University of Massachusetts Amherst
ScholarWorks@UMass Amherst

Masters Theses 1911 - February 2014

January 2008

Deletion of the Bax Gene Severley Impairs Sexual Behavior and Modestly Impairs Motor Function In Mice

Jigyasa Jyotika
University of Massachusetts Amherst

Follow this and additional works at: https://scholarworks.umass.edu/theses

Jyotika, Jigyasa, "Deletion of the Bax Gene Severley Impairs Sexual Behavior and Modestly Impairs Motor Function In Mice" (2008). *Masters Theses 1911 - February 2014*. 158.
Retrieved from https://scholarworks.umass.edu/theses/158

This thesis is brought to you for free and open access by ScholarWorks@UMass Amherst. It has been accepted for inclusion in Masters Theses 1911 - February 2014 by an authorized administrator of ScholarWorks@UMass Amherst. For more information, please contact scholarworks@library.umass.edu.

DELETION OF THE *Bax* GENE SEVERELY IMPAIRS SEXUAL BEHAVIOR AND MODESTLY IMPAIRS MOTOR FUNCTION IN MICE

A Thesis Presented

by

JIGYASA JYOTIKA

Submitted to the Graduate School of the University of Massachusetts Amherst

In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

September 2008

Neuroscience and Behavior

DELETION OF THE *Bax* GENE SEVERELY IMPAIRS SEXUAL BEHAVIOR AND MODESTLY IMPAIRS MOTOR FUNCTION IN MICE

A Thesis Presented

by

JIGYASA JYOTIKA

Approved as to style and content by:

_____
Nancy G. Forger, Chair

_____
Geert J. de Vries, Member

_____
Sandra L. Petersen, Member

_____
Nancy G. Forger, Director
Neuroscience and Behavior Program

ACKNOWLEDMENTS

I would like to thank my advisor Prof Nancy Forger for her patient guidance, insight, contribution to my professional development and tremendous support throughout my graduate study. Thanks also to Prof Geert De Vries for his advice and enthusiasm towards my project.

I am also grateful to my committee member Prof Sandra Petersen.

This study would not have been possible without constant selfless support from my labmate Jill McCutcheon.

Thanks also to postdocs and students in the Forger and De Vries labs - Greta Rosen, Elaine Murray, Dena Jacob, Lauren Ditzian, Theresa Ray, Artie Seeling and to Julie Laroche for their helpful suggestions and comments throughout and for their friendship.

A special thank you to my family for their encouragement and determination always.

ABSTRACT

DELETION OF THE *Bax* GENE SEVERELY IMPAIRS SEXUAL BEHAVIOR AND MODESTLY IMPAIRS MOTOR FUNCTION IN MICE

SEPTEMBER 2008

JIGYASA JYOTIKA, B.SC. (HONS.), MICROBIOLOGY, UNIVERSITY OF DELHI

M.SC., MOLECULAR MEDICINE, TRINITY COLLEGE OF DUBLIN

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Nancy G. Forger

During neural development, nearly 50% of all newly generated neurons undergo cell death after making provisional contact with their target cells. The functional consequences of eliminating this neuronal cell death are not known. Bax, a pro-apoptotic protein, is required for cell death in many neural regions. A null mutation of the *Bax* gene in mice has been shown to increase overall cell number and eliminate the sex differences in neuron number in the anteroventral periventricular nucleus (AVPV) and the principal nucleus of the bed nucleus of the stria terminalis (BNSTp). The aim of my Master's thesis was to study male and female sexual behaviors and motor behavior in *Bax -/-* mice and their wild-type siblings. Animals were gonadectomized in adulthood and provided with ovarian hormones or with testosterone for tests of female and male sexual behaviors, respectively. Wild-type mice exhibited a sex difference in feminine sexual behavior, with high lordosis scores in females and low scores in males. This sex difference was eliminated by *Bax* deletion, with very low receptivity exhibited by both male and female *Bax -/-* mice. Male sexual behavior was not sexually dimorphic among wild-type mice, but mounts and pelvic thrusts were nearly absent in *Bax -/-* mice of both sexes. The

knockouts did not display deficient motor strength or performance at low speeds on a RotaRod apparatus compared to wild type mice. At high speeds, however, *Bax -/-* animals exhibited impairments on the RotaRod. Therefore, developmental cell death may be required for exhibition of male and female sexual behaviors, and for coordination of relatively difficult motor tasks.

CONTENTS

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

CHAPTER 1

INTRODUCTION

Neuronal Cell Death During Development

There is widespread cell death during the normal development of the vertebrate nervous system. About one half of all neurons initially produced die during a period of naturally-occurring cell death after making provisional contacts with targets and afferents. Dying neurons exhibit the characteristic morphological and neurochemical changes of cell death such as cell shrinkage, nuclear condensation, membrane blebbing and fragmentation of nuclear DNA (Bredesen, 1995; Merry et al., 1997). Cell death is thought to numerically match afferent neurons with their target cells (reviewed by Oppenheim, 1991). One reason proposed for the production of excess neurons is that this results in a competition for contacts with cellular partners and, thus, adjusts their numbers to provide optimum innervation of synaptic targets (Hamburger et al., 1982; Pettmann et al., 1998). However, few attempts to test the functional consequences of neuronal cell death have been made. One way to test this is to examine genetically-manipulated mice in which neuronal cell death has been prevented, which is the approach taken here.

The Bcl-2 Family of Proteins and Mechanisms of Cell Death

A family of proteins (called the Bcl-2 family after the discovery of its first member in a *B* *C*ell *L*ymphoma cell line) regulate cell death via an active cell suicide program called apoptosis. The Bcl-2 family of proteins consists of family members

categorized on the basis of structure and function. All proteins in the family share homology in their Bcl-2 homology (BH) domains. Members of the Bcl-2 family are of two kinds depending on whether they inhibit cell death or promote it. Most anti-apoptotic proteins including Bcl-2 and Bcl-xL (*B* *C*ell *L*ymphoma *Ex*tra *L*ong) have four BH domains and a transmembrane domain that facilitates insertion of the protein into the mitochondrial membrane. Pro-apoptotic proteins either have the BH domains 1-3 and the transmembrane domain (e.g. Bax) or only the BH3 domain and sometimes a transmembrane domain. This latter group of proteins are called BH3- only proteins. The exact molecular mechanism through which Bcl-2 family members control apoptosis is not known. As per the "rheostat" model first proposed to explain one possible mechanism, a high Bax to Bcl-2/Bcl-xL ratio in a cell, leads to cell death. In this model, Bcl-2 and Bcl-xL prevent cell death mainly by binding *Bax* through heterodimerization with it and the balance between pro-apoptotic and anti-apoptotic members acts as a checkpoint to determine whether or not a cell will undergo apoptosis (Oltvai et al, 1993; reviewed by Korsmeyer et al., 1999). A more current model proposes that it is the differential subcellular localizations of the Bcl-2 family proteins and the levels of BH-3 only proteins that are critical to the regulation of cell death. In this model, Bcl-2 is primarily localized to the outer mitochondrial membrane and Bax normally resides in the cytoplasm. Upon receiving a death signal Bax translocates to the mitochondria and changes conformation and if unbound by an anti-apoptotic Bcl-2 family protein, it homo-oligomerizes and causes cytochrome c release from the mitochondria into the cytosol (Valentijn et al., 2003)**.** Release of cytochrome c activates downstream cascades resulting in cell death via one of the possible mechanisms discussed below. BH3-only proteins

(singly or in combination) disrupt this equilibrium by binding and inactivating antiapoptotic Bcl-2 proteins, and indirectly promoting *Bax* homo-oligomerization and its downstream sequelae. Other BH3-only proteins directly bind and activate Bax. So the level of BH3-only proteins determines how much Bcl-2 or Bcl-xL is available to bind Bax in the cell and prevent Bax homo-oligomerization and whether or not Bax will be able to bring about cell execution (Harris and Johnson 2001; Putcha et al., 1999, 2002).

In many tissues, Bax as well as another full-length pro-apoptotic Bcl-2 family protein, Bak, are required for cell death and the genes for both proteins must be deleted to prevent cell death (Lindsten et al., 2000). In developing neurons however, Bak in its full-length form is reported to be markedly reduced or altogether absent (Sun et al., 2001). This may be why Bax is so critical for neuronal cell death.

Two alternative theories exist to explain how Bax oligomers cause cell death. Bax oligomers forms may form channels in the outer mitochondrial membrane through which cytochrome c can escape into the cytosol and bind apoptotic protease-inhibiting factor 1 (Apaf-1). This complex recruits and activates caspase 9, which then activates other downstream caspases. Caspases (*C*ystein *Asp*artic Acid Prote*ases)* are downstream death effector molecules that degrade cellular proteins to cause cell death. Another theory about what happens when a cell death signal is received is that Bax alters the activity of pre-existing channels called Permeability Transition Pores (PTPs) between the inner and outer mitochondrial membranes. When Bax homo-oligomers form at the mitochondria, the activity of the PTPs is altered and cytochrome c is released. In support of this theory, blocking PTP inhibits Bax induced apoptosis (Zoratti et al., 1995; reviewed by Gross et al, 1999).

