Title: Behavior state-dependent lateralization of dorsal dentate gyrus c-Fos expression in mice

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Hemispheric lateralization is a fundamental organizing principle of nervous systems across taxonomic groups with bilateral symmetry. The mammalian hippocampus is lateralized anatomically, physiologically, and chemically; however, functional asymmetries are not yet well understood. Imaging studies in humans have implicated the left and right hippocampus in specialized processing associated with episodic memory and spatial navigation, respectively. However, it is not clear if different types of input (i.e. external sensory input vs. self motion input) to the hippocampus are processed in each hemisphere. Immediate early gene (IEG) imaging in animals provides a measure of neuronal activity with a resolution at the level of single cells. To further understand functional lateralization of hippocampal processing, we compared interhemispheric expression of c-Fos in the dentate gyrus (DG), a structure involved in encoding new experiences, in mice that ran on a wheel, encoded a novel object, or remained in home cages. We found that wheel running induced the greatest amount of DG c-Fos expression in both hemispheres, with no difference in expression between the hemispheres. Object exploration resulted in left-lateralization of DG c-Fos expression, whereas control mice were not lateralized. This suggests that information regarding novel objects may be preferentially encoded by the left DG.

Lateralization in the brain refers to interhemispheric asymmetry in the presence or dominance of a particular neural substrate (Concha et al., 2012). Many forms of anatomical and physiological lateralization in the hippocampus have been well described; however, understanding functional lateralization has been more elusive. Maguire and colleagues have suggested that the left and right hippocampus in humans are specialized for episodic memory and spatial navigation, respectively (Maguire et al., 1998; O'Keefe et al., 1998; Spiers et al., 2001; Burgess et al., 2002; Maguire & Frith, 2003). Damage to the left hippocampus impairs memory for where, or from whom, a particular object was received (Spiers et al., 2001). Damage to the right hippocampus impairs navigation in a virtual environment (Spiers et al., 2001). Further, activity in the left hippocampus is invariantly associated with recall of recent and remote autobiographical memories, while activity in the right hippocampus degrades with remoteness of autobiographical memories (Maguire & Frith, 2003), suggesting that the left hippocampus provides longer-term storage of autobiographical memories than the right. It has also been reported that while activity is upregulated in both the left and right hippocampus during goal-directed virtual navigation, only right hippocampal activity is correlated with accuracy of virtual navigation (Maguire et al., 1998).

In rodents, spatial memory has been reported to be right-lateralized (Shinohara et al., 2012), while long-term spatial memory storage has been suggested to be left-lateralized (Klur et al., 2009; Shipton et al., 2014; El-Gaby et al., 2016). Hippocampal interhemispheric asymmetries have also been reported in the physiological substrates of learning and memory in rodents (Kohl et al., 2011; Shipton et al., 2014). However, it is not clear if hemispheric asymmetries are due solely to differences in *how* information is processed, or whether there are also asymmetries with

respect to which types of information are processed in the left and right hippocampus reflecting functional differences between the left and right hemispheres.

The cognitive map theory of hippocampal function suggests that externally-originating sensory input that acts as allocentric spatial cues are gated by internally generated motor feedback from the path integration system (i.e. "dead reckoning"), acting as idiothetic positional cues (O'Keefe & Nadel, 1978; McNaughton et al., 1996). In this way, locomotion-associated firing rates during path integration can be modulated by locational cues such as landmarks (McNaughton et al., 1996). Although the original cognitive map theory acknowledges the possibility of hemispheric lateralization in navigation, specific functions of the left and right hemisphere are not discussed (O'Keefe & Nadel, 1978).

Interestingly, although imaging immediate early gene expression (IEG) in animals provides several advantages over human imaging, such as single-cell resolution and the ability to examine activity following freely moving behaviors, no study has compared IEG expression in the left and right dorsal hippocampus. To begin to understand potential hemispheric functional specialization in the rodent hippocampus, we examined how the behaviors of wheel running and exploration of a novel object are associated with IEG expression in the left and right dentate gyrus. Voluntary wheel running increases neurogenesis and other plasticity-related structures in the dentate gyrus and improves hippocampal-dependent learning (Grégoire et al., 2014). The rodent hippocampus is also known to be necessary for encoding non-spatial memories of objects (Cohen et al., 2013). We speculated that these behaviors may also be relevant to the dichotomy between internally-generated information about movement (e.g., wheel running; or self-motion feedback) and external-originating information about the environment (e.g. novel objects; or landmarks) known to be processed in the human hippocampus (McNaughton et al., 1996).

