Research Report

Initial characterization of mice null for Lphn3, a gene implicated in ADHD and addiction

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ABSTRACT

The LPHN3 gene has been associated with both attention deficit-hyperactivity disorder (ADHD) and addiction, suggesting that it may play a role in the etiology of these disorders. Unfortunately, almost nothing is known about the normal functions of this gene, which has hampered understanding of its potential pathogenic role. To begin to characterize such normal functions, we utilized a gene-trap embryonic stem cell line to generate mice mutant for the Lphn3 gene. We evaluated differential gene expression in whole mouse brain between mutant and wild type male littermates at postnatal day 0 using TaqMan gene expression assays. Most notably, we found changes in dopamine and serotonin receptors and transporters (Dat1, Drd4, 5Htt, 5Ht2a), changes in neurotransmitter metabolism genes (Th, Gad1), as well as changes in neural developmental genes (Nurr, Ncam). When mice were examined at 4–6 weeks of age, null mutants showed increased levels of dopamine and serotonin in the dorsal striatum. Finally, null mutant mice had a hyperactive phenotype in the open field test, independent of sex, and were more sensitive to the locomotor stimulant effects of cocaine. Considered together, these results suggest that Lphn3 plays a role in development and/or regulation of monoamine signaling. Given the central role for monoamines in ADHD and addiction, it seems likely that the influence of LPHN3 genotype on these disorders is mediated through alterations in monoamine signaling.

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Keywords: Lphn3, Latrophilin, Addiction, Attention deficit-hyperactivity disorder

1. Introduction

Addiction and ADHD are highly prevalent and costly to our society, but there is a current lack of reliable diagnostic or pharmacogenetic biomarkers for either disorder, and therapeutic modalities need significant improvement. Recently LPHN3 has been identified by linkage and association studies performed in separate cohorts by separate research groups as

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a prime candidate for both addictive phenotypes and ADHD. Vulnerability to addiction is a complex trait with strong genetic influences that are largely shared by abusers of different addictive substances. Initially, alcohol abuse was linked to Chromosome 4 less than 300 kb 5′ of the LPHN3 gene (Long et al., 1998). Another independent study identified marker D4S244 (also on Chromosome 4) linked to alcohol dependence (Reich et al., 1998; Uhl, 2004a,b). Additional evidence was supplied by a third study that utilized whole genome association analysis to identify SNPs with significant allele frequency differences between abusers of illegal substances and control populations of both European-American and African-American ethnicities (Liu et al., 2006). This research group replicated these previously-identified LPHN3 SNPs as well as additional LPHN3 SNPs in several different populations including polysubstance abusers (Bergen et al., 1999), alcohol dependent samples (Johnson et al., 2006; Liu et al., 2006), and methamphetamine dependent samples from Japan and Taiwan (Uhl et al., 2008a,b). Finally, several groups have performed genome wide linkage and association studies for ADHD (reviewed by Franke et al., 2009), and LPHN3 has been recently associated with attention deficit-hyperactivity disorder (ADHD) (Arcos-Burgos et al., 2010). This group has also found that ADHD and Chromosome 4 loci (in the LPHN3 area) are linked to addiction (Jain et al., 2007).

