



PHYSIOLOGY AND PLASTICITY OF SENSORY SYNAPSES IN THE PIRIFORM CORTEX

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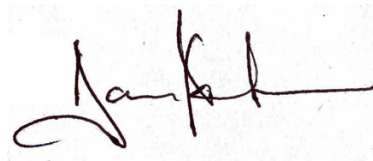
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*This thesis is dedicated to my grandfather, Ray Coppin...
...my idol, my teacher, my advisor, and most of all, my best mate.*

Statement

The work presented in this thesis is, except where specifically acknowledged, my original work performed under the supervision of Professor John Bekkers.

A handwritten signature in dark ink, appearing to read 'Daniel Palmer', with a stylized, flowing script.

Daniel Palmer

June 2024

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First and foremost, I must express my deepest gratitude to my supervisor, Professor John Bekkers. Without his direction, patience and support, this thesis would not have been possible. John is a curious, rigorous, and passionate scientist; however, it is his thoughtfulness and unwavering commitment to his students that sets him apart. The lessons I have learnt from John extend far beyond the lab and will stay with me for life. I consider myself extremely lucky to have had the opportunity to work with him, and can't thank him enough for his guidance and friendship over the past 6 years.

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Abstract

The piriform cortex (PCx) is the first cortical destination of olfactory information. Unlike the six-layered neocortex, the PCx is an anatomically simpler, trilaminar structure, with functional capabilities more closely resembling higher-level associational cortices. Among the most striking features of the PCx is its capacity to encode and store memory, a process thought to primarily manifest through the plasticity of synapses comprising its dense associational network. From the properties and connectivity of the individual cell types that make up the region, through to its higher-level functional capabilities, a detailed understanding of how information is processed within the PCx is being established. Even so, our appreciation of how sensory information is delivered into the PCx, along with how it is transformed and encoded at this level of processing, remains rudimentary. In this thesis, I utilise *in vitro* slice models to investigate the structure, physiology and plasticity of synapses delivering sensory (afferent) information from the olfactory bulb (OB) to the primary excitatory neurons of the PCx, semilunar (SL) and superficial pyramidal (SP) cells.

Given the relationship between postsynaptic morphology and synaptic transmission, I first used enhanced-resolution confocal microscopy to investigate the dendritic structure and distribution of spines on the apical arbours of the two target cell types. SP cells displayed more complex dendritic trees and a higher spine density than SL cells. Interestingly, although arranged at a lower density, spines on the distal apical dendrites of SL cells (where afferent input is received) were significantly larger than those on SP cells. As a result, synaptic opportunity (defined as total spine head surface area per unit dendritic length) was similar for both cell types. In view of the substantial differences in spine structure and distribution, I next combined minimal electrical stimulation with whole-cell somatic recordings to investigate the

properties of afferent synaptic transmission. These experiments showed that SL cells receive significantly larger unitary synaptic inputs from the OB than do SP cells. Variance-mean analysis and strontium-evoked asynchronous EPSCs revealed that these powerful inputs to SL cells are driven by both a higher neurotransmitter release probability (P_r) and higher quantal amplitude (Q). As a result, SL cells can be lifted to action potential threshold with fewer afferent inputs than SP cells.

Next, I asked whether the disparity in afferent synaptic structure and transmission to SL and SP cells extended to their ability to express synaptic plasticity. Using patterned extracellular stimulation, N-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation (LTP) and depression (LTD) could be observed using field recordings. When isolating unitary inputs, however, LTP and LTD could only be induced at afferent-SL synapses, with no reliable plasticity inducible at afferent-SP inputs. Interestingly, afferent-SL LTD was expressed both pre- and postsynaptically, with variable changes in P_r (presynaptic) and Q (postsynaptic) observed. On the other hand, afferent-SL LTP was predominantly presynaptic and appeared to depend on the prior P_r of the stimulated input, such that low P_r synapses could be potentiated while high P_r synapses could not.

In a final set of exploratory experiments, I investigated the excitability of SL and SP cell dendrites. Dendritic NMDA spikes can evoke synaptic potentiation at afferent inputs, so it was important to understand whether the plasticity I observed depended on this mechanism. I made whole-cell recordings from both somas and dendrites of SL and SP cells while systematically increasing the strength of afferent extracellular stimulation. Although putative dendritic electrogenesis was observed at high stimulation strengths, synaptic responses scaled linearly through the minimal stimulation range, indicating that the unitary stimulation strengths used in my plasticity experiments did not evoke active dendritic events.

Overall, the experiments presented in this thesis reveal distinct differences in how SL and SP cells receive and process monosynaptic sensory information from the OB. From dendritic spine structure through to synaptic transmission and plasticity, SL and SP cells are markedly distinct. Given that these cells are the dominant glutamatergic cell types of the PCx, and receive the majority of monosynaptic afferent input from the OB, understanding how they process olfactory input will be critical for our ultimate understanding of how information is encoded and stored within this trilaminar sensory cortex.

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List of Abbreviations

ACSF	artificial cerebrospinal fluid
<i>Aff</i>	afferent (sensory) synapses
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	action potential
<i>Assn</i>	associational (intracortical) synapses
AUC	area under the curve
B	bitufted cell
CAM	content addressable memory
CaMKII	calcium/calmodulin-dependent kinase II
C_m	membrane capacitance
CSF	cerebrospinal fluid
CV	coefficient of variation
DP	deep pyramidal cell
EM	electron microscopy
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
f EPSP	field excitatory postsynaptic potential
G	neurogliaform cell
GABA	gamma-aminobutyric acid
H	horizontal cell
HW	half-width
I	EPSC amplitude
L1/2/3	layer 1/2/3 of the piriform cortex

LFP	local field potential
LFS	low-frequency stimulation
LOT	lateral olfactory tract
LTD	long-term depression
LTP	long-term potentiation
M	multipolar cell
M/T	mitral/tufted cell
mPSC	miniature postsynaptic current
mPSP	miniature postsynaptic potential
N	number of neurotransmitter release sites
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
OB	olfactory bulb
OE	olfactory epithelium
ORN	olfactory receptor neuron
PCx	piriform cortex
PPR	paired-pulse ratio
P_r	probability of neurotransmitter release
PSD	postsynaptic density
Q	quantal amplitude
SEM	standard error of the mean
SL	semilunar cell
SP	superficial pyramidal cell
TBS	theta-burst stimulation

Chapter 1

Introduction

1.1 Overview

Olfaction is a chemosense finely tuned to detect and interpret volatile chemicals in the environment (Pinto, 2011; Wilson and Sullivan, 2011). Given the obvious adaptive advantages of chemical detection – the rapid perception of hazards, pheromones and food, for example – it is not surprising that animals have evolved chemosensory systems that are both sensitive and highly efficient (Buck and Axel, 1991; Malnic et al., 1999; Bargmann, 2006; Niimura and Nei, 2006; Wesson et al., 2008). Despite its critical importance for the development and survival of many organisms, olfaction remains the most enigmatic and poorly understood of the major senses (Keller and Vosshall, 2004; Ache and Young, 2005; Brennan, 2018; Brann and Datta, 2020).

In this thesis I explore the cellular basis of one of the most striking features of olfaction, namely, its reliance upon learning and memory. I begin this chapter with a brief overview of the key role of synaptic plasticity in olfactory processing. Next, I summarise what is known about the anatomy and function of the early stages of the olfactory system, with a focus on the primary olfactory cortex (*piriform cortex*, PCx). Subsequent sections will provide an outline of the properties of synaptic transmission in the PCx, including its plasticity and dependence on dendritic integration. I finish this chapter with a statement of the main aims of the thesis and a summary of my experimental approaches.

1.2 Olfaction and synaptic plasticity

Phylogenetically, chemosensation is the oldest sensory modality, providing the first means of communication between organisms (Hoover, 2010). With its origin in aquatic

ecosystems, vertebrate olfaction can be traced back over 500 million years to the lancelet, a fish-like marine chordate that shares some of the same olfactory receptor genes as modern humans (Niimura, 2012). Given its early evolutionary development, the initial stages of the olfactory system have a relatively simple organisation and cellular architecture compared with the other primary sensory networks. Despite this simplicity, these early stages of olfaction exhibit functional capabilities beyond those of the other primary senses, more closely resembling higher-level association cortices.

There are many factors that differentiate the olfactory system from the other sensory modalities, but perhaps the most unique is the ability to store its own memories. Although the precise mechanisms are yet to be elucidated, the primary cortical structure of the olfactory system, the PCx, displays many of the structural and functional hallmarks of an associative memory system (Haberly and Bower, 1989; Brennan et al., 1990; White, 1998; Wilson and Stevenson, 2003a, b; Zelano et al., 2009). Specific circuit features of the PCx – such as a distributed input layer and a densely interconnected and highly plastic processing layer – have led to the suggestion that the PCx operates as a ‘content-addressable memory’ (CAM) device (Haberly, 1985; Haberly and Bower, 1989). Unlike random access memory, CAM systems have an extra processing layer that associates the content of new input with that of stored information. As well as increasing efficiency, this associative capacity gives CAM systems the ability to use partial or degraded input to access associated memory traces in their entirety (Haberly and Bower, 1989). Although simulations have established the feasibility of such models, it is not known to what extent biological circuits, like the PCx, implement similar mechanisms (Haberly and Bower, 1989; Brennan et al., 1990).

Since the establishment of theoretical models, experimental evidence has supported the idea that the PCx acts as a memory substrate. While not providing a complete biological

description of memory in itself, synaptic plasticity is a necessary component of all memory processes. The excitatory synapses that make up the intracortical network of the PCx are highly plastic, specifically during phases of olfactory learning, suggesting a critical role in processing and storing olfactory information (Hasselmo and Barkai, 1995; Barkai and Hasselmo, 1997; Lebel et al., 2001; Quinlan et al., 2004; Cohen et al., 2008; Chandra et al., 2019). Furthermore, odour representations in the PCx stabilize following repeated exposure, reminiscent of memory “engrams”, further indicating that the system has the ability to retain specific olfactory information (Schoonover et al., 2021). However, piriform representations of odour degrade over time, raising the possibility that the system functions as an efficient, high throughput learning device rather than a stable memory repository (Schoonover et al., 2021).

Substantial progress has been made over the past few decades, but there remain many unanswered questions that directly relate to our understanding of how memories are encoded and stored within the olfactory network. Although we now have a relatively detailed picture of how information is processed within the PCx, and how plasticity manifests within its intracortical network, our understanding of how olfactory information is delivered into the PCx remains rudimentary. The structural and functional properties of synapses delivering sensory information from the olfactory bulb to the PCx will have direct implications for how information is processed within the network. Recently, long-held assumptions regarding the stability of sensory synapses in the PCx have been questioned, with some studies demonstrating robust plasticity at sensory inputs (Franks and Isaacson, 2005; Poo and Isaacson, 2007; Strauch and Manahan-Vaughan, 2017; Kumar et al., 2018; Kumar et al., 2021). Even so, it remains unclear exactly where and under what conditions this form of plasticity is expressed. A complete understanding of olfactory processing and memory must incorporate a more thorough

appreciation of the function of sensory synapses and how plasticity is induced, expressed, and maintained at these inputs.

This thesis will focus on the biophysical properties and plasticity of sensory synapses delivering information from the olfactory bulb into the PCx. My experiments aim to explore synaptic structure, function and plasticity of sensory inputs and, in doing so, will contribute to the ongoing pursuit of the biophysical basis of olfactory memory and the circuit features underlying it.

1.3 Anatomy and function of the early olfactory system

Unlike the other sensory modalities, olfactory information bypasses the thalamus en route to cortical processing, making the broad underlying circuitry relatively uncomplicated and direct (Haberly and Feig, 1983; Shepherd, 2005). The three structures that perform much of the initial processing of olfactory information are the *olfactory epithelium* (OE), located in the nasal cavity, the *olfactory bulb* (OB), and the PCx (*Fig. 1.1*).

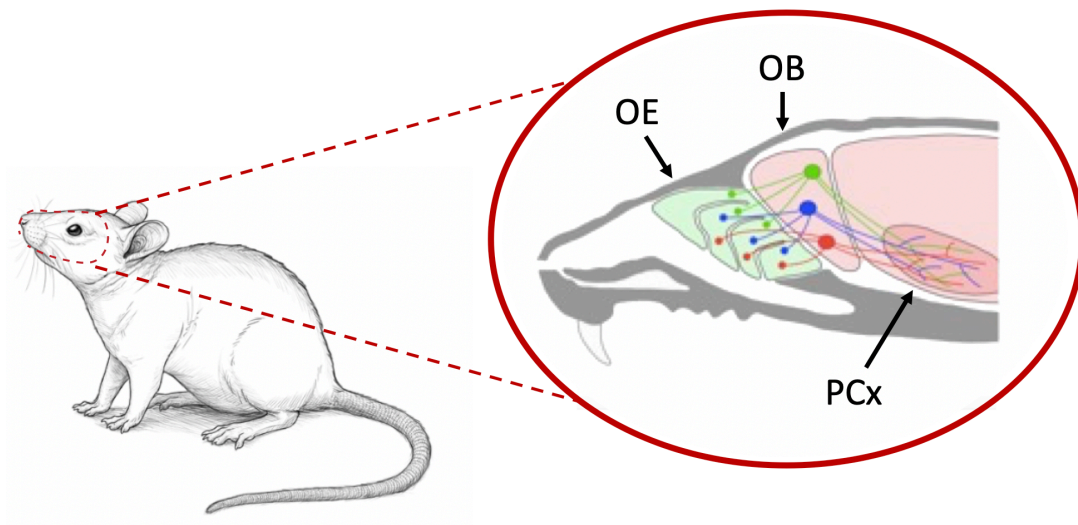


Figure 1.1. Schematic of the initial components of the rodent olfactory system. As an odour is drawn into the nasal cavity, the chemical components of the odour bind to, and are transduced by, olfactory receptor neurons (ORNs) in the main olfactory epithelium (OE). ORNs project directly to the olfactory bulb (OB) where ORNs expressing the same subtype of olfactory receptor converge on small synaptic structures called glomeruli. OB output is sent monosynaptically to the piriform cortex (PCx) where chemical components are reintegrated to form holistic ‘odour images.’

1.3.1 Olfactory epithelium

The OE is the interface of the olfactory system with the outside world, detecting odorants in the environment and transducing them for processing in the brain (Morrison and Costanzo, 1990). Lining the back of the nasal cavity, the OE is comprised of olfactory receptor neurons (ORNs), each expressing a single type of olfactory receptor that responds preferentially to a limited range of chemicals (Dwyer et al., 1998; Billesbølle et al., 2023). In rodents, there are about 1200 functional subtypes of olfactory receptors (Gaillard et al., 2004). Given their exposure to the outside world, ORNs have a short life span (often less than 2

months), and are constantly renewed by stem cells, also present in the OE (Calof et al., 1995). ORNs are unusual among primary receptor cells in having their own axon that directly projects information to the brain (Nedelec et al., 2005).

1.3.2 Olfactory bulb

In the OB, axons projecting from ORNs expressing the same subtype of olfactory receptor converge onto small (100-200 μm -wide) synaptic structures called *glomeruli* (Mombaerts et al., 1996; Feinstein et al., 2004; Nedelec et al., 2005). Interestingly, the position of a specific glomerulus is symmetric between bulbs and consistent within species, resulting in predictable and stable patterns of activation (Vassar et al., 1994). These patterns are commonly referred to as the “*odotopic map*”, where odour identity is represented through the selective activation of glomeruli (Dudai, 1999; Schoenfeld and Cleland, 2005). Mitral and tufted (M/T) cells are the major output neurons of the OB (*Fig. 1.2*). Given that these two cell types have disparate firing properties and projection targets, they are thought to encode different aspects of olfactory information (Mori et al., 1999; Nagayama et al., 2004). Tufted cells respond to a broad range of odour concentrations and project only to the anterior region of the PCx, whereas mitral cells generally only respond to strong olfactory stimulation and project to all regions of the PCx. In the anterior PCx, each cell type appears to target segregated subregions, further suggesting the possibility that mitral and tufted cells transmit distinct odour information (Igarashi et al., 2012).

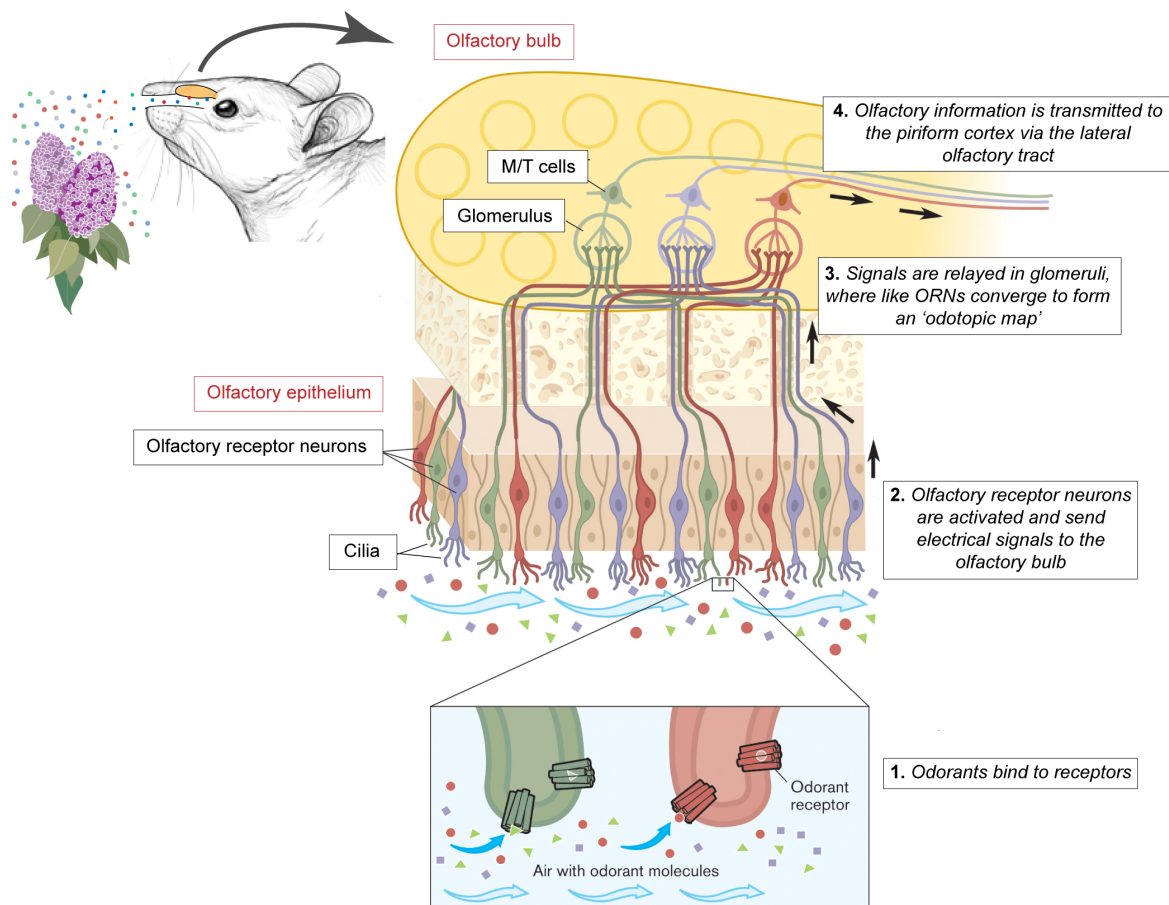


Figure 1.2. Olfactory epithelium and olfactory bulb structure. Schematic representation of a simplified olfactory epithelium and olfactory bulb circuit. Labels 1-4 describe the basic processing stages where odours are transduced by ORNs, represented as an “odotopic map” in the olfactory bulb, then transmitted to the PCx. Modified from: Buck, L. and Axel, R. (1991) *Cell*, 65: 175-187.

1.3.3 Piriform cortex

The PCx is located on the ventral forebrain of the rodent and is the primary cortical structure of the olfactory network (*Fig. 1.3*; Bekkers and Suzuki, 2013). Unlike the typical six-layered columnar organisation of the neocortices, the PCx is a trilaminar *paleocortex* characterised by diffuse internal integration (Suzuki and Bekkers, 2006). As a result, within the piriform circuit odour information is transformed and represented in unique ways (Linster and Hasselmo, 2001; Suzuki and Bekkers, 2006; Bathellier et al., 2009; Howard et al., 2009; Poo and Isaacson, 2009; Stettler and Axel, 2009; Franks et al., 2011; Suzuki and Bekkers, 2011; Wilson and Sullivan, 2011; Suzuki and Bekkers, 2012; Bekkers and Suzuki, 2013).

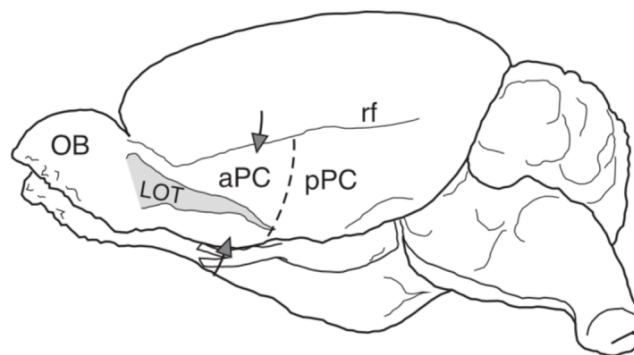


Figure 1.3. *Ventral surface of the rodent brain.* Olfactory bulb, OB; lateral olfactory tract, LOT; anterior piriform cortex, aPC; posterior piriform cortex, pPC; rhinal fissure, rf.

Upon reaching the PCx, the order attained in the OB (odotopic map) is lost, and odours are represented by sparse, irregular patterns of activity (Stettler and Axel, 2009). Within the

densely interconnected associational circuitry of the PCx, segregated OB representations are reconstituted to form holistic *odour images*, which are ultimately what is perceived. These integrated representations of odour stabilize following repeated daily exposure, but become less robust once regular exposure ceases, suggesting that the PCx has the ability to retain specific odour information but remains in flux with the olfactory environment (Schoonover et al., 2021). The posterior region of the PCx robustly represents odour space with features resembling a learned cognitive map (Poo et al., 2021). As such, the PCx concurrently encodes both odour identity and spatial location, forming distributed representations of odour and place. Sparsely distributed representations of the kind established in the PCx are unusual in primary sensory cortices, suggesting unique strategies for processing and coding sensory information (Haberly and Feig, 1983; Haberly, 1985; Haberly and Bower, 1989; Wilson and Sullivan, 2011).

1.3.4 Cellular architecture of the piriform cortex

The three layers of the PCx are populated with a variety of morphologically and functionally distinctive neurons. As shown schematically in *Fig. 1.4*, the most superficial layer, layer 1 (L1), contains two types of sparsely distributed GABAergic interneurons, horizontal and neurogliaform cells. Layer 2 (L2) contains two kinds of densely packed glutamatergic principal neurons, semilunar (SL) cells and superficial pyramidal (SP) cells, as well as sparsely distributed bitufted and neurogliaform interneurons. Layer 3 (L3) contains a more dispersed population of deep pyramidal (DP) cells, as well as neurogliaform and aspiny multipolar interneurons (Suzuki and Bekkers, 2006, 2007, 2010; Bekkers and Suzuki, 2013).

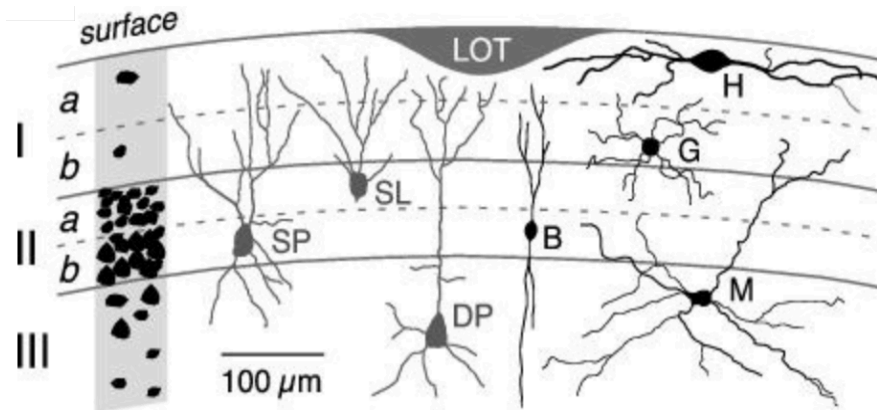


Figure 1.4. Layering, density and distribution of the cells of the piriform cortex. See text for details. Glutamatergic cells: superficial pyramidal, SP; semilunar, SL; deep pyramidal, DP. GABAergic interneurons: bitufted, B; neurogliaform, G; horizontal, H; multipolar, M.

1.3.5 Circuits of the piriform cortex

Two key glutamatergic circuits can be identified in the PCx: the sensory (or *afferent*, *aff*) network which exclusively comprises layer 1a; and the intracortical (or *associational*, *assn*) network which spans layer 1b to layer 3 (*Fig. 1.5*; Suzuki and Bekkers, 2011, 2012; Bekkers and Suzuki, 2013). *Aff* synapses deliver olfactory information monosynaptically from the OB via the lateral olfactory tract (LOT) exclusively to dendrites in layer 1a. Connectivity from the OB to the PCx is surprisingly sparse, with statistical estimates suggesting that principal neurons receive no more than a single input from any given M/T cell (Srinivasan and Stevens, 2019). All three glutamatergic cell types – SL, SP and DP – receive *aff* input from the OB, but SL and SP cells are the primary recipients (Suzuki and Bekkers, 2006, 2011, 2012; Bekkers and Suzuki, 2013). *Aff* input also targets both horizontal and neurogliaform cells in layer 1a,

providing a layer of feedforward inhibition within the *aff* circuitry (Suzuki and Bekkers, 2007, 2010; Suzuki et al., 2022). *Assn* synapses form a densely interconnected network in the deeper layers, which is where deconstructed representations of odour established in the OB are thought to be reintegrated. Along with forming holistic odour images, the *assn* circuitry of the PCx is highly plastic, playing an important role in olfactory learning and memory (Haberly, 1985; Haberly and Bower, 1989; Brennan et al., 1990; White, 1998; Zelano et al., 2009).

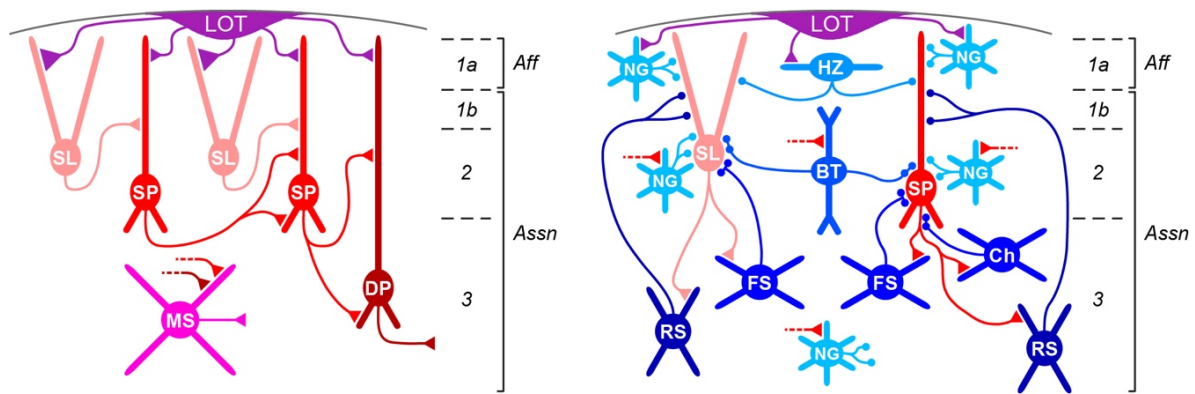


Figure 1.5. Circuits of the piriform cortex. *Left*, schematic glutamatergic (excitatory) circuit diagram of the PCx. *Right*, schematic GABAergic (inhibitory) circuit diagram of the PCx. From: Bekkers and Suzuki (2013).

Current models of PCx processing suggest that *aff* input arriving from the OB is relatively stable, expressing very little synaptic plasticity after the first month of development (Franks and Isaacson, 2005; Strauch and Manahan-Vaughan, 2017). On this view, the *aff* layer of processing merely delivers raw sensory data to the PCx and has minimal capacity to adapt to changing environmental conditions, leaving high-order computations to the more plastic *assn* circuitry. Recently however, there have been strong indications that this description of

aff processing may be inaccurate, with reliable synaptic potentiation demonstrated *in vitro* using a local NMDA-spike induction protocol (Kumar et al., 2021). Although a complete understanding of if or how such plasticity manifests *in vivo* is yet to be established, the possibility of *aff* synaptic plasticity would force us to re-evaluate our understanding of how information is processed and stored within the PCx.

1.3.6 Olfactory coding

The mammalian olfactory system has the capacity to rapidly discriminate thousands of chemical compounds (Uchida and Mainen, 2003; Bushdid et al., 2014; Meister, 2015). This is achieved by implementing an efficient and high capacity *combinatorial coding* scheme (Malnic et al., 1999). As noted in *Section 1.3.1*, the OE comprises a large family of ORNs, with each subtype of ORN sensitive to a defined palette of volatile chemicals (Buck and Axel, 1991). Given that most odours comprise a mixture of many chemicals, any given odour will produce a characteristic pattern of activation across a subset of ORNs (Malnic et al., 1999; Firestein, 2001). The combinatorial nature of this activation pattern, appropriately decoded by higher brain centres, allows relatively few neurons to distinguish a vast number of odour mixtures. In recent years, progress has been made in understanding how the OB and PCx process the information conveyed from the OE (Schaffer et al., 2018; Blazing and Franks, 2020; Brann and Datta, 2020; Pashkovski et al., 2020). However, detailed discussion of this area of research is beyond the scope of this thesis.

1.4 Synaptic structure and function in the PCx

1.4.1 Synaptic populations of the PCx

As mentioned in *Section 1.3.5*, *aff* and *assn* circuits are key components of the PCx. Importantly, the synapses that constitute these circuits are distinct in structure and efficacy, and have different capacities to express synaptic plasticity. These differences both within and between *aff* and *assn* circuits directly influence how information is processed within the PCx.

1.4.2 Dendritic spines and synaptic structure

Chemical synapses, in contrast to electrical synapses, are the most common form of connection between neurons in the mammalian nervous system, with about 1 billion synaptic contacts populating each cubic millimetre of cortical grey matter. Chemical synapses have no structural continuity between pre- and postsynaptic neurons and have three broad components: the *presynaptic element* – a specialised region of the presynaptic neuron's axon called the synaptic bouton *or* axon terminal, which contains vesicles of neurotransmitter; the *synaptic cleft* – the space between the pre- and postsynaptic neuron; and the *postsynaptic element* – a specialised region of somatodendritic membrane on the postsynaptic cell that typically expresses receptors tuned to specific neurotransmitters or modulators which, when activated, mediate postsynaptic currents.

Dendritic spines are small protrusions that stud the dendrites of many neurons and form the postsynaptic element of most excitatory synaptic contacts. Since their discovery, the functional role of dendritic spines has been debated. In 1904, Santiago Ramón y Cajal suggested that spines were well positioned to receive electrical charge and that their presence

substantially increased the receptive surface of the dendritic arbour. More recent calculations have demonstrated that the spines account for about 40% of the total surface area of a typical pyramidal neuron (Peters, 1991). The structure of spines – specifically the surface area of the spine head (which will influence receptor number and density) and the length and diameter of the spine neck (which influences electrical compartmentalisation) – directly affects how chemoelectrical signals are received and propagated through the postsynaptic neuron (Harnett et al., 2012). The physical properties of dendritic spines are often broadly categorised based on their shape (*Fig. 1.6*), although they are malleable and can modify their structure during development and episodes of synaptic plasticity (Borczyk et al., 2019).

Given that the postsynaptic structure of synapses directly influences synaptic strength, understanding the physical properties of spines is critical for understanding synaptic function. Current structural accounts of PCx spines are qualitative in nature, but hint at disparate properties within and between *aff* and *assn* synaptic populations that could help shape important features of information processing in the PCx. As noted in *Section 1.3.5*, *aff* input is exclusively delivered to the distal apical dendrites of principal neurons in the PCx, whereas *assn* input is delivered to proximal apical dendrites and basal dendrites. Distinct differences in spine morphology between cell type and dendritic region (distal/proximal) have been qualitatively observed (Haberly and Feig, 1983). SL cells have large dendritic spines on their distal dendrites, where the *aff* input is received, but much sparser and smaller spines on their proximal dendrites, where *assn* contacts are made. In contrast, SP and DP cells have an apparently uniform arrangement of small spines along the entire dendritic axis. *Chapter 3* of this thesis will address this question in more detail by presenting the first quantitative analysis of spine morphology in the PCx.

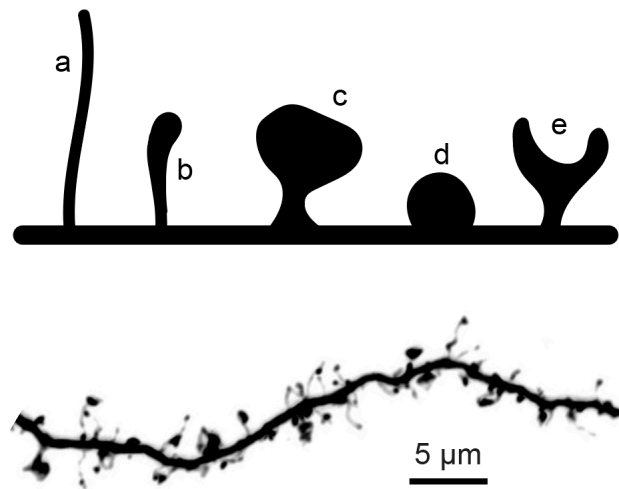


Figure 1.6. Dendritic spine morphology. *Top*, Classical spine types: a, filopodia; b, thin; c, mushroom; d, stubby; e, branched/cup. *Bottom*, Example spine distribution along the apical dendritic axis of a superficial pyramidal cell in the PCx.

In other cell types, spine size correlates with synaptic strength, given that larger spines can accommodate higher numbers of postsynaptic receptors (Nimchinsky et al., 2002; Holtmaat et al., 2006; Arellano et al., 2007; Chater and Goda, 2014; Borczyk et al., 2019). These differences are consistent with electrophysiological recordings in the PCx, that reveal a broad range of unitary *aff* input strengths to excitatory neurons (Franks and Isaacson, 2006). Although unitary *aff* inputs to principal neurons have been reported in the PCx, studies have not differentiated cell type (Franks and Isaacson, 2006), so it is unclear whether the variability in synaptic strength corresponds with differences in spine structure. This question will be addressed in *Chapter 4* of this thesis.

1.4.3 Neurotransmitter release and reuptake

Classic work in the 1950s demonstrated that neurotransmitter is packaged in vesicles located in dense clusters in the presynaptic terminal of synapses (Castillo and Katz, 1954; Palay, 1956). Following an intracellular cascade of events triggered by calcium, vesicles bind to the presynaptic membrane and release their contents into the synaptic cleft, where they can act on postsynaptic receptors (*Fig. 1.7*). When the neurotransmitter contained in a single vesicle (known as a ‘quantum’ of transmitter) is released from the presynaptic bouton, the subsequent response from the postsynaptic site – the miniature postsynaptic current or potential (mPSC/mPSP) – represents the minimal non-zero response possible at that synapse (Bekkers and Stevens, 1995; Bekkers and Clements, 1999). I will elaborate the details of the quantal model of neurotransmission in subsequent sections.

Following release, neurotransmitter is typically recycled for subsequent re-use. Vesicles are recycled through either slow (clathrin-dependent) or fast (clathrin-independent) endocytosis (*Fig. 1.7*). In both cases, due to the action of vesicular ATPase, intravesicle acidity is increased, enabling the proton electrochemical gradient to drive vesicular neurotransmitter transporters (Farsi et al., 2017). During slow endocytosis, membrane retrieval and acidification happen at approximately the same time. In contrast, in fast endocytosis larger structures called endosomes are produced which increase acidity more gradually. From loaded endosomes, synaptic vesicles are then budded off intracellularly. Unlike exocytosis (neurotransmitter release), which can take less than a millisecond, endocytosis can take up to tens of seconds (Gross and Gersdorff, 2016). As a result, persistently active synapses can rapidly deplete neurotransmitter pools.

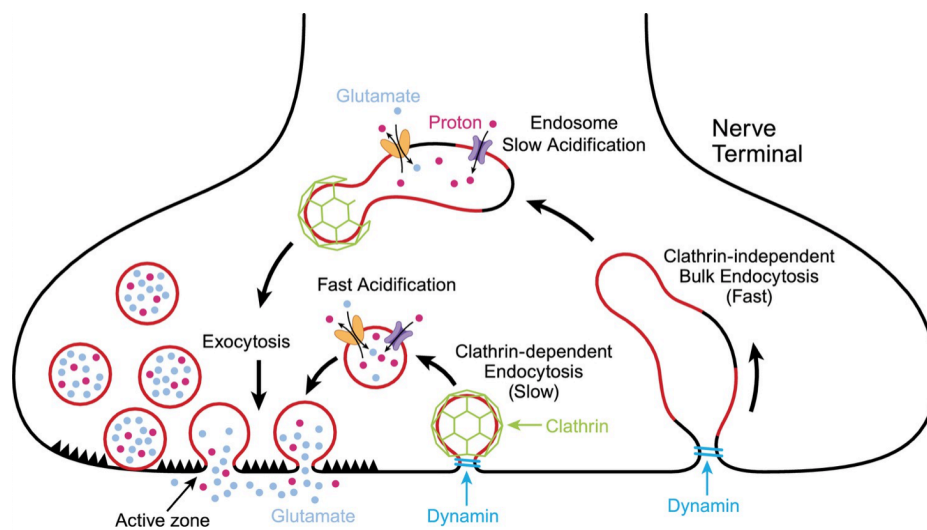


Figure 1.7. Presynaptic structure and vesicle recycling. Schematic representation of both clathrin-dependent (slow) and clathrin-independent (fast) endocytosis. From Gross and Gersdorff (2016).

1.4.4 Synaptic strength and stochasticity

The strength (or weight) of synaptic connections is not fixed and can be influenced by numerous factors such as synaptic and neuronal morphology, prior use, plasticity, and neuromodulation. The cellular mechanisms that underlie such changes in synaptic strength are commonly interpreted using the quantal model of synaptic transmission, introduced by Katz and colleagues based on their studies at the frog neuromuscular junction (Fatt and Katz, 1952; Del Castillo and Katz, 1954), but subsequently generalised by others to encompass a wide variety of synapses (*e.g.* Redman, 1990, for review). The quantal model proposes that postsynaptic responses evoked by presynaptic action potentials are typically compound events that are made up of the probabilistic summation of smaller unitary or ‘quantal’ responses (Redman, 1990). These unitary events, which can be thought of as the building blocks of

postsynaptic potentials or currents, were later shown to be the postsynaptic response to the release of neurotransmitter contained within a single synaptic vesicle (Palay, 1956; Katz, 1962; Südhof, 1995). In the following sections I will briefly summarise some key features of the quantal model that will be used to analyse my results.

1.4.4.1 The quantal model and the coefficient of variation

Three parameters describe the basic framework of the quantal model: the probability of release of a single presynaptic vesicle (P_r), the amplitude of the postsynaptic electrical response to the contents of each vesicle (Q ; also called the quantal amplitude), and the number of synaptic vesicles available for release or, under some interpretations (Walmsley et al., 1998), the number of release sites, each of which can release at most one vesicle (N). The basic theory has been elaborated to include a number of refinements, *e.g.* differences in P_r and Q between different vesicles or release sites, use-dependent changes in P_r , and the non-linear summation of quantal events (Jack et al., 1990; Redman, 1990). However, for the purposes of this thesis, I will consider the simplest version of the theory, the simple binomial model, in which a fixed, homogeneous population of identical vesicles or release sites is assumed.

The binomial model is so called because it proposes that there are two possible outcomes to the arrival of an action potential at a presynaptic release site: either a single synaptic vesicle is released with a probability P_r , or else it is not released with a probability $1 - P_r$. This model also assumes that the N release sites are identical and non-interacting, so their quantal amplitudes, Q , sum linearly. With these assumptions it can easily be shown that the mean synaptic response of these N release sites, when averaged over many trials, is given by the product:

$$I = N P_r Q \quad (\text{Equation 1})$$

where I is the mean response amplitude (taken here to be a current, but it could also be a voltage). Because the synaptic response for each trial depends on a probability, it is not fixed but can vary in size each time an action potential arrives at the N release sites. This variability across trials can be expressed as a variance, Var , which can be shown for this simple binomial process to be given by:

$$Var = N Q^2 P_r (1 - P_r) \quad (\text{Equation 2})$$

The variability across trials can also be expressed by a quantity called the coefficient of variation (CV), which is more convenient because it is unitless. CV is defined as:

$$CV = \text{standard deviation across trials} / \text{mean of all trials} \quad (\text{Equation 3})$$

The standard deviation is just the square root of Var . Hence, equations (1) and (2) can be substituted into the definition of CV (equation 3) to give the following result:

$$CV = \frac{Q \sqrt{N P_r (1 - P_r)}}{N P_r Q} = \sqrt{\frac{1 - P_r}{N P_r}} \quad (\text{Equation 4})$$

The key points here are that, for a simple binomial model of synaptic transmission,

- CV does not depend on the postsynaptic factor, Q (it cancels out)
- CV does depend on the presynaptic factor, P_r

- CV also depends on N , which may be either presynaptic (if it is interpreted as the number of vesicles available for release) or both pre- and postsynaptic (if it is interpreted as the number of structurally distinct release sites, each with an associated postsynaptic density)

This formalism suggests a method for determining the site of expression of synaptic plasticity (presynaptic or postsynaptic). The CV is calculated for a series of trials at the same synapse before and after the induction of plasticity, and the two CV values are compared. If they are equal, an alteration in the purely postsynaptic factor Q is implicated, because the CV is insensitive to changes in Q . On the other hand, if the two CV values are unequal, an alteration in the presynaptic factor P_r and/or the mixed factor N must have occurred. Clearly there is ambiguity in the case where the CV does change, because it could imply either a presynaptic or postsynaptic locus for the plasticity, at least according to one interpretation of N in the theory. No change in CV – implying a change in Q – therefore allows a more robust conclusion (although this, too, has been contested; *e.g.* Korn and Faber, 1991).

