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Prenatal ontogeny of the dopamine-dependent neurobehavioral phenotype in Pitx3 deficient mice

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Abstract

Mouse models with prenatal alterations in dopaminergic functioning can provide new opportunities to identify fetal behavioral abnormalities and the underlying neural substrates dependent on dopamine. In this study, we tested the hypothesis that prenatal loss of nigrostriatal function is associated with fetal akinesia, or difficulty initiating movement. Specific behaviors were analyzed in fetal offspring derived from pregnant Pitx3^{ak}/2J and C57BL/6J dams on the last four days before birth (E15–18 of a 19-day gestation). Using digital videography, we analyzed: (a) Behavioral State, by quantification of high and low amplitude movements, (b) Interlimb Movement Synchrony, a measure of the temporal relationship between spontaneous movements of limb pairs, (c) Facial Wiping, a characteristic response to perioral tactile stimulation similar to the defensive response in human infants, and (d) Oral Grasp of a non-nutritive nipple, a component of suckling in the human infant. Pitx3 mutants showed a selective decrease in Interlimb Movement Synchrony rates at the shortest (0.1s) temporal interval coupled with significantly increased latencies to exhibit Facial Wiping and Oral Grasp. Collectively, our findings provide evidence that the primary fetal neurobehavioral deficit of the Pitx3 mutation is akinesia related to nigrostriatal damage. Other findings of particular interest were the differences in neurobehavioral functioning between C57BL/6J and Pitx3 heterozygous subjects, suggesting the two groups are not equivalent controls. These results further suggest that fetal neurobehavioral assessments are sensitive indicators of emerging neural dysfunction, and may have utility for prenatal diagnosis.

Keywords

Fetal behavior; prenatal movement; Parkinson's disease; Pitx3; dopamine

Prenatal dopamine-dependent behavioral development in the Pitx3 mouse model of Parkinson's disease

Several research models using Prenatal Programming or Fetal Basis of Adult Disease (FeBAD) frameworks (Barker, 1993), have connected changes in the developing dopamine system to the emergence of Parkinson's disease in later life (Calne & Langston, 1983;

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Carvey, Chang, Lipton, & Ling, 2003; Cory-Slechta, Thiruchelvam, Barlow, & Richfield, 2005; Li, 2009; Mattock, Marmot, & Stern, 1988). Research utilizing direct *in vivo* observation of the fetal rat has also revealed that most early emerging behaviors are dependent on the developing dopaminergic system (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). These early behaviors include spontaneous fetal movement and complex response to sensory stimuli. One example is the facial wiping in response to an aversive chemosensory or tactile stimulus (Robinson & Smotherman, 1992), which is highly similar to coordinated defensive responses evoked from human infants (Brazelton & Nugent, 1995). Another example, the oral grasp response to perioral presentation of a non-nutritive artificial nipple (Robinson, Hoeltzel, & Smotherman, 1995) shows striking similarities to suckling, a complex action pattern in mammals, including human infants. Although these studies demonstrate a general dependence of early emerging behaviors on dopaminergic systems and receptor subtypes, the specific circuitry driving the ontogeny of these fetal behaviors has yet to be identified.

One candidate to help uncover the precise neural substrates is the *Pitx3^{ak/2J}* mouse model of Parkinson's disease (The Jackson Laboratory, Bar Harbor). The *Pitx3* aphakia mouse carries a mutation that prevents typical development of the dopamine system, but only in the nigrostriatal pathway. The *Pitx3* model was derived from a spontaneously occurring recessive mutation, first identified as the aphakia mouse due to the retinal degeneration also characteristic of this strain (Varnum & Stevens, 1968). In *Pitx3* homozygous mutants dopaminergic neurons fail to differentiate in the substantia nigra par compacta. By birth, 50% of the dopaminergic neurons are lost in the substantia nigra, while sparing cells in other areas of the dopamine system (Ardayfio, Moon, Leung, Youn-Hwang, & Kim, 2008; Hwang, Ardayfio, Kang, Semina, & Kim, 2003; Maxwell, Ho, Kuehner, Zhao, & Li, 2005). The substantia nigra differentiation relies solely on *Pitx3* signaling, thereby leading to a very selective loss of dopaminergic neurons solely in the nigrostriatal pathway (Nunes, Tovmasian, Silva, Burke, & Goff, 2003; Smidt, Smits, & Burbach, 2003).

We hypothesize that this very specific neural loss should reveal corresponding prenatal deficits in facial wiping and oral grasping responses, if nigrostriatal pathways are involved. In addition to the within litter heterozygous controls, fetal behavior was also observed in the C57BL/6J (C57) background strain. This control group was added to test a secondary hypothesis that C57 background mice and *Pitx3* heterozygous controls have equivalent fetal neurobehavioral developmental phenotypes, based on the practice of researchers using these two control groups interchangeably in studies of adult mouse behavior.

