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Repetitive behavior and increased activity in mice with Purkinje cell loss: a model for understanding the role of cerebellar pathology in autism

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Abstract

Repetitive behaviors and hyperactivity are common features of developmental disorders, including autism. Neuropathology of the cerebellum is also a frequent occurrence in autism and other developmental disorders. Recent studies have indicated that cerebellar pathology may play a causal role in the generation of repetitive and hyperactive behaviors. In this study, we examined the relationship between cerebellar pathology and these behaviors in a mouse model of Purkinje cell loss. Specifically, we made aggregation chimeras between Lc/+ mutant embryos and +/+ embryos. Lc/+ mice lose 100% of their Purkinje cells postnatally due to a cell-intrinsic gain-of-function mutation. Through our histological examination, we demonstrated that Lc/+↔+/+ chimeric mice have Purkinje cells ranging from zero to normal numbers. Our analysis of these chimeric cerebella confirmed previous studies on Purkinje cell lineage. The results of both open-field activity and hole-board exploration testing indicated negative relationships between Purkinje cell number and measures of activity and investigatory nose-poking. Additionally, in a progressive-ratio operant paradigm, we found that Lc/+ mice lever-pressed significantly less than +/+ controls, which led to significantly lower breakpoints in this group. In contrast, chimeric mice lever-pressed significantly more than controls and this repetitive lever-pressing behavior was significantly and negatively correlated with total Purkinje cell numbers. Although the performance of Lc/+ mice is probably related to their motor deficits, the significant relationships between Purkinje cell number and repetitive lever-pressing behavior as well as open-field activity measures provide support for a role of cerebellar pathology in generating repetitive behavior and increased activity in chimeric mice.

Introduction

Repetitive and restrictive behaviors are a major behavioral component of many psychiatric and neurological conditions as well as a variety of developmental disorders, including autism spectrum disorder (ASD; e.g. Bleuler, 1950; Hutt & Hutt, 1970; Bodfish *et al.*, 2000; Militerni *et al.*, 2002). In autism, repetitive behavior may function to control behavior–environment relations such as access to reinforcers, or to gain access to or eliminate particular types of stimulation such as hyperarousal (Ridley, 1994; Kennedy *et al.*, 2000). Hyperactivity often accompanies repetitive behavior in developmental disorders, with approximately 30% of ASD children comorbid for attention deficit/hyperactivity disorder (ADHD; Simonoff *et al.*, 2008).

Although developmental disorders are associated with varied neuropathology, an abnormal cerebellum has been commonly reported. In autism, substantial reductions in the number of cerebellar Purkinje cells have been the most consistent finding in post-mortem brains (Palmen *et al.*, 2004). Pierce & Courchesne (2001) reported that the rate of repetitive behavior in a group of ASD children was negatively correlated with the area of cerebellar vermal lobules VI–VII. They also reported that the ASD children demonstrated increased non-exploratory activity over controls, although a relationship between activity level and cerebellar size was not apparent. However, both functional and morphometric cerebellar abnormalities have been consistently demonstrated in ADHD (Soliva *et al.*, 2009).

We used an animal model to investigate the role of the cerebellum in the occurrence of both repetitive behavior and increased activity by varying the number of Purkinje cells. This was accomplished by making aggregation chimeras between heterozygous lurcher (Lc/+) and wildtype (+/+) embryos. The lurcher mutant mouse is a semi-dominant mutation in the $\delta 2$ glutamate receptor (GRID2) gene that results in the loss of all Purkinje cells between the second and fourth weeks of life (Caddy & Biscoe, 1979; Zuo *et al.*, 1997). The use of the lurcher mutation in mouse chimeras provides a reasonable model system to study cerebellar function (Martin *et al.*, 2003, 2004, 2006).

In this study we tested lurcher, chimeric and wild-type mice in behavioral paradigms designed to assess activity, exploration and repetitive behaviors. Activity was measured as ambulation in an open-field, exploratory behavior was measured by quantifying nose-pokes

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through a hole-board and repetitive behavior was measured as patterns of learned behavior in the progressive-ratio (PR) task.

The PR task is a commonly used lever-press task that programs a systematic increase in the value of the fixed-ratio (FR) requirement following reinforcer delivery. Subjects are free to lever-press as frequently or infrequently as they choose, but must exert progressively more lever-presses to obtain successive reinforcers. As repetitive behavior can take any form and occur in virtually any environment (Kennedy *et al.*, 2000), we reasoned that this task would allow us to explore the role of cerebellar pathology in repetitive behaviors similar to those observed in autism.

We found that open-field activity was negatively correlated with total Purkinje cell number. Similarly, repetitive lever-pressing was negatively related to total Purkinje cell number. These results provide support for a role of cerebellar pathology in generating increased activity and repetitive behaviors.

Methods

Subjects

The original stock of lurcher mice, B6CBACaA^{w-J}/*A-Grid2*^{Lc}, was obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and was maintained at the University of Tennessee Animal Care Facility and the University of Memphis Animal Care Facility. From these mice, three groups of animals were formed: lurcher (gene symbol Lc/+), control (gene symbol +/+) and chimeric mice with varying proportions of cells from the +/+ and Lc/+ genotypes.

Mouse chimeras were made by the standard method of fusing two- four- to eight-cell embryos (Goldowitz *et al.*, 1992). Female +/+ and Lc/+ mice were superovulated with pregnant mare serum (4–5 IU; Sigma, St Louis, MO, USA) followed by human chorionic gonadotropin (Sigma; 4–5 IU) 44 h later. Then, +/+ females were mated with Lc/+ males and Lc/+ females were mated with +/+ males. The following morning, females were checked for the presence of a vaginal plug. All females found positive for a vaginal plug were distinguished as donor females. Two days after the appearance of the vaginal plug, embryos were harvested from the oviducts of the donor females who were overclosed with anesthesia (Averlin, Sigma-Aldrich, St. Louis, MO, USA). The harvested embryos were then subjected to a light pronase treatment to remove the zonae pellucidae, aggregated in pairs, and cultured overnight (37°C, 5% CO_2) in drops of medium (Mullen & Whitten, 1971) covered with paraffin oil. The following afternoon, successfully fused embryos were transplanted into the uterine horns of pseudo-pregnant ICR females. Avertin was administered intraperitoneally as the general anesthetic for the ICR host females prior to transplantation. All surgical procedures and animal care were in accordance with National Institutes of Health guidelines for the use of animals in research and were approved by the institutional animal care and use committees of the University of Memphis and the University of Tennessee Health Science Center. Because the genotype of the embryos at the time of chimera production was necessarily unknown, only half of the progeny were expected to have a truly chimeric, or mixed, genotype (Lc/+↔+/+), while the remaining animals were expected to be entirely composed of Lc/+ or +/+ cells. Of the 81 mice generated, 36 were determined to be truly chimeric in genotype, consistent with these expectations.

In addition to mice generated as chimeras, another group of nine +/+ and nine Lc/+ mice were derived from the breeding colony. Mice in all groups were housed with siblings of the same sex until they reached maturity (< 3 months of age), with food and water available *ad libitum*. They were then transferred to the University of Memphis Animal care facility, individually housed, and then tested following a 2-week period of acclimatization. The animals were maintained under a 12/12-h light–dark cycle (lights on at 06 : 00 h).

Apparatus

The open-field activity and exploration assessments were done in eight identical test environments (Model ENV-515; Med Associates, St. Albans, VT, USA) interfaced to a computer. The open field of each test environment measured 27.9 × 27.9 cm and was enclosed by vertical polycarbonate walls. Three 16-beam infrared arrays detected movement in the *x*, *y* and *z* axes. For the exploration task, a stainless steel insert with four rows of four evenly spaced holes on 10.6-cm centers was placed in each test environment (Model ENV-515HBM). Each hole measured 2.2 cm in diameter with a depth of 1.8 cm.

