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Distribution of parthenogenetic cells in the mouse brain and their influence on brain development and behavior

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ABSTRACT **A systematic analysis of parthenogenetic (PG) cell fate within the central nervous system (CNS) was made throughout fetal development and neonatal and adult life. Chimeras were made between PG embryos carrying a ubiquitously expressed *lacZ* transgene and normal fertilized embryos. After detailed histological analysis, we find that the developmental potential of PG cells is spatially restricted to certain parts of the brain. PG cells are prevalent in telencephalic structures and are largely excluded from diencephalic structures, especially the hypothalamus. These spatial restrictions are established early in development. Behavioral studies with chimeras identified an increase in male aggression when the proportion of PG cells in the brain was high. These studies demonstrate that imprinted genes play key roles in development of the CNS and may be involved in behavior.**

It has been recognized for some time that as a result of genomic imprinting the two parental genomes of mammals are not functionally equivalent (1, 2). Experimentally produced parthenogenetic (PG) and androgenetic (AG) mouse embryos express a wide range of phenotypes, dying between implantation and day 11 of gestation (E11) (1–3); however, PG and AG derived cells can be rescued in chimeras made with normal mouse embryos (N). A major phenotype observed in chimeras involves tissue-specific restriction in the developmental potential of uniparental disomic cells. In PG chimeras, PG cells are selectively excluded from the myogenic lineage, but they participate well in the development of ectodermal tissues such as epidermis and the brain. In such tissues, PG cells can persist into adulthood in significant numbers. In contrast, AG chimeras largely show the reciprocal phenotypes—for example, with high contributions to skeletal muscle and a poor contribution to the brain. A second major phenotype is growth related, with PG chimeras being significantly growth retarded (reviewed in ref. 4).

For PG and AG embryos, the phenotypes described represent the compound effects of several imprinted genes; however, as yet no imprinted genes have been identified that may be implicated in determining the differential distributions of PG and AG cells in a chimera. However, detailed analysis of cell distribution at the histological level will give important insights into the possible functions of imprinted genes and also the possible functions of imprinting for the animal. In the present study, we analyzed cell fate in PG–N chimeras, in which all PG cells were marked by the ubiquitous expression of a *lacZ* transgene. We focused on the anatomical localization of PG cells within the developing central nervous system (CNS) and in the adult brain. We found that PG cells are present in restricted regions of the brain and that this developmental restriction is established very early in gestation.

In the human, aberrant imprinting is increasingly being implicated in the etiology of some human diseases and cancers (5). The restricted developmental potential of PG cells is particularly interesting with respect to imprinted disorders that involve mental retardation and influence behavior. For instance, children with Prader–Willi syndrome inherit both copies of imprinted chromosome 15 from their mothers and suffer sexual dysfunction and obesity. Children lacking portions of maternal chromosome 15 or possessing two copies of imprinted paternal chromosome 15 exhibit mental retardation and puppet-like movements and are prone to seizures as part of Angelman syndrome (6). In mice, genetic complementation analysis has revealed an imprinted region on distal chromosome 2 that, when inherited from only one parent, results in abnormal neonatal behavior and early death (7). Therefore, we also examined whether the presence of large numbers of PG cells within the brain have any consequences for behavior. Finally, we addressed the question of whether the growth retardation phenotype of PG chimeras also applies to growth of the brain.

MATERIALS AND METHODS

Chimeras. Mice used were nonalbino (C57BL/6J × CBA/Ca)F_1 and albino CFLP bred from parent stocks from Harlan Olac (Bichester, U.K.). The transgenic line ROSA26 (kind gift from P. Soriano) was derived on a 129/sv background (8) but was since maintained on a mixed C57BL/6J and CBA/Ca background.

PG embryos were made by electroactivation of ROSA26 superovulated unfertilized oocytes (9) and diploidized as described (10). Chimeras were made by aggregating four-cell stage ROSA26 PG embryos with two-cell stage CFLP embryos. Control chimeras were made by aggregating fertilized ROSA26 with CFLP embryos. Composite embryos were transferred to recipient females on day 3 of pseudopregnancy, counting the day of finding the vaginal plug as day 1 (E1).

