ABNORMAL REPRODUCTIVE FUNCTION IN FEMALE HOMOZYGOUS LEANER MICE

A Thesis

by

NESRIN SERPEDIN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2003

Major Subject: Toxicology

ABNORMAL REPRODUCTIVE FUNCTION IN FEMALE

HOMOZYGOUS LEANER MICE

A Thesis

by

NESRIN SERPEDIN

Submitted to Texas A&M University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Approved as to style and content by:

Louise C. Abbott (Chair of Committee)	Newell McArthur (Member)
Farida Sohrabji (Member)	Timothy Phillips (Member)
Evelyn Tiffany-Castiglioni (Head of Department)	Timothy Phillips (Chair of Toxicology Faculty)

December 2003

Major Subject: Toxicology

ABSTRACT

Abnormal Reproductive Function in Female Homozygous

Leaner Mice. (December 2003)

Nesrin Serpedin, M.D., Carol Davila University, Bucharest, Romania

Chair of Advisory Committee: Dr. Louise C. Abbott

The leaner mouse carries an autosomal recessive mutation in the α1A subunit of neuronal P/Q-type voltage gated calcium ion channels. Due to this mutation, the leaner mouse exhibits severe ataxia, absence seizures and paroxysmal dyskinesia. Mutations in this same gene in humans cause: episodic ataxia type 2, familial hemiplegic migraine, spinocerebellar ataxia type 6 and probably the newly recognized form of human inherited epilepsy.

Decreased amplitude of calcium current in cerebellar Purkinje cells and decreased calcium buffering capacity suggest that failure of calcium homeostasis may lead to the neurodegeneration observed in these mutant mice. Both sexes are affected. Despite their neurological dysfunction, homozygous leaner mice are able to breed and produce viable offspring. The survival rate for these pups is highly correlated with early fostering to normal lactating dams.

This thesis studies the reproductive dysfunction observed in female homozygous leaner mice and is divided into four parts: onset of puberty, estrous cycle, pregnancy and litter assessment, and hormone levels. We have discovered that the onset of puberty is precocious in leaner females compared to age-matched wild type females,

and leaner mice spend more time in estrous than age-matched wild type females. Also, we have observed that leaner mice became pregnant less readily than wild type mice, but once pregnant, female leaner mice produced more pups per litter compared with wild type mice. The number of corpora lutea observed in leaner mice is greater than in wild type mice. In leaner mice, the number of corpora lutea in the ovary corresponding to the uterine horn with the highest number of offspring is larger than the number of corpora lutea found in the ovary corresponding to the other uterine horn. Radioimmunoassays of estradiol hormone levels at postnatal day 28 shows higher levels in leaner compared to age-matched wild type mice. However, at postnatal day 28, the luteinizing hormone levels are similar in both categories of mice.

This study of reproductive dysfunction in leaner mice was performed to gain further understanding about the role of intracellular calcium ion signaling in neuronal regulation of reproductive processes in females.

ACKNOWLEDGEMENTS

I would like to thank Dr. Abbott for her advice, encouragement and continuous support during my graduate studies and research work. I would like to acknowledge and thank each of my graduate committee, Drs. Sohrabji, McArthur, Tiffany-Castiglioni and Phillips, for their support in completing my thesis. I really appreciate my colleagues Drs. Tamy Frank, Sairum Bellum, Bhupinder Bawa, Sang-Soep Nahm, and Ms. Kerry Thuett, my collaborators Kristen Baker, Susan Hernandez, Raul Grajeda, Melanie Krause, as well as Dr. Jill Hiney and Dr. Les Dees for their help in completing this study. Finally, I am deeply grateful to my husband and my parents for their enormous support.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

I. INTRODUCTION AND REVIEW OF THE LITERATURE

Introduction

The nervous system functions as an organ system where neurons and glial cells form a very large network, which integrates all external and internal stimuli and contributes to the elaboration of adequate responses. The fundamental component or organizing unit of this vast network is the synaptic junction. Impairment of synaptic contacts may be due to presynaptic or postsynaptic neurons as well as neurotransmitter-induced receptor activation and electrochemical gradients. Also, processes in the central nervous system are dynamic throughout the entire life of an individual. Despite the recent progress reported in biochemistry and genetics related to the nervous system, many of the molecular mechanisms involved in synapse regression and formation remain unclear.

In addition, infertility in both sexes is one of the big concerns of this century. Despite advances in genetics and technology of *in vitro* fertilization and embryo transfer and implantation, the multifactorial causes that lead to hypofertility and infertility in couples are not yet elucidated. Moreover, while many major insults, which disturb the homeostasis of reproductive function and trigger different pathogenic pathways have already been discovered, the mechanisms through which these react or interact at the molecular level also are not yet completely understood.

This thesis follows the style of the Journal of Neuroscience.

All events taking place in the nervous system utilize a ubiquitous intracellular messenger: calcium (Berridge, 1998, Berridge et al., 2000, Carafoli, 2002). The basic mechanism of calcium signaling is due to periodic and rapid increases in intracellular calcium levels. This elevation is accompanied by extracellular calcium entry through various channels as well as calcium mobilization from intracellular calcium stores (Berridge, 2000). It has been pointed out that hereditary human neurological disorders such as spinocerebellar ataxia type-6, episodic ataxia type-2, familial hemiplegic migraine are linked with various mutations in genes coding for calcium ion channel components such as the α1A subunit or pore-forming subunit of P/Q-type calcium channels (Ophoff et al., 1996, Zhuchenko et al., 1997).

P- and Q-type calcium channels are expressed throughout the brain with the highest expression levels corresponding to regions of high cell density. Until now, cerebellar cortex, hippocampus, olfactory bulb and caudal colliculus have been found to have high numbers of P/Q-type calcium channels (Stea et al., 1994, Tanaka et al., 1995, Volsen et al., 1995, and Campbell and Hess, 1998). However, high neuronal density levels are also present in other brain areas such as cerebral cortex, thalamus and basal ganglia. The large number of neurons present in hypothalamus suggests that there could be a large number of P/Q-type calcium channels in this brain region (Celio, 1990, Gao and van den Pol, 2002). The major physiological functions of mammals including endocrine function have the central regulatory component located in hypothalamus. Thus, it is possible that mutations in P/Q-type calcium channels in hypothalamic neurons could affect reproductive function in both sexes via altered release of neuronal

transmitters, endocrine hormones and/or local factors as well. In other words, reproductive function might be affected due to abnormal functioning of hypothalamic neurons which would influence functioning of the entire hypothalamic-pituitary-gonadal axis.

The leaner mouse carries an autosomal recessive mutation in the α1A subunit of P/Q-type voltage-gated calcium channels (Fletcher et al., 1996). The amplitude of calcium current in leaner cerebellar Purkinje cells is decreased (Lorenzon et al., 1998, Dove et al., 1998). In addition, in the leaner cerebellum calcium–binding protein expression is reduced (Dove et al., 2000). This observation suggests that failure of calcium homeostasis might very well lead to neurodegeneration in these mutant mice.

Further investigation into the relationship between neurologic syndromes and ion channel impairment on one hand, and the reproductive dysfunction on the other hand should improve the diagnosis and permit the development of new therapies for these syndromes. Neurological mutant mice are important tools for studying central nervous system development and function. They are excellent animal models for human diseases.

Calcium Channels in Neurons

Calcium ions play a main role in synaptic transmitter release and membrane excitability (Randall and Tsien, 1995), neurite plasticity and outgrowth (Lipton and Kater, 1989, and D'Angello et al., 1994), gene expression patterns (Starr et al., 1991)

and neuronal survival (Tsien et al., 1991). In the resting state, extracellular calcium concentrations are at least thousands of times larger than the intracellular calcium levels. Calcium pumps and sodium/calcium plasma membrane exchangers maintain the physiological intracellular calcium concentration in the cytosol (Carafoli, 2002).

Calcium homeostasis is important for normal neuronal function whereas uncontrolled increases or decreases in intracellular calcium levels can result in neuronal death. To prevent misregulation of intracellular calcium ion levels that might be detrimental to calcium homeostasis, neurons contain a very complex calcium buffering system. This system includes cytosolic calcium-binding proteins, and several organelles that sequester calcium ions, including the smooth endoplasmic reticulum, mitochondria and nucleus (Carafoli, 2002). In the central nervous system, three major calcium buffering-proteins are expressed in neurons: calbindin, parvalbumin and calretinin (Baimbridge et al., 1992).

Calcium-binding proteins are excellent neuronal markers (Andressen et al., 1993) and they are differently expressed in different types and groups of neurons throughout the central nervous system. Also, it has been suggested that calcium-binding proteins have protective roles in various neurological disease processes such as Alzheimer's disease, Parkinson's disease and epilepsy (Heizmann and Braun, 1992). However, the neuroprotective role of calcium-binding proteins in CNS remains controversial (Airaksinen et al., 1997, Lephart and Watson, 1999). Calbindin D-28k has a very constant and characteristic distribution in association with long-axon neurons (exemplified by projection of neurons in thalamus, nigrostriatum, nucleus basalis of

Meynert, and brain stem), some short-axon cells (represented by interneurons of the cerebral cortex) and is abundant in vegetative centers of hypothalamus (Celio, 1990). Parvalbumin is prominently expressed in the cerebral cortex, hippocampus, cerebellar cortex, cranial nerve nuclei and spinal cord, while it is virtually absent from vegetative centers and pathways (Celio, 1990). Parvalbumin –immunoreactive neurons have a complementary distribution with calbindin D-28k, but in the Purkinje cells of the cerebellum and spinal ganglion neurons both calcium-binding proteins can co-exist (Celio, 1990). Calretinin is expressed mainly in the cerebellum, in granule cells (Kadowaki et al., 1993) and in their parallel fibers located in the cerebellar molecular layer (Rogers, 1989, Arai et al., 1991, Rogers and Resibois, 1992) as well as in other brain areas (Montpied et al., 1995). Calretinin appears to play a key role in influencing presynaptic calcium signaling (Edmonds et al., 2000).

In the nervous system, calcium enters into neurons via voltage-gated calcium channels (VGCC) or ligand-gated calcium channels (Berridge, 1998). Based on electrophysiological and pharmacological characteristics, VGCCs can be high-voltage activated (L, N, P/Q and R-type) or low-voltage activated (T-type). Once entered into neurons, calcium ions stimulate ryanodine receptors "calcium sensors" located on the membranes of the smooth endoplasmic reticulum and trigger calcium-induced calcium release from the endoplasmic reticulum. In addition to this process, the formation of inositol 1,4,5-triphosphate takes place. A rise in intracellular calcium ions induces conformation changes of share proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) that trigger exocytosis of neurotransmitter vesicles into the

synaptic cleft. Calcium ions also regulate gene expression when intracellular calcium increases are large enough to reach the nucleus. If the increase of intracellular calcium ions is prolonged, the morphology and function of synapses, now called synaptic plasticity, is altered.

Voltage-gated calcium channels are membrane proteins composed of a pore-forming/voltage sensing α1-subunit and several regulatory subunits such as α2, β, δ and γ (Catteral, 2000; Ertel et al., 2000). (See Figure 1, taken from Randall et al., 1999).

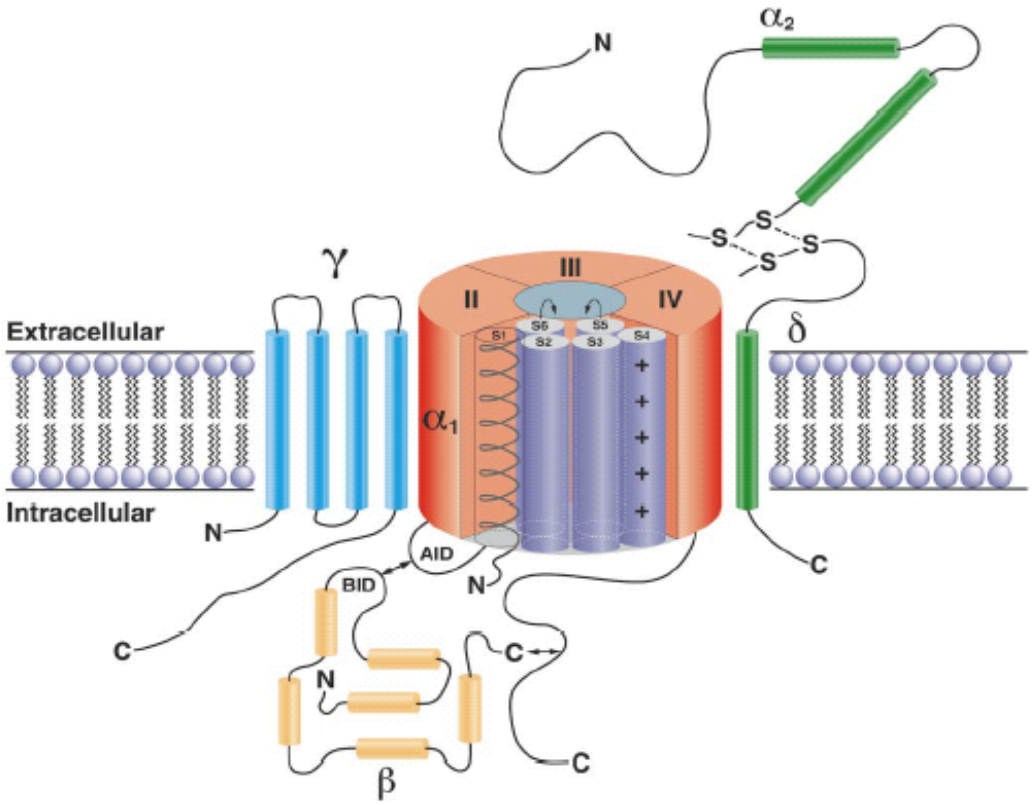


Figure 1. A schematic representation of the voltage-gated calcium channel (Randall et al., 1999).

Stated differently, the α1-subunit forms the pore of calcium ion channels that allows calcium influx into the neuron through the plasma membrane, whereas all the other subunits modulate the pore function (Horn, 2000). Six major types of α1-subunits are currently known: S, A, B, C, D and E (Snutch et al., 1991, Williams et al., 1992). All the voltage-dependent calcium channel α1-subunits have a similar structure with four repetitive domains (I-IV), each consisting of six transmembrane segments (S1-S6) (Catteral, 1995 and Wheeler et al., 1995). Initially, the α1-subunit was considered to be present only in P-type calcium channels but its expression in Xenopus oocytes allowed elucidation of a different calcium channel phenotype (Q-type), a so called "non-P-type calcium channel". The C-terminal of the α1 subunit has a calcium-calmodulin binding site. One pathway of inactivation of the VGCCs, including P/Q type channels, is due to the interaction between the C-terminal of α1 and calcium-calmodulin complex (Randall and Benham, 1999).

