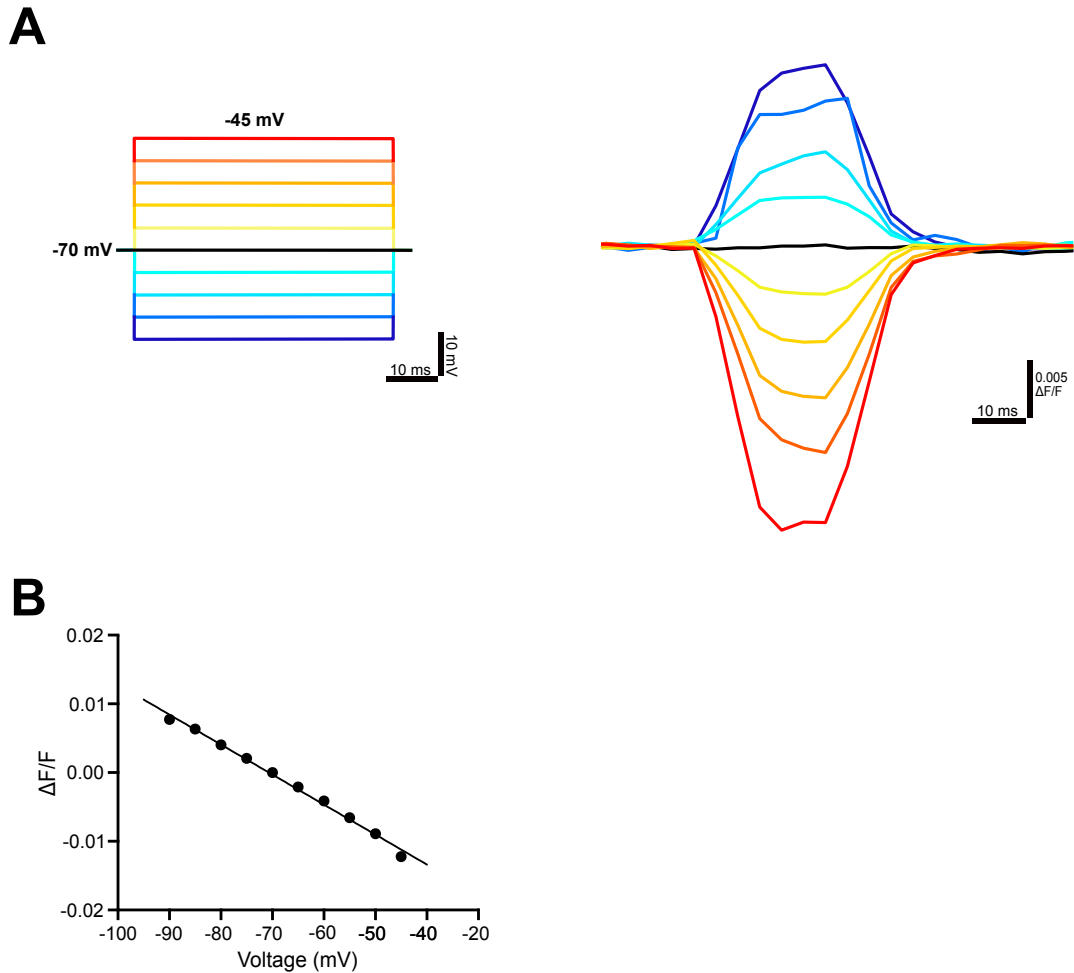


Figure 5.3 ACE-mNeon accurately reports small changes in voltage in acute slices. **(A)** Representative example fluorometric trace for voltage steps in a cell expressing ACE-mNeon. Red traces represent depolarising voltage steps. Blue traces represent hyperpolarising voltage steps. **(B)** $\Delta F/F$ of the maximum response (steady state) as a function of membrane voltage in the representative cell. Black line, linear regression. Average over 3 repeats.

5.2.2 Depolarising and hyperpolarising GABA in the periadolescent cortex

Once I had established that ACE-mNeon had sufficient resolution for resolving small subthreshold PSPs, I proceeded to determine GABAergic polarity at the AIS and soma at the age we observed axo-axonic plasticity (P12-P18).

I prepared acute slices from C57/BL6 mice aged P14-16 that had been electroporated with ACE-mNeon at E15.5. Pyramidal cells in layer 2/3 of both prefrontal and somatosensory cortex were electroporated to maximise the number of electroporated cells. I then proceeded to place the iontophoresis pipette juxtaposed to either the soma or the AIS (Figure 5.4.A-C) of ACE-mNeon+ cells. This pipette contained a solution containing GABA (200mM) and CGP55845, a GABA_B receptor antagonist. CGP55845 was also present in the bath to avoid the activation of extra-synaptic GABA_B receptors (Rinetti-Vargas et al., 2017). To ensure that our signal was not affected by secondary activation of pyramidal cells through disinhibition or ongoing glutamatergic activity, the external ACSF (see chapter 2 for details) contained the NMDA receptor blocker APV and the AMPA receptor blocker NBQX. I ejected GABA from the pipette by giving a 150nA pulse for 20ms. We regularly saw a small artifact that in the voltage imaging responses that coincided with the GABA delivery pulse from the iontophoresis pipette (Figure 5.4.C). A typical response lasted >1.5s and its amplitude at the peak (steady state) varied from 0.02 to 0.08 ($\Delta F/F$) (Figure 5.4.B-C).

I classified the GABA evoked PSPs into hyperpolarising (maximum amplitude > 3x baseline standard deviation), depolarising (maximum amplitude < 3x baseline standard deviation) or putative shunting (maximum amplitude within 3x baseline standard deviation). The results showed that responses tended to be depolarising at the AIS (4/6) and shunting or hyperpolarising at the soma (4/6) (Figure 5.4.E). Similarly, when averaging all the recordings, the AIS and soma presented depolarising and hyperpolarising PSPs, respectively (Figure 5.4.D). When looking at within cell differences between soma and AIS, out of the 5 cells recorded at both sites, 3/5 showed a PSP in the same direction in both compartments (both hyperpolarising/depolarising). Also, in 3/5 cases, events at the soma were more hyperpolarising than at the AIS. (Figure 5.4.D). I then tested whether these responses

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were GABA_AR mediated and not due to a stimulation artefact. For this, I first released GABA at the AIS (Figure 5.5.A) as in the previous section, which generated a strong depolarisation (Figure 5.5.B). I then added the GABA_AR blocker gabazine (SR-95531) to the bath, waited for a 5min incubation and repeated the experiment. The application of gabazine lead to a complete eradication of the evoked GABA depolarisation (Figure 5.5.C), confirming that the observed depolarisations at the AIS were mediated by GABA_AR. Thus, I concluded that GABA mediated depolarisations were possible and more common at the AIS than at the soma in the periadolescent brain.

