The Role of Lis1 in Adult Mammalian System

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THE ROLE OF LIS1 IN ADULT MAMMALIAN SYSTEM

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DEDICATION

This work is dedicated to my loving parents, Hong Dai and Lusheng Gao. Their guidance, love, sacrifices and endless support have been the main driving force behind all of my achievements in my life especially in pursuit of scientific research. Without their emotional support during the last several months, it would not be possible for me to finish up this dissertation.

I would also like to dedicate my dissertation work to my dear Jay Peng. He has been a large part of my motivation and support to make me believe what I have been pursing is all meaningful and is worthwhile years of dedication.
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ABSTRACT

*Lis1* haploinsufficiency in humans results in a “smooth brain” phenotype called lissencephaly, and also causes severe cognitive and motor impairments and epilepsy. Seizure severity and frequency typically worsens with time; patients often die within the first decade due to seizure-induced aspiration and pneumonia. Various mouse models have been used to examine the role of *Lis1* during brain development, and it is clear that *Lis1* regulates a microtubule motor, cytoplasmic dynein. Intriguingly, *Lis1* expression remains high in adult brains indicating that it plays a role in mature systems. Indeed, our group found that *Lis1* and several related proteins regulate dynein-dependent axon transport in cultured adult rat sensory neurons. Here, we hypothesize that loss of *Lis1* in adult mice could lead to neurological disorders or other diseases.

In order to bypass the developmental impact of *Lis1* loss, we first utilized a tamoxifen inducible Cre-mediated recombination system driven by an actin promoter to knock out *Lis1* in adult mice (>8 weeks). We found that loss of *Lis1* in adult mice caused a progressive decline and ultimately death. These phenotypes were not observed in any of a variety of control animals. Analysis of Cre activity revealed it was not uniformly stimulated in all regions of the brain, and is highest in the brainstem, where neuronal processes in regions known to control cardiorespiratory networks were activated. In non-neuronal tissues, high level of
Cre activity was also detected in the hearts of tamoxifen induced Lis1 knockout mice.

Secondly, to determine whether the lethal phenotype is caused by loss of Lis1 in brainstems or in hearts, we generated a cardiomyocyte-specific inducible lis1 knockout mouse model. Deletion of lis1 in hearts did not seem to induce lethality or malaise in adult mice. This strengthened the hypothesis that the death phenotype may be caused by Lis1 loss in neurons of the cardiorespiratory brainstem network.

Lastly, examination of brain sections of experimental mice revealed hallmarks of chromatolysis. Cultured DRG neurons from adult tamoxifen injected experimental animals, but not controls, showed signs of axon pathology and transport defects. Together, our results indicate that Lis1 is essential for adult mammalian system, and its loss of function causes lethal phenotype in adult mice, which may be resulted from dramatic altered neuronal function.
# Table of Contents

**DEDICATION** .......................................................................................................................... iii

**ACKNOWLEDGEMENTS** ............................................................................................................. iv

**ABSTRACT** ................................................................................................................................... v

**LIST OF TABLES** ......................................................................................................................... viii

**LIST OF FIGURES** ....................................................................................................................... ix

**LIST OF SYMBOLS** ....................................................................................................................... x

**LIST OF ABBREVIATIONS** .......................................................................................................... xi

**GENERAL INTRODUCTION** ......................................................................................................... 1

**CHAPTER 1: GENERATION OF AN INDUCIBLE LIS1 CONDITIONAL KNOCKOUT MOUSE MODEL** ......................................................................................................................... 7

**CHAPTER 2: THE EFFECT OF A TAMOXIFEN-INDUCIBLE ACTIN-CRE LIS1 KNOCKOUT IN NERVE SYSTEM** ....................................................................................................................... 23

**CHAPTER 3: THE EFFECT OF AN INDUCIBLE ACTIN-CRE DOUBLE LIS1 KNOCKOUT IN NON-NEURONAL TISSUES AND AN INDUCIBLE MYH6-CRE LIS1 KNOCKOUT MOUSE MODEL** ....................................................................................................................... 60

**CONCLUSIONS AND FUTURE DIRECTIONS** ............................................................................ 84

**REFERENCES** .............................................................................................................................. 89
LIST OF TABLES

Table 2.1 Characterization of phenotypes of inducible Lis1 knockout mice upon high dose tamoxifen injection. ................................................................. 45

Table 2.2 Characterization of phenotypes of inducible Lis1 knockout mice upon low dose tamoxifen injection. ................................................................. 46
LIST OF FIGURES

**Figure 1.1** The three mouse strains used to generate inducible Lis1 knockout mice. ................................................................. 18

**Figure 1.2** Mouse breeding and colony maintenance. ................................................................. 19

**Figure 1.3** Summary of breeding strategy. .................................................................................. 21

**Figure 1.4** Experimental animals and Cre induction. ................................................................. 22

**Figure 2.1** Survival analysis of tamoxifen induced Lis1 knockout mice. ............................... 47

**Figure 2.2** High dose tamoxifen induced Cre activation in brainstem at 3 days following initial treatment ................................................................. 48

**Figure 2.3** High dose and low dose tamoxifen induced Cre activation in brainstem at 5 days following initial treatment ........................................................................ 49

**Figure 2.4** Cre activities were detected in cardiorespiratory control network in tamoxifen induced Lis1 knockout mice. .................................................................................. 50

**Figure 2.5** Lis1 expression level is reduced in brainstem of inducible Lis1 knockout mice. .................................................................................. 51

**Figure 2.6** Neurons in the regions of the nucleus ambiguous show axonal pathology at 3 days following tamoxifen injection. ................................................................. 52

**Figure 2.7** Cultured DRG neurons from adult tamoxifen injected experimental animals, but not controls, show signs of axon pathology ................................................... 54

**Figure 2.8** Lis\(^{fl/l}\);actCreER\(^{TM}\);R26\(^{GT}\) DRG neurons placed in culture after tamoxifen treatment show defective axonal transport. ................................................... 56
Figure 2.9 Heterozygous Lis1fl/+;CreERTM;R26GT mice did not exhibit apparent behavior abnormality. ........................................................................................................... 58

Figure 2.10 Time-course analysis of Cre activation in brains................................. 59

Figure 3.1 Cre activation in various tissues shown in whole mount organs. ...... 76

Figure 3.2 Cre activations in various tissue sections............................................. 77

Figure 3.3 Tamoxifen induced cardiomyocyte-specific Lis knockout mice. ....... 78

Figure 3.4 Cre activation in cardiomyocyte-specific Lis1 knockout mice........... 79

Figure 3.5 Changes in Lis1 levels of tissues from low dose and high dose treated animals. ......................................................................................................................... 80

Figure 3.6 Lis1 expression level changes in tissues from low dose and high dose treated animals........................................................................................................ 81

Figure 3.7 Cre activities in diaphragm and skeletal muscle. .............................. 83
LIST OF ABBREVIATIONS

α-Syn.................................................................................................................α- synuclein
ALS......................................................................................................................amyotrophic lateral sclerosis
AUP...................................................................................................................Animal Use Protocol
CMV..................................................................................................................cytomegalovirous
DHC......................................................................................................................dynein heavy chain
ES.........................................................................................................................embryonic stem cell
IP.........................................................................................................................intrapertitoneal
mT.........................................................................................................................tdTomato
mG.........................................................................................................................EGFP
MT.........................................................................................................................microtubule
MHC......................................................................................................................myosin heavy chain
PAFAH1B1...............................................................platelet-activating factor acetylhydrolase
PD.........................................................................................................................Parkinsons’ disease
PAF.......................................................................................................................platelet-activating factor
PS.........................................................................................................................presenilin
rAAV..................................................................................................................recombinat adenoassociated viral
SOD1....................................................................................................................superoxide dismutase
Lissencephaly is a severe brain developmental disorder, which is also known as “smooth brain”. In patients with this disease, most of the cerebral cortex lacks the grooves and gyrus as a normal brain does. Disorganization of cerebral layers results in devastating consequences- defective cortex function and early death, usually from worsened seizures.

**Lis1 and brain development**

Type I lissencephaly has been attributed to sporadic mutation in the human *LIS1* gene. LIS1 was identified as a subunit of a brain cytosolic isoform of platelet-activating factor (PAF) acetylhydrolase. The first invertebrate LIS1-related gene identified was *nudF* in the mold *Aspergillus nidulans*. Mutations in *nudF* caused a severe defect in the migration of nuclei into and within the hyphal processes, resulting in reduced colony size. This evidence suggested a corresponding function for LIS1 in brain development. Nuclear migration occurs within the processes of proliferating neuroepithelial cells and also some differentiating neurons. Homozygous-null LIS1 mutant mice exhibited early post-implantation embryonic lethality, suggesting a basic cellular role for LIS1. Mice with partial reduction of Lis1 displayed brain disorganization as well as cerebellar defects. Neuronal migration abnormalities were observed in Lis1 mutants both *in vivo* and *in vitro* (Hirotsune et al., 1998). These findings suggested LIS1 played a cell-autonomous neuron-specific role for neuronal migration. The systematic examination of the consequences of dosage reduction of LIS1 in mice revealed defects in interkinetic nuclear migration and neuroblast proliferation (Gambello et al., 2003).
In the hippocampus of Lis1+/- mice, synaptic transmission was severely disrupted, demonstrating hyperexcitability at Schaffer collateral-CA1 synapses and depression of mossy fiber-CA3 transmission (Fleck et al., 2000). A significant alteration in function of inhibitory circuits was found within the malformed Lis1+/- hippocampus. These hippocampal defects could provide a neuronal mechanism for seizures associate with lissencephaly (Jones and Baraban, 2007). It is shown that Lis1 haploinsufficiency can lead to abnormal cell proliferation, migration and differentiation in the adult dentate gyrus (Wang and Baraban, 2007). Lis1 heterozygous mice also displayed abnormal motor behavior and spatial learning and memory (Paylor et al., 1999).

**Lis1/Dynein/Nudel Complex**

It is demonstrated that Lis1 directly interacts with the cytoplasmic dynein heavy chain (DHC) and Nudel, a murine homolog of the *Aspergillus nidulans* nuclear migration mutant NudE (Niethammer et al., 2000). Lis1 and Nudel were found to regulate the distribution of DHC along microtubules. The Lis1/Nudel/DHC complex is seemed to be involved in retrograde axon transport in the adult. This complex may play important roles of cell proliferation, cell survival and/or degeneration (Sasaki et al., 2000). It is speculated that disruption in the Lis1/Nudel/DHC complex may result in functional impairment of axonal transport, which could be responsible for neurological impairment in the devastating disease.
**Roles of Lis1 in cellular functions**

Our group found that Lis1 is enriched in neurons relative to levels in other cell types. Lis1 interacts with the microtubule motor cytoplasmic dynein and may stimulate specific dynein functions in neuronal migration and axon growth (Smith et al., 2000). Dynein and Lis1 play an important role in microtubule advance during growth cone remodeling associated with axonogenesis (Grabham et al., 2007). Besides its role in brain development, Lis1 is also involved in other cellular functions. Overexpression of Lis1 in cultured mammalian cells interferes with mitotic progression and leads to spindle misorientation (Faulkner et al., 2000). Lis1 has also been implicated in dynein-mediated nuclear migration in various organisms (Xiang, 2003). Overexpression of dominant negative dynactin and Lis1 interfered with the rate of cell migration (Dujardin et al., 2003).

There has been controversy over how Lis1 mediates dynein-dependent transport. One group showed that inhibition of Lis1 impaired anterograde movement of cytoplasmic dynein (Yamada et al., 2008). Other group reported that Lis1 inhibition had little effect on lysosomes but greatly inhibited axonal transport of large, but not small vesicles (Yi et al., 2011). Our group previously reported that Lis1 overexpression stimulated retrograde transport, while Lis1 RNAi significantly reduced axon transport of lysosomes moving in axons of adult rat sensory neurons (Pandey and Smith, 2011b). Also, depletion of Lis1 suppressed mitochondrial motility in both anterograde and retrograde directions (Shao et al., 2013).
Axon transport and adult-onset neurodegenerative diseases (AONDs)

Axonal defects have been identified in mouse model of Alzheimer’s disease. In this model, reduction of kinesin motor impaired axon transport enhanced the frequency of axonal defects and increased amyloid-beta peptide level (Stokin et al., 2005). Defective axonal transport of mitochondria was observed in a mouse model of Alzheimer’s disease (Calkins et al., 2011). Axonal transport deficits were found in an ALS mouse model (Bilsland et al., 2010).

Adult-onset neurodegenerative diseases are a group of neurological disorder characterized by a progressive, age-dependent decline in neuronal function and loss of selected neuronal populations. Many major AONDs including Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis (ALS), display axonal pathologies including abnormal accumulations of proteins and organelles (De Vos et al., 2008). Deficits in axon transport have been documented in most AONDs, which commonly result in altered synaptic function and axonal connectivity in these diseases (Morfini et al., 2009). A mouse model postnatally deficient for proteins presenilin (PS) and expressing wild-type human tau exhibited early tau pathology and axonal transport defects, which were associated with reduced neurotrophin signaling, defective learning and memory and impaired synaptic plasticity (Peethumnongsin et al., 2010). Mutations in α-synuclein (α-Syn) cause Parkinsons’ disease (PD). Mutant mice expressing high levels of A53T human α-Syns developed adult-onset neurodegenerative disease with a progressive motor dysfunction leading to death (Lee et al., 2002). In A53T
mice, neurons in brainstem and spinal cord exhibited large axonal swelling, somal chromatolytic changes, and nuclear condensation (Martin et al., 2006).

