COMPETITIVE CELL-SPECIFIC INTEGRATION AND SURVIVAL OF ADULT-BORN NEURONS IN THE MOUSE OLFACTORY BULB

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In several areas of the adult brain, notably the olfactory bulb, new neurons are generated and integrated into existing mature circuits. This observation raises questions about how an ongoing developmental process shapes adult brain circuitry and opens potential treatment avenues for neurological disorders such as traumatic brain injury, ischemia, depression, and degenerative diseases. In the networks where adult neurogenesis occurs, the integration of adult-born neurons is modulated by activity. The experiments in this dissertation are designed to address the hypothesis that adult-born neurons integrate into mature circuits in a competitive, activity-dependent manner.

Specifically, we sought to address the question of whether activity in adult-born neurons directly causes enhanced survival of particular active newborn neurons in a cell-autonomous fashion, or whether survival is a competitive process in which the most active cells in the network have the highest chance of survival. To differentiate between these two hypotheses required manipulating activity both of the adult-born neurons and other neurons within the network. Here we used both virus-mediated reduction of cell intrinsic activity and reduced sensory stimuli to show that survival is a competitive process in which the most active cells in

the network have the highest chance of survival. RNA-based knockdown of Na^+ channels resulted in decreased survival of adult-born neurons from 10-21 d.p.i. We then investigated the role of circuit level activity by inducing sensory deprivation through naris occlusion. Adult-born neurons with normal cell-intrinsic activity showed a reduction in survival in a sensory deprived circuit. But, interestingly, adult-born neurons with reduced intrinsic excitability showed an increased rate of survival in a sensory deprived circuit. These results provide important insights into the role of activity in the integration and therapeutic potential of neural stem cells in mature functional brain circuits.

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PREFACE

Several panels in the introduction are taken from preliminary descriptive work done in our lab on adult-born neurons prior to addressing their integration and survival. They are modified from:

Bagley, J, G LaRocca, D A Jimenez, and N N Urban. "Adult neurogenesis and specific replacement of interneuron subtypes in the mouse main olfactory bulb." ***BMC Neurosci*** **8 (2007): 92-92.**

The work presented within the two research chapters of this dissertation are based on two manuscripts that are in preparation or in the peer review process. These are as follows:

Chapter 2 is a modified version of:

Jimenez, D.A., Oswald, A.M., Interval, E., Osten, P., Urban N.N. Competitive cell-specific survival and integration of adult-born neurons in the olfactory bulb. In Preparation.

Chapter 3 is a modified version of:

Dahlen, J.D., Jimenez, D.A., Gerkin R.C., Urban N.N. Morphological analysis of activity-reduced adult-born neurons in the mouse olfactory bulb. In Review. Frontiers in Neuroscience.

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In graduate school I had the chance to work together with a host of other researchers. They each contributed to the research included in this dissertation and made the lab an inspiring place to work. My thanks to Josh Bagley, Erik Interval, Jeff Dahlen, Anne Marie-Oswald, Greg LaRocca, and Rick Gerkin. Without their help and camaraderie this work would not have been possible.

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LIST OF ABBREVIATIONS

ABN – Adult-born neurons

AP – Action Potential

CB – Calbindin

CR – Calretinin

CRMP-4 – collapsing response mediator protein-4

DAB - 3,3'-Diaminobenzidine

DCX - doublecortin

EGFP – Enhanced green fluorescent protein

EPL – External Plexiform Layer

EsKir2.1 – inward rectifying potassium channel 2.1

FUGW – Flap-Ubiquitin Promoter-EGFP-WRE lentivirus vector

FURW - Flap-Ubiquitin Promoter-tdTomato-WRE lentivirus vector

FU6siGURW - FLAP-Ubiquitin 6 promoter- $Nav_{1.1\text{-}1.3}$ siRNA tdTomato-WRE lentivirus vector

$GABA_A$ – Gamma-aminobutyric acid receptor A

GC – Granule cell (of the olfactory bulb)

GCL – Granule cell layer (of the olfactory bulb)

GFP – Enhanced green fluorescent protein

GLL – Glomerular layer

IP - Intraperitoneal

FLAP – 5-lipoxygenase activating protein

KD – Knock-down

KO – Knock-out

lenti-GFP – adult-born neurons expressing FUGW

lenti-tdTomato – adult-born neurons expressing FURW

lenti-shRNA – adult-born neurons expressing FU6isGURW

MAPK – Mitogen activated protein kinase

MC – Mitral Cell

MCL – Mitral Cell Layer

M/T – Mitral or Tufted Cell

NA – Numerical aperture

$NaChBac^{+}$ - Bacterial voltage-gated sodium channel

NA_v – Voltage gated sodium channel

NC – n-copine

NDS – Normal donkey serum

NMDA – N-methyl-D-aspartate

NMDAr – NMDA receptor

OB – Olfactory bulb

ORN – Olfactory receptor neuron

PB – Potassium buffer

PCNA – Poliferating cell nuclear antigen

PG – Periglomerular cell

RISC – RNA-induced silencing complex

RMS – Rostral migratory stream

ROBO – The roundabout receptor protein

RNA – Ribonucleic acid

shRNA – Short hairpin ribonucleic acid

siRNA - Small interfering ribonucleic acid

SLIT – A family of secreted proteins

SVZ – Subventricular zone

WRE – Wnt-responsive element

1.0 INTRODUCTION

In several areas of the adult brain, notably the olfactory bulb and dentate gyrus of the hippocampus, new neurons are generated and integrated into existing mature networks. This observation raises questions about how the ongoing process of adult neurogenesis shapes adult brain circuitry and opens potential treatment avenues for neurological disorders such as traumatic brain injury (Dash, Mach and Moore 2001, Lowenstein, et al. 1992), ischemia (Liu, et al. 1998, Lichtenwalner and Parent 2006, Greenberg 2007), depression (Santarelli, et al. 2003, Airan, et al. 2007), and degenerative diseases (Sugaya, et al. 2007, Arias-Carrian, et al. 2007, Taupin 2006).

In the networks in which ongoing mammalian adult neurogenesis occurs, the integration of adult-born neurons is modulated by activity (Kempermann 2008, reviewed in Ma, et al. 2009). The experiments in this dissertation were designed to address the hypothesis that adult-born neurons integrate into mature circuits in a competitive activity-dependent manner. Testing this hypothesis will provide important insights into the role of neural activity in the integration of neural stem cells into mature functional brain circuits, as well as their therapeutic potential.

1.1 ADULT NEUROGENESIS

1.1.1 History

> *... the functional specialization of the brain imposes on the neurons two great lacunae; proliferation inability and irreversibility of intraprotoplasmic differentiation. It is for this reason that once the development was ended, the founts of growth and regeneration of axons and dendrites dried up irrevocably. In adult centers the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.*
>
> *-Santiago Ramon y Cajal, 1913*

Throughout the 20th century one of the most persistent tenets of neuroscience was that neurogenesis, the process of creating new neurons, ceased soon after birth in mammals; that the neurons you are born with are the only neurons in your brain as an adult (Ramon y Cajal 1913). This dogma stood unchallenged for over half a century before the first hints emerged of the existence of adult neurogenesis. Beginning in the early 1960s, radioactive labels were used to trace cell lineages and to identify times of cell division. One of these labels was radioactive [H^3]-thymidine which is taken up into dividing cells and incorporated as one of the base pairs of replicating DNA. Joseph Altman began a series of studies showing evidence for neuronal cell division in the hippocampus (Altman and Das 1965) and olfactory bulb (Altman 1969). Due

largely to the popularity of the "no new neurons" dogma these findings were considered a curiosity for the next 30 years.

In the 1980s, researchers began deciphering adult neurogenesis in song birds (reviewed in Nottebohm 2004). Songbirds such as canaries have seasonal bursts of brain growth that coincide with periods of vocal learning. This growth was originally thought to represent solely synaptic growth, but was later shown to represent a large proliferation of adult-born neurons (Nottenbohm 1981, Goldman and Nottebohm 1983). Adult-born neurons in songbirds proliferate in the ventricular zone of the lateral ventricles (Goldman and Nottebohm 1983, Alvarez-Buylla and Nottebohm 1988), after which they migrate to the higher vocal center (Alvarez-Buylla and Nottebohm 1988). Once in the higher vocal center, these ABNs make long distance axonal projections necessary for song production (Nottenbohm et al. 1976). Seasonal periods of adult neurogenesis coincide with these periods of vocal plasticity and the learning of new bird "song" (Alvarez-Bullya et al. 1990, Kirn et al. 1994). This work in song birds prepared the framework and techniques for exploring adult neurogenesis in mammals.

Early work in the 1990s on adult neurogenesis was assisted by the development of two techniques which allowed for clear and straightforward labeling of dividing cells in the brain: 1) bromodeoxyuridine (BrdU), a synthetic thymidine analogue that incorporates into replicating DNA and is used in straight-forward immunohistochemistry staining (Gratzner 1982), and 2) the development of retrovirus' labeling used in lineage tracing (Price, Turner and Cepko 1987, Sanes, Rubenstein and Nicolas 1986). Most retroviruses only infect dividing cells and in neuronal populations in adults they are selective for ABNs. Both of these techniques had the advantage of allowing for double labeling immunoflouresence, which allowed researchers to confirm the neuronal identity of newborn neurons and to test their phenotype for a variety of

immunohistochemical markers such as proteins (Bagley, et al. 2007). However, these two techniques also brought their own difficulties and controversies when missused. (For a discussion in relation to adult neurogenesis see Nowakowski and Hayes 2000, Rakic 2002). Armed with these techiniques and the knowledge gathered in song birds, scientists began exploring adult neurogenesis in mammals.

1.1.2 Mammalian adult neurogenesis

Early work in mammals built on Joseph Altman's body of research using tritiated thymidine to describe the number of ABNs that proliferated and survived in the adult brain (Bayer 1983, Kaplan, McNelly and Hinds 1985, Kaplan and Hinds 1977). This work confirmed Altman's initial contention: that adult neurogenesis continues to occur in the normally functioning central nervous system of adult mammals throughout their lifespan. In mammals, ABNs originate in the sub-granular zone (SGZ) of the dentate gyrus region of the hippocampus and the subventricular zone (SVZ) of the lateral ventrical (see Figure 1, Altman and Das 1965, Altman 1969). The SGZ gives rise to excitatory granule cells, which migrate to the granular zone of the dentate gyrus and extend projections to area CA3 (Kempermann, Song and Gage 2007). The SVZ gives rise to several subtypes of inhibitory interneurons which migrate to the olfactory bulb (OB) and form connections that modulate the odor processing of the principal cells. These cells then communicate odor related information to the CNS (Lim, Huang and Alvarez-Bullya 1997).

Adult-born neurons integrate functionally into the OB. ABNs in the OB display mature morphology (Chapter 3, Kelsch, et al. 2009), phenotype (Whitman and Greer 2007, Bagley, et al. 2007), and physiology (Carlén, et al. 2002, Carleton, et al. 2003, Belluzzi, et al. 2003). In addition ABNs express immediate early indicators of activity, proteins that are expressed in

response to strong levels of neuronal activity such as seizures, learning and memory, and sensory stimuli (Morgan and Curran 1989, Gass, et al. 1992, Brennan, Hancock and Keverne 1992) (Wisden, et al. 1990). Adult born neurons in the olfacatory bulb show expression of immediate early genes in response to sensory stimuli suggesting their functional integration into the olfactory sensory processing circuitry (see Figure 23, Magavi et al. 2005, Miwa and Storm 2005, Alonso et al. 2006).

Adult neurogenesis has also been observed in the human brain. In cancer patients wherein BrdU is used as a quantitative diagnostic measure of the spread of cancer, the BrdU is incorporated into the hippocampus by proliferating ABNs (Eriksson, et al. 1998). Additionally, research shows that ABNs are produced in the human SVZ (Doetsch, García-Verdugo and Alvarez-Buylla 1997, Curtis, et al. 2003, Quiñones-Hinojosa, et al. 2006) and migrate along a human rostral migratory stream (RMS) (Curtis, Kam, et al. 2007). But in contrast, researchers have taken advantage of the time dependence of radioactive ^{14}C in the environment due to nuclear testing in the 1950s and 1960s to test the age of cells in the brain (Spalding, et al. 2005). The level of environmental ^{14}C is well defined, and its concentration in cortical brain regions leads to the conclusion that adult-neurogenesis does not occur in the human cortex (Bhardwaj, et al. 2006)

Although not discussed at length in this dissertation humans and other mammals also have additional areas where adult neurogenesis occurs in disease states such as strokes, seizures, or Huntington disease (reviewed in Parent 2003). Seizures have the effect of increasing cell proliferation in the hippocampus and SVZ (Parent et al. 1997, Parent et al. 2002). Within the hippocampus, these new seizure-induced ABNs have accelerated incorporation rates relative to normal ABNs (Overstreet-Wadiche et al. 2006). Following induced stroke in animal models

neurogenesis occurs in the striatum (Arvidsson et al. 2002, Parent et al. 2002) and in the cortex under certain conditions (Jin et al. 2003, Leker et al. 2007). Patients with Huntington disease possess a greater number of cells expressing PCNA, a marker of cell-proliferation in the SVZ that is additionally correlated with disease stage (Curtis et al. 2003, Curtis et al. 2005). On a more hopeful note the existence of adult neurogenesis in human disease states indicates that the human brain may have an endogenous ability to repair itself.

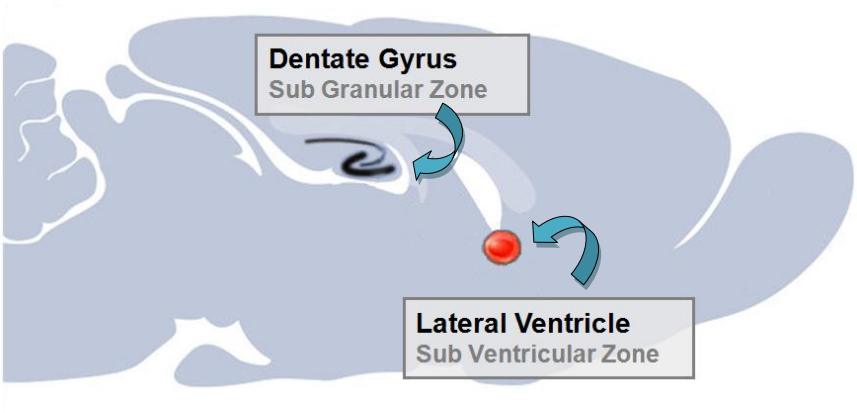

Figure 1. Adult neurogenesis in the mouse.

Neural stem cells exist and produce adult-born neurons in two neurogenic niches in mammals: The subgranular zone of the hippocampal dentate gyrus and the SVZ region lining the lateral ventricle. Adapted from (Taupin and Gage 2002).

1.1.3 Adult neurogenesis in the olfactory bulb

The mammalian OB receives a steady stream of ABNs throughout life (Kaplan, McNelly and Hinds 1985). These neurons proliferate from the subventricular zone (SVZ) of the lateral ventricle and migrate several millimeters along the rostral migratory stream (RMS) until they reach the OB, a process that takes 4-14 days (Lois and Alvarez-Buylla 1993, Lois and Alvarez-Buylla 1994, Doetsch and Alvarez-Buylla 1996). Upon reaching the OB, ABNs differentiate into

two classes of GABAergic interneurons. The vast majority of these interneurons, 90-97%, become axonless granule cells (GC) in the granule cell layer (GCL), where they form dendrodendritic synapses with the principal cells of the OB, mitral and tufted cells (see section 1.2.5, Petreanu and Alvarez-Buylla 2002). The remaining ABNs become periglomerular cells (PG) in the glomerular layer (GLL), where they form synapses with mitral cell dendrites and olfactory receptor neuron axons. After entering the correct layer of the OB, ABNs extend dendrites, form dendritic spines, and begin to receive functional input (See section 1.2.4, Belluzzi, et al. 2003, Carleton, et al. 2003).

These cells have highly dynamic dendritic morphologies and synapses when compared to the overall population or to mature cells of the same type (Mizrahi, et al. 2006, Mizrahi 2007). ABN synaptic connections represent functional integration into the network as ABNs express activity-dependent genes in response to odor exposure (Chapter 2.4.3, Magavi, et al. 2005). They also take on morphological and electrophysiological characteristics of mature neurons over several months (Carlén, et al. 2002, Belluzzi, et al. 2003, Carleton, et al. 2003) and express a mature pattern of calcium binding proteins (Bagley et al. 1997). In addition, ABNs exhibit a uniquely high capacity for plastic changes, such as high levels of synaptogenesis and response to novel odors (Mizrahi 2007, Livneh, et al. 2009, Magavi, et al. 2005). These properties have led to hypotheses concerning the role of ABNs in some types of olfactory learning (Lledo and Saghatelyan 2005).

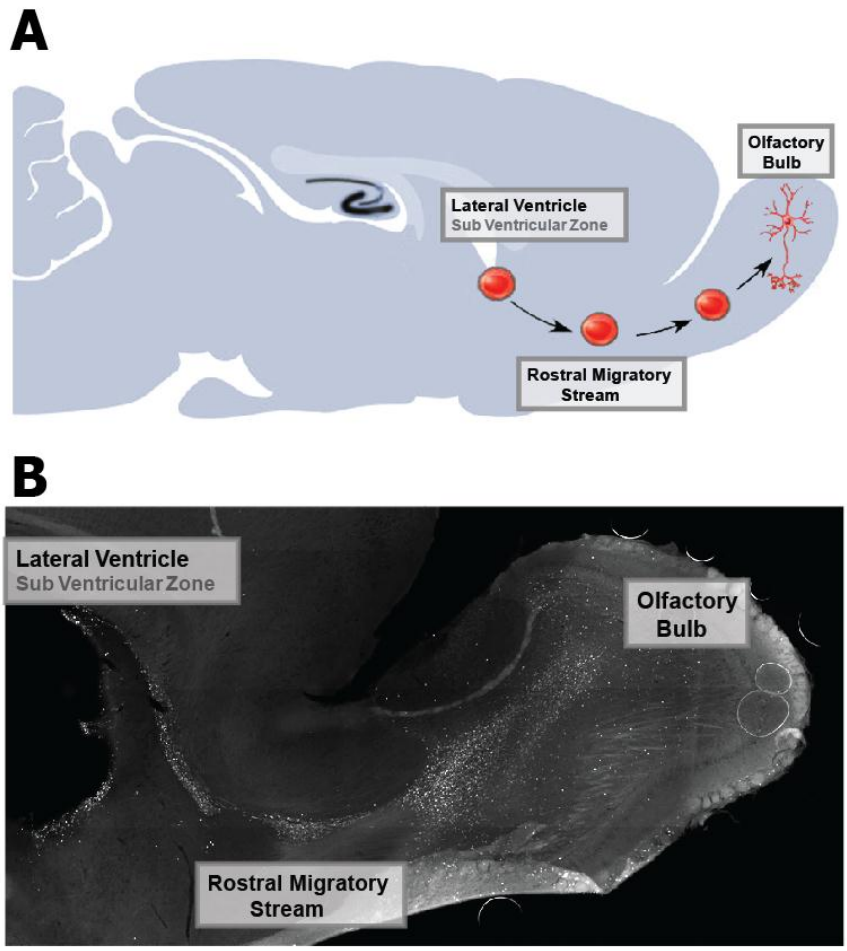

Figure 2. Adult neurogenesis in the adult olfactory bulb.

(A). Neural stem cells in the SVZ produce neuronal precursors which migrate along the rostral migratory stream to the olfactory bulb. Once in the OB these cells migrate radially and differentiate into mature neurons. (**B)**. Sagittal section of the adult mouse brain containing the lateral ventricle, rostral migratory stream, and olfactory bulb. White spots are BRDU positive ABNs which underwent cell division 7 days prior to when the animal being sacrificed. These trace the pathway for ABNs along the SVZ, RMS, and OB. **A** is adapted from (Taupin and Gage 2002).

To summarize the process of adult neurogenesis in the olfactory bulb as it appears in the proceeding sections: The SVZ gives rise to migrating neuroblasts (see section 1.1.3.1), which migrate several millimeters along the RMS (see section 1.1.3.2), then proceed to migrate radially to two distinct layers of the OB (see section 1.1.3.3). ABNs in the OB differentiate into identifiable subtypes which can be determined through their protein expression and

morphological phenotype (see section 1.1.3.4). The majority of OB ABNs then die over the first few weeks of their life; a process of survival that is regulated by several factors (see section 1.1.3.5).

1.1.3.1 Subventricular zone proliferation

The subventricular zone (SVZ) is a neurogenic niche located along the wall of the lateral ventricle in adult mammals. The cells in this niche have been classified into three main cell types: A, B, C (Doetsch, García-Verdugo and Alvarez-Buylla 1997). Type B cells form the neurogenic niche in the SVZ ensheathing the neural progenitors and neural precursor cells and promoting neurogenesis. Type B cells display morphological characteristics of astrocytes and express astrocyte-specific proteins such as glial fibrillary acidic protein (GFAP) (Lois, Garcia-Verdugo and Alvarez-Buylla 1996, Doetsch, García-Verdugo and Alvarez-Buylla 1997). Type C cells, known as transiently amplifying cells, are small and are typically located in clusters of rapidly dividing immature cells (Doetsch, García-Verdugo and Alvarez-Buylla 1997). They give rise to migratory neuroblasts known as Type A cells, which are born along the whole of the lateral ventricle (Doetsch, García-Verdugo and Alvarez-Buylla 1997). Type-A cells born in the SVZ eventually proceed to migrate in through the RMS to the OB (Lois, Garcia-Verdugo and Alvarez-Buylla 1996, Wichterle, Garcia-Verdugo and Alvarez-Buylla 1997). There exists a sequence where Type B cells divide slowly into Type C cells, which then rapidly divide into Type A cells. Endothelial cells in the SVZ play an important role in promoting adult neurogenesis and providing soluble factors that are an important part of the neurogenic niche (Leventhal, et al. 1999, Shen, et al. 2004, Walton, et al. 2006).

1.1.3.2 RMS Migration

Once ABNs enter the RMS they proceed to migrate in chains to the OB (see Figure 3, Lois, Garcia-Verdugo and Alvarez-Buylla 1996, Wichterle, Garcia-Verdugo and Alvarez-Buylla 1997). This distance can stretch several millimeters in the mouse and take on the order of 4-14 days (see Figure 2). Type A cells, migratory neuroblasts produced in the SVZ, are the major cell type that undergoes this migration. The process is regulated by the expression of the microtubule-associated protein DCX and the axon guidance protein CRMP-4 (Francis et al. 1999, Nacher et al. 2000). GABA and calcium signaling modulate the speed of migration (Bolteus and Bordey 2004). Secreted SLIT proteins and ROBO receptors are important for directing the migrating neuroblasts anteriorly towards the OB. A SLIT gradient from the posterior plays a chemorepulsive role (Hu 1999, Wu, et al. 1999) while their associated ROBO receptors are expressed in the SVZ and RMS, creating a permissive gate (Nguyen Ba-Charvet, et al. 1999). Once reaching the end of the RMS, migrating chains of type A cells require a signal to detach from one another and to begin radial migration. Several proteins expressed by cells in the OB, including Tenascin R (Saghatelyan, et al. 2004), reelin (Hack, et al. 2002), and prokineticin-2 (Ng, et al. 2005) induce detachment of type A cells from the chains. Interestingly, expression of Tenascin R is reduced following naris occlusion, a manipulation which broadly reduces sensory stimulion to the OB. This result may point to a mechanism for neuronal activity to regulate recruitment of migrating ABNs to active areas of the OB (Saghatelyan, et al. 2004).

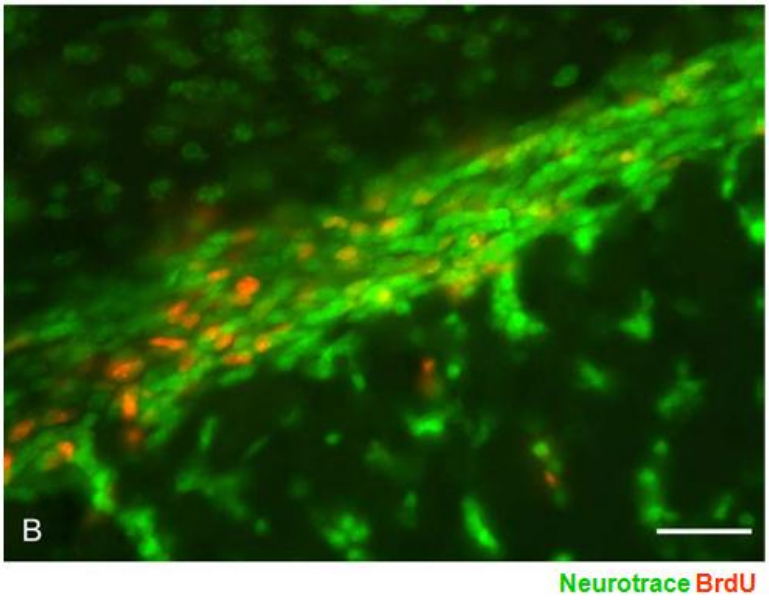

Figure 3. Adult-born neurons migrate through the rostral migratory stream to reach the olfactory bulb. Fluorescent immunohistochemistry image showing BrdU positive adult-born neurons traveling along the RMS. Red channel is stained for BrdU injected 12 hours prior to sacrifice. Green channel is stained for the neuronal cell division termination indicator NeuroTrace. Scale bar 37 μM. Figure panel reproduced from Bagley et al. 2007

1.1.3.3 Radial migration

After completing the migration from the SVZ to the RMS ABNs proceed to migrate radially into layers of the OB. OB ABNs can be grossly categorized into two categories: A small number are PG cells (~5% of the total population) that migrate into the outermost layer of the OB (see section 1.2.4). The majority (~95%) become GC in the GCL of the bulb (see section 1.2.5) that migrate radially to different depths of the GCL. The extent of radial migration within the GCL appears to be regulated by an ABN's birthplace within the SVZ (Merkle, Mirzadeh and Alvarez-Buylla 2007, Kelsch, Lin and Lois 2008). An emerging body of work indicates that this likely relates to the expression of developmental transcription factors that are also expressed in adult SVZ precursor cells. Pax6-null ABNs do not migrate to the superficial GCL instead

remaining in the deep layers of the GCL (Kohwi et al. 2005). While a different population of ABNs expressing the homeobox transcription factor Emx1 reaches the superficial GCL (Kohwi et al. 2007).

Radial migration begins as soon as ABNs reach the bulb and continues over the first 4 weeks after an ABNs birth (see figure 4). By 45 d.p.i. essentially all ABNs have exited the RMS and migrational regions of the bulb such as the anterior commisure (See figure 4, Mizrahi 2007). Work has also shown that different pools of stem cells lead to pools of ABNs that migrate different amounts radially in the OB (Kelsch, et al. 2007, Merkle, Mirzadeh and Alvarez-Buylla 2007). Therefore in order to interpret results that include cell counts in the olfactory bulb both d.p.i. and radial position is important, we have attempted to measure the number of ABNs in migrational zones in interpretting the results of survival experiments and have probed radial distance in the bulb to look for differential effects on disctinct populations of ABNs.

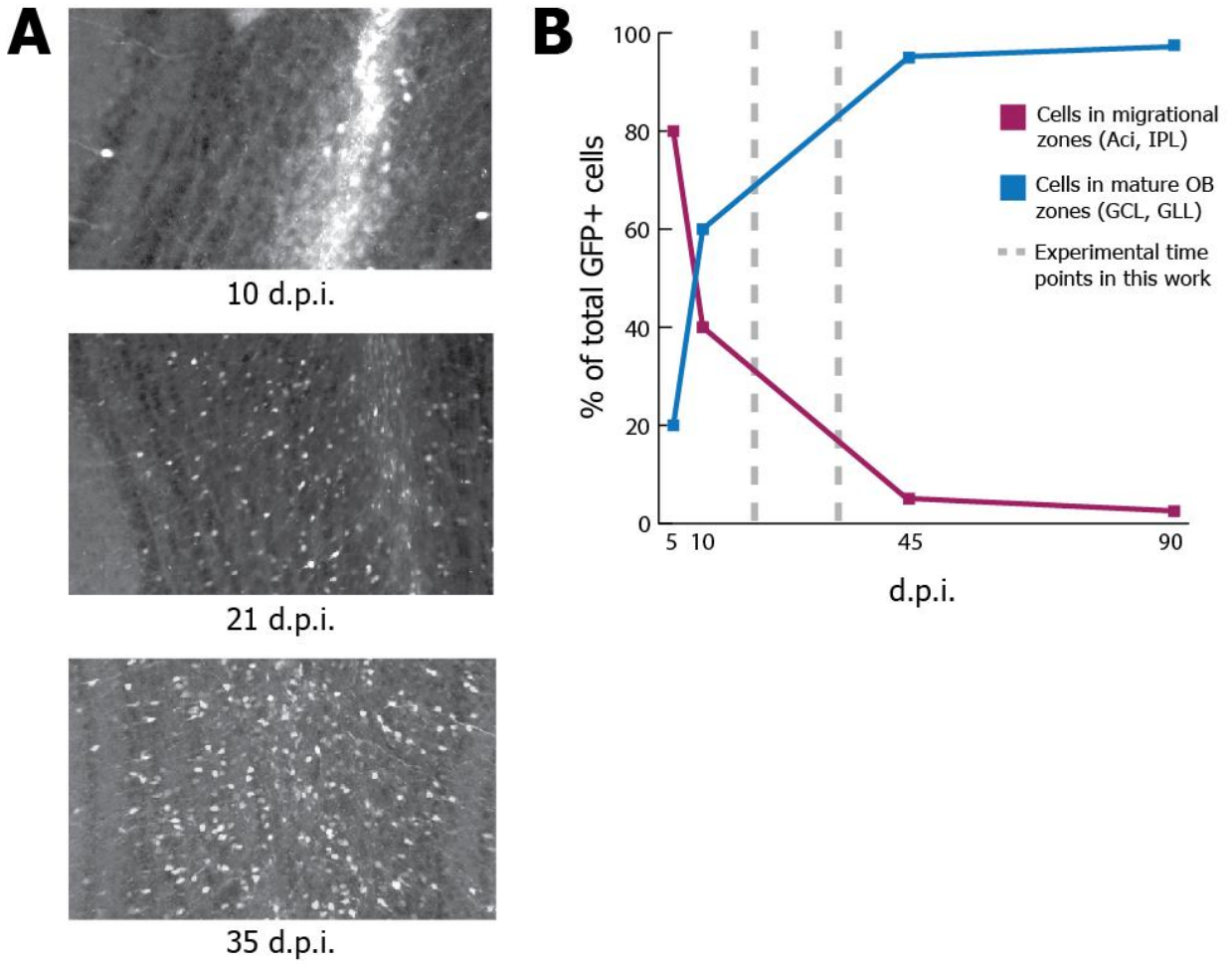

Figure 4. Radial position in the olfactory bulb as a function of time

Migrating ABNs are located in different radial positions of the bulb over the time points sampled in this work. (**A**) fluorescent images showing representative radial positions of neurons at 3 experimental time points. At 10 days many GFP+ ABNs are located in central zones of the OB at the end of the RMS (along the anterior commissure and internal plexiform layer). At 21 days there are still some residual neuroblasts along this region (faint glow in vertical zone to the right of image). By 35 d.p.i. ABNs have migrated radially from the anterior commisure and are located largely in the GCL (anterior commisure visible as white matter region in center of the image). (**B**) Conceptual curves showing the relative fraction of ABNs that reside in the migrational zones versus the GCL & GLL over time. ABNs are more likely to be found in migrational zones early after their birth and overtime migrate radially to the GCL & GLL. At the time points sampled in this work some ABNs are expected to be in migrational zones. Curves created from data found in Mizrahi 2007.

ABNs exhibit a remarkable diversity of their migration patterns and migration endpoints within the OB. ABNs can become PG cells or GCs. Adult-born GCs can reside in the

superficial GCL or deep GCL. In addition to this diversity in location ABNs also exhibit diversity in their expression of proteins. The next section will examine this additional subtype diversity.

1.1.3.4 Subtype differentiation

ABNs in the olfactory bulb show a large amount of subtype diversity. One important aspect of neuronal diversity is expression of calcium binding proteins. Neurons in the olfactory bulb express a range of calcium binding proteins that have limited overlap and thus mark separate populations (Kosaka, et al. 1995, Parrish-Aungst, et al. 2007, Bagley, et al. 2007, De Marchis, et al. 2007). Although their functional role is not clear, calcium binding proteins buffer intracellular calcium and are important regulators of neuronal activity and firing patterns (Burgoyne 2007). Their differentiation and expression are also regulated by neuronal activity (Philpot, Lim and Brunjes 1997, Caggiano and Brunjes 1993). ABNs in the OB can express the calcium binding proteins calbindin, calretinin, N-copine, and parvalbumin (Kosaka, et al. 1995, Nakayama, Yaoi and Kuwajima 1999, Parrish-Aungst, et al. 2007). Some PG ABNs can also express the dopaminergic marker tyrosine hydroxylase (Kohwi, et al. 2005). For ABNs in the OB, the expression of subtype markers changes rapidly over the first 14-40 days of its life (Bagley, et al. 2007) before ultimately maturing into similar subtypes as the overall OB population.

At 40-75 days after birth, expression patterns of ABNs are indistinguishable from the overall OB population (see Figure 4). In previous work (Bagley, et al. 2007), we identified BrdU positive cells in the GLL and then determined the number and percentage of these cells that were also positive for CR (28 of 123 cells; 23 ± 2%), CB (22 of 169 cells; 14 ± 3%), TH (34 of 149 cells; 23 ± 4%), GABA (129 of 215 cells; 60 ± 2%), and NC (11 of 99 cells; 12 ± 2%). The same

analysis was performed on the BrdU positive neurons in the GCL, except that, as stated above, TH-positive cells were not analyzed. The numbers and percentages of GCL neurons expressing the different markers are as follows: CR (302 of 3502 cells; 9 ± 1%), CB (69 of 4127 cells; 2 ± 0%), GABA (319 of 504 cells; 62 ± 6%), and NC (50 of 229 cells; 22 ± 2%).

The factors controlling ABNs eventual subtype are not currently well understood. Differentiation into a subtype of ABNs expressing calretinin, a calcium binding protein, is regulated by expression of the zinc finger transcription factor Sp8 in neuronal precursor cells within the SVZ (Waclaw, et al. 2006). In another subset of ABNs, expression of the transcription factor Pax6 and restricted expression of the transcription factor Olig2 is correlated with an ultimate phenotype of dopaminergic PG neurons (Kohwi, et al. 2005). As mentioned in the previous section a different subset of ABNs express the homeobox transcription factor Emx1 associated with expression of the calcium binding protein calretinin (Kohwi et al. 2007). In early research, there were hints that subtype fate could be regulated by activity in the OB itself (Philpot, Lim and Brunjes 1997), it now appears likely, however, that protein expression levels rather than ABN subtype identity are regulated by OB and cell-intrinsic activity (Nakayama, Yaoi and Kuwajima 1999, Brunjes 1994). We address the role of cell-intrinsic activity in the subtype fate of adult-born GCs in Chapter 2.

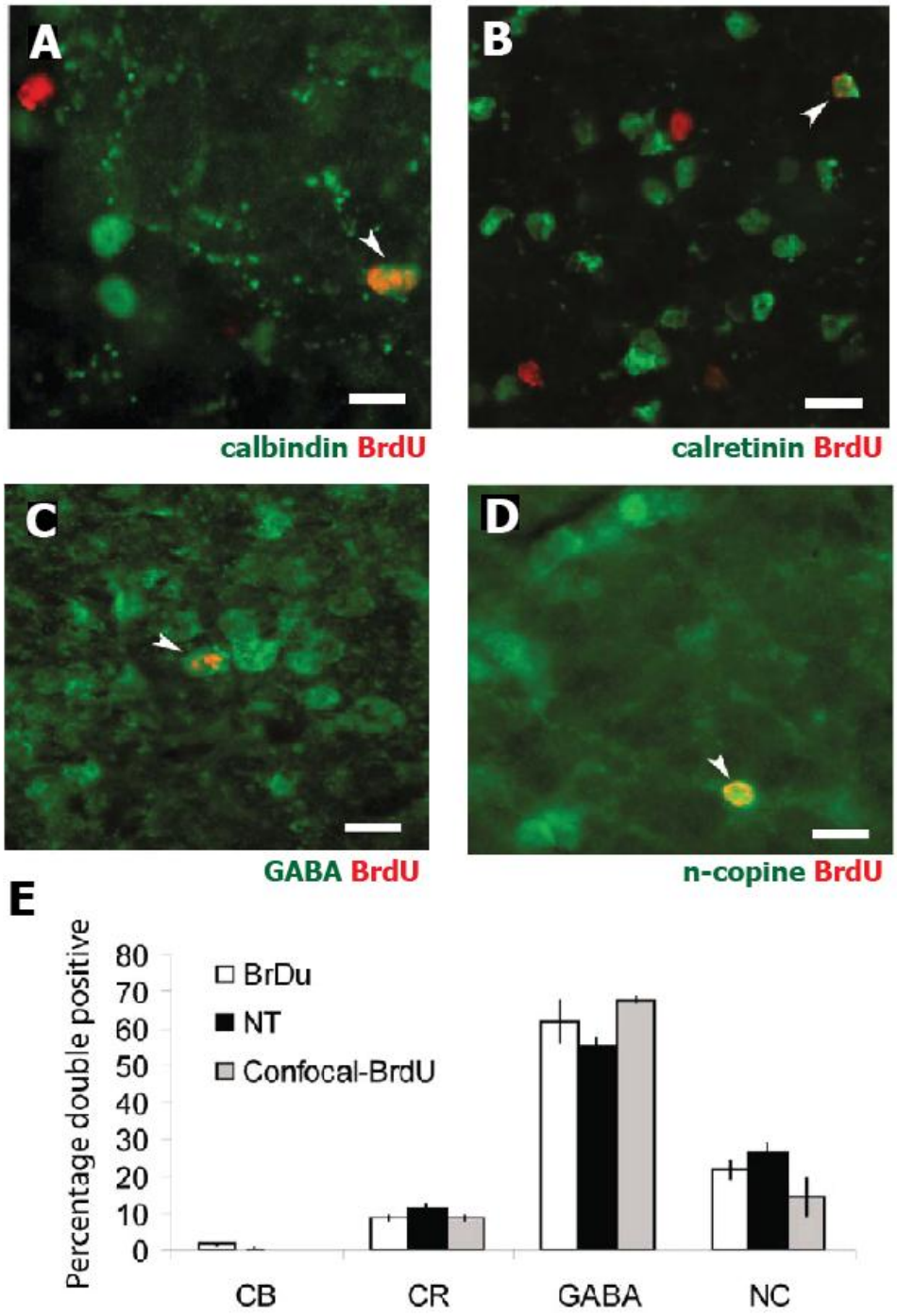

Figure 5. Adult-born neurons differentiate into several distinct subtypes.

Once reaching the OB ABNs differentiate into several identifiable subtypes as evidenced by their expression of several distinct types of calcium binding proteins such as (**A**) calbindin, (**B**) calretinin, and (**D**) n-copine, as well as the inhibitory neurotransmitter GABA (**C**). As indicated by their expression of the cell division markers BrdU or NT, (**E**) 40 days after their final division the population of ABNs express subtypes of calcium binding proteins in the GCL. Scale bars 37 μm. Figure panels reproduced from Bagley et al. 2007.

1.1.3.5 Survival

In rodents, the OB grows slowly over an animal's life. In the GCL, the number of neurons doubles between 3 and 31 months of age (Kaplan, McNelly and Hinds 1985). This is largely due to the addition of tens of thousands of new ABNs generated every day. Even with this slow increase in size, the enormity of this influx requires that a substantial amount of cells die every day to keep the system in balance. Indeed, ABNs have a high rate of cell death, with 50-70% of them undergoing apoptosis within their first few months of life (Petreanu and Alvarez-Buylla 2002, Winner, et al. 2002). Several important factors regulate ABN cell death: sensory activity (Corotto, Henegar and Maruniak 1994, Yamaguchi and Mori 2005, Rochefort, et al. 2002, Alonso, et al. 2006, Miwa and Storm 2005), cell-intrinsic activity (Lin, et al. 2010), neurotransmitters (Cooper-Kuhn, Winkler and Kuhn 2004, Kaneko, Okano and Sawamoto 2006), and neurotrophins (Zigova, et al. 1998, Benraiss, et al. 2001).

This dissertation focuses on the role of neuronal activity, both sensory and cell-intrinsic, in ABN survival (see Chapter 2). Section 1.6 of the introduction is devoted to the role of activity in regulating OB adult neurogenesis.

1.1.4 Adult neurogenesis in the hippocampus

Since its initial description in the 1960's a considerable body of evidence has confirmed adult born neurons are born daily in the mammalian dentate gyrus (Altman and Das 1965, Cameron, Woolley, et al. 1993, Kempermann, Kuhn and Gage 1997, Gould, Reeves, et al. 1999). The presence of these adult born neurons has generated significant interest because of the possibilities of using alternative approaches to treat hippocampal disorders such as depression and degeneration (Doetsch and Hen 2005). And eventually the prospect of the use of stem cell

therapies to treat a host of psychiatric disorders, for which the basic mechanisms may best be worked out in this native neuronal stem cell population (Emsley, et al. 2005).

1.1.4.1 Adult born neurons are born in the dentate gyrus of the hippocampus

Hippocampal adult born cells are derived from a population of stem cell-like radial glial cells which express GFAP and nestin. The nestin expressing radial glial cells divide to produce cells that express the microtubule-associated protein doublecortin (DCX), an early marker of cells committed to a neuronal phenotype. These doublecortin expressing cells initially act as transit-amplifying cells (Seri, Garcia-Verdugo, et al., Astrocytes give rise to new neurons in the adult mammalian hippocampus 2001, Kronenberg, et al. 2003, Seri, Garcia-Verdugo, et al., Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus 2004), dividing over several days to produce additional neuronally committed cells. This population continues to express doublecortin for up to 4 weeks (Brown, et al. 2003) while extending dendrites into the perforant path, extending axons into area CA3, and functionally integrating into the hippocampal circuit. The broad time period during which doublecortin is expressed is partially overlapping with two other developmentally regulated neuronal specific promoters PSA-nCAM (Ambrogini, Orsini, et al. 2004) and POMC (Overstreet, et al. 2004). This time period during the first month after the birth of adult born neurons encompasses the whole period during which adult born neurons are initially integrating. The neurons begin to receive excitatory GABAergic (<1 week) and glutamatergic (2-3 weeks) input quickly, and start exhibiting action potentials as quickly as 4 days after birth. During this period they also exhibit increased measures of plasticity such as LTP and in all likelihood increased dendritic plasticity (Mizrahi and Katz 2003, Mizrahi 2007).

1.1.5 Functional role of hippocampal adult neurogenesis

Deficits in neurogenesis have been implicated in hippocampal dependent tasks as varied as sensitivity to treatment with selective serotonin reuptake inhibitors and spatial memory tasks. Young adult born neurons have distinct physiological behaviors compared to mature granule cells already present in the hippocampal circuit. Immature adult born granule cells display a lower threshold for long term potentiation (LTP) relative to mature cells in the outer layers of the DG (Wang, Scott and Wojtowicz 2000, Schmidt-Hieber, Jonas and Bischofberger 2004). Pairing stimulation with subthreshold Ca^{2+} spikes also leads to long term depression (LTD) in young adult born neurons but not mature granule cells (Schmidt-Hieber, Jonas and Bischofberger 2004). Adult born neurons also seem to effect the expression of plasticity in the dentate gyrus. Loss of adult born neurons following ablation by irradiation leads to the loss of a specific type of LTP between the perforant path and the dentate gyrus (Snyder, Kee and Wojtowicz 2001, Saxe, et al. 2006). During their immature developmental stages ABNs express a different proportion of channels than mature granule cells. Higher levels of T-type calcium channels lead to more frequent dendritic Ca^{2+} spikes and relief of the Mg^{2+} blockade of NMDA receptors (Ambrogini, Cuppini, et al. 2000, Schmidt-Hieber, Jonas and Bischofberger 2004). Lower Na^{+} channel density in immature adult born neurons leads to delays in the generation of action potentials in response to depolarization, but resulting action potentials show a prolonged time course and are more likely to occur (Overstreet-Wadiche, Bromberg, et al., GABAergic signaling to newborn neurons in detate gyrus 2005). Importantly, adult born granule cells also depolarize in response to GABAergic signaling, similarly to neurons early in development. Thus young adult born neurons have a lower threshold for the induction of LTP and LTD, and are not inhibited by

GABAergic input. The net effect of these physiological differences seems to be the facilitation of broad single action potentials.

1.1.5.1 Adult hippocampal neurogenesis and learning

Evidence from several lines of experiments suggests a correlation between adult neurogenesis and learning. In male song birds the main function of adult neurogenesis is the seasonal acquisition and expression of learned songs (Goldman and Nottebohm 1983, Nottbohm 1985). Also in birds, seasonal fluctuations in adult neurogenesis in the hippocampal region of black-capped chickadees is positively correlated to engaging in spatial learning behaviors through seed storage (Barnea and Nottebohm 1994). In mammals there is a consistent correlation between the survival of adult born neurons and activities that involve spatial exploration and learning such as a spatially enriched environment, wheel running, and spatial water maze learning (Kempermann, Kuhn and Gage 1997, Van Praag, et al. 1999, Gould, Reeves, et al. 1999, Ambrogini, Cuppini, et al. 2000). In fact, in spatial learning on the Morris water maze task there is a correlation between performance and the level of adult neurogenesis in different strains of mice (Kempermann and Gage 2002). There is also a consistent correlation between manipulations that cause a reduction in the level of adult neurogenesis and performance in hippocampal dependent learning tasks such as stress, aging, and aversive prenatal experience (Luine, et al. 1994, Gould, Tanapat, et al. 1998, Kuhn, Dickinson-Anson and Gage 1996, Mirescu, Peters and Gould 2004). The most powerful evidence for the role of adult born neurons in mammalian learning may come from deficits produced by methods which ablate continuing adult neurogenesis. Ablation has caused deficits in hippocampal dependent tasks such as trace eyeblink conditioning, contextual fear conditioning, long term spatial memory, and a delayed non-match to sample task in the Morris water maze (Shors, et al. 2001, Winocur, et al. 2006, Saxe, et al. 2006, Snyder, Hong, et al. 2005).

1.2 ORGANIZATION OF THE OLFACTORY BULB

The OB is a chemosensory processing center of the brain located in the verterbrate forebrain and 1st cranial nerve. It bypasses the thalamus and sends output directly to the olfactory cortex (Shepard, Chen and Greer 2004). Its function is to translate the binding of a myriad of odors to hundreds of unique chemoreceptors and finally into unique neuronal patterns of activity. Its accessibility, distinguishable laminar structure, and unusually rapid turnover of both peripheral sensory neurons and inhibitory interneurons make it an attractive system in which to research adult neurogenesis.

1.2.1 Sensory stimuli and olfactory receptor neurons

Sensory stimuli reach the olfactory bulb through the nasal cavities. In mammals, the main pathway for odors is an air pathway through the nostrils (a.k.a. naris, or the plural nares). There are additional secondary pathways, found in the posterior nasal cavities which connect to the pharynx and allow for some amount of air exchange between either side of the skull. For the purposes of this work, it is assumed that occluding the nostrils reduces the airflow to the nasal cavity but does not eliminate it (Philpot, Foster and Brunjes 1997). Once odors travel into the nasal cavities, soluble odors dissolve into the nasal mucus after which they diffuse to of a multitude of unique odorant receptors (>1,000 in mice) (Buck and Axel 1991). Odorant receptors are transcribed from unique genes and each unique receptor has a unique odorant binding profile. Odorant receptors are expressed in olfactory receptor neurons (ORNs) in a 1:1 ratio; That is, only one type of unique receptor is expressed per olfactory receptor neuron. ORNs project their axons, along with axons from other ORNs expressing the same receptor, to receptor-

specific glomeruli in olfactory bulb. Glomeruli thus receive input from only a single type of olfactory receptor. Notably, ORNs have a short life span, on the scale of weeks; as such, the OB is constantly creating connections with new ORNs and removing connections from expired ORNs (reviewed in Schwob 2002).