1.4.4.2 The variance-mean method

The variance-mean (VM) method, which is also based on the equations derived for the simple binomial model (*Equations 1 and 2*), was first developed for ion channels (Sigworth, 1980) then later extended to synaptic transmission (Clements and Silver, 2000). For the VM method, the mean synaptic response amplitude, I (*Equation 1*), and the variance of the response amplitude, Var (*Equation 2*), are measured as for the CV method. These measurements are then repeated for the same synapse after experimentally altering P_r , *e.g.* by changing the concentration of Ca^{2+} in the bath solution. Recordings made with about half a dozen well-spaced P_r values typically suffice (Ikeda and Bekkers, 2009). Var is then plotted against its

corresponding I in each P_r condition. The shape of this plot can be predicted from the binomial model by eliminating P_r from *Equations 1* and *2*:

$$Var = QI - I^2/N \quad (\text{Equation 5})$$

This equation describes an inverted parabola for which the initial slope gives Q and the curvature gives N . Knowing Q and N , the value of P_r corresponding to each I value can then be calculated by substituting into *Equation 1*. By repeating the entire analysis both before and after the induction of synaptic plasticity, this method can be used to find which quantal parameters change as a result of the plasticity (*e.g.* Reid and Clements, 1999). The VM method can also be used to compare the quantal parameters between different synapses; this will be done in *Chapter 4* of this thesis. Note that the VM method depends upon the same assumptions about the simple binomial model of quantal analysis as does the *CV* method, although elaborations of the simple theory have been described (Clements and Silver, 2000; Ikeda and Bekkers, 2009).

1.4.4.3 The paired-pulse ratio method

The paired-pulse ratio (*PPR*) method also draws upon the concepts of the quantal model, although in a qualitative way. Moreover, it depends implicitly on the assumptions of the simple binomial model (Korn and Faber, 1991). The *PPR* for a synapse is calculated by evoking two synaptic responses in quick succession (*e.g.* with an interstimulus interval of 10-50 ms) then dividing the mean amplitude of the second response by that of the first; this provides a simple measure of the amount of short-term plasticity that occurs. For many synapses, short-term plasticity is expressed presynaptically (Regehr, 2012; Jackman and Regehr, 2017). Moreover, *PPR* commonly varies inversely with P_r . That is, if P_r is lowered

(e.g. by reducing the extracellular Ca^{2+} concentration) then the PPR increases, probably because a smaller initial P_r gives more scope for P_r to increase on the second stimulus (Regehr, 2012). Regardless of the details of the mechanism, it is clear that any kind of synaptic manipulation which modifies P_r will also modify the PPR. However, the sensitivity of the PPR to this modification is not known *a priori*, limiting the reliability of this method (Korn and Faber, 1991).

In summary, all three methods described in this section draw upon the key concepts of the quantal model of synaptic transmission. All have been widely used in studies of synaptic plasticity (e.g. Baxter et al., 1985; Bekkers and Stevens, 1990; Malinow and Tsien, 1990; Isaac et al., 1995; Reid and Clements, 1999; Stricker et al., 1999; Clements and Silver, 2000; Emptage et al., 2003; Enoki et al., 2009). However, these methods must be used with caution because of the assumptions of the simple binomial model on which they are based. In this thesis, these methods will be used with the above provisos kept in mind.

1.4.5 Synaptic nonlinearity

Complex information processing systems depend on nonlinearities that can discriminate and modulate information flow. The efficacy of synaptic transmission is dynamic, resulting in nonlinear input-output functions at synapses. Along with dendrite-driven nonlinearities like action potential backpropagation or local NMDA spikes, there are also built-in nonlinearities that emerge from the characteristics of the synaptic hardware.

As discussed, when neurotransmitter is released it must be cleared from the synaptic cleft and repackaged into vesicles. Depending on factors like activation frequency and presynaptic structure, this process can result in varying levels of release-ready

neurotransmitter, causing nonlinear postsynaptic responses. When activated, synaptic input increases conductance and decreases the membrane time constant. As a result, when postsynaptic responses increase in amplitude, the driving force across the membrane reduces until it disappears when the local membrane potential is equal to the reversal potential for the synapse in question. Synaptic saturation of this kind is another example of synaptic nonlinearity that dynamically regulates information flow through neuronal circuits.

1.4.6 Synaptic integration

Dendrites can receive thousands of synaptic inputs. In most neurons, a single synaptic input in isolation is unable to drive neuronal output. As such, inputs must be integrated to drive the membrane potential toward, or away from, action potential threshold. Summating synaptic inputs is not merely a passive process; neuronal dendrites also possess active (voltage-dependent) mechanisms allowing complex synaptic interactions throughout the dendritic arbour (*Fig. 1.8*; London and Häusser, 2005).

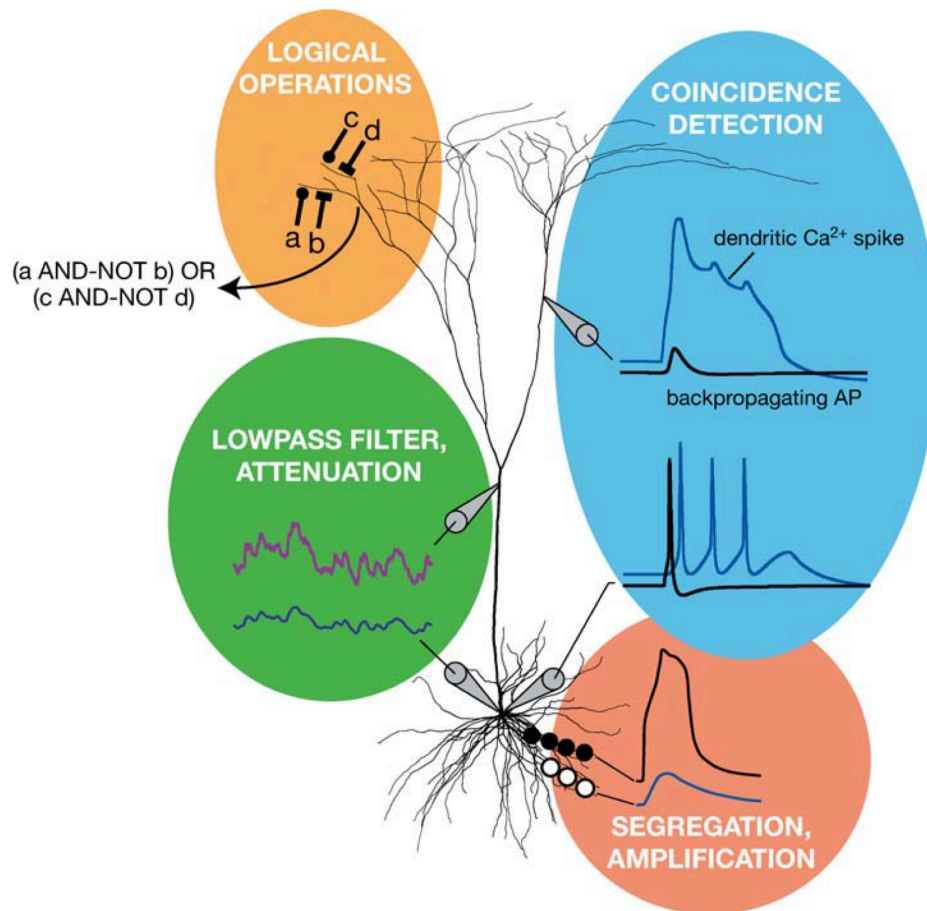


Figure 1.8. Dendritic mechanisms. Computational “toolkit” dendrites employ to transform and integrate synaptic input includes both passive (voltage-independent) and active (voltage-dependent) mechanisms. From: London and Hausser (2005).

Passive dendrites act as low-pass filters, attenuating and smoothing postsynaptic potentials as they propagate through the dendritic tree. Attenuation distance will directly influence the shape of postsynaptic potentials at the soma, in effect ‘labelling’ inputs from different parts of the dendrite (Rall, 1964; London and Hausser, 2005). Furthermore, the shape of postsynaptic potentials directly affects somatic output trains, meaning that equivalent input from different parts of the dendritic tree can have varying influences on neuronal output (Fetz

and Gustafsson, 1983). Active dendrites have the capacity to amplify and segregate certain types of synaptic input through mechanisms like synaptic scaling (Magee, 2000), subthreshold boosting (Migliore and Shepherd, 2002), and local and global dendritic spiking (Golding and Spruston, 1998; Poirazi and Mel, 2001; Williams and Stuart, 2002). It has also been proposed that basic logical operations, equivalent to those used in computer systems, are implemented at dendritic branch points through specific spatial and temporal combinations of excitatory and inhibitory inputs (Koch et al., 1983; Koch and Segev, 2000; London and Hausser, 2005).

Due to their relatively small diameter, studies directly probing active dendritic processes in the principal neurons of the PCx have been limited and contradictory. An initial report from Bathellier and colleagues, using direct patch-clamp recordings, suggested that principal dendrites in the PCx are only weakly active (Bathellier et al., 2009). Although small and fast local dendritic spikes were observed, there was no evidence of dendritic Ca^{2+} or NMDA spikes. In contrast, more recent studies by Kumar and colleagues have reported that local NMDA spikes can be induced at distal principal dendrites in the PCx, serving to amplify OB inputs and impose combination selectivity among them (Kumar et al., 2018). Furthermore, repetitive activation of NMDA spikes has been shown to induce robust synaptic potentiation of OB inputs, suggesting a critical role in the processing and storage of olfactory information (Kumar et al., 2021).

The apparent contradiction between these findings may be due to different experimental conditions. Both groups used *in vitro* preparations. However, Bathellier and colleagues (2009) recorded directly from principal dendrites and activated synaptic input through extracellular stimulation of M/T cell axons via the LOT, whereas Kumar and colleagues (2018, 2021) recorded putative dendritic spikes from the soma and stimulated proximal to the distal dendritic arbour of principal cells, making clustered stimulation of excitatory inputs more likely.

Although this stimulation method is more artificial, the experiments of Kumar and colleagues (2018, 2021) show that the distal dendrites of principal neurons in the PCx have the intrinsic capacity to produce NMDA spikes. However, whether they occur in the live animal and under what conditions remains unclear. In *Chapter 6* of this thesis I will explore this question by making targeted whole-cell recordings from the distal dendrites of SL and SP cells *in vitro* under *in vivo*-like conditions.

1.5 Synaptic plasticity in the PCx

Synaptic plasticity is an activity-dependent phenomenon that describes the ability of chemical synapses to modify their strength. The process of adapting synaptic strength is not only necessary for regulating information flow through neuronal circuits, but is also a critical feature of learning and memory.

Traditionally, plasticity research has focused on glutamatergic synapses in the hippocampus, which are particularly sensitive to synaptic enhancement (Malenka, 1994). However, we now know that synaptic plasticity is a general feature of synapses across brain regions and cell types (Malenka and Bear, 2004). In the PCx, the densely connected *assn* circuitry is highly plastic, an important feature for the integration and storage of olfactory information (Kanter and Haberly, 1990, 1993; Kapur and Haberly, 1998; Poo and Isaacson, 2007; Strauch and Manahan-Vaughan, 2017). In contrast, *aff* synapses are far more stable, with early reports suggesting that synaptic potentiation cannot be expressed later than the first month of development (Franks and Isaacson, 2005; Poo and Isaacson, 2007). Recently, however, robust potentiation has been demonstrated at *aff* synapses, emphasising the need for a more detailed analysis of the induction, expression and maintenance of *aff* plasticity in the PCx (Kumar et al., 2021).

1.5.1 Short-term plasticity in the PCx

With a timescale from seconds to minutes, short-term plasticity is bidirectional, resulting in either a temporary increase or decrease in the strength of synaptic transmission. As well as playing a role in particular types of short-term memory, plasticity of this kind allows for the dynamic regulation of information flow through neuronal circuits.

In the PCx, *aff* inputs delivered to SL and SP cells have distinct short-term plasticity profiles (Suzuki and Bekkers, 2006, 2011; Bekkers and Suzuki, 2013). In contrast to SL cells, synaptic inputs to SP cells strongly facilitate during short-latency repeated activation (<100 ms). Traditionally, synaptic facilitation of this kind is thought to reflect the baseline vesicle release probability at the synapse (Jackman and Regehr, 2017). Low release probability inputs are typically enhanced for a short period following an initial activation due to residual presynaptic calcium and a higher number of readily releasable vesicles of neurotransmitter (Katz and Miledi, 1968; see *Section 1.4.4.3*). In contrast, high release probability inputs tend not to facilitate, and may even depress, after an initial activation due to a depletion of neurotransmitter following the high-release episode (Oleskevich et al., 2000). Although short-term synaptic facilitation is a presynaptic phenomenon, postsynaptic contributors cannot be ruled out (*e.g.* short-term synaptic enhancement through the relief of polyamine blocked Ca^{2+} permeable AMPA receptors; Rozov and Burnashev, 1999). The disparate effects at major input streams to the PCx may indicate that *aff* synapses to SL and SP cells are distinct, both pre- and postsynaptically, and as a result may be processing or representing different types of olfactory information. This will be explored in *Chapter 4* and *5* of this thesis.

1.5.2 Long-term plasticity in the PCx

Canadian psychologist Donald Hebb first proposed the idea that coincident activity between a pair of neurons is required for long-term plasticity, a process he supposed might underlie learning and memory (Hebb, 1949). Many forms of long-term synaptic plasticity in the brain are now known to involve NMDA receptors (Lüscher and Malenka, 2012). Given that NMDA receptor activation requires both the binding of glutamate (typically delivered by presynaptic vesicles) and sufficient depolarisation of the postsynaptic membrane, the NMDA receptor is perfectly suited to detect coincident pre- and postsynaptic activity of the kind proposed by Hebb.

The processes involved in classical NMDA receptor-dependent long-term plasticity are well understood (Malenka and Nicoll, 1993). As well as being permeable to monovalent cations, NMDA receptors allow Ca^{2+} to flow into the postsynaptic neuron (*Fig. 1.9*). Ca^{2+} then interacts with calcium/calmodulin-dependent kinase II (CaMKII) which prompts an intracellular cascade leading to either the phosphorylation and insertion of additional α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, leading to long-term potentiation (LTP), or the removal of pre-existing AMPA receptors from the postsynaptic density, leading to long-term depression (LTD). This change in AMPA receptor density affects the sensitivity of the subsynaptic membrane to synaptically released glutamate and results in a persistent increase or decrease in synaptic strength (Chater and Goda, 2014). Structural changes, like postsynaptic receptor density, can have long-lasting effects on synaptic efficacy spanning hours, days, and possibly even the life of the organism.

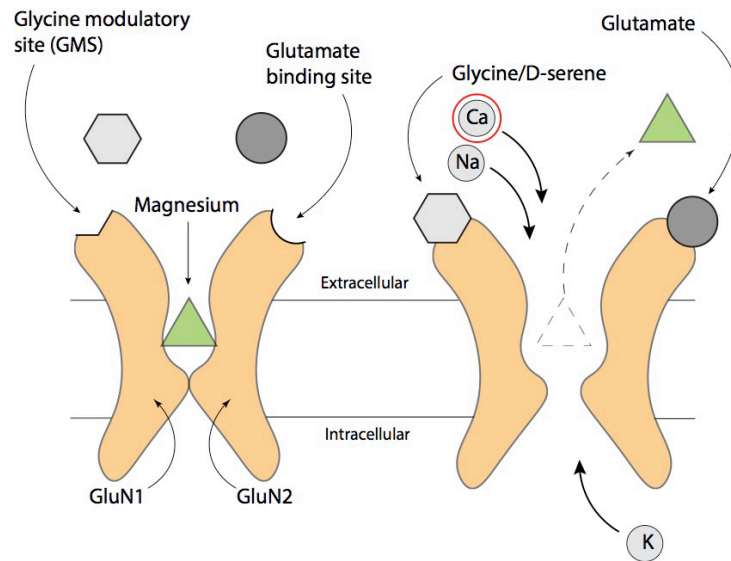


Figure 1.9. Schematic of the NMDA receptor and its key molecular interactions. NMDA receptor activation requires the binding of glutamate and glycine (or D-serine) to extracellular binding sites, as well as postsynaptic depolarisation to relieve the Mg^{2+} block. Given the requirement of both pre- and postsynaptic interaction, the NMDA receptor is well positioned to detect coincident activity between neurons. Once activated, NMDA receptors allow the flow of Na^+ , K^+ , and Ca^{2+} across the cellular membrane.

NMDA receptor-dependent LTP is an important property of *assn* synapses in the PCx, contributing to olfactory learning and memory as well as the salience of odour responses (Jung et al., 1990; Kanter and Haberly, 1990, 1993; Barkai and Hasselmo, 1997; Lebel et al., 2001; Linster and Hasselmo, 2001; Quinlan et al., 2004; Mukherjee et al., 2014; Strauch and Manahan-Vaughan, 2017). Using *in vitro* field recordings, Kanter and Haberly (1990) were among the first to demonstrate LTP in the PCx, showing NMDA receptor-dependent LTP at both *aff* and *assn* inputs. In a subsequent study, an associative form of LTP was demonstrated by coactivating *aff* and *assn* synapses, which terminate on the distal and proximal dendrites of principal neurons respectively. Although homosynaptic plasticity was reliably induced, associative LTP at both input streams could only be evoked when $GABA_A$ receptors were

locally blocked, suggesting inhibitory regulation of associative LTP (Kanter and Haberly, 1993). More recent studies by Canto-Bustos and colleagues demonstrate that disinhibitory circuits mediated by VIP interneurons gate synaptic plasticity in the PCx during the formation of odour representations, further highlighting the role of inhibition in certain forms of PCx plasticity (Canto-Bustos et al., 2022). Synaptic plasticity within the PCx is also sensitive to neuromodulation. In studies combining electrophysiology and biophysical network simulations, cholinergic modulation has been proposed as a key regulator of associative memory performance. By enhancing both LTP and LTD within the *assn* fiber system, it has been hypothesised that acetylcholine increases the stability of odour representations (Hasselmo and Barkai, 1995; Barkai and Hasselmo, 1997).

These and other experiments confirm that the PCx is a highly plastic system. However, most of the work in this field has focused on the *assn* network. Early sensory experience, and therefore *aff* synaptic transmission, is critical during the development of excitatory circuits in the PCx (Franks and Isaacson, 2005). To understand developmental effects on *aff* potentiation, Poo and Isaacson (2007) used *in vitro* patch clamping to measure *aff* LTP across the first month of post-natal development. The level of LTP induced at *aff* synapses continually declined over this period, such that *aff* connections failed to express any long-term plasticity four weeks after birth. Studies by Franks and Isaacson (2005) showed that the critical period coincided with changes in the constituents of *aff* synapses. Importantly, an experience-dependent down regulation of the NMDA receptor contribution to synaptic transmission, coupled with a higher number of AMPA receptors, increased the threshold for *aff* LTP induction as the animal matured. These findings provide a mechanism explaining the regulation of *aff* LTP through early development. However, they do not offer a satisfactory explanation for more recent reports of *aff* LTP beyond the critical period.

In freely behaving animals, Strauch and Manahan-Vaughan (2017) demonstrated that *aff* synapses could be reliably potentiated following 2 weeks of sensory deprivation (naris closure). The structure of NMDA receptors was found to be stable over these 2 weeks, with no changes in the expression of NMDA receptor subunit protein. However, the halfwidth of action potentials did increase, suggesting that a greater neuronal sensitivity to depolarisation may account for the changing propensity to express synaptic plasticity (Strauch and Manahan-Vaughan, 2017). LTP has also been demonstrated *in vitro* following repetitive induction of dendritic NMDA-spikes stimulated using strong extracellular stimulation (Kumar et al., 2021). Although highly artificial, these are informative findings, suggesting that *aff* synapses in the PCx are not intrinsically fixed after development but, rather, are regulated by other cellular or circuit features. These ideas will be further explored in *Chapter 5* of this thesis.

1.5.3 Presynaptic expression of long-term plasticity

Classical long-term plasticity involves modifications in the density of postsynaptic receptors. However, enduring changes in neurotransmitter release from the presynaptic terminal also provides a powerful means to modify neuronal circuits (Bekkers and Stevens, 1990). Although less is known about presynaptic plasticity, it appears to be characterised by many of the same features as canonical postsynaptic plasticity, like associativity, structural changes and bidirectionality. The induction mechanisms of presynaptic changes are distinct from those driving postsynaptic plasticity, with evidence for both intrinsic mechanisms within the presynaptic terminal as well as retrograde signalling from the postsynaptic side (Yang and Calakos, 2013).

Given that electrophysiological recordings are generally made from the postsynaptic cell during plasticity protocols, finding reliable indicators of presynaptic modifications in synaptic transmission requires subtle analysis (Yang and Calakos, 2013). The first examples of presynaptic long-term plasticity were revealed in hippocampal mossy fibre and cerebellar parallel fibre synapses using coarse indicators like paired-pulse ratio (Staubli et al., 1990; Zalutsky and Nicoll, 1990). The phenomenon has since been demonstrated across brain regions and cell types using more sophisticated techniques. In the CA1 region of the hippocampus, it is well known that postsynaptic activity triggers LTP, however the locus of expression has long been debated. By analysing synaptic variability and the failure rate of synaptic transmission, Malinow and Tsien (1990) identified a significant enhancement of transmitter release following LTP induction. Furthermore, the induction of LTP, but not its expression, was blocked by the prolonged dialysis of the postsynaptic cell, demonstrating postsynaptic involvement, potentially through retrograde transmission, in presynaptic LTP induction (Malinow and Tsien, 1990). Optical quantal analysis has also revealed a presynaptic locus of LTP at Schaffer-associational synapses in the hippocampus. By imaging synaptically evoked Ca^{2+} transients before and after LTP induction, it was found that the probability of a single stimulus evoking a postsynaptic Ca^{2+} transient was significantly increased post LTP induction, providing compelling evidence for presynaptic enhancement at these inputs (Emptage et al., 2003).

We now know that long-lasting forms of presynaptic plasticity are ubiquitous throughout the nervous system, contributing to the computational repertoire of neuronal circuits (Kullmann and Lamsa, 2011; Yang and Calakos, 2013; Debanne et al., 2019). As a result, understanding the role presynaptic modifications may play in information processing and memory is essential in a complete theory of circuit function. *Chapter 5* of this thesis will

provide compelling new evidence for presynaptically-expressed LTP at specific synapses in the PCx.

1.6 Overall summary and aims

From the initial transduction of odour components in the nasal cavity, through to integrated representations in the cortex, we now have a remarkable understanding of how odours are transformed and processed within the early stages of the olfactory network. Among primary sensory areas, the PCx has a unique cellular architecture, displaying functional characteristics typically found in higher-level associational regions. As a result, elucidating how the PCx processes, encodes and stores sensory information will not only deepen our understanding of olfaction, but may also reveal fundamental features of information processing in distributed circuits.

Over the past half-century, electrophysiological investigations of the PCx have been fruitful. We now know, for example, that the PCx integrates sensory input from the olfactory bulb to form holistic representations of odour that are thought to be stored through selective synaptic potentiation within its dense *assn* network. Despite our growing understanding of processing within the PCx, models of how sensory information is delivered into this system remain rudimentary. We know that powerful, yet sparse, sensory inputs from the OB target principal neurons in the PCx, but the structural basis and specific cellular targets of these inputs is unknown.

Until recently, sensory synapses in the PCx were thought to be ‘hard-wired’, showing very little scope for plasticity after a critical period in early development. However, it appears this view has substantial limitations, with more recent data suggesting that persistent synaptic

potentiation can be induced at sensory synapses following repetitive local NMDA spikes. Even so, these experiments use strong extracellular stimulation to evoke plasticity which may not accurately represent the sparse inputs these synapses receive *in vivo*. As a result, understanding what types of sensory plasticity are expressed in the PCx and under what conditions it may manifest *in vivo* will require more subtle analysis. Overall, it is clear that an ultimate understanding of how the PCx processes and stores olfactory information will critically depend on a more thorough appreciation of how sensory information is delivered into the PCx system.

The primary focus of this thesis is to elucidate how sensory information from the olfactory bulb is delivered to its primary targets in the PCx, semilunar (SL) and superficial pyramidal (SP) cells. As such, I had 4 broad aims:

Aim 1: *To quantify the dendritic structure and the distribution of dendritic spines along the apical axis of SL and SP cells (Chapter 3).*

Identified SL and SP cells will be filled with biocytin, fixed, then imaged using high-resolution confocal microscopy. Cells will then be traced and reconstructed in 3-dimensions to obtain detailed measurements of dendrite and spine morphology. Given existing qualitative descriptions of SL and SP cell morphology, I hypothesise that the two cell types will differ markedly in spine morphology and distribution, with SL cells possessing larger and fewer spines than SP cells.

Aim 2: *To extend the findings from Aim 1 by examining the functional properties of synapses delivering information to SL and SP cells (Chapter 4).*

This will be achieved using whole-cell patch clamping of SL and SP cell somas to measure postsynaptic responses to minimal extracellular stimulation, isolating unitary *aff* and *assn* inputs. I hypothesise that the powerful *aff* input previously reported will be specifically observed in SL cells, associated with the large dendritic spines that have been observed on these cells. As a result, I expect that fewer *aff* inputs will be required to lift SL cells to action potential threshold than SP cells, suggesting distinct roles in olfactory coding at the first layer of PCx processing.

Aim 3: *To establish the intrinsic ability of sensory synapses targeting SL and SP cells to express synaptic plasticity (Chapter 5).*

Synaptic plasticity will be induced using patterned extracellular stimulation, and synaptic responses will be measured using both local field potential and whole-cell voltage-clamp recordings *in vitro*. Unlike previous plasticity studies at these inputs, I will isolate unitary inputs using minimal stimulation. As well as more accurately replicating sparse OB input, these experiments will allow for the differentiation of pre- and postsynaptic modifications, elucidating subtle, physiologically relevant plasticity mechanisms. I hypothesise that SL and SP cells will exhibit distinct plasticity rules given their expected differences in synaptic structure, function and connectivity within the circuit.

Aim 4: *To explore the electrical properties of SL and SP cell dendrites (Chapter 6).*

This will be done by making whole-cell somatic and dendritic recordings from identified SL and SP cells while incrementally increasing synaptic input using extracellular stimulation to the LOT. Given the increased excitability provided by intracortical circuitry, I hypothesise that synaptically-driven active events will be easier to induce when both *assn* and *aff* circuits are intact.

Chapter 2

Methods

2.1 Animals

The mice used for this research were bred under specific pathogen-free conditions by the Australian Phenomics Facility at The Australian National University. All experiments used 30-40 day-old positive (+/-) or negative (-/-) GAD67-GFP (Δ neo) transgenic mice bred on a C57BL6/J background (Tamamaki et al., 2003). All mice were housed on a 12-hour light-dark cycle and had free access to food and water.

2.2 Slice preparation

Mice were anaesthetised using 2% isoflurane in oxygen and then rapidly decapitated, using procedures approved by the Animal Experimentation Ethics Committee of The Australian National University (Protocol A2018/37). The brain was removed with care to avoid damaging the ventral surface and was immediately placed in ice cold slicing solution comprising (mM) 87 NaCl, 3 KCl, 0.25 CaCl₂, 3 MgCl₂, 25 NaHCO₃, 1.25 NaH₂PO₄, 25 D-glucose, and 75 sucrose.

Most experiments used coronal slices of the anterior PCx. To prepare for coronal slicing, the brain was positioned with the ventral surface facing up. The caudal portion was then removed with a single coronal cut approximately 1 mm above the cerebellum (*Fig. 2.1A*, black dashed line). The rostral portion of the brain was then glued, ventral end down, to the stage of the slicer (7000 smz, Camden Instruments, Loughborough, UK) with the ventral surface facing the blade. With the brain secured, the slicing chamber was immediately filled with ice cold slicing solution (as above) and was continually bubbled with carbogen (5% CO₂/95% O₂). Beginning 1 mm below the OB (*Fig. 2.1A*, red dashed line), 300 μ m thick

coronal slices were prepared through the anterior PCx (*Fig. 2.1A*, pink highlight). Coronal slices were immediately hemisected (*Fig. 2.1B*, vertical dashed line).

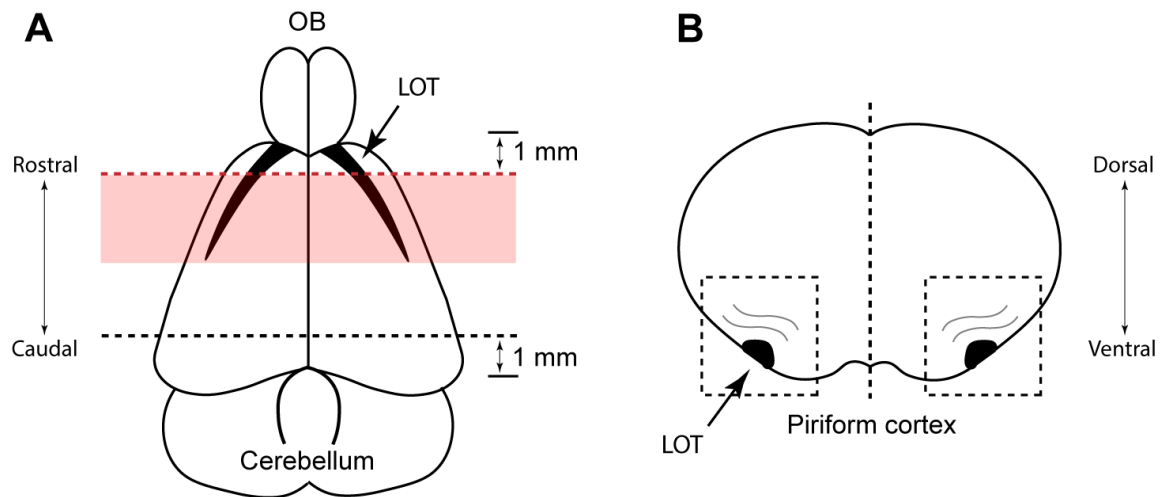


Figure 2.1. Preparing coronal slices of the anterior PCx. *A)* Schematic of the ventral surface of the mouse brain. The caudal portion of the brain was resected 1 mm above the cerebellum (black dashed line). Coronal slices (300 μ m thick) were taken starting from 1 mm below the base of the olfactory bulb (OB; red dashed line) through the length of the anterior PCx (pink highlight). *B)* The PCx is located on the ventral surface of coronal slices (dashed boxes). Coronal slices were hemisected (vertical dashed line). Lateral olfactory tract, LOT; olfactory bulb, OB.

Some experiments used parasagittal slices. To prepare for parasagittal slicing, the caudal portion of the brain was removed (as for coronal slices). A second cut was made parallel to, and approximately 5 mm below, one of the lateral olfactory tracts (LOTs) projecting from the OB (*Fig 2.2A*, blue dashed line). The cut side was then glued to the slicing stage with the rostral end facing the blade. With the brain secured, the slicing chamber was immediately filled with ice cold slicing solution and was continually bubbled with carbogen (5% CO₂/95%

O₂). Beginning slightly above the chosen LOT, 300 µm thick slices were prepared through the width of the LOT (*Fig. 2.2A*, pink highlight).

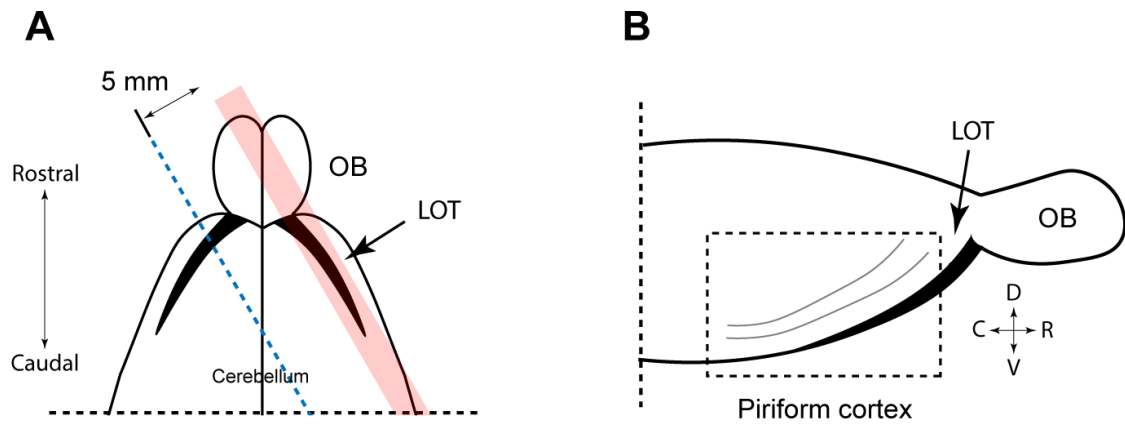


Figure 2.2. Preparing parasagittal slices of the anterior PCx. *A)* Schematic of the ventral surface of the mouse brain. The caudal portion of the brain was resected 1 mm above the cerebellum (as per coronal slice protocol, *Fig. 2.1*). A second cut was made approximately 5 mm below, and parallel to, the chosen LOT (blue dashed line). Slices (300 µm thick) were taken starting above, and extending through, the width of the LOT (pink highlight). *B)* The PCx is located on the ventral surface of parasagittal slices (dashed box). Lateral olfactory tract, LOT; olfactory bulb, OB; dorsal, D; rostral, R; ventral, V; caudal, C.

To allow for recovery, slices were incubated at 34°C for 45 min in carbogen-bubbled artificial cerebrospinal fluid (ACSF) comprising (mM) 125 NaCl, 3 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄·H₂O, 2 CaCl₂, 1 MgCl₂, 25 D-glucose, 2 (+)- sodium L-ascorbate and 3 sodium pyruvate. Following incubation, slices were maintained at room temperature until use.

2.3 Morphology

2.3.1 Filling cells

For morphological reconstructions, internal solution comprised (mM): 135 KMeSO₄, 4 KCl, 1 NaCl, 10 HEPES, 0.1 EGTA, 5 phosphocreatine Na₂, 2 Mg₂ ATP, 0.3 Na₃ GTP, 10 D-sorbitol, 0.4% biocytin and KOH for pH 7.2. Whole-cell configuration was maintained for at least 30 min when filling a cell with biocytin, then the electrode was carefully withdrawn, allowing the membrane to seal over. The slice was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hour, then stored in PBS at 4°C until processing. Only one cell was filled per slice.

2.3.2 Neuron processing and imaging

Standard techniques were used for processing the filled cells (Suzuki et al., 2014). Slices were placed in permeabilization solution (0.3% Triton X-100 plus 1% bovine serum albumin in PBS) for 1 hour, then incubated in streptavidin-Alexa-594 (Thermo Fisher Scientific, Australia; diluted 1:1000 in the permeabilization solution) for 18 hours on a shaker at room temperature. After being washed in PBS, slices were then mounted in Vectorshield (Vector Laboratories, Newark, USA) on glass slides. A Zeiss LSM 800 Airyscan with a Zeiss Observer Z1 inverted microscope was used to image the filled cells (*Fig. 2.3*; Carl Zeiss Australia, Sydney, Australia). Low magnification images of the entire neuron used a Zeiss 10x/0.3 EC Plan-Neofluar objective to acquire a z-stack of 1024 x 1024 pixel (638.9 x 638.9 μ m) frames at 6.46 μ m intervals, averaging 4x per frame (pixel size 0.62 μ m). High magnification images of selected dendritic segments in layers 1a and 1b used a Zeiss 63x/1.2 water immersion C-Apochromat objective and AiryScan imaging mode to acquire a z-stack of

1464 x 1464 pixel (67.6 x 67.6 μm) frames at 0.25 μm intervals without averaging (pixel size 0.05 μm ; example, *Fig. 2.3*). The power of the laser (561 nm) was kept low (0.25-0.3%) to minimize bleaching, and the gain and offset were carefully adjusted to optimize the dynamic range without saturation. At the end of data acquisition, Airy processing was applied to yield the high-resolution image stack in CZI format that was used for spine analysis (Huff, 2015).

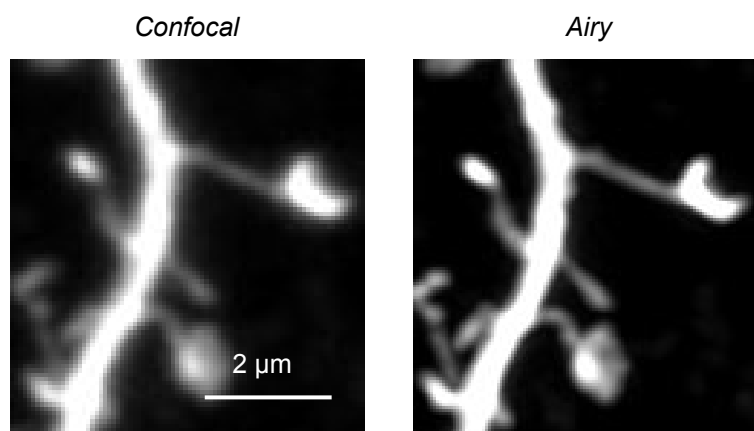


Figure 2.3. Airyscan processing yields higher resolution images. The same field of view imaged in regular confocal mode (left) and with Airy processing (right) on a Zeiss LSM 800 Airyscan microscope. Both images were acquired with a 63x/1.2 water immersion objective (Zeiss C-Apochromat). The confocal image is the average of 2 scans, the Airy image is a single scan. This dendritic segment was from a superficial pyramidal cell in layer 1a of the piriform cortex.

Neurolucida 360 version 2019.1.2 (64-bit) was used to perform all morphological tracing and reconstruction (MBF Bioscience, Williston, USA). Software was run on an Intel (R) Core (TM) i7-4770 CPU processor at 3.40 GHz on 8 cores, 16 GB installed memory (RAM), AMD Radeon HD 8570 video card and 1 GB video memory. CZI image files were

opened in the Neurolucida 3D environment, where all dendrite tracing and spine identification was completed. Display adjustments were made to enhance images using the ‘gamma’ and ‘white point’ toggles; ‘black point’ was always set to zero. Dendrites were traced using the user-guided directional kernels method, after which manual adjustments were made in three dimensions to ensure best fit. Dendritic spines were first detected automatically, then individually checked for fit and exclusion. Detected spines with no distinct spine head were batched as filopodia and were excluded from analysis because they were too thin to be measured reliably. Analysis of filopodia would require higher-resolution imaging which was not feasible in the studies presented. Detector sensitivity was adjusted as necessary for individual spines to correct for under- or over-detection. Once confirmed, dendrite and spine renderings were smoothed and data were exported for further analysis.

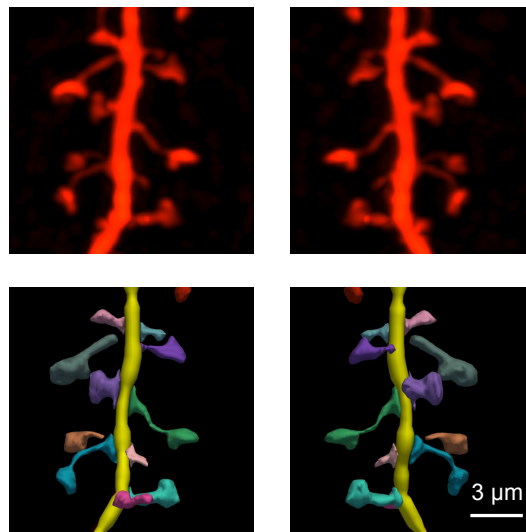


Figure 2.4. *Images were reconstructed in 3-dimensions using Neurolucida 360. Top, Typical dendritic segment (here, from a superficial pyramidal cell in layer 1a) as it appears after imaging with the Airyscan confocal microscope. The same segment is shown before and after rotating by 180°. Bottom, Reconstruction of the segment shown above.*

2.4 Electrophysiology

The experiments presented in this thesis include both local field potential (LFP) and whole-cell patch clamp recordings made *in vitro*. Slices were secured in the microscope chamber using a platinum harp with fine nylon strings. Infrared video microscopy using a differential interference contrast microscope (Olympus BX50WI) was used to image slices for all experiments. For the duration of each experiment, slices were continually perfused with carbogen-bubbled ACSF (2-3 mL/min), warmed to $32^{\circ}\text{C} \pm 2^{\circ}\text{C}$ to approximate physiological conditions. For synaptic transmission experiments, ACSF comprised (mM): 125 NaCl, 3 KCl, 25 NaHCO_3 , 1.25 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2 CaCl_2 , 1 MgCl_2 , 25 D-glucose. For synaptic transmission experiments with strontium, ACSF comprised (mM): 125 NaCl, 3 KCl, 1 CaCl_2 , 4 SrCl_2 , 2.5 MgCl_2 , 25 NaHCO_3 , 1.25 NaH_2PO_4 , 2 ascorbic acid and 3 pyruvate. For LFP experiments, ACSF comprised either (mM): 125 NaCl, 3 KCl, 25 NaHCO_3 , 1.25 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2 CaCl_2 , 1 MgCl_2 , 25 D-glucose; or, (mM): 125 NaCl, 3 KCl, 25 NaHCO_3 , 1.25 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2 CaCl_2 , 0.5 MgCl_2 , 25 D-glucose. For unitary input synaptic plasticity experiments, ACSF comprised (mM): 125 NaCl, 3 KCl, 25 NaHCO_3 , 1.25 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2 CaCl_2 , 0.5 MgCl_2 , 25 D-glucose. For somatic NMDA-spike experiments, ACSF comprised (mM): 125 NaCl, 3 KCl, 25 NaHCO_3 , 1.25 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2 CaCl_2 , 1 MgCl_2 , 25 D-glucose. For dendritic recordings, ACSF comprised (mM): 125 NaCl, 3 KCl, 25 NaHCO_3 , 1.25 $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2 CaCl_2 , 0.5 MgCl_2 , 25 D-glucose.

