The Role of Second-Order Interneurons in the Maintenance and Organization of Olfactory Bulb Circuitry

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Master of Science in Biology by Weston Burnett Davini

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LIST OF ABBREVIATIONS

OB – Olfactory Bulb
MT – Mitral and Tufted
OSN – Olfactory Sensory Neuron
GC – Granule Cell
PGC – Periglomerular Cell
GL – Glomerular Layer
MCL – Mitral Cell Layer
GCL – Granule Cell Layer
TeNT – Tetanus Toxin
Cre – Cre Recombinase
GFP – Green Fluorescent Protein
RMS – Rostral Migratory Stream
PC – Piriform Cortex
P# - Postnatal Day #
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ABSTRACT OF THE THESIS

The Role of Second-Order Interneurons in the Maintenance and Organization of Olfactory Bulb Circuitry

by

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The olfactory bulb serves as an excellent template for evaluating the role of activity in the organization and maintenance of neural networks. Being one of two known sites of neurogenesis in the brain makes it an ideal model for examining the integration of adult born neurons into preexisting circuitry. With regard to the role of activity, we report here that stably expressing tetanus toxin specifically in olfactory sensory neurons has
little impact on the organization of the bulb, whereas tetanus expression in the second-order mitral and tufted (MT) neurons of the mouse olfactory bulb leads to a collapse of the discrete layering of the bulb. The widespread disorganization observed is attributed to the inability of granule cells to establish or maintain connectivity with the MT neurons under these conditions, which eventually leads to dramatically increased cell death within the granule cell layer. The morphological deficits observed appear to be due to their inability to reach a depolarization threshold, specifically through mitral and tufted cell activation of NMDA receptors. Inhibition of apoptotic pathways fails to rescue the granule cells, which suggests that reaching this threshold of activity is necessary for their maturation and survival. For the first time, a central role for second-order interneurons in the organization and maintenance of the olfactory sensory network has been demonstrated.
Introduction

Our interactions with the environment around us are governed by the complex sensory networks of the central nervous system. Upon activation of the first order neurons of our various sensory organs, environmental cues are integrated and interpreted by these circuits, eventually leading to a behavioral response. But we are still learning the basic principles of how those environmental cues become interpreted to induce extremely variable behavioral responses. Between the initial activation of the sensory neuron and the subsequent action, our understanding of the roles of the diverse neuronal subtypes through which the signal passes remains limited. How they organize themselves into the complex circuits that allow us to discern pleasant and dangerous odors, consonant sounds from dissonant ones, and the various colors of a painting relies in a large part on activity-dependent mechanisms.

Activity, as it is referred to here, implies the initiation of an electrochemical signal in one neuron, propagation of the signal down the length of its processes, and its transmission across a synapse, onto another neuron. The propagation of the signal between neurons depends on a number of components, the most obvious of which is its vessel: the synapse. A neuron becomes active when an action potential is triggered by depolarization, and this signal is transmitted between the pre- and post-synaptic bodies of the synapse, typically from one neuron to another. This action potential may be triggered by an upstream signal, or it may occur spontaneously. Transmission of the signal across
the synapse is accomplished by neurotransmitters, neuropeptides, and other signaling molecules. Passage of the signal across the synapse is concluded once these signaling molecules activate their respective receptors on the downstream neuron and activate a depolarization event. The matter of which neuron is downstream is complicated by retrograde signaling, in which signaling molecules pass from the post- to pre-synaptic cell. This is a very simplified picture of the actual series of events, but serves the purposes of this discussion. Together, these components comprise neuronal activity, and it is from the variability of these components that the complexity of our nervous systems originates.

The formation of a functional nervous system requires specific neurons from an enormously diverse pool of neuronal subtypes to locate each other and form stable synaptic connections. What makes sensory systems such attractive models for studying the fundamental principles governing the development and organization of neural networks is their well defined inputs and the ease with which these inputs can be manipulated. This aspect of sensory systems is also what makes them ideal templates for examining the role of activity in establishing and maintaining the neural circuits of the nervous system.

Goodman and Shatz (1993) very succinctly break down the mechanisms of neuronal connectivity into two broad categories: those which are activity-independent and activity-dependent. During the initial development of the nervous system, activity-independent mechanisms guide neurons and neural progenitors with a great deal of specificity to the networks into which they are eventually integrated. Establishing
connections and the fine-tuning that occurs within each network relies largely on activity. These activity-dependent mechanisms persist long after the developmental stages, and will be the focus of subsequent discussion (Purves, et al, 1986).

Activity not only serves to establish new connections, but also to maintain and remodel preexisting synapses depending on the state of neurotransmission in the vicinity. These mechanisms of adapting to changes in activity underlie the foundation of memory formation. In the sensory systems, adaptive changes to sensory stimuli underlie the behavioral changes that are observed following re-exposure to the stimuli, e.g. habituation. In the olfactory system, for example, a novel odorant very quickly evokes a highly dynamic response in the odor receptive fields of the cortex (Wilson, 2000). Stabilizing these adaptive changes in synaptic connectivity encodes the olfactory memory, which allows for future recognition of the previously novel stimulus.

The most compelling demonstration of the role activity plays in establishing and maintaining complex neural circuits are studies in which it is blocked. In sensory systems, this is most commonly accomplished by sensory deprivation, or by specifically targeting the peripheral sensory neurons. The path of transmission of sensory input follows a path that is mirrored in a general sense across the various sensory networks. Sensory activation of peripheral sensory neurons leads to transmission of the signal to second-order interneurons, which project to higher-order brain regions. A great number of other neural subtypes contribute to the propagation of the signal, but this generalized description of the pathway serves the purposes of this discussion for the time being. The effects of sensory input blockade are typically evaluated at the two downstream
junctions: the first-order/second-order intersection, and the connections between the projections of second order neurons and cortical targets.

Deprivation of sensory input via monocular lid closure in macaques leads to a drastic reorganization of the fibers that comprise the ocular dominance columns, discrete columns of neurons and their axonal projections in the visual cortex (Hubel, et al, 1977). The shift of “inactive” neuronal processes no longer receiving input from the sensory neurons of the sensory-deprived eye to the columns downstream of the active eye is mimicked in the rat barrel cortex when all but one whisker is clipped (Margolis, et al, 2012).

Interestingly, ocular dominance columns are present at birth, without the animals being exposed to light, and are maintained long after birth in animals whose eyes have been removed and retinal (sensory) input blocked (Horton and Hocking, 1996; Crowley and Katz, 1999). These data suggest an organizational role for spontaneous activity between the cells comprising the ocular columns and input from their neighbors in the cortex.

The Crowley and Katz study presents another method of manipulating activity: physically preventing the first order, sensory neurons from receiving sensory input, and thus blocking sensory-dependent activation of these neurons. This method can by applied to the olfactory system by naris occlusion, in which the naris is either cauterized shut or otherwise physically blocked. In these studies, the architecture of the bulb is only subtly perturbed, although there is a noticeable reduction in size (Brunjes, 1994; Cummings et al, 1997). Others have blocked odorant activation of sensory neurons in the olfactory epithelium by inactivating the olfactory cyclic-nucleotide-gated (CNG) channel. In these
mice, OSN targeting is left nearly completely unperturbed (Brunet, et al, 1996; Lin, et al, 2000; Zheng, et al, 2000). These results suggest that activity correlated with sensory input may not be necessary for establishing and maintaining connectivity, implying that spontaneous activity may play an important role.