The progressive-ratio task was carried out in four identical operant chambers (Model ENV-307; 15.9 cm long, 14 cm wide, 12.7 cm tall; Med Associates). Each was equipped with a retractable lever (Model E23-07) positioned on the short wall of the chamber such that the lever, when extended, was 1.0 cm from the wall and 2.5 cm above the grid floor. A liquid dipper presented reinforcement consisting of 0.02 mL of evaporated milk sweetened with 0.2% sucrose solution into a food magazine centered on the short wall and adjacent to the lever. The duration of dipper presentation was 7 s throughout training and testing. An infrared photobeam positioned inside the food magazine was used to detect entries into the magazine. A white light located within the food receptacle was used to signal reinforcement delivery. An additional infrared photobeam, used to detect locomotor activity in the rear of the chambers, was located 2 cm above the cage floor and 2 cm from the rear of the chamber (opposite to the wall housing the food receptacle). The chambers were equipped with a house light, located on the rear wall opposite the food magazine and lever, which served as a stimulus to signal when the experimental contingencies were in effect. Each experimental chamber was enclosed within a sound-attenuating Melamine cubicle with a small exhaust fan to provide continuous airflow and background noise. Data were collected with a microcomputer (Spider; Paul Fray Ltd, Cambridge, UK) which controlled the delivery of the liquid reinforcer, and recorded lever-pressing, entries into the food magazine and locomotor activity in the test chamber with a resolution of 0.01 s.

Open field monitoring

Mice were individually placed into the center of the open field and allowed to explore the quadrant freely for 60 min. The movements of each mouse were detected by 16 photobeams on both the *x* and the *y* axes of the floor grid positioned 2.2 cm above the floor creating 17 activity quadrants. In addition, vertical movements were detected by an additional set of 16 photobeams positioned in the *z* axis 7 cm above the floor. Med Associates software was then used to analyse the data and generate counts of photocell beam breaks, vertical jumps, clockwise and counterclockwise rotations, in addition to total jumping time, ambulatory time, resting time, distance traveled and average velocity.

Exploration task

One week after the completion of the open field monitoring, mice were individually placed into each test environment with the hole-board insert covering the open field and allowed to explore freely for 60 min.

Exploration of each hole was detected by the *x* and *y* photobeams and recorded as a nose-poke. Data from six mice simultaneously tested in this task were lost due to a computer malfunction.

Assessment of hunger motivation

Because PR tasks are often used to assess differences in motivation, we tested mice in a probe aimed at determining the presence of any difference between groups in their hunger motivation. In this probe, mice were gradually food-deprived until they weighed between 85 and 90% of their baseline body weight and then allowed to free-feed on the 0.2% sucrose/evaporated milk solution for 40 min in order to familiarize them with the food and weighing procedures, and to prevent food neophobia and reduce handling stress. Each mouse was weighed immediately prior to the beginning of the free-feed session and again after 20 and 40 min. Mice were then tested on two separate days (Probes 1 and 2). Increases in the body weight of each mouse after feeding were used as an index of hunger motivation.

Progressive ratio procedure

Food deprivation was maintained throughout PR testing with a restricted diet of Purina mouse chow (approximately 3 g daily) given after each daily test occurred. Using procedures described in Skjoldager *et al.* (1993), all mice were trained to lever-press. Once all animals reliably obtained 100 reinforcers during 15 min, FR 1 sessions, they began PR training. During each daily PR session, subjects were reinforced for lever-pressing under an arithmetically increasing FR 5 schedule of reinforcement (i.e. 5, 10, 15, 20, etc., lever-presses were necessary to obtain reinforcement), using a procedure similar to that described by Hodos & Kalman (1963). An experimental session terminated when the subject failed to lever-press for five consecutive minutes. The lever-press that completed each FR within the PR schedule was reinforced at lever release. Thus, upon activation of the liquid dipper, the house light was extinguished and the light within the food magazine was illuminated for 1 s. During this 1-s time out, lever-press responses were counted but had no scheduled consequences. All subjects were tested for 17 daily sessions, a time frame in which asymptotic levels of performance were achieved in our own pilot studies.

Dependent measures

In addition to total Purkinje cell numbers, a total of eight behavior-dependent measures were compared among wild-type control, chimeric and lurcher (Lc/+) mice. Breakpoint was defined as the value of the last completed (reinforced) ratio. Breakpoints are directly related to the total number of lever-presses, although there are only minimal temporal constraints on responding (Hodos, 1961; Hodos & Kalman, 1963; Mello *et al.*, 1984; Winger & Woods, 1985; Roberts *et al.*, 1989a,b). Several measures of the temporal characteristics of lever-pressing were also obtained for each completed ratio. A post-reinforcement pause (PRP) was defined as the time interval between the end of the 1-s reinforcer delivery period and the first lever-press following reinforcement. Mean inter-response times (IRTs) were computed by summing for each ratio the time from the offset of a lever-press to the onset of the subsequent lever-press for all responses within that ratio. The summed IRTs were then divided by the response requirement minus 1 to obtain the mean IRT for each ratio. Average response durations were computed for each ratio by summing the time the lever was held in the downward position (switch closure) and dividing by the number of responses in the ratio.

Several measures of task-related behavior were also obtained for each completed ratio. A food entry was counted whenever a mouse blocked the photocell at the opening of the food magazine. In addition, food entry latencies were obtained following each reinforcer delivery. Time-out responses were those lever-presses that occurred during the 1-s time-out period that followed activation of the liquid dipper. An activity 'count' was recorded when the photocell beam at the rear of the operant chambers was broken. Food entries, time-out responses and activity counts were recorded for each completed ratio.

Mean IRTs, response durations and food entry latencies obtained for each ratio were then rank ordered and the median values were computed. For these measures, the median, instead of the mean, was used as a measure of central tendency for these values because the median was less affected by relatively extreme values.

Histology

Following the completion of behavioral testing, all chimeric mice were overdosed with anesthesia (Avertin) and transcardially perfused with physiologic saline followed by acetic acid/95% ethanol fix (1 : 3) for 20 min. Heads were removed at the cervical vertebrae and immersion-fixed overnight in the same fixative. Heads were then placed in 70% ethanol before the brains were dissected out and dehydrated with increasing concentrations of ethanol. Mid-sagittal sectioned brains were then cleared with a series of xylenes and infiltrated with paraffin, followed by embedding in paraffin blocks. Paraffin-embedded brains were sectioned in the sagittal plane using a microtome set for 8 μm thickness. A set of eight chimeric brains was sectioned in the coronal plane at the same thickness. Every 25th section was then mounted on Superfrost+ slides. Immunocytochemistry using an anti-Calbindin antibody (Chemicon, Bellerica, MA, USA) was then performed on all slides to enable the identification of Purkinje cells. After incubation in primary antisera, slides were exposed to an anti-rabbit biotinylated secondary antibody and subsequently visualized using the ABC reaction (Vectastain kit; Vector Labs, Burlingame, CA, USA). Purkinje cells were identified by the brown reaction product of diaminobenzidine. Slides were then counterstained with cresyl violet and dehydrated through ascending alcohol concentrations before being cleared with xylenes. Glass coverslips were applied with Permount.

Purkinje cell counts

Purkinje cell nuclei were identified with the aid of a standard brightfield microscope equipped with 10× eyepieces and a 25× objective. The nuclei are easily identifiable at this magnification due to their light appearance in comparison with the surrounding darkly stained cytoplasm. Purkinje cell nuclei in every 25th section throughout the entire cerebellum were counted with the exception of the paraflocular lobe. Total numbers of Purkinje cells for the entire cerebellum were then estimated from this sampling and corrected for split nuclei using the Abercrombie correction factor (Abercrombie, 1946).

Data analysis

For the open-field monitoring tasks, each dependent measure was analysed using one-way ANOVA with group (control, chimeric or lurcher) as the between-subjects factor. In order to evaluate group differences in the behaviors emitted by the PR test subjects, each behavioral dependent measure was subjected to a two-factor ANOVA (Winer, 1971). In each of these analyses the between-subjects factor

was group (control, chimeric or lurcher) and the within-subjects factor was day (1–17). When appropriate, Dunnett's '*t*' tests were used to further analyse group differences in behavior.

