Serotonin Depletion and Barrel Cortex Development: Impact of Growth Impairment vs. Serotonin Effects on Thalamocortical Endings

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Converging evidence supports a role of serotonin (5-hydroxytryptamine; 5-HT) in barrel cortex development. Systemic administration of 5-HT-depleting drugs reduces cross-sectional whisker barrel areas in the somatosensory cortex (SSC) of neonatal rats. Here we assess the relative impact on barrel pattern formation of (i) 5-HT depletion and (ii) decreased brain growth, which is often associated with pharmacological 5-HT depletion, by comparing the effects of 5-HT-depleting drugs with those of reduced protein intake. Left hemisphere 5-HT levels in the SSC and right hemisphere whisker barrel areas were assessed at postnatal day 6 (P6) in the same animal following injection of *p*-chloroamphetamine (PCA) or *p*-chlorophenylalanine (PCPA) at P0. Both drugs significantly reduced cortical 5-HT content and mean barrel areas at P6, but also body and brain growth. Differences in brain weight accounted for 84.4% of the variance in barrel size, with negligible contributions by cortical 5-HT content. PCPA-treated animals sacrificed at P14 yielded similar trends, albeit less pronounced. Finally, reduced protein intake resulted in lower body weight and cortical 5-HT levels at P6, but yielded no change in brain weight or mean barrel area. Barrel formation therefore appears markedly less sensitive to 5-HT depletion *per se* than to drug-induced growth impairment.

Increasing attention has been recently focused upon putative roles for monoaminergic neurotransmitters in brain development [for review see (Levitt *et al.*, 1997)]. Serotonin (5-hydroxytryptamine; 5-HT) involvement in the fine-tuning of synaptic connections has been well established by classical studies on invertebrates, including *Aplysia* (Glanzman *et al.*, 1990; Bailey *et al.*, 1992). These data suggest that 5-HT may exert 'morphogenetic' or 'neurotrophic' effects on specific neurons. Much less is known about 5-HT contributions to development in the mammalian neocortex.

The rodent somatosensory system, with its one-to-one correspondence between each vibrissa and its cortical barrel-like projection area, represents an ideal model to assess the impact of serotoninergic manipulations on brain development and plasticity [for review see (Rice, 1995; Killackey *et al.*, 1995)]. Furthermore, transient barrel-like distribution of 5-HT (Fujimiya *et al.*, 1986; D'Amato *et al.*, 1987; Rhoades *et al.*, 1990; Blue *et al.*, 1991; Bennett-Clarke *et al.*, 1991, 1994a; Dori *et al.*, 1996), of 5-HT_{1B} and 5-HT_{2A} receptors (Leslie *et al.*, 1992; Bennett-Clarke *et al.*, 1993; Mansour-Robaey *et al.*, 1998) and of the 5-HT transporter (D'Amato *et al.*, 1987; Lebrand *et al.*, 1996; Mansour-Robaey *et al.*, 1998) [for review see (Fuchs, 1995)] in layer IV of neonatal rodent somatosensory cortex (SSC) spur further interest into 5-HT involvement in the development of thalamocortical pathways.

Initial support for neurotrophic roles of 5-HT in mammalian somatosensory pathways has come from pharmacologically induced 5-HT depletion (Blue *et al.*, 1991; Bennett-Clarke *et al.*, 1994b; Osterheld-Haas *et al.*, 1994). Systemic administration of a variety of 5-HT-depleting agents has been shown to produce alterations of barrel-like patterns, best described as a delay in barrel pattern maturation (Blue *et al.*, 1991; Osterheld-Haas *et al.*, 1994) or as a reduction in cross-sectional areas of whisker barrels (Bennett-Clarke *et al.*, 1994b).

Recent support for the involvement of 5-HT in thalamocortical development has come from transgenic models. MAO-A knockout mice are devoid of cortical barrels, which reappear when 5-HT synthesis is inhibited by *p*-chlorophenylalanine (PCPA) (Cases *et al.*, 1996). Furthermore, mice devoid of the plasma membrane 5-HT transporter (Bengel *et al.*, 1998) display significantly thinner barrels in layer IV of the postero-medial barrel subfield (PMBSF) and the absence of barrel-like patterns in the other subfields of the primary somatosensory cortex (Persico *et al.*, 1998).

Further interest in these data, underscoring 5-HT roles in cortical neurodevelopment, is spurred by developmental changes in brain 5-HT content and 5-HT synthesis capacity demonstrated in rhesus monkeys (Goldman-Rakic and Brown, 1982) and in humans (Chugani *et al.*, 1999). Moreover, these results are in line with *in vivo* evidence of transiently increased neonatal 5-HT levels in other brain regions of non-rodent species, possibly related with 5-HT regulation of synaptogenesis (Okado *et al.*, 1989, 1993; Chen *et al.*, 1994; Niitsu *et al.*, 1995) and with previous *in vitro* studies showing 5-HT effects on cortical synaptogenesis, neurite branching, myelination and glial proliferation in tissue culture (Chubakov *et al.*, 1986; Sikich *et al.*, 1990). More recent *in vitro* studies focused on thalamocortical neurons suggest that 5-HT may influence both neurite outgrowth and synaptic transmission in neonatal rats (Rhoades *et al.*, 1994; Lieske *et al.*, 1999). Interestingly, 5,7-DHT-induced barrel pattern alterations *in vivo* appear to be tetrodoxin (TTX)-insensitive (Rhoades *et al.*, 1998).

Although both 5-HT depletion studies and assessments of brains from knockout mice provide converging support for 5-HT roles in somatosensory thalamocortical neurodevelopment, caution is raised by systemic 5-HT-depleting drug treatments inevitably inducing some degree of growth impairment, possibly through malnutrition, which has been shown to delay whisker barrel pattern formation *per se* (Vongdokmai, 1980). Our study was undertaken to provide an estimate of the impact of (i) 5-HT depletion and (ii) delayed body growth on development of the whisker barrel cortex, following systemic 5-HT-depleting drug treatments. To this aim, we systemically administered at birth either the 5-HT terminal-selective neurotoxin *p*-chloroamphetamine (PCA) (Miller *et al.*, 1970; Baumgarten *et al.*, 1982; Commins *et al.*, 1987; Haring *et al.*, 1994) [for review see (Azmitia and Whitaker-Azmitia, 1995)] or the 5-HT synthesis inhibitor *p*-chlorophenylalanine (PCPA) (Koe and Weissman, 1966), whose effects on cortical 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) levels, on whisker barrel cross-sectional areas, and

on body and brain weight were assessed in the same animal for the first time in this study. Furthermore, in order to better define protein deficiency contributions to drug-induced delays in barrel formation, we assessed with the same methodology the offspring of pregnant females fed with either a hypoproteic or a normoproteic diet starting just prior to the expected birth date and throughout the first week of neonatal life of their pups. Our results clearly show that when pharmacological interventions yield both 5-HT depletion and neonatal growth retardation, the latter factor is largely responsible for altered somatosensory cortical development. Moreover, drug or dietary treatments that do not reduce brain weight also do not have an impact on barrel size, despite yielding variable extents of cortical 5-HT depletion.