Borosilicate glass capillaries (GC150F, Harvard Apparatus, Holliston, USA) and a micropipette puller (P-97, Sutter Instrument, Novato, USA) were used to make electrodes. For synaptic transmission experiments, LFP experiments and whole-cell plasticity experiments, electrode resistance was 4-8 $\text{M}\Omega$ when filled with internal solution. For whole-cell dendritic

recordings, electrode resistance was 30-50 M Ω when filled with internal solution. Electrodes were mounted in a pipette holder attached to a micromanipulator (MP-285, Sutter Instrument). A chlorided silver wire within the pipette holder provided contact to the internal solution, and the circuit was completed with a separate chlorided wire in the bath solution. To ensure electrical stability, internal and bath wires were freshly chlorided using 1M KCl before each experiment and voltage offset was confirmed to be ± 2 mV at the end of each recording.

2.4.1 Local field potentials

LFP recordings were used to measure activity from synaptic populations (*Fig. 2.5*). For these experiments, recording electrodes were filled with 1M NaCl internal solution. LFP data was acquired using a MultiClamp 700A amplifier (Molecular Devices, San Jose, USA) operating in current clamp mode. Data was amplified (100-200x), filtered at 10 kHz, then digitised at 50 kHz by an ITC-18 interface (Instrutech/HEKA, Reutlingen, Germany) under the control of AxoGraph (AxoGraph Scientific, Sydney, Australia).

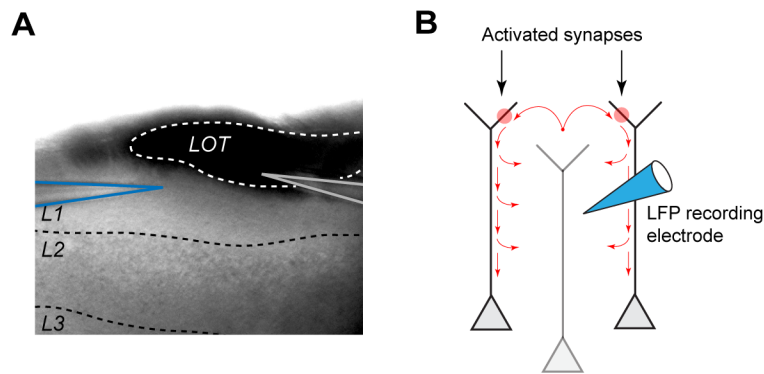


Figure 2.5. Local field potential recording configuration and theory. *A)* Afferent synapses were stimulated by placing a stimulating electrode (grey) in the LOT. LFP recording electrode (blue) was placed in L1a, where afferent synapses are exclusively located. *B)* When excitatory synapses are activated in the dendrites, an influx of ions depolarises the postsynaptic membrane at the site of activation. Dendritic membranes are porous and as a result, there is a persistent leakage of ions as the signal propagates towards the soma. These ion fluxes result in changes in the extracellular potential, which is detected by LFP recording electrodes. The voltage gradients in the extracellular space from which LFP recordings are taken will be influenced by the activity of all nearby cells; thus, the LFP reports the activity of a population of neurons/synapses surrounding the recording pipette.

2.4.2 Whole-cell patch clamp

Standard whole-cell patch clamp techniques were used to measure the activity of targeted cell types. Recording electrodes were filled with internal solution comprising (mM) 135 KMeSO₄, 4 KCl, 1 NaCl, 10 HEPES, 0.1 EGTA, 5 phosphocreatine Na₂, 2 Mg₂ ATP, 0.3 Na₃ GTP, 10 D-sorbitol and KOH for pH 7.2. When filling cells for morphological reconstruction, the internal solution also contained 0.4% biocytin. This solution was filtered (0.2 µm Millipore) immediately before use. Before immersing recording electrodes into the bath solution, positive pressure (3-4 kPa) was applied to the interior of the electrode to prevent blockage of the pipette tip. Upon entering the bath, pipette offset was zeroed, and the electrode

was lowered to contact the target neuron (soma or dendrite). Positive pressure was then released and suction (5-7 kPa) was briefly applied to form a tight seal ($\geq 1 \text{ G}\Omega$) on the membrane. Following the formation of a giga-seal, holding potential was set to -70 mV, and the cell was held in this formation for approximately 60 seconds to allow the seal to settle. Gentle pulses of suction were then applied to break the small piece of membrane beneath the electrode tip, allowing access to the interior of the neuron (*Fig. 2.6*). Access resistance of patch electrodes ($< 20 \text{ M}\Omega$ for somatic recordings; $< 100 \text{ M}\Omega$ for dendritic recordings) was monitored for stability throughout.

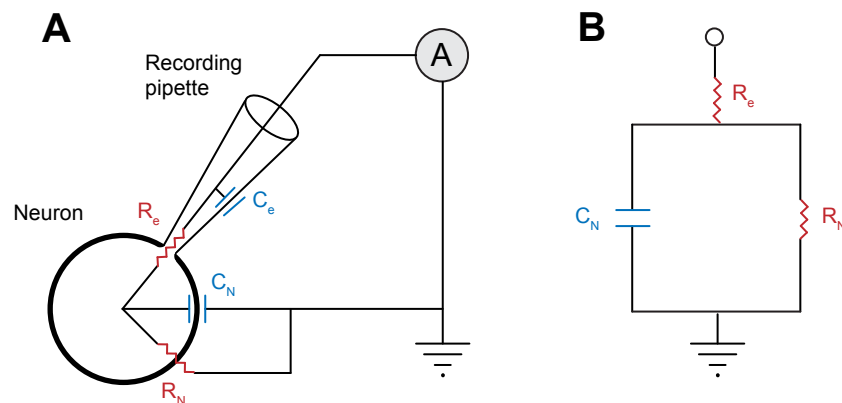


Figure 2.6. Whole-cell circuit configuration. *A)* Primary circuit properties include: electrode capacitance (C_e); electrode resistance (R_e); neuron capacitance (C_N); and, neuron resistance (R_N). The electrode within the glass pipette is connected to a MultiClamp 700A amplifier (A), and the circuit is completed with a grounding electrode in the bath. *B)* Simplified R-C circuit diagram for whole-cell recordings (excluding C_e).

2.5 Pharmacology

The preparation and concentration of the drugs used in the studies presented are summarised in *Table 2.1*. Baclofen is an agonist for the GABA_B receptor, which is selectively expressed presynaptically at *assn* synapses in the PCx. Hence, baclofen was added to the bath solution to suppress the *assn* circuitry and isolate *aff* synapses. Biocytin was loaded intracellularly for *post hoc* neuronal imaging. Gabazine is an antagonist of the GABA_A receptor and was used in the bath solution to globally block GABA_Aergic synaptic inhibition in the PCx. MK-801 is a non-competitive NMDA receptor antagonist and was used in the bath solution to globally block NMDA receptors. QX-314 was used intracellularly, via the internal solution, to block voltage-gated sodium channels in the patched neuron. Strontium was used to replace calcium in synaptic transmission experiments as it has been shown to evoke asynchronous neurotransmitter release from activated synapses (Bekkers and Clements, 1999). 4-AP is a non-selective blocker of voltage-gated potassium channels and was used in the bath solution to globally increase the excitability of the PCx. Further details about the rationale for using these compounds will be given in the Results.

Drug (powdered)	Stock solvent	Final concentration in ACSF
Baclofen	ACSF	100 μ M
Biocytin	Internal solution	0.4%
Gabazine	ACSF	20 μ M
MK-801	ACSF	20 μ M
QX-314	Internal solution	10 mM
Strontium	ACSF	4 mM
4-AP	ACSF	2 mM

Table 2.1: List of drugs used, including solvent used and final concentrations used in experiments.

2.6 Cell identification

The whole-cell recordings presented required the accurate identification of semilunar (SL) and superficial pyramidal (SP) cells. Although transitional cell types occur across layer 2 of the PCx, SL and SP cells can generally be distinguished by their soma shapes and locations, along with their intrinsic electrical properties (Suzuki and Bekkers, 2006, 2011). The experiments presented in this thesis targeted somas at the extreme ends of layer 2 to avoid the ambiguity of transitional cell types, which are more commonly found in the middle of layer 2. SL cells were targeted in layer 2a and have circular somas (*Fig. 2.7*), whereas the somas of SP

cells are found in layer 2b and have more elliptical or pyramidal shaped somas. Under *in vitro* conditions, SL cells have a more depolarised resting potential (approximately -60 to -70 mV), whereas SP cells are typically more hyperpolarised at rest (typically between -70 and -80 mV). SL and SP cells also differ in their input resistance, with the smaller SL cells having input resistances $>150\text{ M}\Omega$ and the larger SP cells $<100\text{ M}\Omega$. Finally, the distinct firing properties of SL and SP cells can also be used to differentiate them. Notably, SL cells have a regular firing pattern and no after-depolarising potential (*Fig. 2.7A*), whereas SP cells fire irregularly and display strong afterdepolarising potentials (*Fig. 2.7B*). To assess this, action potentials were initiated immediately after gaining whole-cell access by applying a series of 1-second-long current steps, beginning at -80 pA and increasing in 40 pA steps until action potential threshold was exceeded (*Fig. 2.7*).

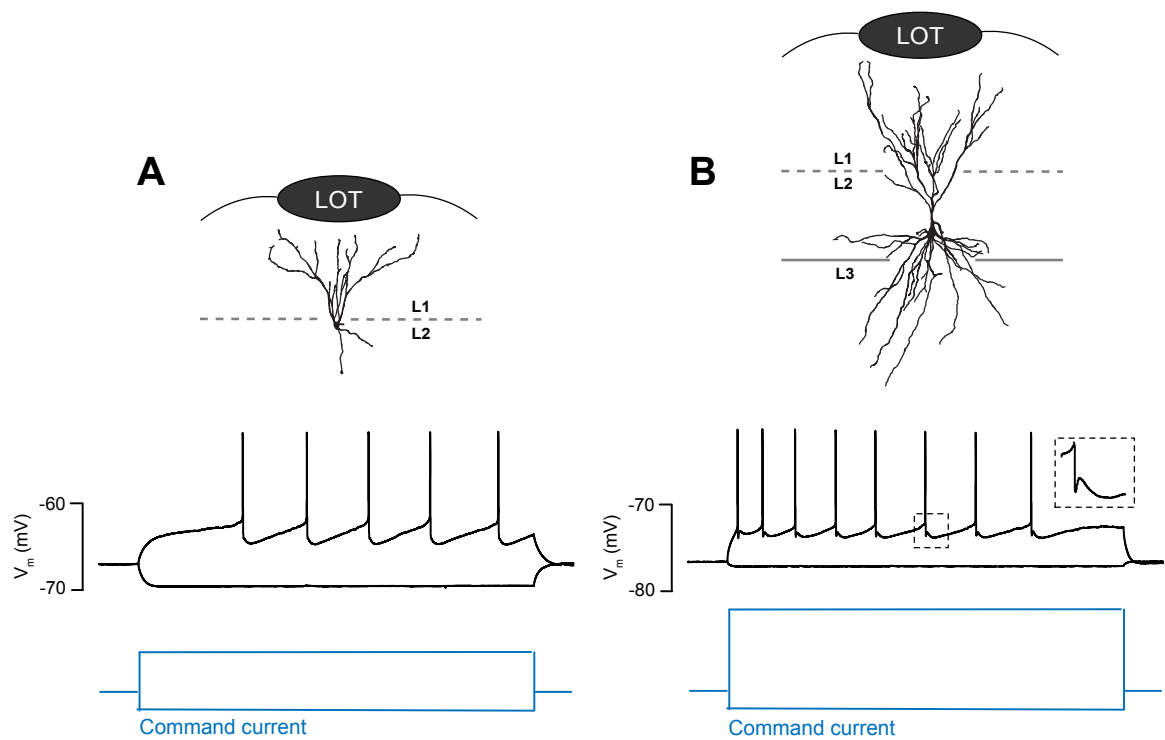


Figure 2.7. Identification of SL and SP cells. *A) Top*, SL cells are located in L2a, have circular somas, and typically only have apical dendrites. *Bottom*, SL cells have a typical resting potential of between -60 and -70 mV, have a delayed onset to first action potential at threshold, do not display after depolarisation, and fire in a regular pattern. *B) Top*, SP cells are located in L2b, have elliptical somas, and have both apical and basal dendrites. *Bottom*, SP cells have a typical resting potential of between -70 and -80 mV, display strong afterdepolarisation (dashed inset), and fire in an accommodating pattern.

2.7 Stimulation protocols

2.7.1 Extracellular synaptic stimulation

Stimulating electrodes were made from glass pipettes (tip diameter of 2-5 μm) wrapped with copper wire and coated in silver paint. Stimulating pipettes were filled with 1M NaCl which provided electrical connection between the internal wire and external conductor to act as a concentric bipolar stimulator (Bekkers and Clements, 1999). An isolated stimulator unit (DS2, Digitimer, Welwyn Garden City, UK) was used to deliver electrical pulses with fixed at 100 μs and adjustable amplitude ranging from 0-100 V. The experiments presented required the stimulation of either *aff* or *assn* inputs in the PCx, which was achieved by placing the stimulation electrode in either the LOT or in layer 2 of the PCx respectively.

2.7.2 Test pulse stimulation protocol

Unless otherwise specified, test pulse stimulation comprised two 100 μs pulses delivered 50 ms apart at 5 second intervals (*Fig. 2.8*). To monitor series resistance, membrane resistance and membrane capacitance, a hyperpolarizing voltage step was applied to the cell near the start of the sweep.

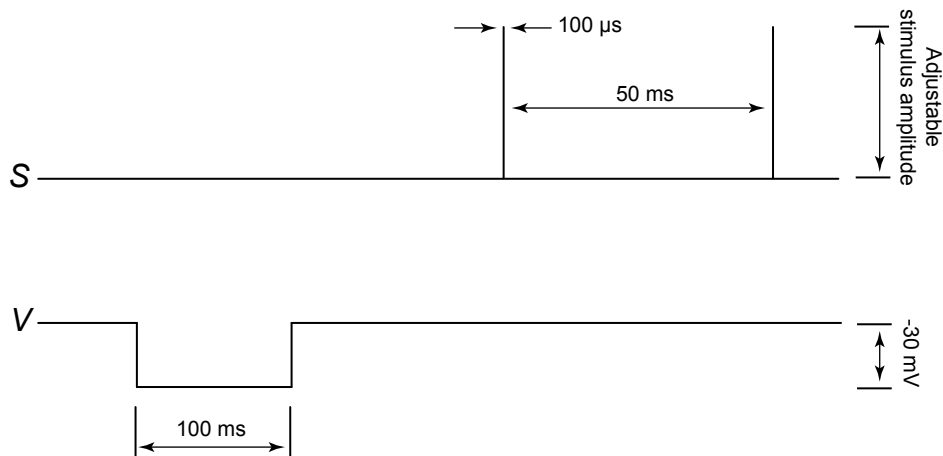


Figure 2.8. Test pulse stimulation protocol. The test pulse protocol used throughout this thesis had two components. *Top* ('S'), Stimulation applied *via* extracellular stimulator electrode. Paired pulses (100 μ s duration) were delivered 50 ms apart (unless otherwise specified) and stimulus amplitude was adjusted on the isolated stimulator unit. *Bottom* ('V'), Voltage clamp command step applied to the recorded cell, used to monitor series resistance, membrane resistance and membrane capacitance throughout the experiment. This had a fixed amplitude of -30 mV, duration 100 ms.

2.7.3 Long-term depression stimulation protocol

For local field potential (LFP) long-term depression (LTD) experiments, test pulses were presented until *f*EPSP amplitude and variability were stable for at least 10 minutes (amplitudes were monitored in real time using the online analysis function in AxoGraph). For whole-cell LTD experiments, test pulses were presented until EPSC amplitude and variability were stable for at least 5 minutes. Once a stable baseline had been established, test pulses were stopped and a low frequency stimulation (LFS) protocol was administered to induce LTD. The LFS protocol used for all experiments comprised a single pulse, presented at 1 Hz, for 15

minutes (900 episodes). During LFS, stimulus strength was unchanged from the baseline setting. To date there has been very little work on synaptic depression in the PCx. For this reason, I chose a classic depression protocol that has been widely used in other brain areas (Mulkey and Malenka, 1992; Kemp and Bashir, 2001).

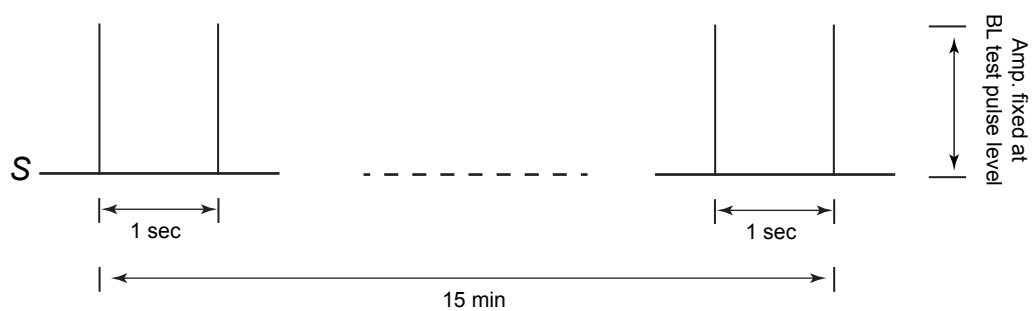


Figure 2.9. Long-term depression stimulation protocol. LTD induction involved single pulses, delivered at 1 Hz, for 15 minutes (900 episodes). Stimulus amplitude was fixed at the level chosen for baseline test pulse stimulation. Pulse width was set at 100 μ s for all experiments.

2.7.4 Long-term potentiation stimulation protocol

For LFP long-term potentiation (LTP) experiments, test pulses were presented until f EPSP amplitude and variability were stable for at least 10 minutes (amplitudes were monitored in real time using the online analysis function in AxoGraph). For whole-cell LTP experiments, test pulses were presented until EPSC amplitude and variability were stable for at least 5 minutes (cells where a stable baseline could not be established within 10 minutes of breaking

in were discarded to avoid washout effects). Once a stable baseline had been established, test pulses were stopped and a theta-burst stimulation (TBS) protocol was administered to induce LTP. The TBS protocol used in my experiments had episodes comprising 10x 10-pulse bursts separated by a start-to start interval of 200 ms; *i.e.* the bursts repeated at 5 Hz, which is the theta frequency. Each 10-pulse burst was delivered at 100 Hz and each pulse had a width of 100 μ s (*Fig. 2.10*). In the experiments presented, the TBS episode was repeated 5 times at 20 second intervals. TBS is a common stimulation method used to induce LTP and is particularly relevant for my purposes, as the theta frequency corresponds directly with the exploratory sniffing frequency of the rodent (Kanter and Haberly, 1990) and it mimics typical OB output *in vivo* (Suzuki and Bekkers, 2006).

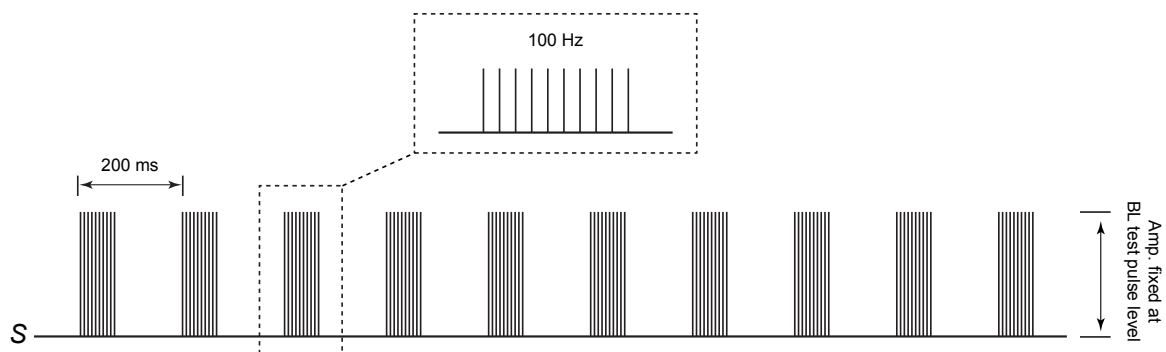


Figure 2.10. Long-term potentiation, theta-burst stimulation protocol. LTP induction involved episodes comprising 10 bursts of 10 pulses delivered at 5 Hz. Each 10-pulse burst was delivered at 100 Hz. For each TBS induction period, this episode was repeated 5 times with 20 seconds recovery between. Stimulus amplitude was fixed at the level chosen for baseline test pulse stimulation. Pulse width was set at 100 μ s for all experiments.

2.7.5 Minimal stimulation protocol

Minimal stimulation was used for synaptic transmission and whole-cell synaptic plasticity experiments (*Fig. 2.11*). Minimal stimulation is an extracellular stimulation technique used to isolate unitary inputs synapsing onto the recorded neuron (Raastad et al., 1992). Using the standard test pulse protocol (*Section 2.7.2*), I achieved unitary axon stimulation as follows. Stimulus intensity was initially set below postsynaptic response threshold, then the intensity was systematically increased in small increments until the first postsynaptic response amplitude and failure rate remained constant over a range of stimulus intensities. Further increase in stimulus strength produced a markedly larger response (*Fig. 2.11*, region c). The stimulus strength was then reduced slightly to ensure the response was reliably within the plateau region (*Fig. 2.11*, region b). This ‘response plateau’ is an indication that a single axon projecting to the target cell is reliably stimulated (Allen and Stevens, 1994).

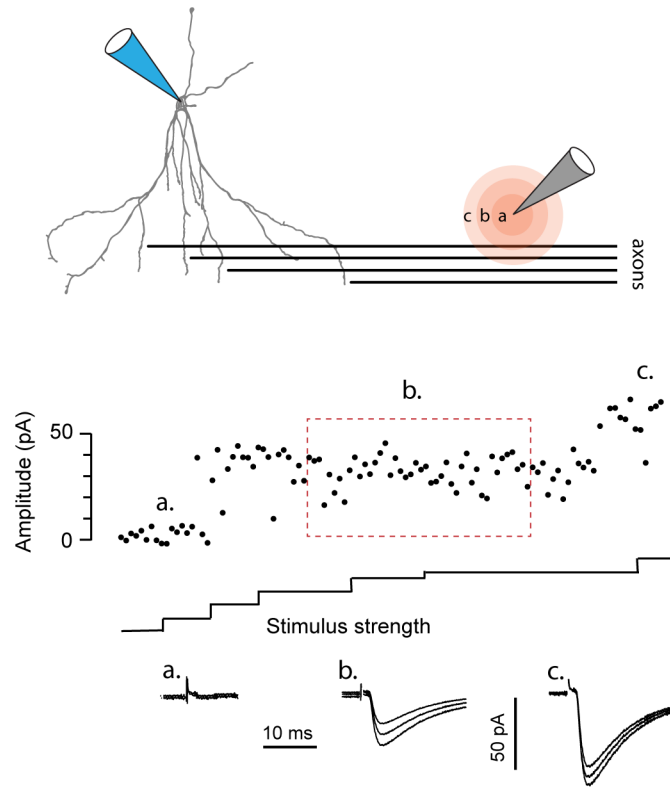


Figure 2.11. Minimal stimulation protocol and theory. *Top*, to achieve minimal stimulation, a stimulating electrode (grey) is placed into the extracellular space. Starting below threshold for a postsynaptic response (*i.e.* no axons projecting to the patched neuron have been activated; *a*, stimulation strength is gradually increased until the response amplitude stabilises over a range of stimulus intensities (*b*, red dashed box), indicating that only a single axon projecting to the recorded neuron has been activated. Further increase in stimulus intensity produces an increase in the amplitude of the recorded response, indicating that additional axons projecting to the recorded neuron have been recruited, *c*. The minimal stimulation experiments targeted a stimulus intensity around the middle of the first response plateau.

2.7.6 Paired-pulse ratio stimulation protocol

For paired-pulse ratio (PPR) experiments, I used a stimulation protocol similar to my test pulse protocol (*Section 2.7.2*) but varied the latency of the second pulse. These experiments included 10 different latencies (ms): 10, 30, 50, 70, 100, 150, 250, 400, 600, and 900. For each experiment, pulses at all latencies were presented 5 times using strong stimulation. As for the test protocol, a hyperpolarizing voltage step was presented at the start of each sweep to monitor series resistance, membrane resistance and membrane capacitance. See *Section 1.4.4.3* for PPR theory.

2.7.7 Somatic NMDA spike stimulation protocol

For somatic NMDA spike experiments, I used either a 3- or 5-pulse burst presented at 20 and 50 Hz in current-clamp mode (*Fig. 2.12*). Again, protocols of this kind have commonly been used in other brain areas to induce non-linear events (Schiller et al., 2000). To monitor series resistance, membrane resistance and membrane capacitance, a hyperpolarizing current step was injected into the cell near the beginning of each sweep. For these experiments, stimulus intensity was incrementally increased through the range of stimulus intensities (0-100 V), or until non-linear spiking occurred.

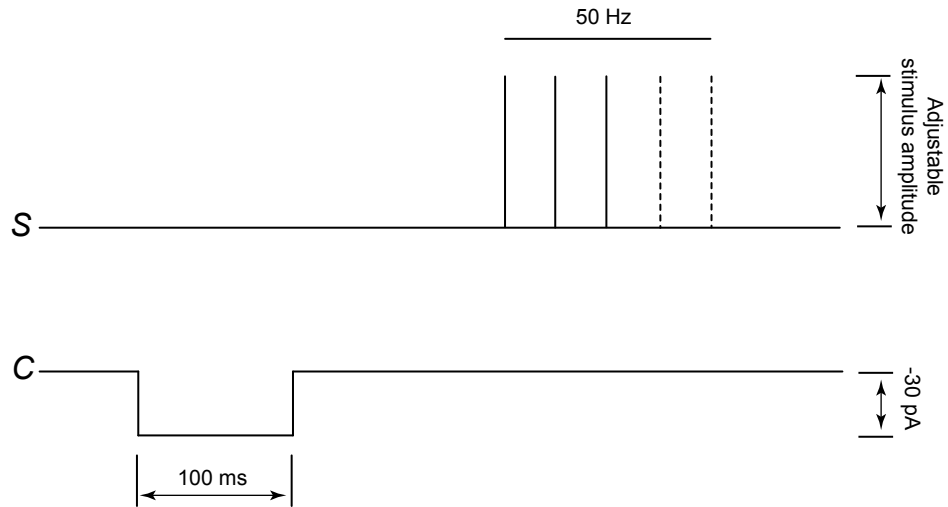


Figure 2.12. NMDA spike stimulation protocol. Top ('S'), 3 or 5 pulses were delivered at 50 Hz every 5 seconds, with increasing stimulus intensity. Bottom ('C'), a hyperpolarizing current injection was used to monitor series resistance, membrane resistance and membrane capacitance throughout. Stimulator pulse width was set at 100 μ s for all experiments.

2.7.8 Dendritic spike stimulation protocol

Dendritic recordings used the same protocols as somatic NMDA spike experiments (Section 2.7.7). Distal apical dendrites of SL and SP cells were patched in layer 1a (Fig. 2.13). Like somatic NMDA spike experiments, *aff* synapses were stimulated via the LOT and stimulus strength was incrementally increased through the range of intensities (0-100 V). Unlike somatic recordings, dendritic responses were recorded in both current- and voltage-clamp modes.

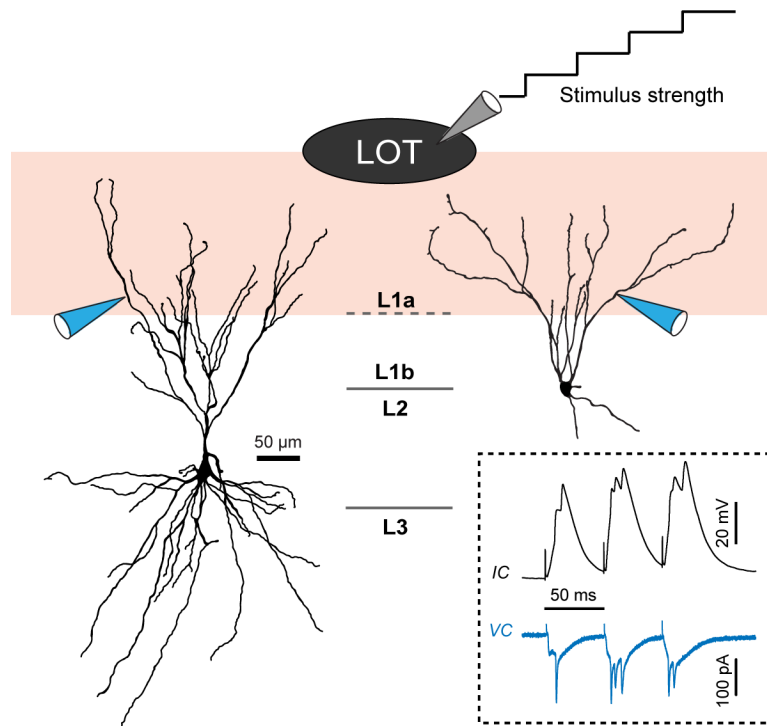


Figure 2.13. Dendritic recording protocol. Apical dendrites of SP (left) and SL (right) cells were patched in layer 1a (blue electrodes). Extracellular stimulation was applied to the LOT (grey electrode) and was incrementally stepped up through the range of stimulus intensities (0-100 V). Dendritic recordings were made in both current- and voltage-clamp modes (dashed box). Stimulation protocols followed those in *Fig. 2.12*.

2.8 Neuronal modelling

Modelling was done using custom code written in *hoc* running under NEURON 8.1 (<https://neuron.yale.edu>). A typical SL cell and SP cell were loaded with biocytin as described in *Section 2.3*, then the dendritic shafts (no spines) were traced with NeuroLucida (MBF Biosciences) and the 3D morphology files imported into NEURON. To correct for the extra membrane area due to spines, I used the following estimates for the measured total spine area per micron length of dendrite, based on the spine analysis performed elsewhere in this project (*Chapter 3, Fig. 3.3D*): SL cells – layer 1a, $2.8 \mu\text{m}^2$ per micron length; layer 1b, $0.7 \mu\text{m}^2$; SP cells – layers 1a and 1b, $2.5 \mu\text{m}^2$; basal dendrites, $2.5 \mu\text{m}^2$. These extra membrane areas were incorporated into the smooth dendrite models using the ‘folding factor’ approach to scale up the dendritic diameter within each segment (Major et al., 1994). Models used a specific membrane capacitance (C_m) of $1 \mu\text{F}/\text{cm}^2$ and axial resistivity (R_a) of $150 \Omega\text{cm}$, consistent with previous work (Major et al., 1994). Specific membrane resistance (R_m) was adjusted until the simulated input resistance matched the reported value for SL cells ($221 \text{ M}\Omega$; Suzuki & Bekkers, 2011) and SP cells ($55 \text{ M}\Omega$; Suzuki & Bekkers, 2011), giving R_m estimates of $21,000 \Omega\text{cm}^2$ and $26,000 \Omega\text{cm}^2$, respectively. The simulated synaptic conductance used a difference of two exponentials, $g = \text{amp} * ((1 - \exp(-(t - \text{onset})/\tau_0)) - (1 - \exp(-(t - \text{onset})/\tau_1)))$, where $\tau_0 = 0.3 \text{ ms}$, $\tau_1 = 3 \text{ ms}$. Simulations were performed with $25 \mu\text{s}$ time steps, resting potential -70 mV and temperature 37°C . The basic results were confirmed using simplified ‘stick models’ of SL and SP cells, and also with a more complex version of the accurate morphological models that incorporated active membrane conductances (data not shown).

2.9 Data analysis

Morphological data were exported from Neurolucida 360 to Excel and subsequently analysed using R. Electrophysiology data were analyzed using Axograph or Matlab (Mathworks, Natick, USA), compiled in Excel, and tested statistically using R (next section).

2.9.1 Cluster analysis for spine morphology

Hierarchical and k-means cluster analyses were both performed on five spine morphology parameters provided by Neurolucida 360: spine volume, surface area, backbone length, head diameter and backbone length/head diameter. Measurements of the spine neck diameter were excluded because they were deemed unreliable at the current spatial resolution. For hierarchical cluster analysis the parameters were normalized to vary between 0 and 1; for k-means the raw values were used. Hierarchical cluster analysis used an unsupervised tree clustering algorithm based on Euclidean distances with Ward's linkage criterion. The k-means cluster analysis set the desired number of clusters to the number identified from the hierarchical cluster analysis, *i.e.* 2 clusters for SL layer 1a, SL layer 1b and SP layer 1b, and 3 clusters for SP layer 1a. All cluster analysis was done using the statistics and machine learning toolbox in Matlab R2020b (Mathworks).

2.9.2 Synaptic transmission analysis

EPSC/EPSPs were adjusted to a 5 ms-long baseline window ending 1 ms before the stimulus time, then amplitudes were measured as averages ± 0.2 ms around the peak or, when measuring subthreshold responses, around the time of the averaged suprathreshold peak EPSC/EPSP. Amplitude histograms were calculated for both the subthreshold period and a

window spanning the putative unitary step in amplitude, then a sum of two Gaussians was fitted to the histograms using the Simplex algorithm implemented in Axograph, which minimized the sum of squared errors. A unitary step was identified if the means of the Gaussians differed by ≥ 2.5 standard deviations of the baseline distribution.

2.9.3 Variance-mean analysis

For variance-mean (VM) experiments, unitary inputs to SL and SP cells were isolated in ACSF with 2 mM Ca^{2+} , measured under voltage clamp with a holding potential of -70 mV. Bath Ca^{2+} concentration was then systematically varied across the chosen range ((mM): 0.1, 0.3, 0.8, 2, 4) while recording 420-850 episodes at a constant stimulus interval of 5 s. Sometimes the same Ca^{2+} concentration was revisited for the same cell. EPSCs were baseline-adjusted and the peak values were measured as described in *Section 2.9.2*, then a plot of EPSC amplitude *versus* time was used to visually identify contiguous regions within which bath Ca^{2+} concentration had stabilized, judging by the stability of the mean EPSC amplitude. The mean and variance of these contiguous EPSC amplitudes were measured ($n = 20-84$ events across all concentrations and cells). Background ‘noise’ variance was estimated for each bath Ca^{2+} concentration and each cell by repeating the amplitude measurements on the same traces but in regions 500 ms after the stimulus. This baseline variance was subtracted from the corresponding variance measured immediately after the stimulus to give a corrected variance. For each cell, the corrected variance was then plotted *versus* the corresponding mean EPSC amplitude ($n = 4-8$ data points per cell), and *Equation 5* (see *Section 1.4.4.2*) was fitted to the points. For technical details, please refer to *Section 1.4.4*.

2.9.4 Strontium asynchronous EPSC analysis

With 4 mM strontium in the bath, weak stimulation was applied to the LOT to evoke EPSCs using the test pulse protocol (single pulse). Traces were digitally filtered at 2 kHz, then the template-matching detection algorithm in Axograph X was used to detect all EPSCs following the stimulus. Use of a lower detection threshold (3) ensured that smaller EPSCs were captured. For each cell, event counts were compiled into a peristimulus time histogram that was fitted to a single exponential plus a constant, where these two components represent the numbers of asynchronous (Sr-evoked) and spontaneous miniature EPSCs, respectively. EPSC amplitudes were sorted into two groups according to their latency from the stimulus (12-162 ms and 1000-1150 ms), then a histogram was calculated for the EPSC amplitudes in each group (bin width 1 pA). The later histogram was subtracted from the earlier one, then the mean of the difference was calculated, yielding an estimate of the amplitude of EPSCs that originate from synapses excited by LOT stimulation.

2.9.5 Local field potential analysis

Peak field EPSP amplitude was used as an indicator of synaptic strength in LFP experiments. After subtracting the baseline, peak amplitude was found by averaging ± 0.2 ms around the peak of the negative-going LFP response (which follows the stimulus artefact and presynaptic volley; *Fig. 2.14*). Paired-pulse analysis was reported as the paired-pulse ratio (PPR), calculated by dividing the amplitude of the second pulse by the amplitude of the first. For clarity, PPR figures were presented by normalising to the PPR during the baseline test pulse period. For each LFP experiment, measured amplitudes for every stimulation (first pulse) were plotted against time. For each condition, time series plots were normalised to the pre-induction baseline, combined, then averaged. Standard error of the mean (SEM) was calculated

as a measure of the variability between experiments. Presynaptic volley amplitude was measured and plotted (as above) as an indicator of stimulation stability. For clarity, figures show the data after smoothing with 5-point decimation; however, all statistics were performed on raw data points.

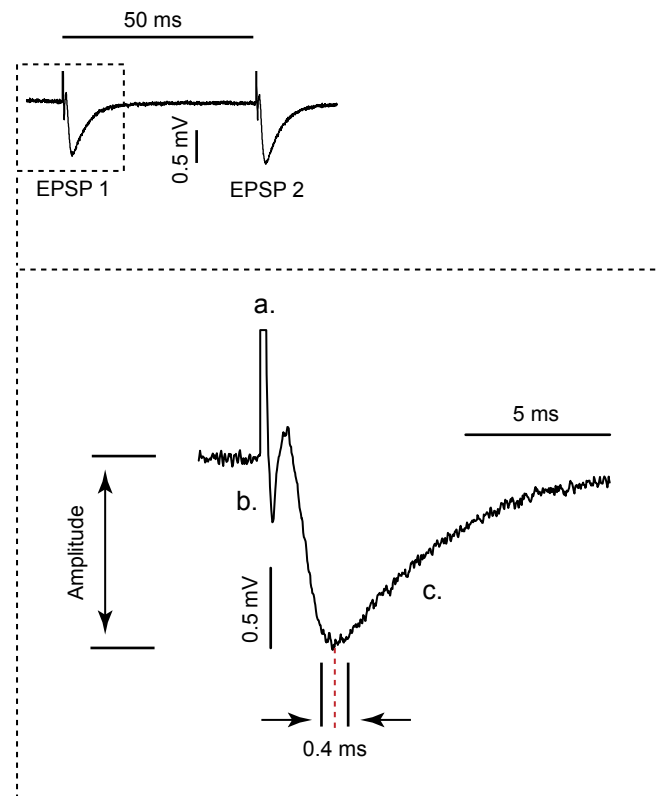


Figure 2.14. Field EPSP measurement and analysis. *Top*, Paired pulses were delivered 50 ms apart. *Bottom*, Amplitude was measured from baseline (subtracted) and was averaged ± 0.2 ms around the peak of the EPSP. *a*, stimulus artefact (truncated); *b*, presynaptic volley; *c*, EPSP.

2.9.6 Whole-cell plasticity analysis

All whole-cell plasticity experiments were performed in voltage-clamp mode at minimal stimulation strength. Once the first response plateau was achieved over a range of stimulus strengths, a stimulus strength mid-plateau was fixed for the duration of the experiment. EPSC amplitudes were used as an indicator of synaptic strength. After subtracting the baseline, peak amplitude was found by averaging ± 0.2 ms around the peak of the negative-going (inward) EPSC (which follows the stimulus artefact; *Fig. 2.15*). Paired-pulse analysis was reported as the PPR, calculated by dividing the amplitude of the second pulse by the amplitude of the first. For clarity, PPR figures were presented by normalising to the PPR during the baseline test pulse period. For each whole-cell plasticity experiment, measured amplitudes for every stimulation (first pulse) were plotted against time. For each condition, time series plots were normalised to the pre-induction baseline, combined, then averaged. SEM was calculated as a measure of the variability between experiments. Series resistance was derived from postsynaptic responses to a brief voltage injection (see *Section 2.7.2*) and was plotted (as above) as an indicator of recording stability. For clarity, figures show the data following 5-point decimation; however, all statistics were performed on raw data points.