A second set of analyses was conducted to determine potential relationships between the total number of cerebellar Purkinje cells and all behavior-dependent measures in the open-field, exploration and PR tasks. Additionally, the relationship between PR breakpoint and the other seven variables on the PR task was also evaluated. Both analyses involved the calculation of Pearson product-moment correlation coefficients using a two-tailed alpha level set to $P < 0.05$. Although the same mice were used in both the open-field and the exploration tasks, data from six mice in the exploration task were unavailable for analysis due to mechanical error of the testing equipment. The PR data analyses included behavioral measures averaged over the final four test days. Log values were used for the temporal measures of IRT and response duration in order to normalize these data. Twenty-four Lc/+↔+/+ chimeras and eight +/+↔+/+ wild-type control animals that underwent histological assessment were included in this analysis. One +/+↔+/+ wild-type control was excluded from this analysis because it was found to have a mean breakpoint more than three standard deviations above the control group mean.

Results

Two different sets of mice were used. The first set consisted of a total of 35 mice which were used in the open-field monitoring experiments. The second set of mice consisted of 64 mice which were trained and tested in the PR task; a subgroup of 22 of these mice was assessed for hunger motivation. Following histology, the mice generated as chimeras were assigned to either the chimeric, lurcher or wild-type groups based upon Purkinje cell number. Additional lurcher and wild-type mice were generated from the breeding colony. Of the 35 mice in the first set, 10 were found to be composed of both mutant and wild-type neurons (Lc/+↔+/+) in that they possessed cerebellar Purkinje cell numbers intermediate between those of +/+ and Lc/+ animals. Of the remaining mice, histology revealed that 12 had no cerebellar Purkinje cells (Lc/+ lineage), while 13 had normal numbers of cells (+/+ lineage). For the set of 64 mice used in the PR task, 26 were determined to be Lc/+↔+/+ chimeras, 20 were Lc/+ and 18 were +/+ (including nine Lc/+ and nine +/+ mice from the breeding colony). It should be noted that two chimeric mice with very few Purkinje cells (fewer than 1000) were dropped from the PR experiment (and all analyses) because they demonstrated gross motor impairment (i.e. ataxia) indistinguishable from that of the Lc/+ mice.

Histological analysis

Figure 1 shows the differences in cerebellar Purkinje cells amongst Lc/+, chimeric and control mice. Purkinje cell numbers of the mice used in the PR task were compared across three cerebellar lobule groupings (I–V, VI–VII, VIII–X) and between right and left sides of the cerebellar cortex. Purkinje cells were found to have an apparently equal distribution pattern across all three lobular groupings (data not shown), but differences in Purkinje cell number were evident between hemicerebella of a single animal, most notably in low-percentage chimeras (Table 1).

Open-field monitoring and hole-board exploration

Of the nine dependent measures obtained through the open field monitoring, ANOVA only revealed significant group differences in clockwise (Group; $F = 6.085$, d.f. = 2.32, $P = 0.006$) and counterclockwise rotations (Group; $F = 3.192$, d.f. = 2.32, $P = 0.05$). Dunnett's *t*-tests showed that Lc/+ mice had significantly more clockwise rotations than chimeric and wild-type mice, and significantly more counterclockwise rotations than wild-type mice. ANOVA of the number of nose-pokes during the hole-board exploration task revealed a significant difference between groups (Group; $F = 8.137$, d.f. = 2.26, $P = 0.002$). Dunnett's *t*-tests showed that chimeric mice (mean = 156) had more nose-pokes than both control (mean = 105) and lurcher (mean = 76) mice.

Relationships between total Purkinje cell number in chimeric and wild-type mice and each dependent measure of the open-field and hole-board exploration experiments were examined via Pearson correlations. Negative relationships were found between total Purkinje cell number and photocell beam breaks ($r = -0.549$, d.f. = 21, $P = 0.007$; Fig. 2A), ambulatory time ($r = -0.527$, d.f. = 21, $P = 0.010$), distance traveled ($r = -0.543$, d.f. = 21, $P = 0.007$), jump counts ($r = -0.452$, d.f. = 21, $P = 0.031$; Fig. 2B) and counterclockwise rotations ($r = -0.567$, d.f. = 21, $P = 0.005$; Fig. 2C) during open-field monitoring. Probably related to the negative relationship between Purkinje cell number and measures of ambulation, there was also a positive relationship between total Purkinje cells and resting time ($r = 0.478$, d.f. = 21, $P = 0.021$). Pearson correlation also demonstrated a negative relationship between total Purkinje cell number and the number of nose-pokes ($r = -0.640$, d.f. = 15, $P = 0.006$; Fig. 2D) during the 60-min testing session using the hole-board. An analysis of each 10-min testing block revealed that this relationship was strongest during the first 10 min of the task ($r = -0.722$, d.f. = 15, $P < 0.001$).

Assessment of hunger motivation

All groups of mice demonstrated significant gains in body weight after both 20 and 40 min in the free-feeding probe sessions. Group differences were found in the amount of weight gain following each 40-min session (Group; $F = 5.34$, d.f. = 2.19, $P < 0.05$; Fig. 3). Dunnett's *t*-tests revealed that Lc/+ mice gained significantly more weight than control and chimeric mice following this probe.

Progressive-ratio analyses

All groups of mice rapidly acquired PR responding (Fig. 4). As shown in Fig. 4A, average breakpoint increased with repeated testing in all the groups (Day; $F = 2.98$, d.f. = 16.944, $P < 0.001$). There were, however, consistent group differences in breakpoint when considered over the 17 test days (Group; $F = 20.61$, d.f. = 2.59, $P < 0.001$). Dunnett's *t*-tests revealed that chimeric mice lever-pressed significantly more than controls, with an average breakpoint of 145.5 in comparison with the mean control breakpoint of 113.5. Lc/+ animals, in comparison, averaged significantly lower breakpoints (mean = 60.7) than wild-type controls.

Related to these group differences in breakpoint, there were group differences in the topography of lever-pressing. All groups learned to press the lever more efficiently with repeated testing (Day; $F = 17.84$, d.f. = 16.944, $P < 0.001$). The duration that the response lever was held in the depressed (closed) state declined from 0.54 s on the first day of testing to 0.34 s during the final session (Fig. 4B). Groups differed in how much they changed over testing (Group × Day; $F = 2.04$, d.f. = 32.944, $P < 0.001$). Durations declined the least in Lc/+ mice and declined the most in chimeric animals, in comparison

