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Neuroanatomical changes in a mouse model of early life neglect

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Abstract

Using a novel mouse model of early life neglect and abuse (ENA) based on maternal separation with early weaning, George et al. (*BMC Neurosci* 11:123, 2010) demonstrated behavioral abnormalities in adult mice, and Bordner et al. (*Front Psychiatry* 2(18):1–18, 2011) described concomitant changes in mRNA and protein expression. Using the same model, here we report neuroanatomical changes that include smaller brain size and abnormal inter-hemispheric asymmetry, decreases in cortical thickness, abnormalities in subcortical structures, and white matter disorganization and atrophy most severely affecting the left hemisphere. Because of the

similarities between the neuroanatomical changes observed in our mouse model and those described in human survivors of ENA, this novel animal model is potentially useful for studies of human ENA too costly or cumbersome to be carried out in primates. Moreover, our current knowledge of the mouse genome makes this model particularly suited for targeted anatomical, molecular, and pharmacological experimentation not yet possible in other species.

Keywords

Mouse models; Brain asymmetry; Diffusion tensor imaging (DTI); Early life neglect and abuse (ENA); Maternal separation

Introduction

Early life neglect and abuse (ENA) is a common problem in the USA with no discrimination for race, gender or socio-economical status. Yearly more than 20 out of every 1,000 children are abused or neglected within the first year of life. In 2008 alone approximately 6 million children were maltreated and of these, about 772,000 were victims of abuse or neglect (US-Government-Report 2008).

Although not all victims develop adverse effects, suggesting that many different factors modulate the consequences of early life experience, many exposed individuals do develop psychiatric maladies that manifest and persist into later life (Lange et al. 1999; De Bellis 2005). These problems include, among others, depression (Bhatia and Bhatia 2007; Widom et al. 2007a), anxiety (Kuhar 1986; Mathews et al. 2008), drug and alcohol abuse (Kaufman et al. 2007; Trent et al. 2007; Widom et al. 2007b), attention deficit hyperactivity disorder (ADHD) (Ouyang et al. 2008), and delinquency (Maxfield and Widom 1996; Widom and Maxfield 1996; Hildyard and Wolfe 2002; Kotch et al. 2008). All of these conditions have been associated with neuroanatomical changes in diverse but significantly overlapping brain structures, perhaps mirroring the overlapping symptoms of these deficits. For instance, among the structures implicated in depression are the amygdala (Sheline et al. 1998), cortex, hippocampus, basal ganglia, and thalamus (Rama Krishnan 1992). In anxiety, the structures implicated include cingulate gyrus, hippocampus, amygdala and frontal lobes (Kuhar 1986). Many of the same structures in the limbic midbrain and forebrain are also associated with consumption of alcohol (Myers and Privette 1989) and drugs of abuse (Garavan et al. 2000). Similarly, prefrontal cortex (PFC) and cingulate gyrus are associated with ADHD (Marsh and Blair 2008; Marsh et al. 2008; Finger et al. 2008) and white matter (WM) abnormalities have been linked to the pathophysiology of mood and anxiety disorders (Buchsbaum et al. 2006; Bruno et al. 2008; Phan et al. 2009; Tham et al. 2010).

Hence, because of the variety of brain structures that are involved, the different degrees to which each structure could be affected and the inherent normal variability among individuals, the purpose of this study was to describe gross anatomical changes that could be consistently observed that are relevant to the behavioral abnormalities seen in survivors of ENA. Here, we determine whether maternal separation with early weaning (MSEW) causes changes that are observable at the level of the whole brain, whole hemisphere, gray matter, or WM. In particular, since there is growing recognition that changes in WM are associated with a variety of psychiatric conditions and because WM provides a means to functionally unify the different gray matter structures that may be altered, we investigated the global integrity of the WM using histochemistry and diffusion tensor imaging (DTI). Further, we begin to explore changes in WM at a cellular level using stereology to quantify changes in Olig2, a marker of oligodendrocyte precursors.

Some previous studies in mice and rats have been successful in demonstrating behavioral abnormalities after early life stress, but protocols used to study maternal separation in rats have been unable to produce a reliable phenotype in mice in other studies (Millstein and Holmes 2007; Pascual and Zamora-Leon 2007; Pascual et al. 2007). We developed the MSEW model in an effort to reliably reproduce, in an animal model, some or all of the anatomical abnormalities associated with ENA. Such a model could allow for a better understanding of the mechanism of the behavioral abnormalities that result from ENA and opens a window of opportunity for targeted anatomical, molecular, and pharmacological research of potential benefit to survivors of ENA.

Materials and methods

Animals

C57Bl/6J (B6) mice were used in this study. Experimentally naïve animals were obtained from Jackson Laboratory (Bar Harbor, ME), and subsequently bred in house. Upon birth, entire litters were randomized to control ($n = 26$) or MSEW ($n = 26$) conditions. Details regarding animal handling, maternal separation procedures, pup thermoregulation, nutrition, and light cycles have previously been published (George et al. 2010). In short, control animals were left undisturbed and weaned at postnatal day (P) 23. MSEW animals were separated from the dam for 4 h per day on P2–5, and 8 h per day on P6–16, and weaned on P17. After weaning, animals were group housed with same sex littermates and sacrificed at P10 ($n = 4$), P17 ($n = 4$), P30 ($n = 10$), or P75–P90 ($n = 34$). Comparisons between MSEW and controls were done exclusively on male animals killed at the same age.

