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L-Dopa reverses behavioral deficits in the Pitx3 mouse fetus

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Abstract

Studies of fetal rodents have provided evidence that early emerging behaviors, such as the suckling response, are dependent on the developing dopaminergic system. Although connections have been made between manipulations of dopamine and altered behavioral responses, the specific neural pathways involved have yet to be discovered. In this study we examined the neurobehavioral output of the nigrostriatal pathway, using the Pitx3^{ak}/2J mouse model (Pitx3). Used extensively in the study of Parkinson's disease, the Pitx3 mouse has very specific prenatal loss of dopaminergic neurons solely in the nigrostriatal pathway. Due to this specificity, we hypothesized that behavioral deficits specific to the nigrostriatal pathway would be reversed with administration of the dopamine precursor 3,4-dihydroxyphenylalanine (L-Dopa). To test this hypothesis, homozygous mutant and heterozygous control fetal subjects were administered one of four doses (0, 25, 50, or 75 mg/kg) of L-Dopa on the day before birth. Quantification of fetal behavior was scored from video recordings of behavioral observations. The behavioral measures used were: (a) spontaneous movement activity, (b) state organization, from quantifications of high and low amplitude movements, (c) Interlimb Movement Synchrony, a measure of limb coordination, and (d) Oral Grasp, similar to a newborn infant suckling response. Specific behavioral deficits observed in the Pitx3 mutants were reversed by L-Dopa administration in a dose-dependent manner. However, different deficits required dissimilar doses for reversal, suggesting that some early emerging behaviors may be more sensitive to the administration of L-Dopa. Taken together, this study provides valuable information about prenatal behaviors dependent on the nigrostriatal pathway.

Keywords

dopamine; substantia nigra; suckling; fetal movement; prenatal behavior

Research investigating the mechanisms and etiology of Parkinson's disease have identified the homeobox gene Pitx3 and the human homolog PITX3 as crucial to dopamine neuron development, as well as contributing to the pathophysiology of Parkinson's disease in humans (Bergman et al., 2010; Bidinost et al., 2006; Fuchs et al., 2009; Gui et al., 2012; Liu et al., 2012; Tang et al., 2012). The aphakia (ak) or Pitx3^{ak}/2J mouse, which is deficient in

the transcription factor Pitx3 in homozygous mutants, models the selective nigrostriatal dopamine loss in Parkinson's disease (Hwang, Ardayfio, Kang, Semina, & Kim, 2003; Hwang et al., 2005; van den Munckhof, Gilbert, Chamberland, Levesque, & Drouin, 2006; van den Munckhof et al., 2003). This spontaneously occurring mutation follows a recessive pattern of inheritance and is mapped to the region of chromosome 19 containing Pitx3 (Semina, Murray, Reiter, Hrstka, & Graw, 2000). During development of both mouse and man, Pitx3 (PITX3) is restrictively expressed in the lens and midbrain, playing a nearly solitary role in dopamine neuron differentiation (Nelander, Hebsgaard, & Parmar, 2009; Nunes, Tovmasian, Silva, Burke, & Goff, 2003; S. M. Smits & Smidt, 2006).

In the genetically altered Pitx3 mouse, over 50% of substantia nigra dopaminergic neurons fail to develop prior to birth, while sparing cells in the remainder of the dopamine system (Hwang, et al., 2003; Maxwell, Ho, Kuehner, Zhao, & Li, 2005; Nunes, et al., 2003; Smidt et al., 2004; van den Munckhof, et al., 2003). This prenatal loss leads to an 80% reduction in striatal dopaminergic functioning, with accompanying behavioral deficits in adulthood (P. Ardayfio, Moon, Leung, Youn-Hwang, & Kim, 2008; Hwang, et al., 2003; Kas et al., 2008; van den Munckhof, et al., 2006; van den Munckhof, et al., 2003). These functional deficits in adult mice are reversed following administration of the dopamine precursor 3,4-dihydroxyphenylalanine, L-Dopa (Beeler et al., 2010; Espadas et al., 2012; Hwang, et al., 2005; van den Munckhof, et al., 2006), demonstrating a clear connection between the functional deficits and dopamine depletion in the Pitx3 mouse (Hornykiewicz, 2002).

Due to the specificity of this dopaminergic insult, which is restricted prenatally to the nigrostriatal pathway, the Pitx3 mouse offers a unique opportunity to study the prenatal functioning of this important neural circuitry. Reports that dopaminergic neurons in the midbrain and striatum appear to have a selective vulnerability to insult (Fearnley & Lees, 1991; Greene, Dingledine, & Greenamyre, 2005; Hirsch, Graybiel, & Agid, 1988), also emphasize the importance of determining the functional significance of the nigrostriatal pathway in terms of diagnostic utility. Numerous studies examining prenatal manipulation of the dopamine system in fetal rodents have uncovered clear patterns of behavioral responses that are dopamine-dependent (Andersen, Gazzara, Robinson, & Smotherman, 1992; Andersen, Robinson, & Smotherman, 1993; Becker & Smotherman, 1996; Dawes, 1986; Moody, Robinson, Spear, & Smotherman, 1993; Robinson, Moody, Spear, & Smotherman, 1993; Smotherman, Moody, Spear, & Robinson, 1993; Smotherman & Robinson, 1995; Varlinskaya, Petrov, Robinson, & Smotherman, 1994, 1995). For example, blocking D₁ and D₂ receptors differentially alter oral responses, such as mouthing movements in fetal rats (Smotherman & Robinson, 1995). These behaviors are part of a larger action pattern known as the Oral Grasp Response, similar to newborns suckling behaviors (Robinson et al., 1992). Deficits in this behavioral response could lead to failure to thrive (Ramsay, Gisel, McCusker, Bellavance, & Platt, 2002), emphasizing the functional importance of the dopaminergic system during prenatal development, and the need to identify disruptions to these processes as early as possible (DiPietro, 2005).

Even though most early emerging fetal behaviors are thought to be dopamine dependent, little is known about how specific neural pathways contribute to the development of behaviors such as coordination during movement or the suckling response. Consequently,

we hypothesized that functional deficits observed in the *Pitx3* mouse fetus were due to dopamine depletion in the nigrostriatal pathway. This hypothesis was tested by reversing those behavioral deficits through administration of the dopamine precursor 3,4-dihydroxyphenylalanine (L-Dopa) directly to the *Pitx3* fetus on the day before birth. Assessment of neurobehavioral functioning was accomplished through direct observation of the fetus, a technique routinely used in the study of fetal rodent behavior (G. A. Kleven & Ronca, 2009; Smotherman & Robinson, 1991).

Method

Subjects

Pitx3^{ak}/2J and C57BL/6J vivarium mice were obtained from Jackson Laboratory (Bar Harbor, ME). Female mice were housed three to a polycarbonate cage with cob bedding and Tek-Fresh nesting material. Rodent chow and water was provided ad libitum and cages were maintained in temperature and humidity controlled rooms on a 12-hr light-dark regime. A breeding colony of heterozygote females were created by crossing female C57BL/6J mice with *Pitx3* homozygous (mutant) males. The fetal subjects for this study were the offspring from 16 pregnancies pairing these heterozygous females to mutant males. Because the *Pitx3* mutation is recessive, heterozygous to homozygous pairings produce relatively equal number of mutant and heterozygous offspring. Only heterozygote controls were included in this study because prior work on fetal behavior in the *Pitx3* mouse revealed only negligible differences between heterozygote and wild-type offspring (G. A. Kleven, Joshi, Voogd, & Ronca, 2013). Conception (E0) was determined by the presence of sperm plugs, which were checked on a daily basis during a 5-day breeding period. On day 6, the male was removed, and the time mated females remained housed together until day 17 (E17) of a 19 day gestation. Females were then individually housed until testing on the day before birth (E18). At all times, both adult and fetal subjects were cared for in accordance with animal care guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research, 2011). All procedures, husbandry, and care for the mice in this study were reviewed and approved by the Wake Forest University School of Medicine Animal Care and Use Committee.