The Central Role of *Bax*

Bax is widely expressed in the nervous system and is present at high levels during the peak of developmental cell death in several brain structures (Vekrellis et al., 1997). The role of Bax has been demonstrated in several types of neuronal cell death. Deletion of *Bax* eliminates much of the neuronal death that occurs in both the PNS and CNS during development (e.g. cranial and retinal ganglion cells, spinal motoneurons, brainstem, cerebellar and hippocampal neurons; White et al., 1998). In one study, sympathetic ganglion cells from *Bax*-/- mice were cultured for more than 21 days in the absence of any exogenous growth factor (Deckwerth et al., 1996), showing that trophic factor deprivation-induced death depends on Bax.

Gonadal Steroid Hormones Organize the Structure of the Central Nervous System in Vertebrates

Several sex differences in neuron number have been identified in the rat central nervous system. Males have many more neurons than females in the sexually dimorphic nucleus of the preoptic area (SDN-POA), principle nucleus of the bed nucleus of the stria terminalis (BNSTp) and in the spinal nucleus of the bulbocavernosus (SNB) (Gorski et al., 1980; del Abril et al., 1987; Guillamon et al., 1988; Breedlove et al., 1980). Females have more neurons in the anteroventral periventricular nucleus (AVPV) of the hypothalamus (Sumida et al., 1993). Phoenix et al first proposed that the differential gonadal steroid hormone exposure of male and female brains during a developmental critical period permanently organizes neural structures and circuits to establish sex

differences in function in the nervous system. Exposing female guinea pigs to testosterone *in utero* permanently interfered with the animals' tendency to show female reproductive behaviors in adulthood. Treating adult females with testosterone had a transient effect, or none at all on these behaviors (Phoenix et al., 1959). The study led to the formulation of the organizational/activational hypothesis - that sex hormones act during the fetal or neonatal stage of a mammal's life to organize the nervous system in a sex-specific manner, and that during adult life, the same hormones may have transitory, activational effects. Male rats have higher testosterone levels than females from day 18 of gestation till day 5 after birth (Weisz et al., 1980; Pang et al., 1979).Early exposure to testicular hormones masculinizes brain structures. Castrating neonatal male rats significantly reduces the volume of the SDN-POA and BNSTp in adulthood whereas exposing developing female rats to testosterone increases the size of these structures in adulthood (Dohler et al., 1984; Guillamon et al. 1988).

In mice, differentiation of the testes is thought to be completed by embryonic day 14.5, and males experience a testosterone surge around day 17 of gestation and a secondary rise just after birth (reviewed by Burns-Cusato et al., 2004). BNSTp volume and cell number is sexually dimorphic in adult C57BL/6 mice as it is in rats (Forger et al.,2004). The difference in volume and cell number emerges between postnatal day P7 and it has been assumed that females have more cells in AVPV based on volume and cell densities in females (Gotsiridze et al., 2007, Zup et al., 2003). AVPV cell number differs between male and female mice too with higher cell numbers in females (Forger et al., 2004). As in rats, male mice exhibit sexual differentiation in the SNB too with higher

SNB motoneuron numbers in males than in females measured (Wee et al., 1987; Jacob et al., 2005).

Testosterone Acts by Increasing or Decreasing the Rate of Apoptosis

Theoretically, sex differences in the nervous system could result from differences in neurogenesis, migration, phenotypic differentiation or hormonally regulated cell death (DeVries and Simerly, 2002). So far, cell death is the best established mechanism and has been implicated in the sexually dimorphic development of the BNSTp, the SNB and the AVPV of mice and rats (reviewed in Forger, 2006). Testosterone (or one of its hormonal metabolites) causes sex differences in neuron number by increasing or decreasing the rate of apoptosis in several neural regions. Apoptosis in these regions can be measured by quantifying dying cells based on their characteristic morphological appearance or by detecting the orderly fragmentation of DNA. However, little is known about the molecular basis of the mechanisms which underlie hormonally controlled cell death or survival.

Masculinization and Feminization

Male rats undergo two developmental processes, masculinization and defeminization, that affect their adult behavior. Masculinization refers to development of the underlying neural circuitry and behavioral patterns that are exhibited either exclusively or to a greater degree by males than females. Male copulatory behaviors are masculinized by androgens during development and also require androgens for activation during adulthood. Defeminization in males reduces the likelihood that males will display

female-typical sex behaviors in adulthood – such as the display of lordosis (a posture adopted by a sexually receptive female during mating). In contrast, females in the absence of testosterone undergo feminization and demasculinization to exhibit female sex behavior (most importantly lordosis) and not exhibit male-like sex behavior. Depriving males rats of their testes, or steroids produced by the testes, during the critical developmental period results in their demasculinization and feminization (Arnold et al., 1984; McEwen et al., 1983).

The testosterone produced by the testes is converted to estradiol in the brain by the enzyme aromatase (Jost et al., 1983). After aromatization, estradiol (E2) binds to two known estrogen receptors (ERs) - ERα and ERβ. Some evidence suggests that ERα is primarily involved in masculinization, while ERβ has a major role in defeminization of sexual behaviors (Kudwa et al 2005; 2006). Testosterone can also bind androgen receptors itself or after conversion to the non-aromatizable androgen, dihydrotestosterone. Activation of androgen receptors is also required for normal masculinization of brain and behavior (Zuologa et al., 2007. Bodo et al., 2006; 2008).

Deletion of *Bax* eliminates sex differences in overall neuron number in some sexually dimorphic regions in the brain involved in sex behavior

To test whether *Bax*-dependent cell death is responsible for causing sex differences in the brain and spinal cord, the SNB, AVPV and BNST were examined in *Bax-/-* mice. A null mutation of the *Bax* gene in mice leads to an increase in cell numbers in both sexes of *Bax*-/-animals in the BNSTp and AVPV and eliminates sex differences in both these regions. (Forger et al., 2004). In another study, dying cells were counted in

the BNSTp on P6 (one of the days of maximum cell death) in *Bax*-/- mice and their *Bax* +/+ siblings. *Bax* gene deletion nearly abolished dead cells in the BNSTp of both sexes supporting the interpretation that the sex difference in BNSTp cell number seen in adulthood was due to *Bax*-dependent, sexually dimorphic cell death during the first week of life in mice (Gotsiridze et al., 2007). In another study done to test the role of the *Bax* gene on motoneuron death in the SNB, motoneuron numbers were measured using immunolabeling for motoneuron-specific marker or retrograde labeling with a fluorescent tracer. By both methods, increased SNB cell numbers were observed in female *Bax*-/- mice. Further, this sex difference was eliminated in *Bax-/-* mice (Jacob et al.,2005).

The phenotypic differentiation and function of the projections formed by the "extra" rescued neurons after *Bax* deletion has not been studied. Whereas the AVPV of *Bax*-/- animals is super-feminized, their BNST is super-masculinized with respect to cell number. So the brains of the *Bax*-/- mice are mosaic with respect to sexual differentiation. It is unknown whether normally sexually dimorphic behaviors are affected by *Bax* deletion. There is evidence that some of the neurons rescued by *Bax* deletion in non-sexually dimorphic areas are atrophic and are not necessarily part of functional neural circuits (Merry et al., 1997; Sun et al., 2003). In the *Bax*-/- mice, the mean cell size in the BNST and AVPV is reduced and this may have functional consequences (Forger et al., 2004). Also, though cells in the AVPV, BNST and SNB have been counted, multiple brain areas would have been affected by the absence of *Bax* (both in sexually dimorphic and monomorphic areas) (Forger et al., 2004; Jacob et al., 2005). Unexpected compensatory or redundancy mechanisms might also be activated because of the gene

deletion (Nelson et al., 1997). Thus, difficult to predict behaviors have been studied in adult mice with extra neurons before. Hu-Bcl-2 transgenic mice (mice which over-express human Bcl-2 under the control of a neuron-specific promotor) show the same performance levels as wild type mice on tests of equilibrium, basic motor coordination and muscular strength as measu red on a hang test, but deficient performance on a complex motor coordination task – running on a rotating rod (RotaRod) at 20 to 40 rotations per minute (rpm) (Rondi-Reig et al., 1999; Rondi-Reig et al., 2002). Most parameters of motor development and function tested have been found to be completely normal in *Bax*-/- mice (Buss et al., 2006). However, sexually dimorphic behaviors have not been studied in these mice.

Together these observations raised the question of how behaviors and in particular sex behavior were affected by deletion of *Bax*. The aim of this project was to test how deletion of *Bax* affected male and female sex behavior and motor function in *Bax*-/- mice.

Female and Male Sex Behavior

In gonadally intact female rats and mice, the sequential release of ovarian estradiol and progesterone integrates sex behavior or sexual receptivity (estrus) with ovulation. In ovariectomized rats and mice, progesterone-facilitated feminine sexual behavior occurs following priming with exogenous estradiol benzoate. More specifically, this treatment is activational in adulthood and maximizes the probability that the female will assume the lordosis posture when mounted by a male. (Lisk 1960; Meyerson, 1972). Lordosis is the sustained dorsiflexion of the spinal column and elevation of the hind

quarters observed in mating female rodents and allows intromission by the male. Lordosis is quantified by the Lordosis Quotient (LQ) – a numerical measure of female receptivity.