The role of spontaneous vs. sensory-dependent firing adds another dimension to consider in evaluating the role of activity in the organization and maintenance of networks (Yu, et al, 2004). Yu and colleagues (2004) sought to examine the role of spontaneous activity by genetically blocking synaptic vesicle release in olfactory sensory neurons via conditional expression of tetanus toxin, as well as by reducing their excitability through the overexpression of and inward rectifying potassium channel (Kir2.1), which hold neurons in a hyperpolarized state. In mice in which nearly all OSNs express TeNT or Kir2.1, OSN targeting to the glomeruli, the small spherical structures within the outermost layer of the olfactory bulb, was left largely unperturbed. However, when only small, defined subpopulations of neurons expressing only one odorant receptor expressed TeNT or Kir2.1, the subpopulations projected to inappropriate targets, and ultimately disappeared. Additionally, expression of TeNT in a small subpopulation beginning in adulthood, after the neurons have established stable connection, causes them to become disorganized and eventually disappear. These results suggest spontaneous activity plays an important role in establishing and maintaining stable connections (Yu, et al, 2004).

The great number of components involved in neurotransmission provide a large pool of targets for investigators to further elaborate the organizational role of activity. The examples discussed thus far have examined activity-dependent plasticity at the first
two junctions in sensory networks, as well as the significance of spontaneous versus sensory-dependent activity. But all of these examples have focused on only one half of the picture. What is missing is a close examination of the role post-synaptic activity plays. Hata and Stryker tackled this question by infusing one hemisphere of the cat visual cortex with muscimol, a GABA$_A$-receptor agonist which blocks post-synaptic activity (Hata and Stryker, 1994). They then deprived the contralateral eye of sensory input and, in a complete reversal of what is observed in simple sensory-deprivation experiments (no muscimol), they observed a shift in ocular dominance towards the deprived eye. These results suggest a very significant role for post-synaptic activity in the behavior of pre-synaptic afferents, and subsequently the general organization of the network.

Even many of the individual components of the synapse are modulated in an activity-dependent manner. For example, long-term potentiation (LTP) and long-term depression (LTD), mechanisms of plasticity by which memory formation is thought to occur, are enabled and defined by acquisition of AMPA receptors at the synaptic membrane (Petralia, et al, 1999). In addition to receptors, some adhesion molecules involved in the targeting of neural projections are modulated in an activity-dependent manner. In zebrafish motor neurons, Plazas and colleagues observed a strong correlation between calcium signaling and that of the adhesion molecule, plexinA3, in axon targeting (Plazas, et al, 2012). In the mouse olfactory bulb, certain adhesion molecules are up- or down-regulated in sensory neurons upon cation influx (Serizawa, et al, 2006).

Even gene expression can be induced in an activity-dependent manner. This is made most obvious by the common use of immediate early genes (c-fos, zif) as markers
of active neurons. Others have observed that neurons are able to switch fates in the absence of stimulation. Yu and colleagues postulated that the disappearance of a silenced sub-population of OSNs was not due to their dying, but rather their change of fate via expression of another olfactory receptor (Yu, et al, 2004). Others demonstrated that this phenomenon does occur, albeit at low frequency, and that OSNs expressing mutant receptors do so with greater likelihood, implying that activity is even capable of reversing the most stringent of gene expression patterns (Shykind, et al, 2004).

As the fate of neurons can be changed in the absence of activity, the same is also true when neurons experience too much input. In addition to neurogenesis, induced seizures generate an extensive, stable remodeling of neurons in the hippocampus (Parent, et al, 1997).

Altogether, these results paint a very central role for activity in shaping neural networks. From the drastic shift of entire populations within the visual cortex following sensory deprivation in one eye, to the reversal of gene silencing to induce a change in cell fate within olfactory sensory neurons, neurotransmission is clearly a necessary component of connectivity and plasticity in the nervous system (Hubel, et al, 1977; Shykind, et al, 2004). Certainly, this examination the role of activity fails to address the contribution of activity-independent mechanisms in the formation of neural networks. But the data presented on cell-adhesion and axon-targeting molecules, as well as the changes observed in AMPA receptor density in LTP suggests that the line dividing processes which depend on activity and those which do not, is quite a blurry one (Petralia, et al, 1999; Plazas, et al, 2012; Serizawa, et al, 2006).
The Olfactory Bulb

The current study examines the organizational and sustaining role of activity within certain populations within the circuitry of the olfactory system. The majority of evidence presented above comes from studies of the visual system because of the breadth and depth of studies conducted in that area, however, the circuitry of the olfactory system is remarkably similar. Several characteristics of the olfactory system make it an ideal template for examining neural network organization and maintenance. For one, sensory input and subsequent activation is very easily manipulated, either by physical ablation via nostril closure or blockage, or by inactivation of the sensory neurons. The olfactory sensory neurons (OSNs) project to the olfactory bulbs (OB), the where sensory input is first integrated and processed in a spatially defined manner to produce a sensory map. This physical representation of sensory stimuli is shared between the visual and olfactory systems. The OB are small, ovoid protrusions at the rostral end of the mammalian brain. That these initial sites of OSN integration and processing within the central nervous system are confined within structures which are easily isolated makes the study of their circuitry much simpler. They contain well-defined inputs and outputs, which further contributes to this simplicity. Still, the circuitry is quite complex, comprised mostly of non-canonical synapses seen in very few other sites within the CNS. The final aspect that makes the olfactory network such an attractive site for investigating the fundamental aspects of network engineering is that it is one of only two sites of adult neurogenesis in the CNS.
The well-defined, layered architecture of the olfactory bulbs simplifies their study to some extent, but also introduces a great deal of complexity. Examining the roles of the individual interneuron subpopulations within the bulb, for example, requires creative approaches due to the limited number of techniques available. Recent advances, however, have allowed scientists to target specific neural subtypes, including some in the olfactory system.

The olfactory sensory neurons send their projections to the outermost layer of the OB, the glomerular layer (GL). The GL is defined by a ring of spherical structures 100-200 μm in diameter called glomeruli (Purves, et al, 2001). Each OSN in the olfactory epithelium expresses only one type of olfactory receptor (OR), and OSNs expressing the same type of receptor project to the same glomeruli. OSNs expressing the same OR typically project to two glomeruli in each bulb. Bellucio and Katz observed that the location of glomeruli innervated by OSNs with the same OR are bilaterally symmetrical between bulbs of an individual, as well as between individuals (Bellucio and Katz, 2001). These defined projection patterns form the basis of an olfactory map, which spatially encodes sensory information. The heterogeneity among OSNs between glomeruli enables a greater degree of organization within the odorant receptor map by the clustering of glomeruli whose OSNs express receptors, which respond to structurally similar odorants (Serizawa, et al, 2006; Matsumoto, et al, 2010). OSNs, along with other populations of olfactory neurons discussed below, are replaced throughout the life of the animal. This neurogenesis and replacement of neurons is a unique feature of the olfactory system, which is not observed in any other sensory system.

Within each glomeruli, OSN axons form synapses with the dendrites of second-
order interneurons, called mitral and tufted (MT) cells. Although the two can be distinguished electrophysiologically, and to a lesser extent, by their projection patterns, mitral and tufted cells will be grouped together for the purposes of this discussion unless stated otherwise (Igarishi, et al, 2012). MT cells are the principal interneurons of the bulb, providing the path by which a signal generated in OSNs travels to the cortex (Nagayama, et al, 2004). MT cell spatial orientation within the bulb varies according their birthdate, however, they are organized to a much lesser degree than OSNs (Imamura, et al, 2011). Unlike OSNs, MT cells are all born during the embryonic stages of the animal, and are retained throughout the animal's lifetime (Blanchart, et al, 2006). Glomeruli are innervated by approximately 25,000 OSN axons which synapse with the apical dendritic processes of approximately 25 mitral cells, 50 tufted cells and 25 other interneurons, called periglomerular juxtaglomerular neurons. Periglomerular cells (PGCs) and juxtaglomerular cells (JGCs) are primarily distinguished by the number of glomeruli that they innervate. While PGCs typically only innervate one glomeruli, JGCs project to two or more. The great number of connections within each glomeruli likely enables the network to filter out “uncorrelated noise” and ensure that the system remains faithful, allowing only strongly evoked signals pass through to higher brain regions (Purves, et al, 2001).