It should be noted that for the analysis of iontophoresis traces, a different signal processing algorithm was used to the one described in the previous section. This was necessary due to the long GABA responses (>1.5s). When fitting a double exponential function for bleach correction, the points in which the evoked event happens need to be ignored to avoid overfitting artifacts. However, if the event takes a large percentage of the total recording time, as is the case for long GABA responses, the fit will suffer from underfitting (i.e poor fitting of bleaching), making this approach undesirable. This can be solved by leaving a proportional longer baseline after the end of the GABA event. However, this would increase bleaching, which needs to be avoided for voltage imaging. As an alternative, I intercalated stimulation trials, where GABA is applied (Figure 5.6.A), with no-stimulation trials, where images are acquired but no GABA is applied (Figure 5.6.B). The bleaching correction fit could then be trained from the no-stimulation trials (Figure 5.6.C). This approach proved very effective and better than the direct subtraction of not-stimulated trials from stimulated trials which does not account for trial-trial difference in fluorescence levels due to bleaching (e.g Figure 5.6.D). As it can be observed in Figure 5.6.C, the algorithm eliminated effect of bleaching as shown by the similar $\Delta F/F$ value before and after the stimuli. It should be noted that after removing the effect of bleaching, a short hyperpolarising phase preceding the long depolarising phase could be observed in some occasions (e.g Figure 5.6.C). The potential reasons for this feature are discussed in section 5.5.2.

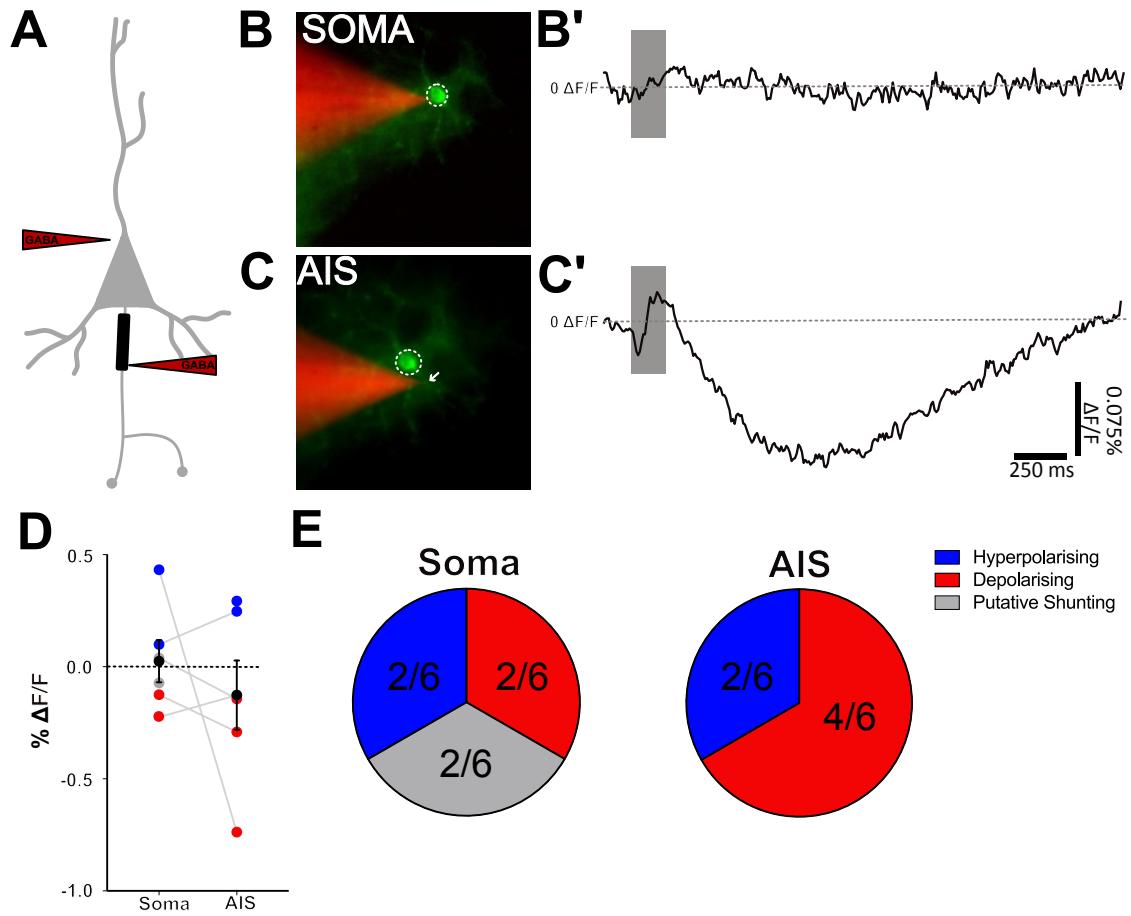


Figure 5.4 GABA evoked depolarisations at both soma and AIS in the periadolescent cortex. (A) Iontophoresis pipettes containing GABA 200 mM were placed in cells expressing ACE-mNeon at either the AIS or soma (B-C) GABA evoked responses at the soma and AIS. Left, images showing cells expressing ACE-mNeon in green and the iontophoresis pipette in red. Dashed circle indicates the ROI from which the fluorescence was measured. White arrow points at the position of the AIS. (B'-C') $\Delta F/F$ signals from cells in (B-C). Artifact generated by the ejecting pulse in grey. (D) Steady state response for all the cells at soma and AIS. Red are depolarising, blue hyperpolarising and gray putative shunting. When recordings were made at both soma and AIS dots are joined by a gray line. In black the average $\Delta F/F$ signal of all cells (mean $\pm$ S.E.M.). (E) Pie chart showing the classification of all recordings at soma and AIS. n= 6 neurons, n = 2 mice.

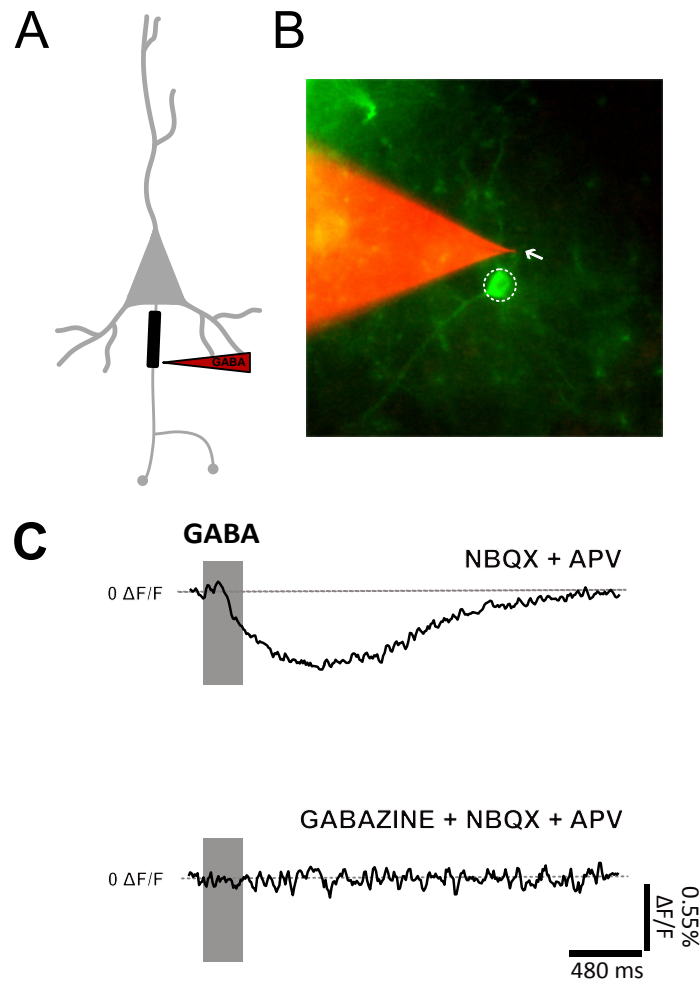


Figure 5.5 GABA evoked depolarisations are blocked by gabazine. (A) An iontophoresis pipette was placed next to the AIS (B) ACE-mNEON+ cell (green) with iontophoresis pipette (red). Dashed circle indicates the ROI from which the fluorescence was measured. White arrow points at the AIS of the imaged cell. (C) GABA evoked response before and after the addition of gabazine.