Preparation of Subjects—Pregnant mice ($n=16$) were prepared for fetal observation on the last day of gestation (E18), using standard procedures developed for the direct observation of the rodent fetus (G. A. Kleven & Ronca, 2009; Smotherman & Robinson, 1991). Under brief isoflurane anesthesia, spinal anesthesia in the form of a chemomyelotomy was administered by injecting 10 μ l of 100% ethanol into the spinal canal of the pregnant female between the first and second lumbar vertebrae. This procedure produces permanent anesthesia, eliminating sensation in the lower half of the body. Following spinal anesthesia, the female was secured in a Plexiglas holding device and immersed to chest level in a buffered isotonic saline bath of Lock's solution, maintained at body temperature (37.5 $\pm$ 0.5 $^{\circ}$ C). The uterus was then externalized into the bath through a midline laparotomy after which the female was left undisturbed for 20 minutes to ensure both the female and fetal subjects had fully recovered from the anesthesia.

Following recovery and acclimation, four fetal subjects were selected for observation from each pregnant female. When possible, the subjects were chosen in equal numbers from each uterine horn. Within a single pregnancy, fetal subjects selected were either all heterozygous (control) or homozygous (mutant). This design permitted all four dose manipulations to be administered to the same genotype within a single pregnancy. The genotype of the fetus was easily identified due to defective retinal development in the mutant offspring (Rieger, Reichenberger, McLean, Sidow, & Olsen, 2001), and was readily visible through the semitransparent uterine wall. For each of the four treatment groups, $n=7$ fetal subjects were observed from the $n=15$ pregnancies. The extra pregnancy was used to replace two heterozygous subjects removed from the study due to hypoxia.

Fetal subjects to be observed were externalized through a small ($<5\text{mm}$) incision in the uterine wall. Once externalized into the bath, both chorion and amnion membranes were removed, in order to facilitate direct access to the fetal subjects. During the observations of spontaneous movement, fetal subjects were allowed to rest within a curved holding device on a platform supporting the externalized uterus. The fetal subjects remained coupled to the pregnant female through intact placental connections the entire observation period. The preparation was monitored throughout the procedures for any possible sign of distress on the part of the dam or fetus.

Drug Administration—In order to determine which behavioral deficits of the *Pitx3* fetus were dopamine-dependent, the dopamine precursor 3,4-dihydroxyphenylalanine (L-Dopa, Sigma Aldrich) was administered by intra-peritoneal injection to both heterozygous and mutant E18 fetal subjects. L-Dopa administration was staggered across the 42 min observation period, in order to create separate video records for each fetal subject. Four doses of L-Dopa (0, 25, 50, and 75 mg/kg) were prepared by dilution with 0.9% buffered saline, resulting in a 10 ml/kg volume for injection. Administration was based on an estimated average E18 fetal mouse weight of 1.15 g. The four dose levels were determined from previous studies with fetal and infant rats (McCrea, Stehouwer, & Van Hartesveldt, 1994; Robinson & Kleven, 2005; Sickles, Stehouwer, & Van Hartesveldt, 1992; Van Hartesveldt, Sickles, Porter, & Stehouwer, 1991). Doses of 100 mg/kg and higher were used in these studies of fetal and newborn rats, in order to induce a stepping response, similar in appearance to fictive locomotion. Because the purpose of this study was to increase dopamine only to levels reversing behavioral deficits, doses starting below this threshold, which would invoke the stepping response, were chosen (e.g., 25, 50, and 75 mg/kg). All doses were administered in a randomized manner within each litter. However, a single fetal subject received only one dose, and each dose was administered once in a litter.

Behavioral Testing and Data Analysis

Fetal behavior was assessed using videography of direct observations. These observations included behaviors known to be affected by manipulation of the dopamine system, such as spontaneous fetal movement (Smotherman, et al., 1993; Varlinskaya, et al., 1994), during which no manipulation was made by the investigator, and the Oral Grasp of a non-nutritive nipple (Andersen, et al., 1992; Smotherman & Robinson, 1995), similar to human infant suckling responses. Spontaneous movement was further investigated by assessing state

organization, a quantification of low and high amplitude movement periodicity, and Interlimb Movement Synchrony, a measure of temporal organization in spontaneous movement. Behavioral testing occurred across the time line as depicted in Figure 1: (a) 1 min baseline recordings of spontaneous movement, (b) administration of L-Dopa, (c) 16 min delay, in order to allow time for the drug to take effect, (d) 4 min video recording of spontaneous movement, and (e) 1 min of artificial nipple presentation and videography of the fetal response. Baseline, spontaneous movement, and Oral Grasp observations were recorded on DV tape for later playback and scoring with JWatcher video event recorder (Blumstein et al., 2006).

Spontaneous Movement and State—Spontaneous or naturally occurring movement of the fetus was observed and quantified. Fetal limb activity, defined as any movement of a forelimb or hindlimb, was quantified for both the baseline and spontaneous movement observation periods. Scoring of limb movements was performed during normal playback speed (30fps) on a 22" diagonal screen Hewlett Packard LCD Monitor. Limb movements were standardized by the observation time, reported as rates/min, and were analyzed by 2-way ANOVA.

Movements were further characterized by state, in order to determine nigrostriatal involvement in behavioral state. State organization was quantified by identifying low and high amplitude periods of movement, as previously reported for E18 fetal mice (G. A. Kleven & Ronca, 2009). Low amplitude movements were defined as small myoclonic or twitch-like movements, occurring only during muscle atonia, and resembling active sleep (Blumberg & Lucas, 1996; Karlsson & Blumberg, 2002). High amplitude movements were characterized as grand and coordinated in appearance, such as peddling, stretching, or other movements where upper body tone was observed. These movements resemble awake-like behaviors. Low and high amplitude states were quantified by total time, mean duration, and proportion of time observed. Statistical analyses were run as repeated measures ANOVA, with State (low and high) as a within subjects factor, and Type (heterozygous and mutant) and Dose (0, 25, 50, and 75) as between subjects factors.

Interlimb Movement Synchrony—Interlimb Movement Synchrony is a quantification of the temporal organization of spontaneous limb movements. Prior work has demonstrated Interlimb Movement Synchrony to be a sensitive indicator of neurobehavioral functioning in developing mammalian species (G. A. Kleven, 2005; G. A. Kleven, Key, L., Lane, M.S., Lauer, K., & Robinson, S.R., 2001; G. A. Kleven, Lane, & Robinson, 2004; G. A. Kleven & Ronca, 2009). Spontaneous limb movements were defined as any active movement of a limb without regard to the direction or duration. No distinction in scoring was made between gross or fine limb movements. For this study, forelimbs were scored, based on the possible interference of stepping responses in the hindlimbs after L-Dopa administration (McCrea, et al., 1994; Robinson & Kleven, 2005; Sickles, et al., 1992; Van Hartesveldt, et al., 1991). Movement of the limbs were further defined as either low amplitude, small myoclonic twitch-like movements (Blumberg & Lucas, 1996; Karlsson & Blumberg, 2002), or high amplitude, grand movements which are voluntary in appearance. Individual forelimbs were scored from video using JWatcher event recorder (Blumstein, Daniel, & Evans, 2006),

during normal playback of DV recordings. Each limb was scored from a separate pass of the same video segment.