Materials and Methods

Animals

Experiments were performed on newborn Lewis rats (Charles River, Calco, Italy) during the first 15 days of postnatal life. In this study, P0 is defined as the first 24 h after delivery. Pregnant female rats were singly housed and maintained on a 12 h light/12 h dark schedule (lights on at 7:00 a.m.), with free access to food and water.

Systemic Administrations of PCA and PCPA

All animals were injected between 3 and 6 h after birth. PCA experiments were carried out on 17 pups belonging to two litters; PCPA experiments were performed on a separate set of 47 animals belonging to four litters. Each litter was divided into an experimental and a control group. Pups were weighted and briefly anesthetized using hypothermia. Experimental animals from the first two litters were injected s.c. with either 3.5 or 14 mg/kg PCA (0.01 ml/g of a 0.35 or 1.4 mg/ml solution in saline, respectively); experimental animals from the remaining four litters received PCPA (300 mg/kg s.c.; 0.01 ml/g of a 30 mg/ml solution). Control animals were injected with equivalent volumes of sterile saline solution. After the injection, pups were quickly warmed using an infrared light and returned to their cage.

Manipulation of Dietary Protein Intake

Since PCA and PCPA administration produced prominent decreases in body and brain growth (see Results), possible contributions of early-postnatal hypoproteic dietary intake to altered barrel development were assessed in 12 pups, belonging to two separate litters. The diets administered to pregnant females were identical to those adopted by Vongdokmai (Vongdokmai, 1980) in mice. Two female Lewis rats undergoing timed pregnancies were fed with a normoproteic 28%-casein diet throughout their pregnancy. Two days prior to the expected delivery date, one of the two females was switched to a hypoproteic 8%-casein diet, including increased amounts of carbohydrates to maintain constant the total caloric intake. Both animals were fed *ad libitum* with either the normoproteic or the hypoproteic diet, until their pups reached P6.

Assessments of Brain Weights and Cortical Dissection

Animals were killed by decapitation either at P6 (PCA- or PCPA-treated vs. saline; hypoproteic vs. normoproteic diet) or at P14 (PCPA-treated vs. saline). Brains were quickly dissected and the hindbrain removed with a coronal cut separating dorsally the superior and inferior colliculi, and reaching ventrally the mamillary bodies (Glowinski and Iversen, 1966). Brains were immediately weighted, the hemispheres separated with a scalpel and the cortices dissected from the rest of each hemisphere. Whisker barrel fields were punched out from left hemispheres using a template (Strominger and Woolsey, 1987), immediately frozen on dry ice (<4 min after sacrifice), stored at −70°C for a maximum of 2 weeks and then placed in liquid nitrogen until later assessments using HPLC. The whole controlateral cortex was quickly placed free-floating in 4% paraformaldehyde dissolved in phosphate buffer (PB, pH 7.4) at 4°C for 12–16 h, flattened, cryoprotected with 30% sucrose dissolved in PB (pH 7.4) for 36–48 h, cut into 50 μm slices using a cryostat and processed for acethylcholinesterase (AchE) staining (see below).

Assessment of Neurotransmitter Levels in the SSC

5-HT, 5-HIAA and norepinephrine (NA) were simultaneously determined utilizing a reverse-phase HPLC procedure coupled with electrochemical detection (Kempf and Mandel, 1981; Cabib and Puglisi-Allegra, 1994). On the day of analysis, frozen samples were weighted and homogenized in 0.1 N $HClO_4$ containing 6 mM Na-metabisulphite and 1 mM EDTA. To compensate for variations in sample size, the extraction solution was added to each sample at a concentration of 10 μl/mg of sample weight. Homogenates were centrifuged at 10 000 *g* for 20 min at 4°C. Supernatants were removed and stored in the dark on ice until a 15 μl aliquot of each sample was transferred to the HPLC system, consisting of a Waters 460 electrochemical detector with a glass carbon working electrode and a pump (Waters 510). The potential was set at 800 mV (vs. Ag–AgCl reference electrode). A Bondapak C18 column (10 mm particle size, 300 × 3.1 mm i.d.) purchased from Waters Assoc. (Millipore, Milford, MA) was employed. The flow rate was 1.1 ml/min. The mobile phase consisted of 8% methanol in 0.1 M Na-phosphate buffer (pH 3.0), 0.01 mM Na_2EDTA and 1.1 mM L-octane sulphonic acid sodium salt (Aldrich, Milwaukee, WI); 3,4-dihydroxy-benzylamine hydrobromide (Aldrich) was used as internal standard.

Labelling Thalamocortical Terminals by AchE Histochemistry

AchE has been shown to transiently label somatosensory thalamocortical afferents in neonatal rodents [for review see (Fuchs, 1995)]. Histochemistry for AchE was performed according to the method of Hedreen *et al.* (Hedreen *et al.*, 1985), achieving on these post-fixed brains results qualitatively similar to those routinely obtained from brains of perfused animals (Fig. 1). Briefly, slices were rinsed in 0.1 M sodium acetate (pH 6.0) and incubated for 5 h at 37°C in a medium containing 50 mg of acetylthiocholine iodide, 65 ml of 0.1 M sodium acetate (pH 6.0), 4 ml of 0.1 M sodium citrate, 10 ml of 0.03 M cupric sulfate ($CuSO_4 5H_2O$), 21 ml of distilled water and 100 μl of a 10^{-2} M solution of tetraisopropyl-pyrophosphoramide (final concentration 10^{-5} M) for 100 ml of total medium volume. Slices were then rinsed with agitation for 10 min in 0.1 M sodium acetate (pH 6.0), incubated for 10 min in 2% potassium ferrocyanide at room temperature and rinsed twice in 0.1 M sodium acetate (pH 6.0).

Measurements of Whisker Barrel Areas and PMBSF Size

AchE-stained sections were used to measure single barrel cross-sectional areas and total PMBSF surface using the Kontron Imaging System KS100 (Kontron Elektronik, Eching b. München, Germany). Following image acquisition, contrast and smoothing were adjusted to yield as well-defined and uniform barrel boundaries as possible in each preparation. All whisker barrels within the PMBSF of the primary somatosensory cortex were circled and areas were measured by one of the authors, who was blind to the treatment group. The following whisker barrel areas were used for statistical analyses: A1–A4, B1–B4, C1–C7, D1–D8, E1–E8. Data from barrels α, β, γ, δ, A5 and higher-order barrels from rows D and E were not included in the analyses, due to interindividual inconsistencies in barrel presence or to frequent unreliability of barrel boundaries. The cortical surface devoted to the PMBSF was measured as (i) 'PMBSF area' expressed in mm^2, encompassing all of the barrels listed above together with barrels α, β, γ, δ; and (ii) 'total line length' expressed in mm, obtained connecting the barrel centroids of the four most caudal vibrissae in each row, as described previously (Bennett-Clarke *et al.*, 1994b). 'PMBSF area' thus provides a direct measure of cortical surface devoted to AchE-stained vibrissa-related patterns, whereas 'total line length' yields a relative estimate of PMBSF area, controlling for differences in the extent of arborization present in the outermost barrels, as first suggested by Riddle *et al.* (Riddle *et al.*, 1992, 1995).