P- and Q-type calcium currents are differentiated principally on the basis of pharmacology. P-type calcium channels were first identified in Purkinje cells and they mediate calcium currents blocked by ω-Agatoxin (Llinas et al., 1989). Q-type calcium channels were first described in cerebellar granule cells and they are less sensitive to ω-Agatoxin (Randall and Tsien, 1995). P/Q-type calcium channels share the α1A subunit as their pore-forming subunit and they are involved predominantly in synaptic transmission. Although P- and Q-type calcium channels are observed predominantly in the cerebellum and moderately in hippocampus and caudal colliculus, it is supposed that

many of the neurons of the entire central nervous system present this type of calcium channel (Campbell and Hess, 1998).

Calcium Channel Mutant Mice – Models for Human Inherited Neurological Disorders

In the early 1960's, Sidman and Green (1965) suggested the existence of some mutations in ion channels and that these mutations might be responsible for many neurological disorders. In 1996, Fletcher et al. identified the first calcium channel mutation in tottering and leaner mice. Recent studies in molecular genetics have shown that many diseases in humans are related to ion channel mutations. These ion channel dysfunctions, known as channelopathies, are mainly due to channels that control entry of Na^{+}, K^{+} and Ca^{2+} ions into neurons and/or glial cells. Channelopathies in humans cause a diversity of symptoms such as ataxia, migraine, epilepsy and many other neuromuscular disorders.

Many spontaneous calcium channel mutant mice have been identified: tottering and leaner (Fletcher et al., 1996, Doyle et al., 1997), rolling Nagoya (Mori et al., 2000), rocker (Zwingman et al., 2001), lethargic (McEnery et al., 1998), ducky (Barclay et al., 2001) and stargazer (Latts et al., 1998). The first three mutant mice are allelic mutants, which carry mutations in the α1A subunit (See Figure 2).

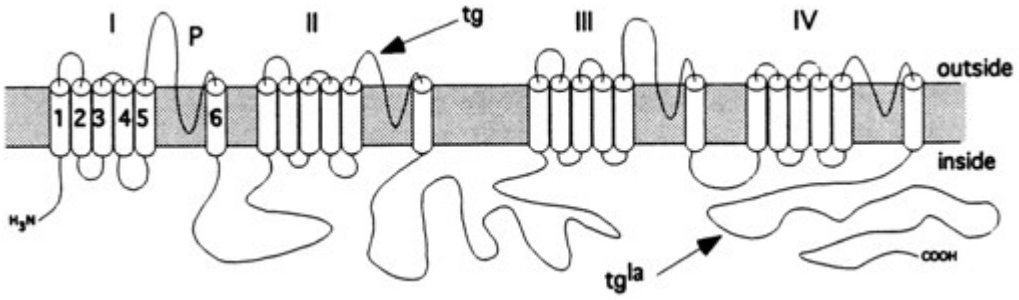


Figure 2. Transmembrane topography of the α1A subunit mutation (Fletcher et al., 1996). tg = tottering, tg la =leaner mice.

In addition to mice that carry spontaneous mutations, many transgenic mice have been obtained by "knocking out" or over expression of specific subunits of calcium channels (Muth et al., 2001).

α1A calcium channels mutant mice exhibit different grades of severity of several phenotypes: ataxia, paroxysmal dyskinesia and absence seizures. The severity of different phenotype is probably a consequence of the mutation site.

Recent studies have linked mutations in the α1A subunit of P/Q-type voltage dependent calcium channels with three human neurological conditions such as familial hemiplegic migraine, episodic ataxia type-2 and spinocerebellar ataxia type-6 (Ophoff et al., 1996, 1998). Also, a newly recognized form of human inherited epilepsy is suggested to be correlated with an α1A mutation (Burgess and Noebels, 1999, Cooper and Jan, 1999). Thus, the human spinocerebellar ataxia type-6 and leaner mouse mutations both disrupt the intracellular carboxyl tail of the calcium channel (Fletcher et

al., 1996, Zhuchenko et al., 1997) resulting in a debilitating ataxia associated with severe cerebellar degeneration (Herrup and Wilczynski, 1982, Heckroth and Abbott, 1994). Familial hemiplegic migraine and episodic ataxia type-2 mutations and the leaner mouse mutation alter the transmembrane and/or pore-region of the channel (Fletcher et al., 1996, Ophoff et al., 1996) resulting in episodic attacks of neurological impairment without major changes in cerebellar morphology (Isaacs and Abbott, 1994). The genotypic and phenotypic similarities between these human disorders and the mutant mice suggest these mice are a useful model in determining cell-type specific regulation of calcium channel subtype expression and function in the mammalian brain (Campbell and Hess, 1998).

Morphological Abnormalities in α1A Gene Mutant Mice

Major morphological abnormalities in the CNS of mice with α1A-gene mutations are relatively absent when observed by light microscopy (Meier and MacPike, 1971, Levitt, 1988, and Isaacs and Abbott, 1992). The most important abnormalities in leaner mice have been detected by routine histopathology (Meier and Mac Pike, 1971, and Herrup and Wilcyznski, 1982). The first notable central nervous system abnormality is cerebellar atrophy and reduction in cerebellar cortical neuron density (Meier and Mac Pike, 1971, and Herrup and Wilcyznski, 1982, Lau, 1999). Both major types of cerebellar cells (Purkinje and granule cells) are lost in leaner mice (Herrup and Wilcyznski, 1982, Lau, 1999, Frank et al., 2003). This loss of cells as well

as the existing surviving cells, which do not function properly, results in profound cerebellar dysfunction. The reduction of cerebellar volume and weight in the tottering mouse is due to a decrease in the cerebellar molecular layer dimensions (Isaacs and Abbott, 1992). The cerebellar molecular layer contains parallel fibers and varicosities, Purkinje cell dendritic arborizations, and glial cells. The major components of the molecular layer are parallel fibers, which are granule cell axons. Loss of granule cells in the leaner mouse begins in the early juvenile period. It was observed that greater than 50% of cerebellar granule cells degenerate in leaner mice by 6 months of age (Herrup and Wilcyznski, 1982).

In addition, the loss of Purkinje cells and Golgi cells in leaner mice is significant, and starts around the 25^{th} day of age (Frank et al., 2003). Some subtypes of Purkinje cells are sensitive to α1A-channel mutations and die, while some other subtypes are not affected and survive. Immunohistochemical staining with Zebrin II among surviving Purkinje cells suggests a potential correlation between cerebellar cortical circuitry and Purkinje cell resistance to the effects of the mutation (Eisenman and Hawkes, 1993).

Moreover, the surviving Zebrin II-positive Purkinje cells of leaner mice present high levels of tyrosine hydroxylase (TH) expression (Hess and Wilson, 1991, Austin, 1992, Heckroth and Abbott, 1994, and Mori, 2000). TH is a rate-limiting enzyme that converts tyrosine to dopamine in catecholaminergic neurons. Normal adult Purkinje cells do not show TH expression. However, the mechanism of abnormal TH expression in these mutant mice has not been elucidated yet. The presence of tyrosine hydroxylase

expression in the Purkinje cells of the mutant mouse cerebellum is possibly due to impaired neuronal maturation. It was supposed that aberrant tyrosine hydroxylase expression in leaner Purkinje cells might be correlated with the abnormal phenotype of these mice (Hess and Wilson, 1991). Sawada and Fukui (2001) suggested that involvement of climbing fiber input and changes in calcium channel expression could determine TH expression impairment in mutant mice, specifically rolling Nagoya mutant mice. In addition, they observed higher expression of corticotropin releasing factor (CRF) in climbing fibers near TH positive Purkinje cells. Ogasawara et al. (2001) reported a similar pattern. Thus, leaner Purkinje cell survival may be correlated with signal input from the olivary nucleus or with the anterograde transport of insulin-like growth factor l (Nahm et al., 2003). Also, Fluoro-Jade, an anionic fluorochrome, which selectively labels degenerating neurons, detects degenerating Purkinje cells in the leaner cerebellum (Frank et al., 2003).

Differential sensitivity of Purkinje cells is unanimously accepted to occur in mice with α1A-gene defects, but this pattern does not elucidate why the Purkinje cell loss occurs only in leaner mice. No loss of glial cells has been detected in the molecular layer of leaner mice (Meier and MacPike, 1971, and Isaacs and Abbott, 1992). Also, neuronal atrophy in some areas of the olivary nucleus and deep cerebellar nuclei of leaner mice has been observed (Heckroth and Abbott, 1994). In conclusion, the leaner α1A mutation alters the major components of the cerebellar circuits.

Electron microscopic evaluation of the cerebellum shows different abnormalities especially in synaptic junctions between granule cell axonal varicosities (parallel fibers)

and Purkinje cell dendritic spines (Rhyu et al, 1999). In leaner mice, some parallel fiber varicosities form synapses with multiple Purkinje cell (4-9) dendritic spines. These synaptic contacts occur just before or at the time of onset of phenotypic neurological disorders. In addition, decreased density of parallel fiber varicosities within the molecular layer seems to occur (Walker, 1999). Enlarged varicosities are seen in leaner mice as early as the 20^{th} –25^{th} postnatal day (Rhyu et al, 1999, Kano et al, 1995).

Calcium–binding proteins are poorly expressed in leaner cerebellum (Dove et al., 2000). Immunohistochemical studies have shown that calbindin is present in Purkinje cells, calretinin in cerebellar granule cells (Rogers, 1989) and parvalbumin is located in Purkinje cells as well as in basket and stellate cells (Celio, 1990). Decreased levels of calbindin and calretinin expression in thalamic nuclei and substantia nigra were observed in genetically epilepsy prone rats (Montpied et al., 1995). These reductions of calcium binding proteins in animal models of epilepsy may increase susceptibility to the initiation of seizures (Montpied et al., 1995). In leaner mice, decreased intracellular buffering capacity in Purkinje cells (Dove et al., 2000) and decreased expression of calretinin in granule cells suggest an adaptative process to maintain calcium homeostasis (Nahm et al., 2002). This process probably takes place in all neurons with altered P/Q-type calcium channels.

Also, infrequent, aberrant Purkinje cell dendritic spines can be observed in leaner mice. The axonal compartment of some Purkinje cells also can contain other atypical characteristics such as torpedo-like swellings (Rhyu et al, 1998). An increase in

microvascularity of the leaner cerebellar cortex may occur in relation to regionally accelerated synaptic plasticity and/or activity (Black et al, 1990, Isaacs et al, 1992).

General Considerations about Leaner Mice

Dickie first described the leaner mice in 1962. These mutant mice were named "leaner" after their movement disorder. Leaner mice lean against the wall of their cage in order to prevent their falling down.

Leaner mice carry an autosomal recessive mutation (Sidman and Green, 1965). This mutation is localized in the calcium channel α1A-subunit gene, which is situated on chromosome 8. This mutation is a glycine to alanine splice site mutation at the 5'end of the unspliced intron in the gene. The truncation of the carboxy terminal of the normal protein determines two α1A-splice variants (Fletcher, 1996, and Doyle, 1997).

The characteristic phenotype of leaner mice includes expression of a severe ataxia, generalized absence seizures that occur throughout the life of the mice and paroxysmal dyskinesia (an intermittent movement disorder). Both male and female leaner type mice are affected. Initially, the leaner mouse was classified as cerebellar malformation mutant due to the reduced cerebellar size as well as the loss of cerebellar neurons that are observed in these mice.

The leaner mouse is very ataxic beginning with postnatal day 10 and this condition continues to grow worse until postnatal day 40-50 (Sidman and Green, 1965). Due to this fact, the leaner mouse viability is reduced during this early postnatal period.

To improve the viability of these pups, some research laboratories used to feed leaner pups with infant food formula four times daily (Dr.Abbott's laboratory, until 1998). Current husbandry practice to ensure survival of leaner pups is to foster the pups to surrogate dams that are lactating. Swiss White Webster mice are often used because they are excellent foster dams. The average life expectancy of the leaner mouse is normal (two years) once they survive past postnatal day 50.

Leaner mice do not show "tremor" (Sidman and Green, 1965). Fletcher et al. (1996) described a lack of "motor seizures" in these mice. Lau (1998) and Rhyu (1999a) described an "intermittent movement disorder" present in these mice. These differences might be due to a variety of definitions, which were used by researchers to characterize the movement disorders that these mice exhibit. These differences in descriptions also could be due to severe ataxia that leaner mice experience, which might be responsible for masking other motor dysfunctions.

The leaner mutation does not appear to significantly alter postnatal cerebellar development. Cerebellar granule cell death via apoptosis begins at postnatal day 10 with a peak at postnatal day 20 (Lau, 1999). Starting with postnatal day 21 loss of Golgi cells is observed in leaner mice (Wilcynzki and Herrup, 1982). Purkinje cell loss begins at postnatal day 25 with a peak at postnatal day 40-50 and continues to be observed until postnatal day 80, which is the latest age examined (Frank et al., 2003). The region of greatest cell loss in the cerebellum is the anterior lobe. Leaner mice also show an aberrant tyrosine hydroxylase expression in Purkinje cells (See Morphological Abnormalities in Leaner Mice Chapter).

Electrophysiological studies on leaner P/Q type calcium channels revealed functional changes due to the leaner mutation. Calcium current though P-type calcium channels is reduced more than 60% (Dove et al., 1998, Lorenzon et al., 1998, and Nakamori, 1998). However, ataxia might be caused not only by the reduction of calcium levels in Purkinje cells but also by disinhibition of deep cerebellar nuclei after reduction of Purkinje cell activity. It also is likely that a similar reduction in calcium current takes place in granule cells. Calcium current reduction probably occurs in neurons from many other areas of the brain due to the wide expression of P/Q type calcium channels. Ogasawara et al. (2001) described delayed responses to nociceptors in leaner mice due to decreased ability on initiating action potential in dorsal root ganglion. Also, Ayato et al. (2000) observed higher threshold for initiating cortical spreading depression and reduced glutamate release in neocortex neurons of leaner mice. Thus, it is likely that a decrease in calcium current density in homozygous leaner mice would most probably attenuate synaptic transmission at synapses between many cell types (Lorenzon et al., 1998).

Alterations in calcium homeostasis due to a decreased calcium uptake into endoplasmic reticulum after a decrease in calcium influx and reduced calcium buffering capacity (Dove et al., 2000) are compensatory mechanisms by the cell to keep calcium concentration in normal limits in order to maintain initiation of calcium signaling cascades (Murchinson et al., 2002).

Neurotoxicity and Reproductive Toxicity

Early in the 1970's, descriptive toxicology was replaced by a new focus concerned mainly on the mechanisms of processes that occur in biological systems exposed to toxicants. The development of new analytical methods, biomathematics and statistics as well as the latest concepts in biochemistry and genetics transformed toxicology into a multidisciplinary research area.

Acute as well as chronic exposures have a varying impact on all organs and systems of the human body. However, the nervous system is one of the most vulnerable regions to toxicants. The effects of toxicants can be observed not only in the central nervous system but also in the peripheral nervous system, in both an acute or chronic manner (Abou-Donia, 1992). At the molecular level, a toxicant might interfere with protein synthesis, affect neurotransmitter production, and disrupt the oxygen support to the nervous system. In addition, a toxic substance might alter the flow of Na^{+}, K^{+} and Ca^{2+} ions across membranes, disrupts transmission of nerve impulses and thereby might lead to cell death (Anthony et al., 1996).