Perfusions and tissue collection

Mice were anesthetized with chloral hydrate (1,500 mg/kg in sterile saline, IP) and transcardially perfused with ice-cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Whole brains were collected and postfixed in 4% PFA at 4°C for 24 h and then cryoprotected in 30% sucrose. Subsequently, they were stored at –20°C for later sectioning. Brains were cut in the coronal plane at 40–50 μm in a freezing microtome. Sections were either processed immediately or stored at –20°C in anti-freezing media (0.05 M PB, 25% glycerol, 30% ethylene glycol) for later processing. For DTI, brains were stored in 4% PFA in PBS at 4°C for 2 weeks. One hour before the DTI scans, brains were washed three times for 10 min in 10 mL PBS to remove the PFA solution. Then, the brains were placed into a custom-built MRI-compatible tube filled with Fluorinert, an MRI susceptibility-matching fluid (Sigma-Aldrich, Inc., St. Louis, MO).

Tissue staining and immunohistochemistry

To distinguish between the left hemisphere (LH) and the right hemisphere (RH) when processing bulk floating sections, the RH was notched. For on-slide processing, cut sections containing both hemispheres were collected and immediately mounted, preserving left–right orientation; slides were then labeled accordingly.

To aid in the observation of gross organization, selected brains were processed with Golgi staining ($n = 3$ controls and $n = 3$ MSEW). These brains were directly moved from 4% PFA to Golgi fixative and the instructions for the FD Rapid GolgiStain™ were followed (FD Neurotechnologies, Catonsville, MD). Sections were cut in the coronal plane at 200 μm on a vibratome.

The bulk of histologically based observations of WM changes were done using Black Gold II myelin staining ($n = 9$ control and $n = 9$ MSEW). For this, series of coronal sections were stained using Black Gold II (Millipore) following the manufacturer's instructions with minor

modifications. Sections were washed with PBS and stained at 60°C until staining of cortical regions was visible (approximately 35 min). After washing with PBS, sections were fixed with 1% sodium thiosulfate, washed again, mounted on gelatin-coated slides, dehydrated in an alcohol series, cleared in Xylenes, and covered with DPX mounting media. Adjacent sections were mounted on coated slides and developed for Nissl using standard cresyl violet histological procedures.

Tissue sections for immunohistochemistry were transferred from freezing media to PBS and processed floating. Sections were washed with PBS and incubated in blocking solution (3% BSA, 3% normal goat serum, 0.7% triton-X 100). For development using 3-3'-diaminobenzidine tetrahydrochloride (DAB), sections were pre-blocked with 3% hydrogen peroxide in PBS for 10 min before putting them into blocking solution. Sections were incubated overnight at 4°C in a primary antibody against Olig2 (1:2,000; Abcam). The following day, sections were washed with PBS, incubated with a biotinylated or a fluorescent conjugated secondary antibody (1:400; Jackson ImmunoResearch) for 4 h at 25°C, washed again, and then either mounted and covered with Vectashield (Vectorlabs) for immunofluorescence or incubated in ABC solution (Vectorlabs) followed by development in DAB solution (Vectorlabs SK-4100). After DAB, sections were mounted on gelatin-coated slides and treated as described for Black Gold II staining.

Microscopy, morphometry, and stereology

Section outlines and cell plotting were done using a fully motorized Zeiss Axioskop microscope and interfacing to a Dell computer running StereoInvestigator® and Neurolucida software (MicroBrightField Inc., Williston, VT). Outlines and fiduciary marks were drawn at 5×. Differences in hemisphere size were computed by comparing hemispheric perimeters. Differences in hippocampal size were similarly computed. The mediolateral extent was measured from the enclosed hippocampal perimeters using Neurolucida Explorer. Each cortical thickness measurement was calculated, dorsally only, as the average of five measurements taken from the cortical outline to the border of the corpus callosum (cc) for the left and right hemispheres individually. The five measurements spread more or less equidistantly from the cingulum crest of the corpus callosum to about 45° laterally, which corresponds to approximately mid-areas of S1. The extent of the cortical areas where the cortical thickness measures were obtained is illustrated by white dotted lines in Fig. 1b. The same extent, although not indicated in Fig. 1a, was also obtained at rostral levels. The thickness of the corpus callosum was obtained at the midline as an average of three independent measures per section at 10× magnification using the measure line function of Neurolucida. Cell counts were done in bright field using a Zeiss Plan-Neofluar 20× objective lens with a 0.5 optical aperture. Regions of interest were drawn following a mouse brain atlas (Paxinos and Franklin 2001). Stereological analysis was performed by systematic random sampling using an optical fractionator probe (West et al. 1991; Gundersen et al. 1999). Optimal sampling parameters were obtained experimentally. Once a positive labeled cell was encountered, its position was plotted using one of the symbols provided by the program. Morphometric data (perimeters, areas, volumes, etc.) were obtained with Neurolucida Explorer.

Assessment of white matter content and organization

WM content was quantified histologically by corpus callosum thickness. The presence or absence of myelinated fiber bundles was assessed using Black Gold II myelin staining. To normalize the staining intensity, control and MSEW brain sections were processed in pairs using the same staining solutions, and for the same length of time.

Myelination was assessed qualitatively as well as quantitatively. For the qualitative assessments we relied on the fact that myelinated fiber bundles normally have a characteristic anatomical placement with reference to surrounding structures as well as a characteristic orientation at particular rostrocaudal and dorsoventral locations. Presence or absence of a bundle, or thinning of a bundle, could be clearly observed and was recorded. All observations were based on a series of adjacent sections containing the structure. Organization or disorganization of fibers was initially judged qualitatively by reference to hundreds of fibers at specific locations which allowed subsequent comparisons between control and MSEW samples. These qualitative observations were followed by observer independent measurements of fractional anisotropy based on DTI imaging.

