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BRAIN GROWTH TRAJECTORIES IN MOUSE STRAINS WITH CENTRAL AND PERIPHERAL SEROTONIN DIFFERENCES: RELEVANCE TO AUTISM MODELS

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Abstract—The genetic heterogeneity of autism spectrum disorders (ASDs) suggests that their underlying neurobiology involves dysfunction at the neural network level. Understanding these neural networks will require a major collaborative effort and will depend on validated and widely accepted animal models. Many mouse models have been proposed in autism research, but the assessment of their validity often has been limited to measuring social interactions. However, two other well-replicated findings have been reported in ASDs: transient brain overgrowth in early postnatal life and elevated 5-HT (serotonin) levels in blood platelets (platelet hyperserotonemia). We examined two inbred mouse strains (C57BL/6 and BALB/c) with respect to these phenomena. The BALB/c strain is less social and exhibits some other autistic-like behaviors. In addition, it has a lower 5-HT synthesis rate in the central nervous system due to a single-nucleotide polymorphism in the tryptophan hydroxylase 2 (*Tph2*) gene. The postnatal growth of brain mass was analyzed with mixed-effects models that included litter effects. The volume of the hippocampal complex and the thickness of the somatosensory cortex were measured in 3D-brain reconstructions from serial sections. The postnatal whole-blood 5-HT levels were assessed with high-performance liquid chromatography. With respect to the BALB/c strain, the C57BL/6 strain showed transient brain overgrowth and persistent blood hyperserotonemia. The hippocampal volume was permanently enlarged in the C57BL/6 strain, with no change in the adult brain mass. These results indicate that, in mice, autistic-like shifts in the brain and periphery may be associated with less autistic-like behaviors. Importantly, they suggest that consistency among behavioral, anatomical, and physiological measures may expedite the validation of new and previously proposed mouse models of autism, and that the construct validity of models should be demonstrated when these measures are inconsistent. **© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: brain growth, blood, autism, postnatal, 5-HT, hippocampus.

Autism spectrum disorders (ASDs) represent a wide range of genetically heterogeneous conditions (Betancur, 2011; Casey et al., 2012) that share similar behavioral symptoms. This suggests that the reported gene mutations may affect various nodes in a specific neural network. The identification of this network and its dysfunction will require a major collaborative effort due to the expected complexity of its connections and dynamic properties. Because of the limitations of human experimentation, this research will have to increasingly rely on a small number of well-validated and widely accepted animal models.

To date, many rodent models have been proposed that replicate some aspects of ASDs, including their genetics, central and peripheral physiology, and behavioral phenotypes (Kahne et al., 2002; McNamara et al., 2008; Carter et al., 2011; Ey et al., 2011; Spencer et al., 2011; Eagleson et al., 2011; Lewis, 2011; Mercier et al., 2011; Bangash et al., 2011; Stephenson et al., 2011; Wöhr et al., 2011). One of the major challenges in the field is to ensure the construct and predictive validities of these models. ASDs are characterized by a maladaptive, inflexible interaction with conspecifics and the environment, as well as impaired social communication. Therefore, mouse models of autism are typically tested by assessing these and related behaviors (Silverman et al., 2010; Fairless et al., 2011). However, a direct comparison between human and rodent social behaviors may be misleading because of the sensitive dependence of the social behavior of a species on its ecological niche. For example, desert gerbils tend to lead solitary lives, whereas gerbil species inhabiting regions with cold winters tend to be highly social (Macdonald, 2006). Likewise, the social mating systems of some vole and deer mouse species show remarkable variability (Insel and Shapiro, 1992; Macdonald, 2006; Robinson et al., 2008; Turner et al., 2010). It suggests that behavioral tests should be used in conjunction with other biological measures that are altered in many autistic individuals and that are likely to be directly comparable across mammalian species. Two such findings are the transiently accelerated brain growth in early postnatal life and the blood hyperserotonemia. Both of these phenomena may be related to abnormalities of the central and peripheral 5-HT (serotonin) systems (Anderson et al., 1987; Jacobs and Azmitia, 1992; Gershon and Tack, 2007; Janušonis, 2008). The central serotonin system has been so well conserved in vertebrate evolution that it allows direct comparisons not only among mammalian orders (e.g. primates and rodents), but also between fishes and mammals (Lillesaar, 2011). Likewise, the general mechanisms of brain development are highly conserved in all vertebrate radiations

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Abbreviations: ASD, autism spectrum disorder; REML, restricted maximum likelihood estimation; *Tph2*, tryptophan hydroxylase 2.

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(Butler and Hodos, 2005) and are virtually independent of the species' position in its ecological system.

Autistic brains tend to transiently overgrow normally developing brains during the first years after birth, but by adulthood autistic and normal brains no longer differ in size (Courchesne et al., 2001, 2003, 2011). Evidence suggests that the brain growth trajectory can be affected by perturbations of the central 5-HT system (Altamura et al., 2007; Vitalis et al., 2007; Wassink et al., 2007). Also, a large subset of autistic individuals has elevated 5-HT levels in blood platelets. This phenomenon was first reported in the early 1960s (Schain and Freedman, 1961) and in the subsequent 50 years has been replicated by many groups of researchers in populations with diverse ethnic backgrounds (Hanley et al., 1977; Anderson et al., 1990; Cook, 1996; McBride et al., 1998; Mulder et al., 2004; Hranilovic et al., 2007; Melke et al., 2008). While the causes of the platelet hyperserotonemia of autism remain unclear (Janušonis, 2008; Anderson et al., in press), several genes have been associated with elevated platelet 5-HT levels (Weiss et al., 2004, 2006; Janušonis et al., 2006; Coutinho et al., 2007). The importance of blood hyperserotonemia in mouse models of autism and anxiety has been emphasized by some groups (Carneiro et al., 2008, 2009; Carter et al., 2011; Veenstra-Vanderweele et al., 2012), including ours (Janušonis et al., 2006).