Just below the glomerular layer is a layer largely devoid of cell bodies, called the external plexiform layer (EPL). This cell-poor region is composed primarily of the lateral dendritic projections of the MT cells, and the apical dendritic projections of granule cells (GCs). The extensive dendro-dendritic synapses that form within the olfactory bulb, and most abundantly within the EPL, are a defining feature of OB circuitry (Isaacson and
Below the EPL can be found the thin band of mitral cell bodies, which comprise the mitral cell layer (MCL). The cell bodies of most tufted cells, on the other hand, reside just deep of the glomerular layer, at the most superficial border of the EPL, although a small population resides within the MCL. Mitral and tufted cells are projection neurons which provide the output for sensory information entering the OB. They project their axons deep into olfactory cortical regions. The sensory map is dramatically reorganized among the axonal projections of MT cells, without any sort of conserved projection pattern into the higher brain regions, even between MT cells innervating the same glomeruli (Ghosh, et al, 2011).

Another layer largely devoid of cell bodies, just below the mitral cell layer, called the internal plexiform layer (IPL), distinguishes the MCL from the largest layer in the bulb, the granule cell layer (GCL). This layer forms the core of the OB, and contains the largest population of cells within the bulb.

The granule cells (GCs) can be divided generally into two populations based on the location of their cell bodies within the layer. The cell bodies of superficial GCs lie out the outer edges of the GCL, closest to the IPL. The somata of some superficial GCs even cross into the mitral cell layer. Deep granule cells, on the other hand, lie closer to the center of the bulb. Subpopulations of granule cells, as well as other interneurons, can be further subdivided based on their expression of certain markers, although this method of classification remains inconclusive.

Granule cells, which are born throughout the lifetime of mammals, form dendro-dendritic connections primarily with the dendritic projections of MT cells, but also
extensively with other granule cells. Their role is an inhibitory one, and they are primarily GABAergic neurons. Glutamatergic input from MT cells leads to GC excitation and subsequent release of GABA onto the cell from which it received the glutamatergic input, as well as other GCs and MT cells nearby. This mechanism of synaptic communication is termed lateral inhibition. The glutamate-driven excitation of GCs is primarily mediated by NMDA receptors on the granule cells, rather than by AMPA receptors, which is the canonical pathway of glutamate-mediated excitation elsewhere in the central nervous system (Isaacson and Strowbridge, 1998; Schoppa, et al, 1998).

**Neurogenesis in the Olfactory Bulb**

One of the most unique features of the olfactory bulb is that it is one of only two well defined sites of adult neurogenesis, from rodents to humans (Altman, J. 1969; Lois and Alvarez-Buylla, 1994; Curtis, et al, 2007). Thus, the olfactory bulb is not only an excellent template to study the role of activity in the maintenance and organization of neural circuitry, but also to study how neurons become integrated into preexisting circuitry. This aspect makes studies in the olfactory bulb particularly applicable to those conducted in the field of stem cell research, where the problem of getting implanted stem cells to properly integrate into their new environment remains a central one.

Besides the hippocampus, the subventricular zone (SVZ), is the only other well established site of adult neurogenesis in the central nervous system (Gage, 2000; Lois and Alvarez-Buylla, 1994). From the SVZ, neural progenitors undergo an impressive migration from the lateral ventricle to the granule cell layer of the olfactory bulb by way
of the rostral migratory stream (RMS). Doetsch and colleagues demonstrated that astrocytes within the SVZ give rise to the neural progenitors that migrate along the RMS and eventually integrate into OB circuitry. Neural precursor cells are generated from the astrocytes, and eventually give rise to neuroblasts, which mature into neurons (Doetsch, et al, 1999).

The adult born neurons of the olfactory bulb are primarily granule cells, although a small portion of the progenitor population becomes periglomerular cells (Lois and Alvarez-Buylla, 1994). The research presented in this thesis focuses on granule cells, and the effects of activity on their development and integration. After completing their tangential migration, granule cells migrate radially within the granule cell layer to their final destination deep or superficial in layer. Developing granule cells are divided into five classes. The first class, which is distinguished by its long body and prominent growth cone on a short leading projection, is found only within the rostral migratory stream. Transition of the GC into class 2 cells is marked by a diminishing growth cone, which is no longer observed in class 3 cells. Once in this third class, the GCs have a noticeably larger cell body and a single projection towards, but not crossing, the mitral cell layer. In class 4 cells, the apical dendrite has developed complex branching patterns and crossed into the external plexiform layer, but lacks the synaptic density which defines class 5 cells (Petreanu and Alvarez-Buylla, 2002).

The reason for constantly replenishing the population of granule cells within the olfactory bulb remains elusive. Estimates put the number of embryonic-born GCs that survive 21 months at less than 10%, while the size of the bulb increases until it plateaus around 20 months of age. GCs continue to become incorporated into the bulb as others
die. The general consensus is that this dynamic process provides another dimension of adaptability to changing environments (Kaplan, et al, 1985).

The role of activity in the olfactory bulb

Studies investigating the role of activity in shaping and maintaining the circuitry of the olfactory bulb have traditionally relied on naris occlusion, in which the nostril is cauterized shut, or otherwise physically blocked. What is generally observed in these experiments is an increase in cell death, primarily within the granule cell layer, as well as a noticeable reduction in the size of the bulb. The architecture of the bulb, however, remains faithful and little perturbation is seen in the circuitry of the OB. These experiments have drastically increased our understanding of activity-dependent processes in the bulb, but many questions remain. As discussed above, the number of components involved in neurotransmission is quite large. Studies utilizing naris occlusion allow scientists to investigate the importance of correlated activity from the sensory neurons, but the roles of spontaneous activity, as well as the contribution of other cell types to the organization and maintenance of the circuitry cannot be addressed (Brunjes, 1994; Cummings et al, 1997; Bastien-Dionne, et al, 2010). Additionally, whether naris occlusion completely blocks odorant access to the sensory neurons has remained a caveat of these studies.

More recently, genetic techniques have allowed researchers to begin to investigate these unanswered questions. By knocking out the cyclic nucleotide gated channels on OSNs to generate anosmic mice, or genetically silencing these sensory neurons using
tetanus toxin or inward-rectifying potassium channels, scientists have been able to elucidate the role of spontaneous activity in the development of the spatial map of the olfactory bulb (Brunet, et al, 1996; Lin, et al, 2000; Zheng, et al, 2000; Yu, et al, 2004). Interesting, only silencing OSNs in a competitive environment, or blocking their excitability seems to have any effect on the targeting of their axonal projections to the glomerular layer. This would suggest that spontaneous activity plays an important role in the development of the spatial map. Still, the contribution of mitral and tufted cells to the effects observed in the granule cell layer remains unaddressed. Philpot and colleagues observed an increase in uncorrelated activity in MT cells following naris occlusion (Philpot, et al, 1997). This would suggest that MT neurons are able to compensate, to a limited degree, for the loss of OSN-mediated activity.

The generally subtle effects of sensory deprivation and sensory neuron silencing on the organization of the circuitry within the OB is in stark contrast to its effects in other sensory systems, as described above. In this experimental paradigm of sensory deprivation within these other sensory systems, dramatic reorganization of downstream circuitry is typically observed, particularly within the spatial maps of the cortex (Hubel, et al, 1977; Margolis, et al, 2012). In the olfactory system, on the other hand, very little is known about the role of activity in the cortex.

Our experiments in the olfactory system sought to address these unanswered questions. To do so, we employed two mouse models in which the OSNs or MT neurons express tetanus toxin (OSN-T animals, or MT-T animals, respectively), synaptically silencing them through tetanus cleavage of a vesicle associated protein (VAMP2) involved in the exocytosis of vesicles, thus preventing them from signaling to
downstream neurons. With this non-intrusive, very specific experimental system, we asked what are the respective roles of activity in OSNs versus MT neurons in the development and maintenance of the olfactory system? As it turns out, the second-order mitral and tufted neurons play a very integral role in the organization and maintenance of the circuitry within the olfactory bulb. What we observed was a perturbation in the bulbs containing silenced MT cells so severe that the normally distinct layers became nearly indistinguishable, whereas the bulbs in which OSNs were silenced looked nearly normal. The general collapse of the architecture of the bulb in MT-T animals was determined to be a extensive disorganization and cell death within the granule cell layer. Adult-born granule cells appear to be entirely dependent on top-down input from MT cells for their maturation and integration into the preexisting circuitry of the olfactory bulb.