To assess hippocampal activity during these tasks, we examined c-Fos expression in adult male (n=14) and female (n=11) C57Bl6 mice. Mice were divided into three groups: 1) wheel running (WR, n= 5 males, 3 females), 2) exploration of a novel object in the homecage (OB, n= 4 males, 3 females), and 3) maintenance in the homecage (CON, n= 5 males, 5 females). All mice were singly housed in standard shoe-box cages in a vivarium colony. In addition, we sought to emphasize the relative salience of internally-generated motor information versus externally-originating environmental cues by testing the wheel-running group in the dark.

Training and testing was conducted in a small, dedicated behavior testing room. WR mice were first trained to run on a wheel for one day. WR mice were brought to the behavior room in their home cage and within a few minutes placed in a cage identical to that of their home cage except containing a wheel. They were permitted 2 hours of housing in the wheel-cage, during which time they all ran to varying extents. They were then transferred back to their homecage, housed in the behavior room overnight, then returned to the vivarium. For WR mice, all training and housing in the behavior room was conducted in the dark. OB and CON mice were not pre-trained prior to testing.

Mice were individually transported in their homecage to the behavior room and kept in the dark for 24 hours before the experiment. The next day, WR mice were transferred from their homecage to the wheel-running cage, allowed 30 minutes of running, and then transferred back to their homecage. Seventy-five minutes after being placed in the wheel-running cage, mice were transported to a perfusion room and sacrificed (see Detailed Methods). For OB mice, on the day of the experiment, the behavior room light was turned on and then a novel object (small PVC pipe) was placed in their homecage. After 30 minutes of exposure, the object was removed and the light was turned off. Seventy-five minutes after the introduction of an object, the mice were

transported to a perfusion room and sacrificed. CON mice were transported in their home cages to the behavior room and housed for 24 hours in the dark as were the other groups. They remained in the dark undisturbed time-matched to the behavioral experiments of the WR and OB groups, and then transported to a perfusion room and sacrificed. Individual time spent wheel running and exploring the object were not quantified.

Mice were euthanized and perfused with 4% paraformaldehyde (see Detailed Methods for more information). Brains were post-fixed, sunk in 30% sucrose in PBS, cryosectioned at 18 or 30 microns, and mounted onto + charged slides. Immunohistochemistry for c-Fos expression and cell quantification was performed as described in Detailed Methods.

We first compared the number of dorsal DG c-Fos expressing cells across treatment groups, between males and females, and between hemispheres (three-way ANOVA: treatment, sex, with repeated measure of hemisphere). We found a main effect of treatment (Figure 1, df=2, F=34.17 P<0.001). Although females had higher mean numbers of c-Fos-expressing cells than males in all treatment groups and both hemispheres, the differences between females and males were not significant (df=1, F=2.894, P=0.106), presumably due to the variability in c-Fos counts across groups resulting from the high levels in the wheel runners. There were no interactions between sex and treatment or hemisphere or both. There was also no overall difference between hemisphere across both sexes and the three treatments (df=1, F= 1.745, P=0.203) and no interactions among any of the variables.

To evaluate post-hoc pairwise comparisons focused on potential hemispheric differences, we combined males and females and ran a two-way ANOVA among the three treatment groups with the repeated measure of hemisphere. As expected from the three-way ANOVA, we found a significant main effect of treatment but not hemisphere (Figure 2). We then compared values for

like-hemispheres across treatments. Numbers of c-Fos expressing cells in both left and right hemispheres in the WR group were significantly greater than in the left and right hemispheres, respectively, than in both the CON and OB groups (Tukey's HSD, $P<0.05$). In the OB group, c-Fos values in left hemisphere were significantly greater than those in the left hemisphere of the CON group (Tukey's HSD, $P<0.05$). There was no significant difference in c-Fos expression between the OB group right hemisphere and the CON group right hemisphere ($P>0.05$).