Statistics

Statistical analyses were performed with Analyze-it v2.12 for Microsoft Excel. The distributions of the data were examined to check for normality and homoscedasticity and analysis of variance (ANOVA) was used to compare means among normally distributed populations with equal variance. In some cases paired sample *t* tests were used to compare control and MSEW animals. Results are presented as the mean $\pm$ SEM.

Diffusion tensor imaging

The MR data sets were obtained on a 9.4 T horizontal bore magnet (Bruker, Billerica, MA, USA) with custom-made ^1H radio frequency coil. 20 contiguous coronal slices of 0.5 mm thickness were acquired for both anatomical and DTI experiments. The anatomical images were acquired using a spin-echo sequence with a repetition time (TR) of 3,000 ms and an echo time (TE) of 10 ms. Twelve averages of 256×256 images were acquired for each slice, resulting in an in-plane resolution of $100 \mu\text{m} \times 100 \mu\text{m}$. The DTI experiments were performed using the Stejskal-Tanner spin-echo diffusion-weighted sequence with a diffusion gradient of 5 ms and a delay between the two diffusion gradients of 15 ms. The TR was 2,000 ms and the TE 25.1 ms. Two Shinnar-Le Roux (SLR) pulses of 1 ms each were used for excitation and inversion, respectively. Eighteen averages were acquired for each slice and the 128×64 images were zero-filled to 256×256 , resulting in an in-plane resolution of $100 \mu\text{m} \times 100 \mu\text{m}$. Sixteen different images were acquired for each slice, fifteen corresponding to various non-collinear diffusion weighting directions with the same $b = 1,000 \text{ s/mm}^2$ and one with no diffusion weighting.

DTI data processing and analysis—The six elements of the diffusion tensor were calculated for each voxel from the intensities of the 16 diffusion-weighted images. The tensor eigenvalues (λ_1 , λ_2 and λ_3) and the corresponding eigenvectors were obtained by matrix diagonalization (Jones et al. 1999; Hasan et al. 2001). The fractional anisotropy (FA) was calculated by the following equation:

$$\text{FA} = \sqrt{\frac{3}{2} \frac{(\lambda_1 - \lambda_{\text{avg}})^2 + (\lambda_2 - \lambda_{\text{avg}})^2 + (\lambda_3 - \lambda_{\text{avg}})^2}{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}}$$

where $\lambda_{\text{avg}} = (\lambda_1 + \lambda_2 + \lambda_3)/3$. The composite FA images were generated using BioImage Suite (<http://www.bioimagesuite.org/>) as follows. First smoothed versions of the FA images (Gaussian smoothing sigma = 0.2 mm) were non-rigidly registered to a single smoothed FA map (a single control mouse not otherwise included in the study) using a non-linear intensity-based warping parameterized in terms of a tensor b-spline grid with uniform control point spacing of 3 mm (Papademetris et al. 2004) as was done in previous work (Chahboune et al. 2009). Next, the unsmoothed FA maps were warped to this common

coordinate space and re-sampled to isotropic resolution (0.1 mm), following which average FA maps for each group were computed as shown in Figs. 2g–h and 4.

Results

Coarse neuroanatomical changes observed in MSEW animals

Coarse structural alterations in diverse brains structures were observed in none of the controls ($n = 26$) and in 25 of 26 MSEW animals studied. These deficits were visible under different types of tissue processing including nissl, golgi, and myelin-stained material, indicating that they were independent of the visualization method. However, the degree of change noticed in any particular structure varied in different cases. Although in MSEW animals there was widespread disorganization, and sometimes absence of myelin fibers, the most striking and easy-to-identify differences occurred in the LH in which visible abnormalities were most prominent as compared with the RH (of the same section) and to controls. Figure 1a–d shows differences in hemispheric size (the LH is smaller than the RH) at both rostral and caudal levels and in golgi (a, b) and myelin (c, d)-stained material. a, b also illustrate differences in cortical thickness most strikingly clear in the dorsal LH (#1). c–f illustrate, in Black Gold II myelin-stained material, both structural changes and myelin deficits in the MSEW animals. Among the abnormalities observed are (#2) a very marked difference in the mediolateral extent of the hippocampus between LH and RH of MSEW animals; (#3) a very salient deficit in the thickness, at this rostrocaudal level, of the corpus callosum as well as the amount of myelinated septofimbrial fibers of MSEW as compared with control; (#4) reduced myelinated fibers in the internal capsule and stria terminalis and (#5) a thinner stria medullaris and absent fornix in the LH of the MSEW as compared with its RH counterpart or to the corresponding controls, and (#6) decreased myelin content in areas such as those surrounding the basolateral amygdala. Quantification of differences in hemispheric and hippocampal size as well as cortical and corpus callosum thickness is described below. However, quantification of additional deficits in myelinated tracks observed in histological preparations was not performed because of the differences in severity of such deficits between the LH and RH of MSEW cases as well as the amount of variability in the structures themselves and the difficulty in accurately delineating borders, for instance, the stria terminalis and internal capsule or the stria medullaris and fornix. Hence, deficits in these structures were demonstrated using DTI imaging methods (see below).

White matter deficits observed by histology were most prominent in the LH as compared with the RH of MSEW cases. Less robust septofimbrial fibers were observed in 7 of 12 MSEW cases and in none of the controls and reduced myelinated fibers in the internal capsule and stria terminalis were observed in 6 of 12 MSEW cases. In one control case an apparent reduction in the LH internal capsule was observed as compared with its RH counterpart. A thinned stria medullaris was seen in 8 of 12 MSEW cases and in none of the controls. A completely missing fornix was never observed in control animals but was seen in the LH of two MSEW cases and in two more MSEW cases it was reduced in volume by more than 50%. In the same cases, the RH fornix was present. Decreased myelin content in areas surrounding the basolateral amygdala was clear in three MSEW cases and in none of the controls.