LPHN3 (latrophilin 3) is a member of the latrophilin subfamily of secretin G protein coupled receptors (GPCR) (Matsushita et al., 1999). LPHN1 and LPHN2 are receptors for latrotoxin (black widow spider venom) (Ichtchenko et al., 1999), which interacts with neuronal GPCRs to stimulate exocytosis of neurotransmitters (Krasnoperov et al., 1997; Lelianova et al., 1997). Structurally, LPHNs appear to be chimeras between cell surface receptors and intracellular signaling molecules (Krasnoperov et al., 2002); however, the endogenous ligands for all three LPHNs are unknown, and all functional studies of LPHNs to date have relied on the interaction of latrotoxin with LPHN1 and LPHN2. Currently, no functional polymorphisms within LPHN3 have been identified that might explain disease susceptibility. A recent mutational analysis of the entire coding region of LPHN3 in a cohort of 139 ADHD subjects and 52 controls was conducted in an attempt to identify susceptibility or protective haplotype alleles. Although some novel polymorphisms were identified, none were associated with obviously significant coding region changes, or canonical splice site alterations (Domené et al., 2011). This suggests that non-coding variations determining the quantity and/or quality of LPHN3 isoforms are likely contributors to ADHD, but the mechanisms by which LPHN3 might influence ADHD susceptibility (or, indeed, any LPHN3 mechanisms at all), are essentially unknown. To address these significant data gaps in our knowledge of the functions of this potentially very important gene, we generated a Lphn3 null mutant mouse, in the hope that characterization of this mutant would enhance our understanding of the pathophysiology of both ADHD and addiction. Here we report preliminary cellular, neurochemical, and behavioral characteristics of these mice.

2. Results

2.1. Lphn3 mutant mouse generation

We utilized gene trap clone FHCRC-GT-S17-5H1 generated with the ROSAFARY vector (Chen et al., 2004) to generate mutant mice; 129S4/SvJae and C57 mix. A schematic representation of this clone (Fig. 1A) depicts both the transcript structure and the location of the trap based on RACE sequence. We generated genomic sequence confirming an insertion which is predicted to interrupt the mucin stalk domain in the middle of the gene. Such a mutation is predicted to obliterate any intracellular signaling from Lphn3, if not all functions. We injected the clone into blastocysts and produced germline mutations. The nulls were viable and appeared physically indistinguishable from

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**Fig. 1** – Lphn3 gene-trap and RT-PCR. A: Screen shot of mouse Lphn3 genomic region and NCBI transcript Lphn3 showing exon and intron structure. Below the transcript line is the raw 3′ RACE sequence alignment from the gene-trapped ES cell clone used to generate the Lphn3 deficient mice. B: RT-PCR verifying knockout of Lphn3 mRNA in null mice.
wild type littersmates, with no evident mortality or morbidity (up to 5 months). Body weight data from a subset of the mice (obtained at 1 and 2, and in some cases 3 months of age from the same mice: ns=14 (10) null, 20 (13) heterozygous, and 14 (5) wild type; numbers in parentheses indicate ns for which body weights were recorded at 3 months) showed no differences between genotypes at any age (one-factor ANOVAs, Fs<1.42, ps>.25). Normal sex ratios and Mendelian relative genotype ratios of 1:2:1 were observed after heterozygous crossings (N=201 pups). Disruption of the Lphn3 transcript was verified with RT-PCR probes generated to the gene-trap insertion site (Fig. 1B).

2.2. Gene expression analysis

Because both ADHD and addiction have been linked to altered monoaminergic signaling, we examined the expression of genes involved in differentiation, survival, and function of dopaminergic and serotonergic neurons and neurotransmitter function in experimentally naïve wild type and Lphn3 null mice (n=6 mice/group at P0). ADHD candidate genes were included as well, as were loci previously suspected to interact with Lphn3. Specifically, we looked for changes in expression levels of the genes for Nurr1, Ncam, TH, DAT, DA receptors (Drd2, 4, and 5), serotonin transporter (5-Htt), serotonin receptor 2A, and Gad67 by Q-RT-PCR. Dopamine transporter and receptors are key for both dopaminergic function and as ADHD candidate genes. Dat1 and Drd4 are the most highly replicated ADHD candidate genes. Drd2 has been implicated in both alcoholism and ADHD. Drd5 is also an ADHD candidate gene. Th is required for synthesis of DA. Nurr1 plays a key role in the maintenance of the dopaminergic system of the brain and is essential for generation of midbrain dopamine cells during embryonic development.

We observed relative statistically significant differential expression between wild type and null mutants based on Lphn3 genotype for: 5-Htt, 5-Ht2a, Dat1, Drd4, Ncam, Nurr1, and TH (Fig. 2 and Table 1) (all p<0.01). Verification of RT and Q-PCR results were obtained after repeating the RT and gene expression assays a second time on the same RNA samples.