1.2.2 The gross anatomy of the olfactory bulb

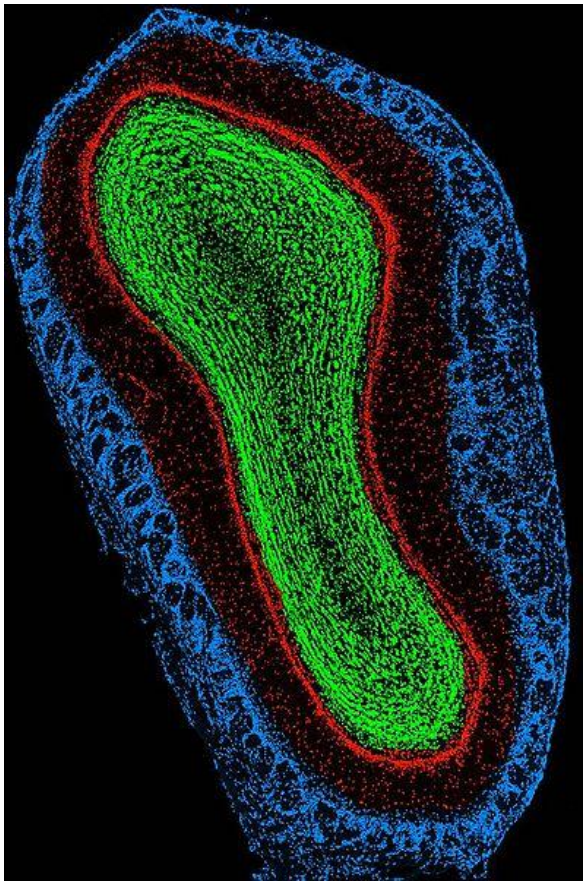

Figure 6. Gross anatomy of the olfactory bulb

A coronal section through the main olfactory bulb of an adult male mouse (strain: C57BL/6j). Image taken with a confocal microscope visualizing all cell nuclei with stain TOTO-3. False color indicates the three main anatomical layers: Blue - GLL containing periglomerular cells, Difuse Red – EPL containing dendrites of Mitral & Granule cells, Concentrated Red – MCL containing cell bodies of mitral and tufted cells, Green - GCL containing granule cell bodies (M. Valley 2006).

The OB is a laminar structure in the forebrain. It receives odorant information from ORNs in the nasal cavity, processes it, and projects it directly to the cortex. The OB has several well defined layers that participate in processing odorant information (see Figure 5). From the outer surface to the interior they are: glomerular layer (GLL), external plexiform layer (EPL), mitral cell layer (MCL), granule cell layer (GCL). Odorant information enters the OB into specialized spheres of neuropil in the GLL called glomeruli. ORNs project axons encoding information from unique receptors to specific glomeruli where all of the axons terminating are from ORNs expressing the same unique odorant receptor (Mombaerts, et al. 1996). ORN axons terminate on dendrites of mitral cells (see section 1.2.3) and periglomerular cells (see section 1.2.4).

1.2.3 Mitral cells

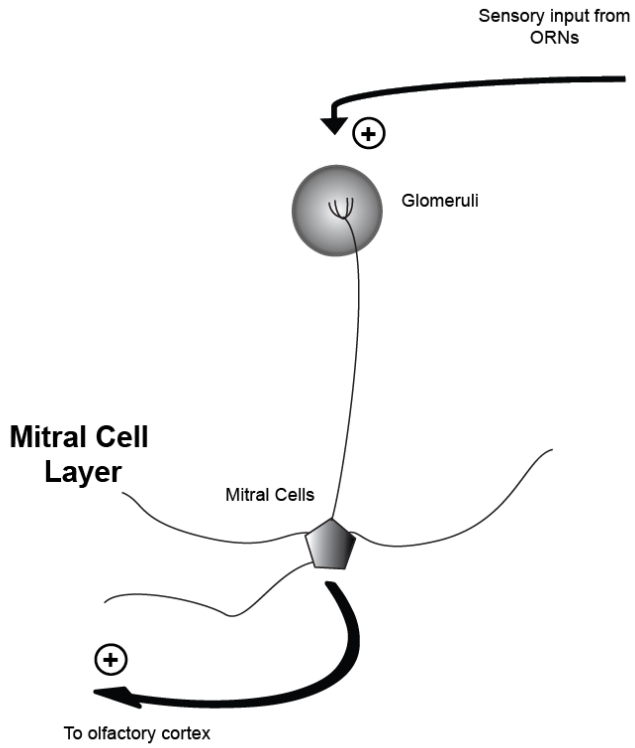

Figure 7. Mitral Cells in the OB circuit.

Mitral cells (MC or M/T) are the principal cells of the olfactory bulb. (Tufted cells are a related population of principal cells with a slightly different anatomy. For the purposes of this dissertation they may be considered collectively). The mitral cells receive excitatory sensory information through a primary apical dendrite that projects to a single glomeruli. Their cell bodies reside in the MCL. After processing the information from the glomeruli they project axons directly to the olfactory cortex, to collateral regions of the GCL in the OB, and to the GCL of the OB in the opposite hemisphere. In addition to the primary dendrite extend to the

glomeruli, MCs have several lateral dendrites that extend outwards into the external plexiform layer. These lateral dendrites make connections with GCs (see section 1.2.5)

1.2.4 Periglomerular cells

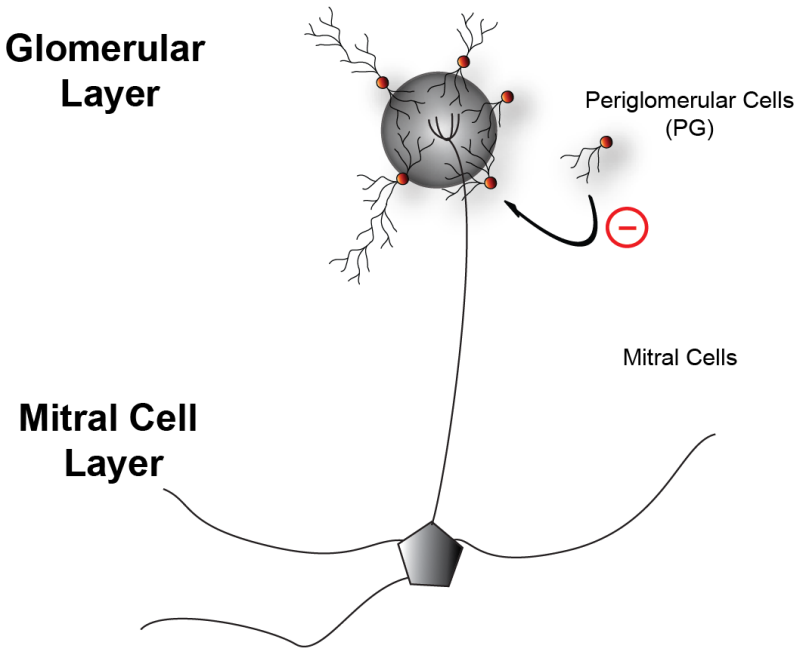

Figure 8. Periglomerular cells in the OB circuit.

Periglomerular (PG) cells are small neurons that surround individual glomeruli. They are largely GABA-ergic and inhibitory. The PG cells send short bushy dendrites into the neuropil, where they receive axonal input from ORNs. They receive additional dendrodendritic input from MCs; MCs have excitatory terminals on their dendrites that can release neurotransmitters onto PG cells. Similarly, PG cells make dendrodendritic synapses onto MC dendrites, releasing GABA onto them and inhibiting MC dendrites.

Of note PG cells continue to be born in adult animals (Altman 1969, Lois and Alvarez-Buylla 1994, Bagley et al. 2007) the majority of PG cells are born postnatally (Bayer 1983). These newborn PG cells exhibit a distinctive form of plasticity: although 3% of the total population of PG cells every month is newborn, the overall number does not change, thus necessitating whole neuronal turnover (Mizrahi et al. 2006). PG cells also exhibit high diversity in subtype and express one of several types of calcium binding proteins (see section 1.1.3.4). Additionally, some have a dopaminergic subtype and express tyrosine hydroxylase.

1.2.5 Granule Cells

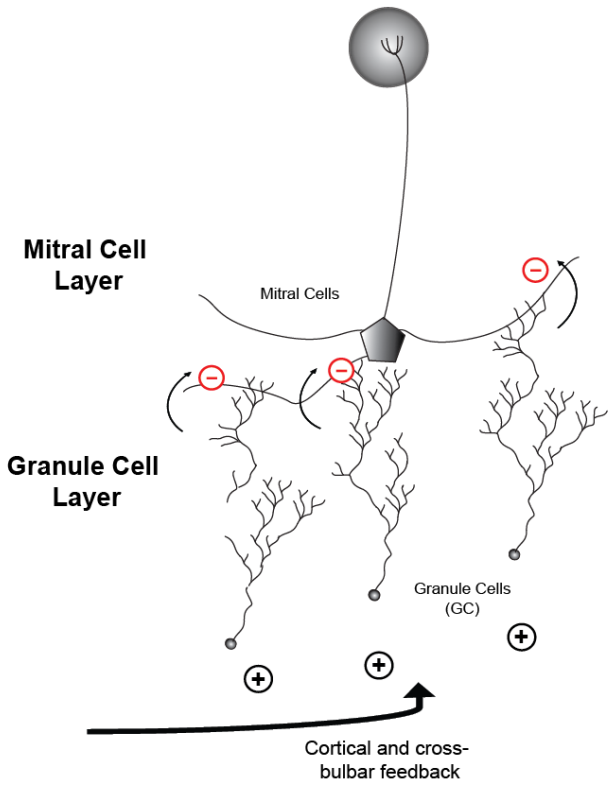

Figure 9. Granule Cells in the OB circuit.

Granule cells (GCs) are small inhibitory neurons located in the granule cell layer (GCL). They are axonless, possessing one main dendrite that extends outwards and branches into the external plexiform layer (EPL). In the EPL, they make connections with MCs, forming reciprocal dendrodendritic connections with MC lateral dendrites. GCs release GABA onto MCs when their synapses are depolarized, which can happen globally within the GC (Egger, Svoboda and Mainen, Mechanisms of lateral inhibition in the olfactory bulb: efficiency and modulation of spike-evoked calcium influx into granule cells 2003, Zelles, et al. 2006), locally within the GC dendrite (Egger, Svoboda and Mainen 2005, Pinato and Midtgaard 2005), or even within a single spine (Isaacson 2001, Egger, Svoboda and Mainen 2003). When a GC becomes sufficiently depolarized it will initiate a back-propagating action potential that can cause GABA to be released onto MCs its connected to (Egger, Svoboda and Mainen 2003). As shown in Figure 9, excitatory input from cortical and cross-bulbar projections causes depolarization at GC synapses and inhibitory release onto MC lateral dendrites. GCs also receive extensive connections in the GCL from ipsilateral MCs linking the mirror glomeruli within the OB of a single hemisphere (De Olmos, Hardy and Heimer 1978, Lodovichi, Belluscio and Katz 2003). Additionally, centrifugal fibers from the olfactory cortex, anterior olfactory nucleus, locus ceruleus, basal ganglia, and basal forebrain typically synapse onto GCs in the GCL (Luskin and Price 1983, Shipley and Ennis 1996).

Voltage gated sodium channels are expressed in GCs and mediate backpropogating sodium membrane potentials and transmitter release. Application of the voltage-gated sodium channel inhibitor, tetrodotoxin, attenuates elicited action potentials in granule cells, suggesting that GC action potentials are primarily driven by voltage gated sodium channels even though they possess no axons (Isaacson and Strowbridge 1998). But, notably showing a reduced role for

other voltage dependent channels in GC currents. Additionally there are hints that voltage gated sodium channels within dendrodendritic spines could assist in depolarization sufficient to activate voltage dependent calcium channels and cause transmitter release (Isaacson 2001, Egger Svoboda and Mainen 2005). Voltage gated sodium channels mediate dendritic sodium spikelets and subsequent low-threshold calcium spikes in GCs (Pinato and Midtgaard 2005). They are thus involved intimately with input signalling as well as output signalling and control a large portion of the membrane depolarization that GCs experience.

Tens of thousands of GCs are born every day in adult mice. In fact the majority of GCs in adult mice are born postnatally (Bayer 1983). These newborn neurons represent a significant amount of novel inhibitory input to MCs and may represent a dynamic form of plasticity that can adapt to changing odor demands (Lledo 2007, Egger and Urban 2006).

1.2.6 Self inhibition of mitral cells

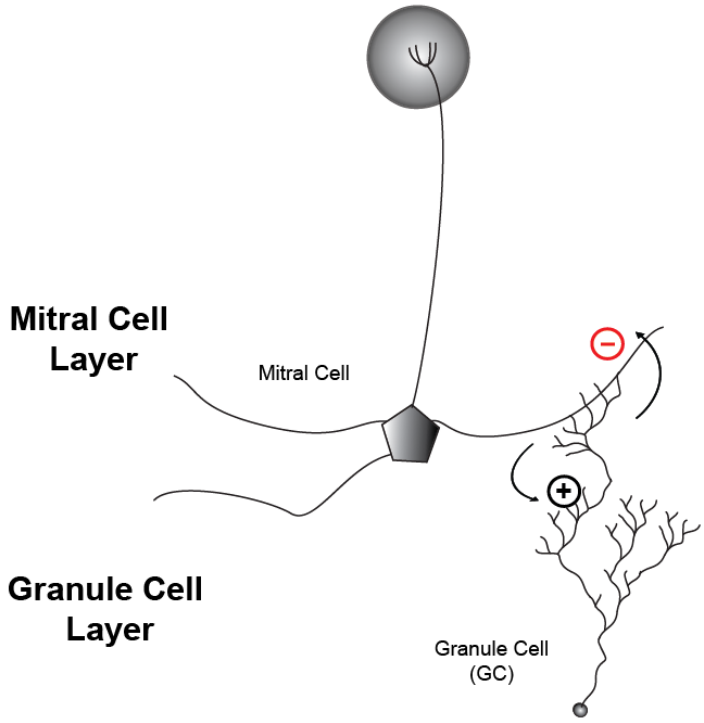

Figure 10. Self inhibition of mitral cells mediated by granule cells in the OB circuit.

GCs form dendrodendritic connections with MCs in the external plexiform layer (Price and Powell 1970). These synapses contain bi-directional synaptic machinery on both the MC and GC. Looking at the kind of isolated circuitry shown in Figure 10, it is possible to imagine a situation where the release of glutamate by excitatory MCs would lead depolarization at the GC synapse, thus causing inhibitory release of GABA from the GCs onto the MCs. In this way, MCs utilize GC mediate self-inhibition on their own lateral dendrites when these lateral dendrites become depolarized.

This type of synapse is a primary source of inhibition in the olfactory bulb. There are approximately 100 times as many GCs as MCs in an adult mouse and each GC makes

approximately 100 dendrodendritic synapses in the EPL onto MCs (Shepard, Chen and Greer 2004). A few simple calculations indicate the presence of 10,000 inhibitory inputs for each MC, compared with a single glomerulus of excitatory input.

1.2.7 Lateral inhibition of mitral cells

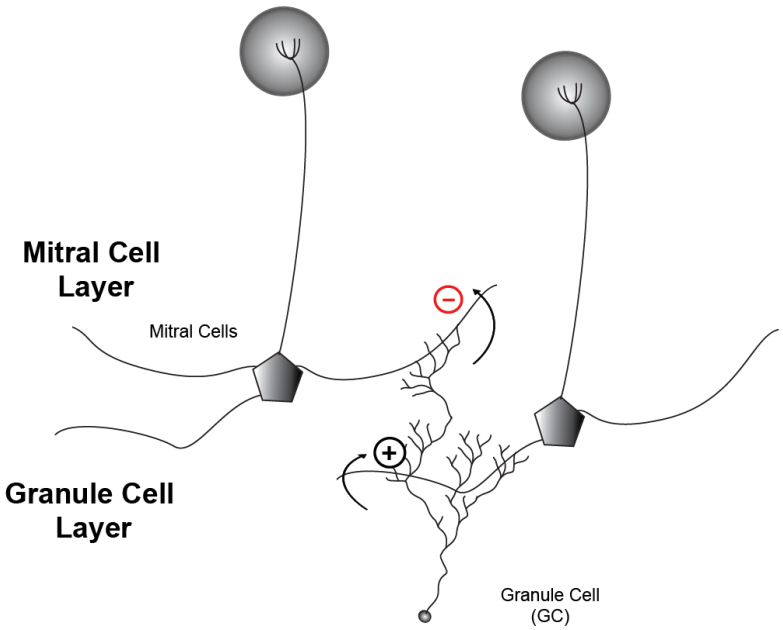

Figure 11. Lateral inhibition of mitral cells mediated by granule cells in the OB circuit.

As summarized in the preceding section the MC-to-GC dendrodendritic synapse is the primary source of output inhibition in the olfactory bulb. Because GCs form synapses with multiple MCs, this type of synapse can also drive inhibition between MCs by way of lateral inhibition (Margrie, Sakmann and Urban 2001, Urban and Sakmann 2002). As seen in Figure 11, excitation of a GC by one mitral cell will drive a membrane depolarization that can, in turn, trigger inhibitory GABA release at a different dendrodendritic synapse, with a different MC.

Furthermore, because GCs make 50-200 synapses with MCs, the resulting lateral inhibition can activate GABA release onto numerous MCs through the excitation of a single GC (Shepard, Chen and Greer 2004). Lateral inhibition from GCs onto MCs can facilitates several modifications of odor information propagating from MCs to central brain regions. Resulting in activity dependent decorellation (Arevian, Kapoor and Urban 2008), synchonization (Galán, et al. 2006, Giridhar, Doiran and Urban 2011), and a sharpening of odor-evoked responses from MCs (Yokoi, Mori and Nakanishi 1995).

Functionally, increasing the inhibition of MCs by GCs increases the speed of discrimination between structurally and pschophysically similar odors in mice, while decreasing this inhibition slows discrimination (Stopfer, et al. 1997, Abraham, et al. 2010). The effect on odor discrimination through increased lateral inhibition of MCs by adult-born GCs may mediate one of the functions of adult neurogenesis in the OB (Gheusi, et al. 2000, Alonso, et al. 2006, Cecchi, et al. 2001). It should be noted that this sample circuit oversimplifies possible effects. Under normal conditions, inhibition can, for example, be permissive based on its timing. For a more complete discussion of these permissive effects see (Urban 2002).

1.2.8 The effect of adult-neurogenesis on inhibition in the olfactory bulb

The most obvious effect of adult neurogenesis on inhibition in the olfactory bulb is the dramatic increase in the number of GCs over an animal's life. The number of GCs in the OB doubles over a rodent's life (Kaplan, McNelly and Hinds 1985), a development that dramatically increases the inhibitory load on MC lateral dendrites.

As compared to mature neurons, adult-born GCs in the olfactory bulb show increased expression of the immediate early gene c-fos when exposed to odors presented to them during

their integration (Magavi, et al. 2005). This may indicate that GC synapses integrate most strongly into olfactory odor patterns presented to them during their formation and are most efficient in response to those odor patterns. OB ABNs have been proposed to encode a sort of olfactory memory enabling the OB circuit to adapt to novel sensory challenges (Lledo and Saghatelyan 2005, Lledo, Alonso and Grubb 2006).

An additional property of ABNs is their transient nature in the OB. Between 50-70% of ABNs die in their first three months after reaching the olfactory bulb (Petreanu and Alvarez-Buylla 2002, Winner, et al. 2002). During this time, they have a high degree of synaptogenesis and dendritic growth and retraction (Mizrahi 2007, Livneh, et al. 2009). The high degree of sampling of available synaptic connections, coupled with the expendability of the majority of ABNs, may allow the eventual survivors to select for the optimal inhibitory connections within the OB during the period of their integration.

A recent well controlled study by Brenton-Provencher and colleagues looked at the effect of the ablation of OB adult neurogenesis on the inhibitory inputs to MCs, physiology of the OB, and odor related behaviors (Breton-Provencher, et al. 2009). Consistent with adult neurogenesis providing a major source of inhibition onto MCs in the OB they saw a decrease in the number of GABAergic synapses onto MCs on their lateral dendrites, a corresponding decrease in the number of spontaneous mIPSCs, and a decrease in the evoked IPSCs to MCs. They also saw a reduction in gamma frequency network oscillations. Behaviorally the ablation of SVZ neurogenesis led to a decrease in detection threshold of odors (but see Lazarini, et al. 2009), and reduced certain forms of odor memory previously shown to be effected by adult neurogenesis (Rochefort, et al. 2002, Mechawar, et al. 2004). It did not however effect spontaneous odor discrimination.

To test the effect of adult neurogenesis on olfactory dependent tasks and odor memory another recent study utilized an inducible genetic ablation technique that stopped adult neurogenesis in both the hippocampus and OB (Imayoshi, et al. 2008). Genetic ablation of adult neurogenesis led to no new neurons reaching the OB after 21 days and decreased density of neurons in the OB over 6-12 weeks. Mice with a genetic ablation of adult-neurogenesis were able to spontaneously discriminate between three sets of enantiomers and retained that odor memory for at least 6 months. This demonstrated that adult neurogenesis is not necessary for a set of difficult odor discriminations or the storage of the associated olfactory memory traces. However, other work using radiation to ablate neural precursors in the SVZ have seen deficits in social interactions mediated by the olfactory system (Feierstein, et al. 2010) and measures of olfactory fear conditioning (Valley, et al. 2009). And Imayoshi and colleagues acknowledge that more difficult measures of odor discrimination or odor memory could depend on adult neurogenesis (Imayoshi, et al. 2008). The role of adult neurogenesis in the normal function of the OB therefore remains an open question; based on ablation studies at the very least we can say that it is not absolutely necessary for most regular functions of the OB but seems to be involved in olfactory circuitry and behavior in a measurable way.

1.3 LENTIVIRAL MANIPULATION OF GENE EXPRESSION

The development of viral labeling and gene transcription manipulations accelerated the field of adult neurogenesis in the 1990s (Naldini, et al. 1996, Yoon, et al. 1996, Lois, et al. 2002, Petreanu and Alvarez-Buylla 2002, van Praag, et al. 2002). Retroviruses can now be used to label ABNs that arise in the SVZ and subsequently migrate to the OB. Importantly, retroviruses

can drive expression of several thousand base pairs of genetic material introduced into host cells and their daughters, allowing for the expression of fluorescent markers like GFP, novel or native proteins of interest, or genetic manipulators of protein expression such as short-hairpin RNA (shRNA).

1.3.1 Lentivirus

Lentivirus is a subtype of retrovirus that is capable of delivering large amounts of chosen genetic material to the infected host cell. Some well-known types of lentiviruses include the simian and human immunodeficiency viruses: SIV and HIV, from which replication incompetent lentivirus vectors have been developed (Zhao 2007). Lentiviruses are unique among classes of retrovirus in that they can infect and replicate within both dividing and quiescent cells (Coffin, Hughes and Varmus 1997). Once the lentivirus' genetic information is transduced into the host cell in the form of single stranded RNA, it undergoes reverse transcription, is converted into DNA, and is finally incorporated into the host genome (see Figure 10). This allows for stable transcription of introduced genetic material by the host cells and any daughter cells (Lois, et al. 2002). Studying adult neurogenesis in the OB (an area several millimeters away from the injection site) has an advantage in that infected ABNs must migrate via the RMS to the area of study. Because of this observed long distance migration neurons flourescently labeled in the OB after migration are known to be ABNs from the SVZ (Petreanu and Alvarez-Buylla 2002).

1.3.2 From shRNA expression to protein expression knockdown

After a lentivirus delivers genetic material to the host cell, this information is permanently incorporated into that cells genome. An experimenter can choose to introduce full genes with chosen promoters, thus allowing for control of transcriptional activity. Gene expression can be limited to specific cell types, or to periods when animals are treated with exogenous small molecules, it can also be expressed ubiquitously in any infected cell (Dittgen, et al. 2004, Komai, et al. 2006). In the case of neuronal progenitors in the SVZ, strong unspecific promoters such as the ubiquitin promoter can be used (Komai, et al. 2006). Promoters can be paired with the genes of fluorescent proteins like EGFP or red fluorescent proteins to induce the expression of a visible marker of lentivirus expression (Lois, et al. 2002, Dittgen, et al. 2004, Komai, et al. 2006). In addition other DNA sequences of interest can be introduced to manipulate gene expression and proteins, as we show in Chapters 2 and 3 (Dittgen, et al. 2004, Komai, et al. 2006). Our later experiments rely on expression of RNA strands that are introduced into the host genome via a lentivirus and then translated into short hairpin RNA (shRNA, see Figure 10). In shRNA, a single strand of RNA is bent back onto itself. The straight sequences at either end are bent back onto each other and are designed to be complementary. The shRNA is first exported from the nucleus via the exportin 5 complex (Yi, Qin, et al. 2003), then recognized by the Dicer complex, which cleaves shRNA in the cytoplasm, leaving a short piece of RNA known as small interfering RNA (siRNA). This siRNA is designed to be complementary to an mRNA of interest. The Dicer complex then facilitates the transfer of a single stranded siRNA to the RNA-induced silencing complex (RISC), which pairs a strand of the siRNA with the mRNA of interest and causes the target mRNA to be degraded (summarized in the schematic in Figure 12).

Over-expression of shRNA through viral vectors has been associated with toxicity (Jackson and Linsey 2010, Manjunath, et al. 2009). In the mouse liver over-expression of shRNA via an AAV vector was shown to overwhelm the Exportin 5 transporter and lead to cell death, this effect could be rescued through over expression of Exportin 5 (Grimm, et al. 2006, Yi, Qin, et al. 2003). Other studies saw accumulation of shRNA strands in the cytosol as well as the nucleus implicating potential saturation of the Dicer complex as well (Boudreau, Monteys and Davidson 2008). Other proteins involved in the pUbquitin promoter or the cleavage of shRNA strands could be affected similarly (Lund, et al. 2004, Sontheimer 2005, Castanotto, et al. 2007). Care must be taken to interpret cell death results resulting from a manipulation using a shRNA vector.

The experiments described in this dissertation will utilize a lentivirus to deliver the genetic code of two sequences of shRNA along with fluorescent protein markers. All three sequences are transcribed within infected neuronal progenitors in the SVZ and subsequently within their daughter ABNs in the OB. The shRNA sequences are designed to be complementary to three homologous sequences of mRNA of interest that encode the protein of voltage gated sodium channels $Na_v1.1$, $Na_v1.2$, and $Na_v1.3$ (see Figure 12). After the introduced shRNA is cleaved into siRNA it causes the degradation of $Na_v1.1$-1.3 mRNA. Over extended time periods, this reduction in mRNA causes the reduced expression of $Na_v1.1$-1.3 protein in a fluorescently labeled population of ABNs (see Figure 17, Komai et al. 2006).

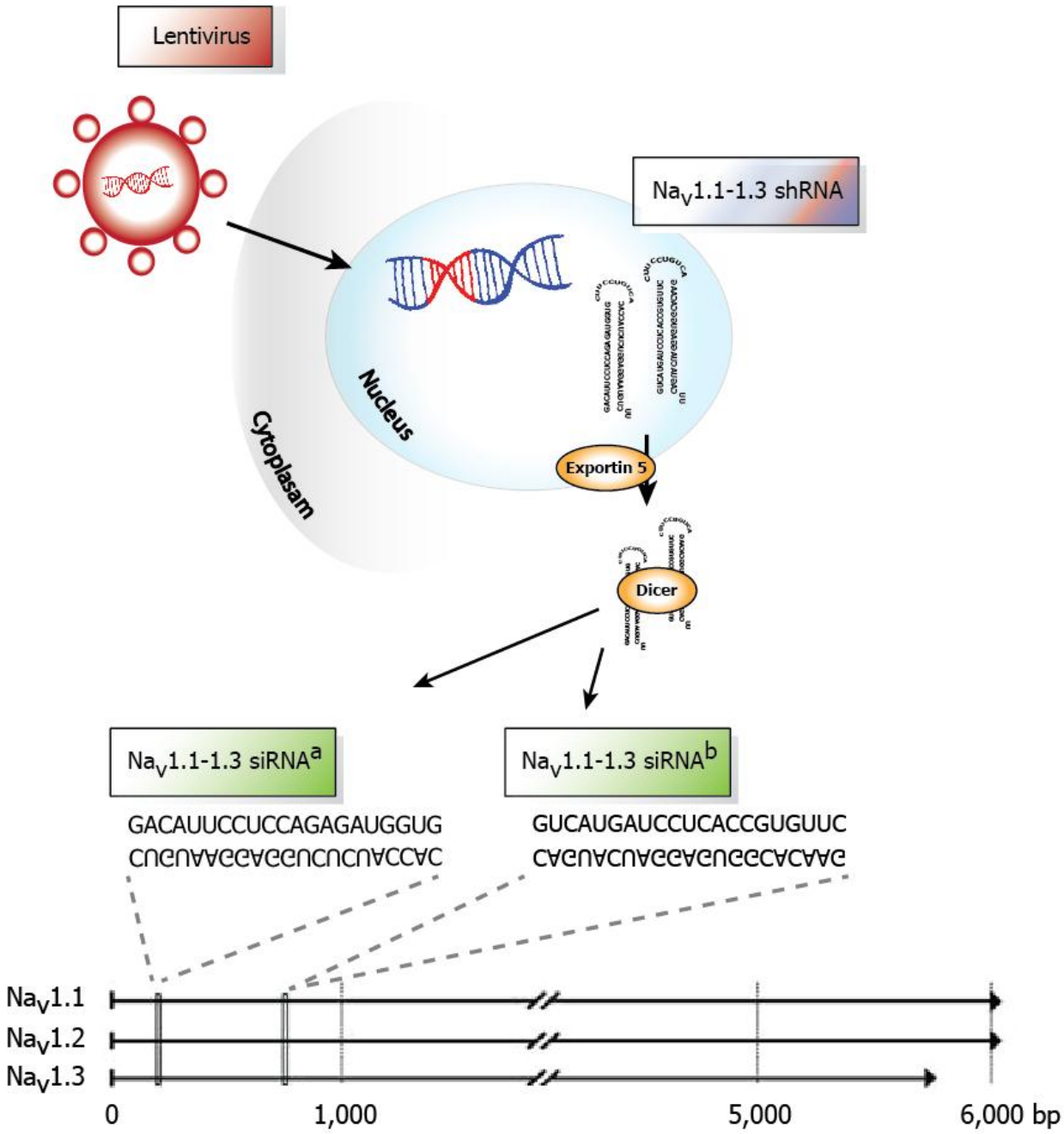

Figure 12. Lentiviral delivery of shRNA constructs drives expression of siRNA and interference with targeted proteins.

Lentivirus delivers viral DNA to target ABNs in the sub-ventricular zone. The viral DNA is randomly incorporated into the host cells genome where it undergoes transcription under an introduced promoter. Introduced shRNA sequences are transcribed from the host's genome and exported into the cytoplasm via Exportin 5. Once in the cytoplasm they are cleaved into siRNA by the Dicer complex. The siRNA is then free to interfere with target protein mRNA where it may lead to its removal from the transcription process and reduce overall expression of the target protein. Figure adapted from (Cojocari 2009) and (Komai, et al. 2006).

1.4 DEVELOPMENT OF CHANNELS REGULATING ACTIVITY IN ADULT BORN NEURONS OF THE OLFACTORY BULB

Manipulations of the level of intrinsic cell-specific activity used in this work rely on the reduction of a particular set of voltage gated sodium channels (Dittgen, et al. 2004, Komai, et al. 2006) . Understanding these channels, their effects on neuron development and survival, and compensatory changes that may occur from manipulation of their expression are important for interpreting the results show in this dissertation.

1.4.1 Voltage gated sodium channels

Voltage gated sodium channels are responsible for the depolarization event of action potentials and are triggered by membrane depolarization. The mammalian genome contains genes encoding nine functional voltage-gated sodium channel primary subunits (Goldin 1995). Four of these are expressed widely in the CNS $Na_v1.1$, $Na_v1.2$, $Na_v1.3$ & $Na_v1.6$ (Meisler, O'Brien and Sharkey 2010).

This work utilizes a genetic manipulation to reduce expression of $Na_v1.1$-1.3 protein selectively in adult born neurons and understanding their natural expression phenotype of the CNS VGSC is especially relevant to the results and conclusions drawn. $Na_v1.1$ is expressed across the CNS early in development and in parvalbumin fast spiking neurons in adults (Beckh, et al. 1989, Ogiwara, et al. 2007). $Na_v1.2$ is expressed across the CNS early in development and had a reduced expression profile in adults. $Na_v1.3$ is expressed largely in unmyelinated axons of the fetal CNS and gives way to $Na_v1.6$ expression in adults (Beckh, et al. 1989, Catterall 2012).

$Na_v1.6$ is expressed largely in myelinated axons and drives action potentials, it is the main VGSC in excitatory neurons.

1.4.2 Voltage-dependent calcium channels

Voltage-dependent calcium channels mediate an influx of calcium across the cell membrane in response to membrane depolarization (Lcinova and Hofmann 2001). They are especially important in neurons where they regulate synaptic vesicle release, immediate early genes, and intracellular calcium receptor proteins (Murphy, Worley and Baraban 1991, Simon and Llinas 1985, Bauer, Schafe and LeDoux 2002). They can produce membrane depolarizations and "spikes" of membrane potential that are visible electrophysiologically (Hagiwara and Byerly 1981). These are believed to facilitate the voltage dpendent currents in ABNs seen following administration with tetrodotoxin (Isaacson and Strowbridge 1998).

1.4.3 Participation in neuronal development

Proliferation of ABN precursors is regulated through neurotransmitters and activity dependent mechanisms. Precursors of ABNs express voltage channels similar to those expressed embryonically (Schmidt-Hieber, Jonas and Bischofberger 2004, Espósito, et al. 2005). Glutamate, acting through an NMDA dependent receptor reduced the amount of proliferation of precursor cells in the DG (Cameron, McEwen and Gould 1995). Serotonin upregulates proliferation of precursor cells in the SVZ and dentate gyrus (Banasr, et al. 2004, Brezun and Daszuta 1999). The level of activity in the hippocampus has been shown to regulate proliferation (Deisseroth, et al. 2004). And physical activity, learning tasks, and enriched

environments, thought to involve the hippocampus, have been shown to increase proliferation (Van Praag, et al. 1999, Brown, et al. 2003, Kempermann, Kuhn and Gage 1997).

Daughter cells from SVZ progenitors migrate up to 5mm tangentially along the RMS to reach the OB in mice (Lledo and Saghatelyan 2005). Though this process occurs prior to the expression of voltage gated sodium channels (Petreanu and Alvarez-Buylla 2002, Carleton, et al. 2003) it is none-the-less regulated by neuronal activity. GABA and GLU_{K5} receptors , with associated Ca^{2+} influxes, reducing the speed of migration (Bolteus and Bordey 2004, Platel, et al. 2008). Additionally the protein Tenascin-R recruits migrating neuroblasts in an activity dependent manner to the OB and binds extracellular to voltage gated sodium channels (Saghatelyan, et al. 2004, Srinivasan, Schachner and Catterall 1998).

The establishment of the dendritic tree is a dynamic process under complex regulation, including regulation by neuronal activity (Konur and Ghosh, Calcium Signaling and the Control of Dendritic Development 2005). This is especially true for ABNs in the olfactory bulb which show high levels of dynamic outgrowth after reaching the OB (Mizrahi, et al. 2006, Mizrahi 2007). During development of somatosensory and visual systems afferent activity leads to high levels of dendritic outgrowth and the formation of synapses (Wise, Fleshman and Jones 1979, Wu, et al. 1999). Increasing the level of afferent activity through enriched environments leads to increased dendritic branching and synapses (Greenough, Volkmar and Juraska 1973, Volkmar and Greenough 1972, Holloway 1966). While reducing activity through pharmacological block, severing the afferent path, or reducing sensory stimuli leads to a deficits in dendritic growth (Wiesel and Hubel 1963, Riesen and Coleman 1968, Rajan and Cline 1998, McAllister, Katz and Lo 1996). The voltage gated sodium channels that we manipulate throughout this work have been postulated to regulate dendritic outgrowth through calcium-dependent regulation of

CamKII and CREB (Redmond, Kashani and Ghosh 2002, Konur and Ghosh, Calcium Signaling and the Control of Dendritic Development 2005). Sensory dependent calcium entry regulated by NMDA receptors has also been shown to be important for dendritic outgrowth (Konur and Yuste 2004, Miller and Kaplan 2003). This bears particular importance given NMDA drives activity necessary for the survival of ABNs in the hippocampus (Tashiro, et al. 2006). Overall, the literature points to the importance of voltage gated channels, and especially calcium entry, as important for the formation of dendrites, synapses, and ultimately neuronal survival.

1.4.4 Compensatory responses to a reduction in expression of voltage gated sodium channels

Reducing the expression of voltage gated sodium channels can be expected to drive a compensatory response in these cells (Rosati and McKinnon 2004).

In humans, epilepsy conditions caused by a miss-sense mutation of Nav1.1 lead to GABAergic interneurons in the hippocampus that are unable to sustain action potentials (Yu, et al. 2006, Catterall 2012). These parvalbumin fast spiking neurons rely on $Na_v1.1$ for the majority of their sodium current and their inhibition leads to runaway seizure activity in infancy driven by the freed excitatory neurons (Ogiwara, et al. 2007). Excitatory neurons in this condition, as well as other interneurons in other areas, have multiple other voltage gated sodium channels to rely on (for instance $Na_v1.6$ is largely expressed in myelinated axons of excitatory neurons). Interestingly, expression of mutant $Na_v1.1$ in this study did not produce a compensatory increase in the expression of other neuronal alpha subunits $Na_v1.2$, $Na_v1.3$, or $Na_v1.6$ (Ogiwara, et al. 2007) though $Na_v1.1$ represents only 10% of basal neuronal sodium channel expression (Gordon, et al. 1987).

During early fetal development caudal areas of the brain switch from $Na_v1.2$ to $Na_v1.1$ and $Na_v1.3$ expression, while initially high reduces after birth (Beckh, et al. 1989) . Indicating that brain areas can express multiple VGSC variants and change expression patterns in response to developmental factors. Knock-out of $Na_v1.1$ in mice drives a compensatory increase in expression of $Na_v1.3$ in GABAergic inhibitory interneurons in the hippocampus (Yu, et al. 2006). Other voltage gated channels can see similar compensatory responses with L-type calcium channels becoming over expressed in the heart following $Ca_v1.2$ knockout (Rosati, Yan, et al. 2011). Combined these results show that compensatory changes in channel expression can follow manipulation of channel expression in neurons.

1.5 EXPERIENCE DEPENDENT PLASTICITY AND THE EFFECT OF SENSORY DEPRIVATION ON THE DEVELOPMENT OF NEURONAL CIRCUITS

Work in this thesis draws on a rich history of study of activities role in the development of neuronal circuits. Hubel and Wiesel pioneered this work in the cat visual cortex for which they received the 1981 Nobel Prize in Physiology or Medicine (Wiesel and Hubel 1963). Following their work other areas of the brain such as somatosensory cortex (Fox and Wong 2005) and olfactory bulb (see proceeding section) have been explored.

1.5.1 A critical period for experience dependent plasticity in the visual cortex

Hubel and Wiesel pioneered the investigation of experience dependent plasticity using the monocular deprivation technique to investigate visual receptive fields in the cat visual cortex

(Wiesel and Hubel 1963, Wiesel, Hubel and Lam 1974). These changes are activity dependent, as binocular enucleation at birth, which removes retinal input from the process, spares normal visual fields (Crowley and Katz 1999). Monocular deprivation of visual input causes shrinkage of the thalamic axonal arbors corresponding to the deprived eye (Antonini and Stryker 1993, Antonini and Stryker 1996). And, dendritic branching and cortical responses in corresponding visual fields of the visual cortex are also reduced following monocular deprivation (Kossel, Lowel and Bolz 1995, Gordon and Stryker 1996, Mataga, Mizuguchi and Hensch 2004, Wallace and Bear 2004). These effects are strongest early in development with significant depressive effects at p23 of rodents and ceding to a potentiating only effect at p30 (Fagiolini, et al. 1994, Gordon and Stryker 1996). The effects of monocular deprivation in the visual cortex are largely independent of neuronal survival of cortical neurons as the process is reversible within the critical period (Movshon 1976, Blakemore and Van Sluyters 1974).

1.5.2 Critical period plasticity in the somatosensory cortex

Denervation of sensory nerves projecting to adult somatosensory cortex lead to a reduction in the cortical fields representing skin fields in a process that potentiates the responses of spared surrounding fields (Merzenich, et al. 1983, Glazewski, et al. 1996). In young mice (<6 months old) whisker pruning also leads to a depression in whisker input into the somatosensory cortex (Fox, Wallace and Glazewsk 2002, Glazewski, et al. 1996). Because this depression does not occur in adult somatosensory neurons it holds particular interest in olfactory neurogenesis where the excitatory scaffolding is largely stable and young, highly plastic, inhibitory neurons are innervating the OB (Mizrahi, et al. 2006). Dendritic spines in the somatosensory cortex exhibit less motility following whisker trimming, highlighting the role of sensory activity in driving

synapse formation in young neurons (Lendval, et al. 2000). Dendritic spine loss is also seen in young somatosensory cortex neurons following whisker trimming (Zuo, et al. 2005).

1.6 THE INFLUENCE OF ACTIVITY ON ADULT NEUROGENESIS IN THE OLFACTORY BULB

Every day tens of thousands of ABNs are added to the rodent OB. This unique form of plasticity adds new inhibitory units to the OB that are able to form new combinations of synapses with mature neurons. This entire process is exquisitely regulated by neuronal activity (see below, reviewed in Kempermann 2008).

1.6.1 Extrinsic activity

One of the earliest observations about the role of activity in regulating OB adult neurogenesis was that odor deprivation is a negative regulator of ABN survival. Unilateral naris occlusion reduces the survival and integration of ABNs (Corotto, Henegar and Maruniak 1994, Cummings, Henning and Brunjes 1997, Yamaguchi and Mori 2005). Genetically anosmic mice show reduced survival and increased ABN cell death (Petreanu and Alvarez-Buylla 2002). The regulation by reduced sensory activity effects the survival and maturation process once ABNs reach the OB but does not effect the proliferation of Type A cells in the SVZ, or migration of ABNs through the RMS (Petreanu and Alvarez-Buylla 2002). Interestingly, the regulation of ABN survivals has a critical period centered around just after ABNs reach the OB and begin

forming synapses from 2-4 weeks after birth (See Chapter 2, Yamaguchi and Mori 2005, Lin et al. 2010).

Conversely, ABN survival is increased by exposure to enriched odor environments and rich enviromental olfactory tasks. Enriched sensory activity and olfactory learning has been shown to increase the number of ABNs surviving in the olfactory bulb (Rochefort, et al. 2002, Alonso, et al. 2006, Moreno, et al. 2009) but for a more naunaced view see (Mandairon et al. 2006); decrease the number of ABNs expressing markers of cell death (Woo, et al. 2006); and promote synaptogenesis (Livneh, et al. 2009). In addition to the positive regulation of survivial by sensory activity, ABNs are recruited into neural circuits for odorants they were exposed to shortly after reaching the olfactory bulb: 2-3 weeks after birth, during the period when they are initially forming synapses (Magavi, et al. 2005, Moreno, et al. 2009)

Radial migration by ABNs in the OB is effected by manipulations that regulate neuronal activity. The receptor binding protein Tenascin-R in particular has been shown to be differentially expressed following reduction in sensory stimuli through naris occlusion, suggesting that activity may play a role in the migration of ABNs to the OB (Saghatelyan, et al. 2004). This result suggests the intriguing possibility that neural activity could recruit for radial migration of ABNs as they enter the bulb, with areas of high activity recruiting additional inhibitory ABNs.

1.6.2 Intrinsic activity

Many manipulations of ABN intrinsic activity have utilized a lentivirus delivery system to drive expression of a membrane channel. Genetic knockout of the endogenous NMDA receptor drastically decreases survival of ABNs in the hippocampus (Tashiro, et al. 2006). This

decreased survival translates to decreased ABN survival in the olfactory bulb (Lin, et al. 2010). Expression of the non-rectifying potassium channel ESKir2.1 also reduces neuronal activity in OB ABNs and leads to their decreased rate of survival (Lin, et al. 2010).

Recently, Lin and colleagues performed an elegant study utilizing a bacterial sodium channel $NaChBac^{+}$ to increase the intrinsic activity in ABNs (Lin, et al. 2010). This bacterial sodium channel activates in a prolonged manner with small depolarizations and is thus believed to increase intrinsic activity. When expressed in ABNs through a lentivirus delivery system $NaChBac^{+}$ causes an increase of survival of 22% at 21 d.p.i. Remarkably, $NaChBac^{+}$ expression completely rescued the decrease in survival caused by genetic knock out of the NMDA receptor and reduction of sensory stimuli by occlusion of the naris. In a separate study, Kelsch and colleagues described the effect of $NaChBac^{+}$ expression on synaptic development of adult-born GCs in the OB (Kelsch, et al. 2009). In this case, $NaChBac^{+}$ expression did not change normal synaptic development but rescued changes caused by sensory deprivation.

It's clear that there exists and interplay between extrinsic and intrinsic activity in the regulation of OB adult neurogenesis. What is not as clear is how the interplay between extrinsic and intrinsic sources of activity drives survival and integration of newborn neurons into the adult brain. The following research chapters of this dissertation describe the experiments and their results which attempt to address these important questions.

1.7 DATA CHAPTERS INTRODUCTION

In this introduction, I have presented a broad outline of the field of mammalian adult neurogenesis in the olfactory bulb. I have necessarily limited the subject of this discourse but

there is a rich field of study in species outside of mammals, in cultured neural stem cells, and in treatments and disease states. If the reader should be interested in learning more many of these fields are reviewed in the text-book "*Adult Neurogenesis*" (Gage, Kempermann and Song 2008). The proceeding sections of this dissertation address the interplay between extrinsic and intrinsic neuronal activity in the regulation of survival (Chapter 2) and functional integration (Chapter 3) of ABNs once they reach the mouse OB. It briefly concludes by discussing general implications and possible future directions (Chapter 4).

1.7.1 Hypothesis and expected results

The hypotheses in the following chapters center around the interplay in intrinsic activity and extrinsic activity in the survival and integration of ABNs in the olfactory bulb. The following tables are included to help conceptualize the results that have been proposed.

	Lowered intrinsic activity	**Lowered extrinsic activity**	**Lowered intrinsic & extrinsic activity**
Survival rate at 21-35 d.p.i.	Decreased	Decreased	Increased
Spine density	Decreased at proximal syanpses	No measured effect	Not assessed
Dendritic complexity	Reduced volume proximal to soma, reduced branching proximal to soma	No measured effect	Not assessed

Table 1. Expected results for experimental conditions and measurements

2.0 COMPETITIVE SURVIVAL OF ADULT-BORN NEURONS IN THE MOUSE OLFACTORY BULB

2.1 CHAPTER SUMMARY

Adult-born neurons are generated and integrate into the olfactory bulb throughout life. This process is regulated by sensory input and cell-intrinsic activity but the interaction between them remains unknown. Here we use both virus-mediated reduction of cell intrinsic activity and reduced sensory stimuli to show that survival is a competitive process in which the most active cells in the network have the highest chance of survival. RNA-based knockdown of Na^+ channels resulted in decreased survival of adult-born neurons from 10-21 d.p.i. We then investigated the role of circuit level activity by inducing sensory deprivation through naris occlusion. Adult-born neurons with normal cell-intrinsic activity showed a reduction in survival in a sensory deprived circuit. But, interestingly adult-born neurons with reduced intrinsic excitability showed an increased rate of survival in a sensory deprived circuit. These results demonstrate that survival of adult-born neurons in the olfactory bulb follow a competitive, activity-dependent process in which the activity of new neurons themselves as well as the activity of the mature existing brain circuits vie to determine which neurons will survive.

2.2 INTRODUCTION

The adult mammalian olfactory bulb and hippocampus both receive a steady supply of newly-born neurons throughout life (reviewed in Ming & Song, 2005). In the olfactory bulb, these new neurons are produced by neural stem cells in the subventricular zone (SVZ) of the lateral ventricle. After birth, they migrate several millimeters along the rostral migratory stream (RMS) from the anterior horn of the lateral ventricle to the olfactory bulb (OB). Finally, within the OB, they migrate radially and ultimately become inhibitory neurons in either the granule cell layer (GCL, ~95% of new neurons) or the glomerular layer (GLL, ~5% of new neurons) (Lois and Alvarez-Buylla 1994, Petreanu and Alvarez-Buylla 2002).