The LH anterior commissure (ac) of MSEW animals often appeared shrunken or incomplete in its robustness and it was commonly surrounded by demyelinated fibers or partially devoid of fibers (Fig. 2a). A hollow space between the surrounding neuropil and the commissural bundle was often encountered. This was very different from the common splitting of the ac often seen in controls (Fig. 2b). Differences in myelination were seen not only in myelin-stained material but were also easily detected with nissl staining. Figure 2c–d illustrates

using myelin and in cresyl violet nissl staining (adjacent sections) clear differences between LH and RH. In d, black arrowheads indicate the presence of lemniscal fibers in the RH and white arrowheads indicate the absence of the corresponding bundle in the LH. Also noticeable in Fig. 2c–d is the difference in hippocampal size between the RH and LH. These differences were demonstrated quantitatively using histological and DTI imaging methods (Fig. 2e–g) and are described in further detail below.

Brain size and inter-hemispheric asymmetry is affected in MSEW mice

Brains of MSEW mice were smaller than those of corresponding age- and gender-matched controls (Fig. 3). In adult male mice, the perimeters of a total of 46 control and 50 MSEW brain sections, matched at rostrocaudal levels, were drawn and measured (range 4–10 sections per brain; controls $n = 8$; MSEW $n = 7$; all sacrificed at P75–90). LH and RH of the control brains had similar perimeters (means in μm : LH = 12,853 and RH = 12,640; $p = 0.4$). However, the LH and RH of the MSEW cases had perimeters (means in μm : LH = 12,002 and RH = 12,444) which were statistically different ($p = 0.03$). Brains of MSEW cases were hemisphere by hemisphere smaller than the corresponding controls. However, comparison of the corresponding RHs did not reach statistical significance ($p = 0.34$) while comparison of the LHs indicated a very significant difference in size ($p = 0.0008$; see Fig. 3b). When compared to the mean of the LH and RH perimeters from control animals, the perimeter of the LH of MSEW animals was $94.2 \pm 2.52\%$ of control ($p = 0.04$) and the RH of MSEW animals was 99.2 ± 1.37 of control, and not significantly changed (Fig. 3c).

A small number of cases, not statistically evaluated, were also investigated at P17 ($n = 4$; 20 sections) and P10 ($n = 4$; 20 sections) to determine if hemispheric size differences were detectable at younger ages. By P17 hemispheric asymmetry was already substantially beyond the normal range in MSEW animals in which the LH was smaller than the corresponding RH. This difference in size between the hemispheres was not apparent at P10, although at this age the whole brain was smaller than age- and gender-matched controls. Hence, up to at least P10, both hemispheres of the MSEW cases appeared to be about the same size and whole brains were $\sim 12\%$ smaller than matched controls. By P17, only the LH of MSEW animals appeared to be smaller and up to adulthood it remained, on average $\sim 6\%$, smaller than its RH counterpart or any hemisphere of controls. Of course, due to the low number of animals evaluated per condition these results are only preliminary, and these findings will require confirmation using larger samples at additional post-natal ages.

In MSEW animals the LH hippocampus is significantly smaller than its RH counterpart

The LH and RH hippocampal perimeters of control ($n = 7$) and MSEW ($n = 7$) adult mice (P75–90) were drawn at $5\times$ using Neurolucida software. A total of 58 (29 LH and 29 RH) control and 64 (32 LH and 32 RH) MSEW hippocampi were drawn. To get an accurate measure of how the size of the LH hippocampus compared to its RH counterpart we calculated the percent difference between the two for every individual section. While in every control case the LH and RH hippocampi were very close in size (as seen in #2, Fig. 1f), in most of the MSEW cases studied the LH hippocampus was smaller than the RH one (as seen in #2, Fig. 1d or Fig. 2c–d). In controls, the difference between the LH and RH hippocampi per section was on average $3.4 \pm 1.4\%$ in the ML extent and $2.8 \pm 1.4\%$ in the DV extent. The difference in perimeters reached $3.3 \pm 1.5\%$. On the other hand, the change in the MSEW cases was on average $26.8 \pm 2.4\%$ for the ML extent, $9.5 \pm 3.2\%$ for the DV extent, and $22.8 \pm 2.7\%$ for the corresponding perimeter (Fig. 3e). The differences in the ML extent ($p = 0.000002$) and the perimeters ($p = 0.000041$) were statistically very significant. The difference in DV extent was just short of reaching statistical significance ($p = 0.08$).

Cortical thickness of LH MSEW animals is decreased as compared with RH or controls

The cortical thickness was measured dorsally in sections from rostral (e.g., as in Fig. 1a) to mid-rostrocaudal levels (e.g., as in Fig. 1b). LH and RH cortical thickness of control ($n = 8$) and MSEW ($n = 8$) adult mice (P75–90) was obtained from drawings of the cortical outline and underlying corpus callosum. These were drawn at 5 $\times$ using Neurolucida software. A total of 88 (44 LH and 44 RH) control and 74 (37 LH and 37 RH) MSEW coronal sections were drawn. The percent difference in thickness of the dorsal LH cerebral cortex as compared with its RH counterpart was calculated for every single section. As discussed above, while in control cases we did not notice differences in the thickness of the LH and RH dorsal cerebral cortex, in the MSEW cases this difference was usually clear (#1 in Fig. 1a, b). Our quantitative analyses corroborated these findings. In controls, the difference in cortical thickness between the LH and RH was on average $1.1 \pm 1.6\%$, whereas the difference in the MSEW animals was $10.3 \pm 2.2\%$ (Fig. 3d). The average difference in LH cortical thickness between controls and MSEW animals was statistically significant ($p = 0.004$). The cortical thickness of the control and MSEW cortical RH was not different ($p = 0.85$).