Thus far, no attempts have been made to demonstrate in a mouse model the triad of autistic alterations: the impaired social behavior, the transiently accelerated postnatal brain growth, and the persistent blood hyperserotonemia. Motivated by these considerations, we conducted a case study in which we used two inbred mouse strains, C57BL/6 and BALB/c. Compared with the C57BL/6 strain, the BALB/c strain is known to show a low level of sociability and some other autism-related behaviors (Brodkin, 2007; Fairless et al., 2008). These strains also carry different alleles for a single nucleotide polymorphism (C1473G) in the gene coding for tryptophan hydroxylase 2 (*Tph2*), the rate-limiting enzyme in the brain 5-HT synthesis pathway. The C57BL/6 strain is homozygous for the C-allele and has a higher brain 5-HT synthesis rate compared with the BALB/c strain that is homozygous for the G-allele (Zhang et al., 2004). This difference in the alleles appears to be insufficient to affect the 5-HT tissue levels in the adult forebrain of C57BL/6 and BALB/c mice congenic for the *Tph2* locus (Siesser et al., 2010). Also, it has no effect on the 5-HT tissue levels in the developing forebrain of the two strains (Yochum et al., 2010). Few studies have directly assessed tissue and/or extracellular 5-HT levels in autistic brains. The results of a recent immunohistochemical study suggest that 5-HT tissue levels may be considerably higher in young autistic brains compared with age-matched control brains (Azmitia et al., 2011), but the evidence for possible alterations of extracellular 5-HT levels in autistic brains remains inconclusive (Narayan et al., 1993; Adamsen et al., 2011), mostly due to severe technical limitations.

In the brain, virtually all tissue 5-HT is stored in serotonergic varicosities (Jacobs and Azmitia, 1992), and tissue 5-HT levels may not reflect the availability of free, extracellular 5-HT that can act on 5-HT receptors. ASDs may be associated with an altered brain 5-HT synthesis rate (Chugani et al., 1999, 2002) rather than altered 5-HT tissue levels. The C57BL/6 and BALB/c strains differ on this measure (Zhang et al., 2004) and also show related differences in the expression of *Tph2* (Bach et al., 2011).

These considerations show that the C57BL/6 and BALB/c strains make an instructive case study because the C57BL/6 strain is often used as the baseline strain against which new models are compared, the BALB/c strain shows some autistic-like behaviors, and some information is available about the genetics of the serotonin system in the two strains. We show that while the BALB/c strain is relatively more autistic-like on behavioral measures, the C57BL/6 strain is relatively more autistic-like on two major biological measures: postnatal brain growth and blood 5-HT. These results (i) demonstrate that care should exercised in choosing the baseline mouse strain and (ii) suggest that postnatal brain growth, blood 5-HT levels, and social behavior, assessed together, may facilitate the validation and ranking of mouse models of autism.

EXPERIMENTAL PROCEDURES

Animals

Timed-pregnant C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed individually on a 12:12 light-dark cycle (lights on at 7:00, off at 19:00) with free access to water and food. The *Tph2* alleles of the two strains (C/C and G/G, respectively) were analyzed by PCR genotyping and were confirmed to be consistent with Zhang et al. (2004). Dams were inspected before noon, and the delivery day was considered to be postnatal day (P) 0. The litters were not perturbed or culled before tissue collection. All procedures have been approved by the UCSB Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and their suffering.

Brain mass

A total of 156 postnatal (P3, P7, P14) brains and 36 adult female brains were used in the analysis. Adult females were used because it allowed us to increase sample sizes by including the dams that had produced litters analyzed at other developmental times. In another study, we have found no significant sex differences in the brain mass, hippocampal volume, and blood 5-HT levels of the CD-1 strain (Albay et al., 2009). At P3, P7, and P14, five litters were analyzed from each of the two strains at each of the three time-points (a total of 30 litters). Animals were weighed, immediately decapitated, and their whole brain (from the rostral pole to the cervicomedullary junction) was dissected from the skull into phosphate-buffered saline (PBS; 0.1 M, pH 7.4) with a fine forceps under a dissecting stereoscope (P3 and P7) or with a fine rongeurs (P14 and adult). Any liquid attached to the surface of the brain was carefully removed by bringing the brain into contact with a piece of Parafilm, and the brain was immediately weighed on a precision (±0.0001 g) balance. In order to ensure temporal independence, each litter was used for only one developmental time-point.

Blood collection

At P3, P7, and P14, one pup from each litter was decapitated, and its trunk blood was collected into a 25-μl drop of 5% Na_2EDTA on

a piece of Parafilm. The blood from adult animals was collected in the same way. Blood samples were visually inspected for blood clots, and their volume was measured with a 200-μl precision pipette. Samples were stored in 1.5-ml microcentrifuge tubes at −75 °C until further analysis.