The acute toxic effects on the CNS can be expressed from mild symptoms such as depression, stupor, tremor, headaches, confusion, agitation, drowsiness or dizziness to severe symptoms like coma or death. Sometimes the effects on the CNS are a combination of depression and stimulation, suppression of anxiety with an increased intolerance to pain or slurred speech with lack of coordination. The participation of hypothalamus is suggested by behavioral changes, which appear as first indicators of CNS damage to toxic agents. The extreme age groups are more vulnerable to toxicants.

The developing nervous system is particularly sensitive in children. Because the capacity to respond and compensate for toxic effects declines with age, and the usage of combinations of multiple drugs that interact and affect the nervous system adversely, elderly people also are considered to be at increased risk.

Among many chemical compounds that are detected as neurotoxic for the human body, heavy metals are placed on the top of the list. On the basis of chemical speciation, mercury is presented in three forms: elemental, organic and inorganic, and each of these forms might undergo environmental transformation. All forms of mercury cross the placenta to the fetus because of lipid solubility (Cox et al., 1989) and result in increasingly severe effects in the neonatal period when nursing on contaminated breast milk (Grandjean et al., 1994).

Methylmercury is of major toxicological significance. It induces neurotoxicity through increase in production of reactive oxygen species (Belletti et al., 2002) and oxidative DNA damages (Le Bel et al., 1992). At the molecular level, methylmercury intoxication alters calcium homeostasis through binding to a variety of enzyme systems including those of mitochondria, microtubule depolymerization, and lisosomal damage alone (Madsen and Christensen, 1978, Atchinson et al., 1994) or in conjunction with neuronal apoptosis (Limbrick et al., 1995). The toxic effect of methylmercury is detected not only in neurons but also in glial cells, especially in astrocytes. Also, methylmercury shows a greater sensitivity in the developing brain (Clarkson, 1983). Exposure of the fetus *in utero* to this toxicant results in abnormal neuronal migration

and improper organization of brain nuclei, males being more affected than females in animals as well as in humans (Sager et al., 1984, Choie et al., 1978).

Fertility in both sexes presumes that complex molecular processes with many levels of regulation occur not only in the hypothalamus but also in the pituitary gland and reproductive organs as well. Numerous commonalties in human and mouse physiology are evident through the study of reproductive dysfunction using genetic mouse models (Burns and Matzuk, 2002). Feedback loops among the hypothalamus, pituitary and ovary can be disrupted by toxicants as well as by alterations in the levels of endogenous hormones such as estrogens (Thomas, 1996). Therapeutic drugs such as anesthetics, sedatives and analgesics as well as drugs of abuse (marijuana) cause temporary disruption in fertility by affecting gonadotropin secretion (Colborn et al., 1993). Lead is associated with sterility and neonatal death. Gametotoxic effects have been demonstrated in both male and female animals (Stowe and Goyer, 1971).

Clinical studies performed in females with epileptic syndromes show an increased incidence in reproductive dysfunction, such as polycystic ovary syndrome, hypothalamic amenorrhea, premature menopause and hyperprolactinemia. Not only the epilepsy but also the drugs used in epilepsy treatment may affect reproductive endocrine function (Cramer et al., 1991). Epileptic seizures are correlated with increased secretion of pituitary hormones, especially prolactin and gonadotropins. Hypersecretion of luteinizing hormone (LH) and a transient hyposecretion of the follicle-stimulating hormone (FSH) determine increase in levels of androgens with characteristic changes in ovary associated with ovarian acyclicity (Yen et al., 1999). In the reproductive

dysfunction of an epileptic person an important role is played by “an inadequate initial physiologic arousal response” (Morell, 1998, Yen et al., 1999).

Reproductive Function in Female Mice

Sexual reproduction in mammalian species takes place after puberty when the female is in a special physiological condition known as estrous.

In young, prepubertal female mice, the epithelium of vagina is bounded together and the orifice is not visible. The onset of puberty occurs at approximately 35 days in mice (Engle et al., 1927). Opening of the vaginal orifice is, in general, coincident with the first estrous and ovulation. Sometimes, the onset of puberty precedes the first estrous but not by more than 4 days (Mirskaia, 1930).

Copulation in mice is accompanied by the formation of a vaginal plug. The plug is formed by a mixture of secretions from the vesicular glands and coagulating glands of the male mouse. In mice, vaginal plugs fill the whole vagina from the cervical canal to the vulva and persist 18 to 24 hours. In many mammalian species, copulation is followed by ovulation.

At the time of copulation, the epithelium is stratified in three distinct regions, most of the cells being cornified. By the fourth day after copulation, the cornified layer of the vagina is lost. If the animal is pregnant, the cornified layer is not regenerated until parturition. Implantation would fail if copulation did not inhibit the next estrous. If the animal failed to become pregnant, the estrous cycle returns to normal after 8-16 days.

The first fertile mating can vary a great deal, but usually occurs at 7 to 10 weeks. Maturity in females occurs in the same time as males or some times somewhat earlier. The normal length of time breeding in mice is 10 to 12 months in females and probably several months later in males. The estrous cycle includes all changes in reproductive organs between two consecutive estrous stages. The estrous cycle can be subdivided into five stages: proestrous, estrous, metestrous I and II, and diestrous. The changes that occur in reproductive organs during estrous cycle are summarized in Table 1.

The simplest way to determine if a female mouse is in estrous stage or not is the vaginal smear or vaginal wash. In the estrous stage, vaginal smear contain more than 90% cornified epithelial cells. In this stage, leukocytes are absent (Figure 3). In all other estrous cycle stages, vaginal smears exhibit variable numbers of leukocytes as well as non-cornified epithelial cells in different stages of development (see Figure 4).

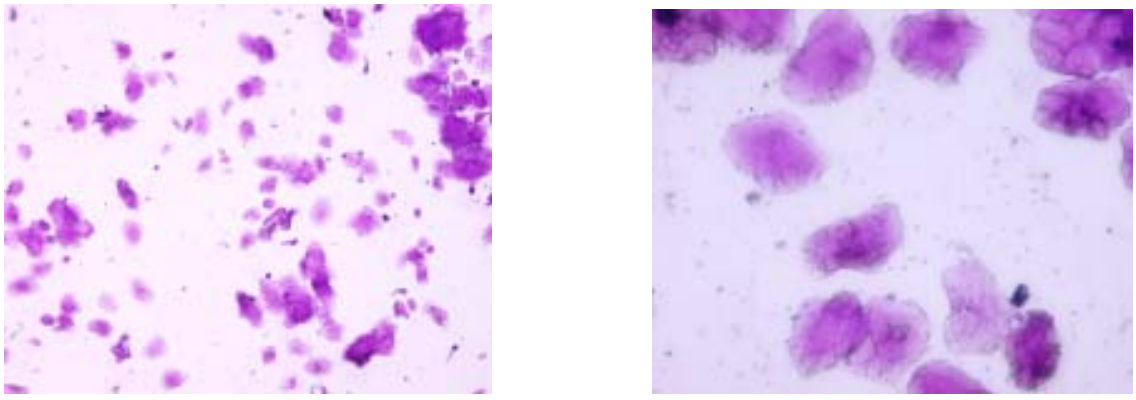


Figure 3. Vaginal smear in estrous stage of estrous cycle. Microscopic view of vaginal smear in estrous stage: lower magnification (4x, left) and higher magnification (40x, right) (Diff-Quik staining).

Table 1. Estrous cycle and the characteristic changes in the reproductive organs of mice (adapted from Bronson et al., 1966)

Stage	**Smear**	**Histology of vaginal epithelium**	**Uterus**	**Ovary and Oviduct**
Proestrous	ECL to EC	Many cell layers (10). Outer 4 nucleated. Active mitoses Few L	Hyperemia and Distension Active mitoses. Few L	Follicles large Few mitoses in germinal E
Estrous	EC to C+	Superficial layer lost C layer superficial Mitoses decreasing L absent	Mitotic activity reach the peak then decrease No L	Ovulation occurs followed by distension of the upper end of oviduct Active mitoses
Metestrous I	C++	C layer delaminated L start to appear	Distension decreased L in E	Early CL present Eggs in oviduct Many atretic follicles
Metestrous II	C++EL++	4-7 E cell layers Many L in outer layer	Walls collapsed Rare mitoses Many L	Growing CL Eggs in oviduct Few mitoses
Diestrous	EL Mucus	4-7 E cell layers with L Growth begins towards end of diestrous	Walls collapsed E with many L Secretion of uterine glands	Follicles start rapid growth towards end of period

E= epithelial cells, C= cornified cells, L= Leukocytes, += many cells, ++= very many cells. The smears given are typical. Some variation may occur.

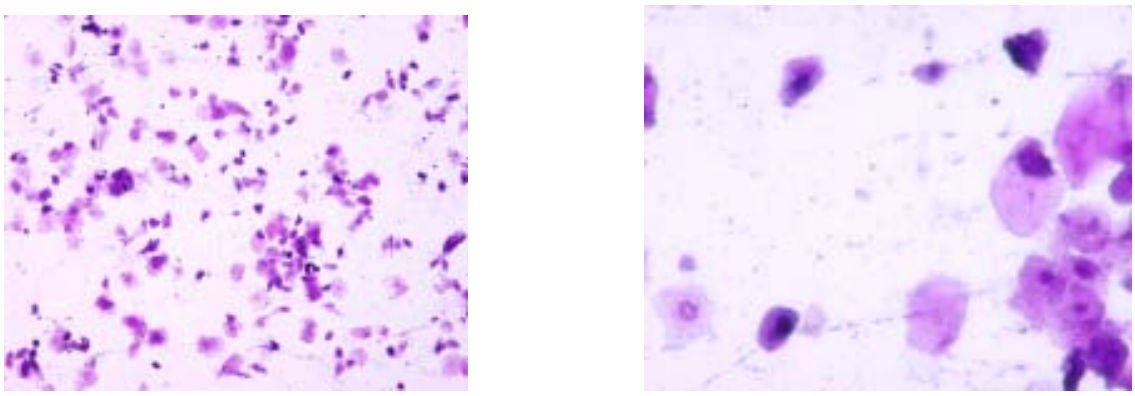


Figure 4. Vaginal smear in non-estrous stage of estrous cycle. Microscopic view of vaginal smear in non-estrous stage: lower (4x, left) and higher (40x, right) magnification (Diff-Quik staining).

The first estrous cycle is typically longer than usual. The onset of estrous usually occurs during the dark cycle. Estrous as well as ovulation is a cyclic process. In young virgin females, ovulation does not occur at every estrous. Also, an estrous may not coincide with each ovulation. This occurs because estrous is dependent on gonadal hormones, whereas ovulation is directly dependent on gonadotropin. The estrous cycle is normal in the absence of the uterus, uterus and cervix or the mammary gland (Long and Evans, 1922, Snell et al., 1940). The normal estrous cycle ceases after ovariectomy. Moreover, the absence of estrous cycle after the transplantation of ovary proves the failure of surgical intervention (Fox et al., 1984).

In the non-suckling mouse, the gestation period is between 19 and 20 days. Birth of litters frequently occurs during the nighttime. An estrous usually occurs about 20

hours after parturition. Nursing females lactate for three weeks. Lactation and a subsequent gestation period might proceed simultaneously. Some authors also believe that matings could occur during pregnancy. However, how many matings are accompanied with ovulation is still unknown (Crew et al., 1930). Ovulation does not occur during pregnancy or lactation. Inhibition of ovulation in the early part of pregnancy is due to copulation or cervical stimulation but in the later period of pregnancy must be due to fetal hormones (Fox et al., 1984).

Following ovulation, the ruptured follicles undergo changes that transform them into corpora lutea (CL). On the basis of the sexual history of female mice, there are four types of corpora lutea (ovulation, copulation, pregnancy and lactation). Corpora lutea of ovulation are corpora formed during an ordinary estrous cycle if mating does not occur. These corpora lutea might persist three-four cycles without significant degeneration (Allen, 1922). Corpora lutea of pseudopregnancy are corpora lutea that result from a sterile mating. Prolonged life of these CL determines a lengthened diestrous. Corpora lutea of pregnancy are formed following a fertile mating. The number of CL of pregnancy is, in general, equal with the number of oocytes ovulated. But the number of litters, mother's body weight as well as mother age can determine some variations in the number of these CL (MacDowell et al., 1929). Differences in numbers of CL also occur between strains and within strains. Corpora lutea of lactation develop in nonpregnant nursing mice from follicles that ovulate at the first postpartum estrous.

Normally, the number of litters has a range, which varies from 5 to 10 (Fox et al., 1984). The sex ratio at birth is 1:1. Litter size can vary greatly with the strain, age

and condition of the mother, as well as with the order of litters. In general, the first-born and the last two-three pups weigh less than the other pups in the same litter.

Mice are born hairless. The sex is distinguished from birth: males have the larger genital papilla and they have a greater distance between genital papilla and anus than females. At postnatal day 3 the external ears open. The eyes open at day 12. The conspicuous nipples appear at postnatal day 9.

The Regulatory Mechanisms of Reproductive Function

The regulatory mechanisms of the reproductive system are very complex, and include neural as well as hormonal pathways. The main role in the regulatory process is played by hypothalamus, which regulates virtually all body organs (Martin, 1988). Also, behavioral needs such as feeding, mating and drinking are regulated by hypothalamus. The endocrine system of females is more complex and dynamic than the male endocrine system.

The hypothalamo-pituitary-gonadal axis regulates the serum hormone levels with a complex feedback mechanism. Gonadotoxic agents act on neuroendocrine processes in the brain (hypothalamus or in other regions of central nervous system which are directly connected with hypothalamus), pituitary or target organ (gonad) (see Figure 5).

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are glycoproteins secreted and released by basophilic cells of the pituitary gland.

