ENVIRONMENTAL ENRICHMENT: ENHANCING NEURAL PLASTICITY, RESILIENCE, AND REPAIR

EDITED BY: Amanda C. Kentner, Anthony J. Hannan and S. Tiffany Donaldson
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ENVIRONMENTAL ENRICHMENT: ENHANCING NEURAL PLASTICITY, RESILIENCE, AND REPAIR

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The collection of articles in this eBook focuses on important issues related to environmental enrichment including standardization, neurobehavioral and physiological effects across the age axis, neuroprotection and plasticity, and implications for translation. Evaluation of key parameters and issues related to standardization is important for promoting species-typical behavior and broader adaptation and translation to clinical settings. Furthermore, understanding seminal mechanisms contributing to the effects of environmental enrichment in both biological sexes is also important for the application of this housing condition to preclinical models of neurological and psychiatric disorders. Taken together, this body of work points to the relevance of enriched housing environments in laboratory practice and the potential for translation to clinical populations.

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EDITORIAL
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Editorial: Environmental Enrichment: Enhancing Neural Plasticity, Resilience, and Repair

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Keywords: environmental enrichment (EE), neuroprotection, rehabilitation, standardization, reproducibility, animal welfare, translational

Editorial on the Research Topic

Environmental Enrichment: Enhancing Neural Plasticity, Resilience, and Repair

INTRODUCTION

Up until the middle of the twentieth century, neuroscience dogma emphasized the fixed, immutable nature of the brain. In the 1960s, however, in what was arguably one of the most revolutionary research programs in neuroscience, a team of researchers at UC Berkeley generated data revealing the dynamic characteristics of mature neural systems. Working with Edward Bennett, David Krech, and Mark Rosenzweig, Marian Diamond (1926-2017) documented the neuroanatomical changes in rats exposed to enriched environments—findings that threatened prior notions of brains being "fixed in stone" (Bennett et al., 1964). In an interview (with KL) several years ago, Diamond described her experience presenting these unexpected results for the first time at a professional conference. After delivering her talk, she heard an audience member shout, "young lady, that brain cannot change!"

Although Diamond confirmed that she had replicated the findings, perceptions about the changing nature of the brain were initially met with resistance and skepticism (personal communication, October, 2009). Not to be dissuaded, Diamond continued her neuroanatomical explorations, verifying that rats exposed to novel and complex environments experienced equally complex neural changes, especially in the cerebral cortex. Although hints of neural plasticity had been introduced by individuals such as Charles Darwin and Donald Hebb prior to Diamond's research, she has been credited for providing tangible evidence of neuroplasticity. One of her colleagues at the University of California at Berkeley, George Brooks, wrote in her obituary, "Dr. Diamond showed anatomically, for the first time, what we now call plasticity of the brain" (Sanders, 2017). She was also remembered as the neuroscientist who gave a new meaning to the idea of "use it or lose it," as she shattered previous notions of a static unchangeable brain that simply degenerated throughout life (Smith, 2017). Through her pioneering work with enriched environments, Marian Diamond most certainly enriched the field of neuroscience, as the notion of neuroplasticity has become a corner stone of contemporary neuroscience. Moreover, her research has inspired many other important investigations into the translational nature of environmental enrichment.

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Dedicated to Dr. Diamond, the current Research Topic focuses on environmental enrichment in terms of neuroprotection/rehabilitation and the use of this system in the laboratory to promote species-typical behavior and generalizable, rigorous investigation into the effects of experience on behavior and physiology.

STANDARDIZATION AND ENVIRONMENTAL ENRICHMENT

Concerns that environmental enrichment may increase variability in experimental endpoints, affecting reproducibility, have impeded the expansion of its use in research. Data described by Bailoo et al. demonstrate that this may be an unfounded assumption. The coefficient of variation across housing conditions did not differ on measures of behavior, growth, or stress physiology. Additionally, the more enrichment provided, the more engagement mice had with their environment. Similarly, Neal et al. report that enhanced social environments facilitate affiliative behaviors and appear to promote problem solving behaviors. Together, this underscores the importance of enrichment for healthy species-typical behaviors.

PHYSIOLOGICAL AND NEUROBEHAVIORAL EFFECTS OF ENVIRONMENTAL ENRICHMENT

Morano et al. demonstrated that removing female rats from environmental enrichment mimics an experience of "loss," leading to increased passive coping behavior, hyperphagia, and evidence of HPA axis dysregulation. These behavioral and physiological changes are associated with an array of psychiatric disease states, suggestive of the utility of this model for understanding loss-related dysfunctions. In contrast, Cutuli et al. review the effects of enrichment exposure across critical periods of development, particularly on parental care and offspring outcomes. Moreover, they provide a specific emphasis on the importance of pre-reproductive vs. post-reproductive experiences and transgenerational effects, highlighting some of their own research findings.

While environmental enrichment can be stressful to some male animals, McQuaid et al. show that post-weaning enrichment increases demonstrations of social behavior, buffered corticosterone, and central cytokine levels, and elevated brain-derived neurotrophic factor in the prefrontal cortex of male mice, following stressor exposure in adulthood.

ENVIRONMENTAL ENRICHMENT AND CLINICAL/NEUROPROTECTION

Environmental enrichment has been demonstrated to induce therapeutic effects in a wide range of preclinical models of neurological and psychiatric disorders. Various articles in this Research Topic have addressed this important issue, providing new insights into molecular and cellular mechanisms of experience-dependent plasticity, and identifying novel candidate targets for enviromimetics (Nithianantharajah and Hannan, 2006).

The first demonstration that EE can be beneficial in a genetic model of a human disorder utilized a transgenic mouse model of Huntington's disease (Van Dellen et al., 2000). Zajac et al. have now demonstrated that one of the molecular effects of EE, even when delivered for a relatively short period (2 weeks), is transcriptional modulation of components of the serotonergic system. Furthermore, these investigators compared EE with an exercise (voluntary wheel running) intervention and found key differences between these two environmental interventions, as well as interesting region-specific and sexually dimorphic effects on gene expression. Finally, they were able to demonstrate functional consequences of the EE effect, via behavioral pharmacology.

The effects of EE on a different mouse model, with haploinsufficiency in the brain-derived neurotrophic factor (BDNF) gene, were described by Grech et al. The authors use a model in which they combine the two negative factors (heterozygosity for the BDNF null allele and chronic administration of the stress hormone corticosterone) to create a "two hit model" of relevance to specific neurodevelopmental and psychiatric disorders. Interestingly, these authors also found sexually dimorphic effects of EE, and were able to correlate their behavioral data with expression levels of the neurotrophin receptor TrkB (and its signaling, via quantification of phospho-TrkB) and specific NMDA receptor subunits, reinforcing the evidence for a role of neurotrophic and glutamatergic signaling in mediating the therapeutic effects of EE.

Another key aspect of EE, which has been often overlooked, is enrichment of the nest conditions in rodent models. Mason et al. have addressed this via a form of nesting enrichment called "closed nest boxes." This form of nesting enrichment was shown to induce beneficial effects in a model of neonatal hypoxia-ischemia. Interestingly, molecular correlates included BDNF and glial-derived neurotrophic factor (GDNF), and sexually dimorphic effects were observed, which has become a recurring theme in EE studies.

Finally, the combination of EE with other therapeutic approaches is an area that has been under-explored and has substantial translational potential. Bhaskar et al. have combined EE with deep-brain stimulation (DBS) and found that the combination increased anxiolytic effects relative to the same DBS intervention performed in standard-housed animals. Combining EE with other interventions, both pharmacological (e.g., enviromimetics; Nithianantharajah and Hannan, 2006) and non-pharmacological (in this case a model of medical device), could reveal additive and synergistic effects that greatly enhance therapeutic efficacy in a wide range of neurological and psychiatric disorders.

ENVIRONMENTAL ENRICHMENT AND AGING

Environmental enrichment may help protect against the effects of cognitive decline. As discussed in Leon and Woo, stimulation

offered through sensory enhancement may also offset the effects of aging on not only the brain, but on multiple body systems significantly impacted by time.

Rapley et al. measured C-type Natriuretic Peptide (CNP) to determine its expression across normal aging and its interaction with the environment. Enrichment housing transiently increased CNP availability in young, but not older rats. This may be suggestive of age-related losses in sensitivity to environmental stimulation.

TRANSLATION

The benefits of environmental enrichment are reported in preclinical settings with little focus on translation. McDonald et al. describe many obstacles to application of environmental enrichment paradigms to patients experiencing stroke. The work highlights the variation between animal and clinical models of enrichment, suggesting that greater alignment is required to improve translation.

FUTURE DIRECTIONS

The potential for the environment to influence behavior has a long history, and subsequent work implicating correlative brain changes were pioneered by Dr. Marian Diamond. The contributions to this Research Topic offer findings regarding enrichment promoting healthy species-typical behavior in the lab and reversing insult. It is the hope of the editors that this evidence will support wider adoption of enrichment in normal housing protocols.

Moreover, it is important to devote attention to the aspects of enrichment in preclinical settings that promote plasticity, to enable the recreation of these components in clinical settings. As McDonald et al. intimate, this may very well present an opportunity to translate successful interventions to clinical populations. Additional work that advances understanding of the mechanisms underlying the benefits of enrichment in healthy and injured/adverse populations is also warranted (Kentner et al., 2019).

We also hope that further work is accomplished that delineates *when* and *how long* enrichment is needed for activating benefits and promoting plasticity across the lifespan. Since many disease states show gender disparities, another line of inquiry should include a focus on biological sex differences in enrichment paradigms. Enrichment shows distinct sex-specific benefits following early insult (Grech et al.; Mason et al). Few researchers are attending to the intergenerational benefits of enrichment (Cutuli et al.) and variation across the age axis, so continued efforts in these areas are also necessary.

After more than six decades of work on environmental enrichment, we applaud the advances in understanding relevant biological parameters and key mechanisms, and emphasize its potential for adaptation to clinical populations, across a broad spectrum of neurological and psychiatric disorders.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the editorial. AK and SD facilitated the concept, design, and overall development of the manuscript.

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ORIGINAL RESEARCH
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Effects of Cage Enrichment on Behavior, Welfare and Outcome Variability in Female Mice

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The manner in which laboratory rodents are housed is driven by economics (minimal use of space and resources), ergonomics (ease of handling and visibility of animals), hygiene, and standardization (reduction of variation). This has resulted in housing conditions that lack sensory and motor stimulation and restrict the expression of species-typical behavior. In mice, such housing conditions have been associated with indicators of impaired welfare, including abnormal repetitive behavior (stereotypies, compulsive behavior), enhanced anxiety and stress reactivity, and thermal stress. However, due to concerns that more complex environmental conditions might increase variation in experimental results, there has been considerable resistance to the implementation of environmental enrichment beyond the provision of nesting material. Here, using 96 C57BL/6 and SWISS female mice, respectively, we systematically varied environmental enrichment across four levels spanning the range of common enrichment strategies: (1) bedding alone; (2) bedding + nesting material; (3) deeper bedding + nesting material + shelter + increased vertical space; and (4) semi-naturalistic conditions, including weekly changes of enrichment items. We studied how these different forms of environmental enrichment affected measures of animal welfare, including home-cage behavior (time–budget and stereotypic behavior), anxiety (open field behavior, elevated plus-maze behavior), growth (food and water intake, body mass), stress physiology (glucocorticoid metabolites in fecal boluses and adrenal mass), brain function (recurrent perseveration in a two-choice guessing task) and emotional valence (judgment bias). Our results highlight the difficulty in making general recommendations across common strains of mice and for selecting enrichment strategies within specific strains. Overall, the greatest benefit was observed in animals housed with the greatest degree of enrichment. Thus, in the super-enriched housing condition, stereotypic behavior, behavioral measures of anxiety, growth and stress physiology varied in a manner consistent with improved animal welfare compared to the other housing conditions with less enrichment. Similar to other studies, we found no evidence, in the measures assessed here, that environmental enrichment increased variation in experimental results.