Hippocampal volume and cortical thickness

Following the collection of a blood sample, the brain was dissected from the skull and immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH. 7.4) at 4 °C overnight. It was cryoprotected in 30% sucrose in PB at 4 °C overnight, embedded in 20% gelatin (type A; 275 bloom), immersed in formalin with 20% sucrose for 3 h at room temperature, and sectioned coronally on a freezing microtome at 50-μm thickness into PBS in 96-well trays. Every lost section was recorded, and the percentage of missing sections was not allowed to exceed 5%. The sections were mounted onto glass slides coated with 0.5% gelatin and 0.05% chromium (III) potassium sulfate dodecahydrate (Pappas, 1971), allowed to air-dry, Nissl-stained with 0.25% Thionin, dehydrated in a graded series of ethanols, cleared in xylenes, and coverslipped with Permount. Since fixation and embedding can alter tissue volume (Baker, 1958; Blinkov and Glezer, 1968), care was taken to ensure that all brains and sections were exposed to the reagents for the same length of time.

Images of serial sections were captured with a Zeiss Axio Imager Z1 equipped with a color digital camera (AxioCam HRc Rev. 2) using a 1× objective, imported into the *Reconstruct* software (version 1.1; http://synapses.clm.utexas.edu), and the outline of hippocampal complex was traced on one side from the most rostral section containing the hippocampal pyramidal layer to the most caudal section containing the granular layer of the dentate gyrus. In this rostro-caudal block, the traced regions included the hippocampus proper, the fimbria, the dentate gyrus, and the subiculum. If a section was missing, the thickness of each of the two neighboring sections was digitally increased by 50%. The quality of tracing was assessed by rotating the 3D-reconstruction and visually inspecting it for discontinuities. No digital smoothing was used. Cortical thickness was measured at the level of the barrel field of the primary somatosensory cortex (S1) from the top of layer I to the underlying white matter. The mean thickness of two nonadjacent sections was used.

Whole-blood 5-HT

Whole-blood 5-HT levels were assessed with high-performance liquid chromatography (HPLC) as described in our published studies (Janušonis et al., 2006; Albay et al., 2009). Briefly, 100 μl of 25% ascorbic acid, 100 μl of 5% sodium metabisulfite, and 10 μl of 10 ng/μl *N*-methylserotonin (NMS, internal standard) were added to frozen whole-blood samples; after thawing and mixing, 75 μl of 3.4 M perchloric acid was added; the samples were kept on ice for 10 min, centrifuged, and the supernate was stored at −80 °C until further analysis. Analysis was performed by HPLC using the following conditions: a 25×.46 cm Altex Ultrasphere column heated to 40 °C was eluted with a mobile phase consisting of 70% 0.1 M NaH_2PO_4 (pH 4.7) containing 150 mg/L octyl sulfate and 20% methanol. Compounds were detected with a Shimadzu RF 10-AXL fluorometer, with excitation and emission wavelengths of 285 and 345 nm, respectively, quantitated by comparing peak heights ratios (analyte/NMS) with those observed for standards, and concentrations expressed as ng per μl. The neurochemicals were determined with typical intra- and interassay coefficients of variation of less than 5% and 10%, respectively. In the calculations, the dilution of the collected blood in the Na_2EDTA solution was factored out (i.e. the reported values represent 5-HT concentrations in the undiluted whole blood).

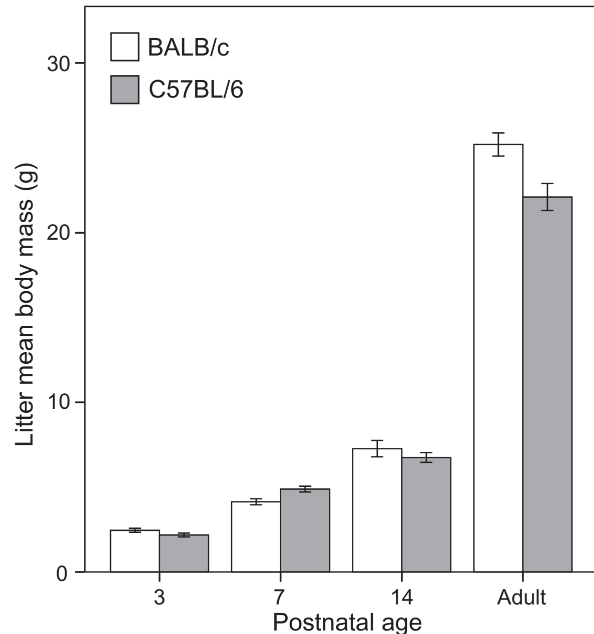

Fig. 1. The body mass of BALB/c and C57BL/6 mice at postnatal days (P) 3–14 and in adulthood. At P3–14, each data point is the mean of a litter (i.e. the height of a bar indicates the mean of the litter means). Error bars are standard errors of the mean.

