

DIFFERENTIAL REQUIREMENTS FOR MITOCHONDRIA
DURING NEURONAL MIGRATION

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This thesis is dedicated to my parents, Dr. Rudolf Hendel and Dr. Catherine Lin-Hendel, and my husband, Christopher Dengler with love and gratitude.

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ABSTRACT

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During the development of the cortex neurons must migrate from their sites of birth to their final destinations. There are two main types of migration in the developing cortex, the radial migration of pyramidal neurons and the non-radial migration of cortical interneurons. There are limited studies directly comparing these two migration processes, and thus our understanding for perturbations that are selective to either non-radial versus radial migration is limited.

Understanding causes of disruption of interneuron migration specifically is clinically relevant due to several interneuron specific developmental diseases. There is a growing body of literature observing a link between interneuron disorders and mitochondrial dysfunction, and thus regulation of mitochondria and their functions are promising targets for identifying selective effects on interneuron development. Mitochondria are organelles serving multiple functions in cells, including energy production, calcium buffering, redox homeostasis, and regulation of cell death. Although mitochondria are known to play essential roles in maintaining neuronal health and function in the adult brain, the importance of mitochondria during neuronal development is poorly understood.

Using *in vitro* mouse brain explant and slice culture systems, we observed distinct localization patterns of mitochondria between interneurons and pyramidal neurons. Mitochondria in migrating interneurons move cyclically throughout the cell during the migration process while

remaining in front of the nucleus in migrating radial neurons. Fluorescence immunohistochemistry confirmed these localization patterns in embryonic mouse brains *in vivo*.

We then applied pharmacologic tools to disrupt mitochondrial oxidative phosphorylation and discovered that interneuron migration is profoundly sensitive to these disruptions compared to radially migrating pyramidal neurons. This was confirmed by examination of a genetic model of compromised oxidative phosphorylation (Ant1). Interneurons with compromised mitochondrial function exhibit decreased migration, increased changes in direction, increased trailing process length, changes in branching behavior, and shifts in centrosome positioning.

We then utilized a dominant negative form of Miro1, a protein involved in mitochondrial trafficking to alter mitochondrial trafficking behavior. We also observed increased direction changes and reduced ability of interneurons to invade the cortex. However, interneuron migration rate was unaffected. The changes in cell migration behavior suggest that mitochondria play a central role in maintaining interneuron cell polarity to allow for progressive interneuron migration during their long journey.

These data collectively support a model in which mitochondrial perturbations have a selective effect on interneuron migration. This work suggests that neuronal deficits occurring in mitochondrial disorders are not exclusively due to neuronal metabolic insufficiency in postnatal stages, but can also have developmental origins.

INTELLECTUAL CONTRIBUTION

The experiment in Chapter 2, figure 7B showing absence of Ant1 expression in mutant mice were done by Meagan McManus in the lab of Doug Wallace. The confirmation of normal radial migration in Chapter 2, Figure 10B were done by Erika Lin-Hendel and Ginam Cho in the laboratories of Stewart Anderson and Jeffrey Golden. The constructs utilized for Chapter 3 experiments, pCAG-IG-Miro1 and pCAG-IG-Miro1K208, were generated by Ginam Cho in the lab of Jeff Golden.

All other experiments in Chapter 2 and all of the experiments in Chapter 3 and 4 were done by Erika Lin-Hendel in the laboratories of Jeffrey Golden and Stewart Anderson.

Chapter 2 is published in the journal Cell Reports March 31, 2016 “Differential Mitochondrial Requirements for Radially and Non-radially Migrating Cortical Neurons: Implications for Mitochondrial Disorders.” and is available via open access here:

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At the time of submission data from Chapter 3 and Chapter 4 are unpublished results

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CHAPTER 1: GENERAL INTRODUCTION

Neurodevelopmental disorders (NDDs) are genetic or acquired diseases that result in childhood-onset of brain dysfunction. NDDs manifest with a broad spectrum of phenotypes. When severe enough, these disorders present with conditions that include epilepsy, intellectual disability (ID) and autism spectrum disorder (ASD). NDDs impose a significant financial and social toll, in part due to their early onset. Unfortunately, treatment options for these diseases are limited. Thus understanding the neurobiological basis of these disorders is paramount to identify new preventative and interventional therapies. Over the past decade, GWAS studies, and more recently, whole exome and genome studies have elucidated the genetic basis in a number of clinical patients. From this data, a fascinating common thread is emerging implicating metabolic dysfunction in many of these disorders. As mitochondria are crucial to cellular metabolism, there is great interest in understanding the role these organelles play in the development of the nervous system.

Epilepsy, ASD, and ID are considered to be disorders of the cerebral cortex, and of cerebral connectivity. The proper formation of the cerebral cortex is dependent on several processes, consisting of proliferation, differentiation, migration, axon and dendrite formation, synaptogenesis, and pruning and stabilization of neuronal circuits. The role of mitochondria has been explored in many of these processes, but our understanding of the importance of mitochondria during neuronal migration is sparse. Deficits in neuronal migration, especially interneurons, are postulated to play a role in many NDDs. Thus, I have focused my thesis work on investigating the role of mitochondria during the process of neuronal migration in cerebral cortical development, and how this role informs our understanding of the consequences of mitochondrial dysfunction in disease.

Development of the Cerebral Cortex

The mammalian neocortex processes environmental stimuli and information into higher cognitive behaviors such as planning and learning. It is a complex structure with six horizontally organized layers made up of networks of neurons. The cortex consists of two basic cell types: excitatory glutamatergic pyramidal projection neurons, and inhibitory GABAergic interneurons. The circuits established by these two cell types maintain a delicate balance between excitatory input, synchronization, and inhibitory tone that are essential for normal cortical function (Freund and Katona 2007, Le Roux, Amar et al. 2008, Xue, Atallah et al. 2014). The formation of these circuits depends on a series of complex neurodevelopmental processes, involving neurogenesis, differentiation, neuronal migration, axon targeting, synaptogenesis, and coordinated apoptosis.

During the early stages of development, the forebrain emerges from cranial bulges of the embryonic neural tube. The telencephalic region is specified during the rapid proliferation of neuroepithelial cells (NECs) lining the neural tube with the expression of *Foxg1* (Tao and Lai 1992, Shimamura and Rubenstein 1997). This region will generate the future neocortex, hippocampus, basal ganglia, and olfactory bulbs. At this point, the developing nervous system consists of two layers. The inner layer consists of a highly proliferative pseudostratified ventricular zone (VZ), and the external or superficial layer is a relatively cell-sparse, process-rich region known as the marginal zone (MZ)(Super, Soriano et al. 1998). There are also distinct developmental regions of the early nervous system; a dorsal pallium which develops into the cortex and hippocampus, and the ventral subpallium, where the future basal ganglia will reside.

NECs divide, generating a first wave of pioneer projection neurons out to form the basal preplate (Rickmann and Wolff 1981). Subsequently, during the splitting of the preplate, the earliest born layer 6 neurons migrate outward to establish the early subplate, or future cortical

plate (CP) (Del Rio, Martinez et al. 2000). During this process, NECs lose epithelial characteristics and transition into radial glial cells (RGCs), the precursor of excitatory projection neurons (PNs) (for review, see (Gotz and Huttner 2005, Franco and Muller 2013)). These RGCs reside in the VZ adjacent to the ventricles and send long projections towards the basal pial surface. In addition to self-renewing and producing PNs, RGCs produce short neural precursors (SNPs) and intermediate progenitor cells (IPCs). SNPs also reside in the VZ (Gal, Morozov et al. 2006), while IPCs migrate outwards and create a distinct region of proliferation referred to as the sub-ventricular zone (SVZ)(Haubensak, Attardo et al. 2004, Miyata, Kawaguchi et al. 2004, Noctor, Martinez-Cerdeno et al. 2004). During this process, a cell-sparse zone, known as the intermediate zone (IZ) can be recognized separating the CP from the VZ/SVZ, populated by afferent and efferent axons from pioneer neurons (Denaxa, Chan et al. 2001). As development progresses, the dorsal pallium and ventral subpallium become markedly distinct from one another morphologically. The subpallium develops dense protuberances that are identifiable at E11.5 known as the lateral, medial, and caudal ganglionic eminences. These distinct pallial and subpallial regions serve as the sources of different neuronal cell type populations. Pyramidal neurons are born in the proliferative zones in the VZ lining the dorsal pallium (Malatesta, Hartfuss et al. 2000, Dehay and Kennedy 2007). In contrast, inhibitory interneurons (INs) of the forebrain are born in the ventral pallium, in the medial, lateral, and caudal ganglionic eminences in addition to the preoptic area (MGE, LGE, CGE) (Anderson, Eisenstat et al. 1997, Butt, Fuccillo et al. 2005). These morphological regions have been further characterized by the expression of transcription factors that contribute to fate determination of the variety of IN subtypes found in the forebrain (Sussel, Marin et al. 1999, Stoykova, Treichel et al. 2000, Flames, Pla et al. 2007).

The birth of neuronal populations in specific regions of the developing brain presents a unique challenge to the developing embryo to create heterogeneous circuits throughout the cortex. Each of these populations must migrate from their origins from these proliferative zones to properly incorporate into their final positions in the brain. There are three primary types of neuronal migration that have been described in the mammalian forebrain; the radial migration of cortical pyramidal cells (Nadarajah and Parnavelas 2002, Noctor, Martinez-Cerdeno et al. 2004), the non-radial or tangential migration of cortical interneurons (Anderson, Eisenstat et al. 1997, Wonders and Anderson 2005, Yokota, Gashghaei et al. 2007), and the chain migration of interneurons migrating to populate the olfactory bulbs (Lois, Garcia-Verdugo et al. 1996, Pencea and Luskin 2003). The migration of pyramidal neurons (PNs) and cortical interneurons (INs) occur primarily during neurodevelopment. Chain migration, also referred to as the rostral migratory stream (RMS), has been observed as early as E15 and continues in the adult brain (Lois, Garcia-Verdugo et al. 1996, Pencea and Luskin 2003). Although these modes of migration each have unique characteristics, the distinct regulation of each mode from one another has not been well studied.

PNs are produced from dividing RGCs in the VZ, or from intermediate progenitor cells (IPCs) located in the SVZ of the dorsal pallium (Tabata and Nakajima 2003, Noctor, Martinez-Cerdeno et al. 2004). The RG cells extend processes towards the pial surface and subsequently, the nucleus moves cyclically within the cell soma between the borders of the VZ, dividing at the ventricular border to produce IPCs or postmitotic PNs (Taverna and Huttner 2010, Wu, Gu et al. 2014). When the neocortex is thin, PN cells can exhibit a migratory behavior known as somal translocation (Nadarajah, Brunstrom et al. 2001, Nadarajah and Parnavelas 2002), in which the the cell soma of the neuron moves rapidly towards the pial surface through shortening of the pial process. As the cortex thickens, post-mitotic PNs (likely both from RG and IPG divisions)

undergo a brief multipolar stage within the intermediate zone, and exhibit lateral movement within the VZ and SVZ and IZ (Tabata and Nakajima 2003, Jossin and Cooper 2011). As the PNs prepare to invade the cortical plate, they shift to a bipolar morphology, migrating radially utilizing the RG processes as supportive scaffolding to reach their final destinations and at times utilizing somal translocation at the end of their journey (Nadarajah and Parnavelas 2002). After pre-plate splitting creates layer 6, the formation of the gross laminated structure of the cortex forms in an inside-out fashion; subsequent waves of cells migrate past deeper layers to establish the more superficial cortical layers (Figure 1) (Berry and Rogers 1965, Frantz and McConnell 1996, Kandel ER 2000).

Inhibitory interneurons of the cortex are born in the germinal zones of the MGE and CGE and migrate long distances in a process called tangential migration (Anderson, Eisenstat et al. 1997, Wichterle, Turnbull et al. 2001). Like in the dorsal pallium, interneurons born are born in the proliferative VZ/SVZ starting as early as 9.5 (Sussel, Marin et al. 1999, Butt, Sousa et al. 2008). Although the dynamics and behaviors of intermediate progenitors in the ventral pallium are not as well studied as those in the cortex, it is thought that there are similarities in which RGCs within the VZ produce IPCs in the SVZ, and these progenitor pools give rise to postmitotic interneurons (Ross 2011). After their birth, newborn interneurons exit these proliferative zones responding to local chemorepulsive cues and motogenic factors (Faux, Rakic et al. 2012, Marin 2013). Cortical interneurons must then navigate dorsally through the ventral pallium to reach the cortex (Figure 1). This process is dependent on the creation of permissive corridors with a combination of repellent cues to prevent migration into the striatum, attractive cues directing cells towards the cortex, and motogenic factors that stimulate migration (for review, (Guo and Anton 2014)). At E12.5, these migrating interneurons begin to invade the cortex in streams from the cortical notch (Marin and Rubenstein 2003). INs migrate across the processes of the radial glia,

moving freely through the cellular milieu of the cortex in a dorsomedial fashion, migrating towards the dorsal curve of the cortex, and into the future hippocampus of the mouse. During this time, INs exhibit highly dynamic migratory behavior; they are able to migrate between streams and reverse direction. Whole brain explant cultures have indicated interneurons utilize this random walk behavior, dispersing them throughout the cortex dorso-caudally. (Figure 1) (Ang, Haydar et al. 2003, Metin, Baudoin et al. 2006, Tanaka, Maekawa et al. 2006, Tanaka, Yanagida et al. 2009). INs also exhibit a radially-directed migration phase, in which they leave their tangentially migrating streams, turn towards the ventricular surface, contact the ventricles, and migrate radially outwards into the cortical plate (Figure 1) (Nadarajah, Alifragis et al. 2003). It is theorized that INs utilize this radially directed migration as a final step reach their positions in the cortical plate (Nadarajah, Alifragis et al. 2002). Although it has been thought that INs migrate independently of RGCs, live imaging studies simultaneously imaging migrating INs and RGCs suggest that interactions with RGCs regulate this radially directed stage of IN migration (Yokota, Gashghaei et al. 2007).

During migration, INs exhibit dynamic changes in cell morphology. Migrating INs extend a leading process in the direction of migration that extends, retracts, and branches (Lysko, Putt et al. 2011, Lysko, Putt et al. 2014). The centrosome, coupled with the Golgi apparatus moves forward into a thickening within the leading process (Bellion, Baudoin et al. 2005). Nucleokinesis follows through a combination of molecular motors pulling on a microtubule cage surrounding the nucleus, and actinomyosin contractions from the rear of the cell (Bellion, Baudoin et al. 2005, Martini and Valdeolmillos 2010).

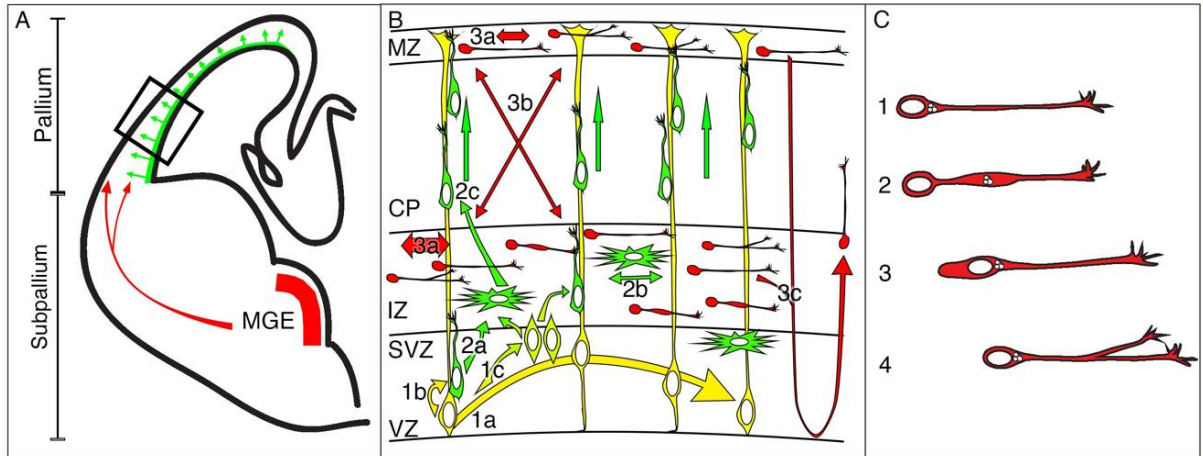


Figure 1. **Development of the cortex**

(A) Projection neurons (PNs) are born in the ventricular zone (VZ) of the dorsal pallium (green line), and migrate radially to their cortical positions in an inside-out fashion (green arrows). Cortical interneurons (INs) are born in the VZ of the ventral pallium in the medial ganglionic eminence (MGE; red line). They then migrate non-radially towards the cortex, invading the cortex in two streams (red arrows). (B) The embryonic cortex is divided into the proliferative VZ and subventricular zone (SVZ). Radial glia cells (RGCs, yellow) undergo cell somal translocations between the ventricular surface and SVZ border (1a), and give rise to PNs (green) (1b) and intermediate progenitor cells (IPCs, light green) that reside in the SVZ. (1c). Both PNs and IPCs can become multipolar cells (2a) that move throughout the SVZ and intermediate zone (IZ), (2b) before transitioning to bipolar radially migrating PNs that invade the cortical plate (CP), utilizing RGCs as scaffolding to guide their migration. Migrating INs invade the cortex in two streams in the IZ, and MZ. These cells migrate tangentially to the RCG scaffolding (3a), and can move across the CP between streams (3b). Additionally, these INs will move toward the ventricles, and undergo a radially directed migration to reach their final destinations in the cortex (3c). (C) Interneurons undergo morphological changes throughout their migration. Centrosomes are located in close association with the nucleus in the cell soma (1). The centrosomes move

forward into the leading process, creating a thickening (2). During nucleokinesis, the forward movement of the nucleus and cell soma toward the centrosome results in the formation of a trailing process (3). The trailing process retracts, completing the migration cycle (4).

Disorders of neuronal migration

Genetic mutations that interfere with the radial migration of PNs often result in gross abnormal cortical structure. The most severe of these disorders, Lissencephaly, causes an absence of the folds, or gyri of the brain, resulting in a smooth brain. The majority of cases of this disorder are caused by defects in *Lis1* and *DCX*, genes that regulate microtubule dynamics (Pilz, Matsumoto et al. 1998).

Defects in interneurons' migration can result in the reduction or absence of interneurons within the cortex. Because interneurons comprise approximately 20% percent of the neurons in the cortex, loss or reduction of these cells does not impact gross cortical structure. However, the loss of these cells has significant impacts on cortical function. Reduction, absence, improper synapse formation, or abnormal behaviors of interneurons are linked to various neurological disorders, termed interneuronopathies (Kato and Dobyns 2005, Rivero, Selten et al. 2015, Marsh, Nasrallah et al. 2016).

Mitochondrial functions within the cell

Mitochondria are complex self-replicating, double membrane organelles found within the eukaryotic cells. Mitochondria are known as the “powerhouse” of the cell (Philip Siekevitz 1957), generating significant amounts of ATP. In addition to their role in energy production, mitochondria play key roles in the regulation of redox homeostasis, steroid synthesis, calcium buffering, and apoptosis (McBride, Neuspiel et al. 2006).

The inner and outer mitochondrial membranes create distinct regions into which mitochondrial functions can be separated. A prime example is the generation of ATP by mitochondrial oxidative phosphorylation (OXPHOS). Glucose is imported through the cell membrane via glucose transporters. Cells generate 2 ATP via glycolysis in the cytosol, converting

glucose to two molecules of pyruvate (Figure 2). Subsequently, pyruvate diffuses through the outer mitochondrial membrane where the mitochondrial pyruvate carriers (MPC1/2) transport it into the inner mitochondrial matrix (Bricker, Taylor et al. 2012, Herzig, Raemy et al. 2012). Within the inner mitochondrial matrix, pyruvate is converted into Acetyl-CoA, and these two molecules enter the TCA cycle, producing reducing equivalents of NADH, and FADH₂. The electron transport chain, consisting of various protein complexes in the inner mitochondrial membrane, harnesses the oxidation of NADH and FADH₂ to pump hydrogen ions into the intermembrane space (for review, see (Saraste 1999)). This creates an electrochemical gradient with which ATP synthase utilizes to generate ATP. This ATP must be transported out of the matrix via the adenine nucleotide transporter (Ant) into the intermembrane space, to allow ATP generated from OXPHOS to be released into the cytosol for use by the cell (Levy, Chen et al. 2000, Klingenberg 2008). ATP diffusion through the cytosol is thought to be insufficient to meet high local energetic demands of the cell (Clegg 1984). This is overcome by energy shuttling pathways, such as creatine kinase/creatine phosphate and adenylate kinase (Ames 2000), clustering of glycolytic enzymes (Beeckmans, Van Driessche et al. 1990), and localization of mitochondria to areas of need (Zinsmaier, Babic et al. 2009).

Although mitochondria are central to various cellular functions the differences in mitochondrial biology, such as genetic regulation, proteomic expression, and localization behavior between different cell types and developmental time points, has not been well studied. Proteomic differences of mitochondrial enzymes have been detected between tissue types, (Johnson, Harris et al. 2007) establishing evidence for the heterogeneity of mitochondria within vertebrates. Recent studies examining the mitochondrial proteome during brain development have revealed drastic changes in mitochondrial protein expression, suggesting changes in

mitochondrial function and behavior may be a strategy throughout development for differentially regulating aspects of cell physiology and behavior (Nakai, Taniuchi et al. 2000).

Mitochondria in Neurons

Neurons are considered to be one of the body's most energetically demanding cell types: the CNS consumes 20% of the body's resting metabolism (Silver and Erecinska 1998). As previously discussed, cells can generate energy via glycolysis or oxidative phosphorylation. *In vitro* comparison of cytosolic vs. mitochondrial ATP production has suggested that embryonic neurons have mostly glycolytic metabolism, and postnatal neurons utilize oxidative phosphorylation (Surin, Khiroug et al. 2012). The main failing of these studies is the generalization of neuronal cell types; only hippocampal pyramidal cultures were assessed. Considering the substantial differences between PNs and INs, it is possible that their metabolic profiles are distinct.

Previous histological studies noted mitochondrial localization in particular areas of neurons, including dendritic shafts and spines (Cameron, Kaliszewski et al. 1991, Popov, Medvedev et al. 2005), Nodes of Ranvier (Berthold, Fabricius et al. 1993, Fabricius, Berthold et al. 1993), axon growth cones (Hollenbeck and Saxton 2005), and synapses (Palay 1956), suggesting that mitochondria are targeted to particular regions of high energetic demand and are likely important for neurologic function.

Neuronal mitochondria are incredibly dynamic structures, with both static and motile populations (Hollenbeck and Saxton 2005, Zinsmaier, Babic et al. 2009) undergoing fusion and fission (Alexander, Votruba et al. 2000, Lee, Sterky et al. 2012, Berthet, Margolis et al. 2014). Kinesin and dynein allow for the anterograde and retrograde movement along the microtubules while various proteins anchored to the mitochondrial membrane regulate their attachment to these

molecular proteins (Hirokawa and Takemura 2005, Pilling, Horiuchi et al. 2006). Some of these adaptor proteins, such as syntabulin, have dual functions in mediating vesicular transport in addition to trafficking mitochondria (Cai, Gerwin et al. 2005). The adaptor protein Milton facilitates the interaction between the mitochondrially bound protein, mitochondrial RhoGTPase (Miro) and kinesin (Liu and Hajnoczky 2009, Chen and Sheng 2013). In response to elevated Ca^{2+} , it is hypothesized that Miro releases kinesin, and binds syntaphilin (SNPH), a protein responsible for anchoring axonal mitochondria (Kang, Tian et al. 2008). This system allows for regulation of mitochondrial localization during synaptic activity (Yi, Weaver et al. 2004).