Scored files of forelimb movements were interleaved and quantifications made between synchronous limb movements in successive 0.1 s intervals with a program written by author M. Voogd. This analysis program was based on previous work developing quantifications of Interlimb Movement Synchrony (G. A. Kleven, 2005; G. A. Kleven, Lane, et al., 2004; G. A. Kleven & Ronca, 2009). Synchrony frequencies at every interval (0, 0.1, 0.2, 0.3, 0.4, and 0.5 s) were standardized by the limb movement rates for each subject, creating relative frequencies of Interlimb Movement Synchrony rates.

Relative rates of synchrony at each interval were analyzed in 3 and 4-way repeated measures MANOVA, using the *Pillai-Bartlett Trace* multivariate test. State and Interval (0, 0.1, 0.2, 0.3, 0.4, and 0.5 s) were within subjects factors, with Type and Dose as between subjects factors.

Oral Grasp Response—The Oral Grasp Response was elicited by presentation of an artificial nipple to the perioral area of the fetus, immediately following the spontaneous movement observation. The non-nutritive nipple was sculpted from vinyl material, in the shape of a mouse mammary nipple. Immediately before testing, fetal subjects were gently restrained by placing a soft strap across the thorax, which was secured to the sides of the holding apparatus (Figure 1). The typical response to presentation of a non-nutritive nipple is a grasp, defined as a firm clenching of the jaw around the nipple by the fetus. This Oral Grasping Response emerges prenatally and shows a clear developmental pattern in rodents (Robinson, et al., 1992; Smotherman, 2002; Smotherman, Goffman, Petrov, & Varlinskaya, 1997). Disruption of this important response prenatally may greatly compromise a pup's ability to receive sustenance from its mother after birth. This deprivation, in turn could impair growth, leading to developmental delays or even death. Fetal response to a non-nutritive nipple presentation was quantified in terms of: (a) latency from first presentation of the nipple until the first grasp response, (b) duration of the grasp, (c) frequency of oral events, such as mouthing and licking of the nipple, (d) frequency of aversive and lateral head movements, and (e) frequency of forelimb and hindlimb movements. Scoring of the oral grasping response was accomplished by slow-motion and frame-by-frame video playback using JWatcher event recorder (Blumstein, et al., 2006). Statistical analyses were performed in a series of 2-way ANOVA of Type, Dose, and Type x Dose interactions. Post hoc pairwise comparisons were adjusted with a Bonferroni correction, due to their large number.

All statistical analyses in this study were run with Statistica software, version 10 (StatSoft Inc., 2011). The level of significance for analyses was set at $p < .05$. Unless otherwise stated, post hoc pairwise comparisons of significant main effects and interactions were analyzed using Fisher's PLSD test. Effect sizes were calculated using partial η^2 (n_p^2).

Results

Spontaneous Movement and State

We hypothesized that alterations in spontaneous movement rates for mutant subjects would be reversed by L-DOPA administration in a dose-dependent manner. Baseline movement rates did not differ among the treatment groups by Type (heterozygous and mutant), $F(1, 48) = 0.061$, $p = .806$, $n_p^2 = .001$; Dose (0, 25, 50, and 75 mg/kg), $F(3, 48) = 0.694$, $p = .561$, $n_p^2 = .044$; or Type x Dose interaction, $F(3, 48) = 0.507$, $p = .680$, $n_p^2 = .033$. Group means and 95% CIs ranged from 27.266 – 47.928 /min [-3.150 – 76.740]. Similarly, post-observation weights of fetal subjects revealed no differences between Type, $F(1, 48) = 0.455$, $p = .503$, $n_p^2 = .009$; Dose, $F(3, 48) = 1.701$, $p = .179$, $n_p^2 = .093$; or Type x Dose interaction, $F(3, 48) = 0.549$, $p = .651$, $n_p^2 = .032$. Group means and 95% CIs ranged from 1.149 – 1.267 g [1.040 – 1.404]. Based on these weights and the L-Dopa administrations, effective doses were calculated. Means and 95% CIs for heterozygous subjects were 24.928 mg/kg [22.357 – 27.498], 47.684 mg/kg [43.449 – 51.920], and 68.882 mg/kg [61.079 – 76.685], and for mutant subjects were 23.908 mg/kg [22.157 – 25.659], 45.464 mg/kg [42.498 – 48.430], and 71.407 mg/kg [64.360 – 78.454].

Movement rates were significantly elevated, for some treatment groups, 15 min after administration of L-Dopa (Figure 2). Analyses revealed a significant main effect of Dose, $F(3, 48) = 15.875$, $p < .001$, $n_p^2 = .498$; but no effect of Type, $F(1, 48) = 0.075$, $p = .786$, $n_p^2 = .002$; or Type x Dose interaction, $F(3, 48) = 0.800$, $p = .500$, $n_p^2 = .048$. Pairwise post hoc comparisons revealed significant elevation of movement rates between the 75 mg/kg subjects (both heterozygous and mutant) and all other groups.

In order to determine if state organization was impacted by administration of LDopa, analyses of total time, mean duration, and proportion for each State (low and high) consistently revealed only main effects of State, with no other factors or interaction terms significant. Similar to prior work with mice, fetal subjects were observed to alternate between periods of low and high amplitude state, with the majority of time spent in low amplitude behaviors (G. A. Kleven & Ronca, 2009; Smotherman & Robinson, 1991). Figure 3 depicts this behavioral cyclicity, and the low amplitude proportions for the main effect of State, $F(1, 48) = 109.14$, $p < .001$, $n_p^2 = .695$. No significant effects were found for Type, $F(1, 48) = 1.030$, $p = .314$, $n_p^2 = .021$; Dose, $F(3, 48) = 1.060$, $p = .375$, $n_p^2 = .062$; Type x Dose, $F(3, 48) = 1.080$, $p = .365$, $n_p^2 = .063$; State x Type, $F(1, 48) = 0.050$, $p = .825$, $n_p^2 = .001$; State x Dose, $F(3, 48) = 1.871$, $p = .149$, $n_p^2 = .113$; or State x Type x Dose interactions, $F(3, 48) = 0.160$, $p = .923$, $n_p^2 = .010$. Confirming this lack of interaction, pairwise comparisons revealed significance only between States for each Type and Dose.

Interlimb Movement Synchrony

All fetal subjects were observed to perform synchronous movements, particularly during low amplitude twitch-like states. We hypothesized that depressed rates of Interlimb Movement Synchrony observed in mutant subjects would be reversed in a dose-dependent manner by L-DOPA administration. A 4-way over-all MANOVA revealed main effects for Dose, State, and Interval, with all factors involved in one or more interaction term (Table 1).

Parsing the data by State, similar results were detected for both low and high amplitude states of movement. However, because fetal subjects from all groups and doses were observed a vast majority of the time to be in a low amplitude behavioral state, the remainder analyses of the Interlimb Movement Synchrony were run on low amplitude data only.