Methods

Subjects

Fetal subjects ($n=7-8$ each group at each age) were the offspring of C57BL/6J (*mus musculus*) pairing, or heterozygous *Pitx3* female mice mated to homozygous mutant males. This heterozygous to homozygous mating of the *Pitx3* mice produces litters of approximately 50:50 heterozygous to homozygous (mutant) offspring, providing within litter controls. Mutant offspring were easily identified and differentiated from heterozygous controls because of the retinal defect caused by the *Pitx3* mutation. This defect results in a substantial reduction in size of the *Pitx3* retina as compared to those in heterozygous subjects (Semina, Murray, Reiter, Hrstka, & Graw, 2000). During the breeding process, groups of three females were housed together with a male for about 5–7 days in polycarbonate cages lined with cob bedding. For environmental enrichment a small amount

of nesting material Tek Fresh (Harlan Teklad) was also provided. The mouse colony was maintained at a constant temperature of 21 ± 1 °C and humidity with a regulated 12:12 light/dark cycle. All mice had [*ad libitum*] access to food and water. For estimation of accurate gestational age, adult female mice were time mated with adult males. Conception (E0) was determined each morning by the presence of a sperm plug. Mice with positive results were singly housed until the day of testing, on one of the last four days of gestation (E15–18). At all times, the mice were cared for in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (Bakeman & Gottman, 1997). All the protocols and procedures were reviewed and approved by the Animal Care and Use Committee at Wake Forest School of Medicine, Winston Salem, NC.

Preparation of subjects

A female pregnant mouse was prepared for observation on the day of testing by brief exposure to isoflurane anesthesia (2% in 100% O₂), administered with a rodent non-rebreathing vaporizer. Spinal anesthesia known as a chemomyelotomy was performed on the unconscious dam which involved injecting 10 µl of 100% ethanol into the spinal canal between the first and second lumbar vertebrae (L₁–L₂). This method of anesthesia eliminates sensation in the lower half of the female's body without suppressing fetal activity. These advantages over general anesthesia have led to its extensive use in rodent research (Coppola & Millar, 1997; Kleven & Ronca, 2009; Robinson & Smotherman, 1992; Smotherman, Richards, & Robinson, 1984). After the administration of the spinal anesthesia, the pregnant female was gently secured to a holding apparatus made of Plexiglas using Velcro straps and immersed chest deep in a buffered isotonic saline bath (Locke's solution) maintained at 37.5 ± 0.5 °C. Reflex tests (hindlimb and tail pinch) were performed at 15 min intervals, in order to confirm the effectiveness of the spinal anesthesia. A small drape was lowered over the upper body of the female, providing a visual barrier between the female and manipulations made by the experimenter. The uterus was then externalized through a midline laparotomy of ~1.5 cm, after which both the female and the fetus were kept undisturbed for 15–20 min, ensuring full recovery from the isoflurane anesthesia.

After recovery and acclimatization, small incisions (~5 mm) were made in the uterine wall in order to externalize each fetal subject, one at a time, into the saline bath solution. Two subjects (one heterozygous and one mutant) were externalized from each *Pitx3* dam, and a single fetus was chosen from each pregnant C57 female. Gender of the fetal subjects was not determined. Therefore, both male and female subjects were included in this study. Externalization sites were alternated between right and left uterine horns. Careful removal of the embryonic membranes (the amnion and chorion) was performed, after which each fetus was allowed to rest on a submerged platform while remaining tethered to the placenta via the umbilical cord. At all times throughout the procedure the fetus and the dam were monitored closely for any signs of distress. Bright pink coloration of the fetal skin and absence of hypoxic behavior were used as positive signs of fetal wellbeing. Acute hypoxia can result from umbilical cord occlusion, and is readily identified by suppression of fetal activity followed by a unique pattern of limb and trunk movements (Smotherman & Robinson, 1986, 1988b). Any subjects exhibiting signs of distress or hypoxia are routinely removed from study.

Neurobehavioral Assessments

Externalized fetal subjects were digitally videographed during one of three behavioral assessments: (a) Spontaneous movement activity (5–10 min), during which time no external manipulations were made by the experimenter, (b) Tactile stimulation (1 min), involving a light stroking of the lateral vibrissal pad near the oral opening with a von Frey bristle (3.7g

force) (Smotherman & Robinson, 1990), or (c) presentation of a non-nutritive nipple (2–3 min) made of vinyl material.

Reports from (Golani & Fentress, 1985; Smotherman & Robinson, 1988a) reveal that exposure to tactile stimulation in the normal mouse fetus induces a unilateral wiping response, similar to adult rat or mouse grooming behavior. This type of coordinated limb movement is similar to the defensive response observed in the human infant (Brazelton & Nugent, 1995). Presentation of a non-nutritive nipple typically produces an oral grasping response in fetal rodents, bearing resemblance to suckling behavior in human newborns (Kleven, 2005; Kleven, Lane, & Robinson, 2004; Robinson et al., 1992; Smotherman, 2002; Smotherman, Goffman, Petrov, & Varlinskaya, 1997). After completion of neurobehavioral assessments, the dam and fetal subjects were euthanized.

Behavioral State—We previously reported in fetal mice (Kleven & Ronca, 2009) patterns of state-like behavior similar to those reported for infant rats (Blumberg & Lucas, 1994, 1996; Karlsson, Gall, Mohns, Seelke, & Blumberg, 2005; Seelke, Karlsson, Gall, & Blumberg, 2005). We defined state-like movements in mouse fetuses as either: (a) High Amplitude, smooth seemingly coordinated movements such as stretching or peddling, during which upper body muscle tone was observed, or (b) Low Amplitude, small myoclonic twitch-like movements, thought to indicate active sleep, and occurring only during muscle atonia. Periods of continuous high amplitude or low amplitude movements, durations, and intervals between successive states were quantified from archived digital video using JWatcher Video software version 1.0 (Blumstein, Daniel, & Evans, 2006) as an event recorder. Means corresponding to state frequency, duration, proportion, and interstate intervals were analyzed in a series of 3-way ANOVAs. Follow up pair wise comparisons of significant main effects and interactions were analyzed using Fisher's PLSD. Effect sizes were calculated using partial η_p^2 . An alpha of $p < 0.05$ was set for all statistical tests which were run using Statistica software Version 10 (Hailman & Hailman, 1993).

