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# *In vivo* magnetic resonance images reveal neuroanatomical sex differences through the application of voxel-based morphometry in C57BL/6 mice

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## Abstract

Behaviorally relevant sex differences are often associated with structural differences in the brain and many diseases are sexually dimorphic in prevalence and progression. Characterizing sex differences is imperative to gaining a complete understanding of behavior and disease which will, in turn, allow for a balanced approach to scientific research and the development of therapies. In this study, we generated novel tissue probability maps (TPMs) based on 30 male and 30 female *in vivo* C57BL/6 mouse brain magnetic resonance images and used voxel-based morphometry (VBM) to analyze sex differences. Females displayed larger anterior hippocampus, basolateral amygdala, and lateral cerebellar cortex volumes, while males exhibited larger cerebral cortex, medial amygdala, and medial cerebellar cortex volumes. Atlas-based morphometry (ABM) revealed a statistically significant sex difference in cortical volume and no difference in whole cerebellar volume. This validated our VBM findings that showed a larger cerebral cortex in male mice and a pattern of dimorphism in the cerebellum where the lateral portion was larger in females and the medial portion was larger in males. These results are consonant with previous *ex vivo* studies examining sex differences, but also suggest further regions of interest.

### Keywords

Voxel-based morphometry; Sexual dimorphism; Magnetic resonance image; Atlas-based morphometry

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## Introduction

The difference in brain size is one of the oldest and best known sexual dimorphisms in neuroscience. In fact, studies have demonstrated that sex differences exist in the central nervous system (CNS) at virtually every level of anatomical detail (Arnold, 2004) and it has become increasingly evident that considering sex differences is vital to understanding the brain (Beery and Zucker, 2011; Clayton and Collins, 2014; McCarthy and Arnold, 2011). Historically, in biological research, sex differences were deemed insignificant and experiments performed in only one sex. This frequently led to an overemphasis of results in one sex while neglecting possible differences that could illuminate our understanding of biological processes. Current research suggests that sex differences are substantial and may bias conclusions if both sexes are not represented (Arnold, 2010).

Neuroimaging studies in humans have shown sex differences in whole brain (Blatter et al., 1995; Luders and Toga, 2010) as well as numerous neuroanatomical structures including the cerebral cortex (Schlaepfer et al., 1995), amygdala (Goldstein et al., 2001), and hypothalamus (Swaab et al., 1985). Gray and white matter volumes were reported as larger in males (Blatter et al., 1995; Luders et al., 2002), but when gray matter was computed as a percentage of total brain volume, females showed a larger gray matter ratio (Gur et al., 1999). These studies demonstrate that sexual dimorphism in the brain extends beyond anatomical structures that are involved in reproductive behavior, in fact occurring throughout the brain (Luders et al., 2004).

Clinically significant sexual dimorphisms in brain function are also well documented (Cahill, 2006). They are found in learning and memory (Andreano and Cahill, 2009), nociception (Berkley, 1997), and analgesia (Gioiosa et al., 2008) to name a few. In cognition, males have been shown to have an advantage in some spatial measures (Lejbak et al., 2011; Voyer et al., 1995) and females to have an advantage in some verbal (Norman et al., 2000; Weiss et al., 2006) and object-location memory measures (Tottenham et al., 2003; Voyer et al., 2007). This cognitive sexual dimorphism raises the possibility that these effects may be connected to structural brain differences between the sexes.

Interest in examining neuroanatomical sex differences has been stimulated by the recognition of widespread sexual dimorphism in disease (Beery and Zucker, 2011). For instance, males show much higher incidence of schizophrenia and developmental disorders (Fombonne, 2005; Gonthier and Lyon, 2004). Females, however, are more susceptible to neurodegenerative disorders and mood disorders (Barnes et al., 2005; Hebert et al., 2013; Piccinelli and Wilkinson, 2000; Zhao et al., 2016). Even when no preponderance is observed, disease progression or response to treatment may vary (Arnold, 2010). Sex differences have been shown in the incidence and course of numerous neurological disorders such as multiple sclerosis (Whitacre et al., 1999), Parkinson’s disease (Shulman and Bhat, 2006), and Alzheimer’s disease (Webber et al., 2005). The incidence and progression of some psychiatric disorders have also been shown to exhibit sex differences. For example, males suffer from schizophrenia more than 2.5 times more often than females. Males also experience an earlier onset of schizophrenia and exhibit more structural brain abnormalities

than females. Relapses are more severe and their response to neuroleptic medication is less favorable (Castle and Murray, 1991).

The underlying mechanisms of sex differences in brain structure are not entirely clear. The organizational-activational hypothesis emphasizes the importance of sex hormones and the timing of their activity in brain architecture (Arnold, 2009b; Morris et al., 2004; Phoenix et al., 1959). Recently, more emphasis has been placed on factors outside of gonadal hormones in the formation of neural sex (Arnold, 2009a). For example, sex chromosomes, epigenetics, and environmental factors have all been shown to produce sex differences in the brain (McCarthy and Arnold, 2011; Raznahan et al., 2013). Striving to uncover the basis of neural sex differences will lead to an improved understanding of sexually dimorphic behavior and disease.