The corpus callosum is thinner in MSEW animals as compared with controls

The borders of the corpus callosum are most clear at the midline, where the fibers are compact, compared with laterally, where many fibers are entering and leaving. Therefore, we limited our measurements of corpus callosum thickness to the midline aspect of the structure. These measurements were made using 21 sections of MSEW animals ($n = 7$) that were matched with respect to rostrocaudal level to 21 sections from control ($n = 7$) animals. Then, for every pair of sections, the thickness of the corpus callosum of controls was set to 100% and the percent difference of the corresponding section for the MSEW case was calculated. On average the MSEW sections had a thinner corpus callosum than controls, at least in the rostral and mid-rostrocaudal levels, where the measurements were taken. The range in the thickness differences was wide (3.7–27.8%) with a mean value of $14.6 \pm 3.4\%$ which indicates that the thickness of the corpus callosum of MSEW animals is just ~85% that of controls on average (Fig. 3f; $p = 0.0048$ by paired t test).

DTI imaging demonstrates extensive decreases in fractional anisotropy due to MSEW

DTI fiber tracking was performed to assess the global integrity of WM (Alexander et al. 2007). Our imaging results strongly agree with and extend our histological observations (see Figs. 2g–h, 4). On average there was widespread decreased FA in multiple areas as a result of MSEW (Fig. 4). These differences reached statistical significance for the ac (0.29 ± 0.06 in control vs. 0.21 ± 0.04 in MSEW; $p < 0.03$) and the thalamus (0.20 ± 0.04 in control vs. 0.15 ± 0.03 in MSEW; $p < 0.04$). Other regions with a substantially higher FA in control versus MSEW cases were the cc (0.26 ± 0.06 vs. 0.22 ± 0.06), septofimbria (0.24 ± 0.06 vs. 0.19 ± 0.05), cingulate gyrus (0.27 ± 0.05 vs. 0.23 ± 0.05), basolateral amygdala (0.21 ± 0.05 vs. 0.16 ± 0.05), and middle and deeper cortical layers (0.22 ± 0.04 vs. 0.18 ± 0.04). Heterogeneity and fiber spread in these regions precluded a detailed statistical analysis. However, future improvements in DTI spatial resolution and larger samples should enable better global and regional comparisons of FA values.

Stereological analysis indicates that Olig2-positive cell density is most severely affected in the LH of MSEW animals

To further study the effects of MSEW on white matter, we used immunolabeling for Olig2 to obtain cell numbers and densities using stereological analysis with an optical fractionator probe (West et al. 1991; Gundersen et al. 1999). Olig2-positive cells were plotted in the motor (M1+M2) and PFC (infra limbic) cortices and in the genu of the cc. The motor cortex

was chosen because of the previously observed thinning of the cortex in this area; PFC because of its involvement in circuitry relating to many of the symptoms of ENA, and the cc because of its involvement in inter-hemispheric communication. We sampled five brains from MSEW cases and five age- and gender-matched controls. Each case was from a different dam and sire. Sections were 50 μm thick when cut and after processing had an average thickness of 18.4 μm with no significant differences in shrinkage between MSEW and control cases. A total of 50 coronal sections were drawn, 5 per brain. Of these, in every case, all five sections contained motor cortex, two contained the PFC and three the genu of the cc. Since each hemisphere was analyzed individually we compared 100 motor regions (50 controls; 50 MSEW); 40 PFC regions (20 controls; 20 MSEW), and 60 cc regions (30 controls; 30 MSEW).

Overall, in the control cases we plotted 21,771 and in the MSEW 21,022 Olig2-positive cells. These 42,700 plotted cells represent a population of 7.48×10^6 Olig2-positive cells with a coefficient of error (Gundersen $m = 1$) < 0.02 (Gundersen et al. 1999).

The mean number of Olig2-positive cells was higher in the PFC and cc of the MSEW cases than in the controls and the differences reached statistical significance. In the motor cortex no significant differences were observed. However, because of the previously detected changes in brain size between MSEW and matched controls and because of the differences in size between LH and RH of the MSEW cases, we did not investigate the number of cells (that could be biased by regional size), but instead, the density of Olig2-positive cells per mm^3 (Fig. 5). In the motor cortex of the controls, the Olig2 density was higher (both hemispheres mean: $64,274 \pm 3,147/\text{mm}^3$; (all values are mean $\pm$ SEM) than that in the MSEW cases (mean: $54,081 \pm 3,702/\text{mm}^3$). This difference was larger if comparisons were made between the LH of the MSEW cases, which had the lowest density ($49,251 \pm 4,949/\text{mm}^3$), and any other hemisphere. The density in the MSEW RH was $58,910 \pm 5,052/\text{mm}^3$. In the PFC cortex of controls, the density of cells was similar in the two hemispheres (mean: $87,803 \pm 9,851/\text{mm}^3$) and different to the density in the RH of the MSEW cases (mean: $54,014 \pm 3,745/\text{mm}^3$), but these densities were significantly lower than that of the MSEW LH (mean: $129,196 \pm 17,518/\text{mm}^3$). In the genu of the cc the density of Olig2 cells in the controls (both hemispheres, mean: $282,398 \pm 18,658/\text{mm}^3$) was lower than in the MSEW cases (mean: $362,443 \pm 44,929/\text{mm}^3$). The control LH (mean: $302,122 \pm 9,330/\text{mm}^3$) and RH (mean: $262,674 \pm 24,473/\text{mm}^3$), and the RH of the MSEW cases (mean: $267,877 \pm 38,069/\text{mm}^3$) all had significantly lower Olig2-positive cell densities than the LH of the MSEW cases (mean: $457,010 \pm 56,239/\text{mm}^3$). Statistical differences ($p < 0.05$) were found only when comparing the LH of the PFC or cc with any other hemisphere in the same region.

