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Static and Dynamic Aspects of Olfactory Processing Circuits

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Neurosciences

by

Kiely Noel James

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2013
The Dissertation of Kiely Noel James is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California, San Diego
2013
DEDICATION

This work is dedicated to my parents, Michael and Stephanie Martinez.
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LIST OF ABBREVIATIONS

AON Anterior olfactory nucleus

AON-pE Anterior olfactory nucleus, pars exerna

AON-pP Anterior olfactory nucleus, pars principalis

EPL External plexiform layer

GC granule cell

GCL granule cell layer

GL glomerular layer

LOT lateral olfactory tract

MCL mitral cell layer

MT cell mitral or tufted cell

MT-T MT-TeNT (Pcdh21-CRE crossed to lox-stop-lox TeNT-GFP). Vesicular release is blocked in mitral and tufted cells.

OSN Olfactory sensory neuron

OSN-T OSN-TeNT (OMP-CRE crossed to lox-stop-lox TeNT-GFP) Vesicular release is blocked in OSNs.

aPC anterior piriform cortex

PC piriform cortex

PGC periglomerular cell
**RMS** rostral migratory stream

**TeNT** Tetanus neurotoxin

**OB** olfactory bulb

**OSN** olfactory sensory neuron

**WT** wild-type
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ABSTRACT OF THE DISSERTATION

Static and Dynamic Aspects of Olfactory Processing Circuits

by

Kiely Noel James

Doctor of Philosophy in Neurosciences

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Professor Kristin Baldwin, Chair
Professor Jeffry Isaacson, Co-Chair

Neuronal circuits must balance adaptation to new developmental stages and a changing environment with the stability necessary to maintain percepts, memories and learned competencies for long periods of time. Studies of the role activity plays in neuronal survival, morphology and connectivity illustrate these two opposing forces. In the highly dynamic olfactory bulb (OB), which integrates newly born neurons throughout
adulthood, we compare the roles of first and second order neuronal signaling on the survival and morphology of several types of OB interneurons. We find that loss of first-order OSN signaling leads to a subtle decrease in OB area due to elevated cell death. Loss of second order MT cell signaling, on the other hand, leads to a more dramatic loss of area and decreased density of several interneuron populations. Surprisingly, MT cells are unaffected by OSN signaling blockade. They also survive in normal numbers when their own signaling is blocked and many their synaptic partners die. While previous studies have demonstrated that the survival of newly migrated granule cells (GCs) is sensitive to activity levels, we demonstrate that all granule cells, regardless of their birthdate, depend on activity for their long-term survival.

To show that the death and morphological defects that we observe in GCs are not due to cooperative effects of apoptotic signaling, but rather to a cell-autonomous requirement for MT cell excitatory input, we recapitulated a blockade of synaptic excitation in sparse GCs. Both knockdown of NMDAR-mediated excitation and chronic hyperpolarization led to similar morphological phenotypes as those we observe under MT cell signaling blockade.

Finally, in some sensory systems that maintain spatial mappings from the periphery into higher processing areas, activity sculpts axonal branching and synapse formation or maintenance. MT cell projections do not maintain the OB’s spatial map, but instead
branch diffusely within cortical target regions. Using single MT cell axonal tracing, we show that neither OSN nor MT cell signaling is required for the gross branching patterns of MT cells or formation of appropriate numbers of presynaptic structures. This may reflect a mechanism for maintaining perceptual stability in the absence of stereotyped spatial maps.
Chapter 1
Introduction to the role of neuronal activity in the olfactory bulb and other circuits

Introduction and global inhibition of activity

A fundamental question in neuroscience concerns role of neuronal activity in neuronal wiring and maturation. In one sense, this question can be answered succinctly: the overarching consensus is that genes set up ultrastructural wiring patterns, and activity (both spontaneous and sensory-evoked) refines connections by modulating synaptic strength, by pruning or growing new dendritic branches or spines, and sometimes by inducing to programmed cell death (Katz and Shatz 1996, Verhage, Maia et al. 2000). However, it is now clear that depending on which circuit or cell type is being queried, and on which particular trait is being quantified, activity and genes have different roles to play and differential importance.

Rather than studying “activity” as a monolithic construct, it is helpful to think in mechanistic terms about what happens when a neuron is active, and to then ask which of these components is causally responsible for generating the developmental events that are assayed. To begin with, we can draw a distinction between pre- and postsynaptic activity. Within the presynaptic neuron, the components of activity could include cell-intrinsic effects of depolarization and downstream signaling (including calcium signaling), the release of presynaptic vesicles, the activity-dependent presynaptic release of molecules such as growth factors, and responses to retrograde signaling (e.g. postsynaptic release of
growth factors or binding of pre- and post-synaptic molecules such as cell adhesion molecules). In the postsynaptic cell, “activity” could include the activation of transmitter receptors and downstream cell signaling, effects of depolarization or heightened calcium in the dendritic spine, and cell-wide effects of active, propagating depolarization.

The experimental tools that are used to block or otherwise perturb activity may target different components of activity. For example, some surgical methods of blocking sensory input such as ocular or naris occlusion may leave first-order sensory neurons intact and able to release neurotransmitters or other signaling molecules. On the other hand, surgical methods to achieve the same end, such as sensory neuron axotomy, can lead to the degeneration and death of primary neurons, which could lead to different effects. Chronic hyperpolarization of a presynaptic neuron (by overexpression of a potassium channel, often Kir2.1) will have cell-intrinsic effects in addition to reducing the probability of action potential-mediated synaptic signaling to postsynaptic partners. By contrast, tetanus toxin (TeNT) expression, which we use in this study, allows the presynaptic neuron to respond to presynaptic inputs and even fire action potentials, but prevents vesicular release, and thus synaptic signaling to postsynaptic partners. It is important to hold these mechanistic differences in mind when interpreting the findings of related studies.
In this introduction, we will first briefly survey the widely varying effects of perturbing activity in several sensory systems. Next, I will introduce the architecture of the olfactory system in some detail and review what is known about activity’s role in its development and maintenance. Finally, I will focus on a peculiar feature of the olfactory system—the ongoing integration of adult-born neurons into the olfactory bulb—and discuss the role that activity plays in the maturation and survival of these neurons.

*Activity in sensory systems*

Sensory systems provide excellent models for elucidating the role of activity in neurodevelopment. There are two important reasons for this: first, it is possible to define and control the input to these circuits, either through deprivation or presentation of particular stimuli, or through surgical or genetic means. Second, many sensory systems contain spatial maps, some of which are topographic, i.e. corresponding to one or more dimensions of the sense in question. For example, within the visual system, eye-segregated regions and retinotopic maps persist across two synapses to form eye-specific layers (LGN) and columns (visual cortex). In the auditory system, ordered tonotopic subregions can be found in the cochlear nucleus, the inferior colliculus, and auditory cortex. The somatosensory system of rodents contains topographic representations of the whiskers across several synapses, terminating in layer 4 of somatosensory cortex.
Sensory deprivation studies shed light onto the ways in which different kinds of activity are important for the formation or maintenance of these maps. More broadly, the ability to manipulate or block different types of sensory input allows experimentalists to finely dissect the roles of different components of activity.

Vision

The development of the visual system is exceptionally well studied, at both the cell and circuit levels. Within the eye, studies on the role of activity have focused on the synapse between bipolar cells and retinal ganglion cells. The vertebrate retina contains a highly stereotyped synapse of bipolar cells onto retinal ganglion cells (RGCs), in which ON bipolar cells synapse onto ON and ON-OFF RGCs, and OFF bipolar cells synapse onto OFF RGCs, in discrete layers that stratify during development. If bipolar cells are prevented from releasing glutamate synaptically, the RGCs that lie postsynaptic to them are not able to correctly prune their dendritic arbors into discrete layers (Bodnarenko and Chalupa 1993). In transgenic mice expressing TeNT in ON but not OFF bipolar cells, the ON BCs form the correct synaptic connections (to ON and ON-OFF retinal ganglion cells), but fewer of them (Kerschensteiner, Morgan et al. 2009). This decrease in synapse number is due to a lower rate of synapse formation rather than a higher rate of synapse elimination. These two studies show that neurotransmission from bipolar cells to RGCs is
important for the formation of appropriately high levels of synapses and synaptic structures, as well as postsynaptic dendritic arbor pruning.

RGCs in turn project to the dorsal lateral geniculate nucleus (LGN) and LGN neurons project to layer 4 of primary visual cortex. In both LGN and layer 4 of primary visual cortex, axonal projections are broad and highly overlapped early in development, and later they segregate into eye-specific layers (in LGN) or columns (in visual cortex). In the RGC to LGN synapse, this segregation occurs before eyes open. This eye-specific segregation depends on spontaneous, pre-sensory activity; it can be prevented by TTX infusion during late embryonic development (Sretavan, Shatz et al. 1988). However, while it has been demonstrated that the spontaneous, pre-sensory activity contains eye specificity, receptive field type and retinotopic information, thus far it is not clear whether this early activity is instructive (i.e. the patterning of the signals instruct spatial structures) or merely permissive (rev. in (Huberman, Feller et al. 2008).

In the 1960s, Hubel and Wiesel published a series of groundbreaking papers describing the thalamocortical visual projections that originate in the LGN. Eye-specific neurons in LGN in turn project axons to eye-specific ocular dominance columns in primary visual cortex. Within visual cortex, monocular deprivation during an early window of time (termed the critical period) leads to loss of territory for the deprived eye. Yet under binocular deprivation, there is no effect on column structure (LeVay, Wiesel et
al. 1980). A later study found that selectively blocking postsynaptic activity within visual cortex (via chronic infusion of muscimol, a GABA-B receptor agonist) during monocular deprivation causes a large and striking shift toward increased area occupied by thalamic axons corresponding to the closed eye (Hata and Stryker 1994). Taken together, this evidence suggests that at this synapse, sensory-evoked activity is instructive for the maintenance of spatially mapped axonal projections, and that it normally involves competitive interactions between axons. Interestingly, neither dark-rearing nor monocular deprivation affects the dendritic fields of RGCs, which are upstream of the affected LGN (thalamic) neurons (Lau, So et al. 1990).

Studies of the effects of activity on RGC projections in several non-mammalian systems have focused not on the maintenance of spatial maps, but on the morphological development of RGC axons. In zebrafish embryos, RGC axonal projections into optic tectum (a structure analogous to superior colliculus) respond to either electrical silencing (Kir2.1 overexpression) or a molecular block of presynaptic vesicle release (dominant negative VAMP expression) with slowed rates of branch formation, growth and elimination. Measures of axon arbor complexity and total length are also diminished for both populations at 5 but not 3 days after fertilization (Hua, Smear et al. 2005), but these effects disappear when neighboring axons are also silenced; i.e., when the element of competition was removed.
Later work on the same synapse in Xenopus determined that TTX activity blockade in retina increases the rate of RGC axonal branch addition and subtraction (Cohen-Cory 1999) and ultimately increases arbor complexity. When TeNT is sparsely expressed in Xenopus tadpole RGCs, their axonal arbors increase in size, though not branch number (Ben Fredj, Hammond et al. 2010). The TeNT-expressing RGC axons also have an unusually high number of filopodia. As in other systems, this effect is attenuated when competition between axons is removed. In particular, when the experiment is altered so that many RGCs express TeNT, the dense filopodia phenotype persists, but RGC axonal arbors are pruned to a normal size (Ben Fredj, Hammond et al. 2010). Thus, when many RGCs express TeNT (i.e. are prevented from releasing synaptic vesicles), there is no measurable effect on RGC axons. The contrast between this result and the effect of intraocular TTX injection (Cohen-Cory 1999) suggests that RGC intrinsic activity (rather than synaptic vesicle release) regulates RGC axonal branching.

Somatosensation

For mice, somatosensation begins with the whiskers, which are arranged into rows and columns. The mouse actively moves its whiskers along the surfaces around it, and the information that each whisker signals is maintained for three consecutive synapses, ending in the thalamocortical projections into somatosensory cortex. These
projections are organized into barrels whose topography maps onto that of the whiskers. In 1973, Van der Loos and Woolsey showed when a single whisker follicle is ablated in a newborn mouse, the corresponding cortical barrel disappears entirely, and the surrounding barrels increase in size to fill the space. (Van der Loos and Woolsey 1973) Interestingly, blocking postsynaptic, NMDAR-dependent activity (via chronic exposure to APV) prevents this loss of thalamic axonal territory (Schlaggar, Fox et al. 1993). Conversely, a recent study showed that a 67% knockdown of neurotransmission in all thalamocortical axons leads to a loss of barrel-like structure in the postsynaptic cells’ dendritic distribution, while the thalamocortical axons themselves maintain a normal barrel structure (Narboux-Neme, Evrard et al.). Finally, an organotypic slice culture study of rat thalamocortical projections demonstrated that electrically silencing either thalamic projection neurons or their postsynaptic target neurons in sensory cortex (with Kir2.1 overexpression) causes decreases in axonal arbor branching (Yamada, Uesaka et al. 2010).

On the whole, these data support a model in which cortical, postsynaptic neurons rely on thalamocortical sensory input to develop barreloid dendritic field, while the thalamic axons may rely on both intrinsic activity and some kind of postsynaptic feedback in order to branch normally.
**Audition**

The primary sensory neurons of the auditory system are called inner hair cells. They synapse onto neurons of the spiral ganglion, which in turn synapse onto neurons of the ventral cochlear nucleus. When the inner hair cells are prevented from firing action potentials (Born and Rubel 1988) or cannot release glutamate (VGluT3 knockout), the volume of the spiral ganglion and VCN are selectively decreased by P10 (Seal, Akil *et al.* 2008) due to death of the postsynaptic target cells. Short-term application of TTX to the auditory nerve to prevent sensory signaling from hair cells to the cochlear nucleus subtly increases the amount of presynaptic axonal branching (endbulbs of Held) onto each postsynaptic cell body (Niparko 1999). It also seems to cause the postsynaptic cell bodies to shrink slightly in size. In a related study of the same region, a different group of hair cell axonal terminals, called modified (small) endbulbs, showed a decrease in size in congenitally deaf cats (Redd, Pongstaporn *et al.* 2000). While little is known of the role of activity in the formation and maintenance of tonotopic maps in the brainstem and other auditory centers, the tonotopic mapping of projections from the cochlear spiral ganglion to the cochlear nucleus in cats does broaden (i.e. grow less precise) in neonatally deafened cats (Leake, Hradek *et al.* 2006).

In the auditory system, then, primary sensory neuron activity not only regulates presynaptic terminal morphology but also seems to control survival of postsynaptic target...
neurons within the cochlear nucleus. Depending on the specific pre- to postsynaptic synapse, loss of activity can either increase or decrease fine axonal arborization.

*Cortical callosum and cortical interneurons*

Over the last five or six decades, studies of the role of activity in development have focused not just on sensory systems, but also on several other cell types and synapses. I will briefly review two such sites: callosal projections in cortex, and the cortical interneurons.

Several papers have examined the role of synaptic transmission in the formation of callosal axonal projections and synapses. In utero electroporation of Kir2.1 or TeNT into mouse somatosensory cortex results in aberrant callosal projections (Wang, Zhang et al. 2007). There are abnormalities in both gross region targeting of axons and in fine-scale, layer-specific targeting, with axons overshooting their target layer at higher percentages. Over time, TeNT-expressing neurons fail to maintain their axonal projections across the callosum and into contralateral cortex. In a similar study that was situated in visual cortex, Kir2.1 electroporation of projection neurons results in abnormal projection densities in some cortical layers (Mizuno, Hirano et al. 2007). By contrast, when half of the target postsynaptic cells were electroplated with a Kir2.1 construct there is no effect on axonal projections.
Finally, a recent study explored the cell-intrinsic effects of selectively silencing late-born cortical interneurons using electroporated Kir2.1 constructs driven by an interneuron-specific promoter, Dlx5/6 (De Marco Garcia, Karayannis et al. 2011). In response to this hyperpolarization, calretinin- and reelin-positive silenced interneurons have smaller, less complex axonal arbors, while VIP-positive interneurons are unaffected. The Kir2.1-expressing neurons also show aberrant, reduced migration that depended on activity between P0 and P3. The cell-type specific migration effect (but not morphology) can be mimicked by expression of a dominant-negative version of ELMO1, a Dlx-regulated gene, and rescued by ELMO1 expression.

This overview of sensory and other systems reveals that rather than following several axioms that hold true across all neurons or synapses, activity’s developmental role is highly cell and circuit dependent. For example, disruption of neurotransmission within a neuronal class (which we will use as our primary tool to perturb the olfactory system) does not affect cell-intrinsic axonal branching patterns in Xenopus RGCs (Ben Fredj, Hammond et al. 2010) or somatosensory thalamocortical projections (Narboux-Neme, Evrard et al ), but it does lead to axonal degeneration and disappearance somatosensory callosal projection neurons (Wang, Zhang et al. 2007). Similarly, chronic hyperpolarization affects the axonal arbors of some but not all cortical interneurons (Bergami, Rimondini et al. 2008).
As I will describe in more detail below, the olfactory bulb contains a number of reciprocal synapses that blur the distinction between pre- and postsynaptic partners. The majority of studies focus either on the cell-intrinsic role of activity or of the role of presynaptic activity. The importance of postsynaptic activity (on presynaptic partners) is less well understood. As mentioned above, hyperpolarizing postsynaptic somatosensory cortical neurons has no effect on presynaptic axonal projections (Yamada, Uesaka et al. 2010). This contrasts with a study in which cerebellar Purkinje cells were hyperpolarized via overexpression of a chloride channel (Lorenzetto, Caselli et al. 2009). In response, the presynaptic climbing fibers, which are usually pruned until a single climbing fiber innervates each Purkinje cell, show aberrant multiple innervation patterns, as well as aberrant subcellular targeting.