Statistical Analyses

A one-way analysis of variance (ANOVA) was performed to assess systemic PCA-induced alterations in each neurochemical, histological and

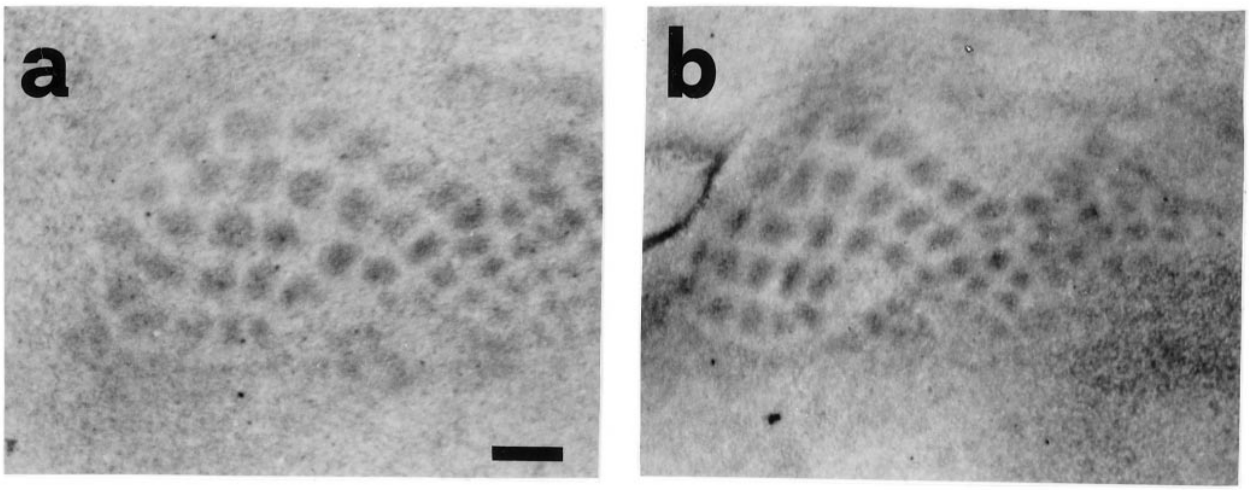

Figure 1. AchE staining of whisker barrel fields from animals injected with (*A*) saline or (*B*) PCA (14 mg/kg) at P0 and sacrificed at P6. Scale bar, 500 µm.

physical parameter. When significant treatment effects were detected, pairwise *a priori* contrasts were performed, testing the hypothesis that PCA would reduce cortical 5-HT content, barrel areas and body growth, as expected on the basis of previous work (Blue *et al.*, 1991; Osterheld-Haas *et al.*, 1994). Independent *t*-tests were used to compare systemic PCPA vs. saline and hypoproteic vs. normoproteic diet effects on each neurochemical, histological and physical parameter.

Correlation coefficients between single variables and stepwise multiple regressions were finally performed to estimate how much of the variance in mean whisker barrel area is explained by differences in brain weight and how much stems from cortical 5-HT content (Tabachnick and Fidell, 1989). Entry and removal of independent variables into the regression equation were defined setting *F*-to-enter = 1 and *F*-to-remove = 0, respectively. The percentage variance in mean whisker barrel area explained by changes in brain weight and/or by extent of 5-HT depletion was derived from the change in R^2 brought about by the entry of each of the two independent variables into the regression equation.

Statistical significance was set at $\alpha < 0.05$ also for *a priori* contrasts. Sample sizes were established as much as possible prior to experiments, using power analyses performed with the POWER program (Dupont and Plummer, 1990). Parameters were set at $\alpha < 0.05$, and $\beta > 0.80$, while interindividual variability was estimated from available literature or original preliminary data. Results are reported as mean ± SEM.

Results

Effects of Neonatal Systemic PCA Administration on Monoamine Levels in the SSC

Single injections of PCA (3.5 or 14 mg/kg s.c.) performed at P0 yielded statistically significant 49.0 and 58.9% reductions in somatosensory cortical 5-HT content, respectively, at P6 (Fig. 2). In these same animals, 5-HIAA tissue levels were decreased by 24.4–40.0%, yielding significantly increased 5-HIAA/5-HT ratios (Fig. 2). The 14 mg/kg dose did not yield changes in 5-HT content, 5-HIAA content and 5-HIAA/5-HT ratio significantly more profound than those produced by the 3.5 mg/kg dose. No significant effect on NA tissue content was recorded.

Effects of Neonatal Systemic PCA Administration on Body and Brain Weight

Statistically significant differences in body weight between PCA- and saline-injected animals were recorded at P6 but not at P0, due to different growth rates (Fig. 2). Compared with saline -injected controls, PCA-treated animals displayed significantly reduced mean body weight [25.7–32.1% for lower and higher dose, respectively; one-way ANOVA: $F(2,16) = 8.30$, $P < 0.001$] and brain weight [9.6% decrease for the higher dose; one-way ANOVA: $F(2,16) = 5.89$, $P < 0.05$] before sacrifice at P6.

Effects of Neonatal Systemic PCA Administration on Whisker Barrel Areas

Single PCA injections at P0 yielded significant (9.0–21.4%) reductions in mean whisker barrel areas, with the higher dose significantly more effective than the lower dose [one-way ANOVA: $F(2,16) = 18.92$, $P < 0.001$; *a priori* contrast (3.5 vs. 14 mg/kg): $T(14) = 6.15$, $P < 0.001$; Fig. 2]. Assessments of single whisker barrel rows indicate that all barrel rows were affected to the same extent (data not shown). Significant reductions in cortical surface devoted to the AchE-stained vibrissae-related pattern were also recorded, measured either as 'PMBSF area' [one-way ANOVA: $F(2,16) = 12.58$, $P < 0.001$] or 'total line length' [one-way ANOVA: $F(2,16) = 7.47$, $P < 0.01$; Fig. 2]. The correlation between 'PMBSF area' and 'total line length' was significant (Pearson $r = 0.84$, $P < 0.001$).

Effects of Neonatal Systemic PCPA Administration on Monoamine Levels in the SSC

Since PCA may also exert direct toxic effects on thalamocortical terminals, which transiently express the 5-HT transporter (Lebrand *et al.*, 1996) (see Discussion), the next series of experiments was undertaken to indirectly estimate the 'weight' of reduced growth rates and 5-HT depletion on barrel formation, while eliminating the potential for direct thalamocortical neurotoxicity.

Single injections of the selective tryptophan-hydroxylase inhibitor PCPA (300 mg/kg s.c.) (Koe and Weissman, 1966), performed during the first 6 h after delivery, produced massive mean decreases in somatosensory cortical 5-HT and 5-HIAA levels (93.4 and 83.3%, respectively) measured at P6 (Fig. 3). As with PCA, systemic PCPA was more effective in reducing 5-HT than 5-HIAA levels, yielding 5-HIAA/5-HT ratios increased by ~150% (Fig. 3).