Statistics

Statistical analyses were performed in R 2.14.1 (the R Foundation for Statistical Computing) and in IBM SPSS 19 (IBM Corporation, Armonk, NY, USA). In ANOVAs, residuals were tested for homogeneity of variance (with the Levene test). If a violation of homogeneity was detected, the data were analyzed with the R *nlme* package (Pinheiro et al., 2011) by choosing the *varIdent* variance structure (Zuur et al., 2009) that allows each experimental group to have a different variance. The *nlme* package was also used in mixed-effects analyses. These statistical approaches are superior to traditional statistical tests that emphasize mathematical convenience (rather than biological relevance) and are strongly recommended for experimental research in neuroscience (Lazic, 2010; Nakagawa and Hauber, 2011). By following a well-established procedure (Zuur et al., 2009), statistical models were built sequentially by using restricted maximum likelihood estimation (REML) and maximum likelihood estimation (ML) as follows: (i) first, a "beyond optimal" model was constructed that included all relevant fixed effects; (ii) the structure of variances and random effects was optimized by comparing nested REML-fitted models with ANOVA and testing the significance of the likelihood ratios (L) (in mixed-effects analyses, the significance of L was calculated "on the boundary," as recommended by Zuur et al. [2009]); (iii) the structure of fixed effects was optimized by comparing nested ML-fitted models with ANOVA and testing the significance of L (the non-significant fixed effects were removed); (iv) the final model was refitted with REML and validated (tested for normality and homogeneity of residuals). In all tests, the significance level was set at 0.05.

RESULTS

Postnatal growth of brain mass

The postnatal growth of body mass in the C57BL/6 and BALB/c strains is shown in Fig. 1. A two-way ANOVA

revealed no significant difference between the strains ($F_{1,58}$=1.29, P=0.26) and no significant interaction between strain and age ($F_{3,58}$=2.34, P=0.083). If adulthood was considered separately, the BALB/c body mass was significantly larger than the C57BL/6 body mass (t_{34}=2.97, P=0.005). However, the analysis of the same set of animals showed that the brain mass of the two strains was virtually identical in adulthood (t_{34}=1.41, P=0.17; the 95% confidence interval of the difference between the BALB/c and C57BL/6 strains: [−0.003, 0.017 g]). This effectively pegged the brains of both strains at the same endpoint and allowed us to directly compare their postnatal growth trajectories.

First, we analyzed postnatal brain mass, disregarding which litter the pups came from (Fig. 2A). A two-way ANOVA revealed a significant interaction between strain and age ($F_{3,184}$=16.8, $P<10^{-8}$). A simple effects analysis showed that the mass of the C57BL/6 brain was not significantly different from that of the BALB/c brain at P3 and in adulthood ($F_{1,184}$=0.17, P=0.68; $F_{1,184}$=2.41, P=0.12, respectively). However, at P7 and P14, the mass of the C57BL/6 brain was significantly greater than that of the BALB/c brain ($F_{1,184}$=51.6, $P<10^{-10}$; $F_{1,184}$=21.5, $P<10^{-5}$, respectively). We next calculated the *z*-scores of the C57BL/6 brain mass by using the BALB/c means and standard deviations at the respective time-points (Fig. 2B). This analysis confirmed the accelerated brain growth at P7–P14, which was similar to early postnatal autistic trajectories presented in the same format (Courchesne et al., 2003). The interaction between strain and age remained significant if only the means of the litters were used (Fig. 2C; $F_{3,58}$=5.57, P=0.002).

While the presented ANOVA analyses are relatively simple, their interpretation poses problems. If all pups are pooled irrespective of the litter, one runs the risk of pseudoreplication due to possible litter effects (Lazic, 2010). If the means of the litters are used, one analyzes the growth of the mean litter brain mass, which is not the same variable as the individual brain mass. Finally, using only one pup from each litter is impractical because of the large samples required to reliably detect alterations in nonlinear growth trajectories. All of these problems are solved by mixed-effects modeling (Zuur et al., 2009; Nakagawa and Hauber, 2011), which we describe next.

First, a "beyond optimal" linear model was constructed that included strain, age, and the interaction between strain and age as the fixed factors. Next, the random component was optimized to account for litter effects. A random intercept greatly improved the model (L=61.4, $P<10^{-14}$). A random slope for either strain or age did not further improve the model ($L=10^{-4}$, P=1.00; L=0.86, P=0.50, respectively), so the final random component contained only the random intercept. The interaction between strain and age was tested in the presence of the random intercept and was retained because its deletion resulted in a significantly worse model (L=19.4, df=3, P=0.0002). The final model was REML-fitted and validated (Fig. 2D–F). The parameters of the model are presented in Table 1. It indicates that there is no overall difference between the brain mass of the two strains, but that at P7 the mass of the C57BL/6 brain is significantly greater than that of the BALB/c brain (by 11%; P=0.007). This result confirms that the C57BL/6 brain transiently overgrows the BALB/c brain by the end of the first postnatal week.

We note that body mass was not included in the model because, at a given developmental time, body mass is a poor predictor of brain mass due to its high sensitivity to environmental factors unrelated to brain growth (Epstein, 1978; Woods and Epstein, 1979). Body mass can be useful as a proxy for litter effects, but the mixed-effects approach allowed us to model general (not only body mass-related) litter effects directly.

Postnatal growth of hippocampal volume and cortical thickness

Alterations in the growth of brain mass do not necessarily reflect changes in the volume of specific brain structures. Therefore, we performed a high-precision volumetric analysis of the hippocampal complex in C57BL/6 and BALB/c brains at P3, P7, P14, and in adulthood (Fig. 3A–C). We also measured the thickness of S1 in these brains at the same time-points (Fig. 3D). The hippocampal volume of C57BL/6 pups was significantly larger than that of BALB/c pups (Fig. 3C; $F_{1,33}$=10.9, P=0.002). The interaction between age and strain was not significant ($F_{3,33}$=1.22, P=0.32). The S1 thickness of C57BL/6 pups was not significantly different from that of BALB/c pups ($F_{1,33}$=2.33, P=0.14), and there was no significant interaction between age and strain ($F_{3,33}$=1.07, P=0.38). However, if tested separately at P7, the S1 thickness of C57BL/6 pups was significantly larger than the S1 thickness of BALB/c pups, even after the Bonferroni correction for the four developmental times (t_9=3.77, P=0.004). In all of these analyses, only one brain from each litter was used, and no violation of the assumption of homogeneity of variance was detected (the Levene test: $P>0.2$). Therefore, the inferences of the standard statistical models were considered to be valid.