Keywords: environmental enrichment, variation, animal welfare, mice, behavioral phenotypes

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INTRODUCTION

The mouse, *Mus musculus*, is the most widely used animal in research, and yet surprisingly little is known about how its behavioral biology relates to the social and physical aspects of current laboratory housing conditions (Latham and Mason, 2004). Consequently, the Committee updating the US Guide for the Care and Use of Laboratory Animals identified a critical lack of empirical evidence on "space and housing needs" and "enrichment, exercise and human contact" (Garber, 2011; National Research Council, 2011). We have recently reported on a systematic assessment of the effects of space allowance on measures of animal welfare in laboratory mice (Bailoo et al., 2018); here we report on the effects of cage enrichment on behavior and measures of welfare in female laboratory mice.

Housing conditions for laboratory mice have been shaped primarily by economics (minimal use of space, equipment and labor), ergonomics (ease of handling, visibility of animals), hygiene (easy to sanitize) and standardization (minimization of variation; see Olsson and Dahlborn, 2002; Baumans and Van Loo, 2013, for further details). Typically, mice have been kept in transparent "shoe-box" cages with bedding, food and water. Such housing conditions lack sensory and motor stimulation and may prevent mice from performing species–typical behaviors, such as nest building (Würbel, 2001; Latham and Mason, 2004). Consequently, such housing conditions are associated with signs of impaired welfare, including abnormal repetitive behavior (Garner et al., 2004a,b, 2011; Garner, 2005; Würbel, 2006; Gross et al., 2012) and anxiety (Chapillon et al., 1999; van Praag et al., 2000; Würbel, 2001). Based on such evidence, Switzerland has declared environmental enrichment (i.e., nesting material) mandatory (The Swiss Federal Council, 2008), while the EU Directive 2010/63/EU (European Parliament Council, 2010) and the US Guide for the Care and Use of Laboratory Animals (2011) only recommend enrichment of rodent cages. Thus, an increasing number of researchers are using nesting material, shelters, gnawing sticks and other enrichment items with the goal of improving the welfare of laboratory rodents (The Swiss Federal Council, 2008; European Parliament Council, 2010; Baumans and Van Loo, 2013).

Environmental enrichment is used to increase sensory and motor stimulation, to facilitate species–typical behavior, and to provide the animals with some degree of control over their environment (Dawkins, 1988; Newberry, 1995; Olsson and Dahlborn, 2002; Nithianantharajah and Hannan, 2006; Gross et al., 2011b; Bennett et al., 2018). Current evidence indicates that even the addition of simple forms of enrichment to standard laboratory cages can improve the welfare of laboratory mice, as shown by reduced abnormal repetitive behavior (DeLuca, 1997; Würbel et al., 1998; Nevison et al., 1999a; Latham and Mason, 2010; Tilly et al., 2010; Bechard et al., 2011; Gross et al., 2011b, 2012) and reduced measures of anxiety (e.g., Chapillon et al., 1999; Roy et al., 2001; Benaroya-Milshtein et al., 2004; Binder et al., 2004; Görtz et al., 2008; Sztainberg and Chen, 2010), although not all studies have confirmed these findings (van de Weerd et al., 1994; Nevison et al., 1999a; Zhu et al., 2006).

In most studies, cage enrichment adds several resources, environmental complexity and sometimes novelty to the standard housing conditions. However, one study has found that within standard laboratory cages neither complexity nor novelty of enrichments had beneficial effects beyond those of nesting material alone (Gross et al., 2011b). Arguably, nesting material is the only enrichment that has consistently been found to be beneficial for mouse welfare (for review, see Olsson and Dahlborn, 2002).

Nesting material is an important resource for laboratory mice as it allows for the expression of species–typical nest building behavior, facilitates thermoregulation and provides shelter, if provided in sufficient quantity (Bult and Lynch, 1997; Sherwin, 1997; van de Weerd et al., 1997; Olsson and Dahlborn, 2002; Smith and Corrow, 2005; Gaskill et al., 2009, 2011, 2012; Gross et al., 2011b). Mice are highly motivated to construct nests (Nicol et al., 2008), and prefer nesting material to nest boxes (van de Weerd et al., 1998). Furthermore, for resting and maintenance behavior, mice prefer much higher ambient temperatures (30–32°C) than are common in animal facilities (20–26°C)—without nesting material, laboratory mice are at a higher risk for experiencing chronic cold stress (e.g., Johnson et al., 2017).

Other forms of enrichment, by contrast, have produced inconsistent effects (for reviews, see Jennings et al., 1998; Olsson and Dahlborn, 2002; Benefiel et al., 2005; Smith and Corrow, 2005; Whittaker et al., 2012; Baumans and Van Loo, 2013). For example, in some studies the provision of shelters has been associated with increased levels of aggression in male mice (McGregor and Ayling, 1990; Haemisch et al., 1994; Howerton et al., 2008), but this seems to depend strongly on the strain of mouse (van de Weerd et al., 1994; Chapillon et al., 1999; Nevison et al., 1999a).

The aim of the present study was to replicate and extend the results of previous studies on the effects of cage enrichment on mouse behavior and measures of welfare. We varied cage enrichment across four levels, including cages: (i) without any enrichment (Barren, B); (ii) with nesting material (Nesting, N); (iii) with deep bedding, shelters and additional vertical space besides nesting material (Enriched, E); and (iv) large pet cages attached to a laboratory cage, offering multiple resources and different items each week for active engagement (Super-Enriched, SE). To increase the generality of our findings, we studied an inbred and an outbred strain of mouse. However, similar to other proof-of-concept studies on environmental enrichment in mice (Van Loo et al., 2003; Wolfer et al., 2004), we only studied female mice as some forms of enrichment are associated with escalating aggression in male mice. Our primary outcome measures of animal welfare were stereotypy performance in the home–cage and measures of anxiety in behavioral tests, as these two measures were most consistently found to be improved by environmental enrichment. Additionally, a range of secondary outcome measures covering different domains of animal welfare were also included: home-cage behavior, measures of growth, endocrine stress responses, brain function, and emotional state. We tested the hypothesis that the welfare of mice increases with increasing degrees of

cage enrichment across our four treatment groups, following the prediction: B < N < E < SE.

Since environmental enrichment renders the animals' environment more complex, concerns have been raised that cage enrichment might lead to higher variability in experimental results. Although empirical evidence does not support these concerns (Augustsson et al., 2003; Wolfer et al., 2004; Baumans et al., 2010; van de Weerd et al., 2010) they seem to persist (e.g., Toth et al., 2011). Therefore, we additionally assessed how cage enrichment affected variability in all measured outcomes.

MATERIALS AND METHODS

Experimental Design, Animals and Housing Conditions

We used a 4 (cage enrichment) × 2 (mouse strain) factorial design conducted in two consecutive batches of equal size; the second batch began 3 weeks after the end of the first batch. Subjects were 192 female mice, 96 each of the inbred strain C57BL/6JRj (C57) and the outbred strain RjOrl:SWISS (SWISS) from Janvier Labs, France. Each batch was comprised of 48 newly weaned non–sibling mice (21–25 days old at delivery) per strain. All mice were ear-tattooed for identification by the same two experimenters (JB and EM) on the day following arrival at the laboratory.

A review of the literature, comparing variation in behavioral outcomes between barren cages and cages with nesting material alone, yielded an effect size range of 0.85–1.0 (Cohen's *d*) resulting in a sample size of $n = 12$ per treatment group. Expecting some of our behavioral measures, such as the behavior in the elevated plus–maze, to be more variable and thus yield a smaller effect size, and to accommodate for possible attrition, we adjusted our sample size to $n = 24$ per experimental condition per strain.

Mice were randomly allocated to the four housing conditions, B, N, E and SE, described in further detail in **Supplementary Table S1**. They were housed in groups of three per cage, with four replicate cages per strain and treatment in each batch (see **Supplementary Table S2**). Each batch of mice was delivered in four boxes, two per strain, containing 24 animals each. Each box of mice was allocated to cages and treatment groups sequentially to minimize individual differences in behavior between cages. Animals were housed in two housing rooms, located on either side of the test room (**Supplementary Figure S1**), with half of the animals per strain per batch in each room. Cage height on the rack was counterbalanced by strain and room between batches.

The B, N and E housing conditions consisted of a Makrolon® Type three cage, which besides food (Kliba Nafag #3430, Switzerland) and tap water *ad libitum*, contained either bedding only (B; Lignocel® select, see **Supplementary Table S1** for depth), bedding and nesting material (N), or deeper bedding, nesting material, a tunnel, a shelter and increased vertical space (E). To increase the generality of our findings, we used two different types of nesting material (10 g of Sizzle Pet® or three paper tissues), tunnels (rat tunnel Plexx EU #13104 or rat retreat Plexx EU #13154) and shelters (arch Plexx EU #13244 or hut Plexx EU #13169), counterbalanced across cages.

The SE housing condition consisted of a Makrolon® Type three cage connected to a Savic™ Mickey XL pet cage, by a clear polycarbonate tunnel 6 cm in diameter. The layout of the SE system and the enrichments used are displayed in **Supplementary Figures S2, S3**. Briefly, the Type three cage contained a paper tunnel (Plexx EU #14152), a paper shelter (Plexx EU #13244), and nesting material (10 g Sizzle Pet® and 3× paper tissues). The Mickey XL cage contained an elevated platform made of polycarbonate (410 cm^2 in floor area, with a 5 cm wall), with a wooden ladder leading up to it and a wooden shelter on top of it. A plastic hammock was attached to the cage lid above the elevated platform where a secondary source of food and water was also accessible. Two wooden coconuts attached to the cage lid and joined by a suspended bridge made of rope and wood, a paper shelter, and a paper tunnel were also provided. These items in the Mickey XL cage were present throughout the study. To stimulate activity and exploration further, and to increase behavioral diversity, additional items were rotated in the SE cage on a weekly basis (see **Supplementary Table S3**).

Husbandry Procedures

Animals were kept on a reversed 12:12 light/dark cycle with lights on at 19:00 h. A red light emitting diode (LED) remained on throughout the entire cycle. Temperature was maintained at 22 ± 1°C, with an average humidity of 40%. Husbandry procedures were conducted weekly (see **Supplementary Methods**).

Because some outcome measures involved long periods of testing and manipulation by the experimenter, all mice were habituated to being handled beginning upon arrival at the laboratory (see **Supplementary Methods**).