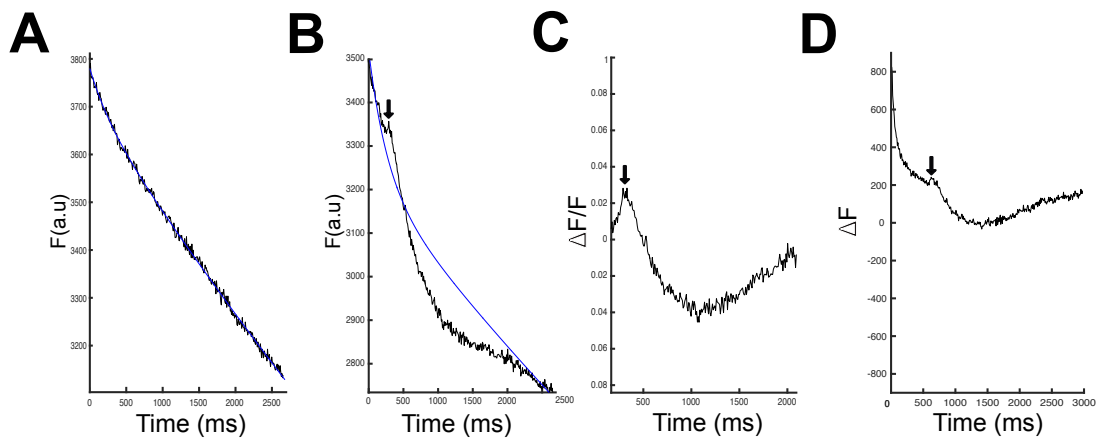


Figure 5.6 Processing of voltage imaging traces with long PSPs. (A,B) Raw fluorescence traces from no-stimulated and stimulated trials, respectively. Blue line, double exponential fit with constants obtained from (A) **(C)** $\Delta F/F$ Bleach corrected fluorescence trace resulting from (B). **(D)** ΔF Trace resulting from the direct subtraction of the raw traces in A and B. Black arrow indicates start of GABAergic event.

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5.2.3 Chandelier cell evoke GABAergic depolarisations in the periadolescent brain.

After establishing in the previous section that the E_{Cl} at the AIS of periadolescent mice could be depolarised with respect to its resting membrane potential, I next tested if the direct activation of a chandelier cell could also lead to depolarisations in pyramidal cells. This was important for two reasons: (1) Our iontophoresis experiments released a large amount of GABA than may diffuse to areas outside of the AIS and (2) we could not exclude the possibility that GABA was acting in regions of the AIS where no axo-axonic synapses are present but still contain GABA_A receptors.

I electroporated ACE-mNeon in Nkx2.1/tdTomato mice and prepared acute slices at age P15-P19. I patched chandeliers in the electroporated areas (somatosensory and prefrontal cortex), where the axonal arbours of the chandeliers were clearly overlapping the electroporated cells (Figure 5.7.A). NBQX and APV were present in the bath to block glutamatergic neurotransmission as in the iontophoresis experiments. To increase our chances of detecting a synaptic event, I delivered a train of 5 action potentials at 50Hz (Figure 5.7.B). Although I could often see several ACE-mNeon+ cells within the reach of an individual ChC axon (Figure 5.7.C), these were often in different Z planes and could not be imaged simultaneously.

When classifying the responses, I used the same classification system as in the previous section (depolarising, hyperpolarising or putative shunting). I classified an event as significantly depolarising or hyperpolarising if its peak during the stimulation period was larger than 3x the standard deviation of the baseline in either direction (e.g Figure 5.7.D). This is traditionally considered in normally distributed samples to be equivalent to $P < 0.01$. Classifying responses as putative shunting synapses was inaccurate here as I could not guarantee that a cell that did not show a significant PSP was connected to the ChC that was being stimulated. Therefore, non-significant potentials were classified as not-connected/putative shunting (Figure 5.7.E).

The results showed that chandelier cells could elicit depolarising PSPs that were timelocked to the stimulation (e.g Figure 5.7.D). The evoked depolarising PSPs lasted

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50-100ms, as expected for an individual GABAergic IPSP at the AIS (see chapters 3 and 4).

Out of the 22 cells imaged, we only detected measurable responses in 5 cells. Importantly, all 5 of these responses were depolarising. The remaining 22 traces showed no detectable response. As mentioned previously, this might be due to the lack of a connection with the stimulated chandelier or to a shunting synapse where E_{GABA} is the same as the cell resting membrane potential. Given the high connectivity of chandelier cells at this age, even after the slicing procedure (Chapter 4: 52.8%), we could infer that near half of the 22 cells were shunting (Figure 5.7.E).

Based on these results I concluded that ChCs can be depolarising during the P12-P18 period, suggesting that the plasticity described in Chapter 4 might follow a homeostatic direction.

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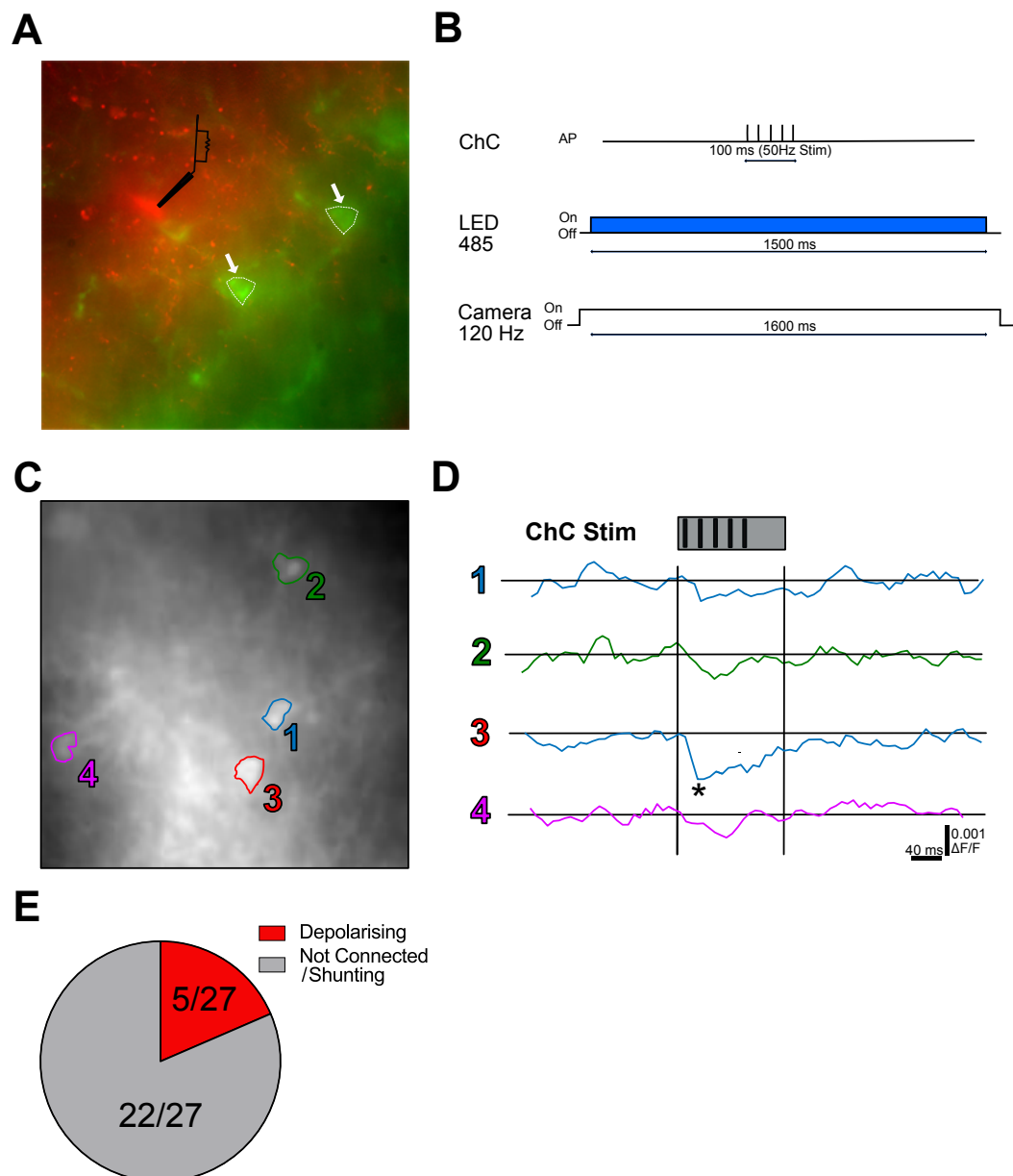