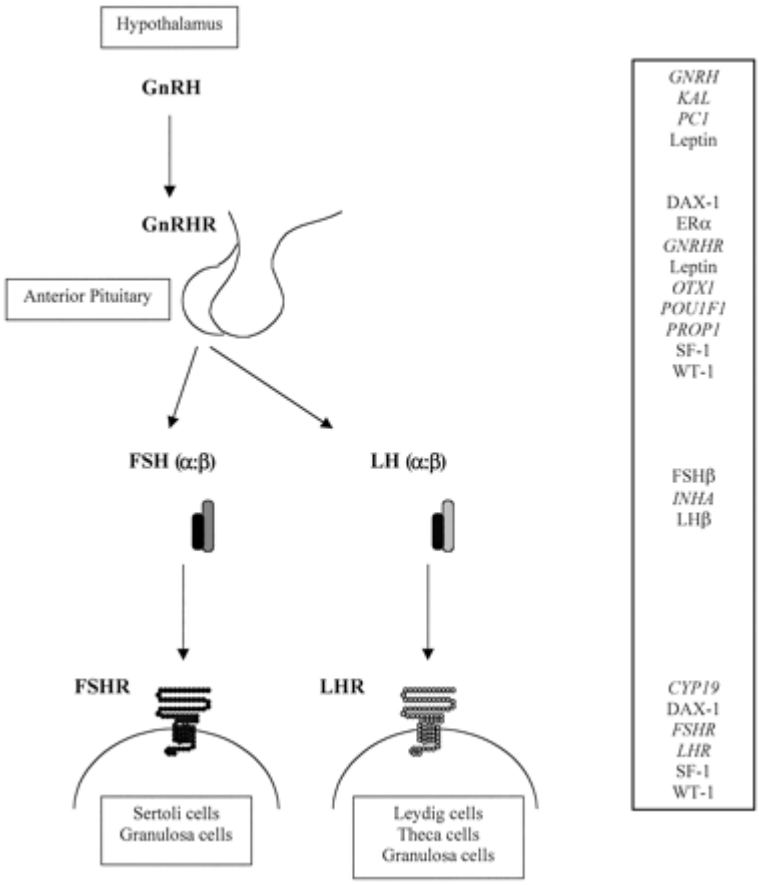


Figure 5. The regulatory mechanism of hypothalmo-pituitary-gonadal axis in the reproductive function (Burns and Matzuk, 2002).

Hypothalamic neuroendocrine neurons synthesize specific releasing or release-inhibiting factors into the hypothalamo-hypophyseal portal system and through blood they reach to adenohypophysis. Here, these factors stimulate or inhibit the anterior pituitary hormone levels. Because of the similarity between luteinizing hormone-releasing hormone (LHRH) and follicle stimulating-releasing hormone (FSHRH), it has been proposed to name this factor gonadotropin-releasing hormone (GnRH). The native

form as well as the synthetic form of LHRH stimulates the secretion of FSH and LH. Recently, a second GnRH II has been identified, which was found in the amygdala, caudate nucleus, hippocampus and thalamus, and in other regions outside of the brain such as kidney, bone marrow, endometrium and ovary (White et al., 1998). It is possible that GnRH II might have a role in influencing behavior.

In the hypothalamus, GnRH control involves GABA, glutamate, neuropeptide Y, neurotensin, angiotensin II, norepinephrine, 5-hydroxytryptamine as well as interleukine 1 and 2 (Martini et al., 1997). Dopamine stimulates the secretion of GnRH (Mac Hadley, 2000).

FSH and LH trigger intracellular signaling pathways by binding to their transmembrane receptors, which are coupled with G-proteins (FSHR and LHR). In females, LH stimulates the follicular development, ovulation and steroidogenesis (progesterone and estrogen secretion) (Burns et al., 2002). FSH promotes gametogenesis in both sexes. Estrogens stimulate the proliferation of uterine mucosa and muscle, the development of mammary gland as well as the secondary sexual characteristics. Also, steroid hormones determine negative feedback on gonadotropins. In addition to steroid hormones, the gonads secrete peptide endocrine factors such as inhibins, activins, which suppress or increase the FSH synthesis (Shelling et al., 2000).

Also, the bidirectional gonadotrope-gonad communication among nongonadotrophe anterior pituitary cell lineage and their hormones play an important role in establishing the physiologic pattern of reproduction. Dysfunction of the thyroid hormones (Stahl et al., 1999), growth hormone (Danilovich et al., 1999), insulin-like

growth factor-1 (IGF-1) (Baker et al., 1996) and leptin (Swerdloff et al., 1978 and Considine et al., 1996) levels as well as their specific receptors (Clement et al., 1998) lead to abnormalities in the complex regulatory cascade of reproductive syndromes. Interestingly, every day there are many new compounds found, that can influence the hypothalamus activity and indirectly the hormonal secretions. Thus, recently it was shown that vitamin D plays an important role in estrogen biosynthesis (Burns and Matzuk, 2002).

The onset of puberty starts with increased levels of gonadotropins. The onset of puberty trigger is not yet clearly understood but it is supposed to be due to GnRH, which signals an elevation of LH levels. Stated differently, the onset of puberty is a central nervous system phenomenon and the role of the gonad in this process is not thought to be major. Also, many other factors such as heredity, race, climate, athletic activity and obesity play important roles with respect to the onset of puberty in human females.

Loss of FSH signalling causes infertility in women (Layman et al., 2000). In contrast, loss of isolated LH signaling function shows effects only in males.

Rationale for This Study

"Reproductive health is not merely the absence of disease or disorders of the reproductive process, but rather, it is a condition in which the reproductive process is accomplished in a state of complete physical, mental and social well-being." (NICHHD,

2003). Like digestion or breathing, reproductive function is a characteristic of life. Successful completion of puberty as well as normal estrous cycles in females is prerequisite for reproduction. The control of reproductive function is very complex, involving both neural and hormonal factors. The onset of puberty is triggered by the activation of hypothalamo-pituitary-gonadal axis, which determines development of secondary sexual characteristics, accelerated growth velocity and bone maturation (Martin, 1988, Burns and Matzuk, 2002), while premature activation of hypothalamo-pituitary-gonadal axis is responsible for precocious puberty (Kempers and Otten, 2002). In addition, the onset of puberty is correlated with pulsatile release of LHRH (Levine, 1997), a phenomenon that cannot occurs in the absence of intracellular calcium ion oscillations (Moore et al., 2002). Moreover, calcium ions are required for LHRH activity (Yu et al., 2002). Although many roles of the calcium ion has been established, very little is known about calcium signalling with respect to reproductive function.

Nowadays, because of numerous commonalties in human and mouse physiology, animal models are used to gain understanding concerning the mechanisms that bring about reproductive impairment. Among the potential mouse models that can be used is the spontaneous mutant mouse named “leaner”. We assume that the impairment of calcium homeostasis due to this mutation as well as disruption in signaling pathways. We hypothesis that the leaner mutation is involved in producing the abnormal reproductive function observed in female leaner mice. Because in humans, reproductive dysfunction is more frequent in females than males, we focused our study on female leaner mice.

To test this hypothesis, we divided our study into four parts, which represent the main stages of sexual development in female leaner mice. We used normal female wild type mice as controls. The four parts of our study are:

1). Onset of puberty

2). Estrous cycle

3). Timed matings and gestational period

4). LH and estradiol hormone levels

This study will be a starting point for further investigation about the role of calcium signaling in neuronal regulation of reproductive function associated with spontaneous mutations that occur in calcium channels. Better understanding of pathogenic mechanisms, which are implied in these reproductive syndromes, will improve diagnosis and the corresponding treatments.

II. MATERIALS AND METHODS

Animals

Male and female control (+/+) and heterozygous leaner mice (+/tg^{la}), on the C57BL/6J background, originally obtained from The Jackson Laboratory (Bar Harbor, ME), were housed and bred to produce control (+/+) and homozygous leaner (tg^{la} / tg^{la}) offspring in the Laboratory Animal Research and Resource (LARR) Facility at Texas A&M University. Mice were maintained in a constant temperature (23-24°C), constant humidity (45-50%) room and with a 12 hours light-dark cycle. Mice were allowed access to food (Wayne rodent chow) and deionized water *ad libitum*. Tg^{la} / tg^{la} mice showed reduced viability when they were left with their natural dams (usually they die at approximately 18-20 days after birth). Death is due to starvation and/or dehydration as a result of severe ataxia, which prevents tg^{la} / tg^{la} pups from adequately interacting with their dam. We obtained better survival if we fostered leaner pups to Swiss White Webster dams, which are excellent foster dams. Beginning at postnatal day 10 leaner pups became very ataxic. Due to this fact, all homozygous leaner mice were supplemented with moistened Purina rodent chow in addition to water (via a water bottle and sipper tube located very close to the bottom of the cage) and dry rodent chow. The moistened rodent chow was changed daily. Swiss females are bred to Swiss males so the Swiss females can be used as foster dams.

Onset of Puberty

To study the onset of puberty, 17 female leaner and 28 female wild type mice were used. Onset of puberty was determined by daily examination for vaginal opening. Daily examination began at postnatal day 21 and continued until opening of the vagina was observed.

Estrous Cycle

For the estrous cycle study, 12 leaner and 20 wild type female mice were used. The estrous cycle was studied in mice that were two- to six- months of age because it takes several weeks after the onset of puberty before the estrous cycle becomes regular. The stage of estrous cycle was established through vaginal washes, which were performed using a lavage method. Glass Pasteur pipettes were fire polished and drawn to a rather fine, smooth point. After sterilizing the pipettes in 70% ethanol, each pipette was dried and then a small amount (approximately 100 microliters) of sterile saline was drawn up into the tip of the pipette. Then the tip of the pipette was inserted into the vagina, the sterile saline was ejected into the vagina and then immediately sucked back up into the pipette. The saline with its cellular contents was deposited onto a glass microscope slide and allowed to dry on a slide-warming tray, set at 60°C. When the slide dried completely the cells located on the slides were stained with Diff-Quik (VMR). To determine the stage of estrous cycle daily observations of the proportion of

cornified versus non-cornified vaginal epithelial cells were made (Long et al., 1922; Bacha et al., 1990) a minimum of 90% cornified cells signifies estrous. The estrous cycle study was performed for a minimum 28 continuous days for each mouse.

Timed Matings and Gestational Period

Timed matings were conducted in which a male mouse was housed overnight, during the dark part of the light cycle, with two or three females of the same genotype. In the morning the male was removed and the females were checked for the presence of a vaginal plug, indicating that copulation had occurred the night before. The day the plug was observed was considered day 0 of pregnancy and on day 18 of pregnancy (gestation in C57BL/6 mice is 18.5-19 days) these mice were anesthetized with isofluorane inhalant anesthetic, sacrificed via cervical dislocation and the uterus was removed. Our study was performed on 11 wild type mice and 10 leaner pregnant mice at the 18^{th} day of pregnancy. We selected this day to be sure that we examined the litters of pregnant mice before their normal delivery. For timed matings the number of plugs was counted. Also, the initial and the final body weight of each pregnant mouse as well as the gain body weight were recorded. Litter and fetal parameters were observed in addition to the location of the fetuses in left or right horns, the number and the location of resorbed embryos or stillborn fetuses and the number of corpus lutea on each ovary. Each fetus was weighed, the gender determined, crown-rump length measured and any external abnormalities / congenital defects noted.

Hormone Assays

Trunk blood was collected with and without anesthesia by decapitating and exsanguinating mice, according to the approved Animal Use Protocol (2003-149). Anesthetics were not used for some of the mice when blood was collected because of the possible side effects on the hormone levels of these chemical compounds. All the samples were analyzed and noted whether use of isofluorane had any affect on the hormone levels that were observed. Trunk blood was obtained from 45 wild type and 30 female mice at 28 days of age and the serum was used to determine concentrations of LH and estradiol (E_2). The postnatal age of 28 days was chosen because of the early onset of puberty in leaner females versus wild type females. In order to separate the serum from the formed elements of the blood, the blood was centrifuged at 4,000 rotations per minute for 4 minutes. All blood collection was performed between 1:00PM to 5:00PM, when the concentration was considered high and without diurnal fluctuations. To perform each analysis we needed 100µl serum. The obtained serum was refrigerated and stored at -70°C until used. Luteinizing hormone was measured in using a RIA procedure. The rat luteinizing hormone antiserum (NIDDK-anti-rLH-S11), antigen (NIDDK-rLH-I-9), and reference preparation (NIDDK-rLH-RP-3) were all provided from National Hormone and Pituitary Program, Ogden Biosevices Corporation (Rockville, MD). Results were expressed in terms of LH RP-3 reference standards when 100 µl of serum sample was used. This assay had a sensitivity of 0.07ng/ml and the inter- and intra- assay variations were both less than 10%. The specificity and recovery

rate of each kit was evaluated before spiking mouse serum samples with LH and E2 proteins in the experimental assays. The rat E2 kit was obtained from ICN Biomedical, Inc. (Costa Mesa, CA). The average recovery rate was calculated from three different test assays. All assays were carried out according to the manufacturers instructions. The intra- and inter-assay coefficients of variation are expected to be less than 10% (Nahm et al., 2003).

All animal procedures were approved by the Texas A&M University Animal Use Committee and met the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication no 85-23, revised 1985).

Statistics

Statistical Analysis for Onset of Puberty

A one-way analysis of variance (ANOVA) was used to evaluate the significant differences with respect to the onset of puberty between leaner and wild type female mice. The significance level for this analysis was set at $p<0.05$. Confidence intervals were calculated using SPSS 10.0 software (SPSS Inc. IL, Chicago, USA).

Statistical Analysis for Estrous Cycle

After the stages of estrous cycle was determined, the proportion of days in estrous versus the proportion of days in nonestrous for each mouse was calculated during 28 days. Because the obtained data did not have a normal distribution, the

nonparametric Mann-Whitney test for the statistical analysis was used. The significance level for this analysis was $p<0.05$.

Statistical Analysis for Timed Matings and Gestational Period

The observed maternal and fetal parameters were discussed separately. The number of plugs as well as the body weight gain in pregnancy was evaluated using Student's t-test. The level of significance was set at $p<0.05$. The number of implants, pups and resorptions per litter compared to wild type mice was evaluated using one-way ANOVA and Scheffe's F-test as a post hoc test, $p<0.05$. Because most of the fetal parameters did not show a normal distribution, a nonparametric test (Mann-Whitney test) to check for significance was used. In these cases, the p-value was less than 0.05. Confidence intervals were calculated using SPSS 10.0 software (SPSS Inc. IL, Chicago, USA).

Statistical Analysis for Hormone Assays

Determination of significance for the hormone assays was performed using one-way and two-way analysis of variance (ANOVA). The significance level for this analysis was set at $p<0.05$. Confidence intervals were calculated using SPSS 10.0 software (SPSS Inc. IL, Chicago, USA).

III. RESULTS

Onset of Puberty

To examine puberty onset, 17 female homozygous leaner and 28 female wild type mice were used. Puberty onset was determined by daily examination for opening of the vagina (see Section II, Materials and Methods). The mean of puberty onset in homozygous leaner females was 30.9 days±0.6 (SEM), with a standard deviation of 2.5 (see Table 2). The range for puberty onset in leaner mice varied from 26 to 38 days. The mean of puberty onset in wild type female mice was 34.1 days±0.7 (SEM), with a standard deviation of 3.4. In this latter group, the range for puberty onset varied from 29 to 41 days. In the wild type group, one female exhibited the puberty onset at 56 days. This female mouse was clearly an "outlier", as indicated by the fact that puberty onset at 56 days is more than the mean plus three standard deviations. This single wild type female was not included in our study. ANOVA showed a statistically significant difference in puberty onset between these two groups ($p<0.05$). The onset of puberty in leaner mice was precocious relative to age-matched wild type females (see Figure 6).