Outcome Variables

Outcome variables covered a range of measures related to animal welfare. Our primary outcome measures were: (i) stereotypy performance in the home–cage; and (ii) measures of anxiety in two behavioral tests (elevated plus–maze test and open field test). In addition, we assessed a range of secondary outcome measures covering different domains of animal welfare, including measures of: (i) home–cage behavior (time budget, use of enrichments); (ii) growth (food and water intake, body weight); (iii) endocrine stress responses (glucocorticoid metabolites in fecal boluses, adrenal weight); (iv) brain function (inhibitory control of behavior as measured by recurrent perseveration in a two–choice guessing task); and (v) emotional state (judgment bias in a spatial Go/No-Go task; **Figure 1**).

For assessing recurrent perseveration and judgment bias, one focal animal from three of the four cages per treatment, strain and batch were randomly selected ($n = 48$ in total). Cage, treatment and strain were counterbalanced across three experimenters (EM, MB-S, JB) such that: (1) no two

experimenters tested animals from the same treatment at the same time; (2) no experimenter tested animals from the same treatment consecutively; and (3) no experimenter tested animals from the same strain consecutively. For judgment bias testing, one experimenter was replaced (MB-S by SH) towards the end of the training period due to an emergency leave of absence in batch 1, and for batch 2, mice from one experimenter (EM) were shaped for two sessions by the other two experimenters (JB and MB-S) due to illness.

Stereotypy Performance in the Home–Cage and Other Home–Cage Behavior

Cage enrichment has previously been reported to reduce abnormal repetitive behavior (DeLuca, 1997; Würbel et al., 1998; Nevison et al., 1999b; Tilly et al., 2010; Bechard et al., 2011), and one study has found that nesting material is associated with a reduction of stereotypic behavior in particular (Gross et al., 2012). To evaluate the effects of increasing degrees of enrichment on the incidence of stereotypic behavior, all cages were recorded for 24 h each, prior to the end of the experiment.

Video-recordings were scored separately for stereotypic behavior and other home–cage behavior using Noldus Observer XT (version 10.5) by EM, CB and JB. From each cage all animals were observed and their behavior scored, although, to evaluate the relation between stereotypic behavior and recurrent perseveration, data from focal animals were used (the same ones tested in the guessing task). Videos of two E cages (all intervals) and one SE cage (one interval) were unavailable for scoring due to equipment failure (see below for a description of the intervals).

Stereotypic behavior was scored using a previously validated ethogram (Novak et al., 2016; see also **Supplementary Table S4**). Mice were observed for 15 min, within four 1-h time windows, distributed across the dark phase (07:30–08:30, 09:30–10:30, 12:30–13:30 and 15:30–16:30). These time windows had been determined by pilot observations to represent two time intervals of high activity and two of low activity. Stereotypic behavior and general activity were sampled using one–zero sampling with 15 s intervals, yielding 240 data points per mouse. Stereotypy performance is reported here as a proportion of active time. Ten percent of all videos were rescored for assessment of intra- and inter-rater reliabilities, which were high throughout (Jansen et al., 2003), κ = 0.94 and 0.96, respectively.

In addition to stereotypic behavior, we also assessed the extent to which the mice engaged with the cage environment, including engagement with enrichments, and how the provided resources within the cage affected general daily activities and facilitated species–typical behavior (**Supplementary Table S5**; category, environmental manipulation). Of note, in terms of engagement with the cage environment, we evaluated manipulation of bedding (e.g., digging) in the B cage, while we additionally evaluated engagement with the provided enrichment items in the N, E and SE cages. Thus, engagement with the cage environment was quantified even in the barren cage, where no enrichments were present. We did not categorize time spent on the lid as engagement with the environment, although these data were analyzed and are described in "Anxiety in the Elevated Plus–Maze Test and Open Field Test" section in our analysis of time budget.

To evaluate engagement with the cage environment, we used the ethogram described under **Supplementary Table S5**. Mice were observed for 30 min, within six 1-h time windows, four in the dark phase (the same as above) and two in the light phase (20:30–21:30, 03:00–04:00), using instantaneous sampling at 1-min intervals and yielding 180 data points per mouse. Ten percent of all videos were rescored for assessment of intra- and inter-rater reliabilities, which were high throughout (Jansen et al., 2003), κ = 0.85 and 0.90, respectively.

Anxiety in the Elevated Plus–Maze Test and Open Field Test

Besides reduced stereotypy performance, cage enrichment in mice has most consistently been associated with reduced measures of anxiety in behavioral tests such as the elevated plus–maze test or the open field test. Both elevated plus–maze behavior (Pellow et al., 1985; Carola et al., 2002) and open field behavior (Denenberg, 1969; Carola et al., 2002) have been validated for differences in anxiety in both rats and mice. On the elevated plus–maze, less time spent on, and fewer entries into, the open arms, as well as reduced locomotion reflect higher levels of anxiety (Walf and Frye, 2007). In the open field, longitudinal assessment of the pattern of locomotor behavior in the open field across repeated exposures has been demonstrated to provide information about how animals behaviorally cope with a stressor and how the hypothalamus–pituitary–adrenal (HPA) axis differentially operates between groups of animals (Whimbey and Denenberg, 1967; Bailoo et al., 2013). Specifically, reduced levels of habituation (i.e., reduced exploration and/or time in the center across days of testing) and/or increased levels of sensitization (i.e., greater exploration and/or time in the center across days of testing), between groups of animals, are associated with increased anxiety and vice versa.

In the present experiment, we used three elevated plus–mazes and three open field arenas. Elevated plus–mazes were made of polycarbonate with infrared (850 nm) backlit floors. Each maze consisted of four arms, each 30 cm in length and 6 cm wide, and a center square measuring 6 × 6 cm. Two arms opposite to each other were open, with a small lip around the perimeter 0.5 cm high, while the remaining two arms were enclosed, with walls 15 cm high. The open field arenas were made of polycarbonate with dimensions 45 × 45 × 45 cm^3 with infrared (850 nm) backlit floors.

For both tests, mice from each cage were randomly assigned to one of three experimenters (EM, MB-S and JB). The test order of cages was counterbalanced by treatment and strain in blocks of four across eight blocks, whereby all four treatments were represented in each block, with two treatments per strain. For consecutive blocks, the strain associated with treatment in the previous block was alternated.

Behavior on the elevated plus–maze was assessed in a single session while behavior in the open field was assessed across four consecutive days; all tests were conducted between 10:00 h

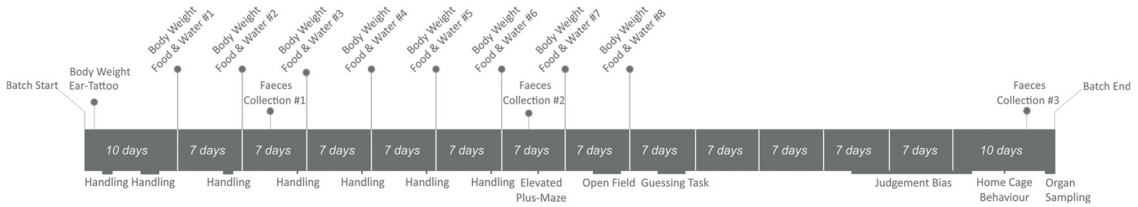

FIGURE 1 | Experimental timeline for a single batch of animals.

and 13:00 h. The test duration for both tests was 5 min. The outcome variables of interest in the elevated plus–maze test were: (1) distance traveled; and (2) time spent in the open arms. In the open field test changes in: (1) distance traveled; (2) time in the center; and (3) time in the corners, across 4 days of testing was evaluated.

For each test session, the cage to be tested was brought to the test room and the overhead lights (120 lux) were turned on. The three animals were then removed from their cage and placed into the apparatus by the assigned experimenter. At the end of the test, the animals were replaced into the home–cage, and the cage returned to the housing room. Between test sessions, the arenas were cleaned with 70% isopropanol. Outcome measures were scored live by Noldus EthoVision XT (version 11.5). Accuracy of video tracking was subsequently evaluated by JB from video recordings ensuring issues associated with automated tracking were eliminated (Bailoo et al., 2010). The detection settings for tracking were selected so that both the percentage of samples in which the subject was not found and the percentage of samples skipped were less than 1% per trial.

Growth: Body Weight and Food and Water Intake

Longitudinal assessment of food and water intake and body weight can provide information about the different types of experienced stress, since acute stress is associated with a loss of and chronic stress with a gain in body weight (Klok et al., 2007; McEwen, 2007; Torres and Nowson, 2007; Lutter et al., 2008). Food and water intake are coupled and thus expected to be positively correlated.

Body weight of each mouse and food and water intake at the cage level, were recorded at weekly cage changes. Food and water intake were assessed for each cage by subtracting the weight of the remaining food and water from that of food and water provided at the last cage change. Thus, we measured food disappearance rather than food intake, as particulates of food dropped in the bedding and spilled water remained unaccounted for. For analysis, food and water intake were corrected for the number of animals per cage when there were fewer than three animals in the cage (see "Attrition" section, for further details).

Endocrine Stress Responses: Fecal Glucocorticoid Metabolites and Adrenal Weight

Non-invasive methods of quantifying circulating levels of glucocorticoids, a primary product of the activation of the HPA stress system, are preferable to invasive methods such as blood sampling, because they do not elicit a stress response. In mammals, glucocorticoids are metabolized by the liver and are excreted in both urine and feces. A validated method of analyzing glucocorticoid metabolites in mice (Touma and Palme, 2005) was used as a measure of endocrine stress responses prompted by the different housing conditions.

Feces were collected, with a minimum of six boluses per mouse per cage, at three time points in the dark phase under red light approximately 24 h after cage changes. Because the transit time of by-products of corticosterone secretion in feces is between 8 h and 10 h (Touma et al., 2003; Touma and Palme, 2005), it is important to note here that we are measuring, in part, arousal/stress as a consequence of the husbandry procedures associated with cage change (Balcombe et al., 2004). Samples were immediately frozen at −20°C and later processed (blinded to experimental treatment, JV and RP) to assess the concentration of 5α–3β, 11β–corticosterone metabolites (ng/0.05 g feces) as described by Touma et al. (2003, 2004). In total 563 samples were processed; four samples were missing due to attrition (see "Attrition" section), and nine samples were not processed because of insufficient sample material.

At the end of the experiment, animals were killed by anesthesia with 5% isoflurane followed by asphyxiation with CO_2, performed by JB. Within 2 min, the animals were transported to the dissection laboratory. Dissections were performed by KH and CG. Animals were first weighed, then the adrenals were extracted and weighed using a precision scale to the nearest 10,000th of a gram (Mettler AE 160). Chronic exposure to stress has been associated with higher levels of circulating glucocorticoids and heavier adrenal glands (van de Weerd et al., 1994; McEwen, 2007). Other organs (brain, heart, kidney, liver, spleen) were also extracted and weighed using a precision scale to the nearest thousandth of a gram (Mettler Toledo ME 802) to obtain additional outcome measures to assess treatment-dependent variability in the data. Organ weights were corrected by body weight.

Brain Function: Recurrent Perseveration in a Two–Choice Guessing Task

The expression of stereotypic behavior has been found to correlate with recurrent perseveration, a form of impaired inhibitory control of behavior, both in humans (autistic children)

and in captive mammals and birds (Turner, 1997; Garner et al., 2003, 2011; Gross et al., 2012). A positive correlation between stereotypy levels and recurrent perseveration has also been observed in mice, notably in C57 mice (Garner et al., 2011), but other studies have yielded mixed results (Latham and Mason, 2010; Gross et al., 2011a, 2012; Novak et al., 2016).