2.3. Neurochemical analysis

In a separate experiment involving an experimentally naïve cohort of mice (n=6 null, 13 heterozygotes, and 10 wild type), we evaluated levels of dopamine and serotonin in the dorsal striatum at 4–6 weeks of age. One-factor ANOVA revealed main effects of genotype for both dopamine (F(2, 26)=3.36, p=.05) and serotonin (F(2, 25)=5.05, p<.05). Post-hoc comparisons (Bonferroni contrasts) showed that Lphn3 null mice had significantly higher DA and 5-HT levels than WT mice (p<.05), but no other differences between groups were significant (Fig. 3).

2.4. Locomotor activity

We evaluated male and female mice (n=15 null, 19 heterozygous, and 14 wild type) for activity levels in an open field arena at 4, 8, and 12 weeks of age. A multi-factor ANOVA (genotype×sex×age) was used to compare data from four activity measures (horizontal activity, vertical activity, stereotypy activity, and center time). On the horizontal activity measure, there was a main effect of genotype (F(2, 42)=14.92, p<.01), but no main effects or interactions involving age or sex (ps>.20) (Fig. 4A). Post-hoc comparisons revealed that null mice showed significantly more horizontal activity than both heterozygous and wild type mice (ps<.01), but that heterozygous and wild type mice did not differ. On the vertical activity measure, there was a main effect of age (F(2, 84)=8.74, p<.01), but no other main effects or interactions involving sex or genotype (data not shown, ps>.11). On the stereotypy measure, there was also a main effect of age (F(2, 84)=4.36, p<.05), as well as a main effect of genotype (F(2, 42)=7.01, p<.01), but no other significant effects (ps>.17). Post-hoc comparisons between genotype revealed that, as with the horizontal activity measure, null mice showed significantly

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**Table 1 – Q-RT-PCR results comparing fold changes in expression of specific genes between Lphn3 wildtype and null mice and their statistical significance levels.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold diff</th>
<th>t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mm5-Htt</td>
<td>2.192468</td>
<td>0.000725</td>
</tr>
<tr>
<td>MmDrd2</td>
<td>0.957235</td>
<td>0.43198</td>
</tr>
<tr>
<td>MmDrd5</td>
<td>1.374059</td>
<td>0.158794</td>
</tr>
<tr>
<td>Mm5-Ht2a</td>
<td>2.544708</td>
<td>1.94E-05</td>
</tr>
<tr>
<td>MmDat1</td>
<td>2.749919</td>
<td>4.79E-05</td>
</tr>
<tr>
<td>MmDrd4</td>
<td>2.921976</td>
<td>6.48E-06</td>
</tr>
<tr>
<td>MmGad</td>
<td>3.150445</td>
<td>0.003392</td>
</tr>
<tr>
<td>MmNcam</td>
<td>2.937998</td>
<td>0.001139</td>
</tr>
<tr>
<td>MmNurr</td>
<td>2.937998</td>
<td>0.001139</td>
</tr>
<tr>
<td>MmTh</td>
<td>3.366177</td>
<td>0.001188</td>
</tr>
</tbody>
</table>

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![Differential Gene Expression](image-url)
more stereotypy activity than both heterozygous and wild type mice (p < .05), but that heterozygous and wild type mice did not differ (data not shown). On the time in center measure, there was a significant interaction between age and sex (F(2, 84)=3.64, p<.05) such that males spent more time in the center of the open field than females at 8 and 12 weeks of age. The main effect of genotype approached statistical significance (p=.07, with null mice showing greater time in center than wild type), but there were no other significant main effects or interactions (data not shown, ps>.16).