Table 2. Onset of puberty in leaner and wild type mice

Genotype	Number of mice	Mean (Days)	SEM	SD	Range (Days)
Leaner	17	30.9	0.6	2.5	26-38
Wild type	27	34.1	0.7	3.4	29-41

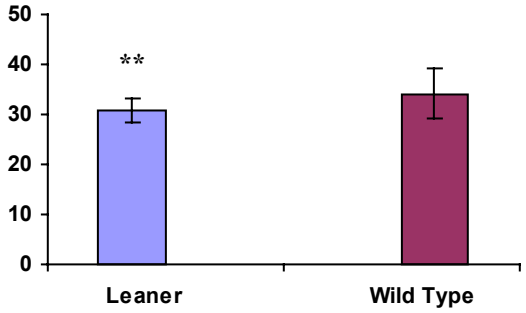


Figure 6. The onset of puberty. Average number of days in leaner and wild type mice. The (**) indicates that leaner mice showed an earlier onset of puberty compared to wild type mice, $p<0.05$.

Estrous Cycle

For analysis of the estrous cycle, we examined daily vaginal smears, which were taken at the same time of day, for both homozygous leaner and wild type mice (see Section II, Material and Methods). Each mouse was examined for a minimum of 28 continuous days and the number of days in estrous and the number of days of nonestrous for both genotypes were determined. After these data were collected, the probability for mice of each genotype to be in estrous was calculated. The probability to be in estrous for homozygous leaner female mice was 0.38, with a standard deviation of 0.08. On the other hand, the probability to be in estrous for female wild type mice was 0.30 with a standard deviation of 0.06 (see Table 3). The range for the total number of

days in estrous for the homozygous leaner mice varied from 7 to 16 (out of a total of 28 days). The range for the total number of days in estrous for wild type female mice varied from 5 to 14 out of a total of 28 days. A Mann-Whitney test indicated a significant difference between these two groups. Specifically, the probability to be in estrous for homozygous leaner mice was significantly greater than age-matched wild type females ($p<0.05$) (See Figure 7).

Table 3. The probability to be in estrous in leaner and wild type mice

Genotype	Number of mice	Probability mean	S D
Leaner	12	0.38	0.08
Wild type	20	0.30	0.06

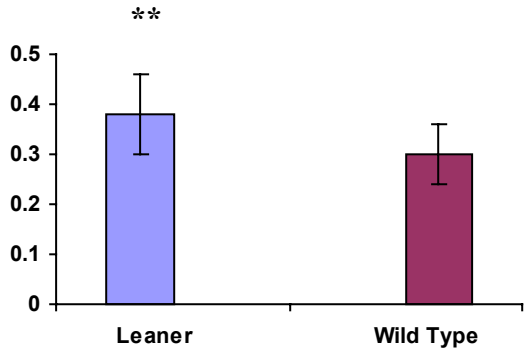


Figure 7. The probability to be in estrous for leaner and wild type mice. The (**) indicates that leaner showed a higher proportion of time in estrous compared to wild type mice, $p<0.05$.

Timed Matings and Gestational Study

We performed timed matings as described in Section II, Material and Methods were performed. The first observation to be made was that the reproductive success of homozygous female leaner mice was significantly diminished relative to the reproductive success of age-matched wild type female mice. Over an initial three-month time period timed matings were performed with 11 female wild type mice and 10 leaner mice. While 10 out of 11 female wild type mice became pregnant (91%) and carried litters to term, only 4 out of 10 leaner mice carried litters to term (40%) during that three month time period (see Table 4).

Table 4. Leaner mice present a reduced rate of fertility (number of pregnancies) in comparison with wild type mice

Genotype	Number of mice	Number of pregnancies	Percentage
Leaner	10	4	40%
Wild Type	11	10	91%

In addition, the average number of plugs observed in leaner mice (2.9±0.88 SD) was significantly higher than the number of plugs observed in the age-matched wild type mice (1.18 ± 0.4 SD; see Table 5). The p-value was less than 0.05 (See Figure 8).

Table 5. The number of plugs in leaner compared to wild type mice

Genotype	Number of mice	Number of plugs	SD
Leaner	10	2.90	0.88
Wild Type	11	1.18	0.40

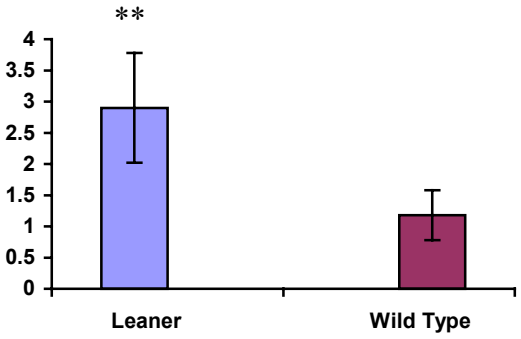


Figure 8. The number of plugs in leaner compared to wild type mice. The (**) indicates that leaner mice showed a larger number of plugs compared to wild type mice, $p<0.05$.

The second aspect of this study was divided into two parts: maternal parameters and fetal parameters. It should be noted that the first set of female mice used for this study resulted in 10 (10 out of 11 mice) wild type mice carrying their pregnancies to term, while only four of 10 homozygous leaner mice carried pregnancies to gestational day 18. In order to have a large enough sample size to compare various maternal parameters and fetal parameters between homozygous leaner mice and wild type mice, timed matings were carried out using additional female homozygous leaner mice until a total of 10 leaner mice were obtained that carried a litter to gestational day 18. We also added an additional timed mating in a wild type female, to bring the total number of timed matings in wild type mice examined to 11.

In the maternal analysis, the age of the dams at the time of pregnancy, dam body weight at gestational ages 0 and 18, dam body weight gain during pregnancy and the number of corpora lutea in each ovary at gestational age day 18 were examined. No significant difference in ages of dams at the time of pregnancy (115.2 days ±10.82 (SD) in wild type versus 148.7 days ±10.63 (SD) in leaner mice) was observed. Also, no statistically significant difference was observed in the initial and final body weights (22.17g ±3.56 (SD) and 35.82 g ±4.59 (SD) in wild type female mice compared to homozygous leaner mice (23.37g ±2.28 (SD) and 39.3g ±3.57 (SD)). All statistical analyses were performed using the Student's *t*-test ($p<0.05$).

The number of corpora lutea in each ovary at gestational age day 18 did not show a statistical difference between the right ovary (5.6 ±2.5 (SD)) and the left ovary (4.3 ±2.0 (SD)) in leaner mice compared with wild type mice (4.5 ±0.8 (SD) and 3 ±1.0

(SD)), respectively. Moreover, no significant difference was observed between the number of corpora lutea located on right and left ovaries for each genotype. However, only the total number of CL at gestational age 18 in leaner mice showed an increasing tendency relative to wild type mice ($p<0.1$). For the study of numbers of corpora lutea a two-way ANOVA ($p<0.05$) statistical test was performed (see Table 6).

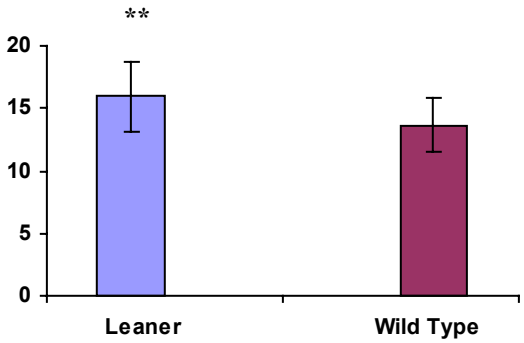
Table 6. The number of corpora lutea in each ovary at gestational age 18 in both categories of mice

Genotype	C L Right	Range (R)	C L Left	Range (L)
Leaner	5.6± 2.5	2-10	4.4±2.1	2-8
Wild Type	4.54± 0.82	3-6	3.0±1.0	2-5

A statistically significant difference in body weight gain during pregnancy was observed. The homozygous leaner mice (15.93g±2.85 (SD)) exhibited a larger weight gain compared to wild type mice (13.65 g ± 2.2 (SD)) (see Table 7 and Figure 9).

Table 7. Body weight gain during pregnancy in leaner compared to wild type mice

Genotype	Number of mice	Mean gain of body weight in pregnancy (g)	SD
Leaner	10	15.93	2.85
Wild type	11	13.65	2.20



F**igure 9. Body weight gain in leaner compared to wild type mice.** The (**) indicates that leaner mice showed a larger weight gain compared to wild type mice, $p<0.05$.

Once the female leaner mice finally became pregnant, they produced more pups per litter compared to wild type female mice (8.9 ±0.87 (SD) and 7.4 ±1.57 (SD),

respectively (see Figure 10). The one-way ANOVA test showed that these values were significantly different ($p<0.02$).

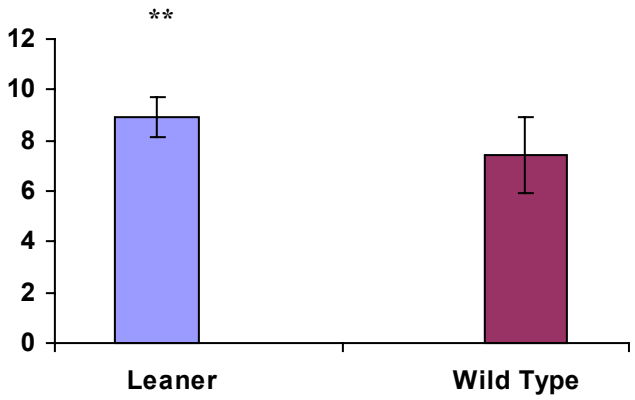


Figure 10. The number of pups in leaner compared to wild type mice. The (**) indicates that leaner mice showed a larger number of pups compared to wild type mice, $p<0.05$.

Also, the average total number of implants observed in the uteri from leaner mice (10.1±1.2 (SD)) was significantly greater than the control, the total number of implants observed in wild type female mice (7.8±1.6 (SD)) (See Figure 11).

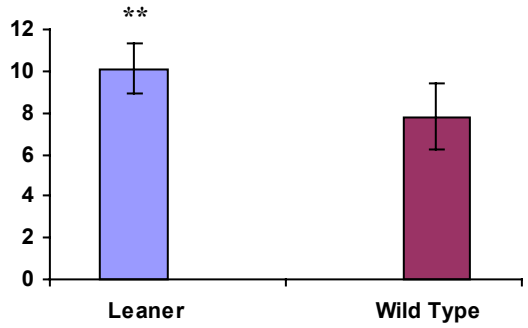


Figure 11. The number of implants in leaner compared to wild type mice. The (**) indicates that leaner exhibited a larger number of implants per litter compared to wild type mice, $p<0.05$.

Only one stillborn leaner pup was observed and no stillborn wild type pups were seen. The number of resorptions showed a tendency towards a slight increase in leaner mice (1.1±1.45 (SD)) compared to wild type mice (0.36± 0.50 (SD)); however, the p-value was > 0.05 and <0.10 so it could not be considered statistically significant (see Figure 12).

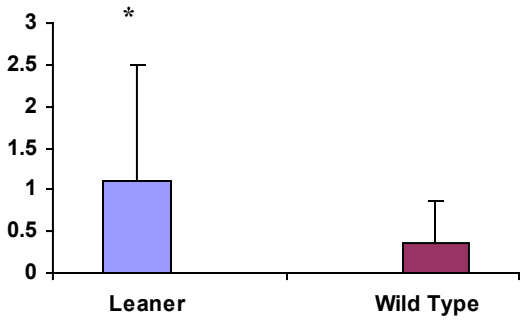


Figure 12. The number of resorptions in leaner compared to wild type mice.

Figure 12. (Continued) The (*) shows that leaner mice presents a larger number of resorptions per litter compared to wild type mice, $p<0.08$.

All the numbers of implants, pups, resorptions and stillborns for both categories of mice are contained in Table 8. Scheffé's F-test was used as post hoc test. P-value was set at 0.05. Also, Figure 13 presents the number of implants, pups and resorptions in leaner compared to wild type mice.

Table 8. The number of implants, pups, stillborns and resorptions in leaner and wild type mice

Genotype	**Number of implants** (Mean)	**Number of pups** (Mean)	**Number of resorptions** (Mean)	**Stillborn** (Mean)
Leaner	10.1	8.9	1.1	0.1
Wild type	7.82	7.45	0.36	0

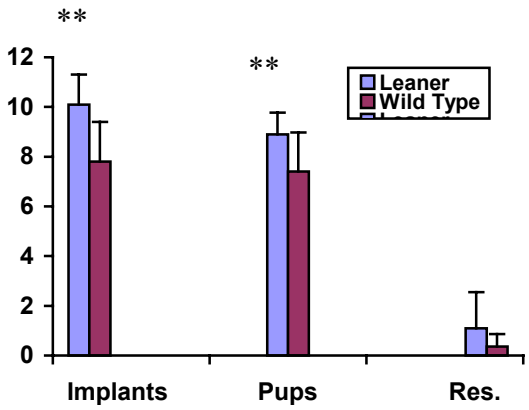


Figure 13. The number of implants, pups, and resorptions in leaner compared to wild type mice. The (**) indicates that leaner mice presented a larger number of implants, pups, and resorptions per litter compared to wild type mice, $p<0.05$.

Also, the number of implants in each horn for both genotypes of mice was recorded. For wild type mice, the number of implants in the right horn was almost equal with the number of implants in the left horn. In leaner mice, the number of implants varied from one horn to the other. We did observe a significant difference ($p<0.03$) between right horn implants in leaner (6.2 ± 2.1 (SD)) compared to wild type mice (4.36 ± 1.43 (SD))(see Table 9 and Figure 14).

Table 9. The number of implants from one horn versus the other horn in both categories of mice

Genotype	Right Horn	Left Horn
Leaner	6.2±2.15	3.8±2.25
Wild Type	4.36±1.43	4.36±1.43

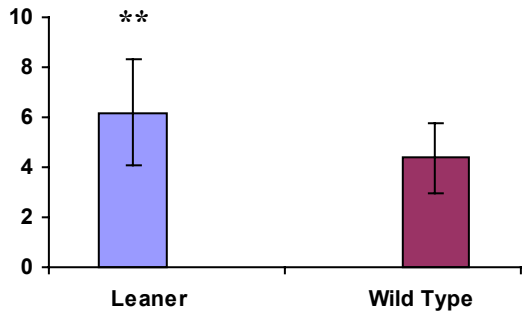


Figure 14. The number of implants from right uterine horn in leaner versus the number of implants in wild type mice. The (**) indicates that leaner mice showed a larger number of implants in the right uterine horn compared to wild type mice, $p<0.05$.

For the analysis of fetal parameters, body weight and crown-rump length (CRL) for all fetuses, their gross anatomy, gender, and any macroscopic malformations were determined. No significant difference was observed with respect to the proportion of male pups versus female pups in both genotypes.