**Lis1 knockout mouse models**

Homozygous-null Lis1 mice are lethal at E7, while deletion of a single allele of Lis1 exhibited hippocampal abnormalities, loosely paced granule cells and neuronal migration abnormalities (Hirotsune et al., 1998). Multiple doses of Lis1 had defects in interkinetic nuclear migration and neuroblast proliferation (Gambello et al., 2003). Lis1+/− mice showed adult hippocampal neurogenesis, abnormal cell proliferation, migration and differentiation in the adult dentate gyrus (Wang and Baraban, 2007). Lis1fl/fl;Pax2-Cre mice that had Lis1 deletion in midbrain-hindbrain E8 did not survive past 0. Lis1fl/fl;Wnt-Cre mice had neuronal loss with embryos dead E13.5. Lis1fl/fl;hGFAP Cre mice that had Lis1 deletion in neuronal precursor cells of the neocortex and hippocampus at E12.5-13.5 survived to P5, but missing the hippocampus. They also had defects in apical spindle orientation in the ventricular zone (Yingling et al., 2008). Lis1fl/fl;K14-Cre mice showed epidermal barrier function in desmosomes, which resulted in perinatal lethality (Sumigray et al., 2011). Adolescent (4weeks) Lis1+/fl;CamKCre mice exhibited deficits in social interaction and dendritic protrusion density (Sudarov et al., 2013).
Cardiorespiratory control network

In this study, we utilized a tamoxifen inducible Cre-mediated recombination system to determine if loss of Lis1 in adult mice could result in any neurological diseases. We found out Lis1 depletion in adult mice leads to progressive decline and ultimate death. We speculate that this death phenotype could be due to defects in cardiorespiratory network in brainstem, which controls heart rate and breathing. Medulla oblongata is located in lower part of the brainstem, which carries out life sustaining function such as breathing, swallowing and heart rate. The pons sits directly above the medulla, connecting upper and lower parts of the brain. A network of neurons located within the brainstem are involved in regulating cardiorespiratory activity (Smith et al., 2007). The circuitry is located in the dorsal respiratory group and ventral respiratory column(VRC) of the medulla oblongata as well as in the dorsolateral pons(Bianchi et al., 1995) (Feldman et al., 2003; Richter and Spyer, 2001). Cardiac vagal pre-ganglionic neurons of the nucleus ambiguous receive inhibitory inputs during inspiration as well as excitatory inputs during post-inspiration(Feldman and Del Negro, 2006). The VRC is responsible for generating the respiratory rhythm. Retrotrpezoid nucleus group and pre-Botzinger complex have been identified as principal components of rhythm generation(Feldman and Del Negro, 2006; Feldman et al., 2003). Neuronal circuits within the medullary pre-Botzinger complex generates the basic rhythm of breathing, and then relay the respiratory pattern to cranial and spinal motor neurons controlling respiratory muscles(Eisele and Jain, 1971; Hayashi and McCrimmon, 1996). The vagus nerve is controlling the heart. Cardiac vagal
pre-ganglionic motoneurons are located in dorsal vagal motonucleus and within the nucleus ambiguous of the medulla oblongata (Jones, 2001; Spyer, 1994). Inhibition of cardiac vagal motoneurons allows stimulus that enhances inspiration increases heart rate, while inputs that suppress ventilation or prolong expiration lower heart rate via cardiac vagal activation (Hyland et al., 2001).
CHAPTER 1

GENERATION OF AN INDUCIBLE LIS1 CONDITIONAL KNOCK OUT MOUSE MODEL
1.1 Introduction

Our lab has previously demonstrated that perturbing Lis1 and its regulators impacts dynein-dependent transport of lysosomes and mitochondria in mature sensory neurons (Pandey and Smith, 2011a). Disruption in Lis1 regulatory pathway could contribute to worsening lissencephaly symptoms, and may be relevant to other neurological disorders.

It is difficult to determine how Lis1 pathways function in the mature nervous system because Lis1 is so important in embryonic development. In order to bypass the embryonic impact observed in conventional Lis1 knockouts, Lis1 should be removed from mature neurons. However, in cultured sensory neuronal system, removing neurons to tissue culture can recapitulate developmental programs rendering the neurons distinct from those in the intact animal. We therefore generated a tamoxifen-inducible Cre mouse homozygous for a floxed Lis1 allele. In this way Lis1 can be silenced at a chosen time after major developmental processes have taken place. This not only allows us to determine the importance of Lis1 in mature neuron, it provides a model for potential therapeutic intervention to counteract symptoms of lissencephaly that may arise post-developationally as a result of transport defects.

Conditional Lis1 knockout was accomplished by inducing Cre-mediated recombination post-developationally to delete a floxed region of the Lis1 gene. Lis1 is most abundantly expressed in nervous system while its expression is very low in non-neuronal tissues, thus, we chose to use β actin-driven promoter Cre to
ensure all neurons lose Lis1. Also, it would give the best chance of observing a phenotype from an induced knockout and not restrict knock out to specific subsets of neurons. The caveat is that this approach would also knock out Lis1 in other adult tissues. However, as expression levels were much lower we expected to observe a brain phenotype, if any.

There strains of commercially available mice will be crossed to generate animals that are homozygous for a floxed Lis1 allele and hemizygous for an inducible Cre recombinase allele and a Cre-reporter allele. The current mouse model for lissencephaly, which carries a heterozygous deletion of Lis1 has substantially milder phenotype than humans with Lis1 haploinsufficiency. Although the Lis1 mutant mice appear to have slow or delayed neuronal migration with an onset near E17.5, Lis1 het mutants have no apparent gross phenotype compared to wild type mice except mild learning deficits in spatial and motor learning (Paylor et al., 1999). Our transport studies indicate that Lis1 dosage can dramatically impact the number, speed, and run lengths of organelles moving retrogradely in axons, and we suspect this contributes to increased pathology in the longer human axons. Therefore, a homozygous knockout of Lis1 serves the best model to study the impact of Lis1-dependent transport defects in mice. On the other hand, the mild phenotype in the current developmental model may be caused by compensatory mechanisms arising because of the early loss of Lis1. If this is the case, knocking out a single allele in mature neurons could result in a more severe adult phenotype. We are able to
examine the impact of both heterozygous and homozygous conditional Lis1 knockouts and determine the effect neuronal survival, morphology and function.

1.2 METHODS AND MATERIALS:

1.2.1 Animals: The three mouse strains used to generate inducible Lis1 knockout mice were purchased from Jackson lab(Figure 1.1).

a) Lis1+/fl (or 129S-Pafah1b1tm2Awb/J-JAX#008002): These mice possess loxP sites flanking targeting vector containing PGKneo. This construct was electroporated into 129S6/SvEvTac derived TC-1 embryonic stem (ES) cells. Correctly targeted ES cells were injected into recipient blastocysts. Mice that are homozygous for this allele are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities. When these mutant mice are bred to mice that express Cre recombinase, resulting offspring will have exons 3 through 6 deleted in the cre expressing tissue(s).

b) Cre-ER™ (or Tg(CAG-cre/Esr1*)5Amc/J-JAX #004453): These transgenic mice have a tamoxifen-inducible cre-mediated recombination system driven by the chicken beta actin promoter/enhancer coupled with the cytomegalovirus (CMV) immediate-early enhancer. The transgene insert contains a fusion product involving Cre recombinase and a mutant form of the mouse estrogen receptor ligand-binding domain. The mutant mouse estrogen receptor does not bind natural ligand at physiological
concentration but will bind the synthetic ligand, 4-hydroxytamoxifen. Restricted to the cytoplasm, the Cre/ESR1 protein can only gain access to the nuclear compartment after exposure to tamoxifen. When crossed with a strain containing a loxP site flanked sequence of interest, the offspring are useful for generating tamoxifen-induced, Cre-mediated targeted deletions. Homozygous mice are not viable or fertile. Heterozygous mutant mice are viable, fertile, normal in size and do not display any gross physical or behavioral abnormalities.

c) R26\textsuperscript{GR} (Gt(ROSA)26Sor\textsuperscript{tm4(ACTB-tdTomato,EGFP)Luo}\textsuperscript{/J}-Jax\#007576): The mT/mG (membrane-Tomato/membrane-Green) targeting vector was designed with a CMV enhancer/chicken beta-actin promotor driving loxP-flanked tdTomato protein sequence. An N-terminal membrane-tagged, enhance green fluorescent protein sequence is distal to the second loxP site. The entire mT/mG construct was inserted into the Gt(ROSA)26Sor locus via electroporation of (129X1/SvJ x 129S1/Sv)F1-derived R1 embryonic stem(ES) cells. mT/mG homozygotes can be identified by tail or whole body epifluorescence. When bred to Cre recombinase expressing mice, the offspring have the mT cassette deleted in the cre expressing tissues, allowing expression of the membrane-targeted EGFP (mG) cassette located just downstream. The CTB promotor allows stronger and persistent expression of the fluorescent proteins compared to the endogenous Gt(ROSA) locus alone. This double-fluorescent system allows direct live visualization of both recombined and non-recombined
cells at single cell resolution, offering an internal control for phenotypic analysis of Cre-induced mosaic mutants.

1.2.2 Mouse breeding and colony maintenance

The University of South Carolina. The experimental protocols were approved by the Animal Use Protocol (AUP) committee. All the transgenic mice and knockout mice were maintained on its original genetic background. However, when generating inducible Lis1 knockout mice by crossing three different mouse strains, mixed genetic background could not be avoided. To minimize variation in the genetic background, the males (Lis1^{fl/fl};actin-CreER^{TM}; R26^{GT}) and females (Lis1^{fl/fl};R26^{GT}) from the same generation were crossed to produce inducible Lis1 knockout mice, and mice from the same litter were compared. However, in order to gather enough samples for statistical analysis, tissues from different animals with the same genotype at different ages were pooled.

Mice were marked by ear puncture at weaning (3-4 weeks old), and genomic DNA was extracted from tail for routine genotyping.

1.2.3 Genotyping:

Mice tails were digested in Tail buffer plus Protease K overnight at 55°C. 2µl of tail digest was used as DNA templates for PCR reaction. Primers for Lis1 flox/flox: Sequence5'->3' TGA ATG CAT CAG AAC CAT GC; CCT CTA CCA CTA AAG CTT GTT C. Primers for Cre : Sequence5'->3' CTA GGC CAC AGA
ATT GAA AGA TCT; GTA GGT GGA AAT TCT AGC ATC ATC C. Primers for R26GT : Sequence5'->3' CTC TGC TGC CTC CTG GCT TCT; CGA GGC GGA TCA CAA GCA ATA ; TCA ATG GGC GGG GGT CGT.

A standard PCR reaction was set up as followed: For each tail DNA sample, to make 25µl total volume, 6.5µl of H2O, 5.5 µl Master Mix 2.5µl each primer and 2µl Tail DNA were added to PCR tubes. The reaction was performed on a PCR thermocycler as follows: 94°C for 3 min for hotstart; 94 °C for 30s, 60 °C for 1 min, 72 °C for 1min. Repeat steps above for 35 cycles. 10µl of the reaction product was run on 5% polyacrylamide gel at 120V for 30 min. Gel was stained with EB for 10min. DNA bands could be detected under UV light.

1.3 RESULTS:

1.3.1 Verifying Lis1+/fl , actin-Cre ER™, R26GT mice by PCR

Lis1+/fl, Lis1 fl/fl mice were confirmed by PCR reactions. After gel electrophoresis, we expected to see heterozygotes with a mutant band at 210 bp and a wild type band at 167 bp; homozygous with one mutant band at 210 bp; wild type with one band at 167bp(Figure 1.2B).

Heterozygous actin-Cre ER™ mice were identified by PCR using primers: Sequence5'->3' CTA GGC CAC AGA ATT GAA AGA TCT; GTA GGT GGA AAT TCT AGC ATC ATC C. A positive band at 100bp was identified as an actin-Cre ER™ mouse. A negative band indicated a wild type allele for actin-Cre ER™ gene(Figure 1.2C).
R26<sup>GT</sup> mice were used a Cre reporter strain. It is reported that heterozygous R26<sup>GT</sup> is sufficient to identify red fluorescence without Cre recombination. Homozygous R26<sup>GT</sup> was detected with a single mutant band at 250 bp on 5% polyacrylamide gel(Figure 1.2D). Heterozygous R26<sup>GT</sup> was identified with one mutant band at 250 bp and one wildtype band at 330bp. One single band at 330 bp indicated wild type alleles for R26<sup>GT</sup>.

1.3.2 Summary of breeding strategy:

Heterozygous Lis1+/fl mice were intercrossed to produce Lis1 fl/fl mice(Figure 1.3). One quarter of the offspring were homozygous for floxed Lis1. Double floxed Lis1 mice were then crossed with Cre ER<sup>TM</sup>+/- mice to obtain heterozygous lis1 flox; Cre ER<sup>TM</sup> Heterozygous floxed Lis1; Cre ER<sup>TM</sup> mice were backcrossed with homozygous Lis1 flox mice. One quarter of the offspring were homozygous for Lis1 flox and heterozygous for Cre ER<sup>TM</sup>. Another quarter of the offsprings were homozygous Lis1 flox without Cre ER<sup>TM</sup>, which served as a control. Tissues from these mice were harvested to examine levels of proteins of interest.

In order to generate inducible Lis1 knockout with Cre-reporter gene, homozygous Lis1 flox mice were crossed with homozygous R26GT reporter strain to obtain 1/4 offsprings carrying heterozygous Lis1 flox; R26GT. Lis1 flox+/-; R26GT+/- mice were then mated to produce homozygous Lis1 flox;R26GT strain. This strain were backcrossed with homozygous Lis1 flox;
Heterozygous Cre ER\textsuperscript{TM} to generate homozygous Lis1 flox;Heterozygous Cre ER\textsuperscript{TM} and R26GT stain. One half of the offspring were Homozygous Lis1 flox; heterozygous R26GT without Cre ER\textsuperscript{TM}, which were used as controls.