Female rodent sexual behavior is sexually differentiated - wild type female mice tested for female sex behavior display lordosis at significantly higher levels than wild type males even after gonadectomy in adulthood and equalization of hormone levels by administration of estradiol benzoate and progesterone to activate female sex behavior in both sexes (reviewed by Kudwa et al., 2006).

It is less clear whether male sexual behavior is differentiated in mice. Wild type male mice of some strains display significantly higher frequencies of mount, intromissions and ejaculations when tested for male sex behavior than do wild type females (even after gonadectomy in adulthood and equalization of hormone levels by administration of testosterone to activate male sex behavior in both sexes) (Wersinger et al., 2000). However, there are studies where females that were gonadectomized and administered testosterone in adulthood exhibited the same or even higher levels of male sex behavior than their male counterparts (Edwards et al., 1971; Bodo et al 2007; Wesinger et al., 2000).

Motor Tests

Developmental neuronal cell death is considered important for matching the number of afferents to targets. Approximately 50% of the motoneurons initially generated during development die between embryonic days E13-19 after motoneurons contact their muscle targets (Lance-Jones, 1982; Yamamoto et al., 1999). In a study done on the neuromuscular system of *Bax*-/- mice, rescued cells were seen to persist in adult animals,

develop a neuronal phenotype, and project axons into peripheral nerves. Complete rescue of motoneurons from apoptosis was seen with an increase in the number of retrogradely labeled facial motoneurons after tracer injections into target regions. An overall reduction in the size of motoneuron cell bodies and axons was seen however. virtually all rescued motoneurons appeared to project axons in close proximity to muscle targets (Buss et al., 2006).

Motor behavior tasks like the RotaRod require the animal to make fine adjustments in the synchronization of muscle movements involved in walking. It is possible that the deficit in motor timing ability is due to lack of neuronal cell death during development in one or more of brain regions affected. The RotaRod test is a sensitive and quantitative measure of motor function or impairment as first described by Dunham et al in 1957 (Dunham et al., 1957; Jaworski et al., 2005, Benn et al., 2005, Chennathukuzhi et al., 2003). The rotating rod tests the mice for their ability to coordinate balance and movement to stay on the rod as it rotates. No sex differences have been reported in RotaRod or Hang Test performance in wild type animals (Rondi-Reig et al., 1999; Slamberova et al., 2006).

Hypothesis and Overall Predictions for All Tests

The hypothesis behind the female and male sex behavior tests was that sex differences in cell number in sexually dimorphic brain regions underlie sex differences in male and female sex behavior. If so, then deletion of the *Bax* gene should reduce the magnitude of (or eliminate) the sex difference in male and female sex behaviors in *Bax*-/- mice.

The hypothesis underlying the RotaRod and Hang tests was that natural neuronal cell death during development contributes to optimal motor function and the overall prediction for the outcome of these tests was that deletion of the *Bax* gene should impair motor function in *Bax*-/- mice.

CHAPTER 2

EXPERIMENTAL DESIGN AND METHODS

Animals and Overall Design

Two cohorts of animals were used. Each cohort had 40 animals (10 each of male and female and *Bax*-/- and wild type mice) obtained from our *Bax* breeding colony. In addition, we used 10 wild type teaser females per cohort, obtained from our C57Bl6/J colony, for tests of male sexual behavior, and 16 CD1 stud males obtained from Charles River Breeding Laboratories for test of female sexual behavior. Animals were housed singly, and maintained on a reversed 14 hour light/10 hour dark photoperiod (lights on at 9:00 PM and off at 11:00 AM EST).The temperature in the room was regulated to remain between 68-72°F and Purina mouse chow and water was available *ad libitum.*. All tests were conducted between 12 noon and 4 pm (i.e. in the early dark phase of the cycle.)

Low levels of male sexual behavior were observed in all groups during the first three tests: only five wild-type animals (3 male; 2 female) and no *Bax -/-* animals exhibited mounts or mounts with thrusts. A "social housing" paradigm (specifically, exposure of singly-housed animals to male or female conspecifics for short periods of time prior to testing) was used previously to increase male sexual behavior (Rissman et al., 1997; Wersinger and Rissman, 2000; Burns-Cusato et al., 2004). So, following the third test for male sexual behavior, animals were housed for two days with a female mouse; the fourth test was done one week later.

Gonadectomies and Hormone Treatments

Animals were gonadectomized in adulthood at more than 6 weeks of age under isoflurane anaesthesia. One week was allowed for recovery from surgery before any treatment was administered. Mice of both sexes were treated with a 20µg/0.1ml of estradiol benzoate (EB) dissolved in sesame oil followed by 500µg/0.1ml progesterone (P) dissolved in sesame oil 51 and 3 hours prior to testing, respectively, to test for female sex behavior. Teaser females were administered the same doses of EB and P as above both when performing practice sessions with CD1 stud males and for male sex behavior tests.

A separate cohort of animals was implanted with a Silastic capsule (1.02 mm ID X 2.16mm OD) packed with 5 mm of crystalline T under the skin between the shoulder blades to test for male sex behavior.

Animals were tested in a quiet dark room with red lighting. Sex behavior tests were conducted in clear Plexiglass testing cages (18 X 38 cm) placed on wooden support boxes installed with mirrors.

RotaRod and Hang Test Apparatus

The Rotarod Apparatus (Columbus Instruments Inc) used consisted of a plastic horizontal rod (50 cm long, 3 cm in diameter) separated by plastic dividers into four compartments. Each compartment was equipped with a timer which was stopped by the weight of the animal when it falls off the rod (Rondi-Reig et al., 1999). A variation of the Hang Test as first described by Tislon et al. (1978) was designed in the lab using a wire (approximately 25 cm long, 0.3 cm in diameter) hung on a wooden rod for support at the

top and looped at the bottom to allow mice to hang on to it by their forepaws. Forelimb strength was assessed by lowering the mouse onto the looped part until it grasped the wire with its front paws. In a variation of this test, in which the mouse used both its forelimbs and hindlimbs to maintain its grip, the mouse was placed on a modified cage lid (with duct tape placed around the edges), and the lid inverted (Hamann et al., 2003; Krieger et al., 2004). The lid was shaken gently for 30 seconds and time recorded. Both pieces of apparatus were located 1.5 m above a thick carpet of bedding (to cushion falls).

Female Sex Behavior

Gonadally intact stud males of the CD1 strain were presented to wild type ovariectomized, EB and P primed practice females once every week for 3 weeks before female sex behavior testing began (to acclimate to the testing and eliminate any studs that did not perform.) The same stud males were then presented to the experimental animals when testing for female sex behavior. Stud males were put into the testing arenas 30 minutes before testing began to allow them to become familiar with the space. At the end of this period, the experimental, hormonally-primed animals were introduced into the arenas. The animals were observed for either a total of 20 minutes or when 20 mounts were made, whichever came first, and the number of mounts and pelvic thrusts by males were noted to calculate the Lordosis Quotient (LQ). In the lordosis posture, female mice do not arch their backs in a strong lordosis posture as do rats, but they remain immobile for males to thrust and so LQ was defined as = # thrusts / # mounts X 100. Latency to mount and intromit was also noted as a measure of female sexual attractivity. A mount was scored if the male placed his forepaws on the back of the female in an attempt to

mate with her (and not as part of play or grooming behavior). A thrust was scored if the stud male attempted to insert his penis into the genital area of the EB and P primed test animals of either sex. The testing arena was fitted with mirrors to allow careful observation of mounts and whether or not a pelvic thrust was really attempted.

Male Sexual Behavior

Ten animals of each genotype and sex were implanted with a T capsule following gonadectomy at 7 weeks of age. Experimental animals from Cohort 2 were implanted with a T capsule to allow chronic release of testosterone and minimize stress to the skin from injections when tested for male sex behavior. Male sex behavior was tested after 2 weeks of capsule implantation to allow the animals both rest and sufficient testosterone levels in their circulation. Ovariectomized wild type females given EB and P injections were used as teaser females. Animals were tested every week for 3 weeks and observed for 40 minutes. The number of mounts, intromissions and ejaculations, as well as the latency to show these behaviors was noted. Criteria for counting mounts and intromissions were the same as above.

RotaRod and Hang Tests

Experimental animals in both cohorts were tested for motor agility and coordination using a RotaRod test. Animals were placed on an accelerating rod that attains a desired speed gradually. Animals were given at least 1 practice trial lasting 90 seconds (on a portion of the RotaRod which was textured with thin rubber strips to allow mice to get a better grip on the rod while it rotated). After a week, animals were subjected

to experimental trials performed at two different maximum speeds of 11 and 22 rpm for a total of 10 trials lasting 300 seconds each on non-textured portions of the rod. Mice got at least 30 minutes of rest between trials on the RotaRod to prevent fatigue. The latency of the animals to fall off the rod was recorded and plotted versus trials.

In the hang test, animals from Cohort 2 were hung by their forepaws on a wire and the latency of the animals to fall off the wire recorded for a maximum of 120 seconds over 2 trials as an indication of their motor strength. In the cage lid variation of this test, the animals were allowed to grasp the cage lid for a few seconds before it was gently inverted and shaken from side to side. The latency of the animals to fall off the lid was recorded for a maximum of 60 seconds over 2 trials.