To characterize our experimental models, we employed a tracing technique to quantify the effects within the granule cell layer. Tracing the most mature, superficial granule cells within our experimental animals, we were able to quantitatively analyze the morphological deficiencies in MT-T mice. No such deficiencies were observed in OSN-T animals. Close examination of the cortical targets of MT cells revealed no change, which is in stark contrast to the effects of sensory deprivation on the cortical regions of other sensory systems.

To recapitulate the effect observed in MT-T animals in their WT counterparts, and elucidate a mechanism for the observed effect, we forced the expression of an inward-rectifying potassium channel (which essentially holds the membrane potential at the resting potential) in the granule cells of mice not expressing tetanus toxin via injection of lentiviral constructs in the SVZ. We also conditionally knocked out a subunit
of the NMDA receptor in granule cells. In these experiments, we were able replicate the sensory-deprived morphology. Finally, to see if we could rescue the ill-fated granule cells, or if a depolarization threshold exists which prevents GC maturation if the cells do not meet the threshold, we employed RNA-knockdown of p53, and over-expression of Bcl2 to block apoptosis within the granule cells via injections of lentiviral constructs into the SVZ.

The research presented here paints a very central role for activity in shaping neural circuits, particularly in the development and integration of adult born neurons. The implications of this research include not only a better understanding of the mechanisms that guide the development and maintenance of neural networks, but also potential foundations for treating neurodegeneration if we can identify a means of reversing the fate of granule cells destined to die in the olfactory bulb. But even more importantly, this research will provide a better understanding of how the brain organizes and maintains such complex networks of neurons.
Materials and Methods

Mice

MC TeNT mice were generated by crossing mice from the Pcdh-21-IRES-CRE (M/T cell Cre) line, generated by Dr. Kristin Baldwin (Boland, et al. 2009), with mice from the TeNT-GFP line, provided by Dr. Martin Goulding (Zhang, et al. 2008). OSN TeNT mice were generated by crossing OMP-IRES-CRE (OSN Cre) mice, developed in the Axel laboratory (Eggan, et al. 2004), with mice from the TeNT-GFP line. Wild type mice used in all injection experiments were litter-mates containing either the Cre or TeNT gene. Conditional NRI knock-out mice were obtained from the laboratory of Susumu Tonegawa (Tsien, et al. 1996a; Tsien, et al. 1996B; Jax #005246). The genotypes of all mice used in each experiment was determined by PCR of the transgenes.

Cloning

Modified versions of the FUGW shuttle vector were used in the design of lentiviral constructs (Lois et al., 2002). Lenti-eGFP, lenti-Kv12.1-SCP-GFP, lenti-Bcl2-SCP-GFP and lenti-VAMP2-Venus were provided by Dr. Anton Maximov. VAMP2-Venus is expressed as a fusion protein, driven by the UbC promoter. The rat VAMP2 component shares the same sequence as its murine counterpart, while the venus
component is a modified GFP. Expression of lenti-Kv12.1-SCP-GFP, whose murine Kv12.1 sequence was cloned by T. Jegla (Zhang et al., 2009), is driven by a synapsin promoter. FLAG-HA-GFP was developed by W. Harper (Sowa et al., 2009) and cloned into the lentiviral shuttle vector Addgene plasmid 22612 by Dr. Kiely James. Lenti-CRE-GFP, whose CRE is driven by the PGK promoter and GFP driven by the UbC promoter, was generated by T. Jacks; Addgene plasmid 20781. Lenti-p53 shRNA-eGFP, in which p53 shRNA expression was driven by the H1 promoter and eGFP expression by the UbC promoter, was cloned by M. Boland.

**Virus Production**

Lentivirus was produced by transfecting HEK 293 cells at approximately 70-80% confluency using the calcium phosphate method. The packaging vectors, pCMVΔ8.9 (22.5 µg) and pVSVG (2.5 µg), along with the shuttling vector (25 µg), were mixed with 2M CaCl₂ (186 µL) and water to total volume of 1.5 mL. This mixture was added drop-wise to 1.5 mL of 2X HBS and incubated for approximately 3 minutes before being added to HEK 293 cells in a 150 cm² flask. 24 hours after transfection, the media was replaced with fresh media (DMEM + 10% Fetal Bovine Serum). 48 hours after transfection, the virus was collected by ultracentrifugation of the supernatant at 25,000 rpm for 2 hours and resuspended with 300 µL 1X PBS. Aliquots were kept at -80 °C.

**Brain Injections**
P0 or P1 mouse pups were anesthetized by being placed on wet ice for approximately 2 minutes. 0.5-2 µL of virus was injected into the lateral ventricles using a glass micropipette (~10 µm tip) connected to a 5 mL syringe by tapered plastic tubing (2 mm – 1mm). The location of the lateral ventricles was determined by drawing a line from the bregma to the eye and injecting 1/3 of the way down to the eye at a depth of 1-2 µm. The pups were then tattooed and allowed to recover under a heat lamp before being returned to their cage.

Transcardial Perfusion and tissue sectioning

At various time points, animals were anesthetized by inhalation of isoflurane and a transcardial perfusion was performed using 4% paraformaldehyde (PFA) in Phosphate Buffered Saline (PBS). The brains were then dissected and postfixed in 4% PFA overnight. 80-µm serial coronal sections of the olfactory bulbs were taken using a vibrotome (Leica VT1000S) after the brains were mounted in agarose, or 25-50-µm serial coronal sections were taken using a cryostat machine (Leica CM3050 S cryostat) after the brains were mounted in O.C.T. Compound (Tissue-Tek).

Immunohistochemistry

Tissue sections were permeabldized in 0.1X Triton-x in PBS (permeabilization buffer) for at least one hour before being treated with blocking buffer (5% Fetal Bovine
Serum in permeabilization buffer) for 30 minutes. Sections were then incubated with primary antibody diluted 1:500 in blocking buffer overnight at 4 °C. The next day, sections were washed three times for five minutes in PBS before incubating them for 2 hours at room temperature in secondary antibody diluted 1:1000 and DAPI (Life Technologies P36935 or P36934) diluted 1:10,000 in blocking buffer. After this incubation step, the sections were washed three times for five minutes in PBS before mounting them on glass slides (cryosections already mounted on slides), drying them for 10 minutes (~1 hour for cryosections), and coverslipping with Prolong Gold anti-fade reagent.