Visual inspection of the data for the 0 mg/kg dose revealed a typical synchrony profile for heterozygous subjects (Figure 4, top left). Similar to other studies of fetal mice and rats, heterozygous subjects receiving the dose of 0 mg/kg had elevated relative frequencies of synchronous movements at lower intervals (0 and 0.1 s). Mutant subjects, in contrast, displayed a profile that was significantly depressed at the 0.1 s interval. For the 25 mg/kg group, these findings are reversed, with heterozygous subjects depressed, both relative to the 0 mg/kg group and the mutant 25 mg/kg subjects (Figure 4, top right). Doses of 50 and 75 mg/kg reveal depressed synchrony profiles in both groups, relative to the typical profile. Post hoc pairwise comparisons confirm the differences between groups at the 0.1 s interval for the 0 and 25 mg/kg doses, $p = .001$ and $.011$ respectively (Figure 4, bottom).

Oral Grasp Response

Fetal subjects from all groups and doses exhibited characteristic oral grasping responses to presentation of a non-nutritive nipple (Table 2). We hypothesized that administration of L-DOPA would reduce in a dose-dependent manner the longer latency to the first grasping response observed in mutant subjects. Analysis of the latency to first grasping response after nipple presentation revealed a significant Type x Dose interaction, $F(3, 48) = 4.44$, $p = .007$, $n_p^2 = .214$; but no main effects of either Type, $F(1, 48) = 0.689$, $p = .410$, $n_p^2 = .014$; or Dose, $F(3, 48) = 0.414$, $p = .744$, $n_p^2 = .025$ (Figure 5). Post hoc pairwise comparisons revealed a significantly longer latency in the 0 mg/kg mutants relative to heterozygous subjects, $p = .001$; and to 75 mg/kg mutants, $p = .004$. Neither frequency of grasps, total time grasping, nor the proportion of time spent on the nipple (Figure 6) was significantly different for either group, or at any dose. However, analysis of the mean grasp duration revealed a main effect of Type, $F(1, 48) = 5.155$, $p = .028$, $n_p^2 = .095$; but no main effect of Dose, $F(3, 48) = 0.597$, $p = .620$, $n_p^2 = .035$; or Type by Dose interaction, $F(3, 48) = 2.005$, $p = .125$, $n_p^2 = .109$. Post hoc pairwise comparisons were significant only between groups at the 75 mg/kg dose, $p = .004$, with heterozygous subjects significantly elevated relative to mutants. In contrast, analysis of oral events/min while attached to the nipple (e.g., licking and mouthing movements) revealed a main effect of Dose, $F(3, 48) = 3.762$, $p = .017$, $n_p^2 = .187$; but no main effect of Type, $F(1, 48) = 2.641$, $p = .111$, $n_p^2 = .051$; or Type by Dose interaction, $F(3, 48) = 2.053$, $p = .119$, $n_p^2 = .112$. Post hoc pairwise comparison of mutant subjects receiving the 0 mg/kg dose revealed significant elevation of oral behaviors relative to heterozygous 0 mg/kg group, $p = .004$; and both mutant and heterozygous groups receiving 25 mg/kg, $p = .004$, and $p = .004$; and 50 mg/kg, $p < .001$, and $p < .001$; respectively.

In contrast to the grasps and oral behaviors, analysis of upper body movements such as forelimb (Figure 7, top) or head (not shown), revealed no differences for any factor or interaction. Hindlimb quantification, on the other hand, revealed main effects for both Type, $F(1, 48) = 5.643$, $p = .021$, $n_p^2 = .103$; and Dose, $F(3, 48) = 11.897$, $p < .001$, $n_p^2 = .421$; but

not the Type x Dose interaction, $F(3, 48) = 1.738$, $p = .171$, $\eta_p^2 = .096$ (Figure 7, bottom). Post hoc pairwise comparisons confirm mutant 0 mg/kg rates elevated relative to heterozygous, $p = .003$, and both heterozygous and mutant 75 mg/kg subjects as elevated relative to those receiving 25 mg/kg, $p < .001$ and $p < .001$; to those administered 50 mg/kg, $p < .001$ and $p < .001$; and to heterozygous receiving 0 mg/kg, $p < .001$.

Discussion

The findings of this study provide clear evidence that behavioral deficits in the *Pitx3^{ak/2J}* mouse are reverse by L-Dopa. More importantly, these results are the first to document the connection between specific components of prenatal behavioral development and the nigrostriatal pathway. Taken together, there are two significant conclusions from this study of fetal neurobehavior: (a) the nigrostriatal pathway contributes to specific identifiable features of early emerging behaviors, and (b) reversal of dopamine-dependent behavioral deficits is both dose and behavior specific.

The nigrostriatal pathway contributes to specific identifiable features of early emerging behaviors

Similar to results from studies of adult *Pitx3* behavior (P. Ardayfio, et al., 2008; P. A. Ardayfio et al., 2010; Beeler, et al., 2010; Espadas, et al., 2012; Hwang, et al., 2003; Hwang, et al., 2005; Kas, et al., 2008; van den Munckhof, et al., 2006; van den Munckhof, et al., 2003), specific behavioral deficits were identified during fetal testing of *Pitx3* mutants and heterozygous controls in the 0 mg/kg groups. Most notable were the decreased relative rates of Interlimb Movement Synchrony (Figure 4, top left), and increased latency to the first grasping response of an artificial nipple (Figure 5). Both these deficits can be described in terms of difficulty initiating coordinative movements, or akinesia, a hallmark symptom of nigrostriatal damage (Calne & Langston, 1983; Jankovic, Chen, & Le, 2005; Parkinson, 1817). However, many observation results were indistinguishable between *Pitx3* mutants and heterozygous controls, suggesting that nigrostriatal circuitry is not a contributing factor in these behaviors, or plays such a minor role that the measures used are not sensitive enough to detect miniscule differences. Considering that the behavioral quantifications used in this study have been found of sufficient sensitivity to detect functional deficits even in a NOEL (No Observable Effects Level) dose of neurotoxin (G. A. Kleven, 2005; G. A. Kleven, Queral, & Robinson, 2004), suggests that lack of sensitivity is not a factor. Rather, the nigrostriatal pathway likely plays a prominent or dedicated role in the latency to grasp and Interlimb Movement Synchrony behaviors. For the other behaviors observed, this circuitry may have no involvement, or may provide redundant function.

Conversely, several results appear contrary to nigrostriatal involvement, most notably the elevated limb movements (Figure 3) and mouthing rates (Table 2) of mutant subjects relative to those of heterozygous controls (0 mg/kg groups). The sporadically elevated activity levels found in this study appear to be characteristic of the *Pitx3* mouse model, because adult behavioral testing has revealed similar results (Hwang, et al., 2005; Kas, et al., 2008), suggesting a form of stereotypy. Investigations into this unique model have uncovered hyper-innervation of the dorsal striatum (S. M. Smits, Noorlander, C.W., Kas,

M.J., Ramakers, G.M., & Smidt, M.P., 2008), which based on normal development of serotonin projections, likely takes place in the mouse several days before birth (Johnston & Coyle, 1981). The proposed theory is that lacking dopaminergic connections from substantia nigra, developing projections of serotonin have no competition in the dorsal striatum. This hyper-innervation then becomes a type of compensation for the missing nigrostriatal pathway; increasing activity levels and masking the loss of dopaminergic functioning (S. M. Smits, Noorlander, C.W., Kas, M.J., Ramakers, G.M., & Smidt, M.P., 2008). By extension, this theory would suggest that other prenatal insults to midbrain dopamine circuitry should also create hyper-innervation of serotonergic pathways in the dorsal striatum.

