EXPRESSION OF Npc1 IN GLIAL CELLS CORRECTS STERILITY IN Npc1+/− MICE:

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A Thesis Submitted to The Honors College
In Partial Fulfillment of the Bachelor’s degree
With Honors in
Molecular and Cellular Biology
THE UNIVERSITY OF ARIZONA
May 2009

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Niemann-Pick type C1 (NPC) disease is an autosomal recessive neurodegenerative disorder. One feature of the mouse model of NPC1 is their infertility. We have made transgenic mice which express the Npc1 protein exclusively in fibrillary astrocytes using the glial fibrillary acidic protein (GFAP) promoter. This selective expression of Npc1 corrects sterility in GFAP-Npc1<sup>E</sup>, Npc1<sup>−/−</sup> mice. Counts of acidophils in the pituitary of GFAP-Npc1<sup>E</sup>, Npc1<sup>−/−</sup> mice as compared to Npc1<sup>−/−</sup> mice, and measurements of dopamine-receptor 2 mRNA in the pituitary suggest mechanisms for fertility enhancement. We conclude that the correction of sterility in GFAP-Npc1<sup>E</sup>, Npc1<sup>−/−</sup> mice is a result of restoring hypothalamic control of the pituitary.
INTRODUCTION

Niemann Pick Type C1 disease (NPC) is an autosomal recessive genetic disorder that has an incidence of about 1 in 100,000. It has been shown that the NPC1 protein's function is to transport cholesterol derived from low-density lipoproteins between intracellular compartments; without NPC1, unesterified cholesterol is stored in lysosomes (Pentchev et al., 1985; Patterson et al., 2001). The mouse model has been the primary model of NPC1 as the neuropathology of the disease is very similar. In this model of NPC1, as in humans, cholesterol accumulates in every part of the body, including in neuronal cell bodies, but the total neuronal cholesterol content is normal because of decreased amounts in axons (Karten et al., 2002).

One feature of Npc1+/- mice is their infertility. Fertility is dependent on the generation and the continuous production of sex hormones, which are essential for physiological regulation. Cholesterol serves as a precursor for steroid hormone synthesis. Cholesterol can be obtained in three different ways: de novo synthesis, through endocytosis of low-density lipoproteins (LDL), and through the selective uptake of cholesterol through high-density lipoproteins (Gwynne and Strauss, 1982). The mutation in Npc1 causes cholesterol derived from low-density lipoproteins to build up in the cell, but the other two sources of cholesterol also provide the sterol needed for hormone synthesis. While a decrease in testosterone levels has been reported (Roff et al., 1993), lack of Npc1 was not found to decrease available levels of cholesterol substrate for steroid hormone synthesis (Xie et al., 2006).

There have been multiple investigations as to the cause of female infertility in the Npc1+/- mouse. It has been found that treatment with gonadotrophs induces ovulation and restores the function of steroidogenic proteins in the BALB/c Npc1+/- mouse (Gevry et al., 2006). This group reported a marked increase in the dopamine-D2 receptor in the pituitaries of Npc1+/- mice. They concluded that the Npc1 mutation affects the ovarian-pituitary-hypothalamic feedback loop by
interfering with estrogen production and demonstrated that chronic treatment with estrogen in the Npc1" mouse restored prolactin expression (Gevry et al., 2006).

We find that fertility is corrected in the Npc1" mouse solely with changes in the nervous system (NS). Our group, (Zhang et al., 2008) found that controlled expression of the NPC1 protein with directed-expression of Npc1 in astrocytes using the glial fibrillary acidic protein (GFAP) promoter lowered cholesterol storage in neurons and corrected sterility (Zhang et al., 2008). We have now studied how fertility is corrected when Npc1 is only expressed in fibrillary astrocytes. A reduction in prolactin, which normally induces expression of LH receptors in follicular cells in the ovary (Gafvels et al., 1992), has been shown to lead to sterility in Npc1" mice (Gevry et al., 2006). If mice with only CNS expression of Npc1 are fertile, as with the GFAP-Npc1E, Npc1" mice, then their regulation of pituitary hormone expression must be corrected.