Interlimb Movement Synchrony—Interlimb Movement Synchrony is the temporal organization between the spontaneous movement of two limbs (Kleven, Lane, et al., 2004). In order to create profiles of Interlimb Movement Synchrony, spontaneous movement was scored one limb at a time during normal playback speed (30fps) on a 22" diagonal screen Hewlett Packard Monitor. JWatcher Video Software version 1.0 (Blumstein, et al., 2006) was used as the event recorder. To insure synchronization of the event records for each limb, the digital video was cued to the identical frame. Because prior work indicated that forelimb movements are an excellent indicator of overall development or delay (Kleven, Lane, et al., 2004; Kleven & Ronca, 2009; Robinson & Smotherman, 1988), only the forelimbs were scored for this study. Spontaneous movement of the forelimbs was defined as any active movement of a limb without regard to direction or duration. Scoring reliability was established for each coder, with a kappa-adjusted intra-rater correlation between multiple event files of the same limbs of 0.95, and a kappa-adjusted inter-rater correlation of 0.90.

Scored event files were prepared for analysis with computer software created specifically for examination of Interlimb Movement Synchrony. Scored files for the individual forelimbs were combined, and the lag time between successive movements of limbs within the pair calculated and rounded to the nearest 0.1 s. These intervals of synchronous movements were quantified in bins of 0.1 s intervals from 0.0 s (perfect interlimb synchrony) to 0.5 s (one half second lag between right and left limb movement). Prior work using this measure in fetal rats revealed the majority of synchronous movements to be clustered within this time period (Kleven, Lane, et al., 2004). Frequencies for each bin were then standardized by movement rates on a subject by subject basis, in order to produce relative rates of Interlimb synchrony that were not affected by the activity level of individual subjects.

The resulting profiles of Interlimb Movement Synchrony were analyzed in a 4-way repeated measure MANOVA, using the *Pillai-Bartlett Trace* multivariate test criteria, with State and synchrony Intervals treated as repeated measures, and strain Type, (C57, heterozygous, or mutant), and Gestational Age (GA) treated as between-subjects measures. Post hoc pairwise comparisons restricted to significant main effects and interactions were analyzed using Fisher's PLSD. Effect sizes were calculated using partial η_p^2 . An alpha of $p < 0.05$ was set for all statistical tests which were run using Statistica software Version 10 (Hailman & Hailman, 1993).

Facial Wiping—Studies have previously described the typical response of the fetal rat to tactile stimulation of the oral area as a Facial Wiping response. This coordinated action pattern is similar in nature to grooming responses seen in adult animals, and has been found to be a sensitive indicator of neural dysfunction in the rodent fetus (Kleven, Queral, & Robinson, 2004; Simonik, Robinson, & Smotherman, 1993; Varlinskaya, et al., 1994). Behavioral responses to tactile stimuli were scored during frame-by-frame playback using JWatcher Video software version 1 (Blumstein, et al., 2006). Evoked behaviors were quantified as: (a) Flails, a movement of the limb that does not make contact with the peri-oral region, (b) Single Wipes, single limb movements that make contact with the peri-oral region, (c) Conjugate Wipes, a simultaneous wipe of facial area with both forelimbs, and (d) General Movements of the body or head. Latency from the beginning of tactile stimulation until the first fetal behavioral response was also quantified (Kleven, 2005; Kleven, Queral, et al., 2004). Results were analyzed using a series of 2-way ANOVAs. Follow up pair wise comparisons of significant main effects and interactions were analyzed using Fisher's PLSD. Effect sizes were calculated using partial η_p^2 . An alpha of $p < 0.05$ was set for all statistical tests which were run using Statistica software Version 10 (Hailman & Hailman, 1993).

Oral Grasp—Presentation of a non-nutritive artificial nipple to a fetal rat typically produces an Oral Grasp response that is most fully expressed in altricial rodents a few days before birth (Robinson, et al., 1992). Oral grasp responses in fetal mice were scored during frame-by-frame playback using JWatcher Video software version 1 (Blumstein, et al., 2006). Behaviors were quantified as follows: (a) Latency to first Grasp, the delay between the first presentation of the artificial nipple and the first Grasp, (b) Grasp, closing of the jaw with the nipple in the mouth, (c) Oral events, licking and mouthing movements, (d) General Movements, includes aversive movements of the head or body/trunk, and (e) Limb Activity, forelimb or hindlimb movement. Results were analyzed as described for Facial Wiping.

Results

Behavioral State

Similar to prior findings with E18 fetal mice (Kleven & Ronca, 2009), alternation of High and Low Amplitude behavioral states were observed at all ages for the three groups (C57, heterozygous, and mutant). Figure 1-A depicts the frequencies of transition between the two states for each age observed. Analyses revealed a main effect of Gestational Age (GA), $F_{3,75} = 707.945$, $p < .001$, $\eta_p^2 = .904$, but no effect of Type, $F_{2,75} = 0.377$, $p = .687$, $\eta_p^2 = .010$, or GA $\times$ Type interaction, $F_{6,75} = 0.472$, $p = .827$, $\eta_p^2 = .036$. Post hoc evaluation revealed significantly elevated transition frequencies on E16 relative to later ages for all groups, with C57 subjects elevated relative to all other ages (E15, E17, and E18). In contrast, 3-way analyses of state duration revealed no main or interaction effects of GA, $F_{3-6,75} = 0.663 - 1.496$, $p = .222-.680$, $\eta_p^2 = .049 - .095$ (Figure 1-B). Significant effects were seen in the main effects of both Type, $F_{2,75} = 8.042$, $p = .001$, $\eta_p^2 = .177$, and State, $F_{1,75} = 28.531$, $p < .001$, $\eta_p^2 = .276$, with a significant State $\times$ Type interaction, $F_{2,75} = 5.550$, $p = .006$, $\eta_p^2 = .036$.