2.5. Locomotor response to cocaine

To determine whether Lphn3 null mice were differentially sensitive to the locomotor stimulant properties of psychostimulants compared to wild type littermates, we evaluated the effects of acute administration of two doses of cocaine using a within-subjects design (n=14 null and 13 wild type mice that had been previously tested for locomotor activity). Analyses were focused on the horizontal activity measure, as the effects of genotype were most robust on this measure. As in the assessment of locomotor activity, null mice displayed significantly greater horizontal activity than wild type mice during the pre-drug habituation period (two-factor ANOVA (genotype × time bin), main effect of genotype, F(1,23)=12.32, p<.01, data not shown). To account for the elevated baseline activity levels in null mutant mice, we subtracted baseline activity counts (in the final time bin of the habituation period) from the counts in each time bin following drug administration. A three-factor ANOVA (drug × genotype × time bin) conducted on these normalized data revealed a main effect of drug (F(2, 46)=6.81, p<.01), such that cocaine administration elevated locomotor activity, and bin (F(11, 253)=11.79, p<.01), such that across groups, activity decreased over the course of the test session. Importantly, there was a 3-way interaction between drug, genotype, and bin (F(22, 506)=1.64, p<.05), such that Lphn3 null mice showed a greater increase in activity in response to the 20 mg/kg dose of cocaine than their wild type cohorts (Fig. 4B). There were no significant main effects or interactions involving sex.

3. Discussion

The LPHN3 gene has been linked to both ADHD and addictive disorders, but the basic functions of the protein product of this gene are almost entirely unknown. The experiments presented here represent an initial attempt at characterization of this gene’s function using mice with a null mutation in the Lphn3 gene (hence, we are more literally characterizing the functional consequences of deletion of Lphn3 in these mice). The resulting alterations in gene expression, neurochemistry, and behavior suggest that Lphn3 plays a role in development and/or regulation of monoaminergic signaling. This suggests in turn that the link between LPHN3 and ADHD/addiction in humans may be mediated by altered monoamine signaling.

Genes in the Lphn family have been implicated in neurotransmitter release due to their affinity for latrotoxin (Sudhof, 2001), but unlike the other members of this family (Lphn1 and 2), Lphn3 does not bind latrotoxin. To date, altered expression levels of Lphn3 have been seen after brain ischemia (Bin Sun et al., 2002) and during development of the adrenal gland (Xing et al., 2009). Lphn3 has also been implicated as a tumor suppressor based on
identified copy number losses and various protein-altering missense mutations in human cancers, particularly lung cancer (Kan et al., 2010). The spatial and temporal expression of LPHN3 supports its role in the pathogenesis of ADHD. In humans, LPHN3 is temporally and spatially dynamic such that its expression decreases as the brain matures, and ultimately remains detectable primarily in regions of the brain independently implicated in ADHD pathogenesis (Arcos-Burgos et al., 2010). We have also identified Lphn3 expression in the developing embryonic mouse brain, as well as in the adult mouse hippocampus and thalamus (data not shown). It is also of note that the dosage of the LPHN3 susceptibility haplotype in humans varies inversely with the ratio of NAA/Cr in the right medial and lateral thalamus (Arcos-Burgos et al., 2010). Further, The NAA/Cr ratio increases monotonically in the right medial and lateral thalamus in relation to the number of copies of the protective haplotype. The ratio of NAA/Cr is a measure of the neuronal number thought to be abnormal in ADHD. Finally, the same LPHN3 variant associated with susceptibility to ADHD is also associated with response to stimulant medication (Arcos-Burgos et al., 2010). Unfortunately, the published literature does not indicate any mechanism of action for LPHN3 nor a potential role in behavior.