Clearly, there are many elements contributing to differences in neural anatomy. In fact, numerous conflicting results exist in human studies describing opposing effects (Ruigrok et al., 2014). For this reason, mice are excellent models for identifying sex differences (Arnold, 2009a; Kovacevic et al., 2005). And although sex differences in mice may not be a perfect proxy for sex differences in humans, mouse models may yield some insight into sex differences without the multiple confounds found in human studies. For example, males and females of the same mouse strain have the same genetic makeup and are housed in similar environments, and controlling for as many variables as possible may allow observed differences in male and female brain structure to be attributed strictly to sex. However, only a few prior studies have provided a thorough analysis of neural sex differences in mice (Corre et al., 2014; Raznahan et al., 2013; Spring et al., 2007). These watershed studies provide invaluable information about sexual dimorphism in the brain, but it is also important to validate and expand this work with carefully-controlled, well-powered studies with meaningful differences in image acquisition and analysis.

Voxel-based morphometry (VBM) is a well-established and well-validated image analysis technique (Ashburner and Friston, 2000, 2005; Good et al., 2001; Luders et al., 2009). It can provide an unbiased and comprehensive assessment of anatomical differences throughout the brain. As such, sexual dimorphism of structures that would not have been expected can be identified. If sex differences across the brain are not homogenous, then regional measures, like VBM, may detect greater local change than measures averaged across the whole brain and thus provide more sensitive indices of change. VBM has been used extensively in humans and is now being used to evaluate gray matter changes in the mouse brain (Keifer et al., 2015; Sasaki et al., 2015; Sawiak et al., 2009, 2013).

In this project, we examined differences in the neural anatomy of 30 female and 30 male C57BL/6 mice. Voxel-based morphometry was used to identify regions where gray matter volume differed significantly. For our analysis, we generated new *in vivo* C57BL/6 tissue probability maps (TPMs) that improved image registration and segmentation in this mouse strain. Our analysis allowed us to identify regions that are sexually dimorphic and can be used to inform further investigation of sex differences in the living brain.

# Methods

## Animals and Study Design

60 C57BL/6J mice ranging from 8–12 weeks old (30 male/30 female) bred (3 generations or less) at UCLA from stock purchased from the Jackson Laboratories (Bar Harbor ME) were analyzed using voxel-based morphometry (VBM). Animals were maintained in a 12 h dark/light cycle with access to food and water *ad libidum*. All procedures were performed in accordance to the guidelines of the National Institutes of Health and the Chancellor's Animal Research Committee of the University of California, Los Angeles Office for the Protection of Research Subjects.

## Image Acquisition

All animals were scanned *in vivo* at the Ahmanson-Lovelace Brain Mapping Center at UCLA on a 7T Bruker imaging spectrometer with a micro-imaging gradient insert with a maximum gradient strength of 100 G/cm (Bruker Instruments, Billerica, MA). An actively decoupled quadrature surface coil array was used for signal reception and a 72-mm birdcage coil was used for transmission. For image acquisition, mice were anesthetized with isoflurane and their heads secured with bite and ear bars. Respiration rate was monitored and the mice were maintained at 37° C using a circulating water pump. Each animal was scanned using a rapid-acquisition with relaxation enhancement (RARE) sequence with the following parameters: $TR/TE_{eff}$ 3500/32 ms, ETL 16, matrix: 256 × 192 × 100, voxel dimensions: 100 × 100 × 100 $\mu m^3$. Total imaging time was 93 minutes. Images were acquired and reconstructed using ParaVision 5.1 software.

## Creating C57BL/6 in vivo tissue probability maps

Magnetic resonance images were processed using statistical parametric mapping 8 (SPM8, http://www.fil.ion.ucl.ac.uk/spm) and the SPMMouse toolbox (Sawiak et al., 2009, 2013) within MATLAB 2013a (Mathworks, Natick, MA). The standard preprocessing steps for VBM were carefully adapted as described below to accommodate the analysis of the C57BL/6 mouse brain images acquired *in vivo*.

The *in vivo* tissue probability maps provided with SPMMouse were created from R6/2 mice (Sawiak et al., 2009, 2013), therefore some spatial mismatch between these prior TPMs and the brains of the C57BL/6 mice used in the current study is to be expected. Thus, to avoid potential bias from systematic differences between the different mouse strains, customized TPMs were created for the current sample as described previously in humans (Kurth et al., 2015; Luders et al., 2004). For this, the acquired images were skull-stripped using hand-drawn masks by one rater (CEM), bias corrected, and spatially transformed into the same space as the R6/2 TPMs provided with SPMMouse by applying affine transformations using SPM routines. All images were subsequently resliced to match the R6/2 TPMs with respect to orientation and resolution. As this new "native" space is consistent with the reference space, new TPMs can directly be obtained using these resliced images (Kurth et al., 2015; Luders et al., 2004). More specifically, the registered and resliced images were segmented using SPMMouse to obtain gray matter (GM), white matter (WM), and cerebrospinal fluid (CSF) segments without any linear or nonlinear deformations that would encode the shape

of the tissue probability maps provided with SPMMouse. Subsequently, these tissue segments were averaged over all 60 animals and smoothed with a Gaussian Kernel of 200 μm full-width-half-maximum (FWHM). This resulted in a new sample-derived set of TPMs. However, seeing that the first segmentation step was performed using the TPMs provided with SPMMouse that were obtained from a different strain, we repeated the process once more with the newly created TPMs. That is, the new TPMs were used in a second iteration to align the skull-stripped and bias-corrected images to the reference space and tissue segment these images into GM, WM, and CSF. Once more, these resulting segments were averaged and smoothed with a Gaussian Kernel of 200 μm FWHM to generate the final set of TPMs for *in vivo* image analysis of C57BL/6 mice (Fig. 1). We named the space defined by these TPMs "Mortimer Space" and they are available for download at http://www.bmap.ucla.edu/portfolio/atlases/MSA.