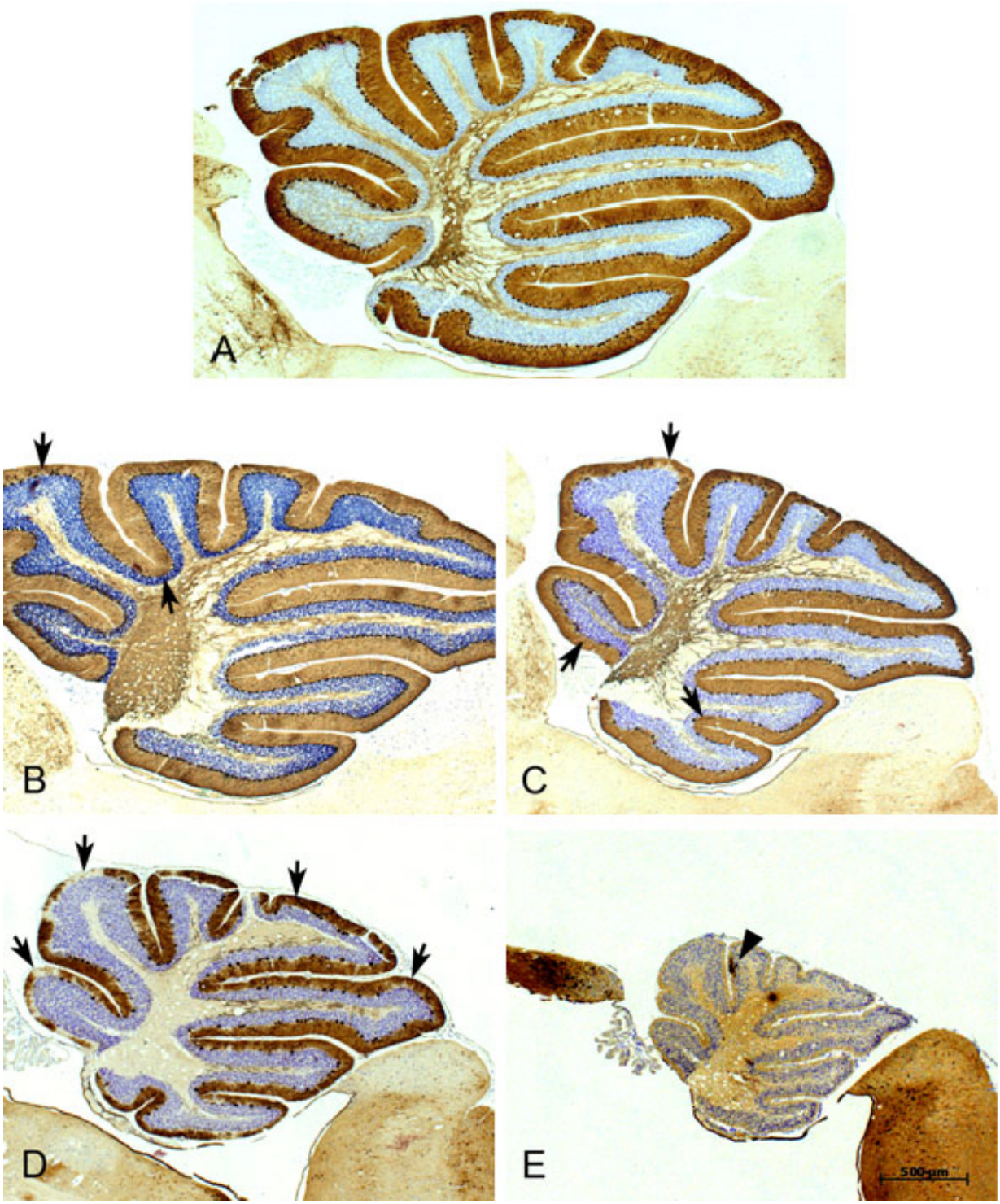

FIG. 1. Examples of the cerebellum from the spectrum of chimeras that were studied. Material is stained for anti-Calbindin to highlight Purkinje cell bodies, their dendritic arborizations into the molecular layer and their axons which pass out through the white matter en route to the cerebellar nuclei. (A) This midline section is from a cerebellum that had normal numbers of Purkinje cells and is thus considered a 100% +/+ chimera. Note the large size of the cerebellum as well as the full complement of Purkinje cells aligned without interruptions along the internal granule cells. The arbors of the dendritic trees highlight the molecular layer. (B) The cerebellum from this chimera was estimated to be about 85% wild-type and the relative normality of the cerebellum is indicated by a total size that is only slightly smaller than the 100% wild-type cerebellum and has only a few gaps in Purkinje cells (arrows). (C) This cerebellum is from a chimera that was estimated to be a 50% chimera. Note the smaller size of the cerebellum and the larger gaps in the Purkinje cell layer (PCL) that are void of Purkinje cells (arrows show a few examples). (D) Although over-stained for immunocytochemistry this cerebellum is obviously smaller than the cerebella in A–C and there are large gaps of Purkinje-cell-free regions in the PCL. One can also see a dramatic reduction in the Calbindin-positive axons in the white matter. This cerebellum is from a 80% lurcher chimera. (E) From a virtually 100% lurcher chimera showing the diminished size of the cerebellum and the virtual lack of anti-Calbindin staining within the cerebellum except for one Purkinje cell (arrowhead) found in this midline section. The rest of the brain stains normally for the antibody, indicating the specific loss of Purkinje cells. The scale bar applies to all images.

TABLE 1. Numbers of Purkinje cells in the cerebella of all Lc/+↔+/+ and +/+↔+/+ chimeras used in the PR study, and in four +/+ mice

	Numbers of Purkinje cells in the cerebellum		
Brain number	Left	Right	Left + right
Lc/+↔+/+ chimeras			
10	460	224	684
25	804	0	804
80	6615	8851	15 466
29	6268	12 418	18 686
26	9446	11 303	20 749
86	16 095	14 545	30 640
53	*	*	33 422
110	21 042	19 093	40 135
82	18 246	22 548	40 794
104	26 438	24 616	51 054
60	*	*	51 970
126	32 041	23 150	55 190
24	28 886	26 432	55 317
8	34 999	23 166	58 165
108	25 033	33 893	58 926
106	28 751	31 501	60 252
58	*	*	66 189
57	*	*	70 096
11	*	*	81 122
52	*	*	87 043
59	*	*	96 094
27	51 946	47 529	99 475
56	*	*	106 164
23	50 965	66 224	117 189
65	47 144	70 619	117 763
7	65 549	64 606	130 155
+/+↔+/+ Chimeras			
113	73 161	64 018	137 180
5	72 238	65 601	137 838
6	68 038	71 191	139 229
68	68 961	74 228	143 189
4	63 802	81 657	145 459
84	73 694	77 981	151 676
69	75 063	82 488	157 552
36	83 291	75 414	158 705
61	87 534	74 980	162 515
+/+ Mice			
206	†	68 390	136 780*
201	77 093	68 649	145 742
205	72 440	76 947	149 386
204	78 586	84 856	163 442

As can be seen in the low-percentage chimeras, the precursor pools that establish left and right Purkinje cell populations can be unique, although in Chimera 10, it appears that a single progenitor cell contributes Purkinje cells to both sides of the cerebellum. The two ataxic chimeras that were excluded from the behavioral analysis are shown at the top. +/+↔+/+ chimeras are shown towards the bottom in bold type. *Brains sectioned coronally in which only total Purkinje cell numbers were determined. †The left side of the cerebellum was damaged during processing and the total number was estimated by doubling the undamaged right side.

with controls. This pattern resulted in lurchers showing significantly longer durations during test days 15 and 16, while chimeras had a significantly shorter duration on day 4 (Dunnett's *t*-tests).

Associated with the relatively inefficient pattern of lever-pressing in Lc/+ mice, they also exhibited significantly longer inter-response intervals, when considered over the course of testing (Fig. 4C). In comparison with mean control IRTs of 3.15 s, Lc/+ mice averaged 6.57 s between lever-presses (Group; $F = 17.72$, d.f. = 2.59, $P < 0.001$). Lurchers also showed a different pattern of change across the test days (Group × Day; $F = 3.55$, d.f. = 32.944, $P < 0.001$). IRTs lengthened during the first four test days and then began to decline with repeated testing. This resulted in significantly longer IRTs in Lc/+ mice during most of the first eight test days, in comparison with controls that showed a steady decline in IRTs over test days. Although IRTs were shorter in chimeric mice, they did not differ significantly from controls on this measure.

As shown in Fig. 4D, there were also significant group differences in PRP time (Group; 22.99 s, d.f. = 2.59, $P < 0.001$) that remained constant with repeated testing (Group × Day; $F = 1.39$, d.f. = 32.944, P = n.s.). Dunnett's *t*-tests indicated that, in comparison with controls (average PRP = 18.4 s), Lc/+ mice maintained consistently longer PRPs over the 17 test days (average PRP = 34.89 s).

Figure 5 depicts other behaviors that occurred during the acquisition of PR responding. As mice in all groups learned the PR task, the number of times per trial that they entered into the food magazine declined significantly (Day; $F = 41.47$, d.f. = 16.944, $P < 0.001$). In comparison with controls, Lc/+ animals showed a significantly slower rate of decline in entries (Group × Day, $F = 1.82$, d.f. = 32.944, $P < 0.005$), which accounted for significant group differences between test days 3 and 7 (Fig. 5A). The performance of chimera and control mice was very similar. Associated with the elevated number of food entries in lurchers, these mice were consistently slower to enter the food magazine once reinforcement had been delivered (Group; $F = 51.74$, d.f. = 2.59, $P < 0.001$). Thus, over the 17 test days entry latencies averaged 0.76, 0.84 and 1.59 s, respectively, in control, chimeric and Lc/+ mice (Fig. 5B). As shown in Fig. 5C, activity in the rear of the test chamber declined significantly in all groups with repeated testing (Day; $F = 11.21$, d.f. = 16.944, $P < 0.001$). There were relatively small group differences in activity over the test days (Group × Day; $F = 1.84$, d.f. = 32.944, $P < 0.005$). Specifically, Lc/+ mice had higher levels of activity on test days 3 and 4, in comparison with controls. Unlike the decline in activity, time-out responses (lever-presses that occurred in the 1 s following reinforcement) remained relatively constant over testing (Fig. 5D) although there was a consistent group difference (Group; $F = 5.49$, d.f. = 2.59, $P = 0.007$). When considered over the 17 test days, lurchers exhibited fewer time-out responses than chimeric animals, although neither group differed significantly from wild-type control animals.