The alterations observed in the Lphn3 null mutant mice are consistent with a role for this gene in monoaminergic signaling. Newborn null mutant mice showed elevated whole brain expression of several genes involved in dopamine (Dat1, Drd4, Th) and serotonin (5-Htt, 5-H2a) signaling, as well as neurodevelopment of these systems (Ncam, Nurr). Interestingly, many of these genes have also been linked independently to ADHD and/or addiction, (Anastasio et al., 2011; Bobb et al., 2005; Eells, 2003; Matzel et al., 2008; Wallis et al., 2008; Werne et al., 2003) suggesting a further link between Lphn3 and these conditions. In addition to changes in monoaminergic gene expression, alterations in monoamine neurochemistry were also observed in null mutant mice. Specifically, at 4–6 weeks of age, null mutants showed elevated levels of both dopamine and serotonin compared to wild type, while heterozygotes showed an intermediate phenotype. This elevation in tissue monoamine content would seem to run contrary to the elevations in expression of genes for both dopamine and serotonin transporters also observed in the null mutants, although it is possible that the upregulation in monoamine transporter genes represents a compensatory response to elevated monoamine synthesis (which would be consistent with the upregulation in Th expression). Given the different timepoints and brain regions sampled, more work will be needed to determine the spatial and temporal specificity of these effects and their relationship to one another. The fact that 9 of 10 of these genes assessed by Q-RT-PCR were overexpressed, coupled with the increases in both of the neurotransmitters DA and 5-Ht, suggests that there are indeed problems with neurotransmitter homeostasis and neuronal differentiation, development, and/or function as we predicted in these mutant mice. This suggests that Lphn3 deficiency results in disruptions of homeostasis for these genes and neurotransmitters, although the mechanisms of this disruption are as of yet unknown.

At the behavioral level, null mutant mice showed a robust elevation in horizontal locomotor activity relative to both heterozygous and wild type mice, which was present across multiple timepoints beginning at 4 weeks of age (a period corresponding to early adolescence in the mouse). This same pattern of behavior was also evident in measures of vertical activity (rearing) and stereotypy (short, repeated movements), although the effect in vertical activity did not reach statistical significance. Null mutant mice also showed a trend toward more time spent in the center of the open field; such an increase in center time could be interpreted as decreased anxiety, but it is difficult to separate such effects from the hyperactivity also present in these mice.

There was no statistical evidence for an age-dependent decrease in hyperactivity in null mutant mice (i.e., no interaction between age and genotype); however, visual inspection of the data (Fig. 4A) suggested that hyperactivity in the null mutant mice may have begun to decrease by the third test period (12 weeks of age), and exploratory post-hoc analyses revealed that the difference between null mutant and wild type mice no longer reached statistical significance at this time point. It is not clear whether this potential decrement represents a true age-dependent decrease in the hyperactive phenotype or a habituation to the locomotor testing environment. Such an attenuation would be consistent with the age-dependent decrease in ADHD symptoms observed in some cohorts (Biederman et al., 1996; Hill and Schoener, 1996). However, it is important to note that at 4 months of age (when cocaine testing took place), null mice still displayed hyperactivity during the baseline, pre-drug period, indicating that the hyperactive phenotype is still present well into adulthood.

The hyperactivity displayed by the Lphn3 null mutant mice is consistent with the elevated striatal monoamine (particularly dopamine) content observed at 4–6 weeks of age. Indeed, elevated striatal dopamine is associated with locomotor hyperactivity in other mutant mouse lines, including the dopamine transporter knockout mice (Gainetdinov et al., 1999). Notably, these Dat1 mice have been proposed to model some features of ADHD (Davids et al., 2003; Milé, 2007; Russell, 2011; Russell, 2007; van der Kooij and Glennon, 2007), suggesting that the Lphn3 null mutant mouse may have some utility in this regard. In contrast to these other models, however, (in which stimulant drugs reverse the hyperactive phenotype), Lphn3 null mutant mice showed an enhanced locomotor response to stimulant drug (cocaine) administration compared to wild type controls, indicating that the relationship between monoaminergic dysfunction, hyperactivity, and response to stimulant drugs is complex.