At P14, somatosensory cortical 5-HT and 5-HIAA levels of PCPA-injected animals displayed more modest mean reductions (28.7 and 35.5%, respectively), with no significant variation in

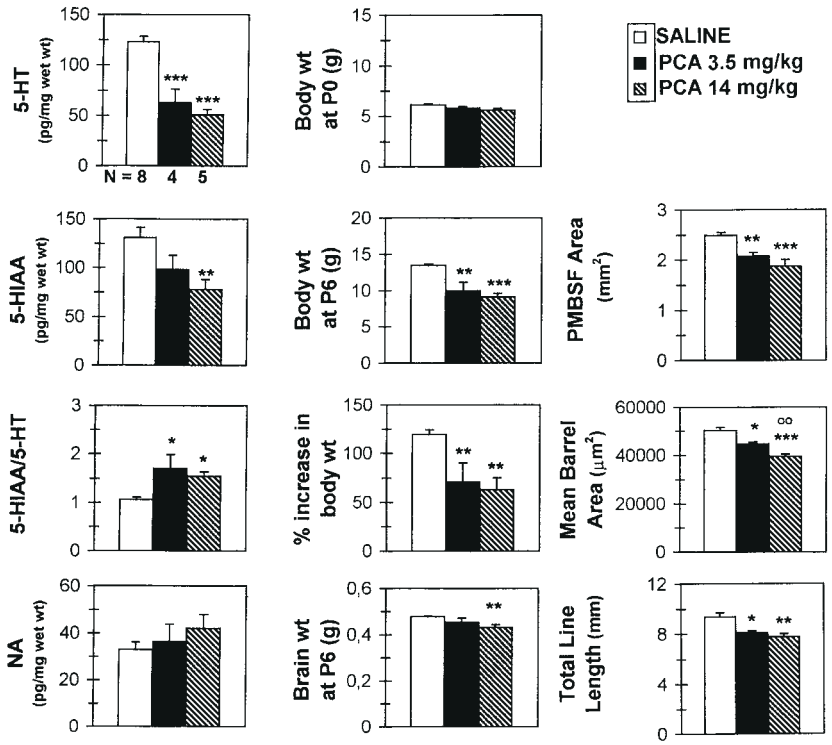

Figure 2. Effects of systemic PCA (3.5 or 14 mg/kg s.c.) or saline injected at P0 on somatosensory cortical tissue content of 5-HT, 5-HIAA and NA (left column); body weights (wt) recorded at time of first injection (P0) and time of sacrifice (P6), percentage increase in body weight between the two measurements and brain weights recorded at the time of sacrifice (P6) (middle column); mean whisker barrel areas, 'PMBSF area' and 'total line length', measured at P6 as described in Materials and Methods (right column). Sample sizes for each group are reported below the 5-HT content histogram. * = saline vs. PCA ° = PCA 3.5 mg/kg vs. PCA 14 mg/kg.

5-HIAA/5-HT ratio (Fig. 4). No significant change in NA levels was recorded at either P6 or P14 (Figs 3 and 4).

Effects of Neonatal Systemic PCPA Administration on Body Growth and Brain Weight

PCPA again produced relevant effects on body and brain weight, assessed at P6 (Fig. 3). PCPA-injected animals displayed even more prominent (37.6%) decreases in body weight, with striking (71.2%) reductions in body growth rate compared with saline-injected controls. Reductions in body weight and growth rates recorded at P6 were accompanied by significant (22.5%) reductions in brain weight as well (Fig. 3).

PCPA effects on body growth were less pronounced though still significant at P14, with 21.1% reductions in body weight and growth rates decreased by 30.8% (Fig. 4). Brain weights were practically identical, with only a 3.6% mean decrease in PCPA-treated animals compared to saline-injected pups (Fig. 4).

Effects of Neonatal Systemic PCPA Administration on Whisker Barrel Areas

Systemic PCPA administration at P0 produced highly significant (19.4%) reductions in mean whisker barrel areas at P6, coupled with 17% and 9.7% reductions in 'PMBSF area' and 'total line length', respectively (Fig. 3). Decreases of similar extent were recorded in all barrel rows, ranging from ~16% for barrels in rows A and B to 21% for rows D and E.

At P14, modest (6.6%) reductions in mean barrel size of PCPA-injected pups were not statistical significant (Fig. 4). Also, non-significant 7.2% decreases in 'PMBSF area' were associated with measures of 'total line length' practically superimposable on those of saline-treated animals (Fig. 4).

Correlations between PCPA Effects on Cortical 5-HT Levels, Brain Weight and Whisker Barrel Areas

Since PCPA does not possess a potential for direct neurotoxic effects, it was possible to estimate the impact of the two independent variables, namely brain weight and 5-HT content in the SSC, on the dependent variable, mean whisker barrel area, using correlation and stepwise multiple regression analyses. Results of these analyses are summarized in Table 1. Mean whisker barrel area values of animals for the entire P0–P6 sample, including 13 PCPA- and 15 saline-injected pups, are significantly correlated with brain weight (Pearson $r = 0.82$, $P < 0.001$), but not with cortical 5-HT content ($r = -0.21$, n.s.). Saline-treated animals show expected positive correlations between mean whisker area and brain weight (Pearson $r = 0.60$, $P < 0.05$), but not with cortical 5-HT content ($r = 0.36$, n.s.). These data derive from two distinct replicas of the P0–P6 experiment performed on two separate litters (litter A: $n = 16$, nine PCPA and seven saline; litter B: $n = 12$, six PCPA and six saline), whose results are displayed in Figure 5. PCPA-treated animals from both litters clearly show a positive linear correlation between mean whisker barrel area and brain weight values, and no correlation between barrel areas and cortical 5-HT content (Fig. 5). No significant correlation is consistently found in saline-treated animals from both litters sacrificed at P6 (Fig. 5).

Similarly, animals sacrificed at P14, including 11 PCPA- and eight saline-injected pups, display mean whisker barrel area values significantly correlated with brain weight (Pearson r =