The relationship of Purkinje cell number and PR measures

Purkinje cell number in chimeric and wild-type mice was related to two of the eight behavioral measures assessed in the PR task. Figure 6A shows the negative relationship between breakpoint and Purkinje cell number. Pearson correlations indicated that this relationship was significant ($r = -0.439$, d.f. = 31, $P = 0.012$). Thus, as the total number of Purkinje cells increased, breakpoint declined. As indicated in Fig. 6B, post-reinforcement pause and Purkinje cell number were significantly related ($r = 0.369$, d.f. = 31, $P = 0.038$) in that mice with lower numbers of Purkinje cells had shorter PRPs.

Discussion

Recent evidence suggests a role for the cerebellum in both repetitive and hyperactive behavior. This evidence includes reports of stereotyped repetitive behaviors in children with prenatal cerebellar malformations (Boltshauser *et al.*, 1996; Hottinger-Blanc *et al.*, 2002), structural and functional magnetic resonance imaging data

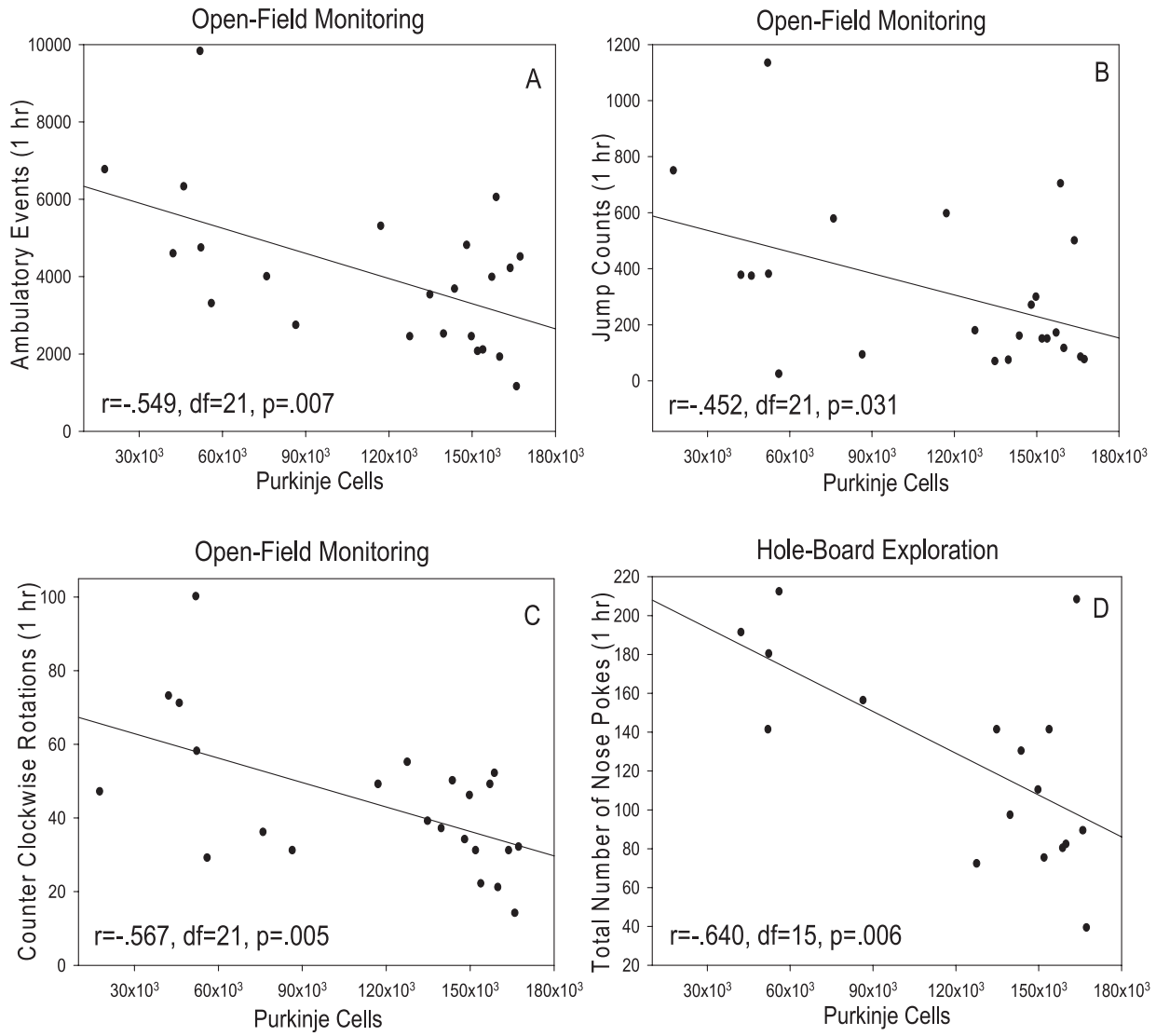

FIG. 2. Scatterplots showing the relationship between total cerebellar Purkinje cell number and some of the measures of ambulation during open-field monitoring and the total number of hole pokes during hole-board exploration. Total Purkinje cell number was negatively related to ambulatory events (A; $r = -0.549$, d.f. = 21, $P = 0.007$), total jump counts (B; $r = -452$, d.f. = 21, $P = 0.031$) and counter clockwise rotations (C; $r = -0.567$, d.f. = 21, $P = 0.005$) as determined by Pearson correlations. (D) Total Purkinje cell number was also negatively related to the total number of nose-pokes during the 60-min session of hole-board exploration ($r = -0.640$, d.f. = 15, $P = 0.006$). Data from six mice tested in this task were unavailable due to computer malfunction.

linking cerebellar abnormalities with ADHD (Soliva *et al.*, 2009), as well as a study demonstrating an inverse relationship between the area of the cerebellar vermis in ASD children and rates of repetitive behavior (Pierce & Courchesne, 2001). Repetitive or stereotyped patterns of behavior and interests are one of the core symptoms of ASDs. Hyperactivity, although not required for diagnosis, is also often observed in ASD children. Thus, given that the most consistent finding in post-mortem autistic brains is pathology of the cerebellum (Palmen *et al.*, 2004) we investigated the role of the cerebellum in hyperactivity and repetitive behavior. To this end, we compared Lc/+↔+/+ chimeric mice and +/+ control mice in open-field monitoring, hole-board exploration and the PR task.

Developmental aspects of Purkinje cell lineage

Through our quantitative analysis of Purkinje cell number, we found that the group of 26 chimeras in this study involved the full range of Purkinje cell number from about 1000 total Purkinje cells in our lowest-percentage chimeras to near normal numbers of Purkinje cells (130 000) in our highest-percentage chimeras.

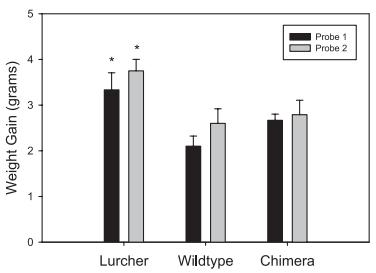

FIG. 3. Mean weight gain of Lc/+, +/+ and Lc/+↔+/+ chimeras in each of two 40-min sessions of the hunger motivation task. Dunnett's *t*-tests revealed that Lc/+ mice gained significantly more weight than control and chimeric mice following these probes (*$P < 0.01$).