Discussion

In this study, we describe gross anatomical changes in the brains of MSEW mice. We report that these neuroanatomical changes include smaller brain size and abnormal inter-hemispheric asymmetry, changes in cortical thickness, abnormalities in subcortical structures, and WM disorganization and atrophy most severely affecting the LH. These changes are concomitant with behavioral abnormalities (George et al. 2010) and changes in gene expression (Bordner et al. 2011).

Human ENA has been associated with changes in multiple brain areas including altered cortical asymmetry in the frontal lobes (Carrion et al. 2001) and superior temporal gyrus (De Bellis et al. 2002) and volume loss and other abnormalities in the prefrontal cortex (Bremner 2006a, b; Tomoda et al. 2009), anterior cingulate gyrus (De Bellis et al. 2000), hippocampus (Bremner et al. 1997; Stein et al. 1997; Driessen et al. 2000; Vythilingam et al. 2002, 2005),

and cc (Teicher et al. 2004). This variety of affected brain areas has made animal models of ENA difficult to obtain (Teicher et al. 2006). Such models are, however, very important. In humans, it cannot be unequivocally proven that alterations in brain anatomy are the consequence of ENA. For example, some authors have argued that neuro-anatomical abnormalities are a risk factor for abuse rather than a consequence of abuse (Davies 1978). Animal models such as MSEW in which animals are randomized to ENA or control conditions allow causality to be rigorously established and are therefore of great value for further research.

That there is hemispheric laterality in animals and that such laterality is affected by early experience (Denenberg 1981) may not have been widely accepted a few decades ago. However, today, there is overwhelming evidence that brain hemispheric asymmetry is the rule of normal neurodevelopment in different species including several mammals and these processes can be affected by environmental and genetic factors. Understanding these asymmetries is critical to our understanding of brain function and provides a host of possibilities to use animal models not only to study those environmental and genetic events that may determine laterality but also to study how laterality relates to human disease (Narr et al. 2007).

The two hemispheres of the human cerebral cortex are anatomically and functionally asymmetric (Galaburda et al. 1978a). Hemisphere dominance has been studied and demonstrated in a multitude of tasks in relation to motor, language, analytical, mathematical, musical, and many other skills and abilities (Gordon 1975; Galaburda, et al. 1978b; Annett and Kilshaw 1982; Schlaug et al. 1995; Toga and Thompson 2003). A well-known example of hemispheric functional asymmetry is the fact that approximately 90% of the population is right handed and language dominance in about 97% of right-handed people is localized to the LH (Sun et al. 2005), the hemisphere regulating movement of the right side of the body. Human left–right specializations are the result of early developmental asymmetric events, and several candidate genes involved in the evolution of human cerebral cortical asymmetry have been identified (Sun et al. 2005, 2006; Sun and Walsh 2006). Asymmetry between cerebral hemispheres has been shown in a host of non-human mammalian species including mice, rats, cats, and rabbits (Kolb et al. 1982), and in all cases the LH is smaller than the RH. Because MSEW exaggerates these asymmetries in the mouse it may prove useful in understanding the molecular determinants of hemispheric asymmetry.

Normal differences in LH versus RH size are small. For instance, in rat, the difference is only approximately 1% as assessed by weight and length. In the mouse the differences are also small based on hemispheric weights (Kolb et al. 1982). Importantly, in these previous studies differences were not detected in structures such as the hippocampus. In our case, using perimeters, we detected a difference in size between the LH (smaller) and RH of the MSEW animals of about 6%, while the difference between LH and RH in controls was on average about 2%. We have previously assessed whether malnutrition is a factor in the effects of MSEW and have excluded this possibility based on analyses of body weight and serum metabolites sensitive to nutritional status (George et al. 2010).

We found the most striking anatomical effects of MSEW within the LH rather than the RH. These differences included a smaller hippocampus, reduction of cortical thickness, and deficits in myelination although the latter was shared between the two hemispheres in midline regions. Deficits were also observed in the RH of MSEW animals as compared with controls, but these were difficult to ascertain in histological material and were much clearer in DTI, which allows a global view.

The strong effects on the LH of MSEW animals may make the model potentially useful to study deficits in speech and language, skills most characteristically human, and associated with the human LH. Similar to language dominance in humans, LH dominance for vocalization also occurs in mice and other mammals and even in frogs and birds (Corballis 2003). Future studies using the MSEW animal model can therefore investigate the effects of ENA on the neurobiology of circuits governing speech and language.

A substantially smaller hippocampus in the LH of MSEW animals was a striking feature. The hippocampus is extremely vulnerable to prolonged stress (Sapolsky 1985a, b; Sapolsky et al. 1985, 1990) and studies have shown reductions on the order of 15% in the volume of the left hippocampus of human adults who suffered childhood trauma (Bremner et al. 1997; Vythilingam et al. 2002). Other studies have found reductions in hippocampal size bilaterally (Driessen et al. 2000).