What happens to the presynaptic partner if the postsynaptic target cell is entirely gone? In the pcd (Purkinje cell degeneration) mouse line, the cerebellar granule cells respond to the degeneration of their postsynaptic target by dying in large numbers (Triarhou 1998). However, a population that lies presynaptic to the granule cells (bipolar neurons of the vestibular ganglion) are unaffected by the death of their postsynaptic cells, either in the pcd line or in the weaver line (Baurle and Guldin 1998), which directly causes death of granule cells (Smeyne and Goldowitz 1989).
Cells and circuits of the olfactory system

The basic structure of the olfactory system is remarkably similar in vertebrates from insects to fish to humans, suggesting that it is a flexible and effective solution to the evolutionary problem of detecting food, danger and mates in one’s environment. This similarity, likely due to convergent evolution rather than homology (Strausfeld and Hildebrand 1999) only adds weight to the mystery of the olfactory circuit. The olfactory bulb, a protuberant structure at the front of the brain, contains a spatial map whose coordinates defy simple description, and accommodates a constant stream of newborn neurons that even in adult animals are produced at rate 2-8 times higher than that observed in the hippocampus (Alvarez-Buylla, Garcia-Verdugo et al. 2001, Kaplan, McNelly et al. 1985, Lois and Alvarez-Buylla 1994). The bulb is radially symmetrical: sections cut in any direction reveal the same distinctive layers of cell bodies and neuronal processes. When regarded in this way, the olfactory bulb seems simple, but this apparent order disguises great complexity. I will therefore pause from my discussion of neuronal activity in neurodevelopment to describe what is known about the organization of the olfactory system, paying special attention to the OB neurons that are central to my work: the mitral and tufted cells, and the granule cells.

The olfactory sensory neurons (OSNs) are the olfactory system’s primary sensory neurons: they are the cells that directly transduce olfactory stimuli into electrical signals
and carry those signals into the brain. The OSNs, along with several types of support cells, form the olfactory epithelium (OE), a sheet-like structure that lines the nasal cavity. Each OSN expresses just a single olfactory receptor (OR) gene (Chess, Simon et al. 1994, Vassar, Ngai et al. 1993), of which there are ~1300 in the mouse genome (Buck and Axel 1991). In the OE, the expression pattern of OSNs expressing a given OR within the OE is zonal, with four DM-VL zones (Ressler, Sullivan et al. 1993, Vassar, Ngai et al. 1993). Within an OR's given expression zone, it can be found in an apparently random spatial pattern of neurons. In contrast to the canonical CNS neuron, which is generated early in development and survives throughout the individual’s lifetime, OSNs die and are born continuously during an animal’s lifetime, with each OSN living an average of six weeks.

The OR superfamily is composed of 7-transmembrane G-protein coupled receptors (Buck Axel) that bind odorant molecules with differing affinity. Odorant binding cause a conformational change in the OR which in turn activates G-olf, the G protein that is specifically expressed in OSNs. Active G-olf stimulates adenylyl cyclase to produce cAMP, and cAMP binds to cyclic nucleotide-gated channels, which then open and depolarize the OSN.

OSN axons course through the bony cribiform plate to the olfactory bulbs (OBs). When odorant-OR binding on the cilia of the OSN culminates in an action potential, that
signal propagates down the axons to the next synaptic waystation. In the OB, OSN axons synapse with the dendrites of the OB's principal neurons (mitral and tufted or MT cells) as well as at least one interneuron population, in spherical bundles of neuropil called glomeruli. Remarkably, each glomerulus contains OSN axons expressing only a single OR (Mombaerts, Wang et al. 1996, Ressler, Sullivan et al. 1994, Vassar, Chao et al. 1994). The location of OR-defined glomeruli is stereotyped with fairly high accuracy (Belluscio and Katz 2001) between individuals and shows mirror symmetry within each individual's two OBs. Along the dorsomedial-ventrolateral axis, glomerular position is correlated to the D-V position of OSN cell bodies in the OE, and this mapping is generated by classical developmental signaling pathways including the ephrins (Cutforth, Moring et al. 2003). However, along the anterior-posterior axis, glomeruli rely on differing, activity-dependent expression levels of cell-surface molecules such as Npn-1, which lead to repulsive interactions between non-cognate OSN axons (Imai, Suzuki et al. 2006). Most ORs have two cognate glomeruli per bulb, making the total number of glomeruli around 2000 per bulb in mice. OSNs converge onto mitral cells at a ratio of ~1000:1 and onto tufted cells at ~500:1 (Shepherd 2004). This equates to approximately 25 mitral and 50 tufted cells per glomerulus. This high level of convergence may reflect a need for reliable signal transduction when odorants are present at low concentrations.
Most tufted cells, called middle and external tufted cells, lie directly below the glomeruli. The remaining tufted cells (internal tufted cells) and the mitral cells form a thin layer (the mitral cell layer or MCL) separated from the external tufted cells by a cell-poor layer called the external plexiform layer. Mature MT cells possess one apical dendrite with a small, complex tuft that fills its target glomerulus uniformly. MT cells also extend lateral dendrites into the EPL, up to ~1000 µm long (McTavish, Migliore et al. 2012). There, they form reciprocal dendrodendritic synapses with the granule cells, which outnumber them 100-fold (Greer 1987, Mori 1987).

Tufted cells and mitral cells have long been known to extend axonal projections differentially to higher brain areas (Igarashi, Ieki et al. 2012, Ramón y Cajal 1909); this difference is widely thought to indicate distinct functional roles for these two cell types. Tufted cells form synapses primarily in the olfactory tubercle while mitral cells extend far into piriform cortex (PC) and other more caudal brain areas (Nagayama, Enerva et al. 2010). In the anterior olfactory nucleus (AON), MT cell main axons and branches maintain some degree of spatial stereotypy: anterior-posterior positioning within the OB is translated to dorsal-ventral positioning within the AON (Ghosh, Larson et al. 2011). Within PC, each mitral cell axon extends processes broadly, so that there is no obvious re-mapping of OR-defined signals (from the glomeruli in the OB) onto PC (Ghosh, Larson et al. 2011, Miyamichi, Amat et al. 2011, Sosulski, Bloom et al. 2011). Thus the
odorant code within PC remains elusive at present. In the amygdala (more specifically, in the posterolateral cortical nucleus of the amygdala), there is some degree of spatial segregation based on glomerular identity (Sosulski, Bloom et al. 2011).

In addition to these axonal projections to higher brain areas, both external (Lodovichi, Belluscio et al. 2003) and internal tufted cells (Ghosh, Larson et al. 2011) also extend intrabulbar axonal projections, synapsing onto granule cells directly below their paired, OR-cognate glomerulus. These projections may be involved in integrating signals from the two "isofunctional" glomeruli. The mechanism underlying these precise connections is not well understood, although presynaptic activity levels can affect them somewhat, as I will discuss below.

Granule cells (GCs) are small, axonless interneurons whose somata lie still deeper than the MCL, in the granule cell layer (GCL). They are GABAergic, whereas MT cells are glutamatergic. The dendrodendritic network of MT and granule cells is thought to mediate both feedback and lateral inhibition of MT cells. GCs can be divided into deep and superficial granule cells, which differ in the location of their soma within the GCL and in their synaptic targets. Deep GCs form reciprocal synapses largely with mitral cells while superficial GCs synapse with tufted cells (Orona, Scott et al. 1983).

Besides the granule cells there are a number of other types of interneurons present in the olfactory bulb. In a morphological schema, interneurons of the glomerular layer
can be split into periglomerular cells (PGCs), which innervate a single glomerulus, and juxtaglomerular cells, which innervate more than one glomerulus. The PGCs have been divided into non-overlapping, immunohistochemically defined populations expressing calretinin, calbindin or tyrosine hydroxylase (indicating dopamine production). Of these three PGC subtypes, the calbindin- and tyrosine hydroxylase-expressing cells are mainly GABAergic while the calretinin-expressing cells are mostly non-GABA-ergic (Parris-Aungst, Shipley et al. 2007). All three periglomerular subtypes are born both embryonically and postnatally, including into adulthood. However, fate-mapping reveals that the bulk of TH-expressing PGCs are born embryonically, while CR-expressing cells are born at continually increasing rates during development (Batista-Brito, Close et al. 2008) and CB-expressing cells are mostly born between E15.5 and P0. A recent paper challenged the identity of TH-expressing interneurons: using single cell tracing they showed that this population innervates several or even tens of glomeruli (Kiyokage, Pan et al. 2010), and argued that TH-expressing cells should be classified as short-axon, juxtaglomerular cells rather than periglomerular cells.

In the arena of the EPL, efforts to link immunohistochemical identity to morphological subtypes of interneurons have been only partially successful. Morphological studies have described Van Gehuchten cells (aspy, with short and simply branched dendrites), multipolar cells, and superficial short-axon cells. The great
majority of somatostatin-expressing cells in the EPL appear to be Van Gehuchten cells morphologically. This class partially overlaps with the immunohistochemically defined parvalbumin (PV)-expressing cells of the EPL (Lepousez, Csaba et al. 2010). PV+ interneurons connect with MT cells and are born during a window from late embryonic to early postnatal stages (Batista-Brito, Close et al. 2008). Finally, there are several immunohistochemically defined interneuron populations in the MCL and GCL, including the very rare, CB-expressing Blanes cells, which inhibit GCs and are largely embryonic in origin, and GCs marked by expression of either CR or 5T4 (discussed below).

From E11 to E13, newly generated MT cells migrate rostrally within the telencephalon to reach the prospective OB. From E14 to E16, the somata orient radially as the OSN axons reach the bulb periphery (Blanchart, De Carlos et al. 2006). MT cell dendritic extension and refinement occurs from E17 to adulthood (Blanchart, De Carlos et al. 2006). OSNs take the lead in glomerular formation, coalescing into protoglomeruli beginning at E17. Only just before birth do the MT cells begin to segregate into these discrete structures. At P0 nearly all (94%) mitral cells innervate multiple glomeruli with dendritic tufts (Lin Axel 2000). By P6, 71% of MCs innervate a single glomerulus, and 24% innervate two glomeruli, leaving only 5% that innervate more than two glomeruli.
Odor coding

As described above, each glomerulus is innervated by the axons of OSNs bearing a single OR and the dendrites of a relatively small number of MT cells. Since each MT cell receives direct inputs from the OSNs of just one glomerulus, the ensemble that consists of the OSNs and MT cells innervating one glomerulus can be thought of as a functional unit, whose activity has most successfully been assessed within the glomerulus itself. The techniques used to assay glomerular responses to odorants include intrinsic imaging (Rubin and Katz 1999), genetically encoded sensors of calcium levels or synaptic release (Kato, Chu et al. 2012, Petzold, Albeanu et al. 2008) or electrophysiological methods. Some such studies, particularly those that employ methods in which the measured responses have been shown to correspond to OSN or even glial activity rather than MT cell activity (Petzold, Albeanu et al. 2008). In these, the activity patterns reported may not accurately reflect the functional output of the OB, which is thought to be carried exclusively by MT cell axons. Nevertheless, these studies have consistently demonstrated what has been referred to as a "combinatorial code" (Malnic, Hirono et al. 1999). That is, odors at physiological concentrations tend to reliably activate specific combinations of glomeruli across trials and even across individuals (Belluscio and Katz 2001). A recent study showed that natural odorants at natural concentrations produce dense glomerular representations compared to what has been reported with more
controlled odorants and delivery (Vincis, Gschwend et al.). In summary, each OR (and thus each glomerulus) can be activated by multiple odorant molecules, and each odorant can activate multiple ORs (and glomeruli).

The odor code in the main cortical olfactory area, PC, is much less clear. MT cells project broadly and diffusely in PC, and cells in PC are in general more broadly tuned (i.e. responsive to a greater percentage of odorants) than the MT cells (Stettler and Axel 2009). Lesions to piriform cortex in rats demonstrate that the PC is necessary for complex odor discrimination. On the other hand, rats with PC lesions are still able to learn to discriminate between simple odors (Staubli, Schottler et al. 1987).

*Synaptic dynamics of the olfactory system*

In contrast to canonical neuronal signal propagation, which relies on axodendritic synapses and on Cajal’s “law of dynamic polarization”, circuit activity in the olfactory bulb is rather less linear. Dendrodendritic synapses are the norm in the bulb, and in many cases, OB neurons form reciprocal synaptic sites, where neuron A releases neurotransmitter onto neuron B and at a closely adjacent site, neuron B releases neurotransmitter onto neuron A.

The best studied, and most numerically important of the OB reciprocal dendrodendritic synapses is formed between the glutamatergic MT cell and the
GABAergic granule cell. As mentioned above, the granule cells lack axons entirely. In their mature state they have a radially extending primary dendrite with some higher order branching, and dendritic spines. When a mitral cell is depolarized and releases glutamate, the granule cells to which it is synaptically connected will in turn release GABA. In cases of reciprocal connection, either through immediately adjacent reciprocal synapses or through synaptic connections on other dendritic spines or branches of the same two neurons, feedback inhibition of the active mitral cell will result. The dendritic morphology and connectivity of MT cells and GCs would predict that MT cells closest to each other would be most likely to experience what is termed lateral inhibition, and that such inhibition would fall off in strength and likelihood with increasing distance. This prediction was challenged by a study showing that mitral cells form sparse connections with just a few other glomerular inputs (Fantana, Soucy et al. 2008).

MT cell dendrodendritic inhibition is mediated by NMDA and non-NMDA receptors on the GCs part, although NMDA receptor activation plays a larger role (Isaacson and Strowbridge 1998). In response to activation of these glutamate channels, GC voltage-gated calcium channels open and GABA is released onto MT cell GABA-A receptors. Importantly, both feedback and lateral inhibition in MT cells are blocked by TTX, demonstrating that both types of inhibition can be accounted for by dendritic glutamate release. Simultaneous recordings from pairs of mitral cells revealed functional
connectivity (via an interposed GC) in about 10% of pairs. In addition to MT cell presynaptic input, the GCs receive both excitation and inhibition from short-axon cells and from centrifugal sources (Shepherd 2004). These centrifugal inputs include piriform (olfactory) cortex, AON, horizontal limb of the diagonal band (ACh), raphe nucleus (5HT), locus coereleus (NE) (Boyd, Sturgill et al. 2012, Shepherd 2004).

MT cells can self-excite as they release glutamate from dendritic presynaptic sites (Isaacson 1999, Salin, Lledo et al. 2001, Schoppa and Westbrook 2001), and they can also effect lateral excitation of other MT cells via both synaptic (Pimentel and Margrie 2008) and electrical (gap-junction mediated) means (Christie and Westbrook 2006, Schoppa and Westbrook 2002). This lateral excitation appears to be present only in MT cells that innervate the same glomerulus (Pimentel and Margrie 2008). There is some evidence that mitral cell excitation may rely substantially on lateral excitation from tufted cells, rather than direct OSN stimulation (Gire, Franks et al. 2012).

GABAergic periglomerular cells can inhibit OSN glutamate release at the axonal terminals within glomeruli (Murphy, Darcy et al. 2005). Some PGCs can also self-inhibit and laterally inhibit one another via dendrodendritic synapses (Murphy, Darcy et al. 2005, Toida, Kosaka et al. 1996). Both CB- and TH-expressing PG cells have been shown to form inhibitory synapses onto MT cells. In the case of CB-PG cells, these synapses are reciprocal, while evidence suggests that TH-PG cells form what has been
termed serial synapses, in which they receive excitatory input from a MT cell (or OSN) and in turn inhibit another MT cell (Toida, Kosaka et al. 2000). CB- and CR-PG cells appear not to be directly postsynaptic to OSNs (Toida, Kosaka et al. 2000); instead, MT cells provide their primary presynaptic excitation. TH-PGCs receive most of their excitatory synaptic input from OSNs, and a small fraction of excitatory input from MT cells.

Like the GCs, the PV-expressing INs of the EPL receive excitatory connections from MT cells and form inhibitory connections onto them, sometimes at directly adjacent reciprocal synapses (Toida, Kosaka et al. 2000).

The role of activity in the development of the olfactory system

Nearly every study of the role of activity during olfactory system development has reduced or eliminated activity the level of the OSNs, the gatekeepers of the olfactory sense. The main goal of these studies has been to determine how activity affects the formation and maintenance of the OSNs’ stereotyped glomerular map. The ways in which activity was reduced differed in mechanistic terms, with distinct effects. This allows us to make mechanistic claims about which aspects of “activity” are indispensible for various developmental events.
Methods to attenuate or prevent OSN signaling include genetic repression of OSN firing through knockouts of the CNG channel (Brunet, Gold et al. 1996, Lin, Wang et al. 2000, Zheng, Feinstein et al. 2000), inhibition of OSN vesicle release via genetic expression of TeNT (Yu, Power et al. 2004), and hyperpolarization through Kir2.1 expression (Yu, Power et al. 2004). One CNG knockout line shows no effect for OSN targeting (Lin, Wang et al. 2000), while a second shows correct gross targeting but mild defects in glomerulus formation for 1 of the 2 ORs examined (Zheng, Feinstein et al. 2000). TeNT or Kir2.1 expression in OSNs expressing a single OR leads to a gradual loss of glomerular targeting (Yu, Power et al. 2004). In the case of one OR, silenced axons disappear altogether, and for two other ORs, Kir2.1 hyperpolarization leads to multiglomerular innervation (Yu, Power et al. 2004). On the other hand, expressing Kir2.1 in all OSNs seems to have no effect on OSN targeting. Altogether, these results reveal a cell-intrinsic role for subthreshold or spontaneous activity in OSN axonal growth and refinement. This competitive effect for activity mimics what has been found in similar studies in the visual system (Ben Fredj, Hammond et al. 2010, LeVay, Wiesel et al. 1980).

Although the glomerular map remains largely faithful when sensory-evoked firing is circumvented in the CNG knockout mouse line, the OSNs do exhibit a reversible decrease in number and contraction in glomerular size, at least as assessed for the P2
OSNs (Cummings and Belluscio 2010). Since the CNG channel gene lies on the X chromosome, the CNG KO line provides the opportunity to study stochastic silencing of ~50% of OSNs through X-inactivation in females (Zhao and Reed 2001). Intriguingly, the labeled, silent OSNs decrease in number over the span of months, but naris occlusion reverses this phenotype. This suggests that either OSNs normally compete for survival with less active OSNs more likely to die, or that naris occlusion promotes high probabilities of OSN survival (such that the death of CNG KO OSNs is overcome).

While most studies of OSN activity perturbations focus on the glomerular map, there is some limited data available on the impact of OSN silencing on the other cells and synapses of the olfactory bulb. When odor-dependent activity is disrupted in the OSNs through naris occlusion or genetic knockout of the CNG channel, tufted cell intrabulbar axonal projections become broader in a reversible fashion, though their gross targeting was unaffected (Marks, Cheng et al. 2006). The gradual refinement of MT cell glomerular tufts during early life is delayed in anosmic CNG KO mice, but by adulthood, mice of this line have mitral cell dendritic tufts that were indistinguishable from those of WT mice (Lin, Wang et al. 2000). These findings indicate a subtle role for sensory-evoked (correlated) activity in MT cell dendritic and intrabulbar axonal refinement.