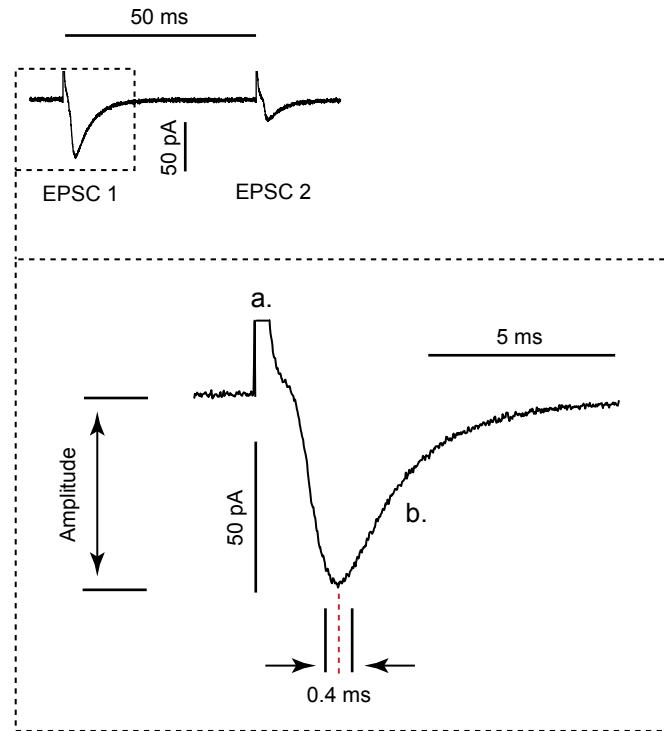


Figure 2.15. Unitary EPSC measurement and analysis for plasticity. *Top*, Paired pulses were delivered 50 ms apart. *Bottom*, Amplitude was measured from baseline (subtracted) and was averaged ± 0.2 ms around the peak of the EPSC. *a*, stimulus artefact (truncated); *b*, EPSC.

2.9.7 Somatic NMDA spike recordings

Somatic NMDA spike recordings were made in current-clamp mode. Analyses were performed on the EPSP generated following the last pulse in the stimulus train (either 3 or 5; *Fig. 2.16*). After subtracting the baseline prior to the first pulse, peak amplitude was found by averaging ± 0.2 ms around the peak of the EPSP, half-width (HW) was measured at 50% of peak amplitude, and area under the curve (AUC) was measured within a fixed window for each condition. To estimate deviation from linearity, amplitude, HW and/or AUC was plotted against the corresponding stimulus setting (*e.g. Fig. 6.2B₁*). A straight line was fitted to the first 3-5 data points and the quality of fit was estimated from the R^2 value. An abrupt decrease in the R^2 value, associated with visually identified sudden deviation of the n th point from the straight line fitted to the first $n - 1$ points, was taken as the onset of a non-linear response.

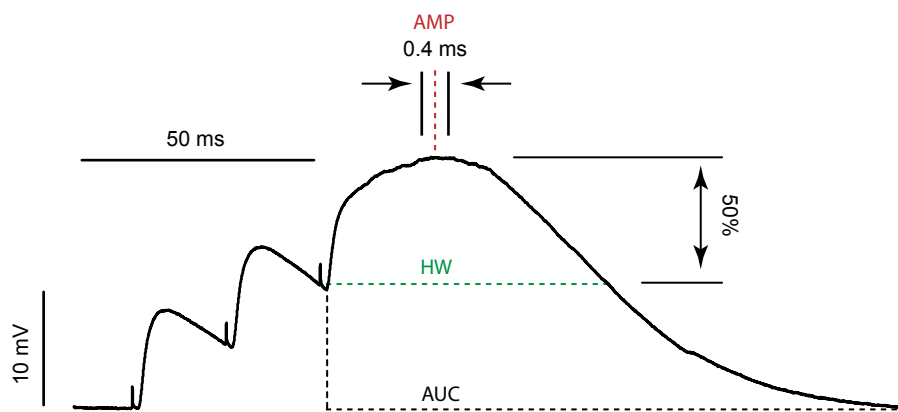


Figure 2.16. Somatic NMDA spike analysis. All analyses were performed on the last EPSP generated following the stimulus train. Amplitude (AMP, red) was measured by averaging amplitudes 0.2 ms either side of the peak, half width (HW, green) was measured at 50% max amplitude, area under the curve (AUC, black) was measured in a window capturing the final EPSP.

2.9.8 Dendritic recordings

Dendritic recordings were made in both current- and voltage-clamp modes. Analyses were performed on the EPSP/C following the last pulse in the stimulus train. After subtracting the baseline prior to the first pulse, peak amplitude was found by averaging ± 0.2 ms around the peak of the EPSP, HW was measured at 50% of peak amplitude, and AUC was measured within a fixed window for each condition (as above, *Section 2.9.7, Fig. 2.16*). Non-linear responses were identified using the method outlined in the previous section (*Section 2.9.7*).

2.10 Experimental design and statistical analysis

The R statistical program (R Core Team, 2017), running under RStudio version 1.1.456, was used for all statistical analyses. Normal distribution of data was confirmed using the Shapiro-Wilk test. Student's two-tailed t-tests (paired or unpaired depending on the data structure) were performed for simple pairwise comparisons and time-series plasticity analysis. One-way ANOVA was used to compare three independent categories (using '*lm*' followed by '*anova*' and '*emmeans*' or '*glht*' (*as appropriate*) for multiple comparisons in R). For field plasticity analysis, 60 contiguous stable data points were chosen in baseline and post-induction for comparison. For presynaptic volley and series resistance analysis, 10 contiguous data points were chosen close to induction to be sure induction protocols did not influence recording stability. All results are reported as the mean change (% change from baseline in plasticity experiments) $\pm$ SEM. Reported *n*-values represent the number of experiments or cells included in the given analysis, and significance is indicated using standard R conventions.

Chapter 3

SL and SP cell morphology

In the PCx, SL and SP cells are the primary recipients of synaptic input from the olfactory bulb. Given the direct relationship between morphology and the intrinsic electrical properties of cells, synaptic integration and ultimately neuronal output, I first wanted to characterise the relevant morphological features of the target cell types.

3.1 Qualitative features of dendrite and spine morphologies of SL and SP cells

For these experiments, slices were taken from the anterior PCx (*Fig. 3.1A, B*). SL and SP cell somas were patched in whole-cell mode with internal solution containing biocytin. The tissue was then fixed and the apical dendrites were imaged using enhanced-resolution confocal microscopy (63x/1.2 NA objective, Zeiss LSM 800 Airyscan). For each cell, apical dendrites were imaged in superficial layer 1 (layer 1a, distal to the soma) and deep layer 1 (layer 1b, proximal to the soma), which receive *aff* input from the OB, and *assn* input from ipsi- and contralateral PCx, respectively (*Fig. 3.1C, D*).

Qualitatively distinct dendritic structure, as well as spine morphology and density, were apparent in the different sublaminae and cell types (*Fig. 3.1C, D*). SL dendrites were less complex, with SP cells branching more frequently along the apical axis (*Fig. 3.1C, D, middle*). There were also obvious layer-specific differences in spine size and distribution. In layer 1a, SL cells displayed particularly large spine heads compared with spines on SP dendrites (*Fig. 3.1C, D, right*). Conversely, in layer 1b, spines appeared to be similar in size between the two cell types, but were clearly sparser in SL cells (*Fig. 3.1C, D, left*).

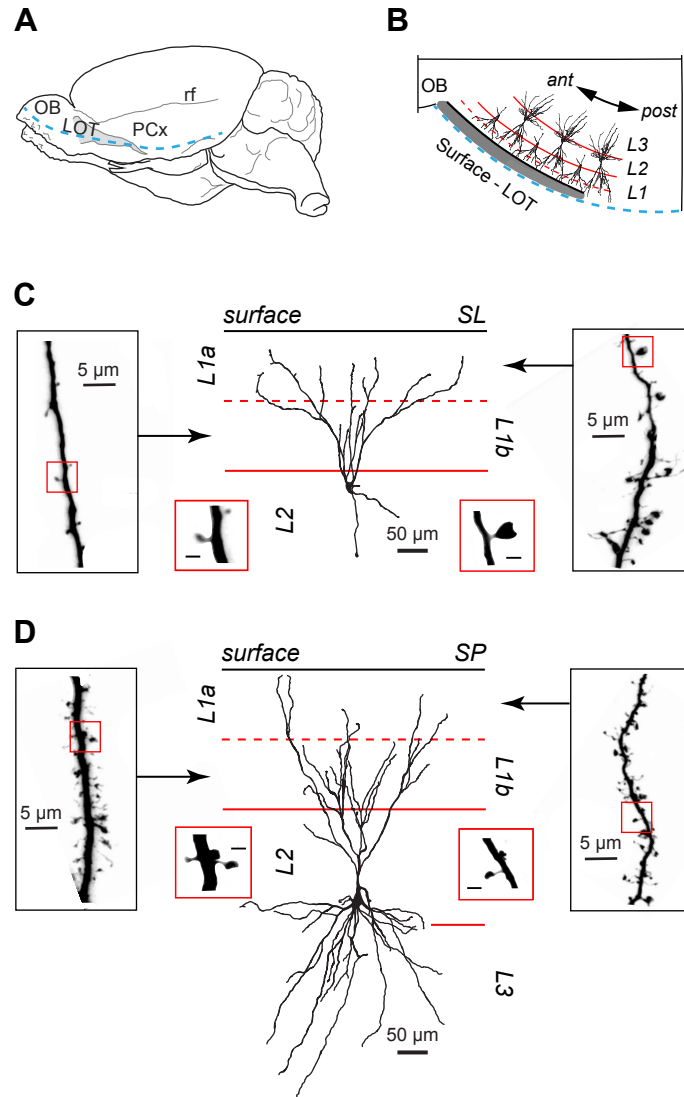


Figure 3.1. *SL and SP cells have distinct dendrite and spine morphologies.* *A)* Schematic of the mouse brain showing the locations of the olfactory bulb (OB), lateral olfactory tract (LOT), piriform cortex (PCx), and rhinal fissure (rf). Blue dashed line represents the location of the schematic parasagittal slice shown in panel *B*. *B)* Schematic parasagittal slice through the PCx (not to scale). SL cell somas are located in superficial layer 2, SP cell somas in deep layer 2. Blue dashed line corresponds to the blue line shown in panel *A*. Red lines correspond to laminar boundaries shown in panels *C* and *D*. *C-D)* Middle, z-projected confocal images of a typical SL cell (*C*) and SP cell (*D*). *Right* and *left* panels show, at higher magnification, example dendritic segments from layers 1a and 1b, respectively, from each neuron. Spines in red boxes on the dendrites are shown at higher magnification in the insets. Inset scale bar, 1 µm.

3.2 SP cells have complex apical dendrites and high spine density

The physical properties of the dendritic tree directly influence how synaptic information is processed and delivered to the soma. To quantify the differences observed in the apical dendrites of SL and SP cells, I extracted data from 3-dimensional reconstructions made in Neurolucida 360 (see technical details in *Section 2.3.2*). Although SL cells regularly branch more than SP cells proximal to the soma, Sholl analysis revealed longer and more complex apical dendrites in SP cells overall (*Fig. 3.2A*), consistent with previous findings (Bathellier et al., 2009). This result was supported by branch point analysis which showed that SP cells branch significantly more along the apical axis than SL cells (*Fig. 3.2B*). As expected, both SL and SP dendrites tapered distally, although the average dendrite diameter in SP cells remained significantly larger than in SL cells in proximal regions (*Fig. 3.2C*). Notably, the arrangement of dendritic spines along SL and SP dendrites was markedly different. SP cells displayed a dense assortment of spines along the entirety of the apical axis, while SL cells, on average, had less than half the number of spines per 10 μm dendritic length (*Fig. 3.2D*; e.g. for layer 1a, SP: 9.6 ± 0.8 per 10 μm dendrite length, $n = 11$ cells; SL: 3.3 ± 0.3 per 10 μm , $n = 8$; $p < 0.001$, 2-way ANOVA).

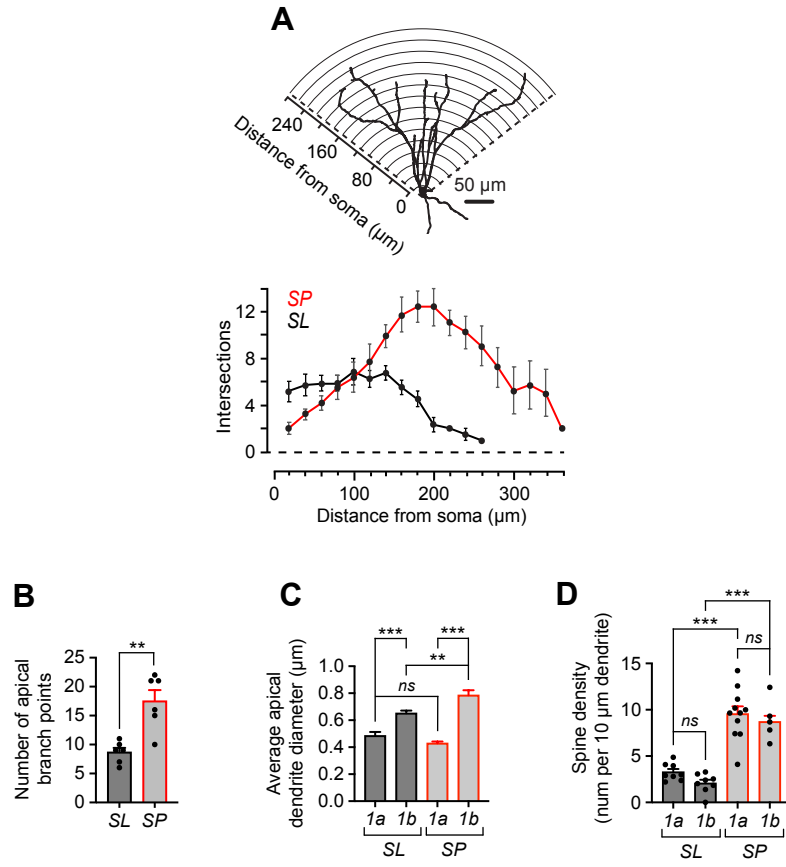


Figure 3.2. SP cells have more complex apical dendrites and a higher spine density than SL cells. *A) Top*, Example two-dimensional Sholl analysis (SL cell). Intersections were counted at 20 μm concentric intervals from soma. *Bottom*, Averaged Sholl analysis by cell type. *B)* Average number of apical branch points by cell type. Points represent individual cells. *C)* Average apical dendrite diameter by cell type and layer. *D)* Spine density (number of spines per 10 μm of dendritic length) by cell type and layer. Points represent separate dendritic sections. In all panels data is from $n = 6$ SL cells and $n = 6$ SP cells. Error bars show $\pm\text{SEM}$. ***, $p < 0.001$; **, $p < 0.01$; ns, not significant

3.3 SL cells have particularly large dendritic spines on distal apical dendrites

I next turned to an analysis of spine shapes. Using reconstructed apical dendrites and spines, I measured the volume and surface area of the spine head, as well as the spine length (measured from the mid-point of the dendrite, along the spine neck, to the top of the spine head), to quantify potential layer- and cell-specific differences in spine morphology.

Spines on the distal dendrites of SL cells were significantly larger on all measures than those found on proximal SL dendrites, or in either region on SP cells (*Fig. 3.3A-C; left, summary; right, distribution of spine volumes in layer 1a*). The total spine head surface area per 10 μm dendrite length in each layer was also calculated as a measure of the opportunity for synaptic connections to be made in each region ('synaptic opportunity'; *Fig. 3.3D*). As expected, given their minimal *assn* connectivity, layer 1b SL dendrites had significantly lower synaptic opportunity than all other analysed regions. Interestingly, however, despite the significant disparity in spine size and density between SL and SP cells in layer 1a, there was no difference in total spine surface area between SL and SP cells in this layer (*Fig. 3.3D*). That is, although SL cells have much larger spine heads in layer 1a, they are fewer in number. In contrast, SP cells achieve the same total spine area by having a larger number of small spines.

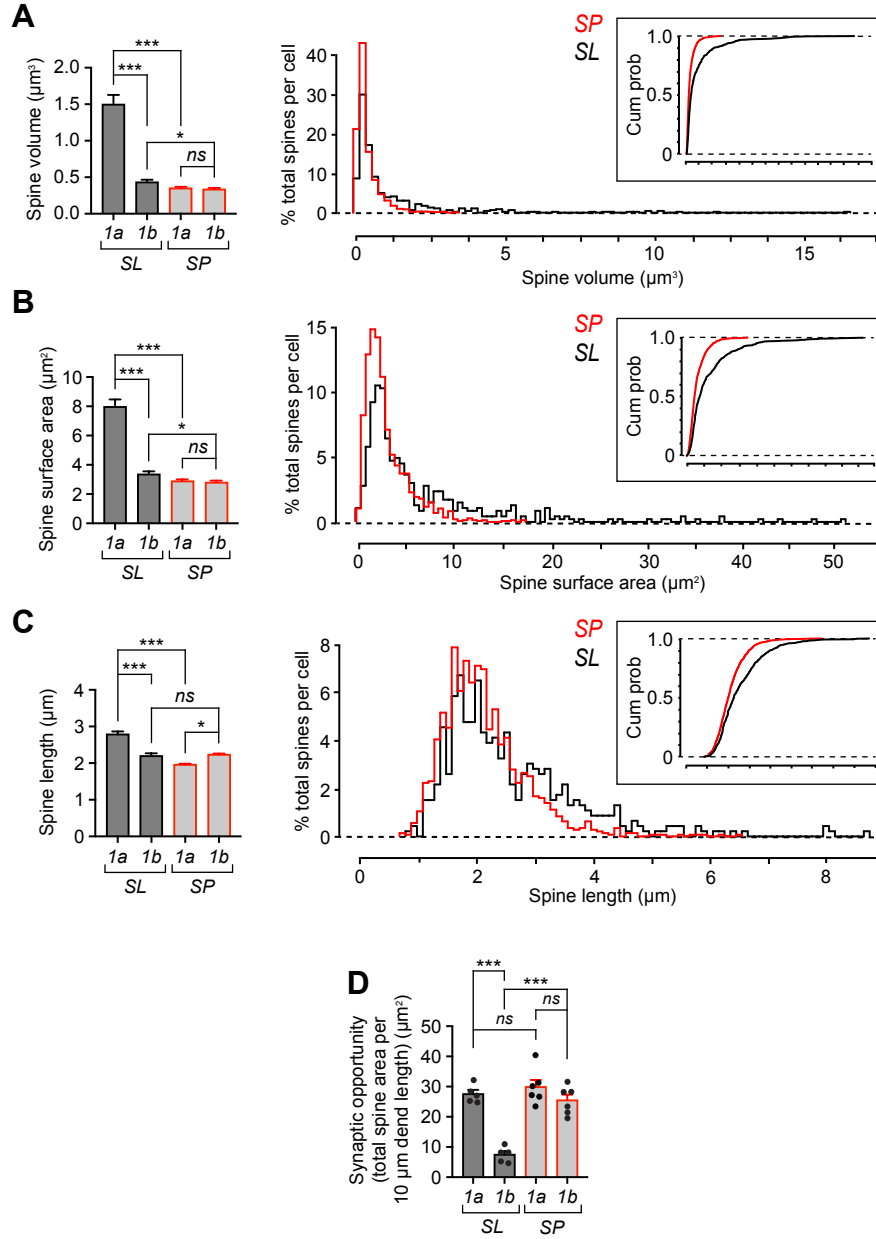


Figure 3.3. SL cells have large spines on distal apical dendrites. *A-C*) Left, Average spine volume, surface area and length, respectively, by cell type and layer. Right, Histograms plotted as percentage of total for each measurement by cell type. Insets, Corresponding cumulative histograms. SL: $n = 5$ cells, $n = 289$ L1a spines, $n = 175$ L1b spines; SP: $n = 6$ cells, $n = 709$ L1a spines, $n = 733$ L1b spines. *D*) Total spine head area (synaptic opportunity) by cell type and layer. Error bars show $\pm$ SEM. ***, $p < 0.001$; *, $p < 0.05$; ns, not significant.

3.4 Continuum of spine shapes along the apical axis of SL and SP cells

Given the distinct differences observed in spine size and distribution, I wondered whether distinct spine types could be discerned in the data (*e.g.* stubby, mushroom, *etc*; see *Section 1.4.2*). To test this, I first used unsupervised hierarchical cluster analysis on 5 morphological parameters (spine volume, surface area, length, head diameter and the ratio length/head diameter) for each of the 4 combinations of layer and cell type (*Fig. 3.4A-D*). This analysis suggested 2-3 broad categories, but these clusters seemed to segregate by overall spine size rather than the particulars of spine shape.

I then turned to k-means clustering, which confirmed that hierarchical clusters were determined primarily by spine size rather than shape. As shown in *Fig. 3.5A-D*, among all parameters there were no clearly delineated groups. These results suggest that spines along the apical axis of SL and SP cells exist on a continuum of shapes and sizes, consistent with the histograms in *Fig. 3.3A-C* which lack modal structure.

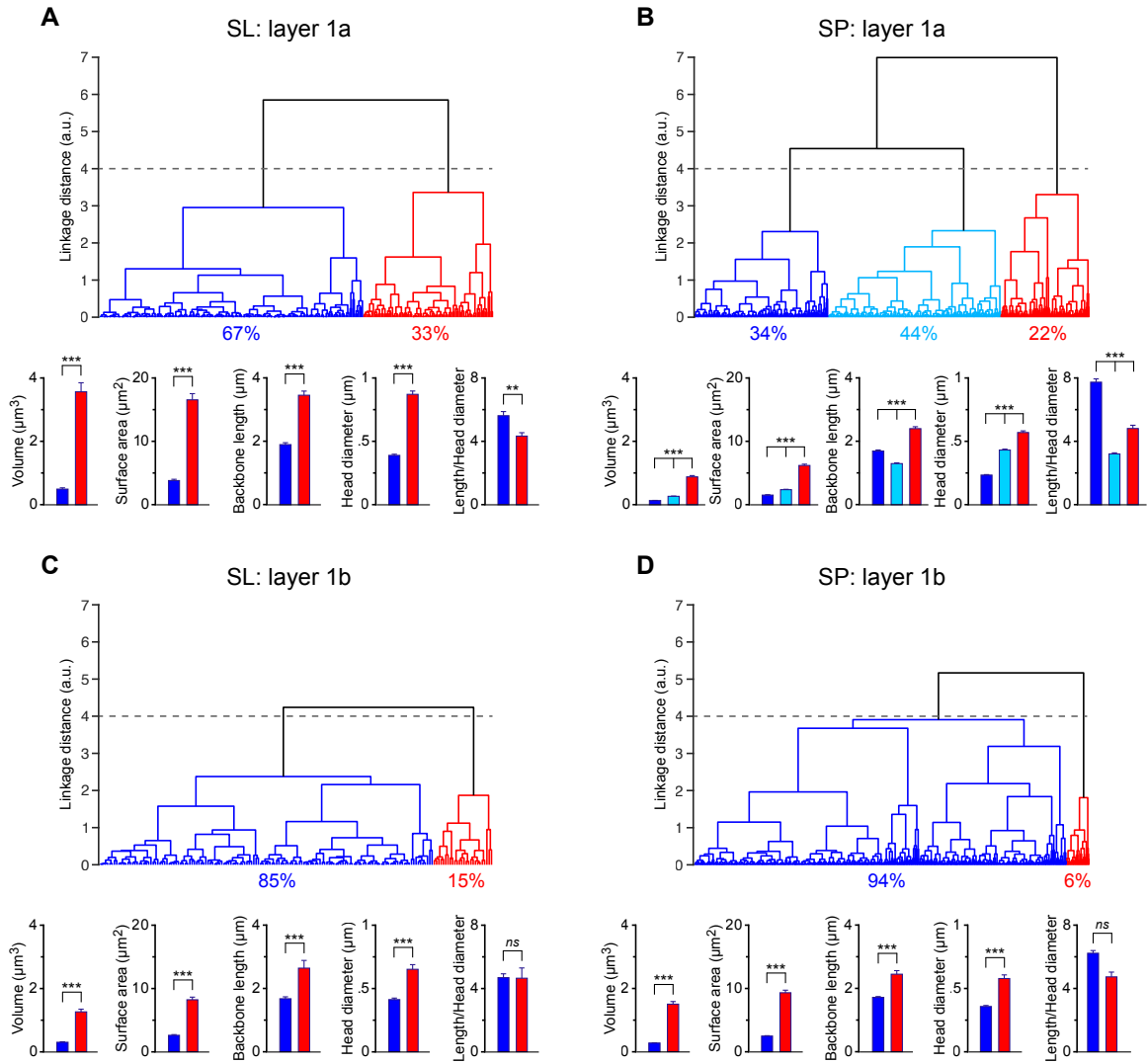


Figure 3.4. Hierarchical cluster analysis applied to spine morphology parameters identifies 2 or 3 classes of spines with different sizes. *A-D*) Unsupervised hierarchical cluster analysis applied to the morphologies of spines in each of 4 dendritic regions (*A*, SL L1a; *B*, SP L1a; *C*, SL L1b; *D*, SP L1b). Each panel shows (*top*) the dendrogram calculated using Ward's linkage criterion; and (*bottom*) the mean $\pm$ SEM of the indicated morphological parameters for each colour-coded cluster of spines. Colour-coded clusters were identified by setting an arbitrary threshold for linkage distance (threshold = 4, horizontal dashed lines). The fraction of spines in each cluster, expressed as a percentage of the total number of spines in each dendritic region, is shown below each cluster. These results show that spines are broadly distributed into “large” (red) and “small” (blue, cyan) categories, with the highest percentage of “large” spines occurring in SL layer 1a. *ns*, not significant; **, $p < 0.01$; ***, $p < 0.001$.

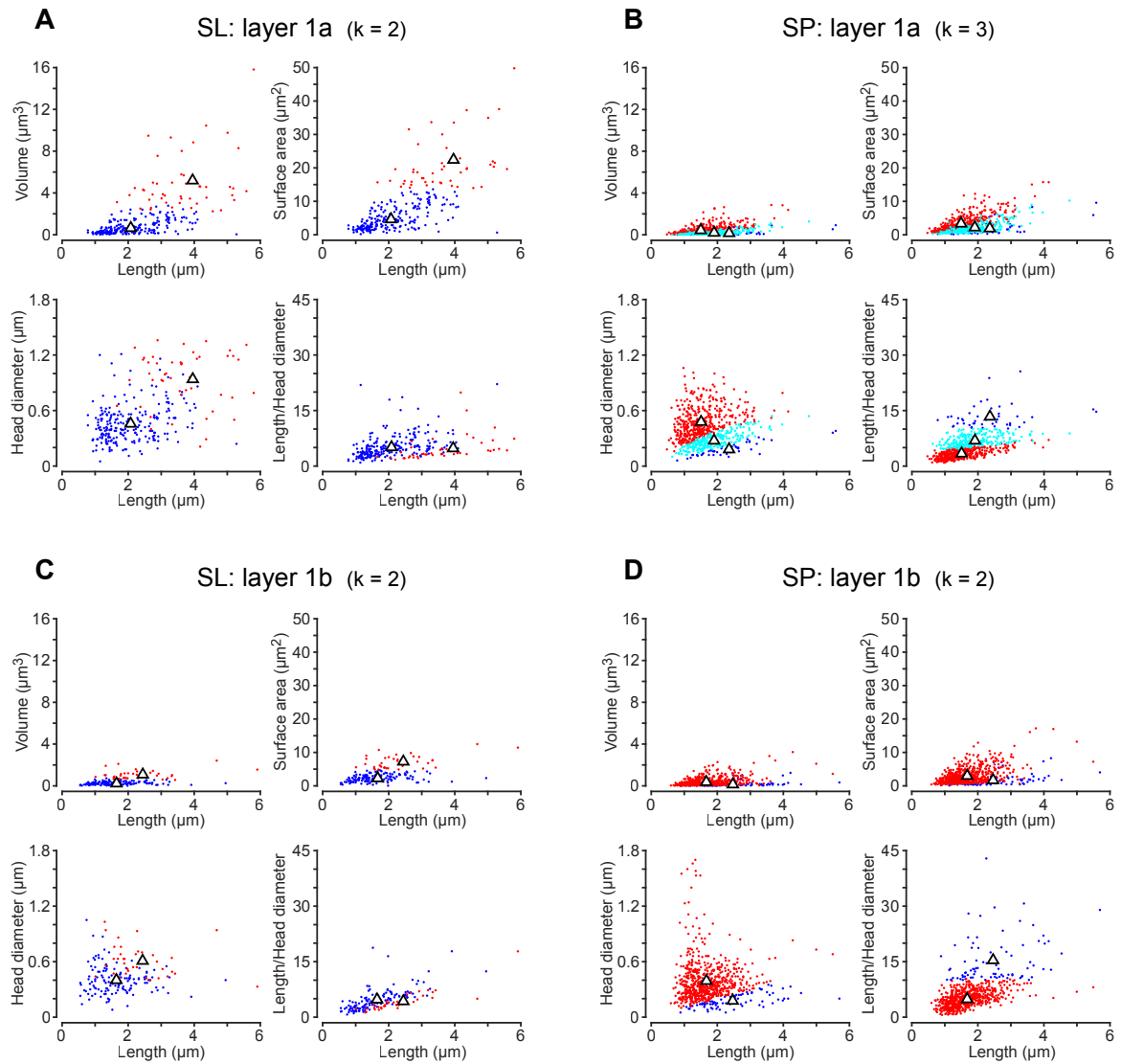


Figure 3.5. *K-means cluster analysis applied to spine morphology parameters suggests that the borders between clusters is ill-defined.* **A-D)** K-means cluster analysis applied to the morphologies of spines in each of 4 dendritic regions (**A**, SL L1a; **B**, SP L1a; **C**, SL L1b; **D**, SP L1b). The expected number of clusters, k , was set to the number identified using hierarchical cluster analysis (*Fig. 3.4*). Each panel shows four scatter plots of spine parameters vs. spine backbone length, colour coded by cluster membership. Diamond symbols show the mean of the surrounding cloud of points within each cluster. These results are broadly consistent with the hierarchical cluster analysis (*Fig. 3.4*) but show that the boundaries between clusters are not clearly defined.

3.5 SL and SP cell morphology: Summary of key findings

The aim of these experiments was to understand the morphology of SL and SP cells, with a particular focus on the structure and distribution of dendritic spines. Differences in the size of SL and SP spines has previously been reported (Haberly and Feig, 1983) but has never been rigorously quantified. Using 3-dimensional neuronal reconstructions, I have detailed the physical properties and distribution of spines along the apical dendritic axis of SL and SP cells, revealing significant differences in both spine size and distribution (*Fig. 3.6*).

The most striking finding from my morphological experiments is the exceptionally large dendritic spines found on SL dendrites exclusively in layer 1a, where *aff* input is delivered from the OB. Despite significant differences in spine size between SL and SP cells in this region, the two cells have equal opportunity for receiving *aff* input from the OB (judging by total spine head area per unit length of dendrite). On the other hand, the opportunity for synaptic connections in layer 1b, where *assn* synapses are formed, is much lower in SL cells, which is consistent with the reported weakness of *assn* synaptic input to SL cells (Suzuki and Bekkers, 2011; Choy et al., 2017).

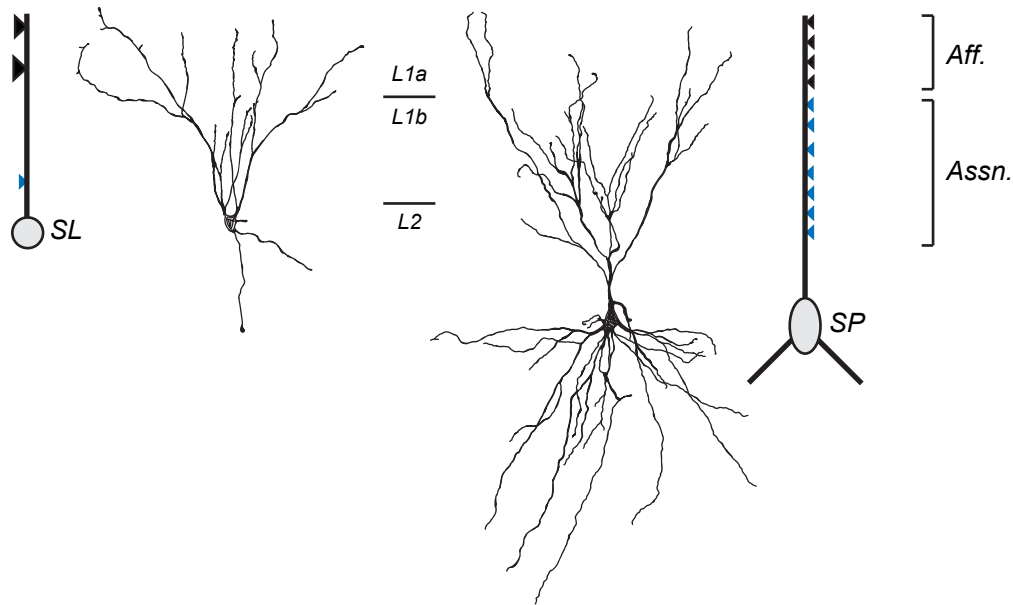


Figure 3.6: Schematic summary of main findings: SL and SP cell morphology. Tracing of SL cell (left) and SP cell (right) with corresponding schematics representing spine size and distribution. SP cells have more complex apical dendrites, as shown through Sholl and branch point analysis (see Fig. 3.2). Schematics represent laminar differences in spine morphology and density between SL and SP cells (see Fig. 3.3). SL cells have particularly large dendritic spines in layer 1a and fewer smaller spines in layer 1b. SP cells have a relatively uniform distribution of small spines along the entire apical axis. In layer 1a, where afferent input is received, SL and SP cells have equivalent synaptic opportunity (total dendritic spine surface area per unit length of dendrite). *Aff.*, afferent input layer (layer 1a); *Assn.*, associational input layer (layer 1b).

3.6 SL and SP cell morphology: Discussion of key findings

3.6.1 Greater dendritic complexity in SP cells may increase computational capacity

As discussed in *Section 1.4.6*, neuronal dendrites implement a range of linear and non-linear mechanisms that allow for elementary computations (London and Hausser, 2005). One such mechanism is the ability to perform basic logical operations (‘*and*’, ‘*or*’, *etc.*) at dendritic branch points that can significantly influence neuronal output (Ferrante et al., 2013).

Compared with SL cells, SP cells have significantly more apical branch points and a higher density of spines, resulting in more opportunities to implement basic logical transformations. Furthermore, local dendritic Ca^{2+} spikes can amplify synaptic input, directly affecting neuronal output. Dendritic NMDA spikes can also be evoked through the spatial and temporal clustering of synaptic input (Antic et al., 2010). Given the dense arrangement of dendritic spines and therefore excitatory synaptic input on the apical axis of SP cells, there may be more opportunity for the appropriate clustering of input, increasing the potential for non-linear synaptic amplification.

3.6.2 Distinct spine sizes may reflect different propensities to express synaptic plasticity

Dendritic spine size can be modified as a result of synaptic plasticity (Matsuzaki et al., 2004). As such, the distinct differences in spine size between SL and SP cells in L1a may reflect distinct abilities of each cell/synapse type to express plasticity. As I will show in *Chapter 4*, SL cells receive stronger unitary *aff* input than SP cells, due to significantly higher quantal amplitudes and release probabilities at these synapses. Furthermore, the exploratory sniffing frequency of the rodent is around 5 Hz, a stimulation frequency often used to induce

synaptic potentiation (*Section 2.7.4*). It may be the case that the higher input strength at SL synapses, combined with consistent activation through exploratory respiration, results in many SL synapses being in a constantly enhanced state.

We observed a continuum of spine shapes along the apical axis of SL and SP cells with no obvious clustering, as assayed using hierarchical cluster analysis and k-means analysis. Similar results have been observed in other brain areas using similar techniques (Yuste and Bonhoeffer, 2004; Berry and Nedivi, 2017; Pchitskaya and Bezprozvanny, 2020; Ofer et al., 2021). Together these findings suggest that clear categories of spines cannot be quantitatively identified using these methods. However, future work using more sophisticated clustering analysis techniques, which are still under development and beyond the scope of this thesis, might reveal clusters that were not resolved here.

3.6.3 Does spine size correspond to ultrastructural synaptic features?

My experiments are limited to optical measurements of postsynaptic morphology, leaving open questions about the structure of presynaptic terminals and the postsynaptic density (PSD). Spine size is often correlated with the area of the PSD, the number of postsynaptic receptors and the size of the readily-releasable pool of synaptic vesicles (Konur et al., 2003; Arellano et al., 2007). However, whether this relationship holds across the spectrum of spine sizes and cell types is not well understood. In the *stratum radiatum* of the hippocampus, for example, PSD surface area and PSD-core volume do not correlate with spine volume in approximately one-third of dendritic spines (Borczyk et al., 2019). Furthermore, although PSD surface area and PSD core volume consistently increase following LTP in this region, the spine heads themselves only grow if they contain smooth endoplasmic reticulum, demonstrating that LTP can substantially alter the volume/PSD ratio in some spines (Borczyk et al., 2019).

Although in many cases it appears that dendritic spine size correlates with ultrastructural synaptic features, more experiments using electron microscopy (EM) are needed to test whether these relationships hold at the spines on SL and SP cells.

3.6.4 Limitations and future directions

The major limitation of my morphological studies was the spatial resolution. As just noted, EM would reveal ultrastructural features of these synapses, giving a more complete structural understanding of the diverse spines I have described. Furthermore, my methods could not reliably reveal the features of the spine neck, which could provide useful information about electrical and chemical compartmentalisation, as well as voltage regulation (Cornejo et al., 2021). Future studies could also investigate the functional consequences of differences in dendritic complexity. Having two morphologically distinct cell types with a common input source and location on the dendritic tree may provide an interesting model for studying the consequences of certain morphological features on dendritic integration.

In the next chapter I turn to a detailed study of the electrophysiological differences between the excitatory synapses received by SL and SP cells.

Chapter 4

Biophysical properties of excitatory synaptic inputs to SL and SP cells

Having quantified differences in spine morphology for SL and SP cells in the previous chapter, in this chapter I explore the electrical properties of four types of excitatory synaptic inputs onto these cells: *aff* inputs to SL and SP cells (*aff*-SL and *aff*-SP synapses, respectively), and the two corresponding *assn* inputs (*assn*-SL and *assn*-SP synapses). Given the different spine morphologies observed at each of these inputs (*Chapter 3*) – notably the very large spines at the *aff*-SL cell input – I hypothesised that differences in the electrical properties of these inputs might also be observed.

4.1 SL cells receive powerful unitary *aff* synaptic inputs

I made whole-cell somatic recordings from identified SL and SP cells *in vitro* and applied a minimal stimulation protocol to the LOT (for *aff*-SL or *aff*-SP synapses) or L2 (for *assn*-SL or *assn*-SP synapses) (*Fig. 4.1A*). Using this method, I was able to evoke unitary EPSCs (*uEPSCs*) that represent postsynaptic responses to single axon stimulation (*Fig. 4.1B-D*). Summary data showed that the mean *uEPSC* amplitude was largest for *aff*-SL cell inputs, consistent with previous reports (Franks & Isaacson, 2006; Suzuki & Bekkers, 2011) (*Fig. 4.1E*, closed symbols and bars; *e.g.* *aff*-SL: 45.7 ± 3.8 pA, $n = 11$ cells; *assn*-SL: 22.3 ± 3.4 pA, $n = 11$; $p < 0.001$, 2-way ANOVA). Thus, the *aff*-SL cell input that expresses the largest spines also exhibits the largest *uEPSCs*.

If the *uEPSC* amplitudes in *Fig. 4.1E* are due to the release of a single synaptic vesicle, then reducing P_r by reducing bath Ca^{2+} should cause the *uEPSC* amplitude to step randomly between 0 pA (*i.e.* failure to release) and the full *uEPSC* amplitude measured in 2 mM Ca^{2+} . However, this did not occur (*Fig. 4.1F*; similar result in $n = 10$ *aff*-SL cell synapses). Instead, lowering Ca^{2+} resulted in the collapse of the *aff*-SL cell *uEPSC* amplitude toward the amplitude

of spontaneous miniature EPSCs (*mEPSCs*) in the same cell, suggesting the presence of multiple sites of neurotransmitter release even when a single axon was being stimulated.

These results suggest that the distinct layer-specific structural differences between SL and SP synapses are also reflected in synaptic strength. Furthermore, it appears that the particularly powerful ‘unitary’ *aff* inputs to SL cells involve multiple sites of vesicle release. Given these broad findings, I next aimed to understand the synaptic properties of *aff*-SL and *aff*-SP inputs in greater detail using more sophisticated approaches.