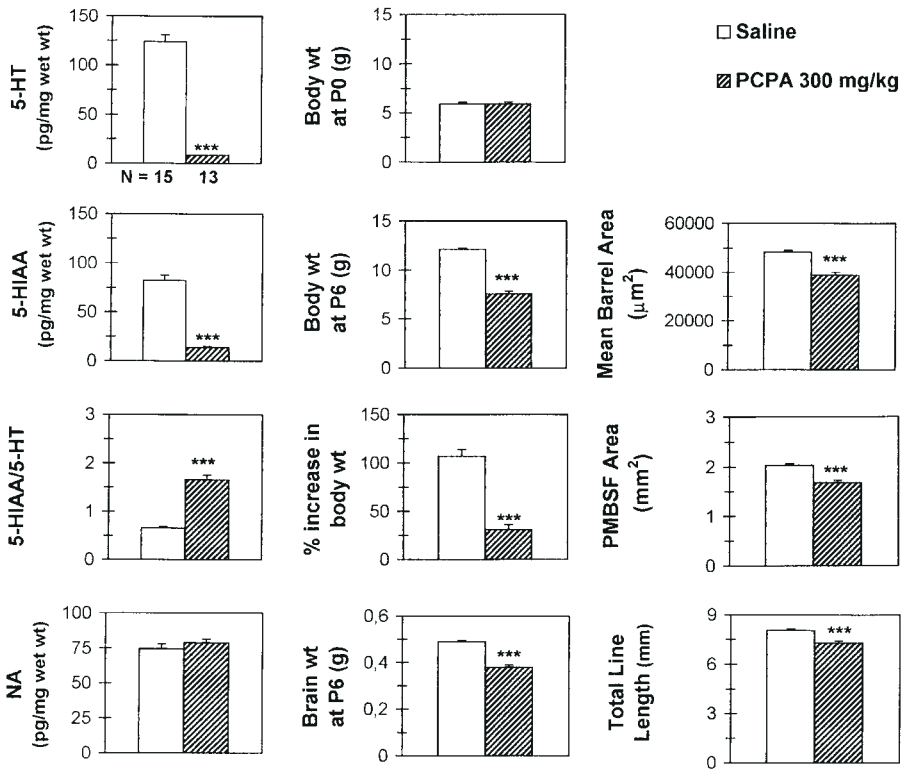

Figure 3. Effects of systemic PCPA (300 mg/kg s.c.) or saline injected at P0 on somatosensory cortical tissue content of 5-HT, 5-HIAA and NA (left column); body weights (wt) recorded at time of first injection (P0) and time of sacrifice (P6), percentage increase in body weight between the two measurements and brain weights recorded at the time of sacrifice (P6) (middle column); mean whisker barrel areas, 'PMBSF area' and 'total line length', measured at P6 (right column). Sample sizes for each group are reported below the 5-HT content histogram. * = saline vs. PCPA.

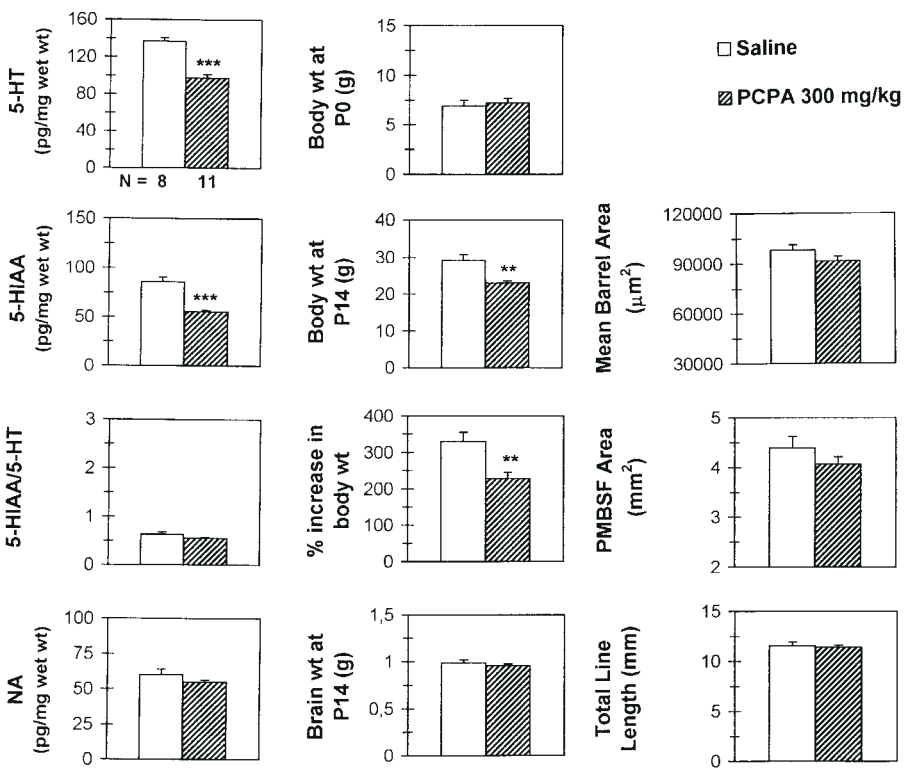

Figure 4. Effects of systemic PCPA (300 mg/kg s.c.) or saline injected at P0 on somatosensory cortical tissue content of 5-HT, 5-HIAA and NA (left column); body weights (wt) recorded at time of first injection (P0) and time of sacrifice (P14), percentage increase in body weight between the two measurements and brain weights recorded at the time of sacrifice (P14) (middle column); mean whisker barrel areas, 'PMBSF area' and 'total line length', measured at P14 (right column). Sample sizes for each group are reported below the 5-HT content histogram. * = saline vs. PCPA.

Table 1
Correlation coefficients between mean whisker barrel area (dependent variable) and brain weight/somatosensory cortical 5-HT content (independent variables), and stepwise multiple regression analyses for animals injected systemically with PCPA (300 mg/kg s.c.) or saline

Litter	Time of injection	Time of sacrifice	*n*		Mean barrel vs. brain weight	Mean barrel vs. 5-HT content	Variance (%) explained by:	
							Brain weight	5-HT content
A+B	P0	P6	28	13 PCPA	$r = 0.82, P = 0.0005$	$r = -0.21$, n.s.	67.9	–
				15 saline	$r = 0.60, P = 0.018$	$r = 0.36$, n.s.	36.4	14.7
							Overall 84.4	–
C+D	P0	P14	19	11 PCPA	$r = 0.75, P = 0.004$	$r = -0.44$, n.s.	56.1	11.0
				8 saline	$r = 0.67, P = 0.035$	$r = -0.76, P = 0.014$	–	58.3
							Overall 51.6	–