If OSNs are prevented from signaling, all layers of the OB decrease in area (Leo, Devine et al. 2000, Marks, Cheng et al. 2006). This is observed in both genetic silencing
(CNG KO) (Marks, Cheng et al. 2006) as well as chronic axotomy by implantation of a Teflon square between the OB and the cribiform plate (Leo, Devine et al. 2000).

A related cluster of studies assesses the effect of genetically ablating MT cells, either from very early embryonic timepoints (through Tbr1 knockout) (Bulfone, Wang et al. 1998) or at a later time point, between P60 and P90 in the pcd mouse (Valero, Weruaga et al. 2007). In the case of embryonic MT cell deletion, the olfactory bulb is much smaller than normal by E16.5. At that point it contains TH-expressing INs, but the MC layer, EPL and GL form an indistinct mass that does not resolve at later developmental timepoints. In the pcd mouse, the authors observe decreased survival in cells of the granule cell layer and EPL, but not the glomerular layer. When they specifically examine CR-expressing populations in the GL and GCL, they find a ~60% decrease in the number of CR-expressing neurons of the GCL, but no decrease in the number of CR-expressing GL neurons (Valero, Weruaga et al. 2007). Importantly, no studies to date have addressed the impact of blocking MT cell activity or signaling, while leaving the MT cells intact within the circuit. The dramatic effects observed in the Tbr1 KO and pcd mice could be due either to a loss of MT cell synaptic input or to the loss of MT cell scaffolding or developmentally important cell signaling interactions.

The work presented in this dissertation builds on what is known about the role of OSN activity in the bulb. It accounts mechanistically for the finding that when OSN
activity or signaling is blocked, the layers of the OB decrease in thickness. For the first time, the cell-type specific effects of disruption of MT cell vesicular release are described and compared with the effects of OSN vesicular release disruption, both within the OB (Chapters 2 and 3) and within cortical target regions (Chapter 4).

*Introduction to adult neurogenesis and the migration and maturation of adult-born OB neurons*

Unlike nearly all other brain areas, the olfactory bulb continues to integrate newborn neurons throughout the adult life of the animal (Altman 1962, Reynolds and Weiss 1992). These adult-born neurons provide an important model for therapeutic strategies to combat neurodegenerative diseases and the effects of brain trauma. By observing and experimentally altering their integration into functioning adult circuits, one can generate new strategies for effectively transplanting or differentiating new neurons, or modifying existing neurons to best fill a niche left empty through injury or illness. This adult neurogenesis produces all the types of OB interneurons discussed above, but not the principal MT neurons. During embryonic development, olfactory bulb interneurons are generated in the lateral ganglionic eminence (LGE). The LGE is the precursor to the subventricular zone (SVZ), from which adult-born OB interneurons originate. The exact percentages of embryonically and adult-derived OB INs in the adult animal are not clear,
and likely change during the lifetime of the animal. Several studies concluded that around 75% of INs are adult-born, while another study puts the upper bound (at least for GCL-residing INs) at 50% (Tucker, Polleux et al. 2006).

It is not clear why the olfactory bulb in particular has evolved to integrate interneurons throughout the animal’s lifespan. Mice lacking adult-born OB INs (through SVZ irradiation or through genetic manipulations) have been reported variously to have deficits in odor discrimination but not olfactory memory (OM) (Gheusi, Cremer et al. 2000), short-term OM but not long-term OM or odor discrimination (Breton-Provencher, Lemasson et al. 2009), long term OM but not short-term OM or discrimination (Lazarini, Mouthon et al. 2009), or no deficit in either odor discrimination or OM (Imayoshi, Sakamoto et al. 2008). Some have suggested that it may be the turnover of GCs (including death of some existing GCs; see below) rather than simply the integration of new GCs, is important for olfactory discrimination (Mouret, Lepousez et al. 2009).

The process of adult OB IN birth, migration and maturation is fairly well characterized. In the SVZ of the adult animal, a population of neural stem cells called type B cells, which resemble glia morphologically and histochemically (Doetsch, Caille et al. 1999), give rise to type C cells. Type C cells divide quickly to form type A cells, which are neuroblasts (immature neurons). These neuroblasts form chains and enter the rostral migratory stream (RMS).
Petreanu et al. (2002) described five discrete stages of adult-born GC development. For the first week after their birth in the SVZ, GCs migrate tangentially along the RMS (class 1). Around P7 (that is, seven days after the birth of the cell rather than the animal) class 2 GCs start to migrate radially within the OB, and their leading and trailing processes increase in length. From P11 onward, class 3 GCs can be found. They can be distinguished by a single, longer process (the developing apical dendrite) extending toward, but not into, the EPL, emergence of a cluster or basal dendrites and loss of the trailing process. Class 4 GCs (P11 onward) extend their apical dendrite into the EPL, and class 5 GCs (P13 onward) have dendritic spines.

Not all adult-born OB neurons survive for the animal’s lifetime. TUNEL and cleaved caspase-3 staining show that about 50% of them undergo programmed cell death between day 15 and day 45 after their birth (Petreanu and Alvarez-Buylla 2002, Winner, Cooper-Kuhn et al. 2002). During this window of “fast cell death” nearly all neurons can be classed as stage 4 or stage 5 based on the morphological criteria listed above, and on their electrophysiological properties (Carleton, Petreanu et al. 2003). Therefore, the neurons that die are thought to have a mature morphology and functional synapses (Petreanu and Alvarez-Buylla 2002). Another study examined this question using a different method. By combining BrdU birthdating with NeuN immunostaining (NeuN labels mature neurons), (Winner, Cooper-Kuhn et al. 2002) showed that one month after
birthdating, 95% of BrdU+ neurons in the GCL are also NeuN positive. The same study demonstrates a loss of \( \frac{1}{3} \) of the total number of cells between 1 and 3 months post labeling. This supports the claim that (at least from one month onward), most GCs that undergo cell death are mature. Of those that survive the early peak in cell death, 50% of adult-born OB neurons are still alive and functional at 19 months (Winner, Cooper-Kuhn et al. 2002).

The synaptic development of adult-born GCs is stepwise and differs in some ways from what is seen in embryonically derived neuronal populations. The GC precursors (class 1 and 2) express AMPA receptors containing a GluR2 subunit, which normally found in mature neurons, and GABA\(_A\) receptors. NMDA receptors, normally found in immature neurons, first appear in some class 2 GCs (Carleton, Petreanu et al. 2003). At class 3, when migration is complete, GCs are subject first to synaptic inhibition and soon after, to synaptic excitation. At class 4 some GCs begin to fire action potentials and at class 5, they are electrophysiologically indistinguishable from surrounding, older GCs. The late appearance of action potentials can be attributed to the timing of Na\(^+\) channel expression and targeting: only in class 4 and 5 GCs can Na\(^+\) channel alpha-subunits be reliably seen at the plasma membrane (Carleton, Petreanu et al. 2003). By contrast, in embryonically born GCs, MT to GC excitation first appears at E17 and the reciprocal inhibitory synapses follow beginning a day later (Hinds 1970).
Most (75%-95%) of adult-born OB neurons are granule cells, (Kelsch, Mosley et al. 2007, Shepherd 2004), and they can be divided into deep and superficial GC subclasses, with early postnatal born GCs more likely to become superficial GCs as compared to late postnatal born GCs (Lemasson, Saghatelyan et al. 2005). The subclass identity of deep- vs superficial GC is a cell-autonomous and genetically predetermined property (Kelsch, Mosley et al. 2007). There is another subtype of GCs, the 5T4-expressing GCs, that seems to be born at a constant rate during the animal’s lifetime (Batista-Brito, Close et al. 2008). 5T4 is a glycoprotein whose expression in the 5T4 GCs mediates their activity-dependent dendritic arborization (Yoshihara, Takahashi et al. 2012).

The distinct PGC subtypes originate from different regions of the SVZ, and in some cases, there are known genetic determinants of cell identity (e.g. Pax6 and Dlx2 for TH+ PGCs (Brill, Snapyan et al. 2008). In addition to possessing distinct spatial and genetic identities, a number of studies indicate that different subclasses of PGCs are preferentially generated at different time points in the animal’s life (Batista-Brito, Close et al. 2008). For examine, among PGCs, TH-PGCs have a very early embryonic peak, CB-PGCs peak around birth and CR-PGCs seem to steadily increase in production rate into adulthood. The parvalbumin-expressing cells of the EPL are born during late embryonic and early postnatal timepoints, while GCs appear to be produced relatively
steadily throughout embryonic and postnatal development. Finally, the CB-expressing
Blanes cells are mostly generated during early embryonic timepoints. The function of
these age-dependent changes in IN subtype production is not known (Lledo, Merkle et al.
2008) (Table 1-1).

Role of activity in the maturation and survival of adult-born OB neurons

Neuronal activity plays an important part in the maturation of newborn, adult-
generated INs. Adult-born neurons are able to migrate and integrate into a mature
neuronal network; they have been shown to enhance sensory and cognitive function
rather than disrupting it. A detailed understanding of activity’s role in their maturation
and survival may lead to advances in therapeutic neuronal replacement in cases of brain
injury or neurodegeneration.

One manipulation of activity, naris occlusion, has been shown to decrease the
survival of newborn GCs as well as their dendritic length and spine density (Corotto,
Naris occlusion also changes the density of postsynaptic (PSD-95) puncta in adult born
GCs: in two subcellular dendritic compartments the density of PSD-95 puncta increases,
while in a third it decreases. On the other hand, naris occlusion uniformly decreases the
density of presynaptic (synaptophysin) puncta (Kelsch, Lin et al. 2009). A timecourse
study combined BrdU labeling with unilateral naris cauterization and demonstrated that
GCs die at elevated rates in response to naris occlusion, but only if that naris occlusion occurred between 14 and 28 days after GC labeling (Yamaguchi and Mori 2005). Furthermore, a similar decrease in survival follows systemic diazepam treatment, again only for treatment periods between P14 and P28. The diazepam could have inhibited GCs directly via their GABA\textsubscript{A} receptors, or indirectly via MT or centrifugal input attenuation. These two manipulations (naris cauterization and diazepam treatment) had additive effects (Yamaguchi and Mori 2005), suggesting that the less excitation GCs experience, the more likely they are to die during that critical period.

All of these results are somewhat muddled by the multiplicity of the effects of naris occlusion on mitral cells, the reciprocal synaptic partners (along with tufted cells) of GCs. In rats, following unilateral, acute naris occlusion, spontaneous mitral cell spiking is significantly attenuated and decoupled from respiration, yet it is still present (Philpot, Foster \textit{et al.} 1997). Therefore, naris occlusion should be regarded as an attenuation, rather than a loss, of both spontaneous and odor-evoked activity. In anosmic mice (CNG KO) whose OSNs cannot fire action potentials (Brunet, Gold \textit{et al.} 1996) no direct recordings of MT cells have been published. However, a comparable manipulation is acute application of lidocaine to the olfactory nerve (Stakic, Suchanek \textit{et al.} 2011), preventing OSN spikes from being transmitted into the OB. Lidocaine application has no effect on the spontaneous firing rate of neurons in the MCL, leading the authors to conclude that
spontaneous MC spiking is either intrinsically generated or attributable to centrifugal inputs to the bulb (Stakic, Suchanek et al. 2011). Therefore, it seems likely that in OSN-silent anosmic mice, MT continue to fire spontaneously at some rate. Bearing this in mind we can ask how the loss of sensory-evoked OSN signaling (but maintenance of spontaneous MT cell firing) affects adult-born GCs.

In CNG KO mice, granule cells proliferate and mature normally, and they survive at the same rate as control mice through P15 (15 days after cell birth). On the other hand, older GCs, assayed at P45, died at a rate four times as great as in control mice (Petreanu and Alvarez-Buylla 2002). The authors concluded that in the absence of sensory-evoked MC activity, granule cells matured normally but then die at higher rates after maturing.

Conversely, if OSN and MT cell activity is increased and synchronized by chronic, broad-spectrum olfactory environmental enrichment (Rochefort, Gheusi et al. 2002), adult born GCs survive at almost double the rate of those of control-housed mice when assayed 30 days after BrdU birthdating. After chronic enrichment with one specific odorant, GCs survive at a higher rate when assayed 25 days after cell BrdU injection (Moreno, Linster et al. 2009). Furthermore, the surviving GCs were more likely to express the immediate-early gene Zif268 in response to the familiar odor than in response to an unfamiliar odor. Another study demonstrated that six days of odor discrimination training (but not unrewarded exposure) administered just prior to sacrifice leads to
increased GC survival at 30 days post-BrdU injection (Alonso, Viollet et al. 2006). In vivo imaging of PGCs reveals that both mature (>40 day old) and immature (14 day old) PGCs dynamically gain and lose postsynaptic sites over the course of hours (Livneh, Feinstein et al. 2009). PGCs show more mature dendritic and spine morphology if they are in high-activity glomeruli, following sensory enrichment with specific odorants. As a whole, this set of occlusion and enrichment studies show that olfactory sensory input from the OSNs promotes GC survival and maturation.

The approaches described above—naris occlusion, genetic silencing of OSNs and olfactory enrichment—all influence adult-born GCs via their synaptic partners, the MT cells. In contrast, a second set of studies have examined the effects of up- or down-regulating the excitability of GCs themselves on their own development. Kelsch et al. (2009) expressed a bacterial voltage-gated sodium channel, NaChBac, in sparse adult-born GCs, upregulating their intrinsic exitability, and saw no effect on pre- or postsynaptic puncta density. However, NaChBac expression is able to rescue the effects of naris occlusion on GC pre- and postsynaptic puncta density (Kelsch, Lin et al. 2009).

The same group extended these findings in a later study (Lin, Sim et al. 2010), showing that sparse expression of Kir2.1 in some adult-born granule cells doubled the likelihood that those GCs would be lost to cell death by 28 days post infection/birth (but not by 14 days, supporting a post-migratory effect of Kir2.1. They also demonstrated that
in addition to its rescuing effect in naris-occluded bulbs, sparse expression of NaChBac enhanced GC survival in control bulbs. A recent study demonstrated a more nuanced effect of activity reduction on adult-born GCs; in it, the authors used siRNA to knock down expression of Nav1.1-Nav1.3 (other voltage-gated sodium channels) in adult-born granule cells from P35 to P70 (Dahlen, Jimenez et al. 2011). They reported a reduction in GC spine density that could be localized to dendritic areas proximal to the soma, and concomitant decreases in dendritic length and number of branches. Electrophysiological data were not shown in this study, so it is possible that the difference in effect size between Nav1.1-1.3 knockdown and Kir2.1 overexpression may be due to differences in the electrophysiological consequences of either manipulation. Lin et al. (2010) also showed that sparse knockout of NR1 (also called GluN1, Grin1), an obligate subunit of the NMDA receptor, caused near-total cell death in NR1 KD GCs compared to mCherry-expressing control GCs.

My dissertation aims to extend these findings on the role that activity plays in the survival and maturation of OB interneurons. Both the naris occlusion experiments and the cell-intrinsic manipulations of GC activity (Kir2.1, Nav1.1-1.3 knockdown, NR1 knockout, NaChBac) hint that typical, physiological levels of synaptic excitation may be important for GC morphological development and even survival. However, to date, there are no published reports of the effects of MT cell activity on other OB neurons. Using
cell-type specific expression of TeNT to block neurotransmission, we compared the roles of OSN and MT cell vesicular release in maintaining not just GCs but a broad panel of OB interneuron populations (Chapter 2). Next, we asked whether the birth date of a GC was predictive of its activity dependence (Chapter 2). Finally, we looked closely at the morphological and synaptic maturity of GCs under both forms of blockade (Chapter 3).
Table 1: Olfactory bulb interneuron subtypes

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>IHC Marker</th>
<th>Soma Location</th>
<th>Birthdate Range</th>
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Chapter 2
A comparison of OSN and MT cell signaling blockade in the olfactory bulb

Abstract

Genetic and surgical blockades of OSN sensory evoked activity lead to relatively mild effects in the olfactory bulb, including decreased layer thickness and increased rates of cell death for adult-born granule cells. Using a CRE-based genetic strategy to express tetanus toxin selectively in neuronal subclasses, we compared the effects of OSN and MT cell vesicular release blockade. While we observed some loss in layer areas under OSN vesicular release blockade, we found that MT cell vesicular release blockade led to greater loss in layer areas, as well as decreases in density for some subtypes of OB interneurons. These decreases were due not to changes in proliferation or migration, but to increased rates of cell death within the OB itself. Granule cells born embryonically were equally dependent on MT cell vesicular release for their survival as those GCs born during early or late adulthood. Surprisingly, MT cells survived in normal numbers under both OSN and MT cell vesicular release. Thus, our comprehensive comparison of the cell-type specific effects of OSN and MT cell vesicular release blockade revealed an important role for MT cell signaling in the maintenance of OB circuitry.
Introduction

Neural circuits are complex assemblies that depend on many kinds of cues in order to develop and maintain their structure during the lifetime of the animal. In general, genetic factors control gross wiring patterns, which are then refined and modulated by neuronal activity. Sensory systems are well suited to studies of the role that activity plays in circuit development and maintenance for two reasons: their input can be tightly controlled, and they sometimes contain intelligible spatial maps or features that are conserved across successive synapses from the periphery to high-order processing regions.

Under conditions of sensory deprivation, spontaneous neuronal activity can still occur in downstream neurons, and propagate in turn to their targets (Krahe and Guido 2011, Philpot, Foster et al. 1997). During early development, blockades of pre-sensory spontaneous activity can prevent normal axonal branching (Sretavan, Shatz et al. 1988). Studies directly comparing the effects of loss of sensory or total activity within a given circuit or cell type are lacking. However, genetic manipulations that chronically hyperpolarize neurons or prevent synaptic vesicle release reveal that a total blockade of activity can have severe consequences on circuit formation, including changes in functional connectivity, axonal or dendritic morphology, and even cell death (Ben Fredj,

Genetic blockades of OSN sensory-evoked depolarization lead to a mild disruption in the glomerular map (Lin, Wang *et al.* 2000, Zheng, Feinstein *et al.* 2000) and chronic hyperpolarization of OSNs through overexpression of a potassium channel leads to a more dramatic disruption of the glomerular map (Yu, Power *et al.* 2004). However, beyond the glomerular map, the effects of OSN activity blockades on other aspects of olfactory circuitry have not been fully addressed. Unlike many other brain regions, the olfactory bulb contains a number of neuronal populations that are born in the adult animal. The primary olfactory sensory neurons (OSNs) are continually regenerated, with an average lifespan of six weeks. Similarly, the many classes of interneurons intrinsic to the OB are born both embryonically and throughout adult life (Batista-Brito, Close *et al.* 2008). These interneurons, which include granule cells (GCs), periglomerular cells (PGCs) and several other less numerous subclasses, are born in the subventricular zone (SVZ), and migrate tangentially through the rostral migratory stream (RMS) into the OB where they move radially to assume their mature position. Around 50% of these new, adult-born neurons die during an early critical period, between 15 and 30–45 days after neuronal birth, during the “wave of fast cell death” (Petreanu and Alvarez-Buylla 2002).