Most of the new neurons in the OB die within 4 weeks of their final division (Petreanu and Alvarez-Buylla 2002, Winner, et al. 2002). Several factors regulate the survival of these adult-born neurons (ABNs), and a great deal of interest has been focused on understanding how activity regulates the integration of ABNs into existing circuits (Carlén, et al. 2002, Rochefort, et al. 2002, Carleton, et al. 2003, Belluzzi, et al. 2003, Saghatelyan, et al. 2005, Kelsch, Lin and Mosley, et al. 2009, reviewed in Lledo and Saghatelyan 2005).

Activity may regulate survival of ABNs through both cell-intrinsic and network mechanisms. In the olfactory bulb and hippocampus, reducing cell-intrinsic activity through cell-specific knock out of NMDA receptor or expression of the ESKir2.1 potassium channel reduces the survival of ABNs (Tashiro, et al. 2006, Lin, et al. 2010). Increasing cell-intrinsic activity through the expression of a bacterial sodium channel with a low activation threshold and slow inactivation increases survival of olfactory bulb neurons (Lin, et al. 2010). Manipulations that reduce network activity such as occlusion of the nares, genetic anosmia, treatment with the $GABA_A$ modulator diazepam, or chronic nicotine exposure decrease survival of ABNs (Corotto,

Henegar and Maruniak 1994, Petreanu and Alvarez-Buylla 2002, Yamaguchi and Mori 2005, Mechawar, et al. 2004). Alternatively, behavioral manipulations that are expected to increase network activity such as sensory driven activity via exposure to a rich set of novel odors increase survival (Rochefort, et al. 2002, Miwa and Storm 2005). As does olfactory discrimination training which likely drives increased neuronal activity via both afferent and sensory pathways (Alonso, et al. 2006). To date it is not known how these two activity-dependent mechanisms of integration interact.

One could propose two mechanisms for activity-dependent survival: 1) A process in which survival of ABNs depends on the absolute level of activity attained in individual neurons, and 2) A process in which the activity of ABNs is relatively compared to other neurons in the circuit to determine survival. Differentiating between these two possibilities requires manipulating the level of absolute activity of individual ABNs and independently manipulating the relative activity of the circuit. Here we use a combination of virus-mediated reduction of cell-intrinsic activity and reduction of network activity through sensory deprivation to investigate the roles of cell-intrinsic versus network activity on the survival of ABNs. We find that a competitive process exists, whereby the most active cells in the network have the highest chance of survival regulates ABN survival in the olfactory bulb.

2.3 MATERIALS & METHODS

2.3.1 Lentivirus preparation

Lentiviruses were produced in the lab of Pavel Osten as described previously (Dittgen, et al. 2004). The shRNA vector was based on FU6siGURW, and expressed EGFP and additionally two short hairpin RNA cassettes against homologous sequences of voltage gated sodium channels $Na_v1.1$, $Na_v1.2$, & $Na_v1.3$ under the control of the human ubiquitin promoter (Dittgen, et al. 2004, Komai, et al. 2006). The EGFP lentiviral vector, FUGW (Flap-Ubiquitin promoter-EGFP-WRE), drove expression of Enhanced Green Fluorescent Protein under the control of the human ubiquitin promoter (Lois, et al. 2002). The tdTomato lentiviral vector, FURW (Flap-Ubiquitin promoter-tdTomato-WRE), drove expression of a monomeric second-generation red fluorescent protein (tdTomato) under the control of the human ubiquitin promoter. The scrambled shRNA vector was based on FU6siGURW and expressed a scrambled sequence of shRNA that did not target any known mRNA (Komai, et al. 2006). All viruses were drawn consistently from lot-specific stocks with viral tires greater than 10^5 infectious units per μl and stored at -80°C in single use aliquots.

2.3.2 Stereotaxic lentivirus injections

Lentivirus injections were performed as previously described (Dittgen, et al. 2004, Cetin, et al. 2006). Briefly, mice were anesthetized by IP injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) in water. Animals were kept deeply anesthetized as assessed by monitoring eyelid reflex, vibrissae movements, and pinch withdrawal. Body temperature was maintained at

37°C with a heating pad with digitally monitored feedback control. Two craniotomies 300-500 µm in diameter were drilled above the SVZ (final coordinates relative to Bregma: anterior -1mm, lateral ±1mm, depth 2.2 mm beneath the pia mater). Pulled glass quartz pipettes were broken to an outer diameter of ≈30 µm and inserted at a 27° angle caudal-rostral into the pia. 500 nL of lentivirus was injected slowly over ≈5 min.

2.3.3 Lentivirus mixtures used to make experimental comparisons

Mice were injected with either single lentivirus constructs (when no cross-hemisphere comparisons were made) or an equal volumetric mixture of two lentivirus constructs (when cross-hemisphere comparisons were made). All mixtures contained an equal volumetric ratio of FURW and either FUGW (**lenti-GFP** condition), FU6siGURW (**lenti-shRNA** condition), or FU6siGURW with a scrambled shRNA sequence that did not target a known protein sequence (**lenti-scrambled-shRNA** condition). FUGW drove expression of solely EGFP. This construct was used to label populations of ABNs without affecting their normal levels of intrinsic activity and is denoted as the **lenti-GFP** condition. FU6siGURW drove expression of EGFP and additionally two short hairpin sequences against homologous sequences of voltage gated sodium channels Nav1.1, Nav1.2, and Nav1.3. This construct was used for ABNs with reduced levels of cell-intrinsic activity and is denoted as the **lenti-shRNA** condition. Another lentivirus utilizing scrambled shRNA along with a sequence encoding GFP was used in control experiments paralleling **lenti-shRNA** and is denoted **lenti-scrambled-shRNA** The last construct drove expression of a monomeric red fluorescent protein tdTomato. This construct was used in the time course cell-intrinsic activity experiments to provide an ABN population with normal levels of activity and a constant viral titer across hemispheres. The two lentivirus mixtures including

red and green fluorescent constructs used in the time course cell-intrinsic activity comparison experiments (see sections 2.4.4 & 2.4.6) were injected into opposite but randomly-selected hemispheres in the same animal. They were prepared as follows: Normal activity condition, referred to as **lenti-GFP**; two viral constructs FUGW & FURW combined in a constant volumetric ratio from consistent preparation lots with consistent viral titers, reduced cell-intrinsic activity condition, referred to as **lenti-shRNA**; two viral constructs FU6siGURW & FURW combined in a constant volumetric ratio (identical to the normal activity condition cocktail) from consistent preparation lots and consistent viral titers. Thus, the exact ratio of red fluorescing FURW was present in both cocktails and served as the common factor in comparisons across hemispheres.

2.3.4 Unilateral olfactory deprivation

Mice were anesthetized by IP injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) in water. After animals became non-responsive as monitored by tail pinch, their external naris was closed by cauterization. A cautery tool (Fine Science Tools, Heidelberg, Germany), consisting of a fine loop of platinum wire that is rapidly heated by passage of electrical current, was placed against the nostril until the skin flap that covers the naris is "melted" onto the inner surface. Immediately following the cautery, the area was swabbed with antibacterial/anesthetic gel and the animal was returned to a warmed recovery cage where it was monitored until recovery 48 hours post procedure. Extent of naris closure was assessed when animals were sacrificed. Only tissue from animals in which the occluded naris remained completely closed was used in immunohistochemistry and analysis.

2.3.5 Odor exposure

Animals were housed within individual cages for 35 days prior to odor exposure. Two and a half hours prior to sacrifice, animals were placed in their individual home cages, an airtight aquarium and exposed to 30 minutes of carbon filtered house air bubbled through mineral oil. This was followed by 30 minutes of carbon filtered house air bubbled through orange essential oil (Of the goddess Ltd., W. Toluca Lake, CA, USA) diluted 1:3 in mineral oil, followed by 90 minutes of carbon filtered house air bubbled through mineral oil. Animals performed investigative behaviors such as rearing during the initial exposure to odor but ceased these behaviors within ~2 minutes. Animals had free access to water but were not provided with food during the odor exposure. Animals were sacrificed directly after the procedure was completed.

Figure 13. Odor exposure experimental setup.

2.3.6 Immunohistochemistry

Mice were anesthetized by IP injection of ketamine (100 mg/kg)/xylazine (10 mg/kg) in water. Once the animals were non responsive, as monitored by tail pinch, they were perfused transcardially with 1% NaCl in phosphate buffer (PB) followed by 4% paraformaldehyde in 0.1M PB. The brains were dissected and post fixed in 4% paraformaldehyde in PB overnight and then sunk in 30% sucrose. Each hemisphere of the brains was sliced using a SM2000R cryostat (Leica microsystems, Bannockburn, IL). Coronal sections from the olfactory bulb were then used for double immunofluorescence labeling. Incubations were performed at room temperature with oscillation. Sections were first incubated in 2% Triton X-100 (Sigma) and 2% normal donkey serum (NDS) (Jackson Immuno Research Laboratories) in PB for 1 hour. The antibodies were added in the presence of 2% NDS and 0.1% TritonX-100 in PB and incubated for 1 hour. Primary antibodies used were: goat anti-GFP at 1:10000 dilution (Abcam, Cambridge, MA, USA), rabbit anti-GFP at 1:1000 dilution (Abcam, Cambridge, MA, USA), rabbit anti-RFP at 1:1000 dilution (Clontech, Mountain View, CA, USA), NeuroMab Mouse anti-Nav1.2 (Antibodies Inc, Davis CA), goat anti-calretinin at 1:1500 dilution (Santa Cruz, Santa Cruz, CA, USA), and mouse anti-n-copine at 1:1000 dilution (Abcam, Cambridge, MA, USA). Secondary antibodies used were donkey AlexaFluor 488 anti-rabbit at 1:600 dilution, donkey AlexaFluor 488 anti-goat at 1:600 dilution, donkey AlexaFluor 594 anti-goat, donkey AlexaFlour 594 anti-rabbit at 1:600 dilution (all secondary antibodies from Molecular Probes, Carlsbad, CA, USA. Sections were then washed three times between each incubation step in PB. After the addition of the first secondary antibody all incubations were performed in the dark.

2.3.7 Imaging procedures

Multiphoton imaging. Sections were imaged in their entirety using a Carl Zeiss LSM 510 Meta NLO two-photon microscope (Carl Zeiss, Jena, Germany). Simultaneous excitation of both EGFP and tdTomato was provided by a Chameleon Ultra-II femtosecond laser (Coherent, Santa Clara, CA) operating at 890nm. Non-descanned detection was provided by two silicon photomultiplier tubes situated on the back of the fluorescence light train of the Axio Observer Z1 inverted microscope stand. A long-pass 680nm blocking filter was placed in front of the detection assembly to prevent any stray two-photon excitation light from reaching them and a 565nm dichroic was subsequently used to split the emission wavelengths between detectors. Individual band pass barrier filters (505-550nm and 575-640nm) were placed in front of each detector for fluorescence emission selection. All images were produced using a 10x 0.3NA EC-Plan Neofluar air objective (0.3NA, Carl Zeiss, Jena, Germany). Z steps for each section were set at 4.0µm (~1 airy unit).

2.3.8 Confocal imaging

100 µm of coronal sections sliced from directly anterior to the anterior olfactory bulb were imaged in their entirety using a Carl Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany). Coronal sections used for cell counts were in most cases 10 µm thick but were sometimes sliced at 300 µm thick if whole cell bodies and dendrites were used for cell reconstructions. In all cases, the section was resolvable completely in all three dimensions using multi-photon imaging. Excitation of 488nm fluorescent antibody conjugates was achieved using a 200mW Argon laser. Excitation of 594 fluorescent antibody conjugates was achieved using a

150mW Argon laser. Descanned detection was achieved using a pinhole width of 65µm. Light detection was provided by two silicon photomultiplier tubes situated on the back of the fluorescence light train of the Axio Observer Z1 inverted microscope stand. Individual band pass barrier filters (505-550nm and 575-640nm) were placed in front of each detector for fluorescence emission selection. All images were produced using a 20x 0.8NA EC-Plan Neofluar air objective (0.8NA, Carl Zeiss, Jena, Germany). Z steps for each section were set at 1.7µm (~1 airy unit). Image stacks were produced in interleaved channels by taking single confocal planes at 488nm and 594nm excitations before moving through the Z plane.

2.3.9 Cell counting procedures

Cell bodies located within the complete volume 100 µm directly anterior to the AOB were counted from ten 10 µM thick coronal sections. The complete volume was imaged using multiphoton imaging (see preceding section). The volume was traced using the Neurolucida software package (MBF Bioscience, Williston, VT), to resolve the GLL and GCL the two anatomical regions of the olfactory bulb in which adult-born neurons survive and mature along with other anatomical regions of the OB such as the MCL and anterior commisure. Cells somata were counted by hand within the GLL and GCL, excluding the subependymal zone/anterior commisure. In order to calculate the measure of survival used in Chapter 2.4.4 and 2.4.6, total cell counts from a single animal we're combined into a Green somata to (Red Somata – yellow somata) ratio. Yellow cells were removed from experimental condition which was consistently encoded by the Red channel. Then the ratio of **lenti-GFP to lenti-shRNA** was calculated for a single measure of survival for that animal. These animal ratios were then used to compare across

experimental conditions. Cell counts from only the GCL were used in analysis of calcium binding protein subtype results (Chapter 2.4.7).

	% Green cells also red	% Red cells also green
21 d.p.i. **lenti-GFP**	0.5% (n=18,864 somata)	45.9% (n=218 somata)
21 d.p.i. **lenti-shRNA**	6.8% (n=3,333 somata)	16.2% (n=1,398 somata)
35 d.p.i. **lenti-GFP**	0.6% (n=23,221 somata)	47.0% (n=315 somata)
35 d.p.i. **lenti-shRNA**	6.1% (n=2,964 somata)	16.9% (n=1,068 somata)

Table 2. Ratio of cells expressing lentiviruses in multiple experimental conditions

2.3.10 $Na_v1.2$ Immunohistochemisty quantification

Expression of $Na_v1.2$ was measured in 60 µm fixed slices immunostained with a primary antibody targeted to $Na_v1.2$ protein. During immunolabeling slices were treated and washed identically within the same well. Intensity values for fluorescence were taken at the widest circumference of each cell using elliptical ROIs in ImageJ 1.3 (National Institutes of Health, Bethesda, MD), several example ROIs are drawn in (Figure 17b). Fluorescent values were normalized based on the average intensity value in the middle 20 µm of each slice.

2.3.11 Electrophysiology procedures

2.3.11.1 *Acute slice preparation*

Slices were prepared as described previously in Urban and Sakmann 2002 and Kapoor and Urban 2006. Briefly, coronal olfactory bulb slices (300–350 µm thick) were prepared from mice injected 21-35 days previously with the lentivirus constructs described in the stereotaxic lentivirus injection section on the day of physiological experiments mice were postnatal day 45-

60. Olfactory bulbs were removed and sectioned on a vibratome (Leica microsystems, Bannockburn, IL) while submerged in ice-cold oxygenated Ringer's solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 25 glucose, 2.5 $CaCl_2$. In some experiments, sucrose substituted Ringer's solution was used for this step containing the following (in mM): 238 Sucrose, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 25 glucose, 2.5 $CaCl_2$, 0.5 ascorbate, 2 *myo*-inositol. Following slicing all tissue was incubated in the Ringer's solution described above at 37°C for thirty minutes after which it was returned to room temperature. All animal care was in accordance with the guidelines of the Institutional Animal Care and Use Committee of Carnegie Mellon University.

2.3.11.2 *Whole cell patch clamp recording*

Slices were superfused with the oxygenated Ringer's solution described above (at 1-2 ml/min) and warmed to 34–36°C. Whole-cell recordings were established using pipettes (resistances of 7-12 M) filled with a solution containing the following (in mM): 120 potassium gluconate, 2 KCl, 10 HEPES, 10 sodium phosphocreatine, 4 MgATP, and 0.3 Na_3GTP, adjusted to pH 7.3 with KOH. Cells were visualized using infrared differential interference contrast (DIC) optics (Olympus BX51WI, 60x 0.9 NA objective) and video microscopy (Dage/MTI). Voltage-clamp and current-clamp recordings were performed using a MultiClamp 700B amplifier (Molecular Devices, Union City, CA). Data were filtered (4 kHz low pass) and digitized at 10 kHz using an ITC-18 (Instrutech, Mineola, NY) controlled by custom software written in IgorPro (Wavemetrics, Lake Oswego, OR). A series of hyperpolarizing and depolarizing current steps (1 second or 250ms duration) was applied to determine the intrinsic sub and supra threshold properties of granule cells. All electrophysiology analysis was performed with custom software written in IgorPro.

Cells were targeted for recordings based on several criteria: 1) Somata were located in the GCL of slices, 2) Somata were fluorescent indicating GFP expression (The fluorescence was confirmed and documented by identifying fluorescent membrane pulled into the pipette), 3) Somata had a single dendrite extending towards the EPL, 4) Dendrites exhibited spines. This phenotype corresponds to Class 5 adult born neurons (Petreanu and Alvarez-Buylla 2002) which have a high level of morphological maturity which does not develop prior to 15-22 days post birth (Petreanu and Alvarez-Buylla 2002, Carleton, et al. 2003).

Slices we're prepared from animals that had been injected with either **lenti-GFP** or **lenti-shRNA** 21-35 days previously. Analyses we're completed on ABNs grouped across these time points except where noted. In experiments that looked at ISI and spike timing, action potentials we're further grouped across current injections. Overall electrophysiological results we're drawn from 332 action potentials across 11 **lenti-shRNA** ABNs and 494 action potentials across 10 **lenti-GFP** ABNs.

2.3.12 Statistics

All comparisons unless otherwise noted were made with a Student t-test. Comparisons of cell counts and fluorescent ratios made across hemispheres and within an animal were made using a 2-tailed paired Student t-test (survival experiments, immunohistochemistry experiments). Comparisons of individual ABN electrophysiological properties were made using individual ABNs from several animals and were tested using 2-tailed unpaired Student t-tests.

2.4 RESULTS

2.4.1 Lentivirus infection causes expression of fluorescent protein and knockdown of voltage gated Na^+ channels

In order to label and manipulate the level of activity within particular ABNs, we utilized three lentivirus constructs to infect neural precursor cells along the SVZ (Figure 12). The first construct drove expression of solely EGFP (**lenti-GFP**). The second construct drove expression of a monomeric red fluorescent protein tdTomato (**lenti-tdTomato**). These two constructs were used to label control ABNs with normal levels of cell-intrinsic activity. The third construct drove expression of EGFP and additionally two short hairpin sequences of RNA (**lenti-shRNA**) directed against homologous sequences of the voltage gated sodium channels $Na_v1.1$, $Na_v1.2$, and $Na_v1.3$ (Dittgen, et al. 2004, Komai, et al. 2006). This construct was used to reduce cell-intrinsic activity in infected ABNs. Expressing **lenti-shRNA** has previously been shown to reduce sodium channel driven excitability in cortical neurons 4-19 days after infection (Komai et al 2006). Granule cells in the olfactory bulb show expression of mRNA encoding voltage-gated sodium channels $Na_v1.1$, & $Na_v1.3$ (Lein et al. 2007), and immunohistochemical staining for $Na_v1.2$ protein (Figure 15a&b).

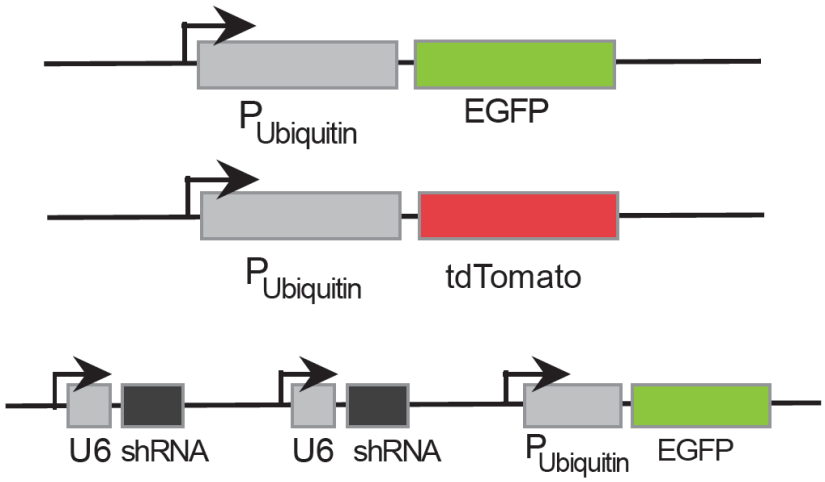

Figure 14. Lentiviral vectors.

Schematic of the lentiviral vectors used in the experiments, lentiviruses express EGFP (top, FUGR), tdTomato (middle, FURW), or a combination of EGFP + two shRNA sequences directed against homologous sequences of $Na_v1.1$-1.3 (bottom, FU6siGURW).

In the range of tens of thousands of lentivirus infected precursor cells migrated to the olfactory bulb. Once reaching the olfactory bulb, these cells migrated radially into the granule cell and glomerular layers, extended complex dendrites, and formed dendritic spines (Figure 15b). At 21 d.p.i., infected ABNs in the granule cell layer had the characteristic morphology of class III, IV, and V olfactory bulb granule cells, as reported previously (Petreanu and Alvarez-Buylla 2002).

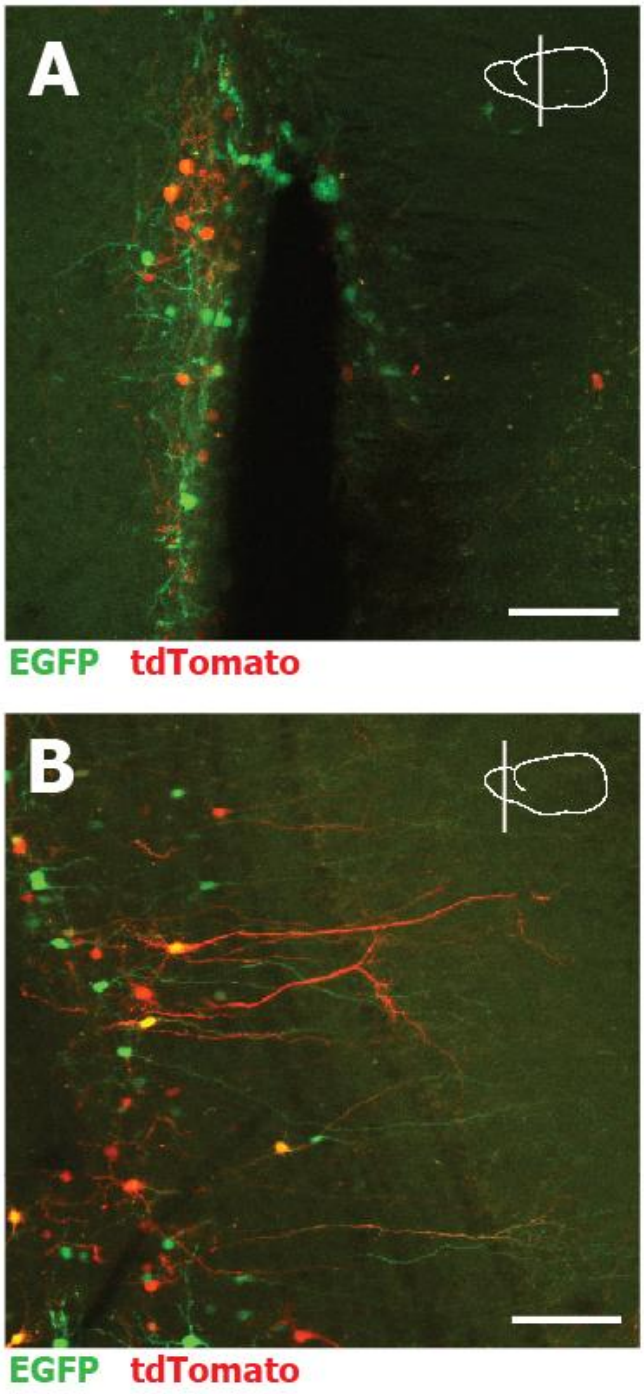

Figure 15. Lentivirus expression drives strong sustained expression of fluorescent proteins.

(**A,B**) Lentivirus constructs drive expression of **lenti-shRNA** and **lenti-tdTomato** and stably infect neural progenitor cells along the (**A**) subventricular zone and (**B**) granule cells in the OB 21 d.p.i. Images are maximum projections of confocal stacks, Green, anti-EGFP; Red, anti-tdTomato; scale bar 100μm.

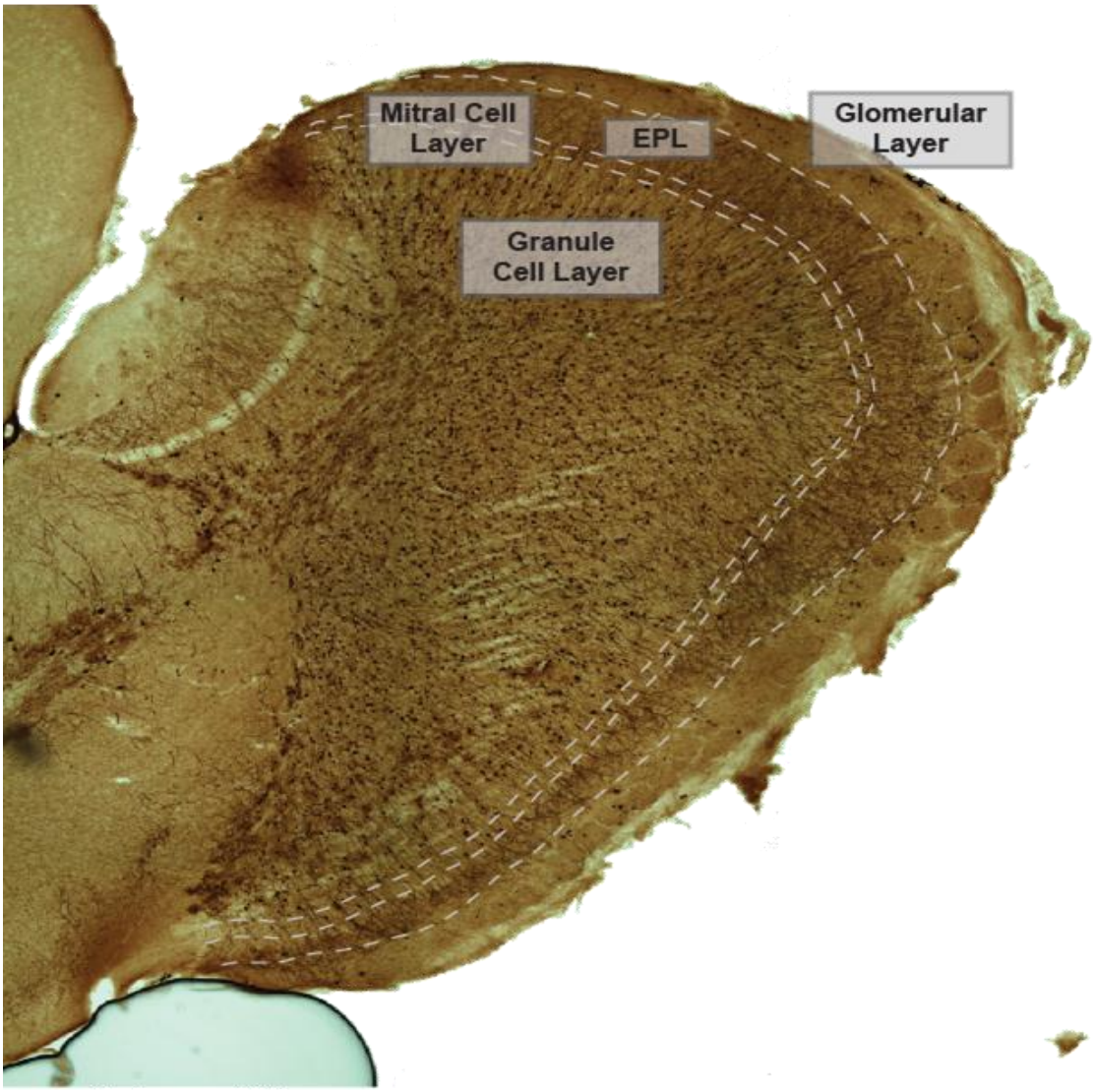

Figure 16. Lentivirus infected ABNs distribute evenly within the OB.

DAB staining against GFP shows lentivirus infected ABNs are distributed evenly across the GCL and GLL of the OB. Somata are easily identifiable throughout the GCL and GLL as dark brown spots.

We next directly compared the expression of sodium channels in **lenti-shRNA** infected ABNs in olfactory bulb slices with antibodies against one of the targeted sodium channels. Importantly $Na_v1.2$ protein was significantly reduced in the cell bodies of GFP^+ neurons expressing **lenti-shRNA** (Figure 15, 78±2.4% reduction, $p<0.001$, n=30 cells).

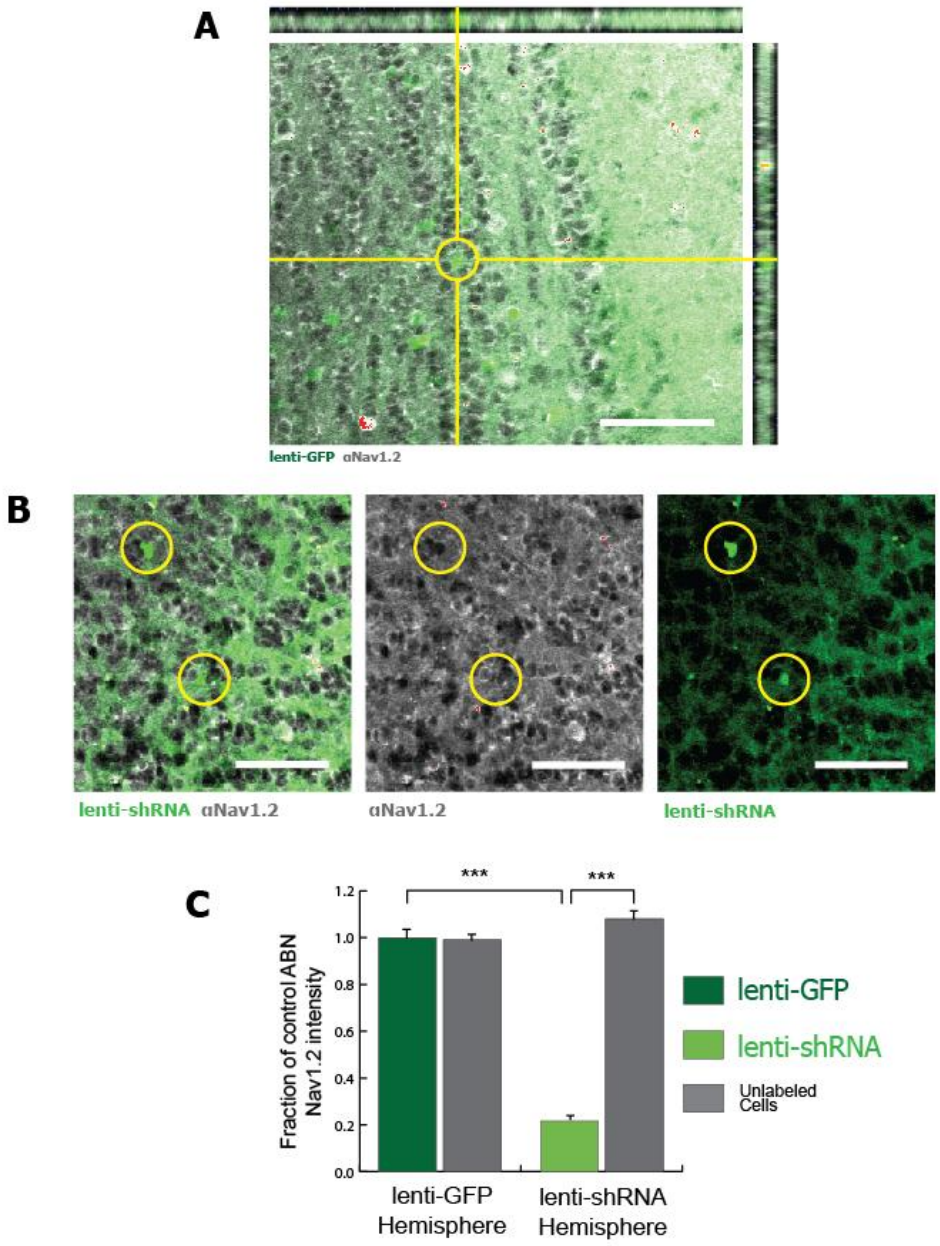

Figure 17. Lenti-shRNA expression lowers expression of voltage gated sodium channel $Na_v1.2$.

(**A**) $Na_v1.2$ immunohistrochemistry exhibits protein expression with a perinuclear staining pattern in the GCL of the OB in the **lenti-GFP** condition. Image is a single plane from a confocal stack with X and Y projections. (B) Sample $Na_v1.2$ immunohistochemistry of two **lenti-shRNA** ABNs. $Na_v1.2$ staining in the white channel has a considerable lowered intensity in **lenti-shRNA** cells which is visible qualitatively. (**C**) $Na_v1.2$ expression is significantly decreased in 21 d.p.i. **lenti-shRNA** adult-born granule cells compared to nearby uninfected cells (79.9% reduction, p value=<0.0001, n=30 cells per group) and **lenti-GFP** control adult-born granule cells in the opposite hemisphere of the same animal (78.2% reduction, p value=<0.0001, n=30 cells from a single animal per group). All scale bars are 50μm.

2.4.2 Knockdown of voltage gated Na^+ channels results in decreased excitability in adult-born neurons

Since our viral constructs significantly reduce Na^+ channel protein levels, we next investigated the effect of the knockdown on cell-intrinsic activity. To label ABNs for targeted electrophysiology we injected shRNA viral construct in one hemisphere (**lenti-shRNA** group) and GFP viral construct (**lenti-GFP**, control group) in the opposite hemisphere. We focused the test on mature ABNs as evidenced by a morphological phenotype consistent with Class 5 neurons (Petreanu and Alvarez-Buylla 2002, Carleton, et al. 2003). Due to this we expect that all ABNs targeted for electrophysiological experiments were at least 14-21 days old. To test whether expression of **lenti-shRNA** sequences caused a functional reduction in excitability we targeted these mature, fluorescently labeled, cells for in vitro whole cell recordings 21-35 d.p.i. and compared responses in the **lenti-shRNA** versus **lenti-GFP** groups. Sample voltage traces from all of the ABNs included in the analysis are included in Figure 17 & 18 and full traces with recorded current are included in Appendix A.

Stimulation of neurons in both groups via whole cell current injection evoked action potentials indicating that sodium channel expression was not completely eliminated (Figure 17 & 18). However, the membrane properties of ABNs in the two groups differed in several respects (Table 1). Consistent with a reduction in expression of voltage gated sodium channels, action potential threshold rose to -19±2.0mV for **lenti-shRNA** infected ABNs (n=9) from -25±3.0mV for **lenti-GFP** ABNs (n=10, p=0.047). Action potential amplitude was reduced by 31% (p=0.017), and the slope of the rise from threshold to peak was reduced by 38% (p=0.035).

Resting membrane potential, action potential width and rheobase, the minimal current amplitude that results in depolarization threshold, did not show significant changes between **lenti-GFP** and **lenti-shRNA** ABNs (Table 3). Most notably the maximum firing rate for **lenti-shRNA** ABNs (37.5±5.7Hz) was reduced by ~40% compared to **lenti-GFP** ABNs (64±5.9Hz, p=0.0028). Plots for action potential frequency versus measured current injections for the two populations showed significant decreases in action potential frequency at higher current injections (Figure 21a, p=0.012 at 75 pA, n=5 **lenti-GFP**, n=5 **lenti-shRNA** and p=0.03 at 84 pA, n=5 **lenti-GFP**, n=3 **lenti-shRNA**). Additionally the heights of action potentials occurring later in spike trains were significantly reduced in **lenti-shRNA** versus **lenti-GFP** ABNs as indicated by a one third reduction in the ratio of the heights of the first to eighth action potentials (Figure 20c, p=0.0025, n=10 **lenti-GFP**, n=9 **lenti-shRNA**).

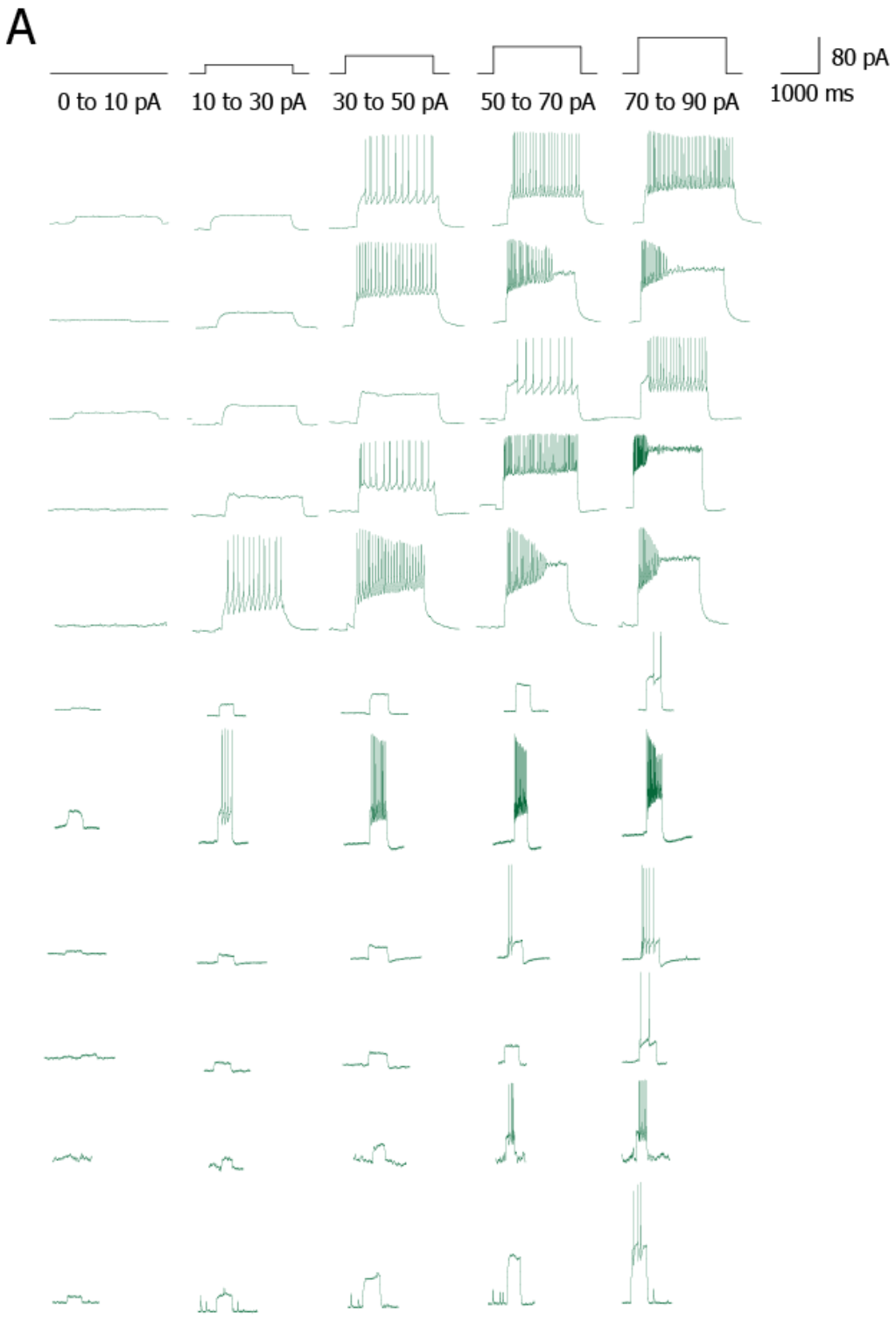

Figure 18. Sample electrophysiology traces from all lenti-GFP included in electrophysiology analysis

Representative voltage traces from 11 **lenti-GFP** ABNs. Rows represent distinct ABNs columns represent ranges of current injection. Full continuous voltage traces and recorded current injections are included in Appendix A.

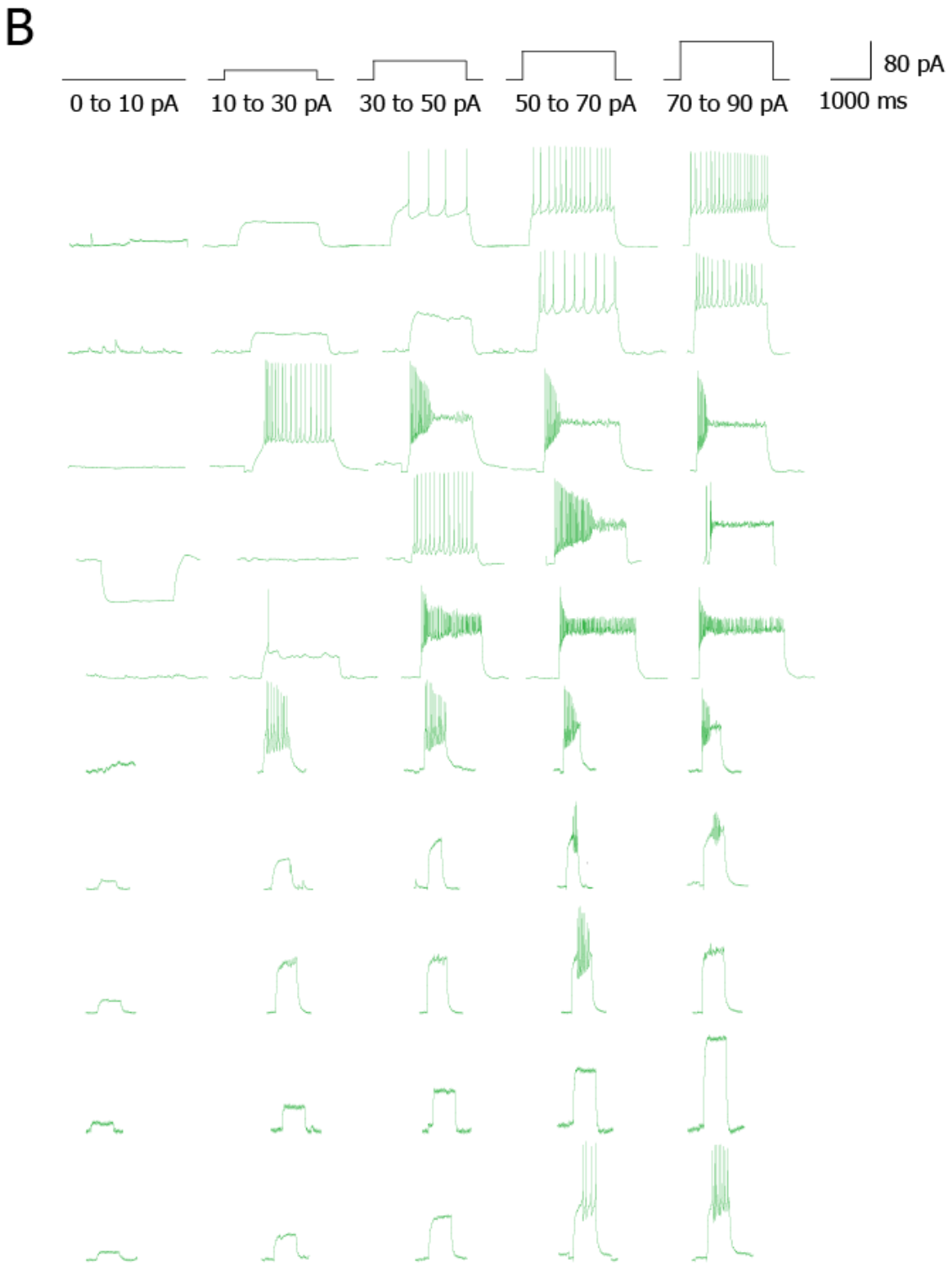

Figure 19. Sample electrophysiology traces from all lenti-shRNA included in electrophysiology analysis

Representative voltage traces from 10 **lenti-shRNA** ABNs. Rows represent distinct ABNs columns represent ranges of current injection. Full continuous voltage traces and recorded current injections are included in Appendix A.

Intrinsic Property	lenti-GFP	lenti-shRNA	P value
Resting Membrane Potential (mV)	-64.0 ± 2.2	-66.3 ± 3.5	0.29
R_{in} (MΩ)	717 ± 83	963 ± 240	0.21
$AP_{threshold}$ (mV)	-25.1 ± 3.0	-18.9 ± 2.0	0.047 (*)
$AP_{amplitude}$ (mV)	51.8 ± 4.8	35.7 ± 4.4	.017 (*)
$AP_{half\text{-}width}$ (ms)	1.06 ± 0.10	1.15 ± 0.15	0.35
Maximum Firing Rate (Hz)	64 ± 5.9	37.5 ± 5.7	0.0028 (**)
$AP_{rise\ slope}$ (mV/ms)	67.3 ± 8.5	41.7 ± 8.4	0.035 (*)
$Current_{threshold}$ (pA)	30.7 ± 9.9	49.9 ± 9.0	0.090
$1^{st}\ AP_{amplitude}$: $8^{th}\ AP_{amplitude}$	0.88 ± 0.04	0.61 ± 0.07	0.0025 (**)

Table 3. ABN biophysical properties *in vitro*

All measures are calculated from whole cell patch recordings from acute coronal olfactory bulb slices injected with a lentivirus construct 21-35 d.p.i. **lenti-GFP**; n=10 cells, n=6 animals. **lenti-shRNA**; n=9 cells, n=6 animals. Data are mean ± s.e.m. P values are calculated using a one tailed, unpaired student t-test (*) indicates $p<0.05$, (**) indicates $p<0.01$.

R_{in} , steady-state input resistance; $AP_{threshold,}$ action potential firing threshold; $AP_{amplitude}$, amplitude of the first action potential initiated as measured from threshold to peak; $AP_{half\text{-}width}$ action potential width measured at half height; 1^{st} $AP_{amplitude}$: 8^{th} $AP_{amplitude}$ Ratio of the height of the first action potential elicited to the height of the 8^{th} action potential elicited as measured at the first current injection with a firing rate of 32 Hz or greater.