The hypothalamic-pituitarian-ovarian feedback loop which is involved in the estrous cycle is of importance since it regulates FSH and LH production and release. If negative feedback from the ovary was altered, and the pituitary did not have a signal to inhibit FSH or LH release, it would potentially have a constantly high level of these hormones, which was reported (Gevry et al., 2006). Also, low ovarian follicle counts lead to a high amount of FSH which has been reported as present in the serum of Npc1" mice. We hypothesize that fertility is corrected by re-establishment of hypothalamic control of the pituitary. We measured LH and FSH levels and acidophils in the pituitary were counted. We also compared levels of mRNA for prolactin and dopamine 2 receptor in the pituitaries of Npc1"", Npc1", and GFAP-Npc1E, Npc1" mice.
RESULTS

As previously reported (Zhang et al., 2008), female and male GFAP-Npc1E, Npc1- mice were kept in the same cage and one female mouse gave birth to a litter of mice. As predicted, all of the mice were Npc1+. When typed for the GFAP-Npc1E transgene, 8/10 mice had the GFAP-Npc1E transgene. It was predicted that 25% of the mice would have two copies of the GFAP-Npc1E transgene which was analyzed using real-time PCR: 5/10 mice had only one copy of the GFAP-Npc1E transgene, and 3/10 had two copies (Zhang et al., 2008).

Using the mice that had two copies of the transgene, a GFAP-Npc1E, Npc1+ x GFAP-Npc1E, Npc1+ line was started, producing all Npc1+ mice that would either have one or two copies of GFAPE. These mice only gave birth to small litters and they usually only could have one litter before their health deteriorated beyond being capable of raising a litter. The average litter size for this line was 5.25 +/− 3 pups with an average of 1 litter per mating pair. The average litter size for the Npc1+ × Npc1+× cross is 6.4 +/− 2.4 pups with a mating life of a female mouse of eight months before litter sizes markedly decrease whereas the GFAP-Npc1E, Npc1+ x GFAP-Npc1E, Npc1+ line was only able to birth healthy litters up to about age 140 days before the onset of symptoms was too severe to raise litters. This age is not representative of the lifespan of the GFAP-Npc1E, Npc1+ line, however. This strain of mice live an average of 172 +/− 23.8 days and the 2x GFAP-Npc1E, Npc1+ line live over 230 days (Zhang et al., 2008). This difference in litter size and mating lifespan of the animal shows that fertility is corrected but only for a limited period of time.

Blood-plasma levels of LH and FSH were compared in Npc1+/+, Npc1− and GFAP-Npc1E, Npc1− mice (Table 1). FSH tended to be lower in Npc1− mice and GFAP-Npc1E, Npc1− were intermediate but all groups were not statistically different. There was a trend of higher LH in the Npc1− mice, but again, the 3 groups were not significantly different.
The pituitary was sectioned ventrally to count acidophils (Fig 1). Enlarged nuclei and vacuoles were noted in the Npc1<sup>−/−</sup> acidophils. Acidophils were counted for the three different types of mice of interest: Npc1<sup>+/+</sup>, Npc1<sup>−/+</sup>, and GFAP-Npc1<sup>E</sup>, Npc1<sup>−/−</sup>. One microscopic field per section was counted at 160x for acidophils (Fig 2). There was a statistically significant decrease in acidophils in the Npc1<sup>−/+</sup> mice which was partially corrected in the GFAP-Npc1<sup>E</sup>, Npc1<sup>−/−</sup> mice.

There was a trend to an increase in message for the Dopamine 2 Receptor (D2R) in the GFAP-Npc1<sup>E</sup>, Npc1<sup>−/−</sup> mice as compared to the Npc1<sup>−/+</sup> mice (Fig. 3). The average amount of D2R mRNA in Npc1<sup>−/+</sup> was 0.0107 +/- 0.0027 relative to GAPDH message. 0.00822 +/- 0.0067 in Npc1<sup>−/+</sup> mice and 0.01022 +/- 0.004 in GFAP-Npc1<sup>E</sup>, Npc1<sup>−/−</sup> mice.