= .129. Post hoc analyses revealed significantly reduced durations of Low Amplitude state on E15 and E18 for *Pitx3* mutant and heterozygous subjects, with correspondingly significant increases in High Amplitude behavior relative to C57 fetal subjects.

Interlimb Movement Synchrony

Rates of spontaneous limb movement changed little across gestation in the three fetal mouse groups (C57, heterozygous, or mutant), with the mutant and heterozygous mice showing reduced rates on the last two days relative to C57 fetuses (Figure 1-C). Statistical analyses revealed a main effect of Type, $F_{2,77} = 6.551$, $p = .002$, $\eta_p^2 = .145$, but no effect of Gestational Age (GA), $F_{3,77} = 0.417$, $p = .741$, $\eta_p^2 = .016$, or GA $\times$ Type interaction, $F_{6,77} = 1.457$, $p = .204$, $\eta_p^2 = .102$. Post hoc pairwise analyses revealed a significant increase in mutant and heterozygous movement rates relative to C57 on E17 and E18. Movement rates at E17 and 18 were also significantly reduced relative to E15 C57 rates.

Overall 4-way ANOVA of Interlimb Movement Synchrony revealed main effects of all factors (GA, Type, State, and Interval) with State $\times$ Interval, and State $\times$ Interval $\times$ GA interactions (Table 1). Forelimb pairs were analyzed separately by State, to determine if high and low amplitude movements contribute differentially to the overall synchrony profiles. Parsed by State, 3-way analyses of Low and High Amplitude synchrony revealed additional interactions of Interval $\times$ GA, and Interval $\times$ Type respectively. Pairwise comparison of Type at each Interval and Age, for Low Amplitude synchrony revealed significant differences between C57 and both mutant and heterozygous subjects, but only at the 0.1 s Interval on E17. All three groups reflect the main effect of Intervals through significantly elevated rates of synchronous movement at the 0.0 and 0.1 s Intervals relative to those 0.2 s on E15 and E18, with significant elevation of only the 0.0 s Interval, relative to all others, on E16–17 (Figure 2-A). This pattern of elevated frequencies at Intervals < 0.2 s is characteristic of Interlimb Movement Synchrony profiles in general (Kleven, Lane, et al., 2004; Kleven & Ronca, 2009). High Amplitude movements, in contrast, produced profiles that were elevated across most Intervals < 0.5 s (Figure 2-B). Pairwise comparison of Type at each Interval and Age, for High Amplitude synchrony revealed significant differences between C57 and both mutant and heterozygous mice, at the 0.0 s Interval on E15, and the 0.5 s Interval on both E16 and E17. Mutant subjects showed additional reduced rates relative to C57 and heterozygous at the 0.2 s Interval on the last two days prior to birth (E17–18).

Facial Wiping

Subjects from all three groups typically responded to perioral tactile stimulation with a flurry of forelimb movements characterized as either Swipes, that make contact with the oral-facial area, or Flails, that are non-contact forelimb movements (Kleven, Queral, et al., 2004; Robinson & Smotherman, 1991). The time period in-between the first contact with the von Frey hair and the first forelimb movement was defined as the Latency to 1st Response (Figure 3-A). A main effect of Type, $F_{2,70} = 27.275$, $p < .001$, $\eta_p^2 = .438$, and a Type $\times$ GA interaction, $F_{6,70} = 2.952$, $p = .013$, $\eta_p^2 = .202$, was revealed by 2-way ANOVA. There was no main effect of GA, $F_{3,70} = 0.632$, $p = .597$, $\eta_p^2 = .026$. Post hoc analysis revealed significantly longer delays to first response for the mutant subjects on E17–18. These times were also significantly longer than those observed on E15–16, revealing increasing delays across development, relative to both control groups. No significant differences or developmental trends were identified for either C57 or heterozygous subjects for this measure.

The flurry of limb responses to perioral stimulation constitutes a single bout of activity. Analysis of bout duration revealed a significant effect of Type, $F_{2,70} = 27.275$, $p < .001$, $\eta_p^2 = .438$, with no effect of GA, $F_{3,70} = 0.632$, $p = .597$, $\eta_p^2 = .026$, or Type $\times$ GA

interaction, $F_{2,70} = 27.275$, $p < .001$, $\eta_p^2 = .438$. C57 controls exhibited very short activity bouts, with mean durations and 95% CIs from E15–18 of 11.575, [4.391, 18.760], 8.127, [3.303, 12.950], 15.644, [7.852, 23.436], and 25.108, [8.9541, 41.263] respectively. In contrast, mutant and heterozygous offspring from Pitx3 dams had significantly longer bouts, up to ten times the length of C57 controls. Mean bout durations and 95% CI for mutant subjects from E15–18 were 90.128, [77.850, 102.406], 110.457, [91.915, 128.999], 100.373, [56.427, 144.319], and 95.838, [81.563, 110.114] respectively. Bout durations for heterozygous subjects did not differ significantly from those of mutant offspring. The means and 95% CI for heterozygous bouts from E15–18 were 78.006, [41.860, 114.151], 81.833, [37.036, 126.630], 167.847, [48.951, 286.744], and 112.972, [84.527, 141.417] respectively.