Fusion and fission of mitochondria are regulation points for mitochondrial morphology, adapting to cellular energetic needs, as well as maintaining mitochondrial health (reviewed in (Bertholet, Delerue et al. 2016)). Mitochondrial fusion is regulated by Mitofusin (Mfn) 1 and 2, which fuse the outer mitochondrial membrane, and Optic atrophy 1 (Opa1), which fuses the inner mitochondrial membrane. Dynamin-related protein 1 (Drp1) and Ganglioside Induced Differentiation Associated Protein 1 (GDAP1) are central regulators of mitochondrial fission (Smirnova, Shurland et al. 1998, Niemann, Huber et al. 2014). Posttranslational modification of Drp1 by phosphorylation (Cribbs and Strack 2007), ubiquitination (Karbowski, Neutzner et al. 2007), and sumoylation (Harder, Zunino et al. 2004) regulates its activity and ability to bind to mitochondria. These proteins have been implicated in neurodegenerative diseases (DuBoff, Gotz et al. 2012, Bertholet, Millet et al. 2013, Niemann, Huber et al. 2014), and their role in neuronal development has only recently been explored (Fang, Yan et al. 2016).

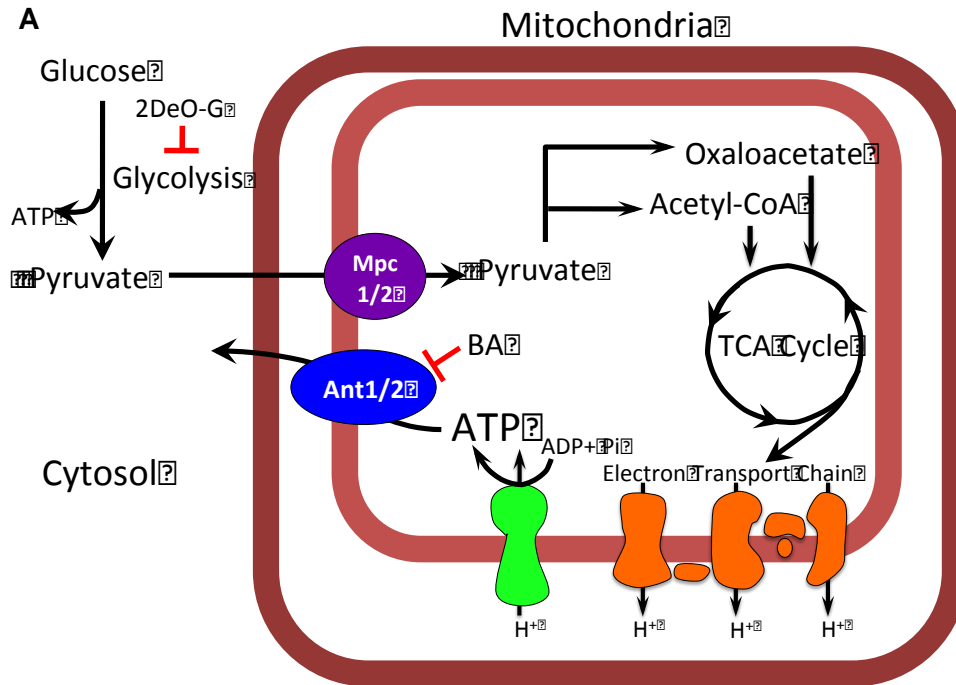


Figure 2. Mitochondrial Oxidative Phosphorylation

(A) Glucose is taken up by the cell, where it can be utilized via glycolysis in a non-reversible process to generate 2ATP and pyruvate. Glycolysis can be inhibited by 2-deoxy-glucose (2DeO-G). Pyruvate is transported into the inner mitochondrial matrix (IMM) via mitochondrial pyruvate carriers (Mpc) 1 and 2. Once within the IMM, pyruvate is converted to Acetyl-CoA, and utilized in the TCA cycle to produce reducing equivalents of NADH and FADH₂. The electron transport chain in the inner mitochondrial membrane utilizes NADH and FADH₂ from the TCA cycle to pump hydrogen ions out of the IMM, resulting in an electrochemical gradient. ATP synthase utilizes this gradient to synthesize ATP. ATP must be transported out of the IMM via adenine nucleotide transporters (Ant) 1 and 2, and diffuse through the outer mitochondrial membrane to the cytosol for utilization by the cell. Ant1/2 are inhibited by Bongkreic Acid (BA).

Mitochondrial disorders in disease

Mitochondrial diseases (MDs) are considered the most common of the inherited metabolic disorders (Schaefer, Taylor et al. 2004). Diagnosis of mitochondrial disorders can be a clinical challenge, as there is much variability in symptomatology; MDs can be single or multisystemic, and can vary in severity and age of onset. One reason for the complicated nature of mitochondrial diseases is that they contain their own mitochondrial DNA (mtDNA). In contrast to the two copies of nuclear DNA, cells contain many more copies of mtDNA, which undergo constant replication. This makes mtDNA more susceptible to generating mutations (Chinnery and Samuels 1999). It is common for individuals with pathogenic mtDNA mutations to have heteroplasmy; a mixture of wild-type and mutated mtDNA. The proportion of wild-type versus mutated mtDNA can vary between patients, organ systems, and even cell types, and have effects on pathogenesis (Chinnery and Schon 2003).

MDs tend to cause clinical manifestations in highly energetic organs, such as brain and muscle. Traditionally, mitochondrial diseases have been studied in the context of the adult nervous system. Mutations in mitochondrial proteins are found in many neurodegenerative disorders, such as Charcot-Marie-Tooth (CMT) subtype 2A, amyotrophic lateral sclerosis (ALS), Parkinson's, Huntington's, Dominant Optic Atrophy, and Alzheimer's disease (Calkins, Manczak et al. 2011, Manczak and Reddy 2012, Chaturvedi and Flint Beal 2013). Although mutated genes in these disorders have varied impacts on ROS regulation, mitophagy (the process by which cells remove damaged mitochondria), OXPHOS and calcium homeostasis, it is suggested that these pathways converge in deficient cellular respiration leading to neuronal loss.

In recent years, interest in mitochondrial dysfunction has expanded from neurodegeneration to include diseases of neuronal dysfunction. Patients with mutations in

mtDNA or other mitochondrial proteins often present with epilepsy; a subset of which are characterized as neonatal or early onset (Khurana, Valencia et al. 2013). Although the mechanism by which mitochondrial mutations cause epilepsy are unknown, it has been hypothesized that increased oxidative stress and energetic compromise disrupt Ca^{2+} hemostasis, modulating synaptic activity and transmission (Chang and Yu 2010). Conversely, epilepsy has also been found to precede mitochondrial dysfunction in clinical and animal models of epilepsy (Kudin, Kudina et al. 2002, Chuang, Chang et al. 2004, Khurana, Valencia et al. 2013). It is thought that seizure activity can down-regulate the expression of OXPHOS related proteins, particularly complex I, and the subsequent energetic deficiencies increase neuronal death, contributing to further pathogenesis (Kunz, Kudin et al. 2000, Sleven, Gibbs et al. 2006, Chen, Chang et al. 2010, Folbergrova, Jesina et al. 2010).

The first evidence of that mitochondrial mutations could result in autism was published in 1999 (Sue, Bruno et al. 1999). Since then, multiple ASD patients have been found to harbor mtDNA mutations and mutations in somatically encoded mitochondrial DNA (reviewed in (Legido, Jethva et al. 2013)). Metabolic abnormalities indicative of mitochondrial dysfunction have also been described in patients, including elevation of lactate, pyruvate, and alanine in blood, cerebrospinal fluid, or brain tissues, and electron transport chain abnormalities in peripheral tissues (Legido, Jethva et al. 2013). There is not yet clear evidence if mitochondrial dysfunction contributes to the pathogenesis of ASD, or if it is an epiphenomenon (Rossignol and Frye 2012). Most hypotheses involving mitochondrial dysfunction and autism suggest that deficits in energy can result in post-natal malfunctioning of neuronal circuits, or that mitochondrial dysfunction can result in immune-mediated deficits resulting in damage to the developing or postnatal brain (Legido, Jethva et al. 2013).

Mitochondria in cell migration

Migration likely places unique metabolic demands on a cell. In non-neural migratory cells, mitochondrial function and localization are essential for normal migration. Upon activation and subsequent chemotaxis, leukocyte mitochondria are observed to mobilize to the posterior uropod (Campello, Lacalle et al. 2006). This process requires a balance between mitochondrial fusion and fission. The inability of mitochondria to target to this region abrogates chemotaxis. Additionally, ATP provided by mitochondrial oxidative phosphorylation is required to phosphorylate myosin II (Campello, Lacalle et al. 2006). Interestingly, during the migration of endothelial cells (EC), PFKFB, a glycolytic activator, localizes to the actin network for lamellipodia. This production of glycolytic ATP is required for EC migration, while oxidative phosphorylation is not required (De Bock, Georgiadou et al. 2013), suggesting that the energetics of cell migration vary depending on cell type.

Abnormal mitochondria have been described in genetic mutants with defects in radial migration. *Lis1* and *DCX* cause lissencephaly, and are known to cause radial and non-radial migration defects. These two factors associate with the perinuclear microtubule cage region, and facilitate nucleus-centrosome coupling during migration (Tanaka, Serneo et al. 2004). *Lis1* regulates cytoplasmic dynein function, resulting in clustering of dynein around the centrosomes in MEF cells (Yamada, Toba et al. 2008, Yamada, Yoshida et al. 2009). Subsequently, mitochondria and β -COP-positive vesicles have a perinuclear localization rather than being widely distributed. However, it is unclear what the contribution to defects in migration mitochondria may have in this model, or if the mislocalization characterized in the MEF cells is present in PN or INs during migration. In *DCX* mutants, mitochondria have abnormal morphology in P0 hippocampal pyramidal cells (Khalaf-Nazzal, Bruel-Jungerman et al. 2013),

but further examination of mitochondrial pathologies during earlier developmental stages, and assessment of mitochondrial function and localization is lacking.

In one model of abnormal radial migration, mitochondrial localization was assessed. Tau1 is a microtubule-associated protein implicated in Alzheimer's, neurodegenerative disorders and ID. (Weingarten, Lockwood et al. 1975, Shaw-Smith, Pittman et al. 2006, Sapir, Frotscher et al. 2012). Reduction of Tau in PNs reduces radial migration, and causes thinning and malformation of migrating PN leading processes (Sapir, Frotscher et al. 2012). In these migrating PNs, mitochondrial mobility into the leading edge of PNs is abrogated, and their processes became vacuolated and abnormal in shape. Although each of these disease models suggests that mitochondria may have a role in radial migration, each of these proteins has additional functions in microtubule dynamics. Thus, it is unclear if these effects on neuronal migration are due to effects on microtubule dynamics, or mitochondrial behavior.

Mitochondria-localized glutamic acid-rich protein (MGARP), was recently characterized as a negative regulator of radial migration (Jia, Liang et al. 2014). Unlike Lis1 and DCX, this protein is localized specifically to mitochondria and is typically lowly expressed in neurons, is significantly upregulated during hypoxia (Li, Lim et al. 2009). Overexpression, or knockdown of this protein in radially migrating neurons using *in utero* electroporation impedes normal migration and interferes with the development of neuronal processes (Jia, Liang et al. 2014). At P0, and in cultured cells, mitochondria displayed rounded morphologies, reduced localization in processes and reduction in mitochondrial numbers. In cultured embryonic neurons, mitochondria also displayed reduced motility within axons. Although this is a more promising indication that regulation of mitochondria is important for neuronal migration, it is unclear how these changes in MGARP levels impact mitochondrial localization during the migration, and if changes in MGARP can impact IN migration. Currently, the function of MGARP is still unclear. It has been

suggested that MGARP is involved in steroidogenesis (Matsumoto, Minegishi et al. 2009), and can impact mitochondrial abundance in addition to its morphology, but it is unknown if additional aspects of mitochondrial function are compromised by MGARP manipulations.

In interneurons, mitochondria have been described in particular locations in migrating interneurons, but not studied in depth. Schaar et. al. utilized presence of mitochondria to assist in identification of the trailing process in electron microscopy preparations of migrating INs *in vitro* (Schaar and McConnell 2005). Additionally, mitochondria have been observed paired with centrosomes and Golgi apparatus within the thickening of the leading process during the forward movement of centrosomes (Bellion, Baudoin et al. 2005). These observations suggest mitochondria may cluster within certain regions of interneurons during the migration cycle, but analysis of mitochondrial localization within migration INs has not been done.

To address this knowledge gap, we, therefore, utilized time-lapse imaging of live cells to observe mitochondrial behavior in actively migrating PNs and INs. We found distinct mitochondrial localization patterns in PNs compared to INs. In PNs, the majority of mitochondria maintain their localization in anterior of the nucleus. In contrast, mitochondria in migrating INs cycle throughout the cell, moving from anterior to the nucleus, forward into the cytoplasmic bleb, and moving back the trailing process during nucleokinesis.

To investigate if there are differential requirements for mitochondrial function in these two migrating populations, we assayed the effect of pharmacological inhibition of mitochondrial oxidative phosphorylation in migrating interneurons. Glycolysis alone is insufficient for IN migration, and INs in which OXPHOS is inhibited, INs exhibit increases in directional changes and slowing of migration. In slice culture, pharmacological inhibition of oxidative phosphorylation reduces IN migration without impacting that of PNs.

We saw the same impact in Ant1 mutants, where the translocation of ATP generated from OXPHOS into the cytosol is compromised. Mutants displayed reduced INs invading the cortex, and alterations in migratory behavior including reduced migration speed and decreased progressive migration, while radial migration was unaffected. Interestingly, Ant1 mutant INs centrosomes were mislocalized, suggesting that centrosome location and IN polarity are sensitive to metabolic perturbations.

We then attempted to alter mitochondrial trafficking behavior by expressing a dominant negative form of Miro1, a protein involved in mitochondrial trafficking during IN and PN migration. Although we did not see changes in IN migration rate, we observed a consistent phenotype of increased direction changes which subsequently reduced the ability of interneurons to invade the cortex. These consistent changes in cell migration behavior suggest that mitochondria are required for the regulation of IN polarity to allow for progressive interneuron migration.

These data collectively support a model of differential requirements for mitochondria between interneurons and pyramidal neurons during migration, and suggests a potential pathway for selective disruption of interneurons during early fetal development.

CHAPTER 2 - DIFFERENTIAL MITOCHONDRIAL REQUIREMENTS FOR RADIALY AND NON-RADIALY MIGRATING CORTICAL NEURONS: IMPLICATIONS FOR MITOCHONDRIAL DISORDERS

Introduction

Mitochondrial diseases (MDs) are the most common inherited metabolic disorder, with an estimated prevalence of 1:5000 (Schaefer, Taylor et al. 2004). Although MDs consist of a spectrum of disorders that can involve single or multisystem presentations, neurological symptoms are common clinical characteristics. In recent years, clinical, genetic, and biochemical studies have revealed an emerging link between mitochondrial dysfunction and neurodevelopmental disorders, including intellectual disability (ID) (Kaplan, Cao et al. 2012, Valenti, de Bari et al. 2014) childhood epilepsy (El Sabbagh, Lebre et al. 2010, Chevallier, Von Allmen et al. 2014) and autism spectrum disorder (ASD) (Giulivi, Zhang et al. 2010, Rossignol and Frye 2012). Interestingly, these conditions have also been associated with interneuron dysfunction (Marin 2012, Olivetti and Noebels 2012). The correlation between MDs and childhood neurological disorders raises the question as to whether interneuron development is particularly dependent on mitochondrial function.

Recent studies have elucidated roles for mitochondria in multiple aspects of neurodevelopment including neuronal differentiation (Wang, Ye et al. 2014), process outgrowth (Cheng, Wan et al. 2012, Kimura and Murakami 2014), and synaptogenesis (Bertholet, Millet et al. 2013). Most of these studies have utilized glutamatergic hippocampal neurons as a model, leaving the contribution of mitochondria to interneuron development relatively unexplored. Since interneurons are thought to be a key factor in the pathogenesis of epilepsy and ASD, we were particularly interested in examining mitochondrial behavior and function during developmental processes for which there are distinct features between glutamatergic projection neurons (PN) and

GABAergic interneurons (IN) in the cortex. During developing PN and IN are derived from distinct locations and take different migration routes: PNs migrate along radial glial fibers from their origin in the dorsal ventricular zone (VZ) and maintain a relatively stable morphology oriented towards the pial surface (Noctor, Martinez-Cerdeno et al. 2004). In contrast, cortical INs take a circuitous path from their origin in the subcortical ganglionic eminences. Along the way, migrating INs frequently pause, change direction, and exhibit extensive branching dynamics of the leading process (Polleux, Whitford et al. 2002, Bellion, Baudoin et al. 2005, Lysko, Putt et al. 2011, Lysko, Putt et al. 2014).

Given the greater distance and dynamic nature of IN migration, we reasoned that it requires more energy than does the migration of PNs. First, we found that the mitochondria within INs have a highly dynamic localization pattern during non-radial migration. In contrast, mitochondria remain more consistently localized in radially migrating PNs. Second, we found migrating interneurons to be exquisitely sensitive to agents that block the utilization of ATP generated through oxidative phosphorylation. Remarkably, glycolysis alone is insufficient for normal IN migration but is able to support the radial migration of PNs. Moreover, the genetic disruption of mitochondrial oxidative phosphorylation (OXPHOS) in mice lacking *Ant1* was associated with dramatic alterations of IN migratory morphology and behavior, including mispositioning of the centrosomes. Conversely, *Ant1*^{-/-} PNs appeared normal in our migration assays. These data suggest that interneuron polarity during migration is particularly sensitive to metabolic disruptions and that OXPHOS is required for normal migration of INs but not PNs. Our results also imply that the symptomatic manifestations of mitochondrial dysfunction and related conditions, including hypoxic injury, on cerebral cortical function may be secondary to their selective impact on cortical interneuron migration.

Results

Mitochondria are highly dynamic during interneuron migration

To examine the role of mitochondria in non-radial versus radial migration, we first sought to characterize the localization of mitochondria in migrating INs and PNs. We classified medial ganglionic eminence (MGE)-derived cells migrating in explant cultures into three morphological classes corresponding to distinct phases of their migration: leading process extension, forward movement of the centrosome, and nucleokinesis/trailing process retraction (Marin, Valdeolmillos et al. 2006). Morphology 1 cells were defined to have slender, tapered leading processes; morphology 2 cells have a bleb or thickening of the leading process; while morphology 3 cells have a clear trailing process (Figure 3A). Each subgroup displayed a distinct distribution of mitochondria (Figure 3B). In morphology 1, mitochondria were concentrated immediately anterior to the nucleus (Figure 3C, $49 \pm 4\%$, % of total mitochondrial area (%TMA) \pm SEM, 5-fold greater % TMA/% total cell area(TCA), $p < 0.05$) (Golden, Zitz et al. 1997, Bellion, Baudoin et al. 2005). In morphology 2 cells, mitochondria were concentrated in the cytoplasmic bleb (Figure 3C, 71 ± 2 , %TMA \pm SEM, 3.9-fold greater %TMA/%TCA than other areas, $p < 0.05$), while in morphology 3, the mitochondria were aggregated in the trailing process and posterior nuclear area (Figure 3C, 29 ± 4 %TMA \pm SEM, $39\% \pm 5$ TMA \pm SEM respectively, 1.7-fold greater %TMA/%TCA than other areas $p < 0.05$) (Bellion, Baudoin et al. 2005).

Although these independent clustering behaviors have been noted in the literature, mitochondrial dynamics during migration have not been studied. To evaluate the subcellular localization of mitochondria in relation to the morphological migratory phases, we next performed time-lapse imaging of fluorescently labeled mitochondria in migrating interneurons *in vitro*. Interestingly, mitochondria displayed consistent positional reorganization during migration,

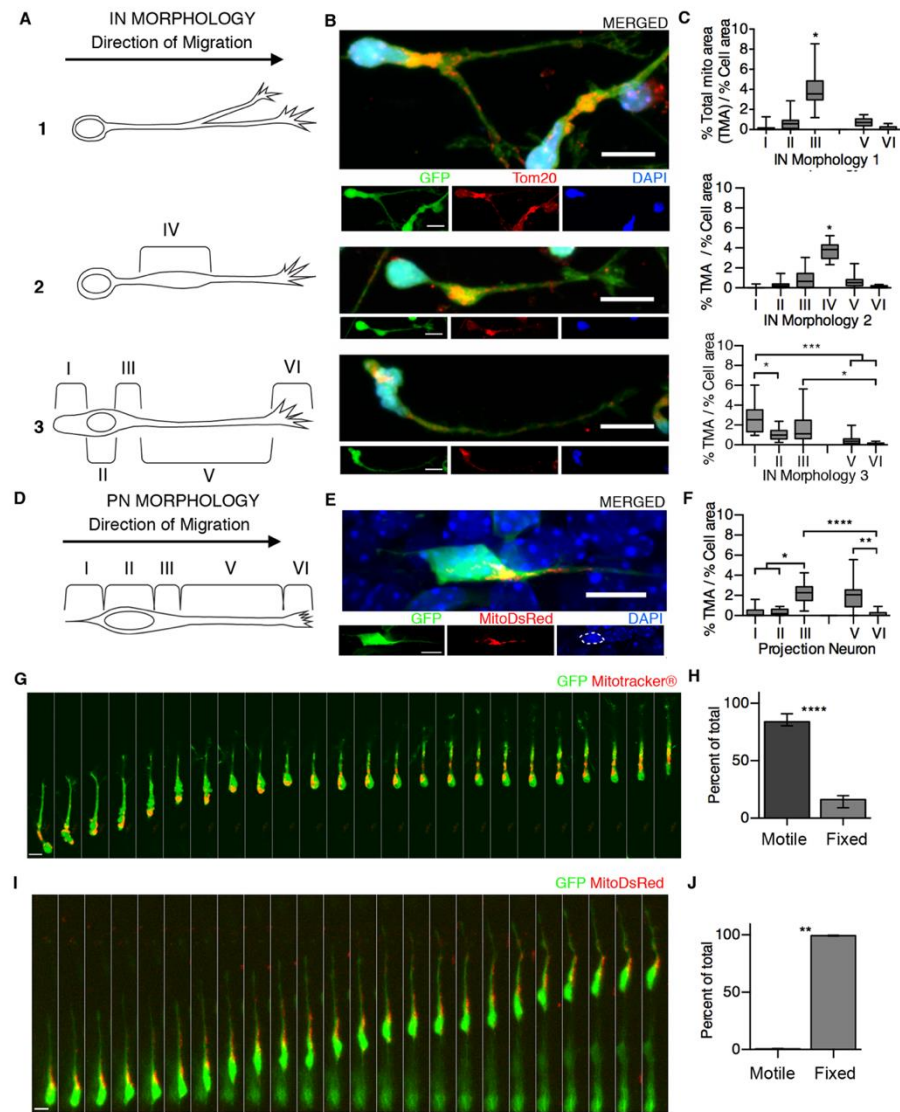


Figure 3. Mitochondrial localization in migrating neurons.