To assess recurrent perseveration, we used a slightly modified apparatus and procedure to the one described previously (Novak et al., 2016; Bailoo et al., 2018). Briefly, all focal mice (n = 48) were trained and tested under red light in three virtually identical apparatuses. Each apparatus consisted of a rectangular arena with two goal-holes at one end and a trapezoid-shaped start-box at the opposite end (see **Supplementary Figure S4**). Animals were first habituated to the apparatus across 3 days and then shaped to retrieve rewards (BioServ chocolate pellets, 20 mg) from both goal holes. Animals were then tested across 2 days, 60 trials/day, where one of the two goals was rewarded with a probability equaling the proportion of responses to the other side in the previous 20 trials (see Novak et al., 2016; see Bailoo et al., 2018 and **Supplementary Figure S5** for further details). Perseveration score (logit [P]), as well as the frequencies of pure repetitions (LLLL, RRRR) and pure alternations (RLRL, LRLR) relative to all possible tetragrams of consecutive choices (n = 16) were calculated for each individual and analyzed.

Emotional State: Judgment Bias in a Spatial Go/No–Go Task

One approach to evaluate the valence (i.e., the positivity or negativity) of emotions in animals is to investigate how decisions in ambiguous situations are biased by the underlying emotional states of the animals—as assessed by cognitive judgment bias tasks (Harding et al., 2004). Previous research evaluating judgment biases in rats indicates that the transfer from standard housing conditions to enriched environments is associated with a relative shift from "pessimistic" to "optimistic" judgments based on the expectancy of non–reward and reward, respectively (e.g., Brydges et al., 2011; Richter et al., 2012). However, no study has investigated the effect of environmental enrichment on judgment biases of mice.

To assess judgment biases, we developed a task which integrates active trial initiation into a spatial Go/No Go task (see Hintze et al., 2018 for further details). Briefly, all focal mice (n = 48), were trained and tested under red light in three virtually identical apparatuses (see **Supplementary Figure S6**). Each apparatus consisted of a rectangular arena with a row of nine goal–holes at one end (five of which were used for this task), and a trapezoid–shaped area containing a nose-poke at the opposite end. Mice were first trained to initiate each trial by nose-poking, and in several subsequent steps, were trained to discriminate that the location of an open goal-hole (at the extreme ends) signaled either reward (Go trial) or non-reward (No–go trial). Once animals had learned this discrimination, ambiguous test trials with open goal–holes at three equidistant intermediate locations between the extreme ends, were interspersed among Go and No–Go trials, across six test sessions. The Go: No–go response ratio to all five goal–holes (positive, three ambiguous, negative) was used as a measure of judgment bias.

Attrition

In batch 1, one C57 mouse housed in the B condition was euthanized prior to the end of the experiment because the animal was favoring the right side of its head (lopsided tilt). Behavioral symptoms indicated mild distress, although post-mortem pathology yielded no diagnosis. In batch 2, one C57 mouse allocated to E condition was found dead at delivery.

Ethical Statement

This study was carried out in accordance with the guidelines of the Swiss Animal Welfare Ordinance (TSchV 455.1). It was approved by the Cantonal Veterinary Office in Bern, Switzerland (permit number: BE16/16).

Statistical Analyses

All statistical analyses were performed using IBM^{TM} SPSS Statistics (version 23), except for the judgment bias task data, which were analyzed using R (version 3.3.2). For parametric models run in SPSS, assumptions of normally distributed errors and homogeneity of variance were examined graphically and, based on these inspections, no transformations of data were needed. Strains of mice were analyzed separately. In SPSS, batch was included in all analyses as a fixed effect—no significant differences were observed and this predictor is not discussed further. P-values less than 0.05 were considered statistically significant for all analyses and are presented as actual values rounded to three decimal places. Raw data for all outcome measures will be made available upon request.

Home-cage and stereotypic behavior was analyzed using Kruskal-Wallis independent sample tests due to the high degree of skewness observed in the data. Data was summed across all observation intervals and expressed as proportions. Significant effects were probed using Kruskal-Wallis pairwise comparisons with a Dunn–Bonferroni correction.

Measures of anxiety—elevated plus–maze and open field behavior—were analyzed using the MIXED procedure. Housing condition and, for the open field only, day of testing, were treated as categorical fixed effects. Mouse nested in cage was added as a random effect. Significant effects were probed with Bonferroni corrected pairwise comparisons.

Food and water intake and body weight were analyzed using the MIXED procedure, with housing condition treated as a categorical fixed effect, and week as a continuous covariate. For body weight, mouse nested in cage was added as a random effect. Significant effects were probed with Bonferroni corrected linear contrasts.

One of our measures of stress physiology—fecal glucocorticoid metabolite concentrations—was analyzed using the MIXED procedure. Housing condition and time point of assessment were treated as categorical fixed effects. Mouse nested in cage was added as a random effect. Significant effects were probed with Bonferroni corrected pairwise comparisons. Adrenal mass between housing conditions was analyzed using

Kruskal-Wallis independent sample tests due to the high degree of skewness observed in the data. Significant effects were probed using Kruskal-Wallis pairwise comparison with a Dunn–Bonferroni correction.

Measures of brain function—recurrent perseveration and patterned responding in a two-choice guessing task—were analyzed using the MIXED procedure. Housing condition was treated as a categorical fixed effect. Significant effects were probed with Bonferroni corrected pairwise comparisons.

Our measure of emotional valence—responding in a judgment bias task—was analyzed using the function glmer of the package lme4 ("family:" binomial, including the "logit" link function). Housing condition, trial type, and their two–way interaction were fixed effects, while trial type nested in session per test day nested in test day, nested in animal ID nested in batch was used as a random effect.

To assess variation between housing conditions, the coefficient of variation (CV), the ratio of the standard deviation to the mean, for each outcome was calculated from the raw data with the exception of home–cage and stereotypic behavior, guessing task and judgment bias.

RESULTS

Engagement With the Housing Environment

Engagement with the housing environment was defined as the mouse being either in contact with an enrichment item (inside, on, or under) or actively manipulating an enrichment item (N, E, SE) or bedding (all groups). The degree of cage enrichment strongly affected engagement with the housing environment in both strains (C57: $H = 116.20$, $p < 0.001$; SWISS: $H = 114.80$, $p < 0.001$, **Figure 2**). *Post hoc* comparisons indicated that in both strains of mice, animals housed in the barren condition were less often observed to be engaged with the housing environment compared to all other groups. Additionally, in both strains, animals housed in the super enriched condition were more often observed engaged with the housing environment compared to animals housed in the nesting condition, and in SWISS mice only, compared to the enriched condition. In the SE housing condition, the mice were engaged with enrichments on average more than 85% of the observed time.

Stereotypic Behavior

The degree of cage enrichment had a strong effect on the expression of stereotypic behavior in the home–cage in both strains (C57: $H = 34.21$, $p < 0.001$; SWISS: $H = 21.77$, $p < 0.001$; **Figure 3**). This was mainly due to greatly reduced stereotypy levels in mice in SE cages compared to mice from all other treatment groups, while there were no consistent differences between mice in B, N and E cages.

The degree of cage enrichment also had effects on specific forms of stereotypies, but these varied with the strain of mouse. The expression of bar-mouthing was affected by cage enrichment in both strains (C57: $H = 21.82$, $p < 0.001$; SWISS: $H = 16.48$, $p < 0.001$; **Supplementary Figure S7**). However, circling on the cage lid was affected by cage enrichment in C57 mice only (C57: *circling*: $H = 20.05$, $p < 0.001$), while back-flipping, route-tracing on the cage lid and twirling were affected by cage enrichment in SWISS mice only (SWISS: *back–flipping*: $H = 20.68$; $p < 0.001$; *twirling*: $H = 13.81$; $p < 0.001$; *route–tracing on the lid*: $H = 11.00$; $p = 0.012$; **Supplementary Figure S7**). These effects were mostly due to higher levels of stereotypies observed in B and N cages compared to E and SE cages, although bar–mouthing in C57 mice was higher in E cages compared to all other cages. However, there were large individual differences in both the form and levels of stereotypy performance, and many 0 values for most forms of stereotypies, which precluded further analysis of treatment effects on specific forms of stereotypies.

Other Home–Cage Behavior

In both strains, variation in home–cage behavior depending on the degree of cage enrichment was mostly determined by the extent of unseen behavior (C57; *active*: $H = 43.28$, $p < 0.001$; *inactive*: $H = 53.05$; $p = 0.001$; *unseen*: $H = 98.14$, $p = 0.001$; SWISS; *active*: $H = 30.48$, $p < 0.001$; *inactive*: $H = 75.42$; $p < 0.001$; *unseen*: $H = 94.78$, $p < 0.001$, **Figure 4**). Behavior was recorded as unseen when enrichments obscured the view of the mice such that the specific pattern of behavior could not be identified unambiguously. That both the relative amount of seen active and inactive behavior decreased with increasing enrichment indicates that engagement with enrichment affected both active and inactive components of behavior. That seen inactive behavior decreased more than seen active behavior further indicates that mice have a stronger preference for shelter when inactive.

Comparing variation among specific patterns of seen active behavior indicated that engagement with enrichments affected specific patterns of active behavior differently. For example, the proportion of grooming decreased with the degree of enrichment across all treatment groups, while the proportion of active behavior on the lid was greatly reduced, and the proportion of active behavior on the floor increased, in SE mice compared to mice from all other groups (**Figure 5**).

Anxiety in the Elevated Plus–Maze Test and Open Field Test

Behavior in the elevated plus–maze test varied with the type of housing condition in C57 but not SWISS mice. However, this effect was only observed with respect to the total distance traveled but not time-in-open arms (*distance traveled*: C57: $F_{(3,28)} = 6.53$, $p = 0.002$; SWISS: $F_{(3,28)} = 0.09$, $p = 0.967$; *time-in-open arms* C57: $F_{(3,28)} = 0.95$, $p = 0.080$; SWISS: $F_{(3,28)} = 2.33$, $p = 0.420$; **Figure 6**). Closer inspection of the data indicated that C57 mice housed in E and SE cages traveled longer distances than those housed in B and N cages, while there were no consistent effects with respect to time-in-open arms.

A secondary *post hoc* analysis evaluating time spent in the center of the maze yielded no differences between our treatment groups (C57: $F_{3,91} = 2.29$, $p = 0.083$; SWISS: $F_{3,92} = 0.46$, $p = 0.709$).