Cleaved caspase-3 (rabbit, CST 9661)  
GABA(rabbit, Sigma-Aldrich A2052)  
GFP (rabbit, Invitrogen A11122 or sheep, Serotec 4745-1051)  
HA (mouse, Covance MMS-101P)  
NeuN (mouse, Millipore MAB377)

Imaging

A Nikon C2 confocal microscope supported by NIS elements was used for all imaging. For granule cell reconstructions (using MBF Neurolucida), large image automated stitching was employed to collect between 8 and 20 z-stacks at 40x. Reconstructions were performed by analyzing z-stacks collected with the Nikon C2 using the tracing program MBF Neurolucida, which allowed us to trace each granule cell in
three-dimensional space. Reconstructable neurons were defined as the one to three most superficial (thus, most mature) granule cells, relative to the mitral cell layer, whose entire dendritic projections were contained within the x, y and z planes collected. In the case of vamp2-venus GC's, the soma must have had detectable native GFP. The dendritic processes were defined as all continuous fluorescent segments connected without gaps to the primary branch. Branches were defined as follows: two branches originating from a shared node must both be at least partially visible in the same plane as the node to prevent projections from other neurons being mistaken for a branch on the tree being traced. Additionally, if a branch projected to another neuron, and was not unquestionably part of the tree being traced, the branch was not included in the tracing. Bifurcating nodes were used in almost all instances, except where two bifurcating nodes lied on the same plane perpendicular to the projection. In these rare instances, a trifurcating node was used. Spines could not have any projections of their own, i.e. no spines and no more than the one terminal puncta. Puncta were defined as any outgrowth directly connected to any branch on the tree, or connected by a spine. Cortical images to evaluate cortical cell densities were collected at 20 or 40x.
**Results**

Though the role of sensory neurons in the olfactory network has been examined extensively, little is known about the contribution of mitral and tufted (MT) neurons to the organization and maintenance of this network. To elaborate the roles of MT neurons versus olfactory sensory neurons (OSNs), we utilized the cell-specific expression of tetanus toxin (TeNT) to conditionally inhibit synaptic input from these cells. Expression of TeNT prevents vesicular release of neurotransmitter by cleaving VAMP, but the cell is still able to receive synaptic input, and even fire action potentials.

The MT conditional knock-out line was generated by crossing mice from the Pcdh-21-IRES-CRE line, a M/T-specific Cre line generated by Dr. K. Baldwin and Dr. K. Eggan (Boland, et al. 2009), with mice containing the TeNT-GFP fusion protein preceded by a lox-stop-lox, provided by Dr. M. Goulding (Zhang, et al. 2008). The olfactory sensory neuron conditional knock-out line (OSN-T) to which they were compared was generated by crossing OMP-IRES-CRE mice, an OSN-specific Cre line developed in the Axel laboratory (Eggan, et al. 2004), with mice from the TeNT line (figure 1A). WT mice in either line expressed either Cre or TeNT alone, between which no differences were observed.

To validate the experimental system, coronal serial sections were stained for the presence of TeNT-GFP in the olfactory epithelium (OE), main olfactory bulb (OB) and the piriform cortex (PC) of WT, OSN-T and MT-T animals (figure 1B). OSN somata are localized in the OE and extend their axons into the glomerular layer (GL) of the OB, whereas MT cell bodies lie within the mitral cell layer and the GL, projecting dendrites
within the GL and external plexiform layer (EPL), and extending their axons to cortical targets, such as the PC. Staining for GFP indicated the proper localization of TeNT-GFP to the OE and OB, and its absence in the cortex of OSN-T animals, which is consistent with OSN projection patterns. MT-T sections stained positive for GFP in the OB and PC in MT-T animals, with an absence of GFP in the OE, consistent with MT projection patterns. These results suggest that the experimental system is functioning as anticipated, and that the correct neuronal populations are being targeted (James, 2013).

To further validate the system, immunohistochemistry was performed on coronal OB serial sections with antibodies against the N-terminal epitope of VAMP2, the vesicle-associated membrane protein cleaved by TeNT, and VGlut2, a glutamate vesicle transporter expressed highly in OSN axonal projections in the GL (figure 2). The reduction in VAMP2 staining in the GL of OSN-T animals compared to WT controls is as would be expected with OSN TeNT expression. In the MT cells of MT-T animals, reduced levels of VAMP2 staining are observed in the GL and EPL compared to WT controls. These data suggest that the TeNT cleavage of VAMP2 is occurring in the appropriate targets (James, 2013).

Immunohistochemical analysis of immediate early gene (IEG) expression was used to test whether blockade of vesicle release was occurring as expected (figure 3). The IEGs c-FOS and Zif268 are both markers of neuronal activity. In the granule cell layer (GCL) of MT-T animals, IEG expression was significantly reduced compared to WT animals, whereas in OSN-T animals, no such reduction was observed. Spontaneous firing of MT cells may account for the normal levels of IEG expression in OSN-T animals, as
increases in uncorrelated MT activity have been reported following naris occlusion (Philpot, et al, 1997). In order to more directly evaluate OSN silencing, immunohistochemical analysis was performed against tyrosine hydroxylase (TH), expressed highly in the periglomerular cells receiving direct input from OSNs. The staining revealed down-regulation of the marker in the GL of both MT-T and OSN-T animals. The correlation between TeNT expression and significant down-regulation of IEGs and/or TH in the surrounding populations of neurons in both MT-T and OSN-T animals, respectively, strongly suggests that physiologically relevant silencing of each cell type in the appropriate animals is occurring (James, 2013).

Beginning around P11, and continuing into adulthood, MT-T animals exhibit an obvious reduction in weight compared to WT littermates. This difference is also noticeable in the size of the olfactory bulbs (figure 4). This stunted growth is in agreement with naris occlusion and OSN cyclic nucleotide gated channel knock-out studies, suggesting that MT neurons play as central a role to sensory processing and bulb development as sensory neurons (Mandairon et al, 2006; Cummings et al, 1997; Zheng, et al, 2000).

Upon closer inspection, the general reduction in whole OB size was quantified by tracing the glomerular, external plexiform and granule cell layers in serial coronal sections, which demonstrated a progressive decrease in the area of the EPL and GCL in both MT-T and OSN-T animals, with a more severe reduction in MT-T animals. Staining for cleaved caspase-3, a marker of apoptotic cell death, revealed that the cause of the layer-specific reductions in MT-T animals was likely due to the significantly elevated levels of cell death in the GCL (figure 6A, B). Interestingly, no such increase in cell
death was observed in OSN-T animals. The decrease observed in the EPL, a cell-poor area primarily composed of MT and GC dendritic projections, can likely be explained the loss of GCs and their processes, at least in MT-T mice. Subsequent staining for NeuN, a marker of mature neurons, yielded a result similar to the cleaved caspase-3 staining (figure 6C, D). A significant reduction in NeuN positive cells was observed only in MT-T animals, whereas NeuN levels in OSN-T animals remained unchanged relative to WT controls.

To investigate the cause of the decreases observed in the GCL among MT-T and OSN-T animals, we developed a lentiviral labeling system in order to analyze GC density and morphology. Injection of lentiviral constructs encoding green fluorescent protein (GFP) into the lateral ventricles at postnatal day 0 (P0) allowed for a close examination of the effect of MT silencing on the morphology of adult born granule cells (GCs) (figure 7). Compared to the bulbs of WT and OSN-T animals, in which GFP positive neurons migrate superficially and project radially into the (EPL), GFP positive cells in MT-T animals fail to project radially, if at all by postnatal day 21. Dendritic projections appear to be shorter on average, with much fewer projections into the EPL. The retention of an immature morphology only in MT-T animals suggests that MT input is required for GC development, whereas sensory input has little impact.

A closer inspection of the bulbs of OSN-T animals revealed an unperturbed morphology compared to WT controls (figure 8). By collecting z-stacks with a confocal microscope, and taking advantage of the tracing program, MBF Neurolucida, we were able to quantitatively analyze individual GCs within tissue sections. Granule cells were selected for tracing based on strict criteria outlined in the Materials and Methods section.
Only the one to three most superficial (and thus, mature) granule cells from each z-stack collected were chosen for tracing. Once traced, a large body of data became available to us. We chose three key parameters to assess morphological complexity: total dendritic length, total number of branches and spine density. Each granule cell that was traced was selected according to strict parameters to ensure an unbiased pool of GCs was generated from each condition.

Utilizing this tracing technique, we were able to quantitatively analyze GC morphology, which revealed no significant difference between WT and OSN-T GCs, according to our pre-determined parameters (figure 9). This is in stark contrast to the morphological deficits that we observed in MT-T GCs (figure 10). In our examination of MT-T GCs, the technique was modified in order to incorporate a fourth parameter, puncta density. In this system, we took advantage of the localization of VAMP2 to synapses, as it is a protein involved in vesicle exocytosis, in order to identify synaptic puncta.