(A) Schemata of interneuron (IN; 1-3) morphologies displayed during migration. (B) Confocal immunofluorescence (IF) images of mitochondria in migrating INs *in vitro* displaying varying localization patterns. Cytosol (GFP), mitochondria (TOM20), nuclei (DAPI). Scale bar = 10 μ m. (C) Quantification of mitochondrial clustering in subcellular locations. Region I: trailing process (TP); II: overlapping nucleus (Nuc); III: 5 μ m anterior to the nucleus (5 μ m AN); IV: cytoplasmic bleb (bleb); V: leading process (LP); VI: leading process tip (LPT). Clustering varied markedly

between IN morphologies. IN morphology 1 clustered in III, * $p = 0.039$. IN morphology 2, clustered in IV, * $p = 0.02$, while IN morphology 3 clustered in I, and II, * $p = 0.0343$, *** $p = 0.0002$. Bars represent median with 25-75th percentiles \pm min/max value of percent total mitochondrial area (%TMA) normalized to region's percent of total cell area (%TCA). $n = 15$ cells each type; Freidman's test with Dunn's correction. (D) Schemata of pyramidal neuron (PN) migration morphology. (E) Confocal IF images of mitochondria in representative migrating PN. Cytosol (GFP), mitochondria (MitoDsRed), nuclei (DAPI). Scale bar = 10 μm . (F) Quantification of clustering mitochondria in migrating PNs. Region I: TP; II: Nuc; III: 5 μm AN; IV: cytoplasmic bleb (bleb); V: LP; VI: leading process tip LPT. III vs. I and II: * $p = 0.0327$; III vs. VI: **** $p < 0.0001$; V vs. VI: ** $p = 0.0064$. $n = 15$ cells; Freidman's test with Dunn's correction. (G) Time-lapse imaging of a migrating *Dlx5/6^{CIG}* IN *in vitro* (cytosol, GFP; mitochondria, Mitotracker® Red CMXRos) shows intracellular movement of mitochondria. 1 frame = 10 minutes. Scale bar = 10 μm . (H) Quantification of INs displaying extensive movement of mitochondrial through the cell compared to cells where cyclical movement was not observed. The majority of INs showed this movement of mitochondria throughout the cell during migration. $p < 0.0001$, unpaired t-test, $n = 5$ independent cultures, 200 cells. Values represent mean \pm SEM (I) Live time-lapse imaging of a migrating PN (cytosol, GFP; mitochondria, MitoDsRed). 1 frame = 10 minutes. Scale bar = 10 μm . (J) Quantification of PNs mitochondrial movement through the cell versus those where the mitochondria remain confined. PNs exhibited fixed localization in front of the nucleus. $p = 0.002$, Mann-Whitney, $n = 6$ independent cultures, 82 cells. Values represent mean \pm SEM.

as their subcellular location changed in concert with the morphology of the migrating cell (Figure 3G and 3H). The mitochondrial localization and changes in location observed in the three IN morphologies were confirmed by characterization of mitochondria in the cortex of E13.5 *Dlx5/6^{CIG}* embryos (Figure 4A, B). The localization of mitochondria in migrating INs was ranked according to morphology as matching or not matching the localization observed in migrating dissociated INs and found to be highly correlated (Figure 4B, C).

In contrast to migrating INs, migrating PNs maintain a relatively consistent migratory morphology after leaving their multipolar phase in the ventricular and subventricular zones (Figure 3D and 3I) (Noctor, Martinez-Cerdeno et al. 2004). During radial migration in the cortical plate, mitochondria were found primarily anterior to the nucleus and in the leading process, showing little change in regional localization (Figure 3E, 3F, 3I, and 3J). These data reveal that the intracellular position, and changes in location, of mitochondrial of INs and PNs are clearly distinguishable and suggest that there may be differences in energy requirements between these two neuronal cell populations during development.

Oxidative phosphorylation is necessary for normal IN migration, but not for radial migration

To determine whether migrating INs and PNs have distinct energetic requirements, we studied their need for mitochondrially-generated ATP in explant and slice cultures. Cells generate ATP through glycolysis in the cytosol and oxidative phosphorylation (OXPHOS) in the mitochondria. To test whether OXPHOS is necessary for normal neuronal migration, we examined cell movement after blocking OXPHOS with either oligomycin or bongkreikic acid (BA). Oligomycin (Olig) blocks mitochondrial production of ATP by inhibiting the ATP synthase (Kulka and Cooper 1962), while BA prevents the translocation of ATP across the inner

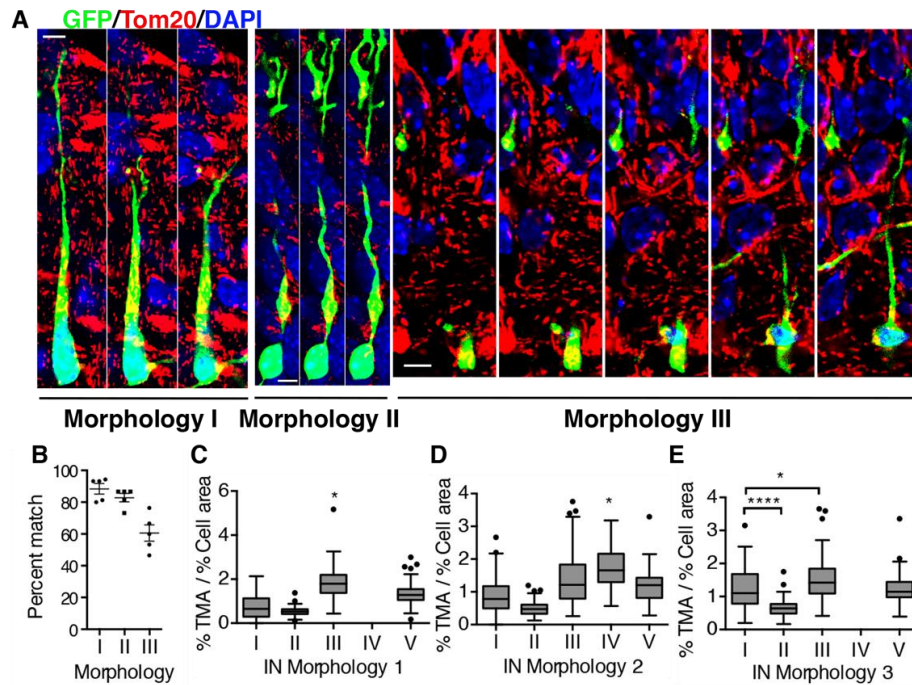


Figure 4. **Mitochondrial localization in interneurons *in vivo***

(A) Representative confocal z-slices of IN morphologies I-III showing mitochondria in INs

within the cortex. Cytosol (GFP), mitochondria (Tom20), nuclei (DAPI). Scale bar = 5 μ m. (B)

Cells were visually scored as matching or non-matching to localization patterns seen *in vitro* (n= 5 individuals, ≥ 50 cells each morphology). (C-E) Quantification of mitochondria in regions.

Region I: trailing process; II: overlapping nucleus; III: 5 μ m anterior to nucleus; IV: cytoplasmic bleb; V: leading process. Similar to *in vitro* data, clustering varied between morphologies.

Morphology 1 clustered in III, * $p \leq 0.029$. Morphology 2, clustered in IV, * $p \leq 0.022$. Clustering within the trailing process of morphology 3 was not as distinct compared to *in vitro* data.

Clustering was higher in region I compared to region II ($p < 0.0001$), most mitochondria clustered in region III ($p = 0.04$). Median with Tukey distribution; dots = greater than 1.5 times interquartile range of the percent total mitochondrial area (%TMA) normalized to region's

percent of total cell area (%TCA). $n \geq 50$ cells each morphology from 5 different individuals.

Freidman's test with Dunn's correction.

mitochondrial membrane by inhibiting the adenine nucleotide translocator isoforms 1 (*Ant1*, also known as *Slc25a4*) and 2 (*Ant2* also known as *Slc25a5*)(Henderson and Lardy 1970). IN migration was exquisitely sensitive to Olig treatment, where 0.02 μM reduced IN migration by 78% (Figure 5A). Treating INs with 2.5 μM BA reduced IN migration by 50% ($p \leq 0.001$) (Figure 6A). These cells showed no reduction in somal translocation (Figure 6C, not statistically significant but trending towards slower) but a significant increase in the time spent paused (Figure 6D). Interestingly, treated cells exhibited elongated trailing processes (Figure 6B, 6F), a higher frequency of trailing processes (Figure 6E) and for more time (Figure 6G) than controls. Furthermore, at low BA concentrations, migrating INs exhibit a 10-fold increase in direction changes (Figure 6H). BA also resulted in significant reduction in the leading process length (Figure 6I). The leading processes also branched less frequently, and the branch lengths were significantly reduced (Figures 6J and 6K).

To determine whether OXPHOS is sufficient to supply energy for IN migration, we removed glucose (GLUC) from the medium or inhibited glycolysis with 2-deoxyglucose (2-DG) and provided the OXPHOS substrate pyruvate (PYR). Alternatively, we substituted GLUC for galactose (GAL). GLUC deprivation reduced migration by ~53% (Figure 5C), while inhibition with 2-DG reduced migration by ~68% (Figure 5C). When INs are supplemented with PYR or GAL, ATP generation is dependent on OXPHOS alone (Marroquin, Hynes et al. 2007, Adeva-Andany, Lopez-Ojen et al. 2014). Both PYR and GAL were sufficient to rescue IN migration fully under conditions of glycolysis inhibition, and this ability to sustain migration was abrogated with the addition of sub-threshold doses of BA (Figure 5C). Therefore, OXPHOS is both necessary and sufficient for normal IN migration.

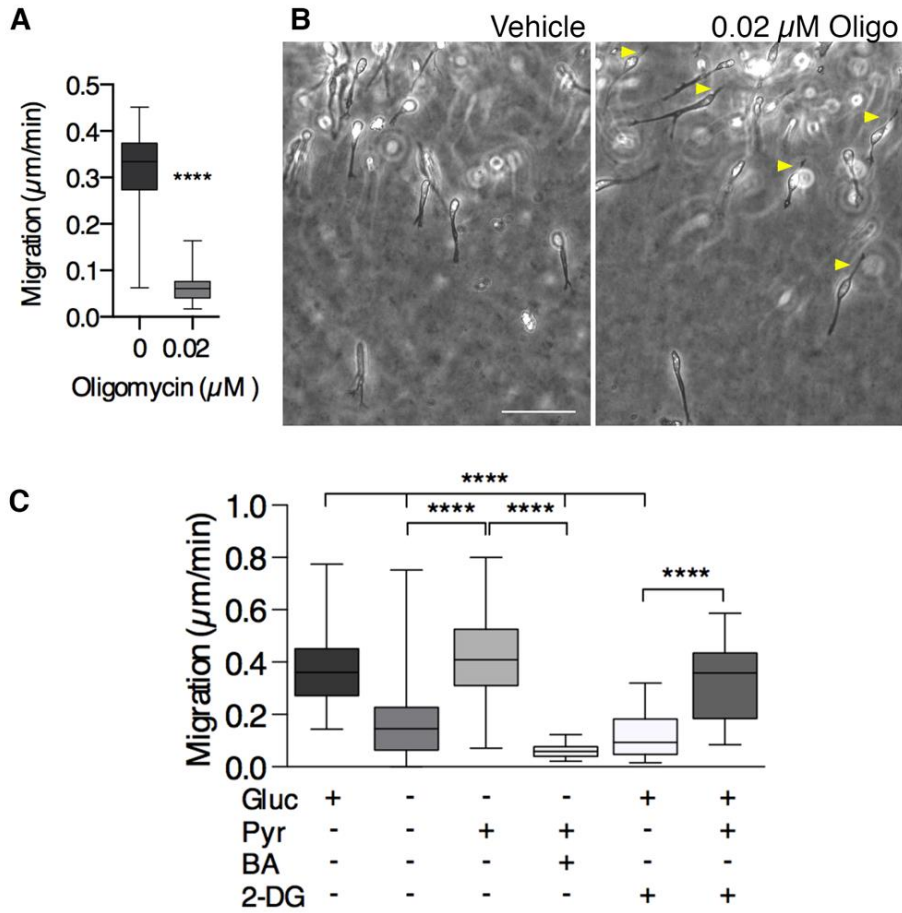


Figure 5. Oligomycin treatment of INs impacts migration

(A) Oligomycin treatment reduced IN migration rates ($\mu\text{m}/\text{min} \pm \text{SEM}$, Oligo: $p < 0.0001$, $N = 3$

independent cultures, 150 cells each, Mann-Whitney) (B) Representative phase image of treated

cells. Yellow arrows = extended trailing processes. (C) OXPHOS substrate pyruvate (Pyr)

rescues decreased migration from glucose deprivation and 2-Deoxyglucose (2-DG) treatment. Pyr

= pyruvate, Gluc = glucose, 2-DG = 2-deoxyglucose, BA = bongkreik acid. $p \leq 0.001$ as

indicated, $n = 5$ independent experiments, >75 cells each, Kruskal-Wallis with Dunn's correction.

Data not shown for Galactose.

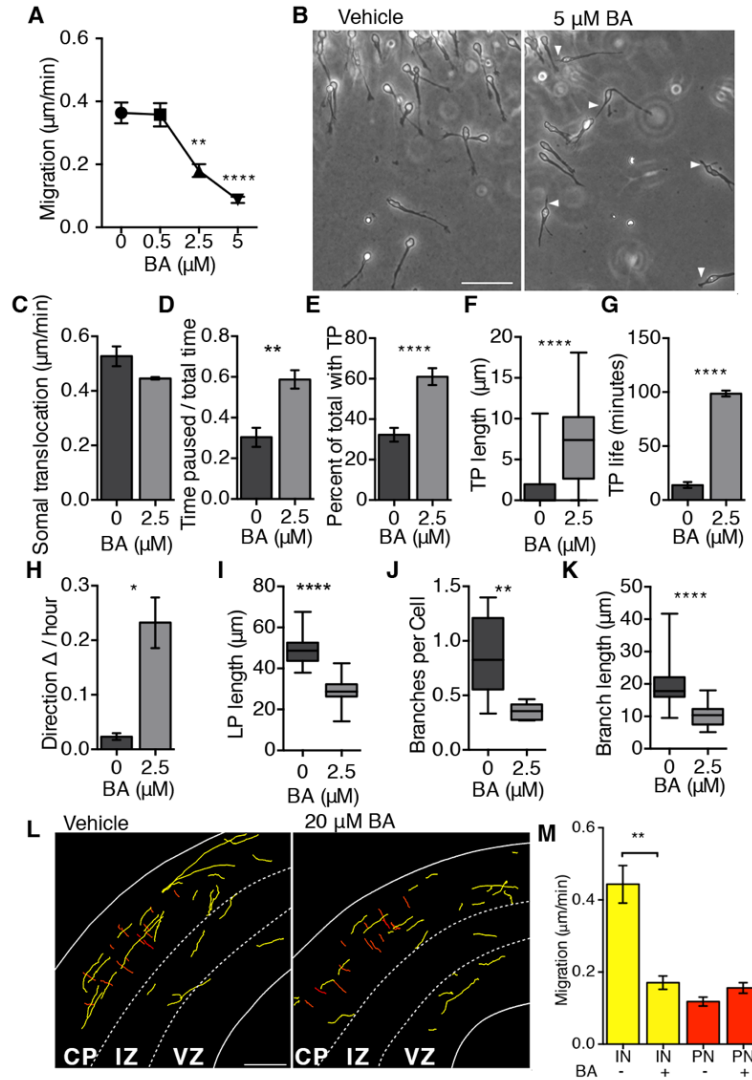


Figure 6. Pharmacological inhibition of OXPHOS reduces IN migration.

(A) Inhibiting OXPHOS with BA decreased IN migration (all in $\mu\text{m}/\text{min} \pm \text{SEM}$; 0 vs. 2.5 or 5, $p \leq 0.001$, $n = 5$ independent cultures, >150 cells each, ANOVA test). (B) Representative phase image of BA treated MGE INs. Arrows identify elongated trailing processes. Scale bar = $50 \mu\text{m}$. (C) Somal translocation for migrating IN, while slightly slower, did not reach significance. ($\mu\text{m}/\text{min} \pm \text{SEM}$; $p \leq 0.075$, $n = 5$ independent cultures, >150 cells each, unpaired t-test test, Welch's correction). (D) BA treated neurons spend more time paused compared to control cells (total time paused/imaging period $\pm \text{SEM}$; $p \leq 0.002$, $n = 5$ independent cultures, >150 cells each,

unpaired t-test test, Welch's correction). (E) Inhibition of OXPHOS had impacts on the trailing process (TP) frequency ($p < 0.001$, unpaired t-test with Welch's correction, mean \pm SEM), (F) TP length ($p < 0.0001$, Mann Whitney, median with interquartile range \pm min/max), and (G) TP life ($p < 0.0001$, Mann-Whitney). For each, $n = 5$ independent cultures, 100 cells each. (H) OXPHOS inhibition also causes increases in direction changes (mean direction changes/hour \pm SEM, $p < 0.0001$; $n = 5$ independent cultures, 100 cells each, unpaired t-test with Welch's correction). (I-K) BA treatment impacted leading process length, branches/cell, and branch length ($p < 0.0001$, $p < 0.001$, and $p < 0.0001$ respectively, Mann Whitney). (L) Sample migration paths of INs (yellow) and PNs (red) in E16 brain slices treated with vehicle or 20 μ M BA. CP = Cortical plate, IZ = Intermediate Zone, VZ = Ventricular Zone. Scale bar = 150 μ m. (M) IN migration rates decreased in slices treated with BA ($p = 0.0023$, $n = 6$ individuals, 20 INs each) whereas PNs were unaffected ($p > 0.05$, $n = 6$ individuals, ≥ 15 PNs in each). Unpaired t-test with Welch's correction, mean \pm SEM.

The requirement for OXPPOS in neuronal migration was further tested using slice cultures where both non-radially migrating INs and radially migrating PNs could be studied simultaneously. We utilized *Dlx5/6^{CIG}* mice in which the INs are genetically labeled with eGFP. At E14, a DsRed expression construct was electroporated into *Dlx5/6^{CIG}* embryos to label the progenitors of radially migrating PNs. Embryos were harvested at E16, and cortical slices from individuals were treated with BA. Remarkably, BA treatment reduced IN migration rates whereas PNs were unaffected (Figure 6L, 6M; IN: $0.289 \pm 0.06 \mu\text{m}/\text{min}$ decrease, $p < 0.002$, PN: $0.03 \pm 0.02 \mu\text{m}/\text{min}$ increase, $p > 0.1$). These data demonstrate that the non-radial migration of cortical interneurons is dependent on OXPPOS, while the radial migration of cortical projection neurons is either not or minimally OXPPOS dependent.

Selective disruption of non-radial IN migration in *Ant1*^{-/-} mutants

We next sought to corroborate our pharmacologic data of the differential effects of OXPPOS on PN versus IN migration in mice lacking the *Ant1* isoform. The genetic removal of *Ant1* reduces the ATP flux from the mitochondria to the cytosol, and *Ant1* is expressed in cortical neurons, including INs (Figure 7A and 7B)(Graham, Waymire et al. 1997, Levy, Chen et al. 2000, Lee, Schriener et al. 2009).

To determine if loss of *Ant1* disrupts IN migration *in vivo*, *Ant1^{+/+}* and *Ant1^{-/-}* brains were sectioned and stained for calbindin at E13.5 to detect a subset of migrating INs. On average, the leading edge of INs from wild-type brains migrated 15% percent farther than that of *Ant1^{-/-}* brains (Figure 8A, 8B), and there was a 30% decrease in total migrating INs invading the cortex in *Ant1^{-/-}* animals (Figure 8C). Additionally, *Ant1^{-/-}* migrating INs displayed aberrant orientation of the leading process with 30% fewer cells oriented in the main migration path (Figure 8G and 8H). Interestingly, *Ant1^{-/-}* INs had longer trailing processes *in vivo* (Figure 8D), a characteristic also

seen in wild-type INs treated with BA *in vitro* (Figure 8C). Neither proliferation or cell death in the MGE or cortex were impacted by loss of *Ant1*^{-/-} (Figure 8E and 8F), excluding these mechanisms as an explanation for the reduction of cells in the mutant cortex. This suggests that a migration phenotype is the primary cause for the IN defect in *Ant1*^{-/-} embryonic cortex.

To confirm the effect of *Ant1* deficiency on IN migration, *Ant1*^{-/-} MGE explants were assayed. *Ant1*^{-/-} INs migrated shorter distances (~40% decrease, Figure 9A and 9B), and more slowly (~42% decrease, Figure 9C) when compared to *Ant1*^{+/+} INs. Interestingly, *Ant1*^{-/-} IN migration was exquisitely sensitive to BA treatment, showing large reductions in IN migration rates at doses that had no effect on *Ant1*^{+/+} cells (Figure 9C) likely due to further blocking of mitochondrial ATP efflux through inhibition of the *Ant2* isoform (Graham, Waymire et al. 1997, Levy, Chen et al. 2000).

In contrast to the clear defects in IN migration, *Ant1*^{-/-} PN migration was normal. E14.5 embryos were electroporated with pCAG-IG and cell positions assayed on E18.5. The loss of *Ant1* did not alter radial migration (Figure 10A and 10B). To further assay the migration of PN we injected EdU to pregnant dams on E14.5 and harvested the embryos on E18.5. Labeling for EdU (E14.5 injections mainly label outer layer neurons) and Tbr1 (a deeper layer neuronal marker) showed normal positioning of cortical neurons between *Ant1*^{+/+} and *Ant1*^{-/-} brains (Figure 10C). Together these data indicate that in marked contrast to the non-radial migration of cortical IN, *Ant1* does not appear to affect the radial migration of PNs.

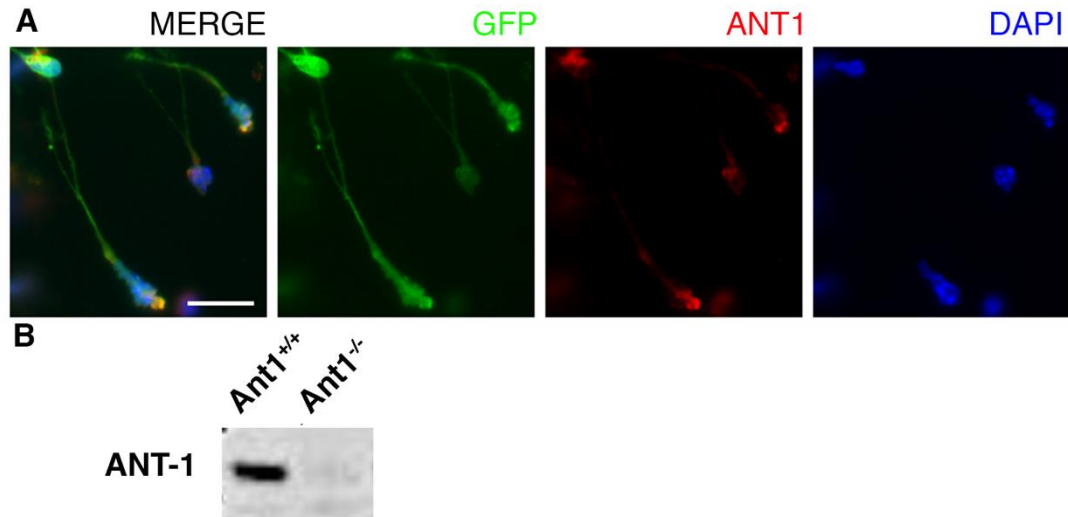


Figure 7. Migrating interneurons express *Ant1*

(A) *Ant1* expression is detectable in migrating *Dlx5/6*^{C1G} INs in explant cultures. Scale bar = 20 μ m. (B) Western blot of whole brain lysate showing specificity of *Ant1* antibody.