Since initial submission of this manuscript and during the review process, some very important and relevant studies on Lphn3 have been published. A zebrafish model has been developed (Lange et al., 2012). Loss of lphn3.1 function causes a reduction and misplacement of dopamine-positive neurons in the ventral diencephalon and a hyperactive/impulsive motor phenotype. The behavioral phenotype can be rescued by the ADHD treatment drugs methylphenidate and atomoxetine. Interestingly, the zebrafish model was generated using morpholinos that caused only transient downregulation of lphn3.1. In addition, as zebrafish contain two orthologs of LPHN3 (due to genome duplication in teleost fish), morpholino knockdown of lphn3.1 (and not lphn3.2) may cause only a hypomorphic reduction in Lphn3 protein, an important consideration for ADHD etiology. While the hyperactivity observed in the zebrafish corresponds to the hyperactivity we observe in the mouse, our data showing alterations of DA and
5-HT in the dorsal striatum at 4-6 weeks of age in the mouse is inconsistent with data indicating no changes in DA in zebrafish larvae brain. This discrepancy may be due to differences in developmental time points between the two species, the fact that we sampled specifically from the dorsal striatum as opposed to global brain tissues, the transient nature of morpholino knockdown, or the fact that zebrafish still have lphn3.2 for potential compensation of the phenotype. A second recent and very significant development was the identification of FLRT proteins as endogenous LPHN ligands (O’Sullivan et al., 2012). This groups reports that the FLRT3 and LPHN3 ectodomains interact with high affinity in trans and conclude that LPHN3 and its ligand FLRT3 play an important role in glutamatergic synapse development. They also show that hippocampal cultures transfected with shRNA against Lphn3 have a decreased number of excitatory synapses.

In summary, the data gathered thus far suggest that Lphn3 null mutant mice display evidence of profound disruption at multiple levels of monoamine signaling. Future research will expand upon these data to further characterize this phenotype at both the molecular (neuroanatomy, gene and protein expression) and behavioral (different drugs of abuse, attention, impulsivity) levels. Given the established links between LPHN3 and ADHD/addiction, such characterization may lead to enhanced understanding of the pathophysiology of these disorders, and potentially yield novel therapeutic targets or biomarkers for diagnostics or prediction of treatment success.

4. Experimental procedures

4.1. Clone expansion, confirmation and microinjection

Gene-trapped murine embryonic stem (ES) cells were thawed and expanded. Sequence verification of the insertion site by means of inverse genomic PCR (IPCR) confirmed the correct gene targeting event within Lphn3. Once the ES cells were expanded and ready for injection, they were trypsinized and re-suspended in 20 mM HEPES) and kept on ice. ES cells were injected into C57BL/6 females (40×40×30 cm, Accuscan Instruments: Columbus, OH) under dim light and white noise conditions. Mice were placed into the chambers, and activity data were collected and saved to a computer file via an array of photobeams that crossed the uterine horn of 2.5 dpc pseudopregnant CD-1 recipients. Pups were born 17 days later and chimeric mice were identified 7-10 days later based on their coat color. High percentage male chimeras were mated to C57BL/6-albino females to test for germline transmission through gene specific PCR genotyping.

4.2. Mice

All animals were genotyped from genomic DNA isolated from tails collected at weaning using Extract-N-Amp (Sigma Aldrich, St. Louis, MO). All mice tested for molecular and behavioral assays were sibling cohorts, and were housed in groups of 5 mice per cage in an animal room at 20-22 °C under a 12-hour light/dark cycle (on at 7:00 h) with ad libitum access to food and water. All procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85–23, revised 1996). Mice were euthanized by CO2 asphyxiation and cervical dislocation prior to tissue harvest.

4.3. Q-RT-PCR

Whole brains were harvested from P0 pups and stored in RNAlater (Ambion, Austin, TX). RNA was extracted from 6 male null and 6 male wild type mice using Qiagen RNeasy kits and quantified. RNA was reverse transcribed with SuperScript III (Invitrogen) and Q-PCR was performed using gene specific TaqMan assays. Gapdh expression was used for normalization. Fold differential expression between mutants and wild types was calculated using the delta–delta Ct method as recommended by the manufacturer (Applied Biosystems, Carlsbad, CA).