We performed injections of lentiviral constructs encoding a fusion protein of VAMP2 and Venus, a GFP variant, into the lateral ventricles of P0 mice. At ages P15.5 and P21, the animals were sacrificed and serial sections of the OBs were stained with an anti-GFP primary, followed by a secondary containing a fluorophore that emitted at 555 nm (red). Localization of the VAMP2-Venus fusion protein to the puncta of infected GCs caused these structures to emit a strong native GFP signal (no amplification). The amplification of the GFP signal using antibodies allowed us to detect the low levels of GFP throughout the rest of the cell. Merging the red and green channels from each image
using confocal microscopy allowed us to trace labeled GCs as before, but also clearly identify synaptic structures to assess the synaptic maturity of the GCs (figure 11, 12).

Quantitative analysis of the tracings revealed a severe reduction in overall complexity at both timepoints (figure 13). In terms of all four parameters used to assess morphology, MT-T GCs failed to escape the early stages of development, and retained an immature morphology. Dendritic length, number of branches, spine density and puncta density were all significantly reduced in the GCs of MT-T animals compared to WT littermates.

Because of the dramatic effect of sensory deprivation on cortical populations in other sensory systems, we wondered whether the morphological deficits observed in the bulb in our system extended to cortical targets of MT neurons. To assess whether MT silencing had a similar effect in the first sites of MT connectivity in the cortex, we stained for NeuN, a marker of mature neurons, GABA, the primary neurotransmitter released onto MT neurons, and cleaved caspase 3 in the piriform cortex (Figure 13, 14). In neither layer one, nor layer two of the piriform cortex, did we observe a significant difference in GABAergic or NeuN positive neurons, or CC-3 activation between any of the three genotypes. This drastic difference between the two main sites of synaptic output of M/T neurons is likely explained by the abundance of additional afferent inputs onto cortical neurons, as opposed to granule cells, which receive input primarily from MT neurons.

From the data presented thus far, there are a number of possible explanations for the morphological differences between MT-T GCs and their WT or OSN-T counterparts. For one, it could be due to a generally unhealthy environment, and a deficiency in growth factors normally released by MT neurons. Another possibility is that an activity threshold
exists within GCs, which is required for development, or for survival. To address these possibilities, we devised three systems to help identify a mechanism for the observed morphological deficiencies in MT-T GCs.

In the first experiment, we co-injected WT animals in the lateral ventricles with a mixture of lenti-HA-GFP (control) and lenti-KV12.1-GFP constructs, which, along with GFP, encodes an inward-rectifying potassium channel (KV12.1) (figure 15). Overexpression of the channel causes the neuron to be held at a resting potential, and thus reduces its excitability. All brain sections were stained using anti-HA primary and a secondary conjugated with a 555 fluorophore against the anti-HA primary, as well as anti-GFP primary and a secondary conjugated to a 488 fluorophore against the anti-GFP primary. In a merged image of the green and red channels, HA-GFP infected controls appear in both channels, and appear yellow in the merged image, whereas KV12.1-GFP infected GCs only appear in the green channel, and remain green in the merged image.

Compared to HA-GFP infected control GCs, the KV12.1 infected cells exhibited a morphology reminiscent of MT-T GCs, with significant reductions in all three parameters (Figure 16). Considering that this was observed in a WT bulb, we can begin to rule out the possibility the changes observed in MT-T animals were due to a generally sick bulb deficient in MT-released growth factors. These data suggest that a checkpoint exists within the path to GC maturation which requires sufficient depolarization.

In order to further elucidate the mechanism by which the marked effect of MT silencing occurs, we targeted the main receptor of MT input onto granule cells. The NMDA receptor is the primary mode of glutamatergic excitation of GCs (Schoppa, et al, 1999). To test whether glutamatergic input on to GCs is the key to their maturation and
integration into the circuitry of the olfactory bulb, we conditionally knocked out the NR1 subunit of the NMDA receptor in adult-born GCs (figures 17, 18). P0 infection of GC precursors in the lateral ventricles with a mixture of the lenti-Cre-GFP construct and lenti-eGFP construct in mice with a floxed NR1 prevented the expression of the integral subunit of NMDA receptors in cells infected with the Cre-GFP construct. As in the KV12.1 experiment, HA-GFP infected controls appear in both the red and green channels, and Cre-GFP infected cells only appear in the green channel. We observed a strikingly similar phenotype in GFP-only cells to that of MT-T granule cells. Knock-out of the NR1 subunit led to a significant decrease in total dendritic length, number of branches and number of spines compared to HA-GFP co-infected controls (figure 19). These data suggest that granule cell maturation is dependent on top-down glutamatergic signaling from MT cells.

To determine if GCs must meet a depolarization threshold for development or survival, we inhibited apoptotic mechanisms in MT-T GCs. To do so, we injected MT-T animals in the lateral ventricles as before, but this time with a combination of lenti-HA-GFP and either lenti-Bcl2-GFP (figure 20), or lenti-p53-shRNA-GFP (figures 21, 22). Both Bcl2 overexpression and p53-shRNA knock-down should inhibit apoptosis by targeting the apoptotic pathway at two separate sites. p53, a pro-apoptotic transcription factor, becomes activated during various instances of cell stress and induces the transcription of other pro-apoptotic factors. One downstream target is the Bax gene, which acts as a pore in the mitochondrial membrane, allowing cytochrome-c to flow into the cytoplasm and further activate the apoptotic cascade. Bcl2 acts to block the Bax pore,
and thus serves to prevent the apoptotic pathway from being activated through that particular mechanism.

In both cases, GC morphology showed no signs of improvement in terms of the three parameters we examined, compared to lenti-HA-GFP infected controls. In figure 23, GCs remain quantitatively similar to VAMP2-Venus MT-T GCs, and appear unable to escape immaturity. The only significant difference that we observed was spine density between VAMP2-Venus MT-T and both p53 infected MT-T GCs, and HA-GFP infected MT-T GCs. This difference may be an artifact of the different experimental systems used, but a larger number of traced granule cells in these experiments will be required for a more conclusive picture. These data suggest that synaptic input from MT neurons dictates the fate of adult-born granule cells. From these data, it seems that only sufficient activity enables GC maturation and survival, and takes precedence over even apoptotic signaling.
Discussion

Once a neuron has arrived in the general neighborhood of its integration, activity-dependent mechanisms take over as the primary determinant of connectivity (Goodman and Chatz, 1993). The plasticity and fine tuning that enable memory formation and the impressive adaptability of our nervous systems is dictated by the varying levels of activity passing through the connections of those networks. Because of the well-defined and easily manipulated input, sensory systems make for an ideal template to examine the organizational and adaptive role activity plays in neural networks. It makes sense then, to not just examine the beginning and endpoints of neurotransmission, but also each of the individual steps between. Though the role of sensory neurons in establishing and maintaining sensory networks has been examined extensively, little is known of what interneurons contribute to these systems, particularly in the olfactory bulb. For the first time, we have demonstrated a very integral role for second-order mitral and tufted neurons in the organization and maintenance of the olfactory bulb.

The marked collapse of the bulb following MT neuron silencing due to extensive disorganization and cell death in the granule cell layer, which was not observed in OSN-T animals, suggests a significant contribution of spontaneous activity in the maintenance of neural networks. The morphology of the MT-T bulb clearly demonstrates the dependence of granule cells on synaptic input from MT neurons. That a much less severe phenotype is observed when MT neurons are receiving little to no synaptic input in OSN-T animals suggests that spontaneous activity alone is at least enough to support normal
integration and connectivity within the bulb. Of course, these conclusions depend entirely on the assumption that our experimental system is thoroughly blocking vesicular release from OSNs and M/T neurons. Though immunohistochemistry showed reductions in VAMP2 in both cases, electrophysiology should be conducted to strengthen the case for silencing (James, 2013). The severity of the MT-T phenotype suggests that, at least in this case, silencing is occurring. Additionally, the less severe shrinkage of the bulb observed in OSN-T animals is in agreement with other studies in which OSNs are genetically prevented from firing (Zheng, et al, 2000; Marks, et al, 2006). Together, these provide at least some assurance that our system is functioning properly.

