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Physiological Properties and Factors Affecting Migration of Neural Precursor Cells in the Adult Olfactory Bulb

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in

Neurosciences

by

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The dissertation of Daniel Paul Darcy is approved, and it is acceptable in quality and form for publication on microfilm:

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ABSTRACT OF THE DISSERTATION

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Newborn neurons are generated and incorporated into existing neural circuitry throughout the lifetime of adult mammals. In rodents, the major targets of neurogenesis are the dentate gyrus of the hippocampus and the olfactory bulb. In the olfactory system, neural precursor cells (NPCs) generated in the lateral ventricle migrate via the rostral migratory stream and radiate into the granule and glomerular layers of the olfactory bulb. Throughout adulthood, NPCs give rise to local inhibitory neurons, granule cells and periglomerular cells, which receive glutamatergic inputs in the bulb. However, the properties of NPCs and the mechanisms that regulate their movement are not thoroughly understood.

Here we show that olfactory bulb NPCs express Ca^{2+}-permeable AMPA receptors (AMPARs), and that activation of these receptors inhibits NPC motility. Glutamate

spillover from distant excitatory synapses in bulb slices is sufficient to activate NPC AMPARs and inhibit their migration. Surprisingly, this effect on migration is independent of Ca^{2+} influx through AMPARs. In many other systems of neural cell migration, intracellular Ca^{2+} signaling plays a critical role in cell motility. Therefore, this Ca^{2+}-independent inhibition of NPC migration is a highly unique aspect of olfactory bulb neurogenesis.

To further test the relationship between intracellular Ca^{2+} and migration in NPCs, we characterize the intrinsic membrane properties of the cells and show that NPCs are capable of firing regenerative Ca^{2+} spikes, and that they express L-type voltage-sensitive Ca^{2+} channels (VSCCs) that underlie spontaneous elevations in intracellular Ca^{2+}. Consistent with our finding that AMPAR-mediated inhibition of migration is independent of Ca^{2+} signaling, modulation of L-type VSCCs in NPCs does not alter migration.

INTRODUCTION

Adult neurogenesis is the production of new neurons in a brain that is no longer undergoing development. It is a topic of significant importance both from the standpoint of understanding the basic functions of the brain, and for the purpose of treating human diseases of the nervous system. For decades following the early histological characterization of the brain, the idea of "no new neurons after birth" was rooted in scientific culture. In fact, this conclusion was drawn from careful evaluation of the available evidence. Today, however, advances in the technology enabling us to confirm and visualize the generation, migration and integration of newborn neurons have informed us that adult neurogenesis is actually commonplace in specific regions of the brain.

This new knowledge challenges conventional notions of static neural circuits, and promises rich avenues of future research into the fundamental principles of circuit organization in the brain. Most importantly, an understanding of the natural processes governing adult neurogenesis may lay the groundwork for clinical applications in which newborn neurons are introduced into an adult brain for the purpose of repair or regeneration of damaged tissue.

Historical background

Early studies of the brain by pioneers of neuroscience such as Ramon y Cajal and Golgi, who used histological techniques to visualize cell morphology and connections in nervous tissue, suggested adult neurogenesis did not occur. Histology provided static snapshots of the brain, and yielded little evidence that neuron turnover, proliferation, or regeneration of neuron processes was present in the central nervous system (CNS) once initial development had completed. In his book on the degeneration and regeneration of the nervous system, Cajal wrote: "Once development has ended, the founts of growth and regeneration ... dry up irrevocably. In adult centres 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." (Cajal 1928)

This assessment of the lost regenerative capability of the adult CNS did not arise from a mere lack of evidence that such regeneration occurs anywhere in the nervous system. Histological observation showed clearly that proliferation of neurons was widespread but confined to embryonic development, or in the case of the cerebellum, shortly thereafter. A related question of new neuron growth was whether existing neurons could repair or reestablish axons that had been lesioned. In his studies of embryos, Cajal observed and named "growth cones," the leading structures at the tip of extending axons. The circuitous route taken by axons during the early wiring of the brain led him to consider the role of an axon's environment in seeking and contacting proper targets, and his ideas helped formulate a hypothesis that has since been strongly confirmed by modern techniques: that the growth cone is an exquisite chemical sensor guiding the axon through the brain via multiple environmental signposts encountered

along the way. In contrast with normal developmental events, lesioned axons in the adult CNS failed to regenerate and produce growth cones, while axons in lesioned sites in the adult peripheral nervous system (PNS) did regenerate in great number, but failed to then navigate to their original appropriate target sites. Thus, the proliferative and regenerative capacity of developing nervous tissue, and partial yet aberrant regenerative potential of the adult PNS, was readily observed at the time of Cajal. None of these features, however, were present in the adult CNS.

Advances in the field

Cajal's conclusion of the "fixed, immutable" nature of the adult brain, well-reasoned and firmly grounded in thorough histological observation across many organisms and many stages of development, saw little challenge for decades. This was to change in the late 1950s when an important new technique for labeling dividing cells was developed: the incorporation of [^{3}H]-thymidine into the replicating DNA of cells in S-phase of the cell cycle (Sidman, Miale et al. 1959). This tritiated thymidine could then be detected with autoradiography, revealing newborn neurons in the brain. Subsequent techniques based on the same principal used a synthetic thymidine analog, bromodeoxyuridine (BrdU), which carries the added advantage of being detectable with standard immunochemistry. With the ability to visualize recently divided neurons in the adult brain, neurogenic regions were identified in the hippocampus (Altman and Das 1965) and olfactory bulb (Smart 1961; Altman 1969). These early reports of newborn neurons were followed by electron micrograph studies that demonstrated the long-term survival of the cells (Kaplan and Hinds 1977) and the presence of synapses along their

somata and dendrites (Kaplan and Bell 1983). Experiments with retrograde tracers that co-labeled newborn cells demonstrated their ability to send axonal projections (Stanfield and Trice 1988).

Although adult neurogenesis was first reported by Altman and Das in mammals, studies in canaries (Goldman and Nottebohm 1983) and other songbirds were primarily responsible for bringing the phenomenon widespread scientific attention and acceptance. Nottebohm and his colleagues demonstrated that neurogenesis in a well-characterized vocal circuit underlay seasonal changes in bird song production, thus establishing important links between new neurons, behavior, and the environment.

Mammalian neurogenesis

Mammalian adult neurogenesis has become a topic of intensive study. In humans, as with rodents, adult neurogenesis has been confirmed in two brain regions, the dentate gyrus of the hippocampus (Eriksson, Perfilieva et al. 1998) and the olfactory system (Curtis, Kam et al. 2007). In rats and mice, the organisms most widely studied, other evidence has been put forth suggesting that neurogenesis may occur in other regions, notably the neocortex (Gould, Reeves et al. 1999) and piriform or olfactory cortex (Pekcec, Loscher et al. 2006). Such reports have not been universally accepted because of technical challenges in interpreting the labeling of newborn cells (Rakic 2002). For the systems where neurogenesis is well-established, behavioral and environmental correlates of new neuron production are beginning to be explored. Sensory experience and behavior regulate the extent of adult neurogenesis that occurs in rodents. For example, voluntary exercise and enriched environment increase cell numbers in the

dentate gyrus (Kempermann, Kuhn et al. 1997; Brown, Cooper-Kuhn et al. 2003; van Praag, Shubert et al. 2005), while the survival (Petreanu and Alvarez-Buylla 2002; Alonso, Viollet et al. 2006) and response (Magavi, Mitchell et al. 2005) of new neurons in the olfactory bulb is modulated by olfactory experience.

The idea of introducing new, functional neurons into an adult brain raises exciting therapeutic possibilities, such as restoring cognitive function lost as a consequence of damage from stroke or neurodegenerative disease like dementia and Alzheimer's disease. A detailed understanding of all phases of naturally occurring adult neurogenesis will be essential to realizing such long-reaching clinical goals.

Neurogenesis and migration in the adult olfactory system

One essential aspect of endogenous adult neurogenesis that has a clear bearing on therapeutic potential is the ability of new neurons to reach their appropriate targets. Neurogenesis in the olfactory system serves as an excellent model for the long-distance migration of newborn cells. In adult mice and rats, migrating neurons may travel 3-5 mm from their site of birth to their targets in the bulb (Lois and Alvarez-Buylla 1994), while in humans the distance traveled is considerably longer (~17 mm) (Curtis, Kam et al. 2007).

Neural precursor cells (NPCs) destined for targets in the olfactory bulb first proliferate in the subventricular zone (SVZ) of the lateral ventricle (Lois and Alvarez-Buylla 1994). They migrate tangentially along the SVZ and move toward the olfactory bulb along a tightly coalescing pathway termed the rostral migratory stream (RMS) (Altman 1969; Kishi 1987; Luskin 1998) (Figure 1). In contrast to the migration of new

neurons during much of cortical development, in which the cells move along radial glial fibers, RMS migration is organized into tightly associated chain-like structures formed by the ensheathing of NPCs by astrocytes (Lois, Garcia-Verdugo et al. 1996). However, these glial sheaths are not required for the formation of chains of NPCs nor the migration of the cells, as NPCs will self-organize into chains and slide past each other in an artificial substrate without glia present (Wichterle, Garcia-Verdugo et al. 1997). NPCs disperse from chains in the subependymal layer (SEL) of the olfactory bulb, then migrate radially into outer layers of the bulb to become GABAergic interneurons in the granule cell layer, glomerular layer, and external plexiform layer (Baker, Liu et al. 2001; Belluzzi, Benedusi et al. 2003; Carleton, Petreanu et al. 2003; Yang 2007) (Figure 2).

The environmental cues that direct NPCs from the ventricle to the bulb are not completely understood. NPCs in the SVZ follow the flow of cerebrospinal fluid established by the beating of motile cilia anchored in the epithelial lining of the ventricle (Sawamoto, Wichterle et al. 2006). This generation of fluid currents may maintain appropriate repulsive chemical gradients by diffuse factors, including the protein Slit, that guide new neurons in their initial phase of navigation out the ventricle (Hu and Rutishauser 1996; Hu 1999; Wu, Wong et al. 1999; Nguyen-Ba-Charvet, Picard-Riera et al. 2004). Since cultured NPCs derived from the SVZ form chains and migrate in the absence of glia or other cell types, in essence recapitulating *in vitro* what is thought to occur *in vivo*, it is possible that signaling via chemical gradients is not essential to maintain the tightly structured coalescence of cells in the RMS or to promote cell movement within this pathway. However, cell adhesion molecules play an essential role in the eventual arrival of NPCs to the olfactory bulb. NPCs express polysialylated neural

cell adhesion molecule (Bonfanti and Theodosis 1994; Rousselot, Lois et al. 1995), the genetic deletion or enzymatic removal of which results in NPC apoptosis (Gascon, Vutskits et al. 2007) and deficits in the cells' migration (Tomasiewicz, Ono et al. 1993; Cremer, Lange et al. 1994; Ono, Tomasiewicz et al. 1994; Chazal, Durbec et al. 2000). Once NPCs have reached the olfactory bulb, reelin acts as a detachment signal permitting the dissolution of chains and the radial fanning of NPCs toward the outer layers of the bulb (Hack, Bancila et al. 2002). However, the final stages of precise targeting by NPCs in the granule cell layer or periglomerular layer of the bulb are poorly understood.

In the experiments presented here, we use current and voltage recordings and imaging of Ca^{2+} indicator dyes to characterize the intrinsic membrane properties of NPCs in the center of the SEL of olfactory bulb slices. NPCs express a diverse array of ion channels and receptors, and we explore the role these play in NPC migration by imaging the movement of the cells deep within the SEL of olfactory bulb slices. In Chapter 1, we explore whether glutamate, the principal excitatory neurotransmitter in the brain, can influence the acute migration of NPCs. We find that activation of AMPA receptors (AMPARs) on the cells inhibits their migration. The subunit composition of these AMPARs renders them permeable to Ca^{2+}, but surprisingly, the effect we see on migration is independent from Ca^{2+} signaling in NPCs. Because Ca^{2+} is such an important intracellular signaling component, and has been implicated in cell migration in other brain systems (Komuro and Rakic 1992; Komuro and Rakic 1993; Komuro and Rakic 1996; Komuro and Rakic 1998), we go on to further explore the uncoupling between Ca^{2+} and migration in NPCs. These experiments form the basis of Chapter 2, in which we demonstrate spontaneous and evoked Ca^{2+} signaling in NPCs and the presence

of high voltage-activated L-type Ca^{2+} channels. Consistent with the Ca^{2+} independent regulation of migration by AMPARs, manipulation of L-type VSCCs does not affect NPC migration.

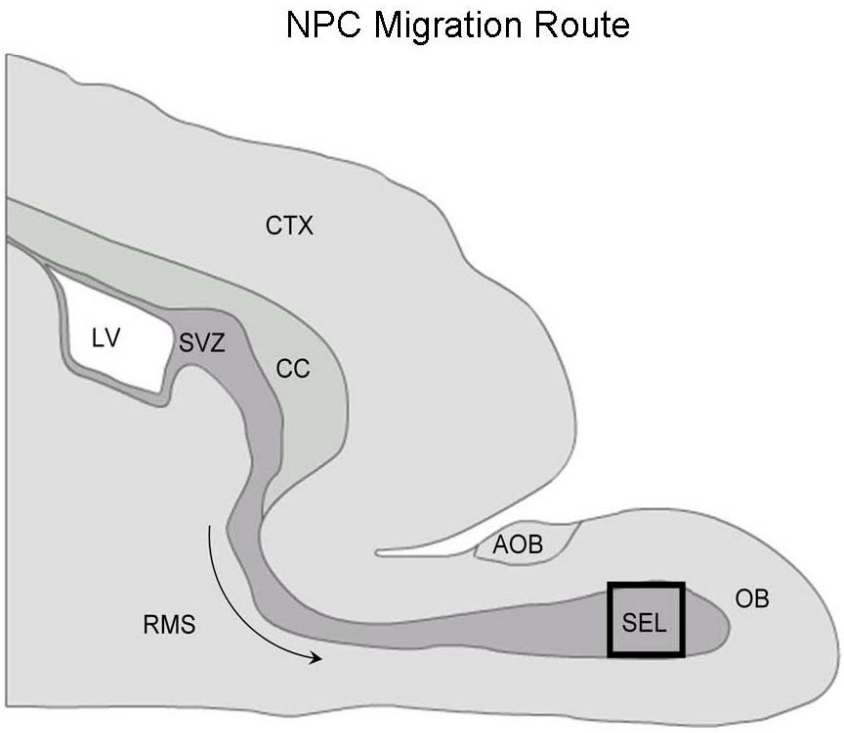

Figure I-1: NPC migration route.

NPCs migrate tangentially from their site of proliferation in the subventricular zone (SVZ), through the rostral migratory stream (RMS) and into the subependymal layer (SEL) of the olfactory bulb (OB). AOB: accessory olfactory bulb; CC: corpus callosum; CTX: cortex; LV: lateral ventricle

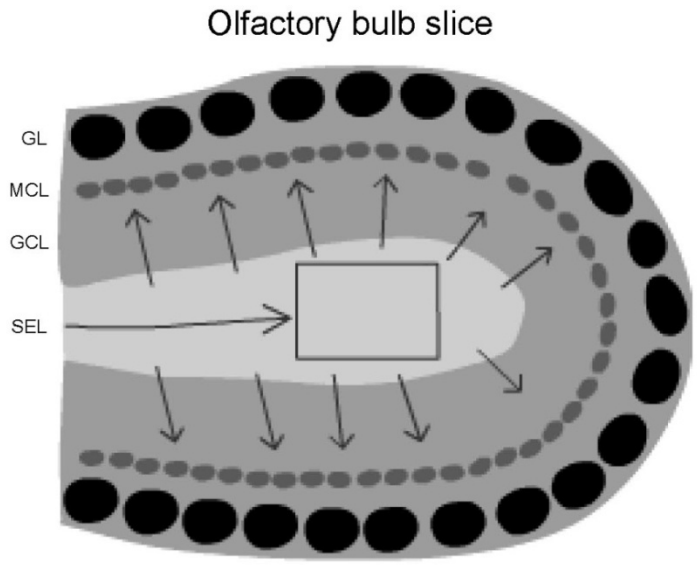

Figure I-2: NPC targets in the olfactory bulb.

NPCs enter the olfactory bulb in the subependymal layer (SEL). They migrate radially from the SEL to become granule cells in the granule cell layer (GCL) or periglomerular cells in the glomerular layer (GL). Box over SEL shows location where all experiments are performed. MCL: mitral cell layer

EXPERIMENTAL PROCEDURES

Slice preparation and electrophysiology

Olfactory bulb slices (350 μm) were prepared from 16-30 day old Sprague-Dawley rats or 1-3 month old FVB/N-Swiss Webster mice in accordance with institutional and national guidelines using standard procedures. Slices were prepared and maintained in aCSF containing (in mM) 83 NaCl, 2.5 KCl, 3.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, 72 sucrose and 0.5 $CaCl_2$ equilibrated with 95% O_2/5% CO_2 at 34º C for 30 minutes and at room temperature thereafter. In the recording chamber slices were viewed using infrared-DIC optics (BX-51W1, Olympus) and superfused with aCSF containing (in mM) 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose and 2.5 $CaCl_2$.