Data Analysis

Data from male and female sex behaviors and motor behavior were analyzed by performing a repeated measures 2-way ANOVA with sex and genotype as the between subjects factors and trial as the within subjects factor. The Mann-Whitney U test was used to analyze male sex behavior.

CHAPTER 3

RESULTS

Female Sex Behavior

Across all six female sex behavior trials, significant main effects of sex ($p < 0.01$), and genotype ($p < 0.0005$), as well as a sex-by-genotype interaction ($p < 0.05$) on the LQ were found. A significant effect of test-session ($p < 0.002$) and a test-by-sex interaction ($p < 0.05$) was also observed. Figure 3 shows the change in pattern of the LQ values with trial for the four groups. The sex difference in female sexual behavior in wild-type mice reported previously (Kudwa et al., 2005; reviewed by Kudwa et al., 2006) was preserved, with significantly higher lordosis quotients in wild-type females than in wild-type males during each of weeks 3, 4, 5, and 6 (all p-values ≤ 0.005). For wild-type females, the LQ rose significantly around the third week of testing and remained high (about 80%) through the end of testing. This was expected from previous studies where wild-type female mice of the C57Bl6J strain when tested for female sex behavior, take 3 trials to show an increase in LQ (Kudwa et al., 2005, Reviewed by Kudwa et al, 2006) . For wild-type male and both male and female *Bax* knockout mice, low levels of female sexual behavior and no significant increase in behavior across test sessions was seen. There also was no sex difference in LQ between female and male *Bax -/-* animals over all six tests combined ($p > 0.30$) or on any single test (all ps > 0.10). LQs in *Bax -/-* animals were lower than in wild-type females ($p < 0.0005$), and did not differ significantly from LQs of wild-type males ($p > 0.05$). So deletion of *Bax* impaired female sex behavior and eliminated the sex difference in this behavior.

Latency of the CD1 studs to mount experimental animals did not differ by genotype or sex ($p>0.05$).

Male Sex Behavior

Table 1 shows the percentage of mice in each group exhibiting mounts or mounts with thrusts. Ten of 20 wild-type animals displayed mounts and/or mounts with thrusts, in contrast to only 1 of 18 *Bax-/-* mice. Before being housed socially with females, no *Bax -/-* mouse displayed any male sex behavior. Fisher's Exact Test confirmed that the proportion of animals mounting ($p < 0.003$) or intromitting ($p < 0.002$) was significantly lower in *Bax -/-* than in *Bax +/+* mice. If all animals were included in the analysis (whether or not they showed any behavior), Mann-Whitney U Test confirmed that *Bax-/-* animals exhibited fewer overall mounts ($p < 0.002$) and mounts with thrusts ($p < 0.001$) than did *Bax +/+* mice. No sex difference in male sexual behavior was observed. There also was no significant effect of sex on the number of mounts or mounts with thrusts ($p = 0.20$ for mounts and $p = 0.153$ for mounts with thrusts, Mann-Whitney U Test).

Motor Behavior

The mean latency to fall off on the cagelid test did not vary by sex or genotype ($p > 0.05$ in both cases), although there was a tendency for wild type mice to perform better ($p= 0.051$; Figure 3). The proportion that successfully completed both the cagelid and hang tests (i.e. hung on for the full duration of 120 seconds on the hang test and for 60 seconds on the cagelid test) did not differ significantly by sex or genotype.

In the RotaRod test (Figure 5A) , there was no significant effect of sex or genotype on latency to fall at the low speed ($p > 0.8$ and $p > 0.06$ respectively.) Mice of all four groups improved with trial when tested at the lower speed ($p < 0.0005$). At higher speeds, *Bax -/-* mice were impaired relative to wild-type controls (Figure 6B, $p = 0.002$). Both genotypes again improved with trial at the higher speed ($p < 0.0005$), but improvements were greater for wild-type than for *Bax* knockout animals ($p < 0.03$ for trial-by-genotype interaction).

CHAPTER 4

DISCUSSION

Bax is abundantly present during developmental cell death in many brain regions (Vekrellis et al., 1997) and deletion of the *Bax* gene alone eliminates most of the neuronal cell death in the PNS and CNS (White et al., 1998). Although the appearance and behavior of *Bax* knockouts are grossly normal (Knudson et al., 1995)**,** sexually dimorphic behaviors have not been studied. It was observed that both the male and female sexual behavior of the knockouts was significantly impaired, and the motor behavior impaired at high speeds on the RotaRod.

Female Sex Behavior

As expected, female sex behavior in wild type mice was sexually dimorphic, with female mice exhibiting significantly higher LQs than the males across the 6 weeks of testing. The knockouts of both sexes showed significantly lower LQs and no increase across trials.

Multi-synaptic circuits in the brain and spinal cord modulate reproductive function. For example the vomeronasal system is a distributed network of sexually dimorphic neuronal populations including the medial amygdala (MeA), BNST, the AVPV, and the ventromedial hypothalamus (VMH). These regions are reciprocally interconnected to integrate olfactory and pheromonal cues with other information to modulate reproductive function (reviewed by Cooke et al., 1998) (see Figure 1). *Bax* deletion increases cell numbers in both sexes of *Bax*-/- animals in the BNSTp, AVPV and

SNB most likely by rescuing neurons that would otherwise die during the perinatal cell death period (Forger et al., 2004; Jacob et al., 2005). The brains of the *Bax* knockout mice studied were mosaic with respect to sexual differentiation within each animal- the AVPV was feminized and the BNSTp masculinized. The lack of lordosis by the knockouts could be because an increase in neuron number in particular brain regions of *Bax*-/- mice inhibited the exhibition of lordosis. In support of this, vomeronasal pathway studies showed that there are more neurons in the VNO structures of male rats in relation to females, which might contribute to the inhibition of the expression of lordosis in males (Segovia et al., 1993).

Also, the BNST has been shown to provide a sexually dimorphic inhibitory (GABAergic) input to the AVPV and the VMH, areas responsible for ovulation (which is coupled with sex behavior) and lordosis respectively, (Polston et al., 2004). So it is possible that the rescued BNST cells serve to inhibit female sex behavior. It has also actually been suggested that the BNST may actually be involved in inhibiting lordosis in rats (Guillamon et al,. 1993).

It is also possible that some of the neurons rescued by *Bax* deletion may not necessarily have formed functional synaptic connections with their targets [although extra SNB neurons in Bax-/- mice have been shown to project to their peripheral target regions](Jacob et al, 2005).

In *Bax*-/- mice, apoptosis is absent in the adult-generated neurons in the dentate gyrus (DG) of the mouse hippocampus resulting in supernumerary neurons which show aberrant migratory patterns showing that loss of programmed cell death may alter guidance signals for the proper migration of cells. These DG neurons also fail to express

the mature DG neuronal marker (though they are differentiated) suggesting that spontaneous programmed cell death in the adult brain may be necessary for the maintenance of the mature neuronal phenotype in the rescued cells (Sun et al., 2004). Recently however, it has been shown in the Forger lab that the cells rescued by Bax deletion in the BNSTp express NeuN, a marker of differentiated neurons, as well as the androgen receptor showing that the rescued neurons may be able to differentiate normally (Holmes and Forger, in preparation).

Since latency of the CD1 studs to mount experimental animals did not differ by genotype, the lower LQs in Bax knockout females are not likely to be because CD 1 stud males did not mount Bax knockouts as readily as the wild type mice.

Male Sex Behavior

Male sex behavior in wild type mice has not been shown to be consistently dimorphic in previous studies. While some studies have shown that wild type males show higher levels of mounting and thrusting than females after gonadectomy and hormone treatment in adulthood (Wersinger et al., 2000), other studies show that normal females of several strains also show high levels of mounting and thrusting in response to a receptive female when treated with T or E in adulthood (Wersinger et al., 1997).

In this study it was found that more wild-type females exhibited male sex behavior than wild type males, although this was not statistically significant. Thus, male sex behavior was not sexually differentiated in the C57Bl/6 strain of mice used for this study.

Overall only 50% of the wild-type animals exhibited mounts or mounts with pelvic thrusts in response to stimulus females. Behavior levels may have been higher if male sex behavior was tested for longer than 40 minutes as has been done in some studies earlier (Wersinger et al., 1997) or by testing animals in their home cages (instead of in a novel test arena which may have been stressful to the animals despite being given time to acclimate to it). Although in a recent study on male sex behavior, similar levels (55-58%) of male sex behavior were elicited by wild type animals gonadectomized and given testosterone treatment even though the animals were tested in their home cages (Bodo et al., 2007).

As for female sex behavior, an increase in neuron number in particular brain regions might have interfered with exhibition of male sex behavior. Increase in neuron number in other sexually dimorphic brain regions (where females have more cells than males) may also have led to suppression of male sex behavior.

Motor Behavior

While *Bax* knockout performance did not vary significantly from that of wild type mice on the hang test, the cage lid test or the low speeds on the RotaRod, performance at the high speed RotaRod test was significantly impaired. A similar pattern is seen with Hu-Bcl-2 mice which show normal performance on tests of equilibrium, basic motor coordination and muscular strength but deficient performance on a complex motor coordination task – walking on a RotaRod at 20 rpm and 40 rpm.