### Voxel-based Morphometry

The magnetic resonance images of all 60 mice were manually registered to the newly created TPMs using 6 parameter linear transformations. The images were bias corrected and tissue segmented into GM, WM, and CSF using the unified segmentation algorithm (Ashburner and Friston, 2005) with the newly created TPMs. The resulting tissue segments were used to create a DARTEL template (Ashburner, 2007) and the individual GM segments were warped to this template and modulated. The size of an optimal smoothing kernel should be on the order of the signal expected (Siegmund and Worsley, 1995) and isotropic smoothing kernels between 5–10 times voxel size are common in the literature (Lerch et al., 2011; Sawiak et al., 2013). We selected a value 6-times our voxel size, or a 600 μm FWHM Gaussian kernel to smooth the normalized and modulated GM segments. The resulting smoothed images constituted the input for the statistical analysis. In addition, bias-corrected images were skull-stripped, warped to the DARTEL template, and averaged to create a mean template for visualization, which we named the Mortimer Space Atlas (MSA). The Mortimer Space Atlas is available for download at http://www.bmap.ucla.edu/portfolio/atlases/MSA.

### Atlas-based Morphometry

A minimum deformation atlas (MDA) was created with the magnetic resonance images from all subjects (30 males and 30 females) used in this study as described (MacKenzie-Graham et al., 2009). The cortex and cerebellum were manually labelled on the MDA using BrainSuite 16a (Shattuck and Leahy, 2002) (http://brainsuite.org/) as described (MacKenzie-Graham et al., 2012). Briefly, our cerebral cortex label was bounded ventrally by the plane inferior to the most anterior point of the corpus callosum at midline. Anterior and posterior boundaries were drawn in the plane before the corpus callosum was no longer continuous across the midline. These boundaries were selected to rely on unambiguous landmarks ensuring that no regions outside of the cerebral cortex were included in our delineations. Cerebellar labels were drawn to include the entirety of the cerebellum, bounded by the inferior colliculus and the vestibular nuclei. The anterior hippocampus label was bounded dorsally by the corpus callosum and ventrally by the most dorsal horizontal section where the anterior commissure was visible. The posterior boundary was the plane where the corpus callosum was no longer continuous across the midline. The posterior hippocampus label was

bound anteriorly by the anterior hippocampus label and extended posteriorly until the semicircular canals were visible. It was bound dorsally by the corpus callosum and extended to the most ventral portion of the hippocampus. These labels were then warped out to the individual images and manually corrected by a blinded operator to correct for discrepancies in the automated registration. BrainSuite was used to calculate the corresponding volumes.

### Statistical Analyses

Sex differences in local GM volume were examined with a general linear model in which the smoothed, normalized GM segments constituted the dependent variable and sex the independent variable. In addition, whole brain volume was included as covariate to account for the variance associated with brain size and to prevent potential effects due to differences in brain size. Within this model significant differences in local GM volume between males and females were determined via student's t-tests. All findings were corrected for multiple comparisons by controlling the false discovery rate (Hochberg and Benjamini, 1990) and significance maps with a threshold at $q \leq 0.05$. Surviving clusters were then overlaid onto the mean template for anatomic localization and visualization.

Whole brain (BR), GM, WM, CSF, and atlas-based morphometry (ABM) data were analyzed in R (https://www.r-project.org/). Volumes were compared with a student's t-test (two-tailed) and reported without multiple comparison corrections. 95% confidence intervals were found by resampling (10,000 bootstraps).

## Results

Analysis of BR, and normalized and modulated GM, WM, and CSF produced by SPMMouse indicated that males had more gray matter and CSF than females (Table 1). However, females demonstrated larger WM volume. Interestingly, although it did not reach statistical significance, males exhibited a statistical trend towards larger whole brain volumes (Fig. 4A). When normalizing for whole brain volume, their comparisons remained statistically significant (Table 2).

Voxel-based morphometry revealed multiple regions of sexually dimorphic gray matter volume throughout the brain (Fig. 2). Several prominent regions were significantly larger in females including the anterior hippocampus, basolateral amygdala, and a dorsal region of the caudoputamen. Females also exhibited larger anatomy in the periaqueductal grey, as well as the paraflocculus and paramedian lobule in the cerebellar cortex.

Males displayed larger cortical volume, as well as in the bed nucleus of the stria terminalis, posterior hypothalamus, inferior colliculus, inferior portion of caudoputamen, and medial amygdala. Several clusters denoting more GM in males also emerged in the thalamus and medial regions of the cerebellar cortex. Nearly all significant differences were bilateral, indicating left-right symmetry in the organization of sexually dimorphic neural anatomy.