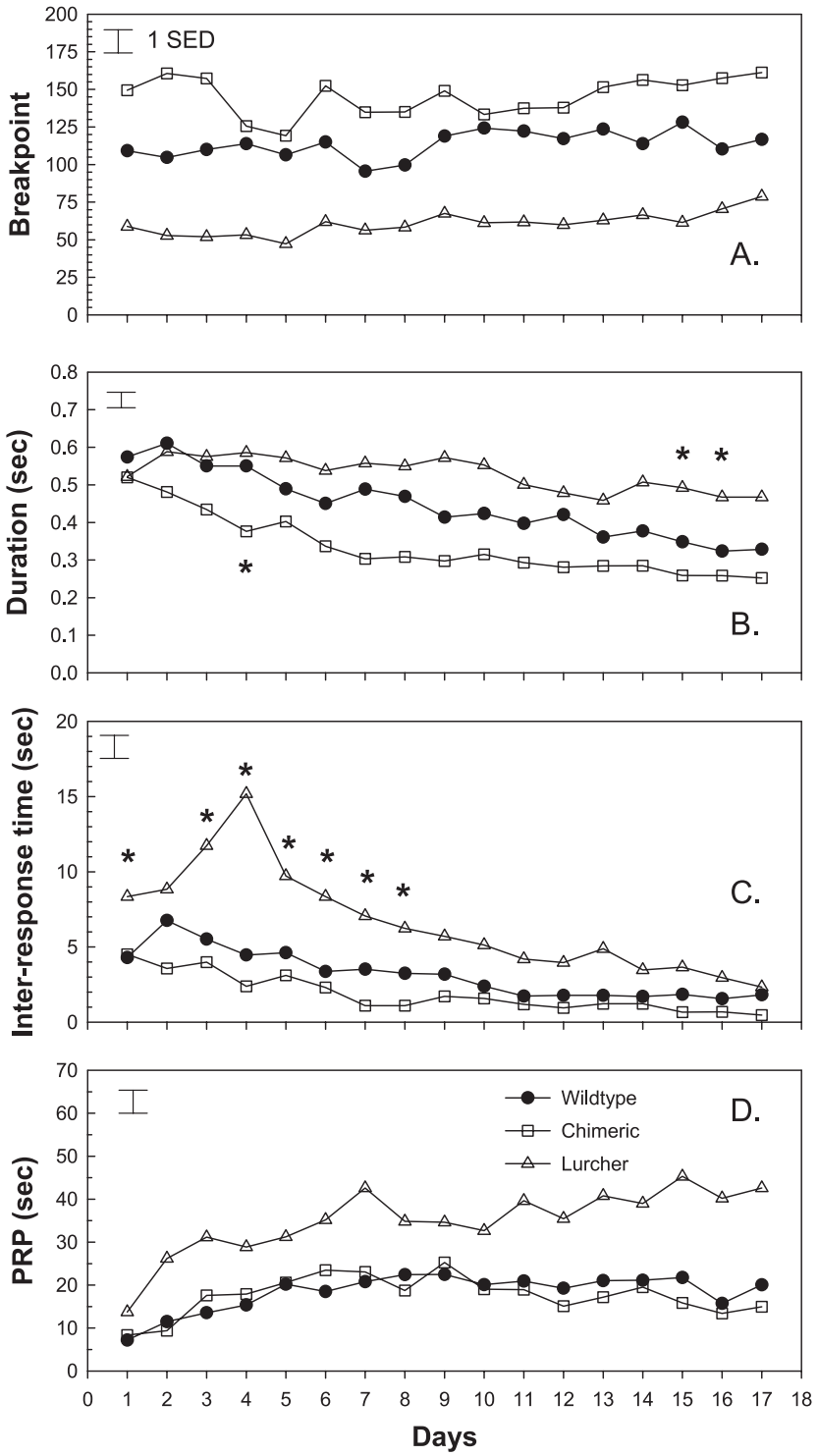

FIG. 4. Mean breakpoint, lever-press duration, inter-response time and post-reinforcement pause of Lc/+, +/+ and Lc/+↔+/+ chimeras in each trial session of the PR task. *Individual trial sessions in which there were significant differences ($P < 0.05$) between Lc/+ and wildtype control mice or chemeric and control mice (Panel B session 4 only). (A) Mean breakpoint increased with repeated testing in all groups (Day; $F = 2.98$, d.f. = 16.944, $P < 0.001$). In addition, analysis of mean breakpoint across all test days revealed that chimeric mice (145.5) lever-pressed significantly more than controls (113.5) while Lc/+ mice (60.7) lever-pressed significantly less (Group; $F = 20.61$, d.f. = 2.59, $P < 0.001$). (B) Mean lever-press duration decreased with repeated testing in all three groups (Day; $F = 17.84$, d.f. = 16.944, $P < 0.001$), but the groups differed in how much they changed over the course of testing (Group × Day; $F = 2.04$, d.f. = 32.944, $P < 0.001$). Lc/+ mice had significantly longer durations during test days 15 and 16, while chimeras had a significantly shorter duration on day 4. (C) Mean inter-response time across all test days differed between groups (Group; $F = 17.72$, d.f. = 2.59, $P < 0.001$). Lc/+ mice (6.57 s) had significantly longer inter-response intervals than control mice (3.15 s), but chimeric mice did not differ significantly from controls. In addition, Lc/+ mice displayed a different pattern of change across test days (Group × Day; $F = 3.55$, d.f. = 32.944, $P < 0.001$). IRTs lengthened during the first four test days and then began to decline with repeated testing, resulting in significantly longer IRTs during most of the first eight test days. (D) Mean post-reinforcement pause differed between groups across all test days (Group; $F = 22.99$, d.f. = 2.59, $P < 0.001$). Lc/+ mice maintained consistently longer PRPs over the 17 test days than controls.