It has been shown that removing sensory-evoked input via naris occlusion further
increases the rate of death for granule cells by ~75% (Frazier-Cierpial and Brunjes 1989, Lemasson, Saghatelyan et al. 2005, Petreanu and Alvarez-Buylla 2002, Saghatelyan, Roux et al. 2005, Winner, Cooper-Kuhn et al. 2002). Naris occlusion also decreases the dendritic spine density of those granule cells that do survive (Kelsch, Lin et al. 2009, Saghatelyan, Roux et al. 2005). Importantly, the granule cells receive direct excitatory input from the MT cells, but not from the OSNs. Therefore, the effects of OSN sensory blockades on GCs are indirect and mediated by MT cells. When OSN sensory-evoked activity is blocked by naris occlusion, MT cells are still able to spontaneously fire action potentials at low rates (Philpot, Foster et al. 1997). It thus seems likely that under OSN activity or signaling blockades MT cells continue to provide excitatory input to GCs, though that input may be highly attenuated. The effects of a total blockade of MT cell signaling on GC survival and maturation have not been described.

Although the granule cells are the most numerous population of OB interneurons, there are many other OB interneuron classes, which differ in location, connectivity and developmental ontogeny. For many of these classes, little is known of their dependence on activity. We wanted to compare the effects of blocking vesicular release, in either OSN or in MT cells, on these neurons as well. Based on the known effects of OSN blockades, we expected to encounter loss of some cell types under OSN vesicular release blockade and potentially under MT cell vesicular release blockade as well. We wished to characterize
the loss for different interneuron populations under each form of blockade. Given their rich variability along several axes, we hoped that this would allow us to understand the predictors of activity dependence. We used viral injections to examine the morphological effects of OSN or MT cell vesicular release blockade on GCs (Chapter 3) as well as MT cell cortical axonal projections (Chapter 4).

Another question that we wished to address is whether the birth date of a GC can predict its dependence on activity. Adult-born granule cells first develop proximal input-only synapses (from either MT cell or cortical axons) and only later form reciprocal synapses with MT cells and begin to fire action potentials (Kelsch, Lin et al. 2008); it is thought that this “listen before speaking” developmental schema may be related to their strong dependence on activity. Embryonically born GCs, on the other hand, form these two types of synapses simultaneously and fire action potentials earlier as well (Carleton, Petreanu et al. 2003, Kelsch, Lin et al. 2008). The embryonically born GCs also seem to lack an activity-dependent critical period for survival (Lemasson, Saghatelyan et al. 2005). Together, this evidence suggests that adult-born GCs may be unusually dependent on sensory-evoked or spontaneous activity, even compared to their embryonically born counterparts. We tested this hypothesis by comparing densities of BrdU-birthdated cells in mice who lacked MT cell vesicular release (MT-T) and their WT littermates.
Materials and Methods

Mouse strains

TeNT-GFP mice were generously provided by Martin Goulding (Zhang, Narayan et al. 2008). The Pcdh21-IRES-CRE (M/T cell CRE) line was generated by Kristin Baldwin while in the Axel laboratory (Boland, Hazen et al. 2009) and the OMP-IRES-CRE (OSN CRE) line was developed in the Axel laboratory (Eggan, Baldwin et al. 2004). WT controls are either CRE or TeNT-GFP positive; we observed no morphological or other differences between CRE and TeNT-GFP controls.

Tissue preparation and immunohistochemistry

Mice P15 and younger were euthanized and brain or olfactory epithelial tissue were post-fixed for one hour (for cryosections) or overnight (for vibratomed sections) in 4% PFA at 4° C overnight. Mice P15 and older were transcardially perfused with 4% PFA in PBS containing CaCl₂ and MgCl₂, and brains were placed in 30% sucrose in PBS (for cryosections) or 4% PFA (for vibratomed sections) at 4° C overnight. For cryosections, brains were embedded in OCT, stored at -80° C and later sectioned at 25 μm with a Leica CM3050 S cryostat. Slides were allowed to air dry for ~1 h, post-fixed for 10 minutes in 4% PFA, rinsed several times with PBS, and treated with blocking buffer (5% heat-inactivated horse serum in PBS-T (PBS + 0.1% TritonX-100)) for 1 h at room temperature. Primary antibodies, diluted at 1:500 (unless otherwise noted) in
blocking buffer, were applied to slides and left on overnight at 4° C. Following three 10-minute rinses in PBS-T, secondary antibodies, diluted at 1:1000 in blocking buffer were applied for 1-2 h at room temperature. Slides were rinsed several times in PBS-T and mounted using ProLong Gold mounting media either with or without DAPI (Life Technologies P36935 or P36934). For vibratomed sections, brains were sectioned at 80 µm using a Leica VT1000S and stained, using the same reagents and timing as above, in 48 well plates. Primary antibodies used:

5T4 (rabbit, Abcam ab129058)
BrdU (1:400, mouse, BD 555627)
Calbindin (rabbit, Swant CB38)
Calretinin (goat, Millipore AB1550)
cFos (rabbit, Calbiochem PC38)
Cleaved caspase-3 (rabbit, CST 9661)
GFP (rabbit, Invitrogen A11122 or sheep, Serotec 4745-1051)
Ki67 (1:300, rabbit, Acris DRM004)
PGP9.5 (rabbit, Abcam ab10404)
Nestin (mouse, MAB5326)
NeuN (mouse, Millipore MAB377)
Parvalbumin (goad, Swant PVG-214)

Sp8 (goat, SCBT C-18)

Tbr2 (rabbit, Millipore AB2283)

TH (rabbit, Pel-Freez P40101-0)

VAMP2/Synaptobrevin (mouse (69.1) Synaptic Systems 104211)

VGluT2 (GP, Millipore AB2251)

Zif268/Egr1 (rabbit, SCBT sc-189)

For BrdU staining, air-dried cryosections or vibratomed sections were rinsed briefly with water and 2N HCl, then incubated in 2N HCl for an hour at 37° C. Sections were then rinsed with PBS-T and processed normally beginning with the blocking step.

For GFP staining of TeNT-GFP, an HRP-conjugated secondary antibody followed anti-GFP primary antibody application. After extensive washes, fluorescein-conjugated tyramide from a TSA kit (PerkinElmer NEL701A001KT) was applied to slides for ten minutes. Finally, after 2-3 additional hours of rinses, Sudan Black dye (0.1% in 70% EtOH) was applied for 10 m to reduce neuronal autofluorescence.

BrdU administration

Bromodeoxyuridine was diluted to 3.125 or 12.5 ug/ul in PBS and injected intraperitoneally into individual mice or pregnant dams at 50 µg/g body weight. For the experiment examining recently born cells in the RMS, mice were injected once, 24 h
prior to euthanization. For the granule cell survival experiments, mice or pregnant dams were injected twice, 24 h apart, and euthanized at later timepoints.

Image collection and image processing

Other than some MT cell/axon reconstructions, all images were acquired on a Nikon C2 confocal microscope, often using automated XY stitching. Image analysis was performed using Nikon NIS-Elements, MBF Neurolucida Explorer and Adobe Photoshop. Cell counts were either automated or done manually depending on the signal strength and background, and normalized to anatomically defined regions using DAPI counterstaining. Several MT cell/axon reconstructions were acquired on an Olympus FluoView 500 confocal microscope.

To calculate the area of each OB layer, we used maximal coronal sections through the olfactory bulb, just anterior to the AOB. Three concentric regions were drawn using DAPI staining as a guide. The first region followed the MCL and the area within it was assigned to the GCL. The second region followed the bottom edge of the GL. From that region’s area, the GCL area was subtracted and the resulting area was assigned to the EPL. Finally, a third region followed the top edge of the GL, excluding the ONL. From that region’s area, the EPL layer was subtracted and the resulting area was assigned to the GL.
For interneuron counts, the regions sampled were as follows. NeuN-GC and Sp8-GC: a superficial section of the GCL (excluding the MCL) extending to approximately ⅓ the radius of the OB and corresponding to an area ~0.04 mm². 5T4-GC and CR-GC: a superficial section of the GCL whose dimensions were chosen to best capture the staining pattern observed in WT animals, corresponding to an area of ~0.055 mm² (5T4) or ~0.07 mm² (CR). CR-PG and CB-PG: a section of the GL corresponding to an area of ~0.1 mm² (CR) or ~0.065 mm² (CB). Blanes(CB)-GCL: either the entire GCL or a section of it that was radially proportional to the whole. PV-EPL: a section of the EPL corresponding to an area of ~0.1 mm². Tbr2-MT: a section encompassing the MCL, EPL and GL corresponding to an area of ~0.2 mm². OB Cleaved caspase-3 staining was measured within the entire GCL.

For BrdU staining, experiments B2-B4 used the entire GCL, or a representative portion of it to exclude regions with artifactually high background. Experiment B1 used a section including the MCL, EPL and the basal portion of the GL that included BrdU+ cells.

All procedures were performed in accordance with the guidelines and standards of the Scripps Research Institutes’ Animal Care and Use Committee.
Results

To compare the effects of vesicular release blockade in OSNs and MT cells, we used knock-in mouse lines in which cell type-specific promoters drive CRE expression: OMP for OSNs and Pcdh21 for MT cells (Boland, Hazen et al. 2009, Eggan, Baldwin et al. 2004) (Fig 2-1). We crossed each of these lines to a third mouse line in which a tetanus toxin-GFP fusion protein (TeNT-GFP) was inserted at the Rosa26 locus, preceded by a stop codon that is flanked by loxP sites. This TeNT-GFP line has been used to disrupt vesicle fusion successfully in a number of specific neuronal populations (Stam, Hendricks et al. 2012, Zhang, Narayan et al. 2008). In cells that express CRE, the loxP sites are recombined and TeNT-GFP transcription can proceed. Below, OMP-CRE x TeNT-GFP mice will be referred to as OSN-T, and Pcdh21-CRE x TeNT GFP mice will be referred to as MT-T.

In order to verify cell type-specific expression of TeNT-GFP, we performed immunohistochemical staining against GFP (Fig 2-2). In OSN-T mice, the cell bodies and dendritic cilia of OSNs in the OE expressed GFP, and at the level of the OB we could detect bright GFP in the glomerular layer, where OSN axons terminate and form synapses onto MT cells and other OB neuronal populations. In the MT-T mice, the MCL, EPL, and GL of the OB expressed GFP, consistent with MT cell expression of TeNT-GFP and trafficking of the fusion protein to dendritic presynaptic terminals. Furthermore, within
two cortical target regions (AON and piriform cortex), GFP was expressed in layer 1, where MT cell axons terminate and form synapses onto cortical neurons.

To confirm whether and where in the olfactory bulb the transgenic tetanus toxin was cleaving VAMP2, we stained for an N-terminal epitope of VAMP2, known to be cleaved by tetanus toxin (Fig 2-3). OSN-T mice showed decreased VAMP2 levels in the glomerular layer, where OSN axons terminate and form synapses. MT-T mice showed decreased VAMP2 in the EPL, where MT cells form reciprocal dendrodendritic synapses with GCs and other interneurons.

To determine the extent of activity decrease in either mouse line, we stained for a panel of immediate early genes (Fig 2-4). In the granule cell layer of the MT-T OB, we observed a significant decrease in both Zif268/Egr1 and cFos expression, while OSN-T granule cells were indistinguishable from wild-type controls in Zif268 and cFos expression. This is consistent with the fact that when OSN sensory signaling is disrupted, MT cells continue to fire at low rates (Philpot, Foster et al. 1997) that are apparently sufficient to maintain resting levels of cFos. In contrast, both OSN-T and MT-T OBs displayed reduced tyrosine hydroxylase (TH) staining. OSN-T OBs showed a dramatic reduction (58%) in TH signal intensity, in agreement with other genetic or surgical models of OSN signaling attenuation (Baker, Cummings et al. 1999, Leo, Devine et al. 2000, Yu, Power et al. 2004). While other OB interneuron subtypes are connected to
OSNs only indirectly, the TH-expressing INs receive the majority of their direct input from OSNs (Toida, Kosaka et al. 2000). The reduction in TH-staining in MT-T OBs probably reflects the effect of blocking signaling from the ~10% of synapses onto TH-PGCs in which MT neurons are presynaptic (Toida, Kosaka et al. 2000).

At P21, we dissected out whole brains of MT-T mice and littermate controls. In MT-T mice, the olfactory bulbs were noticeably smaller than in WT controls (Fig 2-5). This difference was specific to the OB; cortex and cerebellum were comparable in size between the two genotypes.

One common feature of anosmic mice is that they exhibit impaired suckling at birth and or dimished postnatal growth (Brunet, Gold et al. 1996, Zheng, Feinstein et al. 2000). While MT-T mice were normal weight at birth, by P11 they were significantly smaller than their WT littermates (Fig 2-6), and this gap in weight persisted through adulthood. Since MT-T mice survive at the expected Mendelian ratios (data not shown), we believe that MT cell vesicular release likely does not cause a total loss of olfactory function. The smaller size of MT-T mice, however, could indicate partial loss of the ability to smell, and therefore suckle or find food.

Using cell-type specific antibodies for mitral cells (PGP9.5) and OSNs (VGlut2), we observed that OSN-T OBs were typical in their laminar organization, though each layer was slightly thinner than its counterpart in the WT OB (Fig 2-7). This aligns with
what has been observed in other models of OSN signaling attenuation (Leo, Devine et al. 2000, Marks, Cheng et al. 2006). In contrast, MT-T OBs were atypically laminated. Most notably, the cell-poor external plexiform layer (EPL) was irregular in thickness and absent in places. The mitral cells no longer formed a thin ring around the GCL, but instead occupied a wider patch. The glomeruli were irregular in size and shape and aggregated into a wide layer several glomeruli thick.

In order to measure these effects and to determine their developmental timecourse, we quantified layer thicknesses using maximal coronal sections through the OB at three timepoints (Fig 2-8). At P11 the MT-T OB was already smaller than OSN-T or WT OBs and this difference only grew more pronounced at later timepoints (P21 and P125) (Fig 2-9). Strikingly, the MT-T OB shrank in absolute terms between P11 and P125. We found that the overall OB size difference could be largely attributed to differences in EPL and GCL area but not GL area, which was not significantly different between genotypes at P125.

We next wished to examine the effect of loss of OSN or MT vesicular release on several classes of OB interneurons, which vary in their connectivity, birth range, and immunohistochemical signatures. The granule cells, which receive excitatory input from MT cells, are known to die at abnormally high rates when OSN signaling is perturbed through naris occlusion (Frazier-Cierpial and Brunjes 1989, Petreanu and Alvarez-Buylla
To assess the survival of granule cells, we quantified the density of cells within the GCL expressing Sp8, a transcription factor that marks a subset of GCs from migration through maturity (Waclaw, Allen et al. 2006). We found that the density of Sp8-expressing cells in the granule cell layer did not differ from that of WT mice in either vesicular silencing model, although the loss in GCL area/volume (Fig 2-10) in both the OSN-T and MT-T lines allowed us to infer an absolute loss of Sp8-GCs in both models. NeuN is known to mark mature neurons (Park, Baek et al. 2011) and in the OB it is absent from the RMS and from deep regions of the GCL that contain newly born, radially migrating GCs. We observed a decreased density of NeuN-expressing cells in the GCL of MT-T mice. This evidence corroborates our finding that the proliferation and migration of OB INs is unaffected in MT-T (see below) but that at some point after reaching the OB, granule cells begin to die in abnormally large numbers.

Next, we quantified the density of two known sub-populations of GCs: 5T4- and CR-expressing GCs (Fig 2-10) to see if their response to OSN and MT cell signaling blockade followed the pattern of Sp8 or NeuN-marked GCs. 5T4 encodes a membrane protein that promotes dendritic branching in GCs, and it has been shown that blocking OSN sensory signaling leads to a ~50% decrease in 5T4-GC density (Yoshihara, Takahashi et al. 2012). Interestingly, we observed a near total loss of 5T4-GCs in MT-T
mice, but no effect in OSN-T mice. We also observed a dramatic loss in the density of CR-expressing GCs in MT-T mice.

In contrast to TH-expressing INs, the CB- and CR-expressing PGCs in the glomerular layer are excited directly by MT cells but receive no direct input from OSNs (Kosaka and Kosaka 2007). We observed that the loss of MT vesicular release in MT-T mice leads to a ~40% percent decrease in the density of CB-expressing INs of the glomerular layer but has no effect on the density CR-expressing INs (Fig 2-11). We next examined the class of PV-expressing INs, which are situated in the EPL and, like the granule cells, are both pre- and postsynaptic to MT cells (Toida, Kosaka et al. 1996). We observed an almost total loss of PV-INs in MT-T mice, while in OSN-T mice PV-INs were denser than normal, perhaps reflecting normal cell survival within a somewhat smaller layer (Fig 2-11).

Finally, we examined Blanes cells, which are rare, CB-expressing neurons that lie in the GCL and possess a large, ovoid soma (Pressler and Strowbridge 2006). Blanes cells receive excitatory input that can be generated by extracellular electrical stimulation in either the GCL or the GL, suggesting that they may receive MT cell axonal input and/or other centrifugal or local excitatory input. This class of INs was present at normal densities in both OSN- and MT-T mice (Fig 2-11).
We next wished to determine the role of OSN- and MT-vesicular release on the MT cells, using the transcription factor Tbr2 as a marker for MT cells (Mizuguchi, Naritsuka et al. 2012). We hypothesized that the MT cells would undergo increased rates of cell death in both OSN-T mice, where their only known source of excitatory input was attenuated by OSN vesicular release blockade, as well as in MT-T mice, where we observed striking losses of OB interneurons known to be synaptic targets of MT cells. However, to our surprise, we saw no change in Tbr2-expressing cells in OSN-T mice, and we observed an increase in Tbr2 cell density in MT-T animals, which we believes reflects normal survival of MT cells in a compressed EPL (Fig 2-12).