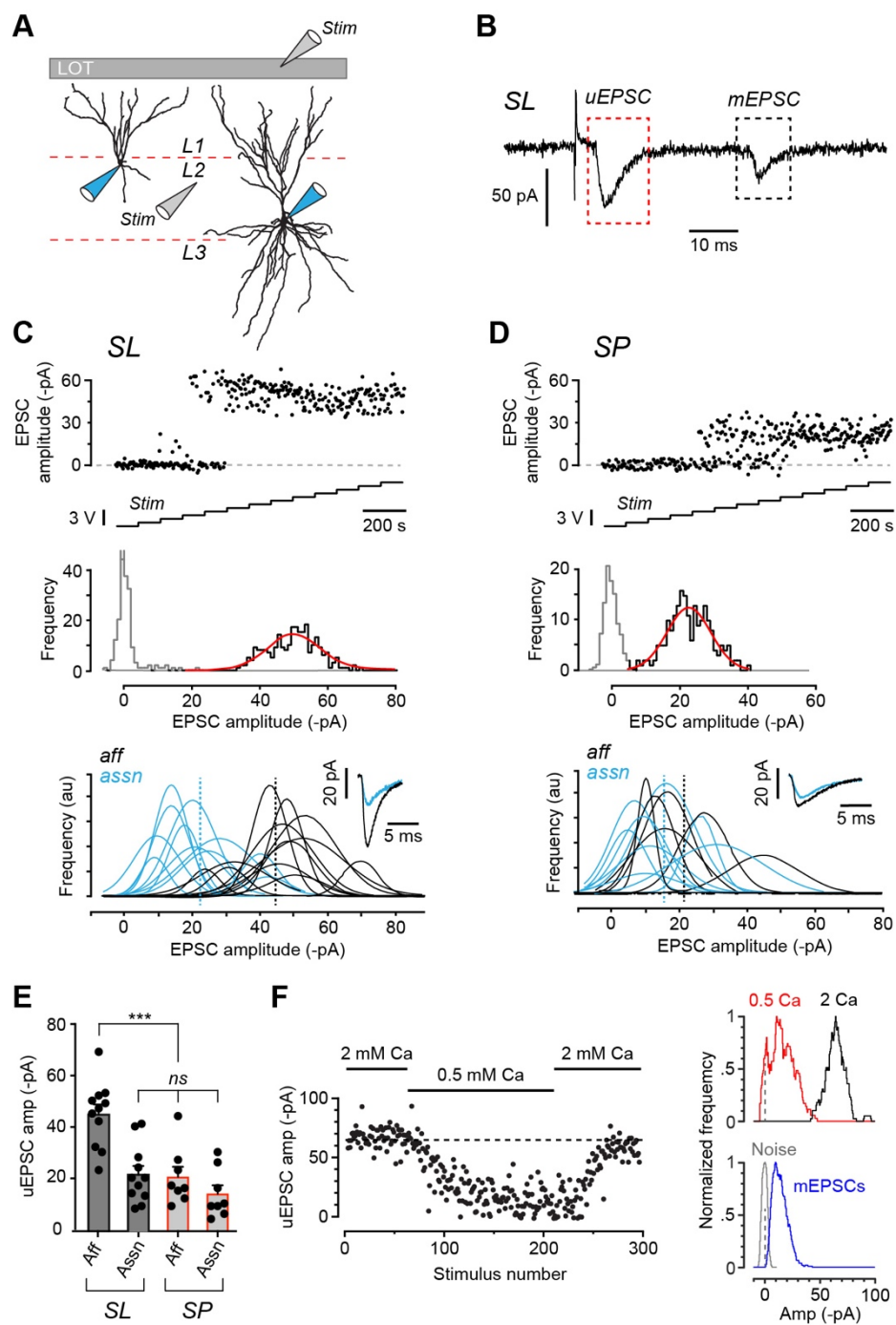


Figure 4.1. (Figure legend is on next page)

Figure 4.1. SL cells receive strong unitary *aff* synaptic inputs that involve multiple neurotransmitter release sites. *A)* Schematic representation of the stimulation and recording configuration. *Aff* synapses were stimulated from the LOT, *assn* synapses were stimulated from layer 2. *B)* Typical unitary EPSC (*uEPSC*, red dashed box) and miniature EPSCs (*mEPSCs*, black dashed box) recorded in an SL cell in ACSF. *C)* *Top*, plot of peak EPSC amplitude *versus* time for a typical experiment in an SL cell in which the stimulus strength (lower trace) is increased by 0.5 V every 100 s. The amplitude reaches a plateau, corresponding to a unitary EPSC, and remains on that plateau over a range of stimulus strengths. *Middle*, histogram of *uEPSC* amplitudes from example data above (*C*, *top*). A unimodal gaussian (red curve) is fitted to the dominant peak. *Bottom*, superimposed fitted gaussians for all SL cells in the dataset, showing results for *aff* (black) and *assn* (blue) inputs. Insets show, superimposed, a typical *uEPSC* for each input. *D)* Similar plots for SP cells. *E)* Summary of all unitary EPSCs evoked at *aff* and *assn* synapses for SL and SP cells. *F)* *Left*, Plot of unitary EPSC amplitudes in 2 mM bath Ca^{2+} and 0.5 mM bath Ca^{2+} . Horizontal bars indicate periods of perfusion of different Ca^{2+} concentrations. *Right, Top*, histogram of EPSC amplitudes recorded in 2 mM Ca^{2+} (black) and 0.5 mM Ca^{2+} (red). *Right, Bottom*, histogram of miniature EPSC amplitudes recorded in the same cell in 2 mM Ca^{2+} . ***, $p < 0.001$; ns, not significant.

4.2 Variance-mean analysis suggests higher quantal amplitude and release probability at *aff*-SL cell synapses

I used VM analysis to estimate the number of neurotransmitter release sites (N), the probability of neurotransmitter release (P_r), and the mean quantal amplitude (Q) associated with *aff* synapses projecting to SL and SP cells (Clements, 2003). In these experiments, unitary *aff* EPSCs were initially evoked in ACSF containing 2 mM Ca^{2+} (as in *Section 4.1*). Then, while holding unitary stimulation, bath Ca^{2+} concentration was systematically varied to alter P_r (*Fig. 4.2A, B, left*). The corrected variance of EPSC amplitudes (σ^2) was plotted *versus* the mean EPSC amplitude (I) at steady-state in each P_r condition, and the data points for each cell were fitted to a parabola (*Section 1.4.4.2; Fig. 4.2A, B, right*).

The estimates of N , P_r and Q for SL and SP cells are summarised in *Fig. 4.2C*. Q was significantly larger at *aff*-SL than *aff*-SP cell inputs (*Fig. 4.2C, left; aff*-SL: $Q = 7.3 \pm 0.7$ pA, $n = 9$ cells; *assn*-SL: $Q = 1.8 \pm 0.3$ pA, $n = 6$; $p < 0.001$, unpaired 2-sided t-test). Estimates of N , which come from the curvature of the fitted parabola, were reliable for SL cells (*e.g. Fig. 4.2A, right*) but less reliable for SP cells, which show much weaker curvature in the variance-mean plot (*e.g. Fig. 4.2B, right*). Nevertheless, it was clear that N was significantly smaller at *aff*-SL than *aff*-SP cell inputs (*Fig. 4.2C, middle; aff*-SL: $N = 12 \pm 2$, $n = 9$ cells; *assn*-SL: $N = 153 \pm 66$, $n = 4$; $p = 0.013$, unpaired 2-sided t-test). Substitution of the fitted Q and N values into *Equation 1* (see *Section 1.4.4.1*) showed that P_r , measured in the standard 2 mM Ca^{2+} ACSF, was significantly larger at *aff*-SL cell inputs (*Fig. 4.2C, right; aff*-SL: $P_r = 0.82 \pm 0.05$, $n = 8$ cells; *assn*-SL: $P_r = 0.23 \pm 0.04$, $n = 4$; $p < 0.001$, unpaired 2-sided t-test). Overall, these experiments suggest that the combination of larger P_r and Q underlies the powerful *aff* inputs received by SL cells.

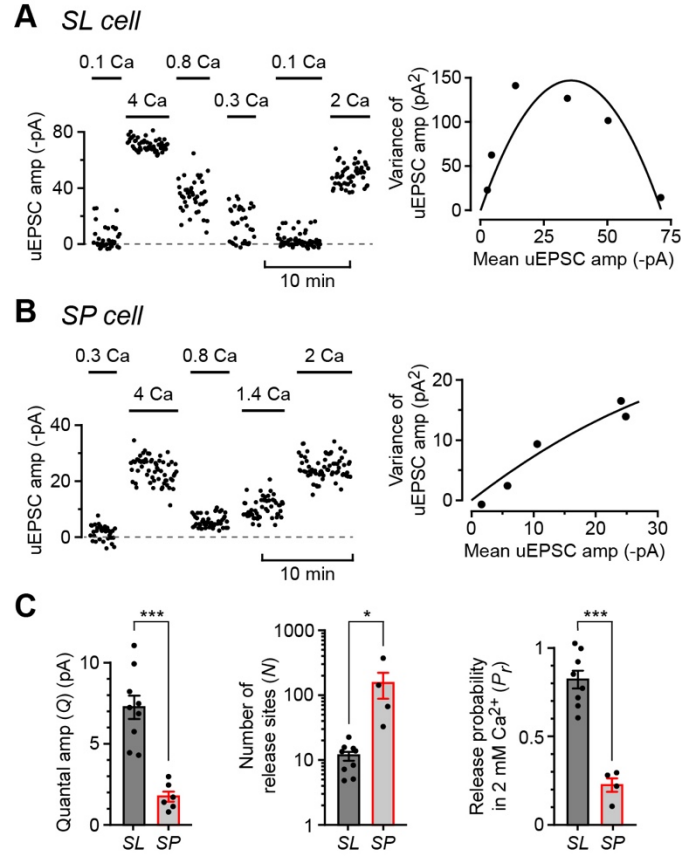


Figure 4.2. Powerful aff input to SL cells is associated with larger mean quantal amplitude (Q) and probability of neurotransmitter release (P_r). *A*) Left, raw data for a typical VM experiment from a SL cell showing periods of different Ca^{2+} concentrations (top) and recorded amplitudes from steady-state periods in each condition. Right, VM plot and parabola fit from the data on the left. *B*) Similar data and VM plot for a typical experiment with a SP cell. *C*) Plots summarising estimates of Q , number of release sites (N), and P_r for aff inputs to SL and SP cells. Data points represent results from individual cells. Error bars represent $\pm$ SEM. SL, $n = 9$; SP, $n = 6$. ***, $p < 0.001$; *, $p < 0.05$.

4.3 Strontium-evoked asynchronous EPSCs confirm larger quantal amplitude at *aff*-SL cell synapses

The strongest finding from the VM analysis (*Section 4.2*) was the significantly larger Q at *aff*-SL cell synapses. To confirm this result, I turned to a more direct method that uses Sr^{2+} to measure postsynaptic responses to univesicular release (Bekkers and Clements, 1999). The presence of 4 mM Sr^{2+} in the bath solution triggers asynchronous EPSCs (*aEPSCs*) which arise specifically from electrically stimulated synapses and which persist for a few hundred milliseconds after the stimulus (*Fig. 4.3A, top panel*, events indicated by red bars *and* blue arrows; *bottom panel*, blue peristimulus time histogram [PSTH] for the same cell). In contrast, if the bath excludes Sr^{2+} these *aEPSCs* are not seen (*Fig. 4.3A, bottom panel*, grey PSTH; data from a different cell). The *aEPSCs* in Sr^{2+} are superimposed on the ongoing background activity of miniature EPSCs (*mEPSCs*), which originate spontaneously from synapses all over the cell (*Fig. 4.3A, top panel*, events indicated by red bars but *not* by blue arrows).

To extract the *aEPSC* amplitude distribution from the mixed *aEPSC/mEPSC* data, I subtracted the amplitude distribution for events occurring in a ‘late’ time window (*Window b*, *Fig. 4.3A, bottom left*), which contains only *mEPSCs*, from the distribution for events occurring in an ‘early’ time window (*Window a*), which contains both *mEPSCs* and *aEPSCs* (*Fig. 4.3A, top right*, red and black traces, respectively), to reveal the *aEPSC* amplitude distribution alone (*Fig. 4.3A, bottom right*, blue trace). The mean *aEPSC* amplitude, obtained from this subtracted amplitude distribution, was 6.0 ± 0.4 pA ($n = 8$ SL cells; *Fig. 4.3C, left dark grey bar*) which was not significantly different from the estimate for Q obtained in the previous section for the *aff*-SL cell synapses using VM analysis (*i.e.* 7.3 ± 0.7 pA, *Fig. 4.3C, right dark grey bar*; $p = 0.18$, unpaired 2-sided t-test).

In contrast to SL cells, in a typical SP cell there were no discernible *aEPSCs* (*Fig. 4.3B, top panel*, and corresponding PSTH, *bottom panel*) and the subtracted *aEPSC* amplitude distribution for this example SP cell was close to zero (*Fig. 4.3C, right bottom*). The mean *aEPSC* amplitude obtained from all Sr^{2+} experiments with *aff*-SP cell synapses was not significantly different from the Q estimated in the previous section using the VM method (*Fig. 4.3C, light grey bars*; mean *aEPSC* amplitude from Sr^{2+} method, 1.4 ± 0.7 pA, $n = 8$ cells; *cf* 1.7 ± 0.3 pA from VM method, *Fig. 4.3C*; $p = 0.82$, unpaired 2-sided t-test). However, the mean *aEPSC* amplitude for SL cells was significantly larger than that for SP cells ($p < 0.001$, unpaired 2-sided t-test). In summary, these Sr^{2+} experiments are consistent with the VM analysis, confirming that the largest mean quantal amplitude is seen at *aff*-SL cell synapses.

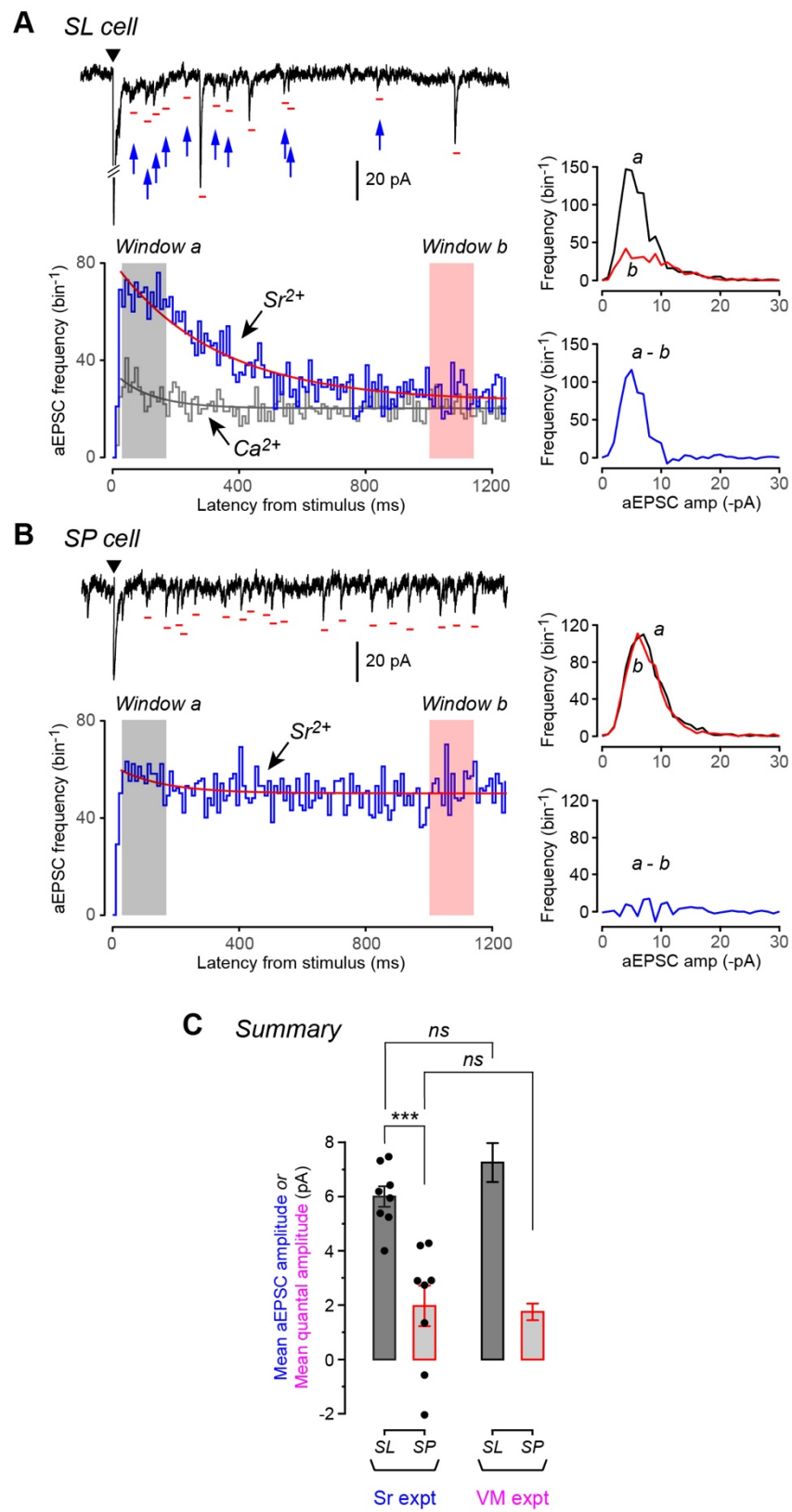


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Figure 4.3. Asynchronous EPSCs in Sr^{2+} confirm larger quantal amplitude for *aff* synapses on SL cells.

A) Example experiment from an SL cell in ACSF containing 4 mM Sr^{2+} plus 1 mM Ca^{2+} . *Left top*, a typical trace showing the large evoked synchronous EPSC (arrowhead above trace), asynchronous EPSCs (red bars with blue arrows) and miniature EPSCs (red bars only). *Left bottom*, peristimulus time histogram for all detected events (*a*EPSCs and *m*EPSCs) from a single experiment. Grey and pink bands indicate early (12-152 ms post-stimulus) and late (1000-1140 ms post-stimulus) measurement windows (labelled *a* and *b*, respectively). *Right top*, superimposed amplitude histograms for events captured in *Window a* (including both *a*EPSCs and *m*EPSCs) and *Window b* (only *m*EPSCs), respectively. Same experiment as in *left* panels. *Right bottom*, histogram *a* minus histogram *b*, representing the distribution of amplitudes for *a*EPSCs alone. **B)** Similar analysis performed for *aff*-SP synapses in a typical SP cell. In this case the *a*EPSCs measured in Sr^{2+} are below threshold for detection. **C)** *Left two bars*, summary of mean quantal amplitudes for *aff*-SL and *aff*-SP cell synapses estimated from Sr^{2+} experiments (SL, mean *a*EPSC amplitude 6.0 ± 0.4 pA, $n = 8$ cells; SP, mean *a*EPSC amplitude 1.4 ± 0.7 pA, $n = 8$ cells). *Right two bars*, mean *Q* values for *aff*-SL and *aff*-SP cell synapses from VM analysis, adapted from Fig. 4.2C, *left*. ***, $p < 0.001$; ns, not significant.

4.4 The measured difference in quantal amplitude between SL and SP cells is not an artefact of dendritic cable filtering

The VM and Sr^{2+} experiments show that the mean quantal amplitude, Q , at *aff* inputs is 3-4 times larger in SL cells than SP cells, when measured at the soma. I wondered if this difference was also present at the synapse, or if the difference observed at the soma was due to cable filtering in the SL/SP cell dendrites. This was addressed by computer modelling.

Dendritic reconstructions for typical SL and SP cells were used in NEURON simulations (Fig. 4.4A, B, left). A model synapse was placed at one of five different locations in the distal apical dendrite (red symbols, Fig. 4.4A, B, left). For each synapse, the peak EPSC amplitude at the soma (calculated under simulated voltage clamp at the soma) was plotted *versus* the peak synaptic conductance at the synapse (Fig. 4.4A, B, right; grey lines are for individual model synapses; black line with error bars shows the average $\pm$ SEM). These plots were used to read off the peak synaptic conductance required to produce the Q that was observed at the soma in the VM experiments (red lines with arrowheads, Fig. 4.4A, B, right): 0.87 nS for *aff*-SL synapses; 0.31 nS for *aff*-SP synapses. Thus, the conductance of *aff*-SL cell inputs, extrapolated back to the synapse, is still about 3 times that of *aff*-SP cell inputs. These results are consistent with the hypothesis that the large spines in SL cells are associated with larger synapses with larger quantal amplitudes.

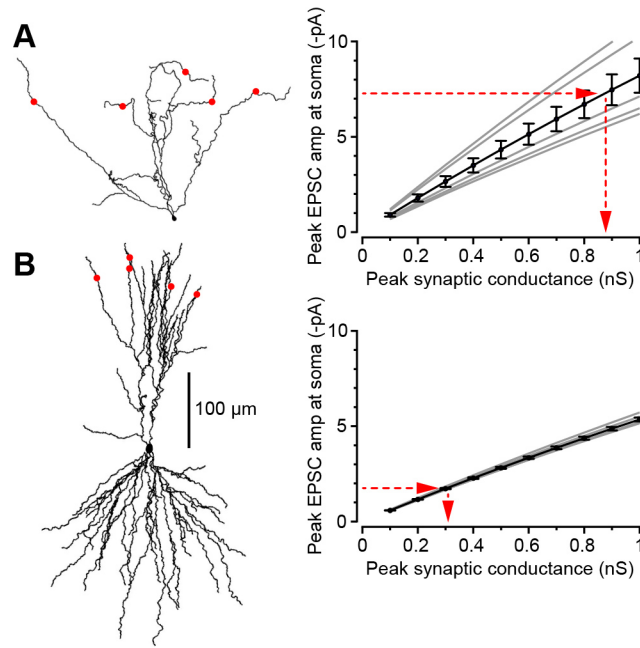


Figure 4.4. *Larger measured Q for *aff*-SL synapses cannot be explained by differential cable filtering in the dendrites of SL and SP cells.* **A)** *Left*, reconstructed SL cell used for simulations showing the locations of 5 simulated *aff* synaptic inputs (red points). *Right*, simulated EPSC amplitudes (at soma) plotted *versus* local synaptic conductance, showing each synaptic input (grey lines) and average $\pm$ SEM of all 5 inputs (black line). To obtain a measured peak EPSC at the soma of 7.3 pA, a synaptic conductance of 0.87 nS is required (dashed red lines). **B)** Similar analysis for a SP cell, showing that a synaptic conductance of 0.31 nS is required.

4.5 SL cells require fewer unitary *aff* synaptic inputs to reach action potential threshold

In a final series of experiments, I wished to broadly understand how *aff* inputs are integrated in SL and SP cells. As previously, minimal electrical stimulation was applied to either the LOT (*aff* inputs) or layer 2 (*assn* inputs), but this time the postsynaptic recording was made in current clamp mode to measure EPSPs. The stimulus strength was then progressively increased in steps to incrementally recruit axons until action potential (AP) threshold was reached (Fig. 4.5A, B, top). A gaussian was fitted to the measured EPSP amplitude distribution for each response plateau to estimate the cumulative strength of each input (Fig. 4.5A, B, bottom).

For *aff* inputs, SL cells exhibited larger increments in EPSP amplitude for each ‘step’ recruitment of axonal input than was the case in SP cells. Thus, an average of only 5.2 ± 0.2 *aff* inputs were required to reach AP threshold in SL cells, whereas 11.7 ± 0.6 *aff* inputs were required in SP cells (Fig. 4.5C, D; $n = 5$ SL cells, $n = 6$ SP cells; significantly different, $p < 0.001$, 2-way ANOVA). These results are consistent with the idea that the high- Q , high- P_r *aff* inputs onto SL cells are more effective at firing these cells than are *aff* inputs onto SP cells.

The summation of synaptic inputs to reach AP threshold depends on other factors in addition to the strength of synaptic input. For example, the intrinsic properties of the dendrites, as well as the location and properties of the spike initiation zone, are also important and typically differ between cell types (Stuart and Spruston, 2015). Therefore, I next focused on comparisons between *aff* and *assn* inputs to the same cell type, starting with SL cells. I found that it was difficult to recruit enough *assn* fibres to fire SL cells because of their low ‘synaptic

opportunity' in layer 1b (*Fig. 3.3D*) and the smaller uEPSCs at this input (*Fig. 4.1E*). I obtained only two SL cells (out of a total of 5) that were able to fire APs with *assn* stimulation before the stimulator reached its maximum setting; the numbers of inputs to reach AP threshold for these 2 cells are shown as filled symbols in *Fig. 4.5D* (bar labelled *assn* SL). Data for the remaining 3 cells that did not reach AP threshold are shown as open symbols in *Fig. 4.5D* and are included in the mean for *assn* SL inputs. Despite the inclusion of some measurements that are underestimates, these results show that significantly fewer *aff* inputs than *assn* inputs are required to fire an SL cell (*Fig. 4.5D*: *aff*, 5.2 ± 0.2 , $n = 5$; *assn*, 7.4 ± 0.9 , $n = 5$; $p = 0.049$, 2-way ANOVA). The same result was obtained when comparing the cumulative number of unitary inputs required to depolarize SL cells towards AP threshold (*Fig. 4.5C*: same data set as in *Fig. 4.5D*; compare continuous and dashed black lines, significantly different, $p < 0.001$, 2-way ANOVA with repeated measures).

A similar analysis for SP cells showed that, opposite to SL cells, *assn* inputs to SP cells were more effective than *aff* inputs at eliciting APs (*Fig. 4.5C*: compare continuous and dashed red lines, $p < 0.001$, 2-way ANOVA with repeated measures; *Fig. 4.5D*: *aff*, 11.7 ± 0.6 inputs to spike threshold, $n = 6$; *assn*, 8.3 ± 0.8 inputs to threshold, $n = 6$, $p = 0.003$, 2-way ANOVA). As demonstrated in *Figs. 3.3/4.1*, SP cells receive high levels of both *aff* and *assn* input that is similar in strength. As such, it makes sense that *assn* input is more effective at driving the cell towards AP threshold because these synapses will undergo less dendritic attenuation before reaching the AP initiation zone in the axon.

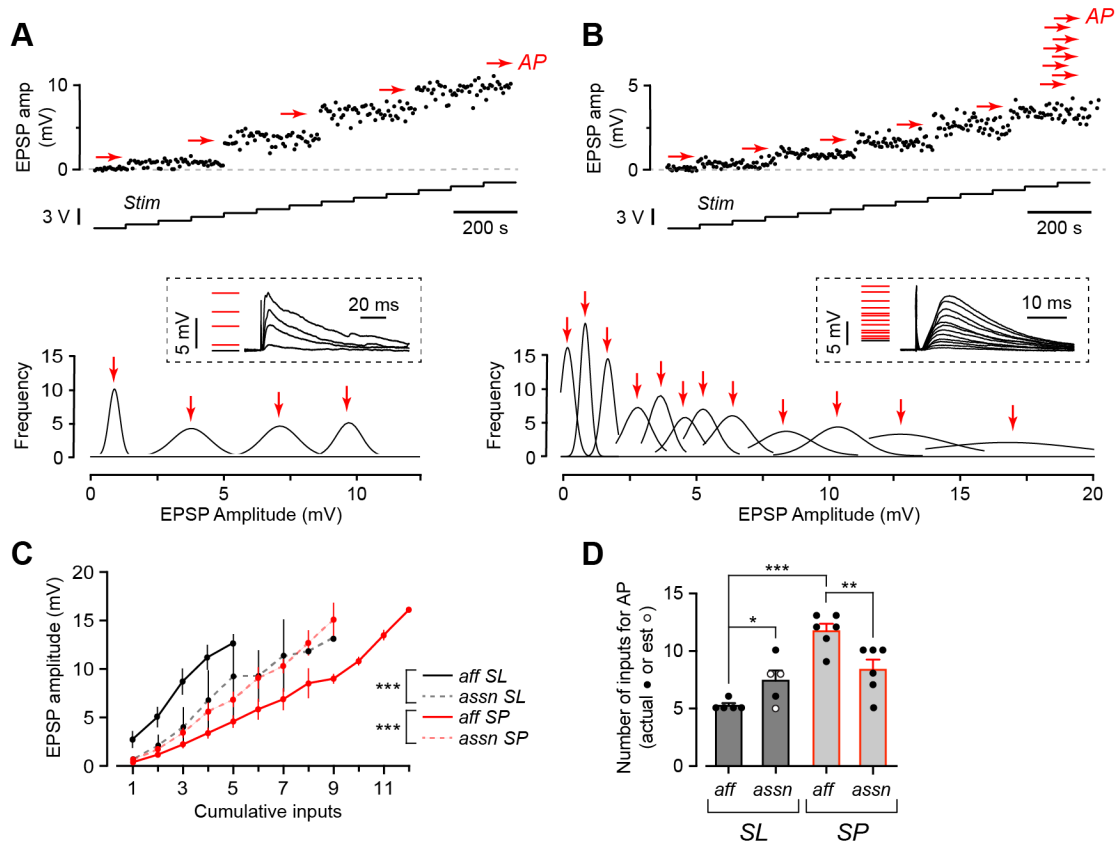


Figure 4.5. SL cells require fewer unitary inputs than SP cells to reach AP threshold. *A)* Top, example plot of EPSP amplitude *versus* time for a typical SL cell while the stimulus strength (lower trace) is increased in steps. Red arrows indicate step-like increases in EPSP amplitude corresponding to the recruitment of unitary inputs until action potential (AP) threshold is reached. *Bottom*, gaussian curves fitted to histograms of peak EPSP amplitude for the data shown above. Insets show, superimposed, typical EPSPs from each of the gaussian regions arrowed below. *B)* Similar data for a typical SP cell. *C)* Summary plot of averaged EPSP amplitude *versus* cumulative number of unitary inputs for each cell type and stimulus layer, from experiments like in panels *A* and *B*. SL: $n = 5$ cells (*aff* and *assn*); SP, $n = 6$ (*aff* and *assn*). *D)* Each point shows either the number of unitary inputs that actually generate an AP in a cell ('actual', filled symbols) or a lower estimate of this number in the case of a cell that did not reach AP threshold ('est', open symbols). Bars show the mean $\pm$ SEM for all points (filled and open) for each cell type and stimulus layer. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; *ns*, not significant.

4.6 Biophysical properties of excitatory synaptic inputs to SL and SP cells:

Summary of key findings

In this chapter I examined the possible electrophysiological correlates of the structural differences between SL and SP spines identified in the previous chapter. I found that SL cells receive powerful unitary *aff* inputs from the OB (layer 1a) which are significantly stronger than all of the other inputs I tested (*assn*-SL, *aff*-SP, *assn*-SP). Using VM analysis and Sr^{2+} -evoked *a*EPSCs, I showed that these strong *aff*-SL cell synapses are underpinned by a larger Q and P_r compared with *aff*-SP cell inputs. Finally, compared with SP cells, SL cells require less than half the number of unitary *aff* inputs to reach AP threshold. Together, these results suggest that SL and SP cells encode different types of *aff* information at the first level of PCx processing, with SL cells more faithfully representing OB output, and SP cells encoding integrated odour representations (*Fig. 4.6*).

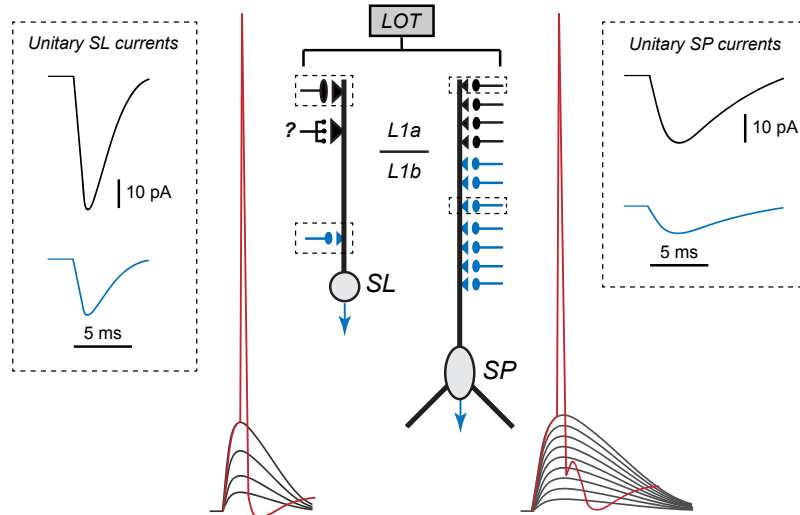


Figure 4.6. Schematic summary of main findings: biophysical properties of excitatory synaptic inputs to SL and SP cells. SL cells receive significantly stronger *aff* inputs from the OB compared with SP cells, primarily due to a larger Q and P_r . As a result, SL cells can be driven to action potential threshold with approximately half as many *aff* inputs as SP cells.

4.7 Biophysical properties of excitatory synaptic inputs to SL and SP cells:

Discussion of key findings

4.7.1 Relationship between spine size and synaptic strength

The most striking finding from this and the preceding chapter was the fact that the strongest *aff* inputs are found in the region of largest dendritic spines – on the distal dendrites of SL cells. I have shown that these strong inputs are, at least in part, due to a large quantal amplitude, Q , and high probability of neurotransmitter release, P_r . In other brain regions and cell types, electron microscopy studies reveal that the postsynaptic density size scales with spine size (Kasai et al., 2003; Holler et al., 2021) and that both AMPA and NMDA receptor-

mediated currents scale with the size of the spine head (Matsuzaki et al., 2001; Noguchi et al., 2005). A similar effect may be occurring at the large spines on SL cells, explaining the large Q at *aff*-SL cell synapses. This explanation assumes that enough neurotransmitter is released from each synaptic vesicle to activate the larger number of postsynaptic AMPA receptors at *aff*-SL synapses.

More surprising is the higher P_r at these synapses. P_r depends on presynaptic factors that determine the probability that a synaptic vesicle will be released in response to an AP in the axon. In this case the axon is a fibre in the LOT. Given that the LOT forms *aff* synapses with both SL and SP cells, P_r must be higher only in those LOT boutons that contact SL cells but not SP cells. This may be achieved by retrograde signalling that matches the properties of a presynaptic terminal to its postsynaptic target, as has been reported at other synapses (Reyes et al., 1998).

4.7.2 *Aff* axonal structure

The VM analysis reports that stimulation of unitary *aff* inputs to both SL and SP cells activates multiple neurotransmitter release sites, with $N \sim 12$ for SL cells and $N \sim 150$ for SP cells (*Fig. 4.2C*). However, my experiments provide no information about the spatial organisation of these release sites. It is known that several release sites can be accommodated on a single presynaptic terminal in the neocortex (Burns and Augustine, 1995; Sudhof, 2012); but, to explain the larger N values I found, it is more likely that unitary LOT axons branch into the PCx, targeting separate dendritic spines. Such branching is likely to be more prolific for *aff*-SP cell synapses to explain their larger N . This hypothesis would need to be confirmed using high-resolution anatomical approaches.

Understanding the structure of these inputs is important in order to establish a complete understanding of *aff* processing in the PCx. Multiple release sites onto a single spine or nearby spines would likely generate local EPSPs that interact and saturate more readily, limiting the amount of depolarisation a unitary axon could deliver (Sun and Beierlein, 2011). On the other hand, if a single axon branches to spatially dispersed spines, although the local responses may be smaller, they will summate more linearly to produce a larger somatic depolarisation. Delivering inputs onto clusters of spines may also increase the likelihood of generating local Ca^{2+} spikes, which have been shown to induce LTP at *aff* PCx synapses (Kumar et al., 2021). As such, understanding the arrangement of *aff* input is important as it can have direct implications for neuronal output and ultimately how information is processed in the PCx.

4.7.3 Transitional cell types

My experiments have shown marked differences in both the structure and function of SL and SP cells. However, layer 2 of the PCx also contains transitional cell types that appear to be graded from strongly SL-like in layer 2a, through to SP-like in layer 2b (Suzuki and Bekkers, 2011). In these experiments I targeted cells at the two extremes of layer 2 to avoid the ambiguity of cells with transitional properties. For this reason, my results may represent the two extremes of a graded continuum of cellular and synaptic properties.

4.7.4 Limitations and future directions

Although my results imply a correlation between the large dendritic spines and powerful unitary synaptic inputs to the distal dendrites of SL cells, these experiments were performed separately. Therefore, I cannot be sure that the large-amplitude synaptic inputs are actually mediated by the larger dendritic spines. In future experiments, it may be possible to

sparsely label OB output neurons (M/T cells) and optogenetically activate individual axons, while recording postsynaptic responses. Using two-photon microscopy, it may be possible to identify the specific spine associated with the recorded EPSC by simultaneously using an intracellular dye in the postsynaptic neuron. Although technically challenging, this approach may allow for more direct study of correlations between spine size and synaptic strength.

I used VM analysis to reveal the synaptic properties underlying *aff* inputs to SL and SP cells (*Section 4.2*). In these experiments, while applying regular test pulses at minimal stimulation strength, I systematically changed the bath Ca^{2+} concentration, shifting the release probability of the activated synapse. In the case of the *aff*-SP cell experiments, even at the highest Ca^{2+} concentration the EPSC variance remained high, resulting in unreliable fits to obtain estimates for N and P_r (see *Fig. 4.2B*). It was not possible to use higher bath Ca^{2+} due to precipitation, but in future work other manipulations might be used to elevate P_r and obtain more reliable estimates for N , *e.g.* short-term potentiation, enhancement of presynaptic AP width or genetic manipulation of the release machinery (Jackman and Regehr, 2017; Dittman and Ryan, 2019).

In the next chapter I delve deeper into the biophysical properties of *aff* inputs to SL and SP cells by studying long-term synaptic plasticity at these synapses.

Chapter 5

Long-term Plasticity at *aff* Synapses on SL and SP cells

Synaptic plasticity has been thoroughly studied at *assn* synapses in the PCx (Kanter and Haberly, 1993; Wilson, 2000; Lebel et al., 2001; Franks and Isaacson, 2005; Poo and Isaacson, 2007; Mukherjee et al., 2014; Strauch and Manahan-Vaughan, 2017). In contrast, *aff* inputs have long been thought to be fixed following the first month of development (Franks and Isaacson, 2005; Poo and Isaacson, 2007). Recently, however, there have been indications that *aff* synapses may express activity-dependent synaptic plasticity into adulthood (Strauch and Manahan-Vaughan, 2017; Kumar et al., 2021), but the exact type of plasticity and context in which it might manifest *in vivo* are yet to be elucidated. The following experiments aim to examine *aff* plasticity in SL and SP cells, and to add detail to current models by exploring the phenomenon at different levels of analysis.

5.1 *Aff* synaptic plasticity: Field recordings

Given the reported difficulty of inducing *aff* synaptic plasticity in the PCx, I decided to begin my investigation using classical LFP recordings (*Section 2.4.1*). The relative simplicity of this technique allowed me to more easily refine the conditions under which synaptic plasticity could be reliably induced at *aff* inputs using standard LTD and LTP protocols (see *Sections 2.7.3-4*).

5.1.1 *Aff* synapses express reliable NMDA receptor-dependent LTD

I began by attempting to elicit LTD at *aff* synapses in the PCx. To isolate *aff* inputs, all experiments were performed in the presence of baclofen (100 μ M), an agonist for GABA_B receptors which are exclusively expressed presynaptically at *assn* synapses in the PCx; thus, baclofen selectively suppresses *assn* inputs, leaving *aff* inputs unaffected (see *Section 2.5*). Paired extracellular stimuli (50 ms apart) were delivered to the LOT at 5 s intervals and the

resultant field excitatory postsynaptic potentials (fEPSPs) were recorded. After establishing a stable baseline using the test pulse protocol for a minimum of 10 min, the low-frequency stimulation (LFS) protocol was administered at the same stimulation strength used for baseline recordings (see *Section 2.7.3*). Following LFS, test pulses resumed for a further 30 min to assess changes in synaptic strength.

This protocol was first performed in normal ACSF, where LTD could not be reliably expressed (*Fig. 5.1A*, black trace; summary in *Fig. 5.1D*, black points; change = $-3 \pm 2.9\%$, $p = 0.381$, paired 2-sided t test, $n = 6$). To enhance the likelihood of inducing LTD, bath $[Mg^{2+}]$ was reduced from 1 mM to 0.5 mM. Under these conditions, LTD was consistently observed at *aff* synapses (*Fig. 5.1B*, black trace; summary in *Fig. 5.1D*, grey points; change = $-21 \pm 1.5\%$, $p < 0.001$, paired 2-sided t test, $n = 6$).

NMDA receptor-dependent LTD depends on an influx of Ca^{2+} through postsynaptic NMDA receptors. To determine whether the LTD observed in these experiments was dependent on NMDA receptors, the above protocol was repeated in bath solution containing 20 μ M MK-801 to block these receptors. Under these conditions, LTD could not be induced in any experiments, confirming that this form of LTD depends on NMDA receptor activation (*Fig. 5.1B*, red trace; summary in *Fig. 5.1D*, red points; change = $+1.0 \pm 2.6\%$, $p = 0.774$, paired 2-sided t test, $n = 4$).

Using paired stimuli allowed me to compare the paired-pulse ratio (PPR) before and after induction of LTD or LTP. A change in the PPR is typically associated with presynaptic changes in synaptic transmission (*Section 1.4.4.3*). If, for example, the second response increases relative to the first after induction (*i.e.* PPR increases) the standard interpretation is that the probability of vesicle release, P_r , has been reduced on the first of the two synaptic

stimuli, leaving more vesicles available for release on the second. The reverse interpretation would hold for a decrease in PPR. As shown in *Fig. 5.1A, B* (left panels), there was no significant change in PPR in any condition in these experiments, suggesting the observed plasticity is purely postsynaptic.

All synaptic plasticity experiments require stable recording and stimulation conditions to be sure that changes in response strength truly reflect changes in synaptic efficacy. To test for stability, I measured the amplitude of the presynaptic volley, which could often be resolved in my recordings. This analysis showed no significant changes in volley amplitude after LFS (*Fig. 5.1C*), confirming the recording stability.

These results (summarised in *Fig. 5.1D*) confirm that *aff* synapses in the PCx have the ability to reliably express NMDA receptor-dependent LTD in adult mice beyond the first month of life. These experiments also show that induction of LTD can be made more reliable by slightly reducing the bath concentration of Mg^{2+} , presumably because relief of Mg^{2+} block of NMDA receptors is then easier to achieve.