Figure 5.7 Chandelier cell activity can lead to GABAergic depolarisations in the periadolescent cortex. **(A)** Widefield image of patched chandelier in chandelier in red, ACE-mNeon+ cells in green. **(B)** Stimulation and Imaging protocol. First line, Chandelier cell (ChC) stimulation train. Vertical lines represent 1 action potential (1 AP). 2nd line, LED stimulation time. 3rd line, camera acquisition rate and time. **(C)** 4x4 binning image of the imaged ACE-mNeon cells which allowed 120Hz imaging. **(D)** Example voltage imaging measurements. Numbers correspond to measurements from ROIs with the same number in (C). Top line indicates, ChC stimulation protocol, 1 vertical line denotes 1 AP. (*) denotes timelocked event 3x > baseline standard deviation. **(E)** Pie chart showing the classification of responses elicited by ChC firing. Red, depolarising. Gray, Putative shunting/ not connected. Blue, hyperpolarising (no events found of this type). n = 27 neurons n = 5 mice.

5.5 Discussion

In this chapter I present a novel way to look at GABAergic polarity by using the newly developed GEVI, ACE-mNeon. First, I showed that I could use this indicator in acute slices to measure subthreshold PSPs. ACE-mNeon could detect changes in voltage as small as 5mV in either a hyperpolarising or depolarising direction. It presented linear responses with increased or decreased voltage, which made within-cell, but not across-cells, comparisons of amplitude possible. Secondly, I used ACE-mNeon to show that GABA was mostly depolarising at the AIS of P12-P18 mice. In comparison, I also showed that at the soma, GABA generated potentials were more varied and tended to be either hyperpolarising or putative shunting during the same period. Finally, I present evidence that the direct activity of chandelier cells could elicit evoked depolarising PSPs in periadolescent mice (<P20). This supports a depolarising role for axo-axonic synapses in the early postnatal brain and suggests a homeostatic role for the axo-axonic plasticity described in chapter 4.

5.5.1 Using ACE-mNeon for measuring GABA polarity

In this chapter, I introduce the use of ACE-mNeon for the measurement of GABA polarity. I first show that it responds linearly and with enough resolution to resolve GABA PSPs in acute slices. I also observed that the ACE-mNeon signal in slices is bright enough to analyse region specific voltage changes in small compartments such as dendrites or the AIS (e.g Figure.5.5.B). To my knowledge this is the first time GEVIs have been used to study GABAergic synaptic polarity and it provides many advantages. In comparison to perforated patches, this approach is considerably more reliable and transferable to *in vivo* preparations. This method could be used for testing GABA polarity in the *awake* mammalian brain during adulthood and development, a very much needed piece of evidence in the field.

However, an important limitation of the probe should be noted. As introduced earlier, this protein is expressed in all types of membranes within neurons, therefore the baseline fluorescence is affected by the fluorescence of non-excitabile membranes such those of organelles. Due to this, comparisons across cells are not possible as the baseline

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fluorescence from different cells will vary depending on the efficiency of targeting to the plasma membrane from one cell to the next, or the presence of different organelles, especially if measuring voltage from a subcellular portion of the cell. Across-cell comparisons of amplitude would require a voltage calibration curve to convert ΔF into voltage for each cell. However, since the probe was linear over the physiological range tested, a potential solution would be to bring the cells to a known voltage, measure the fluorescence signal at that voltage, and normalise other values to that signal. This has been done *in vitro* before with other GEVIs by perfusing the antibiotic gramicidin into the incubation chamber, which raises the resting membrane potential of all neurons to 0 mV (Hoppa et al., 2014).

5.5.2 Depolarising GABA

In section 5.3, I show that GABA tended to have a depolarising effect at the AIS of layer2/3 pyramidal cells during the third postnatal week. This is in agreement with a previous report (Rinetti-Vargas et al., 2017), where they show that on average the AIS has a depolarising E_{GABA} at this age due to an increased expression of NKCC1 transporter in this compartment. As in their study, in our experiments, we also observed that this response was not exclusively depolarising and that in 2/6 cells a hyperpolarising response was also observed. In contrast to their observations, we found two occasions in which GABA was depolarising at the soma. A potential reason behind this difference is that the study by Rinetti-Vargas (2017) do not directly measure E_{GABA} at the soma, but rather proximal dendritic compartment. Therefore, it is possible that this disparity could be due to unknown differences in the E_{Cl} of the soma and dendrites. It is also possible that some GABA, when applied by iontophoresis at the soma, diffused from the iontophoresis pipette onto the AIS. This contaminated signal is more likely to arise in our experiments than those of Rinetti-Vargas et al., since the dendrites they chose were always directly opposite to the AIS and therefore further away from it. However, we were particularly interested in measuring polarity of GABAergic synapses at the soma directly, since this is the compartment targeted by basket cells, which shows a form of plasticity that is opposite to that observed in axo-axonic synapses. Finally, there is a possibility that E_{GABA} might still be depolarising at the soma in a minority of neurons in the P14-P16 period. The general notion is that the GABA-switch occurs around P10 in mice (Ben-Ari et al., 2007), however, there could be a small fraction of pyramidal cells

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where the switch from depolarising to hyperpolarising somatic GABA has not yet happened by P14-P16.