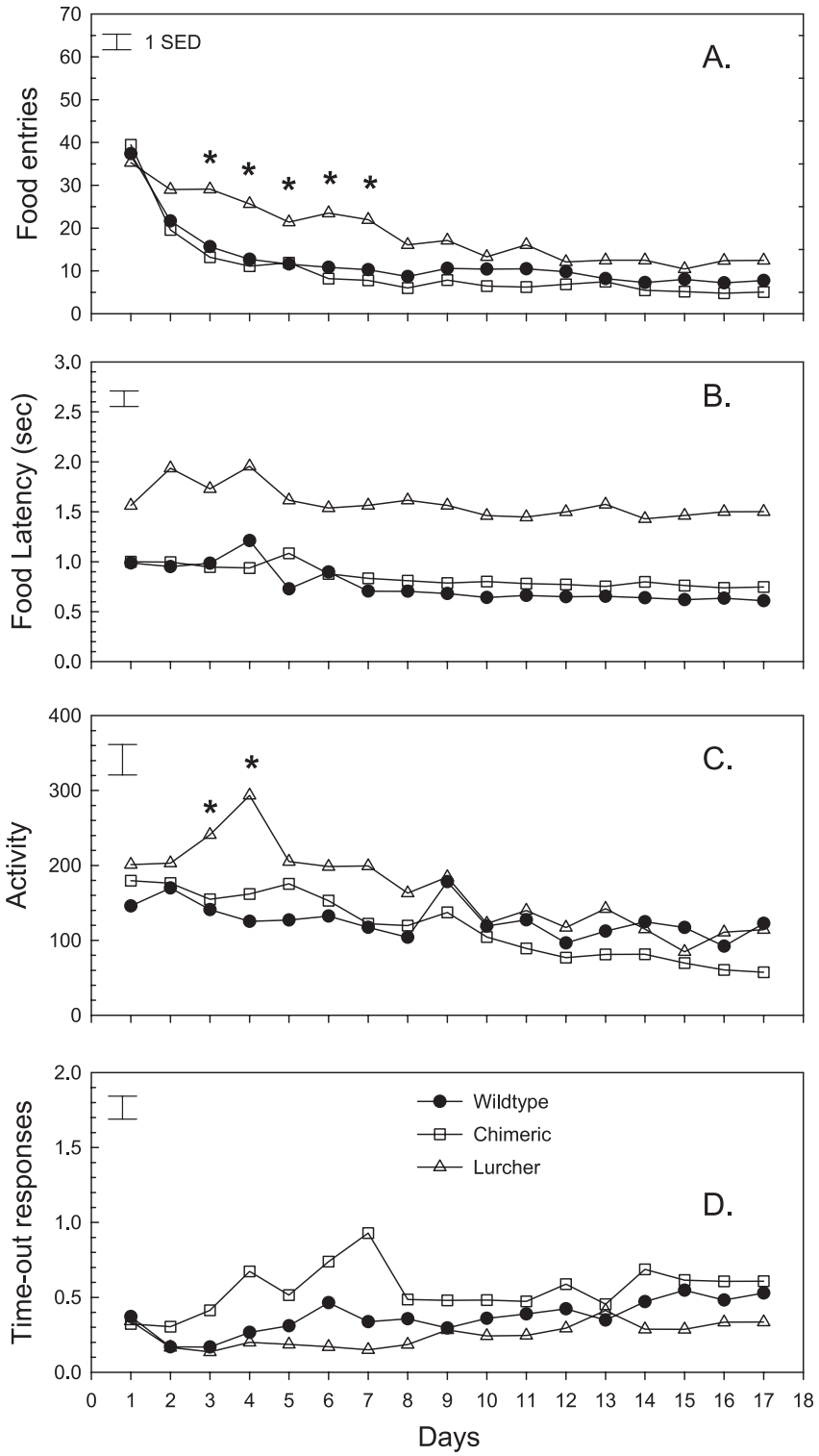

FIG. 5. Mean number of food entries, food latency, off-task activity and number of time-out responses of Lc/+, +/+ and Lc/+↔+/+ chimeras in each trial session of the PR task. *Individual trial sessions in which there were significant differences ($P < 0.05$) between Lc/+ and wildtype control mice. (A) The number of times per trial that each group entered into the food magazine declined significantly across test days (Day; $F = 41.47$, d.f. = 16.944, $P < 0.001$). Lc/+ animals showed a significantly slower rate of decline in entries than control mice (Group × Day, $F = 1.82$, d.f. = 32.944, $P < 0.005$), which accounted for significant group differences between test days 3 and 7. (B) The latency to reach the food magazine once reinforcement had been delivered differed between groups (Group; $F = 51.74$, d.f. = 2.59, $P < 0.001$). Lc/+ mice (1.59 s) demonstrated significantly longer food latencies than control mice (0.76) across all test days. (C) Activity in the rear of the test chamber declined significantly in all groups with repeated testing (Day; $F = 11.21$, d.f. = 16.944, $P < 0.001$). There were relatively small group differences in activity over the test days (Group × Day; $F = 1.84$, d.f. = 32.944, $P < 0.005$) in that Lc/+ mice had higher levels of activity on test days 3 and 4 in comparison with controls. (D) Mean time-out responses differed between groups but remained relatively constant over all test days (Group; $F = 5.49$, d.f. = 2.59, $P = 0.007$). Lc/+ mice exhibited fewer time-out responses than chimeric mice over the 17 test days, although neither group differed significantly from controls.

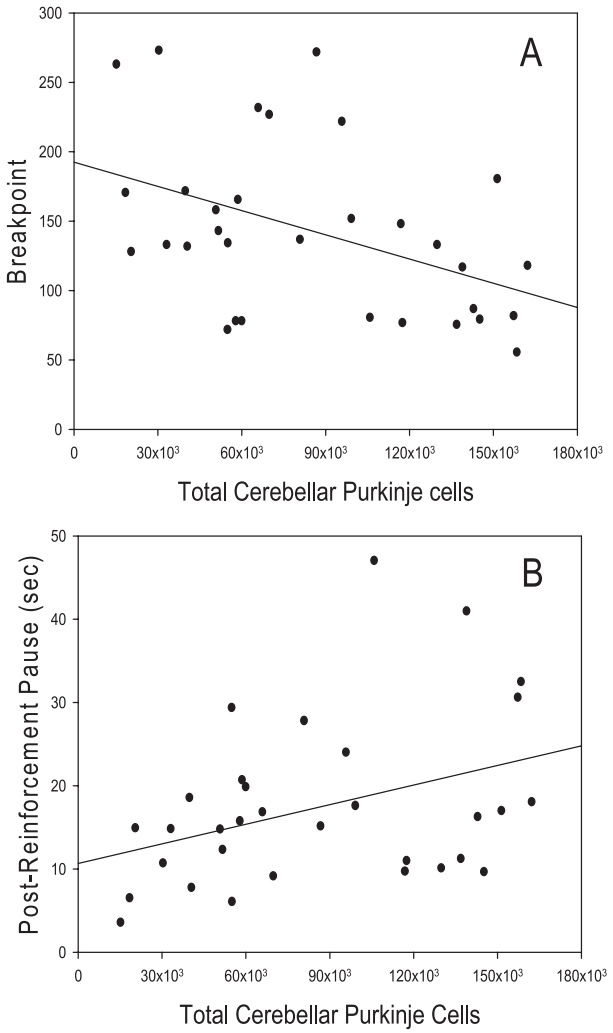

FIG. 6. Scatterplots showing the relationships between total cerebellar Purkinje cell number and both breakpoint and post-reinforcement pause. (A) Total Purkinje cell number was negatively related to breakpoint as revealed by Pearson correlations ($r = -0.439$, d.f. = 31, $P = 0.01$). Breakpoint declined as total Purkinje cell number decreased. (B) Total Purkinje cell number was positively related to post-reinforcement pause ($r = 0.369$, d.f. = 31, $P < 0.05$) in that mice with lower numbers of Purkinje cells had shorter PRPs.

The cerebella of Lc/+↔+/+ chimeric mice have been studied previously in order to gain insight into the development of the cerebellum and determine the progenitor pool size that gives rise to the Purkinje cell population (Wetts & Herrup, 1982a,b). Our comparisons of Purkinje cell number across lobule groupings (lobules I–V, VI–VII, VIII–X) have confirmed previous reports of a random dispersal of Purkinje cell clones throughout the cerebellar cortex (data not shown; Baader *et al.*, 1996; Hawkes *et al.*, 1998). Even in our low-percentage chimeras, Purkinje cells were found in all three lobule groupings; however, differences were evident between left and right hemicerebella, consistent with previous findings (Table 1; Baader *et al.*, 1996; Hawkes *et al.*, 1998). Purkinje cell numbers of our two lowest-percentage chimeras also provide confirmation of the estimated clonal size for a Purkinje cell progenitor and the number of progenitors in the Purkinje cell pool, around 1000 and 100–150, respectively (Baader *et al.*, 1996; Mathis *et al.*, 1997; Hawkes *et al.*, 1998).

Increased activity during open-field and hole-board testing

The results from chimeric and wild-type mice during open-field monitoring demonstrated significant negative relationships between Purkinje cell number and ambulatory events, ambulatory time, distance traveled, jump counts and counterclockwise rotations. These results are probably explained by an inverse relationship between Purkinje cell number and general activity. The positive relationship between Purkinje cell number and resting time supports this conclusion. A significant negative relationship between Purkinje cell number and nose-pokes was also found during the hole-board exploration and, as a group, chimeric mice had significantly more nose-pokes than +/+ mice. Although it is possible that this relationship is also caused by an overall increase in general activity level in mice with fewer Purkinje cells, Pearson correlations failed to demonstrate significant relationships between the number of nose-pokes and any of the dependent measures from the open-field assessment (data not shown). Thus, the increase in nose-poking behavior with decreasing Purkinje cell number may be indicative of increased motivation to explore in these mice. Alternatively, the repetitive nose-poking behavior can be viewed as a repetitive behavior similar to that which was demonstrated by these mice in the PR task.

The role of the cerebellum in exploratory behavior revisited

Previous research has shown that Lc/+ mice and other mice with cerebellar deficits show a significant decrease in nose-poking behavior on a hole-board matrix compared with controls (Lalonde & Botez, 1985; Lalonde *et al.*, 1993; Caston *et al.*, 1998). We also found that Lc/+ mice had fewer nose-pokes than +/+ controls, although this difference was not significant. In contrast, our chimeric mice demonstrated significantly more nose-pokes than controls during this task and the number of Purkinje cells in these mice was negatively correlated with nose-poking behavior (Fig. 2D). These findings cast doubt on previous conclusions suggesting reduced motivation to explore in cerebellar deficient mice and rather suggest that previous results were caused by the motor impairments of the mice (Caston *et al.*, 1998).