Behavior in the open field varied with the type of housing condition in C57 but not SWISS mice. This effect was observed

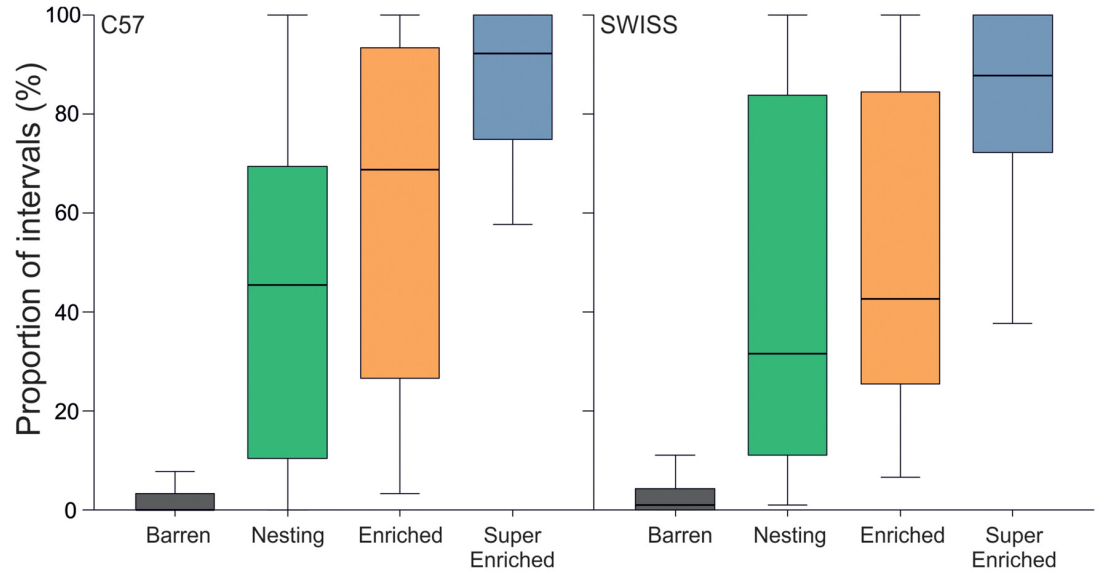

FIGURE 2 | Proportion of intervals across observations (median and IQR) where mice were engaged with the housing environment.

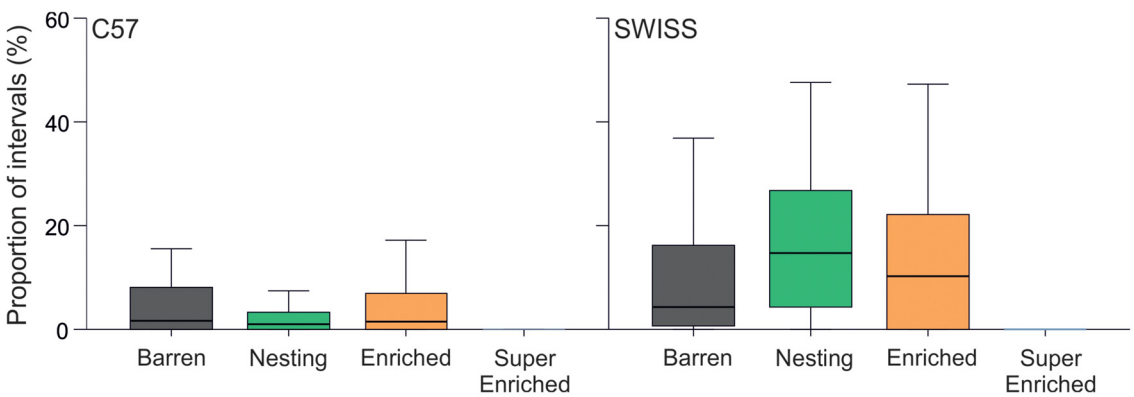

FIGURE 3 | Proportion of intervals across observations (median and IQR) where mice were engaged in stereotypic behavior in relation to housing condition. Note, the *y*-axis is truncated to 60% from 100% to aid with visual clarity, given the low levels of stereotypic behavior.

with respect to both total distance traveled and time in corners, but not time in the center (*distance traveled* C57: $F_{(3,90)} = 8.18$, $p < 0.001$; SWISS: $F_{(3,90)} = 2.106$, $p = 0.105$; *time-in-center* C57: $F_{(3,89)} = 0.11$, $p = 0.234$; SWISS: $F_{(3,88)} = 1.45$, $p = 0.957$; *time-in-corners* C57: $F_{(3,89)} = 3.43$, $p = 0.021$; SWISS: $F_{(3,89)} = 2.25$, $p = 0.088$; **Figure 7**). In both strains, behavior in the open field also varied across days of testing (*distance traveled* C57: $F_{(3,144)} = 100.32$, $p < 0.001$; SWISS: $F_{(3,171)} = 9.44$, $p < 0.001$; *time-in-center* C57: $F_{(3,169)} = 28.65$, $p < 0.001$; SWISS: $F_{(3,173)} = 7.84$, $p < 0.001$; *time-in-corners* C57: $F_{(3,173)} = 68.04$, $p < 0.001$; SWISS: $F_{(3,174)} = 21.95$, $p < 0.001$; **Figure 7**). However, there was no interaction between the type of housing condition and day of testing (*distance traveled* C57: $F_{(9,144)} = 1.64$, $p = 0.109$; SWISS: $F_{(9,171)} = 1.84$, $p = 0.064$; *time-in-center* C57: $F_{(9,169)} = 0.63$, $p = 0.773$; SWISS: $F_{(9,173)} = 0.72$, $p = 0.687$; *time-in-corners* C57: $F_{(9,173)} = 1.62$, $p = 0.114$; SWISS: $F_{(9,174)} = 1.59$, $p = 0.121$; **Figure 7**). Closer inspection of the data indicated that both distance traveled and time in the center decreased while time in the corner increased across the 4 days of testing. Furthermore, C57 mice housed in SE cages traveled shorter distances compared to all other groups.

Growth: Body Weight and Food and Water Intake Per Mouse

In both strains and across all housing conditions, food intake was positively correlated with both water intake and body weight (averaged at the cage level)—except for C57 mice housed in E and SE groups (**Supplementary Table S6**).

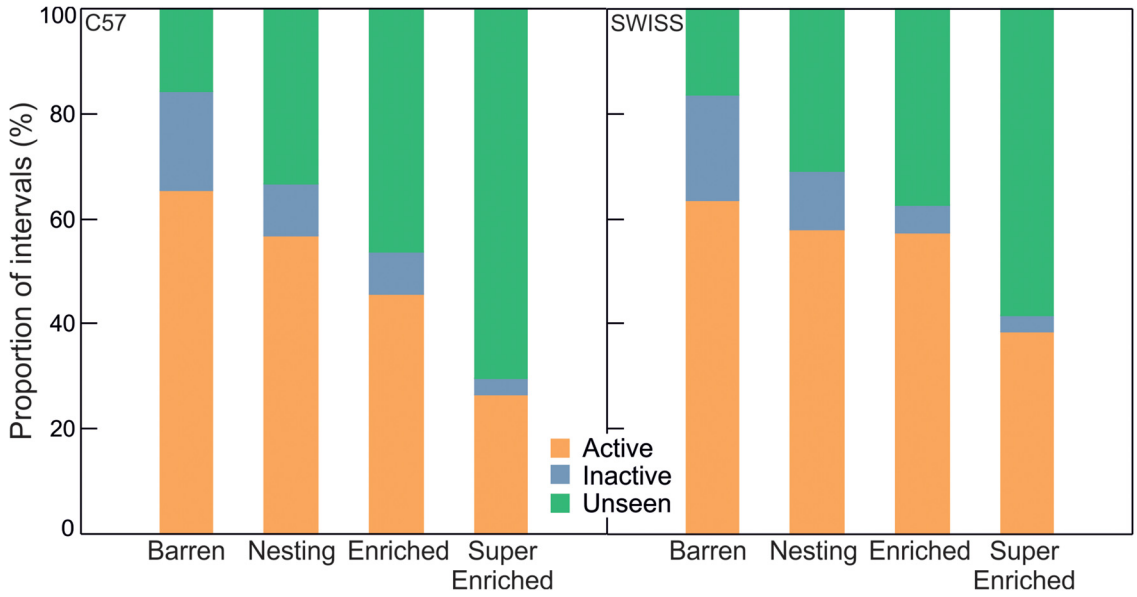

FIGURE 4 | Proportion of intervals engaged in active, inactive and unseen behavior in relation to cage enrichment.

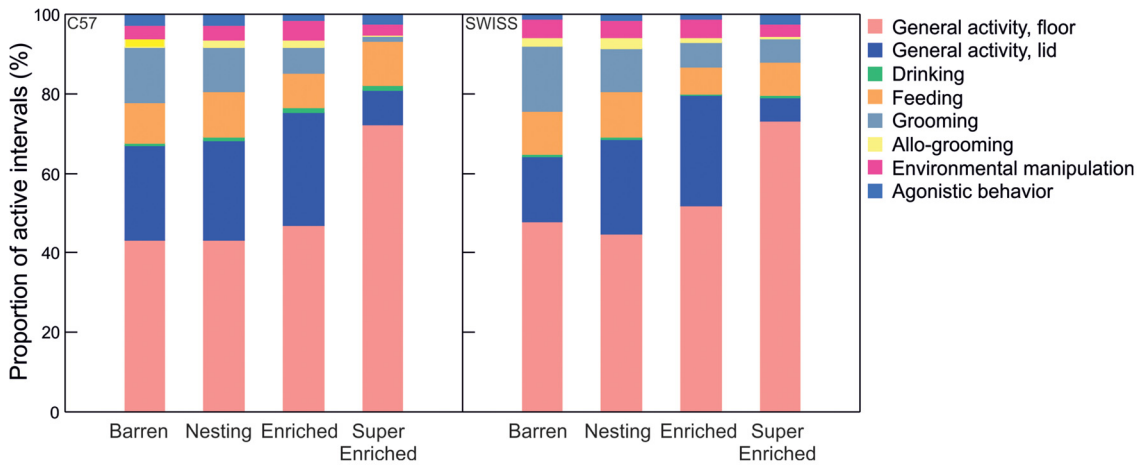

FIGURE 5 | Proportion of intervals engaged in different forms of active behavior in relation to housing condition.

In C57 mice only, water intake was positively correlated with body weight (averaged at the cage level) across all housing conditions—a similar relationship was observed only in SWISS mice housed in the SE condition (**Supplementary Table S6**).

Body weight at arrival did not vary between housing conditions in both strains (C57: $F_{(3,87)} = 0.79$, $p = 0.504$; SWISS: $F_{(3,88)} = 1.55$, $p = 0.208$). Body weight increased with age in both strains (C57: $F_{(8,333)} = 725.88$, $p < 0.001$; SWISS: $F_{(8,348)} = 495.52$, $p < 0.001$; **Figure 8**). Furthermore, body weight varied with the type of housing condition in C57, but not SWISS mice (C57: $F_{(3,99)} = 13.04$, $p < 0.001$; SWISS: $F_{(3,88)} = 0.70$, $p = 0.543$), and there was an interaction between the type of housing condition and week in C57, but not SWISS mice (C57: $F_{(24,333)} = 3.77$, $p < 0.000$; SWISS: $F_{(24,348)} = 1.51$, $p = 0.060$; **Figure 8**). *Post hoc* comparisons indicated that in C57 mice only, mice housed in the SE condition were heavier than mice of all other housing conditions. This difference emerged 1 week after arrival and persisted for the duration of the experiment.