Additionally, to obtain a Cre ER\textsuperscript{TM}; R26GT control, Cre ER\textsuperscript{TM} strain was bred with R26GT strain. Cre ER\textsuperscript{TM}; R26GT controls are important as they carry a Cre recombinase that we would know if activation of Cre upon tamoxifen injection has any effect on mice phenotype.

In the analysis of Cre activation by reporter strain, homozygous and heterozygous R26\textsuperscript{GT} mice were not distinguished for each tissue. R26\textsuperscript{GT} was identified by tail epifluorescence.

1.3.3 Cre induction by tamoxifen:

We chose a tamoxifen inducible Cre system first described by Hayashi, S. & McMahon, A.P (Hayashi and McMahon, 2002b). In the initial characterization of these animals, Cre could be activated in various tissues including central nervous system and in cultured cells. The recombination efficiency is dose dependent. Multiple injections of as highest dose as 9mg of TM did not lead to any obvious change in the behavior of the mice. Triple crosses produced mice with homozygous floxed Lis1 alleles, one Cre-ER allele, and a Cre reporter generated by L. & Luo (Muzumdar et al., 2007). This reporter gene codes for both mTomato (red fluorophore) and GFP, but GFP is only produced if Cre becomes active (i.e., enters the nucleus in response to tamoxifen). Two different
control animals were also generated. The first control carries the floxed Lis1 alleles, but not the Cre-ER allele. The second control carries the Cre-ER allele, but has wild type Lis1. A third control was a mock injection of the triple mutant animal(Figure 1.4).

Adult mice (2-5 months old) were exposed to tamoxifen by intraperitoneal (IP) injections. Different dosages of tamoxifen were used in the experiments with low dose 2mg/day for 5 days, median dose 4mg/day for 5 days or high dose 8mg/day for 2 days. It has been shown that increasing the dosage of tamoxifen injected in the animals increased the frequency of Cre recombination 24h post-injection up to the maximum dose of 9mg of TM(Hayashi and McMahon, 2002a). It revealed that high dose of TM resulted in a more rapid and more efficient induction of recombination. The initial dosage of TM administered in adult mice was determined based on the universal protocol published from Jackson Lab. It states that 2mg of tamoxifen for 5 consecutive days is sufficient to drive Cre activation in adult mice. It is reported that increasing the dosage of tamoxifen injected and multiple rounds of injections would increase the efficiency of Cre recombination. However, in the initial Cre induction studies, the variation in weights of animals was not taken into account.
1.4 Discussion:

The original three mouse strains purchased from Jackson Lab were from different genetic backgrounds. When crossed them to generated inducible Lis1 knockout mouse model, the mixed background was obtained from different types of matings. Different genetic backgrounds may influence the phenotypes of the Lis1 inducible knockout mice. However, we selected the experimental and control animals from the same litter to remove the side effects of different genetic backgrounds. When a R26\textsuperscript{GT} reporter strain was introduced, it allowed a direct visualization of a fluorescent mouse. A single allele of R26\textsuperscript{GT} is sufficient to detect tail fluorescence (Muzumdar et al., 2007).

At least 8 weeks old mice were chosen as adult mice because this is when mouse brain is fully developed and suited for study in adult system. Whether older mice will respond to TM induction differently besides the weight than young mice is unknown. It has been a concern that the blood-brain barrier would prevent access of the drug. However, it has been shown that intraperitoneal injection of TM (3mg/day for 5 days) resulted in efficient Cre recombination including the brain (Hayashi and McMahon, 2002a). Examination of tissues revealed the rapid onset of mG labeling within 24h following Cre induction (Hayashi and McMahon, 2002a). However, there is a concern regarding the lag between GFP expression and Lis1 knockdown. GFP expression may not provide an accurate measure of Lis1 knockdown. Therefore, reduction of Lis1 protein expression level in specific tissues needs to be evaluated by western blot.
This inducible knockout system can be used to remove activity of a gene of interest at a certain stage of postnatal development. This approach would allow us to overcome the developmental effects associated with germ-line ablation. However, a certain gene may play several different roles in different tissues at the same stage. Therefore, a tissue-specific Cre line may be introduced to knock out the gene in a certain tissue. In this case, alternatively, brain specific Cre mouse strain can be used to knock out Lis1 in central nerve system.

In the course of generating double Lis1 knockout mouse model, a heterozygous inducible Lis1 knockout was also generated. It will be valuable to examine the differences between double Lis1 knockout and loss of single allele.
Figure 1.1 The three mouse strains used to generate inducible Lis1 knockout mice. A.) Cre-ER\textsuperscript{TM}: These transgenic mice have a tamoxifen-inducible cre-mediated recombination system driven by the chicken β actin promoter/enhancer. The transgene insert contains a fusion product involving Cre recombinase and a mutant form of the mouse estrogen receptor ligand-binding domain. B.) R26\textsuperscript{GT} reporter: These mice possess loxP sites on either side of a membrane-targeted tdTomato(mT) cassette and express strong red fluorescence in all tissues. mT/mG homozygotes can be identified by tail or whole body epifluorescence. When bred to Cre recombinase expressing mice, the offspring have the mT cassette deleted in the cre expressing tissues. C.) Lis1\textsuperscript{+/fl} These mice possess loxP sites flanking targeting vector containing PGKneo. When these mutant mice are bred to mice that express Cre recombinase, resulting offspring will have exons 2 through 6 deleted in the cre expressing tissue(s).
Figure 1.2 Mouse breeding and colony maintenance.

A.) Heterozygous Lis1+/fl mice (LICCTT) were mated to produce Lis1 fl/fl mice. One quarter of the offspring were homozygous for floxed Lis1. Homozygous R26GT reporter (LLCCtt) mice were intercrossed to maintain the line. 100% of the offspring would be homozygous R26GT mice. Heterozygous Cre mice (LLCcTT)
Figure 1.2 Mouse breeding and colony maintenance.

B.) Lis1+/fl, Lis1 fl/fl mice were confirmed by PCR reactions. After gel electrophoresis, we expected to see heterozygotes with a mutant band at 210 bp and a wild type band at 167 bp; homozygous with one mutant band at 210 bp; wild type with one band at 167 bp. Heterozygous actin-Cre ER\textsuperscript{TM} mice were identified by PCR. A positive band at 100bp was identified as an actin-Cre ER\textsuperscript{TM} mouse. A negative band indicated a wild type allele for actin-Cre ER\textsuperscript{TM} gene. Homozygous R26GT was detected with a single mutant band at 250 bp on 5% polyacrylamide gel. Heterozygous R26GT was identified with one mutant band at 250 bp and one wild-type band at 330bp. One single band at 330 bp indicated wild type alleles for R26GT.
Figure 1.3 Summary of breeding strategy. Heterozygous Lis1+/fl mice (LICCTT) were mated to produce Lis1 fl/fl mice (IIcCTT). One quarter of the offspring were homozygous for floxed Lis1. Double floxed Lis1 mice were then crossed with Cre ER\textsuperscript{TM} +/- mice to obtain heterozygous lis1 flox; Cre ER\textsuperscript{TM}. Heterozygous floxed Lis1; Cre ER\textsuperscript{TM} mice (LLcCTT) were backcrossed with homozygous Lis1 flox mice. One quarter of the offspring were homozygous for Lis1 flox and heterozygous for Cre ER\textsuperscript{TM}(IIcCtt). Another quarter of the offsprings was homozygous Lis1 flox without Cre ER\textsuperscript{TM}, which served as a control. In order to generate inducible Lis1 knockout with Cre-reporter gene, homozygous Lis1 flox mice were crossed with homozygous R26GT reporter strain(LLCCtt) to obtain 1/4 offsprings carrying heterozygous Lis1 flox; R26GT(LICCTt). Lis1 flox+/-; R26GT+/- mice were then mated to produce homozygous Lis1 flox; R26GT strain(IIcCtt). This strain was backcrossed with homozygous Lis1 flox; Heterozygous Cre ER\textsuperscript{TM} to generate homozygous Lis1 flox; Cre ER\textsuperscript{TM} and R26GT strain(IIcCtt). One half of the offspring were Homozygous Lis1 flox; heterozygous R26GT without Cre ER\textsuperscript{TM}(IIcCtt), which were used as controls. To obtain a Cre ER\textsuperscript{TM}; R26GT control, Cre ER\textsuperscript{TM} strain was bred with R26GT strain(LLCCTt).
Figure 1.4 Experimental animals and Cre induction. Triple crosses produced mice with homozygous floxed Lis1 alleles, one Cre-ER allele, and a Cre reporter (lis1\textsuperscript{fl/fl};actCreER\textsuperscript{TM};R26\textsuperscript{GT}). Cre is activated by TM injection. Three different control animals were also generated. The first control carries the floxed Lis1 alleles, but not the Cre-ER allele (lis1\textsuperscript{fl/fl};R26\textsuperscript{GT}). The second control carries the Cre-ER allele, but has wild type Lis1 (actCreER\textsuperscript{TM};R26\textsuperscript{GT}). A third control was a vehicle injection of the triple mutant animal (lis1\textsuperscript{fl/fl};actCreER\textsuperscript{TM};R26\textsuperscript{GT}+corn oil).
CHAPTER 2

THE EFFECT OF A TAMOXIFEN-INDUCIBLE ACTIN-CRE LIS1 KNOCKOUT IN NERVE SYSTEM
2.1 INTRODUCTION

Lis1, the disease-causing gene for a “smooth brain” phenotype in which regions of the cortex of lissencephalies patients are smooth in appearance because of reduced or absent sulci and gyri. Children with lissencephaly have severe mental retardation and other severe neurological abnormalities. They often have feeding problems and hypotonia. A large majority of patients have seizures during the first year. Patients often die due to status epilepticus or pneumonia (Dobyns et al., 1993).

Neurons extend long processes, requiring efficient transport mechanisms to allow communication of survival signals from the distal axon to the cell body (Levy et al., 1985; Perlson et al., 2010). Anterograde axon transport plays a role in supplying proteins and energy to the distal end, whereas retrograde transport is involved in the clearance of misfolded and aggregated proteins form the axon to the soma (2013). Retrograde axon transport is carried out by the multi-subunit motor protein complex cytoplasmic dynein (Schnapp and Reese, 1989).

Defects in dynein-dependent axon transport have been linked to psychiatric disorders and adult-onset neurodegenerative diseases. These diseases are often characterized by cell body inclusions, axonal swellings, and axon terminal degeneration (De Vos et al., 2008; Zuccato et al., 2010). Huntington’s disease, a disorder of the basal ganglia, is caused by polyglutamate repeat expansions in the Huntingtin protein (Zuccato et al., 2010). Huntingtin interacts with dynein and the plus-end directed motor, kinesin (McGuire et al., 2006). The Huntingtin-associated protein 1 (HAP1) binds to mutant Huntingtin,
and to dynactin, a dynein regulatory complex (Engelender et al., 1997; Li et al., 1998). Phosphorylation of Huntingtin by the Akt/PKB kinase pathway in response to IGF-1/PI3K signaling may cause a switch from dynein to kinesin binding to influence the direction of vesicle transport (Caviston and Holzbaur, 2009; Hafezparast et al., 2003; McGuire et al., 2006). Recently basal ganglia defects were reported in a mouse with a point mutation in dynein heavy chain (the catalytic subunit) (Braunstein et al., 2010). Finally, mutations in dynactin that cause cytoplasmic inclusions have been found in eight families with Perry Syndrome, a disorder characterized by early-onset Parkinsonism, depression weight loss and hyperventilation (Strom et al., 2008). Amyotrophic Lateral Sclerosis (ALS) and similar motor neuron disease are also linked to transport. ALS-like symptoms have been observed in mice with dynein point mutations, and dynein binds to a mutant form of superoxide dismutase (SOD1) found in familial ALS, resulting in the formation of large inclusions containing mutant SOD1 (Laird et al., 2008). A mutation in a subunit of dynactin was found in a family with a lower motor neuron disease, and expression of the mutant protein caused motor neuron disease in transgenic mice (Puls et al., 2003).

Impairment of axonal transport has also been related to other neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, and several motor neuron diseases. Several studies reported that transgenic mice overexpressing wild-type or mutant amyloid precursor protein (APP) (Salehi et al., 2006), mutant presenilin1 (PS1) (Lazarov et al., 2007) or wild-type or mutant tau (Zhang et al., 2004) display axonal transport deficits and axonal
swelling. Amyloid plaques are composed of deposits of the amyloid-β-peptide, which is produced by cleavage of the APP by β-secretase and γ-secretase, a multi-subunit protein complex in which PS1 or PS2 is the catalytic subunit.

A recent study showed that Prickle, a mutation of which results in epileptic phenotypes observed in flies, mice, zebrafish, and humans, organizes microtubule polarity and influences both anterograde and retrograde vesicle transport in axons of Drosophila neurons. The seizure phenotype in prickle mutant flies is caused by enhancement of the anterograde transport. Additionally, they showed Prickle heterozygotes exhibit severely reduced vesicle transport, with loss of both alleles of Prickle showing a marked decrease in viability (Ehaideb et al., 2014). This is the first link between defective axon transport and seizures.

Although to date no links between Lis1 and Nudel have been proposed in ALS, huntington’s disease or other neurological disorders, our demonstration that both proteins play a role in retrograde axon transport of lysosomes and mitochondria in mature neurons, coupled with the continued expression in mature brains, suggests this possibility should not be ruled out, and loss of Lis1 post-developmental could result in neurological disorders.