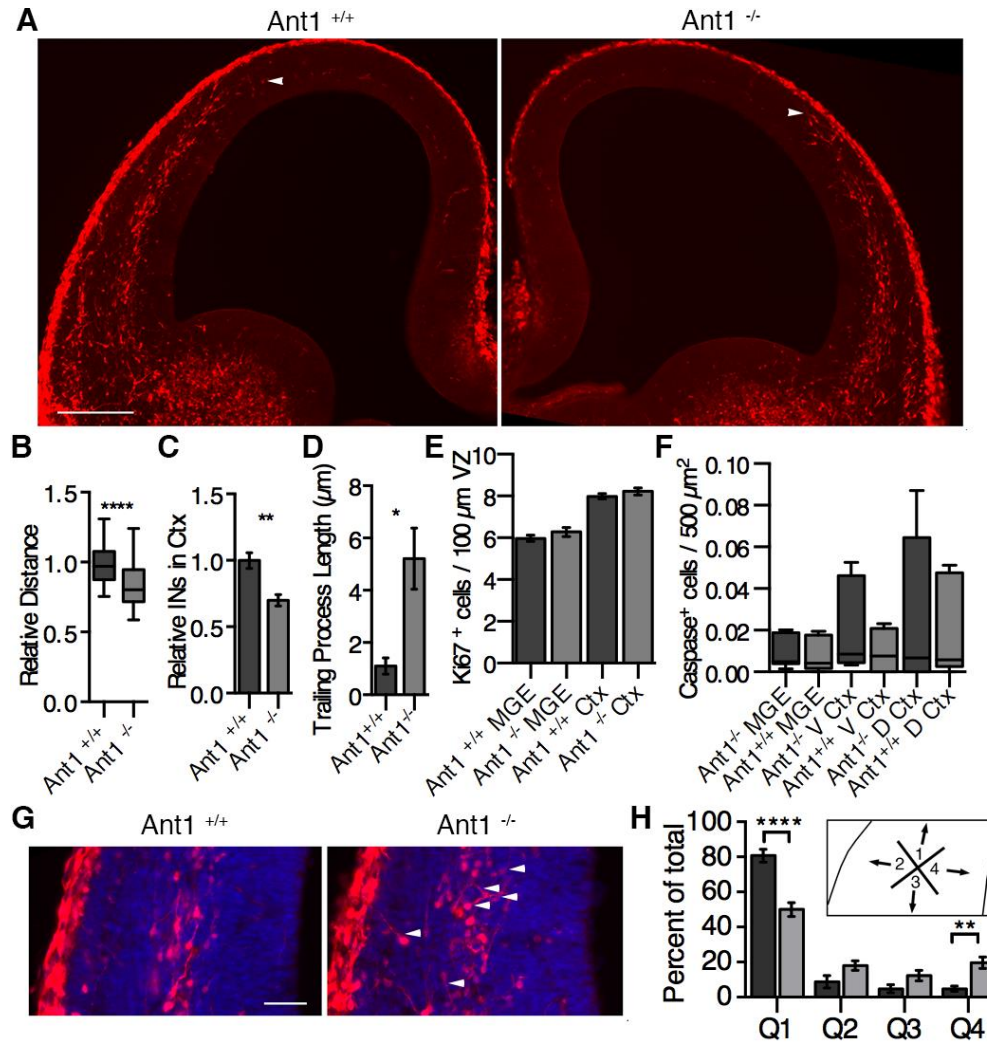


Figure 8. **Interneuron migration is reduced in embryonic *Ant1* mutants.**

(A) At E13.5 migrating INs (labeled by Calbindin immunohistochemistry) have not traveled as far in *Ant1*^{-/-} brains compared to *Ant1*^{+/+} brains. The white arrow = leading migrating INs. Scale bar = 200 µm. (B) Quantification of relative distance of leading cells of migrating INs normalized to *Ant1*^{+/+} (****p < 0.0001, n = 5 individuals, 25 cells each, Mann-Whitney test). Values represent median with interquartile range ± min/max. (C) Quantification of INs in cortex normalized to average of *Ant1*^{+/+} ± SEM, **p = 0.004, n = 5, unpaired t-test with Welch's correction. (D) *Ant1*^{-/-} INs in the cortex have increased trailing process (TP) length (µm ± SEM, *p = 0.022, n = 5 individuals, 25 cells each, unpaired t-test with Welch's correction). (E)

Proliferation in germinal ventricular zones (VZ), indicated by Ki67 immunostaining was not impacted by loss of *Ant1*. Medial ganglionic eminence (MGE) and pallium (Ctx). $p = 0.3$; $n = 5$ individuals, unpaired t-test with Welch's correction. (F) Loss of *Ant1* did not increase cell death, indicated by Caspase 3, in the MGE and the ventral and dorsal pallium (V/D Ctx). $p > 0.99$, $n = 5$ individuals, Kruskal-Wallis, Dunn's correction. Values represent median, 25th-75th percentile \pm min/max. (G) INs in cortex of *Ant1*^{-/-} mice displayed abnormal leading process orientation. Arrowheads = misaligned INs. Scale bar = 75 μ m. (H) Quantification of cortical IN leading process orientation into quadrants: Q1, dorsal; Q2, pial; Q3, ventral; Q4, ventricular orientation. The average percent of IN in quadrant \pm SEM, **** $p = 0.0001$, ** $p = 0.0069$, $n = 5$, ANOVA with Sidak's correction.

Loss of *Ant1* alters centrosome localization in migrating interneurons

To further examine the migration behaviors disrupting IN migration in *Ant1*^{-/-} mutants, we crossed these mice to *Dlx5/6*^{Cre} mice to genetically label forebrain GABAergic neurons. Live imaging of slices from *Ant1*^{-/-} and littermate *Ant1*^{+/+} controls revealed ~31% decrease in migration rates of GFP⁺ cells in the cortex (Figure 9E), and ~3.6 fold increase in direction changes (Figure 9D, 9F and 9G). These data suggest an impaired ability of *Ant1*^{-/-} INs to maintain polarity. To study this further, we examined centrosome localization in MGE explant cultures. In control INs, the centrosome localizes anterior to the nucleus or in the bleb of the leading process. *Ant1*^{-/-} INs displayed markedly aberrant centrosome positions, posterior to the leading edge of the nucleus, or even behind the nucleus (Figure 11A and 11B). To confirm this finding, we also assayed the localization of centrosome after BA. Similar to the findings in BA treated INs, *Ant1*^{-/-} INs also displayed a significant posterior positioning of the centrosome (Figure 11C and 11D). Taken together with the increased direction changes seen after either genetic or biochemical impairment in mitochondrial energetics, these results suggest that IN polarity is particularly sensitive to mitochondrial perturbation.

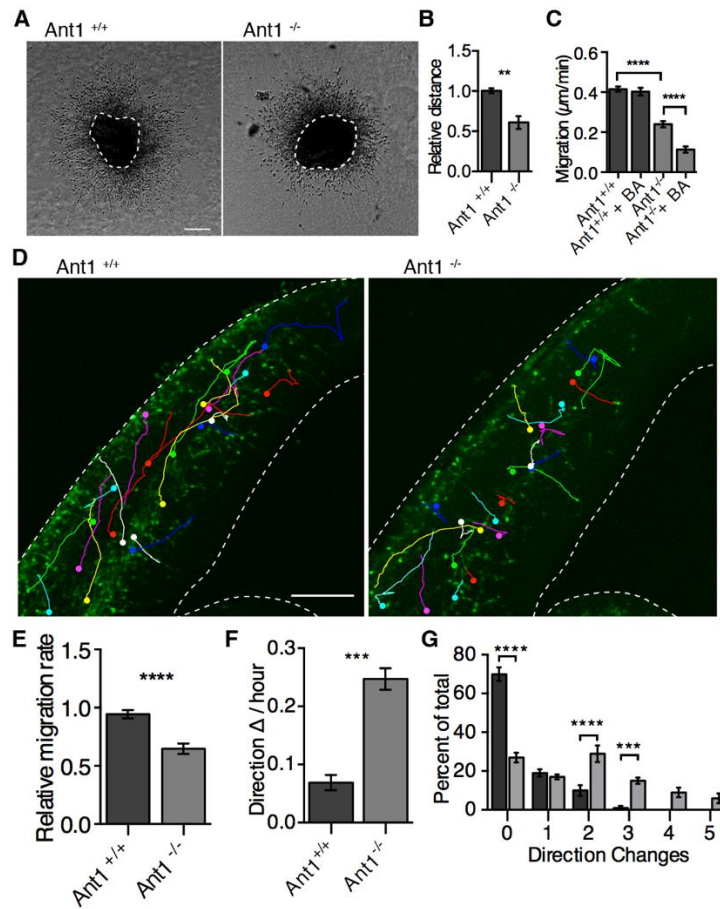


Figure 9. **Abnormal migration by *Ant1*^{-/-} interneurons.**

(A) *Ant1*^{-/-} INs did not migrate out of MGE explant as far as controls (16 hrs). (B) Quantification of IN migration from explants (normalized distance \pm SEM ***p* < 0.005, *n* = 6, 50 cells each, unpaired t-test with Welch's correction). Scale bar = 250 μ m. (C) Quantification of IN migration rates (migration rate in μ m/hr \pm SEM, vehicle vs. 0.5 fM BA in *Ant1*^{-/-}: *****p* < 0.001, *n* = 5, 150 cells each, ANOVA with Bonferonni's correction). (D) Examples of migration path of GFP⁺ INs in brain slice culture. Dots = start; lines = paths. Scale bar = 150 μ m. (E) *Ant1*^{-/-} INs in slices have decreased migration rates (migration rate in μ m/hr \pm SEM *Ant1*^{+/+} vs. *Ant1*^{-/-}: **** *p* < 0.0001, *n* = 5, 20 cell each, unpaired t-test with Welch's correction). (F) *Ant1*^{-/-} INs display increased direction changes per cell/hr \pm SEM (*p* < 0.0001, *n* = 5, unpaired t-test with Welch's

correction).

(G) Frequency plot of IN direction changes; dark grey = *AntI*^{+/+}, light grey = *AntI*^{-/-}. ****p < 0.0001, ***p = 0.0006, n = 5, two-way ANOVA with Sidak's correction.

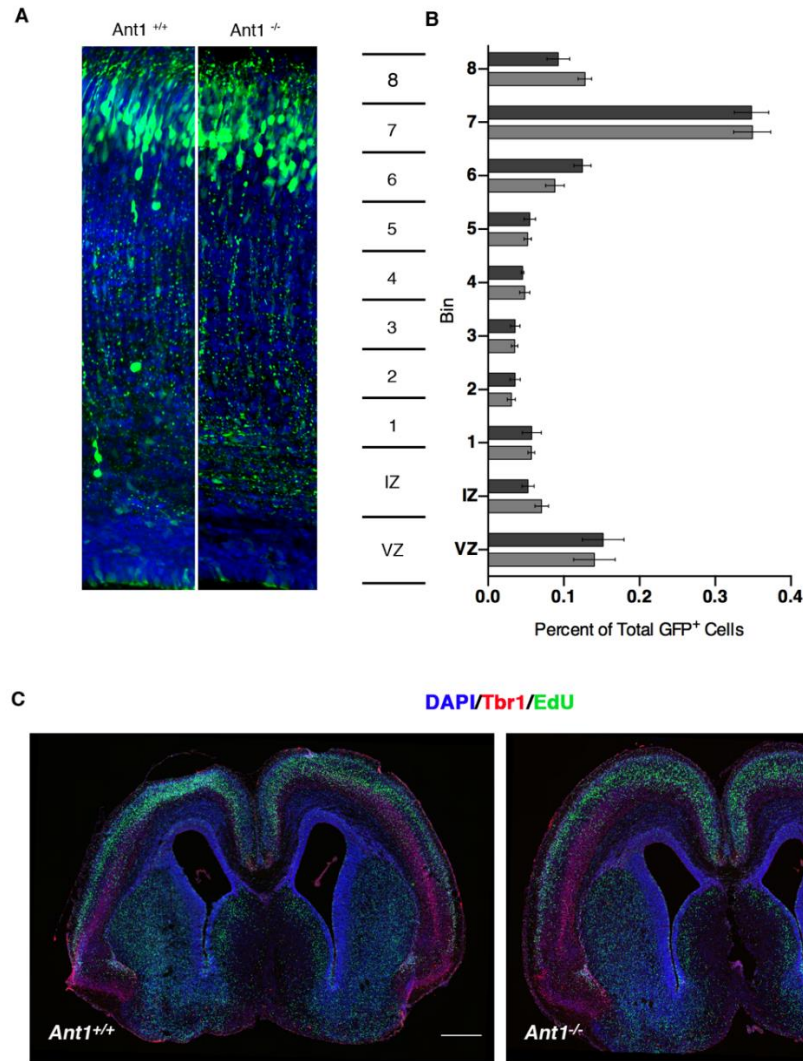


Figure 10. Radial migration is normal in *Ant1*^{-/-} cortices

(A) Confocal max projection of E18.5 cortices, 4 days post *in utero* electroporation with pCAG-IRES-GFP. (B) Quantification of percent total GFP⁺ cells in defined cortical bin: ventricular and intermediate zones, and 8 equal division of the cortical plate (VZ: IZ; 1-8). $p > 0.5$, $n = 5$ individuals each genotype, two-way ANOVA. Scale bar = 250 μm . (C) Representative coronal sections from E18.5 *Ant1*^{+/+} and *Ant1*^{-/-} showing Tbr1 (red) labeled cells normally positioned in the deeper layers compared to outer positioned neurons born on E14.5 (EdU, green). All nuclei stained with DAPI (blue) ($n=3$, scale bar=500 μm).

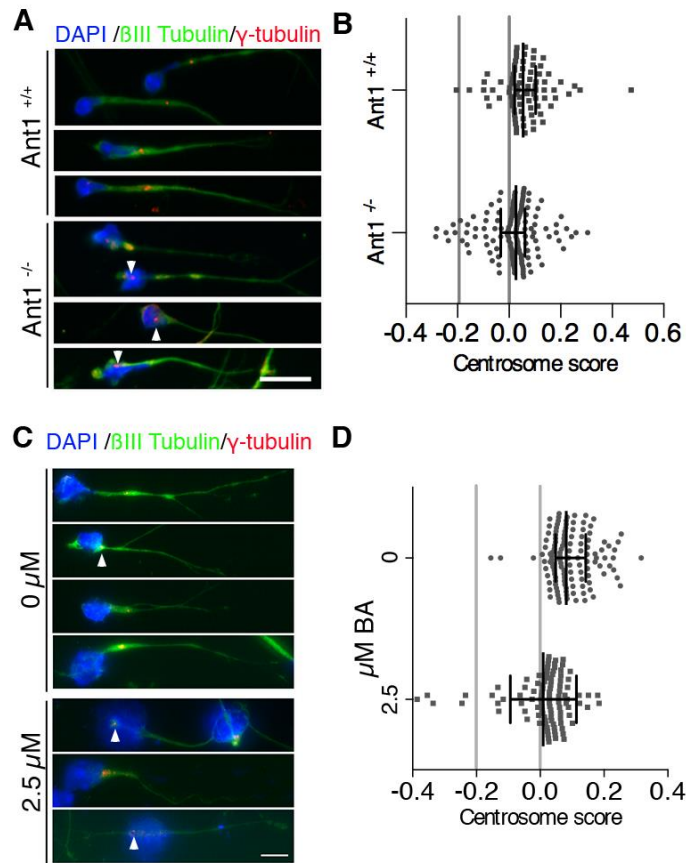


Figure 11. Loss of Ant1 causes shift in centrosome position

(A, C) Sample images of centrosome position in INs show mislocalized centrosome in Ant1^{-/-} INs and BA-treated INs *in vitro*. Gamma-tubulin = red, nucleus = blue, cytoplasm = green. Scale bar = 15 μm. (B, D) Scatter plot of centrosome score of Ant1^{-/-} and BA-treated INs (Grey lines = average nuclear length). Centrosome score ± SEM; p = 0.0005, n = 75 cells each genotype, Mann-Whitney test, median with interquartile range ± min/max.

Discussion

Our results reveal that migrating INs and PNs display major differences in mitochondrial localization. During IN migration, mitochondrial localization is highly dynamic, with the highest density of mitochondria appearing to move between the posterior trailing process, the region anterior to the nucleus, and the cytoplasmic bleb. In contrast, during PN migration, mitochondria are primarily restricted to the region anterior to the nucleus. We also found that inhibition of OXPHOS drastically decreased the migration rates of INs but not PNs. These findings suggest that INs, unlike radially migrating PNs, are highly dependent on mitochondrial ATP production. The reduced migratory rates and increased direction changes observed in INs also suggest that the maintenance of polarity is an energetically vulnerable process and is required for normal IN development. These data link mitochondrial function to the prenatal development of a critical cerebral cortical neuronal subpopulation.

Few studies have addressed mitochondrial localization and trafficking in migrating neurons. Previous work has shown that *Lis1*, *Tau1*, and *DCX*, genes that cause defects in radial migration and IN development, cause mislocalized and altered mitochondrial trafficking (Yamada, Yoshida et al. 2009, Sapir, Frotscher et al. 2012, Khalaf-Nazzal, Bruel-Jungerman et al. 2013). Although this suggests that defects in mitochondrial localization may also impact PNs, each of these genes also regulates microtubule dynamics. Thus in these models, it is unclear if changes in mitochondrial localization contribute to the defects in radial migration or if these genes have direct impacts on mitochondrial function. Our data addresses this issue by investigating mitochondrial localization in both PN and IN populations, and by interfering directly with mitochondrial function. Although mitochondrial dysfunction in addition to other defects may contribute to abnormal radial migration, we provide clear evidence that INs are much more sensitive to OXPHOS deficits.

Mitochondrial contribution to neuronal metabolism has been largely studied in the context of the adult nervous system, focused on how the loss of mitochondrial function results in neurodegeneration and cell death. Recent data has emphasized the importance of mitochondrial energetics in basic neurophysiology. For example, mitochondrial energetics are essential for interneuron regulation of gamma oscillations that are themselves associated with cognitive functions (Kann, Papageorgiou et al. 2014). However, little data exists on the earlier developmental requirements for mitochondrial OXPHOS. Several studies have indicated that regulation of mitochondrial metabolism impacts neurogenesis and differentiation (Bertholet, Millet et al. 2013, Wang, Ye et al. 2014) but the requirement for OXPHOS during neuronal migration had not been studied. Surin et al. suggested that glycolysis is a primary driver of embryonic neuronal metabolism of hippocampal cultures (Surin, Khiroug et al. 2012). Since interneurons comprise only about 6% of the neurons in hippocampal cultures (Benson, Watkins et al. 1994), it is likely the measurements in this study were primarily representative of pyramidal neuron metabolism. This lack of active OXPHOS in embryonic pyramidal cells thus compliments our findings that PN migration is not impacted by OXPHOS inhibition. Our data clearly shows that interneuron migration required OXPHOS, and suggests that distinct neuronal populations have different metabolic requirements during development.

We found that *Ant1* mutant INs exhibit changes in centrosome localization, increased length of the trailing process, and increased direction changes during IN migration. Mitochondria have been implicated in centrosome homeostasis in mitotic cells (Donthamsetty, Brahmabhatt et al. 2014). Additionally, the mislocalization of centrosomes has also been observed in mice lacking mDia1 and 3, proteins of the formin family that regulate cytoskeletal dynamics via Rho-GTPases (Daou, Hasan et al. 2014). Interestingly, IN migration is disrupted in these mutants, but radial migration is not (Shinohara, Thumkeo et al. 2012). In this model, the focus was on

subventricular zone migration of interneurons to the olfactory bulb. Thus, it is unclear whether there are additional phenotypic similarities exist between these models. The similarities in our phenotype, and selective effect on INs suggest that the regulation of centrosomal position and actinomyosin contractions within the trailing process are energetically vulnerable processes and warrant further investigation.

Patients with ASD, and particularly those with combined ASD, ID and epilepsy, commonly have evidence of mitochondrial dysfunction (Giulivi, Zhang et al. 2010, Rossignol and Frye 2012). The manner by which mitochondrial dysfunction contributes to these phenotypes is generally attributed to a deficiency in meeting ongoing neuronal metabolic demands, or increased free radical production resulting in cell death. Our data provide clear evidence for a novel final common pathway into the pathogenesis of ASD, developmental epilepsies, and intellectual disabilities. These clinical phenotypes associated with mitochondrial disorders may not solely arise from energetic deficits or the formation of free radicals during later neuronal function but may be secondary to abnormal IN development.

Materials and Methods

Mice

CD1, or *Dlx5/6^{CIG}* (Stenman, Toresson et al. 2003), and *Ant1^{-/-}* on a C57BL6/NJ (Ronchi, Figueira et al. 2013) of both sexes were used as indicated. The Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia, Philadelphia, PA, approved all studies.

Brain explant and slice cultures

Explant and slice cultures were generated from the indicated embryonic day mouse pups as previously described (Lysko, Putt et al. 2011).

Treatment protocols

For inhibition of oxidative phosphorylation, explants were cultured for 24 hours in DM with 35 mM glucose. Immediately before imaging, media was exchanged with DM with PBS vehicle, Oligomycin (Sigma), or Bongkreikic Acid (Enzo Life Sciences). We found a strain difference in response to treatment to BA. CD1 cells were treated with 0.5, 2.5, 5, and 50 μ M BA, while *Ant1*^{+/+} and *Ant1*^{-/-} were treated with 0.5 fM BA. For glucose deprivation and inhibition experiments, explants were cultured for 24 hours in glucose-free DMEM (Invitrogen) plus N2 supplement (Gibco) with or without 10 mM sodium pyruvate (Sigma) or 5 mM Galactose (Sigma), +/- 2.5 μ M BA. For treatment with 2-DG, explants were cultured for 24 hours with 5 mM glucose DM supplemented with 500 μ M 2-DG (Sigma), with or without 10 mM sodium pyruvate or 5 mM galactose +/- 0.5 μ M BA.

Histology and immunocytochemistry

Brains of E13.5, E16, or E18.5 embryos were processed for histology and immunohistochemistry as previously described (Lysko, Putt et al. 2011). Primary antibodies used included anti-Calbindin D-28k (rabbit; Swant, 1:1000), Caspase-3 (rabbit; Abcam, 1:500), Ki67 (rabbit; Neomarkers, 1:300), anti-Tom-20 (rabbit; Santa Cruz, 1:500), anti-GFP (chicken: Invitrogen, 1:2000). Secondary antibodies included goat anti-rabbit-biotin; Vector Laboratories followed by Streptavidin-Alexa 594; Invitrogen, or anti-rabbit-Alexa 594; Invitrogen anti-chick-Alexa 488; Invitrogen all at 1:2000. Nuclei were counterstained with DAPI. MGE explants were fixed and immunolabeled as previously described (Lysko, Putt et al. 2011) using anti-Tuj1 (rabbit; Neuronal CIII b-tubulin; Covance, 1:1000), anti gamma-tubulin (mouse; 1:200 Sigma), anti-GFP (chicken Invitrogen 1:200), or anti-Ant1 (rabbit) as primary antibodies. Mitochondria

were labeled in migrating interneurons by staining 40 μm thick floating sections from *Dlx5/6^{CIG}* embryos and staining for Tom20, staining expressed GFP within the cytosol with anti-GFP.

Intrauterine electroporations

Embryos at either E14, or E14.5 were electroporated *in utero* as previously published (Nasrallah, Cho et al. 2012) with the following constructs: pCAG-IG (Addgene 11150; 2 $\mu\text{g}/\text{uL}$), pCAG-DsRed (Addgene 11151; 2 $\mu\text{g}/\text{uL}$), pDsRed2-mito (MitoDsRed; Clontech 632421; 0.5 $\mu\text{g}/\text{uL}$). For marking mitochondria in radially migrating neurons, and slice BA treatments, embryos were electroporated at E14 and harvested 48 hrs later. For assessing radial migration, embryos were electroporated at E14.5 and harvested at E18.5.

Live marking of mitochondria in vitro

To image mitochondria in migrating INs, explants from *Dlx5/6^{CIG}* embryos were cultured for 24 hrs. Prior to imaging, cells were treated with 100 nM MitoTracker® Red CMXRos (Invitrogen) for 30 minutes in Opti-MEM (Invitrogen) with 10 mM glucose. Cells were then rinsed with PBS and supplied with fresh DM before imaging.