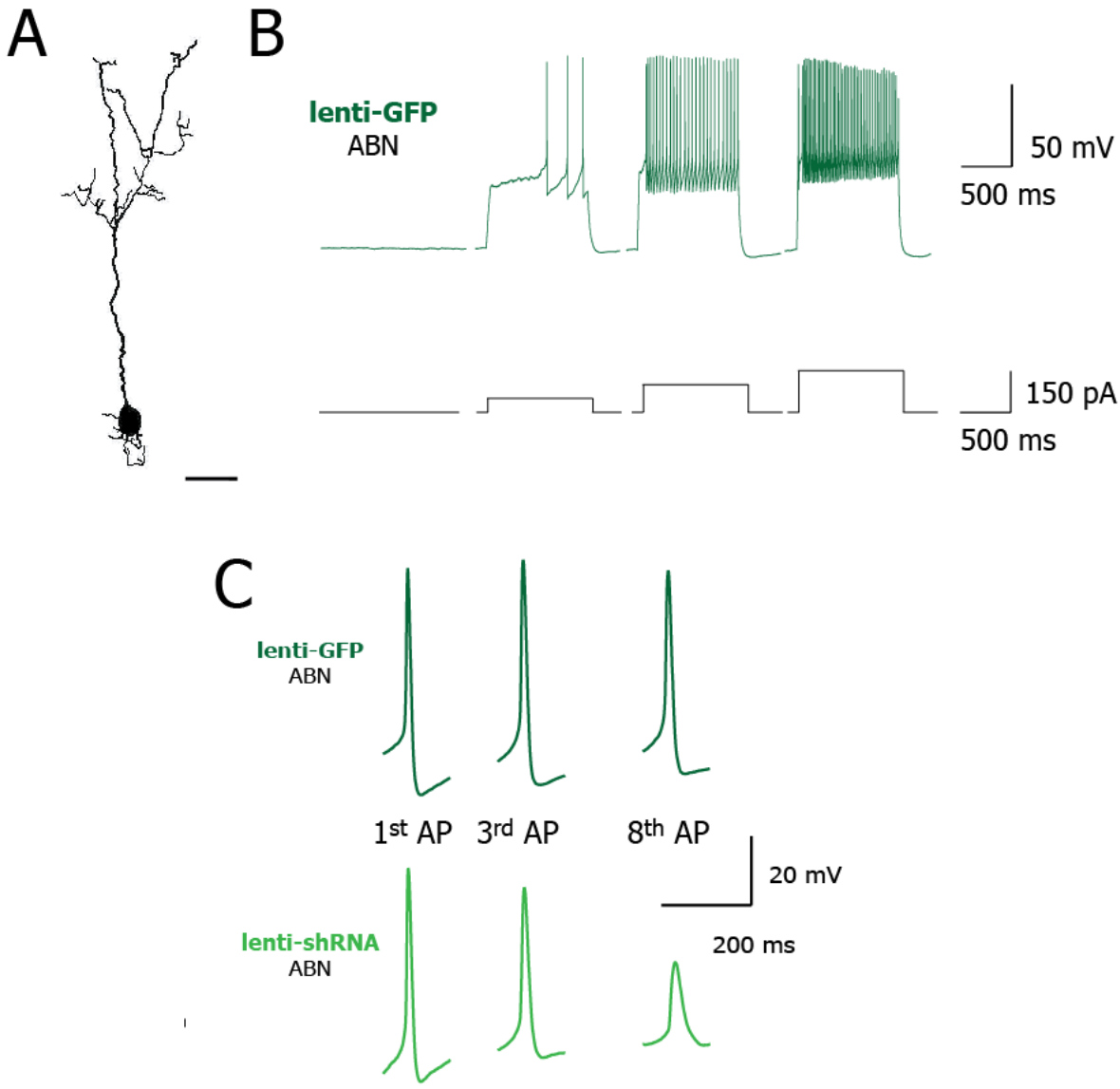

Figure 20. Lenti-shRNA ABNs have reduced excitability consistent with reduced functional voltage gated sodium channels

(**A**) Neurolucida reconstruction of a **lenti-GFP** adult-born granule cell patched for electrophysiology. Scale bar 20µm. (**B**) Sample whole cell patch recordings from ABNs 21 d.p.i. with **lenti-GFP**. Current injections elicit regular well spaced APs in **lenti-GFP** ABNs. (**C**) **lenti-shRNA** ABNs exhibit a reduction in the size of late action potentials in an elicited train. Shown are sample representative action potentials from opposite hemispheres of the same animal from **lenti-GFP** and **lenti-shRNA** ABNs. The amplitude of the eighth action potential elicited from **lenti-shRNA** ABNs shows a 31% reduction when compared **lenti-GFP** expressing cells ($p=0.0025$ using a one-tailed unpaired student t-test, n=8 **lenti-GFP**, n=7 **lenti-shRNA**).

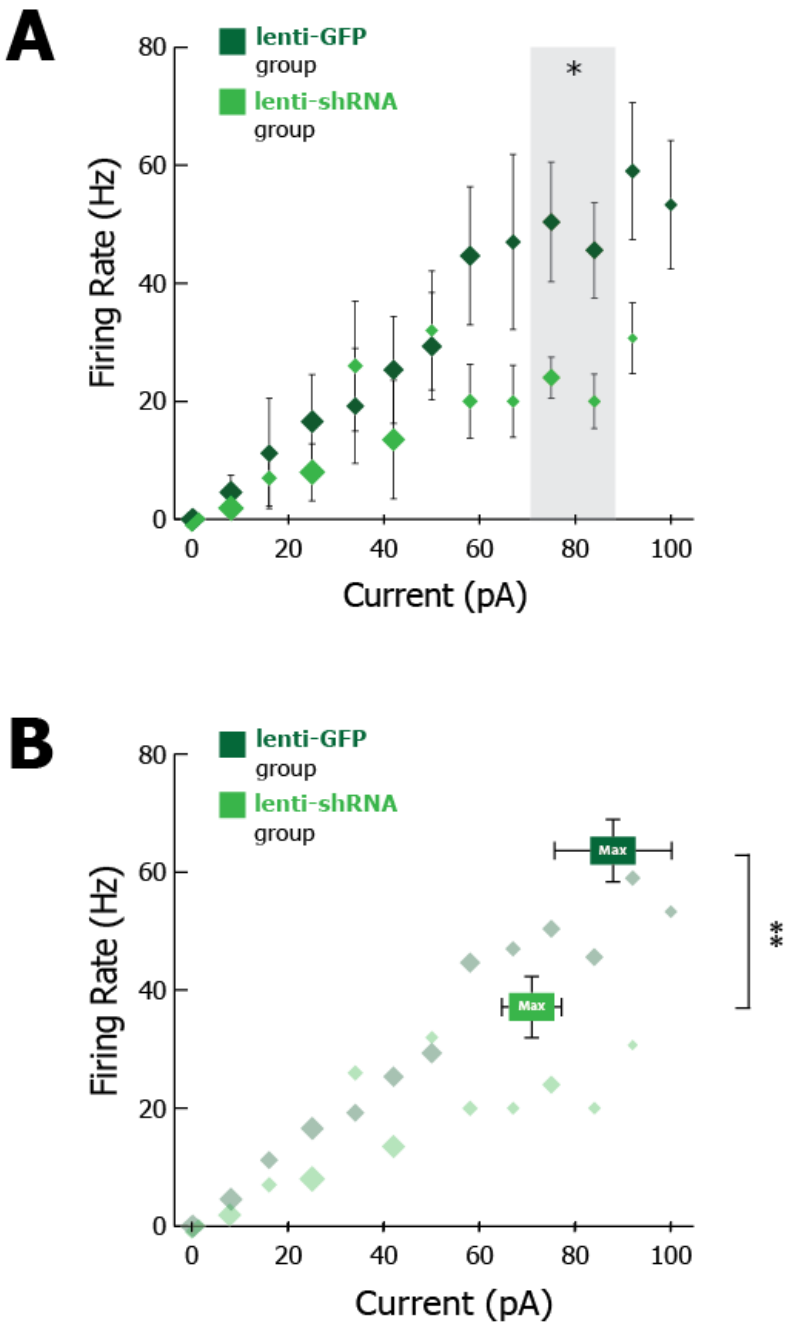

Figure 21. Lenti-shRNA ABNs are less responsive to strong current injections

(**A**) FI curves for the populations show a reduced firing rate for **lenti-shRNA** ABNs when compared to **lenti-GFP** controls at upper current injections. (p=0.01 at 75 mV, n=5 **lenti-GFP**,n=5 **lenti-shRNA** and p=0.03 at 84 mV, n=5 **lenti-GFP**,n=3 **lenti-shRNA**) (**B**) Maximum firing rates for **lenti-shRNA** cells were reduced to 37.5 Hz from 64 Hz in the **lenti-GFP** control condition. (p=0.0028, n=7 **lenti-GFP**,n=8 **lenti-shRNA**). Average firing rates for current injections are duplicated from **A**. Area of plotted data indicates sample size, max = 11 neurons, min = 2. All error bars are the s.e.m., * denotes p<0.05, ** denotes p<0.01 using a Student t-test.

We next sought to test the excitability of **lenti-shRNA** ABNs relative to **lenti-GFP** ABNs in response to stimulus. Because **lenti-shRNA** ABNs displayed a tendency to fire quick bursts of action potentials before going into depolarization block (Qualitative interpretation of Figure 19) we investigated their ISI profile. **Lenti-shRNA** ABNs displayed an ISI profile that was skewed towards faster ISIs relative to **lenti-GFP** ABNs (Figure 22a). This manifested itself into higher instantaneous firing rates early in traces following current injection (Figure 22b). Quantifying this difference revealed a 15% faster rate of action potentials for **lenti-shRNA** ABNs within the first 100ms of stimulus (Figure 22D, 11.5±0.6ms for **lenti-shRNA** versus 13.5±0.6ms for **lenti-GFP**, p=0.027). This difference reversed when looking over the full extent of the stimulus with **lenti-GFP** cells exhibiting 26% faster ISIs over the full stimulus (23.8±0.7ms for **lenti-GFP** versus 32.1±0.2ms for lenit-shRNA, p<0.001). The increase in ISI did not translate into a faster initial action potential as both groups displayed initial action potentials ~120ms after stimulus onset (p=0.48).

We also attempted to look at the age dependence of cells on their ISI profile. In a limited data set, a lenti GFP ABN at 21 d.p.i exhibited 30% faster ISIs within 100ms of stimulus onset versus four **lenti-GFP** ABNs at 26 d.pi. (ISI of 10.4±0.4 ms at 21 d.p.i. versus 14.8±0.7ms at 26 d.p.i.). The 21 d.p.i. ABN exhibited properties similar to the population of measured **lenti-shRNA** ABNs raising the possibility that the **lenti-shRNA** ABNs were sampled at a younger overall time point. This could be caused by continuous production conferred to progenitor cells along with a decreased rate of survival of older **lenti-shRNA** cells (for a more detailed discussion of this limitation see the discussion in 2.5.6). Notably, this analysis was done with a small sample size (n 27 spikes in 1 ABN for 21 d.p.i. and n=65 spikes in 4 ABNs for 26 d.p.i.)

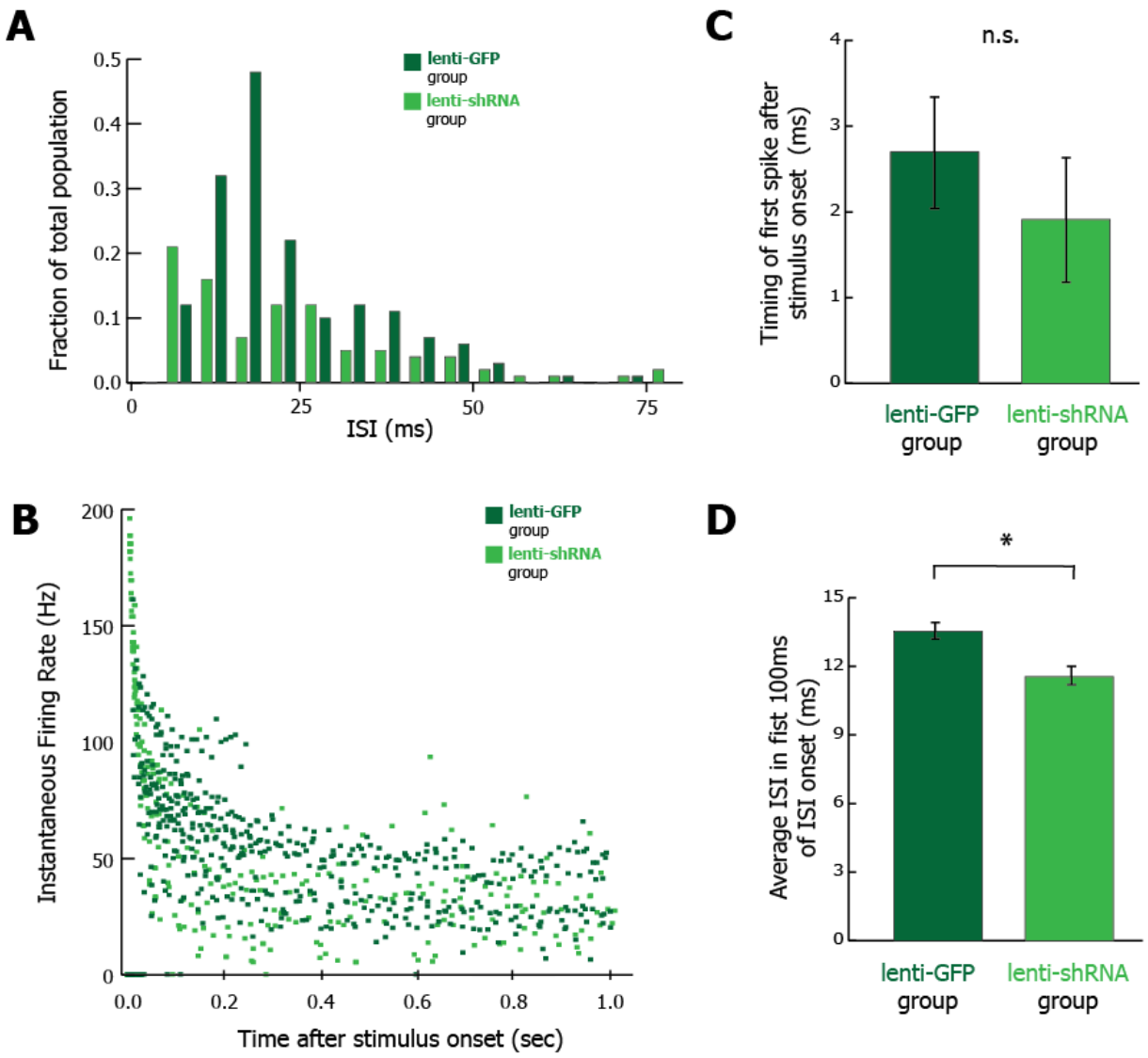

Figure 22. Decreased $Na_v1.1$-1.3 expression causes increased excitability in first 150ms of stimulus onset

(**A**) ISI histogram for experimental groups. **Lenti-shRNA** ABNs display a larger proportion of their action potentials at short ISIs versus controls. (B) Plotting instantaneous firing rate (1/ISI) versus time after stimulus onset shows a propensity for **lenti-shRNA** cells to fire at high firing rates directly after stimulus onset followed by lower firing rates as the stimulus continues. (C) Both experimental groups respond to stimulus onset with the same delay (2.7±0.6ms for **lenti-GFP**, 1.9±0.7ms for **lenti-shRNA**, n.s., p=0.48, n = 20 spikes over 5 animals for GFP, n=41 spikes over 6 animals for shRNA). (D) In the first 100ms after stimulus onset **lenti-shRNA** cells respond with faster firing patterns exemplified by a decreased average ISI (15% faster ISI, 11.6±0.6ms for **lenti-shRNA** versus 13.5±0.6ms for **lenti-GFP**, p=0.031, n=92 spikes over 5 cells for GFP, n=115 spikes over 6 cells for shRNA). * denotes p<0.05 using an upaired Student t-test.

These results indicate that at 21-35 d.p.i. **lenti-shRNA** expression 1) reduces maximum AP amplitude, AP threshold, & AP rise slope consistent with lowered expression of sodium channels, 2) Causes an increase in excitability for a 100ms window immediately after stimuli, 3) reduces overall excitability of ABNs over longer depolarizations (~500ms) 4) reduces the ability of OB ABNs to sustain firing rates greater than ~30Hz in response to strong continued depolarization.

2.4.3 Knockdown of voltage gated Na+ channels results in a reduction of functional response to odor exposure

Given the reduced levels of cell-intrinsic activity in infected GCs, we next investigated whether voltage-gated sodium channel knockdown affected responses to sensory stimuli. It is conceivable that the reduced intrinsic excitability recorded in vitro could be opposed by network effects that still allowed infected ABNs to generate normal levels of activity in response to sensory stimuli.

Using the above approach we performed bilateral injections with either **lenti-shRNA** or **lenti-GFP** in opposite hemispheres. At 35 d.p.i., we exposed these mice to a high concentration of a complex odor mixture for 30 min. Expression of the immediate early gene Egr1 (a.k.a. Zif268) in the OB was used as a measure of functional response to sensory stimuli (Magavi, et al. 2005). **Lenti-shRNA** ABNs were significantly less likely to express the immediate early gene Egr1 in response to odor exposure than **lenti-GFP** ABNs (Figure 18b, 17±3.5% reduction, p=0.008, n=779 **lenti-GFP** in 5 animals, n=592 **lenti-shRNA** in 5 animals). Egr1 is expressed in neurons following strong synaptic activity and is used as an indicator of recent neuronal activity.

The results demonstrate that reduced expression of voltage gated sodium channels not only reduces cell-intrinsic excitability but also decrease the neural response to sensory stimuli. We next examined whether this reduction in cell-intrinsic activity and decreased response to sensory stimuli affected the survival of these ABNs.

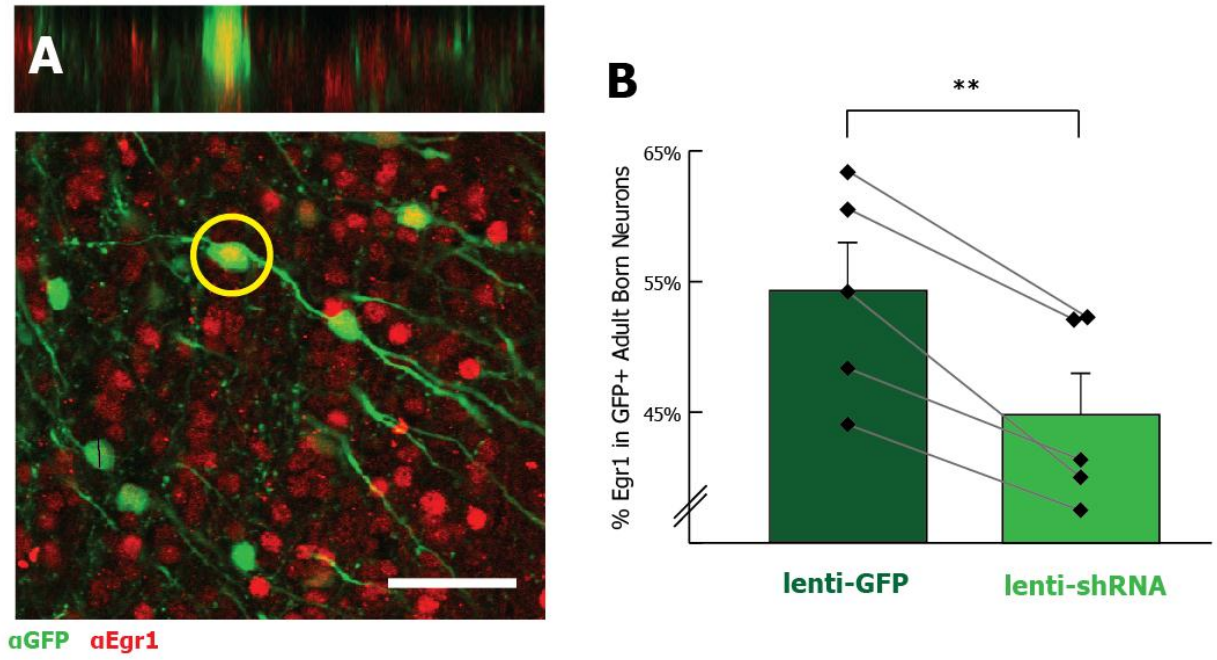

Figure 23. lenti-shRNA ABNs exhibit reduced functional response to odor exposure.

(**A**) Confocal image of the granule cell layer in the olfactory bulb shows Egr1 expression in a **lenti-GFP** expressing ABN following a half hour exposure to a complex odor mixture. Top panel represents the z-plane of an example ABN (circled) expressing both Egr1 and GFP. Scale bar is 30 μm. (**B**) $Na_v1.1$-1.3 knockdown causes a decrease in expression of the immediate early gene Egr1 following odor exposure. Data points represent hemisphere averages for the fraction of GFP^+ adult-born neurons co-labeled for the immediate early gene Egr1 and **lenti-GFP** or **lenti-shRNA**. Lines between data points connect hemispheres from an individual animal. Bars represent group averages for each condition. **lenti-shRNA** ABNs were 17% less likely to express Egr1 following odor exposure ($p=0.008$, n=5 animals, 779 **lenti-GFP**, 692 **lenti-shRNA**). Error bars represent the s.e.m. ** = $p<0.01$.

2.4.4 Cell-specific reduction in survival of adult-born neurons during a narrow critical period

Next, we probed the effect of the cell-specific activity manipulation caused by **lenti-shRNA** expression on ABN survival. To this end, we injected opposite hemispheres of the OB with either FURW + FUGR (**lenti-GFP**) or FURW + FU6siGURW (**lenti-shRNA**), which allowed us to measure the ratio of green to red fluorescent ABNs to control for slight variations in injection amount and location.

In order to measure the fraction of surviving ABNs we then examined the ratio of green to red cells across time. Previous experiments in our lab and others have shown the number of ABNs in the olfactory bulb reaches a maximum at 10-14 d.p.i. with the large majority reaching the OB by 7 d.p.i. (Petreanu and Alvarez-Buylla 2002, Carleton, et al. 2003, Belluzzi, et al. 2003, Bagley, et al. 2007). We thus measured survival at 21 and 35 d.p.i. post injection as the ratio of green to red ABNs normalized to the ratio at 10 d.p.i. when the maximum number of ABNs has reached the OB (summarized in Figure 24a).

At 21 d.p.i., we saw a 47±8.5% reduction in the survival of **lenti-shRNA** ABNs as compared to **lenti-GFP** ABNs in the opposite hemisphere (Figure 24b, $p=0.050$, $n=4$ animals). This was also the case with ABNs located solely in the GCL of the OB (45±8.2% reduction across hemispheres, $p=0.049$, $n=4$ animals, data not shown). Survival of **lenti-shRNA** ABNs at 35 d.p.i. was also reduced (Figure 24b, 44±2.8% reduction, $p=0.026$, $n=4$ animals) but the magnitude of this reduction did not increase from 21-35 d.p.i. (n.s., $p=0.39$). Importantly, previous work has indicated that a critical period for activity-dependent survival of ABNs in the OB occurs between 14-28 days after birth, centered on the narrow time window shown here (Yamaguchi and Mori 2005, Lin, et al. 2010).

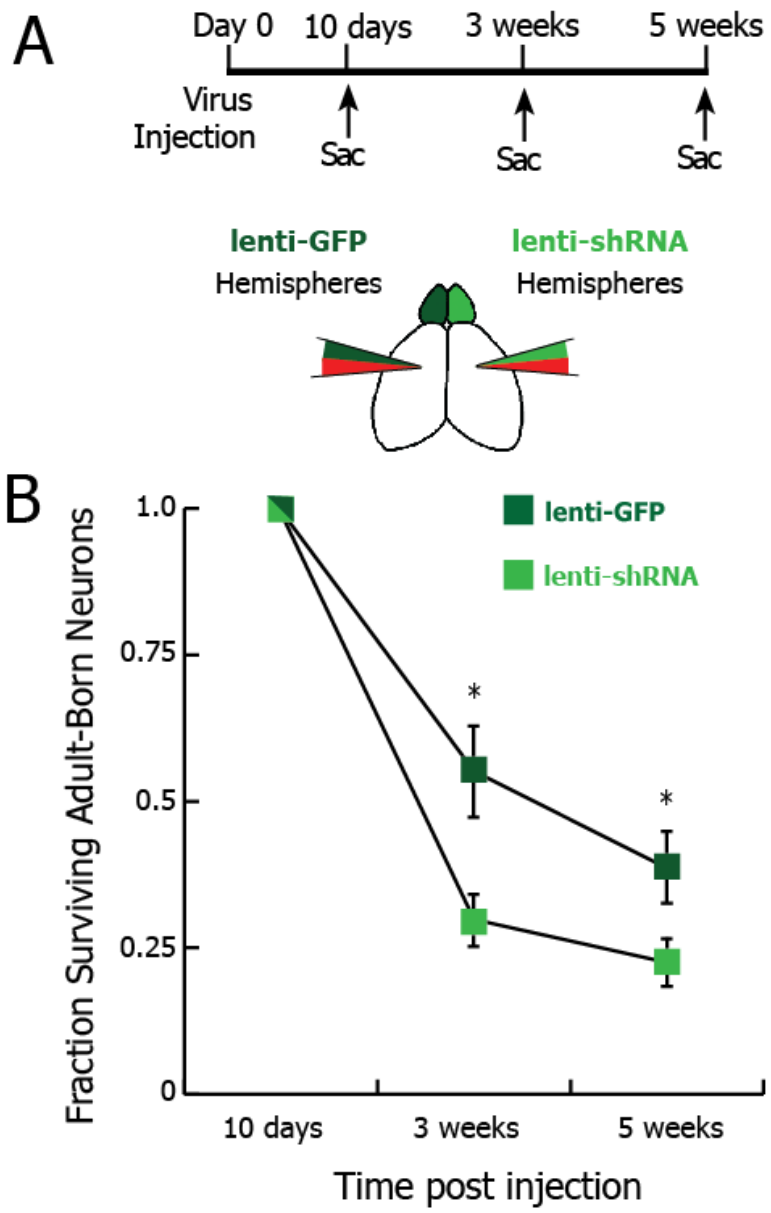

Figure 24. Nav1.1-1.3 knockdown causes reduced survival of ABNs in the mouse olfactory bulb.

(**A**) Schematic of experimental conditions and injection paradigm. Mice were coinjected with 500 nL of lentivirus construct. Each animal was injected in the anterior SVZ of one hemisphere with **lenti-GFP** and **lenti-shRNA** in the opposite hemisphere. All cell counts were normalized based on the number of cells expressing **lenti-tdTomato** (see Section 2.3.3 for normalization process). (**B**) **lenti-shRNA** causes a reduction of the survival of ABNs in the olfactory bulb at 21 d.p.i. and 35 d.p.i.. Data points represent the average of grouped hemispheres normalized to the number of ABNs in the 10 day groups. (n=4 animals per group, p=0.05 and p=0.02 respectively using a two sided paired Student t-test). All error bars represent the s.e.m., =p<0.05.

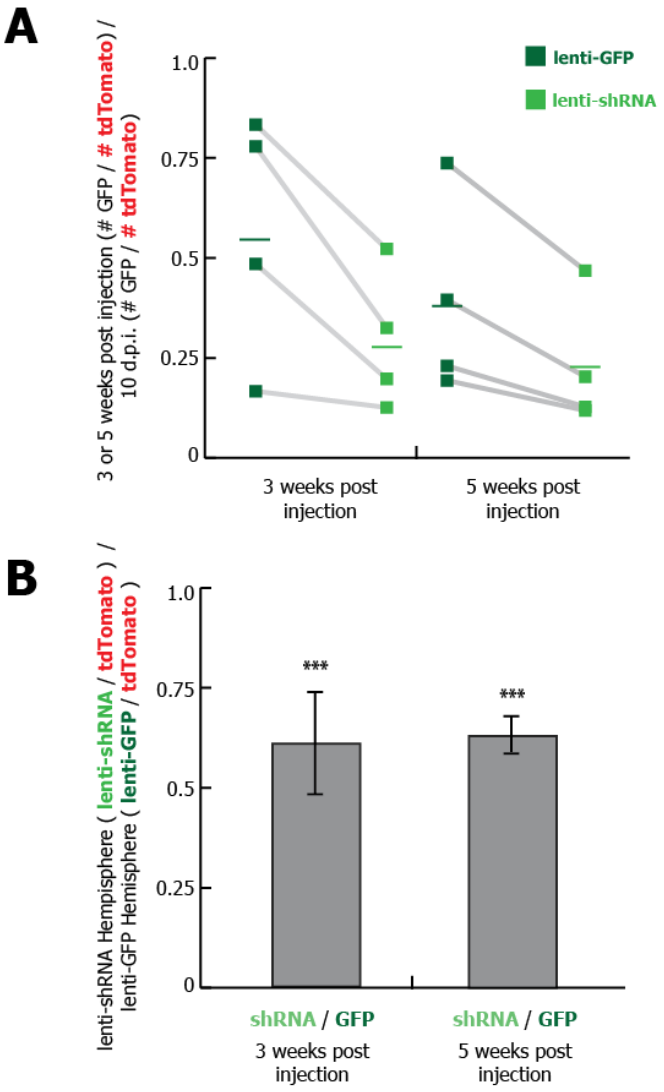

Figure 25. ABN survival is consistently reduced within animals between 10 and 21 days post birth.

At 21 d.p.i. and 35 d.p.i. the survival of **lenti-shRNA** ABNs is consistently lower than the survival of **lenti-GFP** ABNs in the opposite hemisphere of a mouse. Data points are connected to indicate hemisphere pairs within a single animal. The colored line represents the average survival ratio of all hemispheres for the given experimental condition. (B) At 21 d.p.i. the survival of **lenti-shRNA** ABNs is reduced by 47±8.5% when compared to **lenti-GFP** ABNs in the opposite hemisphere and normalized to the number of ABNs at 10 d.p.i. (see Figure 18) when plotting the ratio of the survival the populations are significantly different from 1 ($p=0.0004$ using a Z-test, n=4 hemispheres per condition). The reduced survival ratio of **lenti-shRNA** ABNs compared to **lenti-GFP** cells is maintained to 35 d.p.i. ($p<<0.0001$ using a Z-test, n=4 hemispheres per condition). Data is from the same population as Figure 24. All error bars are the s.e.m., ***=$p<0.001$.

2.4.5 Radial migration of ABNs does not appear to be effected by lenti-shRNA expression

We were interested in testing the radial position of ABNs in our **lenti-shRNA** conditions for two reasons: 1) The reduction in survival seen in 2.4.4 along with continuous production by virally infected progenitor cells could lead to a younger population of ABNs in the **lenti-shRNA** condition, and 2) Previous work describing heterogeneous populations of ABNs stemming from different regions of the SVZ manifested in populations of ABNs that stopped radial migration in different zones of the GCL (Merkle, Mirzadeh and Alvarez-Buylla 2007, Kelsch, et al. 2007). Using the tissue from 2.4.4 we measured the radial distance that ABNs traveled in the GCL (Figure 26). **Lenti-GFP** ABNs at 21 d.p.i. had migrated a shorter distance through the GCL than at 35 days (35% at 21 d.p.i versus 51% at 35 d.p.i.). No difference was seen between the **lenti-GFP** and **lenti-shRNA** population at 35 d.p.i. (51% migration versus 54% migration).

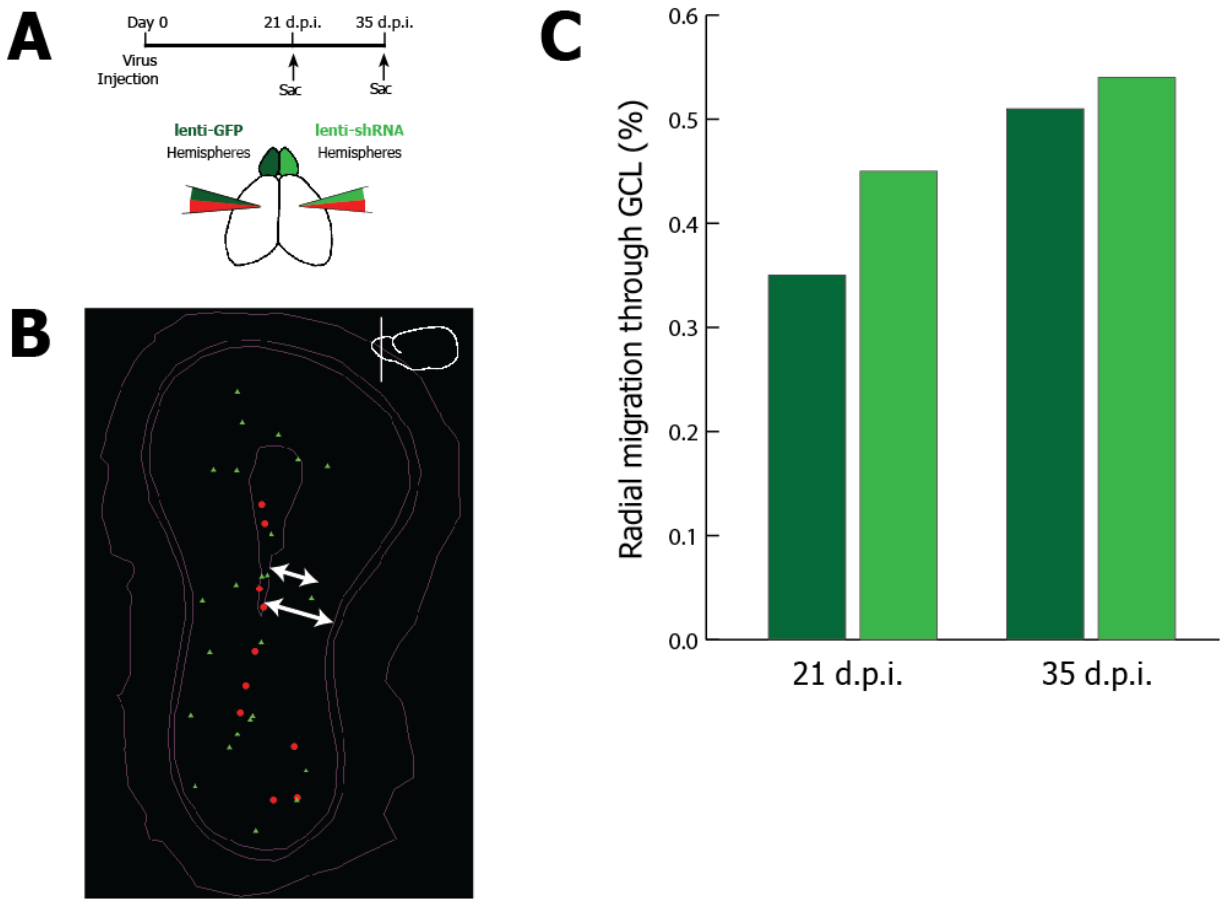

Figure 26. Radial migration of ABNs does not appear to be effected by lenti-shRNA expression

(A) Schematic of experimental conditions and injection paradigm. Tissue used in the measurement of survival in 2.4.4. was analyzed for radial position of fluorescent cell bodies. (B) Neurolucida drawing of a coronal section of the olfactory bulb showing anatomical definitions of OB regions and the position of cell bodies. Migration was measured using a custom IgorPro program that measured the closest 2-dimensional distance between the anterior commissure and the interior boundary of the MCL that intersected the cell body being measured. The fraction that this cell body had traveled along that path was then calculated for each cell body analyzed. (C) Group data for **lenti-GFP** and **lenti-shRNA** populations analyzed. No difference was found across experimental conditions.

2.4.6 Reduction of sensory stimuli causes a competitive reduction in survival in ABNs

We next investigated how survival of ABNs is influenced by the interaction between cell-intrinsic and circuit level activity. Specifically, we were interested in determining whether survival involved the activity of the network as well as the activity of individual ABNs; or if it depends solely on the absolute level of activity in individual ABNs. We examined this by combining the lentivirus mediated reduction in cell-intrinsic activity shown in the previous experiment with unilateral naris occlusion. Unilateral naris occlusion blocks the afferent air pathway to a single nasal cavity sparing the opposite hemisphere. This procedure has been shown to reduce sensory stimuli and circuit level activity in the occluded hemisphere and has been shown to reduce survival of ABNs (Corotto, Henegar and Maruniak 1994, Yamaguchi and Mori 2005).

To manipulate the level of cell-intrinsic activity, we injected the OB bilaterally with **lenti-tdTomato** and either **lenti-GFP** or **lenti-shRNA**. This allowed us to reduce the amount of sensory stimuli via unilateral naris occlusion while maintaining the level of individual ABN's cell-intrinsic activity across hemispheres. The effect of network activity on ABN survival was then made through comparisons across hemispheres at 35 d.p.i. (summary in Figure 27a).

In the **lenti-GFP** animals ABNs in the naris occluded hemisphere showed consistently lower rates of survival than ABNs in the normal hemisphere (-24±4.3%, n=6 animals, p=0.006). This confirms previous results showing that a reduction in ABN survival follows naris occlusion (Corotto, Henegar and Maruniak 1994, Yamaguchi and Mori 2005, Lin, et al. 2010).

However, in the **lenti-shRNA** condition, ABNs in the occluded hemisphere showed an increased rate of survival when compared to non-occluded hemispheres (+24±8.7%, n=6

animals, p=0.01). This indicates that the survival of these ABNs depended not on the absolute level of activity, but on the difference between the activity, of new and existing neurons. Our results suggest that survival is a competitive process, in which survival of ABNs is regulated by the activity levels of individual cells along with the activity levels of neighboring cells.

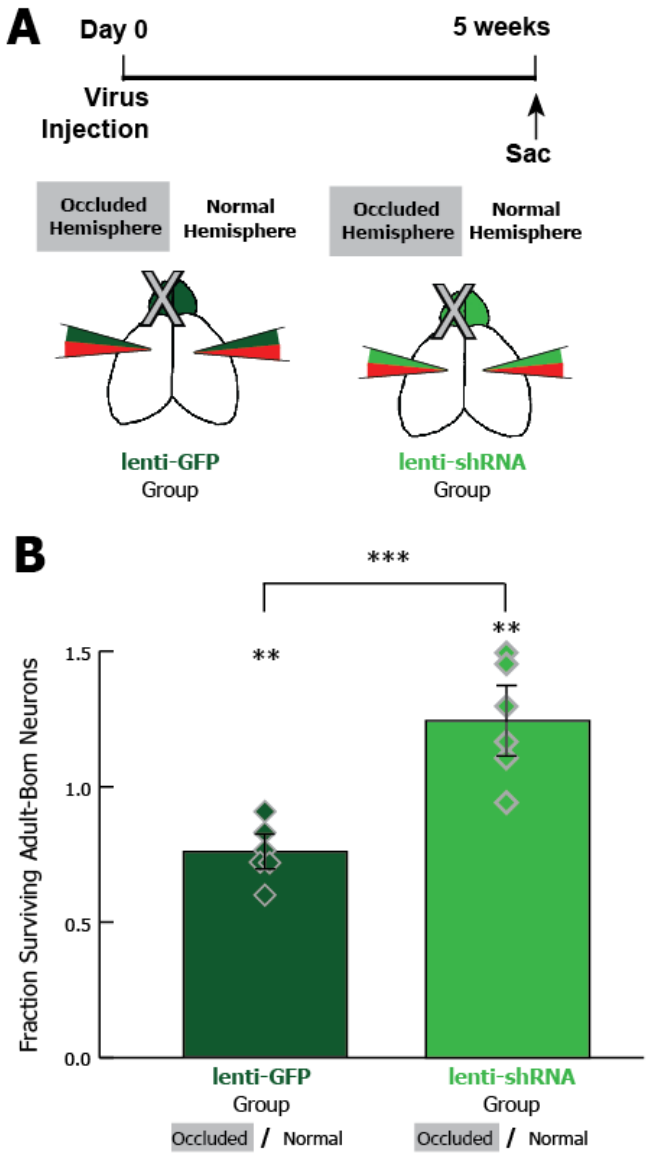

Figure 27. Survival of adult-born neurons is regulated by lowered circuit level activity in a competitive activity-dependent manner.

(**A**) Schematic of experimental conditions and injection paradigm. Two groups of animals were injected bilaterally with one of two lentivirus mixtures (Section 2.3.3). 7 days previous to virus injection animals had a single naris occluded, which reduces air flowing to the olfactory epithelium. After 35 days, the mice were sacrifice and probed for survival. (**B**) Naris occlusion causes a decrease in the survival of adult-born neurons in the mice injected with **lenti-GFP** and conversely an increase in survival in mice injected with **lenti-shRNA**. Bars represents group averages of the number of normalized GFP^+ in the occluded hemisphere divided by the number of normalized GFP^+ positive cells in the non-occluded hemisphere. Both groups are significantly different from 1 using a Z test ($p=0.006$ & 0.01 respectively, $n=6$ animals in both groups) and significantly different from each other ($p<0.001$ using a two sided t-test). Error bars represent the s.e.m.

2.4.7 Expression of a random scrambled siRNA produces a mixed interpretation with no clear result

Strong expression of lentiviral constructs may overwhelm cellular machinery crucially involved in gene transcription (Yi, Qin, et al. 2003, Yi, Doehle, et al. 2005, Zeng and Cullen 2004, Jackson and Linsey 2010). This nonspecific effect can lead to cell death, and even fatality in mice when expressed in the mouse liver (Grimm, et al. 2006). In order to determine whether expressing shRNA resulted in non-specific effects on cell survival we utilized a construct that expressed a random string of nucleotides in a shRNA construct in place of our experimental targeted **lenti-shRNA** (Komai, et al. 2006)**.**

Using a parallel experiment to our assessment of survival after expression of $Na_v1.1$-1.3 shRNA we measured the number of cells surviving of scrambled shRNA cells at a single time point (35 d.p.i.). At 35 d.p.i. ABNss in the control **lenti-GFP** hemisphere cells showed a ratio of 7.54 Green:Red (3,755 **lenti-GFP** ABNs versus 498 lenti-tdTomato ABNs). The experimental **lenti-scrambled-shRNA** hemispheres showed a ratio of 4.70 Green:Red (4292 **lenti-scrambled-shRNA** ABNs versus 913 lenti-tdTomato ABNs). Technical issues and a lack of replacement lentivirus limited us to evaluating survival of adult-born neurons to a single time point. This limitation influenced the possible interpretations that can be drawn from these data. To interpret these data, we considered the follow possible scenarios:

- In the simplest possible case, the titers of the **lenti-GFP** virus and the **lenti-scrambled-shRNA** virus would be similar and so the absolute numbers of GFP-positive cells should be comparable unless the shRNA alters survival.

- Alternatively, if the rate of coinfection of lenti-tdTomato cells is similar, then we should examine the ratio of green to red cells in the hemispheres injected with shRNA versus those with GFP alone.
- Finally, if the titer of **lenti-scrambled-shRNA** was significantly lower than **lenti-GFP** [WHAT SUGGESTS THAT THIS ASSUMPTION IS REASONABLE?] then we would need to know the difference in titer in order to evaluate this data. than there may have been a non-specific effect on survival large enough to explain the results seen in 2.4.4.

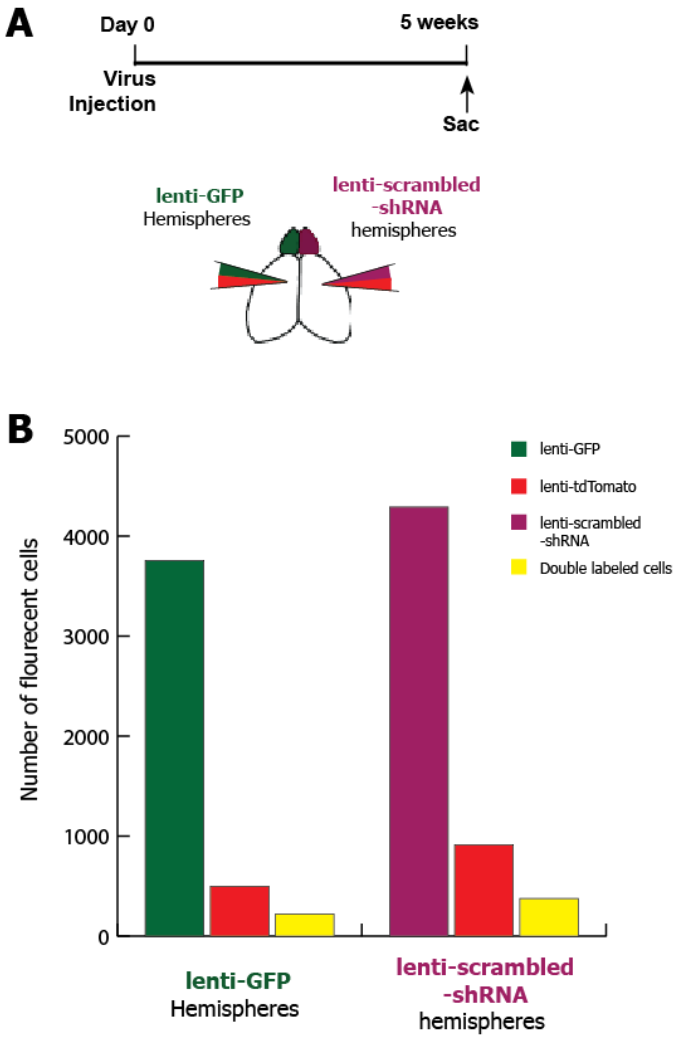

Figure 28. Cell counts for 35 d.p.i. lenti-GFP and lenti-scrambled shRNA hemispheres

(**A**) Schematic of experimental conditions and injection paradigm. Mice were coinjected with 500 nL of lentivirus construct. Each animal was injected in the anterior SVZ of one hemisphere with **lenti-GFP** and **lenti-tdTomato** and **lenti-scrambled-shRNA** and **lenti-tdTomato** in the opposite hemisphere. (**B**) Total group cell counts for experimental groups. Fluorescent ABNs were counted in both hemispheres of 5 animals. Double labeled cells were positive for both GFP and tdTomato and are included in the GFP & tdTomato counts (i.e. double labeled count is not exclusive)

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If titres for the **lenti-GFP** virus and the **lenti-scrambled-shRNA** viruses were similar than the there was a significant reduction of survival of shRNA expression due to expression of an shRNA lentiviral vector but not large enough to explain the reduction of survival seen in

lenti-shRNA tissue. As quantified in table 4, we observed 3,755 ABNs in **lenti-GFP tissue** and 4292 in **lenti-scrambled-shRNA** tissue, normalizing this to the number of **lenti-tdTomato** cells as in our survival experiments yields a multiple of Green:Red cells of 7.5x for **lenti-GFP** and a multiple of 4.7x for **lenti-scrambled-shRNA**. This would indicate a survival rate of 62.3% for cells expressing the scrambled-shRNA string. In this analysis we see a reduction of survival due to the expression of scrambled-shRNA of 47.7% (n=5 animals, p=0.016). Performing the same calculation on cells in the 35 d.p.i. **lenti-shRNA** tissue yields a survival rate of 3.6% (see table 2) a 17 fold lower rate (n=4 animals).

	GFP+ cells (double labeled %)	tdTomato+ cells (double labeled %)
lenti-GFP	3755 (5.9%)	498 (44.8%)
lenti-scrambled-shRNA	4292 (8.7%)	913 (41.1%)

Table 4. Cell counts for 35 d.p.i. tissue used in scrambled shRNA experiments

If the rate of coinfection for lenti-tdTomato and both **lenti-GFP** and **lenti-scrambled-shRNA** is similar than there was an insignificant effect on survival due to expression of shRNA. A fraction of all counted tdTomato ABNs we're also double labeled with GFP. These ABNs can be expected exhibit different rates of survival than the tdTomato+ only positive population due to the expression of the vector containing the GFP. If expressing scrambled-shRNA reduced survival of these double labeled cells we would expect double labeled cells to represent a lower fraction of the tdTomato+ ABNs than in the **lenti-GFP** condition. In the 35 d.p.i. cells measured the two populations exhibited a non-significant difference in double labeling of the tdTomato$^+$ population (n=5 animals, p=0.46). Repeating this analysis in the 35 d.p.i. **lenti-shRNA**

condition yields a significant 2.8x lower rate of survival for double labeled tdTomato+ cells (see table 2, 16.9% green in red for **lenti-shRNA** versus 47.0% green in red for **lenti-GFP**, n=4 animals). Notably for this calculation to be pertinent co-infection rates for **lenti-shRNA** and **lenti-scrambled-shRNA** would have to be similar.

If the titre of **lenti-scrambled-shRNA** was ~17x lower than **lenti-GFP** than the expression of shRNA seen in the **lenti-scrambled-shRNA** would be sufficient to explain the reduction of survival seen in 2.4.4. The calculations outlined above assumed a similar titer for both **lenti-GFP** and **lenti-shRNA**. A similar calculation can be performed that sets the titer 17 times lower that will yield the same reduction in survival seen for our experimental **lenti-shRNA** group at 35 d.p.i. This difference in titer is within the range reported by the laboratory that produced the lentiviral lots used in this work (Dittgen, et al. 2004, Komai, et al. 2006). Given this possibility it is possible that the expression of shRNA could fully explain the reduction of survival seen in the **lenti-shRNA** condition.

2.4.8 Expression of lentiviral delivered Nav1.1-1.3 shRNA does not affect cell subtype fate

ABNs express a variety of protein markers, including calcium-binding proteins that have been used to classify subtypes of inhibitory interneurons in the olfactory bulb. Expression of these proteins changes rapidly postnatally and is regulated by neuronal activity (Philpot, Lim and Brunjes 1997). From p14-p40 expression levels of subtype markers expressed by ABNs reach levels uniform with the overall neuronal population (Bagley, et al. 2007). The development of this expression pattern coincides with the activity-dependent critical period for survival from 14-28 days after birth (Yamaguchi and Mori 2005). This led us to investigate the hypothesis that

activity-dependent survival of ABNs in the granule cell layer of the olfactory bulb is selective for subtypes of inhibitory interneurons.

We compared the level of expression of selected calcium binding proteins in **lenti-shRNA** expressing ABNs to **lenti-GFP** ABNs in the opposite hemisphere 35 d.p.i. Calretinin and n-copine were selected for analysis, as they show the highest levels of expression in the granule cell layer of the olfactory bulb (Bagley, et al. 2007).