Targeted glomerular layer injections of recombinant Sindbis virus encoding fluorescent proteins (Ghosh, Larson et al. 2011) allowed us to visualize the morphology of the MT cells (Fig 2-13). In OSN-T mice, MT cells were qualitatively indistinguishable from those of WT littermates, possessing obliquely angled lateral branches, a single, thick apical dendritic branch whose tuft even filled a single glomerulus. The MT cells of MT-T mice had somewhat thinner lateral dendrites extending into the EPL, and their apical tufts deviated from the normal ovoid shape in some cases, matching the dysmorphism of the glomeruli.

The loss of granule cells and other interneurons could be due to decreased proliferation, migration or survival. We wished to determine which of these possible
causes was at play in the MT-T mice. We ruled out an effect on proliferation and early migration after finding no difference in the density of cells that express Ki67, a marker of mitotic cells, in the RMS (Fig 2-14). RMS immunoreactivity for nestin, a marker for neuronal precursors, appeared qualitatively similar for WT and MT-T mice. We also counted BrdU-labeled cells in the RMS 24 hours after IP injection. Using this method we again saw no difference in the number of newly generated cells between WT and MT-T mice (Fig 2-14). Finally, we examined a more anterior portion of the RMS, within coronal sections that included the AON, for cells immunoreactive to cleaved caspase-3, which is known to be upregulated in dying, apoptotic cells. We observed no difference in the density of CC-3+ cells in the RMS of mice of each genotype (Fig 2-14). These data indicate that proliferation and migration are unaffected in MT-T mice.

To confirm that olfactory INs were dying at abnormal rates after migrating into the bulb, we performed CC-3 cell counts within the OB itself (Fig 2-15). We observed increased cleaved caspase-3 immunoreactivity in the GCL of OSN-T mice in agreement with what others have observed using similar models of olfactory sensory activity loss (Fiske and Brunjes 2001, Petreanu and Alvarez-Buylla 2002, Yamaguchi and Mori 2005), and a further increase in CC-3 cell density in MT-T mice, to a level ~10 times that of WT mice.
Having confirmed that lowered rates of cell survival were responsible for the
decrease in density of a number of OB IN cell types, we next wished to determine
whether neurons born at different developmental timepoints were differentially
susceptible to cell death when MT cell vesicular release was blocked. While it is known
that adult-born GCs show decreased survival under sensory-evoked activity blockade
during an early critical period (Petreanu and Alvarez-Buylla 2002) a role (or lack of role)
for activity in the survival of embryonically-derived granule cells has not been reported.
We birthdated INs by BrdU labeling separate groups of mice at either an embryonic
timepoint (B2; E15.5) or at one of two postnatal timepoints (B3; E10.5 or B4; E30.5)
(Fig 2-16). We examined the OBs and quantified BrdU+ cell density in the GCL for all
groups at a much later timepoint, P125-P135. This timepoint was chosen in order to avoid
any artifacts due to subtle differences in the timing of the early critical period of cell
death. We found that neurons born at all three timepoints in MT-T mice showed very
similar levels of reduction in relation to their WT littermates (Fig 2-17). There were no
significant differences between these groups in the level of reduced survival for MT-T vs
WT GCs (Unpaired t-tests).

We also used BrdU labeling to confirm our finding that MT cells survive at
normal rates in MT-T mice. We injected BrdU within the early embryonic window of
MT cell birth (B1; E11.5) and examined the MCL of injected mice at either P60 or P190
At both timepoints, MT-T animals showed significant increases in MCL BrdU+ cell density. As with the Tbr2 staining, we believe that this increase in density reflects MT cell survival at typical levels within a shrinking EPL.

**Discussion**

Activity drives both the development and the ongoing plasticity of neuronal circuits. In sensory systems, the roles of spontaneous and sensory-evoked activity in circuit development and maintenance can be isolated subtractively through experiments that occlude either evoked or total activity in a class of neurons. In the olfactory system, MT cells receive input from a set of primary neurons (OSNs) that all express the same, single OR, and they provide the sole source of OB output to higher processing areas. We wished to assess the importance of spontaneous and sensory-evoked MT cell signaling in the circuitry of the olfactory bulb and higher cortical areas.

Because MT cells are directly postsynaptic OSNs, blocking sensory-evoked firing in OSNs, either through naris occlusion, or genetic manipulations, will in turn block sensory-evoked firing in MT cells. Several groups have described the effects of such manipulations at the level of the OB. In mice bearing a mutation in the OCNC1 ion channel gene (Zheng, Feinstein et al. 2000), OSNs cannot fire. The OBs of OCNC1-animals are smaller than normal, due to a decrease in the thickness of each layer (Marks,
Cheng et al. 2006). A similar result has been reported in response to OSN axotomy (Leo, Devine et al. 2000), which likely leads to a total blockade of OSN activity. Other studies that focus on adult-born GCs show that removing sensory-evoked activity via naris occlusion leads to increased rates of GC death.

In part, this study represents an endeavor to extend these findings. We wished to look more closely at the structure and distinct neuronal populations within the olfactory bulb to assess the effects loss of OSN synaptic signaling (vesicular release) within the OB. In addition, we hoped to compare the effects of OSN vesicular release blockade (and concomitant MT cell sensory-evoked signaling blockade) with the effects of total vesicular release blockade in MT cells. We accomplished this comparison by crossing two CRE knock-in lines, with specific expression in either OSNs or MT cells, to a third knock-in line that encodes tetanus toxin and allows expression only after CRE excision of an upstream stop codon. This generated a total OSN blockade/sensory MT cell signaling blockade line, (OSN-T) and a total MT cell signaling blockade line (MT-T).

In agreement with previous studies that eliminated either sensory or total OSN signaling, we observed a decrease in layer area for the EPL and GCL in the OSN-T OB. Surprisingly, these layers were yet smaller in area in the MT-T OB, which also showed signs of disorganization. This result hinted at the importance of MT cell activity in the maintenance of OB size and structure.
We next assessed the densities of nine spatially and histochemically defined neuronal populations in the OB in each line at an early adult timepoint (P21). We found that the granule cells, which form reciprocal dendrodendritic synapses with the MT cells, were highly susceptible to MT cell activity blockade. Measures of GC density that were blind to GC birthdate (Sp8 staining) revealed no difference in density between lines, though given the decreased area of the GCL in both the OSN-T and MT-T OB, we can infer a decrease in total number of Sp8-GCs. Measures of mature GCs density (NeuN staining) revealed a decrease in NeuN-GC count in the MT-T OB. This suggests that a loss of MT cell activity is likely to affect mature GCs more profoundly than immature GCs. Changes in cell density can reflect changes in proliferation, migration or survival. This differential effect on mature GCs compared to immature ones provides our first clue that cell survival may be affected. It is also in agreement with previous findings that under genetic knockout of OSN sensory signaling, GCs mature normally and only begin to show decreased survival relative to controls at or after the point that they integrate into the circuit (Petreanu and Alvarez-Buylla 2002).

Two other GC subpopulations also showed decreased density in MT-T OBs. 5T4-expressing GCs form a superficial layer intermingled and just beneath the MCL. There is a nearly complete loss of 5T4-GCs in the MT-T OB (but no change in the OSN-T OB). However, because the expression of 5T4 is known to depend on activity, this may reflect
a downregulation of 5T4 expression rather than a decrease in 5T4-GC proliferation, migration or survival. CR-GCs also show a selective, though more moderate, decrease in density in the MT-T OB.

A second CR-expressing neuronal population in the OB is the class of CR-PGCs. Unlike the CR-GCs, this group showed no decreased density in either the OSN-T or the MT-T OB. Thus, expression of CR is not predictive of susceptibility to either form of signaling blockade. Neither CR-PGCs nor CB-PGCs receive direct inputs from OSNs; instead, their excitatory drive comes from MT cell dendrites, which do receive direct input from OSNs. Yet while CR-PGCs show no decrease in density in OSN-T or MT-T OBs, CB-PGCs are less dense in MT-T OBs. On the other hand, the CB-expressing Blanes cells of the GCL were present at normal densities in all three lines.

Finally, the PV-expressing neurons of the EPL, like the GCs, form dendrodendritic synapses with MT cells. At these synapses, MT cells excite and are in turn inhibited by PV-EPL neurons. We saw a near-total loss of PV-EPL neurons in the MT-T OB, while their density was actually increased relative to WT levels in OSN-T OBs. This latter result probably reflects normal numbers of PV-EPL neurons surviving in a thinner EPL.

In the MT-T OB, CR-GCs were lost and CR-PGCs were spared. Similarly, CB-PGCs were lost but CB-Blanes cells were spared. These results demonstrate that the
expression of a histochemical marker is unrelated to susceptibility to MT cell vesicular release blockade.

A second factor that could predict a neuronal population’s susceptibility could be the ontogenic timecourse of that population, i.e. the distribution of cell birth over developmental time. Unlike many types of neurons, the interneurons of the OB are born both embryonically and postnatally. In some cases, this developmental timecourse extends out to late adulthood. One hypothesis might be that neuronal populations born primarily during embryonic or early postnatal development might be less susceptible to the effects of OSN or MT cell signaling blockade. In support of this hypothesis, GCs born during early postnatal timepoints survive at high rates compared to later born GCs, of which only ~50% survive (Lemasson, Saghatelyan et al. 2005). While the activity dependence of embryonic GCs or other interneurons has not been tested, the fact that they normally mature prior to the onset of normal sensory signaling also lends support to the idea that they could be relatively resistant to blockades of activity.

However, our data do not seem to support this hypothesis. CB-Blanes cells are primarily born embryonically, while CR-GL interneurons are born throughout the animal’s lifetime at a steady rate, yet neither population decreased in density in response to either OSN- or MT-cell signaling blockade. Conversely, PV-EPL interneurons are mostly born just before or after birth, while GCs are born from early embryonic stages
through adulthood, but both populations decrease in density when MT cell signaling is prevented.

A third hypothesis is that a given neuronal population’s response to activity blockade is determined by its identity in the context of the circuit. Indeed, the PV-EPL interneurons and GCs both receive excitatory input from MT cells, and both populations decrease significantly in density when this input is blocked. However, like PV-EPL interneurons and GCs, both CR- and CB-PGCs are thought to receive excitatory input primarily or entirely from MT cells (and not from OSNs). Yet in the MT-T OB, there is no decrease in CR-PGC density, while CB-PGCs show an intermediate decrease in density. Thus, while strong presynaptic inputs from MT cells predicted decreases in density, the opposite did not hold.

Why are CR-PGCs unaffected by MT cell vesicular release blockade, and why is the magnitude of neuronal loss different for subpopulations of GCs and PGCs? There are several potential explanations for these results. First, the current description of the olfactory bulb circuit is likely incomplete. It may be that there are other important excitatory inputs onto the PGCs and GCs that moderate the loss of MT cell excitatory input in the MT-T OB. For example, a recent report described a small population of glutamatergic short-axon cells, whose dendrites ramify in the glomerular layer (Brill, Ninkovic et al. 2009). Noradrenergic centrifugal fibers terminate in several OB layers
including the glomerular layer and the granule cell layer, (2004) and could serve as
another source of excitation for PGCs. Cholinergic fibers terminate mostly in the granule
cell layer and may also provide excitatory input (Shepherd 2004).

Second, it is possible that some of the changes in density that we observe could be
due to a decrease or loss of that immunohistochemical marker’s expression, rather than a
true change in cell number. This certainly seems likely in the case of 5T4, whose
expression is known to be activity dependent. A number of studies in the visual system
have examined the dependence of CR, CB and PV expression levels on sensory activity,
with mixed findings. In some species and regions, CB is reduced while in others it is
unaffected. PV immunoreactivity is reduced in some experimental preparations,
unchanged in at least one, and increased in two others. Calretinin expression is
unchanged in some preparations and increased in others (reviewed in (Kang, Park et al.
2002).

Finally, it may be that some other property intrinsic to each cell type is important
in determining susceptibility to changes in excitatory signaling. For example, it is known
that different cell types can be identified by their distinctive transcriptional states, and
there is some evidence that distinct neuronal populations also bear transcriptional
signatures that persist postnatally (Arlotta, Molyneaux et al. 2005). Through any number
of signaling pathways or other transcription-dependent mechanisms, such a difference in
transcriptional state could render a population more or less vulnerable to blockades of excitatory signaling.

MT cells receive direct excitatory input from OSNs, and no other sources of excitatory input have been reported. We report dramatic decreases cell density for other OB neuronal populations when MT cell vesicular release is blocked. Other studies have also shown that the removal of afferent excitation can lead to the death of postsynaptic cells. Therefore it might be expected that in the OSN-T OB, when that excitatory signaling is blocked, the MT cells might die. However, we see no change in MT cell density, even in very aged mice (data not shown). Furthermore, their morphology appears altogether normal. MT cells are born relatively early, from E11-E13. They migrate and begin to mature before making synaptic contacts with OSNs only shortly before birth. (Blanchart, De Carlos et al. 2006) Perhaps their apparent activity independence can be linked to their status as some of the earliest born OB neurons.

In the context of the MT-T OB, the MT cells do receive excitatory input from OSNs, but many of their postsynaptic partners are missing. In other brain regions, it has been shown that some types of neurons die at elevated rates in response to a loss of postsynaptic targets. Motoneurons that innervate skeletal muscle are perhaps the best-studied such group; others include retinal ganglion cells in response to superior colliculus ablation (Harvey and Robertson 1992) and the neurons of the inferior olivary nucleus in
response to loss of cerebellar Purkinje cells (Chu, Hullinger et al. 2000). However, just as in the case of OSN vesicular release blockade, we see no evidence of increased rates of cell death for the MT cells when their own synaptic signaling is blocked.

We confirmed this finding using BrdU birthdating of MT cells through IP injections of BrdU at E11 and E12, when MT cells are born. At both timepoints, we found increased densities of BrdU+ cells, lying almost exclusively in the MCL and lower GL, where external tufted cells are found. These increased densities likely indicate normal survival rates for MT cells in the context of a shrinking OB.

A second BrdU birthdating study allowed us to examine the relative susceptibility of GCs born at different developmental timepoints to activity blockade-induced cell death. As mentioned above, previous work has shown that GCs born at early postnatal timepoints survive at higher rates than later born GCs (Lemasson, Saghatelyan et al. 2005). Since embryonic GCs develop and mature in the absence of normal sensory-evoked activity (it remains possible that OSNs are activated by molecules present in amniotic fluid) it could be that embryonically born GCs are relatively resistant to signaling blockade dependent cell death. We labeled populations of GCs born at E14-E15, P10-P11 and P30-P31 in MT-T mice and WT littermate controls. When we quantified GC density at a late adult timepoint (P125-P135) we saw a ~60-70% decrease in survival amongst MT-T animals at all three timepoints. Therefore it seems that all GCs
are equally dependent on MT cell signaling for their long-term survival. This result is somewhat surprising given what is known about the difference in activity dependence for early and later adult-born GCs during normal development. It could be that GC death in the MT-T OB is mechanistically different than GC death during the early wave of fast cell death, so that activity dependence in a given IN population for one form of cell death would not predict activity dependence for the other. Morphological data from MT-T GCs presented in Chapter 3 supports the idea that these two forms of cell death are indeed distinct in timing and therefore, mechanism.

What could explain the survival of the remaining ~30-40% of GCs? It could be that they happened to form synaptic connections with one or more mitral cells that express TeNT-GFP at a lower level, allowing some synaptic transmission to occur. Alternatively, perhaps the excitatory inputs that the surviving GCs receive from other brain areas are relatively strong (or early) and can overcome the lack of MT cell excitation.

In summary, the reductions in OB layer area that we observe in the OSN-T and MT-T OB are due to cell death seem to occur in proportion to the decrease in activity levels. Furthermore, our BrdU study indicates that GCs, both newly migrated and mature, both adult-born and embryonically-born, depend on excitatory input in order to survive. At the same time, given the mild area and cell death phenotype in the OSN-T OB, it
seems that many neurons are able to survive when the excitatory input they receive is somewhat reduced, perhaps through homeostatic adjustments in gene expression or connectivity. However, the MT-T phenotype suggests that there is a threshold of excitation below which many OB INs cannot survive.

What might the implications of these findings be for regenerative medicine? They suggest that an important criterion for neurons isolated or differentiated for therapeutic transplantation could be that they have appropriate levels of intrinsic excitability, and are developmentally competent to form postsynaptic specializations. It could be that such features could be introduced deliberately during differentiation through gene expression manipulations or culture conditions.

Chapter 2, in part, comprises a paper in preparation for submission to Neuron entitled “Blockade of excitatory inputs to olfactory processing circuits reveals static and dynamic neural populations.” The dissertation author was the primary investigator and author of this paper.
Figure 2-1: Genetic schema of the mouse lines crossed to generate OSN-T and MT-T mice.

Each CRE line is expressed in a subset of neurons, either OSNs (OMP-IRES-CRE) or MT cells (Pcdh21-IRES-CRE). When crossed with the TeNT-GFP line, the floxed stop codon that precedes the TeNT-GFP open reading frame is excised and TeNT-GFP expression can proceed in the CRE-specified subset of neurons, driven by the Rosa26 locus promoter.
### Figure 2-2: Expression patterns in OSN-T and MT-T mice within the olfactory epithelium, olfactory bulb and several cortical areas.