In both strains, there was no main effect of the type of housing condition on food intake (C57: $F_{(3,28)} = 1.19$, $p = 0.332$; SWISS: $F_{(3,28)} = 2.88$, $p = 0.054$), but food intake varied by week (C57: $F_{(7,66)} = 11.16$, $p < 0.001$; SWISS: $F_{(7,107)} = 9.27$,

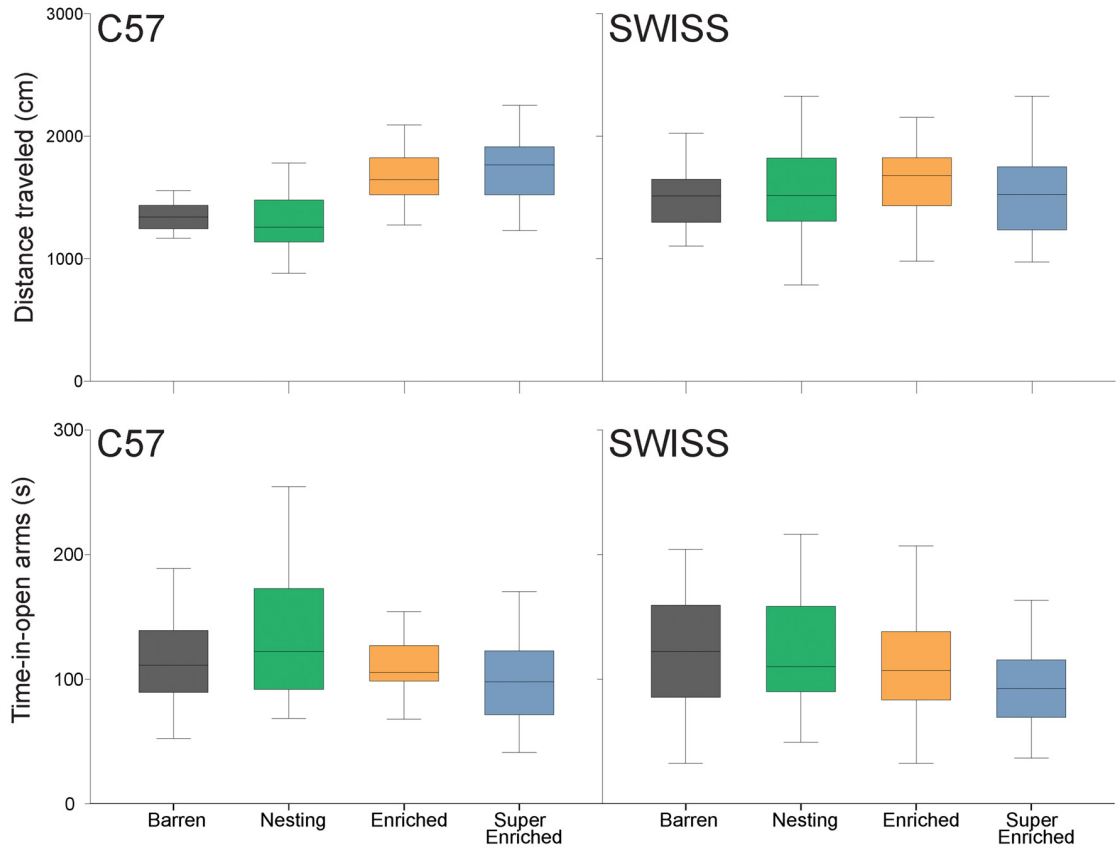

FIGURE 6 | Variation in distance traveled and time-in-open arms (median and IQR) in relation to housing condition.

$p < 0.001$). Furthermore, there was an interaction between the type of housing condition and week in both strains (C57: $F_{(21,66)} = 3.76$, $p < 0.001$; SWISS: $F_{(21,107} = 4.27$, $p < 0.001$; **Figure 9**). Closer inspection of the data indicated that food intake initially increased in both strains and all treatment groups, but flattened or decreased with age, whereby these changes in the time course of food intake differed between treatment groups. Thus, there was a relative decrease in food intake with increasing degree of enrichment at later time points.

Water intake increased with age (C57: $F_{(7,118)} = 5.2$, $p < 0.001$; SWISS: $F_{(7,120)} = 8.66$, $p < 0.001$) and there was a main effect of the type of housing condition on water intake (C57: $F_{(3,28)} = 15.37$, $p < 0.001$; SWISS: $F_{(3,28)} = 3.01$, $p = 0.047$). Furthermore, in SWSS, but not C57 mice, water intake also varied depending on the interaction between the type of housing condition and week (C57: $F_{(21,118)} = 0.880$, $p = 0.606$; SWISS: $F_{(21,120)} = 2.14$, $p = 0.006$; **Figure 9**). *Post hoc* comparisons indicated, in both strains of mice, that average water intake was higher in the SE cages. In C57 mice, water intake was higher in the SE group from week 3 compared to all other groups. In SWISS mice, water intake was higher in the SE group on week 6 compared to B and E groups.

Endocrine Stress Responses: Fecal Glucocorticoid Metabolites and Adrenal Weight

Glucocorticoid metabolite concentration increased across the three time points in SWISS mice, but not in C57 mice (C57: $F_{(2,125)} = 0.52$, $p = 0.596$; SWISS: $F_{(2,142)} = 20.67$, $p < 0.000$; **Figure 10**). Furthermore, there was an effect of the type of housing condition in SWISS, but not C57 mice (C57: $F_{(3,91)} = 0.18$, $p = 0.910$; SWISS: $F_{(3,89)} = 4.38$, $p = 0.006$). *Post hoc* comparisons indicated that glucocorticoid metabolite concentration was, on average, higher in mice housed in B and N cages compared to mice housed in E and SE cages across all three time-points; but only in SWISS mice. There was also an interaction between type of housing condition and time point in C57 mice, but not SWISS mice (C57: $F_{(6,126)} = 2.38$, $p = 0.032$; SWISS: $F_{(6,142)} = 0.64$, $p = 0.687$), but *post hoc* analyses yielded no consistent effects.

Variation in adrenal weight partly reflected the effects found in glucocorticoid metabolite concentrations. Thus, the type of housing condition affected adrenal weight in SWISS mice, but not C57 mice (C57: $H = 2.95$, $p = 0.400$; SWISS: $H = 13.12$, $p = 0.004$; **Figure 11**). However, *post hoc* comparisons indicated a difference in adrenal weight only between mice from N cages compared to mice from E cages.

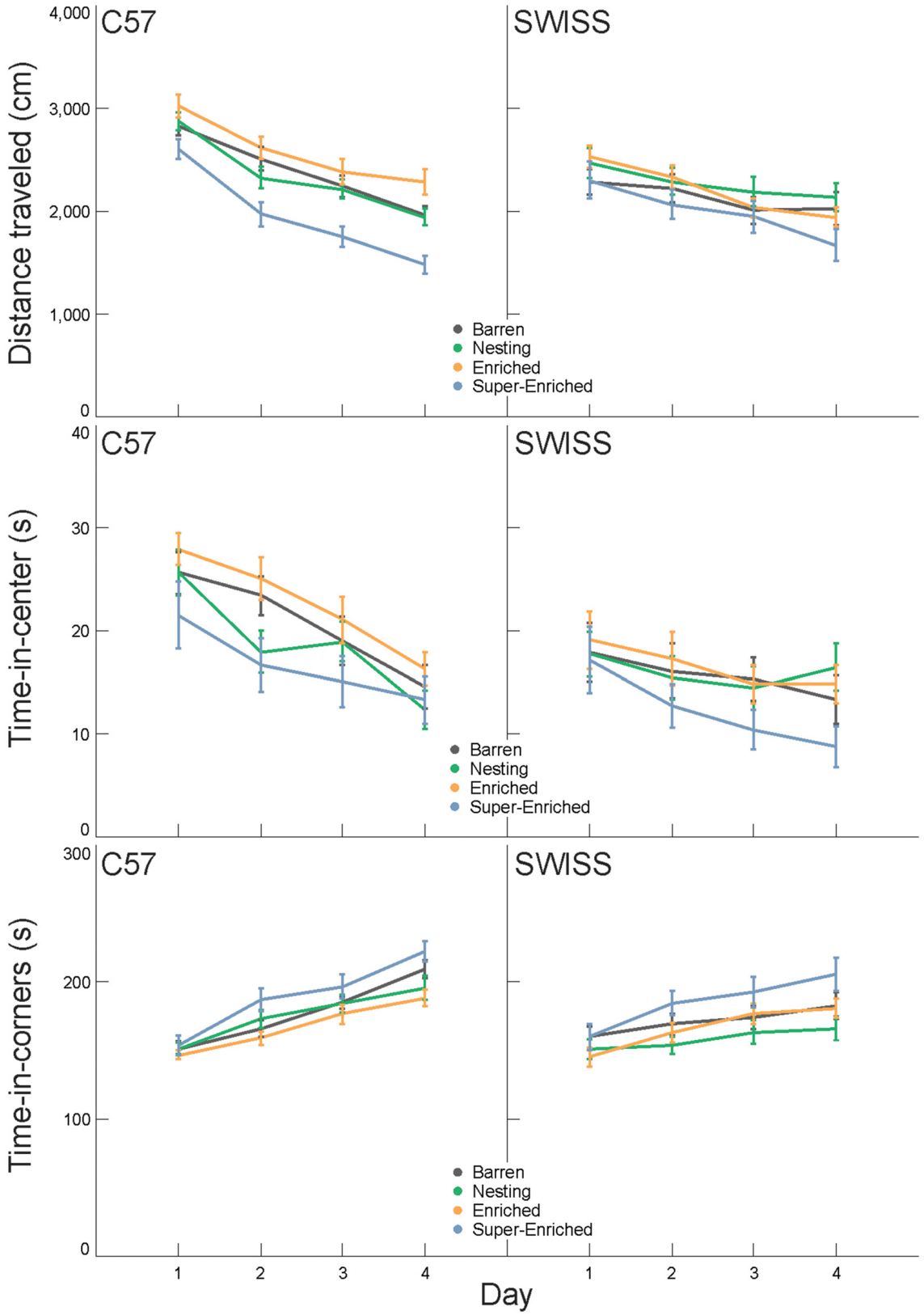

FIGURE 7 | Variation in distance traveled, time-in-center and time-in-corners (mean ± SE) in relation to housing condition and day of testing.

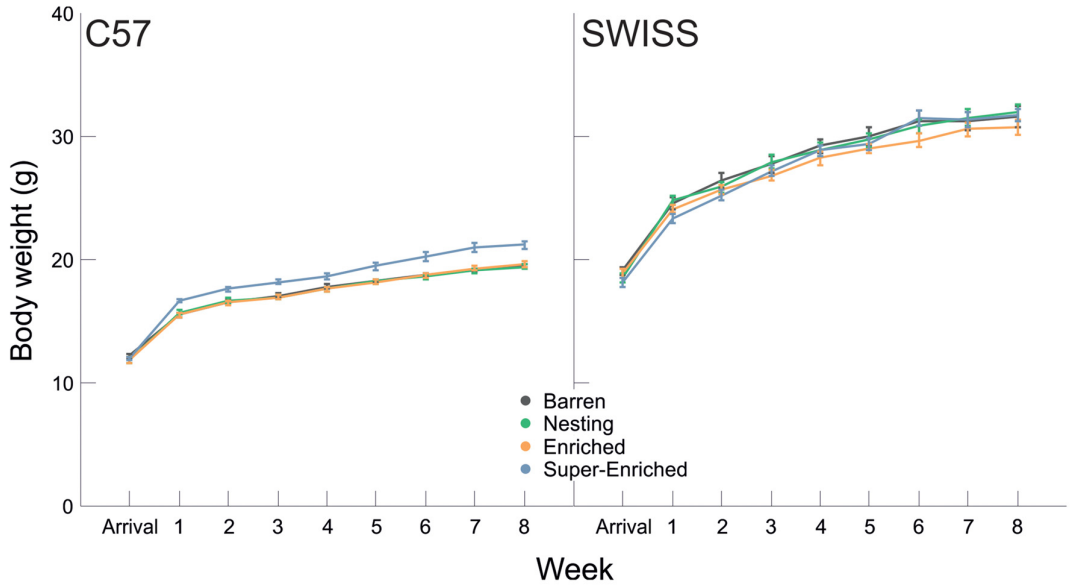

FIGURE 8 | Variation in body weight (mean ± SE) in relation to housing condition and week.