Quantification of Flail and Swipe frequencies within the Facial Wiping bout (Figure 3-B,C) revealed main effects of both GA, $F_{3,73} = 5.214$, $p = .003$, $\eta_p^2 = .176$, and Pattern (Flail vs Swipe), $F_{1,73} = 110.626$, $p < .001$, $\eta_p^2 = .602$, and a GA $\times$ Pattern interaction, $F_{3,73} = 4.236$, $p = .008$, $\eta_p^2 = .148$. There was no effect of Type, $F_{2,73} = 0.412$, $p = .664$, $\eta_p^2 = .011$, Type $\times$ GA, $F_{6,73} = 1.550$, $p = .174$, $\eta_p^2 = .113$, Type $\times$ Pattern, $F_{2,73} = 0.383$, $p = .683$, $\eta_p^2 = .010$, or 3-way interaction of Type, $F_{6,73} = 0.713$, $p = .541$, $\eta_p^2 = .055$.

Oral Grasp

Oral Grasp responses were present in fetuses from all three comparison groups at every age studied. Only two E16 C57 subjects and one E17 mutant fetus failed to grasp the artificial nipple. Figure 4-A depicts the Latency in seconds between the first presentation of the artificial nipple, and the first Grasp. Visual inspection of the data revealed a trend of *decreasing* latencies from E15–E18 for the C57 subjects, and *increasing* latencies across gestation for mutant subjects. A 2-way ANOVA confirmed the visual inspection, revealing a main effect of Type, $F_{2,75} = 57.129$, $p = .034$, $\eta_p^2 = .087$, with no effect of GA, $F_{3,75} = 0.305$, $p = .822$, $\eta_p^2 = .012$, and a Type $\times$ GA interaction approaching significance, $F_{6,75} = 1.964$, $p = .082$, $\eta_p^2 = .136$. Pairwise post hoc analysis revealed significant increases in Latency for C57 at E15, relative to mutant and heterozygous, and also relative to later (E16–18) C57 latencies. In contrast, mutant subjects had significantly longer latencies, relative to other groups, on E16 and E18, with E18 latencies also significantly elevated relative to mutant latency on E15.

There were no main effects of Type, $F_{2,75} = 0.003$, $p = .997$, $\eta_p^2 > .001$, $F_{2,75} = 0.023$, $p = .978$, $\eta_p^2 = .001$, GA, $F_{3,75} = 1.203$, $p = .315$, $\eta_p^2 = .046$, $F_{3,75} = 2.502$, $p = .066$, $\eta_p^2 = .091$, or significant interactions, $F_{6,75} = 0.762$, $p = .602$, $\eta_p^2 = .057$, $F_{6,75} = 0.087$, $p = .997$, $\eta_p^2 = .007$, for either the total or mean duration of Grasp respectively (Table 2). However, analysis of the rate of Grasps, expressed as the frequency of Grasps/min, revealed significant main effects of Type, $F_{2,75} = 7.946$, $p = .001$, $\eta_p^2 = .175$, and GA, $F_{3,75} = 4.250$, $p = .008$, $\eta_p^2 = .145$, but no Type $\times$ GA interaction, $F_{6,75} = 0.808$, $p = .567$, $\eta_p^2 = .061$. Post hoc comparisons revealed that C57 rates on E16–18 were reduced relative to those on E15. Mutant and heterozygous fetuses showed significantly elevated Grasp rates in comparison to C57 on E16–18. Mutant rates on E16 were significantly reduced relative to E15 rates as were E17 heterozygous rates.

General Movements such as Head and Righting movements were also quantified. Analysis of Head movements revealed no main effect of Type, $F_{2,75} = 2.341$, $p = .103$, $\eta_p^2 = .059$, GA, $F_{3,75} = 0.498$, $p = .684$, $\eta_p^2 = .020$, or interaction, $F_{6,75} = 0.577$, $p = .747$, $\eta_p^2 = .044$. Righting movements involve a trunk curl and simultaneous lateral peddling of the forelimbs, similar to those seen in newborn pup righting responses (Pellis, Pellis, & Teitelbaum, 1991). Significant main effects of both Type, $F_{2,75} = 8.313$, $p = .001$, $\eta_p^2 = .181$, and GA, $F_{3,75} = 4.654$, $p = .005$, $\eta_p^2 = .181$, were detected, but no Type $\times$ GA interaction was revealed, $F_{6,75} = 0.823$, $p = .556$, $\eta_p^2 = .062$. Similar to the Grasp and Oral movements/min results, post

hoc analyses revealed that E16–18 C57 rates were significantly reduced relative to E15 C57 and both other groups at all ages. E17 heterozygous subjects also had reduced rates relative to both other groups on E15.

Both forelimb and hindlimb activity were observed during the oral grasping response (Figure 4-B,C). An overall 3-way ANOVA revealed main effects of Type, $F_{2,75} = 4.734$, $p = .012$, $\eta_p^2 = .112$, GA, $F_{3,75} = 8.600$, $p < .001$, $\eta_p^2 = .256$, and Limbs, $F_{1,75} = 16.337$, $p < .001$, $\eta_p^2 = .179$, with significant interactions of Type $\times$ GA, $F_{6,75} = 2.554$, $p = .026$, $\eta_p^2 = .026$, and Limbs $\times$ GA, $F_{3,75} = 7.412$, $p < .001$, $\eta_p^2 = .229$. Neither the Limbs $\times$ Type, $F_{2,75} = 2.833$, $p = .065$, $\eta_p^2 = .070$, or Limbs $\times$ Type $\times$ GA, $F_{6,75} = 1.631$, $p = .150$, $\eta_p^2 = .115$, interactions were significant. Post hoc analyses of hindlimbs revealed significant increases in heterozygous and mutant rates versus C57 on E17–18, and elevated relative to all three groups on E15. Mutant and heterozygous subjects on E17 also had increased rates relative to E16 mutant and heterozygous subjects, while E18 heterozygous rates were significantly elevated relative to all three groups on E16. Similar to hindlimb analyses, post hoc tests of forelimb movements revealed significantly elevated rates for E17–18 heterozygous and mutant subjects compared with other subjects at any age.