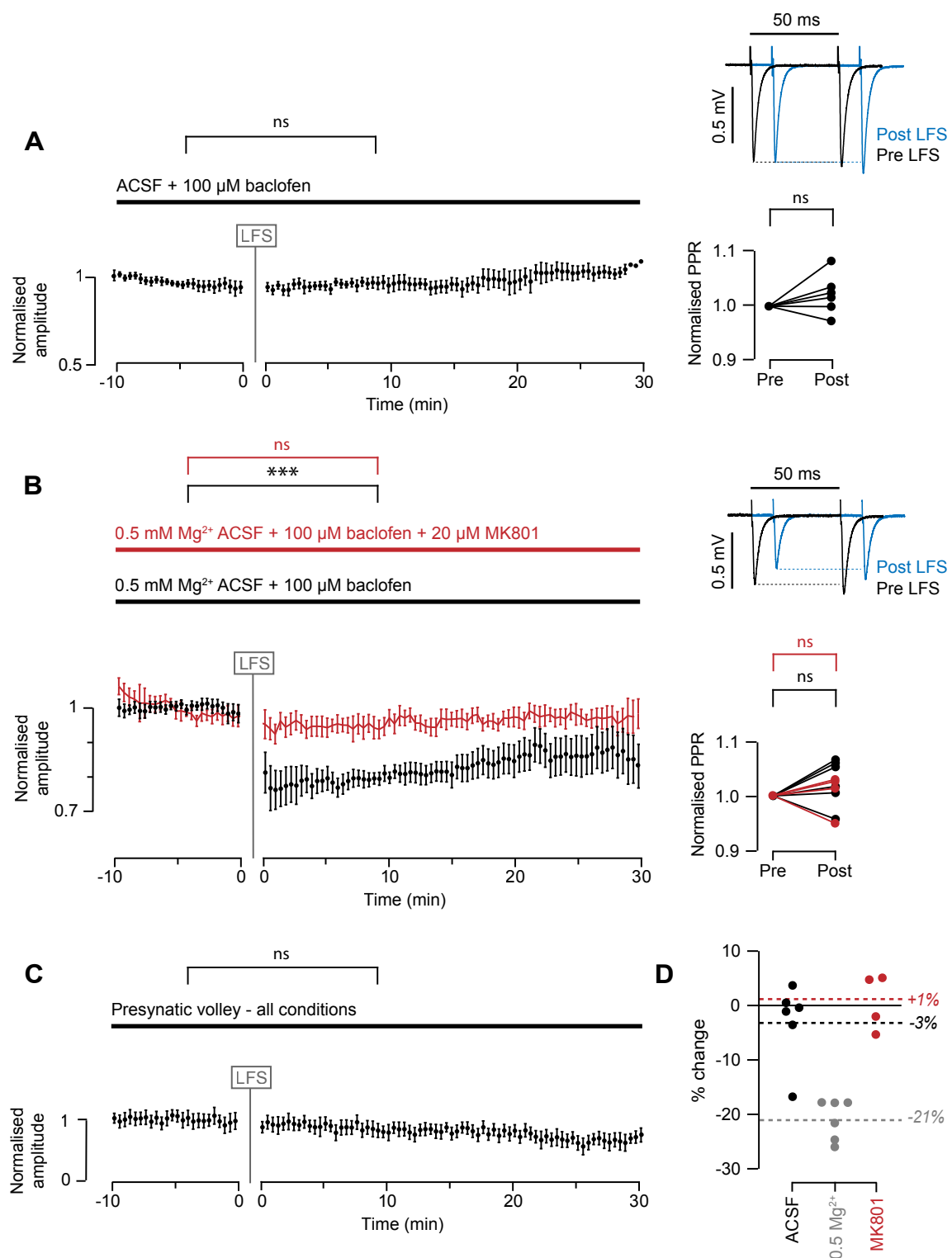


Figure 5.1. (Figure legend is on next page.)

Figure 5.1. *Aff* fEPSPs express reliable LTD which is blocked by MK-801. **A)** *Left*, average time-series plot of normalised fEPSP amplitude shows no change on average following low frequency stimulation (LFS) in ACSF with 1 mM Mg²⁺ ($n = 6$); *Right (top)*, raw traces showing no change in fEPSP amplitude following LFS stimulation (*pre*, black; *post*, blue); *Right (bottom)*, paired-pulse ratio (PPR) was unchanged following LFS. **B)** *Left*, average time-series plot of normalised fEPSP amplitude shows significant reduction following low frequency stimulation in ACSF with reduced (0.5 mM) Mg²⁺ (black, $n = 6$), an effect that is blocked by 20 μ M MK-801 (red, $n = 4$). *Right (top)*, raw traces showing a reduction in fEPSP amplitude following LFS stimulation (*pre*, black; *post*, blue); *Right (bottom)*, there was no change in PPR following LFS in either condition. **C)** *Left*, average time-series plot of normalised presynaptic volley amplitude shows no significant change following LFS in all conditions ($n = 15$), demonstrating stable stimulation. **D)** Summary of all experiments. Filled circles show individual experiments, dashed horizontal lines show mean in each condition. ***, $p < 0.001$; ns, not significant.

5.1.2 *Aff* synapses express reliable NMDA receptor-dependent LTP

Previous work has indicated that LTP cannot be induced at *aff* synapses in the PCx in rodents beyond postnatal 3-4 weeks (Franks and Isaacson, 2005; Poo and Isaacson, 2007). Based on the results for LTD in the previous section, I asked whether *aff* synapses in adult mice are able to express LTP in reduced extracellular Mg^{2+} (0.5 mM).

As with LTD, all LTP experiments were performed in the presence of 100 μ M baclofen with paired extracellular stimuli delivered to the LOT at 5 s intervals to produce *f*EPSPs. After establishing a stable baseline using the test pulse protocol for a minimum of 10 min, the full theta-burst stimulation (TBS) protocol was administered 5 times at 2.5-min intervals at the same stimulation strength as baseline (see *Section 2.7.4*). After each TBS, test pulses resumed to assess changes in synaptic strength.

This protocol was first performed in normal ACSF, in which responses to TBS were variable, with no significant changes on average (*Fig. 5.2A*, black trace; summary in *Fig. 5.2D*, black points; change = $+2.0 \pm 6.2\%$, $p = 0.753$, paired 2-sided t test, $n = 5$). In contrast, with bath Mg^{2+} reduced for 1 mM to 0.5 mM, LTP was consistently observed at *aff* synapses (*Fig. 5.2B*, black trace; summary in *Fig. 5.2D*, grey points; change = $+31 \pm 6\%$, $p < 0.01$, paired 2-sided t test, $n = 5$).

As for LTD, addition of 20 μ M MK-801 to the bath to block NMDA receptors also blocked LTP, confirming that potentiation depends on the activation of NMDA receptors (*Fig. 5.2B*, red trace). There was also no significant change in PPR in any condition tested, and recording stability was confirmed by the absence of significant changes in presynaptic volley amplitude after TBS (*Fig. 5.2C*).

These results (summarised in *Fig. 5.2D*) confirm that *aff* synapses in the PCx have the ability to reliably express NMDA receptor-dependent LTP in adult mice when recordings are done in 0.5 mM extracellular Mg^{2+} .

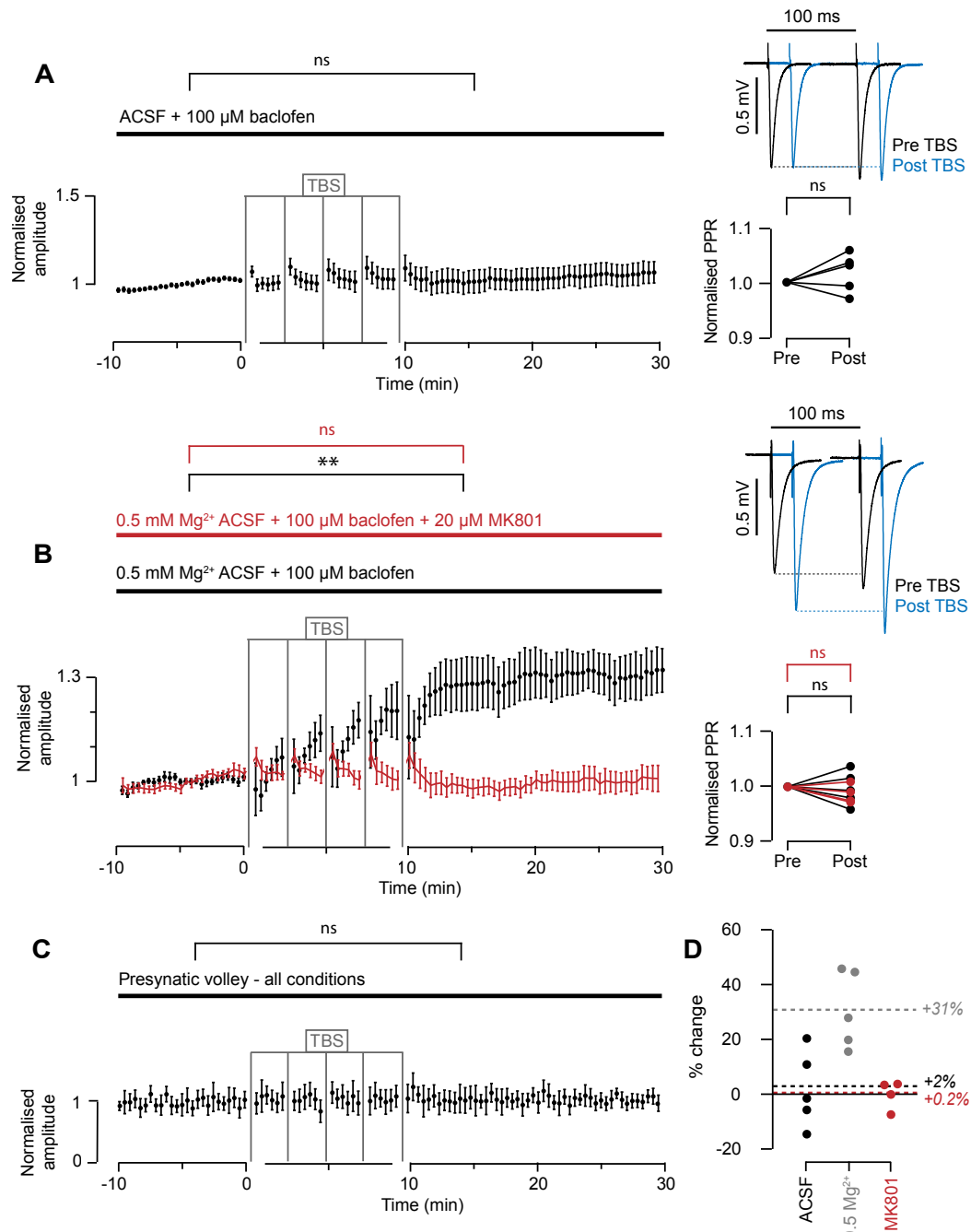


Figure 5.2. (Figure legend is on next page.)

Figure 5.2. Aff *f*EPSPs express reliable LTP which is blocked by MK-801. **A)** *Left*, average time-series plot of normalised *f*EPSP amplitude shows no change on average following theta-burst stimulation (TBS) in ACSF with 1 mM Mg²⁺ ($n = 5$); *Right (top)*, example raw traces showing no change in *f*EPSP amplitude following TBS stimulation (*pre*, black; *post*, blue); *Right (bottom)*, paired-pulse ratio was unchanged following TBS. **B)** *Left*, average time-series plot of normalised *f*EPSP amplitude shows significant increase following TBS in ACSF with 0.5 mM Mg²⁺ (black, $n = 5$), an effect that is blocked in the presence of 20 μ M MK-801 (red, $n = 4$); *Right (top)*, raw traces showing an increase in *f*EPSP amplitude following TBS stimulation (*pre*, black; *post*, blue); *Right (bottom)*, there was no change in PPR following TBS in both conditions. **C)** *Left*, average time-series plot of normalised presynaptic volley amplitude shows no significant change following TBS in all conditions ($n = 14$) demonstrating stable stimulation. **D)** Summary of experiments. Filled circles show individual experiments, dashed horizontal lines show mean in each condition. **, $p < 0.01$; ns, not significant.

5.2 *Aff* synaptic plasticity: Unitary input whole-cell recordings

The results so far indicate that *aff* synapses in the PCx of mice aged 30-40 days have the intrinsic capacity to express synaptic plasticity that persists for at least 30 min. However, the applicability of these findings to plasticity *in vivo* is still uncertain. The strong extracellular stimulation used to elicit *f*EPSPs is likely to be non-physiological. For example, this stimulation is expected to engage strong inhibition which will probably affect the induction of plasticity. Moreover, *aff* input from the OB to the PCx is sparse, with connectivity estimated to be approximately one-to-one between M/T neurons and PCx cells (Srinivasan and Stevens, 2019). The strong stimulation used for LFP experiments will recruit synchronised, multi-axonal *aff* input that does not accurately recapitulate the sparse input likely occurring *in vivo*. To address these concerns, I next turned to whole-cell recordings using plasticity protocols with minimal synaptic stimulation (as performed in *Chapter 4*). Furthermore, given my findings in *Chapter 4* about the distinctive electrical properties of *aff* inputs to SL and SP cells, I examined plasticity in these two cell types separately, starting with SP cells.

5.2.1 Unitary low-frequency stimulation of *aff* inputs does not produce significant LTD in SP cells

All whole-cell experiments were run under the same recording conditions as used in the *f*EPSP experiments (0.5 mM Mg^{2+} , 100 μ M baclofen in the bath). In some initial experiments 20 μ M gabazine was also added to the bath to block GABA_A receptor-mediated synaptic transmission. However, the addition of gabazine had no effect, likely because minimal stimulation strengths did not engage much inhibitory synaptic transmission. The stimulating electrode was placed in the LOT and a whole-cell voltage clamp recording was made from the soma of an SP cell (*Fig. 5.3A*). The stimulus strength was adjusted to achieve

unitary EPSCs that were stable across a range of stimulus intensities ('stimulus plateau', *Section 2.7.5*). As with the LFP experiments, paired extracellular stimuli (50 ms apart) were delivered to the LOT at 5 s intervals. After fixing the stimulus strength and establishing a stable *u*EPSC amplitude for a minimum of 5 min, the amplifier was switched to current clamp mode and the LFS protocol was administered without changing the stimulus strength. The amplifier was switched back to voltage clamp mode, then the paired test stimuli were immediately resumed and continued for at least 20 min (*Sections 2.7.2-3*). All stimuli were applied at the same stimulus setting. At the end of the experiment it was typically confirmed that the *u*EPSCs were still on the stimulus plateau.

Although there were some examples of minor LTD of *u*EPSCs following LFS (*e.g. Fig. 5.3B-C*), when averaged across experiments this change failed to reach significance (summary in *Fig. 5.3H*, black filled circles; change = $-13.0 \pm 5.2\%$, $p = 0.076$, paired 2-sided t test, $n = 6$). MK-801 in the bath abolished any suggestion of LTD (*Fig. 5.3D*, red trace; summary in *Fig. 5.3H*, red filled circles; change = $+4.0 \pm 2.6\%$, $p = 0.225$, paired 2-sided t test, $n = 4$). Series resistance, monitored as an indicator of recording stability, was unaltered across both recording conditions (*Fig. 5.3E*), and there were also no significant shifts in PPR following LFS in either the presence or absence of MK-801 (*Fig. 5.3F*). Lastly, there were no significant changes in the coefficient of variation (CV) of *u*EPSC amplitudes (*Fig. 5.3G*).

In summary, LFS may produce a small LTD of *u*EPSCs in SP cells but, unlike the result for field EPSPs, this effect failed to reach significance (*Fig. 5.3H*). Increasing the number of experiments in the data set will clarify these results.

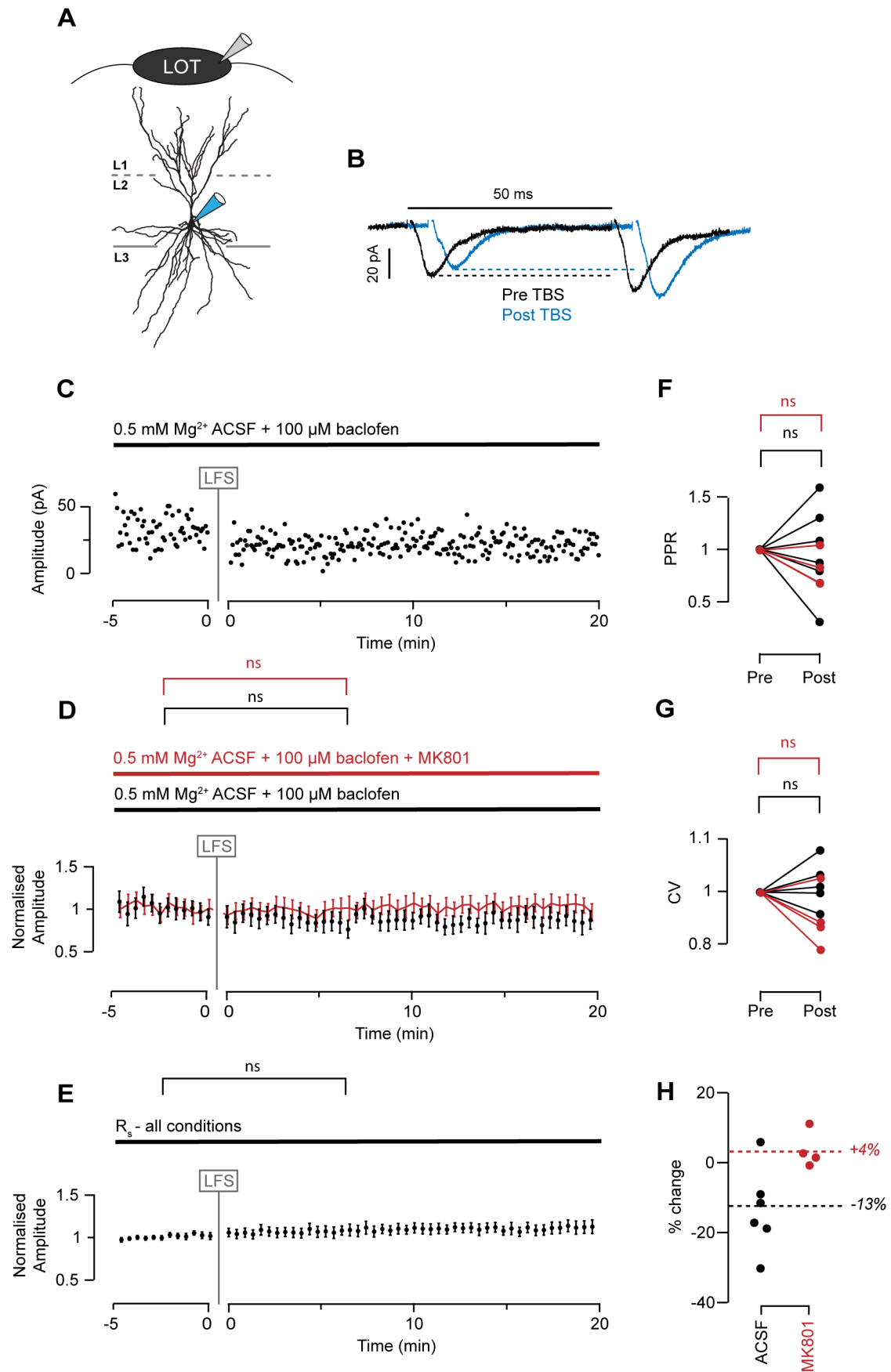


Figure 5.3. (Figure legend is on next page.)

Figure 5.3. *Aff* u EPSCs in SP cells may express weak LTD which is blocked by MK-801. **A)** Schematic showing stimulation and recording set up. Stimulation electrode was placed in the LOT and u EPSC s were recorded from SP cell somas in layer 2b. **B)** Raw traces from a typical SP cell showing marginal depression following LFS. **C)** Time-series plot of u EPSC amplitudes in an SP cell that showed slight depression after LFS. **D)** Average time-series plot of normalised u EPSC amplitudes shows a small but non-significant reduction following LFS (black, $n = 6$). MK-801 prevents any sign of u EPSC amplitude reduction (red, $n = 4$). **E)** Average time-series plot of normalised series resistance shows no significant change following LFS ($n = 7$), indicating stable recording conditions. **F)** Paired-pulse ratio was variable, showing no significant change either with (red) or without (black) MK-801. **G)** LFS did not affect the coefficient of variation of u EPSC amplitudes in either recording condition. **H)** Summary of experiments. Filled circles show individual experiments, dashed horizontal lines show mean in each condition. ns, not significant.

5.2.2 Unitary stimulation of *aff* inputs does not produce LTP in SP cells

I next asked whether LTP could be induced at *aff*-SP cell synapses using the same approach as in the previous section, except that the TBS induction protocol for LTP was used here (*Fig. 5.4A*). As before, stable unitary *uEPSCs* were established, then baseline responses to paired test stimuli were recorded for a minimum of 5 min and a maximum of 10 min to avoid washout (*Section 2.7.2*). The TBS protocol was then administered 5 times at 2.5-min intervals at unitary stimulation strength (*Section 2.7.4*). Following each TBS, test pulses were resumed to assess changes in synaptic strength.

No significant LTP of *uEPSCs* was observed following the repeated TBS induction protocols (example for a single cell in *Fig. 5.4B, C*; average of all cells in *Fig. 5.4D*; change = $-4.0 \pm 2.6\%$, $p = 0.122$, paired 2-sided t test, $n = 5$). Series resistance was stable over time for all experiments (*Fig. 5.4E*) and there were no significant shifts in PPR or the CV of the *uEPSC* amplitude following TBS in either condition (*Fig. 5.4F*).

These experiments suggest that LTP cannot be induced at *aff*-SP cell synapses using minimal stimulation and the TBS induction protocol.

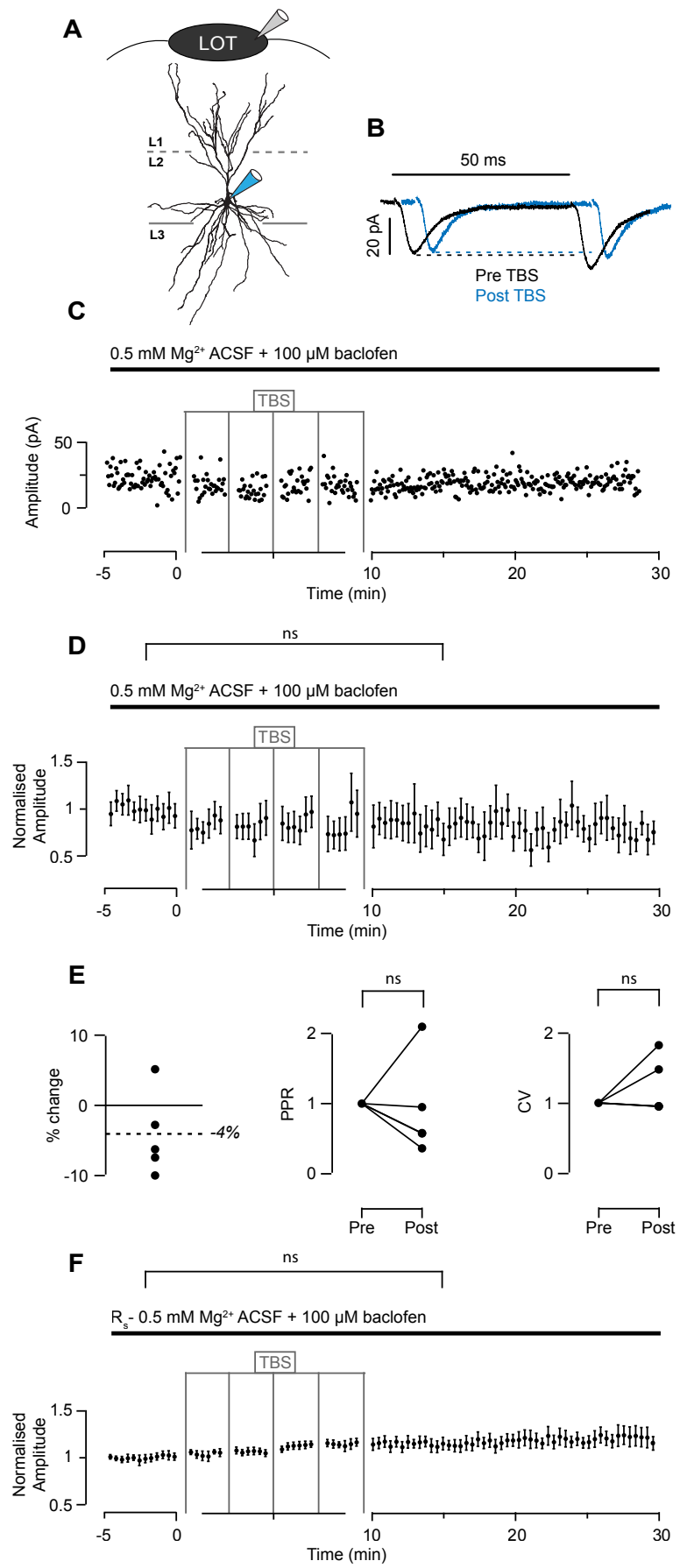


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Figure 5.4. *Aff uEPSCs in SP cells cannot be potentiated using theta-burst stimulation.* **A)** Schematic showing stimulation and recording set up. Stimulation electrode was placed in the LOT, and *uEPSCs* were recorded from SP cell somas in layer 2b. **B)** Raw traces from a typical SP cell showing minimal change following TBS (*pre*, black; *post*, blue). **C)** Time-series plot of typical *uEPSC* amplitudes in SP cells before and after TBS. **D)** Average time-series plot of normalised *uEPSC* amplitude shows no change following TBS (black, $n = 5$ cells). **E) Left**, summary of percent change in *uEPSC* amplitude. Filled circles show individual experiments, horizontal dashed line is average ($n = 5$). **Middle and Right**, normalised paired-pulse ratios and coefficients of variation, respectively, of individual experiments. **F)** Average time-series plot of normalised series resistance ($n = 5$). ns, not significant.

5.2.3 Unitary stimulation of *aff* inputs induces LTD in SL cells

I next turned to an investigation of unitary *aff* plasticity in SL cells, starting with LTD. The experimental design was identical to that for investigating unitary LTD in SP cells (*Section 5.2.1*), except that here the whole-cell recordings were made from visualised SL cell somas in layer 2a. Following LFS, the test stimuli were applied for 20 min. Depending on cell health, at the 20-min mark my standard TBS protocol (as used for triggering LTP) was administered in an attempt to reverse any observed LTD.

Unitary *aff* inputs to SL cells showed significant depression on average following LFS (*Fig. 5.5B*, black trace; *Fig. 5.5D*, black symbols, change = $-32 \pm 6\%$, $p < 0.01$, paired 2-sided t test, $n = 10$). This effect was blocked in the presence of MK-801, confirming NMDA receptor-dependence (*Fig. 5.5B*, red trace; *Fig. 5.5D*, red symbols, change = $-8 \pm 2\%$, $p = 0.143$, paired 2-sided t test, $n = 3$).

In a small number of cases, LTD could be reversed following subsequent TBS, but given a low sample size failed to reach significance (*Fig. 5.5B*, black trace, compare 15-20 min to 25-30 min, change = $+5.0 \pm 8.5\%$, $p = 0.332$, paired 2-sided t test, $n = 5$). Series resistance was stable for all experiments (*Fig. 5.5C*).

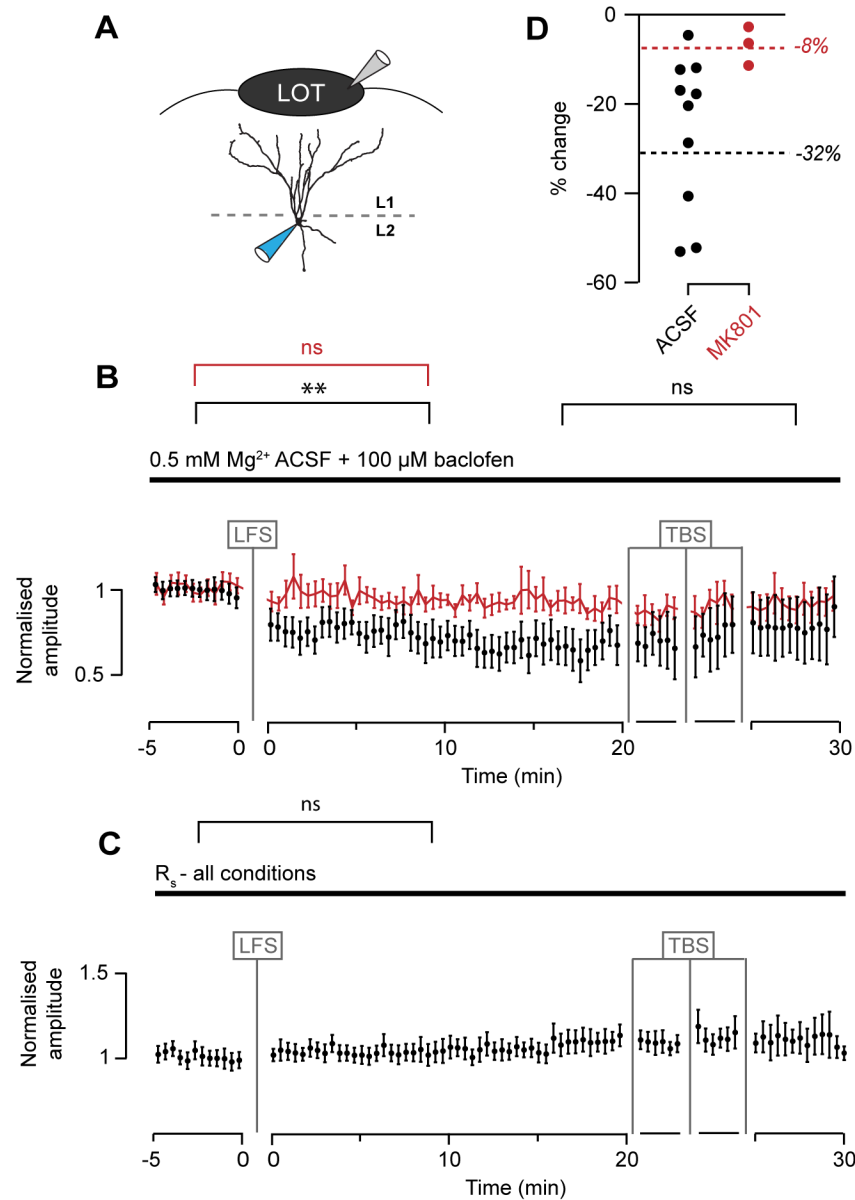


Figure 5.5. Unitary aff synapses to SL cells express variable LTD which is blocked by MK-801.

A) Schematic showing stimulation and recording set up. Stimulation electrode was placed in the LOT and *uEPSCs* were recorded from SL cell somas on the layer 1-2 border. **B)** Average time-series plot of normalised *uEPSC* amplitude shows a strong reduction following LFS (black, $n = 10$), which is blocked in the presence of MK-801 (red, $n = 3$). **C)** Average time-series plot of normalised series resistance from all conditions shows no change following LFS ($n = 9$). **D)** Summary of *uEPSC* amplitude changes that follow unitary LFS. Filled symbols show individual experiments, horizontal dashed lines are averages (black, in ACSF; red, in MK-801). **, $p < 0.01$; ns, not significant.

5.2.4 Unitary *aff* LTD expression is heterogeneous in SL cells

Although most *aff*-SL cell synapses exhibited LTD to some degree, the magnitude of depression was highly variable (*Fig. 5.5D*, black symbols). The origins of this variability became apparent from the amplitude time course plots for individual neurons (examples in *Fig. 5.6A₁₋₆*). In some cells (*e.g. Fig. 5.6A₁*), LFS caused a uniform reduction in *u*EPSC amplitude with little effect on its *CV* (*Fig. 5.6C, left*). According to the standard quantal model (see *Section 1.4.4.1*), synaptic plasticity with no change in *CV* of the EPSC amplitude is indicative of a change in the postsynaptic parameter, quantal amplitude (*Q*). In contrast, in other cells (*e.g. Fig. 5.6A₄*) the LTD seemed to be associated with a reduction in the presynaptic parameter, release probability (*P_r*), together with an increase in the *CV* of the *u*EPSC amplitude (*Fig. 5.6C, right*). There were also some instances in which both *Q* and *P_r* decreased after induction of LTD, suggesting a mixed pre- and postsynaptic mechanism of plasticity (*Fig. 5.6A₆*).

Although the *n*-values are small, it is interesting to note that the ability of the TBS protocol to reverse the previously-induced LTD seemed to depend on the mechanism of expression of the LTD (*Fig. 5.6A₁ cf Fig. 5.6A₄*, red dashed boxes). In cases where the LTD was expressed postsynaptically (change in *Q* and no change in *CV*), TBS had no effect on synaptic strength or *CV* (*e.g. Fig. 5.6A₁* and *Fig. 5.6C, left*). On the other hand, in cases where the LTD was expressed presynaptically (reduced *P_r* and increased *CV*), TBS was able to reverse the LTD and return the *CV* to starting levels or below (*e.g. Fig. 5.6A₄* and *Fig. 5.6C, right*).

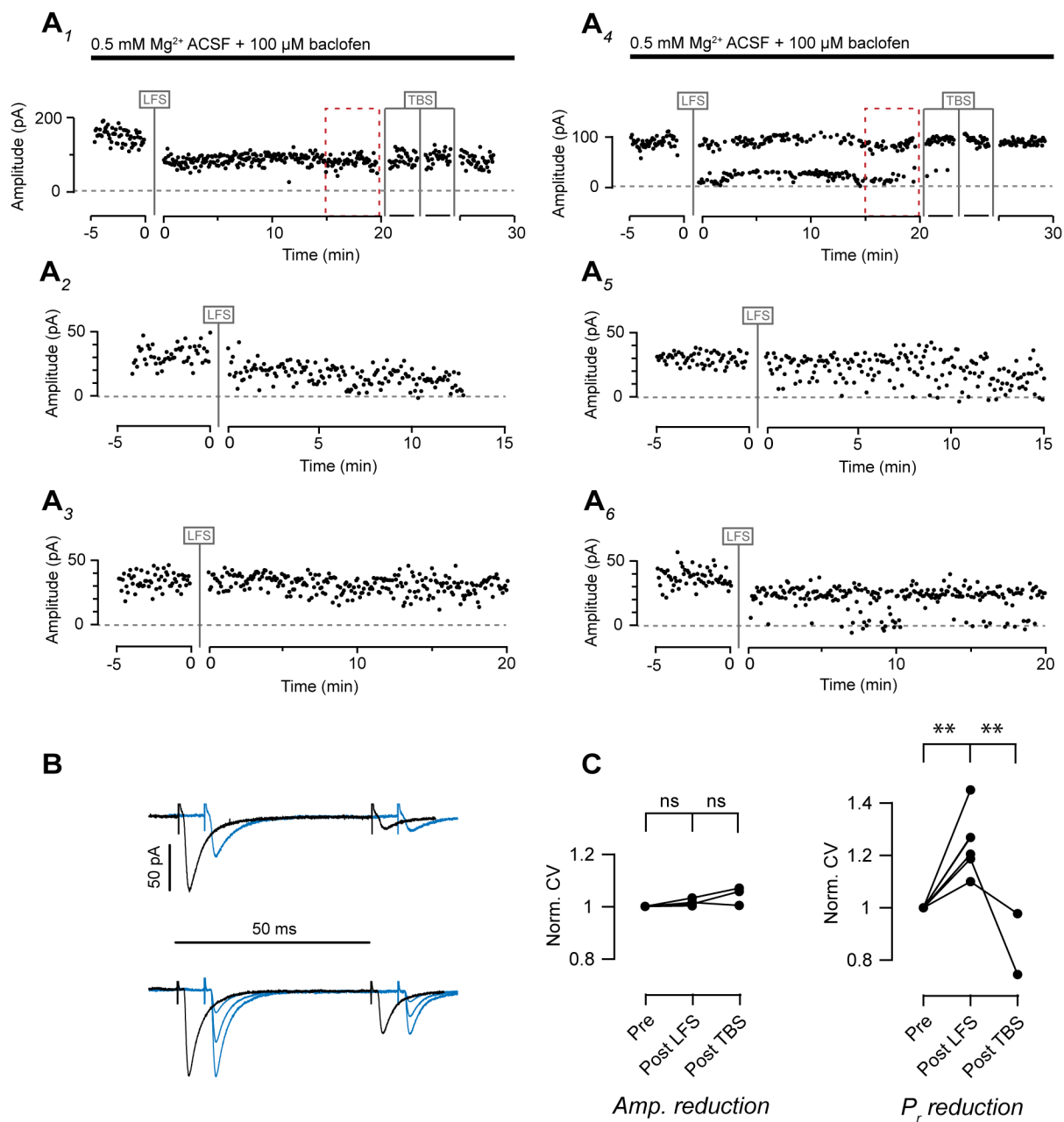


Figure 5.6. (Figure legend is on next page.)

Figure 5.6. Expression of LTD at unitary aff synapses to SL cells is heterogeneous. *A1-6)* Example plots of *uEPSC* amplitude vs time for individual experiments showing different expression profiles. LFS, low frequency stimulation (to trigger LTD); TBS, theta-burst stimulation (to trigger LTP or de-depression). **B)** Example raw traces before LFS (black) and after LFS (blue). *Top*, amplitude reduction like in panel *A1*; *Bottom*, reduction in probability of neurotransmitter release like in panel *A4*. **C)** *Left*, normalised coefficient of variation (CV) of *uEPSC* amplitudes for SL cells with LFS-induced reduction of quantal amplitude. *Right*, normalised CV of *uEPSC* amplitudes for SL cells with LFS-induced changes in release probability. In two of these cells their mean *uEPSC* amplitude was restored by TBS (*e.g.* panel *A4*). **, $p < 0.01$; ns, not significant.

5.2.5 Unitary LTP at *aff*-SL cell synapses depends on the prior state of the synapse

The suggestion in the last section that TBS produced variable synaptic enhancement – depending on the kind of previously-induced LTD – lead me to hypothesise that the ability of *aff*-SL cell synapses to express LTP depends in some way on the prior state of the synapse. To test this hypothesis, I began by using my standard TBS protocol to induce LTP at naïve *aff*-SL cell synapses, *i.e.* without prior induction of LTD. The experimental procedures were identical to those used in *Section 5.2.2* for SP cells.

The effect of TBS on *aff*-SL cell synapses was highly variable. Some synapses substantially increased in strength, apparently driven by presynaptic increases in P_r (*Fig. 5.7A*; note the reduction in failures). Others showed no change (*Fig. 5.7B*) or a reduction in synaptic strength (not illustrated). To test the above hypothesis, I quantified the prior state of the synapse for each experiment by calculating the *CV* of the *uEPSC* amplitude during the baseline period prior to TBS. A plot of percent amplitude change *versus* the calculated baseline *CV* revealed a strong correlation (*Fig. 5.7C*; correlation parameter $R^2 = 0.84$, $p < 0.001$). Thus, *aff*-SL cell synapses with high initial amplitude variability (high *CV*) exhibited strong LTP, whereas synapses with low initial variability (low *CV*) exhibited LTD or no change.

A simple interpretation of these findings is that synapses with more baseline variability have a lower release probability, P_r , so they have greater scope for increasing P_r , thereby generating stronger LTP. In other words, the size of LTP at *aff*-SL cell synapses may depend on the pre-existing probability of transmitter release at these synapses. This idea will be further explored in *Section 5.4* of this chapter.