Lenti-shRNA ABNs showed no differences when compared to **lenti-GFP** ABNs in the opposite hemisphere of animals (Figure 23). Calretinin was expressed in 14±2.0% of **lenti-GFP** ABNs and 12±1.5% of **lenti-shRNA** expressing ABNs with no significant difference across the populations ($p=0.39$, n=5 animals, 1114 **lenti-GFP**, 975 **lenti-shRNA**). N-copine showed expression in 4.7±1.4% of **lenti-GFP** ABNs and 4.7±0.59% of **lenti-shRNA** expressing ABNs ($p=0.99$, n=5 animals, 580 **lenti-GFP**, 454 **lenti-shRNA**). While, these results cannot speak to the 4/5^{ths} of ABNs that do not express Calretinin or N-copine, these results suggest that, at least for this subset of calcium binding protein expressing ABNs, the level of cell-intrinsic activity does not select for the survival of a particular subtype of ABN in the granule cell layer of the OB or influence the expression of Calretinin or Calbindin.

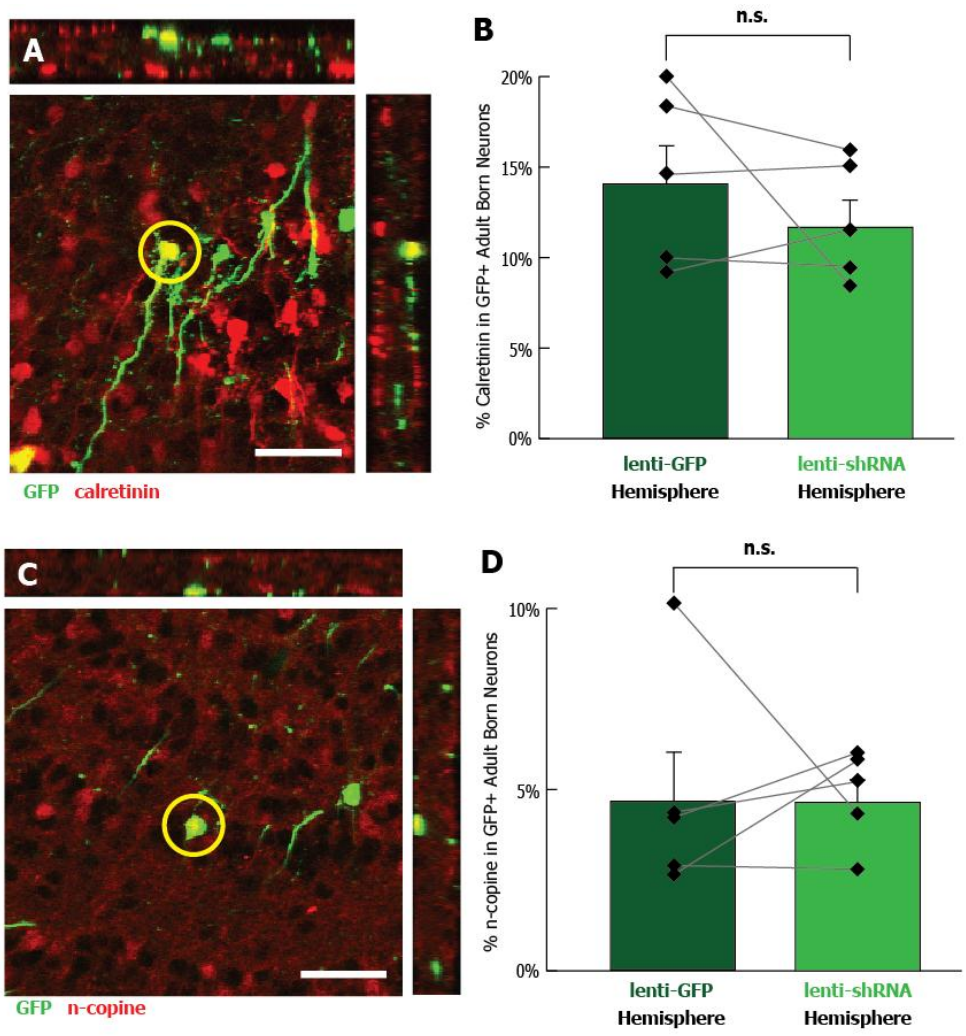

Figure 29 Nav1.1-1.3 knockdown does not affect the expression of Ca^{2+} binding proteins of adult-born neurons 35 d.p.i.

(**A**) Confocal image of the granule cell layer in the olfactory bulb shows calretinin and GFP expression 35 d.p.i. in the **lenti-shRNA** condition. Top and side panels represent the z-plane projection of an example double labeled ABN (circled). Scale bar 30 µm. (**B**) **lenti-shRNA** shows no significant difference in the expression of calretinin when compared to **lenti-GFP** ABNs (p=0.39, n=5 animals in each condition, 1114 **lenti-GFP** cells, 975 **lenti-shRNA** cells). (**C**) Confocal image of the granule cell layer in the olfactory bulb shows n-copine and FP expression 35 d.p.i. in the **lenti-shRNA** condition. Top and side panels represent the z-plane projection of an example double labeled ABN (circled). Scale bar 30 µm. (**D**) $Na_v1.1$-1.3 knockdown shows no significant difference in the expression of calretinin when compared to control GFP expressing adult-born neurons (p=0.99, n=5 animals in each condition, 580 **lenti-GFP** cells, 454 **lenti-shRNA** cells). Data points represent hemisphere averages for the fraction of GFP+ adult-born neurons co-labeled for the Ca^{2+} binding protein of interest in the control **lenti-GFP** or **lenti-shRNA** condition. Lines between data points connect hemispheres from an individual animal. Bars represent group averages for each condition. Error bars represent the s.e.m.

2.5 DISCUSSION

2.5.1 Competitive Activity-Dependent Survival of Adult-Born Neurons

To separate the role of intrinsic and circuit level activity in the survival of ABNs in the OB, we used a combination of shRNA knockdown of voltage gated sodium channels and reduction of sensory stimuli through naris occlusion to manipulate the activity at both the individual ABN and circuit level. Our results show that reduction of cell-intrinsic activity causes a decrease in the survival of ABNs three and five weeks after injection. Confirming previous studies **lenti-GFP** ABNs show a reduction in survival following reduced sensory stimuli (Corotto, Henegar and Maruniak 1994, Yamaguchi and Mori 2005). When combining the two techniques to reduce both cell-intrinsic activity and sensory stimuli we have shown that **lenti-shRNA** ABNs with reduced cell-intrinsic activity are more likely to survive in a sensory deprived circuit than a normally functioning circuit. This suggests that activity-dependent survival of ABNs is regulated by a competitive process where the activity levels of individual cells along with the activity levels of neighboring cells contribute to whether an ABN survives.

Activity has been shown to regulate the integration and survival of ABNs in the OB through both cell-intrinsic and sensory level activity. To date, very little has been done to validate the changes in activity produced by these manipulations in ABNs. Here we describe a shRNA mediated knockdown of voltage-gated sodium channels which was shown to have reduced cell-intrinsic activity overall (but, see increased activity early in response to stimuli) and, importantly, showed reduced expression of an immediate early gene in response to sensory stimuli. This second validation shows that **lenti-shRNA** expression causes reduced participation within a functioning circuit.

Recent results have concluded that there is a minimum threshold of cell-intrinsic activity necessary for ABNs to survive in the olfactory bulb (Lin, et al. 2010). Our work indicates that this is only part of the process. Applying the hypothesis that there is a minimum threshold of cell-intrinsic activity required for survival of ABNs to a situation with reduced sensory activity and reduced cell-intrinsic activity would predict at least as much cell death as with reduced cell-intrinsic activity alone. But, this is not what we have seen when reducing both sensory and cell-intrinsic activity, ABNs in fact show an increased rate of survival when compared to ABNs with reduced cell-intrinsic activity alone. Thus, this process may be competitive; dependent on both cell-intrinsic and also on circuit level activity. Any minimum threshold of cell-intrinsic activity that determines whether ABNs in the olfactory bulb survive would also dependent on the level of activity in the circuit. Our work suggests that ABNs in fact compete with other newborn neurons and mature neighbors in the OB circuit in deciding whether they survive. In a circuit with low sensory driven activity, such as after naris occlusion, the activity threshold for survival is lower. And we would hypothesize that in a circuit with increased levels of network activity the level of cell-intrinsic activity required for an ABN to successfully survive and integrate would be commensurately higher.

2.5.2 Effect of lenti-shRNA on functional activity *in vivo*

In vivo activity of GCs has been lightly studied, as extracellular recording has proven difficult, likely due to their small size and density of the GCL. Several groups have performed intracellular recordings which have shed some light on the natural state of GCs and its relevance to the electrophysiological analysis performed in this work. Our experimental condition, **Lenti-shRNA**, does not show a lower rate of spiking until injected with 80pA of current over 1 second.

Direct intracellular injection of current or stimulation of the sensory tract can reliably elicit this level of spiking in granule cells (Cang and Isaacson 2003, Wellis and Scott 1990). Though most GCs do not display this level of sustained firing in response to odor presentation (Cang and Isaacson 2003, Luo and Katz 2001, Wellis and Scott 1990). Over shorter time periods however GCs can exhibit high frequency spiking as described in this work, for instance at the start of a respiratory inhalation (Wellis and Scott 1990). Given the increased excitability of **lenti-shRNA** cells seen in the first 100ms of stimulus onset this may cause them to be more excitable under natural circumstances. Thus there is a lack of evidence in the literature to support the premise that GCs receive as high of a level of sustained current as we have used in our comparison of firing rates for **lenti-GFP** and **lenti-shRNA.**

There is better evidence to support the premise that granule cells receive high amounts of input over long time periods. Sampling of natural odorants occurs over many seconds and the natural respiratory cycle that drives ORN responses can be measured over seconds. Indeed when recorded in vivo granule cells spike throughout long odor presentations (Wellis and Scott 1990). When exciting mitral cells at high firing rates (50 Hz) in vivo IPSPs from granule cells are not attenuated (Margrie, Sakmann and Urban 2001).

Summarizing, GCs in vivo have average firing rates that are lower than that driven by the currents described here, but do fire at those rates for shorter time periods, and are presented with odors and stimulation over long time periods on the order of those described in this work. Given this difference in the firing rate of *in vivo* GCs and recognizing the limitations of comparing our electrophysiological characterization to the natural activity of GCs our measurement of early activity genes in response to odor is all the more important. The reduction in expression of Egr1

seen subsequent to odor exposure gives us confidence that the expression of **lenti-shRNA** has functional consequences that result in reduced functional integration into the OB.

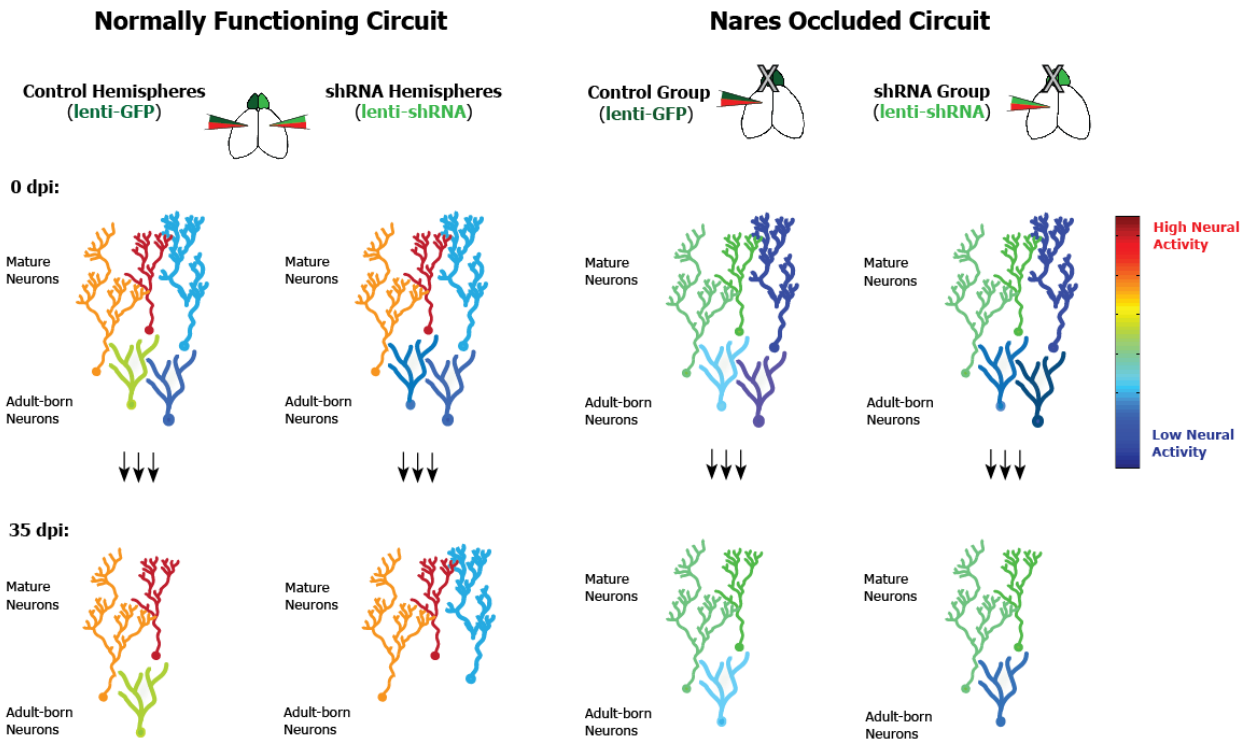

Figure 30. Conceptualization of competitive activity-dependent survival of ABNs in the OB.
Our results show that the survival of ABNs depends not only on the activity of individual ABNs but also on the activity of other ABNs in the circuit. In the conceptual representation of this above the majority of **lenti-GFP** ABNs in a normally functioning circuit die by 35 d.p.i. after their level of neural activity is lower than surrounding mature neurons and other ABNs. When **lenti-shRNA** ABNs are exposed to the same normally functioning circuit they are more likely to die because the reduced activity caused by knock down of voltage gated sodium channels makes them less able to compete with high functioning mature neurons in the same circuit. In a naris occluded circuit sensory stimuli to the olfactory bulb is reduced. This creates a situation where the level of activity between **lenti-shRNA** ABNs and other mature and newborn neurons in the circuit is comparatively closer, leaving them more able to compete for activity-dependent survival factors. The end result is that **lenti-shRNA** ABNs survive at a higher rate in a naris occluded OB than a normally functioning OB.

2.5.3 Reducing expression of naturally expressing voltage gated sodium channels mimics modulation of back propagating sodium spikes

Our work has used lentivirus delivery of short interfering RNA's to knockdown expression of voltage gated Na^+ channels. We believe this manipulation is an improvement over previous gene

expression based methods of reducing cell-intrinsic activity for the following reasons: 1) reducing expression of innate channels does not introduce changes in membrane potential unseen naturally in ABNs, 2) reducing expression of voltage gated sodium channels reduces back propagating sodium spikes which are preferentially caused by input to the cell body and proximal dendrites, 3) reducing voltage gated sodium input reduces overall activity levels, and in particular global activity, which drives back propagating sodium potentials, but should not greatly influence local dendrodendritic communication from granule cells to their dendrodendritic post synaptic partners which is mediated by P/Q and T type calcium channels (Isaacson and Strowbridge 1998, Egger, Svoboda and Mainen 2005). The use of voltage gated sodium channels to manipulate activity in adult-born GCs therefore allows us to specifically reduce back propagating sodium potentials, preferentially associated with cortical input, while maintaining ABNs that can communicate with their post-synaptic partners.

While our method of reducing cell-intrinsic activity shares aspects with other methods regarding compensatory changes in expression of other ion channels, we have shown electrophysiologically that there are not large changes in resting membrane potential or input resistance that one would expect from this kind of compensatory expression did not occur. This is in contrast to significant changes in resting membrane potential and input resistance seen in expression of the inward rectifying potassium channel EsKir1.2 (Lin, et al. 2010). Manipulation of the expression levels of naturally expressing voltage gated channels as seen in this work affect survival without the electrophysiological confounds associated with a foreign protein.

2.5.4 Cell-Intrinsic activity does not contribute to cell subtype fate

The vast majority of ABNs that reach the olfactory bulb become inhibitory interneurons. ABNs in the olfactory bulb can be classified into further subtypes by their expression of calcium binding proteins (Philpot, Lim and Brunjes 1997, Whitman and Greer 2007, De Marchis, et al. 2007, Bagley, et al. 2007). We examined the effect of reduced cell-intrinsic activity on the expression of two calcium binding proteins commonly expressed in adult-born granule cells in the olfactory bulb, calretinin and n-copine (Bagley, et al. 2007). Reduction in cell-intrinsic activity did not lead to changes in the fraction of adult-born GCs expressing either calretinin or n-copine. We have thus concluded that the cell-intrinsic activity level of ABNs does not select for the survival of a particular subtype of granule cell ABN or influence an ABNs subtype fate as measured by the expression of calcium binding proteins, rather a growing body of literature supports the idea that ABNs receive their subtype determination and GCL position determination from their precursor cells (Bagley, et al. 2007, Merkle, Mirzadeh and Alvarez-Buylla 2007, Kelsch, Lin and Lois 2008). In this model, cell-intrinsic activity largely plays a role in regulating the overall survival of ABNs after reaching the OB regardless of their calcium binding protein subtype or the region from which they originated.

2.5.5 Functional implications

Sensory driven activity has been shown to play a role in the refinement of synapses of ABNs following naris occlusion (Saghatelyan, et al. 2005, Kelsch, Lin and Mosley, et al. 2009, and see Chapter 3). Interestingly, Kelsch and colleagues have shown results that the density of glutamatergic synapses along the proximal dendrite of GCs is increased in density following a

reduction in sensory input while distal synapse density is reduced. Proximal synapses should drive activity, such as back propagating sodium potentials that are most affected by our **lenti-shRNA** expression. Furthermore, GCs are the primary target of cortical and cross-bulbar input to the OB, and these inputs synapse in the proximal region of GC dendrites (Price and Powell 1970). The increased cortical signaling associated with increased proximal synaptic density may account somewhat for the increase in survival of **lenti-shRNA** expressing ABNs in a sensory deprived circuit. This suggests that the survival of adult-born GCs may be particularly influenced by cortical and cross-bulbar input to the bulb. This would provide a mechanism for central and cross-bulbar feedback to maintain and refine OB functional connectivity in response to the broad introduction of new inhibitory units to the OB.

2.5.6 Limitations of the work

Several limitations exist in drawing conclusions from the work presented in this chapter. 1) The effect of expressing shRNA has not been fully decoupled from the survival rate of **lenti-shRNA** ABNs in 2.4.4., 2) The age of cells used throughout the experiments cannot be precisely defined due to lentiviral infection of continuously dividing progenitor cells, 3) Complex responses to could be occurring in the electrophysiology of **lenti-shRNA** ABNs in response to lowered expression of $Na_v1.1$-1.3.

The effect of expressing shRNA has not been fully decoupled from the survival rate of **lenti-shRNA** ABNs in 2.4.4. Strong expression of lentiviral constructs is known to overwhelm shRNA machinery crucially involved in gene transcription (Yi, Qin, et al. 2003, Yi, Doehle, et al. 2005, Zeng and Cullen 2004, Jackson and Linsey 2010). And, can lead to cell death (Grimm, et al. 2006). We have attempted to measure the effect of expressing shRNA through the use of a

lentiviral construct expressing a scrambled string of shRNA. This experiment produced results that can be interpreted in multiple ways, one of which is that the expression of shRNA accounts fully for the reduction in survival seen in the **lenti-shRNA** condition. It is important that the results of this work be viewed with the limitation that shRNA expression could be driving the reduction in survival seen relative to control **lenti-GFP** ABNs. Other explanations outlined in 2.4.6, including the expression level of GFP labeled cells and the coinfection rate of lenti-tdTomato come to interpretations that expression of shRNA does not fully account for the reduction in survival displayed in the **lenti-shRNA** condition.). Its important to note that this limitation applies only to comparison in survival between **lenti-shRNA** cells and **lenti-GFP** controls. The increase in survival seen for **lenti-shRNA** cells in a circuit with reduced sensory stimuli are expected to be independent of the expression of shRNA because the conclusions are based on a comparison of two conditions where shRNA is expressed.

We believe the most likely interpretation is that shRNA expression is not driving the reduction in survival seen in ABNs in the **lenti-shRNA** condition for three reasons: 1) Mature cortical neurons expressing lenti-scrambled-siRNA exhibit the same electrophysiological properties and are expressed in the same range of cells per injection (Komai, et al. 2006) 2) Infusion of siRNA into ABNs in the hippocampus does not cause cell death (De Toni, et al. 2008) 3) Work from other laboratories using an independent method, lowering cell-specific activity in ABNs using expression of the inward rectifying potassium channel ESKir2.1 (Lin, et al. 2010) saw a reduction in survival on the same order of magnitude as seen in our **lenti-shRNA** condition (56% reduction at 28 d.p.i. for ESKir2.1 versus 44% reduction at 35 d.p.i. for **lenti-shRNA**

Lentiviral infection of a population of progenitor cells confers continuous production of infected daughter cells. This will have effects on the interpretation of two important areas of this work: 1) It is likely that we have sampled a population of ABNs in the electrophysiological characterization work in 2.4.2 that is skewed toward a younger population of class 5 ABNs, 2) The population of ABNs sampled in the survival experiments in 2.4.4-7 is likely to be skewed towards a younger population of ABNs in the **lenti-shRNA** condition than in the **lenti-GFP** condition. In characterizing the electrophysiology of ABNs we targeted Class 5 ABNs using morphological properties such as dendritic branching and the presence of dendritic spines (Petreanu and Alvarez-Buylla 2002, Carleton, et al. 2003). Given the reported age profile of Class 5 ABNs it is likely these cells are 14 days or older. But, since **lenti-shRNA** cells exhibit a lower rate of survival new entering ABNs would skew our measured population towards younger Class 5 ABNs with an unknown effect on the cells measured. It is likely that at least a portion of the difference seen in the electrophysiciology measurements, though consistent with a reduction in expression of Nav1.1-1.3, is due to this lower aged population. There would be a similar effect on the populations of ABNs probed in the survival and subtype expression experiments. We attempted to address this by limiting our cell counts to the GCL and GLL. When quantified at 45 d.p.i young, migrating, lentiviral infected cells in the anterior commisure area represent <5% of the total population which would lead us to expect a minimal effect on the results seen at 35 d.p.i. (Mizrahi 2007). But, there is a measurable number of neurons that were born days to weeks after the initial lentiviral injection, and importantly in the **lenti-shRNA** condition one would expect this number to be larger as a share of the population given the lower rate of survival seen in 2.4.4. One would expect this to lead to a higher rate of measured survival for 2.4.4, have no effect on the conclusions drawn in the sensory deprived circuit in 2.4.5 due to a

similar effect in the comparison hemispheres, and to have lead to fewer cells expressing subtype markers in 2.4.7 (not the observed result). Thus, in terms of ABN survival we do not expect this confound to have had an effect on the conclusions of this work.

The increase in excitability seen in the first 150ms after stimulus onset could lead to an increase in activity for **lenti-shRNA** ABNs rather than a decrease in activity as proposed. Many of the variables tested electrophysiological would lead to the conclusion that overall levels of activity were lower for **lenti-shRNA** ABNs (reduced AP amplitude, increased AP threshold, reduced maximum firing rate, lowered ISI over long depolarizations, lower firing rates in response to high current injections, reduced 1^{st}:8^{th} AP amplitude ratio, etc.), but the increased excitability within the initial 100ms of a stimulus could lead to increased excitability for these cells in natural conditions. Granule cells recorded in vivo exhibit low firing rates in response to odor presentation (Cang and Isaacson 2003, Luo and Katz 2001, Wellis and Scott 1990). And breathing, especially sniffing, can elicit bursts of spiking on inhalation at roughly this time course (Abraham, et al. 2004, Kepecs, Uchida and Mainen 2005). An increased level of activity would challenge the proposed mechanism for the reduction in survival seen in 2.4.4 and could be a potential mechanism to explain the increase in survival seen in 2.4.6.

3.0 MORPHOLOGICAL ANALYSIS OF ACTIVITY-REDUCED ADULT-BORN NEURONS IN THE MOUSE OLFACTORY BULB

3.1 CHAPTER SUMMARY

Adult-born neurons are added to the olfactory bulb (OB) throughout life in rodents. While many factors have been identified as regulating the survival and integration of adult-born neurons (ABNs) into existing circuitry, the understanding of how these factors affect ABN morphology and connectivity is limited. Here we compared how cell intrinsic (shRNA knock down of voltage gated sodium channels $Na_v1.1$-1.3) and circuit level (naris occlusion) reductions in activity affect ABN morphology during integration into the OB. We found that both manipulations reduce the number of dendritic spines (and thus likely the number of reciprocal synaptic connections) formed with the surrounding circuitry and inhibited dendritic ramification of ABNs. Further, we identified regions of ABN apical dendrites where the largest and most significant decreases occur following shRNA knock down or naris occlusion. In shRNA knock down cells, reduction of spines is observed in proximal regions of the apical dendrite. This suggests that distal regions of the dendrite may remain active independent of $Na_v1.1$-1.3 channel expression, perhaps facilitated by activation of T-type calcium channels and NMDA receptors. By contrast, circuit level reduction of activity by naris occlusion resulted in a global depression of spine number. Together, these results indicate that ABNs retain the ability to develop their

typical overall morphological features regardless of experienced activity, and activity modulates the number and location of formed connections.

3.2 INTRODUCTION

Adult neurogenesis contributes adult-born neurons (ABNs) to the mouse olfactory bulb (OB) throughout life (Altman 1969). These neurons, generated in the subventricular zone (SVZ), migrate to the olfactory bulb, and upon arrival become mature neurons of several different types (Bagley, et al. 2007). A small fraction of these ABNs mature to become periglomerular neurons, but the majority of ABNs become local circuit interneurons morphologically similar to neonatal granule cells (Carleton, et al. 2003, Ninkovic, Mori and Götz 2007, Saghatelyan, et al. 2005) . Following their migration, many factors influence the survival of ABNs in the olfactory system (Alonso, et al. 2006, Lazarini and Lledo 2011). Manipulations that reduce activity – naris occlusion (Corotto, Henegar and Maruniak 1994, Cummings and Brunjes 1997), benzodiazepine treatment (Yamaguchi and Mori 2005), over-expression of potassium channels (Kelsch, Lin and Mosley, et al. 2009) or axonomy of olfactory receptor neurons (Mandairon, Jourdan and Didier 2003) – decrease ABN survival and integration. Conversely, manipulations thought to increase olfactory activity – enrichment of the olfactory environment (Rochefort, et al. 2002), odor discrimination training (Alonso, et al. 2006, Moreno, et al. 2009), or increased excitability (by viral expression of a bacterial sodium channel (Lin, et al. 2010) – have proven to increase rates of ABN survival and thus integration. While our understanding of the regulation of ABN survival by activity has advanced substantially, how these manipulations affect ABN morphology and connectivity is poorly understood (Kelsch, Lin and Mosley, et al. 2009,

Saghatelyan, et al. 2005). Examination of ABN morphology will provide insight into the number and location of connections established by ABNs during their integration into mature olfactory bulb circuitry. Information about these connections will, in turn, indicate how much input ABNs are receiving from the existing network, while also revealing the influence these new cells may have on network activity.

Here we compared how two kinds of activity reduction affect the ability of ABNs to integrate into existing circuitry. Specifically, we asked how reducing either *cell intrinsic* or *network-level* activity influenced the morphology and connectivity of ABNs, as assessed by measures of EGFP-expressing adult-born granule cell morphology. We observed that both modes of activity reduction (intrinsic and network-level) caused reductions in the number of spines formed by ABNs. These changes were widely distributed across regions of the apical dendritic tree, unlike what was observed in a previous report in which activity was reduced by naris occlusion (Kelsch, Lin and Mosley, et al. 2009). Additionally, we observed subtle changes in the morphologies of the apical dendrites of activity-reduced ABNs, evidenced by reductions in branching and apical dendrite complexity. These results suggest that activity-reduced ABNs may be less competent to receive synaptic input and thus integrate poorly when activity is low.

3.3 MATERIALS AND METHODS

3.3.1 Stereotaxic lentivirus injections

All animal protocols were carried out in accordance with the Carnegie Mellon University Institutional Animal Care and Use Committee guidelines. One of two lentivirus constructs,

developed and characterized previously (Komai, et al. 2006), were injected into 2 month old C57B/L6 mice (Taconic Farms). The first viral vector contained an ubiquitin promoter followed by EGFP (**lenti-GFP**) the second viral vector contained a tandem of U6 promoters, each expressing one shRNA, followed by the ubiquitin promoter expressing EGFP (**lenti-shRNA**, see Figure 31). Lentivirus injections were be performed as previously described (Cetin, et al. 2006, Dittgen, et al. 2004). Briefly, mice were anesthetized by IP injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) in water. Animals were kept deeply anesthetized as assessed by monitoring eyelid reflex, vibrissae movements, and pinch withdrawal. Body temperature was maintained at 37°C with a heating pad with digitally monitored feedback control. Two craniotomies 300-500 µm in diameter were drilled above the subventricular zone (final coordinates relative to Bregma: anterior -1mm, lateral ±1mm, depth 2.2 mm beneath the pia mater). Pulled glass quartz pipettes were broken to an outer diameter of ≈30 µm and inserted at a 27° angle caudal-rostral into the pia. 500 nL of lentivirus was injected slowly over ≈5 min.

3.3.2 Unilateral olfactory deprivation

Mice were anesthetized by IP injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) in water. After animals became non-responsive as monitored by tail pinch their external naris was closed by cauterization. A cautery tool (Fine Science Tools), consisting of a fine loop of platinum wire that is rapidly heated by passage of electrical current, was placed against the nostril until the skin flap that covers the naris was "melted" onto the inner surface. Immediately following the cautery, the area was swabbed with antibacterial/anesthetic gel and the animal was returned to a recovery cage where it was monitored until recovery over the following 48 hours. Extent of naris closure was assessed when animals were sacrificed. Only tissue from

animals in which the occluded naris remained completely closed was used in immunohistochemistry and analysis.

3.3.3 Immunohistochemistry

Mice were anesthetized as above and perfused transcardially with cold 1% NaCl in 0.1M phosphate buffer (PB) followed by cold 4% paraformaldehyde in 0.1M PB. The brains were dissected and fixed in 4% paraformaldehyde in PB overnight at 4°C and then sunk in 30% sucrose at 4°C for 24 hours. Each hemisphere of the brain was sliced coronally at 300 µm using an SM2000R cryostat (Leica Microsystems). Incubations were done at room temperature with oscillation. Sections were first incubated in 2% Triton X-100 (Sigma) and 2% normal donkey serum (NDS) (Jackson Immuno Research Laboratories) in PB for 1 hour. Rabbit anti-GFP antibody (1:1000, Invitrogen) was added in the presence of 2% NDS and 0.1% TritonX-100 in PB and incubated for 1 hour. The secondary antibody added was a conjugated Alexa Fluor 488 (1:600, Invitrogen), incubated for 1 hour in 2% NDS and 0.1% TritonX-100 in PB. After the addition of the secondary antibody, all incubations were performed in the dark. Sections were washed three times between each incubation step in PB for 5 minutes.

3.3.4 Imaging procedures

Sections were imaged in their entirety using a Carl Zeiss LSM 510 Meta NLO multi-photon microscope (Carl Zeiss). Excitation of EGFP was provided by a Chameleon Ultra-II femtosecond laser (Coherent) operating at 890nm. Non-descanned detection was provided by two silicon photomultiplier tubes situated on the back of the fluorescence light train of the Axio

Observer Z1 inverted microscope stand. A long-pass 680nm blocking filter was placed in front of the detection assembly to prevent any stray two-photon excitation. An individual band pass barrier filter (505-550nm) was placed in front of the detector for fluorescence emission selection. All images were produced using a 40x 1.3 NA Neofluar air objective (Carl Zeiss). 300 µm thick sections from the first coronal slice directly in front of the accessory olfactory bulb were imaged in their entirety through the Z-plane. Excitation and gain were set to maximize the resolution of dendritic spines in the center of the Z direction. Z-steps for each section were set at 0.8µm.

3.3.5 Granule cell reconstructions

Traced GCs were chosen at random from the center 30% of the Z plane of each slice. The volume used for this randomization was a series of image stacks from the lateral to medial edge of the olfactory bulb. Image stacks were stitched together with the Neurolucida software package (MBF Bioscience). Cell bodies within the center 30% of the Z direction were then numbered and a random number list was generated based on the total number of cell bodies in the volume. The first five granule cells, corresponding to the order generated by the random number list, that met the following criteria were traced: 1) Cell body located in the granule cell layer, between the internal plexiform layer and the subependymal zone; 2) Dendritic tree completely contained within the slice; and 3) Cells sufficiently bright under fluorescence to allow for tracing of all dendritic spines and fine dendritic processes. Cells not meeting all criteria were skipped and replaced by the next randomly numbered cell in the sequence. On average, the first 21.36 (+/- 11.07) neurons from the random number list provided five cells fulfilling all criteria for tracing.

3.3.6 Statistical analysis of traced cells

All slides and images were coded and the analysis was performed with the experimenter blinded to the conditions of the animal and individual brain hemispheres during analysis. Cells were analyzed using the Neurolucida Explorer software package (MBF Bioscience) quantifying aspects of the apical dendrite, such as the location, number, and branching order of spines and dendritic segments, as well as the location of the various lamina of the bulb. Further analysis was done using both Neurolucida Explorer and custom functions written in Igor Pro (Wavementrics). A Wilcoxon Rank-Sum test followed by a Bonferroni correction for multiple measures was used for all statistical tests.

3.4 RESULTS

3.4.1 EGFP-encoding lentivirus labels adult-born granule cells

To visualize ABNs in the olfactory bulb, we injected lentiviruses encoding EGFP into the Subventricular zone (SVZ) of adult mice. This virus infected and labeled neural progenitors, which migrate along the rostral migratory stream (RMS) to the olfactory bulb and integrate as inhibitory granule cells. To measure the effects of manipulating activity on ABN morphology, we considered two groups of animals (Figure 31C&D). The first group (**lenti-GFP** / **lenti-shRNA**, 4 animals, 20 cells; Figure 31C) received a lentiviruses encoding EGFP in one hemisphere (**lenti-GFP**, Figure 31A), and in the opposite hemisphere a lentivirus encoding for EGFP and a short hairpin RNA (**lenti-shRNA**, Figure 31B) that knocked down sodium channel

expression (Komai et al., 2006). These animals were otherwise un-manipulated (no naris occlusion). The second group (**lenti-GFP** / NO, 3 animals, 21 cells; Figure 31D) received injections of **lenti-GFP** only in both hemispheres followed by occlusion of one external naris on the same day (NO). Animals were allowed to survive for 35 d.p.i. – during which expression of sodium channels increases substantially in control cells (Carleton, et al. 2003) – before sacrifice and perfusion (Figure 31E).

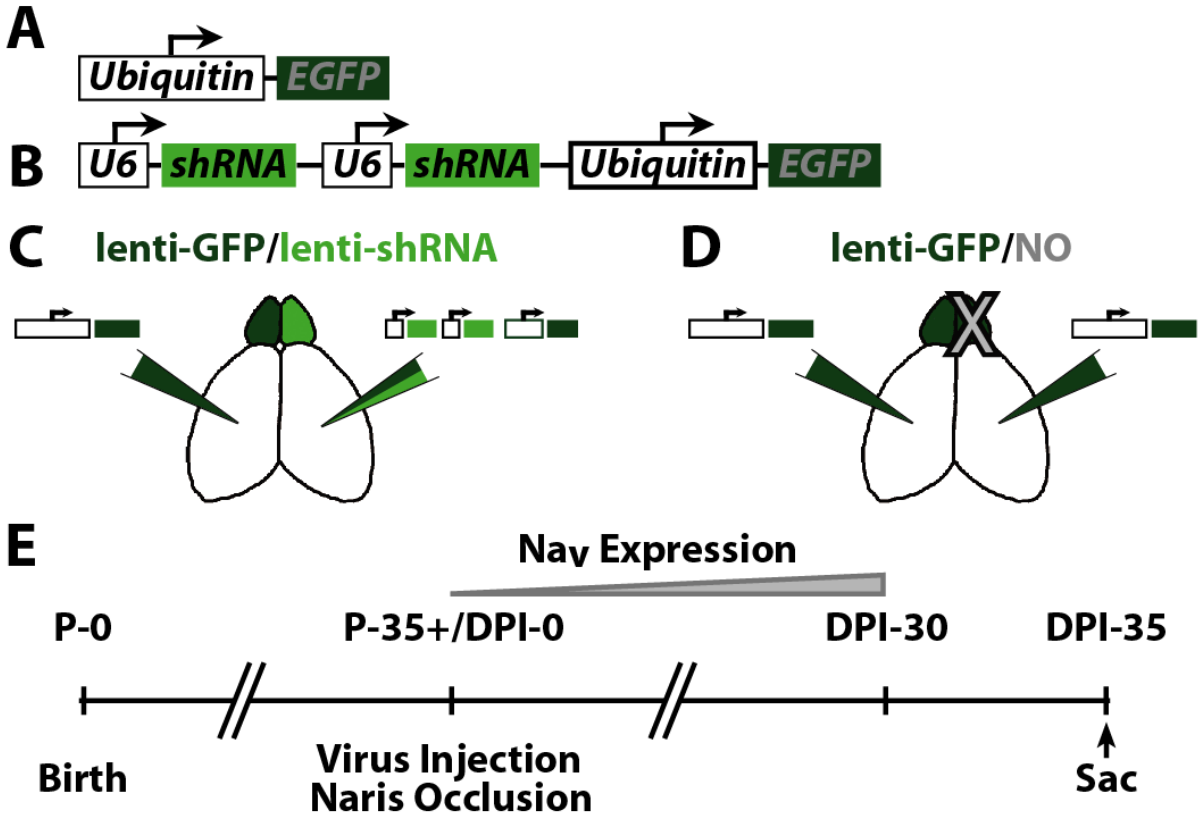

Figure 31. Summary of experimental conditions and injection paradigm.

(**A**) Schematic of EGFP coding lentivirus. EGFP was expressed downstream of the ubiquitin promoter. (B) Schematic of shRNA+EGFP coding lentivirus. Two U6 promoters, each expressing one shRNA against voltage gated sodium channel 1.1-1.3 ($Na_v1.1$-1.3) expression were followed by the ubiquitin promoter expressing EGFP. Each group received the following manipulations: (**C**) **lenti-GFP** (Control, see A) in one hemisphere, **lenti-shRNA** (reduced cell-intrinsic activity, see B) opposite hemisphere. (D) – **lenti-GFP** in both hemispheres, naris occlusion (NO) in one hemisphere. (E) Experimental timeframe. Mice were allowed to survive for 35 d.p.i. and naris occlusion before sacrifice, allowing for expression of Nav in progenitor cells born the day of injection (D.P.I.-0).

Following perfusion, 300µm thick sections were cut and completely contained & labeled ABNs were imaged in their entirety throughout the depth of the section (Figure 32A). Manual three-dimensional cellular reconstructions were performed from the acquired image stacks (Figure 31B, Figure 32).

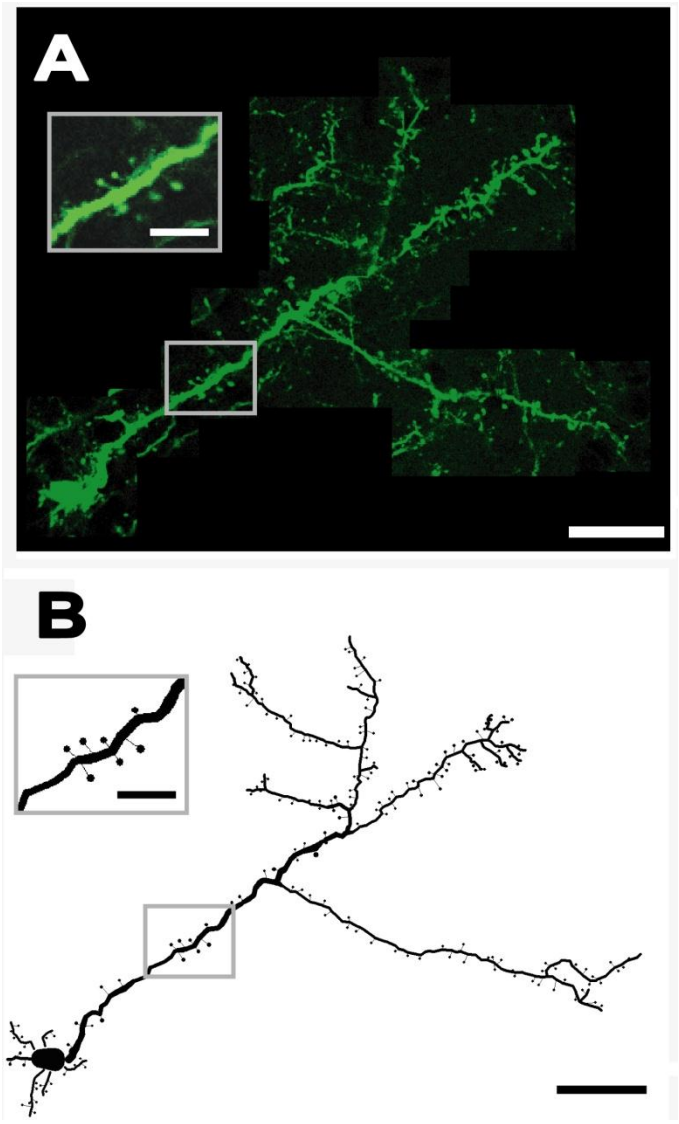

Figure 32. Example 2-photon imaged ABN and morphological tracing.

(A**)** Example neuron from individual slices of an image stack. Scale bar, 100µm. Inset shows resolution of apical dendrite spines. Scale bar, 25µm. **(B)** Example ABN reconstruction from images in (A). Scale bar, 100µm. Inset shows reconstruction of inset in (A). Scale bar, 25µm.

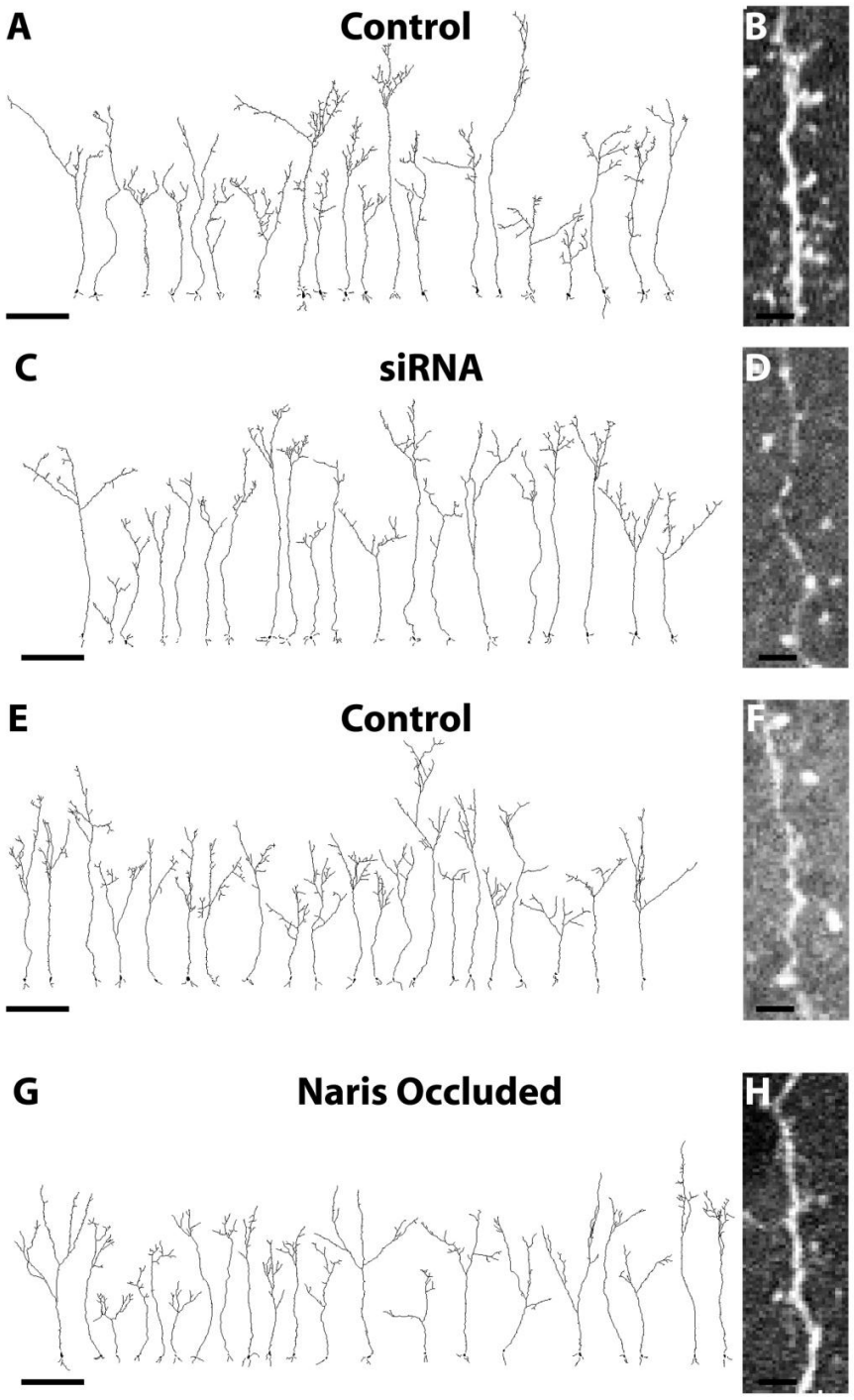

Figure 33. Complete cellular reconstructions of randomly selected EGFP^{+} ABNs.

(A&C) Group 1 GFP+ ABNs; 4 animals, 5 cells per hemisphere, n=20 per condition. Scale bar, 100µm. **(B&D)** Raw image of dendrite and spines taken from image stacks of **lenti-GFP** and **lenti-shRNA** ABNs respectively. Scale bar, 5µm. **(E&G)** Group 2 cells; 3 animals, 7 cells per hemisphere, n=21 per condition. Scale bar, 100µm. **(F&H)** Raw image of dendrite and spines taken from image stacks of control and naris occluded cells respectively. Scale bar, 5µm.

3.4.2 Reductions in spine number

Upon arrival in the OB, ABNs mature into olfactory granule cells (Petreanu and Alvarez-Buylla 2002, Carlén, et al. 2002, Belluzzi, et al. 2003, Carleton, et al. 2003, Saghatelyan, et al. 2005). Olfactory granule cells are inhibitory interneurons which form dendrodendritic connections with olfactory mitral and tufted cells (Rall, et al. 1966) through the formation of dendritic spines (Price and Powell 1970). From our cellular reconstructions, we quantified the number of spines on apical dendrites of mature ABNs to measure the degree to which these cells were able to integrate into mature olfactory bulb circuitry. Genetic reduction of cell intrinsic activity (**lenti-shRNA** mediated knock down of sodium channels) led to significant reductions in the total number of apical dendritic spines formed by integrating ABNs (control: 139.8±11.7; **lenti-shRNA**: 106.5±8.4; p=0.03; n=20; Figure 34A). Network-level reduction in activity (NO) resulted in a similar decrement in total spine number (**lenti-GFP**: 122.9±8.0; NO: 92.9±5.9; p=0.003; n=21; Figure 27A). The two control groups were not significantly different (p>0.31).

We also determined the location along the dendrite where these reductions occurred by plotting the number of spines (spine count) versus the distance from the soma along the dendritic tree (Figure 34B&C). In **lenti-shRNA** (Figure 34B) we saw significant differences in spine count between **lenti-GFP** and **lenti-shRNA** cells beginning as little as 40µm from the soma (at 40µm: **lenti-GFP**: 9.1±0.8 spines/bin, **lenti-shRNA**: 6.9±0.06 spines/bin; p<0.05; at 80µm: **lenti-GFP**: 12.7±1.9 spines/bin, **lenti-shRNA**: 6.9±0.8 spines/bin; p<0.05; n=20 cells). Proceeding distally, in **lenti-shRNA** cells this significant reduction was followed by continued sparseness, although spine counts were similar to control cells in distal segments. Conversely in NO cells, reductions in spine count did not localize to any specific region of the dendrite.