We have generated an inducible Lis1 knockout mouse as described in Chapter 1. In this study, we first analyzed the behavioral consequences of loss of Lis1 post-developmentally in adult mice, given the evidence that Lis1 plays essential roles in many aspects of neuronal development such as neuronal
migration as well as axonal transport. We also examined cytological abnormalities that may indicate neuronal dysfunction in knockout animals.

2.2 METHODS AND MATERIALS

**TM injection and Cre activation**
To induce Cre-mediated recombination, tamoxifen (Sigma-Aldrich) was dissolved in corn oil (Sigma-Aldrich) at a concentration of 40mg/ml and administered to 2-5 month-old Lis1fl/fl, CreERTM, R26GT at different dosages (2mg/d for 5days, 4mg/d for 5days, or 8mg/d for 2days) depending on the experiment settings. Water and food were available *ad libitum*. Experiments were performed on 2-5-month-old male and female littermates after tamoxifen administration. No significant gender differences were observed.

**Tissue preparation and Histology.**
Animals were deeply anesthetized with isofluorane administration and perfused transcardially with ice-cold PBS first, followed by 4% paraformaldehyde (PFA) in 0.1M PBS, pH7.4. For whole-mount organ analysis, organs were isolated and imaged with a CCD camera mounted on a fluorescence microscope(Olympus). For cryosection preparation, brains, hearts, lungs, livers and other tissues were removed and postfixed in 2% PFA+15% sucrose solution prepared in PBS overnight and subsequently cryoprotected by incubation in 30% sucrose solution
for 3-4 hours at room temperature. The cryoprotected brains were submerged in OCT Compound solution and quickly frozen in 2-methylbutane cooled in liquid nitrogen. Sagittal or coronal sections were cut at a 10-μm thickness on a cryostat (Leica) and stored at -80°C before used. Slides were washed three times with PBS, treated with DAPI (Molecular Probes), washed again, mounted in antifade reagent (Invitrogen) and imaged with fluorescence microscopy (Zeiss) or confocal microscopy (Leica).

**Immunoblotting**

The levels of Lis1 protein was evaluated in Lis1 CKO and age-matched control mice by immunoblotting. Mice were deeply anesthetized and decapitated, and the brains and other tissues were removed quickly and frozen on dry ice. Tissue samples were homogenized with Dounce’s homogenizers in ice-cold RIPA buffer with protease inhibitors. Crude homogenates were sat on ice for 20 minutes and then centrifuged at 20,000 g for 1 hour (4°C) to yield soluble supernatant and pellet fractions. Protein concentrations were measured by BCA assay (Thermo scientific) with bovine serum albumin as a standard. Protein samples were subjected to 10% SDS-PAGE and transferred to PVMF membrane. Blots were blocked with 5% nonfact dry milk with 0.1% Tween 20 in PBS and then incubated overnight at 4°C with antibody to Lis1 or dynein intermediate chain. After the primary antibody incubation, blots were washed and incubated with horseradish peroxidate-conjugated secondary antibody, developed with enhanced chemiluminescence(Millipore) and exposed to x-ray film.
To quantify protein immunoreactivity, films were scanned and densitometry was performed using image J. Protein levels were expressed as relative optical density measurements. Immunodensities were normalized to control proteins.

**Quantifying chromatolysis and nuclear eccentricity**

Toluidine blue-stained sections were used as part of the structural analysis. A chromatolytic neuron cell body was taken as one where discrete patches of cytoplasmic basophilia (Nissl bodies) could no longer be resolved in the light microscope over more than half the area of the cell body cytoplasm. An eccentric nucleus was taken to be one where the distance from the center of the nucleus to the cell body periphery was less than the distance from the center of the nucleus to the center of the cell body.

**Primary mouse dorsal root ganglion (DRG) culture**

Primary DRG neuronal cultures were prepared based on the modified protocol for rat DRG culture. Mice were first perfused with sterile ice-old PBS to remove blood and proteases so that nerves can be easily pulled out. Removed the bones surrounds spinal cord and pull out dorsal roots. Dissected out Lumbar 5-6 DRGs and dissociated ganglions in Collagenase for 1 hour, followed by 15 minutes with 0.05% trypsin (Invitrogen). Dissociated neurons were spinned through a 15% sucrose solution. Neurons were plated onto sterile german glass coverslips (Fisher) coated with 10µg/ml poly-D-lysine (Sigma) and 10µg/ml laminin (Millipore).
**Immunostaining**

Neurons were plated onto 12mm coverslips in 24-well plates. Neurons were fixed in 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100 for 5 min at room temperature. Nuclei were visualized with Hoechst and coverslips were mounted on glass slides using ProLong Gold Antifade reagent (Invitrogen). Neurons were observed using an inverted microscope (Axiovert 200, Carl Zeiss Inc.) equipped with Plan-Neo 100x/1.30 objective and a Plan-Apo 63X/1.40 objective. Digital images were acquired using a charge-coupled camera (AxioCam HRm, Carl Zeiss Inc.) linked to AxioVision software (Version 4.7, Carl Zeiss Inc.).

**Fluorescence time-lapse microscopy**

After exposure to 100nM Lysotracker for 20 minutes, coverslips were transferred into fresh medium containing 25mM Hepes, pH7.4, in a water-heated custom-built microscope stage warmed to 37°C using an inverted microscope (Axiovert 200, Carl Zeiss Inc.) equipped with C-Apo 63x/1.2 W/0.2 water-immersion objective. Digital images were acquired using a charge-coupled camera (AxioCam HRm, Carl Zeiss Inc.) linked to AxioVision software (version 4.7, Carl Zeiss Inc.). For tracking lysosomes labeled with Lysotracker-Red (Millipore Inc.) in axons, images were recorded for 2 min at 0.5 second intervals. Each experiment was done three times with different DRG sensory neuron cultures.
Analysis of organelle movement using Image J software

To determine the proportion of organelles that moved in different directions or remained static, kymographs were generated from time-lapse movies using image J software (version 10.2) as described in Miller and Sheetz, 2006 (Miller and Sheetz, 2006). By convention the direction towards the cell body was always to the right, so lines that sloped toward the right at any point with a net displacement of >5µm were categorized as retrograde organelles. Lines that sloped toward the left >5µm at any time during the recording interval were considered anterograde organelles. Lines that showed less than 5µm lateral displacement in any direction during the recording interval were categorized as static.

Statistical analysis

Microsoft Exel and GraphPad Prism software was used for all statistical analysis. A Student’s unpaired, two-tailed t-test, ANOVA or Turkey’s multiple comparison test were used to calculate P values for all datasets. Single standard errors (s.e.m) are shown as bars on each graph.

2.3 RESULTS:

Tamoxifen inducible actin-Cre-mediated knockout of Lis1 in adult mice causes a progressive decline and death as early as second day after tamoxifen injection
In order to induce Cre activation in adult mice, high dose of tamoxifen (8mg/day for two consecutive days) was administrated on the animals to ensure high recombination efficiency (Table 2.1). Adult triple mutant mice at 8-week old injected on two consecutive days with tamoxifen began to exhibit malaise on as early as the second day, with clearly ruffled coats and spinal kyphosis(Figure 2.1A). They were hypoactive and exhibited leg clasping in tail suspension test. In the initial studies experimental animals died on day 3-5 after the first injection (n=11). Control mice without Cre with same amount of tamoxifen injection showed no signs of distress at any time, nor did the mock-injected experimental animals (n=10). In later studies, animals were euthanized on day 3-5 for tissue analysis, and to examine GFP and Lis1 expression in various tissues (n=21). Older mice (2-5 months) injected with the same amount of tamoxifen (8mg/day for 2 days) showed the same behavior changes and died within one week after initial tamoxifen injection (n=3). Older mice tend to exhibit severe malaise at later days compared to younger mice probably due to older mice weigh slightly more than young ones, thus the overall exposure to tamoxifen is less in older mice. In order to determine whether Cre activation by tamoxifen has effect on the observed phenotype, controls without floxed Lis1 (CreER\textsuperscript{TM};R26\textsuperscript{GT}) injected with the amount of tamoxifen were examined and found to be normal, suggesting Cre activation itself does not cause adverse effects in mice (n=24).

Similarly, lower dose of tamoxifen was also administered on adult mice to drive cre activation(Table 2.2). Adult triple mutant mice (8-week old) injected with 2mg tamoxifen per day for 5 consecutive days did not exhibit apparent behavior
changes until day 5 and die eventually between day 9 - 16 (n=6) (Figure 2.1B). The slight difference in survival days is resulted from dose-dependent cre recombination as it is reported that 3mg tamoxifen leads to 50% cells undergoing a recombination event 24 h post injection while 9mg of TM results in up to 80% of cells within a tissue underwent recombination within 24h of TM injection. In total, 35 experimental animals injected with tamoxifen died on day 3-7 or showed very severe malaise between days 3 and 5. Of 52 total control animals, none showed signs of malaise. In later experiments, high dose of tamoxifen (8mgx2) was used to induce Cre activation to ensure high percentage of Cre recombination in mice, unless noted in the experiments.

**Cre activity is not uniformly stimulated in all regions of the brain, and is highest in the brainstem.**

The sudden death of these animals was unexpected, but suggested that either a vital brain function, or a vital function carried out by other organs, was perturbed by Lis1 knockout. It was therefore critically important to determine where Cre was activated at 3-5 days after tamoxifen injection. Examination of green fluorescence of whole brains from perfused animals indicated that Cre was strongly active in the brainstem, and cerebellum (Figure 2.2). Much less GFP was apparent in the cerebral hemispheres at 3 days after tamoxifen. An adult triple mutant mouse with mock injection showed no green fluorescence background under the same exposure time. Brain from inducible Lis1 knockout mice injected
with high dose tamoxifen and sacrificed on 5th day showed stronger GFP in brainstem and cerebellum than it was injected with low dose tamoxifen and euthanized on the same day(Figure 2.3). No significant green fluorescence except a few cells were observed in cortex in high dose tamoxifen treated animals at 5 days after initial injection.

This indicated that we could not easily ascribe the death of knock out mice to cortical malfunction due to Lis1 loss, although this remains formally possible if a knocking out Lis1 in a few cells could lead to death. However, the loss of Lis1 in brainstem, known to be the seat of nuclei controlling to complex cardiorespiratory network, remained a distinct possibility. Saggital sections of an inducible Lis1 knockout brain revealed significant amounts of GFP were present in brainstem and midbrain(Figure 2.4 A,B). Scattered green cells were observed in cortex and cerebellum under low magnification. Activated fibers and axon projections were seen at pons under higher magnification(Figure 2.4 C,D). In order to determine whether and where neuronal cell bodies were activated in the brainstem, coronal sections of brains were used to identify the positions of activated neurons in these animals(Figure 2.4E). Confocal imaging of brainstem sections revealed that significant amounts of GFP were present in what appeared to be neurons in the brainstem(Figure2.4J), and also in neuropil and many fiber tracts, including cranial nerves(Figure 2.4F,H,I). Thus, it was likely that Cre was activated by tamoxifen in neurons that are located in cardiorespiratory control network. Analysis of tissue sections demonstrated that some astrocytes in the cortex and hippocampus and Bergman's glia in the cerebellum also expressed
GFP. These observations are generally consistent with original findings of R26GT mice, however, due to relative short period between Cre induction and analysis, Cre recombination by tamoxifen has not occurred in the majority of the cortex during the 2-day period. It is reported that cell bodies were less prominently labeled compared to cells expressing cytosolic EGFP in brain (Muzumdar et al., 2007).

**Lis1 expression level is reduced in brainstem of inducible Lis1 knockout mice**

To test whether Lis1 level is reduced in brain tissues of adult triple mutant animals upon tamoxifen injection, extracts of brainstem, cerebellum, and cortex from tamoxifen treated of both floxed Lis1 with Cre transgene and floxed Lis1 without Cre mice were probed with polyclonal Lis1 antibody. Brain tissues were collected at 3 days after high dose tamoxifen injection. Lis1 expression level was found to be significant reduced in brainstems of floxed Lis1 with Cre animals compared to controls (Figure 2.5 A). No reduction of Lis1 levels in the cortex was detected from tamoxifen induced Lis1 knockouts at 3 days after initiation of treatment compared to floxed Lis1 without Cre. The changes of Lis1 levels in different brain parts are consistent with Cre activity indicated by the amount of GFP observed in tamoxifen treated Lis1 knockouts. As stated before, low dose tamoxifen treated experimental animals did not exhibit severe behavior abnormality until 7 days after initial treatment. We wanted to compare Lis1 expression patterns in high dose tamoxifen treated Lis1 knockouts and low dose treated animals. Experimental and control mice induced with both doses of
tamoxifen were sacrificed on 5\textsuperscript{th} day after the first injection and brain tissues were harvested on the same time point. Western blot demonstrated that Lis1 expression level had not reduced on 5\textsuperscript{th} day after low dose tamoxifen injection, while its level dramatically decreased at the same time point in high dose treated experimental animals(Figure 2.5B). Fluorescence images of low dose treated Lis1 knockout brains showed weak GFP in brainstem at 5 day after initial treatment(Figure 2.3H), while no significant reduction in Lis1 level was detected in brainstem at the same time point. This observation suggests that there is a latency between visualization of Cre activation by GFP and the actual Lis1 protein knockdown.