Whole-cell electrodes (~6-7 MΩ) for voltage- and current-clamp recordings were filled with a solution containing (in mM) 115.5 KCH_3SO_4, 17.5 KCl, 10 HEPES, 10 phosphocreatine, 3 Mg-ATP, 0.5 Na-GTP, 0.5 EGTA (pH ~7.3, 300 mOsm). For recording Ca^{2+} spikes in current-clamp and isolating Ca^{2+} or glutamate receptor currents, the internal solution was (in mM) 130 D-gluconic acid, 130 CsOH, 5 NaCl, 3 Mg-ATP, 0.2 Na-GTP, 12 phosphocreatine, 10 HEPES, 5-10 EGTA (pH ~7.3, 300 mOsm) and the aCSF included 100 μM picrotoxin (PTX), 1 μM TTX, 1 mM 4-aminopyridine and 4 mM TEA. For glutamate uncaging, 300 μM methyl 1-[5-(4-amino-4-carboxybutanoyl)]-7-nitroindoline-5-acetate was included in recirculated aCSF with (in μM) 1 TTX, 20 BMB

and 100 PTX to block $GABA_A$ receptors and transmitter release. The holding potential was -80 mV unless otherwise noted.

Experiments were corrected for a junction potential (~10 mV). Responses were recorded with an Axopatch 200B amplifier (Axon Instruments), filtered at 2-5 KHz and digitized at 10-20 KHz (ITC-18; Instrutech). Data acquisition and analysis were performed with Axograph 4.9 (Axon) or Axograph X (AxoGraph Scientific) and IGOR Pro 5/6 (Wavemetrics). Ca^{2+} currents were determined using leak subtraction (P/4). Glutamate receptor currents were recorded with 200 μM spermine in the internal pipette solution to prevent polyamine dialysis and 100 μM cyclothiazide in the aCSF to prevent AMPAR desensitization. Current recordings were obtained at room temperature.

Imaging

For imaging of spontaneous and evoked Ca^{2+} transients, olfactory bulb slices were bulk loaded with Oregon Green-1 BAPTA AM (30 μM in aCSF with 1.7% DMSO and 0.05% Pluronic F-127; Molecular Probes) in aCSF at 34º C for 40-60 minutes. Image acquisition (494 nm excitation, 2x2 binning, 2-4 Hz) and analysis were carried out with a cooled-CCD camera system (T.I.L.L. Photonics). Regions of interest (ROIs) were small circles centered on the soma of NPCs.

To study NPC migration, slices were focally loaded in the SEL via 2 minutes of light pressure ejection from a glass pipette with CellTracker Green CMFDA (30 μM, 1.7% DMSO) at 34º C. Slices were stabilized in the imaging chamber for 1 hour before data acquisition. Two-photon laser scanning microscopy (2PLSM) of NPC migration was performed using an ultrafast pulsing Ti:sapphire laser (MaiTai, Newport) coupled via

standard optics (SD Instruments) to a modified laser-scanning microscope (Fluoview 300, Olympus) equipped with photomultiplier tube detectors (R3896, Hamamatsu) in a non-descanned configuration. Three-dimensional timelapses were processed to obtain maximum-intensity projections of NPC migration, and "celltracks" were made by manually tracking all somata that remained visible and displaced at least 20 μm during the course of an experiment. Each celltrack was normalized to its own average velocity during the control period. All migration experiments were performed at 32º C in the presence of (in μM) 1 TTX, 20 bicuculine (BMB), 100 PTX, 50 APV, and 40 MK-801 to block transmitter release in the slice and $GABA_A$ and NMDA receptors on NPCs and other cell types. Experiments examining AMPARs included 100 μM cyclothiazide to prevent receptor desensitization. Experiments in which glutamate was evoked via electrode stimulation were imaged using 2PLSM on slices loaded with Oregon Green 1 BAPTA AM or CellTracker Green CMFDA and included the glutamate uptake blocker DL-TBOA (50 μM) to maximize glutamate spillover into the SEL.

Analysis

Representative traces are the average of five or more consecutive episodes, except where noted. Data are presented as mean±s.e.m. Student's *t*-test was used to determine statistical significance.

CHAPTER 1: Regulation of Neural Precursor Cell Migration in the Adult Olfactory Bulb by AMPA Receptors

Abstract:

Glutamate is the principal excitatory neurotransmitter in the brain, and NMDA receptor (NMDAR) activation has been shown to play a role in neuronal migration in the postnatal development of cerebellar granule cells. In contrast to that finding, we observe that migrating NPCs in the olfactory bulb do not express NMDARs, but do contain AMPAR receptors (AMPARs). While AMPARs found on mature interneurons in the bulb are non-rectifying, NPC AMPARs are rectifying and permeable to Ca^{2+}. Activation of NPC AMPARs by application of glutamate, AMPA, or spillover of glutamate from bulb synapses reversibly inhibits NPC migration. Surprisingly, activation of AMPARs blocks migration in the absence of Ca^{2+} signaling. Thus, NPCs express Ca^{2+}-permeable AMPARs that govern their migration via a Ca^{2+}-independent mechanism.

Introduction:

Many studies have implicated a role for glutamate in the migration of newborn neurons. During embryonic development, activation of NMDARs stimulates the migration of dissociated cortical neurons (Behar, Scott et al. 1999), while other reports provide evidence that tangentially migrating cortical neurons express Ca^{2+}-permeable AMPARs (Metin, Denizot et al. 2000) that can induce neurite retraction following prolonged activation (Poluch, Drian et al. 2001). One well-characterized example in which glutamate receptor activity governs migration is found in the postnatal development of cerebellar granule cells. These cells proliferate in the external granular layer, migrate through the molecular layer (ML) along Bergmann glial fibers, then detach from these fibers, traverse the Purkinje cell layer, and reach their final targets in the internal granular layer (Komuro and Rakic 1998). NMDAR blockade curtails the migration of cerebellar granule cells in slice preparations, while potentiating NMDAR activity accelerates the cells' migration rate. Furthermore, increasing endogenous glutamate levels by blocking astrocytic glutamate uptake in the slice also elevates migration rate (Komuro and Rakic 1993).

NPCs mature into local interneurons of the olfactory bulb that receive glutamatergic input from principal cells (mitral/tufted cells), olfactory nerve receptors, or both. We considered the possibility that NPCs express glutamate receptors well in advance of their synaptic integration, and that these receptors may govern aspects of NPC motility. We find that NPCs in the SEL of young adult rat olfactory bulb slices express Ca^{2+}-permeable AMPARs. These receptors can be activated via endogenous glutamate spillover or perfused application of receptor agonists. Activation of the AMPARs

inhibits NPC migration, but occurs via a mechanism that does not require intracellular Ca^{2+} signaling.

Results:

Previous studies suggest that the majority of cells in the SEL of the olfactory bulb are migrating NPCs (Luskin 1998). To confirm this, we first examined the expression of doublecortin (DCX), a microtuble-associated protein found selectively in migrating newborn neurons (Gleeson, Lin et al. 1999). Acute brain slices from mice expressing GFP driven by the DCX promoter confirmed that virtually all cells in the SEL express this marker of migrating neurons (Figure 1-1).

Since NPCs ultimately mature into local interneurons that receive glutamatergic synaptic input, we next examined the properties of glutamate receptors in the cells. Flash photolysis of caged glutamate (300 μM) evoked large inward currents in NPCs voltage-clamped at -80 mV but elicited little outward current at +50 mV (Figure 1-2; n=6). The NMDAR antagonist APV (50 μM) had no effect on glutamate-evoked responses at either potential (-80 mV: 108.9±7.6% of control; +50 mV: 98.1±14.1% of control). However, currents were entirely abolished by the AMPAR antagonist NBQX (20 μM; -80 mV: 1.2±0.4% of control; +50 mV: -0.8±1.6% of control). These results indicate that NPCs do not express functional NMDARs, and that glutamate-evoked responses reflect the activation of AMPARs. The current-voltage relationship of NPC AMPAR currents was strongly inwardly-rectifying (Figure 1-3; n=11). Thus, NPCs lack the edited GluR2 subtype of the receptor and express Ca^{2+}-permeable AMPARs (Hollmann, Hartley et al. 1991; Hume, Dingledine et al. 1991; Verdoorn, Burnashev et al. 1991; Burnashev,

Monyer et al. 1992). In bulb slices loaded with the Ca^{2+} indicator Oregon Green-1 BAPTA AM (OG1-AM, 30 μM), application of glutamate in the presence of APV, Cd^{2+}, and Ni^{2+} to isolate AMPARs as the only route for Ca^{2+} entry into NPCs confirmed Ca^{2+} permeability of the channels (Figure 1-4; peak response 107% of control; n=1 slice). Nonstationary fluctuation analysis of AMPAR currents yielded a single-channel conductance value of 7.5±0.9 pS (Figure 1-5; n=6), in close agreement with previously reported values of Ca^{2+}-permeable AMPARs (Swanson, Kamboj et al. 1997). In contrast to the receptor properties of NPCs, synaptic stimulation elicited robust NMDAR-mediated synaptic currents and non-rectifying AMPAR responses in mature granule cells as has been previously reported (Isaacson and Strowbridge 1998) (Figure 1-6).

We next examined whether activation of AMPARs can regulate NPC migration. We focally injected the cell-permeable fluorescent dye CellTracker Green CMFDA into olfactory bulb slices and used 2-photon laser scanning microscopy (2PLSM) to collect 3D timelapse data from migrating NPCs. NPC velocity was quantified by measuring the displacement over time of visually distinct cell somata (~10-20 cells/experiment) in the XY plane (Figure 1-7). All migration experiments were performed in the presence of blockers of Na^{+} channels, $GABA_A$Rs and NMDARs to reduce the possibility that non-NPC cell types in the slice, affected by bath applied agonists or antagonists, might release (or stop releasing) factors altering properties of NPC migration. Under these conditions NPC migration proceeded at an average rate of 49.5±2.8 μm/hr (n=420 cells) with an average peak velocity of 112.4±3.7 μm/hr. NPC migration is "saltatory", usually proceeding along small distances with occasional large translocations of the somata (Fig 1-8).

Bath application of 50 μM glutamate (Fig 1-9; n=38 cells, 3 slices) or 50 μM AMPA (Fig 1-10; n=50 cells, 4 slices) reduced markedly the average rate of migration (57.8±9.7% of control for glutamate; 58.6±8.5% of control for AMPA). Migration in most cells gradually resumed following washout of the agonist with the AMPAR antagonist NBQX. In experiments done entirely in the presence of NBQX to prevent AMPAR activation, no such reduction in migration was observed (103.8±10.8% of control), confirming the direct mediation of the effect by AMPARs (Figure 1-11; n=82 cells, 3 slices). This finding demonstrates that activation of AMPARs strongly inhibits NPC migration.

Can glutamate receptors on NPCs detect synaptically released glutamate from the olfactory bulb? To address this question, we stimulated fibers in the granule cell layer in the presence of the glutamate uptake blocker DL-TBOA and monitored NPC responses using fluorescent Ca^{2+} imaging. Under these conditions, a 10 Hz train of stimuli evoked a slowly rising and decaying Ca^{2+} response in NPCs (Figure 1-12A). Stimulation-evoked responses in NPCs were unaffected by APV but abolished by NBQX, confirming they are mediated by AMPARs (Figure 1-12B). The time course of NPC activation exhibited a slow rise and decay consistent with gradual accumulation of glutamate around NPCs (Figure 1-13; n=15 cells). Similar results were seen in 2 other slices. These data indicate that glutamate released from nerve terminals can spillover onto migrating NPCs.

We also tested whether synaptically evoked glutamate could affect the migration of NPCs. We performed experiments in which NPC migration was imaged while periodically delivering a high-frequency burst stimulus to the granule cell layer (20 stimuli at 100 Hz every 20 seconds) in the presence of DL-TBOA. Under these

conditions, approximately half of normally traceable NPCs slowed their rate of migration. We selected and analyzed the migration rates of NPCs that exhibited slowing. The average rate of reduction of selected NPCs was 44.9±6.5% at its peak. Consistent with synaptically evoked glutamate gradually accumulating around NPCs, this inhibition of migration occurs more slowly than with bath application experiments (Figure 1-14; n=80 cells, 10 slices). These results demonstrate that NPC migration can be affected by endogenously released stores of glutamate in bulb slices.

We next examined whether Ca^{2+} influx via AMPARs was responsible for the AMPAR-mediated reduction in NPC migration. We minimized Ca^{2+} in the system by treating slices with the cell-permeable Ca^{2+} chelator EGTA-AM (50 uM) and maintaining them in aCSF containing 1 mM EGTA, 0.5 mM Ca^{2+} and 3.8 mM Mg^{2+}. Under these conditions, NPCs in slices loaded with OG1-AM did not exhibit any detectable rise in Ca^{2+} upon bath application of AMPA for the period of time we also used to examination the effects of AMPAR activation on migration (Figure 1-15). Subsequent wash-in of the normal aCSF Ca^{2+} concentration (2.5 mM) resulted in a large fluorescent signal increase. We observed these results in 3 olfactory bulb slices.

We measured migration velocity using the same conditions to test whether AMPAR-mediated inhibition of migration depends on Ca^{2+} influx. Application of AMPA reduced migration velocity to 60.4±8.6% of control (Figure 1-16; n=49 cells, 3 slices). Unlike experiments in which normal Ca2+ concentrations were present, most NPCs did not recover from this inhibition, suggesting Ca^{2+} may play a role in the resumption of migration following AMPAR activation. These results indicate AMPAR

activation reduces NPC migration via a mechanism independent of an elevation in NPC intracellular Ca^{2+}.

Discussion:

In this study we have demonstrated a potential signaling pathway in which environmental cues could govern NPC migration in the adult olfactory system. All of our experiments are done in the SEL of the olfactory bulb, taking advantage of the fact that this region consists of a homogenous population of cells, the vast majority of which express doublecortin, a microtubule-associated protein and selective marker of newborn migrating neurons. We fluorescently label NPCs in the SEL and use 2PLSM to track their movement beneath the surface of the slice. In our experiments, many NPCs are traceable and migrate with sufficiently rapid rates to observe changes in their velocity over relatively acute time periods (~1 hour).

NPCs in the SEL do not yet receive synaptic inputs, so we used glutamate-uncaging to characterize glutamate receptors on the cells. We found that NPCs express AMPARs, but we never found evidence of current that could be attributed to NMDARs. This is in contrast to another model system of postnatal neurogenesis, the granule cells of the cerebellum, which express NMDARs early in their development (Komuro and Rakic 1993). AMPARs on NPCs are strongly rectifying, suggesting they lack GluR2 subunits, or that if they do contain these subunits they have not undergone the posttranslational editing that confers rectification to the fully formed receptor. A further consequence of AMPARs that rectify current is that they are permeable to Ca^{2+}. To confirm this directly, we imaged NPCs loaded with the Ca^{2+} indicator dye OG1-AM while maintaining slices

in Cd^{2+} and Ni^{2+}, broad-spectrum blockers of VSCCs, and the NMDAR antagonist APV to isolate AMPARs as the only source of Ca^{2+} influx. We then applied glutamate and observed a large increase in intracellular Ca^{2+} in all visible NPCs. We performed nonstationary fluctuation analysis on AMPAR currents in NPCs and derived single-channel conductance values that are in close agreement to other reports of Ca^{2+}-permeable AMPARs. Interestingly, these observations contrast sharply with what we observed in granule cells of the olfactory bulb, the mature cell type most NPCs are destined to become. Mature granule cells express NMDARs and non-rectifying AMPARs. Consequently, we conclude that while NPCs do not express NMDARs and do express Ca^{2+}-permeable AMPARs, they undergo significant changes in their glutamate receptor expression, in both the type of receptor expressed and in the subunit variety of these receptors.

We next performed experiments to test the role of AMPARs in NPC migration. AMPAR activation by glutamate and AMPA reversibly reduced average migration velocity, an effect that was blocked in slices maintained in the AMPAR antagonist NBQX. To test whether NPCs could be activated by spillover of endogenous glutamate stores in the bulb slice, we blocked glutamate uptake mechanisms with DL-TBOA and imaged NPCs loaded with Ca^{2+} indicator while stimulating in the granule cell layer. This caused a slow rise in intracellular Ca^{2+} mediated by AMPARs. We analyzed cells that appeared to respond to granule cell layer stimulation. This analysis was done "blind", with no knowledge of the time the stimulus took place in an individual experiment. This addresses the possibility that cells stopping for reasons unrelated to the stimulus would contribute to our results. The pooled celltracks, aligned to the stimulus onset,

demonstrated that synaptically evoked glutamate inhibited the migration of NPCs, reducing their average velocity more slowly but to a comparable extent of that observed with bath applied AMPA and glutamate.

How are AMPARs coupled to migration in NPCs? These receptors allow a large influx of Ca^{2+}, a known mediator of many cellular events including motility, and thus Ca^{2+} is the obvious candidate for regulation of NPC migration. To test this hypothesis we developed conditions which minimized Ca^{2+} influx while maintaining normal characteristics of NPC migration. NPCs in the simplest condition of aCSF containing no Ca^{2+} do not migrate, possibly because of a Ca^{2+} requirement for extracellular adhesion molecules. Control experiments with BAPTA-AM suggested that this particular chelator did not properly buffer Ca^{2+} in our slice preparation, possibly due to inadequate intracellular loading. NPCs loaded with the membrane permeable Ca^{2+} buffer EGTA-AM, which we determined did buffer Ca^{2+}, and maintained in a solution containing a very low level of free Ca^{2+}, migrate with normal velocities. Under these conditions, we imaged NPCs loaded with Ca^{2+} indicator and verified that AMPAR activation did not lead to a detectable change in intracellular Ca^{2+}. This removal of Ca^{2+}, however, did not prevent AMPAR activation from inhibiting migration, demonstrating the effect is independent from Ca^{2+} signaling through the receptors. Thus, NPCs express Ca^{2+}-permeable AMPARs that inhibit their migration via a Ca^{2+}-independent mechanism.