Differences in gray matter thickness, possibly related to hemisphere-specific specializations, have been detected in humans (Luders et al. 2003, 2006) and rat, mouse, rabbit, and cat (Kolb et al. 1982). We detected abnormal cortical thinning in the dorsal LH of the MSEW animals. Interestingly, children with ADHD show cortical thinning in areas important for attentional control; specifically, worse clinical outcomes are associated with thinning of the left medial PFC (Shaw et al. 2006) and the influence of ENA on the integrity of these structures may therefore be partly responsible for the impaired attention seen in survivors of ENA.

Numerous studies have suggested a connection between disruption of myelin and behavioral changes in psychiatric disorders but the exact mechanisms involved are poorly understood. Most evidence thus far arises from the study of the schizophrenia risk gene Neuregulin 1 (NRG1), signaling through ErbB4 receptors, which has a role in the control of myelination (Lemke 2006). Mice carrying a dominant negative ErbB4 receptor show subtle defects in myelination accompanied by an increase in anxiety, as measured by the elevated plus maze (Roy et al. 2007). Unlike our data, these mice also demonstrate hyperlocomotion in the open field. However, heterozygous knockout of NRG1 results in hyperlocomotion in the open field (O'Tuathaigh et al. 2006). This difference in results may well be explained by the varying anatomical effects of the respective manipulations. For example, white matter deficits in the motor and pre-frontal cortices have been implicated in the expression of aberrant motor movements in ADHD in humans (D'Agati et al. 2010). Myelin deficits due to MSEW could therefore be involved in the hyperactivity and anxiety-like behavior that we observed in mice subjected to MSEW (George et al. 2010). Further work will be required to evaluate this hypothesis.

Changes in the cortex in the form of decreased dendritic neuropil in layer II/III pyramidal neurons of the PFC have been shown after early maternal deprivation (Pascual and Zamora-Leon 2007). Pyramidal neurons constitute the main neuronal pool that contributes myelinated callosal intracortical axons as well as myelinated axons to subcortical structures. Moreover, there is substantial evidence from studies of the hormonal response to early stressors indicating that stress hormones such as cortisol, adrenalin, and vasopressin can induce changes in myelination (Teicher et al. 2006). We find, using DTI imaging, significant alterations in FA in mid and deeper cortical layers. We hypothesized that these and other changes seen at the level of the cc and other WM tracks are due primarily to a disruption of myelin in these areas. There is a good deal of evidence for this model. Since we found normal Olig2+ cell densities and numbers in the RH of MSEW brains and increased numbers in the LH, a model where MSEW causes changes in myelination through decreasing the size of the oligodendrocyte precursor pool can be excluded. Instead, the data are consistent with a model where MSEW causes disruption in oligodendrocyte maturation

such that greater numbers of oligodendrocyte lineage cells remain in an immature, non-myelinating, Olig2+ state. Because the observed differences were robust to normalization for cellular content (i.e., the analysis of cellular densities rather than counts) the observed changes are not an artifact of changes in hemispheric size. Further evidence for this model is provided by Bordner et al. (2011) where markers of mature, myelinating oligodendrocytes were strongly downregulated in the PFC of MSEW animals. The increased density of Olig2+ cells in the LH is in agreement with more pronounced disruption in WM and gray matter structures in the LH as compared with the RH.

The findings presented here warrant further investigation. Deeper histological investigation at higher magnifications should be used in future studies to further confirm the results at the cellular level and to obtain further insights into the mechanisms causing the gross anatomical defects. In addition, future studies should include manipulation of some of the genes found to be associated with MSEW described in Bordner et al. (2011), to determine their role in mediating the effects of MSEW. Such studies are underway and will be the subject of future reports from our laboratory.

In summary, we present evidence that MSEW causes significant alterations in brain structure that resemble those observed in human survivors of ENA. Because of the similarities to the effects of ENA in humans, the MSEW model has the potential to advance our understanding of the mechanisms by which ENA affects brain structure and function. Such work may lead to novel therapies that may be of benefit to children and adults who have experienced ENA.

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Fig. 1.

Hemispheric asymmetry and gross structural changes. Structural and myelination differences can be observed throughout the rostrocaudal extent in coronal sections between the two hemispheres of MSEW animals. **a–d** Differences in hemispheric size, denoted in **a** with a dorsal *red line* contour of the RH whose mirror image does not match the LH. In every case, at least dorsally, the LH is smaller than the RH at both rostral and caudal levels and in nissl (not shown), golgi (**a, b**), and myelin (**c, d**)-stained material. **a, b** Illustrate, in sections from different animals, differences in cortical thickness most strikingly clear in the dorsal LH (#1). The *dotted white lines* in **b** illustrate the general dorsal cortical areas where measurements were taken to compare cortical thickness between the two hemispheres. **c–f** Illustrate, in Black Gold II myelin-stained material, both structural changes and myelin deficits in the MSEW animals as compared with controls. Some abnormalities observed are: (#2) a very marked difference in the mediolateral extent of the hippocampus between LH and RH of MSEW animals; (#3) a very salient deficit in the thickness, at this rostrocaudal level, of the cc as well as the amount of myelinated septofimbrial fibers of MSEW as compared with control; (#4) very poor amount of myelinated fibers in the internal capsule and stria terminalis in the LH of the MSEW as compared with its RH counterpart or to the corresponding controls; and similarly, (#5) a thinner stria medullaris and absent fornix or fornix not detected with myelin staining. Other differences included widespread disorganization of myelin fibers and decreased content in areas such as those surrounding the basolateral amygdala (#6) and the anterior commissure (not shown). In controls, hemisphere size and the sizes and positions of different structures always appear symmetrical and all myelin fibers and other structures appear present. The inter-hemispheric differences in size of different structures in the MSEW case cannot be accounted for by inter-hemispheric difference in the rostroventral axis, i.e., it is not the case that one hemisphere is at a substantially more caudal level than the other. Brain sections from control

cases always appeared more symmetrical than those from MSEW cases at all rostro-caudal levels and in both medio-lateral as well as dorso-ventral directions

Fig. 2.