We then compared left and right hemispheres within each treatment group. Numbers of c-Fos expressing cells did not differ between hemisphere within the CON or WR groups (paired t-test, df 9, $t=-0.45$, $P=0.663$; df 6, $t=0.020$, $P=0.985$). However, there were significantly more c-Fos expressing cells in the left than in the right hemisphere of the OB group (paired t-test, df 6, $t=3.275$, $P=0.017$).

Because amount of c-Fos expression may vary more widely among individuals than between hemispheres, we also calculated a lateralization index (LI), which shows hemispheric asymmetry normalized by the total number of c-Fos cells in each animal (as in Tsoi et al., 2014), and compared LI values across groups.

$$LI = \frac{\text{Right Hemisphere c-Fos+ cells/mm}^3 - \text{Left Hemisphere c-Fos+ cells/mm}^3}{\text{Right + Left c-Fos+ cells/mm}^3}$$

Positive values indicate more c-Fos+ cells in the right hemisphere relative to the left and negative values indicate more cFos+ cells in the left hemisphere.

There was no difference in the Lateralization Index across groups (Figure 3, one-way ANOVA, df=2, $F=2.39$, $P=0.116$). However, this lack of difference was driven by a single left-

lateralized data point in the CON group, without which there was a significant difference among groups (one-way ANOVA, df=2, F=6.56, P=0.006). We also tested whether the degree of lateralization within each group differed from zero. A Lateralization Index of zero indicates bilaterally equal c-Fos expression. We found that the degree of lateralization in the CON and WR groups did not differ from zero (one sample t-test, t=0.319, P=0.757; t=0.087, P=0.934) whereas the Lateralization Index in the OB group was significantly left dominant (one sample t-test, t=-4.457, P=0.004).

Because the statistical lack of hemispheric difference in the CON mice group was due to a single left-dominant subject, we repeated the experimental procedure with 5 additional control animals without additional WR or OB groups. As with the first set of CON, more of these additional control mice similarly had greater c-Fos activity in the right than left hemisphere (n=11 out of 15 were right-dominant); however, the differences in numbers of c-Fos expressing cells between hemispheres was not significant either within this subset or when both control groups were combined (t=-1.509, P=0.206; t=-1.427, P=0.176, paired t-test, respectively).

The cognitive map theory proposes that the hippocampus constructs large scale spatial maps of the environment and is active in navigating throughout such maps. Maguire et al. (1998) found that both hippocampi are recruited during a spatial navigation task in humans. However, these subjects engaged in virtual navigation on a computer screen, and therefore were sitting still, using a joystick to navigate. Therefore, perhaps saccadic eye movements and hand motion-driven navigation is sufficient for navigational motor input or internally generated motor feedback from the path integration system. Alternatively, if mice and humans are processing information similarly perhaps wheel running, albeit stationary, establishes a general "navigational-intention," which is sufficient to activate both sides of the hippocampus in

preparation for storing spatial mapping information in the absence of actual navigation in the WR mice. This is similar to the idea of hippocampal preplay, a phenomenon in which cell assemblies encoding an environment fire in precise temporal orders prior to initial exploration of the environment (Dragoi & Tonegawa, 2011). More simply, running may upregulate hippocampal activity in response to internally generated motor feedback and true navigational processing is not necessary for increased c-Fos expression. Interestingly, in both humans and mice, these tasks induced activity in both sides of the hippocampus.

Although novel object exploration did not affect levels of c-Fos expression in both hemispheres combined compared to controls, it did affect the degree of *lateralization* of c-Fos expression by increasing numbers of c-Fos+ cells specifically in the left hemisphere. This suggests that processing external sensory inputs, such novel object exploration, may be predominantly a left-lateralized activity in the DG. Thus, while self-motion may drive hippocampal activity bilaterally, external sensory input alone may be preferentially processed by the left hemisphere. To our knowledge, this is the first demonstration of lateralized activity specifically in the DG of the rodent dorsal hippocampus. Consistent with the idea of functional hemispheric specialization within the hippocampus, lateralized c-Fos expression has been recently reported in the ventral CA1 and CA3 in rats (Sakaguchi & Sakurai, 2017) such that right-lateralized activity corresponded to measures of increased anxiety.