Delineations of cerebral cortex and cerebellum, as well as anterior, posterior, and whole hippocampus were created for all subjects (Fig. 3) and used to calculate volumes (Table 1). Without spatial normalization, male mice had larger cerebral cortex volumes than females

(Fig. 4B). Cerebellar volumes, however, did not show significant sex differences (Fig. 4C). Male mice had larger posterior and whole hippocampus volumes (Fig 4D & E), whereas female mice had larger anterior hippocampus volumes than males (Fig 4F). When the structure volumes were calculated as percentages of whole brain volume (normalizing for brain volume), male mice had larger GM, CSF, cerebral cortex, and posterior hippocampus volumes than females (Table 2). Females had larger WM and anterior hippocampus volumes than males. These results are consistent with our VBM results, since the cerebral cortex and posterior hippocampus were shown to be larger in males and anterior hippocampus was shown to be larger in females. Interestingly, the lateral cerebellum was larger in females and the medial cerebellum was larger in males, effectively offsetting each other in our ABM results.

## Discussion

Voxel-based morphometry, while extensively used in human neuroimaging research, is novel in rodent image analysis. The use of VBM allowed for an objective and comprehensive analysis of sex differences in C57BL/6 mouse brains. It is not subject to human error or bias and is sensitive to subtle changes that may not be detected with atlas-based morphometry (Ashburner and Friston, 2000, 2001). Additionally, the process is considerably less time consuming than manual analysis and allows for the investigation of all available data (Sawiak et al., 2009, 2013). Our results are consonant and expand upon current literature demonstrating sex differences in the brain (Corre et al., 2014; Dorr et al., 2008; Spring et al., 2007). Though much higher resolution can be achieved in *ex vivo* imaging, our results indicate that VBM can be used to reliably identify sex differences *in vivo*.

In this study, we employed novel tissue probability maps (TPMs) generated from 30 male and 30 female live C57BL/6 mice to examine sex-differences in brain structure using voxel-based morphometry. Since an equal number of males and females were used to create the TPMs, it can readily be applied to either sex in an unbiased fashion. The use of these TPMs improved the accuracy of our VBM examination of *in vivo* C57BL/6 mouse brains.

We observed that males had a statistical trend towards larger whole brain volumes, but this did not reach significance. This is consonant with previous studies in mice, where males have demonstrated 2.5% larger whole brain volumes and human studies where male whole brain volume has been reported as 13–15% larger than females (Gur et al., 1999; Leonard et al., 2008; Luders et al., 2009; Luders et al., 2005) (Table 3). Segment volume analysis revealed that male mice have larger GM and CSF compartments, while females have more WM. Since sex differences in compartment volumes have not previously been explored in rodent studies, it is not clear whether these effects are conserved across strains.

We observed larger volumes in the female anterior hippocampus, which is consistent with the literature (Spring et al., 2007). Sex hormones have been known to alter the anatomy of the hippocampus in rodent models (McEwen and Woolley, 1994; Roof and Havens, 1992), but conflicting results exist on the precise nature of these differences (Corre et al., 2014; Isgor and Sengelaub, 1998). The hippocampus has been heavily implicated in sexually dimorphic behaviors including spatial memory (Jacobs et al., 1990) and fear conditioning

(Gupta et al., 2001). Furthermore, hippocampal dysfunction is sexually dimorphic in several diseases including anxiety and dementia (Lebron-Milad et al., 2012; Murphy et al., 1996). Differences between anterior and posterior hippocampal function and anatomy have been identified in humans in a sex-dependent manner (Chua et al., 2007; Persson et al., 2014) but, again, inconsistencies in literature suggest that the specifics of these differences are not entirely clear (Goldstein et al., 2001; Ruigrok et al., 2014). Despite the variability in the characterization of sex differences in the hippocampus, it is clear differences exist. A larger anterior hippocampus was demonstrated in female mice relative to males using two different analyses (Corre et al., 2014; Spring et al., 2007). This may contribute to hippocampal-dependent sex differences observed in behavior and disease.

Our results also demonstrated that cortical and basolateral amygdala regions appear larger in female mice. This region is known to exhibit a sexually dimorphic pattern of gene expression in humans and mice (Lin et al., 2011). While morphometric differences have been shown in sections of the basal forebrain just anterior to our findings (Dorr et al., 2008; Spring et al., 2007), sex differences have not previously been shown in the more lateral amygdalar regions in mice. This region has been associated with sexually dimorphic behaviors such as pheromone processing, anxiety, and aggression (Akhmadeev et al., 2016). Developing female rats have been shown to display increased rates of cell proliferation (Krebs-Kraft et al., 2010) in the amygdala, but not much is known about the origin of this difference. We also observed a larger medial amygdala and bed nucleus of the stria terminalis in males, consistent with previous findings (Corre et al., 2014; Hines et al., 1992). Both hormones and sex chromosomes have been implicated in contributing to this difference (Corre et al., 2014; Shah et al., 2004). The medial amygdala is known to be involved male reproductive behavior (Newman, 1999) and aggression (Wang et al., 2013), as well as social recognition in both sexes (Ferguson et al., 2001). The results in this study support findings in multiple species, indicating that structural differences in the amygdala are present in a sexually dimorphic pattern.