In an electrophysiological and behavioral study done on *Bax*-/- mice with excess motoneurons, most parameters of motor function tested were completely normal in *Bax*-/-

mice. There was no difference in performance between knockouts and wild types on tests of motor coordination like the RotaRod test or balance tests like beam walking or hole board walking. The Bax-/- mice even showed improved performance on one of the tests of motor strength - the grip strength test possibly because they have more motoneurons (Buss et al. 2006). However, the results from this study may have been based on a single RotaRod trial. From the studies done here and with the Hu-Bcl-2 mice, the *Bax*-/- animals show impairments at the higher speed RotaRod i.e. tasks which require the animal to make fine adjustments in the synchronization of muscle movements involved in walking.

Performance on the RotaRod is known to be dependant on an intact olivocerebellar system (Rondi-Reig et al., 1997). It is possible that perturbations in different elements of the synaptic network involved in motor coordination result in the deficient performance of the *Bax*-/- mice to display robust performance at high speeds.

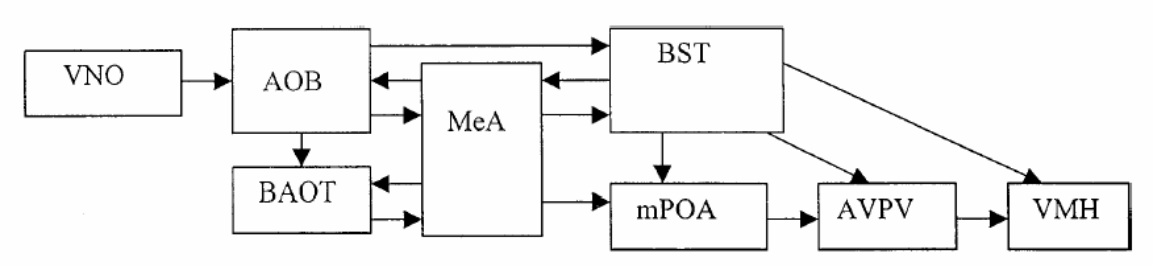

Fig 1 A schematic diagram of the neural circuitry which receives information from the vomeronasal organ (VNO), the receptor for pheromonal signals. Sexual dimorphism has been reported for each of these neural regions in rats. The interconnected nature of this system of nuclei is obvious even though, for clarity, not all the connections between them are depicted. Abbreviations:AOB, accessory olfactory bulb; AVPV, anteroventral periventricular nucleus; BAOT, bed nucleus of the accessory olfactory tract; BST, bed nucleus of the stria terminalis; MeA, medial amygdala; mPOA, medial preoptic area; VMH, ventromedial hypothalamus (Figure and Legend from Cooke et al., 1998)

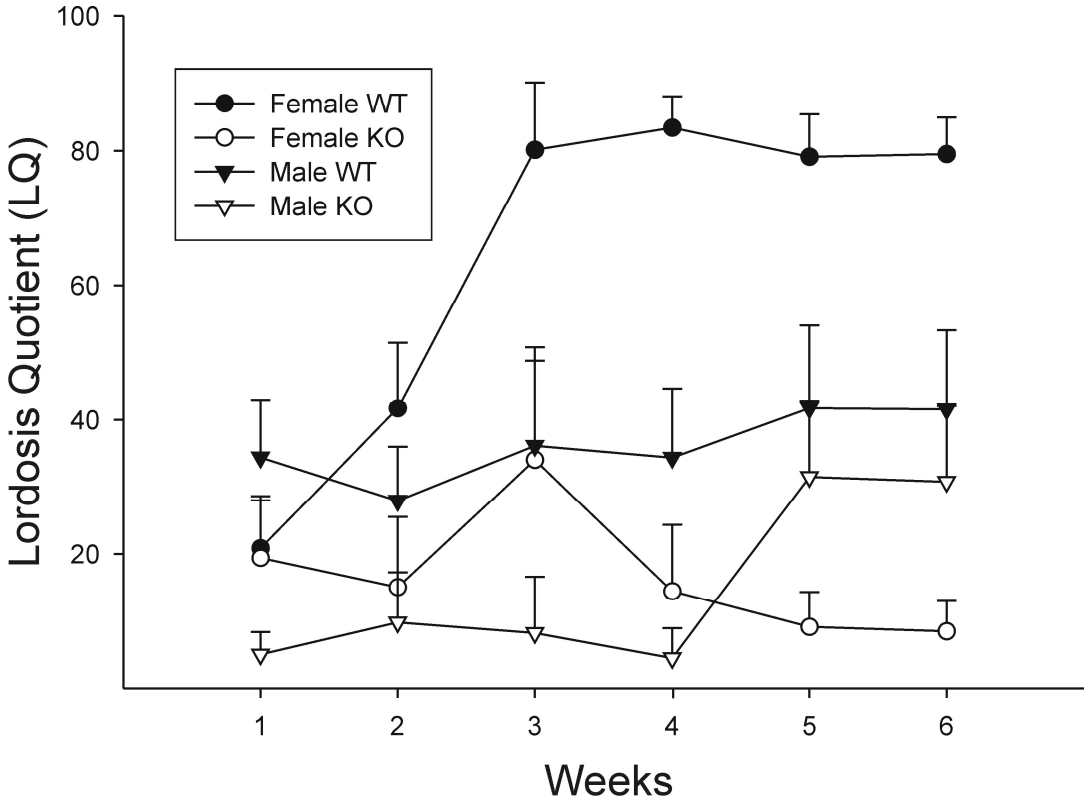

Fig 2 Mean ± SEM lordosis quotients of wild type and knockout mice over 6 weeks of trials. Wild type females show high LQ from weeks 3-6. Knockouts showed significantly lower LQs than wild type females across all 6 trials ($p<0.0005$). Wild type males also showed significantly lower LQs compared to wild type females in weeks 3-6 (all p values < 0.005).

Table 1 Male sex behavior in wild type and *Bax* knockout mice of both sexes showing the percentage of mice in each group exhibiting mounts or mounts with thrusts and the mean number of mounts per animal

	% mounting	% thrusting	# mounts[1,2]
Male *Bax* +/+ (N = 10)	40.0	30.0	9.3 ± 3.7 (4)
Female *Bax* +/+ (N = 10)	70.0	60.0	14.8 ± 2.7 (7)
Male *Bax* -/- (N = 9)	5.5	0	3.0 (1)
Female *Bax* -/- (N = 9)	0	0	--

[1]Includes only those animals that exhibited behavior; number of animals indicated in parantheses.
[2]Means ± SEMs

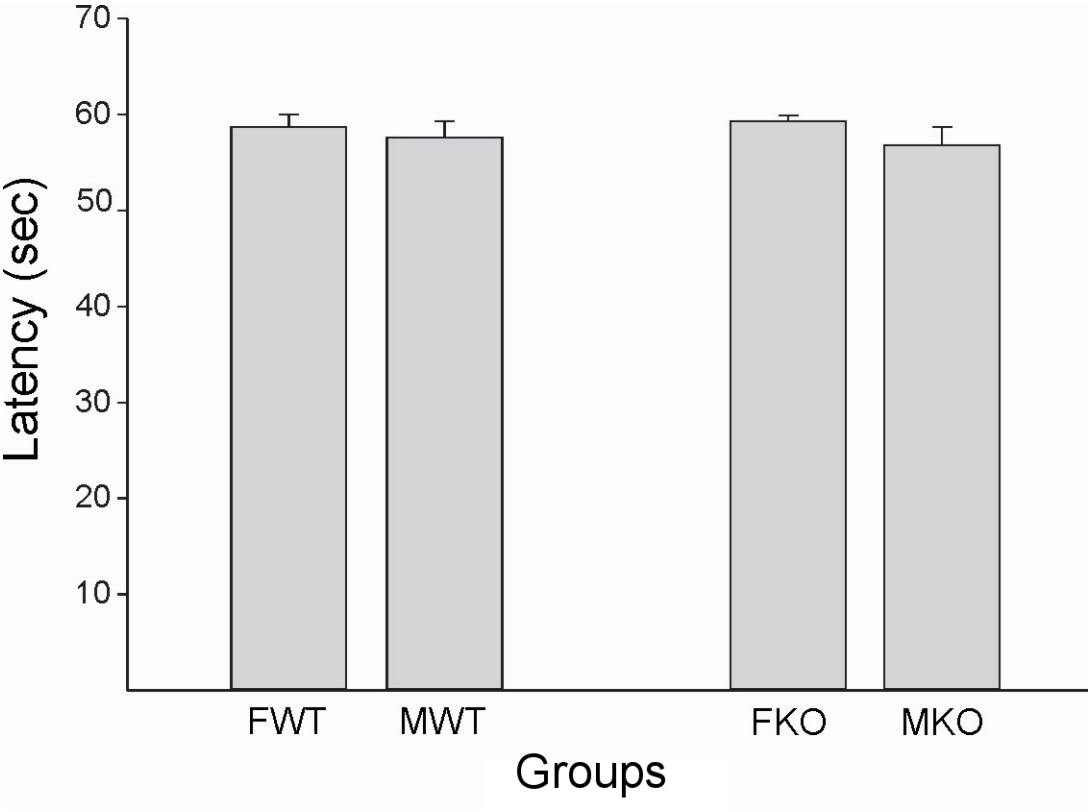

Fig 3 Cagelid test results for wild type and *Bax* knockout mice. There was no difference in performance between wild type and *Bax* knockout mice of either sex ($p>0.7$)