Reversal of dopamine-dependent behavioral deficits is both dose and behavior specific

One of the most striking findings from this study is that, although all behavioral deficits were reversed after L-Dopa administration, various components of behaviors required different doses to return to levels equal those of control subjects. For example, spontaneous forelimb movement, low amplitude Interlimb Movement Synchrony profiles, and oral movements during the Oral Grasp required a minimal dose of 25 mg/kg to reverse deficits observed in the 0 mg/kg groups (Figure 2; Figure 4, top right; and Table 2). However, reversal of hindlimb deficits during the Oral Grasp required a 50 mg/kg dose (Figure 7), and reducing the latency to first grasp of the artificial nipple necessitated a 75 mg/kg dose (Figure 5).

One difference between these findings is that those behaviors requiring only 25 mg/kg of L-Dopa were considerably smaller deficits than those requiring 50-75 mg/kg for reversal. For example, Interlimb Movement Synchrony levels in mutants were reduced by approximately half compared to heterozygous subjects (Figure 4, top left), but mutant subjects showed a 4-fold longer latency to first grasp over controls (Figure 5). Another possible explanation for this seeming inconsistency is that hyper-innervation of serotonergic circuitry to the dorsal striatum may provide compensation for activity related quantifications such as limb movements and Interlimb Movement Synchrony, but is not sufficient to compensate for movement initiation, or akinesia. The addition of LDopa, in this case would return both serotonin and dopamine activity to a balanced state only at very high levels (e.g., 75 mg/kg). It is interesting that at these higher levels of L-Dopa, activity related measures show very elevated responses in all subjects, suggesting that excessive amounts of L-Dopa may be further converted from dopamine to norepinephrine, up regulating activity even further (Chalmers, Baldessarini, & Wurtman, 1971).

Another consideration among these differences in response to L-Dopa is the actual sensitivity of the behavioral measures themselves. Among the quantifications of spontaneous movement, Interlimb Movement Synchrony has been shown sensitive to even small neurotoxin perturbants. Within that context it follows that this measure would be able to differentiate smaller deficits, which might necessitate lower doses of L-Dopa to reverse. Similarly, the 25 mg/kg dose was observed to disrupt the entire synchrony profile for heterozygous subjects (Figure 4, top right), indicating this measure is also sensitive to small changes in neurotransmitter levels.

One final point concerns the use of heterozygote as opposed to wild-type controls. Heterozygous controls were used in this study based on prior findings of only negligible differences between heterozygous and wild-type fetal subjects. Despite similarity of unmanipulated behavior, wild-type response to L-Dopa remains unknown. Future studies comparing wild-type behavior may provide additional insight into these paradoxical findings.

Future studies may also be warranted to examine the role of striatal serotonin in early emerging coordinated behaviors such as Interlimb Movement Synchrony and the Oral Grasping Response. Administration of 5-HT agonists to fetal rats has previously demonstrated elevated rates of movement similar to those observed in the *Pitx3* fetus (Brumley & Robinson, 2005). Of particular interest would be future studies using the administration of 5-HT antagonists to block excessive striatal serotonin.

Taken together, these results document the specific prenatal functional deficits in the *Pitx3* mouse that are nigrostriatal dependent and reversed by direct administration of L-Dopa. Although the mouse model discussed has been used previously to investigate the mechanisms of Parkinson's disease, this study revealed its utility in examining the prenatal development of behaviors dependent on nigrostriatal functioning such as the Oral Grasp Response, similar to newborn suckling behavior (Robinson, et al., 1992). Deficits in this crucial behavioral response lead to malnutrition or even failure to thrive (Ramsay, et al., 2002). The results from this study emphasize the functional importance of the dopaminergic system during prenatal development, providing initial insight into possible early roles of the nigrostriatal pathway.

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References

- Andersen SL, Gazzara RA, Robinson SR, Smotherman WP. Effect of milk on dopamine release in the newborn rat: An in vivo microdialysis study. *Developmental Brain Research*. 1992; 68:286–288. [PubMed: 1394974]
- Andersen SL, Robinson SR, Smotherman WP. Ontogeny of the stretch response in the rat fetus: Kappa opioid involvement. *Behavioral Neuroscience*. 1993; 107(2):370–376. [PubMed: 8387314]
- Ardayfio P, Moon J, Leung KK, Youn-Hwang D, Kim KS. Impaired learning and memory in *Pitx3* deficient aphakia mice: a genetic model for striatum-dependent cognitive symptoms in Parkinson's disease. *Neurobiology of Disease*. 2008; 31(3):406–412. doi: S0969-9961(08)00121-6 [pii] 10.1016/j.nbd.2008.05.017. [PubMed: 18573342]
- Ardayfio PA, Leung A, Park J, Hwang DY, Moran-Gates T, Choi YK, Kim KS. *Pitx3*-deficient aphakia mice display unique behavioral responses to psychostimulant and antipsychotic drugs. *Neuroscience*. 2010; 166(2):391–396. [PubMed: 20026251]

- Becker LA, Smotherman WP. Manipulation of the dopamine system affects fetal and neonatal responses to an artificial nipple. *Developmental Psychobiology*. 1996; 29:139–156. [PubMed: 8919092]
- Beeler JA, Cao ZFH, Kheirbek MA, Ding Y, Koranda J, Murakami M, Zhuang X. Dopamine-dependent motor learning: insight into levodopa's long-duration response. *Annals Of Neurology*. 2010; 67(5):639–647. [PubMed: 20437561]
- Bergman O, Hakansson A, Westberg L, Nordenstrom K, Carmine Belin A, Sydow O, Nissbrandt H. PITX3 polymorphism is associated with early onset Parkinson's disease. *Neurobiol Aging*. 2010; 31(1):1–4. doi: S0197-4580(08)00089-4 [pii] 10.1016/j.neurobiolaging.2008.03.008. [PubMed: 18378359]
- Bidinost C, Matsumoto M, Chung D, Salem N, Zhang K, Stockton DW, Traboulsi EI. Heterozygous and homozygous mutations in PITX3 in a large lebanese family with posterior polar cataracts and neurodevelopmental abnormalities. *Investigative Ophthalmology & Visual Science*. 2006; 47(4):1274–1280. [PubMed: 16565358]
- Blumberg MS, Lucas DE. A developmental and component analysis of active sleep. *Developmental Psychobiology*. 1996; 29(1):1–22. [PubMed: 8719180]
- Blumstein, DT.; Daniel, JC.; Evans, CS. JWatcher Video (Version 1.0). 2006. Retrieved from <http://www.jwatcher.ucla.edu/>
- Brumley MR, Robinson SR. The Serotonergic Agonists Quipazine, CGS-12066A, and α -Methylserotonin Alter Motor Activity and Induce Hindlimb Stepping in the Intact and Spinal Rat Fetus. *Behavioral Neuroscience*. 2005; 119(3):821–833. doi: 10.1037/0735-7044.119.3.821. [PubMed: 15998204]
- Calne DB, Langston JW. Aetiology of Parkinson's disease. *Lancet*. 1983; 2(8365-66):1457–1459. [PubMed: 6140548]
- Chalmers JP, Baldessarini RJ, Wurtman RJ. Effects of L-dopa on norepinephrine metabolism in the brain. *Proceedings of the National Academy of Sciences of the United States of America*. 1971; 68(3):662–666. [PubMed: 5276777]
- Dawes GS. The central nervous control of fetal behavior. *European Journal of Obstetrics & Gynecology and Reproductive Biology*. 1986; 21:341–346. [PubMed: 2873069]
- DiPietro JA. Neurobehavioral assessment before birth. *Mental Retardation and Developmental Disabilities Research Reviews*. 2005; 11(1):4–13. doi: 10.1002/mrdd.20047. [PubMed: 15856447]
- Espadas I, Darmopil S, Vergaño-Vera E, Ortiz O, Oliva I, Vicario-Abejón C, Moratalla R. L-DOPA-induced increase in TH-immunoreactive striatal neurons in parkinsonian mice: insights into regulation and function. *Neurobiology of Disease*. 2012; 48(3):271–281. doi: 10.1016/j.nbd.2012.07.012. [PubMed: 22820144]
- Fearnley JM, Lees AJ. Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain*. 1991; 114(Pt 5):2283–2301. [PubMed: 1933245]
- Fuchs J, Mueller JC, Lichtner P, Schulte C, Munz M, Berg D, Gasser T. The transcription factor PITX3 is associated with sporadic Parkinson's disease. *Neurobiol Aging*. 2009; 30(5):731–738. doi: S0197-4580(07)00348-X [pii] 10.1016/j.neurobiolaging.2007.08.014. [PubMed: 17905480]
- Greene JG, Dingledine R, Greenamyre JT. Gene expression profiling of rat midbrain dopamine neurons: implications for selective vulnerability in parkinsonism. *Neurobiol Dis*. 2005; 18(1):19–31. [PubMed: 15649693]
- Gui Y, Zhao Y, Liu H, Fu J, Xu Z, Hu X. A novel synonymous SNP in PITX3 is associated with Parkinson's disease in Chinese population. *Swiss Medical Weekly*. 2012; 142:w13521–w13521. doi: 10.4414/smww.2012.13521. [PubMed: 22411443]
- Hirsch E, Graybiel AM, Agid YA. Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature*. 1988; 334(6180):345–348. [PubMed: 2899295]
- Hornykiewicz O. L-DOPA: from a biologically inactive amino acid to a successful therapeutic agent. *Amino Acids*. 2002; 23(1-3):65–70. [PubMed: 12373520]
- Hwang DY, Ardayfio P, Kang UJ, Semina EV, Kim KS. Selective loss of dopaminergic neurons in the substantia nigra of Pitx3-deficient aphakia mice. *Molecular Brain Research*. 2003; 114:123–131. [PubMed: 12829322]