\textit{Examination of brain sections for hallmarks of chromatolysis.}

To determine the cytopathology associated with behavior abnormalities and regional Lis1 expression knockdown in brainstem in the inducible Lis1 knockout mice, the brains from experimental and control mice were examined by Nissl staining. As we have seen Cre activity in axonal projections and neuropil in brainstem of animals treated with tamoxifen, we hypothesized that if lis1 disruption causes axonal defects in brains, we were likely to detect neuropathology in the regions that had Lis1 reduction. Axon damage provokes a process termed chromatolysis, characterized by somal swelling, nuclear eccentricity, and reduced Nissl substance (ER and ribosomes). This response may be caused by the disruption of retrograde signaling. Toudine blue staining
was used to visualize somal swelling, nuclear position, and Nissl substance dispersal in brain tissue (Figure 2.6A). A Nissl body can be defined as any aggregate of ribosome ER that could be identified in the light microscope as a patch in the cytoplasm of Toluidine Blue-stained sections (Johnson et al., 1985). Brainstem sections were selected where nucleus ambiguus was present in the coronal sections according to the mouse brain atlas. Landmarks such as fourth ventricle and pyramus granular layers were used to identify the proper sections. Comparison were made from matched sections. Neurons were selected in the regions of nucleus ambiguus, and were scored as having nuclear eccentricity if the perpendicular distance from the NE to the somal edge at any point is less than half the perpendicular distance of the NE to the somal edge at any other point. We measured the ratios of nuclear to soma in inducible Lis1 knockout and control mice and found that experimental mice harboring floxed Lis1 and Cre transgen, had significant larger nucleus versus cytoplasm in brainstem (Figure 2.6C). The ratio of nuclear area versus soma was ~0.5 in tamoxifen induced Lis1 knockout (+Cre) compared to the ratio of ~0.2 in control mice (-Cre). Another conspicuous feature of tamoxifen treated experimental mice, was that nucleus of neurons in were displaced toward the periphery of the soma and the Nissl substance was significant reduced in the soma. We then quantified the percentage of nuclear accentricity based on the criteria described above. Tamoxifen induced Lis1 knockout mice showed as high as 40 percent of neurons having nuclear accentricity, while control mice had very few neurons (10%) showing the same feature (Figure 2.6D).
Cultured DRG neurons from adult tamoxifen injected experimental animals, but not controls, show signs of axon pathology and transport defects.

Because of the strong physical and functional link between Lis1 and the molecular motor protein cytoplasmic dynein, we considered the possibility that Cre activation and subsequent Lis1 loss would result in axon transport perturbations. It is difficult to directly measure transport in the brainstem, so we turned to a culture system that we have used in the past to measure dynein-dependent axon transport. Unlike most adult neurons, DRG neurons can be successfully removed from adult rats and maintained in tissue culture for long periods of time. These neurons extend only axon like processes with polarized microtubules so that retrograde transport is typically thought to be carried out by dynein. On the third day (the day after the 2\textsuperscript{nd} tamoxifen injection) neurons were removed from experimental triple mutants and control animals (no Cre-ER allele) and plated onto laminin-coated coverslips. Only the experimental animals showed GFP fluorescence, while only mTomato fluorescence was detected in control cells(Figure 2.7A). After 24 hours, cultures were fixed and axons examined for morphological changes. Several differences were found between GFP positive axons in from floxed Lis1 animals and Tomato positive axons from floxed Lis1 animals with no Cre. First the length of axon segments between branch points was shorter for GFP axons were shorter(Figure 2.7B). Second there was an increase in the number of varicosities per branch point, and an overall increase in varicosities per mm of axon(Figure 2.7C,D). Varicosities were
often seen at axonal ends, indicating defects in axon transport. These swellings are hallmarks of motor protein disruption in *Drosophila* and have been documented in the corpus collosum of mice believed to have defects in axon transport (Stokin et al., 2005). Therefore, it would be interesting to examine whether axon transport was defective in tamoxifen induced Lis1 knockouts. To do so, DRGs were isolated from adult triple mutant and CreER$^{TM}$;R26$^{GT}$ control mice at 3 days following tamoxifen injection. DRGs were allowed to grow on laminin-coated coverslips for another 24 hours. Lysotracker Red was added to the culture 30 min before live cell imaging. Membrane associated tomato fluorescence was almost gone 3 days after initial treatment. Axon processes can be visualized by GFP labeling via Cre activation. Lysotracker Red has strong red fluorescence that allows us to detect acidic organelles in axons. Time-lapse images were taken at 0.5 s interval for 2 min. Kymographs were generated with Image J software (Figure 2.8A, B). Mice with floxed Lis1 and Cre showed significant reduction in percentage of acidic organelles undergoing retrograde transport compared to mice with wild-type Lis1, while there is no significant difference in anterograde transport with Lis1 perturbation (Figure 2.8C, D, E). The results are consistent with previous study using adult rat DRGs transfected with Lis1 RNAi in culture.
**Tamoxifen inducible heterozygous Lis1 knockout mice do not show apparent behavior abnormality.**

Tamoxifen induced knockout double Lis1 alleles leads to progressive decline and ultimate death in adult mice. We asked whether conditionally knockout single copy of Lis1 in adult mice would result in different pathological consequences than double knockouts using floxed-lis1 and actin-Cre strains. Similarly, adult heterozygous inducible Lis1 knockout and control mice (heterozygous floxed Lis1 without Cre) were injected with 8mg tamoxifen for 2 constitutive days. Heterozygous Lis1fl/+;CreER\textsuperscript{TM};R26\textsuperscript{GT} mice did not exhibit apparent behavior abnormality. This is not surprising as conventional Lis +/- has much milder phenotype than homozygous Lis1 knockouts. What is interesting to us, is that analysis of Cre activity in brain tissues of these animals demonstrated homogenous strong GFP signal in the whole brain, coupled with dramatically reduced red fluorescence(Figure 2.9). We then did a time-course analysis of Cre activation in brains. Three heterozygous inducible Lis1 knockouts were injected with high dose tamoxifen on the same day and were sacrificed individually on day 2, day 7, and day 21 after initial treatment. In a preliminary study, sagittal sections of brains from three animals revealed a gradual increase in the amount of GFP throughout the brain including cortex, cerebellum and brainstem(2.10).
**DISCUSSION:**

Our study of the inducible Lis1 knockout mouse model demonstrates the importance of Lis1 in adult mammals. Tamoxifen induced Lis1 knockout using β-actin Cre recombination system causes rapid death in adult mice. It is by far the first mouse model that studies the pathological consequences of loss of both alleles of Lis1 knockout in adult system. Although it is rather difficult to pin down the pathological causes of the lethal phenotype observed in tamoxifen induced Lis1 knockouts, we have provided several evidences that may be related to neuronal malfunction of cardiorespiratory control network in brainstem.

The death phenotype observed in tamoxifen induced Lis1 knockout mouse model is very consistent. Despite of difference in survival days, all the experimental mice showed sickness, underwent progressive decline and ultimate death, if not euthanized. Adult tamoxifen induced Lis1 knockout mice also exhibited ruffled up fur, hunched posture and leg clasping, indicating impaired locomotion and bad coordination (Connert et al., 2006). However, these severely and progressively disturbed neuromotoric functions were accompanied with the appearance of sings of illness and physical abnormalities observed in tamoxifen induced Lis1 knockout mice. It is possible that disturbed behavior is an indirect result of getting sicker and weaker rather than a direct consequence of loss of Lis1.

Examination of Cre activity in the brains of tamoxifen induced Lis1 knockout mice revealed highest level of GFP expression in brainstem. The loss of Lis1 in brainstem becomes a possible cause of the lethal phenotype. The pre-
Botzinger complex has been identified to be regulating the medullary respiratory network in mammals (Schwarzacher et al., 2011). The presumed pre-Botzinger complex is characterized by an aggregation of loosely scattered, small and lipofuscin-rich neurons, which contain neurokinin 1 receptor as well as somatostatin. The pathoanatomical studies support the view that disruption of the central respiratory network, including the pre-Botzinger complex, contributes to breathing disorders in multiple system atrophy such as Rett syndrome (Weese-Mayer et al., 2006). A study revealed that selective lesions of the nucleus tractus solitarii lead to altered baroreflex control and to cardiac changes that may lead to sudden death (Talman and Lin, 2013). Acute stroke can disturb central autonomic control, resulting in cardiac arrhythmias and ultimately sudden death. Evidence suggests that sudden deaths are probably due to an interaction between cardiovascular and neurological causes (Soros and Hachinski, 2012).

There are other possible neurological explanations for the death phenotype. For example, disruption of Lis1 in myelinating cells could alter neurotransmission. Acute-onset chronic inflammatory demyelinating polyradiculoneuropathy (A-CIDP) features multiple cranial nerve involvement, respiratory failure and skin manifestations (Hantson et al., 2010). Mutation in the tumor-suppressor phosphatase with tensin homology (PTEN) is associated with neurological abnormalities including autism, seizures and ataxia (Fraser et al., 2004; Wei et al., 2006). Pten-deficient neurons in Pten conditional knockout mice revealed enlarged abnormal synaptic structures in the cerebral cortex and cerebellum. Severe myelination defects and weakened synaptic transmission
were observed in these mice (Fraser et al., 2008). Thus, it is possible that loss of lis1 causes defective myelination in cranial nerve, which results in impaired synaptic transmission in the cardiorespiratory control center. Cerebellar cells could also be involved in this network.

Astrocytes have been thought to be required for efficient functioning of respiratory neuronal network activity. Astrocytes have been reported to control breathing through pH-dependent release of ATP (Gourine et al., 2010). Cre activity detected in astrocytes in cortex suggests that loss of Lis1 in these cells may influence the homeostasis of the extracellular space that interfere with Ca+ concentration and neurotransmitter release.

In addition, we found that Lis1^fl/fl^;actinCreER^TM^;R26^GT^ neurons in the brainstem region showed axonal pathology at 3 days following tamoxifen injection. There is a concern that we may have seen magnocellular neurons (large in diameter and spindle-shaped) in one brain section, while paraventricular neurons (small diameter and round-shaped) seen in the other section (Bader et al., 2012). However, note that in the chromatolysis analysis, the neurons we selected had similar size in diameter (Figure 2.6E). Brainstem neurons display a prominent chromatolysis reaction, which is typical of axon injury (Hanz and Fainzilber, 2006). The RNA content and rate of protein production in cell bodies increase in response to axonal injury (Cragg, 1970; Lieberman, 1971). The proposed mechanism is that the rapid ion influx at the injury site generates a number of electrophysiological response and the normal supply of retrogradely transported molecules such as trophic factor signals was interrupted. The
continuous supply of neurotrophic factors from axon terminals to the cell body is critical in maintenance and survival of specific populations of adult neurons (Hanz and Fainzilber, 2006). Recent studies indicate that in ALS, degeneration of motoneuron cell body is late in comparison to degeneration of axons. In the “acute” ALS group, enlargement of the tigroid has been occurring earlier than central chromatolysis. This phenomenon may be related to endoplasmic reticulum stress, disturbed axon transport or functional compensation of the neuronal deficit (Dziewulska et al., 2013). Thus, the chromatolysis occurring in the brainstem neurons from tamoxifen induced Lis1 knockout mouse suggests that axon damage or defects in axon transport has occurred in the brainstem of these mice.