We next asked whether MT silencing has a similar effect on cortical targets as it does on granule cells in the OB. The results from these cortical experiments were not expected, but upon second thought, should have been. Since cortical neurons receive synaptic input from so many sources, it makes sense that MT silencing should have no noticeable affect on them. They likely receive sufficient excitation from neighboring populations to remain at normal densities under both of our experimental conditions.

We then wanted to investigate the causes of GC morphological deficiencies and increased cell death. We postulated that three different models could account for our observations. First, we had simply created an unhealthy local environment, in which there was a deficiency of the growth factors normally released by MT cells. Another model was that an activity threshold exists within the granule cells that must be met for their development. Finally, this threshold may instead be necessary for their survival.
To test whether the inability of granule cells to mature was due to a unhealthy environment, or the existence of a depolarization threshold, we blocked excitation in WT cells. The effect of overexpressing the KV12.1 inward rectifying channel, which holds the membrane potential of infected cells in a hyperpolarized state, mirrored what we observed in MT-T animals. This allowed us to rule out the “sick bulb” model.

In our experiments to recapitulate the effect of MT silencing by targeting the granule cells, our hypothesis that a lack of synaptic input onto GCs was the cause of their degeneration appeared to be confirmed. These experiments provided a mechanism for the aberrant morphology, as targeting the primary receptor for MT signaling onto GCs generated a very similar morphology to MT silencing. Together with the results from the KV12.1 experiments, we can conclude with reasonable confidence that maturation and integration of incoming granule cells is mediated primarily by MT glutamatergic input.

Finally, we asked what happens when we block cell death among MT-T granule cells. If this enabled the GCs to develop and integrate normally, we could conclude that a lack of MT activity drives cell death. If they failed to mature and integrate into the OB circuitry, which is what we observed, this would suggest that only sufficient activity enables proper granule cell development.

This data appear to support the notion that a checkpoint exists along the path of maturation within granule cells. It seems that the inability to meet a depolarization threshold prevents GCs from passing into the later stages of development, and eventually becoming fully integrated into the preexisting circuitry. Failure to meet these basic activity-related requirements eventually leads to GC death, which can’t be averted even through inhibition of apoptotic mechanisms. In the case of OSN-T GCs, only blocking
sensory activity preserves sufficiently high levels of input to enable proper GC
development, though elevated levels of cell death are still observed. This may reflect a
mechanism for adult born GCs to actively seek out the most active MT neurons, and thus
maintain a state of equilibrium.
Figure 1. Genetic schema for tetanus toxin expression in mitral and tufted cells, or olfactory sensory neurons, and validation.

Crossing OMP-IRES-CRE (Cre expressed only in OSNs) or Pcdh21-IRES-CRE (Cre expressed only in MT neurons) mice to mice containing the Rosa-lox-stop-lox-TeNT-GFP transgene produces offspring expressing the tetanus-GFP fusion protein in either OSNs (OSN-T) or MT neurons (MT-T), respectively (A). TeNT-GFP expression in the olfactory epithelium (OE), olfactory bulb (OB), or piriform cortex (PC) of WT animals, OSN-T animals and MT-T animals (B). Blue staining corresponds to DAPI nuclear stain, and green staining corresponds to TeNT-GFP fusion protein. Scale bars correspond to 50 µm in the OE, and 100 µm in the OB and PC.
Figure 2. Reduced VAMP2 staining in GL of OSN-T animals, and EPL of MT-T animals.

OSNs project to the GL and synapse with MT cells in the glomeruli. MT dendritic processes project into the EPL, which shows high VAMP expression in WT and OSN animals. VGlut2, highly expressed in OSNs, labels GL and remains largely unperturbed in all three animals. Scale bar equals 100 µm.
Figure 3. Immediate early gene expression is reduced in MT-T animals, periglomerular cell activity is reduced in OSN-T and MT-T animals.

Expression of immediate early genes (IEGs) cFos and Zif268 is significantly reduced in the GCL of MT-T animals, unperturbed in OSN-T animals. Tyrosine hydroxylase (TH) expression is reduced in both OSN-T and MT-T animals. 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. 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 equals 100 µm. Error bars indicate 95% confidence intervals. *p<.05, ***p<.001; ****p<.0001.
Figure 4. Olfactory bulbs are smaller, body weight is reduced in MT-T littermates.

The brains from P21 littermates are very similar in terms of total length and width, while olfactory bulb size is reduced in MT-T animals. Bodyweight: 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. Error bars indicate 95% confidence intervals. P11, p<.0001. P21, p<.0001. ****p<.0001.
Figure 5. Reduction in OB size appears to be due to reduced EPL and GCL.

Images correspond to the largest coronal OB sections from serial sections of animals 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 bar equals 500 µm. A-D WT=white circles; OSN-T=blue circles; MT-T=red circles. By 11 days, whole bulb volume is significantly smaller in MT-T animals. By day 125-137, whole bulb volume is reduced in ONS-T and more so in MT-T animals (A). GL volume is reduced in ONS-T animals, and unperturbed in MT-T animals (B). EPL and GCL volumes are significantly reduced in OSN-T animals, and more so in MT-T animals (C, D). Error bars indicate 95% confidence intervals.
Figure 6. Increased cell death and decreased density of mature neurons in MT-T GCL.

Images of coronal OB sections stained for cleaved caspase-3 (green), a marker of cell death (A). Quantitative analysis reveals a significant increase in CC-3 positive cells in the GCL of MT-T animals (B). \( n=9 \) (WT), 5(OSN-T) and 5(MT-T), 3-5 sections per animal. WT is \( 7.2 \pm 1.7 \), OSN-T is \( 26.8 \pm 16.4 \), MT-T is \( 52.0 \pm 14 \). 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 equals 200 \( \mu m \). Error bars indicate 95% confidence intervals. *\( p<.05 \); **\( p<.01 \); ****\( p<.0001 \). NeuN is a marker of mature neurons (C, D). Much fewer mature GCs in MT-T animals, while OSN-T animals retain normal densities of NeuN positive 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). WT is \( 5473 \pm 777 \), OSN-T is \( 5978 \pm 950 \), MT-T is \( 1621 \pm 503 \). 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, \( p<.0001 \).
Figure 7. P21 GC Morphology following lenti-eGFP labeling via P0 injection into lateral ventricles.

Whole bulb and granule cell morphology of P21 WT, MT-T and OSN-T animals. Top set is 10X, bottom set is 40X of coronal sections taken from the middle of each bulb. eGFP signal intensified by IHC. OSN-T GCs project radially, like WT GCs. MT-T GCs appear immature with sparse processes if any; many fail to project radially. Scale bars equal 250 µm.
Figure 8. OSN-T GCs appear to develop and integrate normally.

Lentiviral labeling (lenti-eGFP) of GCs in OSN-T and WT littermates reveals normal morphology of OSN-T GCs. Scale bar equals 50 µm.
OSN-T granule cells are not significantly different from WT control in any of the three parameters used to quantify GC tracings. Dendr Length refers to the total dendritic length (A), branches refers to total number of branches per neuron (B), and spines/µm refers to the spine density (C). Scale bar equals 50 µm (D). Asterisks mark GCs shown in figure 8. n=6(WT) and 7(OSN-T). Total dendritic lenth: WT=443.4 ± 52.47; OSN-T= 511.9 ± 76.02; p>0.05. Total number of branches: WT= 14.33 ± 1.585; OSN-T= 14.57 ± 2.419; p>0.05. Spine density: WT= 0.2250 ± 0.01448; OSN-T= 0.1645 ± 0.02338; p>0.05.
Figure 10. VAMP2-Venus labeling reveals morphological deficits in MT-T GCs.