Microscopy

For all experiments, time-lapse images were acquired at indicated intervals for a minimum of 6 hours with an Olympus Fluoview (FV10i) confocal microscope at 37°C, 5% CO². Magnifications were as follows; 10X magnification with 2X zoom for treatment protocols in 5-minute intervals, and 10X magnification in slices in 10-minute intervals. For higher resolution, 60X magnification was used for acquiring mitochondrial localization in migration INs at 10-minute intervals. For slices, Z-stacks of 10 μm each were taken, capturing the full range of detectable GFP⁺ cells or DsRed cells within the slice. Slices were imaged for a minimum of 5 hrs.

Images of fixed explants cells and slices were taken on an Olympus Fluoview (FV10i) confocal microscope at 20x magnification. Mitochondria were localized within individual cells from 40 μ m brain slices by collecting 1.5 μ m z-stacks in using the Olympus Fluoview (FV10i) confocal microscope at 60x magnification. Images of calbindin stained slices and were taken at 5X and 10X magnification every 5 μ m for 15 μ m on a Leica CTR600 fluorescent microscope.

Quantification

In all experiments, cells were selected at random using ImageJ's grid plugin for all experiments unless indicated otherwise. The color-profiler ImageJ plugin was used to generate plots of fluorescence units. For fixed cells, mitochondrial area was calculated by thresholding images using ImageJ's auto-local thresholding plugin (for invitro culture, Bernsen method, 15 pixels), or by color thresholding images for overlapping green and red pixels (for cells in fixed slices). Thresholded images were analyzed using the particle analyzer plugin to calculate mitochondrial area in subcellular regions. The distance of migration along the cortex of the 25 leading cells was measured as a percentage of the distance between the striato-cortical notch and dorsal cortical curve, and normalized to the average distance of wild-type littermates. Relative Migration Distance for explants was calculated from the explant edge to the position of the 10 cells that had migrated the furthest. Values were normalized to averages of *Ant1*^{+/+} littermates. Cell migration speed was calculated as previously published (Lysko, Putt et al. 2011). Leading process orientation was calculated by designating cells into quadrants based on the orientation of their leading processes. Centrosome scores were determined by defining the anterior edge of the nucleus as zero and centrosomes positioned behind the leading edge of the nucleus as negative values. Centrosome position was measured from the posterior of the cell and represented as a percent of total cell length.

Statistics.

Prism 6 software was used for all statistical analysis. Data were tested for normality using either the K-S test or the D'Agostino & Pearson omnibus normality test. If the data were not normal, non-parametric analysis was utilized. $p < 0.05$ were considered significant. All values are represented as mean \pm SEM unless otherwise indicated.

CHAPTER 3: MANIPULATIONS OF THE MITOCHONDRIAL TRAFFICKING PROTEIN MIRO1 IMPACTS INTERNEURON MIGRATION

Introduction

Mitochondria are complex organelles which provide energy, Ca²⁺ buffering, and additional functions within the cell (Lackner 2014). Historically, the localization of mitochondria in discreet regions of neurons generated the hypothesis that specific localization of mitochondria is essential for neuronal physiology (Berthold, Fabricius et al. 1993, Fabricius, Berthold et al. 1993, Hollenbeck and Saxton 2005). Live-imaging of mitochondria in post-mitotic hippocampal neurons revealed highly dynamic behaviors, exhibiting static and motile populations with both retrograde and anterograde movement (Misgeld, Kerschensteiner et al. 2007). The discovery of proteins involved in trafficking mitochondria provided reagents with which to test the significance of mitochondria trafficking for cellular functions (Stowers, Megeath et al. 2002, Brickley, Smith et al. 2005, Fransson, Ruusala et al. 2006). Manipulations of mitochondrial transport proteins have shown that mitochondria indeed have a functional role in these regions of mitochondrial enrichment in neurons, and play central roles in axonal branching, synaptogenesis, plasticity and synaptic maintenance (Guo, Macleod et al. 2005, Kang, Tian et al. 2008, Courchet, Lewis et al. 2013, Spillane, Ketschek et al. 2013). However, the importance of mitochondrial trafficking at earlier neurodevelopmental time points, such as neuronal migration, remains unexplored.

Studies elucidating the molecular mechanisms regulating mitochondrial trafficking have identified several mitochondrial membrane-bound proteins that act as adaptors connecting mitochondria to the molecular motor network within the cell (Figure 12). Atypical Rho-GTPase (Miro), a mitochondrial membrane-bound protein interacts with Milton/GRIF/TRAK, creating an adaptor complex that interacts with the molecular motor KIF5, allowing for anterograde movement of mitochondria in neurons (MacAskill, Brickley et al. 2009). Miro and TRAK also

facilitate the linkage of mitochondria to dynein to drive retrograde migration (van Spronsen, Mikhaylova et al. 2013). Syntabulin has also been shown to act as an adaptor to facilitate interactions between KIF5 and mitochondria for anterograde movement in neurons (Cai, Gerwin et al. 2005), while the proteins FEZ1 and RanBP2 have been suggested as potential candidates (Cho, Cai et al. 2007, Fujita, Maturana et al. 2007). Although these molecular adaptors facilitating mitochondrial movement have been identified, the mechanism of mitochondrial pausing or docking is not completely understood. Calcium binding to the EF hand domains of Miro1 result in the pausing of mitochondria either by resulting in the dissociation of TRAK proteins from KIF5 (Macaskill, Rinholm et al. 2009) or causing the dissociation of KIF5 from microtubules (Wang and Schwarz 2009). The protein syntaphilin has been identified as an additional regulator for mitochondrial docking, acting as an anchor to bind mitochondria to the microtubule network (Chen and Sheng 2013).

We previously described unique and distinct mitochondrial localization behaviors in two migrating neuronal populations: radially migrating projection neurons (PNs) and tangentially migrating interneurons (INs). Mitochondria in migrating INs are much more dynamic in their localization behaviors compared to their PN counterparts (Figure 3). Mitochondria move cyclically during the migration process, moving from anterior to the nucleus, outward into the leading process thickening, and back to the trailing process. We also found that deficits in mitochondrial oxidative phosphorylation selectively disrupted IN migration, while leaving PN migration intact. We hypothesize that IN migration will also be more sensitive to alterations to mitochondrial trafficking compared to the radial migrations of PNs. To test this hypothesis, we manipulated Miro1 due to its localization on mitochondria and its role in mitochondrial trafficking. (Fransson, Ruusala et al. 2006, Macaskill, Rinholm et al. 2009, MacAskill and Kittler 2010) (Figure 12).

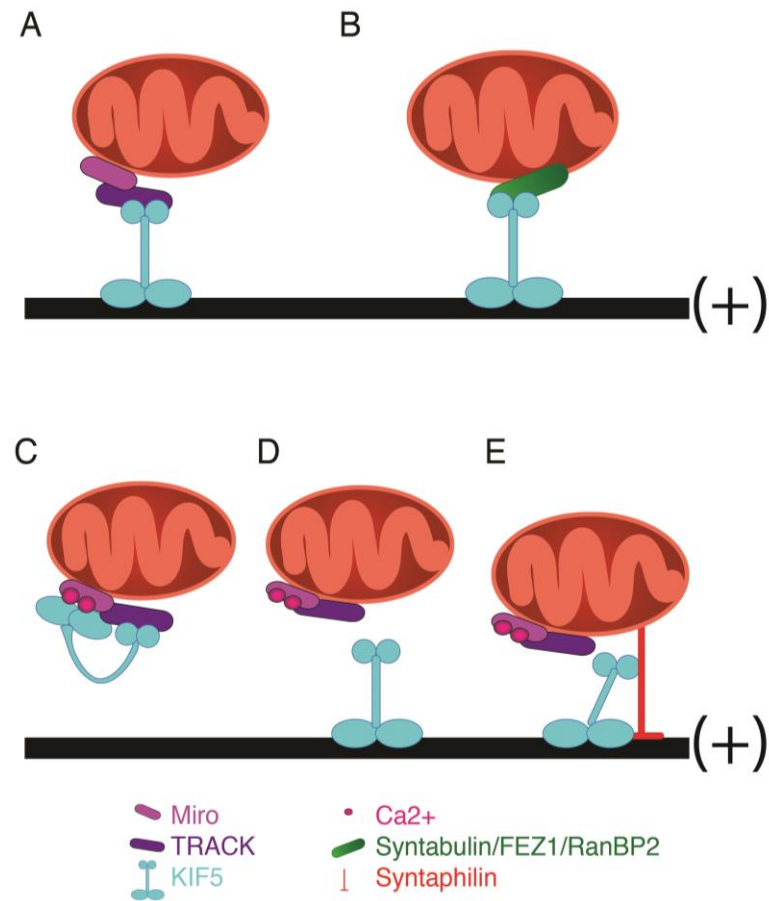


Figure 12. **Proteins involved in mitochondrial trafficking**

(A) MIRO, a mitochondrial membrane-bound protein, with its binding partner GRIF/TRACK, attaches mitochondria to the molecular motor protein KIF5, allowing mitochondria to move along the microtubule network. (B) Syntabulin, FEZ1, and RanBP2 are also candidate proteins for mitochondrial adaptors. (C-E) Binding of calcium to MIRO EF-motifs results in stopping of mitochondria by preventing association of KIF5 with microtubules, TRAK association with KIF5, or by tethering with Syntaphilin.

We utilized a dominant negative form of Miro1, M1K208, which contains paired mutations at its EF binding domains, sites of calcium binding. The expression of this construct in hippocampal cells has been shown to impact calcium-dependent mitochondrial trafficking *in vitro* by preventing calcium-dependent localization to specific parts of hippocampal neurons (Macaskill, Rinholm et al. 2009). As we had observed selective localization of mitochondria to particular regions of interneurons, we chose Miro1 due to its selective impact on mitochondrial trafficking without interfering with vesicular transport (Fransson, Ruusala et al. 2006, Macaskill, Rinholm et al. 2009, MacAskill and Kittler 2010).

We find that IN migration rate is subtly impacted by the expression of a dominant negative form of Miro1. Overexpression of this dominant negative construct in INs *in vitro* does not impact migration rate. However, these cells exhibit a decrease in progressive movement, and change directions more frequently compared to controls. This reduces their ability to invade the cortex in embryonic brain slice culture. Interestingly, we found subtle defects in radially migrating PNs *in vivo*, suggesting that certain aspects of mitochondrial trafficking may play a role in this process. This data supports a model in which changes in mitochondrial behavior and physiology disrupts neuronal migration, with more severe deficits seen in IN migration, elucidating a potential mechanism for the connections between mitochondrial disorders and neurodevelopmental diseases of the cortex.

Results

Expression of a dominant negative Miro1 alters IN migration behavior

Expression of Miro1 in INs was confirmed by western blot of the ganglionic eminences at E14.5, which include migrating INs. Mouse anti-Rhot-1 antibody detected protein in E14.5 whole brain and the embryonic striatum (Figure 13). We then tested our expression constructs to determine if overexpression of Miro1K208 (M1K208) would impact mitochondrial localization in 3T3 cells. We observed perinuclear localization compared to controls that was not seen in

overexpression of wild-type Miro1 (M1) (Figure 14). This correlated with previous results (Fransson, Ruusala et al. 2006).

We then utilized our M1K208 construct to test if mitochondrial localization has an impact on the tangential migration of migrating INs from explant cultures. Migration rates were not significantly affected by overexpression of M1K208 or M1 (Figure 15). There was an extreme effect on the directionality of migration; cells overexpressing Miro1K208 resulted in an increase in direction reversals in migrating cells (GFP: 0.41 ± 0.12 ; M1K208: 2.1 ± 0.24 ; direction changes per cell \pm SEM, $p < 0.01$). This was not seen in cells overexpressing wild-type Miro1 (Figure 15). The consequence of this effect resulted in less outward migration from the explant as compared to GFP controls (Figure 15).

To test if this migration phenotype impacted the ability of Miro1K208 electroporated INs in the context of a complex environment, we utilized whole brains slice electroporation and culturing techniques. The MGE of brain slices from E12.5-E13.5 embryos were electroporated with M1K208 and pCAG-IG and cultured to allow neurons to reach the cortex. Fewer total electroporated cells in Miro1K208 slices invaded the cortex compared to controls (GFP: 20 ± 2 , M1K208: 7 ± 2 , percent of total electroporated cells reaching cortex \pm SEM, Figure 16). This suggests that the increased direction changes of Miro1K208 cells indeed reduce the ability of cells to reach the cortex.

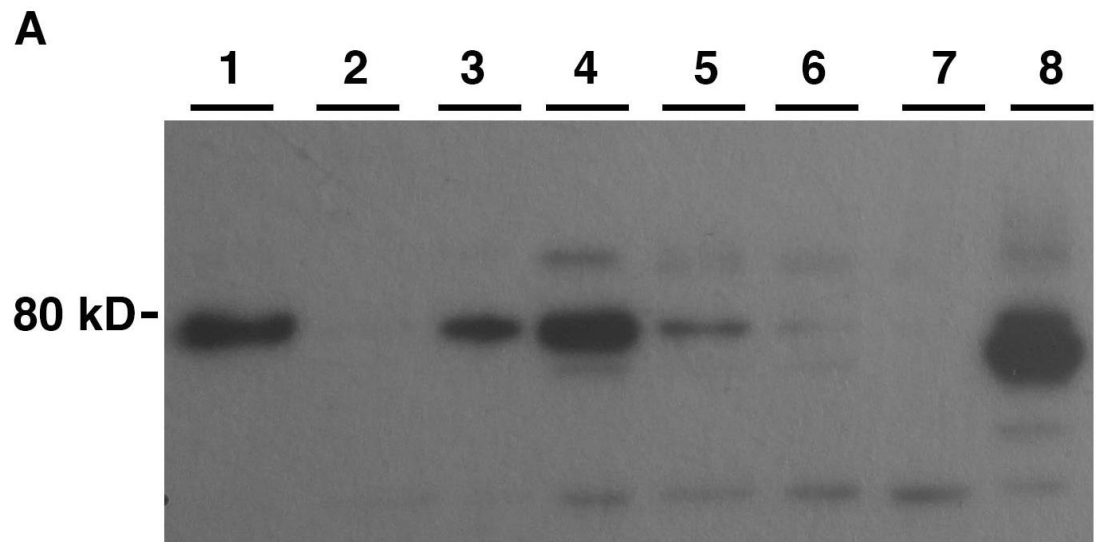


Figure 13. Miro1 protein is detected in E14.5 striatum

(A) Miro1 is detected in E14.5 whole brain and E14.5 striatum, lanes (L) 1 and 3. L1: E14.5 whole brain (WB), L2: WB lysate 3, L3: Striatum, L4: Striatum lysate, L5: L6: L7: L8: COS cells transfected with pRK5HA-Miro1-V13-myc.

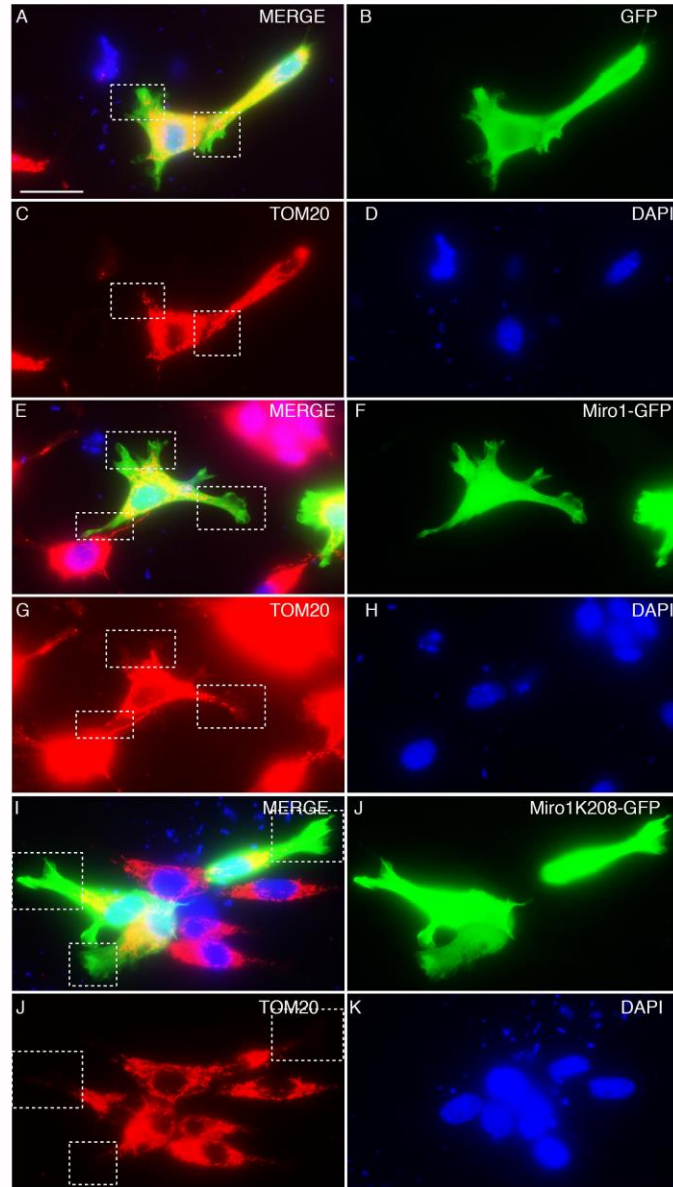


Figure 14. pCAG-IG-Miro1K208 expression in 3T3 cells results in perinuclear localization of mitochondria.

(A-H) Representative images of transfected, fixed 3T3 cells marking mitochondria (Tom20, Red), cytosol of transfected cells (GFP, green) and nuclei (DAPI, blue) in control pCAG-IG (A-D), pCAG-IG-Miro1 (E-H) and pCAG-IG-Miro1K208 (I-K). Dotted lines indicate peripheral edges of transfected cell with mitochondria in control, whereas mitochondria are reduced in those areas with pCAG-IG-Miro1K208.

Expression of dominant negative Miro1 does not alter gross mitochondrial localization behavior

We then wanted to determine if there were observable changes in IN mitochondrial localization with Miro1K208 expression. We have previously observed clustering of mitochondria in distinct regions in INs migrating *in vitro* (Figure 3). INs that had mitochondrial localization correlating to stage of migration (stage 1: tapered leading process with mitochondria concentrated anterior to the nucleus, stage 2: clear cytoplasmic thickening of the leading process with mitochondria concentrated in the thickening, and stage 3: A clear trailing process with corresponding mitochondrial clustering) were scored as normal, and INs in which mitochondria were diffusely distributed, or had mitochondria localized out of synchrony with cell stage were scored as abnormal.

This proved difficult, as electroporated cells are often located further inward from the explant edge, which can reduce antibody penetration. Although a greater number of cells should be assessed, gross mitochondrial localization did not appear to differ significantly between control and Miro1K208 expressing cells (GFP: $87 \pm 1\%$; M1K208: $84 \pm 3\%$, % cells with normally localized mitochondrial pattern \pm SEM, Figure 17). Although gross localization appeared normal, it is possible that Miro1K208 has more subtle effects on mitochondrial trafficking and localization. To determine if Miro1K208 expression altered mitochondrial trafficking rates during migration, we co-electroporated p-CAG-mito-DsRed with p-CAG-GFP and M1K208 constructs into MGE explant culture and examined migrating cells. The detection of the rapid migration behaviors of mitochondria in INs requires imaging techniques with both high-resolution optics, and rapid image capturing rates. First, we assessed imaging capabilities on spinning disk microscopy. We found this required laser powers that could be traumatic to cells, often resulting in cells that did not migrate. In cells imaged, mitochondria exhibited both

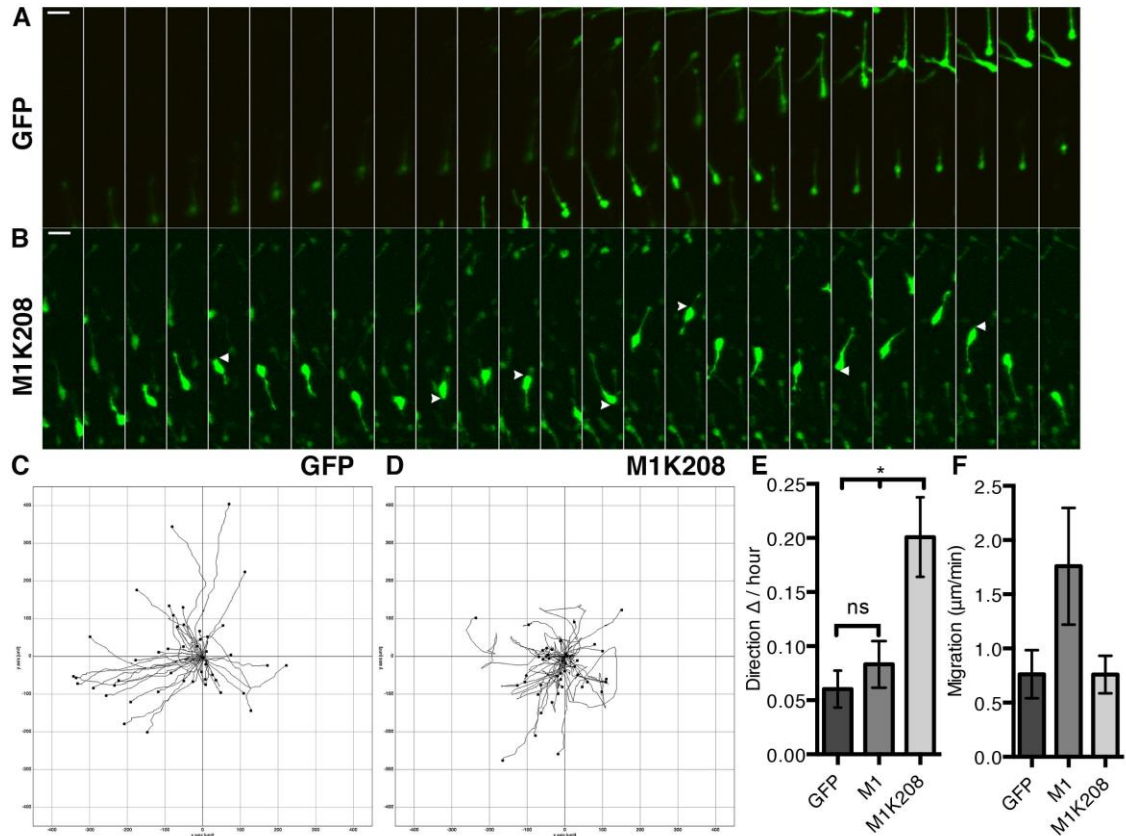


Figure 15. Expression of Miro1K208 increases direction changes in migrating INs *in vitro*.

(A,B) Migrating interneurons electroporated with (A) GFP, or (B) M1K208. White arrows indicate direction changes of the cell. (C, D) Migration tracings of 50 cells each of (C) GFP and (D) M1K208 cells. (E) Quantification of turns per cell for GFP, M1, M1K208 cells $n=3$ replicates, minimum 150 cells total, $p < 0.01$. (F) Migration rate of GFP, M1 and M1K208 expressing cells. $n=3$ replicates, minimum 150 cells total, ANOVA with Bonferroni correction, ns.