In many of the iontophoresis experiments a short byphasic voltage deflection could be observed (e.g Fig 5.4.C') immediately after GABA application and prior to the long (>1.5s) hyperpolarising/depolarising step. These rapid voltage responses were considered artifacts generated by the current applied for the ejection of GABA from the iontophoresis pipette. However, it is also possible that these responses (when followed by a long depolarisation) are not artefactual but actual GABA hyperpolarisations, which triggered potassium mediated long depolarisations (Voipio and Kaila, 2000). This possibility could lead to the mislabeling of K⁺ mediated depolarisations as GABA mediated synaptic depolarisations, confounding the results presented here. Nonetheless, the absence of this byphasic response in the ChC evoked synaptic depolarising potentials (Figure 5.7) and in some iontophoresis responses of similar length (e.g Figure 5.5), suggests that the presence of GABAergic depolarisations at the AIS cannot be entirely explained by this possibility.

It is also important to note that a recent study (Valeeva et al., 2016) has suggested that depolarising GABA might be an artifact of slice recordings. In this study, they record sEPSPs from neocortical pyramidal cells *in vivo* while stimulating interneurons optogenetically. They observe that at all ages sEPSPs frequency is reduced in response to interneuron stimulation, suggesting an inhibitory role of GABA. When they perform the same type of recording in acute slices, EPSP frequency is increased in young animals (<P10) and reduced in adult mice, supporting the developmental depolarising GABA hypothesis. One caveat of the *in vivo* recordings in this study was that they were performed in anaesthetised animals where ongoing cortical activity might have been severely affected, and may also confound their conclusions. Since the depolarising role of GABA is combined with its shunting properties, it is therefore conceivable that in an anaesthetised state, any small depolarisation generated by GABA will lack the necessary ongoing excitatory drive from the network to produce action potentials and corresponding EPSPs. This would reduce GABA function to its shunting properties, hiding its depolarising effects. In fact, another study (Kirmse et al., 2015) has shown that in P3-P4 anaesthetised mice GABA was depolarising in the cortex, but its shunting effect resulted in an overall inhibition of the network. Finally, the measurements carried

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out by Valeeva et al. do not necessarily sample the activity of ChCs. In their experiments they used a transgenic mouse line (GAD2) that will activate all interneuron types, of which the majority will be PV basket cells. This would occlude any effect coming from chandelier cells which would certainly be a minority (Rudy et al., 2011).

5.5.3 Depolarising axo-axonic synapses

In this study, I also present evidence that chandelier cell activity can be depolarising in the periadolescent brain. In fact, we did not observe any clear hyperpolarising response. This goes in accordance with several studies where ChC evoked depolarisations have been observed in P16-P23 rodents (Khirug et al., 2008; Woodruff et al., 2009b, 2011). Interestingly, a seminal study in adult human tissue observed the same effect (Szabadics et al., 2006). However, several studies in rodents have failed to see a depolarising role during adulthood in slice preparations (Glickfeld et al., 2009) and through indirect measures *in vivo* (Lu et al., 2017). A recent study using perforated patch-clamp in acute slices has recently brought these studies together by suggesting that GABAergic synapses switch polarity from depolarising to hyperpolarising at different ages at the soma and the axon. Axo-somatic synapses become hyperpolarising from P7-P10 while axo-axonic synapses stay depolarising until P21 (Rinetti-Vargas et al., 2017). My results agree with this proposal by using a different technique, further strengthening its validity. However, I did not investigate if the depolarising polarity of axo-axonic synapses disappeared at later stages (P21 onwards). Future experiments should investigate this possibility.

5.5.3 Depolarising axo-axonic synapses and homeostatic plasticity

The evidence shown in this chapter supports the hypothesis that the decrease in axo-axonic innervation in response to chronic excitation described in Chapter 4, is in fact an example of homeostatic plasticity. Under this framework, chandelier cells decrease AIS innervation in response to high activity when they are excitatory (during periadolescence) and reduce it when they are inhibitory (in the adult). In this manner, they maintain their impact on the network constant through age. This adds to a growing body of work that has shown that the AIS might serve as a homeostatic hub of the

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neuron that controls excitability via different mechanisms (Grubb and Burrone, 2010; Kuba et al., 2010; Wefelmeyer et al., 2015)

However, fully proving homeostatic plasticity would require evidence that the plasticity described is balancing the system to maintain functionality of the synapse/neuron in their circuit (e.g contribution to network oscillations, tuning to visual stimuli, etc). For example, in the case of PV cells, functionality could be established at the level of gamma oscillations. PV cells are required for gamma oscillations, therefore homeostatic changes in PV should work towards maintaining this function. In the case of chandelier cells this cannot be done unambiguously. As described in the introduction in Chapter 1, a clear functional role of ChCs at the network and behavioral level is still unknown. This is the case in the adult mice and more so during postnatal development. Therefore, without further knowledge, it is difficult to assess to what extent failure to undergo plasticity would impair ChC and circuit function.

5.5.2 Concluding remarks

In this chapter, I have described an alternative method for looking at GABA polarity during development. Using this method, I showed that axo-axonic synapses can be depolarising during the same period in which they are plastic (P12-P18), suggesting a homeostatic role for ChC plasticity.

In the next chapter, I will bring together the findings described in the development, plasticity and function of ChCs made in this thesis and discuss their context in cortical development and function.

Chapter 6 : Summary and general discussion

6.1. Summary of findings

The aim of this thesis was to (1) describe how ChCs develop their axonal arbour and their synapses, (2) to understand if ChCs undergo activity-dependent plasticity during development and adulthood and (3) to determine the polarity of the axo-axonic synapse during axo-axonic synaptogenesis and periadolescent plasticity.

In Chapter 3, I described in detail the formation of the ChC axonal arbour and their axo-axonic synapses. I also provided information on the development of the intrinsic excitability and synaptic electrophysiological features of ChCs. I found that the majority of axo-axonic synapses in L2/3 of the cortex developed during a narrow time window (P12-P18). Similarly, I found that during the same time window ChCs significantly developed their excitability and fast-spiking features. Based on the narrow critical window of axo-axonic development characterised in Chapter 3, in Chapter 4 I explored the activity-dependent plasticity properties of ChCs during this period. I observed that increasing activity from P12 to P18 led to a significant decrease in the number of axo-axonic synapses. The same manipulation in adult ChCs, from P40 to P46, led to the opposite effect, an increase in axo-axonic innervation, suggesting a developmental switch in axo-axonic plasticity. Interestingly, in the periadolescent period I also observed a small significant decrease in AIS length which was associated with a decrease in intrinsic excitability in response to a cell autonomous increase in neuronal activity. Finally, in Chapter 5, I used the recently developed GEVI, ACE-mNeon to assess the polarity of axo-axonic synapses during the P12-

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P18 period. I described that during this period, GABAergic synapses tended to be depolarising at the AIS and hyperpolarising at the soma. This suggests that the plasticity observed in both ChCs and basket cells in chapter 4 may have a homeostatic function.