Maximum intensity projections of GCs from WT (A) and MT-T (B) animals. White boxes in (A) and (C) are magnified in (B) and (D), respectively. Animals were injected P0 and sacrificed P21. Coronal sections were stained with anti-GFP primary raised in rabbit, and anti-rabbit 555 (red) secondary. Green is native GFP. Merged images reveal yellow puncta. Scale bars are equal to 50 µm.
Figure 11. VAMP-Venus GC tracings reveal reduced branching complexity among MT-T GCs.

GCs are arranged in terms of increasing total dendritic length. Spines and puncta not shown. MBF Neurolucida was used to trace the apical dendritic projections of GCs imaged using a confocal microscope. GCs eligible for tracing must have been entirely contained within the z-stacks collected. The one, two or three GCs whose soma were most superficial (i.e. closest to mitral cell layer) among those which met all other criteria in each z-stack were selected for tracing. GCs marked by an asterisk indicate the GCs shown in Figure 10. Scale bar is equivalent to 50 µm.
Figure 12. Quantifications of VAMP-Venus tracings

Tracings from Figure 11 were quantified using Neurolucida Explorer. White bars correspond to WT GCs, red bars correspond to MT-T GCs. “Dendr Length” refers to the total dendritic length, “Branches” refers to total number of branches per neuron, “Spines/µm” refers to the spine density, and “VAMP2 puncta/µm” refers to puncta density. P15.5: n=14(WT), 37(MT-T); P21: 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<.001. 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 13. Cortical neurons are present in normal densities in MT-T and OSN-T animals.

One of the main targets of MT cells in the cortex is the piriform cortex. Coronal sections reveal normal densities of GABAergic neurons (green) and mature NeuN positive neurons (red) in the piriform cortex of MT-T and OSN-T animals. Layer 1 is the top area traced by the white line in the top left image, and layer 2 corresponds to area within the bottom trace. Scale bar equals 250 µm.
NeuN and GABA positive neurons are present in normal numbers in the piriform cortex (PC). NeuN graph corresponds to entire PC (layers 1 and 2). 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, not significant (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. *p<.05. Cleaved caspase-3 a marker of cell death, is not increased in MT-T or OSN-T animals. CC-3 PC layers 1 and 2: 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.
Figure 15. Blocking excitation in WT GCs induces morphology similar to MT-T GCs.

WT P0 pups were co-injected with a mixture of lenti-KV12.1-GFP, which contains the murine KV12.1 inward-rectifying potassium channel gene, and lenti-HA-GFP. Coronal serial sections were stained with anti-GFP primary/488 secondary (green) and anti-HA primary/555 secondary (red) antibodies. Lenti-HA-GFP infected controls stained green and red (A) and (B), whereas lenti-KV12.1-GFP infected GCs stained green only (C) and (D). Both (A) and (C) are merged (green and red channels). Scale bar is 50 µm.
Lenti-KV12.1-GFP infected GCs show morphological deficits reminiscent of MT-T GCs. Compared to their lenti-HA-GFP (WT) infected counterparts in the same bulbs, lenti-KV12.1 infected GCs present reduced length and complexity. N=11 (lenti-HA-GFP), 15 (lenti-Kv12.1-SCPGFP). Total dendritic length: HA-GFP is 606 ± 411, Kv12.1 is 123 ± 68. Unpaired t-test, p<.0001. Number of branches: HA-GFP is 11.0 ± 2.5, Kv12.1 is 1.3 ± .7. Unpaired t-test, p<.0001. Spine density: HA-GFP: .102 ± .016, Kv12.1 .027 ± .021. Unpaired t-test, p<.0001. Error bars indicate 95% confidence intervals. ****p<.0001. Asterisks mark GCs shown in the previous figure (16). Scale bars equal 50 µm.
Figure 17. Schema for NR1 conditional knockout.

GCs infected with lenti-Cre-GFP will no longer express the NR1 subunit of the NMDA receptor.
Figure 18. Blocking input through NMDA receptors prevents maturation and development in GCs.

P0 pups containing a floxed NR1 subunit were co-injected with a mixture of lenti-Cre-GFP and lenti-HA-GFP. Coronal serial sections were stained with anti-GFP primary/488 secondary (green), and anti-HA primary/555 secondary (red). Lenti-HA-GFP-infected control GCs stained green (A) and red (B), whereas lenti-Cre-GFP infected GCs stained green only (C) and (D). Arrows in (A) and (B) identify the same GC, as do the arrows in (C) and (D). Both (A) and (C) are merged (green and red channels). Scale bars equal 50 µm.
NR1-KO GCs in P21 animals exhibit morphological deficits compared to lenti-HA-GFP (WT) infected controls. 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. Asterisks mark GCs presented in figure 18. Scale bar equals 50 µm.

Figure 19. NR1 knockout tracings and quantifications.
Figure 20. Lenti-Bcl2-GFP Images and Quantifications

P0 MT-T pups were co-injected with a mixture of lenti-Bcl2-GFP and lenti-HA-GFP, and sacrificed at P21. In lenti-Bcl2-GFP, Bcl2 and GFP are driven by separate promoters. The bottom-left GC in (A) is infected with lenti-Bcl2-GFP, indicated by its absence in the red channel. The top-right GC is an example of a lenti-HA-GFP control. The tracings of both neurons are marked by asterisks in (B). Scale bars equal 50 µm. In (C), n=9(lenti-HA-GFP) and 6(lenti-Bcl2-GFP) GCs. Total dendritic length: lenti-HA-GFP is 233.6 ± 34.72, lenti-Bcl2-GFP is 136.3 ± 27.26; unpaired t-test, p>0.05 (NS). Total number of branches: lenti-HA-GFP is 8.111 ± 1.775, lenti-Bcl2-GFP is 4.000 ± 1.125; unpaired t-test, p>0.05 (NS). Spine density: lenti-HA-GFP is 0.09501 ± 0.02316, lenti-Bcl2-GFP is 0.05914 ± 0.01064; unpaired t-test, p>0.05 (NS). Error bars indicate 95% confidence intervals.
Figure 21. p53 Knock-Down images.

P0 MT-T pups were co-injected with a mixture of lenti-p53shRNA-GFP and lenti-HA-GFP, and sacrificed at P21. In lenti-p53shRNA-GFP, p53shRNA and GFP expression are driven by separate promoters (H1 and UbC, respectively). GC indicated by arrow in HA-GFP column stains green (GFP) and red (HA). GC indicated by arrow in p53 KD column stains green (GFP) only. Scale bar equals 50 µm.
Figure 22. p53 knock-down tracings and quantification.

N=4(lenti-HA-GFP) and 13(lenti-p53shRNA-GFP) GCs. Total dendritic length: lenti-HA-GFP is 81.63 ± 17.45, lenti-p53shRNA-GFP is 123.5 ± 16.32; unpaired t-test, p>0.05 (NS). Total number of branches: lenti-HA-GFP is 2.000 ± 1.000, lenti-p53shRNA-GFP is 5.615 ± 1.474; unpaired t-test, p>0.05 (NS). Spine density: lenti-HA-GFP is 0.08687 ± 0.05549, lenti-p53shRNA-GFP is 0.1280 ± 0.01471; unpaired t-test, p>0.05 (NS). Asterisks in (B) mark GCs presented in figure 21. Error bars indicate 95% confidence intervals. Scale bar equals 50 µm.
Figure 23. Compilation of quantifications from traced MT-T GCs infected with lenti-HA-GFP, lenti-p53shRNA-GFP, lenti-Bcl2-GFP or lenti-VAMP2-VENUS.

HA-GFP is pooled from p53-KD and Bcl2 experiments (n=13). It is important to note that these quantifications are generated from different assays, and are intended to be used only as a reference. One-way ANOVA was followed by Tukey’s multiple comparisons test. Error bars indicate 95% confidence intervals. The only significant differences are found in spine densities: MT-T VAMP-Venus vs. HA-GFP (p<0.001), and MT-T VAMP-Venus vs. p53 KD (p<0.0001).
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References