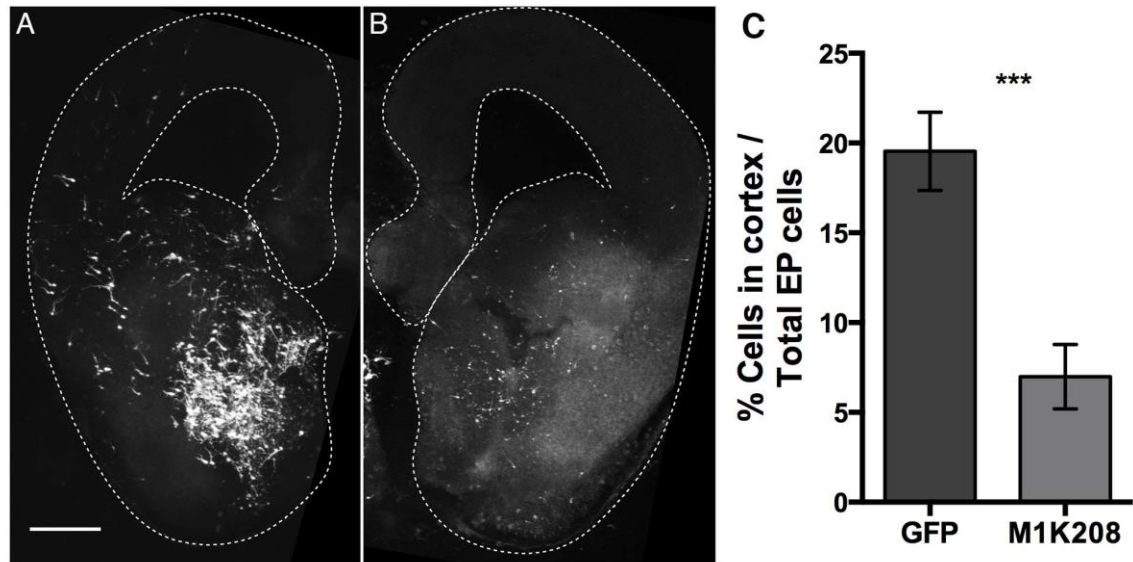


Figure 16. Miro1K208 expression reduces invasion of migrating INs into the cortex in slice culture

(A, B) MGE of slices from E 12.5-13.5 embryos focally electroporated with (A) pCAG-IG, or (B) pCAG-IG-Miro1K208 (M1K208). Scale bar: 300 μ m. (C) Quantification of the percent of total electroporated INs that invaded the cortex. GFP, n = 20, M1K208, n = 9. (***, p<0.001, unpaired t-test with Welch's correction)

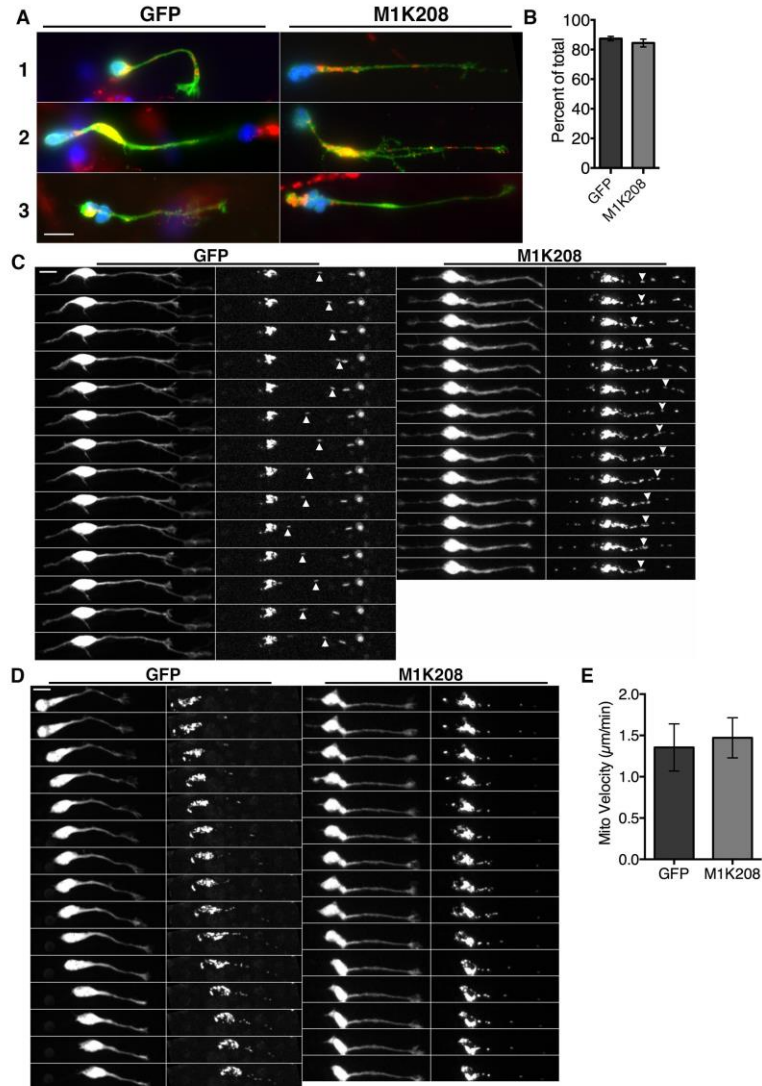


Figure 17. **Expression of Miro1K208 in migrating interneurons does not cause gross mitochondrial localization defects.**

(A) Representative INs electroporated with GFP or M1K208-GFP. Scale bar: 15 μm . (B) Percent of cells with normal distribution of mitochondria $n = 8$ GFP, 3 M1, 6 M1K208. Minimum of 75 cells assessed each construct. (C, D) Imaging of cells co-electroporated with GFP or M1K208 and mtDsRed, 30 sec. per frame. In (C), white arrows in the right panel follow a trackable mitochondria. (E) Mitochondrial velocity in GFP and M1K208 electroporated interneurons. $n=15$ GFP cells, $n=8$ M1K208 cells.

anterograde and retrograde movement of mitochondria within the leading process (Figure 17C and 17D). Preliminary data suggests mitochondria migration rate is not impacted by expression of Miro1K208 (Figure 17E). However, considering these cells were not migrating normally, it is unclear if the mitochondrial trafficking behavior observed matches what would occur during normal migration. Additionally, co-electroporation efficiency with mito-DsRed and pCAG-IG/Miro1K208 was too low to generate enough cells to assess mitochondrial trafficking behavior adequately.

As an alternative method, we imaged migrating neurons with two-photon microscopy. We did find that this imaging system was capable of capturing migrating interneurons with high frequency (as high as 15s intervals) for long periods of time without compromising cell migration and cell health (Figure 18C). However, mito-DsRed was not detectable in this system (Figure 18A, 18B). Mitotracker Red was detectable in this system and allowed for high-resolution tracking of mitochondria. However, electroporated cells are often farther away from the outer edge of the migrating cells and the dye does not penetrate into the explant sufficiently for adequate signal detection. We then examined whether expression of Miro1K208 would interfere with the radially directed migration of PN neurons. Embryonic day 14.5 embryos were electroporated with M1K208, or control constructs. After 4 days, there were increased numbers of Miro1K208 cells in the VZ/SVZ of the cortex, but this was not statistically significant (Figure 19B). We then assessed PNs at P15, after which PN migration is complete. Interestingly, although Miro1K208 cells do reach outer layers, there is a subtle defect, with a 36 percent increase of Miro1K208 cells in bin 7 and a 23 percent decrease of Miro1K208 cells in bin 8 compared to controls (Figure 19D).

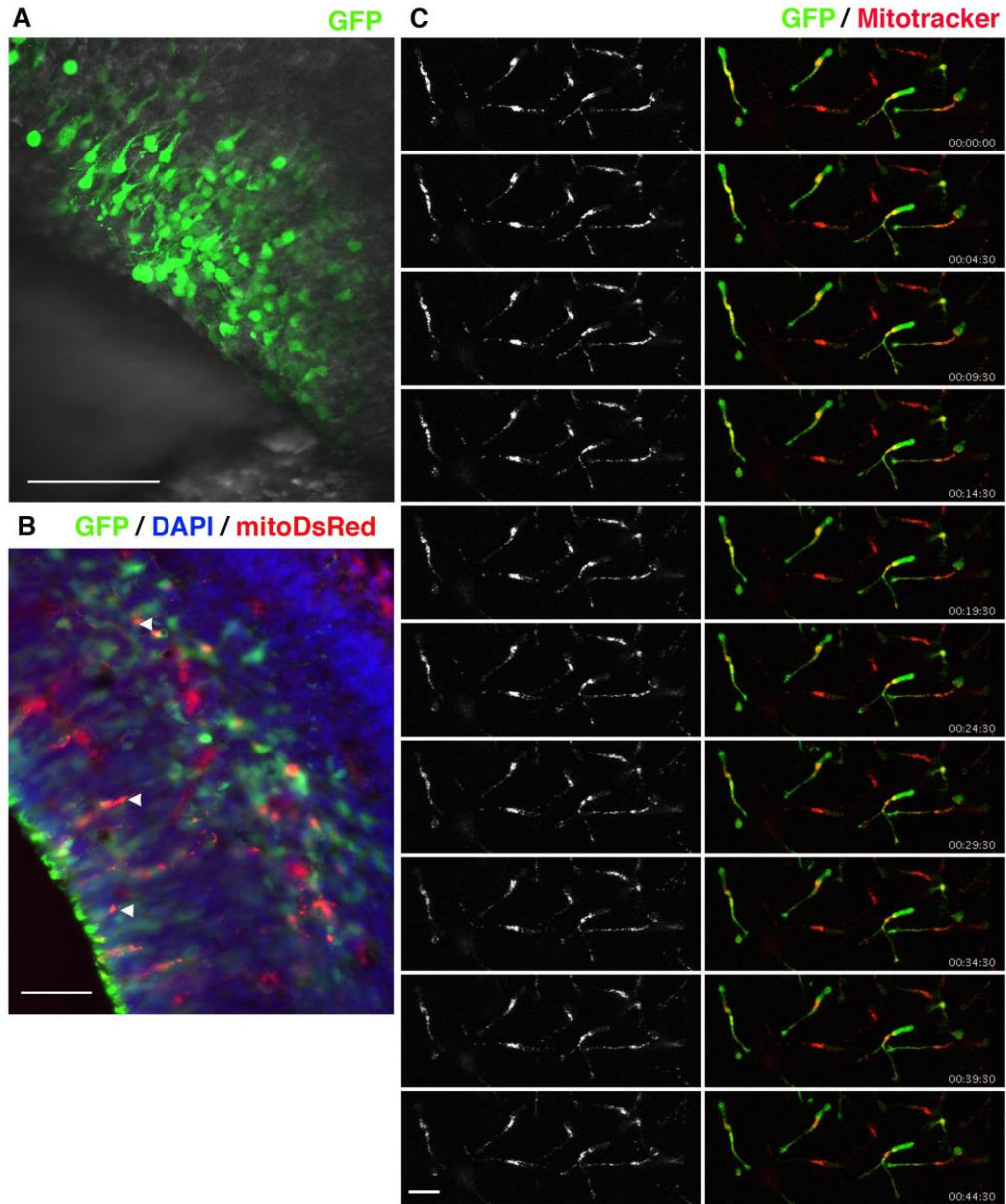


Figure 18. **Imaging techniques examining mitochondria**

(A) Two-photon image of radial cortical cells electroporated with GFP and MitoDsRed. The filters are unable to pick up the signal from DsRed, (B) clearly seen in fixed tissue from co-electroporated brains. (C) Two-photon live imaging of Dlx5/6-Ires-GFP interneurons stained with MitoTracker red displaying normal migratory behavior. Cells were imaged in a single plane, every 30 seconds. Scale: 25 μ m.

Discussion

We found the expression of Miro1K208 compromised interneuron migration by disrupting progressive movement of migrating cells. This disruption resulted in an inability of M1K208 expressing interneurons to reach the cortex in cultured embryonic brain slices. Interestingly, this migration phenotype was observed during the interference of oxidative phosphorylation in migrating INs (Figure 5 and 6).

Due to the similarities between the phenotypes, it would be important to determine if the dominant negative form of Miro1 has any effect on mitochondrial function. A limitation of our experimental model is the difficulty with which to measure the mitochondrial function and respiration in migrating interneurons. The XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA), although uniquely capable of measuring active respiration in cell culture wells rather than cell suspensions, often requires large numbers of purified cells (Wu, Neilson et al. 2007). Multiple attempts to measure the respiration of migrating interneurons from explants utilizing this system were unsuccessful (data not shown). Explant cultures also contain a mixed population of dividing neural progenitor cells, which likely have a different metabolic profile compared to the oxidative phosphorylation of migrating INs. Additionally, electroporation of the MGE is relatively inefficient, and very few cells expressed introduced constructs (approximately 50-200 cells per explant). An alternative would be to express fluorescent ATP sensor constructs targeted to the mitochondria versus cytosol (Tarasov and Rutter 2014). However, these tools have not been validated in interneurons which are a highly complex cell type to electroporate and image. Although we can infer that the impact on mitochondrial ATP production is likely intact due to the normal interneuron migration observed in M1K208 expressing INs (Figure 15F) and it has been shown that Miro1K208 does not impact mitochondrial function in rat hippocampal neurons (Macaskill, Rinholm et al. 2009), we cannot

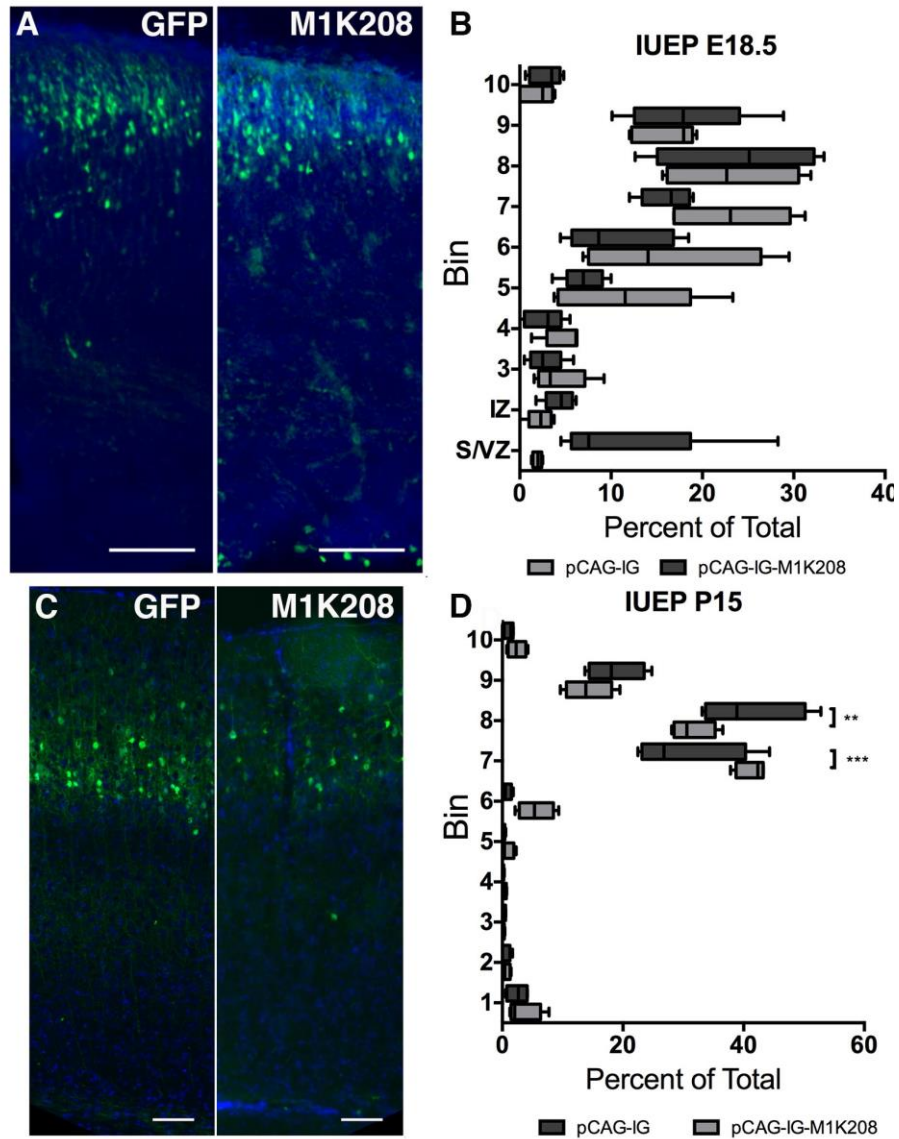


Figure 19. Miro1K208 has subtle effects on radial migration

(A) Fluorescence immunohistochemistry of radially migrating pyramidal neurons (PNs) 4 days after *in utero* electroporation. (B) Quantification of percent of total electroporated cells in cortical bins. $n = 5$ for control, and Miro1k208. Scale bar is $100 \mu\text{m}$. 2-way ANOVA with Sidak's correction. (C) Fluorescence immunohistochemistry of radially migrating pyramidal neurons at P15. (D) Quantification of percent of total electroporated cells in equally divided cortical bins. $n = 5$ for control, and Miro1k208. 2-way ANOVA with Sidak's correction. (A, C) Scale bar is $100 \mu\text{m}$.

definitively conclude that M1K208 has no effect on ATP production from oxidative phosphorylation.

Mitochondria express a dynamic migratory pattern within migrating INs (Figure 3). It is unclear if this subcellular localization of mitochondria plays an important role in IN migration. Although expressing a dominant negative form of M1K208 in hippocampal neurons interferes with mitochondrial trafficking (Macaskill, Rinholm et al. 2009), we were unable to confirm the effect of M1K208 on mitochondrial dynamics in migrating interneurons. Imaging migrating neurons requires a balance between adequate optical resolution, adequate laser power, and high PMT sensitivity. The movement of migrating INs is a process occurring over minutes, while mitochondria can move rapidly in seconds. Often the laser power, z-stack resolution and high-frequency imaging intervals to capture mitochondrial movements resulted in cells that would no longer migrate (Figure 17C and 17D). Additionally, the process of transporting INs to imaging facilities also resulted in cells that would not appear healthy (collapsed leading processes and absence of migration).

Although our preliminary analysis did not detect changes in mitochondrial migration rates, these cells were not exhibiting normal migration behavior and may have been physiologically compromised, which can alter mitochondrial behavior (Chang and Reynolds 2006). However, we did observe mitochondria within an extended trailing process of a Miro1K208 expressing cell, which was not seen in a GFP cell with similar morphology (Figure 17D). The continued presence of mitochondria in this extended trailing process may contribute to the persistence of this structure, but significantly more cells need to be assessed.

Miro1K208 may alter the percent of moving mitochondria, alter behaviors such as pausing and docking, or prevent the exclusion mitochondria from certain regions. The imaging methodology utilized to measure mitochondrial migration did not have sufficient resolution to

assess these behaviors. Thus, the evaluation of mitochondrial trafficking in cells exhibiting normal migration behaviors would be required to categorize the effect of Miro1K208 on mitochondrial trafficking behaviors in migrating neurons.

Two-photon microscopy is ideal for reducing photo-toxicity, and appears optimal for assessment of mitochondria in migrating cells (Figure 18), and we were able to capture GFP positive interneurons treated with mito-tracker at high time resolution without compromising cell migration. However, limited dual excitation of mito-dsred and M1K208-GFP prevented our ability to detect mitochondria in co-electroporated cells (Figure 18) utilizing this imaging methodology. Mito-tracker penetration into electroporated explants is insufficient to reach cells expressing GFP constructs (data not shown). Utilizing ultra-resolution microscopy, or better filters may give the resolution and excitation required to capture subtle changes in mitochondrial dynamics in these cells. Additional genetic tools may also facilitate this process. Genetic mouse models are available with fluorescently tagged mitochondria *Thy1-mitoCFP*, which would allow for tracking of mitochondria in electroporated cells. (Misgeld, Kerschensteiner et al. 2007).

A Miro1 knockout would provide a valuable tool as well. This particular mouse model exists, and although the gross structure of these mutant cortices was normal in Miro1 knockouts (Nguyen, Oh et al. 2014), interneurons were not assessed. MGE explant cultures from Miro1^{-/-} *Dlx5/6Cre-Ires-GFP* paired with vital mitochondrial dyes would allow examination of the effects of Miro1 loss on mitochondrial dynamics in pure cultures of Miro1 deficient migrating interneurons would be useful. If this genetic tool were utilized, experiments assessing interneuron migration would have to be performed, as our prior data was utilizing a dominant negative form of the protein rather than a genetic knockout.

An alternative method for addressing this experimental challenge would be to manipulate other aspects of the mitochondrial-trafficking protein network. The knockdown of Syntabulin

results in a significant reduction of anterograde migration of mitochondria and reduced mitochondria in distal axonal processes (Cai, Gerwin et al. 2005). However, this protein has been associated with the movement of vesicles within neurons (Su, Cai et al. 2004). Knockdown of APC slows Miro/Milton anterograde trafficking of mitochondria in multiple cancer cell lines (Mills, Brocardo et al. 2016). However, APC also has additional functions in microtubule dynamics and thus experiments must be conducted to separate the consequences of these two functions of APC. Overexpression of Syntaphilin results in immobilization of mitochondria in axonal mitochondria, and is a candidate to test a more drastic impact on mitochondrial movement in migrating INs (Chen and Sheng 2013).

Confirming abnormal mitochondrial localization behavior was not successful in this study. Data from Macaskill et al. suggests that M1K208 expression interferes with the ability for hippocampal neurons to target their mitochondria during neuronal firing (Macaskill, Rinholm et al. 2009). Fixed cells electroporated with Miro1K208 and Miro1GFP had grossly normal mitochondrial localization, suggesting that Miro1K208 may cause a subtler impact on mitochondrial localization or dynamics in interneurons. Conclusive evidence of altered mitochondrial dynamics requires live-imaging of mitochondria within electroporated cells, and although preliminary data suggests that mitochondrial migration rates are not affected, higher time resolution studies are required to fully examine changes in mitochondrial dynamics in migrating interneurons.

We previously found that migrating PNs were not impacted by manipulations in oxidative phosphorylation (Figures 6 and 10). However, we did not assess PN at later stages (P15). The alteration of mitochondrial trafficking having subtle impacts on radial migration provides evidence that radial migration can be effected by manipulations in mitochondria. Radially migrating PNs may require mitochondria to be persistently localized in the anterior perinuclear region for local ATP or Ca buffering. Live imaging experiments would provide additional

information if changes in mitochondrial trafficking would be detectable, however adequate time and spatial resolution in slice culture is likely to be a significant challenge. Targeting a protein such as Syntaphilin, which would result in a more severe impact on mitochondrial trafficking behavior may cause a more pronounced phenotype that may be more easily detected.

Although we were unable to confirm disruption of mitochondrial localization with our available resources, this preliminary work is consistent with a model in which migrating interneurons are more sensitive to mitochondrial perturbations compared to their radially migrating counterparts.

Material and Methods

Constructs

pRK5HA-Miro1k208-GFP, Miro1V13-myc and Miro1-GFP plasmids were kindly provided by Drs. P. Appenström and G. Hajnoczky. These base constructs were used to construct pCAG-IG-Miro1 and pCAG-IG-Miro1K208 by PCR cloning. The coding sequences of Miro1 and Miro1K208 with the following primers: FW: accg gaattc cacc atgaagaagacgtgcggatcct. R: acttca acgcgt tcaattgcatcgtcatccttgtaatctcgctgttcaataatgctttgtacatagc. Product was inserted into linearized pCAG-IRES-EGFP by utilizing EcoR1 and MluI sites. Construct fidelity and mutations were confirmed by sequencing.

Cell culture

3T3 or Cos cells were transfected with indicated plasmids utilizing Fugene 6 (Roche), and cultured for 2 days prior to fixation or protein isolation.

Mice

CD1 mice were used for all experiments. Both sexes were used for experiments. The Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia, Philadelphia, PA, approved all studies.

Western Blot

Protein was obtained from E14.5 whole brain, micro-dissected striatum, 3T3 cells, and COS cells transfected with pRK5HA-Miro1V13-myc as a positive control. Protein was run on a 10% SDS page gel. Blots were probed with mouse-anti-Rhot1 (Sigma) at 1:1000, and goat-anti-mouse-HRP (Biorad) at 1:2000, and developed with ECL (Thermoscientific).