- Hwang DY, Fleming SM, Ardayfio P, Moran-Gates T, Kim H, Tarazi FI, Kim KS. 3,4-Dihydroxyphenylalanine reverses the motor deficits in Pitx3-deficient mice: Behavioral characterization of a novel genetic model of Parkinson's disease. *Journal of Neuroscience*. 2005; 25(8):2132–2137. [PubMed: 15728853]
- Jankovic J, Chen S, Le WD. The role of Nurr1 in the development of dopaminergic neurons and Parkinson's disease. *Prog Neurobiol*. 2005; 77(1-2):128–138. doi: S0301-0082(05)00102-4 [pii] 10.1016/j.pneurobio.2005.09.001. [PubMed: 16243425]
- Johnston MV, Coyle JT. Development of central neurotransmitter systems. *Ciba Found Symp*. 1981; 86:251–270. [PubMed: 6121688]
- Karlsson KA, Blumberg MS. The union of the state: myoclonic twitching is coupled with nuchal muscle atonia in infant rats. *Behav Neurosci*. 2002; 116(5):912–917. [PubMed: 12369810]
- Kas MJ, van der Linden AJ, Oppelaar H, von Oerthel L, Ramakers GM, Smidt MP. Phenotypic segregation of aphakia and Pitx3-null mutants reveals that Pitx3 deficiency increases consolidation of specific movement components. *Behav Brain Res*. 2008; 186(2):208–214. doi: S0166-4328(07)00434-2 [pii] 10.1016/j.bbr.2007.08.032. [PubMed: 17919745]
- Kleven, GA. Dissertation. 2005. Patterns of Early Behavioral Development after Exposure to a Prototypical Neurotoxin Methylazoxymethanol (MAM) Dissertation..
- Kleven GA, Joshi P, Voogd M, Ronca AE. Prenatal ontogeny of the dopamine-dependent neurobehavioral phenotype in Pitx3-deficient mice. *European Journal of Neuroscience*. 2013; 37(10):1564–1572. doi: 10.1111/ejn.12184. [PubMed: 23489835]
- Kleven GA, Key L, Lane MS, Lauer K, Robinson SR. A comparative study of movement bout organization in preterm human infants, Norway rats, and spiny mice. [Abstract] *Developmental Psychobiology*. 2001; 38(3)
- Kleven GA, Lane MS, Robinson SR. Development of interlimb movement synchrony in the rat fetus. *Behavioral Neuroscience*. 2004; 118(4):835–844. [PubMed: 15301609]
- Kleven GA, Qeral L, Robinson SR. Prenatal methylazoxymethanol exposure alters evoked responses in fetal rats. *Neurotoxicology and Teratology*. 2004; 26(5):663–671. [PubMed: 15315815]
- Kleven GA, Ronca AE. Prenatal behavior of the C57BL/6J mouse: A promising model for human fetal movement during early to mid-gestation. *Developmental Psychobiology*. 2009; 51:84–94. [PubMed: 18980217]
- Liu H, Wei L, Tao Q, Deng H, Ming M, Xu P, Le W. Decreased NURR1 and PITX3 gene expression in Chinese patients with Parkinson's disease. *European Journal Of Neurology: The Official Journal Of The European Federation Of Neurological Societies*. 2012; 19(6):870–875. doi: 10.1111/j.1468-1331.2011.03644.x. [PubMed: 22309633]
- Maxwell SL, Ho HY, Kuehner E, Zhao S, Li M. Pitx3 regulates tyrosine hydroxylase expression in the substantia nigra and identifies a subgroup of mesencephalic dopaminergic progenitor neurons during mouse development. *Developmental Biology*. 2005; 282:467–479. [PubMed: 15950611]
- McCrea AE, Stehouwer DJ, Van Hartesveldt C. L-dopa-induced air-stepping in preweanling rats. I. Effects of dose and age. *Brain Res Dev Brain Res*. 1994; 82(1-2):136–142.
- Moody CA, Robinson SR, Spear LP, Smotherman WP. Fetal behavior and the dopamine system: Activity effects of D-sub-1 and D-sub-2 receptor manipulations. *Pharmacology, Biochemistry & Behavior*. 1993; 44(4):843–850.
- National Research, C. Guide for the care and use of laboratory animals. 8th ed.. National Academy Press; Washington, DC: 2011.
- Nelander J, Hebsgaard JB, Parmar M. Organization of the human embryonic ventral mesencephalon. *Gene Expression Patterns: GEP*. 2009; 9(8):555–561. [PubMed: 19825428]
- Nunes I, Tovmasian LT, Silva RM, Burke RE, Goff SP. Pitx3 is required for development of substantia nigra dopaminergic neurons. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100(7):4245–4250. [PubMed: 12655058]
- Parkinson, J. An Essay on the Shaking Palsy. Sherwood Neely and Jones; London: 1817.
- Ramsay M, Gisel EG, McCusker J, Bellavance F, Platt R. Infant suckling ability, non-organic failure to thrive, maternal characteristics, and feeding practices: A prospective cohort study. *Developmental Medicine and Child Neurology*. 2002; 44(6):405–414. [PubMed: 12088309]