Levels of prolactin message in the pituitary, however, had a wide range between the three different genotypes. The average amount of prolactin mRNA in Npc1<sup>−/+</sup> was 0.04 +/- 0.003 relative to GAPDH message, 0.00084 +/- 0.0015 in Npc1<sup>−/+</sup> mice and 0.000315 +/- 0.0032 in GFAP-Npc1<sup>E</sup>, Npc1<sup>−/−</sup> mice. Thus, we did not find a correction of prolactin when measuring mRNA levels.
DISCUSSION

There have been multiple studies on the causes of infertility in Npc1−/− mice, both in males and females. In males, Roff, et al. (1993) found decreased levels of testosterone which they attributed to a failure of synthesis in Leydig cells. They concluded that the availability of cholesterol for steroid synthesis in endocrine tissues was deficient. It is not, however, since alternative cholesterol-synthesis pathways, such as de novo synthesis and scavenger receptor class B type I-mediated uptake of HDL cholesteryl ester provide normal amounts (Xie et al., 2006). These pathways are not affected by a lack of Npc1, which inhibits the movement of LDL-derived cholesterol from the endosome to the endoplasmic reticulum (reviewed in Garver and Heidenreich, 2002; Strauss et al., 2002).

Erickson, et al. (2002) found that male sterility in Npc1−/− mice could be corrected merely by changing the genetic background while Fan, et al. (2006) found decreased binding of sperm to the zona pellucida and decreased proteolytic processing of cyritestin. For females, Erickson, et al. (2002) found that creating Npc1−/− compound homozygous recessive with the mdr1a−/− (multiple drug resistance 1a knockout) mice restored fertility. This was interpreted as increasing cholesterol movement to the endoplasmic reticulum, but pregnant mare serum (PMS), human chorionic gonadotrophin (hCG) pretreated Npc1−/− mice showed normal numbers of corpora lutea and levels of progesterone (Erickson et al., 2002). As mentioned, Gevry, et al. (2006) concluded that decreased estrogen synthesis was the primary cause of infertility.

Our data support the hypothesis that the cause of infertility in Npc1−/− mice is a result of loss of hypothalamic control of the pituitary. Re-establishment of hypothalamic control of the pituitary by expression of Npc1 using the GFAP promoter led to fertility in Npc1−/− mice. Although the litters were somewhat smaller and the fertile life-span of the mice was greatly reduced, this is probably due to the continuing neurodegeneration. It is possible that these mice did have more litters but were too sick to care for their young.
Gevry et al. (2006) found LH and FSH to be elevated in the \( N_{pc1}^{+/-} \) mice which they believed to be secondary to decreased steroidogenesis in the corpora lutea (Christenson and Devoto, 2003). We found levels of LH and FSH which were not significantly different between \( N_{pc1}^{+/-} \), \( N_{pc1}^{-/-} \) and GFAP-\( N_{pc1}^{E} \), \( N_{pc1}^{-/-} \) mice. Gevry et al. did not report at what stage of the estrous cycle blood was collected for measurement of hormones. In the present study, blood was collected during diestrus, a point which circulating levels of LH and FSH are low. Thus, the differences between the two studies could relate to differences in timing of sample collection. The lower values of estrogen reported in the \( N_{pc1}^{+/-} \) mice (Gevry et al., 2006) may be due to the hypothalamus not releasing enough Gonadotropin-Releasing Hormone. Alternatively, trophic effects on the number of acidophils may be crucial. We show that acidophils were increased in the pituitaries of the GFAP-\( N_{pc1}^{E} \), \( N_{pc1}^{+/-} \) mice as compared to the \( N_{pc1}^{+/-} \) mice. Acidophils are involved in sex hormone secretion. An increased number of these cells can increase the amount of circulating prolactin in the anterior pituitary, which contributes to follicular production, and increases estrogen release in the ovaries.