We considered other possible couplings between AMPARs and cell motility. AMPARs pass the cations Na^{+} and K^{+} through their ion pore, and the proper equilibrium of these ions is maintained by the membrane-bound Na^{+}/K^{+}-ATPase or sodium-potassium pump. Since this enzyme requires ATP, and ATP may be required by other

cellular processes governing motility, we reasoned that if prolonged activation of AMPARs created an ionic imbalance sufficient to overwork the pump to the point of draining the cells' ATP supplies then other intracellular processes may be disrupted. We attempted to study migration in the presence of ouabain, which simply disables the Na^+/K^+-ATPase, in the hope that ATP stores would not be depleted following AMPAR activation. However, ouabain did not prevent AMPAR activation from inhibiting NPC migration, so we could draw no conclusion from these experiments. We did subsequent experiments substituting Na^+ with choline in the aCSF to prevent Na^+ influx upon AMPAR activation, and experiments in which we maintained slices in elevated K^+ levels to dissociate depolarization from AMPAR activation. Under these conditions, AMPAR activation still led to a reduction in migration velocity. Thus, we were unable to account for the action of AMPARs on migration with a single candidate ion.

Could AMPARs signal via a conformational change to second messenger systems in NPCs? We attempted to block or activate second messenger pathways in the hopes that we would observe any change in the AMPAR-mediated inhibition of migration. We observed migration in slices maintained separately in NEM to block pertussis toxin-sensitive G-protein action, KT5720 to inhibit protein kinase A (PKA) activity, okadaic acid and sodium orthovanadate to inhibit protein phosphatase 1 and 2A and protein tyrosine phosphatase activity, forskolin to activate adenylyl cylcase and increase intracellular cyclic AMP (cAMP), and 8-bromo-cAMP to raise cAMP levels directly. None of these manipulations yielded clear results, either because migration was disrupted directly, or because they failed to prevent a reduction of migration velocity upon subsequence AMPAR activation.

Thus, AMPAR-mediated inhibition of migration remains a highly unique and intriguing aspect of adult neurogenesis in the olfactory system, and warrants much further inquiry and research. We speculate that NPCs may "sense" an environment where glutamatergic activity is widespread, and correspondingly slow their migration to establish whether appropriate targets for synaptic integration in the olfactory bulb have been reached.

ACKNOWLEDGEMENT

Chapter 1, in full, is in preparation under the working title "Excitable Neural Precursor Cells in the Adult Olfactory Bulb: Regulation of Migration by Ca^{2+}-permeable AMPA Receptors." The dissertation author is the primary investigator and author of this paper.

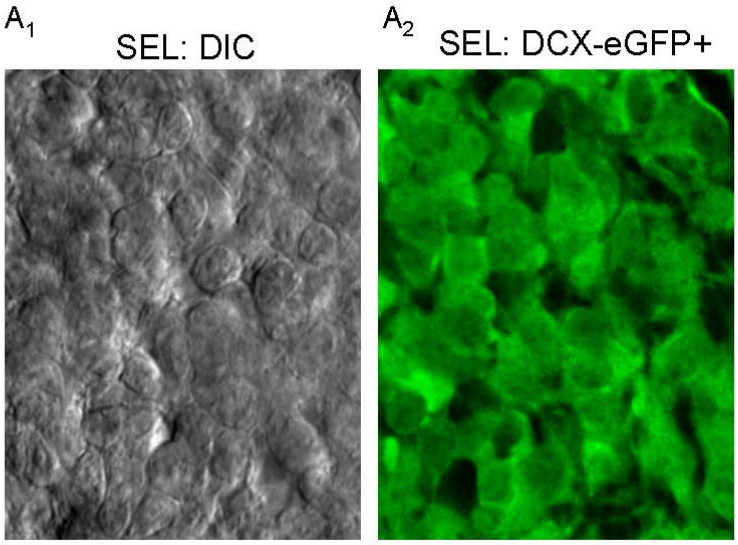

Figure 1-1: NPCs express doublecortin, a marker of newborn migrating neurons.

A_1, DIC and A_2, green fluorescence images from the SEL of an adult mouse expressing eGFP under the promoter for doublecortin. The vast majority of NPCs observed in the SEL were GFP+.

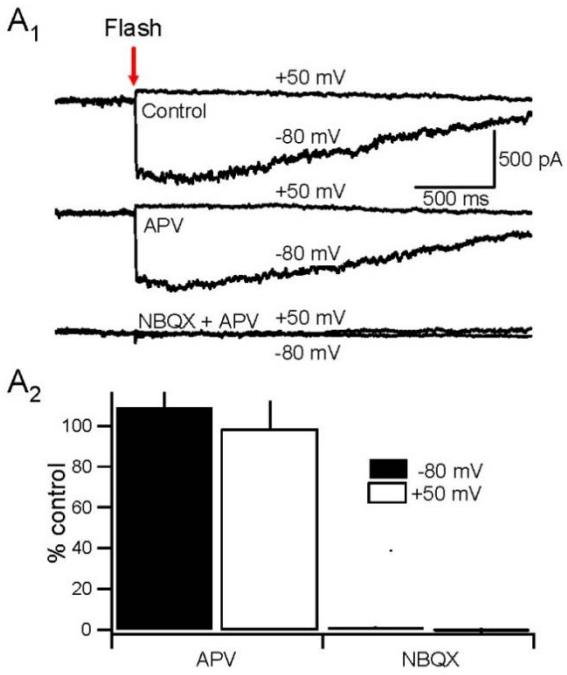

Figure 1-2: NPCs express AMPARs but not NMDARs.

A_1, Example current traces from an NPC held at -80 mV and +50 mV in response to glutamate uncaging under control conditions (top) and after bath application of APV (middle) and NBQX (bottom). A_2, % current remaining after APV and NBQX application at each holding potential (n=6).

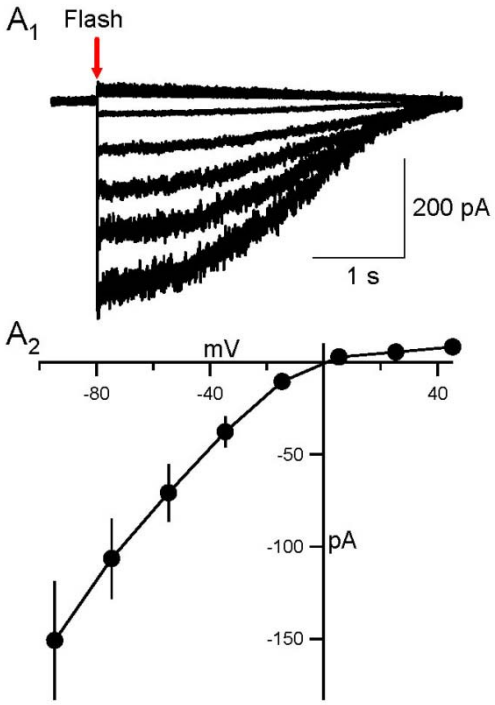

Figure 1-3: NPC AMPARs are rectifying.

A_1, Example AMPAR current responses to voltage steps (-95 mV to +45 mV) and A_2, corresponding IV plot (n=11).

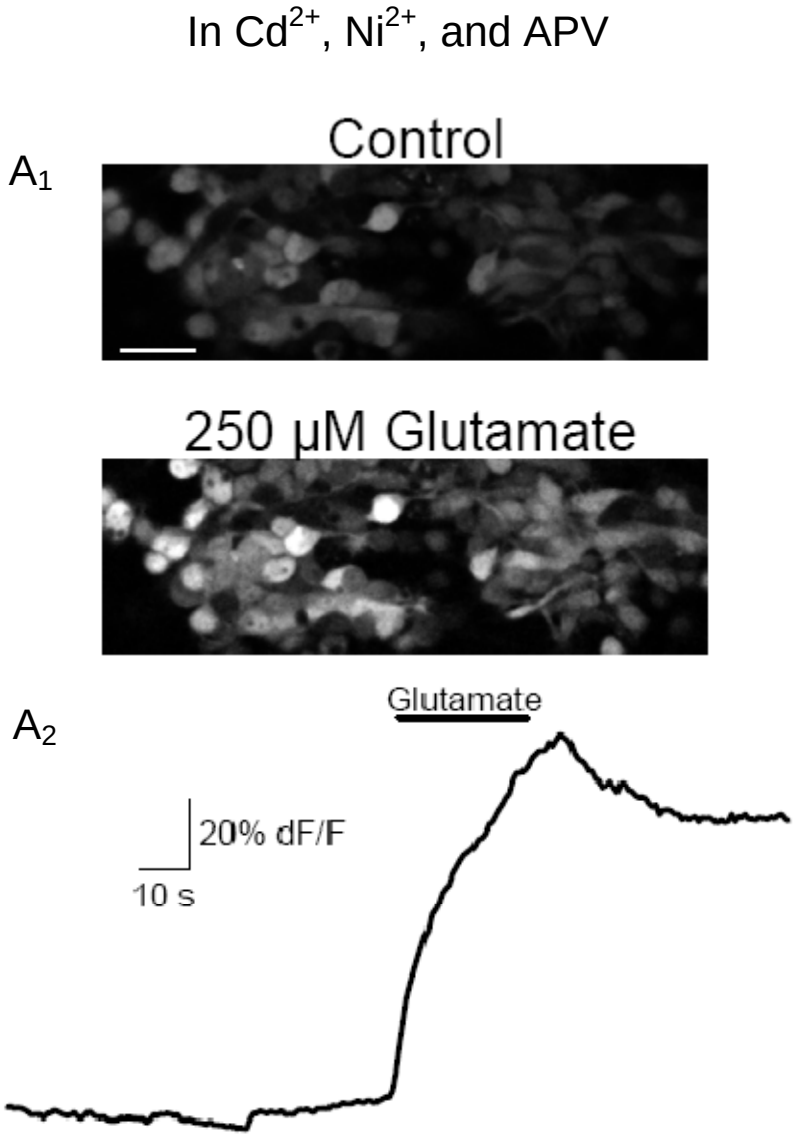

Figure 1-4: NPC AMPARs are Ca^{2+}-permeable.

A_1, Example image frames of NPCs loaded with Ca^{2+} indicator before and after bath application of glutamate and A_2, corresponding wide-field delta F/F trace. Scale bar is 20 µm.

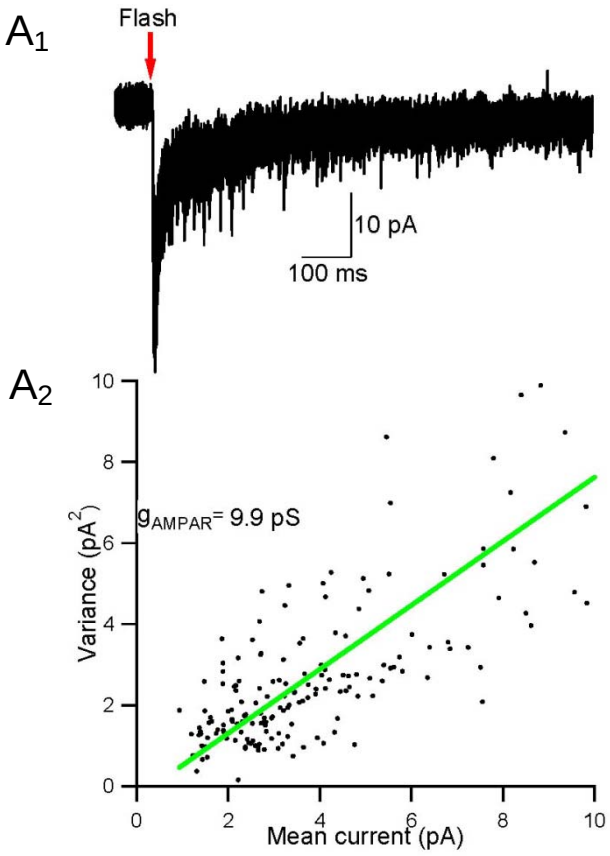

Figure 1-5: Single-channel conductance properties of NPC AMPARs.

A_1, Example current trace and A_2, corresponding nonstationary fluctuation analysis of an NPC AMPAR response.

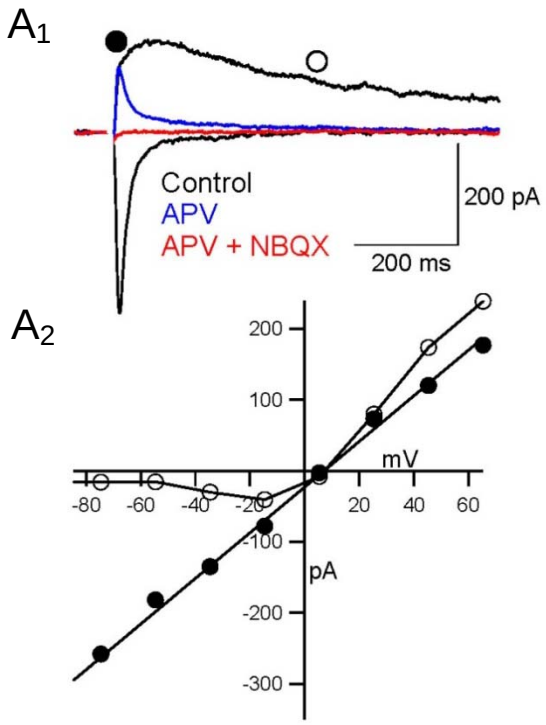

Figure 1-6: Properties of synaptic currents in mature granule cells of the olfactory bulb.

A_1, Example current traces and A_2, corresponding IV plot of synaptically evoked NMDAR and non-rectifying AMPAR currents from a mature granule cell.

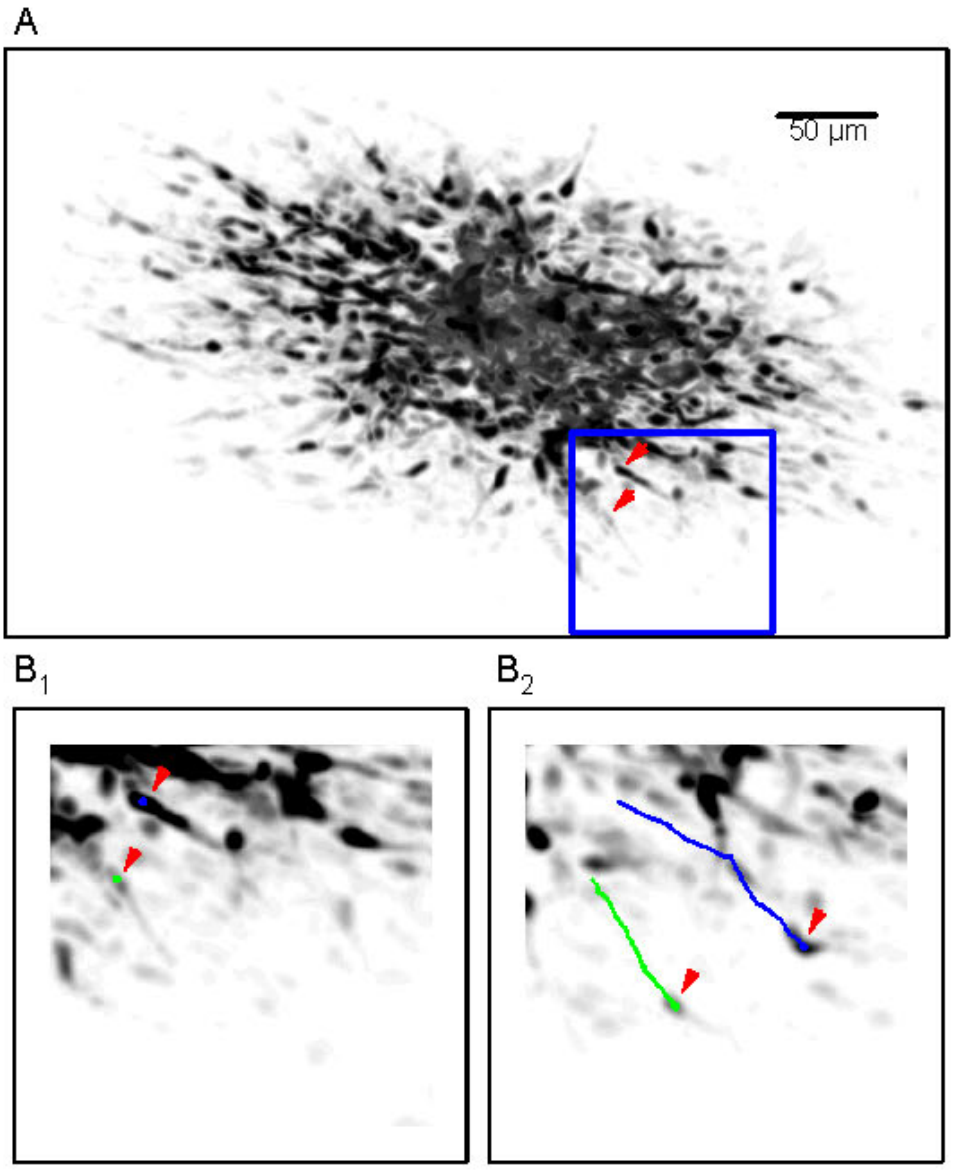

Figure 1-7: Imaging and "celltracking" NPC migration.