Histological Analysis of Chimeras. Whole embryos up to E12 and tissue sections of fetuses, neonates, and adult chimeras were stained in 5-bromo-4-chloro-3-indolyl β-D-galactoside solution for *lacZ* expression as described (11). Whole-mount preparations were sectioned after wax embedding. All sections were counterstained with neutral red. In adult chimeras, the level of chimerism was determined from brain sections, specifically from the parts of the brain that receive a high contribution of PG cells (cortex, striatum, hippocampus). Coat and eye pigmentation could be used only as a guide to which animals were chimeric and did not bear any obvious relationship to brain chimerism. Chimerism was quantitated using the National Institutes of Health IMAGE 1.54 image analysis program. For each chimera, three sections each through the

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Abbreviations: CNS, central nervous system; PG, parthenogenetic; AG, androgenetic; N, normal fertilized; E11, embryonic day 11; VNO, vomeronasal organ.

frontal cortex, the hypothalamus, and the brainstem were analyzed. Constant light intensity was maintained throughout the analysis and the same magnification (×100) was used for each section. The density of blue cells above a constant threshold was measured in absolute terms in a fixed area of 200 × 200 pixels and averaged for each animal.

Measurement of Brain Size. Thirty coronal sections of brains from six chimeras and six nonchimeric CFLP littermate controls (matched for age and sex) were taken at intervals through the forebrain (anterior striatum to posterior hippocampus) and measured using image capture and analysis software (National Institutes of Health IMAGE). The mean brain size for each animal, expressed relative to body weight, was analyzed by discriminant function analysis, a procedure in the general area of multivariate analysis (12). Brains for six further controls (three ROSA26–CFLP and three ROSA26) were also measured and compared with body weight.

Behavior. Chimeric animals and their nonchimeric male littermates (CFLP) were paired with estrous females of known receptivity. Chimeric females were paired with a male of proven sexual performance. All observations were carried out in the test animal's home cage. Inspection, mounting, and intromission were recorded during a 30-min test, which was terminated prematurely if intromission occurred. Each animal was tested on three occasions at intervals of 4–5 days.

Aggressive behavior tests were conducted in a neutral cage into which both experimental male and stimulus male were simultaneously introduced. Inspections, which animal initiated the aggressive interaction, and the latency to perform this were recorded. Tests were of 10 min duration or were terminated after the first aggressive incident. Each experimental male had three tests, each with a different stimulus male. Behavioral data were related to the levels of brain chimerism after histological analysis of all brains. CFLP and ROSA26 males were tested for aggression as additional controls for possible genetic background influences in the chimeras.

RESULTS

Developmental Analysis of PG Cell Contribution to the CNS. In chimeras, all ROSA26-derived cells could be identified by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining for *lacZ* expression. Table 1 describes the number of chimeras produced and analyzed in detail at different stages of development. While the degree of chimerism varied substantially, the patterns of cell distribution were highly consistent between chimeras. In control chimeras, the ROSA26 cells were randomly distributed throughout the brain in the adult and during development, regardless of the level of chimerism.

In PG–N chimeras, PG cells exhibited a differential distribution in the developing brain as early as E10.5. In lower contribution chimeras, there were many PG cells in the mesencephalon, together with numerous columns in the telencephalon, but there was a virtual absence of PG cells from diencephalic areas (Fig. 1*a*). By E12, this patterning was more marked with high cortical levels of PG cells but a complete absence of cells from the hypothalamus and preoptic area (Fig. 1*c*). Sections through E14 revealed the distribution of PG cells to be high in cortex and striatum but low in diencephalon (Fig. 1*b*). The number of PG cells in the cortex also displayed a rostrocaudal distribution, with highest levels in the future frontal cortex and lower levels in the occipital cortex. Coronal sections revealed the contrast of cells in cortex compared with hypothalamus, where they are virtually absent (Fig. 1*e*). High levels can also be seen in the trigeminal ganglia and developing hippocampus on E18 (Fig. 1*e*). Higher magnification through the medial preoptic area shows PG cells to be absent, while high levels were seen in the trigeminal ganglia (Fig. 1*f*).

Table 1. Number and stage of ROSA26–CFLP PG and control chimera analysis

Age	PG chimeras/ total recovered	Control chimeras/ total recovered
E10.5	14/22 (6)	8/12 (4)
E12	13/24 (9)	ND
E14	8/18 (4)	6/8 (3)
E16	6/14 (3)	5/7 (3)
Neonate	11/36 (6)	17/21 (2)
Adult	22/79 (15)	17/21 (6)

Numbers in parentheses indicate chimeras analyzed in detail. ND, not done.