Discussion

This study presents the first quantification of fetal neurobehavior in a genetically altered mouse model. Similar to prior work with other fetal rodents (e.g., Norway rat, spiny mice, C57Bl/6J laboratory mouse), the Pitx3 mouse fetus exhibited characteristic spontaneous and elicited neurobehavioral responses. Although differences in spontaneous behaviors across the three groups were observed, for most of the state and spontaneous movement measures, heterozygous controls were indistinguishable from the Pitx3 mutants. Only when parsing Interlimb Movement Synchrony by high and low amplitude states were distinctions between mutant and control subjects evident. Specifically, during high amplitude movements, mutant fetuses displayed a depressed profile of Interlimb Movement Synchrony rates relative to both heterozygous and C57 controls (Figure 2-B). These reduced rates were particularly evident on the last two days before birth (E17–18) at the 0.1 s interval. At other temporal intervals, rates of synchronous activity in mutant fetuses were similar to those of controls, thus a reduction at the 0.1 s interval may represent a slight shift towards longer latencies between limb movements. Longer latencies in initiating behaviors have also been reported for adult subjects from this mouse model (Hwang, et al., 2003), suggesting difficulty initiating coordinative movements or, akinesia, which is a hallmark symptom of Parkinson's disease.

Quantification of spontaneous behavior patterns such as state durations, state transitions, and limb movements revealed rates and proportions similar to those reported for other mammalian species, including the human fetus (de Vries, Visser, & Prechtl, 1985; de Vries, Visser, & Prechtl, 1988; de Vries, Visser, & Prechtl, 1982; Kleven & Ronca, 2008). However, developmental trends were less evident across gestation, when compared to more precocial species. This outcome may be due to the highly altricial nature of mouse development prior to birth, relative to other mammals (Clancy et al., 2007).

Response to perioral tactile stimulation and to presentation of an artificial nipple revealed striking differences between mutant Pitx3 subjects and both control groups. Mutant fetuses exhibited significant delays relative to heterozygous and C57 group in Facial Wiping (Figure 3-A) and Oral Grasp (Figure 4-A). Notably, these deficits persisted across the last four days of gestation with increasing delays to the first response for mutant Pitx3 subjects. Delayed responding was not due to an inability of the fetus to detect the stimuli since both mutant and control subjects responded with similar flurries of limb activity (Figures 3-B,C

and 4-B,C). However, this type of delayed response to a stimulus is strikingly reminiscent of akinesia, or difficulty initiating movement, is a hallmark symptom of Parkinson's disease (Gui et al., 2012). Taken together, our findings suggest that the akinesia reported herein as well as the altered patterns of synchronous movement in the mutant fetuses are due to the early loss of dopaminergic functioning in the Pitx3 mouse. This conclusion is also consistent with a sizeable corpus of literature examining the neurobehavioral alterations in the fetal rodent following manipulations of dopaminergic and opioid systems (Andersen, Umphress, Robinson, & Smotherman, 1993; Moody, et al., 1993; Smotherman, et al., 1993; Smotherman & Robinson, 1995; Varlinskaya, et al., 1994, 1995).

Although this study demonstrates that neurobehavioral assessment is effective in detecting damage to the developing nervous system that is of genetic origin, the neurobehavioral deficits observed provide powerful information about the developing dopaminergic system, specifically the nigrostriatal pathway. Prior investigations of fetal behavior used global manipulations of dopamine which may have resulted in more generalized outcomes. For example, administration of D₁ antagonists, or a combination of D₁ and D₂ receptor blockades were shown to increase overall movement activity in fetal rats (Moody, et al., 1993). In contrast, the Pitx3 mutants in this study were observed to have activity rates indistinguishable from controls. These D₁ and D₁/D₂ antagonists also increased mouthing and suckling movements, although no such reductions were observed in mutant Pitx3 subjects from this study. However, blocking only D₂ receptors in fetal rats revealed results similar to the present study with no appreciable change in activity, mouthing, or defensive wiping responses. Taken together, these results suggest the possibility that there may be a preferential loss of D₂ functioning in the Pitx3 mouse fetus (Moody, et al., 1993). However, it is also likely that the differences that exist between these studies of global dopaminergic function and the current study reflect the very specific damage to dopamine nigrostriatal circuitry observed in Pitx3 fetus. Specifically, this comparison points to deficits in the nigrostriatal pathway as the principle mechanism leading to fetal delays in initiating behavioral responses.

Finally, this study confirms the sensitivity of fetal neurobehavior in the assessment of neural dysfunction. Both evoked and spontaneous measures of fetal behavior revealed identifiable deficits in this genetically altered mouse model of Parkinson's disease. At the same time, comparison between heterozygous controls and subjects from the C57 background strain revealed few differences. Those differences that were detected were striking, however. Specifically, mean rates of limb movement on the days prior to birth were significantly disparate between the two control groups (see Figures 1-B,C and 4-C), with rates of movement for heterozygous subjects elevated similar to those of the Pitx3 mutants. These results suggest that although similar, the two control groups studied are not interchangeable. Depending on the neurobehavioral measure and age studied, opposite results could be obtained. Taken together this study demonstrates the specificity of fetal observations in the detection of neurobehavioral deficits prior to birth. Further, it highlights the sensitivity of these measures, and potential utility in the study of genetically altered animal models of neurological dysfunction such as Parkinson's disease.