We found that sex differences in the cerebellum were, intriguingly, heterogeneously arranged. The medial portion of the cerebellar cortex was larger in males while the lateral regions, including the paraflocculus and paramedian lobule were larger in females. This heterogeneity was consistent with Spring et al. (2007). Remarkably, these findings were demonstrated in a human study as well (Fan et al., 2010). Fan et al. found that the medial cerebellum, including lobules V and VIIIb, was significantly larger in human males. Human females were shown in this study to have larger lateral cerebellum, including Crus II. These findings demonstrate nearly the exact pattern of sexual dimorphism in our results. The similarity in our findings suggests conservation of sex differences in the cerebellum across species. Little is known about functional sex differences in the cerebellum. There is evidence, however, linking these regions to behaviors that may be functionally relevant to sexual dimorphism (Stoodley and Schmahmann, 2009; Stoodley et al., 2012). Functional MRI suggests lobules IV–V and VII, all of which were shown to be larger in males, are involved in sensorimotor tasks. Language tasks have been associated with Crus II, which is larger in females (Stoodley and Schmahmann, 2009; Stoodley et al., 2012). Further characterizing these differences will be invaluable as human studies have linked sex

differences in cerebellar structure to differences in cognitive function (Gur et al., 1999), social behavior (Wang et al., 2014) and disease (Keller et al., 2003).

Additionally, we found that the dorsal region of the caudoputamen was larger in females while the ventral region, as well as the nucleus accumbens, was larger in males. The striatum is known to have many functional sexually dimorphic properties, but the literature describing structural sex differences in this region is scarce (Becker and Hu, 2008; Bobzean et al., 2014). What literature does exist is inconsistent (Corre et al., 2014; Spring et al., 2007; Wong et al., 2015). Spring et al. (2007) did not find volume differences in the caudoputamen, but did identify sex differences in its shape. Corre et al. (2014) did report, however, that gonadal hormones were associated with volume differences in several regions within the caudoputamen. Regardless of sex chromosome complement, gonadal females displayed regions of greater volume, but the specific pattern of these differences was unclear. Our results demonstrate, for the first time, a robust bilateral pattern of sexual dimorphism in the striatum. These structural differences could contribute to the sexually dimorphic behaviors that are regulated by the striatum including motivation, reward, and impulse control (Becker and Hu, 2008; Bobzean et al., 2014). Sex hormones seem to play a pivotal role in manipulating these behaviors. Estrogens, for example, enhance neuronal excitability in the striatum and increase dopamine receptor availability (Yoest et al., 2014). Females and males react differently to substance abuse as a result of these properties and a better understanding of the associated neural differences may lead to more successful methods of treating the condition.

As a validation of our VBM results, we analyzed the data using atlas-based morphometry. Consistent with our VBM results, ABM revealed that cerebral cortex was larger in males. Interestingly, no sex differences were observed in the volume of the cerebellum. However, given the sexual dimorphic pattern of GM volume in the cerebellum noted with VBM, this is, again, consistent with our findings. VBM indicated that the medial portion of the cerebellum was larger in males, but the lateral portion was larger in females. It is possible that the sex differences offset one another so that the net cerebellar volume is not statistically different between the sexes. This result highlights one of the strengths of this approach, that VBM allows us to perform sophisticated voxelwise statistical analyses. In this report, we used a general linear model to perform the statistical analysis, permitting us to use total intracranial volume (TIV) as a covariate. In this way, each voxel can have its own relationship with the covariates, unveiling the regional cerebellar differences between females and males we observed. Furthermore, the consistency between our results and Spring et al., despite different analysis techniques, is noteworthy and likely represents biologically meaningful effects. There were some differences between our results and other reports in the literature (Corre et al., 2014; Spring et al., 2007), but these discrepancies may be due to differences in imaging resolution and contrast, differences in the nature of the analyses (deformation based morphometry vs. voxel-based morphometry), or even differences in the size of the smoothing kernel used for the analysis.

The tissue probability maps that we generated appear to over-represent white matter in the brainstem. This may limit the ability to detect changes in brainstem nuclei, although

experiments with other, similar TPMs have demonstrated that differences in the brainstem can be visualized (Sawiak et al., 2009).

It is important to acknowledge that sex differences may vary across different mouse strains. In rodents, it seems that both sex hormones and sex chromosomes contribute to sex differences in anatomy (Arnold, 2009a; McCarthy and Arnold, 2011). Furthermore, epigenetics and environmental factors have also been known to alter neural structure (Champagne, 2008; McCarthy and Arnold, 2011). Recently, investigation into the effects of sex chromosomes using the four core genotypes (Arnold and Chen, 2009) has suggested significant differences in the neuroanatomy of mice possessing different sex chromosome complement (XX or XY), but the same gonadal sex (Corre et al., 2014). Considering how anatomical differences in the four core genotypes translate to disease models presents an intriguing line of future research.

Though VBM is a powerful analysis tool, it is unable to determine the basis of morphological differences. We cannot distinguish whether our observed results are due to differences in neuronal number, neuronal size, or even cellular composition. To fully understand the anatomy underlying neural sex differences, future analysis should include a histological component, perhaps with use of modern optical clearing technologies to visualize entire brains (Chung et al., 2013; Spence et al., 2014).

## Conclusion

Voxel-based morphometry has not previously been used to analyze sex differences in mice. Our approach was particularly well suited to the study of sex differences through the use of novel TPMs that equally represented both sexes. Males exhibited larger cerebral cortex, medial amygdala, and medial cerebellar cortex volumes, whereas females displayed larger anterior hippocampus, basolateral amygdala, and lateral cerebellar cortex volumes. The results presented are consonant with previous findings that demonstrated sexually dimorphic brain morphometry (Corre et al., 2014; Spring et al., 2007) signifying a robust effect across studies. Much is still left to be discovered about the basis of anatomical sex differences in the brain, but it is clear that the differences are measurable and therefore cannot be considered negligible. They can, however, inform our understanding of the brain and sexually dimorphic behavior and disease.