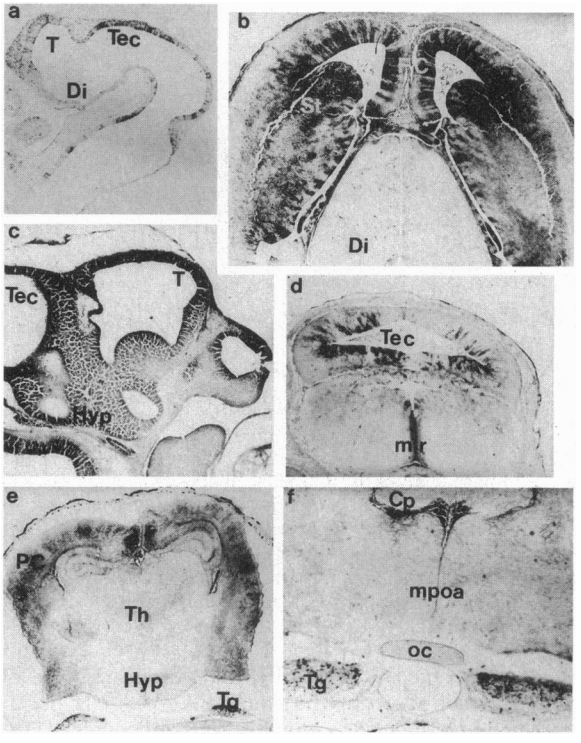

FIG. 1. Sections through the developing brain showing the distribution of ROSA26 PG cells stained for *lacZ* expression and counterstained with neutral red. Photography with a red filter makes PG cells appear black. (*a*) E10.5 embryo; saggital section of head region. (*b*) E14 embryo; horizontal section through forebrain region. (*c*) E12 embryo; saggital section of head region. (*d*) E14 embryo; coronal section through midbrain. (*e*) E18 embryo; coronal section at the level of the hippocampus. (*f*) E18 embryo; coronal section at the level of the medial preoptic area. T, telencephalon; Tec, tectum; FC, frontal cortex; mr, medial raphe; Di, diencephalon; St, striatum; Hyp, hypothalamus; Th, thalamus; oC, optic chiasm; Tg, trigeminal ganglion; PC, parietal cortex; Cp, choroid plexus; mpoa, medial preoptic area. (*a–d* and *f*, ×26; *e*, ×13.)

Coronal sections at the brainstem level showed few PG cells in the pons but a substantial contribution to the midbrain. Interestingly, a thin midline concentration of PG cells accumulated in the region of future serotoninergic neurons (Fig. 1*d*).

Distribution of PG Cells in Adult Brain. The distribution of PG cells in adult brains was consistent with that observed at fetal stages. Quantitative image analysis of sections through PG and control chimeric brains showed that PG cells were significantly excluded from the hypothalamus in comparison to the frontal cortex and brainstem, with the frontal cortex receiving the greatest contribution (Fig. 2*A*). The exclusion of PG cells from the hypothalamus is shown in Fig. 3, in which PG and control chimeras with similar high levels of chimerism in the frontal cortex are compared. Fig. 4 shows the contribution of PG cells to the frontal cortex and striatum (Fig. 4*A*), hippocampus, thalamus (Fig. 4*B*), and midbrain (Fig. 4*C*) in