6.2. Axo-axonic development coincides with a period of depolarising E_{GABA} at the AIS

In Chapter 3, I described that axo-axonic synaptogenesis peaked in the P12-P18 period. At this age GABA is thought to have a depolarising role as suggested by the experiments in Chapter 5 and a previous study (Rinetti-Vargas et al., 2017). However, whether this response is depolarising during 'awake'-like *in vivo* activity has not been established. This is because the resting membrane potential under 'awake' conditions could be higher and therefore closer to E_{GABA} at the AIS (Woodruff et al., 2011). This would diminish any depolarising role of the axo-axonic synapse and promote its shunting properties. The effects of GABAergic innervation not only depend on local E_{GABA} but also on the shunting effect of GABA_AR channel opening. The opening of GABA_AR generates a transient increase in synaptic conductance, which decreases input resistance (R_{in}). Following Ohm's law ($V = IR$) during this transient decrease in R_{in} , a higher amount of current (I) is required to generate a voltage change, making the cell less responsive to other inputs (Isaacson and Scanziani, 2011). Therefore, a mildly depolarising response such as the one that ChCs could generate during this period in awake conditions might still be shunting. Nonetheless, in Rinetti-Vargas et al., 2017, the reported E_{GABA} at the AIS during the P12-P18 period appears to be strongly depolarising ($>20mV$), even supra-threshold in many occasions, and could therefore surpass the GABA_AR mediated shunt. Based on this information, I propose that ChCs are depolarising during the period of synapse formation. Nonetheless, as discussed in the previous chapter, future experiments assessing axo-axonic GABAergic polarity in awake animals, via voltage imaging or gramicidin perforated patches, are needed to fully confirm their polarity.

The presence of depolarising GABA during axo-axonic synaptogenesis may play an important role in this process. In fact, most of GABAergic synaptogenesis from different interneuron types tends to occur during periods of depolarising E_{GABA} . The majority of GABAergic interneurons make their synapses before P10 (Hinojosa et al., 2018), this is also the period at which GABA is believed to be depolarising or even excitatory (Ben-Ari et al., 2007). In fact, several studies indicate that depolarising GABA may play an important role

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in the formation of GABAergic synapses. Increases in neuronal activity have been associated with an increase in both GABA_AR receptor clusters and presynaptic GABAergic boutons (Rannals and Kapur, 2011; Flores et al., 2015). Similarly, blockage of neuronal activity has been associated with a decrease in the number of GABAergic boutons (Hartman et al., 2006). Additionally, a recent study showed that GABA_AR mediated depolarisations mediate GABAergic synapse formation (Oh et al., 2016). Overall, these studies suggest that GABA mediated depolarisation leads to the stabilisation of synapses.

Interestingly in my study I observe that by increasing ChC activity, in a period of depolarising GABA, axo-axonic synapse number is decreased. This suggests that axo-axonic synaptogenesis favours network homeostasis over any pro-synaptogenic role of depolarising GABA. Nonetheless, depolarising GABA might still promote synapse formation at the local level in ChCs. It could be hypothesised that at the local level GABA mediated depolarisations promote synapse formation also in ChCs, however when network activity is increased (as consequence of depolarising GABA or otherwise) homeostatic programs are activated and prioritised, which reduce overall axo-axonic innervation. Hence, it would be the tight balance between local and global effects of GABA depolarisations that determines the final extent of ChC innervation. Future studies comparing the effects of increased GABA release (e.g uncaging (Oh et al., 2016)) at the single cell and network level in pyramidal cells will hopefully shine more light on this question.

6.3. Is the developmental switch in axo-axonic plasticity unique to ChCs?

In Chapter 4, I showed that axo-axonic synapses match the direction of their synaptic plasticity to the polarity of GABA. Can all GABAergic interneurons do the same thing?

Other interneurons such as basket cells also show, like ChCs, depolarising GABA during synaptogenesis (Ben-Ari et al., 2007) but at an earlier period (P5-P10) (Hinojosa et al., 2018). Could these cells undergo synaptic homeostatic adaptations during this period? In favour of this hypothesis is the fact that many interneurons can experience activity-dependent homeostatic changes at least in their mature state (Bartley et al., 2008; Chen et al., 2012; van Versendaal et al., 2012; Keck et al., 2013). However, as mentioned in section 6.2, GABA mediated depolarisations from other interneurons are thought to prioritise and promote synapse formation over network homeostasis (Oh et al., 2016). Nonetheless, the

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manipulations in the latter study were done at the single cell level. As mentioned before, it is possible that interneurons regulate the plasticity of their synapses differently at a local and global level. For example, GABA mediated depolarisations may activate different plasticity mechanisms at the level of individual synapses (promoting synapses formation) than at the network level (promoting homeostasis and reducing GABAergic innervation when GABA is depolarising).

In future studies, it would be interesting to see if by increasing or reducing network activity during the peak of synaptogenesis of other interneurons types we would also observe homeostatic adaptations at a synaptic level like in ChCs.

6.4. The AIS and its synapses form the homeostatic hub of the neuron

The potential depolarising role of axo-axonic synapses in the P12-P18 period, combined with the activity-dependent decrease in axo-axonic synapse number and strength, suggests that this might be a form of homeostatic plasticity.

Under a homeostatic model, in the periadolescent brain, high network activity leads to a decrease in axo-axonic innervation (at this time depolarising), ultimately decreasing overall levels of excitation. This model applies also to the adult brain, since the switch in the direction of the axo-axonic plasticity I describe here, matches the switch in the polarity of axo-axonic synapses (Rinetti-Vargas et al., 2017). Therefore, in the adult brain, an increase in network activity leads to an increase in total axo-axonic innervation (by now hyperpolarising) which may ultimately lead to an increase in total inhibition and a decrease in the overall level of excitation.

A growing body of work proposes the AIS as a major homeostatic regulator of excitability (Grubb and Burrone, 2010; Kuba et al., 2010; Evans et al., 2015; Wefelmeyer et al., 2015). In parallel with the plasticity of the AIS, in this thesis I show that activity-dependent plasticity at axo-axonic synapses, appears to also be working towards this end. Therefore, it is tentative to speculate that the AIS and its synapses might form the homeostatic hub of the neuron. Nonetheless, questions remain regarding how these two forms of plasticity interact with each other. Previous work has shown that the position of axo-axonic synapses along the pyramidal cell axon has important implication in the homeostatic function of AIS

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plasticity (Wefelmeyer et al., 2015). In this thesis, I showed that in response to high network activity both AIS length and the density of axo-axonic synapses decreased. The combination of these two plastic events is more difficult to interpret. Future computational work parametrising AIS plasticity, axo-axonic plasticity and a delayed switch in GABA polarity will hopefully shed more light onto this question.

6.5. Using GEVIs to examine axo-axonic polarity and function *in vivo*

It is worth noting that, so far, the depolarising nature of ChCs in the periadolescent brain, and hence its potential role in homeostatic plasticity, has only been confirmed *ex vivo* in acute slices (Rinetti-Vargas et al., 2017). Recent studies have suggested that measurement of GABA polarity, and hence the role of axo-axonic synapses, could be corrupted in *ex vivo* preparations, the type of preparation where most electrophysiology is done (see Chapter 5 discussion for more details). So far, evidence *in vivo* has always proposed an inhibitory role for axo-axonic synapses (Lu et al., 2017). However, this has only been tested in adult animals where we know that even in *ex vivo* preparations, axo-axonic synapses present a hyperpolarising polarity (Rinetti-Vargas et al., 2017). It is therefore critical to investigate GABA polarity at the AIS *in vivo* in the periadolescent brain, in order to confirm the role of the plasticity observed in this thesis. This has proven very difficult in the past due to the inherent difficulty of gramicidin perforated patches (see Chapter 5.2.1 for more details), which is only exacerbated in *in vivo*. However, the technique described in this thesis, using voltage imaging, is easily transferable to *in vivo* preparations. By expressing a 2-photon excitable voltage indicator such as ASAP2 (Chamberland et al., 2017) in pyramidal cells and simultaneously stimulating ChCs by targeted optogenetic stimulation, we could track the polarity of axo-axonic synapses in individual pyramidal cells throughout postnatal development. This would provide a definite answer to the role of axo-axonic synapses at different ages as well as confirming or disproving the homeostatic role of axo-axonic plasticity proposed in this thesis.