Within the OE, OSNs express GFP only in the OSN-T mice, consistent with OSN expression of the TeNT-GFP fusion protein. In the OB the OSN axons of the OSN-T mice coalesce into GFP-dense glomeruli. In the OB of MT-T mice, on the other hand, there is GFP staining in the MCL, EPL, and GL, consistent with MT cell expression of the TeNT-GFP fusion protein. In piriform cortex of MT-T mice, there is GFP staining in layer 1, where MT cell axons terminate and form synapses onto the cortical neurons. Scale bars, 50 µm OE, 100 µm OB and CX.
Figure 2-3: VAMP2 is reduced specifically in the GL (OSN-T) or EPL (MT-T).
VGlut2 specifically labels the glomerular layer due to its high relative expression in OSNs. In WT OB, VAMP2 staining is most intense in the EPL. In the OSN-T OB, VAMP2 staining is specifically reduced in the glomerular layer, where OSN axons form dense presynaptic terminals. In the MT-T OB, VAMP2 staining is specifically reduced in the EPL. Scale bar, 100 µm.
Immediate early genes Zif268 and cFos are reduced in the GCL of MT-T but not OSN-T. Zif268: n= 6(WT), 3(OSN-T) and 5(MT-T) animals, 3-4 sections per animal. cFos: n= 4(WT), 2(OSN-T) and 3(MT-T) animals, 2-3 sections per animal. In the GL, TH is strongly reduced in OSN-T and somewhat reduced in MT-T. TH:n= 8(WT), 4(OSN-T) and 3(MT-T) animals, 2-4 sections per animal. Zif268: WT is 2002 ± 288, OSN-T is 2301 ± 232, MT-T is 218 ± 217. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, p<.0001. OSN-T vs MT-T, p<.0001. cFos: WT is 567.0 ± 176.3, OSN-T is 437.3 ± 239.4, MT-T is 92.9 ± 85.6. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, p<.001. OSN-T vs MT-T, p<.05. TH: WT is 100.0 ± 15.9, OSN-T is 41.6 ± 11.4, MT-T is 60.0 ± 17.3. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, p<.0001. WT vs MT-T, p<.05. OSN-T vs MT-T, n.s. Scale bar, 100 µm. Error bars indicate 95% confidence intervals. *p<.05, ***p<.001; ****p<.0001.
Figure 2-5: A specific reduction in the size of the olfactory bulb size for MT-T mice. Whole brains dissected at P21. While the two brains are the same size overall, the olfactory bulbs of the MT-T mouse are noticeably smaller than those of the WT littermate. Scale bar, 5 mm.
Figure 2-6: Growth curves for WT and MT-T mice from P0 to P21.
P0: n=28(WT) and 16(MT-T); P5: n=22(WT) and 10(MT-T); P11: n=39(WT) and 21(MT-T); P21 n=22(WT) and 12(MT-T). P0: WT is 1.53 ± .05, MT-T is 1.55 ± .10. P5: WT is 3.48 ± .31, MT-T is 3.11 ± .41. P11: WT is 6.88 ± .36, MT-T is 5.06 ± .58. P21: WT is 11.20 ± .74, MT-T is 7.38 ± 1.29. Unpaired t-tests for WT vs MT-T: P0, n.s. P5, n.s. P11, p<.0001. P21, p<.0001. ****p<.0001.
Figure 2-7: The laminar organization of the OB is disturbed in MT-T mice. In these P21 coronal OB sections, VGlut2 staining in OSN axons reveals the GL and PGP9.5 staining in MT cell somata and lateral dendrites reveal the EPL. The MCL is indistinct and appears to invade the abnormally thin EPL. The glomeruli are irregular in size and shape. Scale bar, 100 µm.
Figure 2-8: Layer-specific changes in the OB during postnatal development. Maximal coronal OB sections at P11, P21 and P125-137. P11: n=3(WT), 2(OSN-T) and 3(MT-T); P21: n=4(WT), 6(OSN-T) and 4(MT-T); P125-137: n=2(WT), 1(OSN-T) and 2(MT-T) animals, 3 sections per animal. Scale bars, 500 µm.
Figure 2-9: Quantification of layer differences illustrated in Figure 2-8.
X-axis indicates postnatal days. Whole OB P11: WT is 3.14 ± .25, OSN-T is 3.23 ± .16, MT-T is 2.37 ± .36. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, p<.01. OSN-T vs MT-T, p<.01. Whole OB MT-T: P11 is 2.37 ± .36, P125 is 1.87 ± .15. Unpaired t-test, p<.05. Error bars indicate 95% confidence intervals.
Figure 2-10: Loss of GCs in OSN-T and MT-T mice examined for several subpopulations.

NeuN is found in mature GCs (n= 5(WT), 3(OSN-T) and 4(MT-T) animals, 2-3 sections per animal). Sp8 is found in GCs at all developmental stages (n=6(WT), 2(OSN-T) and 4(MT-T), 2-3 sections per animal). 5T4 is found in some superficial GCs (n= 5(WT), 6(OSN-T) and 4(MT-T), 1-4 sections per animal). CR marks two distinct populations: a subclass of GCs and a class of PGCs (n= 4(WT), 4(OSN-T) and 4(MT-T), 2-3 sections per animal). NeuN: WT is 5473 ± 777, OSN-T is 5978 ± 950, MT-T is 1621 ± 503. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, p<.0001. OSN-T vs MT-T, p<.0001. Sp8: WT is 7181 ± 569, OSN-T is 6910 ± 1271, MT-T is 6908 ± 1125. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, n.s. 5T4: WT is 1599 ± 240, OSN-T is 1564 ± 239, MT-T is 35 ± 17. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, n.s. CR: WT is 889 ± 111, OSN-T is 937 ± 97, MT-T is 346 ± 122. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, p<.0001. OSN-T vs MT-T, p<.0001. Scale bar, 100 µm. Error bars indicate 95% confidence intervals. ****p<.0001.
Figure 2-11: Other OB IN classes show specific patterns of loss or survival.

CR marks two distinct populations: a subclass of GCs and a class of PGCs (n= 4(WT), 4(OSN-T) and 4(MT-T), 2-3 sections per animal). CB primarily stains a class of PGCs (n= 6(WT), 2(OSN-T) and 3(MT-T), 2-4 sections per animal). CB is also found in sparse Blanes cells within the GCL (n= 6(WT), 2(OSN-T) and 3(MT-T), 2-4 sections per animal). PV marks a population of neurons within the EPL (n= 4(WT), 4(OSN-T) and 4(MT-T), 2-3 sections per animal). CR: WT is 1444 ± 188, OSN-T is 1344 ± 207, MT-T is 1261 ± 135. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, n.s. CB: WT is 696 ± 61, OSN-T is 630 ± 146, MT-T is 394 ± 135. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, p<.0001. OSN-T vs MT-T, p<.01. Blanes: WT is .82 ± .35, OSN-T is .49 ± .36, MT-T is 1.56 ± 1.12. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, p<.0001. OSN-T vs MT-T, p<.0001. Scale bar, 100 µm. Error bars indicate 95% confidence intervals. **p<.01, ****p<.0001.
Figure 2-12: MT cells are present in normal numbers in OSN-T and MT-T mice. Tbr2 staining at P21 (n=7(WT), 3(OSN-T) and 6(MT-T) animals, 2-4 sections per animal) at P21 reveals an increased density of MT cells in MT-T OBs compared to WT and OSN-T OBs. WT is 1880 ± 154, OSN-T is 1737 ± 251, MT-T is 2939 ± 574. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, p<.0001. OSN-T vs MT-T, p<.0001. Scale bar, 100 µm. Error bars indicate 95% confidence intervals. ****p<.0001.
Figure 2-13: Representative images of MT cells in OSN-T and MT-T mice.
Collapsed and aligned z-stacks of MT somata and dendritic tufts after OB injection with Sindbis virus encoding GFP. Glomerular tufts are inset. Note that MT cells in OSN-T and MT-T mice extend lateral dendrite and one apical dendrite whose tuft extends into the GL. The MT-T MT cell seems to have thinner dendrites and may have an abnormally shaped glomerular tuft. Scale bar, 100 μm.
Figure 2-14: Proliferation and migration are normal in MT-T mice.
Sagittal sections through the rostral migratory stream. Nestin staining appeared qualitatively similar in WT and MT TeNT animals. The number of Ki67+ or BrdU+ cells (24 hours after BrdU injection) along a 500 µm length of RMS are not significantly different between WT and MT TeNT animals (Ki67: n=2(WT) and 2(MT-T) animals, 1-2 sections per animal; BrdU: n=4(WT), 3(MT-T) animals, 1-4 sections per animal). In coronal sections that include the AON, the density of cleaved caspase-3+ cells in the RMS was not significantly different between WT and MT TeNT animals. N=7(WT), 2(OSN-T) and 5(MT-T) animals, 1-5 sections per animal. Ki67: WT is 135 ± 29, MT-T is 122 ± 54. Unpaired t-test, n.s. BrdU: WT is 110 ± 21, MT-T is 112 ± 28. Unpaired t-test, n.s. CC-3: WT is 69 ± 34, OSN-T is 23 ± 41, MT-T is 33 ± 36. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, n.s. Scale bar, 500 µm.
Figure 2-15: Increased levels of cell death in the MT-T GCL.
Cleaved caspase-3 staining reveals increased rates of apoptotic cell death in the MT-T GCL compared to the GCL of WT and OSN-T OBs (n=9(WT), 5(OSN-T) and 5(MT-T), 3-5 sections per animal. WT is 7.2 ± 1.7, OSN-T is 26.8 ± 16.4, MT-T is 52.0 ± 14. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, p<.05. WT vs MT-T, p<.0001. OSN-T vs MT-T, p<.01. Scale bar, 200 µm. Error bars indicate 95% confidence intervals. *p<.05; **p<.01; ****p<.0001.
Figure 2-16: Schema of embryonic and postnatal BrdU injections.
Pink and blue arcs above the line represent the developmental windows of cell birth for MT cells (pink) and GCs (blue). The arrows below the line indicate separate experiments that birthdate cohorts of cells: MT cells (dark red), embryonically-born GCs (light blue) and two groups of postnatally-born GCs (dark blue).
Figure 2-17: Embryonically and postnatally derived GCs are lost at equal rates in MT-T mice.

BrdU staining in GCL at >P125 (E15.5 injection: n=2(WT), 5(MT-T) animals, 3-4 sections per animal; E10.5 injection: n=2(WT), 3(MT-T) animals, 2-6 sections per animal; E30.5 injection: n=2(WT), 3(MT-T) animals, 2-3 sections per animal). MT-T GCs were reduced in density by 60-65% compared to WT for all three birthdates (B2-B4). MT-T BrdU density relative to WT. B2/E15.5: 31.7, 95% c.i. are 17-55. B3/P10.5: 30.9, 95% c.i. are 17-51. B4/P30.5: 40.6, 95% c.i. are 21-66. Scale bar, 100 µm. **p<.01; ***p<.001.
Figure 2-18: Presumptive MT cells survive into advanced adulthood in MT-T mice. BrdU staining in MCL/EPL at P60 or P137 (P60: n=1(WT), 1(MT-T) animals, 4 sections per animal; P137:n=4(WT), 3(MT-T), 2-3 sections per animal). MT cells are denser in MT-T OBs. MT-T BrdU density relative to WT. P60: 166, 95% c.i. are 129-222. P190: 391, 95% c.i. are 167-848. Scale bar, 100 µm. **p<.01; ***p<.001.
References


Chapter 3
Morphological features of postnatally generated granule cells depend differentially on activity

Abstract

In Chapter 2, we showed that when MT cell vesicular release is blocked, several populations of OB interneuron, including GCs, die at abnormally high rates. However, in many circuits, perturbing neuronal activity or signaling does not lead to cell death but does affect the morphological development of either the experimental neuron or its pre- or postsynaptic partners. In these experiments, we hoped to determine the extent to which MT-T GCs were able to mature in the absence of afferent input. To answer this question, we used sparse lentiviral labeling to quantify the morphological and synaptic effects of MT cell vesicular release blockade in GCs at two timepoints. By 15.5 we found dramatic deficits in all measures of morphological and synaptic maturity when we compared MT-T GCs to WT GCs. In a separate set of experiments, we used the same sparse infection strategy to address the possibility that GC death was cooperative under MT cell vesicular release blockade. We found that reducing excitatory input or hyperpolarizing sparse GCs resulted in morphological deficits similar to what we observe for MT-T GCs. This suggests that the maturation and survival of individual GCs depends on excitatory synaptic input from MT cells.
**Introduction**

Having established the elevated rates of cell death amongst GCs and other neuronal populations in the MT-T OB, we wished to look more closely at the structure of individual neurons. The wave of “fast cell death” culls ~50% of GCs between 15 and 45 days after they are born. This range of time corresponds to later maturational stages (class 4 and 5). Therefore, during normal development, the majority of GCs migrate and begin to mature. Then at the point when they reach their destination and begin to establish synaptic connections, some percentage of them undergoes cell death.

In our model of reduced MT cell vesicular release (MT-T), we observe that granule cells and several other OB interneuron populations are reduced in density, and that this reduction is due to increased rates of cell death. We wondered if GCs in the MT-T OB follow a typical developmental trajectory, maturing normally and then dying as class 4 or 5 neurons), or if MT cell signaling blockade induced cell death either earlier or later than the peak of fast cell death.

We also hoped to learn about the synaptic development of GCs in the context of the MT-T OB. It is known that granule cells first receive unidirectional synaptic inputs that could correspond to MT cell or cortical excitation (Kelsch, Lin *et al.* 2008). Then at a slightly later developmental point, they form the more distal dendritic spines that are sites of reciprocal synapses with MT cells. Electrophysiological evidence corroborates this timeline. A high percentage of class 3 GCs receive IPSCs or EPSCs (~80% and ~35%
respectively), while Na\(^+\)-mediated spikes are present in a majority of GCs only at the most mature stage (class 5) (Carleton, Petreanu et al. 2003). These data suggest a hypothesis about the formation of GC presynaptic sites. Given that GCs receive synaptic input before developing the morphological and electrophysiological characteristics necessary to form functional synapses onto their postsynaptic partners, it is possible the former (synaptic input onto GCs) is necessary for the latter (development of output spines and/or synaptic sites). We tested this hypothesis in our MT-T model of MT cell vesicular release blockade by examining the density of GC dendritic spines and presynaptic puncta.

We also hoped to elucidate the mechanism underlying the effects of MT-T on GC in more detail. In the MT-T OB, MT cells are prevented from releasing synaptic vesicles, and as a result we see dramatic increases in the rates of cell death in the granule cell layer, as measured by cleaved caspase-3 immunohistochemistry (Fig 2-15). In the field of stroke research, it is known that ischemic strokes lead to an inflammatory response, including release of proteases, cytokines and reactive oxygen species (reviewed in (Yenari and Han 2012) as affected neurons apoptose. These in turn can initiate an apoptotic response in nearby, unaffected neurons, thus broadening the site of the stroke-related injury.

We wondered if a similar positive feedback loop of cell death might be at work in the MT-T olfactory bulb. In that model, elevated cell death and perhaps a loss of GC
scaffolding or signaling increase the probability that newly born migrating GCs will undergo cell death, and a cycle of cell death is continually reinforced in the OB. We can term this a cooperative model of cell death. In a second model, the apoptosis and loss of other GCs does not affect (or plays a relatively minor role in) the survival or death of newly generated GCs. Rather, MT cell vesicular release is required for GC maturation and survival, so that when it is blocked in the MT-T OB, individual GCs undergo death at elevated rates.

To distinguish between these two models, we used two methods to alter levels of excitation for sparse GCs. In the first, we blocked NMDAR-mediated signaling, which constitutes a portion of the glutamatergic excitation from MT cells (AMPA receptors also mediate MT cell to GC glutamatergic signaling). In the second, we overexpressed a potassium channel, which should maintain a hyperpolarized resting potential in affected GCs. By manipulating only sparse GCs in each of these experiments, we could check whether their morphology looked similar to that of MT-T GCs (confirming model 2) or substantially less affected (confirming model 1, the cooperative model of GC death.
Materials and Methods

Mouse strains

MT-T mice are described in the Materials and Methods section of Chapter 2. NR1 mice were generated in the laboratory of Susumu Tonegawa (Tsien, Chen et al. 1996, Tsien, Huerta et al. 1996); Jax stock number 005246.

Tissue preparation and immunohistochemistry

Tissue preparation and immunohistochemistry are as described in the Materials and Methods section of the Chapter 2. Primary antibodies used:

GFP (rabbit, Invitrogen A11122 or sheep, Serotec 4745-1051)

HA (mouse, Covance MMS-101P)

Zif268/Egr1 (rabbit, SCBT sc-189)

Lentiviral constructs

Lentiviral constructs were cloned using modified versions of the FUGW shuttle vector (Lois hong.,baltimore 2002). Unless otherwise noted, they encode proteins driven by the UbC promoter. Three constructs were gifts from A. Maximov: Lenti-eGFP, lenti-Kv12.1-SCP-GFP and lenti-VAMP2-Venus. Murine Kv12.1 was originally cloned by T. Jegla (Zhang et al 2009). In lenti-KV12.1-SCP-GFP, gene expression is driven by a synapsin promoter. In lenti-VAMP2-Venus, rat VAMP2 (whose amino acid sequence is 100% identical to murine VAMP2) and the GFP variant Venus are cloned in frame as a fusion protein, with Venus at the C-terminal. FLAG-HA-GFP was generated in
laboratory of W. Harper (Sowa et al, 2009) and cloned into the lentiviral shuttle vector by K.N.J.; Addgene plasmid 22612. Lenti-CRE-GFP was generated in the laboratory of T. Jacks; Addgene plasmid 20781. Lenti-CRE-GFP encodes GFP driven by the UbC promoter and CRE driven by the PGK promoter.

Production of lentivirus

Shuttle vectors were co-transfected with packaging vectors pCMVΔ8.9 and pVSVg into HEK293T cells using the calcium phosphate method. Virus was collected at 48 h post-transfection, concentrated by ultracentrifugation (2 h at 113,000 g, 4 °C), resuspended in a small volume of PBS and stored at -80 °C.

Intraventricular injection of lentivirus

A simple manual air pressure-based system using a 3 mL syringe, plastic adaptors and thin tubing and was constructed and fitted with a glass micropipette (~10 µm tip diameter). Neonatal mice (P0 or P1) were anesthetized for several minutes on wet ice. The injection site was targeted by drawing a line from bregma (visible beneath the skin) to the top of each eye with a fine-tip marker, then crossing it perpendicularly approximately ⅓ of the way down from the top (bregma). At an injection depth of 1-2 µm relative to the skin surface, a volume of 0.5-2 µl of virus was ejected. Mice were allowed to recover beneath a warm lamp for several minutes and then returned to the home cage.

Neuronal reconstructions
Images of granule cells were acquired on a Nikon C2 as automatically stitched large image z-stacks at 40x. For each image, up to three neurons were chosen to reconstruct, in increasing order of the distance of the soma from the mitral cell layer. Criteria for reconstruction were that the neuron appeared to be completely contained within the section/z stack with no obviously truncated branches at the top or bottom, and that the soma contain some detectable native GFP, indicating a minimum level of VAMP2-GFP expression. Using Neurolucida 6.0 (MBF Bioscience), we reconstructed the granule cell’s dendritic tuft, excluding any trailing dendrite. Spines and (for VAMP2-Venus tracings) presumptive VAMP2 puncta (native GFP puncta on dendritic branches or spines) were marked and attached to the dendritic tracing for density analysis. Dendritic spines were marked separately, based on both red (immunostained GFP) and green (unstained GFP) channel signal.

All procedures were performed in accordance with the guidelines and standards of the Scripps Research Institutes’ Animal Care and Use Committee.