It has been argued that in humans, hemispheric isolation may result in a higher degree of hemispheric specialization. Hippocampal subfields in the rodent brain send both ipsi- and contralateral projections. In contrast, it is widely believed that humans have only ipsilateral projections directly from CA3 to CA1 (Wilson et al., 1987). Though the presence of bilateral projections in humans has been argued for (Phelps et al., 1991; Gloor et al., 1993; Rosenzweig et

al., 2011), this position has not been widely adopted (Lavenex & Amaral, 2007). Thus, hemispheric task specialization may in fact be more prevalent or more important for primate hippocampal function.

Interestingly, a recent study showed that sharp-wave ripples in the rat hippocampus, thought to function in memory consolidation, have coordinated activity within, but not across hemispheres (Villalobos et al., 2017). This suggests that even though information may be shared across hemispheres, cell assemblies corresponding to a particular experience may be intrahemispheric and their information consolidated in isolation to each other. This is consistent with other work indicating that long-term spatial memory may be lateralized to the left hemisphere (Klur et al., 2009, rats; Shipton et al., 2014, mice). Further, neurophysiological studies in mice have identified lateralization in some of the key neural substrates, such as LTP and NMDA receptor subunits, thought to underlie hippocampal function (Kawakami et al., 2003; Kohl et al., 2011; Shinohara et al., 2013; Shipton et al., 2014; Benito et al., 2016). This suggests that even if there are differences in interhemispheric communications between humans and rodents, hippocampal hemispheric specialization may generalize across mammals.

Our data support the idea that rodents are a suitable model for the study of lateralization in hippocampal function, perhaps akin to functional asymmetry in primates and humans. Thus, considering hemispheric differences in rodent models may provide critical insight into the mechanisms of hippocampal function.

Detailed Methods

Animals and Housing: All protocols used here were approved by the Queens College IACUC committee. Adult male and female C57Bl6 mice (Jackson Labs) were group housed (3-4) on a 12hr light/dark cycle with free access to food and water.

Immunohistochemistry: Ninety minutes following the end of behavior, mice were deeply anesthetized with 0.2 mL of Euthasol (Virbac) and perfused intracardially with 0.1M PBS and 4% PFA. The brain was bisected and the dorsal hippocampus was sectioned at 18µm (two CON brains and one OB brain were cut at 30µm) one hemisphere at a time. Cutting order of hemisphere was pseudorandomized. Every third section was mounted directly onto charged microscope slides with a random orientation in both axes such that hemisphere could not be determined by the section orientation. Frozen sections were rinsed once in 0.1M PBS for 10 minutes. A block containing 10% donkey serum and 3% Triton-X in PB was applied for 30 minutes, followed by a 48-hour incubation of primary anti-c-Fos made in rabbit (Millipore, catalog number: ABE457, 1:1000) in block. Sections were then rinsed in 0.1M PBS three times and then incubated with Cy3-conjugated secondary donkey anti-rabbit for 2 hours. Sections were rinsed in 0.1M PBS three times before a serial dehydration in ethanol and xylene and cover slipping in Krystalon (Harleco).

Cell Quantification: The DG was traced under 4x magnification using Neurolucida software coupled to an Olympus BX51. Cy3-labeled cells within the DG were counted by an experimenter blind to condition under 60x magnification using a rhodamine filter. For brains sectioned at 18µm, 4-5 sections were quantified; for brains sectioned at 30µm, 2-4 sections were quantified. Densities of c-Fos expression within hemispheres were calculated by determining the number of c-Fos+ cells within the DG boundary multiplied by the volume sampled.

Statistics:

A three-way ANOVA was used to test the effects of sex, hemisphere, and treatment on c-Fos expression. A two-way mixed-model ANOVA was used to compare c-Fos in the right and left hemisphere between behavioral groups. A one-way ANOVA was used to compare total c-Fos expression across the left or right DG between behavioral groups. Paired t-tests were used to compare numbers of c-Fos+ cells between left and right hippocampi within groups. A one-way ANOVA was used to determine if lateralization indices varied across groups. A one sample t-test was used to determine if c-Fos lateralization indices for particular groups differed from zero (bilateral). P values reported for t-tests are two-tailed.