Our results confirm that reduction of Lis1 in adult neuron causes axonal pathology and defective axon transport. We found that there was an overall increase in varicosities per mm of axon. Mice with floxed Lis1 and Cre showed significant reduction in percentage of acidic organelles undergoing retrograde transport compared to the control mice. Axonal defects have been noted in mouse model of Alzheimer’s disease. Similar axonal defects in the early stages of Alzheimer’s disease have also been seen in humans. It is reported that axonal swellings are consisted of accumulated abnormal amounts of microtubule-associated and motor proteins, organelles, and vesicles. It is shown that reduction of kinesin motor protein impaired axonal transport, enhanced the frequency of axonal defects and increased amyloid-β peptide levels and amyloid deposition (Stokin et al., 2005). There is also evidence showing defective axon
transport of mitochondria, abnormal mitochondrial dynamic and synaptic degeneration in a mouse model of Alzheimer’s disease (Calkins et al., 2011). ALS is a fetal neurodegenerative disease, characterized by the loss of motor neurons in primary motor cortex, the brainstem, and the spinal cord, causing devastated basic, fundamental movements, such as breathing, and typically causes death in most cases (Kim et al., 2014). Deficit in axonal transport is a key pathogenic event in ALS and an early disease indicator of motor neuron degeneration (Bilsland et al., 2010). Brainstem is consisted of several types of motor neurons that are responsible for breathing and heart beating. Axonal defects we have seen in the brainstem and cultured DRG neurons of tamoxifen induced Lis1 knockout mice may indicate that the motor neurons in the brainstem undergo neurodegeneration due to Lis1 loss, which leads to neuronal misfunction. Another possibility we have not examined in these mice is defects in synapse transmission. We could conceivably look at synapses by electronic microscopy or immunofluorescence.
Table 2.1 Characterization of phenotypes of inducible Lis1 knockout mice upon high dose tamoxifen injection.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>8 mg x2 TM</th>
<th>Phenotype</th>
<th>Age at injection (months)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;2</td>
<td>2-3</td>
</tr>
<tr>
<td>Lis1^{fl/fl}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>actCre^{ERTM}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R26^{GT}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>dead within 1 week</td>
<td>2/2</td>
<td>9/9</td>
<td>3/3</td>
</tr>
<tr>
<td>+</td>
<td>Sick and euthanized by endpoint</td>
<td>2/2</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td>-</td>
<td>dead or sick</td>
<td>ND</td>
<td>0/3</td>
<td>0/5</td>
</tr>
<tr>
<td>Lis1^{fl/fl}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R26^{GT}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>dead or sick</td>
<td>0/2</td>
<td>0/7</td>
<td>0/12</td>
</tr>
<tr>
<td>actCre^{ERTM}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R26^{GT}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>Sick and euthanized by endpoint</td>
<td>0/8</td>
<td>0/13</td>
<td>0/5</td>
</tr>
</tbody>
</table>
Table 2.2 Characterization of phenotypes of inducible Lis1 knockout mice upon low dose tamoxifen injection.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>2 mg x 5 TX</th>
<th>Phenotype</th>
<th>2-3 month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lis1&lt;sup&gt;fl/fl&lt;/sup&gt; actCre&lt;sup&gt;ERTM&lt;/sup&gt; R26&lt;sup&gt;GT&lt;/sup&gt;</td>
<td>+</td>
<td>Dead within 3 weeks</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Sick and euthanized by endpoint</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>normal</td>
<td>3/3</td>
</tr>
<tr>
<td>Lis1&lt;sup&gt;fl/fl&lt;/sup&gt; R26&lt;sup&gt;GT&lt;/sup&gt;</td>
<td>+</td>
<td>normal</td>
<td>3/3</td>
</tr>
<tr>
<td>actCre&lt;sup&gt;ERTM&lt;/sup&gt; R26&lt;sup&gt;GT&lt;/sup&gt;</td>
<td>+</td>
<td>normal</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 2.1 Survival analysis of tamoxifen induced Lis1 knockout mice. A) Adult triple mutant mice at 8-week old injected on two consecutive days with tamoxifen (+Cre+TM) began to exhibit malaise on as early as the second day, with clearly ruffled coats and spinal kyphosis. They were hypoactive and did not respond to external stimuli. Control mice (-Cre+TM) showed wild type phenotypes. B) Kaplan-Meier survival curves showing cumulative morbidity - percentage of mice showing sickness. In the initial studies experimental animals injected high dose tamoxifen (+Cre+8mgx2 TM) started showing sickness at day 2 following the first injection (n=30). Experimental mice injected with low dose tamoxifen (+Cre+2mgx5 TM) did not exhibit apparent behavior changes until day 5 (n=10). Control animals (-Cre) injected with tamoxifen did not show any signs of malaise or behavior abnormality (n=31). Log-rank Test *** P<0.001
Figure 2.2 High dose tamoxifen induced Cre activation in brainstem at 3 days following initial treatment. A,B) Whole mount images of control mice Lis1^fl/fl; R26<sup>GT</sup> injected with 8mg tamoxifen for 2 consecutive days showed strong red fluorescence. C,D) with no GFP expression in the brain. G,H) Examination of green fluorescence of whole brains from perfused animals indicated that Cre was strongly active in the brainstem, and cerebellum(under the same exposure time as controls. Much less GFP was apparent in the cerebral hemispheres at 3 days after tamoxifen. E,F) Residual red fluorescence was shown in the images.
Figure 2.3 High dose and low dose tamoxifen induced Cre activation in brainstem at 5 days following initial treatment. A,B) An adult triple mutant mouse with vehicle injection (+Cre+vehicle) showed no green fluorescence background. C,D) The brain from an inducible Lis1 knockout mouse injected with high dose tamoxifen and sacrificed on 5th day showed strong GFP in brainstem and cerebellum. A few green cells were also detected in the cortex. Red fluorescence was greatly reduced in brainstem and cerebellum and remained high in cortex. E,F) Control brain(-Cre+2mgx5 TM) showed strong red fluorescence but no GFP expression. G,H) Low dose tamoxifen treated experimental animal (+Cre+2mgx5 TM) exhibited relatively low amount of GFP expression in brainstem and cerebellum at 5 days following initial treatment.
Figure 2.4 Cre activities were detected in cardiorespiratory control network in tamoxifen induced Lis1 knockout mice. A,B) Saggital sections of an inducible Lis1 knockout brain revealed significant amounts of GFP were present in brainstem and midbrain. Scattered green cells were observed in cortex and cerebellum under low magnification. (n=1) C,D) Activated fibers and axon projections were seen at pons under higher magnification (x40). (n=1) E) Coronal sections of brains identified the positions of activated neurons in these animals. (n=4) F) Confocal imaging of brainstem sections revealed that significant amounts of GFP were present in neurons in the brainstem, and also in neuropil and many fiber tracts. G) Diagram of a saggital brain section showing different regions. H, I) Cre activity was also detected in cranial nerves. (n=1)
Figure 2.4 J Coronal sections of brainstems from tamoxifen induced Lis1 knockout mice. Brains were harvested from 4% paraformaldehyde perfused animals. Tissues were embedded in OCT and sectioned using cryostat. 10µm thick sections were collected and mounted on gelatin-coated slides. Images were taken by a dissecting scope attached with a CCD camera. Images show GFP expression in brainstems at 3-5 days following Cre induction by tamoxifen.
Figure 2.5 Lis1 expression level is reduced in brainstem of inducible Lis1 knockout mice. A.) Extracts of brainstem, cerebellum, and cortex from tamoxifen treated of both floxed Lis1 with Cre transgene and floxed Lis1 without Cre mice were probed with polyclonal Lis1 antibody. Brain tissues were collected at 3 days following high dose tamoxifen injection. Lis1 expression level was found reduced in brainstems of floxed Lis1 with Cre animals compared to controls. No reduction of Lis1 levels in the cortex was detected from tamoxifen induced Lis1 knockouts at 2 days after initiation of treatment compared to floxed Lis1 without Cre. No difference of dynein expression level was detected in tamoxifen induced Lis1 knockout lysates compared to control ones. B.) Cortex, cerebellum and brain stem of experimental animal were collected at Day 5 following tamoxifen treatment. Lis1 expression level was found reduced in brainstems and cerebellum of floxed Lis1 with Cre animals compared to controls. No difference of dynein expression level was detected in tamoxifen induced Lis1 knockout lysates compared to control ones. C.) Quantification of Lis1 expressions in the cortex, cerebellum, and brainstem from 4 animals of each genotype. Values are mean±SD. ** P<0.01, student t-test.
Figure 2.6 Lis1<sup>fl/fl</sup>;actCreER<sup>TM</sup>;R26<sup>Gt</sup> neurons in the regions of the nucleus ambiguous show axonal pathology at 3 days following tamoxifen injection. A.) Toluidine blue staining showed somal swelling, nuclear position, and Nissl substance dispersal in brain tissue. In tamoxifen treated experimental mice, nucleus of neurons in was displaced toward the periphery of the soma and the Nissl substance was significant reduced in the soma (thick arrows). Brainstem sections were selected where nucleus ambiguus was present according to the mouse brain atlas. Comparison were made from matched sections. B.) Diagram shows a coronal section of mouse brain. The region analyzed was reticular formation where contain nucleus ambiguus. C.) Quantification of nuclear area/soma area in inducible Lis1 knockout mice and control mice. Neurons were scored as having nuclear eccentricity if the perpendicular distance from the NE to the somal edge at any point is less than half the perpendicular distance of the NE to the somal edge at any other point. Experimental mice had significant larger nucleus versus cytoplasm in brainstem. Measurements were from 50 neurons from 3 sections of 2 mice per genotype. Values are mean±SD. ***P<0.0001, student t-test D.) Quantification of the percentage of nuclear eccentricity observed in tamoxifen inducible Lis1 knockout mice and control mice. Measurements were from 50 neurons from 3 sections of 2 mice per genotype. P=0.0043, student t-test.
Figure 2.6 Lis1\textsuperscript{fl/fl};actCreER\textsuperscript{TM};R26\textsuperscript{GT} neurons in the regions of the nucleus ambiguous show axonal pathology at 3 days following tamoxifen injection. E.) High magnification images of brainstem neurons from tamoxifen inducible Lis1 knockout mice and control mice. Scale bar=10\textmu m
Figure 2.7 Cultured DRG neurons from adult tamoxifen injected experimental animals, but not controls, show signs of axon pathology A.) Experimental animals showed GFP fluorescence after Cre induction, while only mTomato fluorescence was detected in control cells. Varicosities (large axon swellings at growth cones and axon terminal ends) were seen in the tamoxifen induced DRGs (white arrows). B) Several differences were found between GFP positive axons in from floxed Lis1 animals and Tomato positive axons from floxed Lis1 animals with no Cre. First the length of axon segments between branch points was shorter for GFP axons were shorter. Branch points were defined as a segment of axon between two adjacent growth cones. The first branch was counted from a leading process emerging from the cell body. C) There was an increase in the number of varicosities per branch point, and D) There is an overall increase in varicosities per mm of axon. Measurements were collected from 32 branches from 19 neurons of Lis1 knockouts and 30 branches from 9 neurons of the control. Values are mean±SD. **p=0.0054, ***p<0.001, student t-test.
Figure 2.7 E.) Representative images of axon varicosities seen in inducible Lis1 knockout and control mice. Much smaller swellings were seen in control neurons (short arrow). Only large swellings were counted as varicosities (long arrow). Scale bar=20μm.
Figure 2.8 Lis<sup>fl/fl</sup>;actCreER<sup>TM</sup>;R26<sup>GT</sup> DRG neurons placed in culture after tamoxifen treatment show defective axonal transport. A,B) Retrograde transport was disrupted in mice with floxed Lis1 and Cre compared to mice with wild-type Lis1. C,D,E) There is significant reduction in percentage of acidic organelles undergoing retrograde transport compared to mice with wild-type Lis1, while there is no significant difference in anterograde transport with Lis1 perturbation. Measurements were collected from 24 axons of 3 sets of animals per genotype. Anter: the numbers of organelles that moved toward the cell body, Retro: away from the cell body, Both: bi-directionally, Static: made negligible moves in either direction. Values are mean±SD. Turkey’s multiple comparison test was performed. ***p<0.001
Figure 2.8 Lis\textsuperscript{fl/fl};actCreER\textsuperscript{TM};R26\textsuperscript{GT} DRG neurons placed in culture after tamoxifen treatment show defective axonal transport. F.) Representative images of kymographs showing transport data in mice with floxed Lis1 and without floxed Lis1.
Figure 2.9 Heterozygous Lis1fl/+;CreERTM;R26GT mice did not exhibit apparent behavior abnormality. Analysis of Cre activity in brain tissues of these animals demonstrated homogenous strong GFP signal in the whole brain, coupled with dramatically reduced red fluorescence.
Figure 2.10 Time-course analysis of Cre activation in brains. Three heterozygous inducible Lis1 knockouts were injected with high dose tamoxifen on the same day and were sacrificed individually on day 7, and day 21 after initial treatment. Saggital sections of brains from three animals revealed a gradual increase in the amount of GFP throughout the brain including cortex, cerebellum and brainstem.
CHAPTER 3

THE EFFECT OF AN INDUCIBLE ACTIN-CRE DOUBLE LIS1 KNOCKOUT IN NON-NEURONAL TISSUES AND AN INDUCIBLE MYH6-CRE LIS1 KNOCKOUT MOUSE MODEL
3.1 INTRODUCTION:

Analysis of the actin-Cre driven inducible Lis1 knockout in an adult mice model suggested a previously unidentified but essential role of Lis1 in adult mammalian system, particularly in maintaining normal neuronal function. We have identified evidence of correlations between loss of Lis1 in brainstem and axon defects in tamoxifen induced Lis1 knockout animals. However, whether reduction of Lis1 expression level and the subsequent neuronal pathology in cardiorespiratory center in brainstem directly lead to the lethal phenotype observed in adult mice remains unclear. We could not rule out the possibility that loss of Lis1 upon Cre activation in other tissues such as hearts, lungs, liver etc could contribute to the death phenotype in tamoxifen inducible Lis1 knockouts since β-actin Cre drives Cre expression in most tissues throughout the whole body. Thus, it would be necessary to examine Cre activity in other tissues besides brains.

Although Lis1 expression level is very low in non-neuronal tissues, knockdown of Lis1 in life-threatening organs such as hearts could conceivably lead to ultimate death phenotype in adult mice. Besides its critical role in regulating neuronal functions in embryonic brain development, Lis1 is also involved in regulating other important cell functions. A lot of these regulations involve the interaction between Lis1 and a microtubule motor, dynein. The levels of Lis1 alter the distribution of dynein IC in Cos-7 cells and Lis1+/- fibroblasts exhibit microtubule organization(Smith et al., 2000). Lis1 has also been
implicated in mitotic spindle orientation and chromosome segregation, defects in which, in turn, affect mitotic progression (Faulkner et al., 2000). In mammalian cells, dynein/dynactin recruits Lis1 to kinetochores. Lis1 regulates dynein/dynactin binding to microtubule (MT) (Coquelle et al., 2002; Tai et al., 2002). Interaction between Lis1 and dynein heavy chain is important for membrane transport and possibly other cellular activities (Liang et al., 2004).

Invitro studies showed that Lis1 reduced microtubule catastrophe events, and its interaction with the cytoskeleton may be transducing a signal pathway (Sapir et al., 1997). The yeast homologue of Lis1, Pac1p has been shown to interact with a ubiquitin ligase (Alonso et al., 2012).

To date, there are only a few studies investigating roles of Lis1 in non-neuronal system. The epidermal-specific loss of Lis1 results in dramatic defects in microtubule reorganization. Lis1 ablation also causes desmosomal defects, which contributes to loss of epidermal barrier activity, resulting in completely penetrant perinatal lethality (Sumigray et al., 2011). A most recent study demonstrated that Lis1 is critically required for hematopoietic stem cell function and leukemogenesis. Conditional deletion of Lis1 in the hematopoietic system led to a severe bloodless phenotype, and embryonic lethality (Zimdahl et al., 2014).

Conditional deletion of Lis1 in developing hair cells causes defects in cytoplasmic dynein and microtubule organization, resulting in planar polarity defects without overt effects (Sipe et al., 2013). Although limited, these observations from Lis1 knockout mouse model suggest that Lis1 disruption in other tissues could result in defective organ functions.
Although we do not expect loss of Lis1 in non-neuronal adult tissue would lead to severe phenotypes given its relatively low expressions, if it does so, it would be a previously unidentified yet critical role of Lis1 in adult organs besides the brain.