Brain Function: Recurrent Perseveration in a Two–Choice Guessing Task

Recurrent perseveration was not affected by housing condition, stereotypy level, or by the interaction between the type of housing condition and stereotypy level (C57; *housing condition*: $F_{(3,16)} = 0.57$, $p = 0.645$; *stereotypy level*: $F_{(1,16)} = 0.38$, $p = 0.850$; *housing condition* × *stereotypy level*: $F_{(3,16)} = 0.43$, $p = 0.732$; SWISS; *housing condition*: $F_{(3,16)} = 1.09$, $p = 0.384$; *stereotypy level*: $F_{(1,16)} = 0.38$, $p = 0.549$; *housing condition* × *stereotypy level*: $F_{(3,16)} = 0.71$, $p = 0.558$; **Figure 12**).

Response patterns in the guessing task varied with the type of housing condition in C57 mice, but not SWISS mice (C57; $F_{(9,80)} = 4.31$, $p < 0.001$; SWISS; $F_{(3,16)} = 1.22$, $p = 0.295$; **Figure 13**). Closer inspection of the data indicated that C57 mice housed in N cages made more pure repetitions compared to all other groups, albeit to one side only (LLLL).

Emotional State: Judgment Bias in a Spatial Go/No–Go Task

All C57 mice reached the learning criterion for testing, whereas two SWISS mice were excluded during the Go/No–go Discrimination stage (see Hintze et al., 2018 for further methodological details). Total training duration for all stages (Habituation, Shaping for Trial Initiation, Left–Right Discrimination, Go/No–go Discrimination) was 14.20 ± 1.63 sessions for C57 mice and 15.50 ± 1.79 sessions for those SWISS mice that reached the test criterion.

During testing, the animals' decision as to whether or not to go when confronted with the different trial types varied as an interaction between the type of housing condition and trial type for both C57 and SWISS mice (C57: $\chi^2_3 = 13.46$, $p = 0.004$; SWISS: $\chi^2_3 = 22.97$, $p < 0.001$; **Figure 14**).

Closer inspection of the data revealed no consistent differences in response patterns depending on the type of housing condition. In both strains, the strongest treatment effect was observed in near-negative (NN) trials, as indicated by fewer go responses in mice from B cages in C57 mice, and in mice from SE cages in SWISS mice, compared to mice from all other treatment groups. However, NN trials were also associated with the largest variability in the number of go responses.

Variability in the Measured Outcome Variables

To assess potential effects of the type of housing condition on the variability of experimental results, we calculated the CV for all measured outcome variables, with the exception of home–cage and stereotypic behavior, and for judgment bias and guessing task data where only a subset of animals were tested ($n = 6$ per strain per housing condition). The CV is the ratio of the standard deviation to the mean, which yields a dimensionless, standardized measure of dispersion. It thus allowed us to compare variation estimates directly between the different outcomes measured in this experiment.

CVs varied greatly depending on the measured outcome variable (**Figure 15**, **Supplementary Table S7**). Most CVs were relatively small and there were no consistent relationships between cage enrichment and CV across the range of outcome variables assessed in this study.

DISCUSSION

To study the effects of environmental enrichment on measures of animal welfare in mice, we systematically varied housing conditions across four levels of enrichment and measured animal welfare in a multi-faceted way. Overall, the greatest benefit

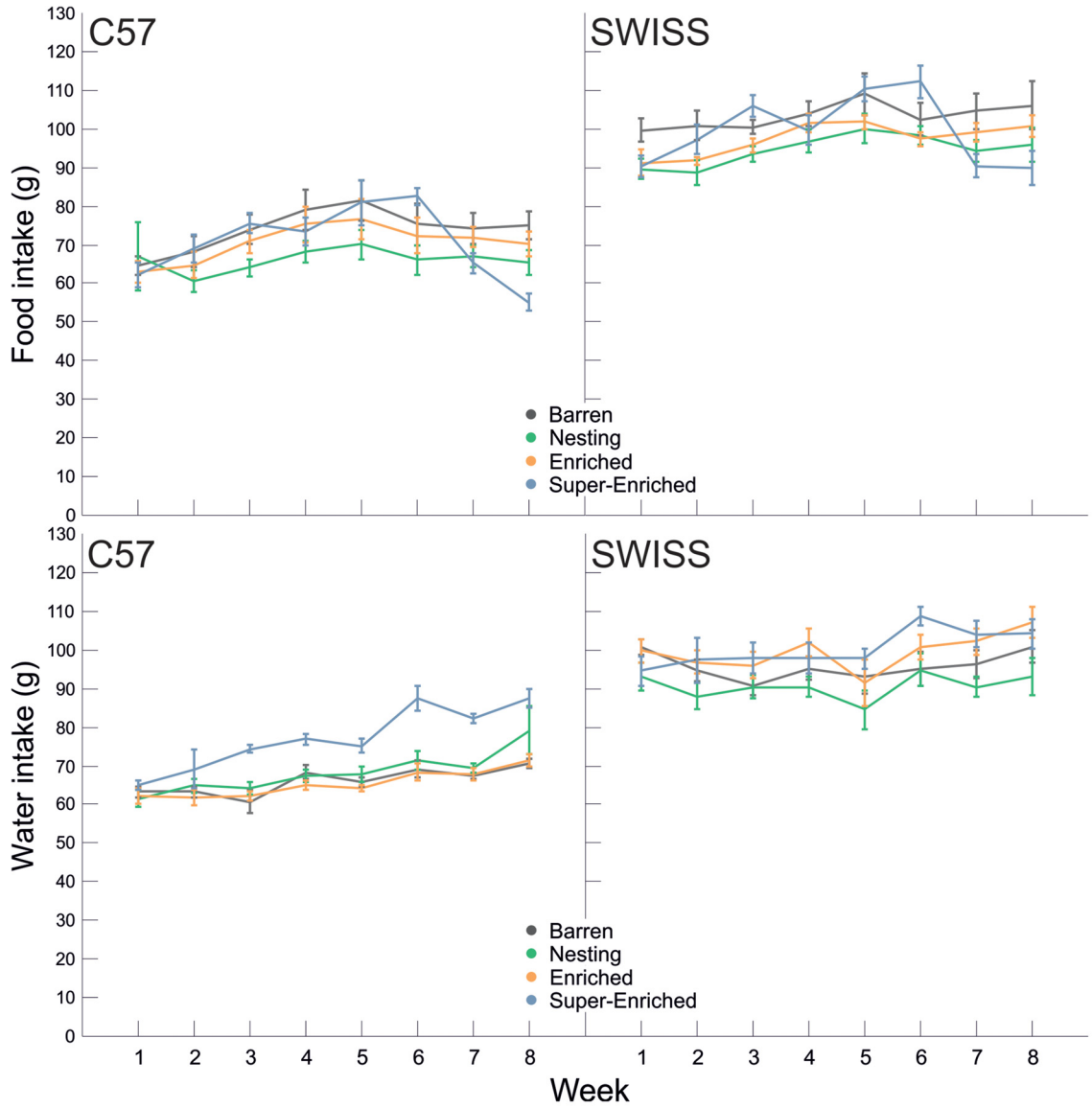

FIGURE 9 | Variation in food and water intake (Mean ± SE) in relation to housing condition and week.

to welfare was observed in animals housed with the most extensive enrichment, the SE housing condition—stereotypic behavior, some behavioral measures of anxiety, growth and stress physiology, all varied in a manner consistent with improved animal welfare. We also assessed the effect of the different housing conditions on variation in outcome measures. Similarly to other studies (Augustsson et al., 2003; Wolfer et al., 2004; Baumans et al., 2010; van de Weerd et al., 2010), we found no evidence that environmental enrichment increased variation in experimental results, for the outcome measures assessed here.

When evaluating whether and how animals engaged with the enrichment items and evaluating the relation to welfare, substantial differences were noted—animals were more often observed to be in, on or under items, and manipulated items as the degree of enrichment increased. One possible explanation for this difference may be that there is a higher probability for contact with different items, simply by chance, as the number of items increased and the "free" floor area decreased. If this explanation were true, we would predict that despite higher levels of engagement with the housing environment, animal welfare would not be improved. This explanation may apply to N and E groups—even though engagement with enrichment increased with the degree of enrichment, no consistent improvements in measures of welfare were observed in comparison to the B housing condition. For example, the expression of stereotypic behavior did not vary in a systematic way between B, N and E housing groups even though engagement with enrichments increased with increasing degrees of enrichment in these groups.

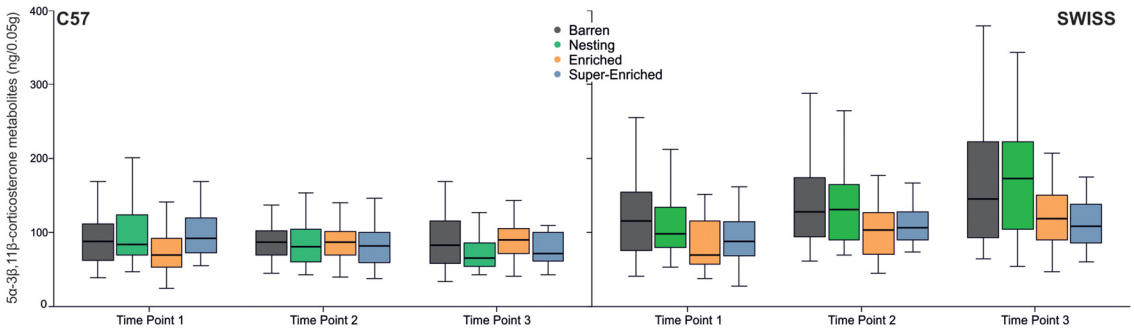

FIGURE 10 | Variation in glucocorticoid metabolite concentrations (median and IQR) in relation to housing condition and time point of measurement.