(Key : FWT – Female Wild Types, MWT – Male Wild Types, FKO – Female Knockouts, MKO – Male Knockouts)

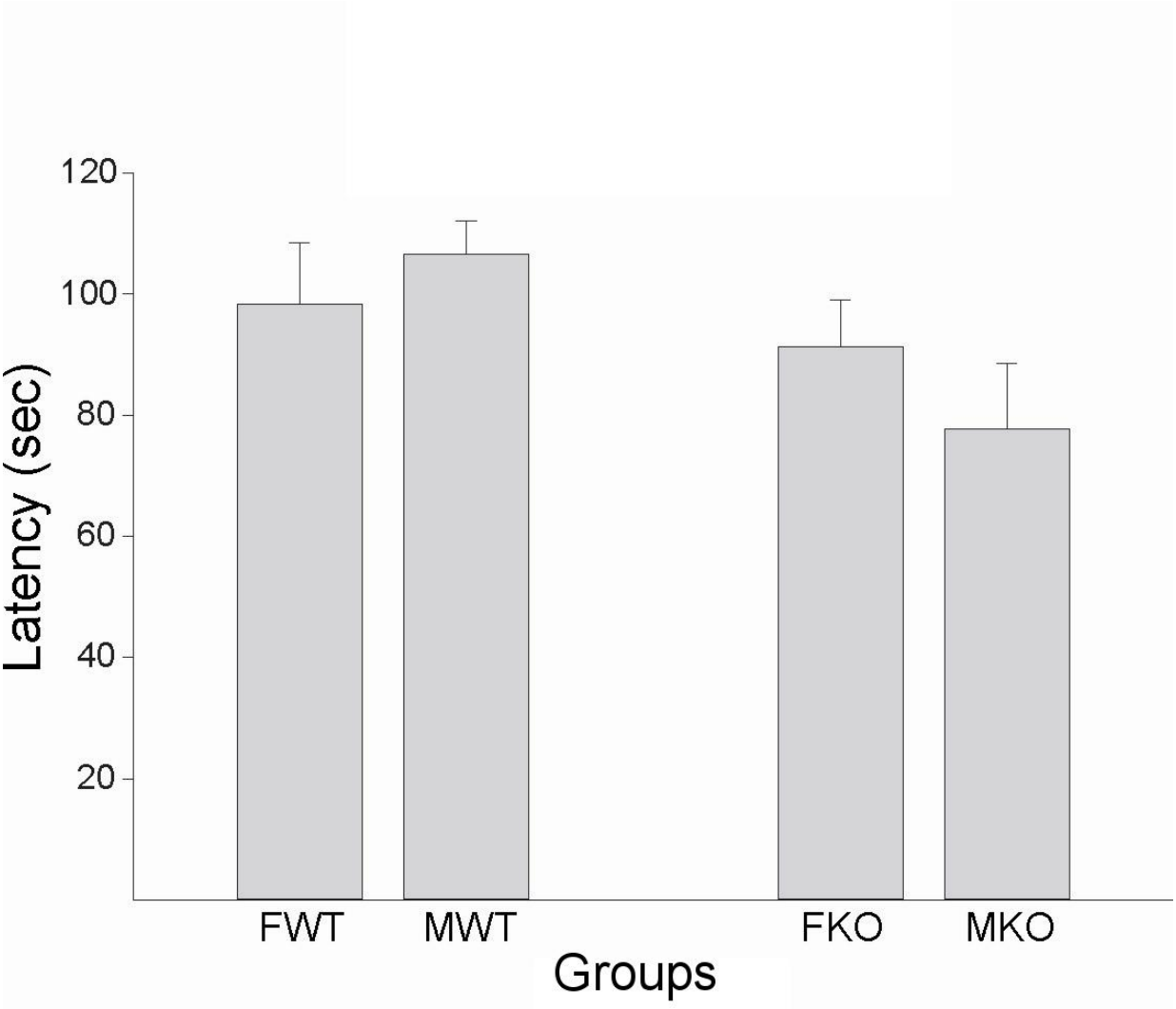

Fig 4 Hang test results for wild type and *Bax* knockout mice. There was a tendency for wild type animals to do better than the knockouts (p=0.051)

(Key: FWT – Female Wild Types, MWT – Male Wild Types, FKO – Female Knockouts, MKO – Male Knockouts)

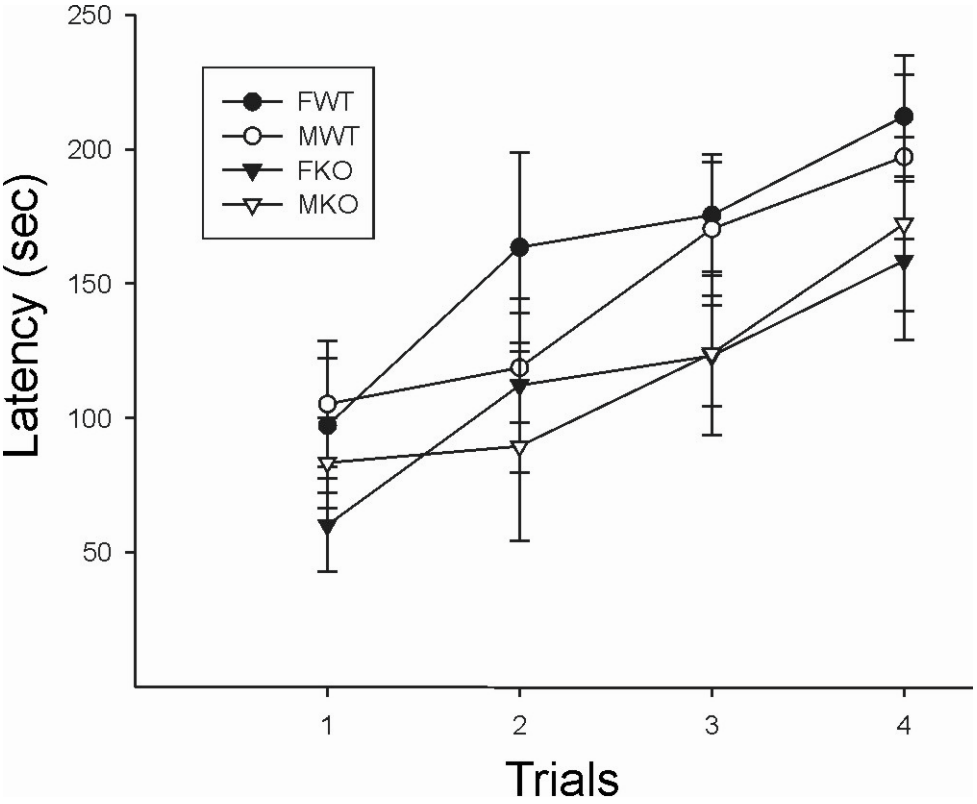

Fig 5A RotaRod Test Results at 11 rpm. There was a tendency for wild type mice to perform better than knockouts across all 4 trials on this test ($p<0.07$)

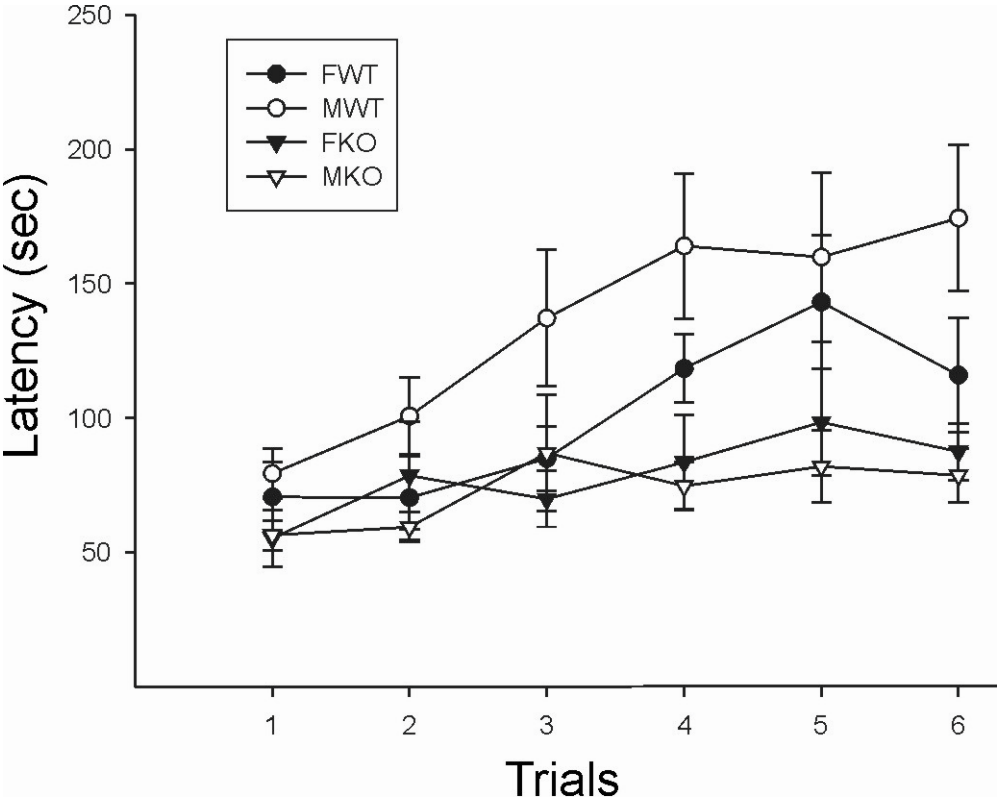

Fig 5B RotaRod Test Results at 22 rpm. *Bax* knockouts exhibited shorter latencies to fall than wild type mice across all 6 trials on this test ($p<0.002$)

REFERENCES

Arnold AP, Gorski RA (1984).Gonadal steroid induction of structural sex differences in the central nervous system.Annu Rev Neurosci.;7:413-442.