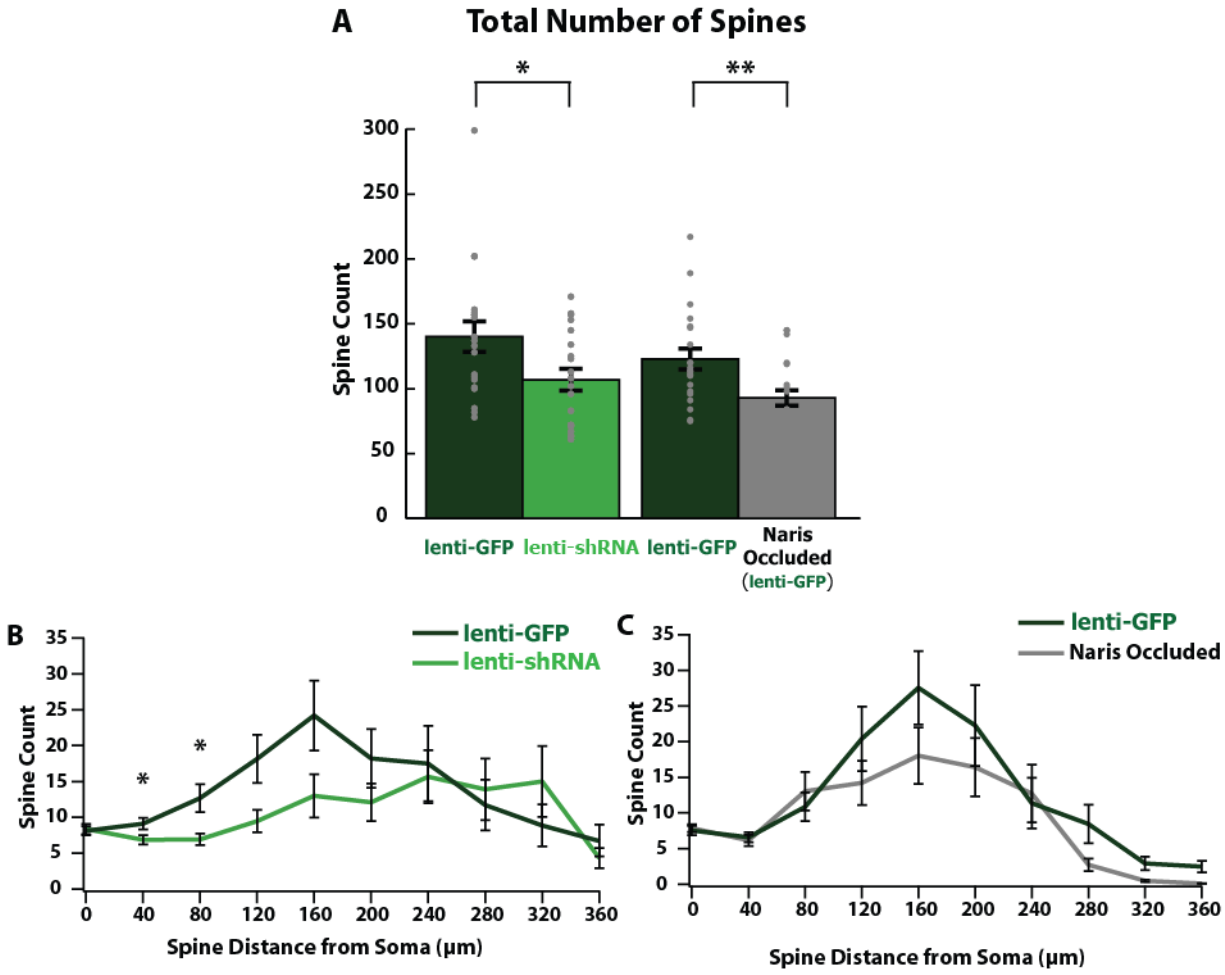

Figure 34. Reducing activity reduces the number of functional synapses formed in ABNs.

(A) Total spines per cell (grey dots) and group means (bars with standard error) for reconstructed ABNs. Both **lenti-shRNA** and naris occlusion showed significant reductions in total numbers of spines compared to controls (**lenti-GFP** / **lenti-shRNA**: p=0.031, n=20; **lenti-GFP** /Naris Occluded: p = 0.003, Wilcoxon Rank Sum test, n=21) in the number of formed synapses. **(B, C)** Distribution of spines plotted as total number of spines per 40µm bin versus distance along apical dendrite path with standard error. Significant differences occurred in early segments (40um-80um) of apical dendrites in **lenti-shRNA** cells (b); ($p<0.05$ and $p<0.05$ respectively, n=20). * = $p<0.05$, ** =$p<0.01$.

We next evaluated whether changes in dendrite morphology could explain the observed decreases in spine count by comparing differences in average spine densities per unit of dendritic length between control and activity-reduced cells (Figure 35A). We also plotted the distribution of spine density in spines/µm of dendrite along the dendritic tree for both control and activity-

reduced cells (Figure 35B&C). This normalization controls for changes in spine density due to changes in dendritic arborization. Comparison of spine density again revealed a small but significant reduction by **lenti-shRNA** (**lenti-GFP**: 0.25±0.01 spines/µm; **lenti-shRNA**: 0.21±0.01 spines/µm; $p<0.001$; n=20) and naris occlusion (**lenti-GFP**: 0.23±0.01 spines/µm; naris occlusion: 0.21±0.01 spines/µm respectively; $p=0.039$; n=21; Figure 28A). In cells from **lenti-GFP**/ **lenti-shRNA** animals, the spine density distribution shows that this reduction is observed throughout the first 200 µm of the somato-dendritic axis (Figure 35B), with large differences in spine density in proximal (at 40µm from soma: **lenti-GFP**: 0.23±0.02 spines/µm; **lenti-shRNA**: 0.16±0.01 spines/µm, $p<0.001$; at 80µm from soma: **lenti-GFP**: 0.28±0.04 spines/µm, **lenti-shRNA**: 0.16±0.02 spines/µm, $p<0.01$; n=20) regions of the dendrite. There was no significant difference in spine density in distal dendritic regions of **lenti-shRNA** cells (Figure 35B). In **lenti-GFP**/naris occluded animals, differences in spine density distribution (Figure 35C) were small and not significant.

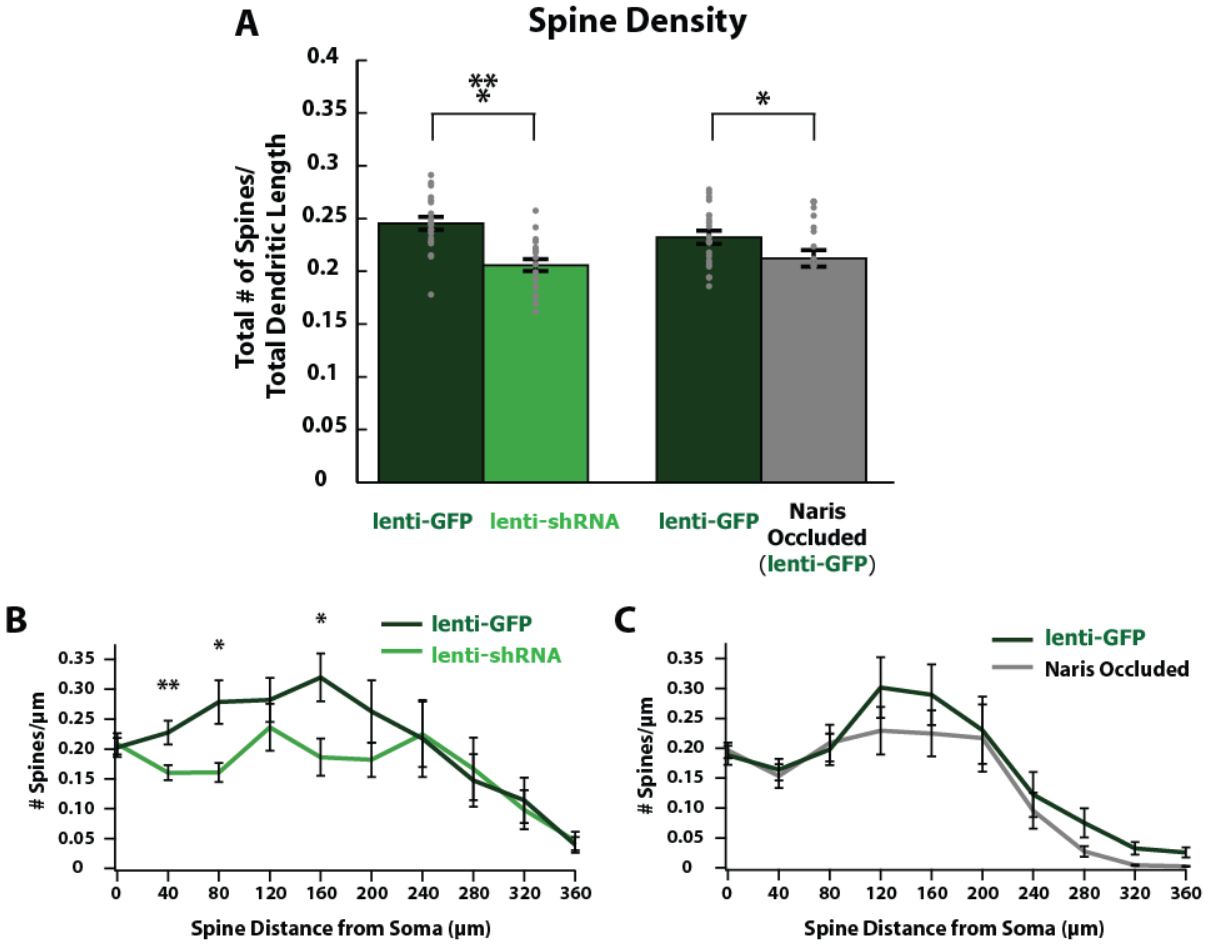

Figure 35. Reducing activity decreases spine densities in specific regions of ABNs.

(A) Spine density per cell (grey dots) and group means (bars with standard error). Spine density calculated as total spines per cell divided by total apical dendrite length. Both **lenti-shRNA** and naris occluded cells show significant overall reductions in spine (**lenti-GFP** / **lenti-shRNA**: $p<0.001$, $n=20$; **lenti-GFP** / Naris Occluded: $p=0.039$, $n=21$), without significant difference in total apical dendritic length (see Table 5 & 6). **(B, C)** Spine density plotted as total number of spines divided by total apical dendrite length in 40µm bins versus distance along apical dendrite path with standard error. **lenti-shRNA** cells **(B)** show significant decreases in spine density in proximal and intermediate regions of the dendrite (at 40µm: $p<0.01$; at 80µm: $p<0.05$; at 160µm: $p<0.05$; $n=20$). No significant differences were observed in naris occluded cells **(C).** * = $p<0.05$, ** =$p<0.01$, ***=$p<0.001$.

3.4.3 Activity deprivation causes reduced dendritic branching

To understand how ABNs integrating into the mature circuit might sample input from potential presynaptic partners, we compared how each of the two types of activity reduction affects the morphology and complexity of the apical dendritic trees of integrating ABNs independent of spine distribution (Figure 36). Extensive and elaborate ramification of dendritic trees into zones of potential synaptic input would suggest successful integration of ABNs. Initial comparisons of total dendritic length, total number of branches, average terminal branch distance, and average terminal branch order showed no significant differences in either group (Table 5 & 6), though in both experimental groups across these measures there was a trend towards reduced dendritic complexity in the activity-reduced groups. While these measures of overall dendritic complexity did not vary significantly, we wanted to carefully investigate the possibility of morphological changes specific to those regions where we saw changes in spine count and spine density.

Property	Lenti-GFP	lenti-shRNA	P value
# Branches	27.85 ± 3.54	20.80 ± 2.10	0.17
Average Terminal Branch Order	5.58 ± 0.30	4.94 ± 0.22	0.16
Dendritic Length (µm)	565.39 ± 41.40	517.55 ± 38.12	0.56
Average Terminal Distance (µm)	245.79 ± 18.60	267.70 ± 16.89	0.20

Table 5. Apical dendrite properties – lenti-GFP versus lenti-shRNA

Property	Lenti-GFP	Naris Occluded	P value
# Branches	28.33 ± 2.45	22.80 ± 1.39	0.09
Average Terminal Branch Order	5.37 ± 0.21	5.11 ± 0.14	0.35
Dendritic Length (µm)	545.91 ± 34.57	464.89 ± 37.07	0.06

Table 6. Apical dendrite properties – lenti-GFP versus naris occluded

To this end, we performed a 3-D Sholl analysis (Figure 36A) to measure the total dendritic length or number of intersections in spheres of increasing radii (Figure 36A1). These measures are reported as a function of sphere radius and are indicative of the degree of branching in neuronal processes (Sholl, 1953), where more complex processes result in more intersections with the "Sholl" sphere as distance increases. Sholl analysis of total dendritic length indicates **lenti-shRNA** cells have reduced dendritic length beginning in medial regions of the dendrite, showing a significant difference at a radius of 120µm (**lenti-GFP**: 42.3±7.1 µm; **lenti-shRNA**: 20.4±1.0 µm; $p<0.01$; $n=20$; Figure 36A2), indicating that control cells contain more or longer branches than siRNA knock down cells. Unlike **lenti-shRNA** cells, Naris Occluded animals failed to exhibit significant differences in any region of the dendrite (Figure 36A3).

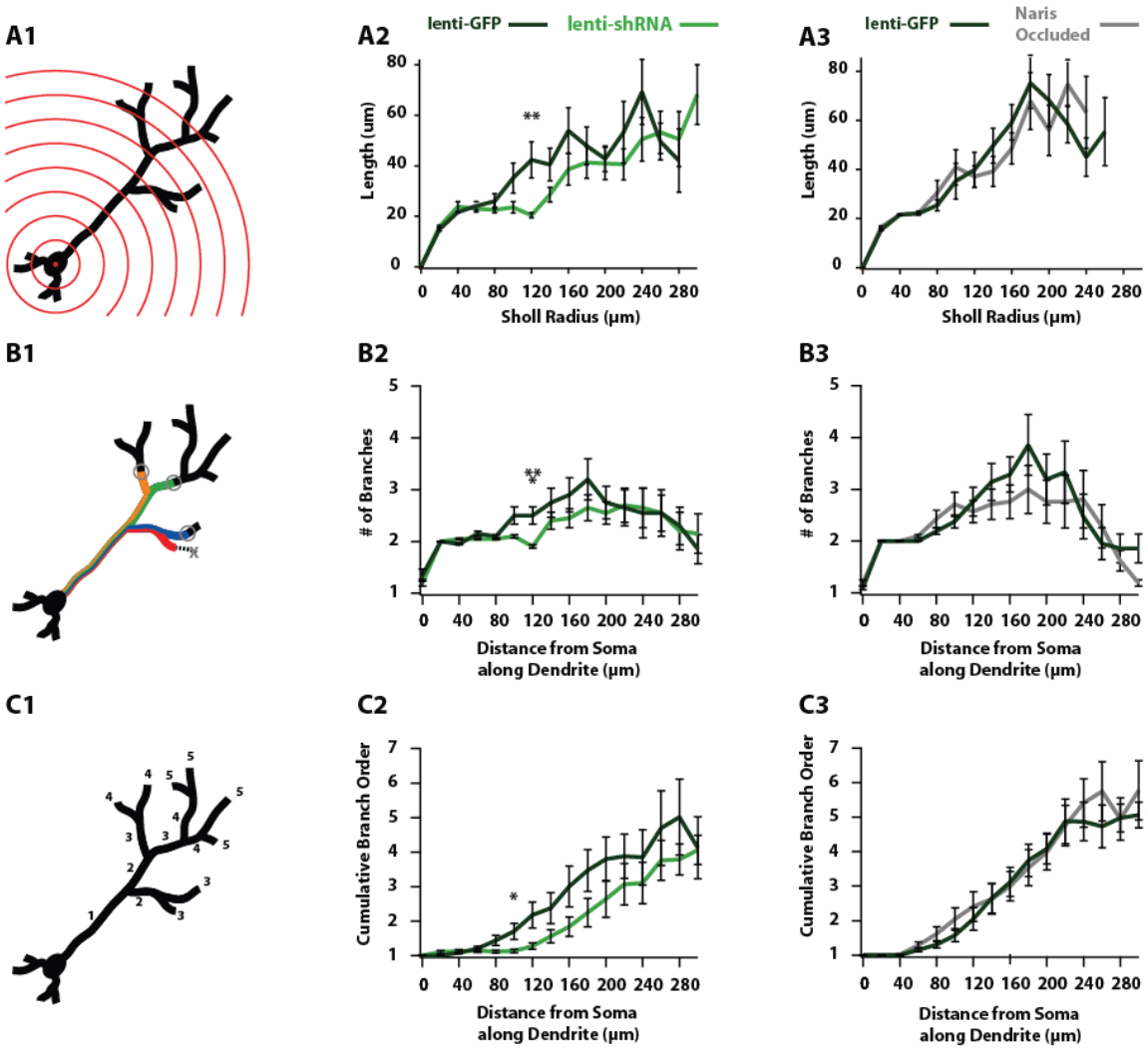

Figure 36. Reductions in activity reduce the overall complexity of newborn ABNs.

(A1) Classical Sholl analysis with radii increasing in 20µm increments. Sholl analysis measures apical dendrite length within each sphere plotted against radius from soma. **(A2)** **lenti-shRNA** cells show a significant reduction in length in proximal-intermediate distances from soma (at 120µm: $p<0.01$, n=20). **(A3)** Naris Occluded cells show no significant differences in Sholl analysis. **(B1)** Modified Sholl analysis of branch intersections. Modified Sholl analysis represents the number of branches at a fixed distance along the apical dendrite (not counting terminated branches). **(B2)** **lenti-shRNA** cells show a significant reduction in the number of branches at proximal-intermediate distances from soma (at 120µm: $p<0.001$, n=20). **(B3)** Naris Occluded cells show no significant differences in modified Sholl analysis. **(C1)** Cumulative branch order along length of apical dendrite. Plotted as the cumulative branch order (where branch order increments by 1 following bifurcation) versus distance from soma along apical dendrite length. **(C2)** **lenti-shRNA** cells show a significant reduction in cumulative branch order at proximal-intermediate distance from soma (at 100µm: $p<0.05$, n=20). **(C3)** Naris Occluded cells show no differences in cumulative branch order. * = $p<0.05$, ** =$p<0.01$, ***=$p<0.001$.

Reductions in the length of dendrites as assayed by the 3-D Sholl analysis may be explained by two scenarios: (1) dendrites in activity-reduced groups are less tortuous than those in the control group, or (2) there is a reduction in the number of branches formed by activity-reduced cells. Qualitatively, there did not appear to be significant differences in the relative tortuosity between control and activity-reduced cells of either group (Figure 33). Therefore, we suspected that the differences observed in the distribution of length could be accounted for by differences in branch pattern, i.e. the reduction in activity resulted in a reduction of high-order branching. To test this, we created modified Sholl plots that showed the number of intersections as a function of the distance from the soma *along the dendritic tree* (Figure 36B1) to form a more accurate picture of the magnitude of branching of the dendritic tree. We observed that **lenti-shRNA** reduced branching at medial distances (at 120µm: **lenti-GFP**: 2.5±0.17 branches; **lenti-shRNA**: 1.9±0.03 branches; $p<0.01$; $n=20$; Figure 36B2). Thus, an exaggerated dendritic tree of a **lenti-shRNA** cell would resemble a control cell stripped of branches at all but the most distal locations. Global activity reduction in naris occluded cells showed no significant difference in branching in our modified Sholl plot (Figure 36B3).

To examine global differences in dendritic complexity between control and activity-reduced cells, we plotted the average branch order against the distance from the soma *along the dendritic tree* (Figure 36C1). Though differences in total branch number and average terminal branch order were insignificant for both activity reduced groups (Table 5), observing these subtle differences together with the distributions of both the standard (Figure 36A) and modified (Figure 36B) Sholl plots suggest differences in cumulative branch number. Consistent with this prediction, there appeared to be an overall reduction of branching in siRNA knock down cells over much of the dendrite (Figure 36C2). Branch order was significantly reduced at 100µm

(**lenti-GFP**: 0.71±0.23; **lenti-shRNA**: 0.14±0.05; $p<0.05$; n=20) although branching recovered at the furthest distal segments (Figure 36C2). However, global activity reduction appeared to have no effect on the distribution of branch order Figure 36C3). Coupled with the modified Sholl plot Figure 36B), these data suggest that while global activity reduction does not compromise branch elaboration, it does cause reductions in branch length.

3.5 DISCUSSION

Alterations of activity can result either promote or inhibit the survival of ABNs in the olfactory bulb (Lin, et al. 2010, Cummings, Henning and Brunjes 1997, Mandairon, Jourdan and Didier 2003, Moreno, et al. 2009, Mouret, et al. 2009, Yamaguchi and Mori 2005). Here we used a viral vector encoding an shRNA to knock down sodium channel expression and naris occlusion to reduce the activity of ABNs that migrate to the olfactory bulb (see Chapter 2). Both methods used to reduce neuronal activity resulted in significant decreases in the total number of spines, overall spine density, and several measures of branching complexity. In **lenti-shRNA** cells, we observed that reductions in spine number, spine density and branching complexity incurred primarily in proximal and intermediate regions of the dendritic tree. However naris-occluded cells did not exhibit significant changes in any specific region along the dendrite but rather changes in dendritic length and branching were distributed along the length of the dendrite.

Previously published results by Kelsch *et al* (2009) reported that naris occlusion resulted in highly localized changes in the number of formed connections of ABNs labeled by a lentiviral vector expressing GFP tagged PSD-95. Specifically, changes seen in ABNs experiencing sensory deprivation by naris occlusion resulted in significant decreases in the number of PSD-95

labeled synapses at distal regions of the dendrite, and significant increases in the number of synapses at proximal regions of the dendrite (Kelsch, Lin and Mosley, et al. 2009). This effect does not appear in our results; however, it is important to note that PSD-95-GFP likely labels glutamatergic inputs onto ABNs rather than GABAergic outputs (Sheng 2001). While Kelsch *et al* (2009) did observe output synapses of ABNs via labeling of synaptophysin (SypG), which localizes to presynaptic neurotransmitter vesicles (Südhof and Jahn 1991), they reported significant changes in the number of SypG+ clusters at distal regions of the dendrite only. Because our experimental paradigm aimed to look at the effect of naris occlusion on the *functional* integration of ABNs into the olfactory bulb, we chose looked at changes in spines, which serve as the primary sites of GABAergic lateral inhibition onto mitral/tufted cells of the olfactory bulb (Panzanelli, et al. 2009). While our dataset appeared to exhibit trends consistent with analysis of SypG+ clustering by Kelsch *et al* (2009) it is unclear why our results did not reach significance.

Conversely, Lin *et al* (2010) observed the effects of genetically manipulating excitability on both survival and morphology of ABNs. They noted that neither genetically decreasing excitability (via expression of *ESKir2.1*) nor genetically increasing excitability (via expression of *NaChBac*) had any effect on morphology of ABNs (Lin et al., 2010). While these results appear to contrast our own, it is important to note that Lin *et al* (2010) altered the excitability of ABNs by inserting ion channels rather than altering expression of endogenous channels. Thus, the degree to which activity was changed may be different in our studies versus the previous work. Alternatively, homeostatic mechanisms may more effectively compensate for reductions in activity not caused by knockdown of endogenous channels. These subtle and specific

manipulations may provide insight into how differences in activity across unmanipulated ABNs can influence their connectivity.

Our results suggest that reduced levels of activity in ABNs adversely affect their ability to integrate into existing OB circuitry. Specifically, fewer spines formed by ABNs experiencing reduced activity indicates fewer connections made with surrounding circuitry, and thus reduced participation in olfactory processing. Further, it appears that these two different manipulations of activity differentially affect spine development of ABN dendrites. While it is believed that naris occlusion results in sensory deprivation (and thus reduction of activity) in the olfactory bulb (Frazier-Cierpial and Brunjes 1989), the effect of knocking down expression of voltage gated sodium channels (Na_v 1.1-1.3) in ABNs is less clear.

In mature olfactory granule cells, lateral inhibition onto mitral cells can be evoked by global action potentials (Carleton, et al. 2003, Petreanu and Alvarez-Buylla 2002) and low-threshold spiking (Egger 2008). Global action potentials result in long-lasting depolarizations, mediated by calcium activated nonspecific cation currents (Egger 2008), and NMDAR-dependent calcium influx. This results in prolonged release of GABA at dendrodendritic synapses, and thus more efficient lateral inhibition onto mitral and tufted cell dendrites when compared to inhibition due to low-threshold spiking (Hall and Delaney 2002). By knocking down Na_V expression in ABNs, we hoped to reduce the number of global action potentials and their resulting activity.

Given the role of Na_V channels in the physiology of olfactory granule cells, we hypothesize that global spiking of ABNs serves as the primary form of activity in proximal regions of the dendrite (Egger, Svoboda and Mainen 2003). However, at distal regions of the dendrite, highly localized calcium transients, due to dense populations of T-type calcium

channels in spines, facilitate NMDA receptor activation (Egger, Svoboda and Mainen 2005) and allow for dendrodendritic inhibition independent of sodium channel activation (Isaacson and Strowbridge 1998, Schoppa, et al. 1998). That is, activity in more proximal regions of the dendrite may be more vulnerable to fluctuations of Na_v channel expression, where activity in distal synapses may be more electrically isolated from the soma and thus depend only weakly on voltage gated sodium channels. This selective change in activity may be relevant to the differences between our results and those of Kelsch *et al* (2009).

One question is whether **lenti-shRNA** changes overall activity of affected olfactory bulbs, including uninfected cells. Since our lentiviral injections selectively labeled neural progenitors, only a small fraction of cells in a given olfactory bulb are infected by the virus; consequently the activity reduction in the infected granule cells is likely to have little effect on overall OB activity. Thus, the overall level of input received by new granule cells in the hemisphere injected with **lenti-shRNA** virus is likely to be comparable to cells in the **lenti-GFP** hemisphere. By contrast, cells in nares-occluded animals experience a reduction of activity via a reduction of synaptic input from sensory neurons to mitral cells. This will directly reduce input to distal dendrites of granule cells and also reduce somatic spiking. Thus, changes in the spatial profile of formed spines may reflect differences in the profile of activity in cells under these two different conditions.

We have focused on differences observed in *mature* ABNs. Therefore, we chose a single time point (35 d.p.i.), which allowed ample time for ABN maturation following birth. As reported previously, ABNs reach "class 5" morphology (full maturation) 15-30 days after birth (Petreanu and Alvarez-Buylla 2002). Cells in this morphological class have been shown to have adult-like membrane properties and sodium channel expression (Carleton, et al. 2003). Given

the typical maturation time of ABNs, we do not expect any time points beyond 35 d.p.i. to show differences from those reported here, but examining earlier time points could identify when activity reduction begins to affect synapse formation.

We have shown that ABNs can develop nearly normal morphological features despite manipulations in their activity levels. That is, these neurons adapt to their altered states or environments by altering specific aspects of their morphologies, while maintaining their gross structural features. Thus, major morphological features of these neurons develop in a manner that is largely insensitive to changes in activity, and observed changes are linked to more subtle and possibly highly-specific changes in input number and location.

4.0 GENERAL DISCUSSION

Ever since Ramon y Cajal's 1913 seminal work "*Degeneration and Regeneration of the Nervous System*," the dogma has been that new neuronal birth stops with development (Ramon y Cajal 1913). The revelation of mammalian adult neurogenesis opened a new way of thinking about, in essence, new born neurons born into the mature scaffolding of the adult brain (Altman and Das 1965, Gage, Song and Kempermann 2008). The study of adult neurogenesis promises to bring insights into disease states (Parent et al. 1997, Arvidsson et al. 2002, Curtis et al. 2005), synaptic development (Kelsch, et al. 2009, Livneh, et al. 2009), and eventual treatments for neuronal repair (Parent, Yu, et al. 1997, Arvidsson, et al. 2002, M. A. Curtis, et al. 2005).

4.1 SUMMARY AND INTERPRETATIONS OF THE FINDINGS

This work focused on the role of both extrinsic and intrinsic neuronal activity on the survival and integration of adult-born neurons in the olfactory bulb. Adult neurogenesis in the olfactory bulb is regulated by extrinsic neuronal activity (Corotto, Henegar and Maruniak 1994, Yamaguchi and Mori 2005) and cell-intrinsic activity (Lin, et al. 2010, W. Kelsch, et al. 2009). This dissertation further examined the interaction between extrinsic and cell-intrinsic activity based on the hypothesis that the survival of ABNs in the olfactory bulb is a function not just of their activity but of the activity of the surrounding neuronal circuit; That the survival and integration of adult-

born neurons entails a competitive process whereby the most active cells in the circuit integrate the most efficiently and are the most likely to survive.

In Chapter 2, we showed:

- Reduction in cell-intrinsic activity driven by lentiviral knockdown of endogenous voltage gated sodium channels causes a reduction in survival of OB ABNs 21 and 35 d.p.i. (Figure 24 & 25).
- ABNs with reduced cell-intrinsic activity in a sensory deprived circuit show an increased rate of survival when compared to ABNs with reduced cell-intrinsic activity in a normally functioning circuit (Figure 27).
- Adult-born granule cells with reduced cell-intrinsic activity show the same expression pattern of two calcium binding proteins that define two distinct population subtypes (Figure 29).

Importantly, we showed that the manipulation we used, lentiviral knock-down of voltage gated sodium channel expression, resulted in a reduction of neuronal activity as measured by several methods including: 1) reduced functional activity in response to odor stimulation exhibited by reduced expression of the immediate early gene Egr1 (Figure 29), and 2) Reduced sodium potential frequency and maximum firing rate in response to current injection (Figure 21).

Adult-born neurons mature over several weeks to exhibit mature neuronal morphology (Petreanu and Alvarez-Buylla 2002, Kelsch, Lin and Lois 2008) and physiological characteristics (Belluzzi, et al. 2003, Carleton, et al. 2003, Carlén, et al. 2002). This process is regulated by neuronal activity (Lin, et al. 2010).

In Chapter 3 we showed:

- Total number of dendritic spines, the morphological counterpart to a glutamatergic synapse, was reduced in adult-born GCs in the OB following reduction of both intrinsic activity (**lenti-shRNA** condition) and sensory driven activity (naris occlusion condition) (Figure 34).
- Adult-born GCs manifesting a reduced intrinsic activity (**lenti-shRNA**) condition showed reduced dendritic length, branching, spine number, and spine density in the proximal region of their dendrites (Figure 34 & Figure 35).

4.1.1 A proposed model for competitive activity-dependent synaptic integration of adult-born GCs in the olfactory bulb

Our results show effects of reduced neuronal activity on the morphology and synaptic integration of adult-born GCs in the olfactory bulb (Chapter 3). Here I attempt to explain our results and others obtained using similar approaches in a single model of synaptic integration.

Our **lenti-shRNA** manipulation reduces the expression of voltage gated sodium channels in adult-born GCs (Figure 17) and reduces the frequency and strength of sodium potentials generated at the soma (Figure 18-22, Table 3). Voltage gated sodium channels are known to be important for the initiation of back-propagating action potentials and communication with MCs (Isaacson and Strowbridge 1998, Egger, Svoboda and Mainen 2005, Pinato and Midtgaard 2005) and the communication of afferent input synapses to the distal output synapses between GCs and MCs (Egger 2008). Importantly, global sodium potentials drive long-lasting depolarizations, which mediate calcium influx (Egger 2008). Calcium influx is known to be important for activation of pro-survival factors (see next section) as well as the formation and maintenance of synapses (Dolmetsch, et al. 2001). It thus may necessary for the stabilization and maintenance of

synapses that are being rapidly formed and retracted in young ABNs (Mizrahi 2007, Livneh, et al. 2009).

In Chapter 3 we showed that **lenti-shRNA** selectively drove reduced synaptic density in the proximal region of adult-born GC dendrites (Figure 35 and see schematic in Figure 37). It is, perhaps, not surprising that by reducing the strength and frequency of action potentials reduces the synaptic density in this region.

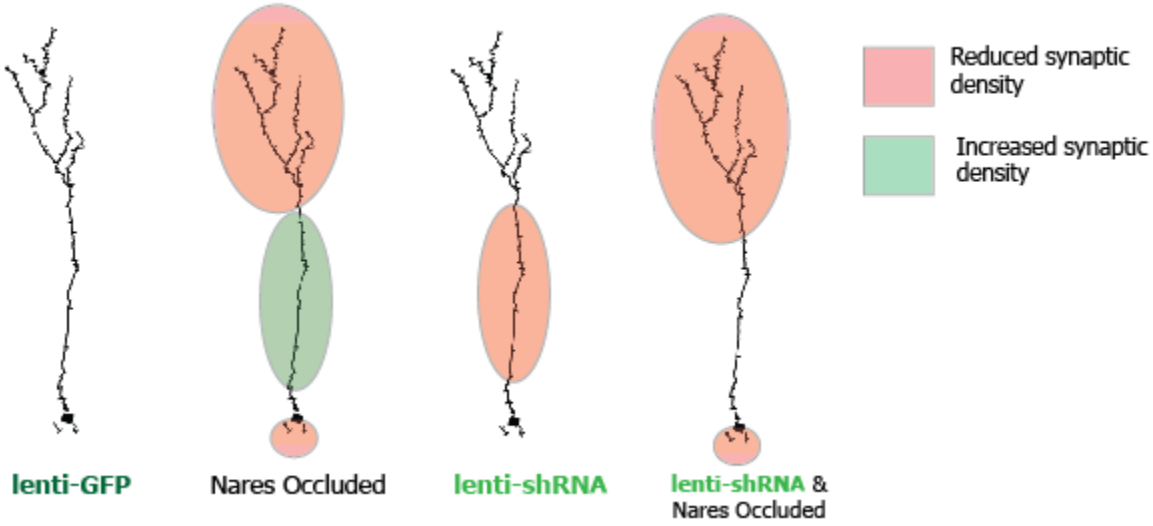

Figure 37. Proposed schematic showing synaptic density changes in adult-born GCs in response to activity manipulations.

The neuronal activity manipulations described in this dissertation drive different synaptic density changes depending on the manipulation. From left to right: ABNs in the **lenti-GFP** condition serve as a control group with normal levels of synaptic density. ABNs in a unilateral naris occluded hemisphere have a global reduction glutamatergic synaptic density (Figure 35a) but show an up-regulation of PSD-95 indicated synapses in the proximal region of their main dendrite (W. Kelsch, et al. 2009). **lenti-shRNA** ABNs show a reduced synaptic density in the proximal region of their dendrite (Figure 35b). And based on the synthesis of our results and (W. Kelsch, et al. 2009) we would hypothesize that the combination of **lenti-shRNA** and unilateral naris occlusion would drive a reduction in distal synapses but a rescue of synaptic density in the proximal region when compared to the **lenti-shRNA** only condition.

The proximal region of GC dendrites receive synaptic inputs from a number of afferent pathways (Shepard, Chen and Greer 2004) summarized in Figure 38. These include; cholinergic input via the Hind limb of the diagonal band, ipsilateral mitral cell axons that mediate mirror glomeruli signaling (De Olmos, Hardy and Heimer 1978, Lodovichi, Belluscio and Katz 2003); input from the contralateral anterior olfactory nucleus; input from piriform cortex; serotonergic input via the Raphe Nucleus; norepinephrine via the Locus coeruleus. The majority of these inputs, including all of the glutamatergic inputs, do not synapse onto MCs (Shepard, Chen and Greer 2004) and thus their synapses onto the proximal region of GC dendrites are their main method of modulating MC input into the cortex. By reducing expression of voltage gated sodium channels in our **lenti-shRNA** condition we are thus removing infected ABNs from participation in the centrifugal pathways modulation of OB output.

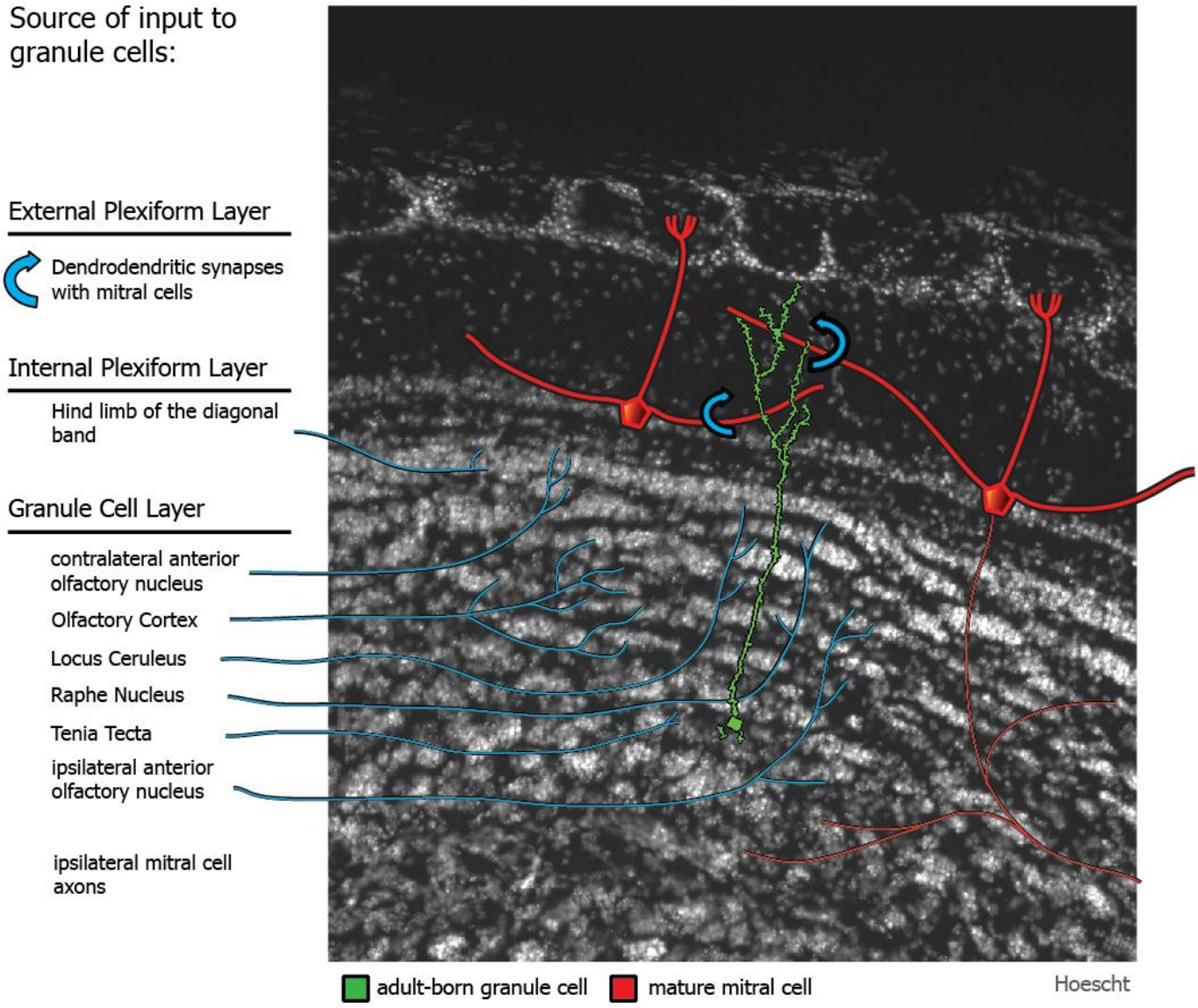

Figure 38. Neuronal input to GCs.

Granule cells receive input in two layers of the olfactory bulb: The EPL and GCL. In the EPL GCs form dendrodendritic connections with MCs; MCs release Glutamate onto GCs and GCs release GABA onto MCs. This is a GC only location for transmitter release. In the GCL and the neuropil surrounding it (the internal plexiform layer) GCs receive input from centrifugal pathways and ipsilateral mitral cell collaterals. These inputs are regionalized within the bulb with some projections preferentially targeting the deep bulb and others projecting more superficially. Figure adapted from (Shepard, Chen and Greer 2004).

Nares occlusion produces effects on synaptic density that are somewhat complementary to the effects observed in our **lenti-shRNA** manipulation. We described a global reduction in

synaptic density of ABNs following unilateral naris occlusion (Figure 34a), Kelsch and colleagues performed a similar study on ABNs in a circuit that had undergone unilateral naris occlusion that used the glutamatergic synaptic marker PSD-95 (W. Kelsch, et al. 2009). They saw not only a reduction in synapses, but additionally saw an increased density of glutamatergic synapses in the proximal region of the dendrite. This increase in glutamatergic synapses likely represents increased signaling from afferent pathways such as the olfactory cortex and contralateral anterior olfactory nucleus. In an interesting possibility, centrifugal pathways may be transmitting increased odorant related information encoded by the functional bulb to the GCs in the naris occluded bulb. The synthesis of the above synaptic density results is represented in the nares occluded schematic in Figure 37. We propose that the complementary changes in synaptic density seen in **lenti-shRNA** ABNs and the changes in a naris occluded circuit would combine to rescue the synaptic loss caused by **lenti-shRNA**. Because of this increase in afferent signaling the ABNs would thus be more able to compete with their neighbors in the occluded bulb than the **lenti-shRNA** ABNs in the normally functioning bulb. This is possible because 1) the **lenti-shRNA** ABNs would have increased synaptic input and 2) the mature GCs surrounding them would be less active, and thus less competitive, than the GCs in the normally functioning circuit.

4.1.2 A proposed model for competitive activity-dependent survival of adult-born neurons in the olfactory bulb

The work in this dissertation suggests that ABNs in the OB are competing for a scarce pro-survival resource in the bulb. That resource is dependent on the level of circuit level activity in the bulb because manipulations that reduce circuit level activity reduce survival of ABNs

(Corotto, Henegar and Maruniak 1994, Yamaguchi and Mori 2005, Mandairon, Jourdan and Didier 2003). That resource must also be dependent on the activity of individual cells because cells with reduced intrinsic activity show reduced survival in a normal activity circuit (Chapter 2, Lin et al. 2010). This dissertation work attempted to address the competitive interplay beween circuit and cell-intrinsic activity using reduced expression of voltage gated sodium channels to manipulate the level of cell-intrinsic activity.

Voltage gated sodium channels mediate back-propagating sodium potentials and communication from afferent inputs. They also induce long-lasting depolarization, which induces NMDA receptor mediated influx of calcium (Egger 2008). Several other manipulations that effected ABN survival in the olfactory bulb involved calcium. NMDA receptors mediate long lasting depolarization and calcium influx (Hall and Delaney 2002) and NMDA receptor knock out drives a drastic reduction in the survival of ABNs in the olfactory bulb (Lin, et al. 2010, Tashiro, et al. 2006). This reduction is rescued by expression of a bacterial voltage gated sodium channel that drives long lasting depolarization and presumably calcium influx (Lin, et al. 2010). Cholinergic innervations also drive long lasting depolarization and calcium influx and strongly regulates ABN survival in the OB (Cooper-Kuhn, Winkler and Kuhn 2004). Ca^{2+} influx is known to drive the pro-survival mitogen-activated protein kinase (MAPK) pathway (Xia et al. 1996, Hetman et al. 1999) and its activation is known to be necessary for odorant induced increases in survival of ABNs in the OB (Miwa and Storm 2005).

This accumulating evidence may point to a system wherein activity-dependent survival is mediated though activation of the MAPK pathway. There are varieties of pathways for activity dependent MAPK activation to occur, which are more fully described in section 2.3. Some possibilities include increases in intracellular calcium (Section 4.2.1.2), increased cholinergic

inputs (4.2.1.1), calcium influx through NMDA receptors (Lin, et al. 2010), increased afferent signaling (4.2.1.4), or neurotrophin action at the synapse (4.2.1.3). The competitive activity-dependent survival discussed within this dissertation would thus be the result of these pathways working in tandem to decide which cells in the circuit can compete most effectively for a limited pool of MAPK activating signals.

4.1.3 Limitations of the work

The conclusions reached in this dissertation would carry even greater weight had prior measures of morphology existed analogous to our experimental condition combining **lenti-shRNA** and naris occlusion. There was a rich description in both the literature and in our experiments of the morphological effects of both reduced cell-intrinsic activity and naris occlusion (Chapter 3, Kelsch et al. 2009). However, being able to directly compare those measures from the same experimental group would reinforce the conclusions.

The use of lentivirus' to infect a dividing pool of progenitor cells brings several caveats to the interpretation of the results. Our lentivirus injections target a self-regenerating pool of astrocytes along the sub-ventricular zone that give rise to daughter cells that were investigated in this work (Doetsch, Caille, et al. 1999, Palmer, Willhoite and Gage 2000). Viral transfection of these progenitor cells causes stable long term expression of introduced genes and confers copies of the incorporated DNA into multiple daughter cells (Naldini, et al. 1996, Levison and Goldman 1993, M. Luskin 1993, Morshead, Craig and van der Kooy 1998). But, Experiments using both BrdU pulses (half-life of several hours) and lentivirus injections show a drop in the number of lentivirus labeled neurons entering the olfactory bulb as soon as 10 days after injection and ~5% of total lentiviral labeled cells actively migrating to the bulb at 45 d.p.i. (Figure 4, Mizrahi 2007).

Additionally, all of the neurons randomly selected in Chapter 3 exhibited class 4-5 morphological characteristics that correspond to a population of neurons that were underwent their final division at least 21 days previously produced (Petreanu and Alvarez-Buylla 2002, Carleton, et al. 2003). For the results looking at survival of adult born neurons expressing Lenti shRNA, if new GFP+ neurons we're entering the OB at high levels one would expect a directionally opposite effect. Given experimental results in other laboratories, the morphological maturity of the GFP+ cells in the GCL at 35 d.p.i seen in Chapter 3, and the directional effect on conclusions reached on survival we do not expect that the conclusions reached in this work were affected by the continued expression of lentiviral genes by progenitor cells though we acknowledge that it is likely that a small % of neurons were born days and even weeks after lentivirus injections.

The lentivirus' used in this work take advantage of crucial cellular machinery involved in transcription of genes into proteins. In normally functioning cells this machinery serves to regulate the expression level of proteins and to protect cells against generation of proteins that should not be transcribed. Utilizing this machinery to reduce the transcription of $Na_v1.1$-1.3 in our **lenti-shRNA** condition could cause multiple non-specific effects by overwhelming the shRNA machinery and limiting or precluding its normal function (Jackson and Linsey 2010). Indeed long term over-expression (2 months) of virus delivered shRNA has been shown to decrease cytosolic levels of native micro RNAs and leads to death in the mouse liver (Grimm, et al. 2006). The transporter protein exportin particularly must bind and transport strands of shRNA across the nuclear membrane in order to activate them and can be expected to be inhibited competitively based on the relative concentration of the introduced shRNA to other shRNAs (Yi, Qin, et al. 2003, Yi, Doehle, et al. 2005, Zeng and Cullen 2004). Other proteins

involved in the pUbquitin promoter or the cleavage of shRNA strands could be affected similarly (Lund, et al. 2004, Sontheimer 2005, Castanotto, et al. 2007). Any effect expression of shRNA in our transfected cells could be expected to be large because of the use of the strong pUbiquitin promoter and multiple copies of shRNAs within a single vector (Komai, et al. 2006).

There could be an additional effect given multiple transfections and the transfection of multiple copies of the introduced gene in observed cells (exhibited most directly in cells expressing both GFP and RFP). One would expect the effect of this limitation may be attenuated in regards to the conclusions drawn however, because it occurs in both experimental and control conditions. Additionally, the ratio of red to yellow cells was not significantly different between 21 d.p.i. and 35 d.p.i in the control-GFP condition (see table 2) so expression of multiple copies of the introduced genes does not impact cells survival on the scale measured in this work.