4.4. HPLC

Whole brain tissue was harvested from 4 to 6 week old male mice (n=6 null, 13 heterozygotes, and 10 wild type). Brains were extracted and snap frozen, slices were made and tissue punches through the striatum were collected. Tissue concentrations of dopamine and serotonin were determined using high pressure liquid chromatography (Kramer et al., 2007, Shah et al., 2005; Sved, 1989). Just prior to assay, the tissue samples were weighed and sonicated in perchloric acid containing dihydrobenzylamine (DHBA; an internal standard, 100 ng/ml: ESA, Chelmsford, MA). The amines were passed through a low volume nylon 0.2-μm filter (Model 8110: Fisher Scientific, Houston, TX) and the resulting supernatant was injected onto a reversed phase C-18 column (Shiseido, 5 cm, Model # A3RE01176: ESA, Chelmsford, MA). The sample amines were eluted using a filtered and degassed mobile phase (Fast Dopamine: ESA, Chelmsford, MA) and then quantified by electrochemistry (Coulouchem II: ESA Chelmsford, MA) using a microdialysis cell (Model 5014B: ESA: Chelmsford, MA). Sample dopamine and serotonin peak heights were compared with external standard peak heights (Sigma Chemical, St. Louis, MO) that were processed in a manner similar to that of the samples. Sample values were expressed as amine concentrations (pg) per mg wet weight of sample tissue.

4.5. Locomotor activity

Fifteen null (6 female; 9 male), 19 heterozygous (9 female; 10 male), and 14 wild type (9 female; 5 male) mice were used to assess locomotor activity. Locomotor activity was assessed during the light cycle in 8 standard activity chambers (40×40×30 cm, Accuscan Instruments: Columbus, OH) under dim light and white noise conditions. Mice were placed into the chambers, and activity data were collected and saved to a computer file via an array of photobeams that crossed the open field at two heights. Activity was measured over 30 min in six bins of 5 min each. Each mouse was tested at 3 timepoints in an identical manner (4, 8, and 12 weeks of age, which corresponds to early adolescence, late adolescence, and adulthood). Four measures of activity were used to assess behavior in the open field: horizontal activity (total number of breaks of the lower set of photobeams), vertical activity (total number of
breaks of the upper set of photobeams—i.e., rears), stereotypy activity (number of repeated breaks of the same photobeam or set of photobeams) and center time (amount of time spent more than 1 cm from the wall of the open field).

4.6. Cocaine administration

Fourteen null (5 female; 9 male) and 13 wild type (8 female; 5 male) mice (ages 4-5 months) used in the locomotor activity tests were also used to assess the effects of acute cocaine administration on locomotor activity. Following a 30-minute habituation period (six 5-minute bins) in the activity chambers, mice were given acute i.p. injections of one of three doses of (-) cocaine HCI (NIDA Drug Supply Program, 0, 5, and 20 mg/kg in physiological saline vehicle, 10 ml/kg) immediately prior to a 60-minute locomotor activity test session (twelve 5-minute bins). Each mouse received each dose of cocaine using a within-subjects design, and the order of doses was randomized by sex and genotype. At least 48 h elapsed between successive injections. The doses of cocaine were calculated as the weight of the salt and were chosen based on the ability to stimulate locomotor activity in mice (Dudek et al., 1991; Phillips et al., 1998).

4.7. Statistical analysis

Gene expression and neurochemical data were analyzed via independent samples t-tests. Data from the locomotor activity tests were analyzed using multi-factor repeated measures ANOVA (sex×genotype×time point). Data from the cocaine administration test were also analyzed using multi-factor repeated measures ANOVA (sex×genotype×drug dose×time bin). Bonferroni post-hoc comparisons were used when appropriate (in the presence of main effects or interactions in the ANOVAs). In all cases, p≤.05 were considered significant.

Acknowledgments

The Texas A&M Institute for Genomic Medicine generated the Lphn3 null mice and provided funding for this project. We thank Alex Ivanov, Colin Vokes, and Rebecca Hofford for technical assistance.

References