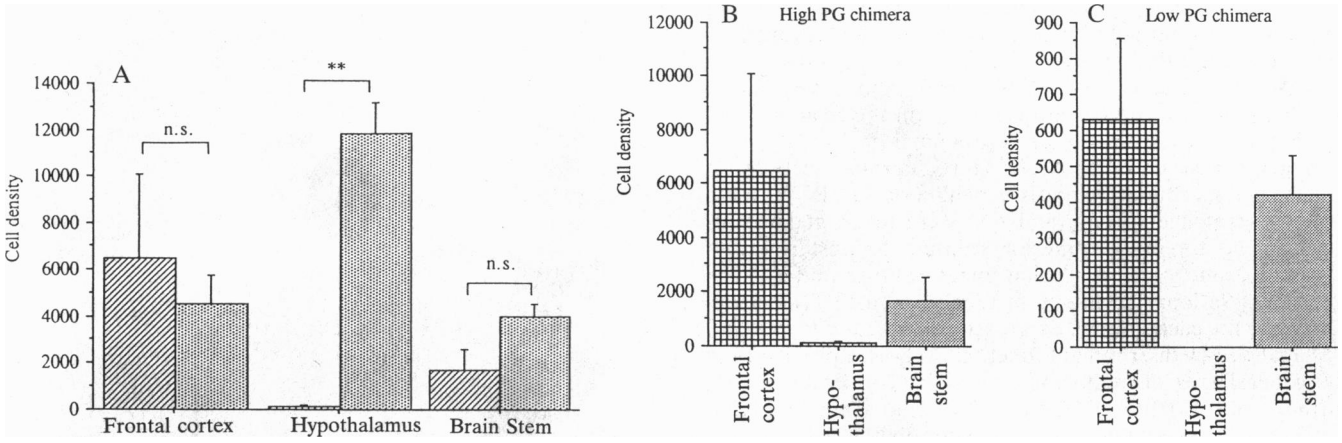

FIG. 2. Image analysis of ROSA26 cell density in chimeras. (*A*) Cell contributions in frontal cortex, hypothalamus, and brainstem between high-level PG ($n = 4$) and control chimeras ($n = 3$). Mean cell density (expressed in pixels) + 1 SE is shown in each region for the two groups of animals. Exclusion of PG cells from the hypothalamus is highly significant ($t = 10.591$; $P < 0.0001$) (indicated by **). Differences between frontal cortex or the brainstem were not significant (n.s.). Differences between brain regions (seen in controls) result from variations in absolute cell density in these regions. (*B* and *C*) Similar pattern of cell distribution, for the same brain regions, between high ($n = 4$) (*B*) and low ($n = 3$) (*C*) contribution PG chimeras (note difference in scale for cell density for high and low chimeric groups) is shown.

the same chimera. Very few PG cells were seen in the hypothalamus, especially medially, while the zona incerta had notable contributions. In the midbrain, the cells observed collected in the superior colliculus and red nucleus. Other brainstem areas to which PG cells contributed include substantia nigra, ventral tegmental area, locus coeruleus, medial raphe, and parabrachial nucleus. Interestingly, the pattern of cell distribution described above was not greatly influenced by the overall level of chimerism. The cell distributions for groups of higher and lower level chimeras in the frontal cortex, hypothalamus, and brainstem are shown in Fig. 2*B*.

Important neural areas outside the brain that receive a high contribution of PG cells include spinal and cranial nerve ganglia, the retina, olfactory mucosa, septal organ, and the vomeronasal organ (VNO). Interestingly, in the olfactory mucosa, the distribution of PG cells among the olfactory receptor neurons was bilaterally symmetrical but regionally different (Fig. 5*a*). This was in contrast to the chemoreceptors in the VNO, where no obvious symmetry or organization of PG cells could be discerned (Fig. 5*b*).

Behavior Analysis of Chimeras. Behavior tests were performed blind with respect to levels of brain chimerism and data were related to chimerism only after histological analysis of brains. In formal sexual behavior tests, neither male nor female

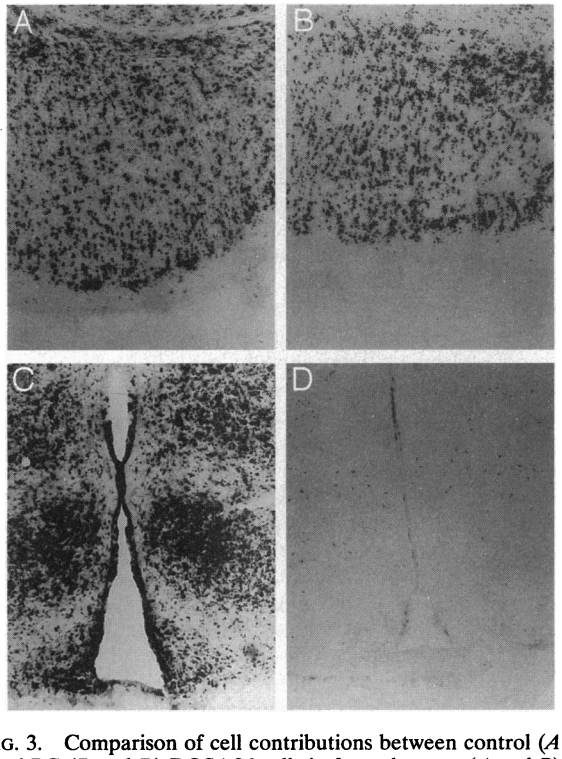

FIG. 3. Comparison of cell contributions between control (*A* and *C*) and PG (*B* and *D*) ROSA26 cells in frontal cortex (*A* and *B*) and hypothalamus (*C* and *D*).