Unilateral naris occlusion reduces the number of olfactory sensory neurons (Farbman, et al. 1988), the number of M/T cell synapses onto adult-born granule cells in the EPL (W. Kelsch, et al. 2009, Saghatelyan, et al. 2005, Benson, Ryugo and Hinds 1984), the expression of tyrosine hydroxylase in dopaminergic PG cells (Baker, et al. 1993, Cummings and Brunjes 1997, Puche and Shipley 1999), and the size and number of neurons in the OB (Fiske and Brunjes 2001, Cummings and Brunjes 1997, P. Brunjes 1985), however, odor stimulation drives c-Fos expression in MCs and PG cells in the naris occluded hemisphere (Jin, Franzen and Baker 1996) suggesting that naris occlusion does not result in complete sensory deprivation but the OB continues to receive activity either through the pharynx or centrifugal pathways. Indeed, mice that have undergone unilateral nares occlusion and a bulbectomy in the contralateral, unblocked, hemisphere can still perform odor guided tasks (Coppola, Coltrane and Arsov 1994). And ORNs from unilateral naris occluded hemispheres show compensatory physiological mechanisms that

potentiate their responses in vitro (Waggener and Coppola 2007). Additionally in response to unilateral naris occlusion the density of PSD95 synapses onto the proximal dendrite of adult-born GCs increases. These synapses are excitatory and presumably from centrifugal sources. Also, unilateral naris occlusion reduces the concentration of dopamine in the OB while sparing levels of norepinephrine (Brunjes, Smith-Crafts and McCarty 1985) . Overall this manipulation leads to a complicated picture of neuronal activity. Sensory activity transmitted by ORNs and transmitted via MCs seems to be reduced, but not eliminated. And compensatory physiological changes as well as the increased inputs from centrifugal pathways make it hard to discern the changes in neuronal activity that ABNs face in the occluded hemisphere.

Another limitation of the work lies in the conclusion about the reduction of activity conferred by the expression of lentiviral vectors. Given that actively diving cells continue to populate the OB with fluorescent cells (discussed above) when targeting GFP+ cells for electrophysiological work, the age of cells cannot easily be defined. We attempted to minimize this confound by targeting fluorescent neurons that exhibited mature Class 5 characteristics (e.g. single dendrite extending to EPL, spines on dendrite, see 2.3.11) that develop after 15-22 days post birth (Petreanu and Alvarez-Buylla 2002, Carleton, et al. 2003). But the experimental groups are expected to differ because of the difference in survival rate seen in 2.4.4. This would have two effects: 1) Our recording period extended from 21-35 days post injection, given that fewer **lenti-shRNA** cells survive to this time period, those that do survive can be expected to have a different, younger, population profile, 2) Lentivirus confers continuous production of ABNs from progenitor cells which would result in an increased sampling of ABNs that had recently reached Class 5 morphology. Given these two confounds there is the possibility that we

have compared a cohort of more mature **lenti-GFP** Class 5 ABNs to a less mature sample of **lenti-shRNA** Class 5 ABNs in our electrophysiological comparison.

There are additional limitations to the interpretation of our electrophysiological experiments even in these populations. As described in 2.5.2 significant differences between the experimental **lenti-shRNA** cells and the control **lenti-GFP** cells were found in high frequency ranges that have not been reported in in vivo GC recordings. We believe this will have important functional implications for **lenti-shRNA** GCs in the OB such as increased excitability at lower current injections. But, electrophysiological differences were found in AP amplitude, AP threshold, and the ratio of the amplitude of the 8^{th} to 1^{st} AP. And **lenti-shRNA** GCs were also less likely to express the immediate early gene Egr1 in response to odor presentation.

The expression of **lenti-shRNA** has the advantage of being what we consider a minimal manipulation at baseline conditions. ABNs are able initiate sodium potentials normally up to ~40 hertz (see FI curve in Figure 21). We did not observe a significant effect when compared to **lenti-GFP** on any of the baseline membrane properties that we measured such as input resistance or resting membrane potential (Table 3). While these observations give us confidence in physiological effects of the **lenti-shRNA** manipulation, they do not rule out compensatory changes in other voltage gated proteins. Possibilities for this include the compensatory expression of other voltage-gated sodium channels, voltage dependent calcium channels, increased expression of NMDAr, down regulation of voltage gated potassium channels, or changes in the buffering of intracellular ions. All of these changes would lead to similar physiological measures as seen in Table 3. This could be especially important in terms of calcium channels, as high voltage gated L-type calcium channels have been identified as mediating membrane depolarization of migrating neuroblasts (Darcy and Isaacson 2009). Such

calcium channels are intimately involved in communication at the dendrodendritic synapse between adult-born granule cells and mitral cells (Reviewed in Egger and Urban 2006, Egger Svoboda and Mainen 2005).

4.2 ACTIVITY-DEPENDENT MECHANISMS WHICH MAY INFLUENCE ADULT-BORN NEURON SURVIVAL AND INTEGRATION

Adult-neurogenesis is exquisitely regulated by neuronal activity in the adult-brain. Many pathways and systems are candidates to drive the activity-dependent survival and integration shown in this dissertation. A few of the most likely candidates are discussed in this section.

4.2.1.1 Neuromodulators

Several centrifugal pathways that synapse onto the proximal region of adult-born granule cells involve the neuromodulators, choline, serotonin, dopamine, and norepinephrine. The most relevant of these to activity-dependent survival and integration is the cholinergic system which projects to adult-born GCs from the basal forebrain via the hind limb of the diagonal band (see Figure 30, Shepard, Chen and Greer 2004).

Cholinergic stimulation has been shown to strongly regulate the survival of ABNs once they reach the OB (Cooper-Kuhn, Winkler and Kuhn 2004, Kaneko, Okano and Sawamoto 2006). Cholinergic fibers from the basal forebrain project via the hind limb of the diagonal band to the olfactory bulb and make synapses with GCs in the GCL and IPL (Shepard, Chen and Greer 2004). These synapses modulate the excitability of granule cells and increase GC inhibition onto

MCs at dendrodendritic synapses (Pressler, Inoue and Strowbridge 2007). Cholinergic fibers represent an intriguing potential source of the proximal synapses that may play an important role in regulating the survival of **lenti-shRNA** ABNs in unilateral naris occluded circuits.

4.2.1.2 Calcium

Levels of intracellular calcium have been identified as an important regulator of neuronal survival (Collins, et al. 1991). In the hippocampus (Tashiro, et al. 2006) and olfactory bulb (Lin, et al. 2010) genetic knock out of the NMDA receptor leads to decreased survival of ABNs. NMDA receptors mediate calcium entry and dendrodendritic signaling between MCs and GCs in the distal region in the EPL (Schoppa, et al. 1998, Isaacson and Strowbridge 1998, Chen, Xiong and Shepherd 2000). In mature cortical neurons calcium entry through L-type calcium channels activates the MAPK pro-survival pathway and contributes to synaptic stability and neuronal survival (Dolmetsch, et al. 2001). Due to the effect of NMDAr KO on survival and its role in mature neuronal health, it is likely that calcium entry and intracellular calcium levels have some effect on the survival of ABNs and the formation and maintenance of their synapses. Our manipulation in the expression of voltage gated sodium channels is germane to calcium influx and intracellular levels of calcium because global sodium spikes lead to calcium influx at distal synapses mediated by T-type calcium channels (Egger, Svoboda and Mainen 2003, Egger 2008). A corresponding decrease in the influx of calcium and its intracellular level may be involved in the decrease in ABN survival and the reduction in synaptic density seen in our **lenti-shRNA** condition.

4.2.1.3 Synapses

Based on the time course of the decrease in survival we've seen in our **lenti-shRNA** cell-intrinsic activity manipulation, regulation of survival by cell intrinsic activity-dependent mechanisms must occur on a short time scale of 21 days. This short window is supported by work using EsKir2.1 where a 56% reduction in survival of ABNs was seen between 2 and 4 weeks (Lin, et al. 2010). Additionally sensory driven activity is known to have a critical period for survival from 2-4 weeks (Yamaguchi and Mori 2005, Lin, et al. 2010). Recently two studies have looked at the development of the output synapses of adult-born granule cells. Kelsch and colleagues looked at the development of synaptophysin:GFP positive clusters which label presynaptic output sites (Kelsch, Lin and Lois 2008). They saw a development in the distal dendritic regions going from a sparse synaptic density to a developed synaptic density from 3 to 4 weeks after the birth of ABNs. Another group utilized a lentivirus delivered channelrhodopsin2 which gave them control over ABN depolarization using light pulses (Bardy, et al. 2010). Adult-born GCs began driving eISPCs in MCs 4-6 weeks after infection which is indicative of a functional synaptic connection. Based on the established critical period for activity-dependent survival that we and others have shown (Chapter 2, Yamaguchi and Mori 2005, Lin, et al. 2010) it is likely that ABN survival is solely regulated by synaptic input and is not significantly affected by synaptic output.

Growth factors such as BDNF and its associated receptor TrkB have been implicated in the survival of ABNs in a variety of systems (Li, et al. 2008, Kirschenbaum and Goldman 1995, Rossi, et al. 2006, Zigova, et al. 1998) and are more generally required for synaptic formation and maintenance (Berghuis et al. 2006, Rico Xu and Reichardt 2002, Gonzalez et al. 1999, Chakravarthy, et al. 2006, reviewed in Lichtman and Colman 2000). TrkB receptors are

expressed by GCs in the olfactory bulb (Deckner, et al. 1993). BDNF also activates the MAPK pathway (Hetman, et al. 1999) which is known to be necessary for odorant induced increases in survival of ABNs in the OB (Miwa and Storm 2005). Their role in regulation of synapse formation, synapse maintenance, and adult neurogenesis suggest that growth factors and Trk receptors could be involved in the competitive activity-dependent survival seen in this dissertation.

4.2.1.4 Cortical and cross-bulbar feedback

GCs in the olfactory bulb receive excitatory input from M/T cells at their distal synapses within the EPL, but also receive excitatory input proximally from axonal inputs from the cortex (De Olmost et al. 1978, Haberly and Price 1978, Shipley and Adamek 1984) and M/T cells (see Figure 30, Price and Powell 1970, Orona, Rainer and Scott 1984). Proximal axonal inputs may be more effective than sensory input in driving depolarization sufficient to initiate sodium spikes and overcome NMDAr blockade at dendrodendritic synapses globally (Balu, Pressler and Strowbridge 2007). Sensory stimulation in rodents under anesthetic *in vivo* elicits sodium spikes in GCs but these tend to be sparse (~2.1 APs per respiratory cycle) and cease after the first respiration cycle (Cang and Isaacson 2003). While gamma frequency input from axonal projections in the GCL readily elicits global sodium spikes via proximal synapses in GCs (Halabisky and Strowbridge 2003). Given the increase in survival of **lenti-shRNA** ABNs in nares occluded circuits, the increase in the density of those proximal synapses after unilateral nares occlusion (W. Kelsch, et al. 2009), and the importance of proximal axonal input to the functioning of GCs in the olfactory bulb, one could hypothesize that increased axonal signaling from cortical regions promotes and is necessary for the survival of adult-born GCs.

4.3 RELEVANCE TO OLFACTORY BULB FUNCTION

Despite the scale of adult neurogenesis in the OB, there is a surprising lack of behaviors associated with the process thus far. Descriptions of functional changes caused by the process are sparse as well. There are, however, two functions that have been described for ABNs in the olfactory bulb: odor discrimination between similar odors and a form of olfactory memory. Both of these have sound theoretical footing, Ceccchi and colleagues describe a mathematical model in which adult-born GCs modulate MC firing rates to maximize the MC output difference between similar odors (Cecchi, et al. 2001). With the addition of additional untrained units this model is then able to learn over time and will perform better on odor discrimination than a model without new inhibitory units. Inherent in this model is a form of olfactory memory as well as trained units to adopt the synaptic firing rates that best discriminate odors presented during their integration into the model.

Behavioral data has supported these predicted observations. 8 weeks after birth adult-born GCs respond most strongly to odors that were presented to animals 2-3 weeks after BrdU injection: a time when their input synapses are forming most rapidly (Magavi, et al. 2005). Animals reared in enriched olfactory environments, a manipulation that increases the survival of ABNs, spend less time investigating a novel odor, a measure of olfactory memory, than control mice (Rochefort, et al. 2002). Odor discrimination is impaired in animals with reduced OB adult neurogenesis (Gheusi, et al. 2000, Enwere, et al. 2004).

We have shown that ABNs with reduced cell-intrinsic activity integrate less fully into the OB circuit as evidenced by reduced expression of the immediate early gene Egr1 (Figure 23). We would hypothesize that neurons with reduced cell-intrinsic activity would be less able to encode olfactory memory information and are less able to contribute to discrimination of similar

odors. Additionally cells that are less active intrinsically than their neighbors would not be able to compete efficiently for the activity-dependent pro survival factors outlined in 4.2 and would be pruned from the circuit. ABNs that were more able to integrate into the circuit however, for instance new neurons that can more efficiently encode odorant information integrating into a circuit that does not adequately maximize the output difference of MCs, would be more likely to survive at the expense of their less efficient neighbors.

4.4 CONCLUSIONS AND FUTURE DIRECTIONS

To conclude, both extrinsic and cell-intrinsic neuronal activities regulate the survival and integration of adult-born neurons in the olfactory bulb. Lowered cell-intrinsic activity in adult-born GCs causes lower rates of survival (Chapter 2) and a lowered number of glutamatergic synapses (Chapter 3). Similarly, reducing sensory activity causes lowered survival (Chapter 2) and a lowered number of glutamatergic synapses (Chapter 3) in adult-born neurons. When we combine manipulations that lowered both the level of extrinsic and cell-intrinsic activity, survival of ABNs increased relative to ABNs with solely lowered cell-intrinsic activity. We conclude that the activity of not just individual ABNs but also the relative activity of other neurons in the circuit is important in determining whether an ABN successfully survives and integrates into the OB.

Previous work has shown that the synaptic distribution of ABNs in the OB develops over a similar time period as the activity-dependent survival of ABNs. (Kelsch, Lin and Lois 2008, W. Kelsch, et al. 2009). We would expect that our combination of lowered sensory activity and lowered cell-intrinsic activity would display an increase in the number of proximal spines near

the cell body relative to the **lenti-shRNA** condition. This increase was seen by Kelsch and colleagues in a reduced sensory activity condition (W. Kelsch, et al. 2009) and may in part account for the increase in survival seen in our combination of **lenti-shRNA** and naris occlusion. Our results suggest that, in order to understand the role of circuit level activity in the regulation of ABN survival and integration, it would be important to more fully understand the contribution of afferent synapses to the proximal region of the adult-born GC dendrite. Future experiments could be designed to selectively reduce afferent inputs to GCs through pharmacology or lesion of the tracts described in Figure 30. In combination with unilateral naris occlusion, manipulation of cell-intrinsic activity, and fluorescent labeling one could then quantify the effect on ABN survival that each afferent pathway provides. But in terms of understanding the factors that regulate survival of ABNs, the recognition that both sensory inputs and afferent inputs interact may prove to be the more important conclusion.

The results shown here suggest that repair or replacement treatments may be most effective in circuits with a low level of circuit activity. From this we would hypothesize that neural stem cell grafts would be at their most effective in integrating new neurons within quiet circuits that have normal innervations of afferent pathways. This is a similar situation to what one would expect following an ischemic stroke. Focal regions of strokes may present an opportunity as SVZ progenitor cells exhibit increased proliferation after middle cerebral artery occlusion (Arvidsson, et al. 2002, Jin, et al. 2001). These progenitors can migrate to the striatum after stroke (Kobayashi, et al. 2006), and may survive long term and produce synaptic machinery (Yamashita, et al. 2006).

The unexpected existence of adult-born neurons has provided us with the surprising potential to understand how an ongoing developmental process shapes adult brain circuitry. It

has provided us with the potential to understand aspects of neuronal replacement and repair far in advance of the development of neural stem cell therapies. And it has opened potential treatment avenues for neurological disorders such as traumatic brain injury, ischemia, depression, and degenerative diseases. Thus, these and future experiments examining the activity-dependent survival and integration of ABNs will provide insight into how neural stem cells become new neurons that successfully integrate into the scaffolding of the adult brain. Neuronal activity promises to be an important consideration and tool in the future of neuronal repair and replacement therapies.

APPENDIX

A. ELECTROPHYSIOLOGY TRACES

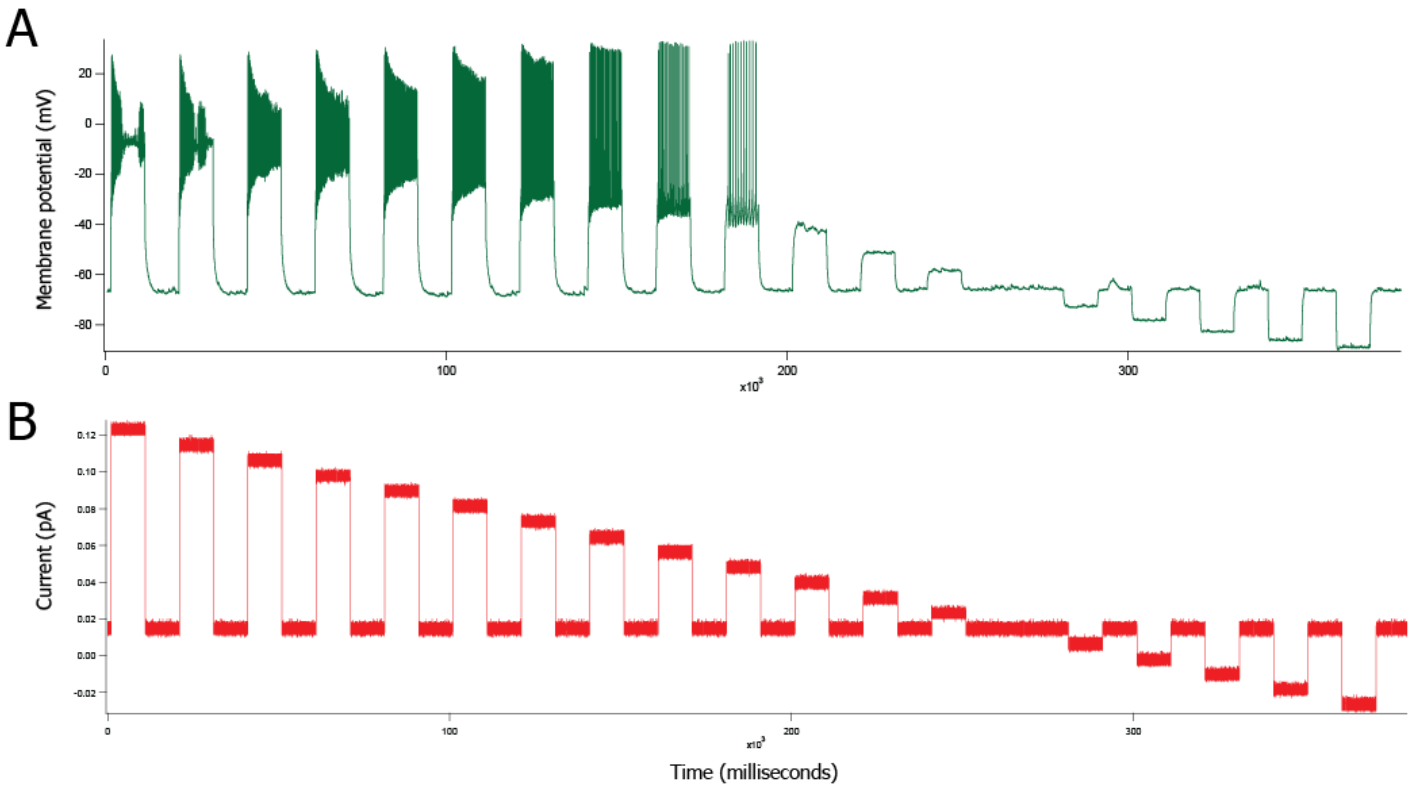

Figure 39. Voltage recording from lenti-GFP cell 1

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

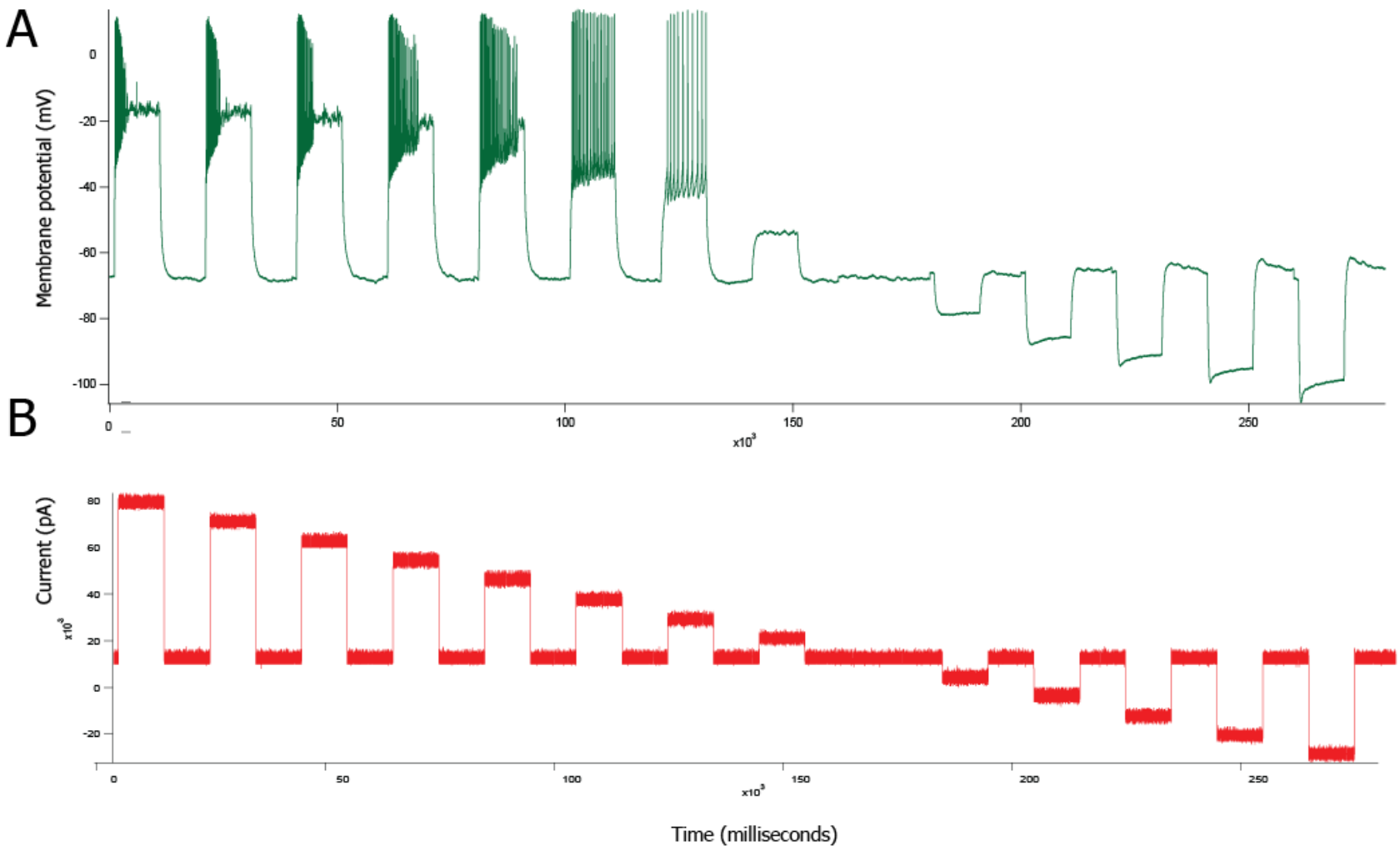

Figure 40. Voltage recording from lenti-GFP cell 2

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

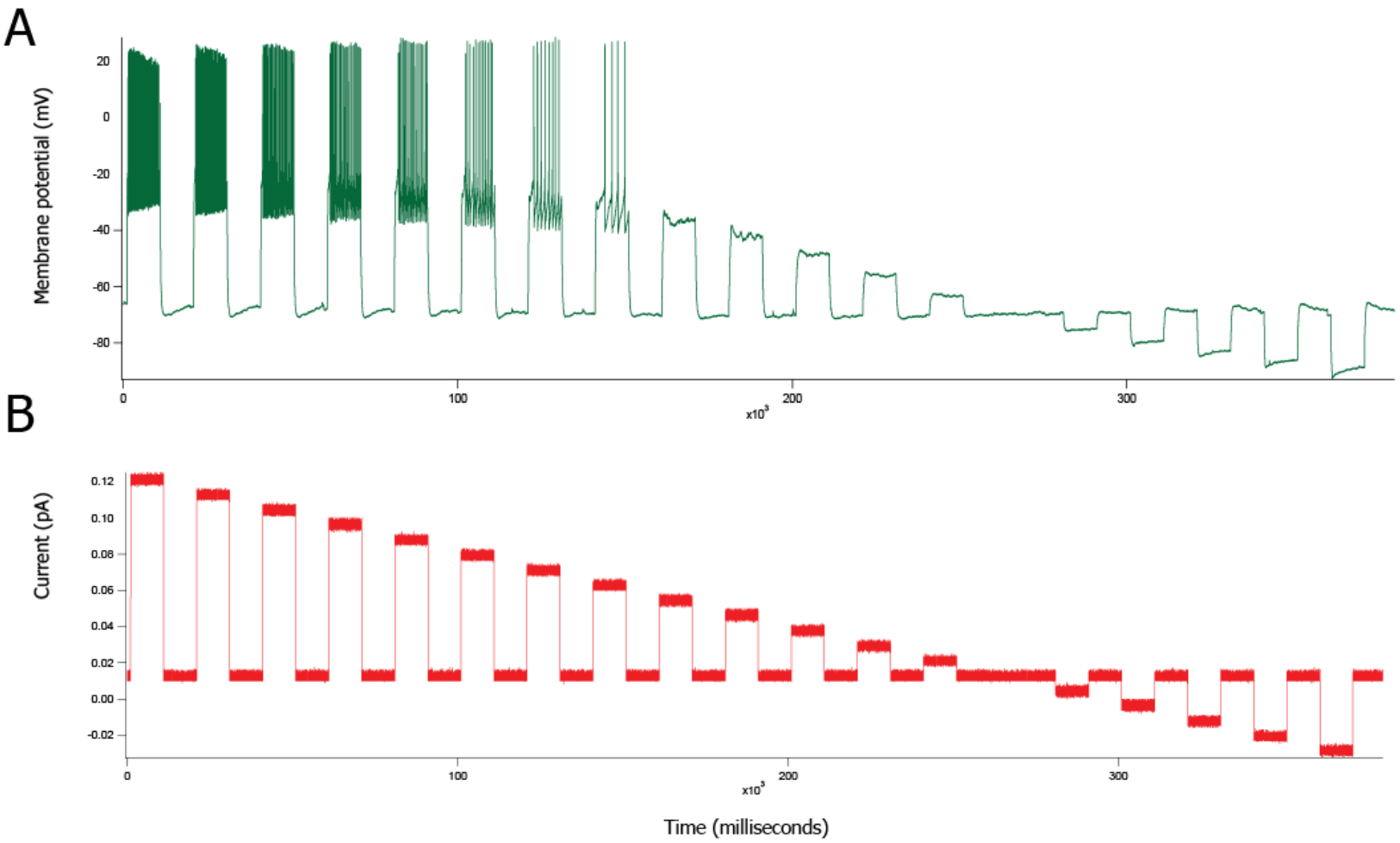

Figure 41. Voltage recording from lenti-GFP cell 3

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

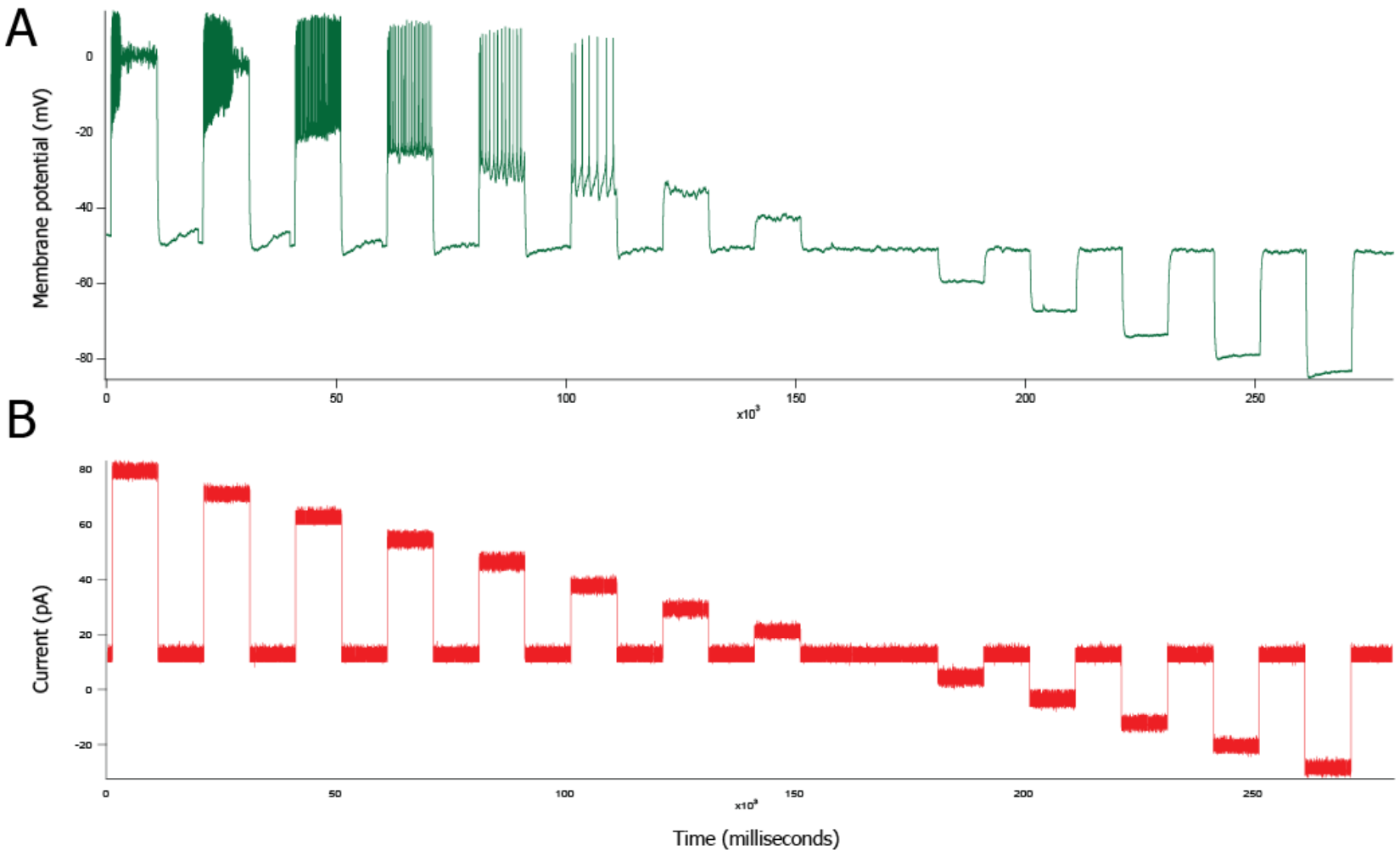

Figure 42. Voltage recording from lenti-GFP cell 4

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

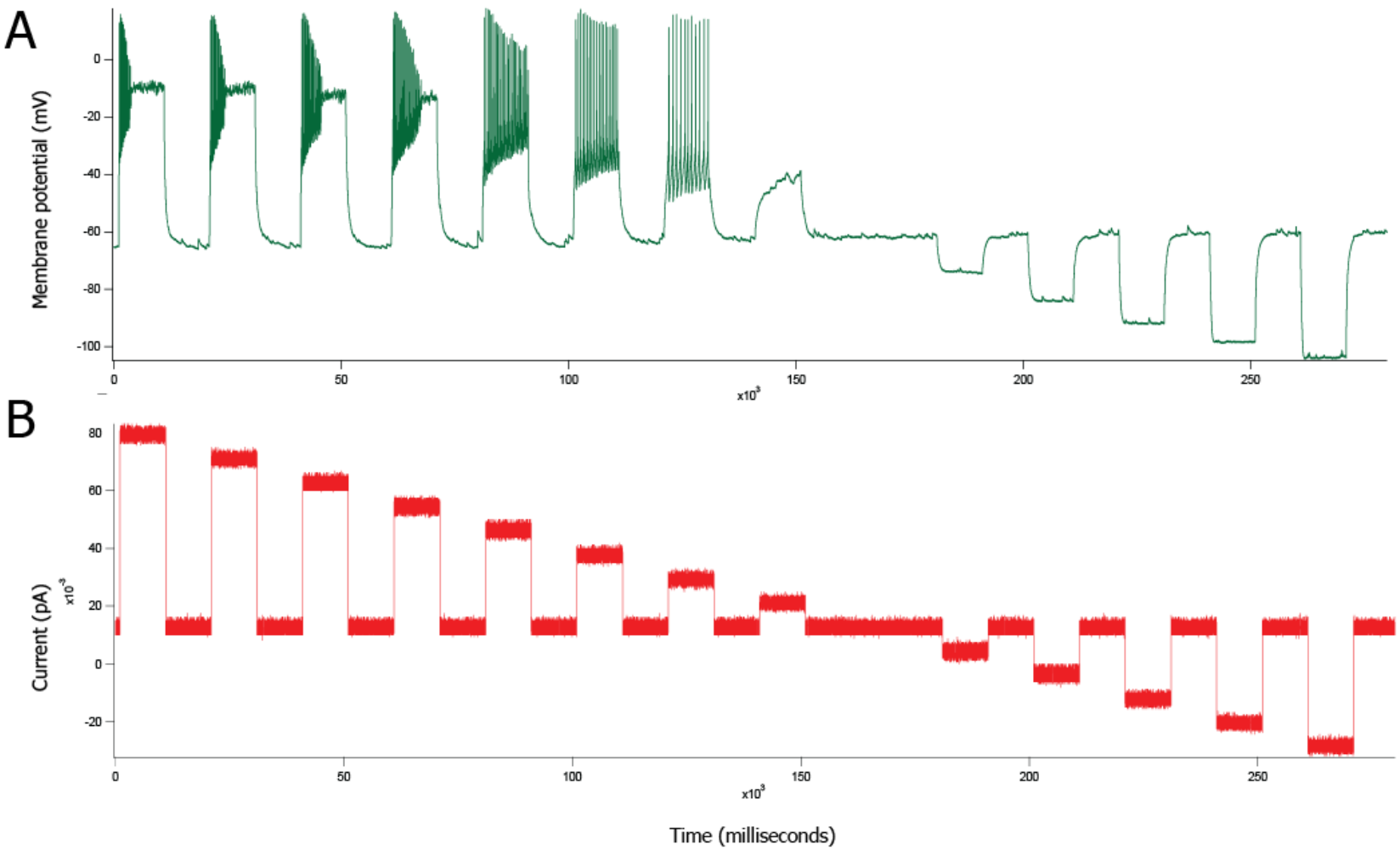

Figure 43. Voltage recording from lenti-GFP cell 5

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

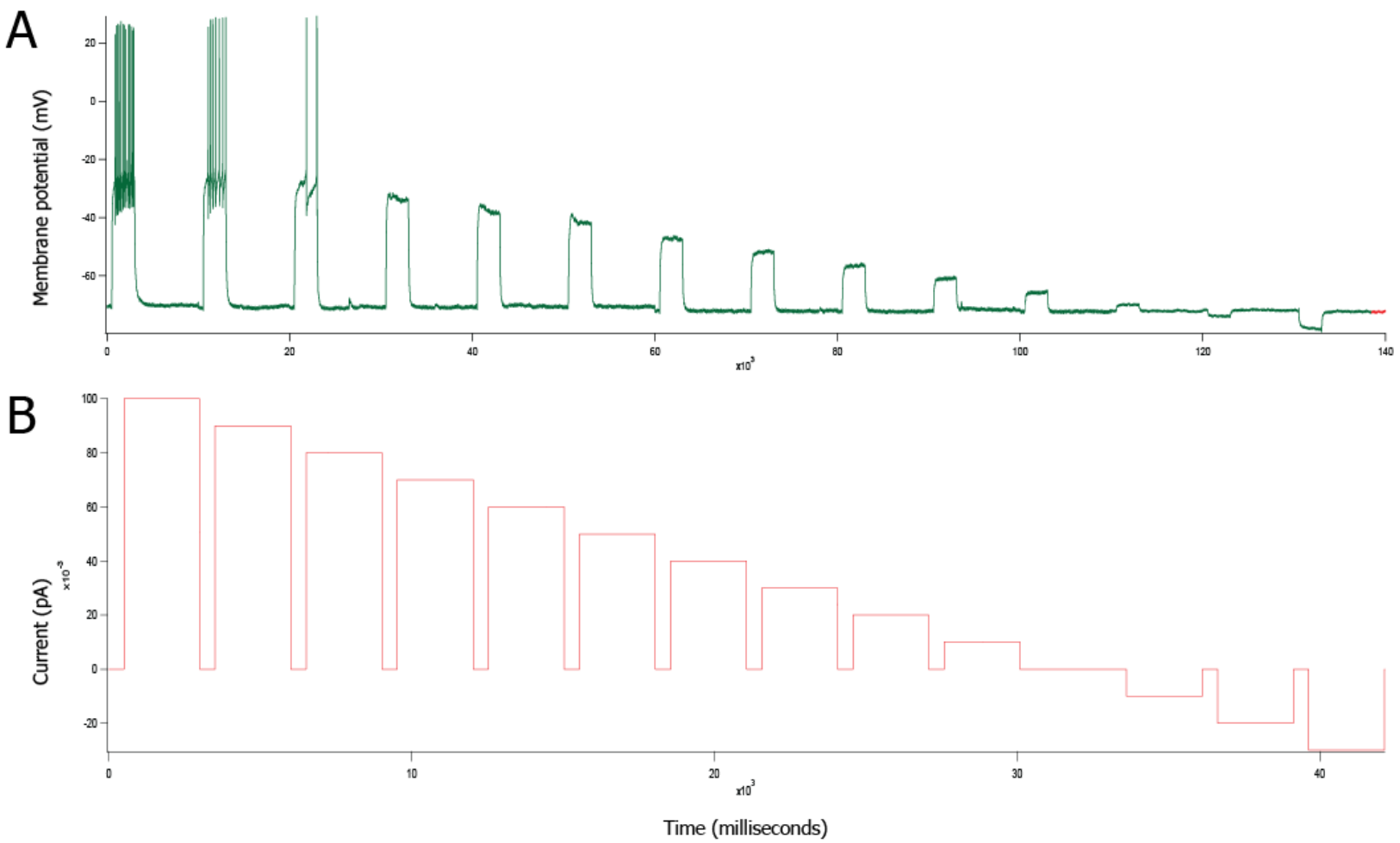

Figure 44. Voltage recording from lenti-GFP cell 6

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections which were written to the record electronically in the trace recorded in (B).

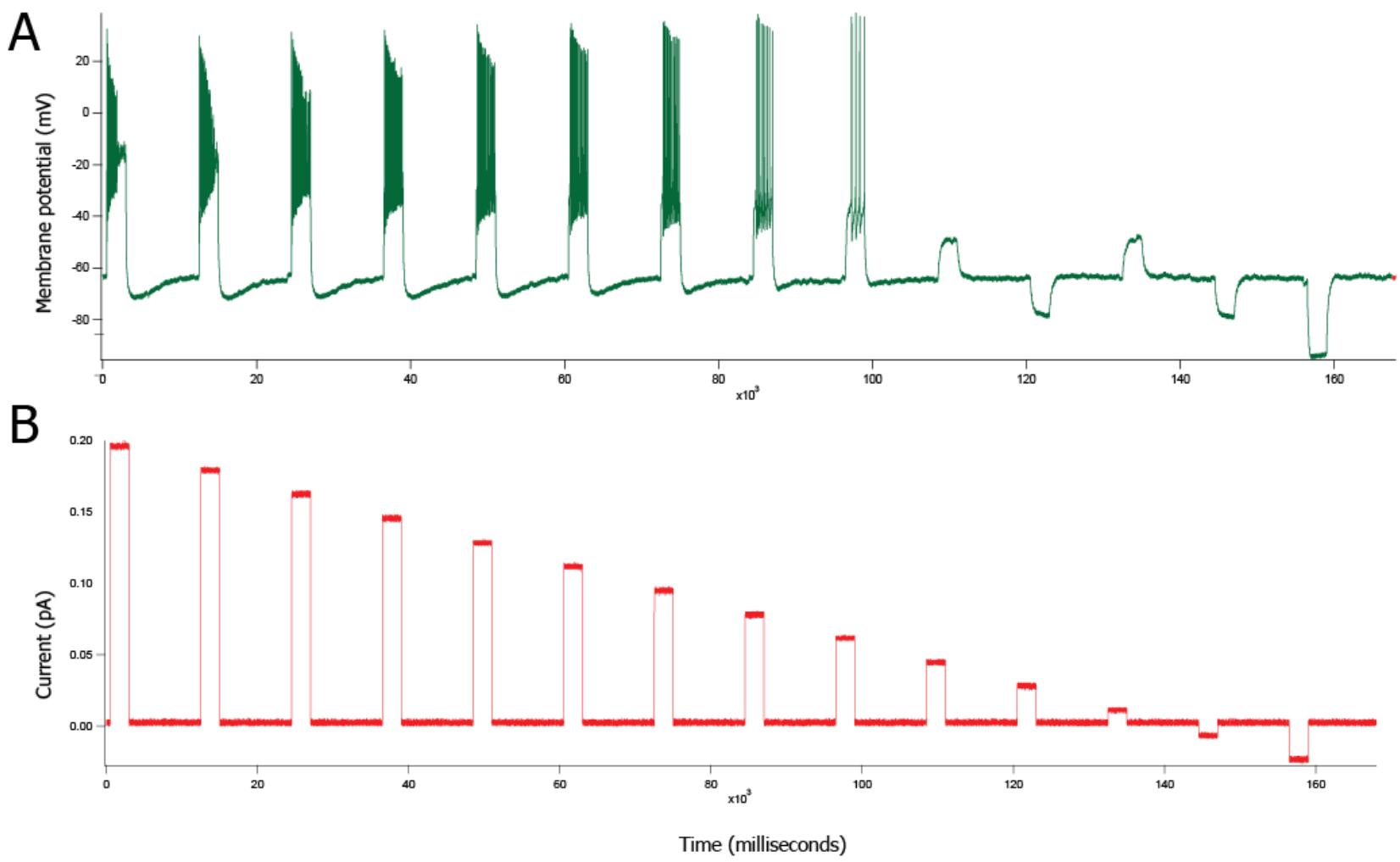

Figure 45. Voltage recording from lenti-GFP cell 7

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

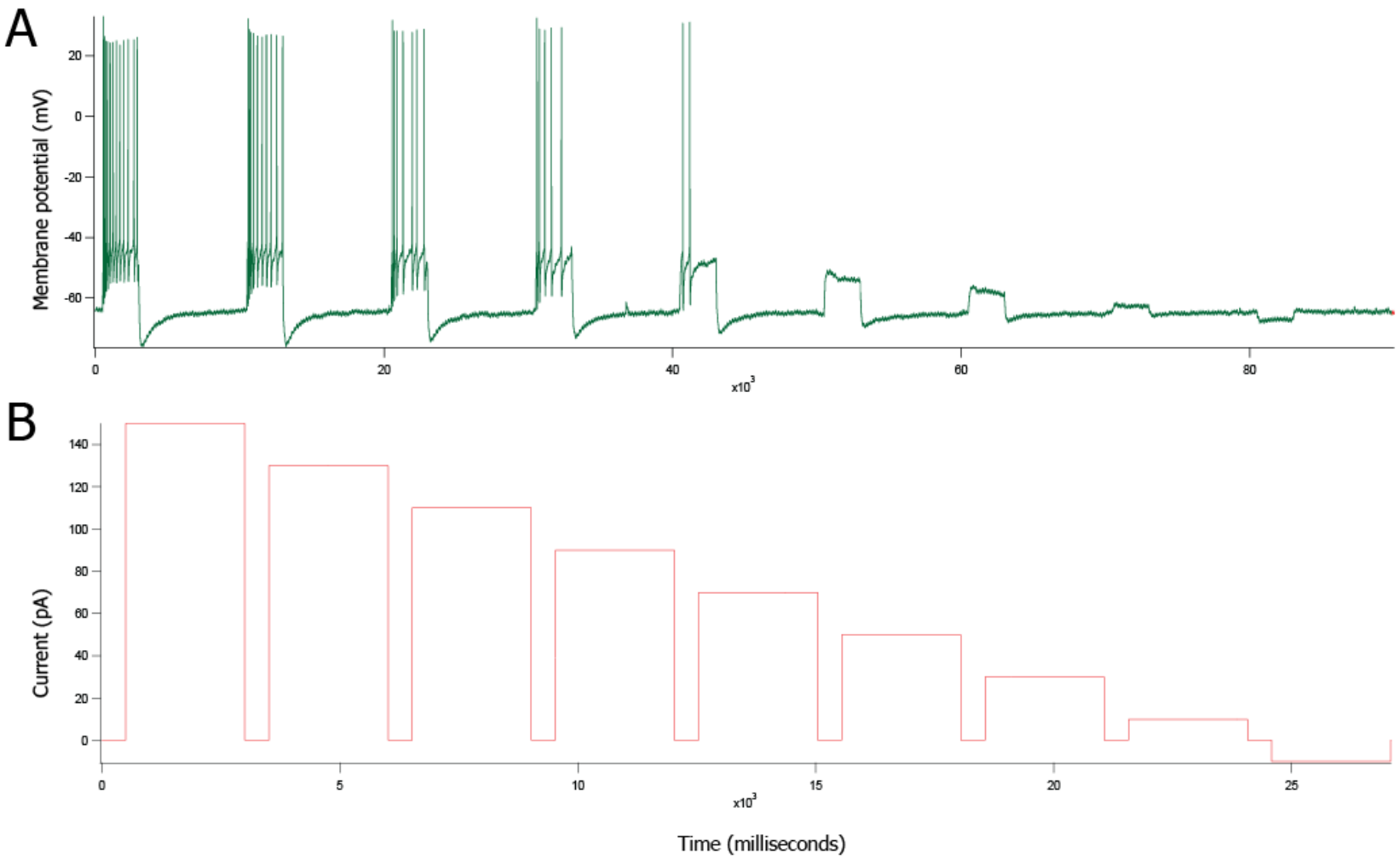

Figure 46. Voltage recording from lenti-GFP cell 8

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections which were written to the record electronically in the trace recorded in (B).

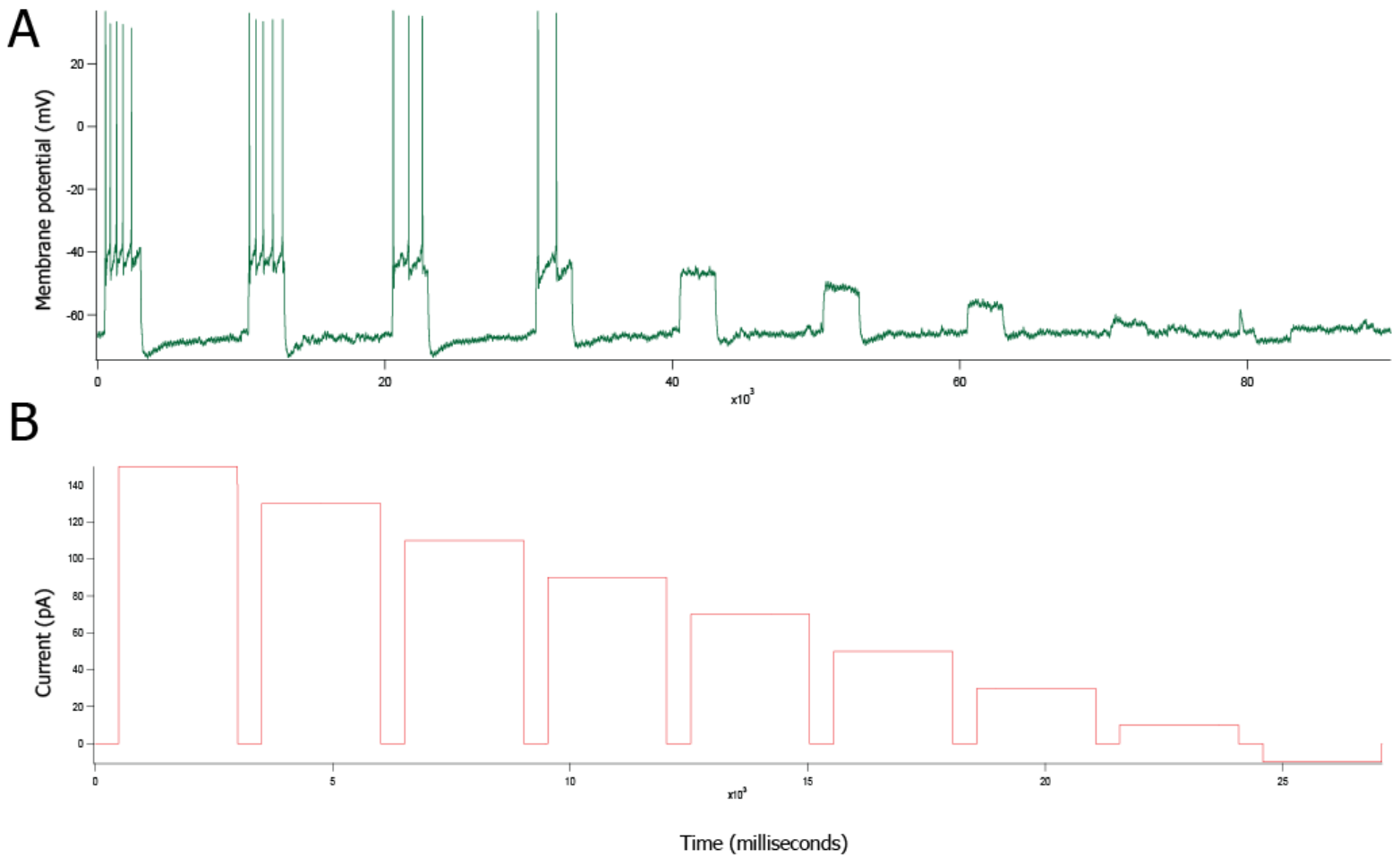

Figure 47. Voltage recording from lenti-GFP cell 9

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections which were written to the record electronically in the trace recorded in (B).