A, NPCs in an olfactory bulb slice focally injected with CellTracker Green CMFDA at t=0' of a typical imaging experiment. Red arrows point to examples of isolated NPC soma. B_1, Enlargement of blue inset in (A) at t=0' and B_2, t=80' with two example celltracks (green and blue lines).

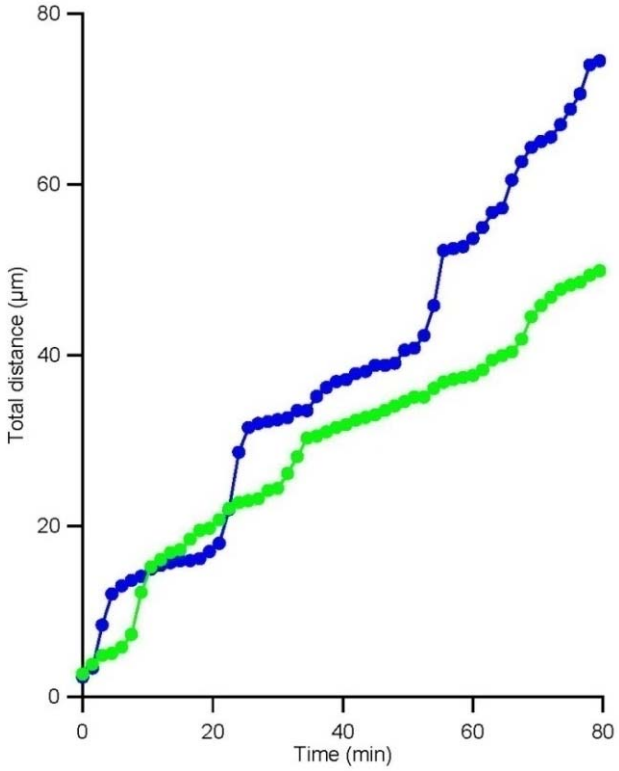

Figure 1-8: NPC migration is saltatory.

Total distance over time plotted for the two NPCs illustrated in Figure 1-7. Slow incremental migration is interspersed with large translocation of somata, resulting in a stepwise procession of total movement.

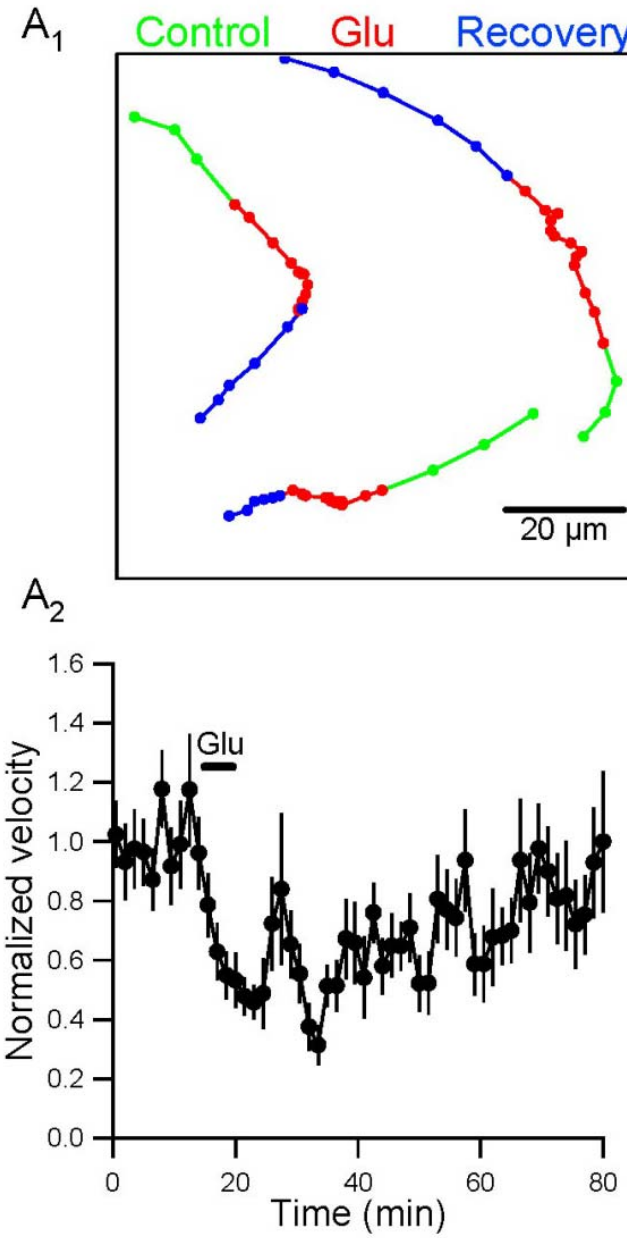

Figure 1-9: Effects of glutamate on migration of NPCs.

A_1, Example celltracks of NPC control migration (green), peak effect during and following bath exposure to glutamate (red), and migration recovery several minutes after subsequent wash-in of NBQX (blue). A_2, Time course of effect of glutamate on normalized NPC migration (n=38 cells, 3 slices).

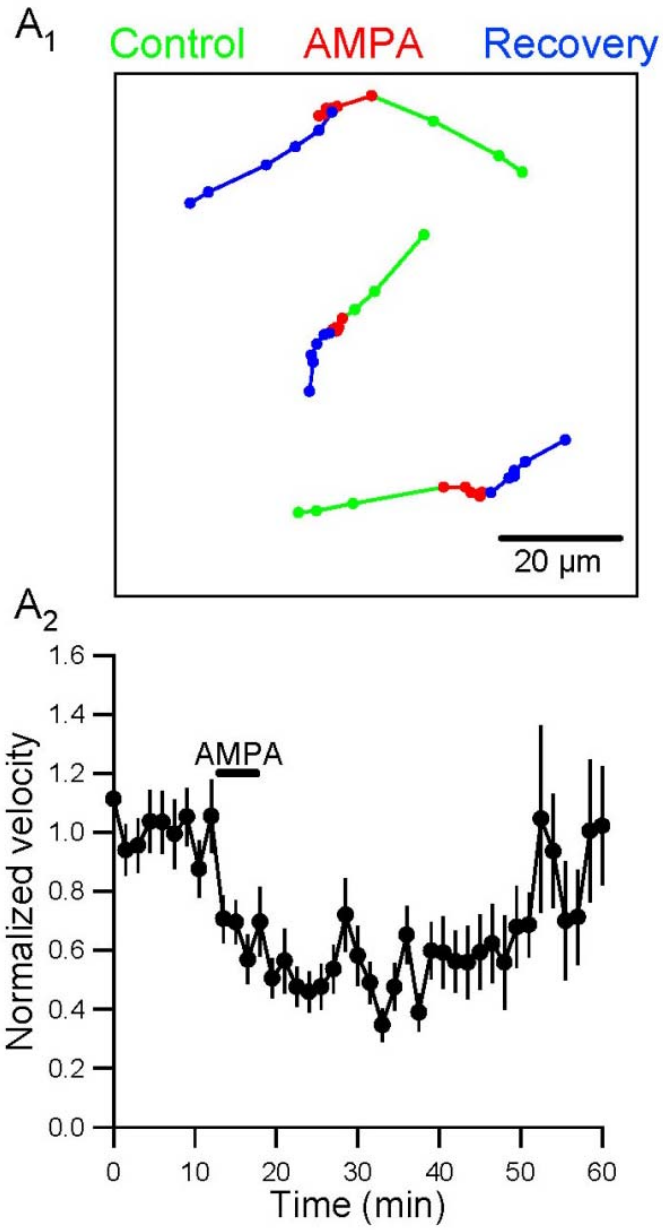

Figure 1-10: Effects of AMPA on NPC migration.

A_1, Example celltracks of NPC control migration (green), effect of AMPA application (red), and recovery of motility (blue). A_2, Time course of effect of AMPA on normalized NPC migration (n=50 cells, 4 slices).

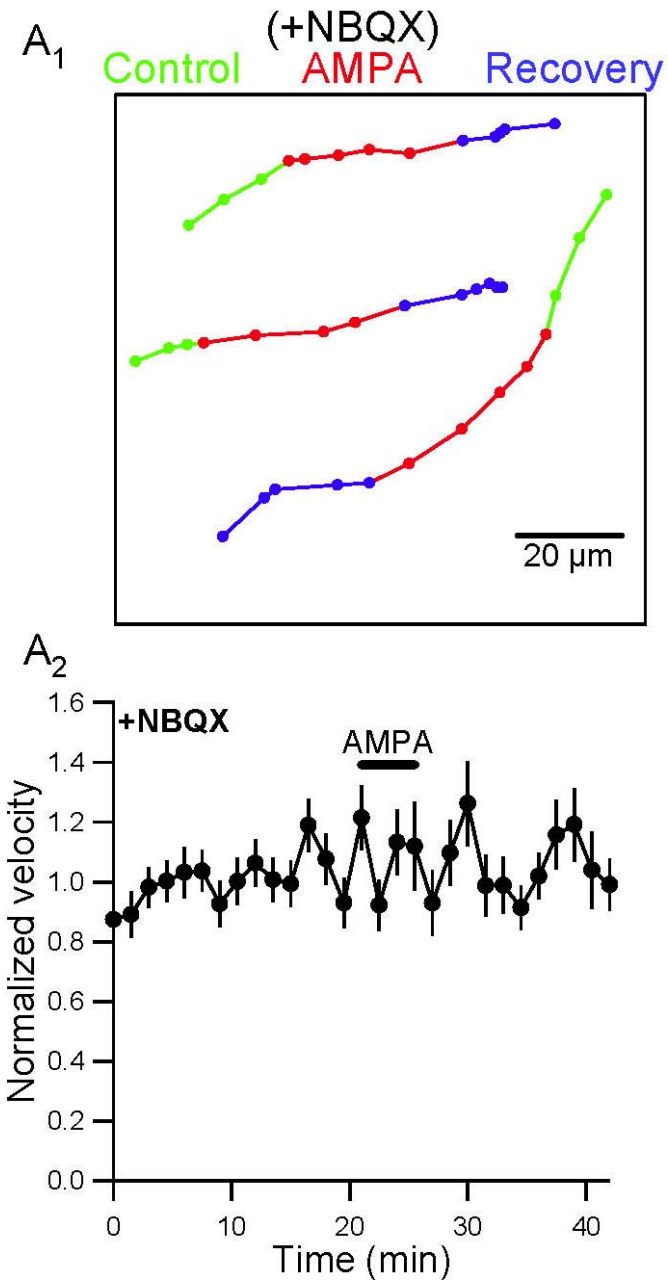

Figure 1-11: NBQX blocks AMPAR-mediated inhibition of NPC migration.

A_1, Example celltracks of NPCs in slices maintained in NBQX to block AMPARs. Control migration (green), effect of AMPA (red), and subsequent recovery (blue) do not differ in average velocity. A_2, Time course of effect of AMPA on normalized NPC migration in slices maintained in NBQX (n=82 cells, 3 slices).

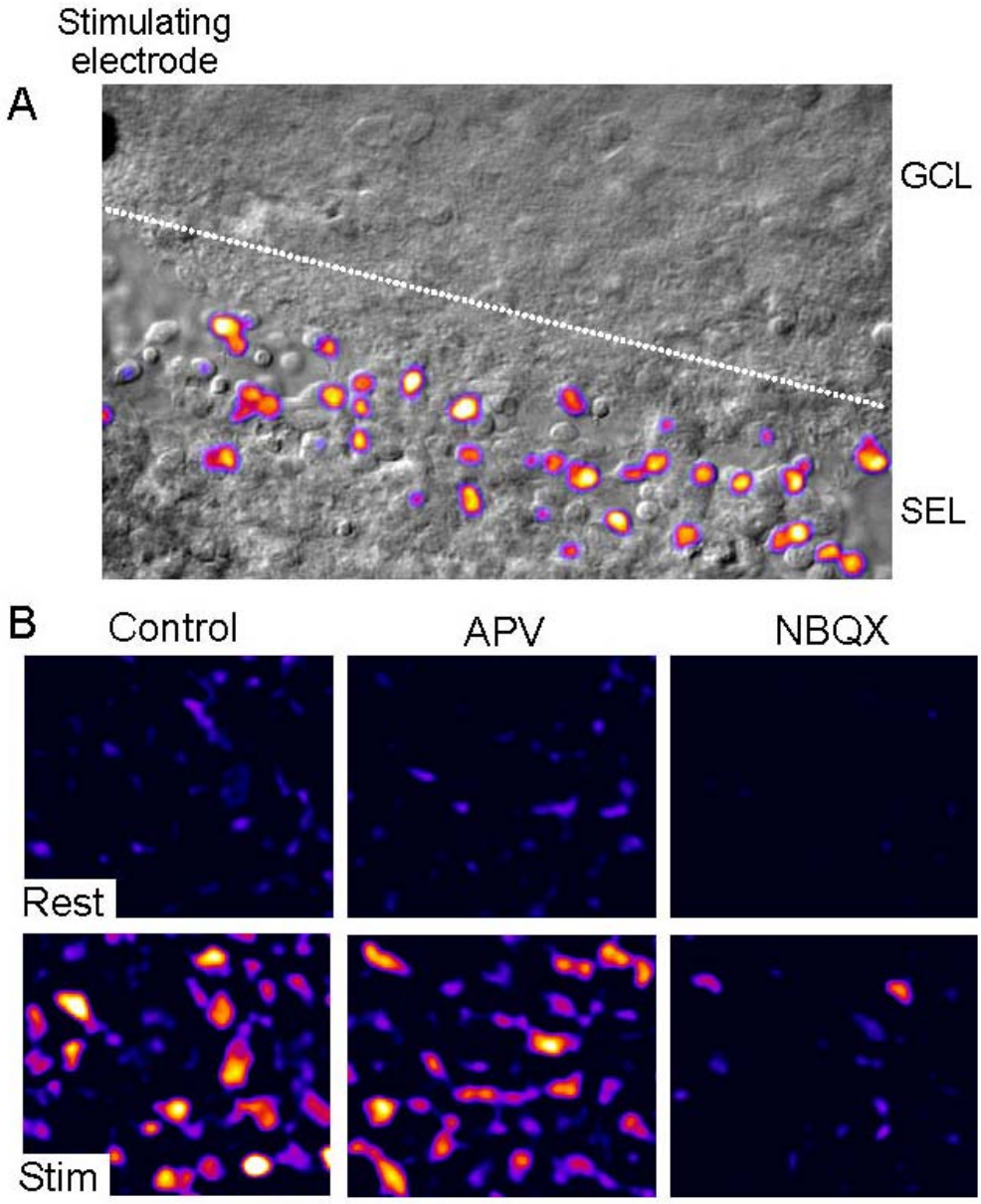

Figure 1-12: Synaptically evoked glutamate excites NPCs.

A, Representative imaging field showing placement of a bipolar simulating electrode (upper-left) and field of imaged NPCs. Pseudocolor overlay shows peak activation of NPCs that responded to glutamate spillover in the presence of the glutamate uptake blocker TBOA. B, Average delta F/F frames of Ca^{2+} activity at baseline ("Rest", top panels) and peak response following a 10 Hz stimulus in the granule cell layer ("Stim", bottom panels). Cells responded under control conditions (left) and after application of APV (middle) but response was diminished by application of NBQX (right).

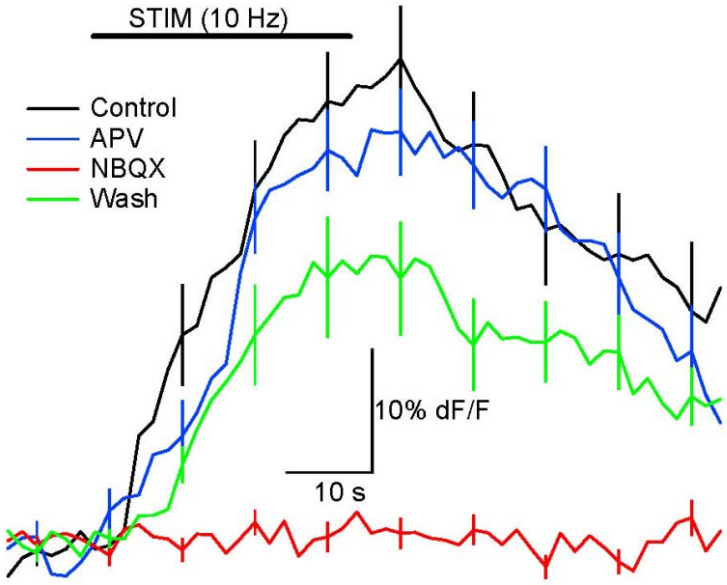

Figure 1-13: Time course of NPC activation by synaptically evoked glutamate.

Delta F/F traces of NPC activation under control conditions in TBOA (black), following application of APV (blue) and NBQX (red), and recovery following washout of receptor antagonists (green) (n=15).