Results

We next wished to examine the morphology of the GCs that migrate into the MT silent OB. Previous work demonstrated that under chronic naris occlusion, GCs exhibit a modest decrease in spine density but no distance in total dendritic length or number of
branches (Dahlen, Jimenez et al. 2011, Saghatelyan, Roux et al. 2005). Given the relative magnitude of cell death and loss of GCL area, we hypothesized that in MT-T mice, individual GCs might exhibit more pronounced changes in morphology compared to GCs in sensory-silenced models. We performed intraventricular lentiviral injections at P0, labeling newborn neurons that subsequently migrated through the RMS and into the OB. This allowed us to visualize individual neurons and later, to quantify their morphological and synaptic properties through 3D tracing and reconstruction of infected GCs.

We first injected lentivirus that encoded GFP under the control of the UbC promoter and at 21 days post-injection, we observed even labeling of a subset of GC in mice of all three genotypes (Fig 3-1). The morphology of GCs in MT-T mice differed dramatically from that of WT or OSN-T GCs. The cell bodies and apical dendrites of WT (and OSN-T) GCs display finely tuned radial orientation. Their apical dendrites ramify within the EPL and bear dendritic spines. In contrast, the GCs of MT-T mice lacked apical dendrites in some cases, and in others they were oriented aberrantly in many directions.

We next performed intraventricular injections of a lentivirus that encoded a fusion of VAMP2 and GFP. By using a primary antibody against GFP followed secondary antibody conjugated to a fluorophore that emitted 555 nm wavelength (red) light, we could detect low levels of GFP that filled the infected neurons, including dendritic spines.
We also collected images in the green channel absent any immunohistochemical amplification, which consisted of puncta that colocalized with the dendrites and dendritic spines (Fig 3-2). We believe that these puncta correspond to presynaptic sites to which the VAMP2 fusion protein was trafficked.

As mentioned above, GCs form reciprocal dendrodendritic synapses with MT cells. It is known that GCs first experience spontaneous synaptic events as class 3 neurons, which are defined as possessing a growing apical dendrite that approaches but has not yet crossed into the EPL (Carleton, Petreanu et al. 2003). Only once the GC extends its apical dendrites into the EPL (class 4) does it begin to spike and in the final stage (class 5) the GC dendrites develop spines. For our reconstructions, we chose VAMP2-GFP GCs in an unbiased manner (imaging up to three cells per section in descending order of somatic distance from the EPL, ensuring that the entire cell lay within the section) (Fig 3-3). We found that the GCs of MT-T animals have drastically reduced total dendritic length and complexity (number of branches) as early as P15.5 (Fig 3-4). We also recorded the density of dendritic spines and VAMP2 puncta, and we observed large losses at P15.5 and at P21. The existence of spines means that at least some MT-T GCs can attain aspects of the final stage of maturity, class 5. The reduction in VAMP2 puncta could be an indirect effect of reduced numbers of spines, or it may be more directly related to the loss of vesicular release in its reciprocal synaptic partners, the
MT cells. This result is reminiscent of the decrease in synaptophysin puncta density seen in GC dendrites following chronic naris occlusion (Kelsch, Lin et al. 2009).

Using the same neonatal lentiviral injection technique to sparsely label GCs, we performed two experiments to explore the cause of GC death and morphological defects. We wished to determine whether the effects we saw could be recapitulated in sparsely affected GCs. Alternatively, if we found that sparse GCs were unaffected, this would suggest that the drastic effects on GCs in the MT-T OB were cooperative, with newly born GCs unable to survive and mature within a milieu of cell death and abnormal cytoarchitecture.

To determine which of these two models was correct, we used a genetic approach to eliminate NMDAR-mediated input onto sparse GCs in an otherwise normal OB. Both NMDA and AMPA receptors are expressed at high levels by GCs (Sassoe-Pognetto and Ottersen 2000) and contribute functionally to GC excitation by MT cells, with NMDA receptors specifically generating the slow component of the GC EPSC (Isaacson and Strowbridge 1998). NMDAR-mediated excitation is first observed in class 2 granule cells, as they begin to migrate radially within the olfactory bulb (Carleton, Petreanu et al. 2003), eliminating the possibility of an effect of NR1 KO during GC migration.

We obtained mice homozygous for the insertion of loxP sites flanking a 12 kB region of the NR1 (Grin1, GluN1) gene that encodes the transmembrane domain and C-
terminal regions (Tsien, Chen et al. 1996, Tsien, Huerta et al. 1996) (Fig 3-5). NR1 is an obligate subunit of the NMDAR (Mori and Mishina 1995), so by severely truncating the NR1 subunit, we should be functionally knocking out all NMDAR-mediated excitation. This conditional NR1 KO line has been used successfully in over 30 publications, including one that targeted olfactory GCs (Lin, Sim et al. 2010). We performed P0 intraventricular injections of a lentiviral construct encoding CRE and GFP separately (lenti-CRE-GFP), mixed with a control lentiviral construct encoding HA-tagged GFP (lenti-HA-GFP). In the sparse GCs infected with lenti-CRE-GFP, expression led to loxP site recombination and a functional knockout of NR1 and thus, NMDAR-mediated excitation (Fig 3-5).

We performed 3D reconstructions of control, lenti-HA-GFP GCs and NR1-KO GCs at P21, in order to assess the effect of loss of NMDAR-mediated excitation on sparse GCs (Fig 3-6, 3-7). In the NR1 KO GCs, we observed a substantial relative decrease in total dendritic length, number of branches, and dendritic spine density (Fig 3-8). The NR1 KO GCs looked similar to the GCs of MT-T mice, although their morphological deficits were not as severe. This intermediate effect could be due to the fact that AMPAR-mediated excitation of GCs is preserved in NR1-KO GCs, whereas in the GCs of MT-T mice, both types of excitation are presumably prevented at MT-GC synapses. The morphological effects that we observe in NR1-KO GCs, which trend in the
direction of our observations in MT-T cells, support a model in which each GC migrates and matures independently, and in which the drastic morphological defects that we observe in MT-T mice stem primarily from the direct effect of a loss of MT cell-mediated excitation of GCs.

We also sparsely infected GCs with a lentiviral construct expressing the voltage-gated potassium channel Kv12.1 (Elk1, Kcnh8), which begins to activate at -90 mV (Zhang, Bursulaya et al. 2009). Kv12.1 was cloned in frame with a fusion of the self-cleaving 2A peptide and GFP, allowing detection of Kv12.1-expressing neurons (lenti-Kv12.1-SCPGFP). Because the lentiviral construct drives gene expression using the synapsin promoter, expression levels in infected neurons should be very high compared to endogenous levels of Kv12.1 gene expression. As a result, infected neurons should be chronically hyperpolarized relative to control neurons, preventing them from experiencing intrinsic or synaptic depolarization and the associated intracellular signaling events.

We injected a mixture of lenti-Kv12.1-SCPGFP and lenti-HA-GFP and examined OB slices at P21 (Fig 3-9). When we quantified levels of Zif268 expression in either group of infected neurons, we observed a dramatic decrease in Zif268 expression in Kv12.1-SCPGFP GCs relative to GFP-HA GCs (Fig 3-10). Amongst reconstructed Kv12.1 GCs at P21, we again saw a drastic effect on GC morphology (Fig 3-11). All
three measures of GC maturity were dramatically reduced Kv12.1-SCPGFP GCs relative to control GCs (Fig 3-12). This experiment provides additional evidence that the blockade of MT cell synaptic signaling in MT-T mice directly causes the death and morphological defects of GCs.

Discussion

In Chapter 2, I presented evidence that MT cell vesicular release blockade (in the MT-T animal model) lead to decreased cell density for some populations of interneurons in the olfactory bulb, and that this decrease was due to high rates of cell death rather than changes in proliferation or migration. This set of experiments in Chapter 3 focused on the morphological effects of MT cell vesicular release blockade on the granule cells, as well as an investigation of the mechanism underlying these changes. For each experiment, we performed neonatal intraventricular injections of several lentiviral constructs designed to label or manipulate certain aspects of individual, newborn neurons. When we section and examine the OB several weeks later, sparse labeled GCs and other interneurons can be clearly visualized.

We first injected a simple construct encoding GFP under the control of the UbC promoter. In OSN-T animals the GCs looked indistinguishable from those in WT animals. In both cases, the GCs had radially oriented apical dendrites that ramified within
the EPL. In MT-T animals, on the other hand, the GCs had very short or even missing apical dendrites with no clear orientation. In the MT-T OB we also observed more speckling or blebs that were GFP+, which may residual dendritic processes from dead GCs.

We wished to quantify these morphological effects as well as potential effects on dendritic spine density and presynaptic terminals. The spines of GCs contain both presynaptic and postsynaptic sites (Shepherd, Chen et al. 2007), so measures of their density cannot distinguish between the two. We particularly wished to see if the lack of MT cell excitatory input would affect the formation of GC presynaptic sites. Adult-born GCs receive synaptic input before they begin to fire action potentials and produce synaptic output. This suggests that the former might be a causal factor in establishing the latter.

We used a lentiviral VAMP2-GFP fusion protein construct to quantify dendritic properties, dendritic spine density and presynaptic puncta density for MT-T and WT GCs. At both P15.5 and P21 we observed sizable decreases in all these measures for MT-T GCs. Nevertheless, many MT-T GCs did extend their apical dendrite into the EPL, a hallmark of class 4 GCs. Similarly, MT-T GCs possessed some dendritic spines, which are found on the most mature GCs (class 5) (Petreanu and Alvarez-Buylla 2002). While this evidence demonstrates that at least some GCs in the MT-T OB attain certain
hallmarks of maturity, they also show drastic reductions in complexity, overall dendritic length, spine density and VAMP2 puncta density. Finally because spine density is affected in the MT-T GCs, we cannot conclude that MT cell excitatory input plays a causal role in promoting GC presynaptic sites. This is because it could be that declining spine density necessarily limits VAMP2 puncta density. Alternatively, it could be that loss of MT cell input does limit the formation of GC presynaptic sites, which in turn causes a decrease in spine density. Our criteria for selecting GCs to be reconstructed favor more mature GCs (those whose somata lie nearest to the EPL), yet in the MT-T OB, the largest and most complex GCs that we reconstructed are qualitatively similar to the smallest WT GCs, at P15.5 and at P21 (Fig 3-3). These results suggest that when MT cell vesicular release is prevented in the MT-T OB, it is not the case that GCs develop to class 5 and then regress. Rather, it seems that MT-T GCs deviate from the typical developmental path and those that survive attain only limited features of mature GCs.

Given the drastic increase in GC death and changes in their branching patterns, we wished to address the extent of cooperativity in the morphological effects of MT cell TeNT expression on GCs. In one model, newborn GCs integrating into the OB are affected by the presence and survival of existing GCs. They may require either the physical scaffolding of normal GC dendritic branches, or some signaling or trophic factor from existing GCs. If such a scaffold or factor is missing in the MT-T OB, the
morphological effects that we observe in GCs could be indirect. Alternatively, they may respond to apoptotic signals in the MT-T OB, creating a positive feedback loop in which GC death is self-perpetuating. In a second model, the migration and maturation of newborn GCs occurs independently of existing GCs, and the loss of branching and presynaptic structures that we observe are directly due to a lack of excitatory input from MT cells.

We injected neonatal, conditional NR1 KO mice with a lentiviral construct encoding GFP and CRE. In the sparse infected granule cells, NR1 was knocked out and presumably NMDARs, which require two NR1 subunits, could not form. GCs normally express both NMDARs and AMPARs, and MT cell glutamate release activates both kinds of glutamate receptors. Thus, this experiment constitutes a partial knockdown of MT cell activity. In these NR1 KO GCs, as in MT-T GCs and Kv12.1 GCs, we observe dramatic defects in dendritic morphology and spine density. One measure, total dendritic length, showed only an intermediate defect in NR1 KO GCs compared to MT-T GCs and Kv12.1 GCs (One-way ANOVA followed by Tukey’s multiple comparisons test: NR1 KO vs Kv12.1, p<.01; NR1 KO vs MT-T, p<.01; Kv12.1 vs MT-T, NS. One-way ANOVA for control GCs from these 3 experiments showed no significant differences). This partial recapitulation may be attributable to the fractional nature of the signaling blockade in this experiment. An important caveat to these results is that centrifugal inputs
from piriform cortex also have an NMDAR-dependent component (Boyd, Sturgill et al. 2012). This sparse GC blockade of NMDAR-mediated excitation will also affect these excitation pathways.

An earlier study used the same NR1 conditional KO line in conjunction with injections of CRE-encoding retrovirus to examine the role of NMDAR signaling in GC survival (Lin, Sim et al. 2010). The authors observed a near total loss of NR1 KO GCs by 28 days post injection. We do not observe a change in the ratio of NR1 KO to control GCs between P21 and P35 (data not shown), although at both timepoints the inter-animal variability in NR1 KO/control GCs was high. Perhaps differences in viral CRE expression levels could account for this difference: it could be that our virus recombines loxP sites in only one NR1 allele in some GCs, allowing enough NR1 expression for them to survive.

In a parallel experiment, we used potassium channel Kv12.1 to chronically hyperpolarize sparse GCs, mimicking the lack of MT cell excitation in the MT-T OB. The GCs that we reconstructed showed similar defects in dendritic morphology and spines as were observed in MT-T GCs. This is further evidence that the loss of MT cell excitation in the MT-T OB affects GCs individually rather than leading to a kind of feedback loop where dysfunction and death beget more dysfunction and death. Furthermore, it suggests that these effects are due to decreased GC activation
(depolarization), as opposed to the loss of a synaptically localized signal such as cell-adhesion molecule mediated signaling.

In sum, these two recapitulation experiments (Kv12.1 and NR1 KO) confirm that the GC phenotype that we observe in the MT-T OB is due to a loss of synaptic transmission (i.e. a blockade of excitatory signaling) from MT cells, rather than any effect on non-synaptic vesicle release, or secondary effects of tetanus toxin expression. They also show that MT signaling blockade is likely to independently affect individual GCs and thus that MT synaptic input is a critical factor in the fate of each maturing GC.

Chapter 3, in part, comprises a paper in preparation for submission to Neuron entitled “Blockade of excitatory inputs to olfactory processing circuits reveals static and dynamic neural populations.” The dissertation author was the primary investigator and author of this paper.
Figure 3-1: Intraventricular injection of lenti-GFP reveals morphological deficits in MT-T GCs.
Note that GC dendrites are short or missing and there is little branching within the EPL.
Scale bars, 100 µm.
Figure 3-2: Examples of lenti-VAMP2-Venus labeled P21 GCs corresponding to starred reconstructions in Figure 3-3.
Indicated dendritic branches are inset. Scale bar, 100 µm.
Figure 3-3: Representative range of GC reconstructions revealed by intraventricular injection of lenti-VAMP2-Venus fusion protein. Neurons within each group are arranged in descending order of total dendritic length. Starred neurons are shown in Fig 3-2. Scale bar, 50 µm.
Figure 3-4: Quantification of morphological and synaptic features of reconstructed GCs.

White bars indicate WT, red bars indicate MT-T. P15.5: n=14(WT), 37(MT-T); P20.5: n=9(WT), 15(MT-T) from 3 animals. Total dendritic length P15.5: WT is 326 ± 133, MT-T is 147 ± 34. Unpaired t-test, p<.001. Total dendritic length P21: WT: 509 ± 175, MT-T 120 ± 34. Unpaired t-test, p<.0001. Number of branches P15.5: WT is 13.9 ± 7.5, MT-T is 6.4 ± 2.3. Unpaired t-test, p<.01. Number of branches P21: WT: 14.1 ± 5.3, MT-T 3.0 ± 1.5. Unpaired t-test, p<.0001. Spine density P15.5: WT is .100 ± .056, MT-T is .018 ± .011. Unpaired t-test, p<.0001. Spine density P21: WT: .135 ± .052, MT-T .014 ± .018. Unpaired t-test, p<.0001. VAMP2 puncta density P15.5: WT is .246 ± .071, MT-T is .108 ± .003. Unpaired t-test, p<.0001. VAMP2 puncta density P21: WT: .234 ± .054, MT-T 3.0 ± 1.5. Unpaired t-test, p<.0001. Error bars indicate 95% confidence intervals. ***p<.001; ****p<.0001.
Figure 3-5: Genetic schema for sparse GC NR1 knockout.
Figure 3-6: Examples of NR1 KO GCs or lenti-HA-GFP expressing GCs.
Scale bar, 50 µm.
Figure 3-7: Representative range of reconstructions of lenti-HA-GFP or NR1 KO GCs.

Starred neurons are shown in Fig 3-6. Scale bar, 50 µm.
Figure 3-8: Quantification of morphological features of reconstructed lenti-HA-GFP GCs or NR1 KO GCs.
N=17 (HA-GFP), 17(NR1 KO) from 3 animals. Total dendritic length: HA-GFP is 556 ± 84, NR1-KO is 248 ± 59. Unpaired t-test, p<.0001. Number of branches: HA-GFP is 8.1 ± 1.9, NR1-KO is 2.1 ± .7. Unpaired t-test, p<.0001. Spine density: HA-GFP: .118 ± .038, NR1-KO .012 ± .006. Unpaired t-test, p<.0001. Error bars indicate 95% confidence intervals. ****p<.0001.
Figure 3-9: Examples of GCs expressing Kv12.1-SCPGFP or HA-GFP. Scale bar, 50 µm.
Figure 3-10: Zif268 levels in lenti-Kv12.1-SCPGFP GCs are drastically reduced compared to lenti-Zif268 levels in HA-GFP GCs.

N=40 (lenti-HA-GFP), 10 (lenti-Kv12.1-SCPGFP) cells, 8 sections, 2 animals. HA-GFP is 100.0 ± 21.8, Kv12.1 is 6.8 ± 9.6. Unpaired t-test, p<.0001. Error bars indicate 95% confidence intervals. ****p<.0001.
Figure 3-11: Representative range of GC reconstructions of lenti-HA-GFP or lenti-Kv12.1-SCPGFP GCs.

Starred neurons are shown in Fig 3-9. Scale bar, 50 μm.
Figure 3-12: Quantification of morphological features of reconstructed GCs expressing lenti-HA-GFP or lenti-Kv12.1-SCPGFP.