Whole-blood 5-HT levels

We next asked whether the transient brain overgrowth in C57BL/6 is accompanied by another consistent finding in autism research, the blood hyperserotonemia that persists into adulthood (Anderson et al., 1990, in press; Mulder et al., 2004; Janušonis, 2008). At all ages, the whole-blood 5-HT levels of C57BL/6 pups were significantly higher than those of BALB/c pups (Fig. 4). A two-way ANOVA revealed a significant strain effect ($F_{1,57}$=16.7, $P<0.001$), but no significant interaction between strain and age ($F_{3,57}$=0.60, P=0.62). However, the assumption of homogeneity of variance across the age-groups was severely violated and could lead to incorrect inferences (the Levene test: $F_{7,57}$=4.90, $P<0.001$). Therefore, we next accounted for this variability by explicitly including it in the statistical model. The validated model is given in Table 2. The overall estimated difference between the strains was 0.40 ng/μl (Table 2). Compared with the BALB/c strain, this increase

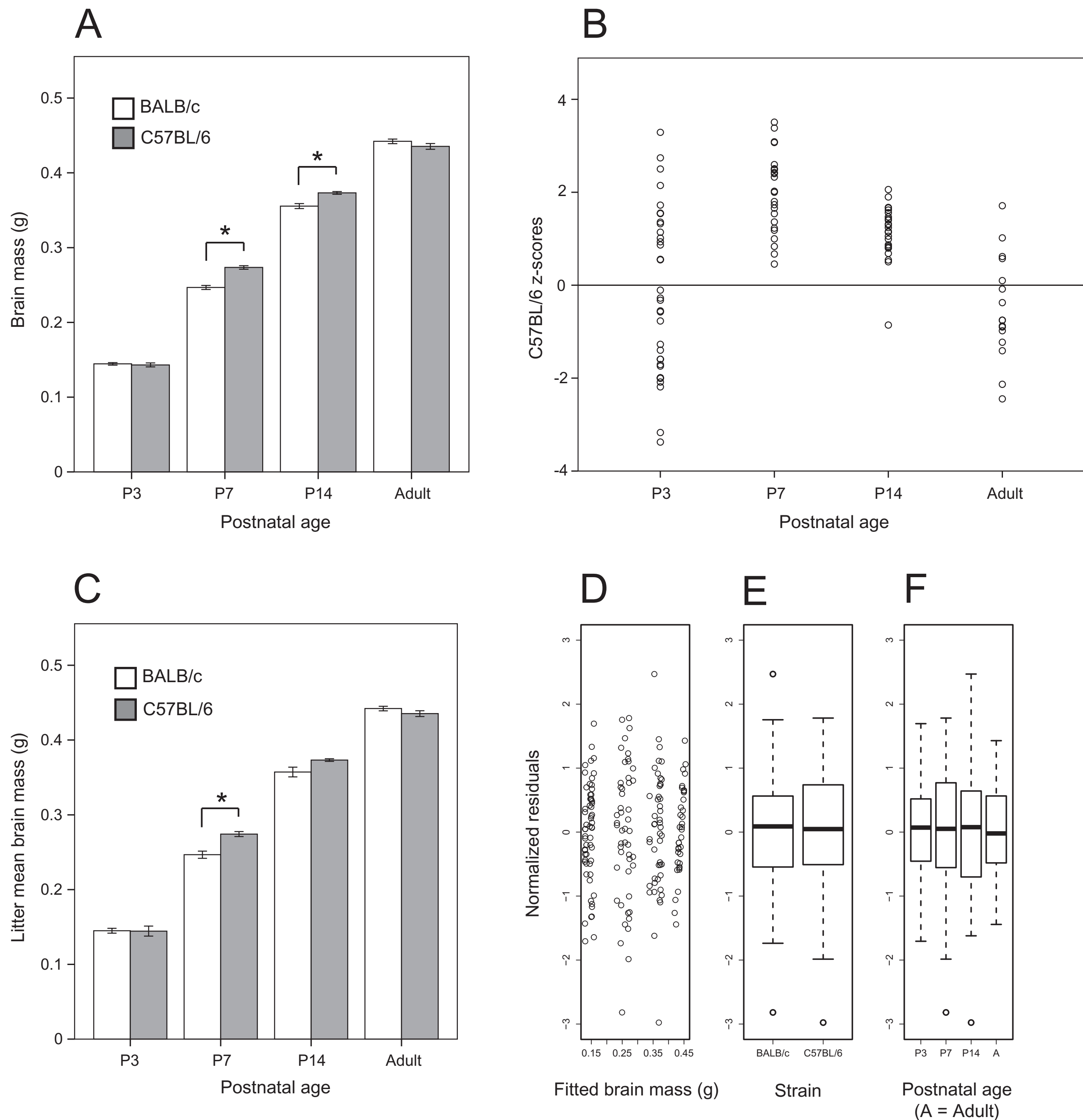

Fig. 2. The brain mass of BALB/c and C57BL/6 mice at P3–14 and in adulthood. (A) All individuals pooled irrespective of the litter. (B) The *z*-scores of C57BL/6 individuals calculated with respect to the BALB/c means and standard deviations at each time-point. This representation has been used to analyze the postnatal growth of autistic brains (Courchesne et al., 2003). (C) The litter means (at P3–14, each data point is the mean of a litter, and the height of a bar indicates the mean of the litter means). (D–F) The validation of the mixed-effects model (Table 1). The residuals are normally distributed (the Shapiro–Wilk test: $P=0.24$) and have homogeneous variances across the groups (the robust [median-based] Levene test: $F_{1,190}=1.27$, $P=0.26$ for the strain groups; $F_{3,188}=2.71$, $P=0.05$ for the age-groups). In (A) and (C), error bars are standard errors of the mean. The asterisks indicate significant ($P<0.05$) simple effects.

was equivalent to 57%, 49%, 18%, and 10% at P3, P7, P14, and in adulthood, respectively.

DISCUSSION

Two mouse strains were investigated with regard to several biological measures that can facilitate the design and validation of mouse models of autism. The obtained results have implications for other mouse strains and models. However, we do not suggest that the biological mechanisms underlying the observed developmental shifts in the two mouse strains are equivalent to those in autistic individuals. Instead, we propose that when such equivalence

Table 1. The brain mass (*bm*) of the C57BL/6 and BALB/c strains from P3 to adulthood. The final mixed-effects model is represented by $bm(s, a)=(C+\gamma)+s+a+s\times a+\varepsilon$. The BALB/c strain and P3 are assumed to be the base categories. For the model validation, see Fig. 2D–F.

Parameter	Groups	Value (g)	SE (g)	*t*	*df*	*P*
Fixed intercept						
C	All	0.1450	0.0050	29.05	126	$<$0.0001**
Random intercept						
γ	Random across litters	*mean*=0 *sd*=0.0105				
Strain						
s	BALB/c	0				
	C57BL/6	−0.0007	0.0070	−0.09	58	0.93
Age						
a	P3	0				
	P7	0.1017	0.0071	14.40	58	$<$0.0001**
	P14	0.2120	0.0071	29.84	58	$<$0.0001**
	Adult (A)	0.2972	0.0059	50.73	58	$<$0.0001**
Strain×Age						
$s\times a$	BALB/c×P3	0				
	BALB/c×P7	0				
	BALB/c×P14	0				
	BALB/c×A	0				
	C57BL/6×P3	0				
	C57BL/6×P7	0.0281	0.0100	2.82	58	0.0066**