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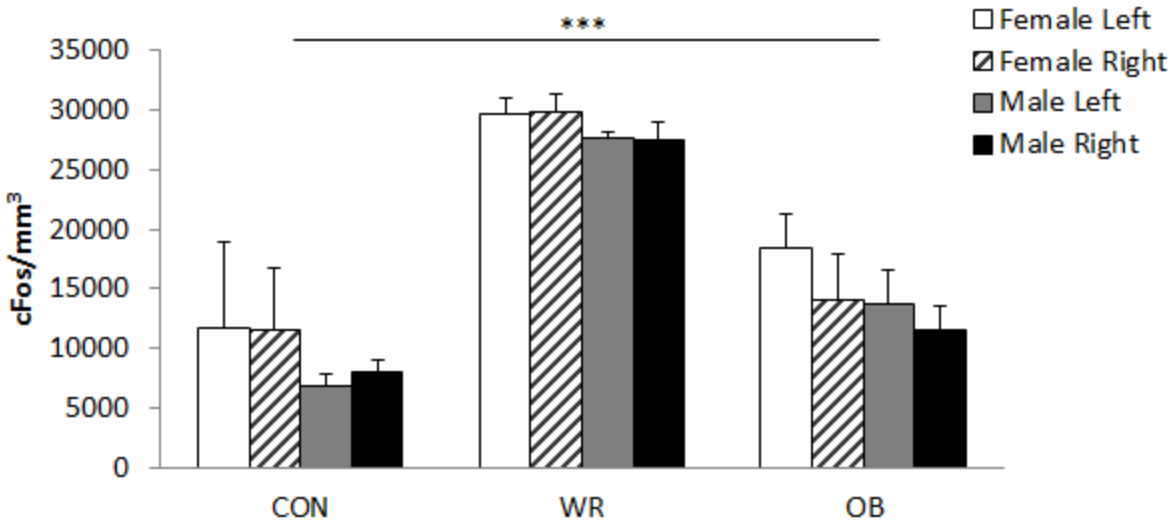

Figure 1. Dorsal DG c-Fos expression in male and female mice across hemispheres and behavioral groups. *** indicates $p<0.001$