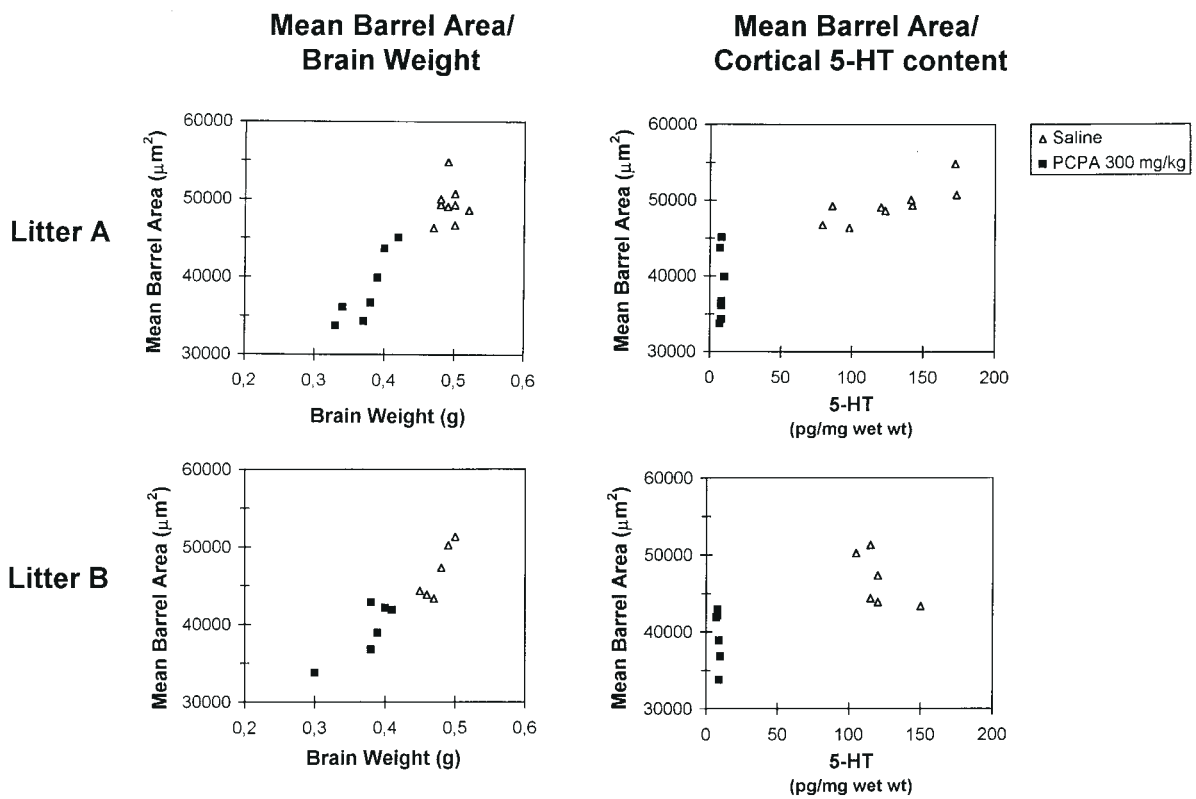

Figure 5. Scatter plots of mean whisker barrel areas by brain weight (left) and by somatosensory cortical 5-HT content (right) in two distinct litters of pups injected systemically with PCPA (300 mg/kg s.c.) or saline.

0.75, $P < 0.01$), but not with cortical 5-HT content ($r = -0.44$, n.s.), as summarized in Table 1. Saline-treated animals again show expected positive correlations between mean whisker barrel area and brain weight (Pearson $r = 0.67$, $P < 0.05$), and also negative correlations with cortical 5-HT content ($r = -0.76$, $P < 0.05$), as more mature brains at this age likely possess more developed barrels and lower 5-HT amounts stored in thalamocortical endings. Also these data derive from two distinct replicas of the same experiment performed on two separate litters injected at P0 and sacrificed at P14 (litter A: $n = 9$, five PCPA and four saline; litter B: $n = 10$, six PCPA and four saline), providing identical outcomes.

Stepwise multiple regression analyses performed on the entire P0–P6 sample of 28 animals indicate that brain weight explains 84.4% of the variance and that somatosensory cortical 5-HT content does not reach the threshold to fit into the model, as it would explain only 0–5% of the variance (Table 1). Both litters A and B, analyzed separately, display identical trends (Fig. 5). Analyses performed on the P0–P14 sample yield a very similar outcome, with brain weight explaining 51.6% of the variance and somatosensory cortical 5-HT content again not reaching the threshold to fit into the regression equation (Table 1).

Effects of Decreased Protein Intake on Neonatal Monoamine Levels in the SSC, Body and Brain Weight, and on Whisker Barrel Areas

Results summarized in the previous sections indicate that PCA and PCPA effects on whisker barrel size may largely stem from drug-induced reductions in body and brain growth rates. In order to assess potential contributions of malnutrition and diminished protein intake to these effects, we appraised the alterations produced by a hypoproteic diet fed to a pregnant

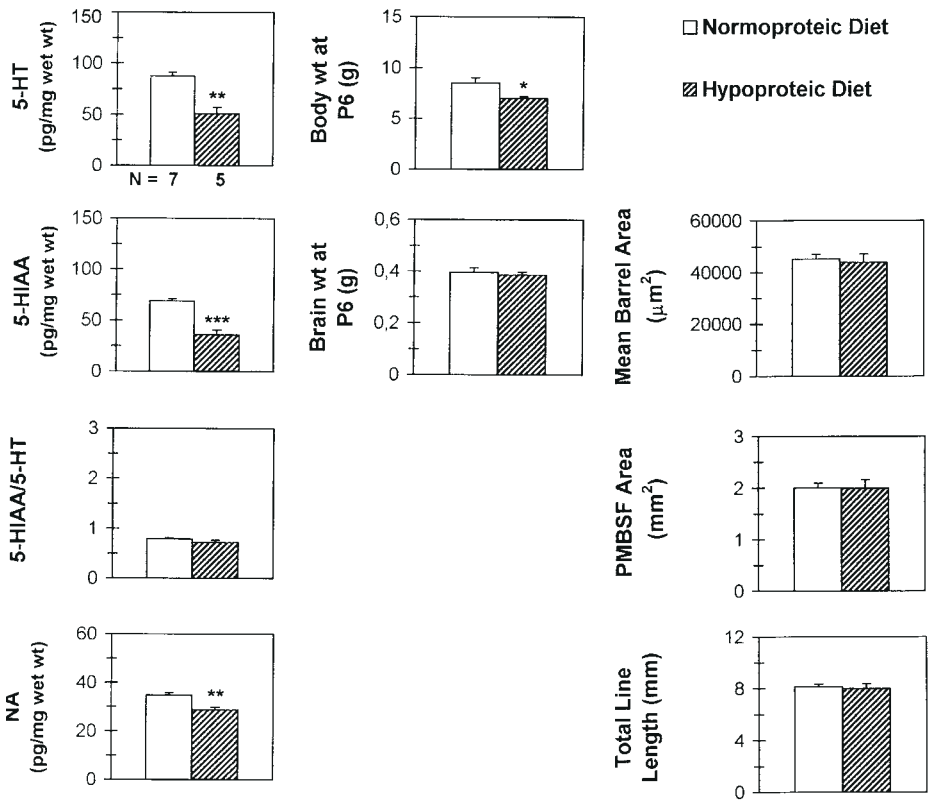

Figure 6. Effects of a hypoproteic or normoproteic diet on somatosensory cortical tissue content of 5-HT, 5-HIAA and NA (left column); body and brain weights (wt) measured at the time of sacrifice (P6) (middle column); mean whisker barrel areas, 'PMBSF area' and 'total line length' (right column), measured at P6. Sample sizes for each group are reported below the 5-HT content histogram. * = normoproteic vs. hypoproteic diet.

female starting 2 days prior to the expected delivery date and throughout the first week of postnatal life of her offspring. This protocol was chosen to ensure that protein intake would be reduced starting at approximately the same developmental stage affected by PCPA inections, i.e. at P0.

Compared with seven pups of a female rat fed the normoproteic-normocaloric diet, five pups of a female fed the hypoproteic-normocaloric diet showed highly significant (41.8 and 47.6%) decreases in somatosensory cortical 5-HT and 5-HIAA levels, respectively (Fig. 6). Also, somatosensory cortical NA levels were reduced by 19.33%. Despite significantly lower cortical 5-HT levels and significant (17.7%) reductions in body weight at P6, negligible (2.6%) decreases in brain weight were interestingly paralleled by similarly negligible (2.9%) reductions in mean barrel area (Figs 6 and 7).