	C57BL/6×P14	0.0169	0.0100	1.69	58	0.096
	C57BL/6×A	−0.0061	0.0084	−0.73	58	0.47
Residual						
ε	Random across individuals	*mean*=0 *sd*=0.0089				

SE, standard error; *sd*, standard deviation; *t*, Student *t*; *df*, degrees of freedom,** $P<0.01$.

can be demonstrated, the studied measures can greatly strengthen the validity and relevance of the mouse model.

Previous research has shown that the BALB/c strain exhibits some autistic-like behaviors (Brodkin, 2007; Fairless et al., 2008). Our results show that it is the C57BL/6 strain that is more autistic-like on two measures: transient brain overgrowth in early postnatal development and persistent blood hyperserotonemia. Taken together, these findings do not imply that either of the strains is an acceptable autism model, but they demonstrate that behavioral tests can be grossly insufficient to demonstrate the relevance of a mouse model to autism research, especially if the model is not supported by construct validity (van der Staay et al., 2009). Currently, construct validity is likely to be based on gene mutations observed in autistic individuals, but future mouse models can be designed to replicate autistic-like shifts in the dynamics of entire neural networks.

When construct validity is not well established (as is often the case in autism research), the researcher may be forced to decide which of the measures should be given more weight in the selection of the most appropriate model. Behavioral measures appear to be immediately relevant, but they are a function of the species' ecological niche. In contrast, neuroanatomical and neurophysiological measures can be highly conserved in phylogenetic radiations and therefore are especially strong where behavioral measures are weak. Based on our results, we suggest that behavioral tests should always be complemented with analyses of brain growth and blood 5-HT levels. While inconsistency among these measures does not automatically invalidate a mouse model, it does indicate that caution should be exercised until more solid evidence becomes available. This is important because the very concept of the autistic-like mouse is based on species-independent assumptions. Also, the proliferation of mouse autism models is becoming unsustainable and hinders large-scale collaborative efforts.

The observed transient increase in the C57BL/6 brain mass might be related to the higher brain 5-HT synthesis rate in this strain due to its *Tph2* allele (Zhang et al., 2004; Bach et al., 2011). However, other genes in the C57BL/6 genome are likely to contribute to this phenomenon. Generally, it is difficult to predict how a major perturbation of the brain 5-HT system will affect the gross anatomy of the adult brain, suggesting that these effects depend on more subtle mechanisms than general 5-HT levels or the activity of a single receptor. Mice lacking serotonin 5-HT_{1A} or 5-HT_4 receptors are grossly normal, including their adult brain mass or size (Heisler et al., 1998; Compan et al., 2004). Ablation of nearly all brainstem serotonergic neurons has little effect on the gross brain cytoarchitecture and cortical thickness of the adult brain (Hendricks et al., 2003). However, the absence of the 5-HT transporter does alter the thickness and cell density in the cerebral cortical layers of the mouse brain. Intriguingly, these effects are strain-dependent (Altamura et al., 2007).