- Rieger DK, Reichenberger E, McLean W, Sidow A, Olsen BR. A double-deletion mutation in the *Pitx3* gene causes arrested lens development in aphakia mice. *Genomics*. 2001; 72(1):61–72. doi: 10.1006/geno.2000.6464S0888-7543(00)96464-4 [pii]. [PubMed: 11247667]
- Robinson SR, Hoeltzel TC, Cooke KM, Umphress SM, Smotherman WP, Murrish DE. Oral capture and grasping of an artificial nipple by rat fetuses. *Developmental Psychobiology*. 1992; 25(8):543–555. [PubMed: 1487080]
- Robinson, SR.; Kleven, GA. Learning to move before birth.. In: Hopkins, B.; Johnson, S., editors. *Advances in Infancy Research: Prenatal Development of Postnatal Functions*. Vol. 2. Ablex; Norwood, NJ: 2005. p. 131-175.
- Robinson SR, Moody CA, Spear LP, Smotherman WP. Effects of dopamine and kappa opioid receptors on fetal responsiveness to perioral stimuli. *Developmental Psychobiology*. 1993; 26:37–50. [PubMed: 8382637]
- Semina EV, Murray JC, Reiter R, Hrstka RF, Graw J. Deletion in the promotor region and altered expression of *Pitx3* homeobox gene in aphakia mice. *Human Molecular Genetics*. 2000; 9(11): 1575–1585. [PubMed: 10861284]
- Sickles AE, Stehouwer DJ, Van Hartesveldt C. Dopamine D1 and D2 antagonists block L-dopa-elicited air-stepping in neonatal rats. *Brain Res Dev Brain Res*. 1992; 68(1):17–22.
- Smidt MP, Smits SM, Bouwmeester H, Hamers FP, van der Linden AJ, Hellemons AJ, Burbach JP. Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene *Pitx3*. *Development*. 2004; 131(5):1145–1155. [PubMed: 14973278]
- Smits SM, Noorlander CW, Kas MJ, Ramakers GM, Smidt MP. Alterations in serotonin signalling are involved in the hyperactivity of *Pitx3*-deficient mice. *Eur J Neurosci*. 2008; 27(2):388–395. [PubMed: 18215235]
- Smits SM, Smidt MP. The role of *Pitx3* in survival of midbrain dopaminergic neurons. *J Neural Transm Suppl*. 2006; ((70)):57–60. [PubMed: 17017509]
- Smotherman WP. Early experience with the artificial nipple. *Developmental Psychobiology*. 2002; 41:1–14. [PubMed: 12115286]
- Smotherman WP, Goffman D, Petrov ES, Varlinskaya EI. Oral grasping of a surrogate nipple by the newborn rat. *Developmental Psychobiology*. 1997; 31:3–17. [PubMed: 9222113]
- Smotherman WP, Moody CA, Spear LP, Robinson SR. Fetal behavior and the endogenous opioid system: D1 dopamine receptor interactions with the kappa opioid system. *Physiology and Behavior*. 1993; 53(1):191–197. [PubMed: 8381974]
- Smotherman, WP.; Robinson, SR. Accessibility of the rat fetus for psychobiological investigation.. In: Shair, HN.; Barr, GA.; Hofer, MA., editors. *Developmental psychobiology: New methods and changing concepts*. Oxford University Press; New York: 1991. p. 148-163.
- Smotherman WP, Robinson SR. Dopamine D-sub-1 and D-sub-2 effects on fetal mouthing responses to milk. *Physiology & Behavior*. 1995; 57(1):15–19. [PubMed: 7878109]
- StatSoft Inc. STATISTICA (data analysis software system) (Version 10). 2011
- Tang L, Zhao S, Wang M, Sheth A, Zhao Z, Chen L, Chen L. Meta-analysis of association between *PITX3* gene polymorphism and Parkinson's disease. *Journal of the Neurological Sciences*. 2012; 317(1-2):80–86. [PubMed: 22429667]
- van den Munckhof P, Gilbert F, Chamberland M, Levesque D, Drouin J. Striatal neuroadaptation and rescue of locomotor deficit by L-dopa in aphakia mice, a model of Parkinson's disease. *J Neurochem*. 2006; 96(1):160–170. doi: JNC3522 [pii] 10.1111/j.1471-4159.2005.03522.x. [PubMed: 16269007]
- van den Munckhof P, Luk KC, Ste-Marie L, Montgomery J, Blanchet PJ, Sadikot AF, Drouin J. *Pitx3* is required for motor activity and for survival of a subset of midbrain dopaminergic neurons. *Development*. 2003; 130(11):2535–2542. [PubMed: 12702666]
- Van Hartesveldt C, Sickles AE, Porter JD, Stehouwer DJ. L-dopa-induced air-stepping in developing rats. *Brain Res Dev Brain Res*. 1991; 58(2):251–255.
- Varlinskaya EI, Petrov ES, Robinson SR, Smotherman WP. Intracisternal administration of SKF-38393 and SCH-23390: Behavioral effects in the rat fetus. *Pharmacology, Biochemistry & Behavior*. 1994; 48(3):741–748.

Varlinskaya EI, Petrov ES, Robinson SR, Smotherman WP. Asymmetrical development of the dopamine system in the fetal rat as indicated by lateralized administration of SKF-38393 and SCH-23390. *Pharmacology, Biochemistry & Behavior*. 1995; 50(3):359–367.

Figure 1.

Schedule of L-Dopa delivery and behavioral observations. Bars depict the experimental timeline for the four fetal subjects observed from each pregnancy. The timing of L-Dopa administrations and behavioral observations were staggered, in order to facilitate separate video recordings for each subject. The gray segments denote the 1-min baseline (left) and 4 min spontaneous movement observation periods (right). The bar to the right of the 1-min baseline indicates the time point of L-Dopa administration. A period of 16-min was allowed for L-Dopa activation, immediately following the administration (white bar). The dark gray bar (far right) indicates the 1-min presentation of a non-nutritive artificial nipple, and the corresponding behavioral observation of the Oral Grasp Response. Inset: A mutant Pitx3 subject is shown gently restrained in a curved holding apparatus during observations of the Oral Grasp. Restraint is achieved by securing a soft strap across the thorax of the fetus at the beginning of the Oral Grasp observation. Subject above is exhibiting a typical grasp of the non-nutritive artificial nipple. Note the microphthalmic eye, visible through the semi-transparent skin. One uterine horn is also visible to the left of the fetal subject. The scale bar is 5 mm.

Figure 2.

Forelimb activity during spontaneous movement. Bars represent the mean frequency/min of forelimb movement during the 4-min spontaneous movement observation period. Fetal subjects from either mutant (mut) or heterozygous (het) groups were administered one of four doses of the dopamine precursor L-Dopa (0, 25, 50, or 75 mg/kg). Broken line denotes the mean level of forelimb activity from het subjects receiving the 0 mg/kg dose (saline controls). Error bars depict the *SEM*.

Figure 3.

State cyclicity and proportion of low amplitude behavior. (A) Sections of the circle depict the mean durations of high amplitude (black), and low amplitude (gray) state for heterozygous controls. High amplitude activity includes awake-like behaviors such as stretching and peddling movements. Low amplitude activity is defined as twitch-like movement occurring during periods of upper body atonia. Periods of quiescence (white) occurred in between high and low amplitude states, with very brief transitions (< 1 s) between low amplitude and high amplitude behavior. Arrows represent the cyclic nature of state organization, with behavior alternating between high and low amplitudes, and transitional periods of quiescence. Subjects typically exhibited 3-4 cycles during the 4 min observation period. (B) Bars represent the mean proportion of low amplitude state activity during the spontaneous movement observation. Fetal subjects from either mutant (mut) or heterozygous (het) groups were administered one of four doses of the dopamine precursor L-Dopa (0, 25, 50, or 75 mg/kg). The dotted line represents the mean level of low amplitude state for the het subjects at the 0 mg/kg dose (saline controls). Error bars depict the *SEM*.