In this chapter, we examined Cre activity in various tissues in inducible adult Lis1 knockout mice. Cre activity was found most abundantly in the heart and skeletal muscle, which are relevant to high expression of β-actin in these tissues. We also analyzed changes of Lis1 expression levels in multiple tissues of tamoxifen induced Lis1 knockouts. Significant reduction of Lis1 in adult heart prompted us to generate an inducible cardiomyocyte- specific Lis1 knockout mouse model. The alpha-MHC-MerCreMer transgene has the mouse cardiac-specific alpha-myosin heavy chain promoter (Myh6) directing expression of a tamoxifen-inducible Cre recombinase to adult cardiac myocytes. Mice homozygous for the Myh6 transgene are viable and fertile prior to tamoxifen administration. Lexow et al reports that a single tamoxifen injection (40mg tamoxifen/kg body weight) resulted in the uniform recombination in cardiomyocyte fibers without the cardiomyopathological effects. (Lexow et al., 2013) One week following tamoxifen injection, Cre activation detected by scattered β-gal expression was observed in atria, and homogenous expression in ventricles(Sohal et al., 2001). Driving Cre expression specifically in the heart with a MHC-6 promoter did not result in observable behavior abnormality.
3.2 MATERIALS AND METHODS:

Mouse breeding and colony maintenance

All the mice used in this project were maintained at the animal research facility in The University of South Carolina. The experimental protocols have been approved by the animal use protocol (AUP) committee. All the transgenic mice and knockout mice were maintained on its original genetic background. However, when generating inducible Lis1 knockout mice by crossing three different mouse strains, mixed genetic background could not be avoided. To minimize variation in the genetic background, the males (Lis1fl/fl;myh-6-CreER™; R26GT) and females (Lis1fl/fl;R26GT) from the same generation were crossed to produce inducible Lis1 knockout mice, and mice from the same litter were compared. However, in order to gather enough samples for statistical analysis, tissues from different animals with the same genotype at different ages were pooled.

Mice were marked by ear puncture at weaning (3-4 weeks old), and genomic DNA was extracted from tail for routine genotyping.

1.2.3 Genotyping:

Primers for genotyping heart-specific Cre are the same as universal Cre. Mice tails were digested in Tail buffer plus Protease K overnight at 55 °C. 2µl of tail digest was used as DNA templates for PCR reaction. Primers for Lis1 flox/flox: Sequence5’-3’ TGA ATG CAT CAG AAC CAT GC; CCT CTA CCA CTA AAG CTT GTT C. Primers for Cre: Sequence5’-3’ CTA GGC CAC AGA ATT GAA
AGA TCT; GTA GGT GGA AAT TCT AGC ATC ATC C. Primers for R26GT:
Sequence 5’->3’ CTC TGC TGC CTC CTG GCT TCT; CGA GGC GGA TCA CAA
GCA ATA; TCA ATG GGC GGG GGT CGT.

A standard PCR reaction was set up as followed: For each tail DNA sample, to
make 25 µl total volume, 6.5 µl of H2O, 5.5 µl Master Mix 2.5 µl each primer and
2 µl Tail DNA were added to PCR tubes. The reaction was performed on a PCR
thermocycler as follows: 94°C for 3 min for hotstart; 94 °C for 30s, 60 °C for 1
min, 72 °C for 1min. Repeat steps above for 35 cycles. 10 µl of the reaction
product was run on 5% polyacrylamide gel at 120V for 30 min. Gel was stained
with EB for 10min. DNA bands could be detected under UV light.

**TM injection and Cre activation**

We used the same amount of tamoxifen to induce Cre-mediated recombination in
heart specific Lis1 knockout mice as the universal Cre. Tamoxifen (Sigma-
Aldrich) was dissolved in corn oil (Sigma-Aldrich) at a concentration of 40mg/ml
and administered to 2 month-old Lis1 \textsuperscript{fl/fl}; myh6-CreER\textsuperscript{TM}; R26GT at 8mg/day for 2
days depending on the experiment settings. Water and food were available *ad
libitum*.

**Tissue preparation and Histology.**

Animals were deeply anesthetized with isofluorane administration and perfused
transcardially with ice-cold PBS first, followed by 4% paraformaldehyde (PFA) in
0.1M PBS, pH7.4. For whole-mount organ analysis, organs were isolated and
imaged with a CCD camera mounted on a fluorescence microscope (Olympus). For cryosection preparation, brains, hearts, lungs, livers and other tissues were removed and postfixed in 2% PFA+15% sucrose solution prepared in PBS overnight and subsequently cryoprotected by incubation in 30% sucrose solution for 3-4 hours at room temperature. The cryoprotected brains were submerged in OCT Compound solution and quickly frozen in 2-methylbutane cooled in liquid nitrogen. Sagittal or coronal sections were cut at a 10-μm thickness on a cryostat (Leica) and stored at -80°C before used. Slides were washed three times with PBS, treated with DAPI (Molecular Probes), washed again, mounted in anti-fade reagent (Invitrogen) and imaged with fluorescence microscopy (Zeiss) or confocal microscopy (Leica).

**Immunoblotting**

The levels of Lis1 protein was evaluated in Lis1 CKO and age-matched control mice by immunoblotting. Mice were deeply anesthetized and decapitated, and the brains and other tissues were removed quickly and frozen on dry ice. Tissue samples were homogenized with Dounce’s homogenizers in ice-cold RIPA buffer with protease inhibitors. Crude homogenates were sat on ice for 20 minutes and then centrifuged at 20,000 g for 1 hour (4°C) to yield soluble supernatant and pellet fractions. Protein concentrations were measured by BCA assay (Thermo scientific) with bovine serum albumin as a standard. Protein samples were subjected to 10% SDS-PAGE and transferred to PVMF membrane. Blots were blocked with 5% nonfakt dry milk with 0.1% Tween 20 in PBS and then incubated
overnight at 4°C with antibody to Lis1 or dynein intermediate chain(). After the primary antibody incubation, blots were washed and incubated with horseradish peroxidase-conjugated secondary antibody, developed with enhanced chemiluminescence (Millipore) and exposed to x-ray film.

To quantify protein immunoreactivity, films were scanned and densitometry was performed using image J. Protein levels were expressed as relative optical density measurements. Immunodensities were normalized to control proteins.

**Immunostaining**

Neurons were plated onto 12mm coverslips in 24-well plates. Neurons were fixed in 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100 for 5 min at room temperature. Nuclei were visualized with Hoechst and coverslips were mounted on glass slides using ProLong Gold Antifade reagent (Invitrogen). Neurons were observed using an inverted microscope (Axiovert 200, Carl Zeiss Inc.) equipped with Plan-Neo 100x/1.30 objective and a Plan-Apo 63X/1.40 objective. Digital images were acquired using a charge-couple camera (AxioCam HRm, Carl Zeiss Inc.) linked to AxioVision software (Version 4.7, Carl Zeiss Inc.).
3.3 Results:

*Mosaic Cre activity was detected in non-neuronal tissues of inducible β-actin Cre Lis1 knockout mice*

The fluorescent images of whole mount organs showed strongest GFP levels in heart tissue, while relatively low level of Cre recombination detected in liver at 3 days following tamoxifen injection. At Day 5, an increase in Cre activation indicated by homogenous green fluorescence detected in the heart and lung, but GFP level in liver remained low at this point (Figure 3.1). Tissue sections of tamoxifen induced Lis1 knockout mice revealed a subset of cells were activated in the heart, lung, liver and kidney(Figure 3.2). These observations are consistent with the original report of actin-Cre ER mouse model that Cre activities in multiple tissues were highly mosaic, with the highest recombination efficiency detected in highly vascular tissue like the heart. The GFP expression patterns detected in lung, liver and kidney suggest that Cre was activated in a certain type of cells. Whether these cells have more β-actin expression than others that do not show green remains unclear. Tissue sections of tamoxifen injected control mice do not have GFP expression in all organs.

*Tamoxifen induced cardiomyocyte-specific Lis1 knockout mice do not exhibit aberrant phenotype*

Although there is significantly less Lis1 in these tissues normally, the death of knock out animals suggested the possibility that Lis1 loss in the heart itself could result in cardiac failure. To test this directly we crossed our double floxed
Lis1/Cre-reporter mice with a mouse in which Cre-ER expression driven by a myosin heavy chain (MHC6) promoter and is restricted to cardiomyocytes. Generated heterozygous floxedLis1;myh-6-Cre;R26^{GT} were backcrossed with double floxed Lis1;R26^{GT} to obtain homozygous floxed Lis1;myh6-Cre;R26^{GT} mice. Mice harboring double floxed Lis1;R26^{GT}; but no myh-6-Cre served as control ones(Figure 3.3). Experimental and control mice were injected with 8mg tamoxifen for two consecutive days. Interestingly, none of the floxed Lis1 animals showed any overt behavior abnormality following tamoxifen injection (n=6). Lis1 expression level was examined by western blot and found reduced in heart tissue. Fluorescence images of whole tissue revealed strong GFP expression in cardiac muscle, with significant red fluorescence reduced. No Cre activation was observed in the whole brain including brainstem in this heart-specific Lis1 knockout mouse model(Figure 3.4). This strengthened the hypothesis that the death phenotype was caused by Lis1 loss in neurons of the cardiorespiratory brainstem network. However, it remains possible that loss of Lis1 in other cell types in the mouse could contribute to the severe phenotype of Lis1 knockout.

**Comparison of Lis1 expression patterns in multiple tissues of low-dose and high-dose tamoxifen induced Lis1 knockout mice**

Analysis of multiple tissue sections revealed that Cre activity was present in non-neuronal tissues as well as brains. However, the degree of Cre activation upon tamoxifen injection varies between tissues. Additionally, low-dose tamoxifen treated Lis1 knockout mice survived longer than high-dose tamoxifen treated
ones. We know this difference in survival is not due to the direct effect of
tamoxifen as actin-CreER\textsuperscript{TM}:R26\textsuperscript{GT} mice with wild-type Lis1 treated with
tamoxifen do not have detectable behavior abnormality. It may reflect different
changes in Lis1 expression patterns in various tissues, which in turn, may result
in different phenotypes or distinct timings of disease progression in adult mice.
Thus, it would be interesting to compare Lis1 levels in various tissues of low-dose
and high-dose tamoxifen induced Lis1 knockout mice. We compared changes in
Lis1 expression patterns in low-dose and high-dose tamoxifen treated animals.
High dose tamoxifen treated animals showed severe malaise on day 5 following
initial treatment. Low-dose treated animals did not exhibit any behavior
abnormality on day 5. Tissues were collected on day 5 to examine changes of
Lis1 levels. We found that there was a significant reduction in Lis1 level in
brainstem from high-dose treated experimental mice compared to the control,
while low-dose treated animals did not have Lis1 reduction in brainstem on day
5(Figure 3.5). Consistently, there was a Lis1 reduction in tamoxifen treated
hearts in both low dose and high dose cases. However, dynein and tubulin
expression levels were very low in the heart, suggesting Lis1/dynein pathway in
the heart may not be as important as it is in the brain. All of these suggest that
loss of Lis1 in brainstem may be the cause of lethal phenotype observed in
tamoxifen induced Lis1 knockout mice.

High dose tamoxifen treated experimental mice die within one week
following initial injection. Most of them showed severe malaise and were about to
die by the endpoint that we euthanized and harvested the tissue for further
analysis. Among those, there was one mouse that showed milder phenotype than other experimental mice. It exhibited leg clasping during tail suspension test, however, it did not have other symptoms such as kyphosis, difficulties walking and breathing like other experimental mice did. Low dose tamoxifen treated experimental mice did not show aberrant phenotype on day 5 following initial injection. We harvested tissues from the high-dose treated mouse that showed milder phenotype and compare them to the tissues from the low-dose treated experimental mice. Tissue lysates were probed with polyclonal Lis1, GFP and tubulin antibodies. After normalizing to tubulin, western blot showed that there is a small decrease (~24.7%) in Lis1 in the cortex of high-dose treated animal compared to the control, while there was no difference in between low-dose treated animal and the control(Figure 3.6). Interestingly, both in low-dose and high-dose treated animals, there were dramatic decreases of Lis1 level in hearts. There was a small reduction in the high-dose treated lung compared to the control. No difference in tamoxifen treated liver tissues compared to control livers. These results suggest that reduction of Lis1 level in cortex may result in behavior abnormality seen in this animal. However, Lis1 reduction in the heart and the lung did not result in severe phenotype that leads to death observed in other tamoxifen induced Lis1 knockout mice. GFP expressions in the tamoxifen induced Lis1 knockout tissues are consistent with fluorescent images of tissue sections shown earlier, with highest level in hearts.
Cre activation in diaphragm and skeletal muscle tissues

Heart specific Lis1 knockout mice did not show any obvious behavior abnormality compared to wild type mice. It suggests that loss of Lis1 in heart does not cause death phenotype. Other possible causes of lethal problems include defects in diaphragm that supports regular breathing. Evidence showed that there was no significant loss of motor neurons or dorsal root ganglia sensory neurons, but a decrease in complexity of neuromuscular junctions (Courchesne et al., 2011). We then examined Cre activity in diaphragm and other skeletal tissues. We detected strong GFP expression in diaphragm and thigh muscle, which is rich in β-actin (Figure 3.7A). However, Lis1 expression in diaphragm is almost undetectable compared to its levels in brains and hearts even after overnight exposure (Figure 3.7 B).