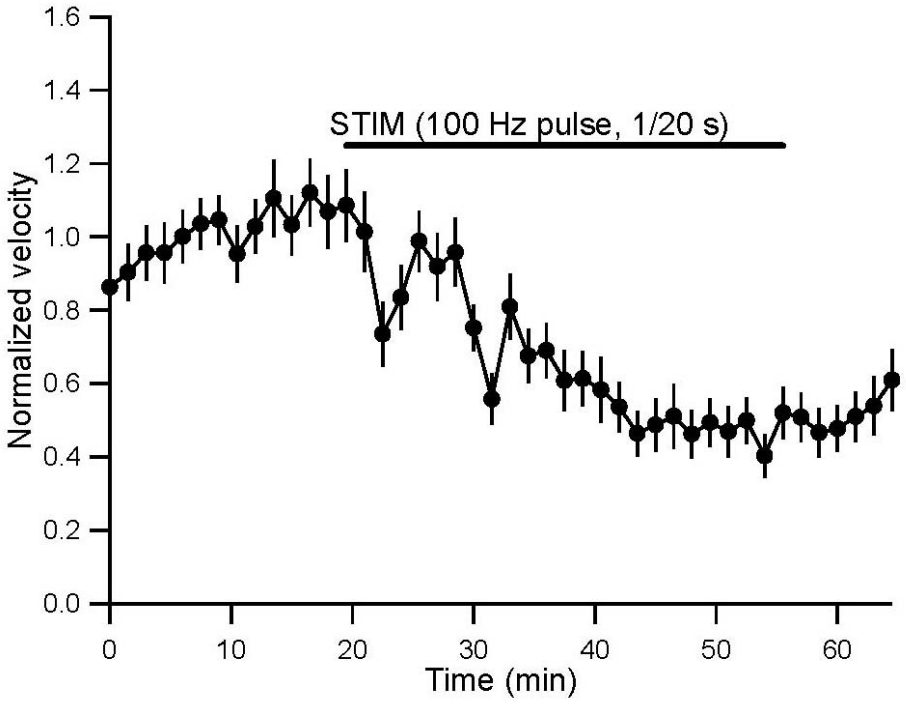

Figure 1-14: Synaptically evoked glutamate inhibits migration of NPCs.

Time course of the effect of glutamate evoked by granule cell layer stimulation (20x 100 Hz, 1/20 sec) on normalized NPC migration (n=80 cells, 10 slices).

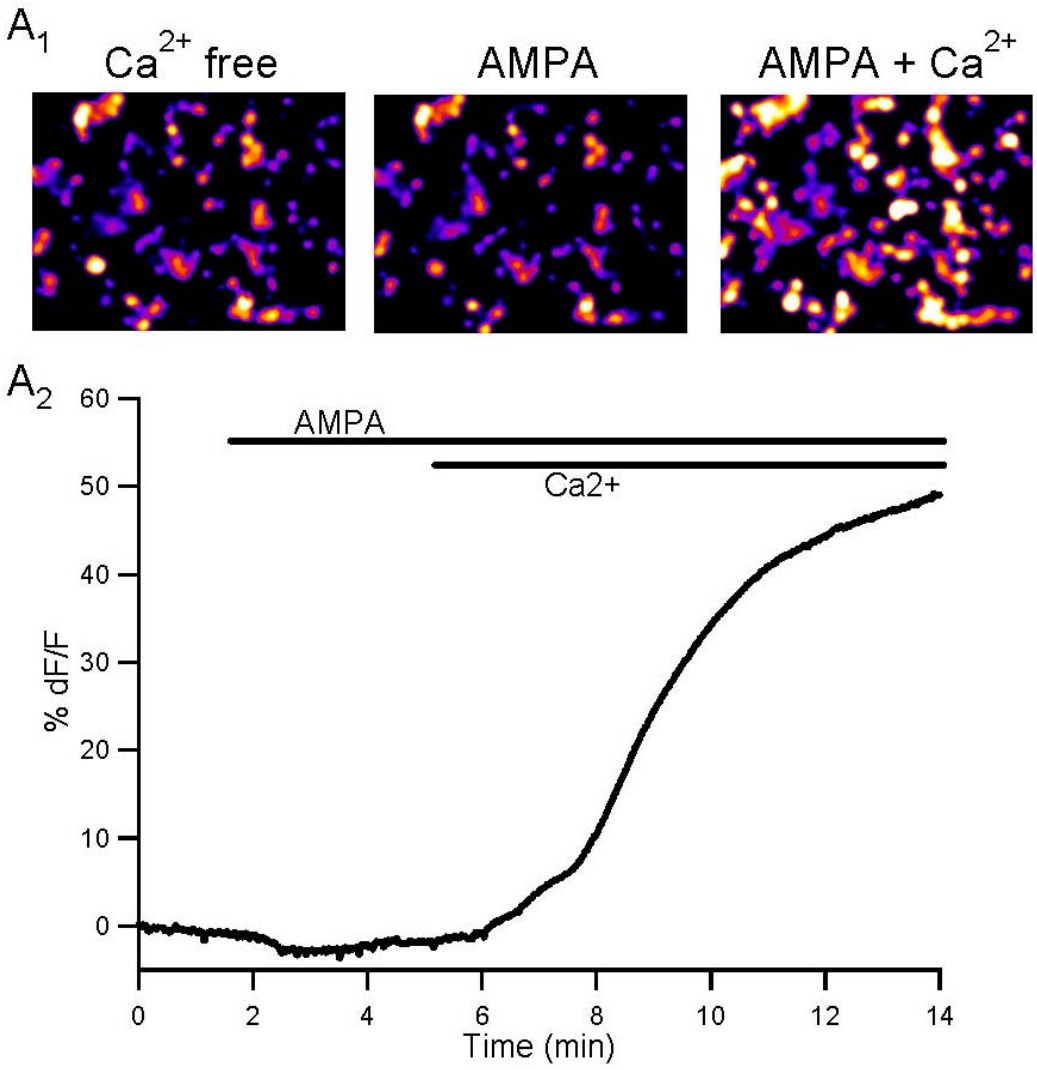

Figure 1-15: EGTA-AM and low extracellular Ca^{2+} prevents an increase in NPC intracellular Ca^{2+}.

A_1, Example delta F/F imaging frames from NPCs loaded with Ca^{2+} indicator under conditions in which Ca^{2+} has been minimized (Ca^{2+} free), following application of AMPA (AMPA), and following introduction of aCSF with normal Ca^{2+} levels (AMPA + Ca^{2+}). A_2, Example delta F/F trace showing time course of application of AMPA followed by normal Ca^{2+} concentration.

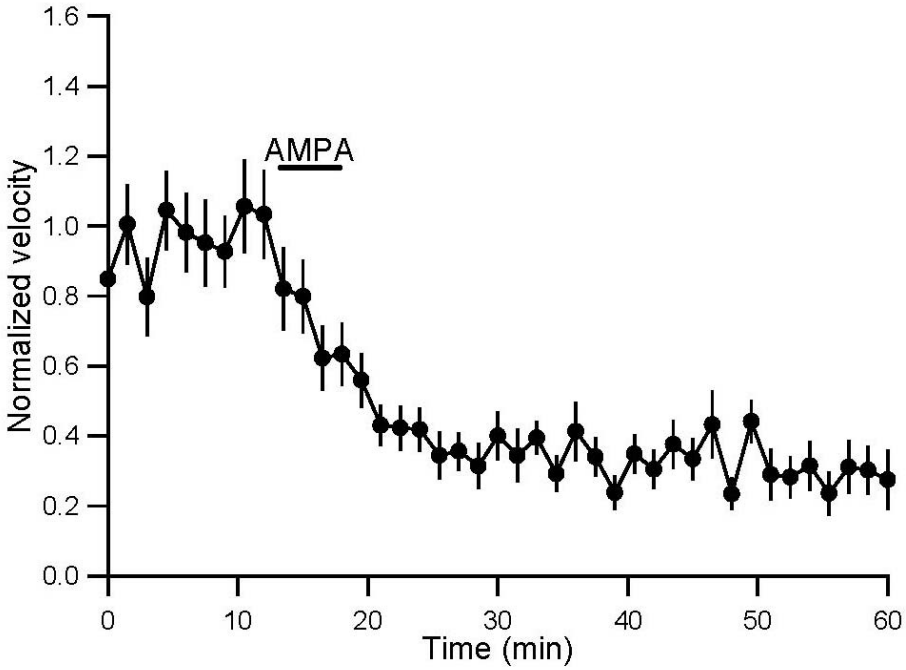

Figure 1-16: AMPAR-mediated inhibition of migration acts through a Ca^{2+}-independent mechanism.

Time course of effect of AMPAR-mediated inhibition of normalized NPC migration under minimized Ca^{2+} conditions (n=49, 3 slices).

CHAPTER 2: Effects of Intracellular Calcium Signaling on Migration in Neural Precursor Cells of the Adult Olfactory Bulb

Abstract:

Ca^{2+} signaling has been extensively implicated in the guidance and migration of neurons. For example, in the postnatal development of the cerebellum, N-type voltage-sensitive Ca^{2+} channels (VSCCs) are critical for the proper migration of granule cells. We have previously shown that activation of Ca^{2+}-permeable AMPA receptors (AMPARs) on neural precursor cells (NPCs) of the adult olfactory bulb inhibits the cells' migration, but that this effect on migration is not dependent on Ca^{2+}. Are there other sources of Ca^{2+} signaling in NPCs, and might these other pathways affect NPC migration? We use a Ca^{2+} indicator dye and electrical recordings from NPCs to demonstrate the presence of L-type VSCCs that underlie spontaneous and depolarization-evoked intracellular Ca^{2+} transients in the cells. Manipulation of these channels does not affect NPC migration. These data confirm that Ca^{2+} signaling and motility are uncoupled in NPCs of the olfactory bulb.

Introduction:

Ca^{2+} is a major component of intracellular signaling that regulates a wide range of biological processes through diverse pathways. Many cell types that migrate, including muscle (Gerthoffer 2007), lymphocytes (Bhakta, Oh et al. 2005), sperm (Publicover, Harper et al. 2007), glia (Lohr, Heil et al. 2005), and neurons (Komuro and Rakic 1998), are dependent on signaling that alters intracellular Ca^{2+} levels. Of particular interest is the migration of neurons within the adult brain, an organ that presents many challenges to repair and recovery following damage from acute injury or disease. In select localized niches in the brain, neuronal birth and migration persists beyond prenatal development, and the role of Ca^{2+} in the migration, targeting and integration of these newly generated cells has become a topic of particular importance.

In the cerebellum, the region where postnatal neuronal migration has been most thoroughly investigated, fluctuations in intracellular Ca^{2+} are tightly coupled to cell movement. Granule cells in the cerebellum proliferate in the external granular layer, migrate through the molecular layer (ML) along Bergmann glial fibers, then detach from these fibers, traverse the Purkinje cell layer, and reach their final targets in the internal granular layer (Komuro and Rakic 1998). Post-mitotic granule cells express N-type voltage-sensitive Ca^{2+} channels (VSCCs) before they begin migrating, and signaling through these channels controls the rate of the cells' movement through the ML (Komuro and Rakic 1992). Further, changes in extracellular Ca^{2+} concentration are positively correlated with migration rates, supporting the hypothesis that VSCC activity that allows Ca^{2+} influx is a key mechanism controlling granule cell movement. Following the discovery of N-type VSCC regulation of migration, it was demonstrated that granule cells

also express NMDA type glutamate receptors. As with the VSCCs, NMDA receptors permit Ca^{2+} entry into the cell, and activity through those receptors is positively coupled to migration in the ML (Komuro and Rakic 1993). Subsequent imaging of granule cells from cerebellar microexplant cultures, labeled with the Ca^{2+}-indicator dye Fluo-3, established the direct link between intracellular Ca^{2+} fluctuations and movement: transient elevations in intracellular Ca^{2+}, whether occurring spontaneously or induced via extracellular depolarization, corresponded with forward translocation of cell somata (Komuro and Rakic 1996).

The clearly established role of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and its positive regulation of migration in postnatally generated cerebellar granule cells is not a universal feature for all systems of neuron motility, illustrated by migration during organism development. For example, examination of mice that do not express functional NMDA receptors failed to reveal a deficit in neocortical development, whether because the migration of these cells is distinct from cerebellar granule cells, or due to intrinsic compensatory mechanisms (Messersmith, Feller et al. 1997). Furthermore, a considerably more complex relationship between $[Ca^{2+}]_i$ and movement has emerged for the guidance of growth cones at the end of developing axon. Experiments in embryonic spinal neurons of *Xenopus* have demonstrated that global increases in $[Ca^{2+}]_i$ in growth cones generally lead to the halting or retraction of neurites from cultured explants or *in vivo* (Gu and Spitzer 1995; Gomez and Spitzer 1999). Conversely, lowering growth cone $[Ca^{2+}]_i$ causes accelerated neurite extension. Other experimental approaches inducing localized elevations in Ca^{2+} within the growth cone can, depending on the type of stimulation, cause growth cone turning toward or away from the site of increase (Zheng

2000; Wen, Guirland et al. 2004). These varied responses to Ca^{2+} have lead to the view that changes within a range of Ca^{2+} levels, or the range of frequency of transient increases in $[Ca^{2+}]_i$, can produce differential effects on growth cone motility. In certain other cultured neuron systems, however, manipulations that modulate $[Ca^{2+}]_i$ do not lead to the expected changes in neurite outgrowth or neuron migration. For example, stimulation of cultured rat superior cervical ganglion neurons leads to an 3-5 fold increase in intracellular Ca^{2+}, but does not affect their rate of growth (Garyantes and Regehr 1992). Another report has shown no effect of intracellular Ca^{2+} on migration rates of rat autonomic motor neurons in organotypic slice cultures (Wetts and Vaughn 2000). These studies raise the possibility that other mechanisms may govern motility independent of Ca^{2+} signaling for some cell types or cell environments.

In rodents, NPCs may travel ~5 mm from their site of birth to their targets in the bulb. What intrinsic and extrinsic factors guide the cells along such a considerable distance? Given the widespread involvement of Ca^{2+} in regulating cell motility, one possibility is that NPCs recapitulate the events observed in migrating cerebellar granule cells, and exhibit a correlation between intracellular Ca^{2+} levels and movement underlying their migration. However, we have previously shown that NPCs express Ca^{2+}-permeable AMPARs that inhibit their migration via a Ca^{2+}-independent mechanism, conflicting with the idea that Ca^{2+} is the key mediator of NPC motility in the olfactory system. In this study, we examine multiple modes of Ca^{2+} signaling in NPCs migrating in the subependymal layer (SEL) of the olfactory bulb and examine how these events relate to the cells' migration.

Results:

We bulk loaded NPCs with the membrane-permeable Ca^{2+} indicator Oregon Green-1 BAPTA AM (OG1-AM, 30 μM) and collected fluorescent timelapse data of fields of SEL cells. We observed spontaneous transient elevations in Ca^{2+} in NPCs (Figure 2-1). These transients were observed in about 25% of all visible NPCs. The frequency of transients observed was usually less than one per minute, and imaging experiments usually proceeded for four minutes, therefore this is an underestimate of the fraction of NPCs in which transients occur. NPCs frequently form tightly clustered groups of cells, and this physical arrangement could enable the cells to signal via gap-junctions. It has previously been shown that coordinated fluctuations in intracellular Ca^{2+} occur in precursor neurons of the ventricular zone via gap junction coupling (Owens and Kriegstein 1998). To explore this possibility in NPCs, we measured the frequency with which Ca^{2+}-transient elevations occurred in adjacent pairs of cells. We counted spikes as simultaneous if they occurred in two adjacent cells during a 2 second time window. This frequency was not greater than when individually measuring coincident activity between each of the cells from an adjacent pair and a random distant cell in the image field (13.6±6.7% coincidence for adjacent cells, 11.9±5.5% coincidence for randomly paired cells; n=22 cells, 1 slice). Thus, despite the dense packing of some NPCs we did not find evidence for Ca^{2+} signaling through gap-junctions in the cells.

What is the underlying source of these intracellular Ca2+ fluctuations? To explore whether neurotransmitter receptors or ion channels may govern spontaneous Ca^{2+} elevations in NPCs, we imaged Ca^{2+} activity in these cells before and after application of

a blocker cocktail containing (in μM) 1 TTX, 50 APV, 20 NBQX and 25 SR-95531 (Gabazine) to block Na^+ channels, NMDARs, AMPARs, and $GABA_A$ receptors ($GABA_ARs$), respectively. Transient frequency was not affected by the blockers (Figure 2-2; transients per minute in control: 0.77±0.09; blockers: 0.72±0.07; n=95 cells, 3 slices). To test for a contribution of intracellular Ca^{2+} stores, we did similar experiments quantifying transient frequency before and after the addition of thapsigargin (5-10 μM). Transients persisted in the presence of thapsigargin (Figure 2-3; transients per minute in control: 1.39±0.13; thapsigargin: 1.61±0.13; n=91 cells, 5 slices), discounting a role for thapsigargin-sensitive Ca^{2+} stores in the generation of Ca^{2+} transients. We next tested whether depolarization of cells would increase the frequency of transients. Elevation of extracellular K^+ from 2.5 mM to 10 mM significantly increased transient frequency (Figure 2-4; transients per minute in control: 0.72±0.11; high K^+: 1.36±0.14; n=57 cells, 3 slices). Hence, while ionotropic neurotransmitter receptors, Na^+ channels, and thapsigargin-sensitive stores do not alter the rate of Ca^{2+} transients, membrane depolarization increases them. This suggests a role for a voltage-sensitive Ca^{2+} channel.