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Abbreviations

C57	C57BL/6J
GA	gestational age
Het	heterozygous
Mut	mutant
SEM	standard error of the mean

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Figure 1.

Behavioral state and forelimb activity. (Panel A) Bars depict the mean frequency of transitions between high and low amplitude behavior for mutant (mut), heterozygote (het), and C57BL/6J (C57) subjects on the last four days before birth. All behaviors were observed to fall into these two categories, with short periods of quiescence marking the transitions between the states. High amplitude was defined as a continuous period during which the fetal movements were smooth, fluid, and of large magnitude. In contrast, the low amplitude state was defined as a continuous period of twitch-like movements. Error bars depict the standard error of measure, *SEM*. (Panel B) Bars depict the mean duration of high amplitude (top) and low amplitude (bottom) behaviors in seconds for fetal mice on each of the last four days of gestation. High amplitude state was defined as a continuous period during which the fetal movements were smooth, fluid, and of large magnitude. In contrast, the low amplitude state was defined as a continuous period of twitch-like movements. Error bars represent the standard error of measure, *SEM*. Asterisks denote significant differences between C57 subjects and both mut and het groups on E15 and E18. (Panel C) Bars represent the mean rates of forelimb movement per min on the last four days before birth. Error bars depict the standard error of measure, *SEM*. Asterisks denote significant differences between C57 and both mut and het subjects on E17 and E18.

Figure 2.

Interlimb Movement Synchrony. (Panel A) Profiles present the mean relative frequency of Interlimb Movement Synchrony during low amplitude movements across the last four days of gestation for mutant (mut), heterozygote (het), and C57BL/6J (C57) fetal mice. Low amplitude movements were defined as those possessing a twitch-like property. Profiles are standardized by activity level of the forelimbs, and expressed as rates per minute at each of the .1 s intervals. Each data point represents the relative rate of movement synchrony for the corresponding interval of time (e.g. .1, .2 s, etc.). Synchrony intervals represent the lag in time between movements within a limb pair. Error bars depict the standard error of measure (SEM). Asterisks denote significant difference between groups at a specific interval. (Panel B) Profiles present Interlimb Movement Synchrony of high amplitude movements across the last four days of gestation for mutant (mut), heterozygote (het), and C57BL/6J (C57) fetal mice. High amplitude movements were defined as those characterized as smooth, fluid, and

of large magnitude. Profiles are standardized by activity level of the forelimbs, and expressed as rates per minute at each of the .1 s intervals. Each data point depicts the relative rate of movement synchrony for the corresponding interval. A synchrony interval is the lag in time between movements within a limb pair. Error bars represent the standard error of measure, *SEM*. Asterisks denote significant differences between groups at a specific interval.

Figure 3.

Behavioral response to an oral/facial tactile stimulus. (Panel A) Bars represent the mean latency in seconds of the first limb response to an oral/facial tactile stimulus (von Frey bristle of 3.7g force) during the last four days before birth in mutant (mut), heterozygote (het), and C57BL/6J (C57) mice. The latency to first response was calculated from the time of stimulus onset until the first observable forelimb movement. Error bars depict the standard error of measure, *SEM*. Asterisks denote significant differences between mut subjects on E17 and E18, and all other groups and ages. (Panel B) Bars depict the mean frequency of fetal swiping responses to the oral/facial tactile stimulus described above on each of the last four days of gestation. Swipes are single wiping strokes where only one paw at a time comes in contact with the facial area. In contrast, both paws wipe simultaneously in a conjugate wipe (not shown). Error bars depict the standard error of measure, *SEM*. (Panel C) Bars depict the mean frequency of flailing responses to the oral/facial tactile stimulus on

each of the last four days of gestation. Flails are noncontact wiping strokes of single limbs. Error bars depict the standard error of measure, *SEM*.

Figure 4.

Behavioral response to a non-nutritive nipple. (Panel A) Bars represent the mean latency in seconds of the first grasping response to a non-nutritive artificial nipple. The stimulus was presented to mutant (mut), heterozygote (het), and C57BL/6J (C57) mice on one of the last four days before birth. A grasp was defined as any closure of the jaw with the nipple in the mouth, including attempts of brief duration. Error bars depict the standard error of measure, *SEM*. Asterisks denote significant differences between subjects of the same gestational age. (Panel B) General hindlimb activity during presentation of a non-nutritive artificial nipple on the last four days of gestation. Bars depict the mean frequency per minute of hindlimb movements during the presentation period. Error bars represent the standard error of measure, *SEM*. Asterisks denote significant differences between C57 subjects and mut and het groups on E17 and E18. (Panel C) General forelimb activity during presentation of a non-nutritive artificial nipple on the last four days of gestation. Bars depict the mean

frequency per minute of forelimb movements during the presentation period. Error bars represent the standard error of measure, *SEM*. Asterisks denote significant difference between het and C57 subjects on E17, and both het and mut subjects and the C57 group on E18.