3.3 DISCUSSION:

The pathological causes of the lethal phenotype seen in tamoxifen induced Lis1 knockout mice are largely unknown. A critical question is whether the pathological consequences arise from the brainstem neuron itself or from non-neuronal cells. Mosaic Cre activity was detected in non-neuronal tissues of inducible β-actin Cre Lis1 knockout mice with high level in the heart. Heart failure is one of the possible reasons that can account for the lethal phenotype. Tamoxifen induced heart specific Lis1 knockout mice did not exhibit any observable behavior abnormality as it is seen in the β-actin Cre inducible Lis1
knockout mice. Given the fact that myh6-Cre only drives Cre activation in cardiomyocytes, we cannot rule out the possibility that loss of Lis1 in other heart cells such as neural crest cells may affect the phenotype in adult mice. Additionally, the original myh6-Cre mouse strain is in B6.FVB(129) mixed background, which may also impact the phenotype observed in these mice. There is no literature that described the role of Lis1 in heart development so far. However, there is a link between Lis1 being a target of miR302/367 and lung development. Lis1 is essential for proliferation and the precise control of mitotic spindle orientation in both neuroepithelial stem cells and radial glial progenitor cells (Yamada et al., 2010; Yingling et al., 2008). Downregulation of miR302/367 in transgenic lung endoderm displayed a disorganized apical layer. Lis1 was found to be associated with miR302/267 for establishment of proper apical-basal polarity of a single-layered endoderm (Tian et al., 2011). However, again, these findings are done in the context of developmental stage. No direct link between Lis1 level and lung function in the adult system has been identified so far.

Western blot analysis of Lis1 levels in various tissues of the tamoxifen induced Lis1 knockout mouse that had much milder phenotype showed highest GFP expression level and significant reduction in the heart compared to other tissues. This suggests that reduction of Lis1 in hearts is not likely to result in the lethality phenotype observed in β-actin Cre Lis1 knockout mice. We also did western blot analysis of Lis1 levels in a tamoxifen induced Lis1 knockout mouse that had severe malaise. It showed that there was a significant reduction of Lis1 in brainstem, while only a slight decrease in other tissues. This preliminary result
strengthens our hypothesis that the death phenotype is resulted from loss of Lis1 in brainstem. However, due to the technical difficulty of harvesting multiple tissues from 4 different treatment groups at one time, a more systematic and detailed tissue analysis will be needed to further confirm the changes of protein levels in various tissues upon Cre induction. Percentage of Lis1\textsuperscript{fl/fl};Cre recombination can also be measured at DNA level using quantitative PCR amplification of Lis1\textsuperscript{fl/fl} allele from genomic DNA isolated from each tissue. Alternatively, changes in mRNA level can be used to estimate the recombination efficiency in various tissues.

The reasons of death phenotype in the inducible Lis1 knockout mouse can not be certain because β-actin Cre is ubiquitously expressed, and many tissues can be affected. Another possibility we have not discussed above is respiratory muscles, like diaphragm. Analysis of Cre activity showed strong GFP expression in diaphragm and other skeletal muscle. Defects in neuromuscular junction in the diaphragm could be correlated with breathing defects that may be responsible for death of the inducible Lis1 knockout mice. However, Lis1 expression in diaphragm is undetectable compared to it is in the brain and the heart. Furthermore, low levels of Cre activity were also detected in the liver and kidney of tamoxifen induced animals. Given the fact that Lis1 plays a important role in mitosis and dynein-dependent receptor recycling and vesicle transport, loss of Lis1 in liver and kidney may affect hormone metabolism through signaling. Although hepatomegaly and edema are not evident, liver and kidney cannot be excluded.
Figure 3.1 The fluorescent images of whole mount organs. A,B,C) No green fluorescence was detected in heart, lung and liver of a control animal(-Cre_injected with tamoxifen. D,E,F) The fluorescent images of whole mount organs showed strongest GFP levels in heart tissue, while relatively low level of Cre recombination detected in liver at 3 days following tamoxifen injection. G,H,I) At Day 5, an increase in Cre activation indicated by homogenous green fluorescence detected in the heart and lung, but GFP level in liver remained low at this point.
Figure 3.2 Cre activations in various tissue sections. 

A,B,C,D) Tissue sections of control mice (-Cre) injected with tamoxifen showed no GFP expression in heart, lung, liver and kidney. 

E,F,G,H) Tissue sections of tamoxifen induced Lis1 knockout mice revealed a subset of cells were activated in the heart, lung, liver and kidney. Shown are cardiac muscle (E), lung alveoli (F), liver hepatocytes (G) and kidney glomerulus (H). Scale bar=200µm
A.) Three mouse strains were used to generate cardiomyocyte-specific Lis1 knockout mice. B.) We crossed our double floxed Lis1/Cre-reporter mice with a mouse in which Cre-ER expression driven by a myosin heavy chain (MHC6) promoter and is restricted to cardiomyocytes. Generated heterozygous floxedLis1;myh-6-Cre;R26\textsuperscript{GT} were backcrossed with double floxed Lis1;R26\textsuperscript{GT} to obtain homozygous floxed Lis1;myh-6-Cre;R26\textsuperscript{GT} mice. Mice harboring double floxed Lis1;R26\textsuperscript{GT}, but no myh-6Cre served as control ones.

Figure 3.3 Tamoxifen induced cardiomyocyte-specific Lis knockout mice.
Figure 3.4 Cre activation in cardiomyocyte-specific Lis1 knockout mice

A.) Fluorescence images of whole tissue revealed strong GFP expression in cardiac muscle, with significant red fluorescence reduced. No Cre activation was observed in the whole brain including brainstem in this heart-specific Lis1 knockout mouse model.

B.) Lis1 expression level was examined by western blot and found reduced in heart tissue.

C.) Quantification of Lis1 expressions in the hearts from 2 animals of each genotype. Values are mean±SD. ** P<0.01, student t-test.
Figure 3.5 Changes in Lis1 levels of tissues from low dose and high dose treated animals.  

A.) Tissues were collected on day 5 to examine changes of Lis1 levels. There was a reduction in lis1 level in brainstem from high-dose treated experimental mice compared to the control, while low-dose treated animals did not have Lis1 reduction in brainstem on day 5. Consistently, there was a Lis1 reduction in tamoxifen treated hearts in both low dose and high dose cases.

B.) Quantification of Lis1 expressions in the brainstem and heart from 3 animals of each treatment per genotype. BS: Brainstem, HT: Heart. Low: 2mg tamoxifen injection per animal. High: 8mg tamoxifen injection per animal. Values are mean±SD.
Figure 3.6 Lis1 expression level changes in tissues from low dose and high dose treated animals. 

A) There was a small reduction in the high-dose treated lung compared to the control. No difference in tamoxifen treated liver tissues compared to control livers. 

B) Western blot of kidney and diaphragm from low-dose/high-dose tamoxifen treated animals. 30μg protein extracts were loaded on each lane. There was a reduction of Lis1 in high-dose treated kidney compared to the control. However, there was no detectable Lis1 present in diaphragm. Commasse gel staining was used as a loading control.
Figure 3.7 Cre activities in diaphragm and skeletal muscle. **A)** Strong GFP expression was detected in diaphragm and thigh muscle, which are rich in β-actin. **B)** Lis1 expression in diaphragm is almost undetectable compared to its levels in brains and hearts even after overnight exposure. **C)** Quantification of Lis1 expressions in the brain, heart and diaphragm from 3 control animals (-Cre). Values are mean±SD. **P<0.01,** ***P<0.001. One-way ANONA test.
CONCLUSIONS AND FUTURE DIRECTIONS
LIS1 is the gene that encodes for the β subunit of platelet-activating factor acetylhydrolase (PAFAH1B1). Heterozygous mutation or deletion in LIS1 causes a severe developmental brain disorder, lissencephaly in humans. Homozygous null mice die early in embryogenesis, while mice with one inactive allele displayed brain disorganization. Moreover, Lis1 has been shown to be regulating dynein dependent organelle transport. In this study, the effect of Lis1 knockout in adult mammalian system has been investigated using a tamoxifen inducible knockout mouse model directed by β-actin Cre. Loss of Lis1 in adult mice caused a progressive decline and ultimately death. These phenotypes were not observed in any of a variety of control animals. Analysis of Cre activity revealed it was not uniformly stimulated in all regions of the brain, and is highest in the brainstem, where neuronal processes in regions known to control cardiorespiratory networks were activated. Deletion of lis1 in hearts did not seem to induce lethality or malaise in adult mice. This strengthened the hypothesis that the death phenotype may be caused by Lis1 loss in neurons of the cardiorespiratory brainstem network. Analysis of brain sections and cultured sensory neurons from adult tamoxifen injected experimental animals revealed axonal pathology and defective axon transport. However, due to the ubiquitous expression pattern of Cre, we could not rule out the possibility that loss of Lis1 in non-neuronal tissues may contribute to the death phenotype observed in tamoxifen induced Lis1 knockout mice. We have noted that some of the tamoxifen induced Lis1 knockout mice had wet fur and enlarged abdomen. Whether these symptoms occur frequently still
needs further examination. This may suggest a bladder and gut dysfunction, which are components of autonomic functions.

By controlling the site of drug delivery, β-actin Cre allows spatial modulation of gene activity. In this case, 4-OH tamoxifen can be sterotaxicically injected in the region of mouse brainstem to knockout Lis1 in mice. Alternatively, recombinant adenoassociated viral (rAAV) vectors expressing Cre recombinase can be microinjected into distinct mouse brain regions to selectively knockout Lis1(Schierberl and Rajadhyaksha, 2013). However, sterotaxical injection of tamoxifen in the mouse brain may not activate the same subset of neurons that are expressing GFP upon IP injection. If loss of Lis1 specifically in brainstem causes lethality in adult mice, it will suggest that depletion of Lis1 in brainstem dramatically alters the neuronal function, which may lead to devastating consequences.

Although we have provided some information on low-dose tamoxifen induced Lis1 knockout mice, further experiments will be needed to characterize the phenotype with loss of Lis1. Moreover, adjusting the dosage of tamoxifen injected in the animal, we can generate different levels of mosaicism which would result in different phenotypes(Espinosa et al., 2014). By identifying regions that have undergone the recombination, the role of Lis1 in these regions can be investigated. Low doses of TM induce recombination in a small set of cells, which may circumvent the lethality observed in high dose tamoxifen induced Lis1 knockout mice.
Because its essential roles in brain development, the majority of Lis1 mutant mice generated have been aimed at elucidating the effects of Lis1 depletion during developmental stage. Our inducible Lis1 knockout mouse is one of the few mouse models that studied the role of Lis1 in mature mammalian system. The progressive decline and lethal phenotype is unexpected but interesting. Axonal pathology observed in presumably motor neurons in brainstem indicated the possible link between neuronal dysfunction and disruption of Lis1/dynein pathway. Given the fact that Lis1 is a dynein regulator, mutation in dynein heavy chain result in progressive motor neuron degeneration in heterozygous animals(Andersen, 2003). Defects in dynein dependent axon transport have been linked to motor neuron degeneration in mice. Thus, it will be reasonable to examine how loss of Lis1 alters Lis1 and dynein interactions, distribution or activity under pathological conditions. Potential changes in interacting proteins can be monitored by IP-Western with the 74.1 DIC antibody. We can also determine if dynein is more or less likely to co-pellet with microtubules from inducible Lis1 knockout brain.

There are several limitations in the current study. First, although Lis1 protein decrease has been demonstrated by western blot in the whole brainstem of mutant mouse, a more detailed description for the specific cell types affected by Lis1 knockout in adult mice has not been examined. This should have been achieved by immunostaining on tissue sections, however, the Lis1 antibody used in the current study is not suitable for immunostaining on tissue sections. Therefore, the pattern of Lis1 knockout is demonstrated indirectly by GFP
expression of R26\textsuperscript{GT} transgene. For future study, we can use a commercially available Lis1 antibody H300, a rabbit polyclonal IgG to accurately examine Lis1 expression in various tissue. Secondly, the axon transport study was only performed on cultured sensory neurons which may not provide direct information on the effect of loss of Lis1 on motor neurons in brainstem. It is difficult to do transport study on tissues. However, an approach has been established to allow single cargo analysis in motor axons in situ. By utilizing a Thy1-MitoCFP transgenic mouse which selectively labels neuronal mitochondria, the flux of fluorescently labeled mitochondria could be measured in intercostal nerves in triangularis sterni nerve muscle explants (Bilsland et al., 2010; Marinkovic et al., 2012). In addition, axonal morphology in brain tissues has not been examined in this study. In human neurodegenerative disorder dystrophic neurites are filled with autophagic vacuolar structures that can be visualized by electron microscopy (Nixon, 2007). Developmental Lis1 heterozygosity resulted in changes in dendrites in the adult hippocampus, but it was not clear whether this reflected a developmental defect or a later onset event (Fleck et al., 2000). We can compare dendritic arbors in inducible Lis1 knockout and control mice using Golgi Staining. Thirdly, the ultimate death of the inducible Lis1 knockout mouse prevents further analysis at later stages. Thus, other Cre lines with more specific expression pattern could be considered in future. For instance, the Camk2a-Cre/ESR1 strain, available through the European Mouse Mutant Archive, carries an inducible Cre recombinase under the control of regulatory elements of the
CAMK2a gene, would permit analysis of neuronal effects in the cortex and hippocampus.

The current study exploits the technique of tamoxifen inducible knockout to study the role of Lis1 in adult mammalian system. To our knowledge, this is the first study that investigates the effects of deletion of double alleles of Lis1 in adult system using a inducible knockout mouse model. The findings from this study demonstrate the importance of Lis1 in adult mammals. We provide a novel model system to explore the function of Lis1 in intact animals and a model for potential therapeutic intervention to counteract symptoms of lissencephaly that may arise post-developmentally as a result of transport defects.
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