The body weight of the fetuses was similar in both genotypes (1.08±0.05 (SD) in wild type and 1.08 ±0.2 (SD) in leaner mice). The fetuses from two leaner females

were not weighed because of their small size at the time the litter was examined. This discrepancy was likely due to the fact that these two leaner females were observed to have vaginal plugs on three consecutive days. It was determined that pregnancy day 0 in these two leaner mice was the first day that a vaginal plug was observed. It was only at the time of litter examination that it was determined that the first day of pregnancy that was chosen was not the real beginning of these two pregnancies. All the pregnant females were sacrificed at the 18th gestational day to ensure that the litters were examined prior to parturition. The fetal body weight range varied in wild type mice from 0.75 to 1.2 g and in leaner mice from varied from 0.91 to 1.43 g (see Table 10).

Also, the length of each fetus was determined, using crown-rump as the method of measurement. No significant difference between the two groups of mice was observed. In wild type mice, the mean fetal length was 21.35 mm ±0.94 (SD) and the range was from 17 to 24 mm. In leaner mice, the average fetal length was 21.44 mm ±1.77 (SD) and the range varied from 19 to 24 mm (see Table 10).

Table 10. The average body weight and length in both categories of mice

Genotype	Body Weight (g)	Range BW	Length (mm)	Range CRL
Leaner	1.08 ± 0.2	0.91-1.43	21.44 ± 1.77	19-24
Wild Type	1.08 ± 0.5	0.75-1.2	21.35 ± 0.94	17-24

Hormone Assays

To perform this study, we completed two different assays: luteinizing hormone (LH) and estradiol.

For testing LH, trunk blood was collected from 20 wild type and 20 leaner female mice and all the steps were followed, as stipulated in Section II: Material and Methods. The necessary amount of serum for each sample was 100 µl. Because one of the leaner samples presented less than this amount, it was eliminated from the study. For each genotype, 10 of the samples were collected from anesthetized mice and 10 of the samples were collected without anesthesia. There was no significant difference in the amount of LH that was present in the samples collected without anesthesia as compared to the samples collected with anesthesia for each genotype. Therefore, the samples were pooled to form a sample size of 20 for each genotype. In each group of wild type and leaner mice, there were two different conditions: mice that had an open vagina at the time of blood collection (8 wild type and 6 leaner mice) and mice that did not have an open vagina (12 wild type versus 13 leaner mice).

The average levels of LH in both wild type groups were 0.78ng/ml ± 0.66 (SD) with 0.23 (SEM) in mice with open vagina, and 0.45ng/ml± 0.23 (SD) with 0.07 (SEM) in mice without onset of puberty. The mean levels of LH in leaner were 1.05ng/ml± 1.12 (SD) with 0.45 (SEM) in mice with onset of puberty versus 0.37ng/ml±0.30 (SD) with 0.18 (SEM) in the others. In the latter group one value was eliminated because it was an outlier (the value was more than the mean plus 3SD). There was no statistically

significant difference in the LH levels in leaner versus wild type before the onset of puberty. Also, the values of LH after the onset of puberty in leaner compared to the values corresponding to the wild type were not significantly different ($p<0.05$). Also, there were no significant differences in the average LH levels in wild type (0.58ng/ml ± 0.47 (SD) and 0.1 (SEM)) compared to leaner (0.59ng/ml± 1.01 (SD) and 0.23 (SEM)). Two-way analysis of variance (ANOVA) test was performed ($p<0.05$). For wild type mice, there were no statistically significant differences in the levels of LH before and after the onset of puberty. However, in leaner mice the LH-levels were statistically significant comparing before and after the onset of puberty (0.37ng/ml±0.30 (SD) with 0.18 (SEM) versus 1.05ng/ml± 1.12 (SD) with 0.45 (SEM), $p<0.05$) (See Table 11 and Figure 15).

Table 11. The average luteinizing hormone levels at postnatal day 28, in correlation with the onset of puberty

Genotype	No	LH Mean (ng/ml)	No after onset	LH after onset (ng/ml)	No before onset	LH before onset (ng/ml)
Leaner	19	0.59±1.01	6	1.05±1.12	13	0.37±0.3
Wild Type	20	0.58±0.47	8	0.78±0.66	12	0.45±0.23

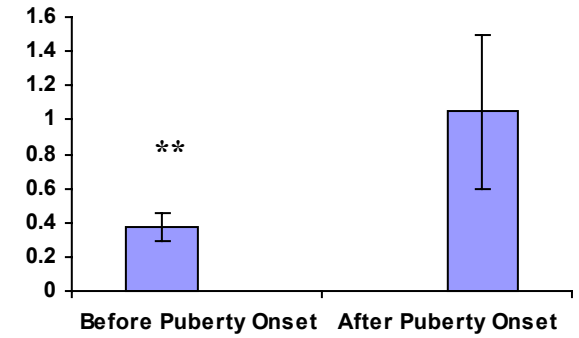


Figure 15. LH levels in leaner mice before and after puberty onset. The (**) indicates that the LH-level before the onset of puberty is lower than the LH-level after the onset of puberty in leaner mice, $p<0.05$.

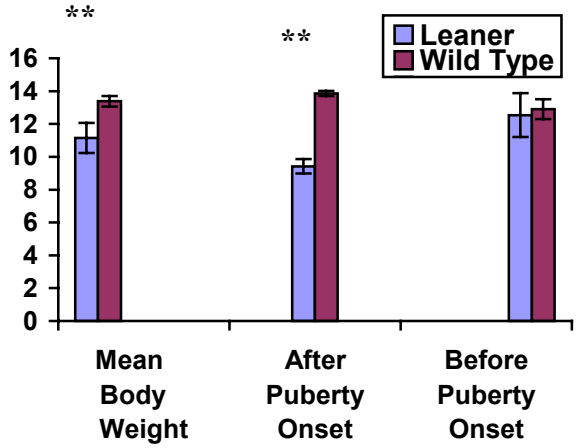


Figure 16. Body weight in leaner versus wild type mice before and after puberty onset. The (**) indicates that leaner mice presented less body weight compared to wild type mice, after puberty onset, $p<0.05$.

The body weight was checked before decapitation. At postnatal day 28, the mean body weight in wild type (13.38g±1.06 (SD) with 0.33 (SEM)) was higher than the weight in leaner mice (11.15g±2.73 (SD) with 0.91 (SEM)). No significant difference in weight in leaner versus wild type mice before puberty onset was observed ($p<0.05$). After puberty onset the body weight in wild type (13.86g±0.34 (SD) with 0.15 (SEM)) was significantly higher than in leaner mice (9.42g±0.89 (SD) with 0.44 (SEM)), ($p<0.01$). While the weight in wild type was constant before and after puberty onset, in leaner this difference was significant (12.54g±2.99(SD) with 1.34 (SEM) versus 9.42g±0.89 (SD) with 0.44 (SEM), $p<0.08$). (See Table 12 and Figure 16).

Table 12. Mean body weight in leaner and wild type mice before and after puberty onset

Type of mice	Puberty Status	Mean	SD	SEM
Leaner	**Before puberty**	12.54	2.99	1.34
Leaner	**After puberty**	9.42	.89	.44
Leaner	**Combined**	11.15	2.73	.91
Wild type	**Before puberty**	12.90	1.36	.60
Wild type	**After puberty**	13.86	.34	.15
Wild type	**Combined**	13.38	1.06	.33

For determining estradiol levels, blood was collected from 15 wild type and 9 leaner female mice in the same manner as the previous radioimmunoassay (See Section II: Material and Methods). Among these samples, four values, two for each strain, presented levels of estradiol that were not detectable. These values were not considered because they were outliers (outside of interval: mean ± 3 SD). In the wild type group four mice exhibited open vaginas but the obtained values of estradiol were not significantly different within the wild type group. The average estradiol level was 20.14pg/ml± 8.46 (SD) with 3.2 (SEM) in leaner and 28.03pg/ml± 8(SD) with 2.21 (SEM) in wild type mice (see Table 13). Using one-way analysis of variance (ANOVA), there was a significant difference ($p<0.05$) between the means of estradiol levels in leaner versus wild type mice (see Figure 17). Also, the mean body weight in wild type mice (12.1g± 0.2 (SD) with 0.1 (SEM)) had a tendency to be higher than in leaner mice (10.5g±2.1 (SD) with 0.71 (SEM)), $p<0.07$.

Table 13. The estradiol levels in wild type and leaner mice

Genotype	Number of mice	E2 level (Mean) (Pg/ml)	SD	SEM
Leaner	7	20.14	8.46	3.2
Wild type	13	28.03	8.0	2.21

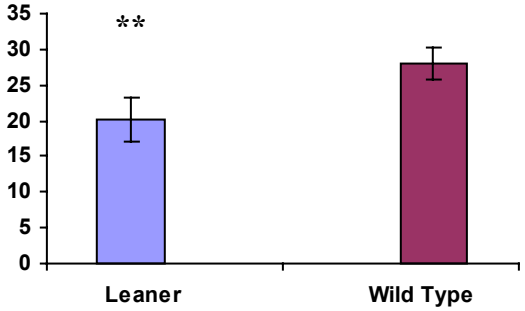


Figure 17. Estradiol levels in leaner compared to wild type mice. The (**) indicates that leaner mice showed a lower concentration of estradiol in serum compared to wild type mice, $p<0.05$.

IV. DISCUSSION

Leaner mice carry an autosomal recessive mutation in the α1A-subunit of neuronal P/Q-type voltage-gated calcium channels. These types of calcium channels are found throughout the entire central nervous system, including the hypothalamus. Due to the leaner mutation, leaner mice exhibit movement disorders similar to ones observed in humans. Calcium ions play the central role in the pathogenic mechanisms of this phenotype. Impairment of calcium homeostasis is due to decreased amplitude in calcium current in neurons (Dove et al., 1998) and a reduced calcium buffering capacity in these cells (Dove et al., 2000). Despite their neurological dysfunction, homozygous leaner mice are capable of breeding and producing viable offspring.

The present study examined the main stages of reproductive female function in leaner mice compared with similar, age-matched wild type mice. The onset of puberty is precocious in female leaner mice compared with wild type female mice. Our observations with respect to the onset of puberty in wild type mice are similar to those previously mentioned in literature (Eagle et al., 1927, Fox et al., 1984). The onset of puberty is a central nervous system phenomenon, and any disruptions that occur in CNS neurons especially in the hypothalamus affect this process. We can hypothesize that the calcium channel mutation in hypothalamus might be responsible for the premature activation of hypothalamo-pituitary-gonadal axis. Once activated, the hypothalamus determines gonadotropin-releasing hormone (GnRH) secretion and its release in the

portal blood. Consequently, GnRH reaches the anterior pituitary gland, where it triggers the LH and FSH secretions.

The estrous cycle study shows that female leaner mice spend more time in estrous compared with age-matched wild type mice. The normal estrous cycle in wild type mice is four days. In leaner mice, the estrous cycle duration is longer than in wild type mice (data are not shown). The sequence estrous versus non-estrous phases in leaner mice is also different from wild type mice. While for wild type mice, one day in estrous is followed by three or four days of non-estrous, for leaner mice the number of days in estrous (two to five) and non-estrous (two to six) are both increased significantly. Regularity of estrous cycles in leaner mice is similar with wild type mice but the number of days in estrous is significantly increased. Proliferation of uterine mucosa and muscle as well as the development of mammary glands and other secondary sexual characteristics is a consequence of an increase in the estrogen levels. Estrogen secretion is under pituitary control through FSH and LH. Perturbations in FSH and LH releases result in dysfunction in the estrous cycle. We presume that FSH and LH hormone levels are increased precociously in leaner mice and this would result in high secretion and release in estrogen levels. However, under normal circumstances the high level of estrogens determines a negative feedback regulation, which inhibits the secretion of GnRH to the pituitary and leads to reduce levels of gonadotropins. Our data suggested that the stimulation of estrogen secretion by FSH and LH occurs as expected while the inhibitory effect of estrogens on the hypothalamus and pituitary is impaired.

This occurrence in leaner mice may be due to dysfunction in hypothalamic physiology as a consequence of calcium homeostasis impairment.

The reproductive success is diminished in leaner mice relative to the reproductive success of age-matched wild type mice. Leaner mice have more difficulty in becoming pregnant than do wild type mice. This is due to several causes. First, leaner mice exhibit several movement disorders, including ataxia, and lack of coordination during timed matings. In addition, it is important to mention the possible behavior impairment due to abnormal neurons in the hypothalamus as well as due to secretion and release of GnRH II from other regions of the brain. It is possible to speculate that GnRH II is secreted in increased amounts because of calcium homeostasis impairment due to the calcium channel mutation. A better explanation would take into account a combination of neuronal factors with inputs from the hypothalamus, which triggers GnRH release, and humoral factors such as LH, FSH, activins, which respond to other stimuli combination (growth hormone, prolactin, insulin-like growth factor-1, leptin) or their specific receptors.

The number of plugs in leaner mice is significantly greater than in wild type mice. This fact is probably compensatory for the decreased reproductive success rate, perpetuation of the species being an aim for all animals. Once pregnant, leaner mice have the same behavior as wild type mice. A significant gain in body weight is observed in leaner compared with wild type mice but this fact is most probably due to the larger number of implants rather than abnormal gestation periods. It is possible that some

hormonal dysfunction also could exist in leaner mice during pregnancy. However, all the proposed pathologic mechanisms are purely speculative at this time.

The number of implants in leaner mice is greater than in age-matched wild type mice. Additionally, the number of pups in leaner mice was larger than in wild type mice. This fact argues with previous observations in favor of a compensatory mechanism to enhance the reproductive rate survival. It is therefore possible that a superovulation phenomenon might determine an overstimulation of the ovaries. In order to provide a quantitative analysis of ovarian activity, we evaluated the number of CL in each ovary for leaner and wild type mice. No significant difference was observed between ovaries in each mouse or between leaner and wild type mice when the total number of CL are compared. The only significant difference was in the number of CL in the right ovaries of leaner mice compared to the right ovaries of wild type mice. Moreover, we noticed that the largest number of CL from one ovary and the horn with the largest number of pups are situated in the uterine horn on the same side of the body. This fact can be rationalized on the basis of superovulation in one ovary and a normal ovulation in the other ovary.

The occurrence of superovulation can be due to hypersecretion of LH and FSH. However, it was not clearly understood why the right ovary was overstimulated more than the left ovary. It will be interesting to learn if there are some preferential pathways in this process.

In addition, the number of resorptions is greater in leaner mice in contrast with wild type mice. It is also possible to speculate that leaner mice presented a limited

capacity to retain the larger number of implants, despite high FSH and LH levels. Probably, the local factors and specific receptors might contribute to this process, but all presumptions are purely speculative.

The proportion of male versus female pups was not significantly different. This observation is in concordance with previous studies (Fox et al., 1984). Also, the size as well as the gross anatomy did not show any significant difference among pups for both of these categories.