Table 1

Interlimb Movement Synchrony 4-way MANOVA					
	df	Pillai-Bartlett*	F	p	η_p^2
<i>Between subjects</i>					
GA	3, 77		30.695	<.001	.545
Type	2, 77		5.703	.005	.129
GA $\times$ Type	6, 77		1.017	.421	.073
<i>Within subjects</i>					
State	1, 77	0.075	6.209	.015	.075
State $\times$ GA	3, 77	0.074	2.054	.113	.074
State $\times$ Type	2, 77	0.018	0.703	.498	.018
State $\times$ GA $\times$ Type	6, 77	0.019	0.246	.959	.019
Interval	5, 73	0.944	244.155	<.001	.578
Interval $\times$ GA	15, 225	0.399	2.300	.005	.057
Interval $\times$ Type	10, 148	0.031	2.744	.004	.032
Interval $\times$ GA $\times$ Type	30, 385	0.429	1.206	.214	.068
State $\times$ Interval	5, 73	0.706	35.011	<.001	.281
State $\times$ Interval $\times$ GA	15, 225	0.188	2.815	.022	.069
State $\times$ Interval $\times$ Type	10, 148	0.127	1.007	.441	.024
State $\times$ Interval $\times$ GA $\times$ Type	30, 385	0.242	0.652	.922	.055
<i>Interlimb Movement Synchrony, High Amplitude 3-way MANOVA</i>					
	df	Pillai-Bartlett*	F	p	η_p^2
<i>Between subjects</i>					
GA	3, 77		3.189	.032	.107
Type	2, 77		0.258	.773	.007
GA $\times$ Type	6, 77		0.566	.756	.0423
<i>Within subjects</i>					
Interval	5, 73	0.762	46.751	<.001	.375
Interval $\times$ GA	15, 225	0.156	.823	.652	.038

Interlimb Movement Synchrony 4-way MANOVA

	df	Pillai-Bartlett*	F	p	η_p^2
Interval $\times$ Type	10, 148	0.246	2.074	.030	.049
Interval $\times$ GA $\times$ Type	30, 385	0.401	1.120	.307	.097

Interlimb Movement Synchrony, Low Amplitude 3-way MANOVA

	df	Pillai-Bartlett*	F	p	η_p^2
<i>Between subjects</i>					
GA	3, 77		9.339	< .001	.267
Type	2, 77		2.684	.075	.065
GA $\times$ Type	6, 77		0.111	.995	.009

Within subjects

Interval	5, 73	0.760	46.331	< .001	.504
Interval $\times$ GA	15, 225	0.312	4.676	.001	.079
Interval $\times$ Type	10, 148	0.225	1.874	.053	.013
Interval $\times$ GA $\times$ Type	30, 385	0.333	0.914	.600	.037

* Pillai-Bartlett Trace multivariate test criteria

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

Variable	E15	E16	E17	E18
Grasp, total time				
mut	37.850 [23.691, 52.009]	30.207 [14.346, 46.068]	36.881 [10.163, 63.598]	30.574 [20.018, 41.130]
het	26.131 [7.955, 44.306]	32.885 [18.652, 47.118]	32.730 [15.962, 49.497]	42.253 [19.026, 65.480]
C57	28.559 [17.344, 39.774]	24.816 [3.058, 46.573]	35.069 [26.513, 43.626]	45.668 [28.963, 62.373]
Grasp mean duration				
mut	16.647 [8.165, 25.130]	15.641 [9.566, 21.716]	22.454 [−6.125, 51.033]	24.629 [10.870, 38.388]
het	12.313 [7.146, 17.480]	15.470 [11.032, 19.909]	22.108 [12.623, 31.594]	26.969 [−1.035, 54.972]
C57	15.105 [8.615, 21.596]	15.763 [5.191, 26.335]	22.124 [15.545, 28.704]	23.168 [17.469, 28.867]
Grasp/min				
mut	2.773 [1.172, 4.374]	1.519 [0.872, 2.166]	2.127 [0.623, 3.631]	1.716 [0.674, 2.757]
het	2.447 [1.196, 3.697]	1.955 [1.153, 2.756]	1.317 [0.898, 1.735]	2.192 [0.694, 3.690]
C57	1.979 [1.060, 2.898]	0.650 [0.043, 1.256]	0.928 [0.678, 1.178]	0.671 [0.406, 0.936]
Oral Movements/min				
mut	3.893 [1.206, 6.580]	5.911 [1.751, 10.071]	8.716 [1.871, 15.561]	10.528 [3.980, 17.076]
het	4.743 [0.822, 8.664]	9.384 [3.616, 15.152]	6.979 [2.323, 11.634]	16.178 [4.016, 28.339]
C57	3.938 [2.012, 5.865]	3.646 [1.222, 6.070]	2.039 [1.125, 2.952]	2.458 [0.923, 3.994]
Head movements/min				
mut	4.419 [1.463, 7.374]	2.396 [0.115, 4.676]	6.829 [−5.309, 18.967]	2.792 [−1.551, 7.135]
het	3.225 [0.910, 5.540]	2.679 [1.187, 4.170]	3.001 [−0.118, 6.121]	3.662 [−0.006, 7.330]
C57	0.990 [0.575, 1.404]	2.381 [0.241, 4.521]	2.023 [−0.034, 4.080]	0.886 [0.207, 1.564]
Righting movements/min				
mut	2.600 [1.315, 3.884]	1.519 [0.872, 2.166]	2.322 [0.672, 3.972]	1.716 [0.674, 2.757]
het	2.447 [1.196, 3.697]	1.955 [1.153, 2.756]	1.317 [0.898, 1.735]	1.746 [0.846, 2.647]
C57	1.979 [1.060, 2.898]	0.650 [0.043, 1.256]	0.928 [0.678, 1.178]	0.671 [0.406, 0.936]

Note. All data are group means for mutant (mut), heterozygote (het), and background C57BL/6J (C57) fetal subjects on the last four days of gestation (E15–E18).