Correlations between Cortical 5-HT Levels, Brain Weight and Whisker Barrel Areas in Malnourished Pups

Mean whisker barrel area values of animals from the entire sample, including five malnourished and seven control pups, were significantly correlated with brain weight (Pearson r = 0.78, $P < 0.01$), but not with cortical 5-HT content ($r = 0.06$, n.s.), as summarized in Table 2. Both malnourished and control pups, analyzed separately, yielded significant correlations between mean whisker barrel areas and brain weight, but no correlation with cortical 5-HT content (Table 2).

Stepwise multiple regression analyses indicate that brain weight explains between 52.1 and 75.4% of the variance, depending on whether only malnourished or control animals are taken into account, or both (Table 2 and Fig. 7). Somatosensory

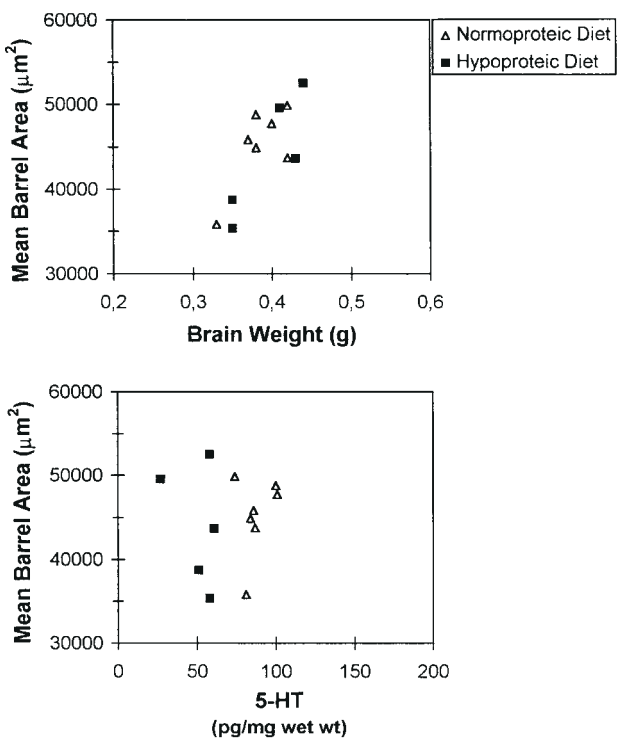

Figure 7. Scatter plots of mean whisker barrel areas by brain weight (top) and by somatosensory cortical 5-HT content (bottom) in two litter of pups sacrificed at P6, whose mothers were fed with a normoproteic or a hypoproteic diet starting 2 days prior to the expected delivery date.

Table 2
Correlation coefficients between mean whisker barrel area (dependent variable) and brain weight/somatosensory cortical 5-HT content (independent variables), and stepwise multiple regression analyses of the offspring of pregnant Lewis female rats fed with a normoproteic (26% casein) vs. a hypoproteic (8% casein) diet starting 2 days prior to the expected delivery date and throughout the first week of postnatal life

Litter	Diet between E1 and E19	Diet between E19 and P6	*n*	Mean barrel vs. brain weight	Mean barrel vs. 5-HT content	Variance (%) explained by:	
						Brain weight	5-HT content
E	26% casein	26% casein	7	$r = 0.72, P = 0.033$	$r = 0.28$, n.s.	52.1	–
F	26% casein	8% casein	5	$r = 0.87, P = 0.026$	$r = -0.34$, n.s.	75.4	11.7
Total (E+F)			12	$r = 0.78, P = 0.002$	$r = 0.06$, n.s.	60.2	–

cortical 5-HT content does not fit into the model (Fig. 7), except for contributing an additional 11.7% of variance in malnourished animals (Table 2).

Discussion

Methodological Issues

The results reported here were achieved by concomitantly assessing body and brain weights, monoamine levels in the SSC and whisker barrel areas in the same animal following systemic drug administration or manipulation of dietary protein intake. Parallel measurements of neurotransmitter levels in the SSC of one hemisphere and of whisker barrel size in the controlateral hemisphere greatly enhance the reliability of our results and their statistical power.

The impact of inter-individual and particularly inter-litter variability was significantly reduced by using inbred Lewis rats instead of outbred strains. Genetic homogeneity has further strengthened the reliability of our findings, by contributing to the minimal variability in monoamine and metabolite levels, barrel and PMBSF size, and physical parameters encountered in this study (see Figs 2–4 and 6).

Systemic PCA experiments employing two distinct doses were performed to verify the coincidence of 5-HT depletion and weight loss using several different 5-HT-depleting drug paradigms; however, only results obtained with PCPA, a selective tryptophan-hydroxylase inhibitor, were used for multiple regression analyses (Table 1 and Fig. 5). Systemic PCA data were excluded from these analyses because of the potential confounding factor of PCA-mediated direct damage of thalamocortical endings, which transiently express both the plasma membrane 5-HT transporter and the vesicular monoamine transporter in neonatal rodents (Lebrand *et al.*, 1996). PCA can be expected to cause massive 5-HT release from terminals of mostly dorsal raphe neurons providing diffuse innervation of the somatosensory cortex (Bennett-Clarke *et al.*, 1991; Lebrand *et al.*, 1996). Favored by PCA-induced MAO inhibition, extracellular 5-HT is believed to be non-enzymatically converted into 5,6-DHT, a neurotoxic compound selectively taken up by 5-HT transporter-expressing terminals; once inside the terminal, 5,6-DHT rapidly autoxidizes forming highly reactive quinones, which spontaneously cross-link intracellular proteins, causing neurite degeneration (Miller *et al.*, 1970; Baumgarten *et al.*, 1982; Commins *et al.*, 1987; Haring *et al.*, 1994) [for review see (Azmitia and Whitaker-Azmitia, 1995)]. The transient expression of the 5-HT transporter and of the vesicular monoamine transporter during the first 2 weeks of neonatal life in rodents could thus expose thalamocortical terminals to neurotoxic damage by drugs, such as PCA, active through uptake-dependent mechanisms. Preliminary results using PCA-delivering elvax chips placed directly over the SSC of neonatal rats indeed provide initial support to this hypothesis (Persico *et al.*, 1997) (unpublished observations).

One practical limitation inherent to our experimental approach is that only one staining can be used to label thalamocortical terminals, when working with manageable sample sizes. Histochemistry for AchE was employed here for its rapidity, reliability and validity. Our histochemical procedure was adjusted to consistently yield, in unperfused tissues fixed only by immersion, results comparable to those obtained using perfused brains (Fig. 1). Furthermore, AchE has been shown to be a valid and reliable marker of thalamocortical terminals in neonatal rats: it is transiently expressed by thalamocortical neurons between P3 and P18, and somatosensory cortical AchE corresponds precisely to areal and laminar distributions of thalamocortical terminals (Kristt, 1979; Kristt and Waldman, 1981; Robertson, 1987). Nonetheless, DiI or tenascin staining should be employed in future experiments similar to those described here, to further strengthen our conclusions.

In malnutrition experiments, dietary protein intake may have been higher than anticipated solely on the basis of the hypoproteic-normocaloric 8%-casein diet, as cannibalism of dead pups by their mother occurs frequently in rodents during the first few days after delivery. Nonetheless, the significant decreases in somatosensory cortical 5-HT and NA levels recorded in malnourished pups (Fig. 6) strongly suggest that the diet effectively reduced amino acid intake and consequently brain monoamine synthesis.