Baum MJ. 1979 A comparison of the effects of methyltrienolone (R 1881) and 5 alpha-dihydrotestosterone on sexual behavior of castrated male rats. Horm Behav.13:165-174

Baum MJ, Tobet SA, Starr MS, Bradshaw WG.1982. Implantation of dihydrotestosterone propionate into the lateral septum or medial amygdala facilitates copulation in castrated male rats given estradiol systemically. Horm Behav. 16:208-223.

Beach FA, Noble RG, Orndoff RK.1969. Effects of perinatal androgen treatment on responses of male rats to gonadal hormones in adulthood. J Comp Physiol Psychol. 68:490-497

Becker JB, Arnold AP, Berkley KJ, Blaustein JD, Eckel LA, Hampson E, Herman JP, Marts S, Sadee W, Steiner M, Taylor J, Young E. 2005. Strategies and methods for research on sex differences in brain and behavior. Endocrinology. 146:1649.

Bodo C, Rissman EF.**A**ndrogen receptor is essential for sexual differentiation of responses to olfactory cues in mice. Eur J Neurosci 2007 Apr;25(7):2182-90

Breedlove SM, Arnold AP. 1980. Hormone accumulation in a sexually dimorphic motor nucleus of the rat spinal cord Science. 210:564-566

Burns-Cusato M, Scordalakes EM, Rissman EF. 2004. Of mice and missing data: what we know (and need to learn) about male sexual behavior. Physiol Behav. 83:217-232.

Buss RR, Gould TW, Ma J, Vinsant S, Prevette D, Winseck A, Toops KA, Hammarback JA, Smith TL, Oppenheim RW. 2006.Neuromuscular development in the absence of programmed cell death: phenotypic alteration of motoneurons and muscle. J Neurosci. Dec 27;26:13413-13427.

Canteras NS, Simerly RB, Swanson LW. 1995 .Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat. J Comp Neurol. 360:213-245.

Chennathukuzhi V, Stein JM, Abel T, Donlon S, Yang S, Miller JP, Allman DM, Simmons RA, Hecht NB.2003. Mice deficient for testis-brain RNA-binding protein exhibit a coordinate loss of TRAX, reduced fertility, altered gene expression in the brain, and behavioral changes Mol Cell Biol.23:6419-6434.

Christensen LW, Nance DM, Gorski RA.1977.Effects of hypothalamic and preoptic lesions on reproductive behavior in male rats. Brain Res Bull.2:137-141.

Cooke B, Hegstrom CD, Villeneuve LS, Breedlove SM.Sexual differentiation of the vertebrate brain: principles and mechanisms. 1998. Front Neuroendocrinol. 323-362.

Deckwerth TL, Elliott JL, Knudson CM, Johnson EM Jr, Snider WD, Korsmeyer SJ. 1996. *BAX* is required for neuronal death after trophic factor deprivation and during development. Neuron.17:401-11.
del Abril A, Segovia S, Guillamon A. The bed nucleus of the stria terminalis in the rat: regional sex differences controlled by gonadal steroids early after birth. 1987. Brain Res. 429:295-300.

De Jonge FH, Louwerse AL, Ooms MP, Evers P, Endert E, van de Poll NE Brain Res Bull.1989 2: 483-492. Lesions of the SDN-POA inhibit sexual behavior of male Wistar rats.

Dohler KD, Coquelin A, Davis F, Hines M, Shryne JE, Gorski RA. 1982. Differentiation of the sexually dimorphic nucleus in the preoptic area of the rat brain is determined by the perinatal hormone environment. Neuroscience Letter, 33: 295-298.

Dohler, K.D. *et al*. Prenatal and postnatal influence of testosterone propionate and diethylstilbestrol on differentiation of the sexually dimorphic nucleus of the preoptic area in male and female rats.1984. Brain Res. 302: 291–295

Dunham NW, Miya TS. A note on a simple apparatus for detecting neurological deficit in rats and mice J Am Pharm Assoc Am Pharm Assoc (Baltim).1957. 46:208-209

Edwards DA.Neonatal administration of androstenedione, testosterone or testosterone propionate: effects on ovulation, sexual receptivity and aggressive behavior in female mice. Physiol Behav. 1971.6:223-228.

Feder HH. Hormones and sexual behavior. Annu Rev Psychol.1984.35:165-200.

Forger NG. Cell death and sexual differentiation of the nervous system. Neuroscience. 2006.138:929-938

Forger NG, Rosen GJ, Waters EM, Jacob D, Simerly RB, de Vries GJ. Deletion of *Bax* eliminates sex differences in the mouse forebrain. Proc Natl Acad Sci. 2004. 101:13666-13671.

Guillamon A, Segovia S, del Abril A.1988. Early effects of gonadal steroids on the neuron number in the medial posterior region and the lateral division of the bed nucleus of the stria terminalis in the rat. Brain Research, 44 : 281-290.

Guillamón A, Segovia S 1993. Sexual dimorphism in the vomeronasal pathway and sex differences in reproductive behaviors Brain Res Brain Res Rev. 1993 Jan-Apr;18:51-74.

Gross, A., X.M. Yin, K. Wang, M.C. Wei, J. Jockel, C. Milliman, H. Erdjument-Bromage, P. Tempst, and S.J. Korsmeyer.1999. Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem 274: 1156-1163

Gorski RA, Harlan RE, Jacobson CD, Shryne JE, Southam AM.1980. Evidence for the existence of a sexually dimorphic nucleus in the preoptic area of the rat. Journal of Comparative Neurology, 193: 529-539.

Gotsiridze T, Kang N, Jacob D, Forger NG. 2007. Development of sex differences in the principal nucleus of the bed nucleus of the stria terminalis of mice: role of *Bax*-dependent cell death. Dev Neurobiol. 67:355-62

Hamann M, Meisler MH, Richter A. 2003. Motor disturbances in mice with deficiency of the sodium channel gene Scn8a show features of human dystonia Exp Neurol. 184:830-838.

Hamburger V, Oppenheim RW. 1982. Naturally occurring neuronal death in vertebrates. Neurosci Commentaries. 1:39-55.

Harris CA, Johnson EM Jr. 2001.BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require *Bax* to induce apoptosis in neurons.J Biol Chem. 12;276:37754-37760

Hennessey AC, Wallen K, Edwards DA. Preoptic lesions increase the display of lordosis by male rats. Brain Res. 1986.370:21-28.

Hockenbery, D., G. Nunez, C. Milliman, R.D. Schreiber, and S.J. Korsmeyer. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature. 348: 334-336

Hsu, Y.T., K.G. Wolter, and R.J. Youle. 1997. Cytosol-to-membrane redistribution of *Bax* and Bcl-X(L) during apoptosis. Proc. Natl. Acad. Sci. USA. 94:3668–3672

Jacob DA, Bengston CL, Forger NG.Effects of *Bax* gene deletion on muscle and motoneuron degeneration in a sexually dimorphic neuromuscular system. 2005. J Neurosci.25:5638-5644.

Jaworski DM, Soloway P, Caterina J, Falls WA. 2006. Tissue inhibitor of metalloproteinase-2(TIMP-2)-deficient mice display motordeficits. J Neurobiol. 66:82-94.

Jost A.1983. Genetic and hormonal factors in sex differentiation of the brain. Psychoneuroendocrinology. 8:183-93.

Kippin TE, Cain SW, Pfaus JG. 2003. Estrous odors and sexually conditioned neutral odors activate separate neural pathways in the male rat. Neuroscience.117:971-979.

Korsmeyer SJ, Gross A, Harada H, Zha J, Wang K, Yin XM, Wei M, Zinkel S. 1999. Death and survival signals determine active/inactive conformations of pro-apoptotic *BAX*, BAD, and BID molecules.Cold Spring Harb Symp Quant Biol. 64:343-350.

Krieger KE, Abbott MA, Joksimovic M, Lueth PA, Sonea IM, Jeannotte L, Tuggle CK Transgenic mice ectopically expressing HOXA5 in the dorsal spinal cord show structural defects of the cervical spinal cord along with sensory and motor defects of the forelimb.Brain Res Dev Brain Res. 2004.150:125-139.