Little is known about how these and other perturbations can affect the trajectory (as opposed to the final plateau) of postnatal brain growth. We surmise that many

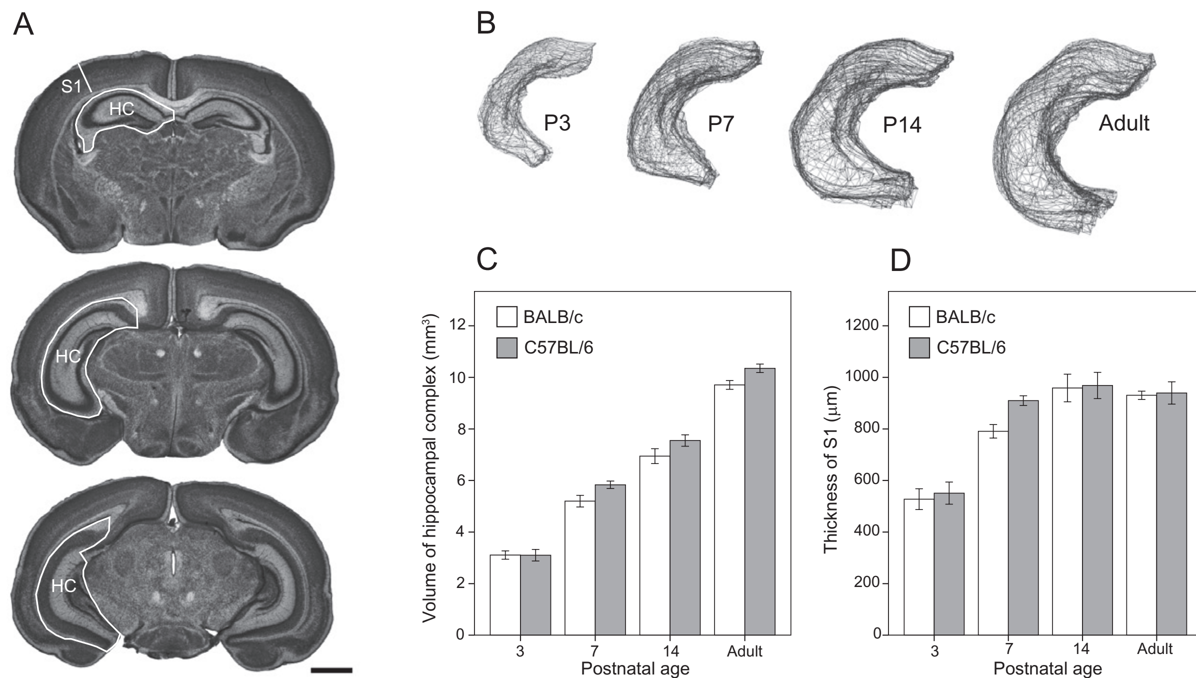

Fig. 3. The volume of the hippocampal complex and the thickness of the primary somatosensory cortex (S1) of BALB/c and C57BL/6 mice at P3–14 and in adulthood. (A) Representative Nissl-stained sections used in 3D-reconstructions of the hippocampal complex (all serial sections were aligned and traced in *Reconstruct*). (B) Representative wire-frame images of the reconstructed hippocampus at all ages (C57BL/6). (C) The volume of the hippocampal complex. (D) The thickness of S1. Error bars are standard errors of the mean.

researchers would be interested in this information, but it would require obtaining large samples and using advanced statistical analyses to account for litter and other random effects. Since presently funding agencies and professional journals place little value on either (Janušonis, 2009; Lazic, 2010; Nakagawa and Hauber, 2011), there is no doubt that many subtle, but revealing, changes in currently existing mouse models remain undiscovered. It is also likely that

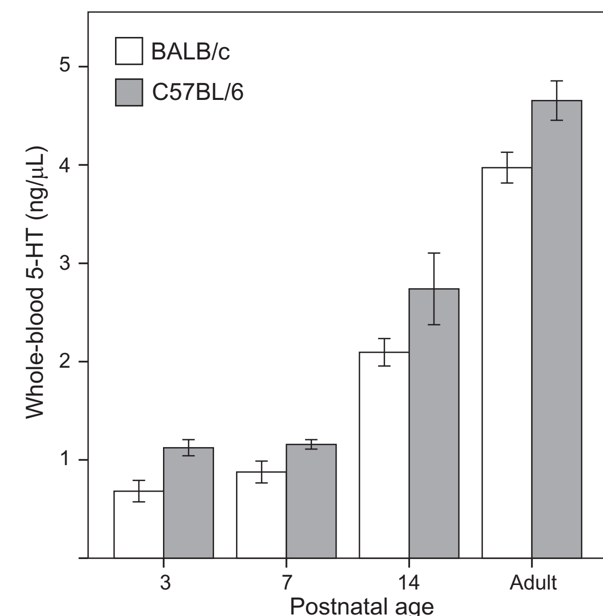

Fig. 4. The whole-blood 5-HT levels of BALB/c and C57BL/6 mice at P3–14 and in adulthood. Error bars are standard errors of the mean.