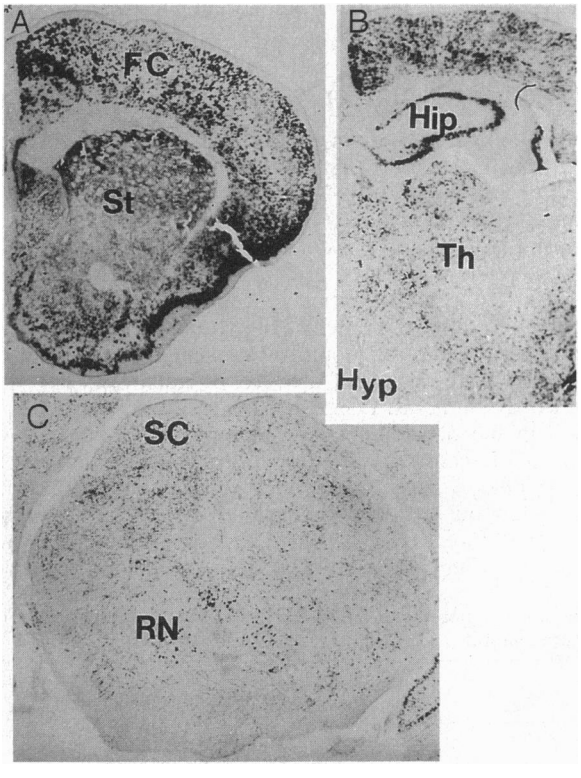

FIG. 4. Coronal sections of frontal cortex, striatum (*A*), hippocampus, thalamus, and hypothalamus (*B*), and midbrain (red nucleus and superior colliculus) (*C*) of the same animal. PG cells are virtually absent from the hypothalamus but make a high contribution to the cortex, striatum, and hippocampus. FC, frontal cortex; St, striatum; Hip, hippocampus; Hyp, hypothalamus; Th, thalamus; SC, superior colliculus; RN, red nucleus. (×5.)

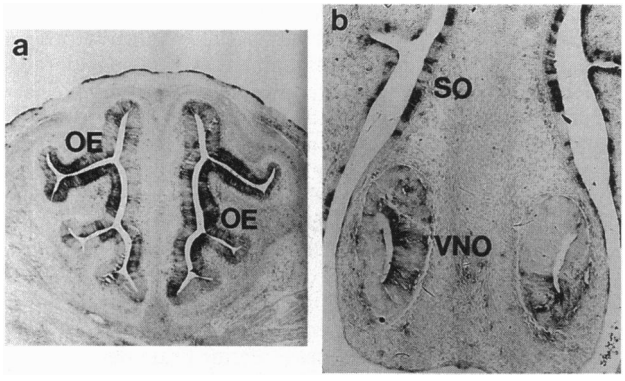

FIG. 5. Coronal section through olfactory mucosa (*a*) and septum (*b*) of day-18 embryo showing organized, symmetrical accumulation of PG cells in olfactory epithelium but asymmetrical accumulation in the VNO. OE, olfactory epithelium; SO, septal organ. (*a*, ×20; *b*, ×40.)

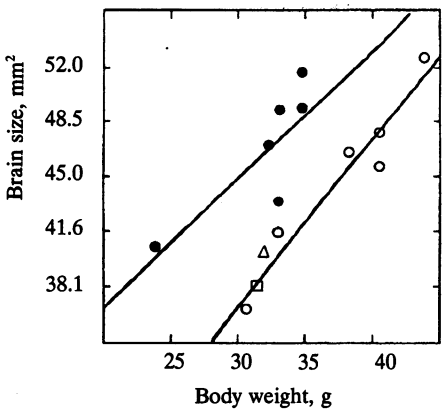

FIG. 6. Comparison of brain size relative to total body weight between adult ROSA26 PG–CFLP chimeras (●) and their nonchimeric (CFLP) littermates (○) (discriminant analysis, $F = 10.95$; $P < 0.003$). For ROSA26 (□), from which PG cells were derived, and control ROSA26–CFLP chimeras (△) matched for body weight with PG chimeras, brain size relative to body weight falls on the same slope as the nonchimeric littermate control brains.