Kudwa AE, Michopoulos V, Gatewood JD, Rissman EF. Roles of estrogen receptors alpha and beta in differentiation of mouse sexual behavior.2006.Neuroscience. 138:921-928.

Kudwa AE, Rissman EF. 2003. Double oestrogen receptor alpha and beta knockout mice reveal differences in neural oestrogen-mediated progestin receptor induction and female sexual behaviour. J neuroendocrinol. 978-983

Kudwa AE, Bodo C, Gustafsson JA, Rissman EF. 2005. A previously uncharacterized role for estrogen receptor beta Defeminization of male brain and behavior, Proc Natl Acad Sci 102 : 4608–4612.

Lindsten, T., Ross, A. J., King, A., Zong, W.-X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K.G., Mahar, P., Frauwirth, K. 2000. Mol. Cell 6:**,** 1389–1399

Lisk RD.1960. A comparison of the effectiveness of intravenous injections as opposed to subcutaneous injections of Progesterone: induction of estrous behavior in the rat. Can J Biochem Physiol 38:1381-1383

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. 1993.Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene.Proc Natl Acad Sci.1;90:11162-11166

Flanagan-Cato LM, Calizo LH, Daniels D. 2001. Synaptic Organization of VMH Neurons That Mediate the Effects of Estrogen on Sexual Behavior Hormones and Behavior. 178-182

Mani SK, Blaustein JD, O'Malley BW. 1997. Progesterone receptor function from a behavioral perspective. Horm Behav. 3:244-55

Matsumoto T, S. Honda and N. Harada. 2003. Alteration in sex-specific behaviors in male mice lacking the aromatase gene, Neuroendocrinology 77:416–424

McEwen BS. 1983.Gonadal steroid influences on brain development and sexual differentiation.Int Rev Physiol. 27:99-145.

Meyerson DH. 1972. Latency between intravenous of progestins and the appearance of estrous behavior in estradiol treated rats Horm Beh. 3:1-9

Miller, M. A., P. E. Kolb, and M. A. Raskind. 1993.Extra-hypothalamic vasopressin neurons coexpress galanin messenger RNA as shown by double in situ hybridization histochemistry. J.Comp Neurol. 329.3:378-84

Motelica-Heino I, D.A. Edwards and J. Roffi. 1993. Intermale aggression in mice: does hour of castration after birth influence adult behavior, Physiol. Behav**.** 53 :1017–1019

Nelson RJ.Horm Behav. 1997. The use of genetic "knockout" mice in behavioral endocrinology research. 3:188-196

Olster DH, Blaustein JD. 1988. Progesterone facilitation of lordosis in male and female Sprague-Dawley rats following priming with estradiol pulses. Horm Behav. 3:294-304.

Oltvai ZN, Milliman CL, Korsmeyer SJ. 1993 .Bcl-2 heterodimerizes in vivo with a conserved homolog, *Bax*, that accelerates programmed cell death. Cell. 27;74:609-619.

Oppenheim RW. Cell death during development of the nervous system. 1991.Annu Rev Neurosci.14:453-501.

Pang SF, Caggiula AR, Gay VL, Goodman RL, Pang CS. 1979. Serum concentrations of testosterone, oestrogens, luteinizing hormone and follicle-stimulating hormone in male and female rats during the critical period of neural sexual differentiation.J Endocrinol. 80:103-110.

Pettmann B, Henderson CE. 1998. Neuronal cell death. Neuron. 20:633-647.

Phoenix CH, Goy RW, Gerall AA, Young WC. 1959. Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology. 65:369-382.

Pfaff D. 2005. Hormone-driven mechanisms in the central nervous system facilitate the analysis of mammalian behaviors. Endocrinology 184, 447-453

Planas, B, Kolb PE, Raskind MA, Miller MA. 1995. Sex difference in coexpression by galanin neurons accounts for sexual dimorphism of vasopressin in the bed nucleus of the stria terminalis. Endocrinology 136;2: 727-733.

Petersen SL, Ottem EN, Carpenter CD. 2003. Direct and indirect regulation of gonadotropin-releasing hormone neurons by estradiol. Biol Reprod. 69:1771-1778.

Polston EK, Gu G, Simerly RB, 2004. Neurons in the principal nucleus of the bed nuclei of the stria terminalis provide a sexually dimorphic GABAergic input to the anteroventral periventricular nucleus of the hypothalamus.Neuroscience. 123:793-803.

Putcha GV, Deshmukh M, Johnson EM Jr. 1999. *BAX* translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. J Neurosci.19:7476-7485.

Putcha GV, Harris CA, Moulder KL, Easton RM, Thompson CB, Johnson EM Jr. 2002. Intrinsic and extrinsic pathway signaling during neuronal apoptosis: lessons from the analysis of mutant mice. J Cell Biol. 157:441-453.

Rhees,RW , Al-Saleh, HN , Kinghorn EW, Fleming DE, Lephart ED.1999. Relationship between sexual behavior and sexually dimorphic structures in the anterior hypothalamus in control and prenatally stressed male rats. Brain Res Bull 50:193-199

Rondi-Reig L, Lohoff A, Y. Lemaigre-Dubreuil, N. Delhaye-Bouchaud, J.C. Martinou, J. Caston and J. Mariani. 1999. Hu-bcl-2 transgenic mice with supernumerary CNS neurons exhibit motor timing impairment. Eur. J. Neurosci. 11 :2285–2290

Rondi-Reig L, Delhaye-Bouchaud N, Mariani J, Caston J. 1997. Role of the inferior olivary complex in motor skills and motor learning in the adult rat. Neuroscience. 955-963.

Ryan BC and J.G. Vandenbergh, Intrauterine position effects, 2002. Neurosci. Biobehav. Rev. 26:665–678.

Slamberova R, Pometlova M, Charousov. 2006 . Postnatal development of rat pups is altered by prenatal methamphetamine exposure. Neuropsychopharmacol Biol Psychiatry. 82-88.

Smith MS, Freeman ME, Neill JD. 1975. The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 96:219–226

Sumida H, Nishizuka M, Kano Y, Arai Y.1993. Sex differences in the anteroventral periventricular nucleus of the preoptic area and in the related effects of androgen in prenatal rats. Neuroscience Letter, 151: 41-44.

Sun YF, Yu LY, Saarma M, Timmusk T, Arumae U.2001.Neuron-specific Bcl-2 homology 3 domain-only splice variant of Bak is anti-apoptotic in neurons, but pro-apoptotic in non-neuronal cells.J Biol Chem.276:16240-7

Sun W, Gould TW, Vinsant S, Prevette D, Oppenheim RW. 2003. Neuromuscular development after the prevention of naturally occurring neuronal death by *Bax* deletion.J Neurosci 7298-7310.

Sun W, Winseck A, Vinsant S, Park OH, Kim H, Oppenheim RW. 2004. Programmed cell death of adult-generated hippocampal neurons is mediated by the proapoptotic gene *Bax*.J Neurosci. 24:11205-11213

Tilmann C and B. Capel. Neuropsychopharmacol Biol Psychiatry. 2002. Cellular and molecular pathways regulating mammalian sex determination. Horm. Res. 57 :1–18.

Tilson HA, Cabe PA, Mitchell CL. 1978 . Behavioral and neurological toxicity of polybrominated biphenyls in rats and mice. Environ Health Perspect. 257-263.

Tsai, M.-J. & O'Malley, B. W. 1994. Annu. Rev. Biochem. 63: 451-486

Vale JR , D. Ray and C.A. Vale. 1973. The interaction of genotype and exogenous neonatal androgen and estrogen: sex behavior in female mice, Dev. Psychobiol. 6:319–327.

Vekrellis K, McCarthy MJ, Watson A, Whitfield J, Rubin LL, Ham J. 1997.*Bax* promotes neuronal cell death and is downregulated during the development of the Nervous system.Devlopment, 124:1239-1249

Wee BE, Clemens LG. 1987. Characteristics of the spinal nucleus of the bulbocavernosus are influenced by genotype in the house mouse.Brain Res.424:305-310.

Wersinger SR, Rissman EF .2000. Dopamine Activates Masculine Sexual Behavior Independent of the Estrogen Receptor α .Neuroscience, 20:4248-4254

Williams CL, Blaustein JD. 1988. Steroids induce hypothalamic progestin receptors and facilitate female sexual behavior in neonatal rats.Brain Res.449:403-407.

White FA, Keller-Peck CR, Knudson CM, Korsmeter SJ, Snider WD .1998. Widesspread elimination of naturally occurring neuronal death in *Bax*-deficient mice. J neurosci, 18: 1428-1439.

Weisz J, Ward IL. Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring. Endocrinology. 1980. 103:306-316

Wilson JD , J.E. Griffin and F.W. George. 1980 .Sexual differentiation: early hormone synthesis and action, Biol. Reprod. 22: 9–17

Zoratti, M. and I. Szabo. 1995. The mitochondrial permeability transition. Biochim. Biophys. Acta 1241: 139-176

Zup SL, Carrier H, Waters EM, Tabor A, Bengston L, Rosen GJ, Simerly RB, Forger NG. 2003. Overexpression of bcl-2 reduces sex differences in neuron number in the brain and spinal cord. J Neurosci.23:2357-62