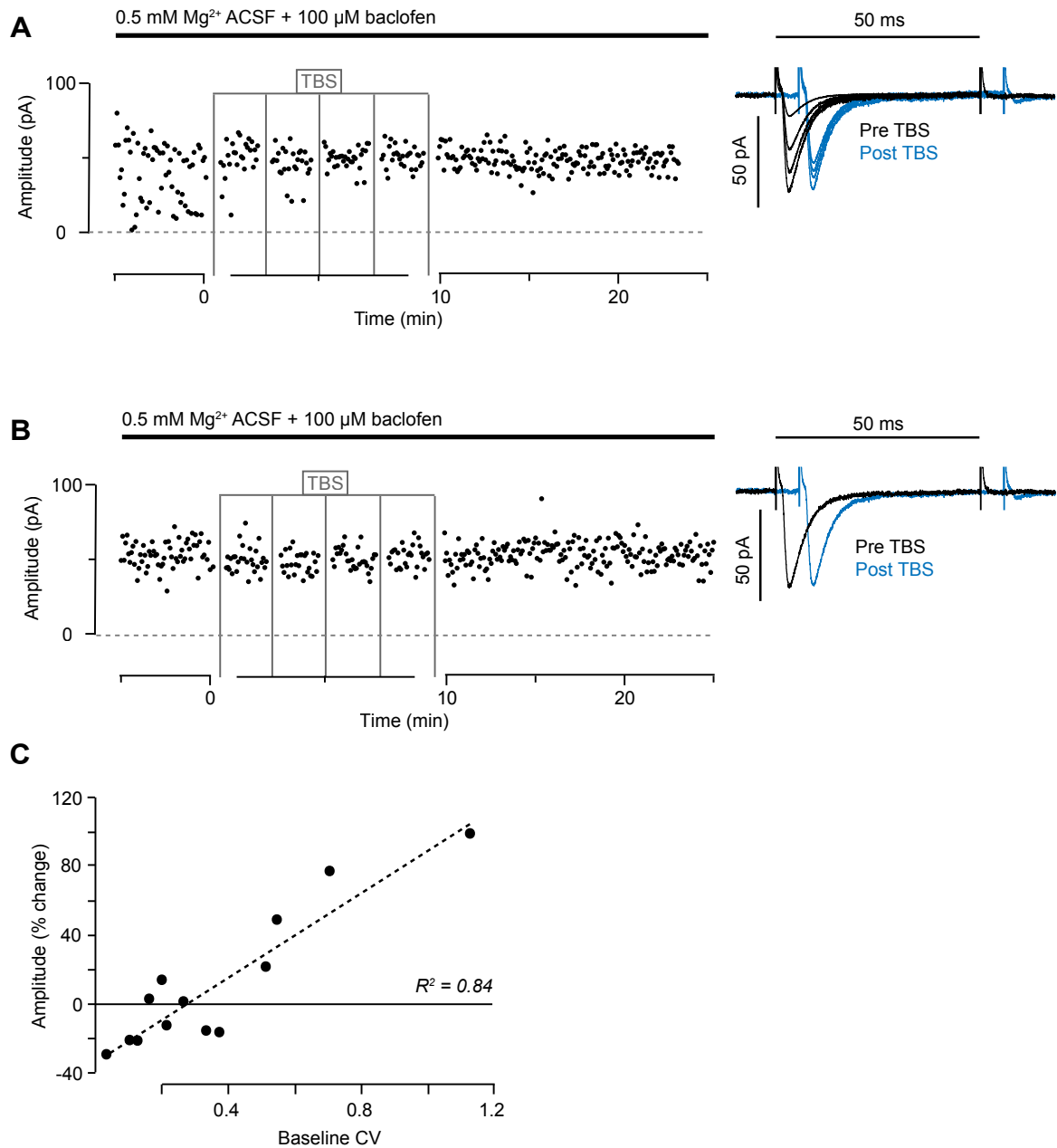


Figure 5.7. Unitary LTP of aff-SL cell synapses depends on the prior synaptic state. *A)* Left, plot of uEPSC amplitude *versus* time for an example aff-SL cell input exhibiting high variability in its baseline amplitude. Vertical lines labelled ‘TBS’ indicate times of theta-burst stimulation. *Right*, illustrative uEPSC traces for this cell (*pre-TBS*, black; *post-TBS*, blue). *B)* Similar plots for a different SL cell exhibiting low variability in its baseline amplitude. *C)* Regression analysis of change in uEPSC amplitude following TBS *versus* average baseline CV in the same cell, showing strong correlation ($R^2 = 0.84$, significant correlation, $p < 0.001$, Pearson’s correlation coefficient, $n = 13$ cells).

5.2.6 Differences in plasticity at *aff*-SL and *aff*-SP synapses may be partially explained by differences in their responses to TBS

The experiments in this section have shown that *aff* synapses onto SL cells exhibit stronger LTP, on average, than *aff* synapses onto SP cells. A possible explanation for the difference is that TBS may more strongly depolarise SL cells, enhancing the induction of LTP. To test this hypothesis, I averaged responses to all TBS trains recorded in SL or SP cells (*Fig. 5.8A*). There was no significant difference between the two cell types based on three different measures of the magnitude and kinetics of depolarisation by TBS (*Fig. 5.8B*; *e.g.* average area under the V_m plot: SL, 138 ± 17 mV.ms, $n = 10$; SP, 105 ± 40 mV.ms, $n = 5$; unpaired 2-sided t test, $p = 0.377$). On the other hand, SL cells are more depolarised at rest than SP cells (*Fig. 5.8C*), so the peak depolarisation achieved during TBS will be greater in SL cells than in SP cells. Thus, it remains possible that LTP induction is more effective in SL cells.

Aff inputs to SP cells are reported to express strong short-term facilitation, whereas those to SL cells do not (Suzuki and Bekkers, 2006). Hence, I was surprised that the response to TBS was similar in SL and SP cells; the latter would be expected to show much more facilitation during this burst of stimuli. In the next section, therefore, I conduct further experiments to understand why this may be the case.

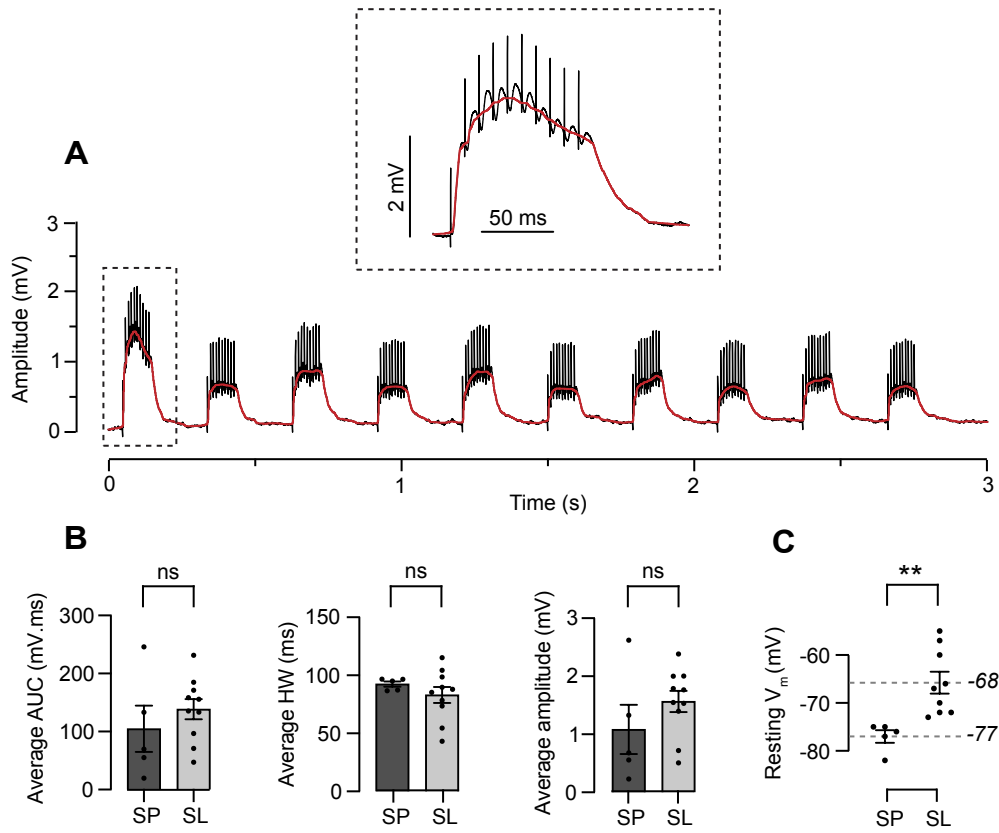


Figure 5.8. TBS-evoked unitary EPSPs in SL and SP cells are not significantly different. *A*) Example combined average TBS traces (black), with median- and low pass-filtered trace overlaid (red). *B*) Summary plots comparing area under curve (AUC), halfwidth (HW) and amplitude for TBS-evoked EPSPs in SL and SP cells. Symbols show averages from all TBS induced EPSPs in individual cells. *C*) Symbols show resting potentials of individual SL and SP cells. **, $p < 0.01$; ns, not significant.

5.2.7 *Aff*-SP synapses express short-term depression, rather than facilitation, in older mice

I systematically explored short-term facilitation at *aff*-SP cell synapses by recording *u*EPSC responses to paired stimuli at a range of interstimulus intervals under various recording conditions. Interestingly, I found that *aff*-SP cell synapses in 35 day-old mice exhibited paired-pulse depression, not facilitation as previously reported in younger (20 day-old) mice (*Fig. 5.9A*, compare black and grey symbols). I also confirmed that this depression in older mice was not affected by the bath Mg^{2+} concentration (0.5 mM used here *cf* 1 mM in Suzuki and Bekkers, 2006; *Fig. 5.9B*, compare blue and black symbols).

Another difference between my experiments and previous work was that 100 μ M baclofen was present in all of my experiments to isolate *aff* inputs without contamination from *assn* inputs, unlike previous reports (Suzuki and Bekkers, 2006). I found that baclofen had minimal effect on short-term plasticity in 35 day-old animals (*Fig. 5.9C*), but converted facilitation to depression in younger animals (*Fig. 5.9D*). This age-dependent effect of baclofen hints at developmental shifts in the expression of GABA_B receptors that may help explain the reduced propensity to express LTP after the first month of life.

In summary, these results confirm that, for the older animals used in my experiments, TBS was similarly effective at producing depolarisation in SL and SP cells, and so the observed differences in long-term plasticity at *aff*-SL and *aff*-SP synapses are more likely to be due to fundamental differences between these two synaptic inputs.

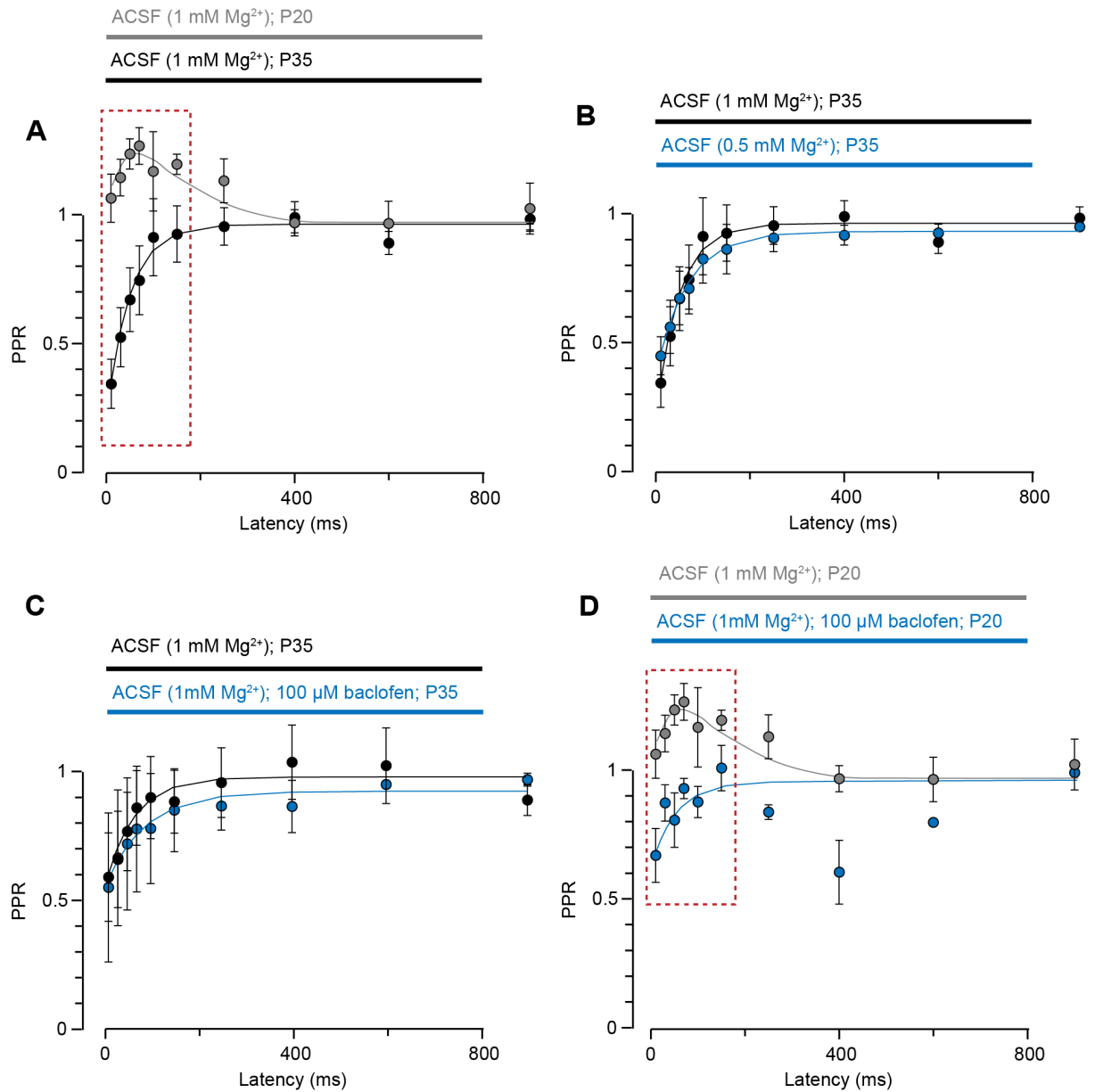


Figure 5.9. *Aff-SP cell synapses show short-term depression, not facilitation, in older mice, and this effect involves $GABA_B$ receptors.* **A)** Average PPR at different latencies 1 mM Mg^{2+} in animals aged postnatal (P) 20 (grey) and P35 (black). **B)** Average PPR at different latencies in 1 mM Mg^{2+} (black) and 0.5 mM Mg^{2+} (blue) in animals aged P35. **C)** Average PPR at different latencies in ACSF without baclofen (black) and in ACSF with 100 μM baclofen (blue) in animals aged P35. **D)** Average PPR at different latencies in ACSF (grey) and with 100 μM baclofen (blue) in young animals (P20).

5.3 Long-term plasticity at *aff* synapses on SL and SP cells: Summary of key findings

Given the distinct structural and functional features of *aff* synapses on SL and SP cells (*Chapters 3, 4*), in this chapter I explored the hypothesis that these synapses may also express long-term synaptic plasticity in different ways. I began by using local field potential recordings to show that reliable LTD and LTP could be induced at *aff* synapses onto a mixed population of SL and SP cells, provided bath $[Mg^{2+}]$ was reduced to 0.5 mM. This plasticity was dependent on NMDA receptors and appeared to be expressed postsynaptically, because the paired-pulse ratio did not change.

I next used whole-cell recordings with unitary stimulation in baclofen to study the properties of LTD and LTP at isolated *aff* inputs onto identified SL and SP cells. *Aff*-SP cell synapses showed little change following either LFS or TBS induction protocols. In contrast, *aff*-SL cell synapses expressed LTD that was generally reliable although variable in its site of expression (presynaptic or postsynaptic). The magnitude of LTP at *aff*-SL synapses was also variable, but was correlated with the *CV* of *u*EPSC amplitudes prior to induction: synapses with higher *CV* strongly potentiated by reducing variability, whereas lower *CV* synapses showed minimal change following TBS, or even depressed in some cases.

In summary, *aff* plasticity can be reliably induced by reducing bath Mg^{2+} and using strong synaptic stimulation in field recordings. *Aff*-SL cell plasticity (both LTP and LTD) can be expressed either presynaptically or postsynaptically. The magnitude of LTP appears to depend on the pre-induction probability of transmitter release at these synapses. *Fig. 5.10* shows a schematic representation of these main conclusions.

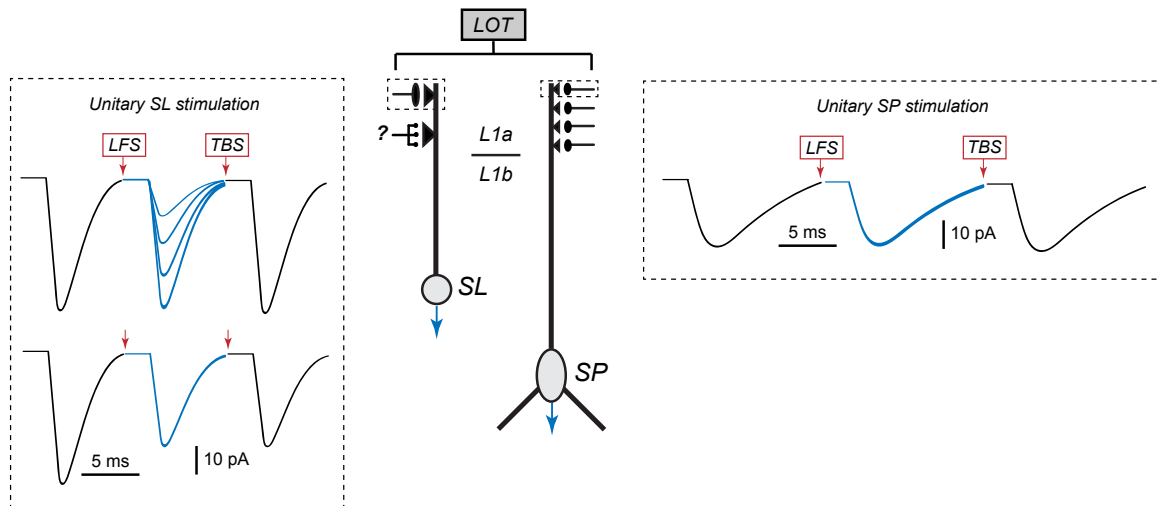


Figure 5.10: Schematic summary of main findings: long-term plasticity at aff synapses on SL and SP cells.

Unlike *aff*-SP cell synapses, that showed minimal LTD or LTP in response to LFS or TBS, respectively (inset in dashed box, *right*), *aff*-SL cell synapses were responsive to both patterns of stimulation (insets in dashed box, *left*). Following LFS, *aff*-SL synapses either increased their amplitude variability (*right, top*) or reduced their amplitude (*right, bottom*). *Aff*-SL synapses with higher baseline variability exhibited larger LTP following TBS, suggesting that LTP magnitude depends on the presynaptic state of this synapse.

5.4 Long-term plasticity at *aff* synapses on SL and SP cells: Discussion of key findings

5.4.1 Relevance of reduced bath Mg^{2+} concentration

Reducing bath Mg^{2+} concentration from 1 mM to 0.5 mM resulted in more reliable induction of LTP and LTD. As well as increasing the overall excitability of the slice, the reduction in Mg^{2+} may also help to enhance the availability of NMDA receptors, which I have shown are required for the induction of plasticity at these synapses.

Although 1 mM Mg^{2+} ACSF is conventionally used for *in vitro* electrophysiology experiments, cerebrospinal fluid (CSF) Mg^{2+} levels fluctuate in the live animal. In C57BL/6J mice, normal Mg^{2+} concentrations in the CSF have been reported at 0.89 ± 0.11 mM (Sun et al., 2009), and in healthy human populations Mg^{2+} levels vary from 0.6 to 1.4 mM (Haensch, 2010). Although low levels of Mg^{2+} in CSF are associated with disorders such as epilepsy, it is clear that there is a broad range of CSF Mg^{2+} concentrations among healthy individuals. Combined with the ability of Mg^{2+} to cross the blood-brain barrier (Ghabriel and Vink, 2011), this data suggests that CSF Mg^{2+} concentration is dynamic.

My plasticity experiments were performed at the low end of the usual range of CSF Mg^{2+} . The plasticity effects I observed could be seen at higher Mg^{2+} concentrations, only less reliably. As such, my experiments show the forms of plasticity that *aff* synapses in the PCx are capable of, but how and when this plasticity manifests in the live animal remains an open question.

5.4.2 Is minimal *aff* stimulation a relevant protocol for synaptic plasticity in the PCx?

Using anatomical and statistical methods, Srinivasan and Stevens (2019) show that, on average, only one synapse is made between a single glomerulus in the OB and any given PCx neuron. This surprising circuit property has been hypothesised to ensure that odour discrimination and sensitivity match circuit size. This sparse connectivity must be considered in plasticity experiments given the activity-dependent nature of synaptic modifications and the differences in postsynaptic depolarisation that would likely result from clustered *verses* sparse input structures. These differences could directly impact the ability of synapses to express plasticity and could influence the mechanism of plasticity induction (Citri and Malenka, 2008; Kastellakis et al., 2015). Even though most odours are complex mixes of different chemical components, and therefore activate multiple glomeruli, minimal stimulation may more accurately resemble the subtle *in vivo* input streams than traditional strong stimulation methods.

5.4.3 *Aff* synapses may have the ability to express different forms of long-term plasticity

Through this chapter, I have demonstrated long-term plasticity at *aff* synapses using both LFP and unitary input recordings. Although in both cases robust synaptic changes were observed, it is likely that the different experimental set ups reveal different forms of plasticity. One indication is that there was no change in PPR in either LFP data sets, suggesting that the plasticity measured in these experiments is expressed postsynaptically. In contrast, at the unitary input level, only SL cells expressed plasticity with evidence of both pre- and postsynaptic expression. Unlike unitary input recordings, which use minimal stimulation, LFP recordings require strong synaptic stimulation to evoke stable responses. As demonstrated by Kumar et al. (2021), strong high-frequency stimulation of *aff* synapses – like that used in my

LFP experiments – may evoke NMDA-spikes in pyramidal neurons, resulting in robust LTP. It could be the case that the strong induction protocol used in my LFP experiments evoked NMDA-spike induced plasticity in large populations of *aff*-SP synapses. Given that SP cells vastly outnumber SL cells in the PCx and receive more *aff* synaptic input (see *Chapter 3*), it may also be the case that the more subtle presynaptic plasticity observed in *aff*-SL synapses is masked by robust postsynaptic LTP evoked by strong stimulation.

5.4.4 Unitary *aff* plasticity is reliably expressed in SL but not SP cells

There was a distinct difference in the ability of SL and SP cells to express plasticity at unitary *aff* synapses. Reliable LTD and LTP was only observed in SL cells, which may be explained by their more depolarised resting potential (*Fig. 5.8*). The activation of NMDA receptors, which is required for the plasticity I observed, requires sufficient depolarisation of the postsynaptic membrane (Clarke et al., 2013). Although my LTP induction protocol had similar kinetic effects on both SL and SP cells (*Section 5.2.6*), the more depolarised resting potential of SL cells would result in greater absolute depolarisation. Given the downregulation of NMDA receptor involvement in synaptic transmission at *aff* inputs after early development (Franks and Isaacson, 2005), the significantly more depolarised resting potential of SL cells may be required to increase the probability of NMDA receptor-mediated plasticity.

5.4.5 Unitary LTP at *aff*-SL synapses correlates with initial synaptic P_r

As demonstrated in *Fig. 5.7*, I uncovered a strong relationship between the initial CV of *aff*-SL synapses and their propensity to express plasticity. CV is an indicator of the probability of neurotransmitter release, such that larger CV values correspond to a lower probability of release. In my analysis, I found that higher baseline CV values (lower P_r synapses) were associated with greater synaptic potentiation. This finding makes sense given that lower release probability synapses would have more scope to potentiate (*i.e.* they are further from saturation, $P_r = 1$).

This finding also raises the possibility that the diversity of SL spine size explored in *Chapter 3* may represent the structural manifestation of synaptic plasticity. As well as shifting the efficacy of synaptic transmission, LTP and LTD can directly alter the size of dendritic spines (Yang et al., 2008; Borczyk et al., 2019). As such, it could be the case that the unusually large spine heads I observed on distal SL dendrites had already undergone maximal LTP. This could help to explain the broad spectrum of P_r , response strengths and propensities to express LTP observed at *aff*-SL inputs.

5.4.6 Unitary input plasticity suggests intrinsic synaptic mechanism

Many forms of synaptic plasticity are thought to rely on large postsynaptic depolarisations through clustered inputs or local NMDA spikes, or the coordination between synaptic activation and action potential backpropagation. In *Chapter 6*, I will confirm that the forms of unitary plasticity described do not depend on active dendritic processes, indicating that the mechanisms driving unitary plasticity are intrinsic to the synapse itself.

This form of plasticity may serve different functions to the broader-scale forms of plasticity often described. Given that unitary plasticity can seemingly be induced in relative isolation from the rest of cell, it may be used to ‘reset’ synapses without having to modify or disrupt others. This would allow for more subtle circuit modifications and introduce the opportunity for different layers of synaptic coding.

5.4.7 Limitations and future directions

My experiments clearly show certain forms of plasticity that *aff* synapses in the PCx have the capacity to express. However, understanding how and when plasticity manifests in the live animal is an open question. As such, the main limitation of these experiments lies in the differences in circuit function between the *in vitro* and *in vivo* situation.

More so than in other brain regions, there is a distinct reduction in the excitability of the PCx *in vitro*, primarily due to the loss of respiratory drive present in the live animal (Oswald and Urban, 2012; Gonzalez et al., 2023). This is an important consideration for plasticity studies in the PCx because the reduction in excitability may substantially impact plasticity induction in a variety of ways. As discussed, NMDA receptor-dependent plasticity relies on postsynaptic depolarisation to relieve the NMDA receptor Mg^{2+} block and allow the influx of Ca^{2+} that is critical for the induction of plasticity (Liu et al., 2004). Also, it has recently been demonstrated that local NMDA spikes can induce synaptic potentiation at *aff* synapses in the PCx (Kumar et al., 2021) and, as I will show in the next chapter, NMDA receptor-mediated active events are more prevalent when slice excitability more closely resembles *in vivo* levels. Furthermore, unpublished work from our laboratory using two-photon microscopy *in vivo* demonstrates that the additional excitability provided through respiration significantly

enhances action potential backpropagation, which is critical for some forms of long-term synaptic plasticity (Dan and Poo, 2006; Letzkus et al., 2006; Kampa et al., 2007).

Baclofen was used in all of my experiments to isolate *aff* synapses (*Section 2.5*), however this is a highly artificial situation. It may be the case that the additional depolarisation provided by *assn* inputs influences *aff* plasticity induction *in vivo*, especially in SP cells that receive substantially more *assn* drive. In layer 5 pyramidal cells, for example, distal synaptic input is amplified when coactive with proximal inputs, significantly shifting neuronal output and properties of synaptic plasticity (Takahashi et al., 2016; Takahashi et al., 2020).

Through the experiments presented in this chapter, I have demonstrated some forms of plasticity that *aff* synapses in the PCx are capable of expressing. The main limitations discussed speak to the difference between circuit features in slices and the live animal. To better understand how *aff* plasticity manifests in the live animal, Ca^{2+} indicators could be sparsely expressed in both OB output neurons and SL/SP cells, allowing visualisation of both the pre- and postsynaptic terminal using two-photon microscopy. By tracking Ca^{2+} fluxes during the application of novel odours, for example, it may be possible to observe synaptic modifications in real time. An experiment of this nature would provide *in vivo* context and confirm that *aff* synapses in the PCx are dynamic, as my data suggests.

In the next chapter I will explore excitability of SL and SP cells dendrites and confirm that minimal stimulation is unlikely to evoke active dendritic processes.

Chapter 6

Dendritic electrogenesis in SL and SP cells

So far in this thesis I have assumed that *aff* synapses are received on purely passive apical dendrites, allowing relatively unambiguous interpretation of the synaptic responses recorded at the soma. A report by Larkum and colleagues provided initial evidence that the dendrites of SL and SP cells in the PCx are, indeed, largely passive (Bathellier et al., 2009). More recently, however, Schiller and colleagues have reported that these dendrites are not passive, and can express NMDA receptor-dependent spikes that may facilitate synaptic potentiation at *aff*-SL and *aff*-SP synapses (Kumar et al., 2021). In this chapter I address the question of whether the stimulation strengths used in my experiments are capable of inducing dendritic electrogenesis.

6.1 Minimal *aff* stimulation is subthreshold for triggering dendritic electrogenesis

There was no evidence of dendritic electrogenesis during my unitary plasticity induction protocols (TBS or LFS), *i.e.* EPSP amplitude scaled linearly (*Methods* 2.9.7; see example in *Fig. 5.8A*). To confirm that dendritic electrogenesis could be detected if it was present, I decided to use a protocol known to induce NMDA spikes at *aff* inputs in the PCx (Kumar et al., 2021).

To do this, I made whole-cell recordings from either the soma (*Fig. 6.1A, top*) or apical dendrite at 80-120 μm from the soma (*Fig. 6.1B, top*) of SL and SP cells. An extracellular stimulating electrode was placed in the LOT and recordings were made in ACSF containing 0.5 mM Mg^{2+} , as per plasticity experiments. Trains of three stimuli (interstimulus interval 20-50 ms) were applied. Stimulus intensity was set below threshold for eliciting a synaptic response then was increased in 2 V steps through the full range of the stimulator (0-100 V), or

until regenerative responses were elicited (see *Section 2.9.7-8*, for details on how regenerative responses were identified). At lower stimulus strengths (<12 V), the EPSP amplitude increased linearly with increasing stimulus strength, whether measured in the soma (*Fig. 6.1A*) or dendrites (*Fig. 6.1B*; superimposed EPSPs in black, middle panels; plot of amplitude vs stimulus strength, bottom panels). Further increases in stimulus strength often elicited an abrupt increase in EPSP amplitude, particularly for the later stimuli in the train (*Fig. 6.1A, B*; red traces, middle panels), suggestive of dendritic electrogenesis. Importantly, however, these active responses required stimulus strengths that were significantly higher than those used for minimal stimulation (*Fig. 6.1C*; mean stimulus used for unitary input, 2.00 ± 0.35 V, $n = 24$ cells; minimum mean stimulus for first eliciting electrogenesis, 24 ± 13 V, $n = 3$, and 76 ± 13 V, $n = 7$, for somatic and dendritic recordings, respectively; $p < 0.001$).

Given that there was no evidence of dendritic electrogenesis during my plasticity induction protocols (analysis not shown), or at unitary stimulation strengths using this protocol which has been previously used to specifically elicit active dendritic events, these results indicate that unitary *aff* inputs are subthreshold for triggering dendritic electrogenesis in SL and SP cells. Hence, the synaptic plasticity observed at unitary *aff*-SL and *aff*-SP synapses in *Chapter 5* most likely does not involve active dendritic processes such as local calcium or NMDA spikes, or backpropagating action potentials.

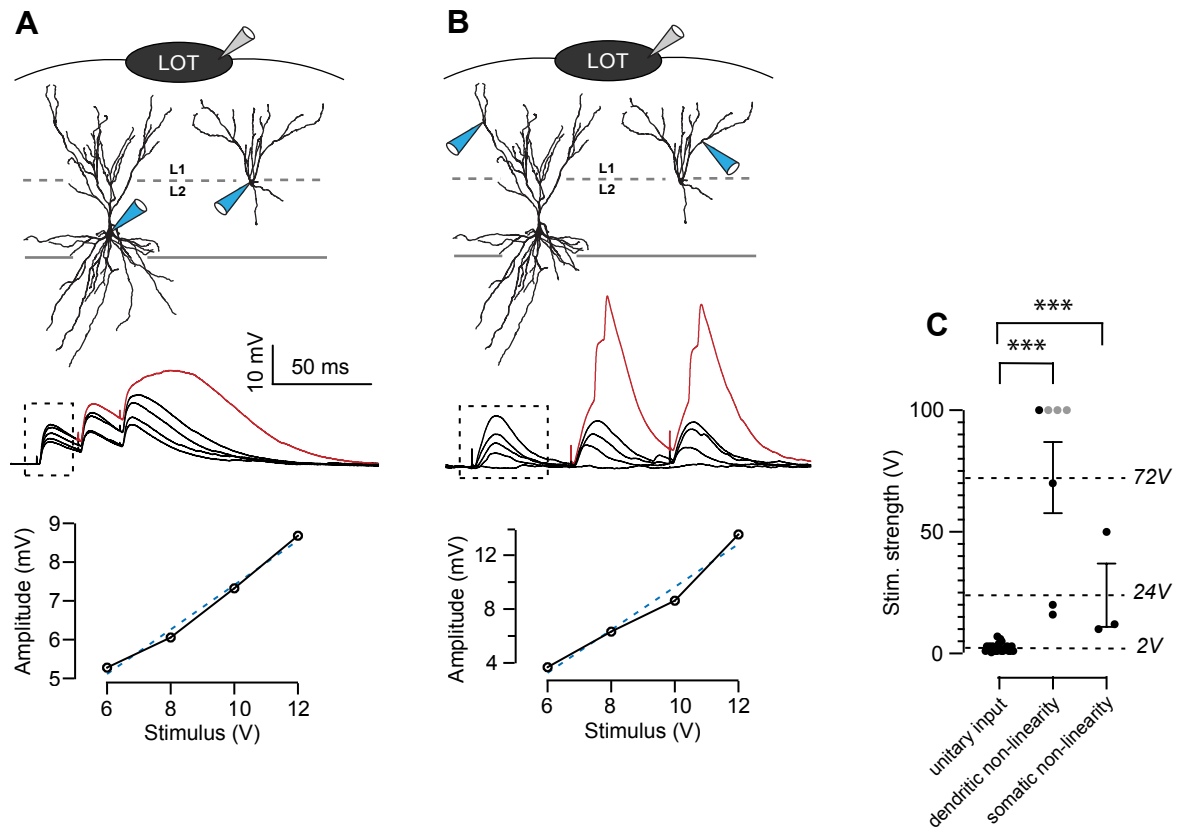


Figure 6.1. Unitary affer stimulation is subthreshold for triggering a nonlinear response when recording from either the soma or the apical dendrite. *A)* Top, schematic of the recording configuration for somatic experiments. An extracellular stimulator was placed in the LOT (grey) and whole-cell current-clamp recordings were made from the soma of either SP or SL cells (blue). Middle, example traces recorded from the soma of an SP cell showing linear scaling of EPSPs at low stimulus intensities (black), and the first non-linear event (red). Bottom, plot of EPSP amplitude for this data shows linear scaling at low stimulus intensities. Blue dashed line is a linear fit to 4 data points. *B)* Similar results for apical dendritic recordings made 100 μm from the soma of an SL cell. *C)* Summary of experiments of this kind, comparing the stimulation strength for achieving a unitary input ($n = 24$ cells) or the first sign of non-linear scaling when recording from the dendrite (grey points indicate no non-linearity at maximal stimulus strength; $n = 7$) or soma ($n = 3$). ***, $p < 0.001$.

6.2 Strong *aff* stimulation can evoke non-linear EPSPs measured at the soma

Although minimal stimulation did not evoke active events, strong stimulation to the LOT did induce non-linear EPSPs that could be measured from the soma in some cells. In these experiments, extracellular stimulation was delivered to the LOT and was increased from 0 to 100 V in 2 V steps, or until non-linear events were evoked. EPSPs were recorded from the somas of SL and SP cells (*Fig. 6.2A*). To confirm that non-linear EPSPs were evoked by *aff* inputs, and not by *assn* inputs, experiments were performed both in ACSF ($n = 3$) and in the presence of 100 μ M baclofen to block recurrent *assn* circuits ($n = 7$). Of the 10 recorded cells, 5 produced distinct non-linearities in half-width (HW), area under the curve (AUC) and amplitude as stimulus intensity increased through the range (example cell, *Fig. 6.2B₁*). Responses from the remaining 5 cells scaled linearly (example cell, *Fig. 6.2B₂*). Non-linear EPSPs were observed in both conditions (*Fig. 6.2C*; in ACSF non-linear events were evoked in 2/3 cells; with baclofen, non-linear events were evoked in 3/7 cells) and there was no difference in the prevalence of non-linear EPSPs between cell types (data not shown).

To confirm baclofen perfusion was effective, I compared EPSP kinetics following the addition of baclofen to the bath. Average EPSP halfwidth significantly reduced under these conditions (*Fig. 6.2D*), confirming that the *assn* synaptic input had been dampened.

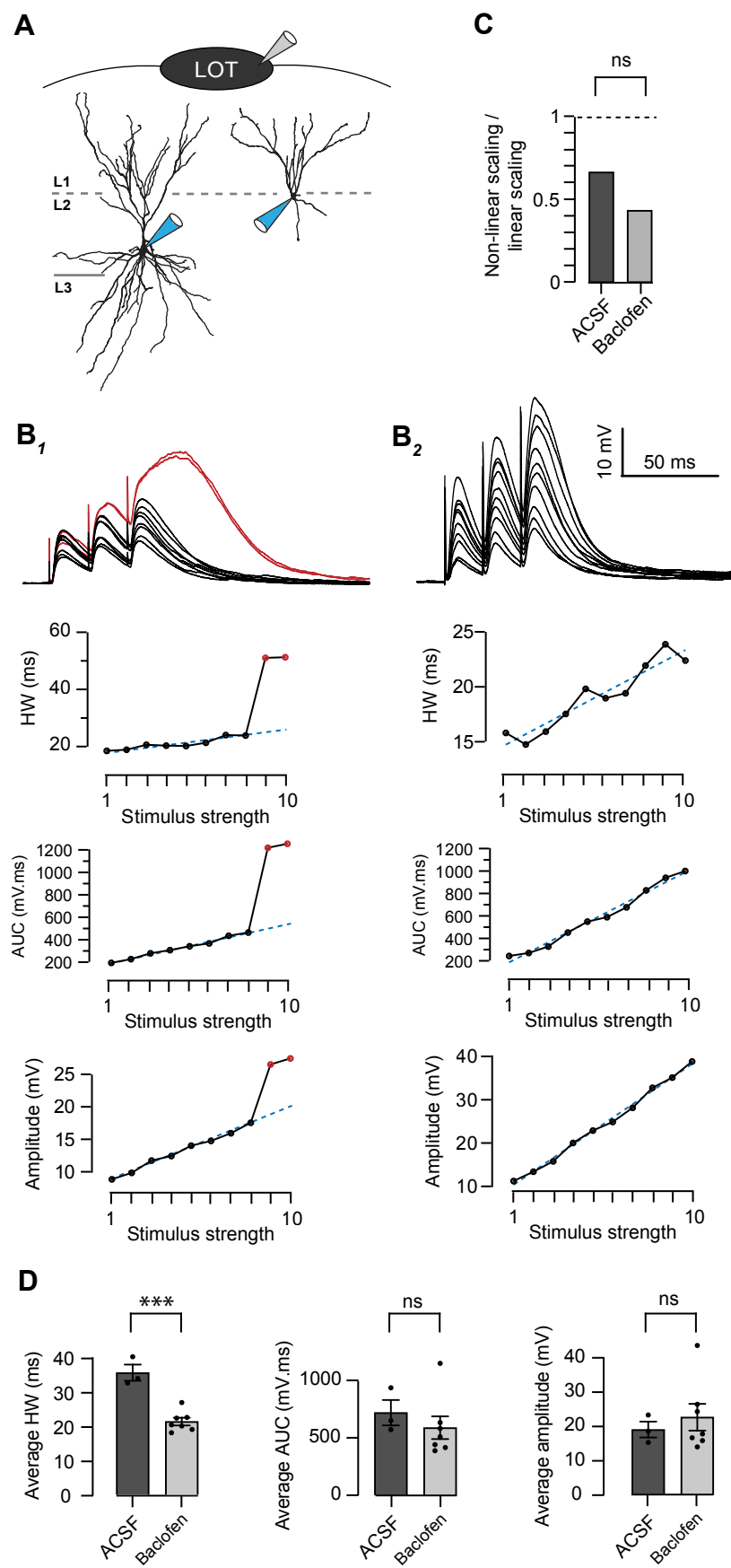


Figure 6.2. (Figure legend is on next page.)

Figure 6.2. Non-linear EPSP responses can be generated by aff synaptic stimulation. *A)* Top, schematic of the recording configuration for somatic non-linear EPSP experiments. An extracellular stimulator was placed in the LOT (grey) and whole-cell current-clamp recordings were made from the soma of either SP or SL cells (blue). *B₁)* Example of a neuron that displayed the abrupt onset of a non-linear response to increasing strength of LOT stimulation. Top, superimposed EPSPs recorded with increasing stimulus intensities, showing a non-linear response (red). Below, half-width (HW), area under the curve (AUC), and amplitude, showing non-linear increases in all measures (red data points). *B₂)* Corresponding data from a different neuron that displayed a linear scaling in EPSP size with increasing LOT stimulation. *C)* Ratio of cells that produced non-linear EPSPs to those that did not, separated by condition (ACSF/baclofen). *D)* EPSP kinetics (HW, AUC, amplitude), measured from third EPSP, for all experiments in ACSF and baclofen. ***, $p < 0.001$; ns, not significant.

6.3 *In vivo*-like conditions increase the prominence of NMDA receptor-dependent electrogenesis

I have shown in the previous two sections that non-linear synaptic events (*i.e.* putative dendritic electrogenesis) can be elicited by strong LOT stimulation in slices under control conditions. However, the situation is likely to be different in the intact animal. *In vivo* recordings from the PCx show that this cortex exhibits high levels of spontaneous excitatory activity (Tantirigama et al., 2017). The ongoing excitatory drive will constitutively depolarise neurons (thereby reducing Mg^{2+} block of the NMDA channel) and increase the availability of extracellular glutamate. Both of these are conditions that increase the likelihood of NMDA receptor activation, possibly leading to NMDA spikes.

To test this hypothesis, I increased the excitability of slices by adding 2 mM 4-AP to the bath solution. For these experiments, cells were patched in the whole-cell configuration and extracellular stimulation was delivered to the LOT (5 pulses delivered at 50 Hz, every 5 seconds). A strong stimulus strength was chosen below action potential threshold in ACSF, then 4-AP was washed into the bath. All experiments were concluded by washing in 20 μ M MK-801 to check for NMDA receptor-dependence. Consistent increases in spontaneous activity were observed in 4-AP (*Fig. 6.3A, bottom, green*) compared with ACSF (*Fig. 6.3A, bottom, black*; change is quantified as variance of fluctuations in *Fig. 6.3B*). Application of 4-AP also significantly increased the AUC of LOT-evoked EPSPs, which was partly reversed by MK-801 (*Fig. 6.3B, left*; control, 1.60 ± 0.18 mV.s; 4-AP, 5.97 ± 0.71 mV.s; 4-AP + MK-801, 2.68 ± 0.43 mV.s; $n = 6$ slices, $p < 0.001$, 2-way ANOVA). These results support my hypothesis, and suggest that enhanced spontaneous excitability is important for the determining the likelihood of EPSP non-linearities, specifically those that are driven by NMDA receptor activation.