One of the important results of this thesis is the level of LH and estradiol in both groups of mice. There was no significant difference in LH level in leaner mice compared with wild type mice. In addition, there was no statistically significant difference in LH levels within wild type group, although there were two different categories: mice at puberty (open vagina) and mice before puberty (closed vagina). There was a significant difference between the LH levels before the onset of puberty and after the onset of puberty in leaner mice. There were no other significant changes in LH levels because it is very difficult to catch a mouse in the "peak" just before the onset of puberty. The peak in LH lasts only a few hours. On the other hand, vaginal washes were not performed on females at the time of vaginal opening, and consequently the stage of the estrous cycle they were in at the time of blood collection was not known. We obtained a trend toward higher average LH levels in leaner mice compared with similar wild type mice but these values were not significantly different.

The level of estradiol in leaner mice was lower than in wild type mice at the days of age. This result has to be analyzed with respect to the functioning of the

hypothalamo-pituitary-gonadal axis. Lower levels of estradiol in leaner mice suggest that up regulation of GnRH is taking place, and consequently, FSH and LH should also be up regulated. Our study showed a tendency towards increased LH levels in post-pubertal leaner mice but did not in wild type mice. This may have been a result of the difficulty of collecting and timing of the process rather than the incorrectness of our hypothesis. High levels of LH and FSH determine increases in estradiol secretion. We found that a low level of estradiol, which might be due to the fact that most of estradiol was used in uterine mucosa and muscle regeneration or was bound to its receptors. Thus, the GnRH down regulation process is affected.

In this study we observed that leaner mice have a decreased body weight when compared to wild type mice. Before the onset of puberty, both types of mice had similar body weight, while after the onset of puberty the differences were more evident. These data suggest that the onset of puberty may correspond to an actual loss of body weight in leaner mice. Precocious puberty usually occurs when the body weight was increased, which result in an increased level of leptin and other anabolic factors. Further investigation is necessary to fully understand the mechanisms of precocious puberty in leaner mice and loss of body weight.

In summary, leaner mice present precocious puberty, spend more time in estrous and become pregnant less readily than wild type mice. In general, the body weight of leaner mice is lower than that of wild type mice. At postnatal P28, the body weight of leaner mice is less than that of wild type mice and decreased after the onset of puberty. The body weight of leaner mice is greater than that of wild type mice only in

pregnancy. In addition, leaner mice present a larger number of plugs, implants and pups as well as CL in the right ovary than wild type mice. Estradiol levels in leaner are decreased compared to wild type mice. In addition, it will be useful to determine the FSH levels in order to clarify this mechanism. It is also possible that other factors might be implicated in these regulations, such as leptin, CRF, IGF-1, prolactin.

Leaner mice usually die at P20 due to starvation and/or severe ataxia because they are not able to feed adequately (Dr. Abbott, personal observation). Possible explanations of this fact are due to inability of the pups to interact with their dams, behavioral abnormalities or lower concentration of prolactin hormone levels. It would be interesting to measure prolactin levels in leaner mice dams.

IGF-1 is produced in the liver and is the primary source of circulating IGF-1 (D'Ercole et al., 1984, Froesch et al., 1985). In the CNS, IGF-1 was observed in the olfactory bulbs, hippocampus, hypothalamus and cerebellum (Rotwein et al., 1988, Garcia-Segura et al., 1991). In mice with the α1A mutation in P/Q-type voltage-gated calcium channels, cerebellar Purkinje cells show a decreased level of IGF-1 (Rotwein et al., 1988) and CRF (Holder et al., 1981). Also, the body weight is in correlation with IGF-1 levels (Nahm et al., 2003). Due to this mutation, it is also likely that leaner mice have decreased levels of these factors in the hypothalamus. In addition, because IGF-1 supplementation in leaner mice partially ameliorated cerebellar dysfunction (Nahm et al., 2003), it might be possible to obtain some positive effects in the reproductive dysfunction of these mice as well. Also, these factors can contribute to dysfunction in hypothalamo-pituitary-gonadal axis regulation.

V. CONCLUSIONS

Calcium channels are widely expressed in the mammalian brain. The hypothalamus contains numerous P/Q-type voltage gated calcium channels (Gau and van den Pol, 2002). Impairment in these channels due to α1A mutations results in alterations in calcium homeostasis and consequently in the functions that are regulated by hypothalamic neurons.

The α1A-voltage-gated calcium channel mutant mice (in our case leaner mice) provide valuable experimental starting points for determining the pathogenic mechanisms and for developing therapies for neurological diseases such as ataxia (Zuchenko et al., 1997), epilepsy (Noebels et al., 1979 and Fletcher et al., 1996) and different forms of episodic migraine (Ophoff et al., 1996 and Miller, 1997). The reproductive dysfunctions associated with these neurological syndromes have not been adequately studied. The leaner mouse offers a model that can be investigated in order to elucidate the pathogenesis of reproductive dysfunction with respect to P/Q type voltage-gated calcium channels.

Abnormal function of the hypothalamus results in impairment in the hypothalamo-pituitary-gonadal axis physiology. Secretion of excessive levels of GnRH causes hypersecretion in LH and FSH levels, which consequently results in increased estradiol levels. Precocious puberty, hyperovulation as well as prolonged estrous activities in leaner mice are consistent with this hypothesis. Moreover, lower levels of

estradiol observed in leaner mice suggest that GnRH is not down regulated due to abnormalities in the neuronal hypothalamic network.

VI. FUTURE APPROACHES

1. In order to better understand the pathogenic mechanism of LH release, which did not shown in our experiment increased level of LH, the uterus of mice with open vaginas should be examined to see in which stage of the estrous cycle they were when blood collection was performed.

2. It will be interesting to learn if other dysfunction exists in regulating GnRH by testing FSH levels.

3. CRF, GH, IGF-1 or thyroid hormone levels might influence the hypothalamo-pituitary-gonadal axis alone or in correlation with other factors. Interactions among them or with the other specific substrates may explain the pathogenesis of reproductive function in these mice.

4. The hypothalamus is an important behavior center. Due to the leaner mutation, these mice might have abnormal reproductive behavior, which might explain the timed mating and/or lactation impairments. Behavioral studies might be useful to support this supposition.

REFERENCES

Abbott LC, Isaacs KR, Heckroth JA (1996a) Co-localization of tyrosine hydroxylase and Zebrin II immunoreactivities in Purkinje cells of the mutant mice, tottering and leaner/tottering. Neuroscience 71: 461-475.

Abbott LC, Rhyu IJ, Walker DB, Sotelo C (1996b) An electron microscopic study of Purkinje and parallel fibers synapses in the cerebellar molecular layer of tottering, leaner and compound heterozygous tottering/leaner mice. Society of Neuroscience Abstracts 647: 7.

Abou-Donia MB (ed) (1992) Neurotoxicology. Boca Raton, FL: CRC Press.

Airaksinen MS, Thoenen H, Meyer M (1997) Vulnerability of midbrain dopaminergic neurons in calbindin-D28k-deficient mice: lack of evidence for a neuroprotective role of endogenous calbindin in MPTP-treated and weaver mice. Eur J Neurosci 9: 120-127.

Allen E (1922) The estrous cycle of the mouse. American Journal of Anatomy 30: 297-371.

Andressen C, Blumcke I, Celio MR (1993) Calcium binding proteins: selective markers of nerve cells. Cell Tissue Research 271: 181-208.

Anthony DC, Montine TJ, Graham DC (1996) Toxic responses of the nervous system. In Casarett and Doull's Toxicology, The science of poisons, 5th ed. Klassen CD (ed). New York: McGraw-Hill. 463-486.

Arai R, Winsky L, Arai M, Jacobowitz DM (1991) Immunohistochemical localization of calretinin in the rat hindbrain. J Comp Neurol 310: 21-44.

Atchinson WD, Hare MF (1994) Mechanisms of methyl mercury-induced neurotoxicity. FASEB J 8: 622-629.

Austin MC, Schultzberg M, Abbott LC, Montpied P, Evers JR, Paul SM, Crawley JN (1992) Expression of tyrosine hydroxylase in cerebellar Purkinje neurons of the mutant tottering and leaner mouse. Brain Res Mol Brain Res 15: 227-240.

Ayata C, Shimizu-Sasamata M, Lo HE, Noebels JL, Moskowitz MA (2000) Impaired neurotransmitter release and elevated threshold for cortical spreading depression in mice with mutations in the alpha 1A subunit of P/Q type calcium channels. Neuroscience 95: 639-645.

Bacha WJ, Bacha L (1990) Female Reproductive System. In Color Atlas of Veterinary Histology, second ed., Philadelphia: Lippincott Williams & Wilkins, 191-244.

Baimbridge KG, Celio MR, Rogers JH (1992) Calcium-binding proteins in the nervous system. Trends Neuroscience 15: 303-308.

Baker J, Hardy MP, Zhou J, Bondy C, Lupu F, Ballve AR, Efstratiadis A (1996) Effects on an Igf 1 gene null mutation on mouse reproduction. Molecular Endocrinology 10: 903-918.

Belletti S, Orlandini G, Vettori MV, Mutti A (2002) Time course assessment of methylmercury effects on C6 glioma cells. Journal of Neuroscience Research 70: 703-711.

Berridge MJ (1998) Neuronal calcium signaling. Neuron 21: 13-26.

Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signaling. Nat Rev Mol Cell Biol 1:11-21.

Black JE, Isaacs KR, Anderson BJ, Alcantara AA, Greenough WT (1990) Learning causes synaptogenesis, whereas motor activity causes angiogenesis in cerebellar cortex of adult rats. Proc Nat Acad Sci USA 87: 5568-5572.

Bronson FH, Dagg CP, Snell GD (1966) Reproduction. In Biology of the Laboratory Mouse, 2nd ed., New York: McGraw-Hill, 187-204.

Burgess DL, Noebels JL (1999) Single gene defects in mice: the role of VDCC in absence models. Epilepsy Research 36: 111-122.

Burns KH, Matzuk MM (2002) Minireview: genetic models for the study of gonadotrophin actions. Endocrinology 143(8): 2823-2835.

Campbell DB, Hess EJ (1999a) L-type calcium channels contribute to the tottering mouse dystonic episodes. Molecular Pharmacology. 55(1): 23-31.

Campbell DB, North JB, Hess EJ (1999b) Tottering mouse motor dysfunction is abolished on the Purkinje cell degeneration mutant background. Experimental Neurology. 160(1): 268-78.

Carafoli E (2002) Calcium signaling: a tale for all seasons. Proc Nat Acad Sci USA 99: 1115-1122.

Catteral WA (1995) Structure and function of voltage gated ion channels. Ann Rev Biochem 64: 493-531.

Catteral WA (2000) Structure and regulation of voltage-gated calcium channels. Ann Rev Cell Dev Biol 16: 521-555.

Celio MR (1990) Calbindin D-28k and parvalbumin in the rat nervous system. Neuroscience 35:375-475.

Choie BH, Lapham LW, Amin-Zaki L, Al-Saleem T (1978) Abnormal neuronal migration, deranged cerebral cortical organization and diffuse white matter astrocytosis of human fetal brain. J Neuropathology Experimental Neurology 37: 719-733.

Clarkson TW (1983) Methylmercury toxicity to the mature and developing nervous system: possible mechanisms, in Sarkar B (ed): Biological Aspects of Metals and Metal-Related Diseases. New York: Raven Press, 183-197.

Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bougneres P, Lebouc Y, Froguel P, Guy-Grand B (1998) A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature 392: 398-401.

Colborn T, von Saal FS, Soto AM (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101(5): 378-384.

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, Caro JF (1996) Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334: 292-295.

Cooper EC, Jan LY (1999) Ion channel genes and human neurological disease: recent progress, prospects and challenges. Proc Nat Acad Sci USA 96: 4759-4766.

Cox C, Clarkson TW, Marsh DO, Amin-Zaki L, Tikriti S, Myers GG (1989) Dose-response analysis of infants prenatally exposed to methylmercury: an application of a single compartment model to single-strand hair analysis. Environ Research 49: 318- 332.

Cramer JA, Jones EE (1991) Reproductive function in epilepsy. Epilepsia. 32 Suppl 6, 19-26.

Crew FAE and Mirskaia L (1930) Mating during pregnancy in the mouse. Nature 125: 564.

D'Angelo E, Rossi P, De Filippi G, Magistretti J, Taglietti V (1994) The relationship between synaptogenesis and development of voltage-dependent currents in cerebellar granule cells in situ. J Physiology 88: 197-201.

Danilovich NA, Wernsing D, Coschicagno KT, Kopchick JJ, Bartke A (1999) Deficits in female reproductive function in GH-R-KO mice; role of IGF1. Endocrinology 140: 2637-40.

D'Ercole AJ, Stiles AD, Underwood LE (1984) Tissue concentrations of SMC: further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. Proc Nat Acad Sci USA 81: 935-939.

Dickie MM (1962) Mouse News. Neurosci Letters 27:37.

Dove LS, Abbott LC, Griffith WH (1998) Whole-cell and single channel analysis of P-type calcium currents in cerebellar Purkinje cells of leaner mutant mice. Neurosci 18: 7687-7699.

Dove LS, Nahm SS, Murchinson D, Abbott LC, Griffith WH (2000) Altered calcium homeostasis in cerebellar Purkinje cells of leaner mutant mice. Journal of Neurophysiology. 84 (1): 513-24.

Doyle J, Ren X, Lennon G, Stubbs L (1997) Mutations in the Cacn11a4 calcium channel gene are associated with seizures cerebellar degeneration, and ataxia in tottering and leaner mutant mice. Mammalian Genome 8: 113-120.

Edmonds B, Reyes R, Schwaller B, Roberts WM (2000) Calretinin modifies presynaptic calcium signaling in frog saccular hair cells. Nat Neuroscience 3: 786-790.

Engle ET, Rosasco J (1927) The age of the albino mouse at normal sexual maturity. Anat Rec 36: 383-388.

Ertel EA, Campbell KP, Harpold MM, Hoffman F, Mori Y, Perez-Reyes E, Schwartz A, Snutch TP, Tanabe T, Birnbaumer L, Tsien RW, Catteral WA (2000) Nomenclature of VGCC. Neuron 25: 533-535.

Fletcher CF, Lutz CM, O'Sullivan TN, Shaughnessy Jr., JD, Hawkes R, Frankel WN, Copeland NG, Jenkins NA (1996) Absence epilepsy in tottering mutant mice is associated with calcium channel defects. Cell 87: 607-617.

Fox J, Cohen B, Loew F (1984) Laboratory Animal Medicine, Academic Press Inc. 36-42.

Frank TC, Nunley M, Ramon R, Abbott LC (2003) Fluoro-Jade identification of cerebellar granule cell and Purkinje cell death in the α_{1A} calcium ion channel mutant mouse. Neurosci. 118:667-680.

Froesch ER, Schmidt C, Schwander J, Zapf J (1985) Actions of IGF-1. Ann Rev Physiology 47: 443-467.

Gao XB, van den Pol AN (2002) Melanin concentrating hormone depresses L-, N-, and P/Q-type voltage-dependent calcium channels in rat hypothalamic neurons. J of Physiology 542: 273-286.