Figure 4.

Interlimb Movement Synchrony during low amplitude behavioral state. Profiles represent the mean relative frequency of Interlimb Movement Synchrony in the forelimbs at each of the 0.1 s intervals during low amplitude behavior. Low amplitude behaviors were defined as those possessing a twitch-like property during upper body muscle atonia. Quantification of synchronous forelimb behavior represents the lag between right and left forelimb movements. Fetal subjects from either mutant (mut) or heterozygous (het) groups were administered one of four doses of the dopamine precursor L-Dopa (0, 25, 50, or 75 mg/kg). The dashed lines reflect the het subject profile at the 0 mg/kg dose (saline control). Error bars depict the SEM.

Figure 5.

Latency to 1st grasp response. Bars depict the mean latency in seconds to the first grasp response after presentation of a non-nutritive artificial nipple. A grasp is defined as any firm closure of the jaw around the nipple, including those of brief duration. Fetal subjects from either mutant (mut) or heterozygous (het) groups were administered one of four doses of the dopamine precursor L-Dopa (0, 25, 50, or 75 mg/kg). The broken line reflects the mean latency to 1st grasp for the het subjects at the 0 mg/kg dose (saline controls). Error bars depict the *SEM*.

Figure 6.

Proportion of observation period attached to nipple. Bars represent the mean proportion of the 1-min observation period during which fetal subjects were attached to the non-nutritive artificial nipple in a grasping response. Grasps were defined as any firm closure of the jaw around the nipple, including those of short duration. Fetal subjects from either mutant (mut) or heterozygous (het) groups were administered one of four doses of the dopamine precursor L-Dopa (0, 25, 50, or 75 mg/kg). The broken line reflects the mean proportion of grasp time for the het subjects at the 0 mg/kg dose (saline controls). Error bars depict the *SEM*.

Figure 7.

Forelimb and hindlimb activity during the Oral Grasp Response. Bars depict the mean rates/min of movement frequency for forelimbs (top) and hind limbs (bottom) while grasping the non-nutritive artificial nipple. Fetal subjects from either mutant (mut) or heterozygous (het) groups were administered one of four doses of the dopamine precursor L-Dopa (0, 25, 50, or 75 mg/kg). The dotted lines reflect the level of limb activity for the het subjects at the 0 mg/kg dose (saline controls). Error bars depict the *SEM*.

Table 1

Interlimb Movement Synchrony 4-way MANOVA

	df	Pillai-Bartlett *	F	p	η_p^2
<i>Between subjects</i>					
Dose	3,48		8.871	<.001	.357
Type	1,48		3.362	.073	.065
Dose $\times$ Type	3,48		1.015	.394	.060
<i>Within subjects</i>					
State	1,48	.266	17.371	<.001	.266
State $\times$ Dose	3,48	.400	10.676	<.001	.400
State $\times$ Type	1,48	.008	0.391	.535	.008
State $\times$ Dose $\times$ Type	3,48	.086	1.509	.224	.086
Interval	5,44	.693	19.880	<.001	.228
Interval $\times$ Dose	15,138	.564	2.132	.012	.169
Interval $\times$ Type	5,44	.256	3.033	.019	.064
Interval $\times$ Dose $\times$ Type	15,138	.252	0.841	.630	.061
State $\times$ Interval	5,44	.691	19.658	<.001	.330
State $\times$ Interval $\times$ Dose	15,138	.673	2.660	.001	.195
State $\times$ Interval $\times$ Type	5,44	.111	1.097	.376	.027
State $\times$ Interval $\times$ Dose $\times$ Type	15,138	.330	3.038	.019	.067

Interlimb Movement Synchrony, High Amplitude 3-way MANOVA

	df	Pillai-Bartlett *	F	p	η_p^2
<i>Between subjects</i>					
Dose	3,48		13.801	<.001	.450
Type	1,48		1.601	.212	.032
Dose $\times$ Type	3,48		1.560	.211	.089
<i>Within subjects</i>					
Interval	5,44	.635	15.284	<.001	.373
Interval $\times$ Dose	15,138	.634	2.466	.003	.189

Interlimb Movement Synchrony, High Amplitude 3-way MANOVA					
	df	Pillai-Bartlett *	F	p	η_p^2
Interval $\times$ Type	5,44	.255	3.005	.020	.070
Interval $\times$ Dose $\times$ Type	15,138	.260	0.872	.596	.061
Interlimb Movement Synchrony, Low Amplitude 3-way MANOVA					
	df	Pillai-Bartlett *	F	p	η_p^2
<i>Between subjects</i>					
Dose	3,48		2.799	.049	.149
Type	1,48		0.370	.546	.008
Dose $\times$ Type	3,48		0.881	.458	.052
<i>Within subjects</i>					
Interval	5,44	.314	4.027	.004	.116
Interval $\times$ Dose	15,138	.599	2.297	.006	.173
Interval $\times$ Type	5,44	.043	.393	.851	.010
Interval $\times$ Dose $\times$ Type	15,138	.273	2.508	.043	.092

* Pillai-Bartlett Trace multivariate test criteria

Table 2
Oral Grasp Durations and Behavioral Response Frequencies [with 95% Confidence Intervals]

Variable	Saline	25 mg/kg	50 mg/kg	75 mg/kg
Grasp, total time				
mut	23.857 [11.662, 36.053]	21.906 [13.391, 30.421]	15.494 [9.278, 21.711]	18.138 [15.235, 21.042]
het	27.794 [18.188, 37.400]	23.484 [8.551, 38.416]	19.738 [12.922, 26.554]	27.747 [17.412, 38.083]
Grasp mean duration				
mut	10.853 [8.125, 13.581]	13.325 [10.000, 16.650]	10.052 [7.294, 12.811]	7.584 [6.594, 5.574]
het	17.952 [5.945, 29.958]	11.796 [3.859, 19.734]	11.446 [7.242, 15.650]	18.560* [8.218, 28.902]
Grasp/min				
mut	12.859 [4.923, 20.796]	17.053 [2.710, 31.396]	26.414 [14.959, 37.870]	12.719 [2.888, 22.549]
het	10.868 [2.033, 19.704]	27.261 [14.953, 69.475]	16.268 [7.949, 24.587]	22.589 [13.912, 31.265]
Oral Movements/min				
mut	8.029* [2.944, 13.115]	2.761 [0.406, 5.115]	0.987 [-0.601, 2.574]	4.100 [0.045, 8.155]
het	2.384 [-1.039, 5.806]	2.077 [-0.235, 4.390]	0.933 [-0.001, 1.866]	4.160 [0.809, 7.510]
Head movements/min				
mut	1.764 [-0.085, 3.613]	0.160 [-0.231, 0.551]	0	0
het	1.076 [-0.386, 2.537]	0.170 [-0.267, 0.608]	0	2.109 [-1.903, 6.121]

Note. All data are group means for mutant (mut) and heterozygote (het) fetal subjects. L-Dopa doses (0, 25, 50, and 75 mg/kg) were randomly administered to either all mut or all het subjects within each litter.

* significant difference between heterozygous and mutant groups, $p = .004$