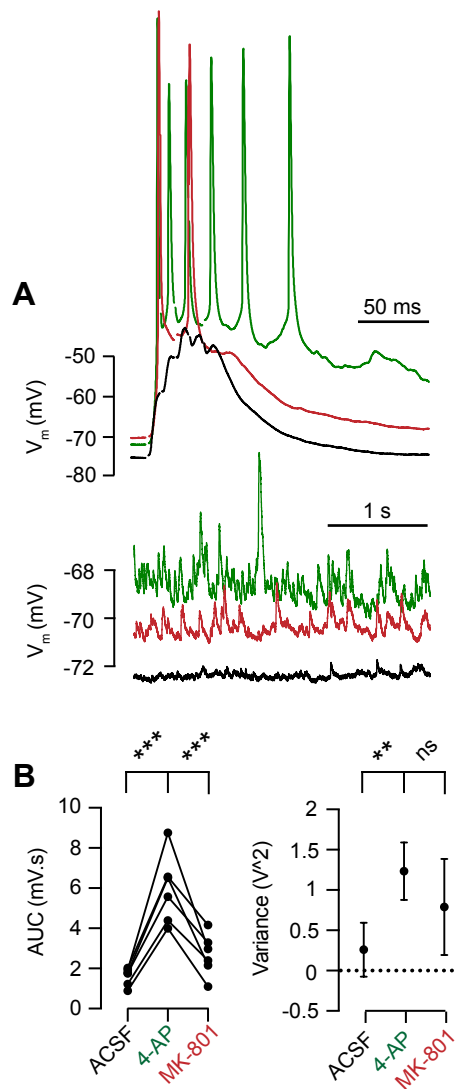


Figure 6.3. *In vivo-like spontaneous activity increases the prominence of NMDA receptor-dependent active events.* **A)** *Top*, example evoked EPSPs in ACSF (black), in ACSF + 2 mM 4-AP (green), and in ACSF + 4-AP + 20 μ M MK-801 (red). *Bottom*, spontaneous baseline activity in ACSF (black), 4-AP (green), and MK-801 (red). **B)** *Left*, AUC of EPSPs in ACSF, 4-AP, and MK-801. *Right*, variance of spontaneous baseline activity in ACSF, 4-AP, and MK-801. ns, not significant; **, $p < 0.01$; ***, $p < 0.001$.

6.4 Dendritic recordings reveal sodium spikes

Although somatic recordings can reveal active processes originating in the dendrites, direct recordings from the dendrites are more discriminating (*e.g.* compare *Fig. 6.1A* and *6.1B*). I therefore returned to whole-cell dendritic recordings from SL and SP cells to better understand the types of dendritic electrogenesis evoked by *aff* synaptic input.

In these experiments, extracellular stimulation was delivered to the LOT, and apical dendrites of either SL or SP cells were patched in layer 1a, 80-120 μm from the soma. As for somatic recordings, stimulus intensity was incrementally stepped up from 0 to 100 V, initially in ACSF and subsequently in ACSF containing 20 μM MK-801. Interestingly, the non-linearities observed were visible when measuring V_m amplitude only, with AUC and HW increasing with stimulus strength in an approximately linear fashion (*Fig. 6.4A, black*). As shown in the raw traces in *Fig. 6.4A (top)*, the non-linear amplitude jump was accompanied by the emergence of small spikes with relatively fast kinetics. These spikes persisted in MK-801 (*Fig. 6.4A, red traces*). Although the addition of MK-801 to the bath solution reduced all measures, these reductions did not reach significance (*Fig. 6.4A, bottom panels; e.g. V_m amplitude: control, 35 ± 8 mV, $n = 3$; MK-801, 26 ± 9 mV, $n = 2$, $p = 0.281$, unpaired t-test*).

Given the fast kinetics of the observed dendritic spikes, I hypothesised that they may be mediated by voltage-dependent sodium channels. To test this, the same protocol was applied with QX-314, a voltage-gated Na^+ channel blocker, in the internal solution (see *Methods, Section 2.5*). No fast dendritic spikes were observed in this condition (*Fig. 6.4B*). Although this experiment was performed in only one cell, these preliminary results suggest that these fast dendritic spikes depend on voltage-gated Na^+ channels.

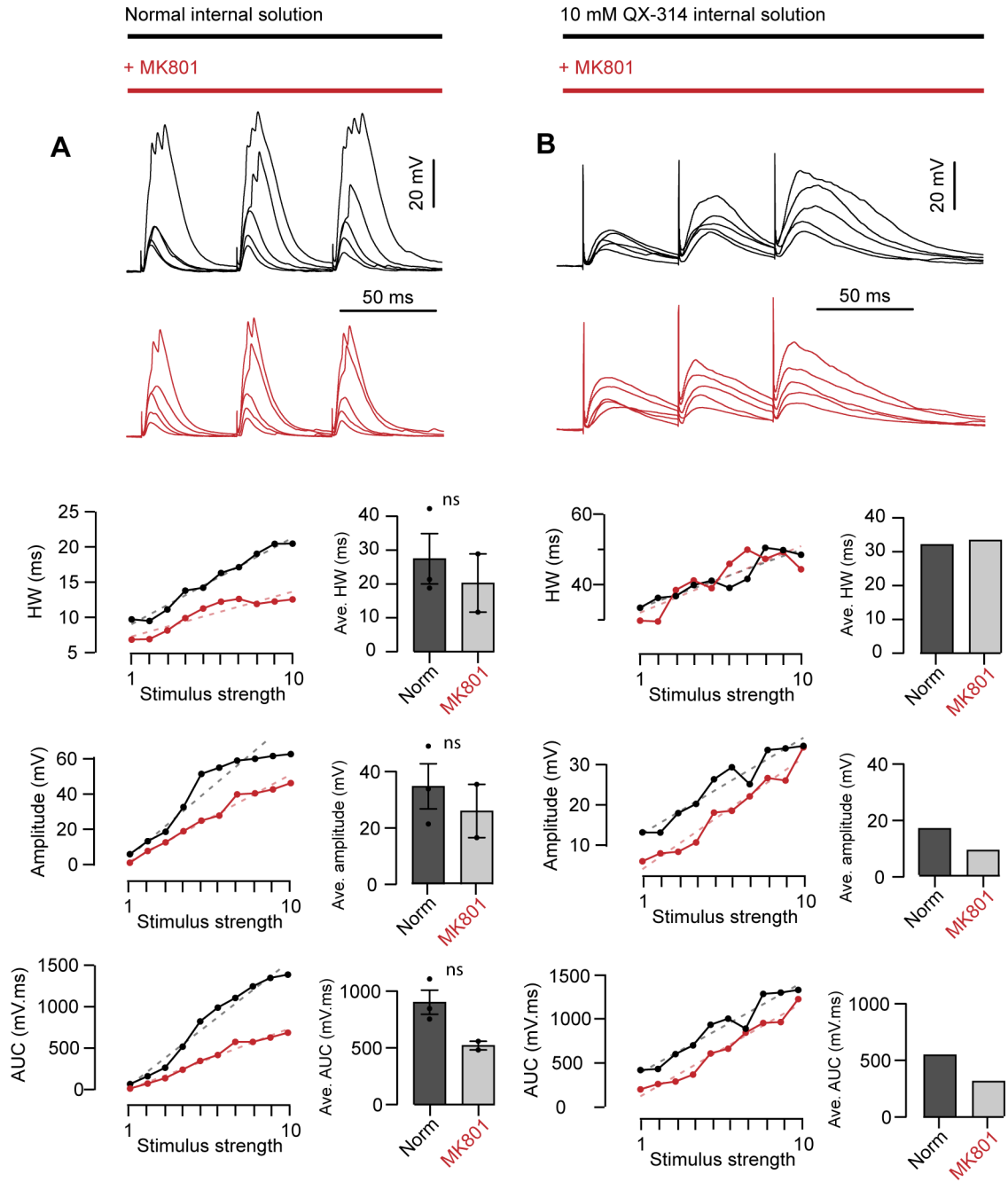


Figure 6.4. Aff synaptic stimulation elicits dendritic sodium spikes. *A)* Example traces recorded from the distal apical dendrites of a SP cell showing small spikes at higher stimulus intensities. *Top*, EPSPs evoked in ACSF; *Bottom*, EPSPs from the same dendrite after MK-801 bath application. *Below left*, HW, amplitude, and AUC, corresponding to the raw traces shown in *A*. *Below right*, average HW, amplitude and AUC of third EPSP, from all dendrites patched under these conditions. *B)* Similar experiment in a SP cell with internal QX-314, showing the absence of small spikes. Summary data are from this single cell. ns, not significant.

6.5 Dendritic sodium spikes cannot be generated through depolarising dendritic current injection

As shown in the previous section, *aff* synaptic input from the LOT can evoke sodium spikes that can be recorded from the dendrites of SL and SP cells. However, these experiments did not establish whether these spikes originate in the dendrite or from action potentials that backpropagate into the dendrites from the axon. Ideally, paired somatic-dendritic recordings would address this question directly, but these were not feasible at the time. Hence, I took a more indirect approach.

In all dendritic recordings, depolarising current steps were injected through the recording electrode for evidence of local generation of dendritic action potentials. *Fig. 6.5* shows an example of a SP cell in which sodium spikes were recorded in the dendrites following strong synaptic stimulation of the LOT (*Fig. 6.5A*). In contrast, spikes could not be evoked in this cell by depolarising current steps through the dendritic patch electrode (*Fig. 6.5C*). Subsequent recording from the same cell with internal QX-314 confirmed that the LOT-evoked dendritic action potentials were sodium spikes (*Fig. 6.5B*). This preliminary experiment suggests that sodium spikes may not be directly elicited in the dendrites, but instead could be driven by backpropagation that requires strong synaptic input.

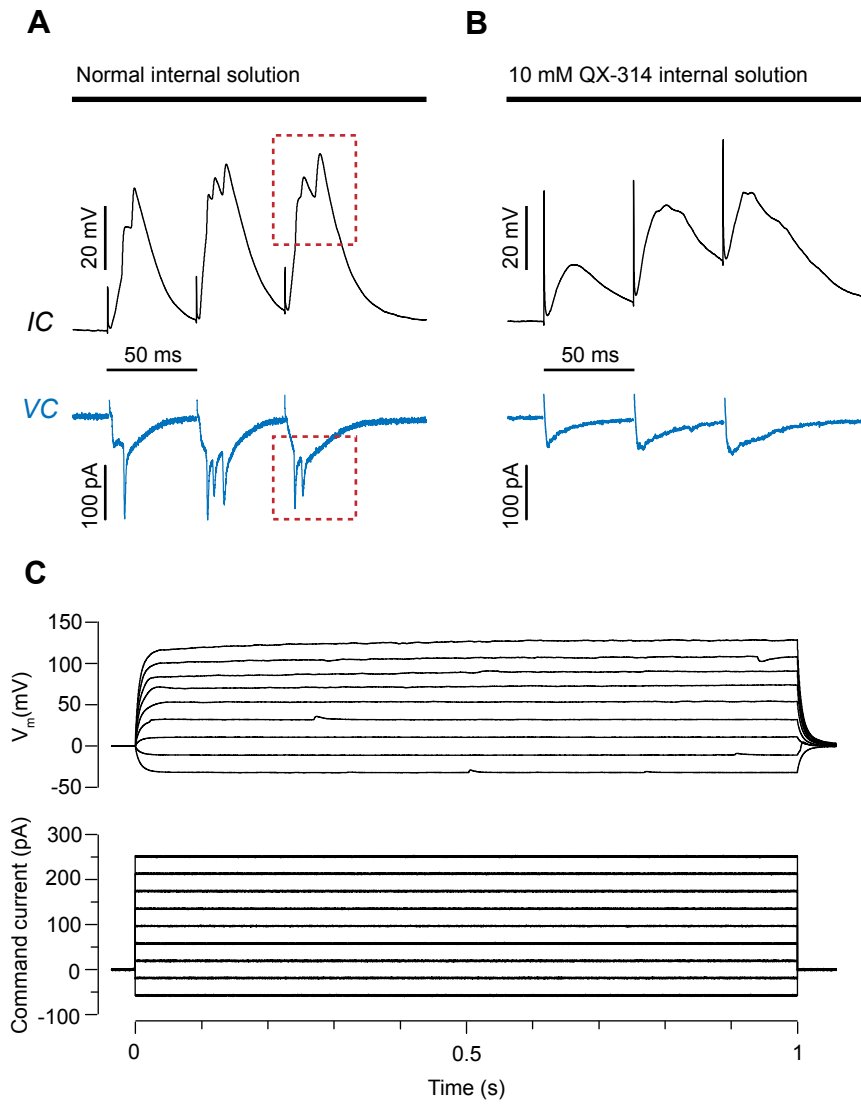


Figure 6.5. Sodium spikes cannot be elicited through depolarising dendritic current injections. *A)* An example dendritic recording from an SP cell showing spikes in both current- and voltage-clamp mode at high stimulus intensities (red dashed boxes). *B)* Dendritic recordings from the same cell as *A*, showing the ablation of spikes with internal QX-314, confirming their dependence on sodium channels. Recordings shown in both current- and voltage-clamp mode. *C)* Voltage responses (*top*) to depolarising dendritic current injections (*bottom*), showing no evidence of locally-generated action potentials.

6.6 Dendritic electrogenesis in SL and SP cells: Summary of key findings

By recording from both the soma and dendrites of SL and SP cells, I found that the minimal stimulation protocols I used for LTP induction (*Chapter 5*) did not elicit active dendritic events (*Fig. 6.1*). This finding suggests that the long-term plasticity I observed at unitary *aff* inputs does not depend on action potential backpropagation or local dendritic electrogenesis, and so is likely to be driven by a mechanism intrinsic to the synapse itself.

At higher stimulation strengths it was possible to elicit non-linear EPSPs (*i.e.* putative electrogenic events) that were visible from both the soma and dendrites of SL and SP cells (*Figs. 6.2-6.4*). In a series of preliminary experiments, I made some tentative findings about these non-linear EPSPs. First, they were inconsistently expressed across different neurons (*Fig. 6.2A, B*) and were only partially suppressed by baclofen (*Fig. 6.2C-F*), suggesting that they were not simply due to runaway polysynaptic excitation in the *assn* inputs. Second, non-linear EPSPs were more prominent when spontaneous activity was enhanced, an effect that was partially reversed by blocking NMDA receptors (*Fig. 6.3*). These findings point to the contribution of NMDA spikes to non-linear EPSPs, especially under *in vivo*-like conditions. Third, dendritic recordings showed that non-linear EPSPs were often capped with flurries of small, fast spikes that were blocked by internal QX-314 (*Fig. 6.4*), suggesting a contribution of sodium spikes. Lastly, depolarising current steps applied to dendrites were unable to elicit local voltage-dependent spikes (*Fig. 6.5*), showing that synaptic input is required to evoke the sodium spikes observed.

Importantly, no difference in the prevalence of non-linear EPSPs was observed between SL and SP cells (*Section 6.2*). Thus, the different spine and dendritic architectures on these

two classes of cells do not substantially impact dendritic electrogenesis. However, more experiments are required to confirm these preliminary results.

6.7 Dendritic electrogenesis in SL and SP cells: Discussion of key findings

6.7.1 Are non-linear EPSPs cell-autonomous or circuit phenomena?

In this chapter I defined non-linear EPSPs by their abrupt increase in amplitude and slowing of kinetics in response to a graded increase in strength of LOT stimulation. This non-linear change is often taken as a hallmark of cell-autonomous electrogenesis (Bathellier et al., 2009; Kumar et al., 2018; Kumar et al., 2021). However, another interpretation is that the non-linearity is due to sudden changes in the electrical activity of the neural circuit in which the recorded neuron is embedded, often associated with seizure-like activity (Paz and Huguenard, 2015; Luo, 2021). This possibility is especially relevant to the PCx, with its recurrent excitatory *assn* circuit that predisposes it to epilepsy (Suzuki and Bekkers, 2011; Cheng et al., 2020).

I used baclofen to suppress *assn* circuits (Tang and Hasselmo, 1994; Franks and Isaacson, 2005; Suzuki and Bekkers, 2011), and I found that non-linear EPSPs could still be elicited, albeit at higher stimulation strengths (*Fig. 6.2*). It is possible that baclofen did not provide a complete block of *assn* circuitry, or had other effects that caused a compensatory enhancement of recurrent excitation (*e.g.* changes in synaptic inhibition; Franks & Isaacson, 2005). On the other hand, the kinetics of the non-linear EPSPs (*Figs 6.1A, 6.2A*) have a more stereotyped smooth shape than EPSPs identified as arising from recurrent excitation (Suzuki & Bekkers, 2011), supporting the idea that they are cell-autonomous phenomena. Future work could definitively address this point by using genetic methods to selectively ablate *assn* circuits in the PCx (Bolding and Franks, 2018).

6.7.2 Ion channels involved in *aff*-evoked dendritic electrogenesis

Assuming that the observed non-linear EPSPs do arise from dendritic electrogenesis, a variety of voltage-dependent ion channels could be responsible (Stuart and Spruston, 2015). I initially focused on NMDA channels because of recent reports of NMDA spikes in the dendrites of SL and SP cells (Bathellier et al., 2009; Kumar et al., 2018; Kumar et al., 2021). In support of NMDA receptor involvement, I found that MK-801 suppressed the prolonged EPSPs that appeared under *in vivo*-like conditions of high spontaneous activity (*Fig. 6.3*). I reasoned that NMDA spikes should be most apparent under these conditions, because the ongoing background activity would provide extracellular glutamate to activate NMDA receptors, as well as constitutive depolarisation to reduce Mg^{2+} block of NMDA channels. On the other hand, I found little evidence of NMDA spikes in my dendritic recordings in normal conditions (*Fig. 6.4*). However, the number of recordings was small and this point needs to be explored further.

Turning to the possible involvement of other voltage-dependent ion channels in generating non-linear EPSPs, I did not examine the contributions of voltage-gated Ca^{2+} channels, in part because previous work has indicated that Ca_v channels play little role in the dendrites of SL and SP cells (Bathellier et al., 2009; Kumar et al., 2018; Kumar et al., 2021). However, I did find evidence for the involvement of voltage-gated Na^+ channels, which are discussed in the next section.

6.7.3 Sodium spikes may reflect local dendritic spikes or backpropagating action potentials

Recordings from dendrites often revealed small spikes that increased in frequency with stimulation strength (*Fig. 6.4*). These spikes were not NMDA receptor-dependent and were blocked intracellularly by QX-314, demonstrating their dependence on sodium channels. As noted above (*Section 6.4*), questions remain about the origin of these spikes – whether they originate in the dendrites or backpropagate from the axon – but revealing their source is likely to be important for understanding how information is processed in these neurons. Although dendritic sodium spikes tend to be compartmentalised to the dendritic segment where they were initiated and often fail as they propagate to lower input-resistance regions, they can deliver substantial charge to the soma and influence axonal output (Golding and Spruston, 1998; Losonczy and Magee, 2006; Katz et al., 2009). Furthermore, dendritic spikes mediated by voltage-gated sodium channels – thought to promote large, transient Ca^{2+} influxes due to their proximity to NMDA receptors and L-type Ca^{2+} channels – are required for LTP in the distal dendrites of hippocampal pyramidal cells (Kim et al., 2015). Backpropagating action potentials are also thought to have important computational functions including the amplification of synaptic input, coincidence detection (Schaefer et al., 2003), and spike timing-dependent plasticity (Dan and Poo, 2004). Thus, the characteristics and functions of dendritic sodium spikes in SL and SP cells is likely to be a rich area of future enquiry.

6.7.4 Limitations and future directions

The primary goal of the experiments in this chapter was to find whether unitary stimulation was capable of eliciting dendritic electrogenesis. I determined that this was not the case. Subsequent experiments aimed to study the properties of the non-linear responses that

appeared with stronger *aff* stimulation. These experiments were exploratory in nature and raised many new questions. Future work could apply the plethora of techniques that have been used in other brain regions to study dendritic electrogenesis, including multi-site electrical recordings from the same neuron, calcium and voltage imaging, and targeted recordings *in vivo* (Stuart and Spruston, 2015).

Chapter 7

General Discussion

Throughout this thesis I have explored the key properties of *aff* inputs received by the two main cortical recipients of olfactory information, SL and SP cells. These findings will help guide our understanding of how olfactory information is received, processed and stored within the PCx. Given that specific discussion has been provided in the Results sections (*Chapters 3-6*), I will now focus on the broader implications of my work. First, I will summarise the main findings of my experiments and briefly discuss them. Then, I will explore some of the more fundamental cell biology and physiology questions that have been raised by my experiments. Finally, I will discuss higher-level phenomena, including potential roles of SL and SP cells in olfactory coding and pattern completion, as well as the broader utility of *aff* synaptic plasticity in the PCx.

7.1 Summary of the main findings

For clarity, I will summarise my key findings with reference to the initial aims laid out in *Chapter 1*. Aim 1 was addressed through the first set of experiments described in *Chapter 3*:

***Aim 1:** To quantify the dendritic structure and the distribution of dendritic spines along the apical axis of SL and SP cells.*

These experiments demonstrate that SL and SP cells are morphologically distinct. Along the entire axis of their apical dendrites, SP cells have a dense arrangement of relatively small dendritic spines. On the other hand, SL cells have significantly fewer but larger spines on their distal apical dendrites, and very few small spines proximally. Despite these differences in size and distribution, synaptic opportunity (total spine head surface area per unit length of dendrite) was approximately equivalent on the distal apical dendrites of both cell types where *aff* input is received. Sholl and branch point analysis revealed that SP cells have significantly more

complex apical dendrites than SL cells. Taken together, these results confirmed that SL and SP cells are morphologically distinct, likely underlying their different functions within the PCx circuitry.

These findings led to my second aim exploring the functional properties of SL and SP synapses, which was addressed through the experiments presented in *Chapter 4*:

***Aim 2:** To extend the findings from Aim 1 by examining the functional properties of synapses delivering information to SL and SP cells.*

By using unitary input stimulation, VM analysis and recordings of asynchronous EPSCs, I explored the biophysical properties of synapses delivering information to SL and SP cells. These experiments demonstrated particularly powerful *aff* input to SL cells, and substantially weaker *aff* input to SP cells. Further analysis revealed that higher P_r and Q underpinned the stronger *aff*-SL inputs, resulting in SL cells requiring less than half as many *aff* inputs to reach action potential threshold than SP cells. These findings further suggest that SL and SP cells represent different types of information at the *aff* input level, and perform different roles within the PCx circuitry.

Given the morphological and functional disparity between SL and SP cells, specifically at the *aff* input level, in *Chapter 5* I asked whether these synapses are also distinct in their ability to express synaptic plasticity:

Aim 3: To establish the intrinsic ability of sensory synapses targeting SL and SP cells to express synaptic plasticity.

Here, I used field recordings to demonstrate that NMDA receptor-dependent LTP and LTD can be reliably expressed at *aff* synapses in the PCx in adult animals. The plasticity could be seen in the standard bath solution, but lowering the bath Mg^{2+} level allowed plasticity to be expressed more reliably; hence, this condition was maintained throughout. By applying the same protocols to unitary inputs, I uncovered significant differences between SL and SP cells in their ability to express LTD and LTP. Only *aff* synapses onto SL cells could be potentiated and depressed reliably. Interestingly, at these inputs there was evidence for both pre- and postsynaptic expression of LTD. The ability of *aff*-SL cell synapses to express LTP was predominantly presynaptic and depended on the prior P_r of the synapse, such that inputs with a lower starting P_r could be potentiated to a greater degree.

Finally, in *Chapter 6*, I performed preliminary experiments investigating the intrinsic electrical properties of SL and SP cell dendrites:

Aim 4: To explore the electrical properties of SL and SP cell dendrites.

These experiments were exploratory and, as such, the results presented are provisional and require further investigation. Firstly, these experiments confirmed that minimal stimulation is sub threshold for eliciting dendritic electrogenesis. This was important to distinguish the plasticity I observed from other known forms of *aff* plasticity in the PCx that rely on dendritic NMDA spikes. At strong stimulation strengths, I did identify non-linearities in EPSP scaling – an indication of dendritic electrogenesis – which were enhanced when the excitability of the

bath was increased. It was confirmed that strong synaptic stimulation elicited sodium spikes that could be recorded in the dendrites, however their exact origin will require further study.

7.2 *Aff* synapses in the PCx may be a useful model for studying dendritic spine structure

Dendritic spines are highly motile, especially during development. They are able to be formed, eliminated, expanded and contracted, ultimately determining the organisation of synaptic networks (Bhatt et al., 2009). Although spine dynamics are under genetic and molecular control, they can also be regulated by synaptic input and plasticity (Kasai et al., 2010; Runge et al., 2020). The structure of dendritic spines on SL and SP cells may prove to be a useful model for studying determinants of dendritic spine morphology, as both cell types receive *aff* synaptic input from the same source, yet have remarkably distinct spine structures. This observation raises interesting questions about what determines spine size, as the type of input alone appears insufficient. Developmental factors could be considered, like the molecular regulation of filopodia and trans-synaptic signalling, which are both known to shape spine development (Bhatt et al., 2009; Ethell and Pasquale, 2005; Yoshihara et al., 2009; Zev and Smith, 1996). Likewise, adaptive factors, like greater LTP at *aff*-SL cell synapses, could explain their unusually large spines.

7.3 Physiological relevance of minimal *aff* stimulation

Many of the experiments described in this thesis have depended on the extracellular stimulation of unitary synaptic inputs (*Chapters 4, 5, 6*). As previously discussed (*Section 5.4.2*), my focus on unitary excitation was motivated in part by evidence that afferent

connectivity from the OB to single PCx neurons is sparse (Srinivasan and Stevens, 2019), suggesting that minimal *aff* stimulation is a plausible model of the situation *in vivo*. Moreover, unitary stimulation is helpful for technical reasons, allowing me to probe the capabilities of *aff* synapses in the simplest situation, without the ill-defined complexities that are likely to result from multi-input excitation (and inhibition).

However, in *Chapter 6* I have also shown that more complex phenomena, like dendritic electrogenesis, can occur at higher stimulation strengths when multiple inputs are recruited. This raises the question: what operational regime is more relevant to the *in vivo* function of this circuit – sparse, unitary synaptic excitation, or dense, multi-input excitation? Complexities like dendritic electrogenesis are implicit in the operation of the PCx and are likely to have consequences for synaptic plasticity and information processing if, as expected, they are engaged under physiological conditions *in vivo* (see *Section 6.3*).

Given the complexities of the olfactory environment, it is likely that the principal neurons in the PCx are exposed to both clustered and sparse input streams. This variability will influence properties of dendritic integration and the type of plasticity induced, adding to the computational repertoire of the system.

7.4 Factors that determine synaptic strength

My analysis revealed striking differences in synaptic strength between *aff* inputs to SL and SP cells. *Aff*-SL cell synapses have fewer release sites on average and larger P_r , while the opposite is the case for *aff*-SP cell synapses (see *Chapter 4*). Future experiments could investigate the structural basis for these differences. For example, identified cells could be filled with an electron-dense label for serial electron microscopy. Using this technique, the

number of release sites in synaptic boutons contacting large spines on SL cells could be identified, and similarly for the smaller SP spines. This could be related to the N values I have determined. This analysis could also be used to measure postsynaptic density size (a measure of number of AMPA receptors), which could be related to the Q values I have measured. Furthermore, uncaging or optogenetic approaches could be used to excite single presynaptic boutons, which would avoid the necessary assumptions of the “unitary” extracellular stimulation approach that I used. This method would give more reliable information about functional properties of single boutons.

7.5 Synaptic plasticity at *aff*-SL and *aff*-SP cell synapses

Under my experimental conditions, I determined that *aff*-SL synapses were more plastic than *aff*-SP synapses, for both LTP and LTD. Given that synaptic plasticity is so widespread and functionally diverse, it can be difficult to determine what underlies these differences. Future experiments could use different induction protocols to investigate necessary signalling cascades involved at each input.

Variable degrees of plasticity at *aff*-SL synapses seemed to depend on the prior state of the synapse, specifically the P_r . Synapses with a low prior P_r (*i.e.* larger CV) could be reliably potentiated, such that P_r increased following TBS. However, if P_r was initially high (*i.e.* small CV), the potential for presynaptic potentiation is very low as the synapse is closer to saturation. Under these conditions, I observed synaptic depression following TBS protocols (see *Fig. 5.7C*). Informed by both my morphology and plasticity experiments, I hypothesised that the larger SL spines are those receiving high- P_r inputs, so are already maximally potentiated. Likewise, I hypothesise that the smaller spines (which are also present on *aff*-SL cell dendrites – see *Chapter 3*) are those receiving lower- P_r inputs and can therefore be

potentiated, perhaps activating structural changes that turn them into large spines. Note that these low- P_r *aff*-SL cell synapses were not picked up in VM analysis because my unitary stimulation approach is biased against them. This hypothesis could be tested using the optical stimulation methods described in *Section 7.3*.

7.6 Dendritic electrogenesis

The experiments presented in *Chapter 6* examining dendritic electrogenesis were exploratory and so the findings I presented are provisional. Nevertheless, these experiments did raise some interesting questions that could direct future investigations. Under my experimental conditions, dendritic electrogenesis could be elicited in the apical dendrites of SL and SP cells through *aff* synaptic stimulation. To ensure the non-linear EPSPs observed were not a result of recurrent recruitment of the *assn* circuitry at high stimulation strengths, baclofen was included in the bath solution. However, given the relative non-specificity of baclofen as a GABA_B receptor blocker, it is possible that elements of the *assn* network remained intact. Future experiments could use more selective *assn* blockers (*e.g.* genetic approach of Bolding & Franks, 2018) to confirm that *aff* synaptic stimulation alone can trigger dendritic electrogenesis.

Isolating *aff* inputs in this way is useful for discerning cellular and circuit features. However, it is important to acknowledge that such models remove potentially important features present in the *in vivo* context. In the live animal, with the entire circuitry intact, respiration provides powerful oscillatory drive, substantially increasing the excitability of the PCx – a feature that could directly impact the propensity of SL and SP cell dendrites to generate active events. Unpublished data from our laboratory using 2-photon imaging shows substantially more excitation and prominent dendritic electrogenesis in the intact animal.

Further experiments, both *in vivo* and *in vitro*, could be performed to explore the mechanisms involved in this dendritic electrogenesis (*e.g.* involvement of different subtypes of voltage-activated Ca^{2+} channels, NMDA channels, *etc.*). Understanding the electrical properties of SL and SP dendrites will have important implications for how information is encoded in these cell types, and for their ability to express certain forms of synaptic plasticity.

7.7 SL and SP cells may encode distinct types of olfactory information

The observation that SL and SP cells are both morphologically and functionally distinct raises questions about their respective roles within the PCx circuitry. It has been suggested that SL and SP cells process olfactory information sequentially, with SL cells receiving the majority of OB input before passing information to SP cells for more complex processing (Bekkers and Suzuki, 2013). However, more recent work reports that SL and SP cells are not hierarchical in this way, but rather process olfactory information in parallel (Nagappan and Franks, 2021). My experiments support this view by confirming that both SL and SP cells are dominant recipients of *aff* input from the OB. However, by revealing novel functional differences between synapses delivering *aff* information from the OB to SL and SP cells, my findings hint at distinctive processing roles within the PCx circuitry.

As a result of their synaptic and dendritic properties, SL cells require fewer OB inputs to reach action potential threshold (*Chapter 4*). Assuming that the unitary inputs received by SL and SP cells originate from one M/T cell belonging to a single glomerulus (which reports the response of a single subtype of odorant receptor; see *Chapter 1*), it might be said that SL cells more faithfully represent the segregated chemical components of odour established in the OB, because fewer inputs are required for SL cells to reach firing threshold. In contrast, SP cells require significantly more *aff* inputs to reach threshold (around twice as many as SL cells,

see Fig. 4.5), suggesting that their output encodes a more integrated representation of odour. Therefore, SL cells likely provide more specific (tuned to a narrower range of odour components) and reliable (powerful, high P_r inputs) source of odour identity at the *aff* layer of processing. This level of ‘ground truth’ from the *aff*-SL inputs may be an important reference point for the highly plastic *assn* circuitry of the PCx, where odour images and memories are established (Haberly, 1985; Haberly and Bower, 1989; Brennan et al., 1990; Jung et al., 1990; Kanter and Haberly, 1990; White, 1998; Lebel et al., 2001; Wilson and Stevenson, 2003a; Chandra and Barkai, 2018).

The hypothesis that SL cells provide a stable layer of odour identity that can be used as a reference point for the highly plastic *assn* circuitry could be tested by segregating SL and SP cells at different stages of olfactory learning. By using a mouse line expressing Cre-recombinase in Ntng1-expressing cells, SL cells can be exclusively targeted in the PCx (Bolding et al., 2020; Nagappan and Franks, 2021). Cre-dependent inhibitory opsins could be used to optogenetically silence SL cells at different stages of olfactory learning. I predict that odours will still be learnt during SL cell suppression, because *aff* input will still be received by SP cells (Nagappan and Franks, 2021). However, representations of odours learnt during SL suppression may drift more rapidly as the highly plastic *assn* circuitry lacks the stable reference points provided by SL cells (Schoonover et al., 2021). This effect could be tracked by measuring PCx responses to the learnt odour over subsequent days using 2-photon Ca^{2+} imaging.

7.8 *Aff* synaptic plasticity may increase processing efficiency and computational range of the PCx

The view that *aff* synapses delivering information from the OB to the PCx are unable to express synaptic plasticity in adult animals has been pervasive (Franks and Isaacson, 2005; Poo and Isaacson, 2007; Strauch and Manahan-Vaughan, 2017). More recent studies, along with those described in this thesis, detail specific forms of robust *aff* plasticity that can be reliably observed *in vitro* beyond early development (Kumar et al., 2018; Kumar et al., 2021). Although more work is required to understand how these forms of plasticity manifest *in vivo*, it may be necessary to revise our understanding of how PCx circuitry processes and stores olfactory information.

Adaptation of sensory input is common in all sensory systems (Wark et al., 2007). Within the olfactory network, sensory adaptation is known to occur through multiple mechanisms. For example, habituation of ORNs in the OE and neuromodulatory feedback to the OB are known to serve this function (Kurahashi and Menini, 1997; Brunert and Rothermel, 2021). Given that the odour space can vary dramatically over short and long timescales, such mechanisms allow the olfactory system to shape its sensitivity to match the demands of the changing sensory environment. The plasticity of *aff* synapses that I demonstrate in SL cells could help refine this sensitivity function by dynamically regulating cortical input.

There are three components of my results that support the hypothesis that *aff*-SL synapses could dynamically track the sensory environment. First, the plasticity I observe at these synapses is activity dependent, so would be modulated directly by sensory input. Second, *aff*-SL cell plasticity does not depend on clustered inputs – it can be elicited and expressed at

a unitary synapse. Given the sparse connectivity between OB and PCx (Srinivasan and Stevens, 2019), this would be a critical feature for accurately regulating sensory inputs. Finally, the *aff* plasticity I observe is bidirectional, which is to say that synapses could be both potentiated and depressed, allowing for the dynamic prioritisation of inputs. Interestingly, my LTD induction protocol used 1 Hz stimulation, which is similar to the respiration frequency in mice (1-3 Hz; Deschenes et al., 2012). Respiration-synchronised excitation from the OB is commonly seen in the PCx *in vivo* (Gonzalez et al., 2023), so it is possible that this form of LTD serves to globally depress *aff*-SL synapses to increase the contrast, and therefore sensitivity, to potentiated inputs. Such a feature could also effectively ‘reset’ these synapses when not involved in sensory encoding.

In summary, the presynaptic *aff* plasticity I have demonstrated may allow sensory synapses to track environmental demands in subtle and dynamic ways. As opposed to having hard-wired inputs, such a mechanism would allow the system to prioritise sensitivity, enhancing the survival strategies of the animal.

7.9 SL cells may enhance pattern completion

The olfactory system provides a tractable model for studying the cellular basis of memory because, unlike the other primary sensory systems, it both processes and stores olfactory information within its own circuitry (Haberly, 1985; Haberly and Bower, 1989; Brennan et al., 1990; White, 1998; Wilson and Stevenson, 2003b, a; Zelano et al., 2009; Chandra and Barkai, 2018). Thought to act as an associative memory system, integrated memory traces are proposed to be generated and maintained through synaptic plasticity within the dense *assn* circuitry of the PCx (Stettler and Axel, 2009; Shakhawat et al., 2014;

Schoonover et al., 2021). *Pattern completion* is a critical feature of associative memory systems, allowing partial or degraded input to activate stored memory traces in their entirety.

Although deep pyramidal cells, as well as horizontal and neurogliaform interneurons receive *aff* information, the majority of monosynaptic input from the OB is delivered to either SL or SP cells. As such, upon smelling a new odour, a particular population of SL and SP cells will be activated, which will feed odour information forward into the *assn* network where memory traces are established and refined. When the odour is detected at a later time, SL and SP cell output will reactivate the stored memory trace. In *Chapter 4*, I demonstrate that SL cells require fewer *aff* inputs than SP cells to reach action potential threshold. Given that a unitary *aff* input corresponds to a single chemical component of an odour mixture, SL cell output will be more robust if an olfactory stimulus is degraded through the loss of chemical components. This may be an important property of the PCx as it could enhance pattern completion and memory recall when the odour stimulus is partial or degraded. This reasoning would be flawed if SP cells were more likely to receive clustered inputs from the glomeruli encoding the same chemical component; however, no such preference was reported in a statistical analysis of OB-PCx connectivity (Srinivasan and Stevens, 2019). In summary, SL cells may be an important component of the associative memory system of the PCx, providing greater sensitivity to degraded input and, therefore, enhance pattern completion capabilities.

This hypothesis could be tested by combining an odour identification task with selective silencing of SL cells. For example, a mouse could be trained to associate a complex odour mixture with a reward (*e.g.* the mouse is trained to lick when it identifies the odour mixture). By systematically deleting one chemical component at a time from the odour mixture, the threshold at which the mouse switches from pattern completion (*i.e.* filling in the missing components) to pattern separation (*i.e.* identifying the modified mixture as a new odour) can

be determined (Barnes et al., 2008; Chapuis and Wilson, 2012). By using a knock-in mouse line expressing Cre-recombinase in Ntn1-expressing cells, combined with cre-dependent inhibitory opsins (Bolding et al., 2020; Nagappan and Franks, 2021), the same experiment could be run under conditions where SL cells are silenced. Under these conditions, I predict that the switch threshold will occur with the loss of fewer components compared to the condition with SL cells intact.

7.10 Is the PCx the ultimate repository for olfactory memory?

The PCx is a key node for olfactory memory, serving critical functions during odour learning. Following difficult olfactory discrimination tasks, the general excitability of the PCx increases for several days, making the system more sensitive to plasticity induction (Lebel et al., 2001). Over this period, accelerated olfactory learning and a greater predisposition to express LTP in both ascending (from the OB) and descending (from the orbitofrontal cortex) inputs have been reported (Barkai and Hasselmo, 1997; Barkai and Saar, 2001; Cohen et al., 2008; Chandra and Barkai, 2018). Using single unit recordings maintained for several weeks during odour learning paradigms, Schoonover et al. (2021) demonstrated that PCx representations of odour are refined and stabilized over this period. However, when regular exposure to an odour ceased, representations of that odour would drift back to baseline levels after around a month. Taken together, these findings raise interesting questions regarding the role of the PCx as a repository of odour memory over extended periods.

If the PCx is the primary substrate of long-term odour memory, there must be a representation of odour that is stable over time. This could be facilitated by downstream regions that have mechanisms to compensate for representational drift, or that are particularly sensitive to invariant features of patterns as they drift over time. It is also possible that the PCx

is not the ultimate repository of olfactory memory. Higher-order regions like insular cortex, orbitofrontal cortex and medial prefrontal cortex receive projections from the PCx and are all implicated in broader memory function (Borghei et al., 2024; Mathiasen et al., 2023). It is possible, therefore, that long-term olfactory memory is distributed among higher-order regions beyond the PCx.

Accounting for the degradation of odour representations in the PCx, ‘scratch pad’ hypotheses of piriform memory have been suggested (Schoonover et al., 2021). Given the history-dependent nature of established odour representations, along with their rapid decay when exposure ceases, it has been posited that the PCx may function as a fast learning system that continually encodes and represents information, but constantly overwrites itself. By increasing the gain of specific sensory inputs during learning, the *aff* plasticity I describe may increase the time for which odour representations can be maintained by the PCx, before ultimately being represented elsewhere. Given the vastness of odour space and the instability of the olfactory environment, a highly adaptable, fast learning system would be evolutionarily advantageous.

7.11 Conclusion

In this thesis, I have used neuronal imaging and various *in vitro* electrophysiology techniques to study the morphology and function of *aff* synapses delivering information from the OB to the primary excitatory neurons of the PCx, SL and SP cells. Ultimately, this work has revealed striking differences in synaptic structure, transmission and plasticity at these inputs, implicating their distinct roles in olfactory processing. Perhaps the most surprising of my results were those demonstrating unitary-input LTP and LTD at *aff* synapses on SL cells. It has been a pervasive view that *aff* synapses in the PCx do not express plasticity beyond development, but my findings show that these inputs do have the intrinsic capacity to express plasticity into adulthood. Further investigations will be needed to understand if and how these forms of plasticity manifest *in vivo*. Overall, the studies presented will inform future investigations into how olfactory information is processed, encoded and stored in the PCx.

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