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## Abbreviations

**ABM** atlas-based morphometry

| | |
|---|---|
| **MDA** | minimum deformation atlas |
| **VBM** | voxel-based morphometry |
| **TPM** | Tissue probability map |

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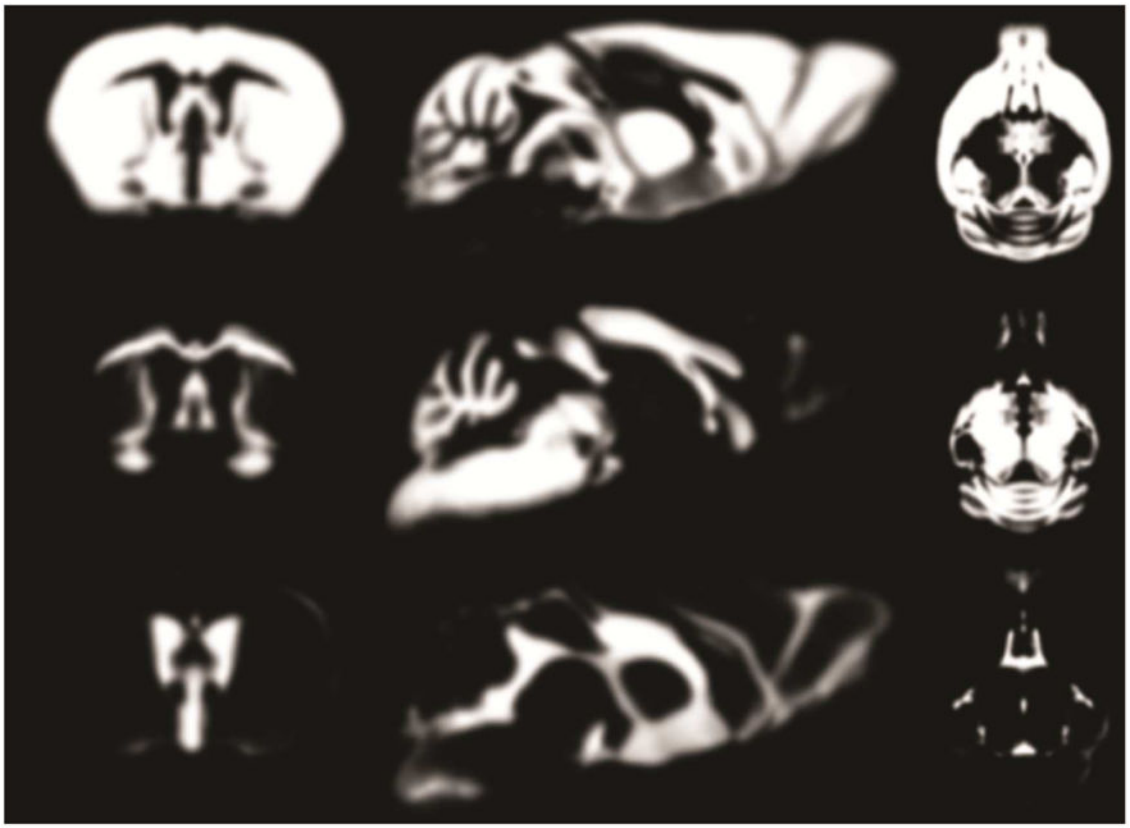

**Fig. 1.**

Tissue probability maps. Tissue probability maps for gray matter (top), white matter (middle), and cerebrospinal fluid (bottom). Coronal (left), sagittal (middle), and axial (right) sections through each map represent the probability that a given voxel belongs to that class of tissue. Maps were created using T2-weighted magnetic resonance images from 30 male and 30 female adult C57BL/6 mice.

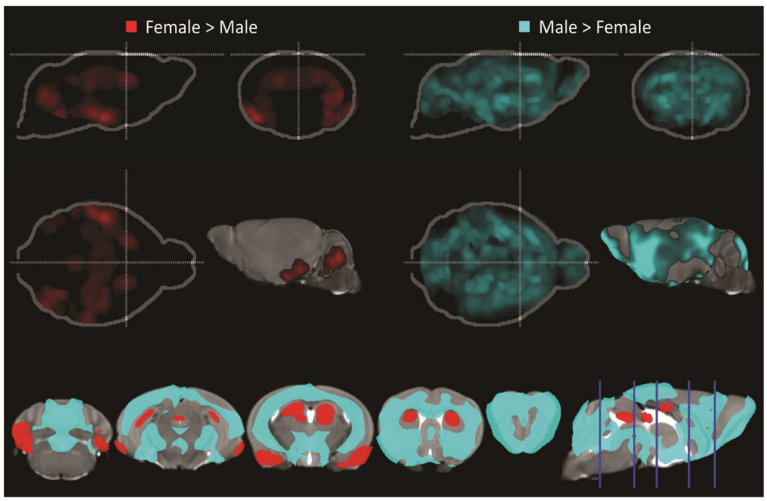


**Fig. 2.**

Localized sex differences in gray matter volume. VBM revealed regions that were significantly ($p \leq 0.05$, FDR corrected) larger in females (red) or males (aqua). Maximum intensity projections are overlain on the Mortimer Space Atlas standard glass brains where the dotted line represents bregma. Surface views of significant regions are shown over a 3D rendering of the mean template. Coronal sections (bottom row) demonstrate regions larger in females (anterior hippocampus, basolateral amygdala, and lateral cerebellar cortex) or larger in males (cerebral cortex, medial amygdala, and medial cerebellar cortex). Blue lines on the sagittal section indicate the position of the coronal slices from left to right respectively.