Finally, most studies performed to date, including ours, focus on cross-sectional barrel areas, which may not represent the most valid and/or sensitive parameter to assess 5-HT modulation of thalamocortical development in the somatosensory system. Direct assessments of neurite arborization patterns, for example, may yield more interpretable results.

Effects of 5-HT Depletion on Neonatal Growth: Comparison between Our Findings and Previous Literature

The involvement of 5-HT in development of the mammalian neocortex is supported by evidence coming from both *in vitro* and *in vivo* studies. Our results confirm prior findings (Blue *et al.*, 1991; Osterheld-Haas *et al.*, 1994; Bennett-Clarke *et al.*, 1994b) indicating that systemic 5-HT depleting treatments produce an overall maturational delay, as supported here by brain weights and cross-sectional areas of whisker barrels being significantly reduced at P6, but not at P14 (Figs 2–4). This study, however, points toward an interpretation of *in vivo* results neither simple nor straightforward, and spurs interest in a reappraisal of *in vivo* studies employing 5-HT depleting drugs, both in neonatal rodents (Blue *et al.*, 1991; Bennett-Clarke *et al.*, 1994b; Chen *et al.*, 1994; Osterheld-Haas *et al.*, 1994) and in other species (Okado *et al.*, 1993; Niitsu *et al.*, 1995).

This study reaches three conclusions: firstly, the body and brain weights of PCA- or PCPA-injected neonatal rats increase between P0 and P6 significantly less than those of saline-treated pups; secondly, when 5-HT depletion and decreased brain growth are present, delayed maturation of thalamocortical pathways induced by impaired brain growth is clearly 'dominant' over selective 5-HT-mediated modulation of thalamocortical pathways (Fig. 5 and Table 1); and thirdly, drug or dietary treatments that produce significant reductions in cortical 5-HT levels without prominently affecting brain weight (i.e. PCPA-injected animals at P14 and neonatal malnutrition) yield minimal or no impact on whisker barrel size (Figs 4, 6 and 7 and Table 2).

The effect of 5-HT-depleting drugs on body and brain weights is believed to stem mostly from decreased food intake, as suggested by 5-HT_{1B} and 5-HT_{2C} receptor-mediated modulation of feeding behaviors, affecting both hypothalamic and extrahypothalamic sites (Lucas *et al.*, 1998). Nonetheless, contributions from altered metabolic rates are supported by the relatively modest impact of the hypoproteic diet on body weight compared with the effect of PCA- and PCPA-treatment.

Studies assessing 5-HT impact on development of thalamocortical pathways using 5-HT-depleting agents such as PCA or 5,7-DHT usually report modest-to-moderate decreases in body weight (Blue *et al.*, 1991; Osterheld-Haas *et al.*, 1994) in the absence of significant decreases in brain weight (Blue *et al.*, 1991; Bennett-Clarke *et al.*, 1994b; Osterheld-Haas *et al.*, 1994). The brain is well known to be one of the organs that least suffers from malnutrition merely in terms of decreased organ weight, possibly due to 'brain growth sparing mechanisms involving enhanced amino acid transport through the blood–brain barrier (Desai *et al.*, 1996). Despite these compensatory mechanisms, malnutrition has been shown to trigger significant alterations in brain metabolism, involving, for example, fatty acids (Marin *et al.*, 1995), 5-HT synthesis and release (Chen *et al.*, 1992; Blatt *et al.*, 1994; Manjarrez *et al.*, 1994, 1996), as well as noradrenergic turnover (Soto-Moyano *et al.*, 1995), which do not necessarily reverse following nutritional recovery (Manjarrez *et al.*, 1994, 1996). Furthermore, the time course of drug-induced maturational delays reported in some studies (Blue *et al.*, 1991; Osterheld-Haas *et al.*, 1994) is superimposable on the time course of delayed thalamocortical development induced by malnutrition (Vongdokmai, 1980). Finally, these studies also pose the problem of potential direct damage to thalamocortical terminals, which cannot be immediately distinguished from modulation of terminal arborization and barrel formation by 5-HT. Direct damage, for example, could readily explain persistent changes in whisker barrel areas that are maintained into adulthood (Bennett-Clarke *et al.*, 1994b), as well as TTX-insensitivity of 5,7-DHT-induced reductions in mean barrel areas (Rhoades *et al.*, 1998).

PCPA doses administered in studies assessing 5-HT roles in synaptogenesis were usually higher than the dose employed here and/or administered repeatedly over several days (Okado *et al.*, 1993; Chen *et al.*, 1994; Niitsu *et al.*, 1995). Treatment protocols of this sort may also yield prominent reductions in body and brain growth, although inter-species differences in sensitivity to drug effects on weight may be present. Our results suggest caution in attributing reductions in synaptic density entirely to decreased 5-HT content, as weight loss may have contributed to this effect or may even possibly account for it, following the most aggressive treatments.

All pharmacological treatments employed here may have produced decreases in cortical 5-HT content below the threshold necessary for serotoninergic modulation of thalamocortical development. However, prior studies have employed either doses higher than the ones administered here (Blue *et al.*, 1991; Osterheld-Haas *et al.*, 1994) or selected animals displaying more profound 5-HT depletions than those recorded in our PCA-treated sample (Bennett-Clarke *et al.*, 1994b). Some of our PCPA-treated animals clearly display very profound 5-HT depletions in the presence of barrel areas within the range of saline-injected pups (Fig. 5).

In conclusion, postnatal brain growth appears to be a major determinant of barrel field development, while postnatal reduction in 5-HT brain levels following pharmacological or dietary interventions *in vivo* appear to exert rather minor direct effects on barrel development, if any. This outcome is remarkably different from the major alterations in somatosensory cortical cytoarchitecture produced by early postnatal exposure to 5-HT excess both in MAO-A and 5-HT transporter knock-out mice (Cases *et al.*, 1996; Lebrand *et al.*, 1996; Persico *et al.*, 1998). Furthermore, the difficulty in disentangling 5-HT neurodevelopmental roles from the consequences of drug-induced growth impairment and from direct neurotoxic damage of thalamocortical terminals may further support the preferential use of transgenic mouse models (Cases *et al.*, 1996; Lebrand *et al.*, 1996; Persico *et al.*, 1998) over pharmacologic 5-HT depletion paradigms as a means to reliably assess 5-HT impact on neocortical development. Interestingly, experiments using 5-HT transporter knock-out animals indicate that the critical period for 5-HT modulation of thalamocortical terminal growth into the somatosensory cortex may be largely limited to the first 48 h after birth in mice (A.M. Persico, F. Keller, K.P. Lesch and D. Murphy, manuscript in preparation), as found for systemic PCA- and PCPA-injected animals (A.M. Persico and F. Keller, unpublished observation). However, in knock-out mice 5-HT excess does appear to modulate thalamocortical pattern formation in the absence of effects on body and brain growth, and of changes in amount of cortical surface devoted to the PMBSF (Persico *et al.*, 1998).

Notes

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