Table 2. The whole-blood 5-HT levels (*wb*) of the C57BL/6 and BALB/c strains from P3 to adulthood. The final statistical model is represented by $wb(s, a)=C+s+a+\varepsilon$. The BALB/c strain and P3 are assumed to be the base strain and age categories, respectively.

Parameter	Groups	Value (ng/μl)	SE (ng/μl)	*t*	*df*	*P*
Intercept						
C	All	0.7047	0.0785	8.97	60	<0.0001**
Strain						
s	BALB/c	0				
	C57BL/6	0.3992	0.0810	4.93	60	<0.0001**
Age						
a	P3	0				
	P7	0.1085	0.0880	1.23	60	0.22
	P14	1.5126	0.2011	7.52	60	<0.0001**
	Adult	3.4030	0.1429	23.81	60	<0.0001**

Since in ANOVA the variance of the residuals (ε) was not homogeneous across the age-groups, the *gls* function with the *varIdent* variance structure was used to estimate the model (the R *nlme* package). The interaction between strain and age was not significant ($L=3.08$, $df=3$, $P=0.38$) and was removed from the final model. The residuals were normally distributed (the Shapiro–Wilk test: $P=0.19$) and had homogeneous variances across the groups (the robust (median-based) Levene test: $F_{1,63}=0.50$, $P=0.48$ for the strain groups; $F_{3,61}=0.34$, $P=0.80$ for the age-groups). SE, standard error; *t*, Student *t*; *df*, degrees of freedom,** $P<0.01$.

many of these changes are time-sensitive, which leads to another deep methodological issue (Janušonis, 2012). It is interesting to note in this regard that the transient overgrowth of the C57BL/6 brain appears subtle, but it is actually larger than the overgrowth of autistic brains (compare Fig. 2B with Fig. 1 of Courchesne et al. [2003]).

In light of our findings, new insights may be gained by testing a larger set of mouse strains that do not differ in their adult brain mass. Some of these strains are likely to show accelerated brain growth during postnatal development. If these same strains always have higher blood 5-HT levels, it may lead to a major reassessment of how the central and peripheral 5-HT systems interact in development. This association may be mediated by factors expressed in both systems, or it may be due to direct 5-HT exchange between the systems. We have hypothesized the former possibility on theoretical grounds (Janušonis, 2005) and have experimentally demonstrated it in mice lacking functional serotonin 5-HT_{1A} receptors. These mice develop blood hyperserotonemia (Janušonis et al., 2006) in addition to developmental brain alterations that result in elevated levels of anxiety in adulthood (Heisler et al., 1998; Ramboz et al., 1998; Parks et al., 1998; Gross et al., 2002). An interaction between the brain and peripheral 5-HT has been recently demonstrated in embryonic mice, the forebrain development of which is affected by 5-HT synthesized in the placenta (Bonnin et al., 2011; Bonnin and Levitt, 2011). It is intriguing that genetic ablation of brainstem serotonergic neurons has no gross effects on brain and cortical cytoarchitecture (Hendricks et al., 2003), but prenatal treatment with DL-*p*-chlorophenylalanine (*p*CPA), a 5-HT synthesis inhibitor, does (Vitalis et al., 2007). One possible explanation is that these effects are caused by the action of *p*CPA in the gut or the placenta (Bonnin et al., 2011), and that the subsequent changes in peripheral 5-HT levels affect the 5-HT levels in the developing central nervous system.

We note that some recent reports (Brodkin, 2007; Yochum et al., 2010) have cited previously published findings (Wimer et al., 1969; Roderick et al., 1973; Wahlsten et al., 1975) that the adult BALB/c brain is significantly larger than the adult C57BL/6 brain. Our large sample data contradict these reports. The cause of this discrepancy is not clear, but the three reports published in 1969–1975 should be interpreted with caution. Wimer et al. (1969) and Roderick et al. (1973) have examined only four to five mice per group and have presented neither standard errors nor pair-wise statistical comparisons. Wahlsten et al. (1975) have used larger groups, but they have compared brains after a long fixation with 10% formalin, which is known to increase measured brain mass by around 8% (Blinkov and Glezer, 1968). Furthermore, the fresh C57BL/6 brain mass obtained by Roderick et al. (1973) is comparable to the fixed BALB/c brain mass obtained by Wahlsten et al. (1975).

In the present study, the hippocampal complex of the C57BL/6 strain outgrew that of the BALB/c strain, and this difference persisted into adulthood. The volume of the hippocampus has been shown to be enlarged in autistic adolescent individuals (Groen et al., 2010). Another study has found the hippocampus to be enlarged (by around 10%) at all studied ages up to adolescence, in contrast to the amygdala that is enlarged only in preadolescent children (Schumann et al., 2004). By comparison, in our analysis, we found the C57BL/6 hippocampus to be larger than the BALB/c hippocampus by 12%, 9%, and 7% at P7, P14, and in adulthood, respectively.

In summary, we recommend that mouse models of autism be consistently evaluated on at least three measures: social behavior, postnatal brain growth, and blood 5-HT levels. It is especially important when the construct validity of a model remains in question. While the consistency among these measures is not sufficient to establish the validity of a model, it can facilitate the selection among several models that appear to be equally autistic-like in behavioral tests. Intriguingly, some currently available mouse models may already exhibit ASD-like changes on all three measures, but to our knowledge no such evaluation has yet been completed.

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