chimeras differed significantly in measures of sexual behavior when compared with controls (Table 2). However, there was a tendency for aggressive behavior to be higher in chimeric males. Two groups of animals were identified, one with high aggression and one that was normal. After autopsy, we found that all animals with high levels of brain chimerism, assessed primarily in the frontal cortex, fell into the more aggressive group. Chimerism in animals from the high and low chimeric groups is shown in Fig. 2*B*.

For the aggressive group, chimeras initiated aggression twice as frequently as their male partners (16 vs. 8 times), while controls initiated aggression at the same rate as male partners (12 vs. 11 times). In addition, the latency of attack on other males was significantly shorter in chimeras (Table 2). This influence of PG cells on aggressive behavior was related to their maternal disomy and not to differences in genetic background (ROSA26 vs. CFLP). In a comparison of the parental strains, ROSA26 males initiated aggression at the same rate as their male CFLP partners (7 vs. 9 times), and the latency of ROSA26 males to attack was no different from CFLP males. The attack latency of both ROSA26 and CFLP controls was significantly different from chimeras ($P < 0.04$; Table 2).

Determination of Brain Size. Although it is well established that a high contribution of PG cells in chimeras results in marked fetal growth retardation that is sustained into adulthood, no measurements have been made previously on brain size. Therefore, we compared brain size with total body weight for adult chimeras and their nonchimeric littermates (CFLP background). Although PG adult chimeras had a lower mean body weight (32.1 g) than controls matched for sex and age (37.8 g), brain size was no different (46.9 mm^2) from that of controls (45.1 mm^2). However, since brain size normally correlates strongly with body weight, both across and within mammalian species (13, 14), we compared brain size relative to body size for the two groups (Fig. 6) and found that the PG chimeras had significantly larger brains for their body size (discriminant analysis, $F = 10.95$; $P < 0.003$). To control for differences in genetic background and for a possible effect of chimerism itself, we also compared brain size with body weight for the ROSA26 donor strain and control ROSA26–CFLP chimeras. These controls, matched for mean body weight (31.6 g compared with 32.1 g), did have significantly smaller brains (36.9 mm^2) than the PG chimeras (46.9 mm^2) (discriminant analysis, $F = 10$; $t = 3.99$; $P < 0.002$). Indeed, the values for the ROSA26 donor strain and control ROSA26–CFLP chimeras fall on the same line as the values for nonchimeric (CFLP) littermate controls for the PG chimeras (Fig. 7).

DISCUSSION

We have studied the compound effects of genetic imprinting on brain development and function by making a detailed analysis of PG cell fate in the developing CNS in chimeric embryos and neonatal and adult mice. In chimeras, PG cells accumulate in telencephalic forebrain regions, leaving parts of the diencephalon almost devoid of cells. The general distribution observed in adult brains is schematized in Fig. 7.

Exclusion of cells from diencephalon is seen as early as E10.5. At this stage, many PG cells are found in the tectal region of the midbrain. The restricted distribution of PG cells becomes clearer by E12 and E14, when the forebrain telencephalic and diencephalic structures become clearly defined. In the adult, PG cells contribute most to the cortex, striatum, and hippocampus and are virtually excluded from the diencephalon, particularly the hypothalamus. This pattern is sustained regardless of the level of chimerism, although a few PG cells can be seen in the thalamus as well as the habenula when the highest levels of chimerism are obtained. Thus, from the earliest stages of forebrain development, a pattern of cell distribution is established that is sustained into adulthood.

Table 2. Analysis of sexual and aggressive behavior in PG–N chimeric and control mice

	Female sexual behavior		Male sexual behavior		Male aggressive behavior	
	Mount latency	Mount frequency	Mount latency	% test with full mating	Inspect frequency	Attack latency
Chimera	5.1 ± 2.5	10.8 ± 4.8	9.5 ± 4.0	22	2.8 ± 1.2	16.9 ± 7.4
Control	6.5 ± 2.7	6.5 ± 5.4	9.1 ± 4.0	42	3.8 ± 1.4*	265 ± 150*
					4.9 ± 2.3†	269 ± 67†
P value	NS	NS	NS	NS	NS	<0.04

P values were determined by Student's *t* test. NS, not significant.
*Nonchimeric littermate (CFLP) control group.
†ROSA26 donor strain control group.