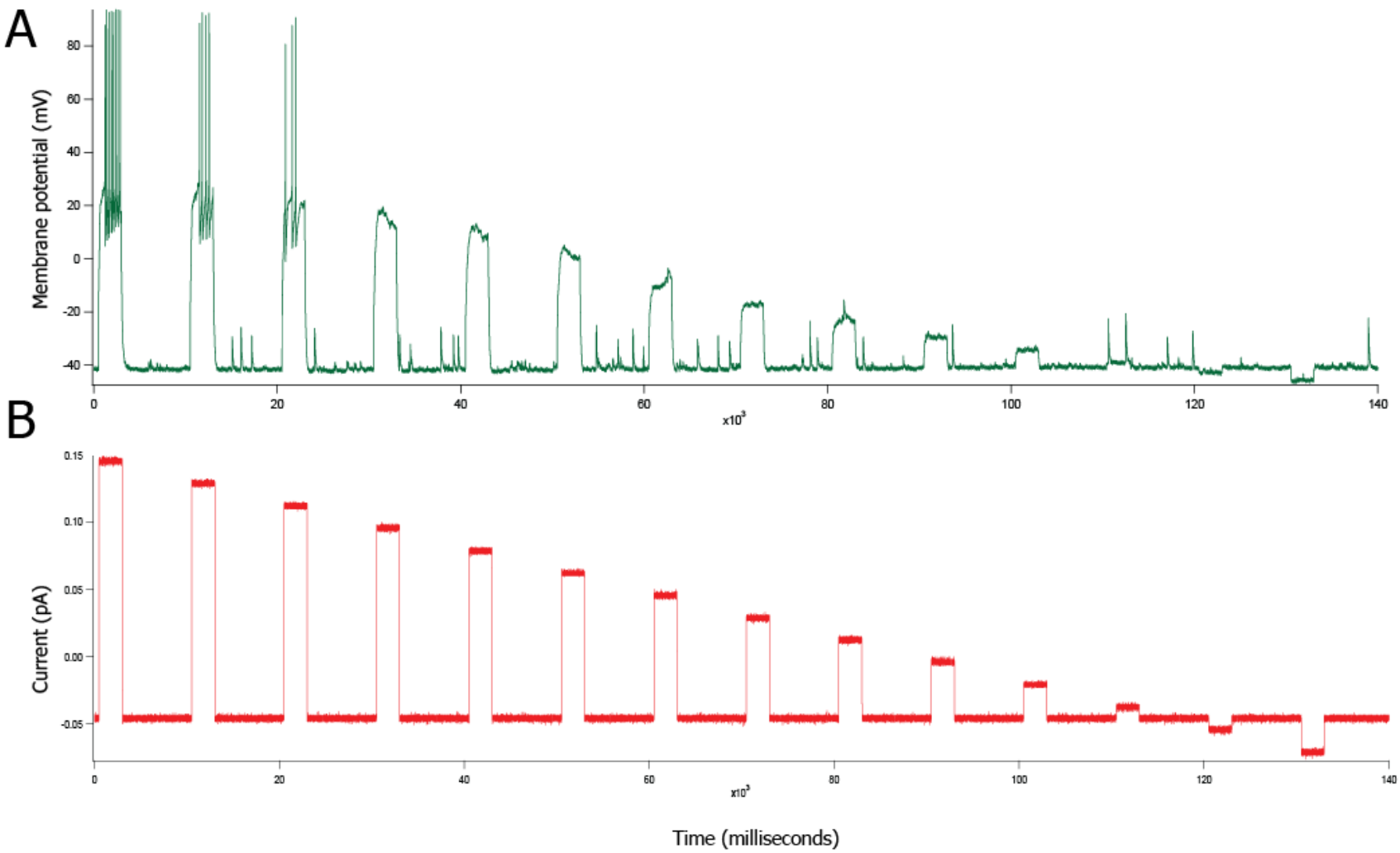

Figure 48. Voltage recording from lenti-GFP cell 10

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

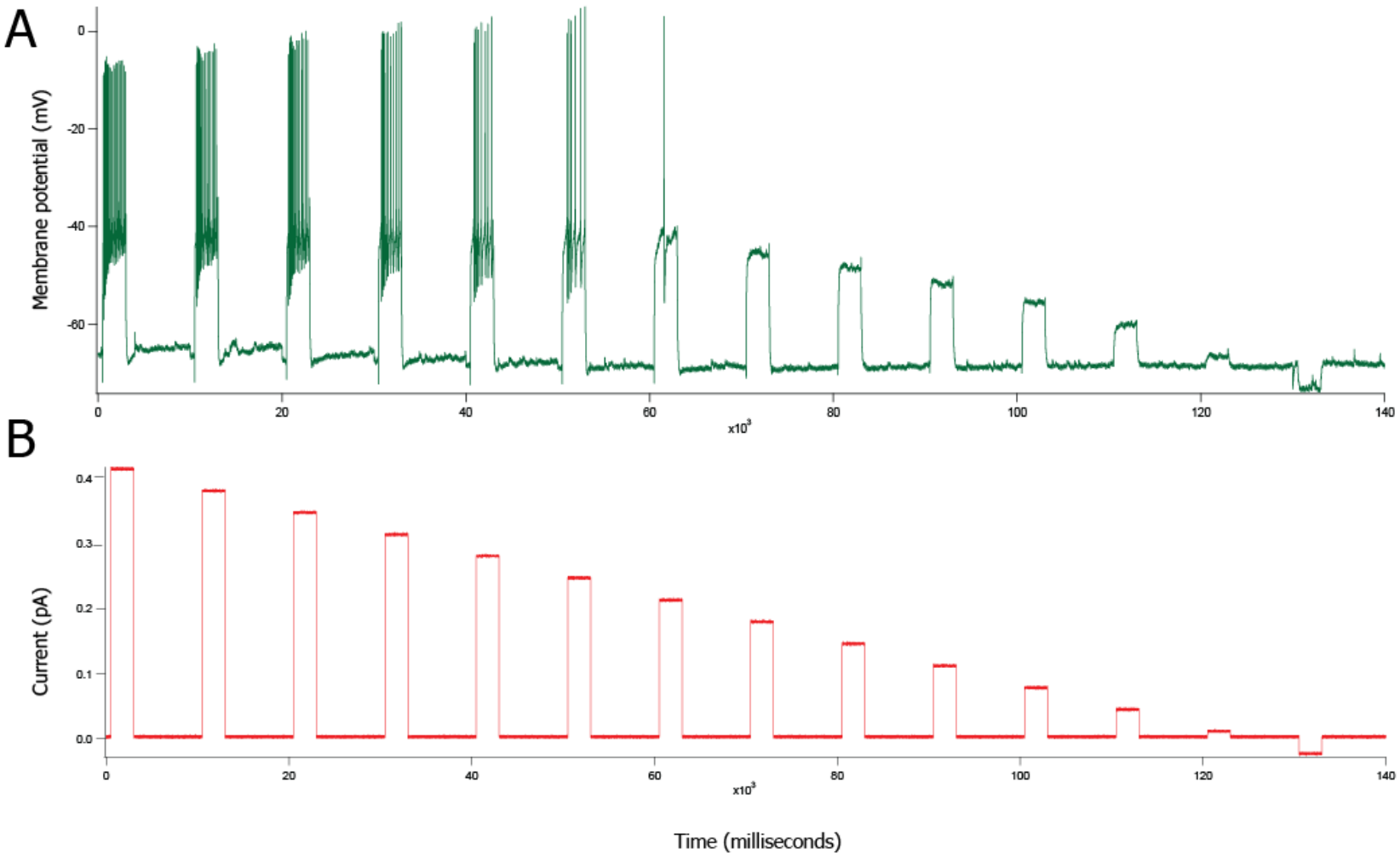

Figure 49. Voltage recording from lenti-GFP cell 11

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

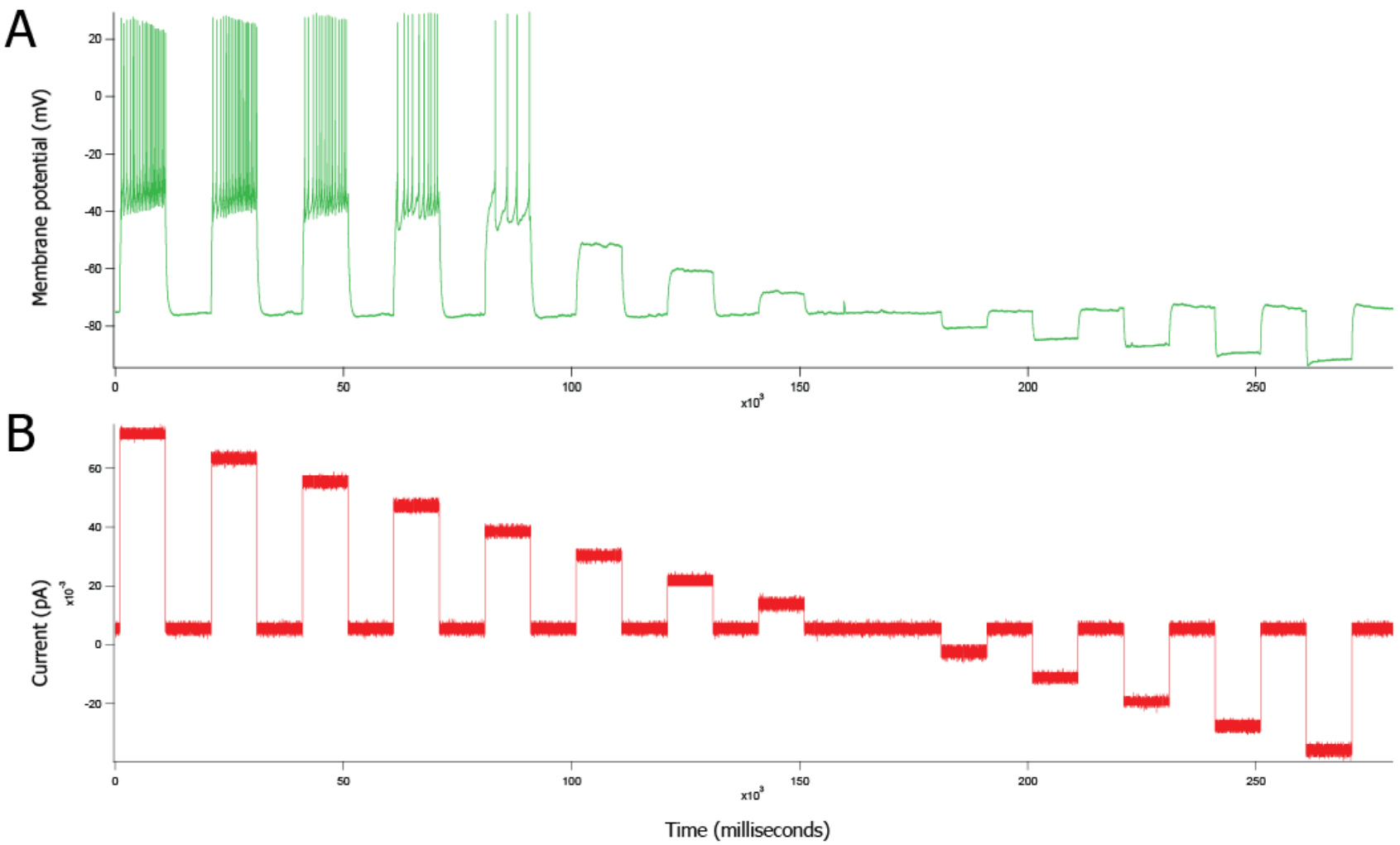

Figure 50. Voltage recording from lenti-shRNA cell 1

(A) Sample sweeps of a **lenti-shRNA** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

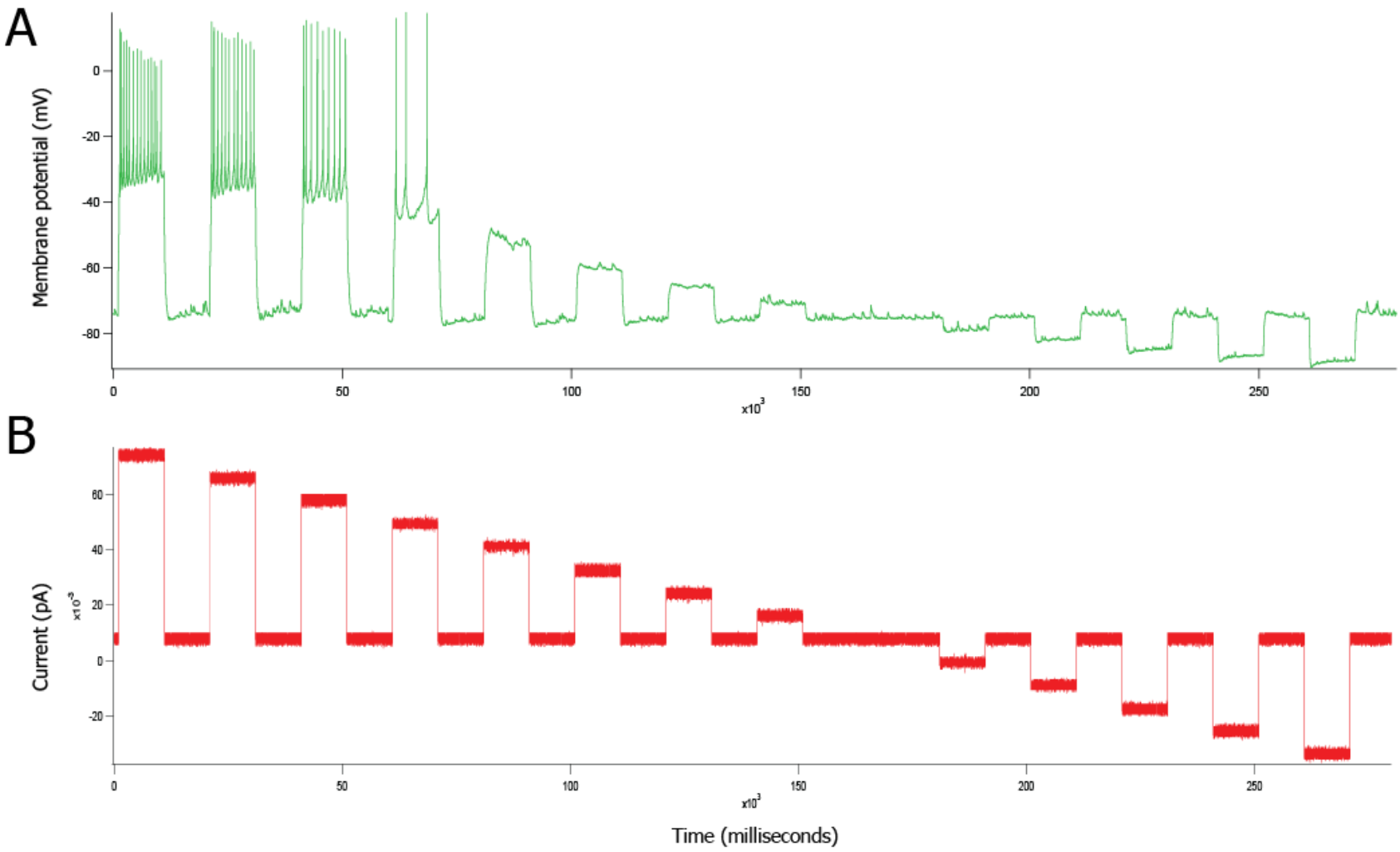

Figure 51. Voltage recording from lenti-shRNA cell 2

(A) Sample sweeps of a **lenti-shRNA** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

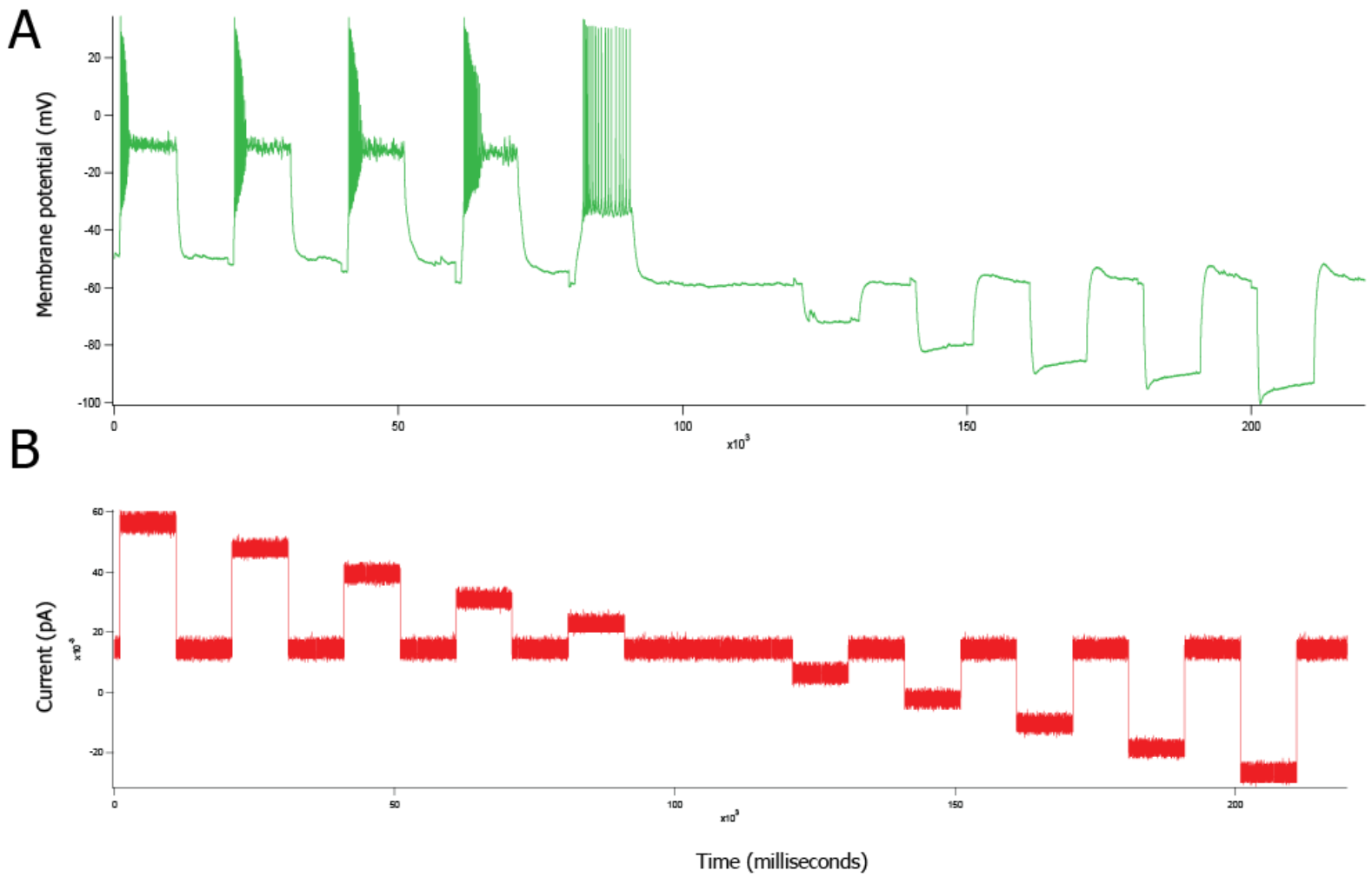

Figure 52. Voltage recording from lenti-shRNA cell 3

(A) Sample sweeps of a **lenti-shRNA** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

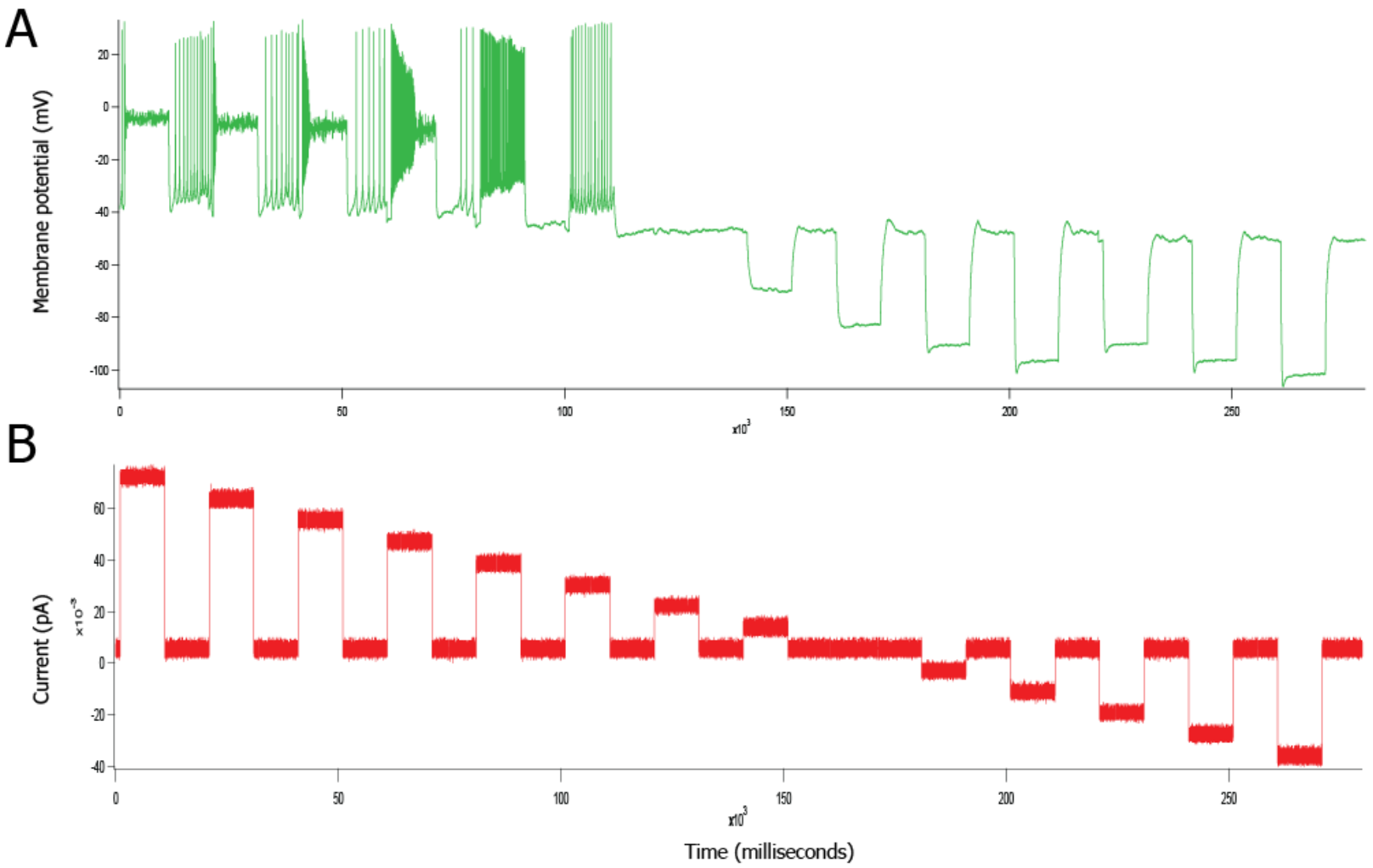

Figure 53. Voltage recording from lenti-shRNA cell 4

(A) Sample sweeps of a **lenti-shRNA** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

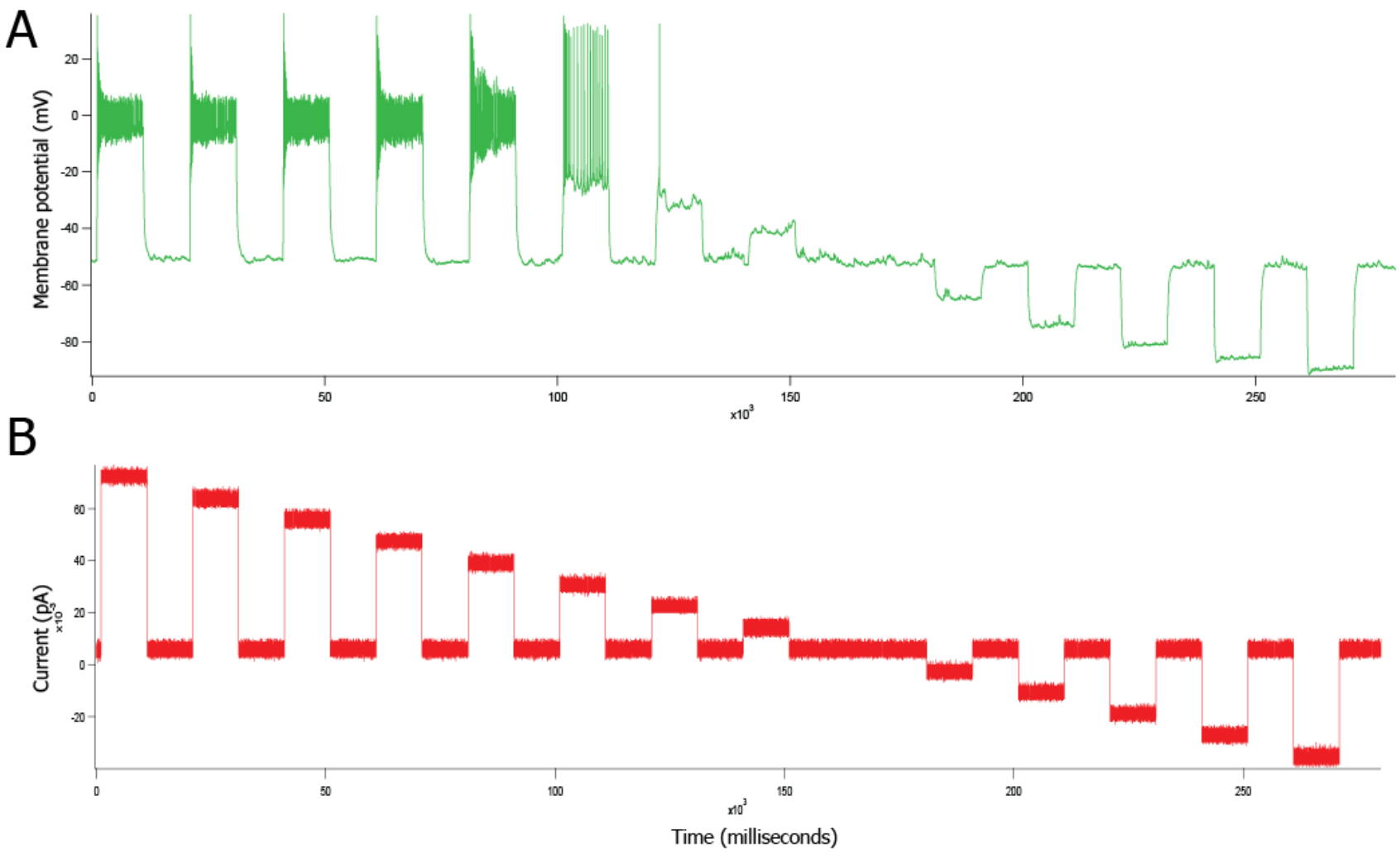

Figure 54. Voltage recording from lenti-shRNA cell 5

(A) Sample sweeps of a **lenti-shRNA** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

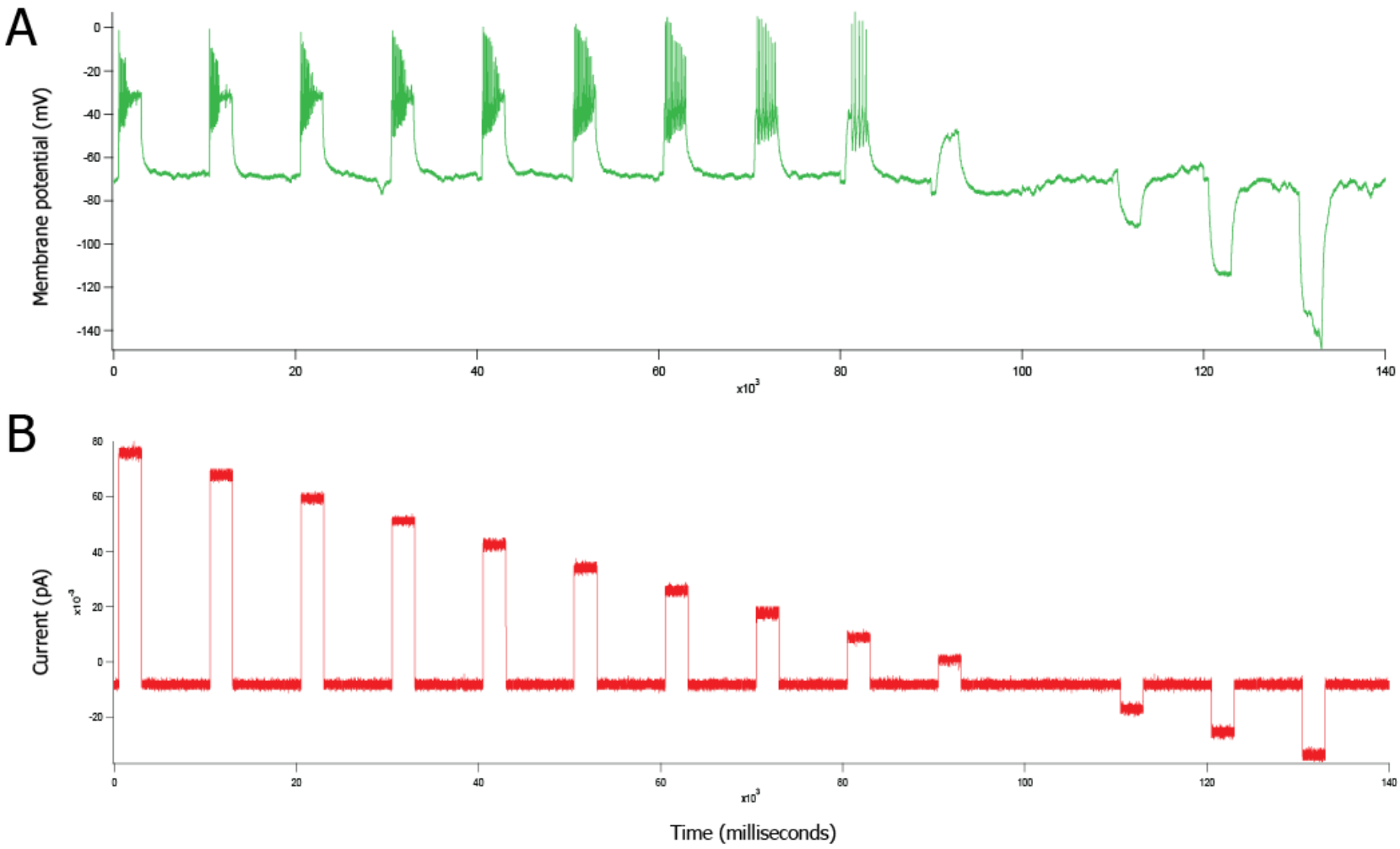

Figure 55. Voltage recording from lenti-shRNA cell 6

(A) Sample sweeps of a **lenti-shRNA** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

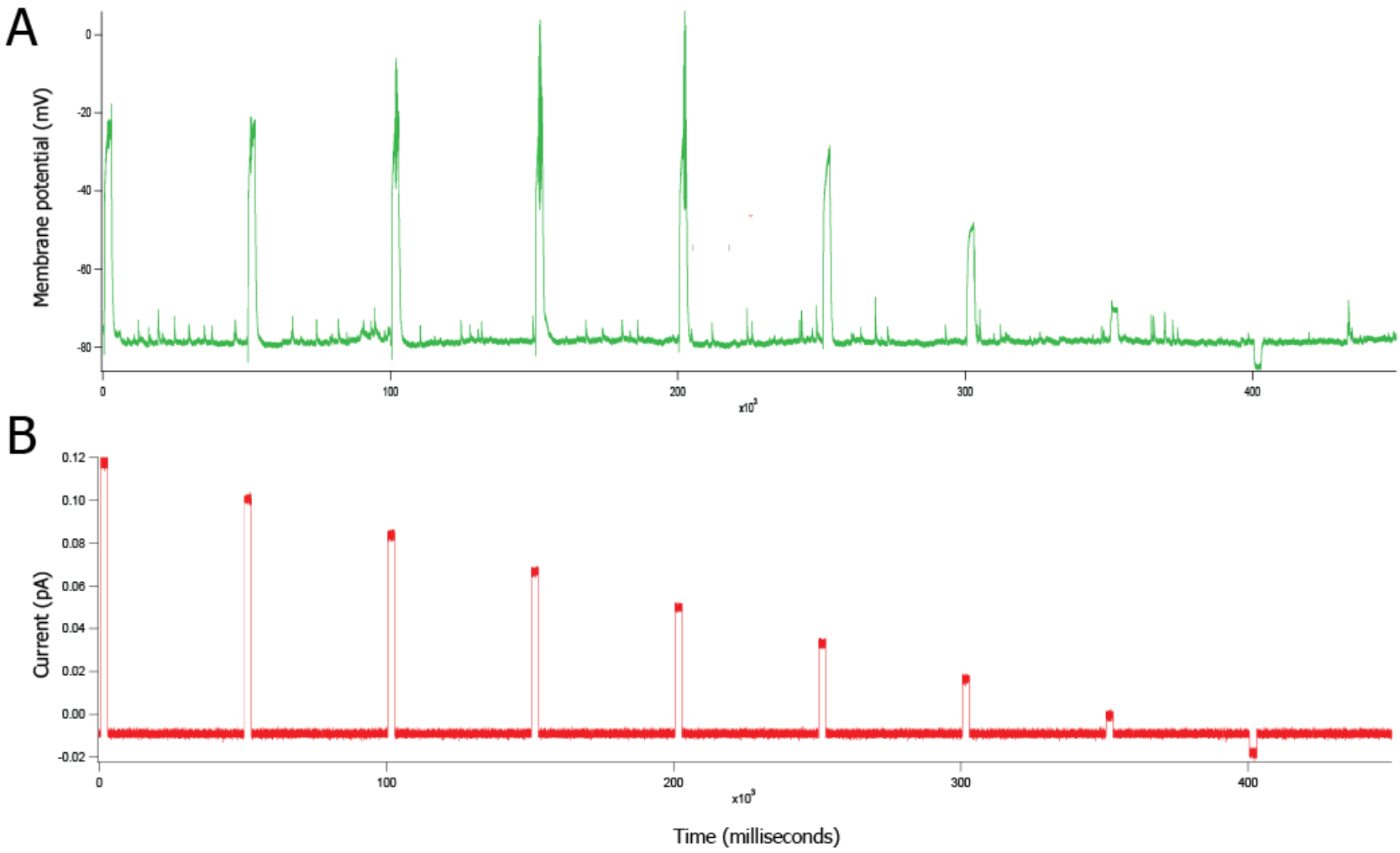

Figure 56. Voltage recording from lenti-shRNA cell 7

(A) Sample sweeps of a **lenti-shRNA** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

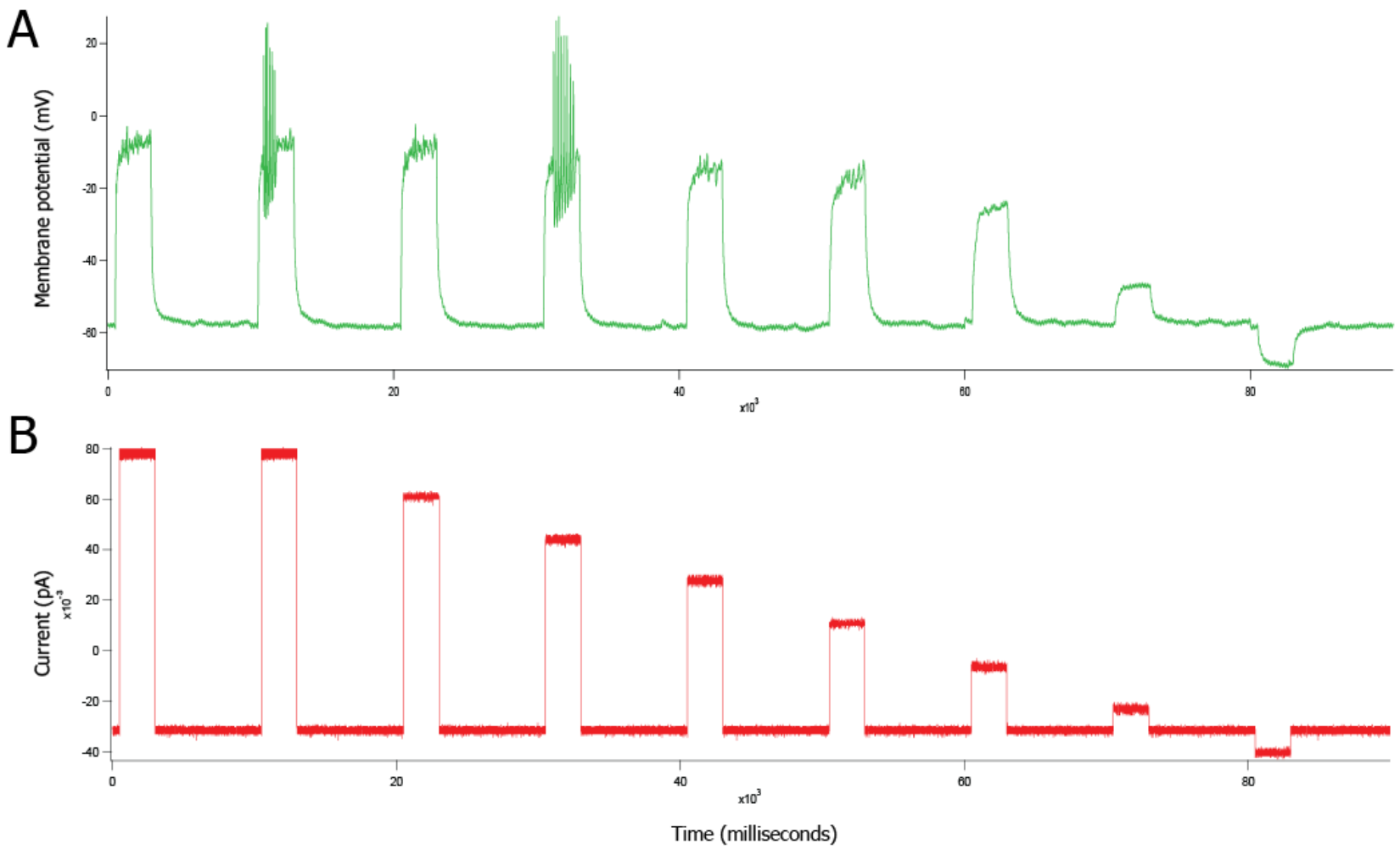

Figure 57. Voltage recording from lenti-shRNA cell 8

(A) Sample sweeps of a **lenti-shRNA** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

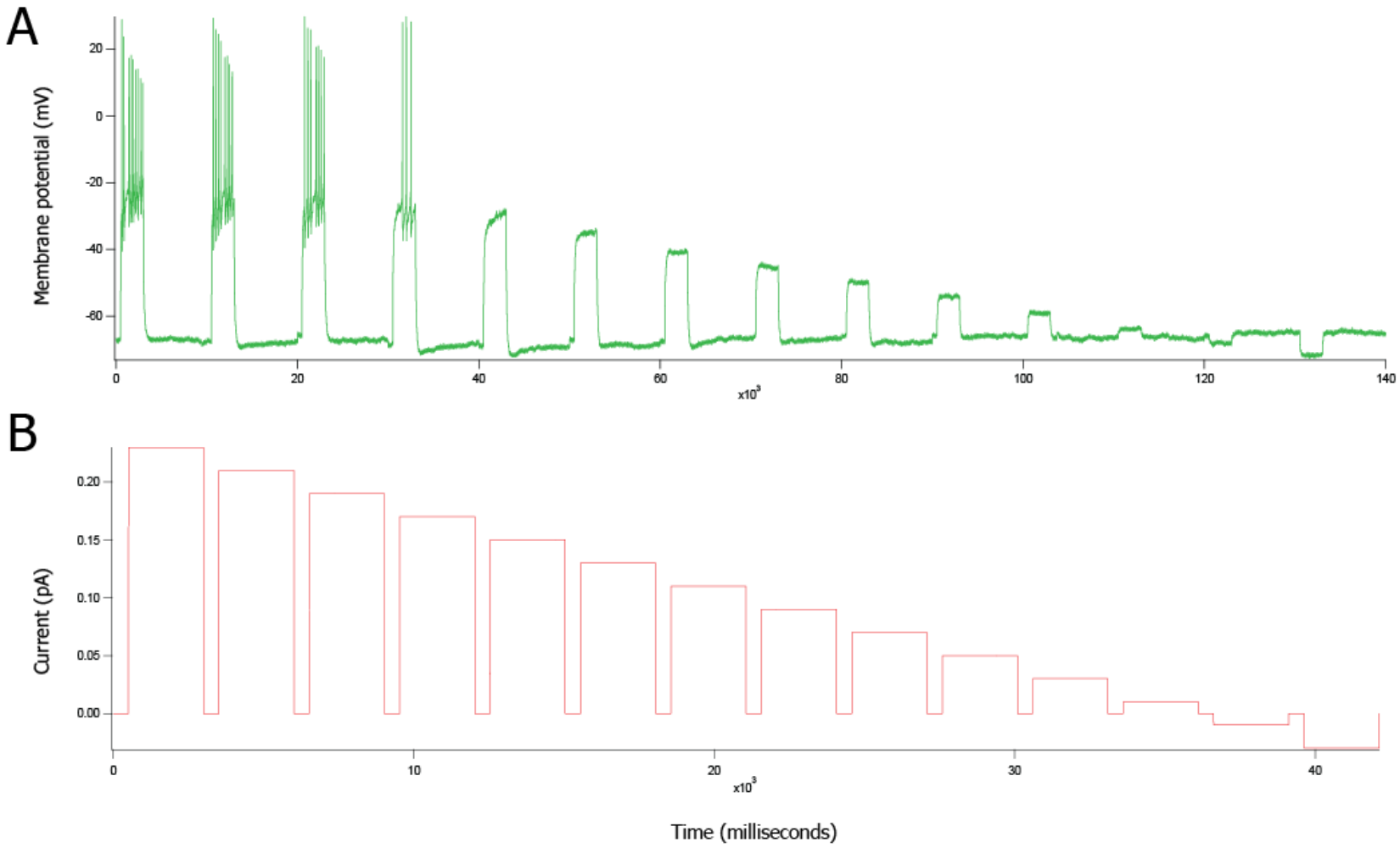

Figure 58. Voltage recording from lenti-shRNA cell 9

(A) Sample sweeps of a **lenti-GFP** ABN held under voltage clamp. Neuron was stimulated by the current injections which were written to the record electronically in the trace recorded in (B).

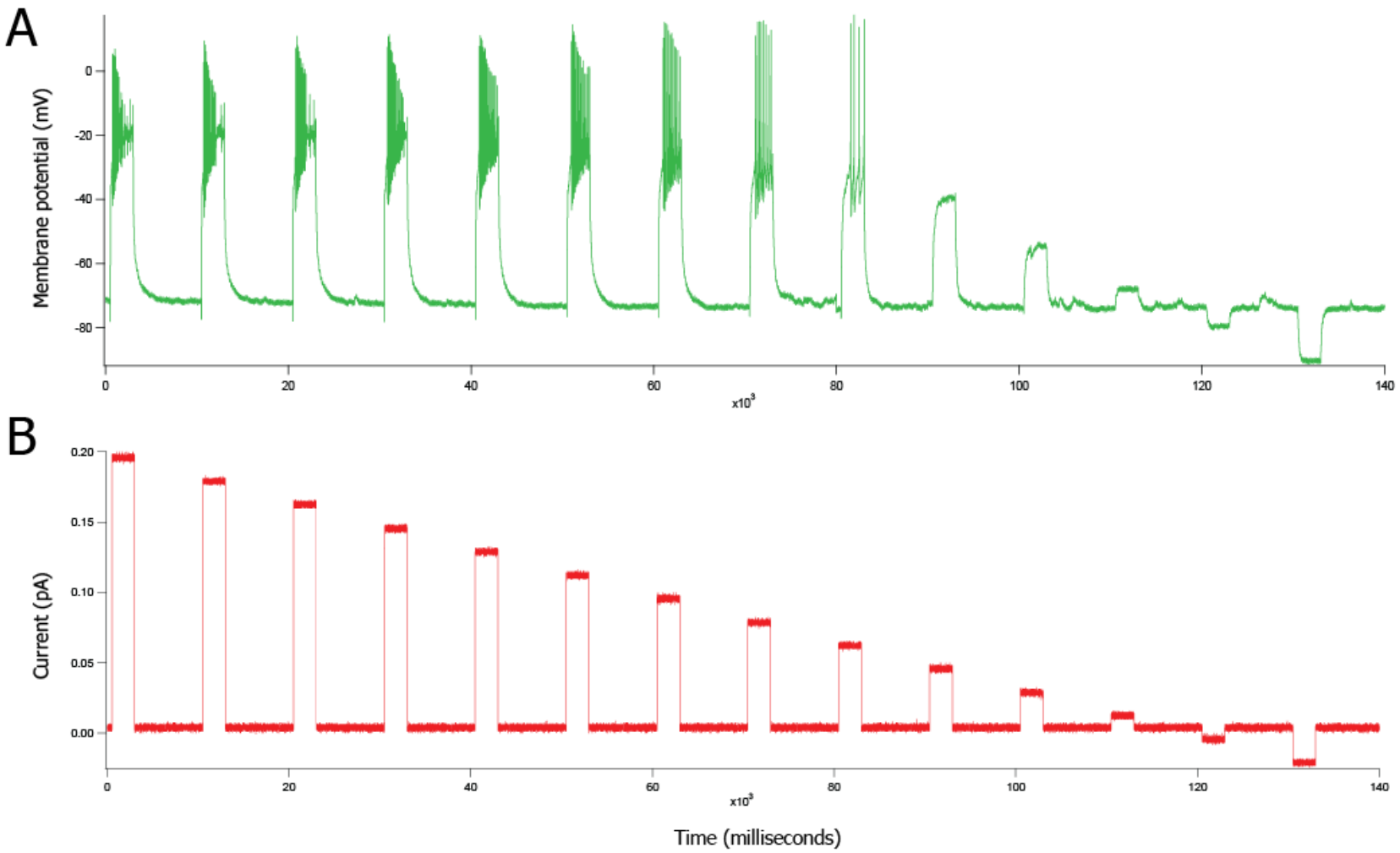

Figure 59. Voltage recording from lenti-shRNA cell 10

(A) Sample sweeps of a **lenti-shRNA** ABN held under voltage clamp. Neuron was stimulated by the current injections recorded concurrently in (B).

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