We then tested the effect of dihydropyridines, selective modulators of L-type Ca^{2+} channels, on spontaneous calcium transients in NPCs (Figure 2-5; n=60 cells, 4 slices). Transients were largely abolished by the L-type Ca^{2+} channel antagonist nimodipine (30 μM). Subsequent washout of nimodipine in the presence of the agonist (S)-(-)-BayK 8644 (5 μM) restored and enhanced spontaneous activity. These results indicate that L-type Ca^{2+} channels underlie spontaneous Ca^{2+} transients in NPCs. Dihydropyridine-sensitive Ca^{2+} transients could also be evoked by depolarization via brief focal application of aCSF containing 45 mM K^+ (Figure 2-6; n=3 slices). Together these

results indicate that both spontaneous and depolarization-evoked rises in intracellular Ca^{2+} are largely mediated by L-type Ca^{2+} channels.

We next made whole cell recordings to characterize the intrinsic electrical membrane properties of NPCs in the SEL. Consistent with previous findings (Belluzzi, Benedusi et al. 2003; Wang, Krueger et al. 2003) these cells have a high input resistance (R_{in}=3.6±0.5 GΩ; n=6). In current-clamp, NPCs failed to generate fast action potentials in response to depolarizing current steps (-10 pA to 40 pA; Figure 2-7; n=8). Although they did exhibit a small, early transient depolarization reminiscent of a nascent spike, these events were not sensitive to block of Na^+ channels (data not shown). In voltage-clamp recordings, membrane depolarization evoked small transient inward currents (Figure 2-7 filled circles and inset) and maintained outward currents (Figure 2-7 open circles). The rapid inward currents were abolished by TTX (1 μM; data not shown), indicating that they are mediated by voltage-gated sodium channels. The maintained outward currents have previously been shown to be generated by voltage-gated K^+ channels (Wang, Krueger et al. 2003). Presumably the small amplitude of Na^+ current relative to K+ current in NPCs prevents the generation of Na^+ channel-mediated action potentials.

In the presence of the Na^+ channel blocker TTX and potassium channel blockers TEA and Cs^+, small depolarizing current injections evoked slow regenerative spikes in NPCs (Figure 2-8A_1; n=3). These events were blocked following application of the broad-spectrum Ca^{2+} channel antagonist Cd^{2+} (200 μM; data not shown). Voltage clamp recordings in the presence of TTX and TEA revealed Cd^{2+} sensitive currents consistent with high voltage-activated (HVA) Ca^{2+} channels (Figure 2-8A_2,A_3; n=7).

We next examined whether modulation of voltage-gated Ca^{2+} channels regulates NPC migration. We focally injected the cell-permeable fluorescent dye CellTracker Green CMFDA into olfactory bulb slices and used 2-photon laser scanning microscopy (2PLSM) to collect 3D timelapse data from migrating NPCs. NPC velocity was quantified by measuring the displacement over time of visually distinct cell somata (~10-20 cells/experiment) in the XY plane. All experiments were performed in the presence of TTX, $GABA_AR$ and NMDAR blockers to reduce the possibility that non-NPC cell types in the slice, affected by bath applied agonists or antagonists, might release (or stop releasing) factors altering properties of NPC migration. Bath application of the L-type Ca^{2+} channel antagonist nimodipine (30 μM; Figure 2-9; n=47 cells, 3 slices) or agonist BayK (5 μM; Figure 2-10; n=47 cells, 4 slices) had no effect on average NPC migration velocity, suggesting that L-type Ca^{2+} channels do not modulate NPC migration in an acute fashion. Thus, despite the presence of Ca^{2+} transients in NPCs and the important role of signaling through VSCCs for migration of other cell types, notably cerebellar granule cells, spontaneous Ca^{2+} transients mediated by L-type Ca^{2+} channels are not coupled to the migration of NPCs of the olfactory bulb.

The absence of an effect on migration following manipulation of Ca^{2+} transients in NPCs is consistent with the AMPAR-mediated inhibition of migration occurring through a Ca^{2+}-independent mechanism. However, it is possible that prolonged Ca^{2+} influx through L-type Ca^{2+} channels might regulate cell motility. Experiments blocking or potentiating spontaneous Ca^{2+} transients, which seldom last longer than one minute even in the presence of BayK, do not address this. We took advantage of the previously reported fact that NPCs express $GABA_ARs$, and that activation of these receptors

depolarizes NPCs (Wang, Krueger et al. 2003). To confirm whether GABAR activation could lead to an increase in intracellular Ca^{2+}, we applied varying concentrations of GABA to NPCs loaded with Ca^{2+} indicator. We observed subsequent rises in intracellular Ca^{2+} as measured by changes in wide-field fluorescence (Figure 2-11, n=5 slices).

We next examined the effects of $GABA_AR$ activation and consequent rise in intracellular Ca^{2+} on NPC migration. We measured the effects on migration for a range of concentrations of both GABA (10-200 μM) and the $GABA_AR$ agonist muscimol (50-100 μM). Each application of $GABA_AR$ agonist was followed by the $GABA_AR$ antagonists bicuculine and picrotoxin. No change in migration was observed for activation or blockade of $GABA_ARs$ in individual experiments, or when pooled together (Figure 2-12; n=79 cells, 8 slices). Because $GABA_AR$ activation leads to an increase in intracellular Ca^{2+} in NPCs, these experiments provide further evidence that sustained Ca^{2+} influx through VSCCs is not coupled to the migration of the cells.

Discussion:

In this study we have examined the intrinsic membrane properties of NPCs and how Ca^{2+} signaling in these cells governs their migration. By loading NPCs of the olfactory bulb with a Ca^{2+} indicator we observed spontaneous, transient elevations in intracellular Ca^{2+}. To determine what governs these Ca^{2+} transients we imaged spontaneous activity before and after application of a cocktail of blockers against Na^{2+} channels, NMDARs, AMPARs, and $GABA_ARs$. These blockers had no effect on the frequency of events. Likewise, thapsigargin did not alter the rate of Ca^{2+} transients,

discounting a role for thapsigargin-sensitive intracellular Ca^{2+} stores in the generation of the events. However, depolarizing the cells by applying aCSF containing 10 mM K^+ did increase transient frequency, suggesting that voltage sensitive Ca^{2+} channels were responsible. In experiments applying dihydropyridines, selective modulators of L-type Ca^{2+} channels, we found that nimodipine largely abolished spontaneous Ca^{2+} transients, while BayK strongly potentiated them. Focally puffing aCSF containing a high K^+ concentration also evoked rapid Ca^{2+} transients sensitive to dihydropyridines in NPCs. These results indicate that L-type Ca^{2+} channels underlie spontaneous and evoked elevations in NPC intracellular Ca^{2+}.

Spontaneous Ca^{2+} activity could originate as a consequence of the extreme electrotonic compactness of NPCs. For an NPC with an input resistance of 3 GΩ, a single L-type Ca2+ channel with 28 pS conductance (Umemiya and Berger 1995) passes 2.8 pA of current given the conservative estimate of 100 mV of driving force on Ca^{2+} ions. Thus, the opening of a single channel effectively changes the membrane potential by around 8.4 mV. This could recruit other voltage-gated Ca^{2+} channels to their open state before depolarizing activity is opposed by K^+ channels.

To further characterize NPC Ca^{2+} signaling, we made whole-cell recordings to measure current and voltage responses of the cells. NPCs express an array of ion channels, and while the dominant voltage-gated conductance in NPCs is a sustained outward current through K^+ channels, many of the cells also express some degree of Na^+ current. However, we never observed fast electrical spikes. Thus, the amplitude of Na^+ current in NPCs appears to be insufficient to overcome K^+ channel-mediated conductances and generate canonical action potentials. With Na^+ and K^+ currents

blocked, we observed slow regenerative electrical spikes and sustained Ca^{2+} currents that activated above -40 mV. Taken together with the presence of spontaneous Ca^{2+} transients in NPCs, these data indicate that the cells exhibit membrane characteristics of 'excitable' cells, and are capable of active signaling through regenerative electrical events.

We next examined whether manipulating L-type Ca^{2+} channels in NPCs affected their migration. We fluorescently labeled NPCs and tracked their migration beneath the bulb slice surface using 2PLSM. Blocking L-type Ca^{2+} channels with nimodipine or potentiating their activity with BayK had no effect on the migration velocity of NPCs. This suggests that, unlike the case for cerebellar granule cells in which spontaneous elevations in intracellular Ca^{2+} directly correlate with forward translocation of cell somata, Ca^{2+} transients in NPCs are not coupled to their migration.

Even in the presence of the L-type Ca^{2+} channel agonist BayK, rises in NPC intracellular Ca^{2+} are still periodic and seldom last longer than one minute before switching off for some time. It is possible that, while transient elevations in intracellular Ca^{2+} do not affect migration, prolonged Ca^{2+} influx could inhibit the cells' movement. To test this, we took advantage of the presence of the $GABA_A$Rs in NPCs. In these cells, $GABA_A$R activation is depolarizing, and using Ca^{2+} indicator dye we confirmed that application of GABA caused a prolonged rise in NPC intracellular Ca^{2+}. In migration experiments, multiple concentrations of GABA or the GABA analogue muscimol had no effect on migration. These data discount the possibility that prolonged Ca^{2+} influx into NPCs alters their migration.

Previously, we showed that activation of Ca^{2+}-permeable AMPARs on NPCs inhibits migration via a Ca^{2+}-independent mechanism. In the present study, we show multiple modes of Ca^{2+} signaling in NPCs that do not alter their migration. Taken together, these data provide multiple lines of evidence for an uncoupling between intracellular Ca^{2+} and migration in NPCs. It is intriguing to speculate that the cells' ability to "ignore" Ca^{2+} events for migration purposes frees Ca^{2+} to play other important roles in their maturation, such as regulating transcription, as these cells migrate toward their final targets in the olfactory bulb.

ACKNOWLEDGEMENT

Chapter 2, in full, is in preparation under the working title "Excitable Neural Precursor Cells in the Adult Olfactory Bulb: Regulation of Migration by Ca^{2+}-permeable AMPA Receptors." The dissertation author is the primary investigator and author of this paper.

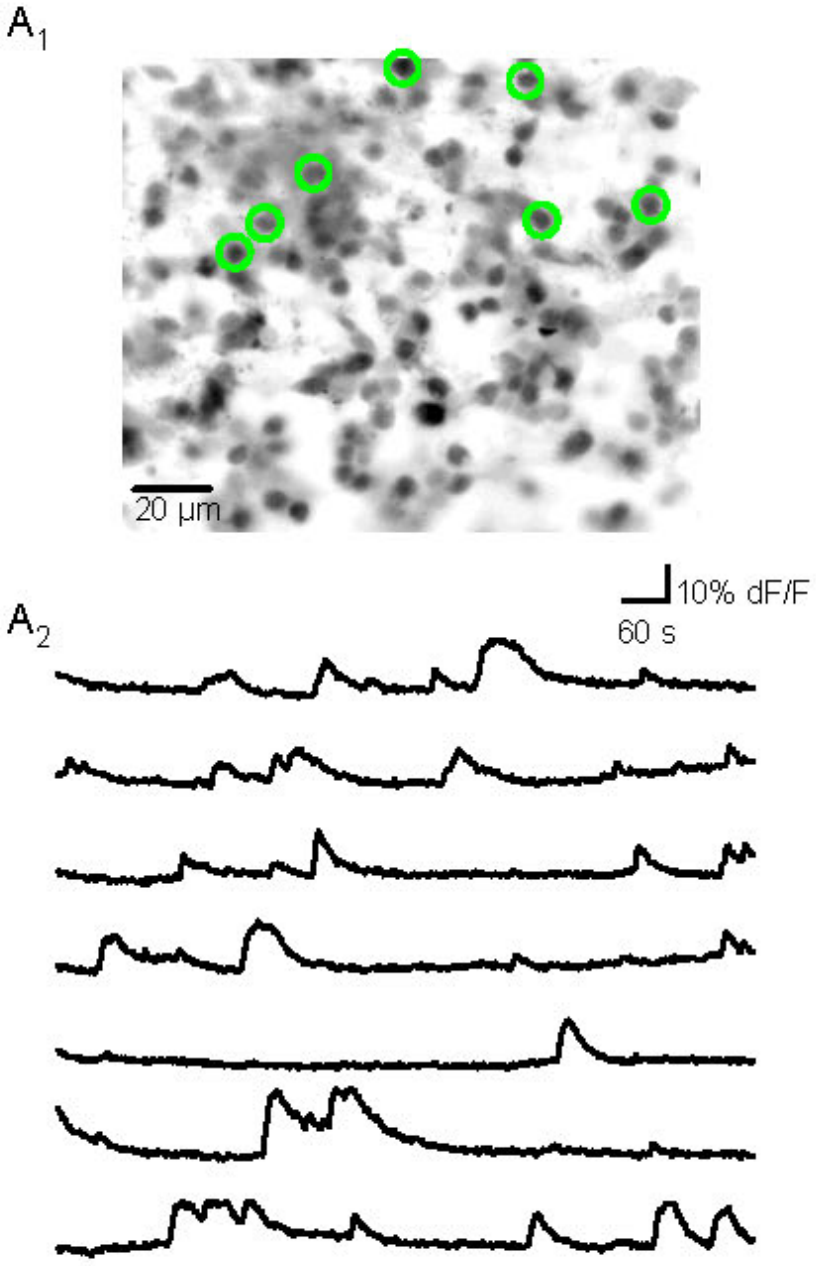

Figure 2-1: Spontaneous Ca^{2+} transients in NPCs in the olfactory bulb SEL.

A_1, NPCs of an acute olfactory bulb slice loaded with Ca^{2+} indicator. Circular ROIs are centered on seven individual cells. A_2, Delta F/F traces of average changes in fluorescence intensity in each ROI demonstrate spontaneous Ca^{2+} transients in NPCs.

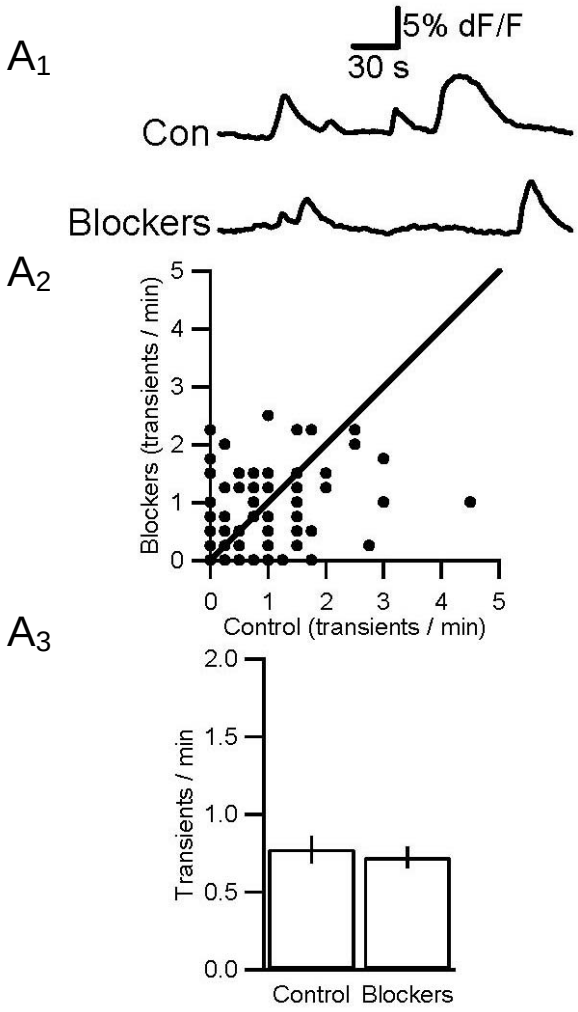

Figure 2-2: Ionotropic receptors and ion channels do not contribute to spontaneous Ca^{2+} transients in NPCs.

A_1, Example delta F/F traces of fluorescence from a representative NPC before and after addition of blockers of NMDARs, AMPARs, $GABA_A$Rs, and Na^{2+} channels. A_2, Comparison of transient frequency before and after application of blockers. A_3, Average transient frequency before and after application of blockers (n=95 cells, 3 slices).

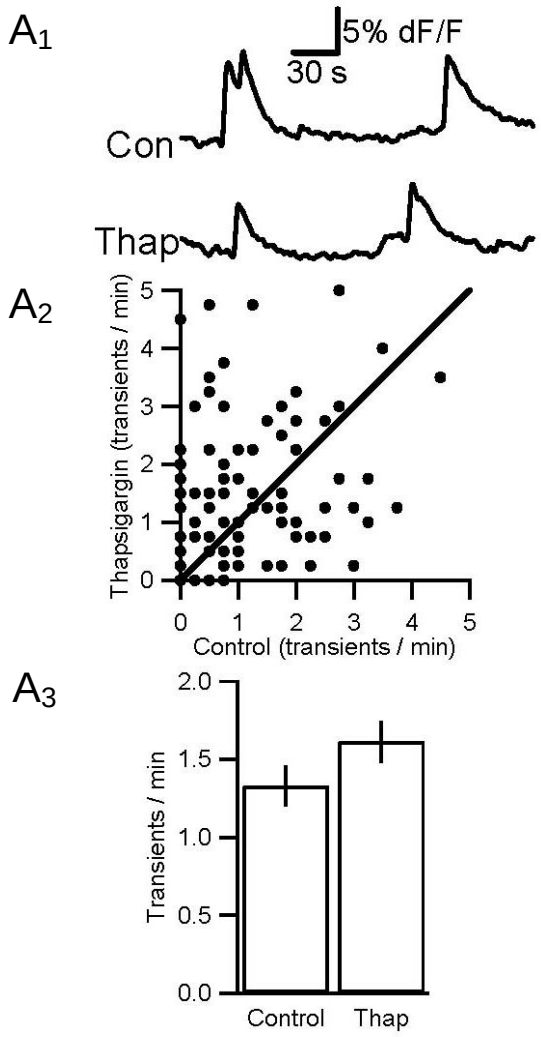

Figure 2-3: Thapsigargin-sensitive Ca^{2+} stores do not contribute to spontaneous transients in NPCs.