Repetitive pattern of responding in the PR task

From the results of Lc/+↔+/+ chimeras, we demonstrated a relationship between Purkinje cells and lever-pressing behavior in the PR task. Breakpoint was significantly higher for the chimeric group than controls and within the group of chimeric mice was significantly and negatively correlated with the number of Purkinje cells in the cerebellum (Figs 4A and 6A).

We consider it unlikely that the higher breakpoint in Lc/+↔+/+ chimeras results from enhanced food motivation. Not only were the chimeric and wild-type mice food-deprived to a similar degree, but they also did not show any group difference in weight gain when allowed to free-feed for a set amount of time (Fig. 3). Instead, we believe that the increases in lever-pressing that resulted in higher breakpoint for mice with reduced Purkinje cell numbers may be related to the repetitive nature of this task. The significant positive relationship between Purkinje cell number and PRP helps to demonstrate the increased focus of these mice on repetitive lever-pressing behavior, as those chimeras with the fewest Purkinje cells, and therefore the highest breakpoints, paused for the shortest amount of time following reinforcement (Fig. 6).

Although chimeric mice demonstrate improved performance in this repetitive task based upon their degree of Purkinje cell loss, it is interesting that Lc/+ mice with complete Purkinje cell loss do not. One possible explanation for this apparent contradiction is the ataxia of the Lc/+ mice. It may be that this PR task is too demanding for these

motor-deficient mice to exhibit the same repetitive pattern of responding as chimeric mice. The presence of repetitive behaviors in these mice may only be detected through ethological observation. Indeed, Lc/+ mice do exhibit a repetitive 'lurching' gait as part of their ataxia.

Influence of motor deficits on the performance of lurcher mice in the behavioral tasks

Lc/+ mice are ataxic, but chimeric mice with Purkinje cell numbers above a threshold of about 1000 cells show no outward signs of motor impairment (Martin *et al.*, 2003, 2004). It is therefore prudent to interpret the behavioral results of Lc/+ mice independently of chimeras. During open-field monitoring, Lc/+ mice had significantly more rotations than chimeric and wild-type mice. This is probably related to the ataxia of Lc/+ mice, as they are often observed moving in circular patterns as they stumble around. In the PR task, Lc/+ mice had more head entries and a slower rate of decline in head entry number over trials compared with controls. These results can again be explained by the ataxia of these mice, particularly with their characteristic 'lurching' movements. The lurching movement involves a forward and backward head motion that may result in increased head entries into the food chamber. Lc/+ mice were also consistently slower to enter the food magazine once reinforcement was delivered. This finding is probably caused by a greater difficulty in moving between the lever and food magazine (Martin *et al.*, 2004).

For the PR task-related behaviors, Lc/+ mice demonstrated a significantly lower breakpoint than control mice. Breakpoint has previously been used as an index of motivation (Schmelzeis & Mittleman, 1996). However, because the motivating factor of hunger seemed to be higher for Lc/+ mice (Fig. 3), and the reward was consistent in each trial (0.02 mL of evaporated milk/sucrose solution), the increase in task demand due to motor impairment is most likely responsible for their lower breakpoint. The higher amount of weight gain observed in Lc/+ mice in the hunger motivation probe may be due to the fact that Lc/+ mice are significantly smaller than controls at baseline. The food deprivation may therefore have a greater impact on these mice.

Lc/+ mice also demonstrated significantly higher post-reinforcement pause times and less efficient lever-pressing than control mice. The increase in rest between trials is another indication of the increased effort demanded from Lc/+ mice by this task. Although the less efficient lever-pressing may be partially explained by the motor impairment of these mice, this deficit may also be related to practice. Lever-press duration and IRT are both measures that improve with practice. This is clear by the gradual decline in latencies of both measures for all groups over the 17 test sessions (Fig. 4) and significant negative correlations between breakpoint and these temporal characteristics of lever-pressing (data not shown). Because of their lower breakpoint, Lc/+ mice were exposed to fewer trials in a session, and therefore received less practice than control and chimeric mice.

The role of the prefrontal cortex in the observed Purkinje cell relationships

We propose that the consistent negative correlations between Purkinje cell number and activity, nose-poking and repetitive lever-pressing demonstrated in this study are driven by a disruption of prefrontal dopamine neurotransmission. Specifically, we have previously reported that brief electrical stimulation of the cerebellar Purkinje cell layer resulted in stimulation time-locked increases in extracellular dopamine levels in the medial prefrontal cortex (MPFC). This increase was absent in Lc/+ mice with a complete loss of cerebellar Purkinje cells, suggesting that Purkinje cell outputs are a critical component of this modulatory cerebellar–MPFC circuit (Mittleman *et al.*, 2008). Although chimeric mice have not yet been investigated in this paradigm, it is reasonable to predict that the magnitude of influence of this circuitry on cortical dopamine transmission will be dependent on the number of cerebellar Purkinje cells. In this vein it is interesting to note that similar to the results of the present study, rats with small neonatal, excitotoxic lesions of the MPFC show increased lever-pressing and significantly increased breakpoints in a food-rewarded PR task (Schwabe *et al.*, 2006). Additionally, both monkeys and humans with lesions within the ventral regions of the frontal cortex in a variety of paradigms display deficits in inhibitory response control that have been described as, 'increased control over behavior by pre-potent stimuli', where 'pre-potent' is defined as stimuli with previous motivational significance (Jentsch & Taylor, 2001). In the PR task the response lever is a pre-potent stimulus as it has acquired motivational significance by contingent association with the liquid food reward. Thus, it appears probable that in the current study reductions in the number of Purkinje cells in chimeric mice leads to a disruption in dopamine transmission in the MPFC, and this in turn leads to increased and repetitive lever-pressing that could be interpreted as a deficit in inhibitory response control. Interestingly, an early study on cats with bilateral cerebellar cortical ablation demonstrated an impaired ability to inhibit bar-pressing during periods of non-reward in an operant task, a structure–function relationship perhaps similar to our findings in cerebellar deficient chimeric mice (Davis *et al.*, 1970).

Conclusion

Stereotypy has been defined by Ridley (1994) as 'the excessive production of one type of motor act, or mental state, which necessarily results in repetition'. The excessive production of lever-pressing by Purkinje cell-deficient chimeric mice in this study seems to fit this definition. However, it is premature to conclude that the Lc/+↔+/+ chimeric mice in this study demonstrated 'stereotypies' analogous to those found in children with ASD. Nonetheless, the superior performance of these mice in a rewarded task demanding repetitive behavior, together with the increased activity and repetitive behavior patterns shown by these mice in the open-field observations, make an intriguing comparison with potential clinical correlates in ASD. Indeed, a previous study found that children with ASD demonstrated a significant increase in activity over controls and also had a significant negative relationship between the rate of repetitive behavior and the size of their cerebellar vermis (Pierce & Courchesne, 2001). Also, one of the proposed causes of ASD is self-generated or maternally derived pathogenic antibodies and several recent studies have shown that antibodies from children with ASD or their mothers target cell populations within the cerebellum including Purkinje cells (Dalton *et al.*, 2003; Singer *et al.*, 2006; Wills *et al.*, 2009). It is therefore of interest that a recent study demonstrated increased activity and repetitive behavior in rhesus monkeys exposed to IgG antibodies from mothers of children with ASD (Martin *et al.*, 2008). Thus, the above findings, coupled with the consistent pathology of the cerebellum demonstrated in ASD, suggest that cerebellar impairment may lead to these maladaptive behaviors in children with ASD.

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Abbreviations

ADHD, attention deficit/hyperactivity disorder; ASD, autism spectrum disorder; FR, fixed-ratio; IRT, inter-response time; MPFC, medial prefrontal cortex; PR, progressive-ratio; PRP, post-reinforcement pause.

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