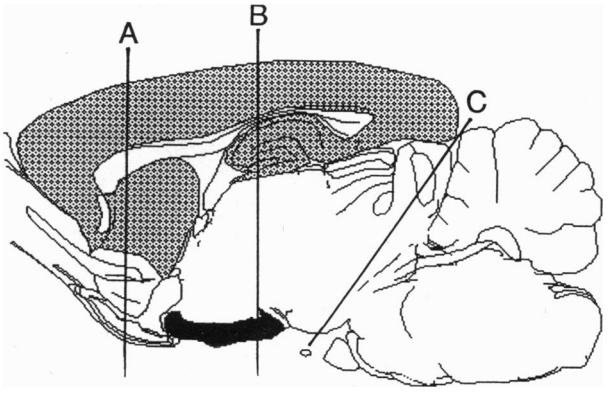

FIG. 7. Line drawing of saggital section through the adult brain. Stippled area delineates regions that show the highest accumulation of PG cells; solid area indicates regions from which PG cells are excluded. Line A, level of sections shown in Fig. 4*A*; line B, level of sections shown in Figs. 3 and 4*B*; line C, level of sections shown in Fig. 4*C*.

In adult telencephalic structures, even generally low level chimeras have a high contribution of PG cells. In contrast, the relatively high numbers of PG cells seen in the brainstem at midgestation produce a relatively small contribution to the adult brainstem, and absolute numbers in this region appear to depend more heavily on the level of chimerism. The sustained presence of PG cells in the forebrain may result from enhanced proliferation of PG cells in this area. Such a possibility is supported by the finding that brain size of PG chimeras is enlarged relative to body weight when compared to controls.

Large numbers of PG cells in the cortex had no overt effect on mouse behavior. However, the tests used were not designed to specifically assess cortical function. The finding that chimeric males were more aggressive was significant. Since aggressive behavior in mice is dependent on olfactory cues (15, 16), the rapid onset of aggressive behavior in PG chimeric males could result from a greater sensitivity to, or more efficient processing of, these cues. The finding of high levels of PG cells among the olfactory receptor neurons and in areas of the brain that process this information to produce behavior (pyriform and frontal cortex) is congruent with this hypothesis.

Uniparental disomy of distal chromosome 2 results in neonatal death with associated behavioral problems, including failure to suckle (7, 17). Interestingly, the most chimeric neonatal mice also failed to suckle and usually died within the first few days of birth, even when competition from larger littermates was removed. In these chimeras, we have noted exceptional PG contributions (up to 90%) in frontal cortical regions of the brain compared with low contributions in the vital organs (e.g., liver, kidney) and complete absence of PG cells in skeletal muscle in the same animals. Thus, neonatal death may be associated with the very high numbers of PG cells present in the brain. The failure to thrive could be associated with the chromosome 2 disomy phenotype. This could involve poor recognition of the mother. In this regard, a possible influence of imprinting on olfaction is worth consideration (18). In the olfactory mucosa, PG cells accumulated with remarkable symmetry over the turbinate bones and were virtually absent from the midline septum except for the septal organ, which also showed a high concentration. This symmetrical organization of PG cells in the olfactory mucosa may reflect the arrangement of olfactory neurons with respect to their separate projections to each olfactory bulb. Whether or not this arrangement of PG cells influences the types of olfactory receptors expressed remains to be determined (18).

These studies have identified functions of imprinted genes that result in specific phenotypes affecting brain development, brain size, and behavior. Of the genes that have now been identified as being imprinted (19), some are expressed in the brain, including *Snrpn* and *U2af bp-rs* in the mouse and PAR1 (*D15S227E*), PAR5 (*D15S226E*), *IPW*, and *ZNF127* from the Prader–Willi region in the human; however, it is not known at present how altered expression levels of these genes may contribute to the developmental or behavioral phenotypes reported here. Clearly, identification of further imprinted genes and analysis of their expression patterns and functions in the brain will be of great interest and need to be understood with respect to the phenotypes established in this study.

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