A_1, Example delta F/F traces from a representative NPC before and after addition of thapsigargin. A_2, Comparison of transient frequency before and after application of thapsigargin. A_3, Average transient frequency before and after application of thapsigargin (n=91 cells, 5 slices).

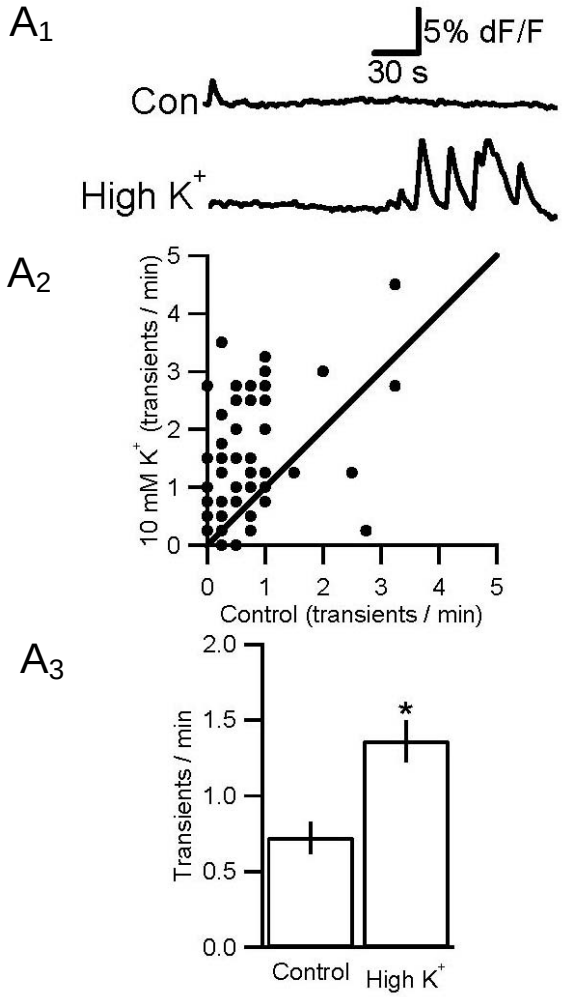

Figure 2-4: Spontaneous Ca^{2+} transients are governed by membrane potential in NPCs.

A_1, Example delta F/F traces from a representative NPC before and after addition of aCSF containing 10 mM K^+ (high K^+). A_2, Comparison of transient frequency before and after application of high K^+ aCSF. A_3, Average transient frequency before and after application of high K^+ aCSF (n=57 cells, 3 slices).

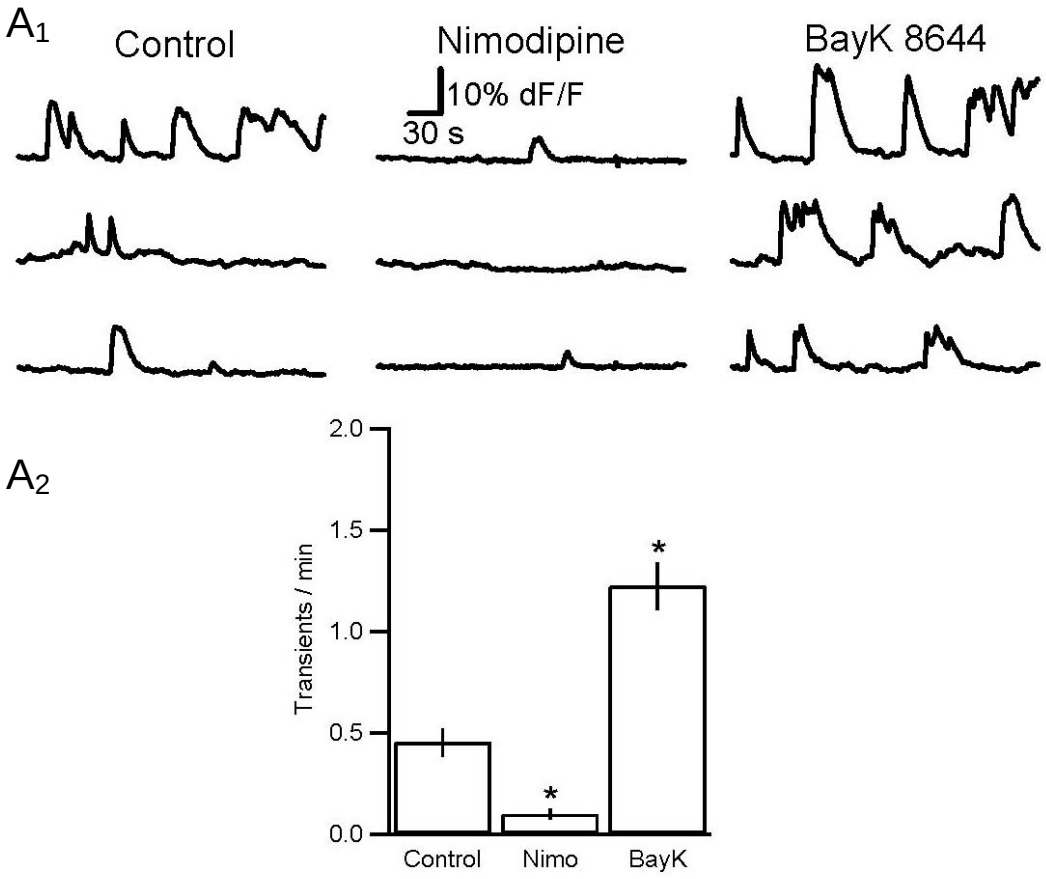

Figure 2-5: L-type VSCCs mediate spontaneous Ca^{2+} transients in NPCs.

A_1, Example delta F/F traces from 3 representative NPCs. A_2, Average change of transient frequency in response to nimodipine and BayK 8644 (n=60 cells, 4 slices).

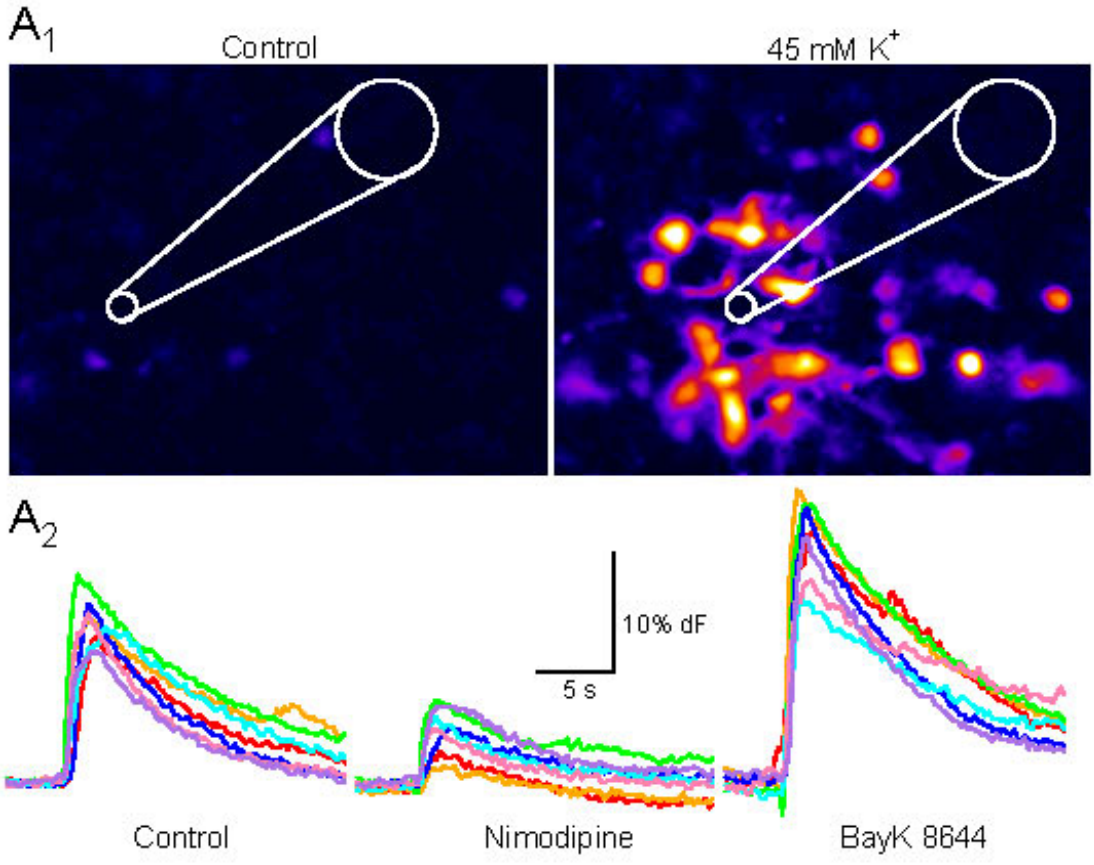

Figure 2-6: L-type VSCCs mediate depolarization-evoked Ca^{2+} transients in NPCs.

A_1, Example delta F/F frames from NPCs before and after a brief puff of 45 mM K^+ aCSF. A_2, Delta F/F traces from several NPCs show reduction of response by nimodipine and potentiation by BayK 8644.

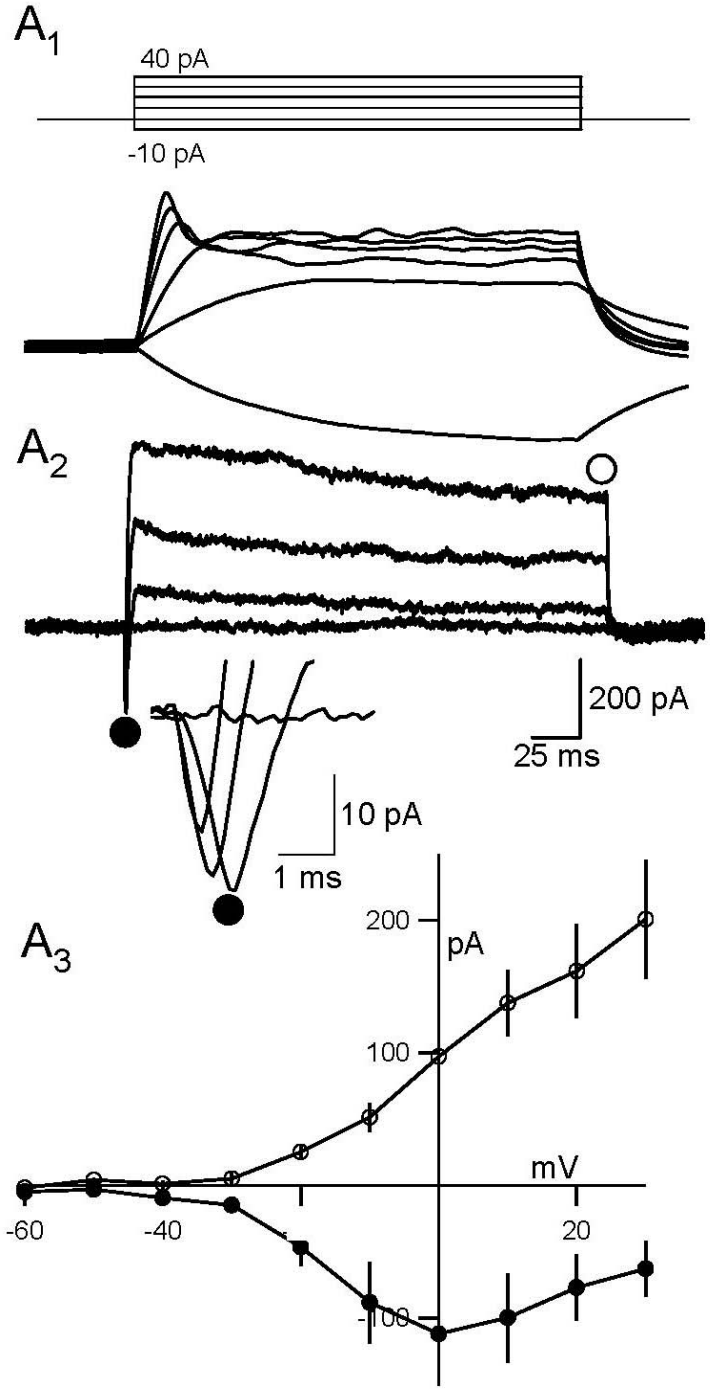

Figure 2-7: NPCs express Na^+ and K^+ channels.

A_1, Traces from an NPC held in current clamp in response to current step injections (V_R=-42 mV). A_2, Voltage-step traces (V_M=-80 mV) from an NPC with mixed Na^+ (filled circles; inset) and K^+ current (open circles) and A_3, corresponding IV plot (n=8).

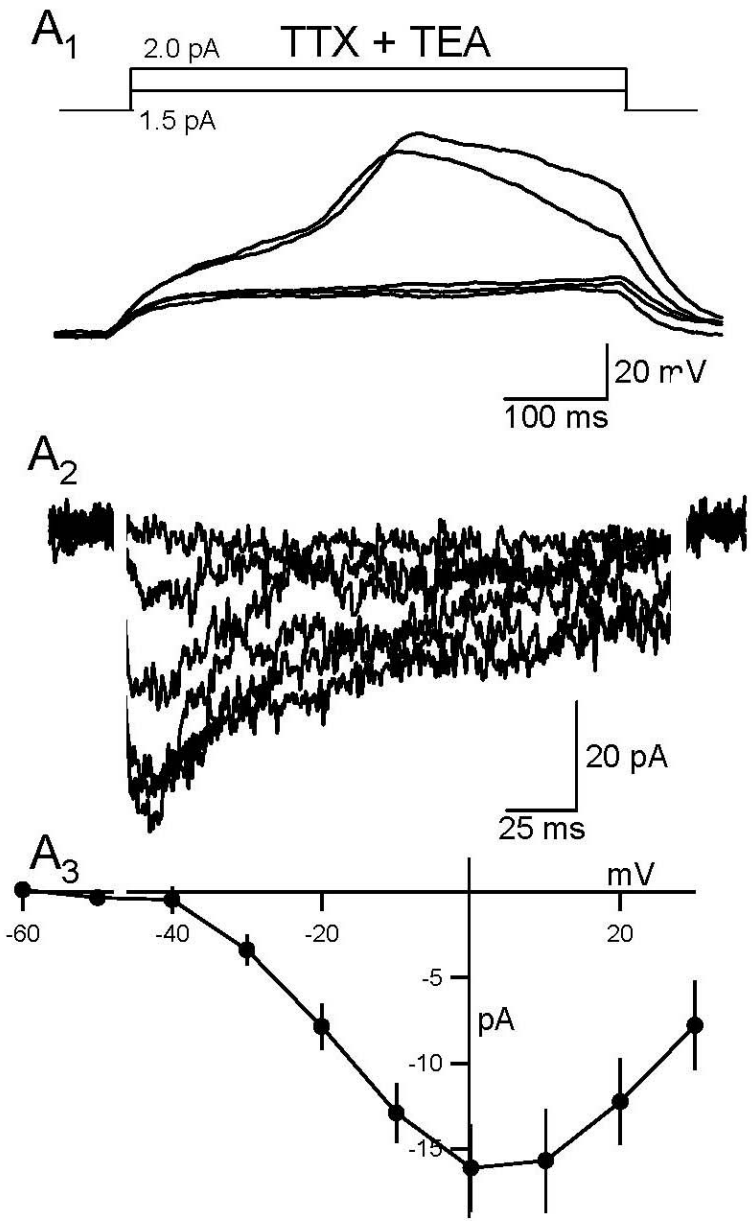

Figure 2-8: Regenerative electrical spikes and Ca^{2+} currents in NPCs.

A_1, Example traces of a regenerative electrical spike elicited by small current injections in an NPC with Na^+ and K^+ channels blocked. A_2, Example traces of Ca^{2+} current elicited by voltage-steps and A_3, IV plot demonstrating HVA Ca^{2+} channel activity (n=7).