Prolactin is well-known to be involved in expression of LH receptors in ovarian follicle cells (Gafvels et al., 1992; Bole-Feyssot et al., 1998). The results of the quantitative-PCR indicate that prolactin expression varies greatly in the different genotypes of mice that were studied but tended to be lower in the \( N_{pc1}^{+/-} \), than in the \( N_{pc1}^{-/-} \) mice and it was not affected by the transgene. The large range of data could be explained by the estrous cycle of mice. Prolactin remains fairly constant throughout the estrous cycle but spikes during the afternoon of proestrous (Smith et al., 1975). The age at times of dissections of the mice were kept constant, however their estrous cycles were not being monitored for the mRNA studies. This could explain some extremely high differences that were seen in the quantitative-PCR. When \( N_{pc1} \) is expressed in glial cells, there is clearly more control over the expression of prolactin as indicated in the restoration of Dopamine 2 receptor levels, which could cause an increase in response to LH and increase estrogen synthesis in the ovaries.
There are many receptors involved in the uptake of dopamine in the pituitary. The D2 receptor, however, when knocked-out, has been shown to greatly reduce fertility (Chen and Zhuang, 2003). Gevry et al. (2006) had found elevated levels of the long and short forms of the D2 receptor in Npc1<sup>i</sup> mice using a semi-quantitative method while we found mildly decreased levels of total receptor using a quantitative method. The expression of the Dopamine 2 receptor message was found to be restored in the GFAP-Npc1<sup>E</sup>, Npc1<sup>−/−</sup> fertile mice. Sustaining normal levels of the D2 receptor has been seen as essential in the regulation of circulating estrogen (Saiardi et al., 1997).

The death of neurons is a key feature of the Niemann-Pick Type C disease. Restoring the Npc1 protein in glia has significant effect on the lifespan and the fertility of which mouse model. The hypothalamus is the source of dopamine that is necessary for regulating prolactin secretion in the mouse (Ben-Jonathan and Hnasko, 2001). The delayed onset of the NPC disease in the GFAP-Npc1<sup>E</sup>, Npc1<sup>−/−</sup> mice, as well as the reversal of sterility, shows that hypothalamic control of the pituitary contributes to fertility as well as to a less severe onset of the disease.
MATERIALS AND METHODS

Animals

The Npc1<sup>−/−</sup> mice are on the BALB/cJ background and Npc1<sup>+/−</sup> were bred by mating Npc1<sup>−/−</sup> mice. Animals were housed in the University of Arizona Animal Care Facility and maintained on a diet containing 6% fat with water ad libitum. Animals were weaned at 21 days of age, ear-tagged, and tail-tipped for genotyping. All animal usage was according to protocols approved by the University of Arizona's Institutional Animal Care and Use Committee. Homozygous Npc1<sup>−/−</sup> mice were identified using PCR analysis of DNA obtained with the following primers: 5' – GGTGCTGGACAGCCAAGTA – 3' and 5' – GACCAACTCTGAAAGGCTITG – 3'. Npc1 was expressed exclusively in astrocytes using the promoter for the glial-acidic fibrillary protein (GFAP) was used (Zhang et al., 2008). Positive transgenics and their progeny were identified by PCR using a GFAP/C-3123 boundary specific forward primer and an Npc1 specific reverse primer: 5' – ACTAGCGGGCCGCGGTACAATTCCG – 3' and 5' – TACCCTGCAGCAGGGGATCCAGAC – 3'.

Determination of LH and FSH in blood-plasma Levels

Blood was collected from mice during diestrus into an Eppendorf tube containing EDTA until a minimum of 150 μl of blood was obtained. Plasma was obtained by centrifugation for 2 minutes at 8000 rpm. The supernatant was removed from the red blood cells and stored at -80 degrees C. Mouse LH and FSH cold reference standards were used (LH, AFP- 5306A; FSH, AFP- 5308D) for the Radio-Immuno Assays of plasma. Primary antibodies were from the NIDDK (LH, anti-rLH-S-11 [AFP C697071P]; FSH, anti-rFSH-S-11 [AFP CO97 2881]) from A. F. Parlow. Goat anti-rabbit whole serum was used as the secondary antibody (from Dr. Cheryl Dyer, Northern Arizona University). Radio-labeled [I<sup>125</sup>] hormone (Rat LH NIDDK rLH-1-10 AFP-
115368, or Rat FSH NIDDK rFSH-1-10, AFP-4251A, also from A.F. Parlow) was added to unknowns and standards. Next, primary antibody was added, and the tubes were incubated overnight at room temperature. Secondary antibody and 2% normal rabbit serum were added, incubated for 5' and the tubes spun at 70,000 g. The supernatant was removed and the pellet counted in a gamma counter. Data was then processed with the Assay Zap calculation program.