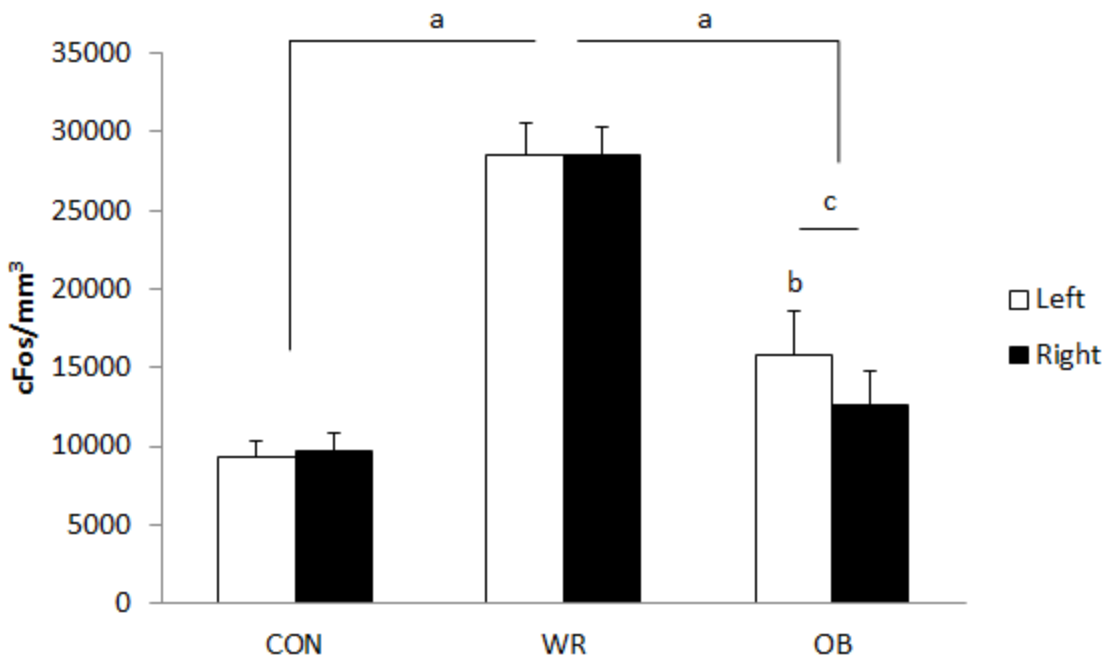

Figure 2. Dorsal DG c-Fos expression across hemispheres and behavioral groups. *a* indicates an overall effect of wheel running on left- and right-hemisphere c-Fos expression as compared to controls and object exploration; *b* indicates an effect of object exploration on left-hemisphere c-Fos expression as compared to controls; *c* indicates a difference between the left and right DG c-Fos expression within the object exploration group. All letters indicate $p<0.05$.

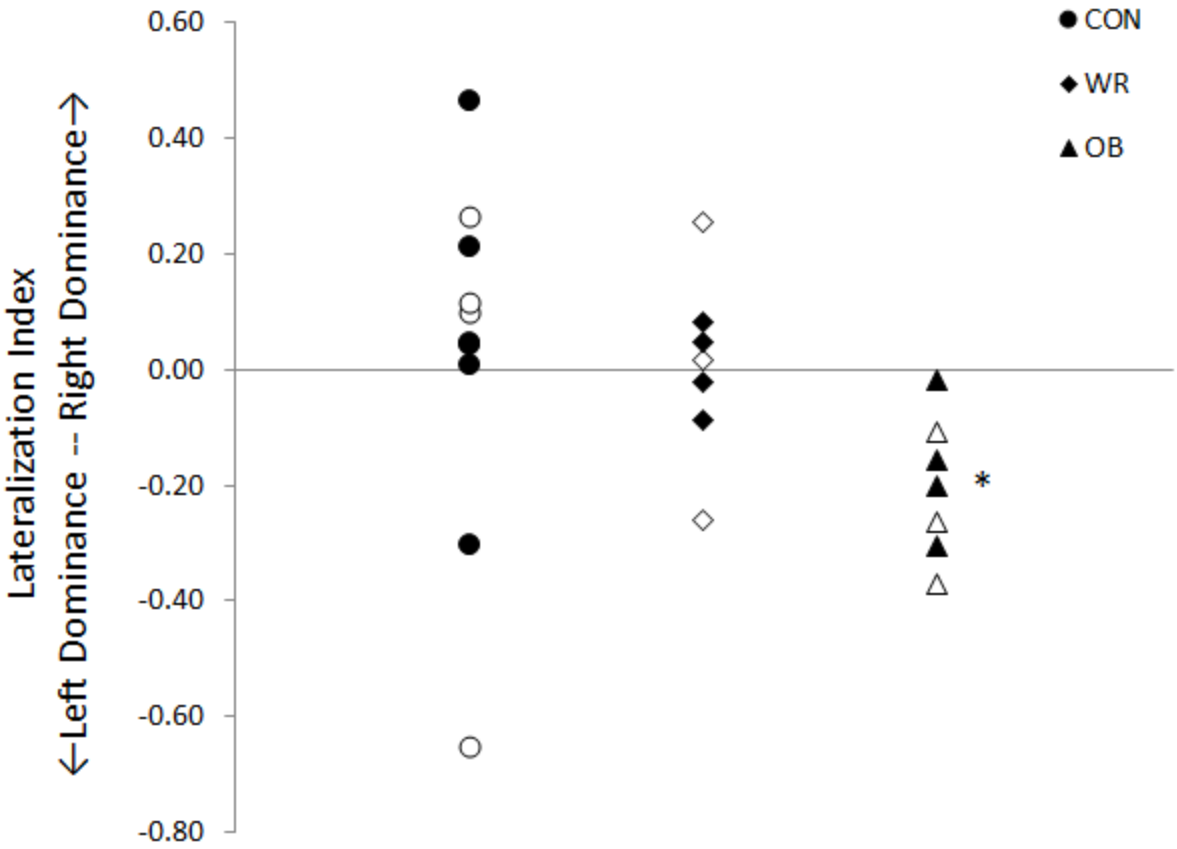

Figure 3. Degree of hemispheric lateralization in within behavioral groups. Open markers indicate females; closed markers indicate males. * indicates a group whose mean lateralization index differs from zero, $p<0.05$