6.6. Potential role of ChC plasticity in disease

Failure to undergo homeostatic plasticity has been associated with the appearance of several neurological disorders (Wondolowski and Dickman, 2013). Could deficits on the activity dependent plasticity of axo-axonic synapses generate any of these pathologies? This

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is currently highly speculative. However, under a homeostatic model of axo-axonic plasticity, failure of ChCs to undergo homeostatic plasticity may generate network dysregulations associated with neurological disorders. In fact, as discussed in Chapter 1, the link between ChC malfunction and the appearance of several diseases such as schizophrenia and epilepsy has been established, however these studies have ignored the plastic mechanisms of these cells and their synapses (DeFelipe, 1999; Fazzari et al., 2010). Future studies will hopefully evaluate ChC plasticity in animal models of schizophrenia (e.g NRG1 or ErBB4 knockout (Jones et al., 2011)) and epilepsy (e.g kainic acid (Ben-Ari, 1985)) and investigate whether this is affected in any manner.

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Appendix 1

SR	7 MOhm	4 MOhm	3 MOhm	SR	7 MOhm	4 MOhm	3 MOhm	SR	7 MOhm	4 MOhm	3 MOhm	SR	7 MOhm	4 MOhm	3 MOhm
31	77.42	87.10	90.32	24	70.83	83.33	87.50	17	58.82	76.47	82.35	10	30.00	60.00	70.00
30.9	77.35	87.06	90.29	23.9	70.71	83.26	87.45	16.9	58.58	76.33	82.25	9.9	29.29	59.60	69.70
30.8	77.27	87.01	90.26	23.8	70.59	83.19	87.39	16.8	58.33	76.19	82.14	9.8	28.57	59.18	69.39
30.7	77.20	86.97	90.23	23.7	70.46	83.12	87.34	16.7	58.08	76.05	82.04	9.7	27.84	58.76	69.07
30.6	77.12	86.93	90.20	23.6	70.34	83.05	87.29	16.6	57.83	75.90	81.93	9.599	27.08	58.33	68.75
30.5	77.05	86.89	90.16	23.5	70.21	82.98	87.23	16.5	57.58	75.76	81.82	9.499	26.32	57.89	68.42
30.4	76.97	86.84	90.13	23.4	70.09	82.91	87.18	16.4	57.32	75.61	81.71	9.399	25.53	57.45	68.09
30.3	76.90	86.80	90.10	23.3	69.96	82.83	87.12	16.3	57.06	75.46	81.60	9.299	24.73	56.99	67.74
30.2	76.82	86.75	90.07	23.2	69.83	82.76	87.07	16.2	56.79	75.31	81.48	9.199	23.91	56.52	67.39
30.1	76.74	86.71	90.03	23.1	69.70	82.68	87.01	16.1	56.52	75.16	81.37	9.099	23.08	56.04	67.03
30	76.67	86.67	90.00	23	69.57	82.61	86.96	16	56.25	75.00	81.25	8.999	22.22	55.56	66.67
29.9	76.59	86.62	89.97	22.9	69.43	82.53	86.90	15.9	55.97	74.84	81.13	8.899	21.35	55.06	66.29
29.8	76.51	86.58	89.93	22.8	69.30	82.46	86.84	15.8	55.70	74.68	81.01	8.799	20.45	54.55	65.91
29.7	76.43	86.53	89.90	22.7	69.16	82.38	86.78	15.7	55.41	74.52	80.89	8.699	19.54	54.02	65.52
29.6	76.35	86.49	89.86	22.6	69.03	82.30	86.73	15.6	55.13	74.36	80.77	8.599	18.60	53.49	65.12
29.5	76.27	86.44	89.83	22.5	68.89	82.22	86.67	15.5	54.84	74.19	80.65	8.499	17.65	52.94	64.71
29.4	76.19	86.39	89.80	22.4	68.75	82.14	86.61	15.4	54.55	74.03	80.52	8.399	16.67	52.38	64.29
29.3	76.11	86.35	89.76	22.3	68.61	82.06	86.55	15.3	54.25	73.86	80.39	8.299	15.66	51.81	63.86
29.2	76.03	86.30	89.73	22.2	68.47	81.98	86.49	15.2	53.95	73.68	80.26	8.199	14.63	51.22	63.41
29.1	75.95	86.25	89.69	22.1	68.33	81.90	86.43	15.1	53.64	73.51	80.13	8.099	13.58	50.62	62.96
29	75.86	86.21	89.66	22	68.18	81.82	86.36	15	53.33	73.33	80.00	7.999	12.50	50.00	62.50
28.9	75.78	86.16	89.62	21.9	68.04	81.74	86.30	14.9	53.02	73.15	79.87	7.899	11.39	49.37	62.03
28.8	75.69	86.11	89.58	21.8	67.89	81.65	86.24	14.8	52.70	72.97	79.73	7.799	10.26	48.72	61.54
28.7	75.61	86.06	89.55	21.7	67.74	81.57	86.18	14.7	52.38	72.79	79.59	7.699	9.09	48.05	61.04
28.6	75.52	86.01	89.51	21.6	67.59	81.48	86.11	14.6	52.05	72.60	79.45	7.599	7.89	47.37	60.53
28.5	75.44	85.96	89.47	21.5	67.44	81.40	86.05	14.5	51.72	72.41	79.31	7.499	6.67	46.67	60.00
28.4	75.35	85.92	89.44	21.4	67.29	81.31	85.98	14.4	51.39	72.22	79.17	7.399	5.41	45.95	59.46
28.3	75.27	85.87	89.40	21.3	67.14	81.22	85.92	14.3	51.05	72.03	79.02	7.299	4.11	45.21	58.90
28.2	75.18	85.82	89.36	21.2	66.98	81.13	85.85	14.2	50.70	71.83	78.87	7.199	2.78	44.44	58.33
28.1	75.09	85.77	89.32	21.1	66.82	81.04	85.78	14.1	50.35	71.63	78.72	7.099	1.41	43.66	57.75
28	75.00	85.71	89.29	21	66.67	80.95	85.71	14	50.00	71.43	78.57	6.999	-0.00	42.86	57.14
27.9	74.91	85.66	89.25	20.9	66.51	80.86	85.65	13.9	49.64	71.22	78.42	6.899		42.03	56.52
27.8	74.82	85.61	89.21	20.8	66.35	80.77	85.58	13.8	49.28	71.01	78.26	6.799		41.18	55.88
27.7	74.73	85.56	89.17	20.7	66.18	80.68	85.51	13.7	48.91	70.80	78.10	6.699		40.30	55.22
27.6	74.64	85.51	89.13	20.6	66.02	80.58	85.44	13.6	48.53	70.59	77.94	6.599		39.39	54.55
27.5	74.55	85.45	89.09	20.5	65.85	80.49	85.37	13.5	48.15	70.37	77.78	6.499		38.46	53.85
27.399	74.45	85.40	89.05	20.399	65.69	80.39	85.29	13.399	47.76	70.15	77.61	6.399		37.50	53.12
27.299	74.36	85.35	89.01	20.299	65.52	80.30	85.22	13.299	47.37	69.92	77.44	6.299		36.51	52.38
27.199	74.26	85.29	88.97	20.199	65.35	80.20	85.15	13.199	46.97	69.70	77.27	6.199		35.48	51.61
27.099	74.17	85.24	88.93	20.099	65.17	80.10	85.07	13.099	46.56	69.47	77.10	6.099		34.43	50.82
26.999	74.07	85.19	88.89	19.999	65.00	80.00	85.00	12.999	46.15	69.23	76.92	5.999		33.33	50.00
26.899	73.98	85.13	88.85	19.899	64.82	79.90	84.92	12.899	45.74	68.99	76.74	5.899		32.20	49.15
26.799	73.88	85.07	88.81	19.799	64.65	79.80	84.85	12.799	45.31	68.75	76.56	5.799		31.03	48.28
26.699	73.78	85.02	88.76	19.699	64.47	79.70	84.77	12.699	44.88	68.50	76.38	5.699		29.82	47.37
26.599	73.68	84.96	88.72	19.599	64.29	79.59	84.69	12.599	44.44	68.25	76.19	5.599		28.57	46.43
26.499	73.58	84.91	88.68	19.499	64.10	79.49	84.62	12.499	44.00	68.00	76.00	5.499		27.27	45.45
26.399	73.48	84.85	88.64	19.399	63.92	79.38	84.54	12.399	43.55	67.74	75.81	5.399		25.93	44.44
26.299	73.38	84.79	88.59	19.299	63.73	79.27	84.46	12.299	43.09	67.48	75.61	5.299		24.53	43.40
26.199	73.28	84.73	88.55	19.199	63.54	79.17	84.37	12.199	42.62	67.21	75.41	5.199		23.08	42.31
26.099	73.18	84.67	88.51	19.099	63.35	79.06	84.29	12.099	42.15	66.94	75.21	5.099		21.57	41.18
25.999	73.08	84.62	88.46	18.999	63.16	78.95	84.21	11.999	41.67	66.67	75.00	4.999		20.00	40.00
25.899	72.97	84.56	88.42	18.899	62.96	78.84	84.13	11.899	41.18	66.39	74.79	4.899		18.37	38.78
25.799	72.87	84.50	88.37	18.799	62.77	78.72	84.04	11.799	40.68	66.10	74.58	4.799		16.67	37.50
25.699	72.76	84.44	88.33	18.699	62.57	78.61	83.96	11.699	40.17	65.81	74.36	4.699		14.89	36.17
25.599	72.66	84.38	88.28	18.599	62.37	78.49	83.87	11.599	39.66	65.52	74.14	4.599		13.04	34.78
25.499	72.55	84.31	88.24	18.499	62.16	78.38	83.78	11.499	39.13	65.22	73.91	4.499		11.11	33.33
25.399	72.44	84.25	88.19	18.399	61.96	78.26	83.70	11.399	38.60	64.91	73.68	4.399		9.09	31.82
25.299	72.33	84.19	88.14	18.299	61.75	78.14	83.61	11.299	38.05	64.60	73.45	4.299		6.98	30.23
25.199	72.22	84.13	88.10	18.199	61.54	78.02	83.52	11.199	37.50	64.29	73.21	4.199		4.76	28.57
25.099	72.11	84.06	88.05	18.099	61.33	77.90	83.43	11.099	36.94	63.96	72.97	4.099		2.44	26.83
24.999	72.00	84.00	88.00	17.999	61.11	77.78	83.33	10.999	36.36	63.64	72.73	3.999		-0.00	25.00
24.899	71.89	83.94	87.95	17.899	60.89	77.65	83.24	10.899	35.78	63.30	72.48	3.899			23.08
24.799	71.77	83.87	87.90	17.799	60.67	77.53	83.15	10.799	35.19	62.96	72.22	3.799			21.05
24.699	71.66	83.81	87.85	17.699	60.45	77.40	83.05	10.699	34.58	62.62	71.96	3.699			18.92
24.599	71.54	83.74	87.80	17.599	60.23	77.27	82.95	10.599	33.96	62.26	71.70	3.599			16.67
24.499	71.43	83.67	87.76	17.499	60.00	77.14	82.86	10.499	33.33	61.90	71.43	3.499			14.29
24.399	71.31	83.61	87.70	17.399	59.77	77.01	82.76	10.399	32.69	61.54	71.15	3.399			11.76
24.299	71.19	83.54	87.65	17.299	59.54	76.88	82.66	10.299	32.04	61.17	70.87	3.299			9.09
24.199	71.07	83.47	87.60	17.199	59.30	76.74	82.56	10.199	31.37	60.78	70.59	3.199			6.25
24.099	70.95	83.40	87.55	17.099	59.06	76.61	82.46	10.099	30.69	60.40	70.30	3.099			3.23