Brain explant culture

Explants were generated from at embryonic day 14.5. Embryonic brains were dissected on ice cold HBSS (Sigma), embedded in 4% low-melt agarose (Lonza) and sliced into 250 μ m thick sections. Explants were then electroporated with constructs at a concentration of 3 μ g/ μ L. The MGE was then microdissected in DFS (DMEM:F12; Invitrogen, with 10% fetal bovine serum, 40 mM Glucose, 40 μ M L-Glutamine) and cut into ~200 μ m cubes, and were placed in a gel of 35% acidic collagen (0.0035 N acetic acid in rat tail collagen; BD Pharmogen), 50% matrigel (BD Pharmogen), 15% DM (DM; glucose free DMEM (Invitrogen), N2 supplement (Gibco), +/- Glucose as indicated) and 5 mM HEPES. Explants and gel incubated at for 30 minutes at 37 °C 5% CO₂ to set, and DM medium added.

Brain slice electroporation and culture

Brains were dissected on ice cold HBSS (Sigma), embedded in 4% low-melt agarose (Lonza) and sliced into 250 μ m thick sections on a Leica VT1200S vibratome, and placed on

filter inserts (Millipore). Slices were covered multiple layers of matrigel collagen mixture (as previously described) incubated at 37 °C 5% CO₂ for 20 minutes to recover. Slices were then placed in pressure injected with ~3 μL of DNA at a concentration of 3 μg/μL and electroporated using an NEPA21 electroporator. Slices were then cultured for 4 days in DM medium before fixation with 4% PFA for 1 hr RT and imaged via confocal imaging.

Immunohistochemistry and Immunocytochemistry

3T3 cells were fixed for 10 mins at RT in 4% paraformaldehyde (PFA), and rinsed in PBS buffer, and permeabilized for 20 minutes with 0.04% Triton. MGE explants were fixed in for 12 minutes in 4% PFA and PHEM (PIPES, HEPES, EGTA, and MgSO₄) buffer at 37 °C. After washing in 1x PBS, explants were stored in 1x PBS at 4 °C until staining. For staining, explants were treated with 1:50 collagenase (Roche) for 15 minutes, permeabilized for 15 minutes with 0.04% Triton. Explants were blocked with 10% normal goat serum for 1 hour at RT. Explants and 3T3 cells were incubated overnight at 4°C with primary antibodies anti-Tom20 (rabbit; Santa Cruz, 1:750), anti-GFP (chicken Invitrogen 1:200).

Intrauterine electroporations

Embryos at E14.5 were electroporated *in utero* as previously published (Nasrallah, Cho et al. 2012) with pCAG-IG (Addgene 11150; 2 μg/μL) or pCAG-IG-Miro1K208. Embryos were electroporated at E14.5 and harvested at E18.5, or at P15. Pups at P15 were anesthetized and perfused with 4% PFA and fixed overnight at 4°C. Brains were frozen and sectioned at 30 μm (E14.5) and 12 μm (P15) sections. For assessment of two-photon microscopy for imaging mitochondria, embryos were electroporated with pCAG-IG, and MitoDsRed, and harvested 24 hours later.

Microscopy

Time-lapse images were acquired at indicated intervals for a minimum of 6 hours with an Olympus Fluoview (FV10i) confocal microscope at 37°C, 5% CO² at 10X magnification with 2X zoom for treatment protocols in 5-minute intervals and 10X magnification in slices in 10-minute intervals. Images of fixed explants INs and IUEP brain sections were taken on a Leica CTR600 fluorescent microscope. Spinning disk microscopy, Z-stacks were at 2-micron intervals with captures every 2 minutes. Two-photon microscopy, with z-stacks of 2.5 microns.

Quantification

Cell migration characteristics.

Cell migration speed was calculated as previously published (Lysko, Putt et al. 2011). Cells were selected at random and followed during the live imaging process using ImageJ Manual Tracking plugin. For *in vitro* experiments, a minimum of 25 cells per n was followed.

To assess cortical migration, the cortical thickness was divided into 10 bins. For E18.5, bin 1 consisted of the VZ/SVZ recognized by DAPI staining, bin 2 consisted of the cell-sparse IZ, and the remaining cortical plate was divided into 8 bins. For P15, the cortex was equally divided into 10 equal bins. Up to three 100 µm alternating regions of interest spanning the thickness of the cortex per section and a minimum of 3 sections per individual were counted. The percent of total electroporated cell per bin was calculated to account for differences in transfection efficiency.

Mitochondrial localization and tracking

Mitochondrial localization was quantified as previously described (Material and Methods, Chapter 1). Mitochondria that were distinguishable were tracked utilizing ImageJ Manual Tracking plugin.

Statistics

Prism 6 software was used for all statistical analysis. Data were tested for normality using either the K-S test or the D'Agostino & Pearson omnibus normality test. If the data were not normal, non-parametric analysis was utilized. $p < 0.05$ were considered significant. All values are represented as mean \pm SEM unless otherwise indicated.

CHAPTER 4: CONCLUSIONS / FUTURE DIRECTIONS

Our data reveals a fundamental difference between the requirements for non-radial versus radial migration in metabolism and has implications in our understanding of the impact of mitochondrial dysfunction to cause neuronal deficits of developmental origin. Contrary to the hypothesis that embryonic neurons are glycolytic in their metabolism (Surin, Khiroug et al. 2012), we show conclusive evidence that mitochondrial oxidative phosphorylation is essential for interneuron migration. Inhibition of mitochondrial OXPHOS resulted in decreased migration rates, increasing direction changes and abnormal centrosome positioning. Cells also exhibited morphological changes including increased trailing process length and life as well as decreasing leading process length, branch length, and leading branches per cell. Collectively, these changes contributed to a reduction in the ability of interneurons to migrate into the cortex.

Mitochondrial OXPHOS impacts IN leading process behavior

In our studies, inhibiting OXPHOS pharmacologically results in decreased leading process length and decreased branching (Figure 5 I-K), a potential indication of leading process and branch instability. A balance of leading process dynamics versus stabilization and appropriate branching behaviors are required for normal interneuron migration (Kappeler, Saillour et al. 2006, Martini, Valiente et al. 2009, Gopal, Simonet et al. 2010, Lysko, Putt et al. 2011). Interneuron leading process dynamics and leading process branching are due to modulation of the actin and cytoskeleton network (Lysko, Putt et al. 2014). Thus, investigating the specific effects of pharmacologic and genetic OXPHOS inhibition on the cytoskeletal network is a logical direction of future research.

Dynamic and stabilized microtubules have been assessed in migrating interneurons with acetylated and tyrosinated tubulin (Gopal, Simonet et al. 2010, Lysko, Putt et al. 2014). In migrating interneurons stabilized microtubules are localized throughout the leading process shaft, while dynamic microtubules are more prominent within the trailing process, leading process tip, and newly forming branches (Lysko, Putt et al. 2014). Preliminary experiments indicate that acetylated tubulin in BA treated interneurons does not extend down to the distal leading process compared to control cells (Figure 20A-C). Although tyrosinated tubulin remains to be evaluated, this reduction of acetylated tubulin warrants further examination of microtubule network regulators. Several microtubule-associated proteins (MAPs) known to contribute to microtubule stability (Tau, DCX, APC, and MAP2) and are expressed along the leading process in migrating interneurons (Lysko, Putt et al. 2014). Of these MAPs we will evaluate Tau, DCX and APC, MAPs that have been linked to mitochondrial behavior, as potential candidates for evaluating microtubule regulation in OXPHOS-inhibited interneurons. We will also discuss DISC1, a protein known to modulate microtubule stability through its interactions with MAP1 (Morris, Kandpal et al. 2003).

Tau has been implicated in the regulation of mitochondrial morphology in radially migrating neurons. Although examination of Tau localization in OXPHOS-inhibited cells is a relevant experiment to conduct, knockdown or genetic ablation of Tau results in abnormal radial migration (Sapir, Frotscher et al. 2012). We did not observe and deficits in radial migration in our previous pharmacologic and genetic interference of OXPHOS, and thus Tau localization and function may not be impacted. Although loss of DCX in migrating interneurons results in decreased branch stability, migration speeds are not significantly impacted. Thus interpretation of any changes in DCX in OXPHOS may not directly contribute to the profound migration phenotype we see in INs (Kappeler, Saillour et al. 2006).

The MAP adenomatous polyposis coli (APC) has been implicated in mitochondrial trafficking in colorectal cancer cells (Mills, Brocardo et al. 2016). Additionally, APC's regulation of the microtubule network is distinct between radial and tangentially migrating cells; APC regulates microtubule (MT) dynamics via the microtubule severing protein p60-katanin in INs, but not PNs (Eom, Stanco et al. 2014). APC deficient cells have increased expression of and phosphorylation of p60-katanin. This subsequently results in increased p60-katanin activity, and increased MT severing, thus decreasing MT stability. Considering our interneuron selective deficit with OXPHOS inhibition, APC is the most attractive candidate of the aforementioned MAPs to evaluate in our experimental models.

DISC1, a protein involved in multiple cellular processes, supports microtubule stabilization by interacting MAP1A and the centrosome (Morris, Kandpal et al. 2003). DISC1 knockdown results in long leading process, the opposite of our phenotype. However, DISC1 is expressed in the leading process tip and the trailing process, and it is possible that its functions are distinct in these two regions. Additionally, DISC1 has also been shown to interact with mitochondrial trafficking adaptors and mitofusins (Norkett, Modi et al. 2016). Thus, considering our distinct trailing process phenotype, DISC1 is still of interest for evaluation in this region.

One way to assess the contribution of microtubule instability within OXPHOS-inhibited cells is through pharmacologic stabilization of microtubules. Several inhibitors of microtubule catastrophe have been tested in migrating cancer cell models, such as indanocine, which results in widespread tubulin acetylation, and thus low levels of this inhibitor may reduce leading process deficits seen BA-treated cells (Kapoor and Panda 2012). This will allow us to evaluate the contribution of leading process instability to the migration phenotype we observed. In addition to microtubule stabilization, microtubule polymerization behaviors may be altered in OXPHOS-inhibited cells. In addition to evaluation of tyrosinated tubulin in fixed OXPHOS-inhibited cells,

live-imaging experiments allowing for real-time analysis of microtubule dynamics should be conducted. Live time-lapse fluorescence microscopy of EB3, a microtubule endcap protein, would allow higher resolution evaluation of microtubule polymerization during active migration and leading process branching behaviors (Steinecke, Gampe et al. 2014).

Interneuron branching can be characterized into branched leading process and interstitial branches that emerge from the leading process (Gopal, Simonet et al. 2010, Lysko, Putt et al. 2011). More in-depth analysis of branching behavior can be pursued in our model of OXPHOS inhibition to better characterized branching deficits observed into interstitial branching versus leading process splitting. This information will also contribute to the characterization of deficits in branch initiation and branch persistence. Branching along the leading process is initiated by filamentous actin protrusions being stabilized by tyrosinated tubulin (Lysko, Putt et al. 2014). We have preliminary data suggesting that inhibition of leading process phalloidin protrusions are reduced (Figure 20C and 20D). Although we did not assess tyrosinated tubulin within the leading process, it is likely that the absence of the actin protrusions along the leading process in treated cells would concurrently result in the absence of tyrosinated tubulin associated with these transient leading process structures. Further investigations into why these actin protrusions are absent from the leading process of OXPHOS-inhibited cells and their significance to changes in branching dynamics should be investigated.

In post-migratory neurons, axon branches originate from actin patches that proceed Arp2/3 targeting and actin filopodia formation (Spillane, Ketschek et al. 2011). In sensory axons, this is associated with localized areas of PI3K activity and linked to actively respiring mitochondria (Ketschek and Gallo 2010, Courchet, Lewis et al. 2013). Although the localization of Arp2/3 in migrating interneurons is unknown, it is a strong candidate to assess if the initiation of leading process filipodia formation is dependent on oxidative phosphorylation. Actin filipodia

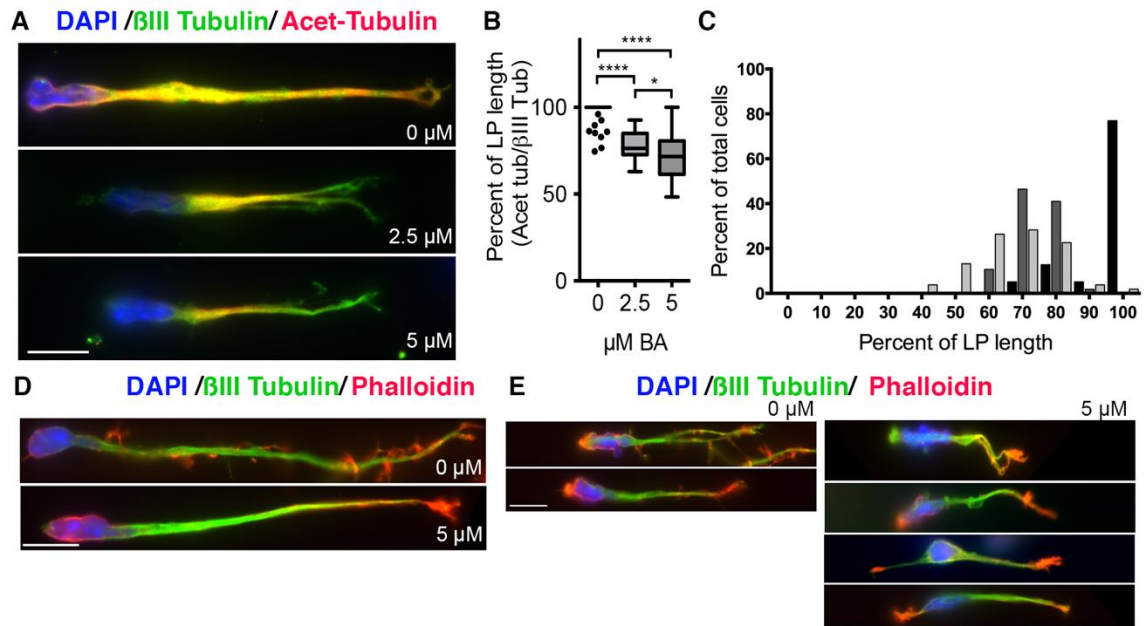


Figure 20. Bongkreikic acid treated cells exhibit changes in acetylated tubulin and F-actin localization

(A) Representative immunofluorescent images of *in vitro* cultured MGE cells treated with BA.

DAPI = blue, BIII-Tubulin = green, and acetylated tubulin = red. (B). Quantification of the

percent of total LP length expressing acetylated-tubulin. $N \geq 34$ cell each. (C) Distribution of

acetylated tubulin expression in the leading process. (D-E) F-actin staining in BA-treated cells.

Note the reduced F-actin protrusions along the leading process of BA-treated cells. (E) F-actin is

present in the trailing process of vehicle treated cells. In BA-treated cells with normal trailing

processes, there were cells seen with no F-actin. Cells with abnormally long trailing processes

have F-actin localized to the tips, similar to the leading processes. F-actin in the leading process

tip does not appear to be impacted. (A-E) Scale bar = 10 μ m.

are subsequently stabilized by formins, like mDia, and it is thought that this protein can also impact RhoA regulation of actin patches in axonal branching (Spillane and Gallo 2014). Indeed, mDia has been implicated in selective defects in interneuron migration in F-actin regulation, especially in the trailing process (Shinohara, Thumkeo et al. 2012).

F-actin at the leading tip is also thought to contribute to the splitting of the leading process into terminal branches (Bellion, Baudoin et al. 2005, Lysko, Putt et al. 2014). OXPPOS inhibition does not appear to effect F-actin localization at the leading process tip of migrating interneurons (Figure 20C and 20D). More refined examination of branching behavior will determine if interstitial branching is selectively impacted with OXPPOS inhibition. If reduced leading process splitting is also observed, it is possible that downstream regulators of leading process branching from the LP tip are impacted (Lysko, Putt et al. 2014).

Branch stability has been previously assessed in models of deficient migration (Gopal, Simonet et al. 2010) by measuring acetylated versus tyrosinated tubulin in the structure of existing branches. Although co-staining of acetylated and tyrosinated tubulin were not successful in preliminary experiments, further analysis of acetylated tubulin within identifiable branches seen in OXPPOS-inhibited cells will be performed.

Regulation of the cytoskeleton consists of a complex regulatory network that regulates the nucleation, extension, stabilization, and dynamics to facilitate the drastic changes in cell morphology observed in migrating interneurons. There are multiple potential studies to dissect the impacts on molecular regulators of the cytoskeletal network and the importance of mitochondria during this process. However, although preliminary data suggests changes in both microtubule and actin exist in interneurons with OXPPOS inhibition, these changes may be

secondary consequences to compromised polarity (reduced LP identity), or guidance signaling rather than direct regulation of the cytoskeleton.

Cell polarity is likely impacted by Inhibition of Mitochondria OXPHOS

Both inhibition of oxidative phosphorylation and the expression of Miro1K208 resulted in significant increases in direction changes and a loss of progressive migration that ultimately reduced the number of interneurons migrating into the cortex (Figure 5, 6, 8, 9, 15 and 16). These consistent abnormal migration behaviors, as well as the profound changes in trailing process behavior, suggest that mitochondria play a key role in trailing process function and interneuron polarity.

The trailing processes of OXPHOS-inhibited cells exhibit behaviors similar to their leading processes: extending, retracting, and even occasionally branching (see supplemental movies in (Lin-Hendel, McManus et al. 2016)). Further characterization of trailing process dysfunction with OXPHOS inhibition is an opportunity for future research. The trailing process is commonly identified by its morphological and cyclical traits; a small ephemeral cytoplasmic domain behind the nucleus opposite to the leading process that is most clearly distinguished during nucleokinesis, and by proteins that localize in this region when it is observed. Consistent with previous publications, we have confirmed the tendency of mitochondria to cluster in this region during nucleokinesis (Bellion, Baudoin et al. 2005). Molecular markers of the trailing process are sparse: f-actin concentrates in this region during nucleokinesis, and it is a site of myosin IIB localization (Martini and Valdeolmillos 2010). Preliminary studies examining the cytoskeletal components of OXPHOS-inhibited cells suggest that some OXPHOS-inhibited cells with normal morphology lack this F-actin localization (Figure 20D). Additionally, cells exhibiting abnormally long trailing processes express characteristic F-actin localization similar to the leading process tip

(Figure 20D). This immunocytochemical data should be confirmed with fluorescence live-imaging techniques utilizing fluorescent actin constructs such as lifeact or RFPP-UtrCH to observe real-time actin condensation in the trailing process in OXPHOS-inhibited cells (Riedl, Crevenna et al. 2008, Martini and Valdeolmillos 2010, Lysko, Putt et al. 2014). Additionally, staining for proteins associated with the leading process enriched in the leading process tip, such as cortactin or DCX would further support that elongated trailing processes in OXPHOS-inhibited cells have more leading process characteristics (Lysko, Putt et al. 2014).

It is possible that mitochondria function within this region by providing energy for myosin IIB phosphorylation and thus towards functional actomyosin contractions. Attempts to examine the status of myosin IIB phosphorylation in interneurons with immunohistochemistry was unsuccessful. Although fluorescence resonance energy transfer constructs exist to visualize phosphorylation of myosin II regulatory light-chain in cells (Yamada, Hirose et al. 2005), they have not been tested in interneurons. It is possible that myosin IIB localization to the trailing processes itself is disrupted during oxidative phosphorylation, and live imaging studies utilizing GFP-NMII mice, which express a GFP-tagged fusion protein of human myosin IIB would be a useful tool in investigating this further (Bao, Ma et al. 2007).

The regulation of interneuron polarity during migration is poorly studied, and there are few publications that describe phenotypes of loss of polarity in migrating interneurons. Thus, each of these studies provides potential insight to the phenotypes observed in our studies with manipulations of mitochondria.

Diap1/2, also known as mDia1 and 3, are Rho-regulated actin nucleators. Deficiency in mDia1 selectively impacts interneuron migration (Shinohara, Thumkeo et al. 2012). These cells exhibit abnormally localized centrosomes. Within these mutant neurons, F-actin condensation and

myosin IIB localization in the trailing process are abnormal. Considering that we see an absence of F-actin in the trailing process of some migrating interneurons treated with BA (Figure 20D), it is possible that as in mDia knockouts, that myosin IIB localization to the trailing process is impeded. The similarities between mDia knockouts and our model support the hypothesis that mitochondrial-generated ATP is required in the trailing process to support its function in migration. Another example of abnormal interneuron polarity during migration has been demonstrated by interfering with p53/Cdk5 phosphorylation of ErbB4 (Rakic, Kanatani et al. 2015). Although these cells exhibit differing branching abnormalities, abnormal polarity of migrating interneurons was observed. Interestingly, Cdk5 has been shown to regulate mitochondrial fission by phosphorylation of DRP1 (Jahani-Asl, Huang et al. 2015). If mitochondrial oxidative phosphorylation is disrupted in interneurons, it is possible that phosphorylation of key proteins involved in trailing process function does not occur, impeding the subsequent contraction of the trailing process to allow for appropriate nucleokinesis.

N-cadherin (N-cad), a cell adhesion molecule, is expressed along the migrating path of both radially and tangentially migrating neurons (Kadowaki, Nakamura et al. 2007). This adhesion molecule functions by homophilic interactions and is expressed in migrating cortical interneurons. Fascinatingly, Luccardini et al. discovered the essential role of N-cad in maintaining interneuron polarity during tangential migration (Luccardini, Hennekinne et al. 2013). Both overexpression of dominant negative N-cad and genetic ablation of N-cad revealed similar abnormalities in phenotype: extended trailing processes with increased polarity reversals, shortened leading processes, and abnormal centriole and Golgi apparatus localization. Additionally, myosinIIB localization was impaired with expression of a dominant negative N-cad construct *in vitro*. Previous studies have also shown that changes in N-cad signaling can result in changes in the primary cilium (Luccardini, Leclech et al. 2015). There are striking similarities to

the phenotypes observed in this work in comparison to interneurons undergoing inhibition of oxidative phosphorylation. It is possible that mitochondria play a key role in normal N-cadherin signaling in addition to potential effects on myosinIIB localization and phosphorylation.

The N-cad pro-migratory signaling cascade in interneurons not fully elucidated. One proposed pathway is via activation of MAPK/ERK signaling, and potentially regulating downstream RhoA activity via Rac1 and Cdc42 activation of p190RhoGAP. RhoA is known to play a role in interneuron migration (Katayama, Imai et al. 2013). Interestingly RhoA and formins have been shown to regulate the distribution mitochondrial localization (Minin, Kulik et al. 2006), providing a link between mitochondrial localization and known functional regulators of interneuron migration that are associated with the trailing process.

Proteins directly responsible for maintaining cell polarity and centrosome positioning are poorly studied in interneurons. Studies on polarity complexes in neurons have emphasized axon and dendrite specification in post-migratory neurons. The Par3/Par6/aPKC complex is thought to affect neuronal polarization by down-regulation of microtubule affinity regulating kinase MAPK2 and by activation of Rac1 via Cdc42 (Chen, Wang et al. 2006). Studies investigating genetic structural abnormalities in human patients revealed overexpression of LIS1, a protein known to impact neuronal migration, displayed aberrant migration and decreased expression of polarity proteins, suggesting that these proteins play a role in maintaining cell polarity during earlier stages of neurodevelopment (Bi, Sapir et al. 2009). In cerebellar radial glial cells, Par6 is necessary for normal migration (Solecki, Model et al. 2004). Although it is unclear if these polarity proteins play a similar role in migrating interneurons, they represent potential candidates to investigate if inhibition of OXPHOS results in a true loss of polarity.