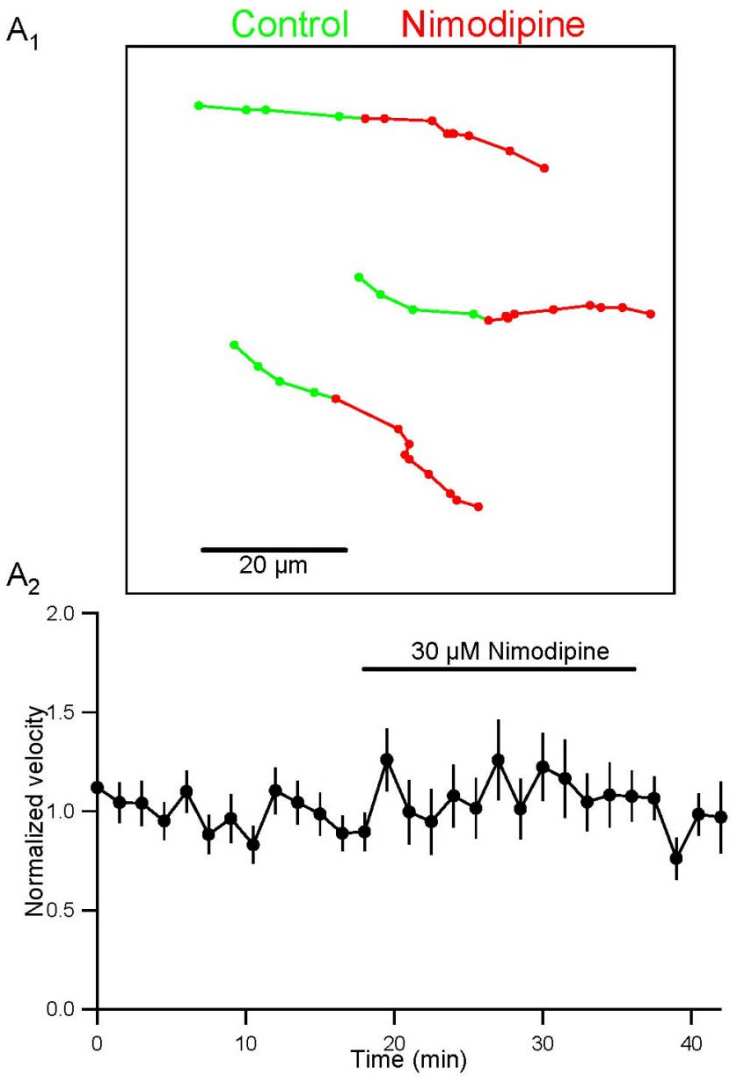

Figure 2-9: Effects of blocking Ca^{2+} transients on migration of NPCs.

A_1, Example celltracks of NPCs migrating under control conditions (green) and during exposure to nimodipine (red). A_2, Average time course of normalized migration with bath application of nimodipine (n=47 cells, 3 slices).

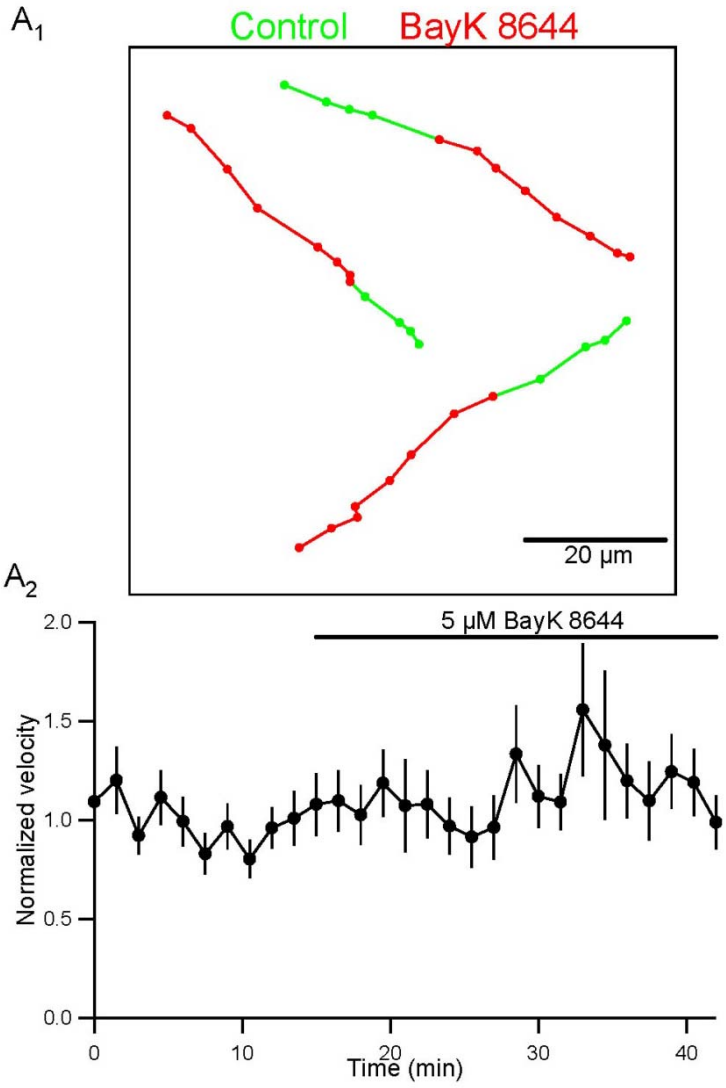

Figure 2-10: Effects of potentiating Ca^{2+} transients on migration of NPCs.

A_1, Example celltracks of NPCs migrating under control conditions (green) and during exposure to BayK (red). A_2, Average time course of normalized migration with bath application of BayK (n=47 cells, 4 slices).

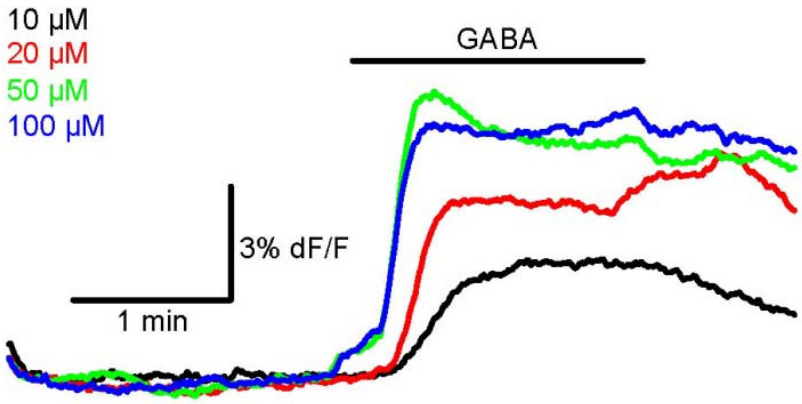

Figure 2-11: $GABA_AR$ activation leads to increased intracellular Ca^{2+} in NPCs.

Example wide-field delta F/F of NPCs loaded with Ca^{2+} indicator demonstrating graded and prolonged responses to different concentrations of GABA.

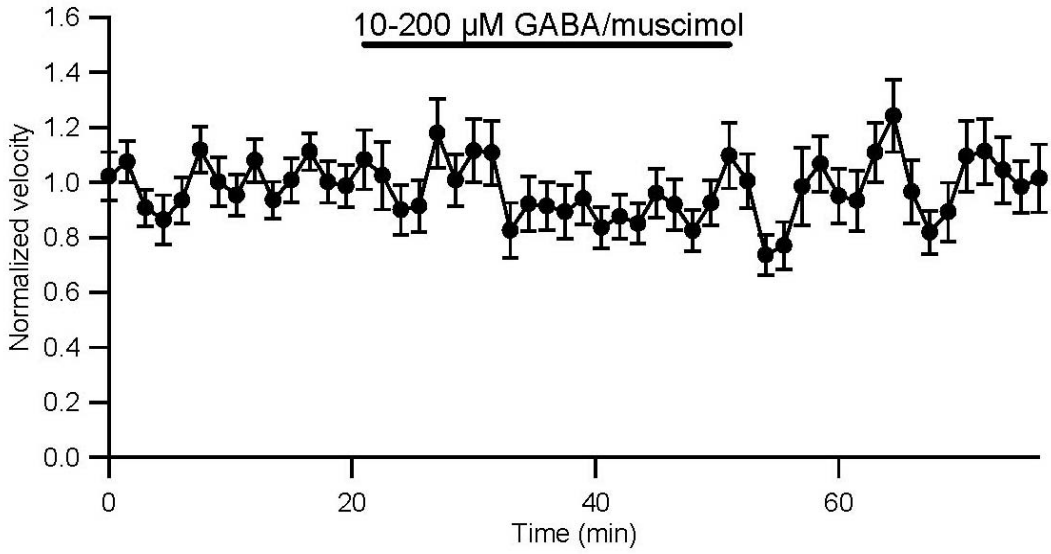

Figure 2-12: Effects of $GABA_AR$ activation on NPC migration.

Average time course of normalized migration with bath application of GABA or muscimol (n=79 cells, 8 slices).

CONCLUSIONS

Advances in technology allowing the visualization of newborn neurons have demonstrated that neurogenesis occurs in the olfactory system and hippocampus of adult mammals, including humans. NPCs migrate several millimeters from the lateral ventricle to the subependymal layer of the olfactory bulb and contribute to the continual turnover of local interneurons in that structure, granule and periglomerular cells (Lois and Alvarez-Buylla 1994). Recent reports have demonstrated that the extent of turnover is tightly regulated, affecting about one-third of periglomerular cells and only a small fraction of glomerular cells over a 9 month period (Ninkovic, Mori et al. 2007). About half of these cells survive in the bulb after one month (Petreanu and Alvarez-Buylla 2002), with changes in an animal's olfactory environment influencing the survival (Petreanu and Alvarez-Buylla 2002; Alonso, Viollet et al. 2006) and response (Magavi, Mitchell et al. 2005) of NPCs. Studies such as these indicate precise ongoing regulation of neurogenesis in the olfactory system, and suggest that NPCs are well-situated to exert a powerful influence on existing circuits. However, the functional role of NPCs, and how they govern signaling within the olfactory bulb, is still unclear.

A detailed comprehension of adult neurogenesis of the olfactory system will synthesize knowledge of NPC proliferation, fate specification, long- and short-term migration, synaptic targeting and integration, and finally circuit- and behavior-level consequences of network integration. Here, we have isolated one aspect of adult neurogenesis and have done experiments to measure whether intrinsic membrane

properties of NPCs are coupled to their short-term, or acute, migration. Specifically, we hypothesized that cell signaling events may govern basic navigational changes such as stopping, speeding-up, or turning. We studied NPC migration using 2-photon laser scanning microscopy to image and track the movement of fluorescently-labeled cell somata beneath the surface of olfactory bulb slices. All of our imaging experiments took place in the center of the SEL of the bulb, a region where the vast majority of cells express doublecortin, a selective marker of newborn migrating neurons. This system made possible rapid experimental manipulations that affected migrating NPCs via ionotropic receptors and ion channels expressed by the cells.

We considered that, since NPCs eventually receive glutamatergic synaptic inputs from olfactory receptor axons or from principle cells in the bulb, glutamate itself may play a role in the migration of new cells entering regions of mature bulb circuitry. First, we characterized glutamate receptors on NPCs by making whole-cell recordings from the cells and uncaging glutamate to observe directly current through glutamate receptors. We found that NPCs do not express the NMDA type of glutamate receptor. This is in contrast with postnatally migrating cerebellar granule cells, which do express NMDARs (Komuro and Rakic 1993).

NPCs of the olfactory bulb, however, do express AMPARs lacking the edited GluR2 subunit. As an expected consequence of the absence of this particular subunit, NPC AMPARs rectify current and are permeable to Ca^{2+}, as demonstrated by imaging NPC intracellular Ca^{2+} in response to glutamate application when AMPARs were isolated. Further, these Ca^{2+}-permeable AMPARs have single-channel conductance properties similar to value reported by others (Swanson, Kamboj et al. 1997).

Interestingly, mature granule cells of the bulb exhibit NMDA and non-rectifying AMPA currents (Isaacson and Strowbridge 1998), indicating that as NPCs mature they eventually change receptor expression patterns, and produce NMDARs and AMPARs containing the GluR2 subunit.

We observed that application of glutamate or AMPA caused a rapid, reversible inhibition of NPC migration velocity. This affect was blocked in slices maintained in NBQX, demonstrating a direct role for AMPARs. Furthermore, we observed that glutamate spillover from synapses in the granule cell layer could activate AMPARs on NPCs, as measured with imaging of intracellular Ca^{2+}, and that synaptically-evoked glutamate also affected migration of NPCs. Taken together, these data provide evidence that glutamate stores in the bulb can provide a signal to NPCs that reduces their migration velocity. Although we have restricted our experiments to looking at large-scale effects of glutamate on migration in the subependymal layer, it is possible that localized glutamate spillover from mature synapses plays a role in the targeting specificity of NPCs that have migrated into the established neural networks found in outer bulb layers.

How are AMPARs coupled to NPC migration? Because Ca^{2+} is a key mediator of many intracellular events and has been shown to play a role cell migration in other brain systems (Komuro and Rakic 1996), we considered AMPAR-mediated Ca^{2+} signaling to be the most likely way migration could be regulated in olfactory bulb NPCs. Other possible ways that AMPAR could couple to migration include non-Ca^{2+} ion flux through the receptors, or a conformational change of the AMPARs themselves. All of these possibilities, including Ca^{2+} signaling, could lead to downstream signaling events that are subject to experimental manipulation.

We exhaustively tested our primary hypothesis that AMPAR-mediated inhibition of migration occurs via a Ca^{2+}-dependent mechanism. To study this, we used Ca^{2+} imaging to develop conditions under which we could activate AMPARs and observe no detectable rise in NPC intracellular Ca^{2+}. Then, using the same conditions while measuring the migration of the cells, we tested the effects of AMPAR activation and saw no difference in the extent of reduction of NPC migration. There was little recovery of NPC migration, unlike what we observed using normal Ca^{2+} concentrations, suggesting that Ca^{2+} may still be an important signaling element for some aspects NPC motility. However, the present results discount Ca^{2+} as the primary mediator between AMPARs and the reduction of NPC migration.

Because Ca^{2+} is known to play a role in the postnatal migration of granule cells in the cerebellum (Komuro and Rakic 1992; Komuro and Rakic 1993; Komuro and Rakic 1996), we wished to explore further the unexpected lack of a requirement for Ca^{2+} in AMPAR-mediated inhibition of NPC migration. To do this, we loaded NPCs with Ca^{2+} indicator and measured their basal intracellular Ca^{2+} levels. Interestingly, we observed spontaneous elevations in intracellular Ca^{2+} in the cells. The frequency of these Ca^{2+} transients was unaffected by blocking Na^{+} channels, AMPARs, NMDARs, or $GABA_A$Rs in the slice, and they persisted following depletion of thapsigargin-sensitive intracellular Ca^{2+} stores. The frequency of the transients increased with membrane depolarization, suggesting a role for a voltage-sensitive Ca^{2+} channel in the generation of transients. Indeed, we found that dihydropyridines, which consist of selective agonists and antagonists for L-type VSCCs, strongly modulated spontaneous Ca^{2+} transient frequency and fast depolarization-evoked responses of NPCs. Consistent with these results, we

recorded Ca^{2+} currents with current-voltage relationships characteristic of high voltage-activated L-type VSCCs, and we also observed small, regenerative electrical events or 'spikes' carried by Ca^{2+} conductances. Thus, NPCs exhibit substantial Ca^{2+}-mediated signaling events even under resting conditions.

Blocking or potentiating spontaneous Ca^{2+} transients in NPCs did not alter their migration velocity. To test whether a sustained influx of Ca^{2+} (as distinct from a transient elevation in Ca^{2+}) would produce a measurable effect on migration, we took advantage of the fact that NPCs express $GABA_A$Rs that depolarize the cells when activated (Wang, Krueger et al. 2003). As demonstrated using Ca^{2+} imaging, $GABA_A$R activation leads to a sustained rise in NPC intracellular Ca^{2+}. However, neither GABA nor the $GABA_A$R agonist muscimol affected NPC migration velocity. These experiments provide further evidence that Ca^{2+} does not carry migration signals in NPCs.

Our results have highlighted numerous interesting and potentially important properties of NPCs and NPC migration in the adult olfactory bulb. We have shown that AMPAR activation on NPCs has a dramatic effect on their migration. While the mechanisms coupling receptors to cell motility are not yet completely understood, the ability of NPCs to "sense" glutamate presents an intriguing scenario. Perhaps NPCs stop or slow near circuitry that is sufficiently active to create glutamate spillover, and it is the high level of glutamatergic activity itself that signals the need for incorporation of inhibitory neurons.

Our experiments with AMPARs and migration also led to the surprising conclusion that, although NPCs AMPARs are permeable to Ca^{2+}, Ca^{2+} itself does not act as a signal for NPC migration velocity in the relatively rapid time course we have

presently examined. We have explored this lack of coupling between Ca^{2+} and migration from multiple experimental standpoints, using dihydropyridines to block or enhance spontaneous fluctuations in NPC intracellular Ca^{2+}, and using $GABA_AR$ agonists to depolarize NPCs and induce prolonged Ca^{2+} influx. None of our manipulations led to a change in NPC velocity, providing multiple lines of evidence that NPCs migrate independently of intracellular Ca^{2+} events. We suggest that Ca^{2+}-independent migration of NPCs may constitute another of their unique 'abilities', because migration that persists without being influence by intra- or extracellular events perturbing NPC Ca^{2+} levels could be more robust. Further, the Ca^{2+}-independence of migration permits intracellular Ca^{2+} signaling to play other roles in NPCs, including regulation of transcription and receptor expression events that, for instance, may underlie the changes observed in glutamate receptor type and subunit composition between NPCs and mature interneurons in the olfactory bulb.

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