Alterations in white matter tracks. Myelination defects are not limited to one hemisphere but are more salient in the LH and at both rostral and caudal levels. **a** The LH ac of a MSEW animal appears shrunk or incomplete in its robustness. Demyelinated and apparently degenerating fibers make the periphery of the fiber bundle appear empty. The extent of demyelination was very clear in 7 of 12 MSEW cases examined and in none of the controls (**b**, also 12 cases examined). Similarly, there are clear differences in myelination between the LH and the RH of a MSEW case at a more caudal level. These differences are obvious in both myelin (**c**) and in cresyl violet nissl (**d**) staining (adjacent sections). In **d**, *black arrowheads* indicate the presence of lemniscal fibers in the RH and *white arrowheads* indicate the absence of the corresponding bundle in the LH. Series of sections were followed in this particular case and the same observation was made in several other MSEW cases (5 out of 12 cases examined). This was not observed in any of the control cases (0 of 12 cases examined). Also noticeable in **c**, **d** is the difference in hippocampal size between RH and LH. **e**, **f** Magnetic resonance (spin-echo) anatomical images from a single MSEW animal show clear differences in size between LH and RH. In the rostral image (**e**) the *dashed line* helps distinguish the difference. At the caudal level the difference in size is so obvious the *dashed line* is unnecessary (there is also a small notch of the LH ventrolateral pole). In an individual case like this it is not possible to ascertain internal morphological details. In **g**, **h** averaged FA maps indicate that, in general, the DTI imaging results agree with the histological results. Here **g** shows asymmetry in the LH ac, which appears smaller, of MSEW mice ($n = 5$), than the corresponding ac in control mice ($n = 5$). At a more caudal level **h** shows higher density of fibers in the RH (*black arrowheads*) than in LH (*white arrowheads*) of MSEW mice ($n = 5$). The FA scale was contracted between 0 and 0.36 (instead of 0 and 1) to allow better visualization of differences between the MSEW and control cases. Additional gross changes are visualized in Fig. 4. Note that histology and imaging were done in different groups of animals

Fig. 3.

Hemispheric and hippocampal perimeters and cortical and corpus callosum thickness are affected in MSEW mice. **a** Example of LH versus RH perimeters in one pair of animals (1 control, 1 MSEW). To facilitate viewing the hemispheres are *color coded* and not all perimeters are shown. Differences in size between LH and RH in the MSEW case are obvious and also easy to see in reference to controls when perimeters are superimposed. **b** The average LH perimeter of MSEW animals is significantly smaller than its RH counterpart and any hemisphere of controls. This difference is statistically significant. **c** The LH of the MSEW animals is approximately 6% smaller than its RH counterpart or any hemisphere of controls. **d** When cortical thickness is compared between LH and RH of controls, the difference is on average only $1.1 \pm 1.6\%$ while the same comparison in MSEW animals indicates a mean difference of $10.3 \pm 2.2\%$. The difference in the means between control and MSEW is statistically significant. **e** When the size of the hippocampus in the LH is compared with the size of the corresponding RH hippocampus in the same section, the mean difference in mediolateral ($3.4 \pm 1.4\%$), dorsoventral ($2.8 \pm 1.4\%$) or whole perimeter ($3.2 \pm 1.5\%$) is less than 5% for every parameter measured. On the other hand, because in all MSEW cases the LH hippocampus is substantially smaller than the RH hippocampus, the same comparisons indicate substantial differences: $26.8 \pm 2.4\%$ for the ML extent, $9.5 \pm 3.2\%$ for the DV extent, and $22.8 \pm 2.7\%$ for perimeter. Comparison of differences between controls and MSEW cases reached statistical significance for the ML extent and perimeter. **f** Since the corpus callosum thickness was taken at the midline between the hemispheres, sections, matched at rostrocaudal levels, between control and MSEW cases were compared. The corpus callosum thickness was made 100% in every control section. The decrease in the thickness of the corpus callosum for MSEW cases was calculated to be on average $14.6 \pm 3.4\%$. All mean values are given $\pm$ SEM, as indicated also by the *error bars*. $**p < 0.05$

Fig. 4.

Diffusion tensor imaging (DTI) indicates reduced FA in MSEW mice. Averaged FA maps for MSEW ($n = 5$) and control mice ($n = 5$) for five contiguous slices, where each slice was 100- μm thick and spaced apart by 200 μm . In all regions identified by the *black arrowheads* the FA values were lower in MSEW cases. These regions include, among others, middle and deeper cortical layers, external capsule, thalamus, basolateral amygdala, and an apparent disconnection between corpus callosum and cingulum. Differences in hemispheric size between MSEW and control cannot be discerned here because unsmoothed FA maps were warped to a common coordinate space. The FA scale was contracted between 0 and 0.36 (instead of 0 and 1) to allow better visualization of differences

Fig. 5.

Olig2 is altered in different regions of MSEW mice. The density of Olig2-positive cells per mm^3 was statistically significantly higher in the PFC and genu of the cc of the LH as compared with its RH counterpart and to the average of both hemispheres in controls. There was little difference in the Olig2 density between hemispheres of control animals, or between these hemispheres and the RH of MSEW animals. In the motor cortex the density of Olig2-positive cells was lower in the LH as compared with the RH, and in the PFC the RH of MSEW had lower density than the average in controls but these differences did not reach statistical significance.