Table 1.1 Series resistance compensation. Percentage values used for 7, 4 and 3 MOhms series resistance (SR) compensation.

Appendix 2

Experiments	Number of animals per condition	Total number of animals in experiment
Chapter 3		
Morphological analysis of ChC development (Fig 3.4 - Fig 3.6)	2-3	21
Intrinsic excitability of ChCs throughout development (Fig 3.7)	3-4	7
ChC-ChR2 characterisation (Fig 3.8)	2-3	5
Electrophysiological analysis of ChC development (Fig 3.4 - Fig 3.6)	4	8
In-vivo imaging of ChC development (Fig 3.10)	NA	3
Chapter 4		
c-Fos expression increases in hM3Dq+ cells at P12 mice following CNO injection (Fig 4.2)	2	4
c-Fos expression increase in hM3Dq+ and hM3Dq-network cells from P18 mice following prolonged activation with CNO (Fig 4.3)	2-3	8
Effects of high network activity in ChC plasticity (P12-P18) (morphology) (Fig 4.4, Fig 4.6 and Fig 4.7)	3-4	11
Effects of high network activity in ChC plasticity (P12-P18) (electrophysiology – synaptic properties) (Fig 4.8)	4	8
Effects of high network activity in ChC plasticity (P12-P18) (electrophysiology – connection probability) (Fig 4.8)	4-9	14
Effects of high network activity in AIS plasticity (P12-P18) (morphology) (Fig 4.5)	3-4	11
Effects of high network activity in AIS plasticity (P12-P18) (electrophysiology) (Fig 4.9)	2	4
Effects of high network activity in basket cell plasticity (P12-P18) (morphology) (Fig 4.10)	3	9
Effects of high network activity in basket cell plasticity (P12-P18) (electrophysiology) (Fig 4.11)	3-5	8
Reversibility of ChC plasticity (Fig 4.12 and Fig 4.14)	2-3	5
Reversibility of AIS plasticity (Fig 4.13)	2-3	5
Effects of high network activity in ChC plasticity (P40-P46) (morphology) (Fig 4.15 and Fig 4.17)	3-5	11
Effects of high network activity in AIS plasticity (P40-P46) (morphology) (Fig 4.16)	3-5	11
Plastic effects of high ChC activity (Fig 4.19 and Fig 4.20)	3	4
Pair recording of ChC-pyramidal cells (Fig 4.21)	NA	2

APPENDIX 1

Chapter 5		
Voltage imaging of GABAergic potentials after GABA iontophoresis (Fig 5.4 and Fig 5.5)	NA	2
Voltage imaging of axo-axonic GABAergic potentials (Fig 5.7)	NA	5

Table 2.1 Number of animals used for the experiments in this thesis.