One defining feature of interneuron polarity is the centrosome's localization anterior to the nucleus and within the leading process (Bellion, Baudoin et al. 2005). Indeed, in our experiments, we saw abnormal localization of centrosomes in *Ant1*^{-/-} interneurons that was exacerbated with additional inhibition of OXPHOS (Figure 11). The centriole associates closely with the primary cilium in migrating interneurons (Baudoin, Viou et al. 2012, Higginbotham, Eom et al. 2012). Conditional deletion of *Arl13b* in migrating interneurons results in erratic cell migration behavior, with decreased progressive migration. Additionally, these mutants display abnormal branches emerging from the rear of the cell similar to what we have characterized as extended trailing processes (Higginbotham, Eom et al. 2012). These studies also displayed abnormal responses to guidance cues, emphasizing a potential for a multifactorial mechanism leading to the abnormal migration observed in our studies.

Guidance Factors

Another possible mechanism for the reduced progressive interneuron migration following mitochondrial manipulations is abnormal responsiveness to guidance signals. As discussed above, it may be a contributing factor if mitochondrial manipulations interfere with the maintenance and function of the primary cilium. Additionally, the leading process itself plays a central role in directing oriented migration of interneurons in response to guidance cues (Martini, Valiente et al. 2009). Thus, we are presented with the possibility that abnormal leading process dynamics reduce the structural capacity of migrating interneurons to respond to directional cues properly or potential defects in primary cilium function result in the output of increased direction changes and impacts on polarity.

In our work, we did not address the effect of inhibition of oxidative phosphorylation on the ability of interneurons to respond to external cues. Although the spatiotemporal landscape of

guidance cues for interneurons *in vivo* is full of contextual variability and the responsible receptors and downstream effectors have not been conclusively defined, several guidance cue candidates have displayed motogenic, repulsive, and attractive cues to interneurons in explant culture. Slits, a secreted signaling molecule expressed in the MGE and LGE are thought to repel interneurons from the proliferative zones towards the dorsal pallium (Zhu, Li et al. 1999). When exposed to a source of Slit in culture, interneurons display a robust directional response. Semaphorins 3A and 3F expressed in the striatum are also known to repel MGE interneurons (Marin, Yaron et al. 2001). Nrg1-IG, a diffusible splice variant of neuregulin has been shown to be a robust attractant *in vitro* (Martini, Valiente et al. 2009). Each of these secreted molecules are potential candidates to investigate the ability of interneurons to respond to extracellular cues under the challenge of oxidative phosphorylation inhibition *in vitro*.

In addition to attractive and repulsive cues, there are a series of secreted factors in the ventral pallium that serve as pro-motogenic factors. Scatter factor (SF) and BDNF are expressed in the MGE (Powell, Mars et al. 2001). In the context of our explant model, these factors should be present, although it is possible the production and secretion of these molecules are impeded by OXPHOS inhibition. Providing these factors at high concentrations with substrates such as beads or with concentration gradients in microfluidic chambers may allow for a categorical assessment of the cells ability respond to external cues in an OXPHOS inhibition model (Higginbotham, Eom et al. 2012).

Additional roles of mitochondria in neuronal physiology

We have shown that the mitochondrial energetics are important for normal neuronal migration. Mitochondria have multiple roles within neurons in addition to acting energy production, including calcium buffering (Kwon, Sando et al. 2016), steroid hormone synthesis

(Osheroff and Hatten 2009), regulation of reactive oxygen species (Hou, Ouyang et al. 2012), apoptosis, and fatty acid synthesis (Kastaniotis, Autio et al. 2016). Each of these areas present avenues of inquiry for the potential complex and interconnected regulation of neurodevelopment. For the purpose of this discussion, we have focused on the role of mitochondria in calcium homeostasis.

Calcium oscillations have been observed in migrating interneurons and are required for normal interneuron migration (Bortone and Polleux 2009, Martini and Valdeolmillos 2010). These oscillations have been linked to GABA_A receptor depolarization and from Ca²⁺ release from the endoplasmic reticulum. Abolishing calcium transients during migration by chelation with BAPTA decreases actin condensations in the trailing process, and reduces myosin IIB phosphorylation (Martini and Valdeolmillos 2010). Manipulation of the potassium-chloride cotransporter, KCC2, directly impacts the frequency of calcium oscillations: high KCC2 abrogates calcium oscillations and results in increased pausing of interneurons (Bortone and Polleux 2009).

MCU, the calcium uniporter localized within mitochondria allows for a rapid uptake of calcium. Further, mitochondria can closely associate with the endoplasmic reticulum to facilitate this process. Interestingly, calcium oscillations have been shown in multiple cell types to support oxidative phosphorylation, as oscillations promote the activation of proteins and generation of substrates required for oxidative phosphorylation (Rizzuto, De Stefani et al. 2012). MCU function is necessary for normal oxidative phosphorylation (Griffiths and Rutter 2009, Mallilankaraman, Cardenas et al. 2015). It is possible that calcium oscillations during the early stages of interneuron migration support the energetic demand during tangential migration, and that this need decreases as interneurons shift towards their final destinations. In addition to determining if interfering with calcium oscillatory behavior interferes with mitochondrial localization, it would also be

fascinating to assess if interneurons expressing higher levels of KCC2 are less sensitive to perturbations in oxidative phosphorylation.

Mitochondrial localization and dynamics

We found that mitochondria display specific subcellular localization that differs between the dynamic localization in tangentially migrating interneurons and the static localization within radially migrating pyramidal neurons. We then sought to determine if manipulations in mitochondrial trafficking without interfering with mitochondrial function would be sufficient to disrupt interneuron migration.

Although we were unable to confirm whether mitochondrial mislocalization causes abnormal interneuron migration, there are examples of proteins known to interfere with interneuron migration that also describe abnormal localization of mitochondria (Yamada, Yoshida et al. 2009, Steinecke, Gampe et al. 2014, Norkett, Modi et al. 2016). It is unclear if the described mitochondrial mislocalization contributes to abnormal migration phenotypes in these genetic models. As discussed in Chapter 3, there are alternative reagents to test our hypothesis that the subcellular localization of mitochondria is required for normal interneuron migration, particularly in maintenance of interneuron polarity and trailing process contraction.

In addition to their trafficking behaviors, mitochondria undergo extensive fusion and fission. Multiple proteins involved in fusion and fission have been implicated in cell migration in the immune system and models of cancer cell migration (Campello, Lacalle et al. 2006, Desai, Bhatia et al. 2013, Zhao, Zhang et al. 2013). Thus, it is possible that these proteins play a role in the migration of neuronal cell types. There are currently studies in the literature examining mitochondrial fusion and fission in migrating neurons. Before manipulation of these proteins is pursued, fusion and fission of mitochondria within migrating interneurons should be assessed.

Mitochondrially targeted photoactivatable-GFP co-expressed with MitoDsRed has been shown to allow for tracking of mitochondrial fusion and fission in mammalian cells in culture (Karbowski, Cleland et al. 2014). If such technically demanding imaging experiments prove unfeasible, the overexpression and knockdown of these proteins are associated with abnormal mitochondrial morphology, and thus may still provide adequate insight into the necessity of mitochondrial fusion and fission during interneuron migration.

Overall, a primary function of the fusion and fission of mitochondria is to maintain mitochondrial homeostasis. Impaired mitochondria are flagged by PINK, which facilitates parkin recruitment and subsequent mitophagy (Vives-Bauza, Zhou et al. 2010). The majority of research of PINK and parkin have been focused on their role in neurodegeneration. However, recent studies suggest that mutations in these proteins result in embryonic changes in neuronal physiology (Villeneuve, Purnell et al. 2016). It would be interesting to explore the role and importance of mitophagy in migrating interneurons. There are new tools available that allow real-time monitoring of mitophagy, a key component to evaluating processes during active interneuron migration. This consists of a tandem RFP-eGFP mitochondrially targeted protein. As RFP is more pH stable, mitochondria targeted to lysosomes become marked in red (Kim, Khan et al. 2013). Utilizing this tool in migrating interneurons and pyramidal neurons may reveal differences in mitochondrial turnover between these populations. If interneurons have less mitochondrial turnover, this may explain their increased sensitivity to mitochondrial perturbations.

The significance of mixed mitochondrial populations

Our understanding of the complexity and heterogeneous nature of mitochondrial populations is evolving. The first evidence that mitochondria are not a uniform population was

the discovery that populations of mitochondria exist in differing stages of membrane polarity (Smiley, Reers et al. 1991, Keil, Funke et al. 2011). This theoretically divides mitochondria into more energetic populations with higher membrane polarity from those that are less so. Interestingly, studies examining mitochondrial membrane potential at the early stages of the developing blastocysts exhibit cell-type specific mitochondrial polarity (Van Blerkom, Cox et al. 2006). Some theorize that the variations in populations of mitochondria are linked with their age and robustness. Lower energy producing mitochondria are thought to be older, and more likely to undergo mitophagy (Jin, Lazarou et al. 2010, Hu, Li et al. 2016). This brings forth the question of whether mitochondria within migrating interneurons and pyramidal neurons are a uniform or mixed population. Utilizing indicators of mitochondrial membrane potential in migrating neurons may reveal differences in the “activity” between these two cell types, or even between mitochondria that localize to particular regions (Smiley, Reers et al. 1991, Keil, Funke et al. 2011). This has brought up fascinating concepts regarding variation in mitochondrial populations and disease models. Cancerous cells are distinguishable from healthier cells by differences in mitochondrial polarization in their populations (Jiang, Fan et al. 2015). Examining these features of mitochondria in cell types with differing metabolic profiles and the potential for dysfunction in disease is likely to be an exciting field of research with implications in neuronal development and disease.

The role of mitochondria in radially migrating neurons

In our initial studies, we saw grossly fixed mitochondrial localization in radially migrating pyramidal neurons (Figure 3). Initial experiments utilizing pharmacological inhibition of OXPHOS with bongkreikic acid in whole brain slices showed normal radial migration rates as compared to non-radially migrating interneurons. This was also seen at early stages (E18.5) in *Ant1*^{-/-} embryos (Figure10). However, we observed a subtle effect on radial migration with

expression of Miro1K208 at postnatal stages after migration should be complete (P15) (Figure 19). Investigating *Ant1*^{-/-} pyramidal neurons electroporated with GFP at these later time points would give an indication if deficits in OXPHOS utilization also result in similar, or more severe migration deficits.

This data also brings forth the question of what impacts we may see on pyramidal neuron development with more severe inhibition of mitochondrial function and localization. Within the literature, there are three examples of abnormal radial migration being associated with mitochondrial localization. Knockdown of *Pdss2*, a protein involved in ubiquinone biosynthesis, results in morphologically abnormal mitochondria and significant impingements on radial migration paired with more severe impacts including reduced cell proliferation and increased cell death (Lu, Lu et al. 2012). As with other studies, it is unclear what component of mitochondrial physiology is impacted, thus it is difficult to place this mutation on a scale of mitochondrial deficits. Ubiquitination is an important component of the regulation mitochondrial fission; thus, it is possible that loss of this protein impedes normal mitochondrial homeostasis. Overexpression of mitochondria-localized glutamic acid-rich protein (MGARP) resulted in a combination of mitochondrial defects including abnormal structure, distribution and mitochondrial motility (Jia, Liang et al. 2014). This subsequently resulted in radial migration deficits. Although mitochondrial function was not assessed in this model, these studies present evidence for later developmental abnormalities in the reduced dendrite and axon formation in cultured neurons with MGARP overexpression, and the reverse with knockdown of MGARP. This emphasizes two intriguing concepts; the potential for additive developmental impacts of chronic/progressive mitochondrial dysfunction, and the potential importance of the appropriate balance of mitochondrial proteins and their functions.

Knockdown of TAU, a microtubule stabilizing protein, induces deficits in mitochondrial localization, radial migration, and dendritic arborization (Sapir, Frotscher et al. 2012). Although further studies are required to both characterize and determine the contribution of mitochondrial dysfunction to the phenotype, it would be interesting to utilize this genetic model to test pharmacological interventions that boost mitochondrial function. As TAU microdeletions are associated with neurological disease in humans, positive results from such studies would provide a foundation for potential therapeutic interventions.

These data from the literature and our subtle migration phenotype observed in radially migrating neurons suggests that there are certain components of radial migration that may be altered with mitochondrial disruption, potentially more severely compared to the manipulations we made in our evaluation of oxidative phosphorylation. The subtle impacts we observed with overexpression of M1K208 in PN cells indicated that effects of less severe mitochondrial manipulations may have impacts later on in the radial migration process. Additionally, mitochondrial proteins have been shown to play a role in proliferation and differentiation (Kim, Shaker et al. 2015), and cell orientation in dividing neuronal stem cells (Mattson and Partin 1999). Thus, there are likely time-dependent requirements for mitochondrial functions such as OXPHOS during radial neuron development, and the severity of pathology is dependent on when these impacts occur during pyramidal neuron development.

Preliminary data utilizing an Emx-Cre conditional deletion of Ant2 on an Ant1 background suggests that cortical development can be profoundly impacted with more drastic interference of OXPHOS. At E18.5, *Ant2^{fl/y}Ant1^{-/-}Emx-Cre⁺* mice have significantly smaller cortices, and expanded cortical ventricular zones (Figure 21). Although preliminary data does not suggest significant differences in proliferation or cell death within the cortex, more individuals are required to do a quantitative comparison. The fact that few GFP electroporated cells at e14.5

emerged from this expanded ventricular zone after 4 days puts for the possibility that these cells have deficits in initiating radial migration or invading the cortical plate. Small numbers of electroporated cells can be seen outside the expanded ventricular zone with abnormally structured and oriented processes (Figure 21D, 21F). This preliminary data suggests that there is a threshold level of OXPHOS function, below which pyramidal neuronal development becomes compromised.

Implications for neurological diseases

Our work emphasizes the importance of exploring the importance of mitochondria at various stages of neurodevelopment. In our studies, we could observe a gradient of impacts; interfering with mitochondrial trafficking with Miro1 did not impact migration rates, but interfering with OXPHOS resulted in more severe phenotypes. Additionally, Ant1 interneurons were hypersensitive to additional inhibition with BA, which likely was due to complete blocking of ATP translocation through inhibition of Ant2. We also see this gradient of effect in the development of pyramidal cells; a population that was only subtly impacted with mitochondrial manipulations during migration becomes profoundly disrupted with ablation of both Ant1 and Ant2. This gradient of impact presents us with a potential for “additive” injury. If the embryo is exposed to additional stressors during stages of interneuron migration, it is possible those with mitochondrial mutations may be more susceptible to developing interneuron-specific deficits. For example, impaired mitochondrial function may increase risk of severe disruption of interneuron migration due to oxygen deprivation.

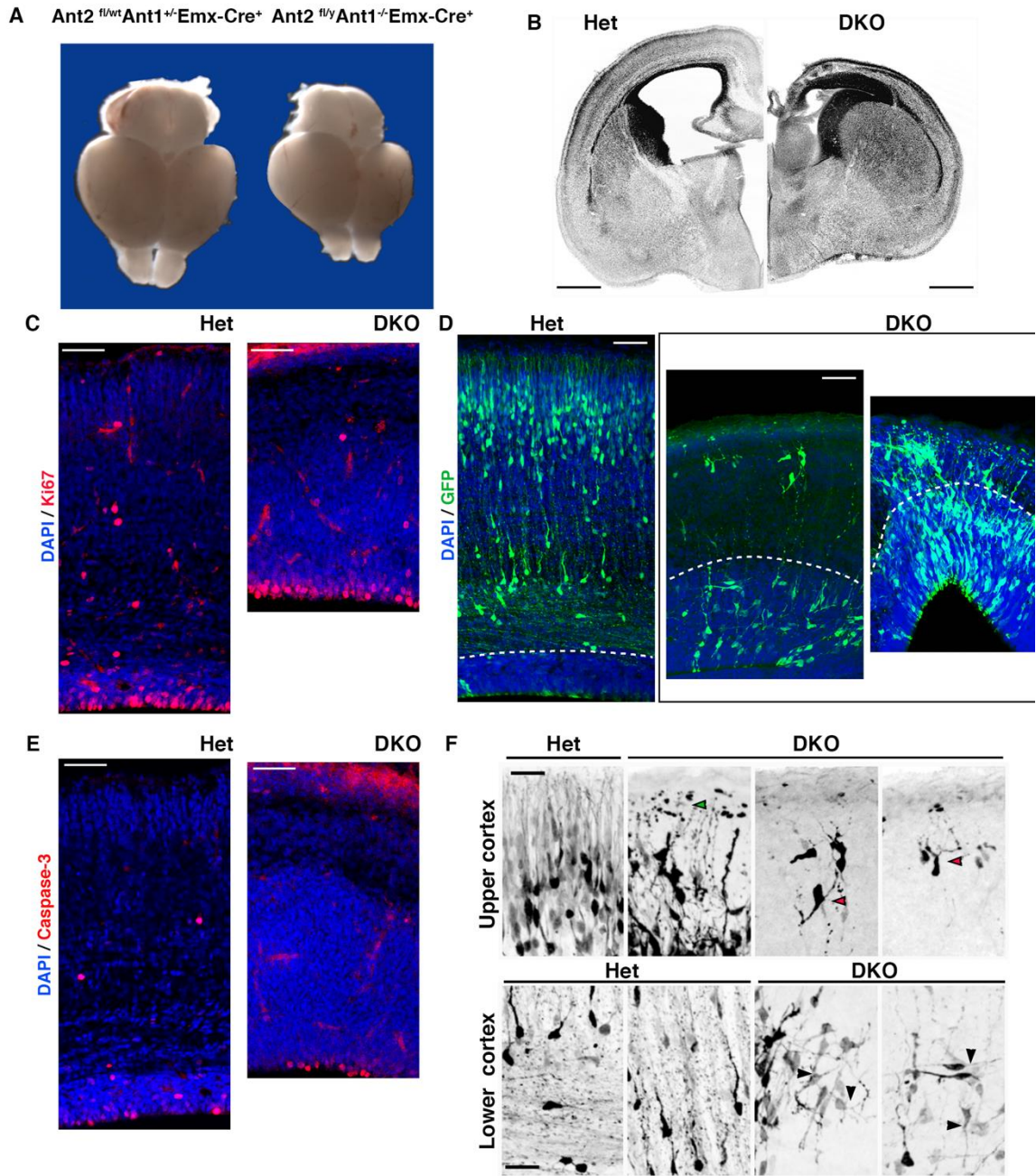


Figure 21. Preliminary data reveals profound cortical abnormalities in Ant1/2 DKO within the mouse cortex.

(A) Conditional ablation of Ant2 in cortical pyramidal cells utilizing Emx-Cre on an Ant1^{-/-} background results in grossly smaller telencephalon. (B) Inverted LUT of DAPI stained cross sections of a DKO and Ant1^{+/-} Ant2 null reveal that 1 copy of Ant1 is sufficient for visually

normal cortical structure compared to severely abnormal cortex of DKO, characterized by an expanded ventricular zone. (C, E) Ki67 and caspase 3 staining appears to be similar, suggesting proliferation and cell death at E18.5 may be unaffected (more individuals required). (D) GFP electroporated cells reveals few cells migrating outwards beyond the expanded ventricular zone. (F) Inverted LUT images of cells from sections shown in (D) show the normal tapered apical dendrite within cells that have reached the upper cortex in Het, while cells in the upper cortex of DKO display abnormal arborization (red arrows, upper right panels), membrane blebbing (green arrow, upper left panel). Cells in the lower cortex show mostly uniform orientation, while DKO cells have putative leading processes oriented in multiple directions (black arrows, lower right panels). N=2 *Ant1^{+/-}Ant2^{-/-}* females, 1 DKO. Scale bar = B: 250 μ m, C-E: 50 μ m, F: 25 μ m.

It would be interesting to utilize the *Ant1*^{-/-} mouse model to test this theory of initial mitochondrial mutations causing increased sensitivity to additional impingements. Interneuron explants can be exposed to hypoxic culture conditions, and the impact to neuronal migration assessed. Although more challenging, it is possible to model intrauterine growth restriction in mice in by uterine artery ligation to reduce oxygenation of embryos. This method could be utilized to subject *Ant1*^{-/-} embryos to hypoxic conditions *in vivo* (Janot, Cortes-Dubly et al. 2014).

Another model of developmental impingements that can result in neuronal deficits include viral infection and inflammation during gestation. Interest is growing in examining the impact of viral infection and inflammation on mitochondria function (Anand 2013, Khan, Syed et al. 2015). These are additional potential areas where combinatorial impingements could result in a gradient of severity of interneuron developmental deficits.

Further exploration of varying levels of mitochondrial functional inhibition at different stages of development may help elucidate if there are important developmental windows where interneuron development can be compromised. One clear future study for our work would be to examine interneuron development of *Ant1*^{-/-} interneurons at later stages in development. It is unclear if these cells will “catch up,” and reach their final destinations in time to form appropriate synaptic connections. Additionally, the terminal differentiation and firing properties of *Ant1*^{-/-} interneurons at postnatal stages should be addressed.

In summary, this work has opened a new arena of research in the field of neuronal migration. As detailed above, our current lines of research could expand into investigations of the molecular mechanisms of the unique phenotypes described in our experimental models, particularly phenotypes involving cytoskeletal dynamics and cell polarity. Additionally, studies examining non-OXPHOS-related functions of mitochondria are likely to provide additional insights into the

roles and requirements of these versatile organelles during neurodevelopment. Further, assessment of the effects of mitochondrial manipulations to both interneuron and pyramidal cells at time points both preceding - and following - neuronal migration could pinpoint key windows in which disruptions to mitochondrial function could precipitate pathologic outcomes during neural development. In conclusion, a comprehensive understanding of mitochondrial contribution to neurodevelopment as a whole is likely to not only to expand our basic understanding of the nervous system, but also to lend insight into possible mechanisms behind and therapeutic avenues towards the treatment of neurodevelopmental diseases.

Materials and methods

Mice

Ant2^{fl/fl}Ant1^{-/-} and *Ant1^{-/-}* mice were kindly provided by Dr. Doug Wallace. *Ant1* mice were bred with *Emx-Cre* mice to generate *Ant1^{+/-}EmxCre⁺* males. *Ant1^{+/-}EmxCre⁺* males were bred with *Ant2^{fl/fl}Ant1^{-/-}* females, and the resulting litters were used for experiments.

All mice were maintained on a C57/BL6 background. The Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia, Philadelphia, PA, approved all studies.

In utero electroporation

Ant1/2-Emx-Cre litters were electroporated with pCAG-IG as previously described at E14.5 and harvested at E18.5.

Immunohistochemistry

Brains from *Ant1/2-Emx-Cre* litters were harvested and fixed as previously described (materials and methods, Chapter 2). 50 micron floating sections were obtained utilizing a

vibratome, and slices were stained with caspase-3 (rabbit; Abcam, 1:500), Ki67 (rabbit; Neomarkers, 1:300), or anti-GFP (chicken: Invitrogen, 1:2000). Secondary antibodies included anti-rabbit-Alexa 594; Invitrogen anti-chick-Alexa 488; Invitrogen all at 1:2000. Nuclei were counterstained with DAPI.

Immunocytochemistry

MGE explants were generated, cultured as previously described (materials and methods, Chapter 2). Tubulin was labeled with anti-Tuj1 (rabbit; Neuronal CIII b-tubulin; Covance, 1:1000), or YL1/2 (Tyr-a-tubulin, Millipore; 1:750), and actin was labeled with Alexa Fluor 594 phalloidin (Molecular Probes; according to manufacturer's instructions)

Microscopy

In vitro cultured MGE neurons were imaged utilizing a Leica CTR600 fluorescent microscope. Imaging of *Ant1/2* brain slices

Quantification

The expression of acetylated tubulin along the leading process of stained interneurons were measured, as well as the total length of the leading process. The expression of acetylated tubulin was evaluated as a percentage of total leading process length for each measured cell.

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