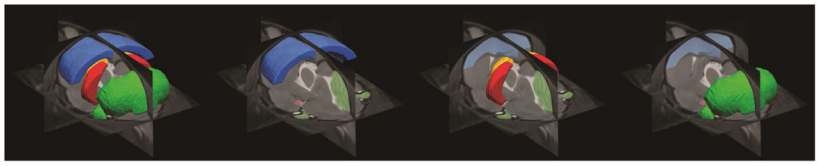

**Fig. 3.**

Minimum deformation atlas with region delineations. Surface views of cerebral cortex (blue), anterior hippocampus (yellow), posterior hippocampus (red), and cerebellum (green) delineations are overlaid on the minimum deformation atlas created from the T2-weighted magnetic resonance images of 30 male and 30 female C57BL/6 mice used in this study.

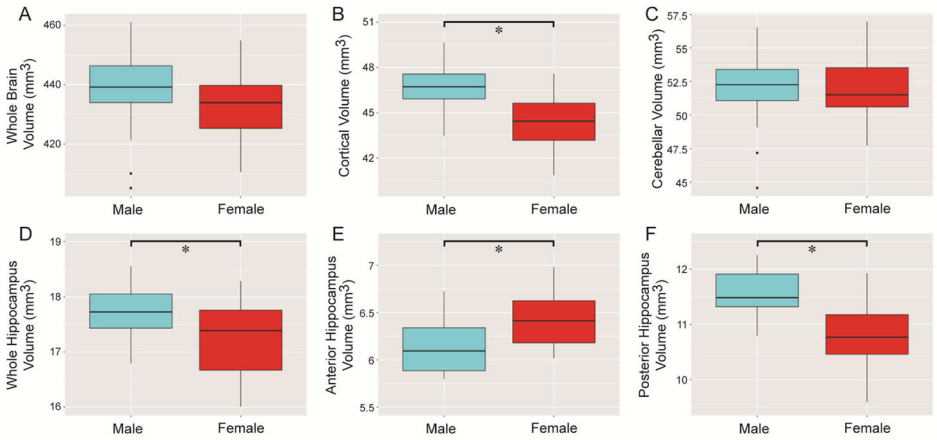


**Fig. 4.**

Atlas-based morphometry supports VBM results. (A) Whole brain volume showed a statistical trend towards being larger in males (aqua) than females (red) ($p = 0.07$). (B) Cortical volume was larger in males ($p = 2.96 \times 10^{-6}$). (C) No statistically significant sex difference was found in cerebellar volume. (D) Whole hippocampus volume was larger in males ($p = 2.47 \times 10^{-3}$). (E) Anterior hippocampus volume was larger in females ($p = 1.71 \times 10^{-4}$) while (F) Posterior hippocampus volume was larger in males ($p = 8.25 \times 10^{-7}$). All p-values are FDR-corrected for multiple comparisons.

## Table 1

Regional volumes for male and female C57BL/6 mice. All reported p-values are FDR-corrected for multiple comparisons. 95% confidence interval in brackets. (n = 30 males, 30 females)

| Region | Mean ($mm^3$) | | Effect Size ($mm^3$) | *P* value |
|---|---|---|---|---|
| | Male | Female | | |
| Whole brain | 438.43 [434.05, 442.42] | 432.48 [428.31, 436.63] | 5.95 [−0.06, 11.65] | 0.07 |
| Gray matter | 262.25 [257.00, 267.26] | 251.00 [247.62, 254.39] | 11.25 [5.01, 17.21] | $1.41 \times 10^{-3}$ |
| White matter | 130.60 [126.93, 133.97] | 143.20 [140.86, 145.53] | 12.60 [16.94, 8.41] | $2.94 \times 10^{-6}$ |
| Cerebrospinal fluid | 45.58 [43.18, 47.98] | 38.29 [36.50, 40.07] | 7.29 [4.37, 10.23] | $4.60 \times 10^{-5}$ |
| Cerebral cortex | 46.72 [46.20, 47.21] | 44.32 [43.67, 44.97] | 2.39 [1.58, 3.24] | $2.96 \times 10^{-6}$ |
| Cerebellum | 51.89 [50.99, 52.73] | 51.84 [51.11, 52.58] | 0.05 [−1.09, 1.17] | 0.93 |
| Whole Hippocampus | 17.71 [17.54, 17.88] | 17.22 [16.98, 17.45] | 0.49 [0.21, 0.78] | $2.47 \times 10^{-3}$ |
| Anterior Hippocampus | 6.13 [6.04, 6.22] | 6.42 [6.33, 6.52] | 0.29 [0.16, 0.43] | $1.71 \times 10^{-4}$ |
| Posterior Hippocampus | 11.58 [11.44, 11.72] | 10.80 [10.60, 11.00] | 0.78 [0.54, 1.02] | $8.25 \times 10^{-7}$ |