Garcia-Segura LM, Perez J, Pons S, Rejas MT, Torres- Aleman I (1991) Localization of IGF1 like immunoreactivity in the developing and adult rat brain. Brain Research 560: 167- 174.

Grandjean P, Jorgensen PJ, Weihe P (1994) Human milk as a source of methylmercury exposure in infants. Environ Health Perspect 102: 74-77.

Griggs RC, Nutt JG (1995) Episodic ataxia as channelopathies. Ann Neurology 37: 607-617.

Hadley ME (2000) The endocrine hypothalamus. In Endocrinology, 5th ed, London Prentice Hall, 111-137.

Heckroth JA, Abbott LC (1994) Purkinje cell loss from alternating sagittal zones in the cerebellum of leaner mutant mice. Brain Research 658: 93-104, 1994.

Heizmann CW, Braun K (1992) Changes in calcium-binding proteins in human neurodegenerative disorders. Trends Neuroscience 15: 259-264.

Herrup K, Wilczynski SL (1982) Cerebellar cell degeneration in the leaner mutant mouse. Neuroscience 7: 2185-2196.

Hess EJ, Wilson MC (1991) Tottering and leaner mutations perturb transient developmental expression of tyrosine hydroxylase in embriologically distinct Purkinje cells. Neuron 6: 123-132.

Holder AT, Spencer EM, Preece MA (1981) Effect of bovine growth hormone and a partially pure preparation of somatomedine on various growth parameters in hypopituitary dwarf mice. J Endocrinol 89: 275-282.

Horn R (2000) A new twist in the saga of charge movement in voltage-dependent ion channels. Neuron 25: 511-514.

Isaacs KR, Abbott LC (1992) Development of paramedian lobule of the cerebellum in wild type and tottering mice. Developmental Neuroscience 14: 386-393.

Jouvenceau A, Eunson LH, Spauschus A, Ramesh V, Zuberi SM, Kullmann DM, Hanna MG (2001) Human epilepsy associated with dysfunction of the brain P/Q-type calcium channel. Lancet 358:801-807.

Kadowaki K, McGowan E, Mock G, Chanders S, Emson PC (1993) Distribution of CaBP mRNAs in rat cerebellar cortex. Neuroscience Letters 153: 80-84.

Kano M, Hashimoto K, Chen C, Abelovich A, Aiba A, Kurihara H, Wantanbe M, InoueY, Tonegawa S (1995) Impaired synapse elimination during cerebellar development in PKCγ mutant mice. Cell 83: 1223-1231.

Kempers MJ, Otten BJ (2002) Idiopathic precocious puberty versus puberty in adopted children; auxological response to gonadotrophin-releasing hormone: agonist treatment and final height. European Journal of Endocrinology 147(5): 609-16.

Lau FC (1999) Apoptosis reduced intracellular free calcium level and altered gene expression in the cerebellum of the leaner mutant mouse. Ph.D. Dissertation, Texas A&M University, College Station.

Lau FC, Abbott LC, Rhyu IJ, Kim DS, Chin H (1998) Expression of calcium channel α1A mRNA and protein in the leaner mouse cerebellum. Brain Res Mol Brain Res 59: 93-99.

Layman LC, McDonough PG (2000) Mutations of follicle stimulating hormone-beta and its receptor in human and mouse: genotype- phenotype. Molecular Cell Endocrinology 161: 9-17.

LeBel CP, Ali SF, Bondy SC (1992) Deferoxamine inhibits methylmercury-induced increases in reactive oxygen species formation in rat brain. Toxicology Applied Pharmacology 112: 161-165.

Lephart ED, Watson MA (1999) Maternal separation: hypothalamic-preoptic area and hippocampal calbindin D 28k and calretinin in male and female infantile rats. Neuroscience Letters 267: 41-44.

Levine JE (1997) New concepts of the neuroendocrine regulation of gonadotrophin surges in rats. Biology of Reproduction 56: 293-959.

Levitt P (1988) Normal pharmacological and morphometric parameters in the noradrenergic hyper innervated mutant tottering mouse. Cell Tissue Research 252: 175-180.

Lipton SA, Kater SB (1989) Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. Trends Neuroscience 12: 265-270.

Llinas RR, Sugimori M, Cherksey B (1989) Voltage-dependent calcium conductances in mammalian neurons: The P channel. Ann NY Academy Science 560: 479-489.

Long JA, McLean-Evans H (1922) The Estrous Cycle in the Rat and Associated Phenomena, volume 6, Berkeley: University of California Press.

Lorenzon NM, Lutz CM, Frankel WN, Beam KG (1998) Altered calcium channel currents in Purkinje cell of the neurological mutant mouse leaner. Journal of Neuroscience 18(12): 4482-9.

Macdowell EC, Allen E and Macdowell CG (1929) The relation of parity, age and body weight to the number of corpora lutea in mice. Anat Rec 41: 267-272.

Madsen KM, Christensen EF (1978) Effects of mercury on lysosomal protein digestion in the kidney proximal tubule. Lab Invest 38: 165- 171.

Martin J (1988) Reproductive Function. In Neuroanatomy Text and Atlas. 2nd ed, Stamford, CT: Appleton & Lange.

Martini L, Motta M, Piva F, Zanisi M (1997) LHRH, LHRF, GnRH: what controls the secretion of this hormone? Mol Psychiat 2: 373-375.

Meier H, MacPike AD (1971) Three syndromes produced by two mutant genes in the mouse: clinical, pathological and ultrastructural bases of tottering, leaner and heterozygous mice. J Hered 62:297-302.

Mirskaia L and Crew FAE (1930) Maturity in the female mouse. Proc. Roy. Soc. Edinburgh 50: 179-186.

Montpied P, Winsky L, Dailey JW, Jobe PC, Jacobowitz DM (1995) Alteration in levels of expression of brain calbindin D-28k and calretinin mRNA in genetically epilepsy-prone rats. Epilepsia 36: 911-921.

Moore J, Shang JP, Wray S (2002) *In situ* GABAergic modulation of synchronous GnRH-I neuronal activity. Neuroscience 22(20): 8932-8941.

Morell MJ (1998) Effects of epilepsy on women's reproductive health. Epilepsia 39 Suppl 8, 32-37.

Mori Y, Wakamori M, Oda S, Fletcher CF, Sekiguchi N, Mori E, Copeland NG, Jenkins NA, Matsushita K, Matsuyama Z, Imoto K (2000) Reduced voltage sensitivity of activation of P/Q-type calcium channels is associated with the ataxic mouse mutation rolling Nagoya. Neuroscience 20: 5654- 5662.

Murkinson D, Dove LS, Abbott LC and Griffith WH (2002) Homeostatic compensation maintains calcium-signaling functions in Purkinje neurons in the leaner mutant mouse. Cerebellum 1: 119-127.

Nahm SS (2002) Analysis of gene and protein expression related to cerebellar neurodegeneration in the calcium channel mutant mouse, leaner. PhD dissertation, Texas A&M University, College Station.

Nahm S-S, Frank TC, Browning M, Sepulvado J, Hiney JC, Abbott LC (2003) Insulin-like growth factor-I improves cerebellar dysfunction but does not prevent cerebellar neurodegeneration in the calcium channel mutant mouse, leaner. Neurobiology of Disease 14 (2): 157-301.

National Institute of Child Health and Human Development (NICHHD)-Reproductive Health for the 21st Century (2003). A strategic plan. Web page address: http://www.nichd.nih.gov/publications/.

Ogasawara M, Kurihara T, Hu Q, Tanabe T (2001) Characterization of acute somatosensory pain transmissions in P/Q-type calcium channels mutant mice, leaner. FEBS Letters 508:181-186.

Ophoff RA, Terwindt GM, Vergouwe MN, van Eijk R, Oefner PJ, Hoffman SM, Lamerdin JE, Mohrenweiser HW, Bulman DE, Ferrari M, Haan J, Lindhout D, van Ommen GJ, Hofker MH, Ferrari MD, Frants RR (1996) Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the calcium channel gene CACNL1A4. Cell 87: 543-552.

Ophoff RA, Terwindt GM, Frants RR, Ferrari MD (1998) P/Q-type calcium channel defects in migraine, ataxia and epilepsy. Trends Pharmacology Science 19: 121-127.

Randall A, Benham CD (1999) Recent advances in the molecular understanding of VGCC. Mol Cell Neuroscience 14: 255-272.

Randall A, Tsien RW (1995) Pharmacological dissection of multiple types of calcium channel currents in rat cerebellar granule neurons. J Neurosci 15: 2995-3012.

Rhyu IJ, Abbott LC, Walker DB, Sotelo C (1999) An ultra structural study of granule cell /Purkinje cell synapses in tottering, leaner and compound heterozygous tottering/leaner mice. Neuroscience 90(3): 717-28.

Rogers JH (1989) Immunoreactivity for calretinin and other calcium binding proteins in cerebellum. Neuroscience 31(3): 711-721.

Rogers JH, Resibois A (1992) Calretinin and calbindin D28k in rat brain: patterns of partial co-localization. Neuroscience 51(4): 843-865.

Rotwein P, Burgess SK, Milbrandt JD, Krause JE (1988) Differential expression of IGF genes in rat CNS. Proc Nat Acad Sci USA 85: 265-269.

Sager PR, Ascher M, Rodier PM (1984) Persistent differential alterations in developing cerebellar cortex of male and female mice after methylmercury exposure. Developmental Brain Research 12:1-11.

Sawada K, Fukui Y (2001) Expression of tyrosine hydroxylase in cerebellar Purkinje cells of ataxic mutant mice: its relation to the onset and or development of ataxia. Journal of Medical Investigation 48: 5-10.

Shelling AN, Burton KA, Chand AL, van EE CC, France JT, Farquhar CM, Milsom SR, Love DR, Gersak K, Aittomaki K, Winship IM (2000) Inhibin: a candidate gene for premature ovarian failure. Human Reproduction 15: 2644-2649.

Sidman RL and Green MC (1965) Catalog of the Neurological Mutants of the Mouse. Cambridge, MA: Harvard University Press.

Snell GD, Fekete E, Hummel KP, Law LW (1940) The relation of mating, ovulation and the estrous smear in mouse to time of day. Anat. Rec. 76: 39-54.

Snutch TP, Tomlinson WJ, Leonard JP, Gilbert MM (1991) Distinct calcium channels are generated by alternative spicing and are differentially expressed in the mammalian CNS. Neuron 7: 45-57.

Stahl JL, Kendall SK, Brinkmeier ML, Greco TL, Watkins-Chow DE, Campos-Barros A, Lloyd RV, Camper SA (1999) Thyroid hormone is essential for pituitary somatotropes and lactotropes. Endocrinology 140: 1884-1892.

Starr TVB, Prystay W, Snutch TP (1991) Primary structure of calcium channels that is highly expressed in the rat cerebellum. Proc. National Acad. Science USA 88: 5621-5625.

Stea A, Tomlinson WJ, Soong TW, Dubel SJ, Vincent SR and Snutch TP (1994) Localization and functional properties of rat brain alpha1A calcium channel reflect similarities to neuronal Q and P-type channels. Proc Nat Acad Sci USA 91: 10576-10580.

Stowe HD, Goyer RA (1971) The reproductive ability and progeny of F1 lead-toxic rats. Fertil Steril 22:755-760.

Swerdloff RS, Peterson M, Vera A, Batt RA, Heber D, Bray GA (1978) The hypothalamic-pituitary axis in genetically obese mice: response to LHRH. Endocrinology 98: 1359-1364.

Tanaka O, Sagagami H, Hondo H (1995) Localization of mRNAs VDCC: four subtypes of $\alpha 1$ and β subunits in developing and mature rat brain. Mol Brain Res 30: 1-16.

Thomas JA (1996) Toxic responses of the reproductive system. In Casarett and Doull's Toxicology, The Basic Science of Poisons, 5th ed. Klassen CD (ed). New York: McGraw-Hill, 547-581.

Tsien RW, Ellinor PT, Horne WA (1991) Molecular diversity of voltage-dependent calcium channels. Trends Pharmacology. 12: 349-354.

Volsen SG, Day NC, McCormack AL, Smith W, Craig PJ, Beattie R, Ince PG, Shaw PJ, Ellis SB, Gillespie A, Harpold MM, Lodge D (1995) The expression of neuronal VDCC in human cerebellum. Mol Brain Res 34: 271-282.

Walker D (1998) Quantitative light and electron microscopic study of cerebellar granule cells and parallel fiber varicosities in adult tottering, leaner and compound heterozygous mice. MS thesis, Texas A&M University, College Station.

Wheeler DB, Randall A, Sather WA, Tsien RW (1995) Neuronal calcium channels encoded by the alpha 1A subunit and their contribution in the CNS. Prog Brain Res 105: 65-78.

White RB, Eisen JA, Kasten TL, Fernald RD (1998) Second gene for GnRH in humans. Proc Nat Acad Sci USA 95: 305-309.

Williams ME, Brust PF, Feldman DH, Patthi S, Simerson S, Maroufi A, McCue AF, Velicelebi G, Ellis SB, Harpold MM (1992) Structure and functional expression of an omega-conotoxin-sensitive human N-type calcium channel. Science 257: 389-395.

Yen SC, Jaffe RB, Barbieri RL (1999) Polycystic ovary syndrome in Reproductive Endocrinology. 4th ed., Philadelphia: JB Lippincott Company, 436-478.

Yu WH, Karanth S, Mastronardi CA, Sealfon S, Dean C, Dees WL, McCann SM, Lamprey J (2002) GnRH-III acts on its putative receptor via nitric oxide to release FSH. Exp Biol and Med. 227(9): 786-793.

Zhuchenko O, Bailey J, Bonnen P, Ashizawa T, Stockton DW, Amos C, Dobyns WB, Subramony SH, Zoghbi HY, Lee CC (1997) Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. Nature Genetics 15: 62-69.

VITA

NESRIN SERPEDIN

HOME ADDRESS: 2902 Meadowbrook Court, College Station TX 77845

OBJECTIVE: Enter into a residency program in family practice medicine or obstetrics.

QUALIFICATIONS: Up to present, my experience includes nine years of extensive training in medical sciences: six-year long MD-program, two-year long training program as a physician in the ER, and one-year long residency program. Also, my experience includes two years of research and training in toxicology.

EDUCATION:

2001-2003: Master of Science in Toxicology, Texas A&M University, College Station.

1999-2000: Residency Program in Obstetrics and Gynecology, Selcuk University, Konya, Turkey.

1991-1997: M.D. (Medical Doctor), University of Medicine and Pharmacy ``Carol Davila'', Bucharest, Romania. Thesis: ``New Therapeutical Approaches in Stomach Cancer". Supervisor: Dr. Octavian Unc. GPA=9.0/10.00

EMPLOYMENT:

1999-2000: Physician in Residency Program and Research Assistant, Selcuk University, Konya, Turkey. Performed training in Obstetrics-Gynecology.

1997-1999: M.D. Training, E.R. Clinical Hospital of Constanta, Constanta, Romania. Performed training for first medical aid.