References


Chapter 4

Cortical projections and target neurons are unaffected by blockades of OSN or MT cell signaling

Abstract

In many sensory systems, activity plays a role in modulating axonal projections to higher processing regions, either through changes in branching or changes in synapse number. In some cases, the survival of target neurons requires neurotransmission from afferent inputs (Born and Rubel 1988, Seal, Akil et al. 2008). We examined olfactory neurons in piriform cortex for signs of elevated cell death or decreased density, under conditions of either OSN or MT cell vesicular release blockade. We found that neither form of blockade affected the survival of cortical target neurons. Next, we used long-range, single MT cell axonal tracing to characterize the roles of OSN and MT cell vesicular release in determining primary branching and axonal bouton densities. In three target cortical regions (AON-pE, AON-pP and aPC), we found that vesicular release from either OSNs or MT cells was dispensable in the development of appropriate MT cell axonal branching patterns and bouton densities. Thus, in contrast to the OB, cortical olfactory areas are surprisingly resistant to either subtle (OSN-T) or drastic (MT-T) reductions in afferent inputs.

Introduction

The role of activity and synaptic signaling, either sensory-evoked or total, in olfactory cortex remains unknown. Past studies using genetic and surgical models of
OSN signaling blockade have focused on the glomerular map and a handful of other features of the OB. No phenotype—or lack of a phenotype—has been reported for higher order sites of olfactory processing such as the anterior olfactory nucleus (AON) or piriform cortex (PC). The MT cells, as the sole class of projection neurons in the OB, send axons into these cortical olfactory regions and form synapses onto both excitatory and inhibitory neurons there.

In the olfactory bulb, the effects of MT cell vesicular release blockade are dramatic, with multiple interneuron populations decreasing in number or density (see Chapter 2). While not every interneuron population that receives most or all of its excitatory input from MT cells decreases in density in the MT-T OB (see CR-PGCs), it is the case that among the cell types we looked at, every population that decreased in density did receive most or all of its excitatory input from MT cells. In olfactory cortical regions, both excitatory and inhibitory neurons receive associational inputs from other olfactory cortical regions, in addition to the excitatory input from MT cells (Shepherd 2004). Therefore, we predicted that the survival of olfactory cortical neurons would not be as drastically affected as the OB interneuron populations. Given the relatively mild effects of OSN vesicular release blockade in the OB, we predicted that olfactory cortical neurons would be unaffected in the OSN-T mouse.

We also wished to examine the axonal projections of the MT cells themselves under conditions of either OSN or MT cell activity blockade. In other sensory systems, a loss of sensory-evoked activity during a critical period can lead to distortions in spatial maps found in cortex even when separated by several synapses (somatosensation: (Stern, Maravall et al. 2001), vision: (LeVay, Wiesel et al. 1980)). However, recent work from
several labs has revealed that in contrast to the visual, auditory and somatosensory systems, the olfactory system does not maintain spatial mapping of inputs within cortical regions (Ghosh, Carnahan et al. 1994, Miyamichi, Amat et al. 2011, Sosulski, Bloom et al. 2011). Rather, MT cells send off primary collaterals in an apparently stochastic pattern, with sister MT cells (innervating the same glomerulus) no more similar in their primary branching pattern than any two MT cells would be. Furthermore, each individual mitral cell axonal arbor branches quite extensively and fills large volumes within multiple regions of olfactory cortex. Since there is no known spatial map in olfactory cortex and due to the extensive and highly variable morphology of single MT cell axons, we could not assess the impact of OSN or MT vesicular release blockade on spatial projection patterns of MT cell axons. Instead, we focused on measures of MT cell axonal branching and connectivity.

In advance, it was difficult to predict how each form of activity blockade might affect these measures of MT cell axon function. In the somatosensory system, when thalamocortical projection neurons were chronically hyperpolarized, their axonal arbors were markedly reduced in size and complexity (Yamada, Uesaka et al. 2010). On the other hand, in the auditory system, infusion of TTX onto the auditory to prevent sensory signaling from hair cells to their cochlear nucleus targets had the effect of increasing hair cell axonal branching (Niparko 1999). Both manipulations are probably nearly complete blockades of signaling, rather than simply blockades of sensory signaling, making them more analogous to MT cell vesicular release blockade (MT-T). And yet, they had opposite effects on axonal branching.
Similarly mixed results have been obtained in studies that examine the
dependence of synapse formation or maintenance on activity or synaptic signaling. In the
retina, when vesicular release was blocked in a class of bipolar neurons, they formed
fewer synapses onto their postsynaptic RGC targets (Kerschensteiner, Morgan et al.
2009). But in the developing hippocampus, chronic activity blockade via TTX infusion
increased the number of excitatory synapses that were formed (Lauri, Lamsa et al. 2003).

One can imagine a reasonable explanation for either phenotype (gain or loss of
branching or synapses). If synaptic communication is impaired or diminished, it could be
that the presynaptic neuron might try to compensate by increasing its output through
either denser axonal branching or greater synapse density. On the other hand, our
knowledge of widespread axonal pruning during development supports the possibility
that the ineffective presynaptic neuron might respond by retracting its branches or
eliminating ineffective synapses. Therefore, in advance, we did not have a strong
prediction for what the effects of OSN or MT cell silencing would be on mitral cell
axonal projections. We used single mitral cell axonal fills, tracing and reconstruction to
assess the role that sensory signaling (OSN vesicular release) or MT cell vesicular release
might play in the primary branching patterns and the formation and maintenance of
presynaptic structures in MT cell axons.

Materials and Methods

Tissue preparation and immunohistochemistry

Tissue preparation and immunohistochemistry are as described in the Materials
and Methods section of the Chapter 2. Primary antibodies used:
Cleaved caspase-3 (rabbit, CST 9661)
GABA (rabbit, Sigma-Aldrich A2052)
NeuN (mouse, Millipore MAB377)
VAMP2/Synaptobrevin (mouse (69.1) Synaptic Systems 104211)
VGluT2 (GP, Millipore AB2251)
Zif268/Egr1 (rabbit, SCBT sc-189)

Olfactory bulb-targeted injection of Sindbis virus tracers

Injections were performed as previously described (Ghosh, Larson et al. 2011) but without reference to a fluorescently labeled glomerulus. Briefly, 3-week-old mice were anesthetized with isoflurane (2% in 100% O₂) and olfactory bulbs were injected with Sindbis virus encoding fluorescent proteins (GFP alone, RFP alone or GFP and RFP in a single construct). Mice were euthanized 48 h after injection.

Image collection and image processing

For cortical reconstructions of mitral cell axons, images were acquired either on a Nikon C2 or an Olympus Fluoview 500 at 20x. Cells to trace were chosen using these criteria: 1) the cell body was large, triangular in shape and lay within the MCL 2) a thick apical dendrite ended in a tuft filling a single glomerulus 3) the axon projected posteriorly through the LOT and remained visible through anterior PC. Images acquired on the Olympus Fluoview 500 were manually stitched in XY space. For all data sets, successive z stacks were manually aligned to make a rostral-caudal superstack in Neurolucida. Brain regions (AONpe, AONpp, aPC) were identified using DAPI nuclear counterstaining. For primary branch analysis, the main axon was traced and branch points where secondary branches emerged were marked and traced for a short distance. For bouton analysis, three
representative images (each corresponding to a z-stack of a single section) per target brain region were analyzed for each animal. A maximal projection of each image was used to trace a stretch of secondary- or higher order MT cell axonal branch and count the number of corresponding boutons.

For cortical cell density counts, the regions sampled were as follows. Zif268 and NeuN: layer 2 of PC. GABA: layer 1 and 2 of PC, separately.

All procedures were performed in accordance with the guidelines and standards of the Scripps Research Institutes’ Animal Care and Use Committee.

**Results**

We wished to determine the role that OSN vesicular release (i.e. sensory-evoked activity) and MT cell vesicular release play in the innervation of cortical areas and the formation of presynaptic structures. In Chapter 2, I demonstrated that in the MT-T mouse, TeNT-GFP could be detected in layer 1 of PC (Fig 2-2). We next examined the AON and aPC to determine whether MT cell tetanus toxin was functioning distally to reduce VAMP2 expression (Fig 4-1). In MT-T mice, we observed a reduction in VAMP2 reactivity in layer 1 of AON and aPC, where MT axons synapse onto the dendrites of their postsynaptic partners. These data indicate that in the MT-T mouse, MT cells successfully traffic TeNT-GFP along the length of their axons (and specifically to higher-order axonal branches that contain presynaptic endings) and that the tetanus toxin is able to cleave VAMP2 there. We expected that neurons in the MT-T PC should experience a drastic reduction in afferent activity, while the OSN-T PC should experience an
intermediate level due to the non-evoked, spontaneous activity of the MT cells. Indeed, we observed this graded decrease in the expression of activity marker Zif268 (Fig 4-2).

Having validated the effectiveness of each model of vesicular release blockade, we used immunohistochemical staining to see the effects of each type of activity blockade on the neurons of these target areas. First we examined levels of cell death using cleaved caspase-3 staining in several distinct regions. We found no increase in cell death in layers 1 and 2 of piriform cortex (Fig 4-3) nor in the LOT or olfactory tubercle (a cortical region primary targeted by tufted cells) (data not shown). We also directly quantified the density of neurons found in piriform cortex, using either a pan-neuronal marker (NeuN) or a marker for inhibitory neurons (GABA) (Fig 4-4). In agreement with the cleaved caspase-3 staining data, we saw no differences from WT in cell density for either population of neurons under blockade of vesicular release in OSNs or MT cells. While OSN-T and MT-T did show significantly different densities of NeuN, neither group differed significantly from WT.

To determine the effects of each form of signaling blockade on MT cell axonal projections, we used glomerular-targeted injections of recombinant Sindbis virus bearing fluorescent protein genes to label sparse MT cells in 3 week-old mice. Using consecutively imaged z-stacks, we created 3D reconstructions of their axonal projections into AON (divided into two sub-regions, AON-pE and AON-pP) and aPC. In both OSN-T and MT-T mice, the LOT appeared typical in location and size, and the sparse labeled axons projected perpendicularly through it as we observe in WT mice (data not shown). Furthermore, the fine, higher-order axonal branches ramify in the correct layer (layer 1 of AON and aPC). We counted the number of primary branches extending from the main
axon in each of the three target regions and found that neither form of vesicular release blockade affected the branching count in any region (Fig 4-5).

Having shown that the gross patterns of MT cell branching were unperturbed by blockades of sensory or general activity, we next wished to examine the effects of these perturbations on the density of axonal boutons. The axons that we reconstructed featured round and ovoid boutons that are known to correspond to presynaptic sites. We eliminated the possibility that these structures are "blebs" related to viral overload based on their size and shape, on their location exclusively on collaterals and not on the main branches running through the LOT and on the fact that we did not observe any disintegration of axonal branches. We calculated bouton densities per axonal length for each target region (AON-pE, AON-pP and aPC), and for each of the three mouse lines. We found that neither blockade of vesicular release affected bouton density (Fig 4-6). Further, we report that MT cell presynaptic bouton density is equal for all three cortical target regions in WT mice (Two-way ANOVA, p=.55). Thus, in contrast to the drastic effects of blocking either OSN- or MT-cell vesicular release within the OB, both the cells of the cortical target regions and the gross branching and presynaptic fine structure of MT axons appear resistant to these manipulations.

Discussion

This set of experiments was designed to explore the effects of OSN and MT cell vesicular release blockade within olfactory cortical regions. Having observed elevated levels of cell death in the OB, we examined cortical target neurons to see if they too were
dying at abnormal rates. We also reconstructed single mitral cell axons and quantified their primary branching and dendritic density.

In piriform cortex, we saw no changes in levels of cell death, nor did we observe changes in the density of total neurons (NeuN) or inhibitory neurons (GABA). One explanation for the cortical olfactory neurons’ insensitivity to activity blockade could be that they receive excitatory input not just from MT cells but also from associational fibers (from neurons in piriform cortex and other cortical olfactory regions) (Shepherd 2004). For OB GCs, the sparse NR1 KO and Kv12.1 experiments suggest that GC death and morphological deficits are caused by decreased excitation(depolarization (see Chapter 3). If this is the case, then it is unsurprising that cell types like those in piriform cortex, which receive substantial excitatory inputs in addition to MT cell inputs, are resistant to OSN or MT cell vesicular blockade.

A second explanation for the differences in cell survival that we observe in the OB and in piriform cortex could be that the olfactory bulb is an unusually dynamic brain structure. Unlike most other areas of the brain, it accommodates the integration of newborn neurons throughout the lifetime of the animal, and concomitantly, it experiences relatively high rates of cell death as incoming adult-born neurons are selected either for integration or elimination. Psychophysical tests in mice revealed that the cell death component of the OBs dynamism is required for optimal odor discrimination(Mouret, Lepousez et al. 2009). Perhaps OB interneuron cell types are unusually dependent on activity for survival in order to ensure the success of the activity-dependent selection that they undergo after arriving in the olfactory bulb.
In a second set of experiments, we used a single MT cell axonal tracing technique developed in our lab to quantify two aspects of MT cell axonal projections within three target areas: AON-pE, AON-pP and aPC. We quantified branch density and axonal bouton density under either model of vesicular release blockade (OSN-T and MT-T) and observed no differences relative to WT controls for either measure.

Given that blockades of activity or vesicular release have been shown to have opposite effects on either measure of axonal morphology, we did not have expectations in advance about how OSN and MT cell vesicular release would affect them. Nevertheless, it was somewhat surprising that we saw no significant difference in either measure, under either form of vesicular release blockade. Organisms depend for their survival on quick and accurate sensory transduction of the environmental stimuli that they encounter. In other sensory systems such as the visual, auditory and somatosensory systems, activity plays an important role in refining axonal branching and synapse number, processes that are thought to optimize sensory processing. In light of the known plasticity of other sensory systems, it is somewhat surprising that neither MT cell axonal primary branching nor bouton density is affected in the OSN-T or MT-T animal.

As mentioned earlier, in contrast to somatosensory, auditory and visual cortical areas, olfactory cortex does not contain known spatial maps; certainly the spatial glomerular map within the OB is not recapitulated in piriform cortex. Through behavioral experiments we know that rodents, like humans, can form salient olfactory associations that can be created through single trials (Cousens and Otto 1998) and that may persist for long periods of time in the absence of exposure to that stimulus (Brown, Gunn et al. 2009). Finally, through lesion studies we know that the olfactory percepts are most
strongly associated with olfactory/piriform cortical activity. In light of the lack of a stereotyped spatial map in piriform cortex, the salience and stability of olfactory memories poses a mechanistic riddle. It could be that the lack of plasticity that we observe in both MT cell projections and target neurons in response to OSN or MT cell vesicular release blockade reflects the need for exceptional stability in perceptual coding within an individual.

Finally, it remains possible that other parameters of MT cell axonal projections do change in response to blockades of OSN or MT cell vesicular release. Such parameters could include total axonal length, branch complexity, volume innervated, synapse strength or other quantitative measures that are beyond the scope of this paper.

Chapter 4, in part, comprises a paper in preparation for submission to Neuron entitled “Blockade of excitatory inputs to olfactory processing circuits reveals static and dynamic neural populations.” The dissertation author was the primary investigator and author of this paper.
Figure 4-1: VAMP2 levels are reduced in layer 1 of AON and aPC in MT-T mice. Scale bar, 100 μm.
Figure 4-2: Zif268 levels are intermediate in OSN-T PC and low in MT-T PC.
N= 6(WT), 3(OSN-T) and 5(MT-T) animals, 3-4 sections per animal. WT is 923 ± 188, OSN-T is 488 ± 200, MT-T is 77 ± 27. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, p<.01. WT vs MT-T, p<.0001. OSN-T vs MT-T, n.s. Scale bar, 100 µm. *p<.05, **p<.01; ****p<.0001.
Figure 4-3: Cell death is unchanged in OSN-T and MT-T PC.
Cell death was assessed with cleaved caspase-3 staining. N= 8(WT), 2(OSN-T) and 5(MT-T) animals, 1-8 sections per animal. Number of cells counted = 62(WT), 22(OSN-T) and 21(MT-T). WT is 2.08 ± 1.26, OSN-T is 2.54 ± 2.06, MT-T is 2.57 ± 1.47. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, n.s. Scale bar, 100 µm.
Figure 4-4: Cortical neurons are present at normal densities in OSN-T and MT-T PC.

NeuN: n=6(WT), 3(OSN-T) and 3(MT-T) animals, 4-9 sections per animal. GABA: n=8(WT), 4(OSN-T), and 5(MT-T), 3-5 sections per animal. NeuN: WT is 4163 ± 373, OSN-T is 4637 ± 429, MT-T is 3787 ± 477. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, p>.05. GABA Layer 1: WT is 59.1 ± 7.7, OSN-T is 58.6 ± 12.8, MT-T is 56.5 ± 20.7. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, p>.05. GABA Layer 2: WT is 113.7 ± 14.8, OSN-T is 110.4 ± 15.9, MT-T is 124.4 ± 24.5. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, p>.05. Error bars indicate 95% confidence intervals. Scale bar, 100 μm. *p<.05.
Figure 4-5: Representative MT cell axonal projections in OSN-T and MT-T mice. Right, representative reconstructions of MT cell axons in three target areas of cortex. R: rostral, C: caudal. Left, images of higher order MT cell axonal branches in piriform cortex. Note boutons. AON-pE: WT is 2.33 ± 0.71, OSN-T is 3.08 ± 1.31, MT-T is 1.82 ± 0.63. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, n.s. AON-pP: WT is 2.00 ± 0.59, OSN-T is 2.08 ± 0.95, MT-T is 1.71 ± 0.54. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, n.s. aPC: WT is 2.13 ± 0.83, OSN-T is 2.83 ± 1.36, MT-T is 1.77 ± 0.89. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, n.s. Scale bar, 50 μm.
Figure 4-6: Primary branching and bouton density are unaffected in OSN-T and MT-T mice.
Quantification of number of primary branches: n= 10(WT), 8(OSN-T) and 12(MT-T) axons analysed from n=(WT), 4(OSN-T) and 5(MT-T) animals. Quantification of bouton density: n= 4-5 animals per genotype, 3 sections/area/animal. AON-pE: WT is .12 ± .03, OSN-T is .14 ± .07, MT-T is .13 ± .02. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, n.s. AON-pP: WT is .11 ± .05, OSN-T is .13 ± .06, MT-T is .12 ± .03. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, n.s. aPC: WT is .12 ± .01, OSN-T is .13 ± .06, MT-T is .14 ± .11. One-way ANOVA followed by Tukey’s multiple comparisons test. WT vs OSN-T, n.s. WT vs MT-T, n.s. OSN-T vs MT-T, n.s. Error bars indicate 95% confidence intervals.
References