**Histological Examination of Acidophils**

Pituitaries were harvested from 3 Npc11/ females, 3 Npc11/ females, and 3 GFAP-Npc1E, Npc11/ females. Pituitaries were fixed in Bouins fixative for 1 hour and then the fixative was replaced with 70% ethanol. Pituitaries were paraffin-embedded and 5 micrometer sections were cut. These were stained using the Herlant-Blue stain for acidophils (Herlant and Pasteels, 1967). Acidophils were examined for any aberrant morphology. They were counted in various fields at 400x. The number of acidophils per unit area was averaged per mouse and the three genotypes of mice were compared using student’s t-Test.

**RNA Isolation and Analyses**

Pituitaries were harvested and stored in RNA-later solution (QIAGEN), and total RNA was extracted obtained using the QIAGEN total RNA extraction kit according to the manufacturer’s protocol. Up to 500 ng of total RNA was reverse transcribed using Oligo dT primers and 1 unit per reaction of SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. Standard PCR of the cDNA was used to determine if any DNA contamination occurred during the reverse transcription and to determine optimal annealing temperatures. Real-time PCR quantified mRNA expression as compared to a GAPDH control (Ponchel et al., 2004) using the following primers: for prolactin, 5' – AGCCCCCGAATACATCCTAT – 3' and 5' – TGGTGCACAAACAGACAGATT – 3'; for dopamine
D2 receptor, 5' – GGATTCACTGTGACATCTTTG – 3' and 5' – GAGATGGTGAAGGACAGGAC – 3'; and for GAPDH: 5' – GTCCTCTGGGTGGCAGTGAT – 3' AND 5' – TTGTGATGGGTGTAACCAC. Expression was analyzed using the $2^{-\Delta\Delta CT}$ (deltadelta CT) method of comparing fold difference to the endogenous control (Livak and Schmittgen, 2001).
ACKNOWLEDGEMENTS

We thank Jessica McVey for administrative support and Patricia Hoyer for comments on the manuscript. This work was supported by NIH (5R01 ED000343-5) and the Holsclaw Family Professorship in Human Genetics and Inherited Diseases.
REFERENCES


FIGURE LEGENDS

Figure 1. Sectioned pituitaries at 100X and 400X. Note the enlarged nuclei and vacuoles in the Npc1" mice versus in the Npc1" and GFAP-Npc1E, Npc1" mice (400X). The decreased number of acidophils can also be seen in these mice (100X).

Figure 2. Counts of acidophils in the three genotypes.

Figure 3. Dopamine 2 receptor expression in GFAP-Npc1E, Npc1" mice compared to Npc1" and Npc1" mice.
Table 1. Levels of LH and FSH in Npc1<sup>−/+</sup> and GFAP-Npc1<sup>E</sup>, Npc1<sup>−/+</sup> mice

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<th>FSH</th>
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<tr>
<td>Npc1&lt;sup&gt;−/+&lt;/sup&gt;</td>
<td>(5) 5.342 ± 1.77&lt;sup&gt;*&lt;/sup&gt;</td>
<td>(5) 8.80 ± 0.67</td>
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<tr>
<td>Npc1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(4) 2.46 ± 0.34</td>
<td>(4) 11.19 ± 1.77</td>
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<tr>
<td>GFAP-Npc1&lt;sup&gt;E&lt;/sup&gt;, Npc1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(5) 3.57 ± 0.49</td>
<td>(5) 8.33 ± 1.12</td>
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<sup>*</sup> (n) mean ± std error