## Table 2

Regional volumes for male and female C57BL/6 mice normalized to male and female mean whole brain volume respectively. All reported p-values are FDR-corrected for multiple comparisons. 95% confidence interval in brackets. (n = 30 males, 30 females)

| Region | Mean Percentage of Total Brain Volume | | Effect Size | *P* value |
|---|---|---|---|---|
| | Male | Female | | |
| Gray matter | 59.82 [58.62, 60.96] | 58.04 [57.25, 58.82] | 1.78 [0.36, 3.16] | 0.02 |
| White matter | 29.79 [28.96, 30.56] | 33.11 [32.57, 33.65] | 3.32 [2.40, 4.32] | $3.72 \times 10^{-7}$ |
| Cerebrospinal fluid | 10.40 [9.85, 10.95] | 8.85 [8.44, 9.27] | 1.54 [0.84, 2.23] | $1.37 \times 10^{-4}$ |
| Cerebral cortex | 10.66 [10.54, 10.77] | 10.24 [10.10, 10.40] | 0.41 [0.22, 0.60] | $2.05 \times 10^{-4}$ |
| Cerebellum | 11.84 [11.63, 12.03] | 11.99 [11.82, 12.16] | −0.002 [−0.004, 0.001] | 0.29 |
| Whole Hippocampus | 4.04 [4.00, 4.08] | 3.98 [3.93, 4.03] | 0.06 [0.01, 0.12] | 0.11 |
| Anterior Hippocampus | 1.40 [1.38, 1.42] | 1.48 [1.46, 1.51] | 0.09 [0.06, 0.12] | $3.74 \times 10^{-6}$ |
| Posterior Hippocampus | 2.64 [2.61, 2.67] | 2.50 [2.45, 2.54] | 0.14 [0.09, 0.20] | $2.23 \times 10^{-5}$ |

## Table 3

Prominent regions displaying sexual dimorphism in this study compared to mouse and human literature.

| Region | Relevant behaviors | This study | Mouse literature | Human literature |
|---|---|---|---|---|
| Cerebral cortex | Cognition, sensory functions, motor functions (Parent and Carpenter, 1996) | Larger in males | Some regions larger in males, some larger in females (Spring et al., 2007) | Larger in males (Giedd et al., 1997; Goldstein et al., 2001) |
| Bed nucleus of the stria terminalis | Fear (Fendt and Fanselow, 1999) | Larger in males | Larger in gonadal males (Corre et al., 2014) | Larger in males (Allen and Gorski, 1990) |
| Posterior hypothalamus | Hormone production and release (Parent and Carpenter, 1996)<br>Sexual stimulation (Caggiula and Hoebel, 1966) | Larger in males | Larger in gonadal males (Corre et al., 2014)<br>Larger in females (Spring et al., 2007) | Whole hypothalamus larger in males (Goldstein et al., 2001; Swaab et al., 1985) |
| Anterior hippocampus | Spatial memory (Jacobs et al., 1990)<br>Fear conditioning (Gupta et al., 2001) | Larger in females | Larger in females (Spring et al., 2007) | Whole hippocampus larger in females (Giedd et al., 1996; Goldstein et al., 2001) |
| Ventral caudoputamen | Motivation, reward, impulse control (Becker and Hu, 2008; Bobzean et al., 2014) | Larger in males | Difference in shape (Spring et al., 2007)<br>Several nuclei larger in chromosomal males (Corre et al., 2014) | Putamen larger in males (Goldstein et al., 2001)<br>Caudate larger in females (Filipek et al., 1994; Goldstein et al., 2001; Murphy et al., 1996) |
| Dorsal caudoputamen | Motivation, reward, impulse control (Becker and Hu, 2008; Bobzean et al., 2014) | Larger in females | Difference in shape (Spring et al., 2007)<br>Several nuclei larger in gonadal females (Corre et al., 2014) | Putamen larger in males (Goldstein et al., 2001)<br>Caudate larger in females (Filipek et al., 1994; Goldstein et al., 2001; Murphy et al., 1996) |
| Medial cerebellar cortex (including lobules II–IX) | Sensorimotor functions (Allen et al., 1997; Stoodley and Schmahmann, 2009; Stoodley et al., 2012)<br>Proximal limb and trunk coordination (Ivry et al., 1988) | Larger in males | Larger in males (Spring et al., 2007) | Larger in males (Fan et al., 2010) |
| Lateral cerebellar cortex (including paraflocculus and paramedian lobule) | Language tasks (Stoodley and Schmahmann, 2009; Stoodley et al., 2012)<br>Motor planning for extremities (Ivry et al., 1988) | Larger in females | Larger in females (Spring et al., 2007) | Larger in females (Fan et al., 2010) |
| Basolateral amygdala | Pheromone processing, Anxiety, Aggression (Akhmadeev et al., 2016) | Larger in females | Larger in females (Spring et al., 2007) | Whole amygdala larger in males (Giedd et al., 1996; Goldstein et al., 2001) |
| Medial amygdala | Reproductive behavior (Newman, 1999)<br>Social Recognition (Ferguson et al., 2001)<br>Aggression (Wang et al., 2013) | Larger in males | Larger in gonadal males (Corre et al., 2014) | Whole amygdala larger in males (Giedd et al., 1996; Goldstein et al., 2001) |