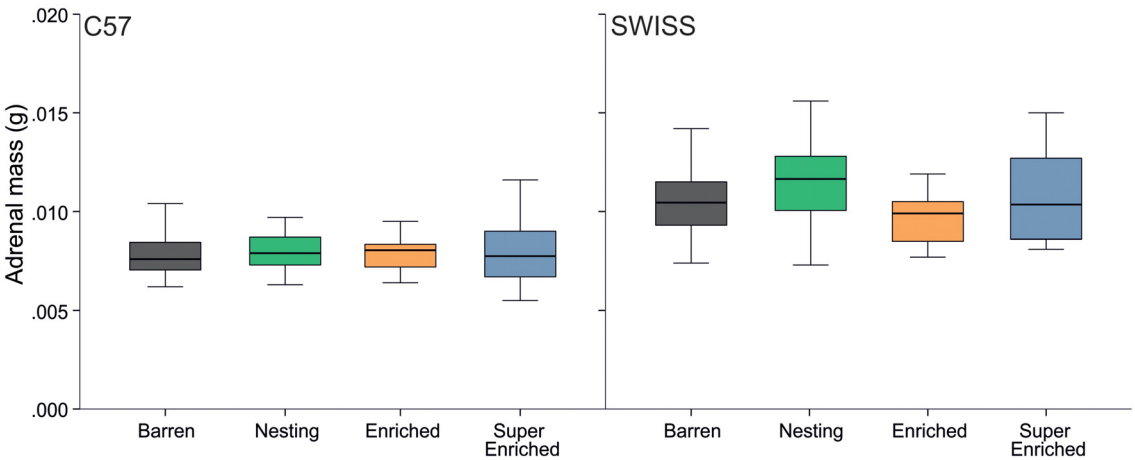

FIGURE 11 | Variation in adrenal mass (median and IQR) in relation to housing condition.

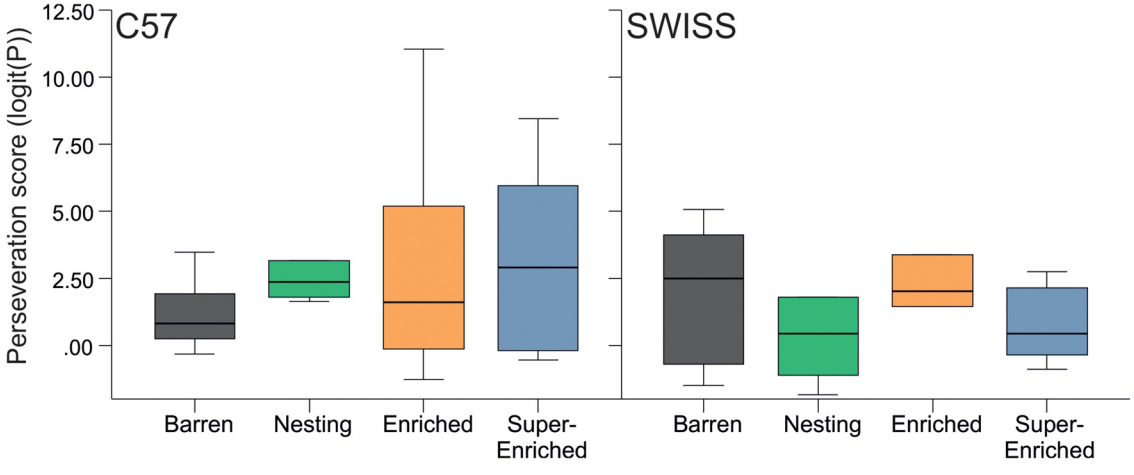

FIGURE 12 | Variation in perseveration score (median and IQR) in relation to housing condition.

However, this does not apply to the SE housing condition. Although the SE condition was the most enriched, it also offered the most "free" floor and lid space. Furthermore, in the SE housing condition, virtually no stereotypic behavior was

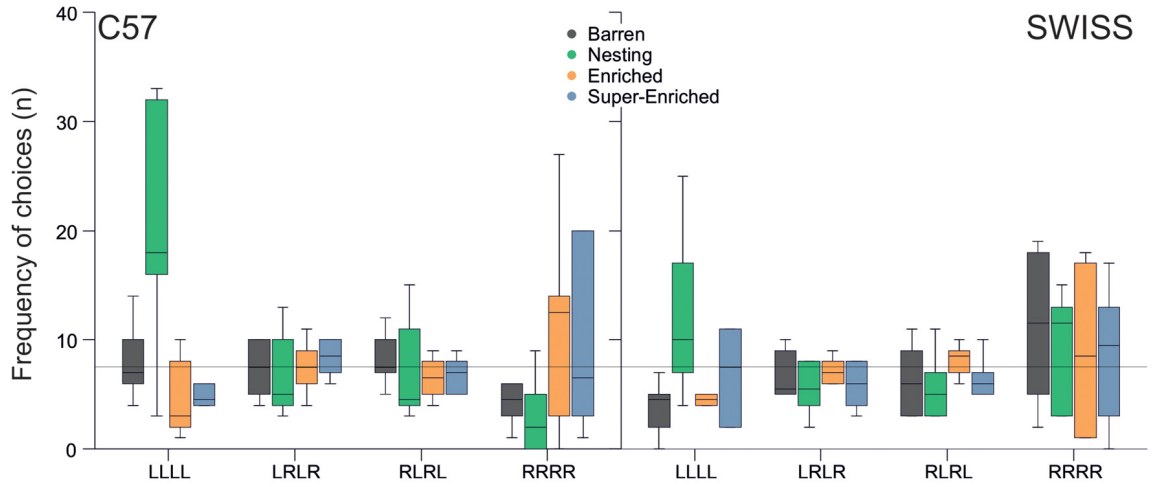

FIGURE 13 | Variation in repetitions and alternations in relation to housing condition (median and IQR). The line represents the expected frequency of choices given a random search strategy.

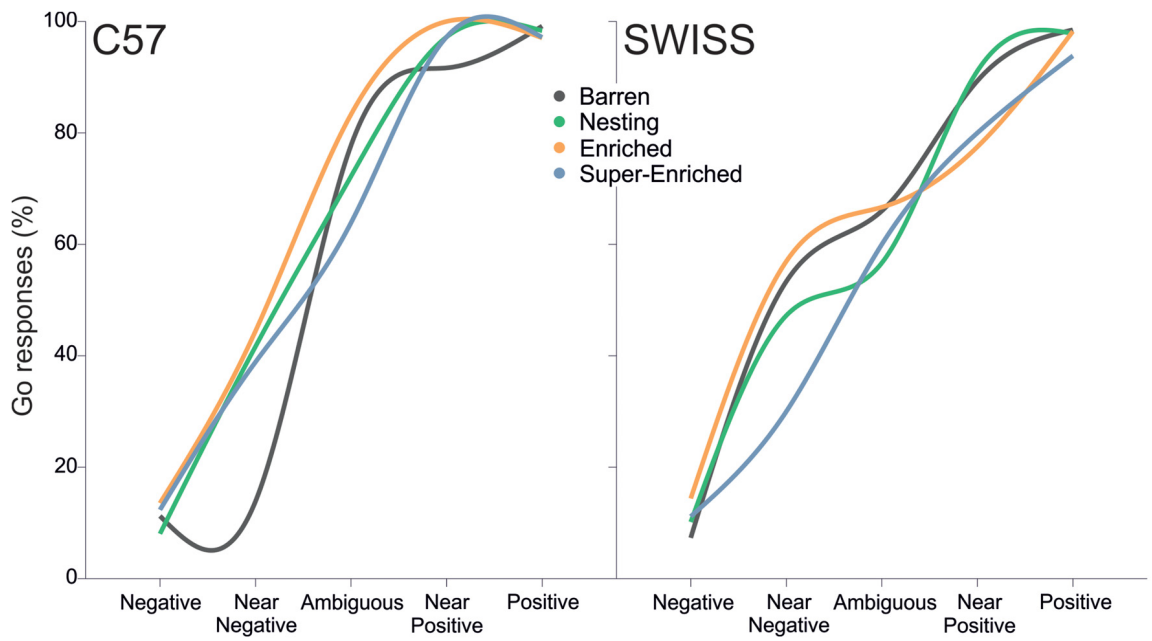

FIGURE 14 | Fitted regression lines showing go responses in relation to housing condition and strain.

observed—suggesting improved welfare in this group (Mason and Latham, 2004). It is possible that the prevalence of stereotypic behavior was underestimated in the SE group, given that the highest levels of unseen behavior were also observed in this group. We find this explanation unlikely given that bar–mouthing, the most commonly reported form of stereotypy in laboratory mice (Würbel, 2006), was observable and recorded since the bars of the cage were not covered by enrichment items. Animals in the SE group displayed extremely low levels of bar–mouthing (on average <1%). It is similarly unlikely that mice were performing any of the other forms of stereotypy categorized here when unseen, given the amount of space that is necessary for the performance of these behaviors; most of the unseen behavior was recorded when mice were under or in enrichment items. Therefore, this difference in stereotypy performance, with respect to housing condition, likely reflects the true incidence of behavioral expression. Underestimation of stereotypic behavior, when animals were unseen, in B, N and E cages is also unlikely given that unseen behavior in these cages was primarily scored when animals were either in the nest or in a

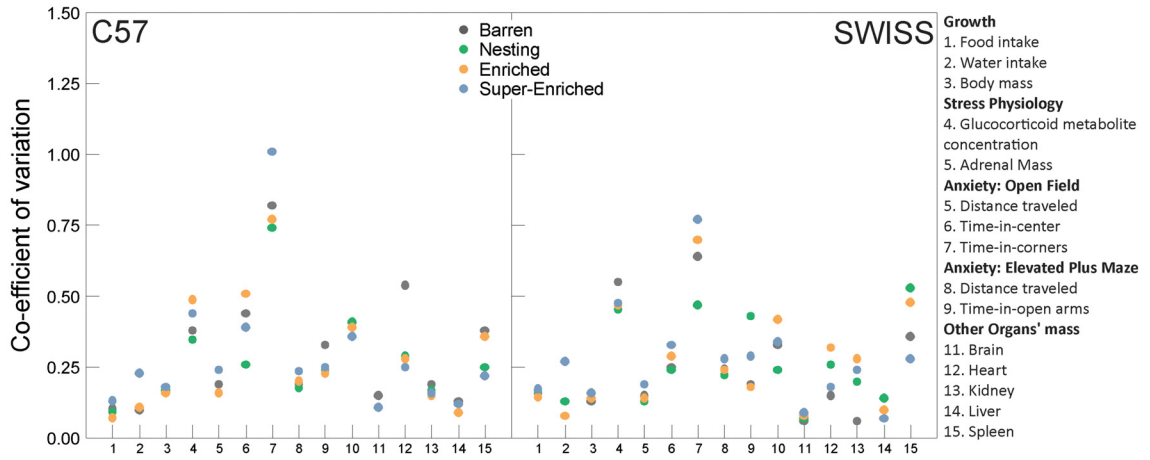

FIGURE 15 | Co-efficient of variation estimates by outcome measure, housing condition and strain.

huddle and where one mouse visibly blocked another. Neither of these two scenarios is likely to have masked the display of stereotypic behavior.

When assessing differences in time budget, some differences were observed—for example, the proportion of grooming and of active behavior on the lid decreased with the degree of enrichment across all treatment groups. In contrast to stereotypic behavior, however, other components of the animals' time budget categorized here could occur when an animal was in or under enrichment items. For example, we frequently observed nesting material shaking in a rhythmic pattern, indicative of grooming or allo-grooming behavior; this behavior was coded as unseen, given that the animals were not visible. Therefore, it is quite likely that our time–budget assessment underestimates the prevalence of specific behaviors and these data should therefore be interpreted with caution. On the other hand, as our data indicated that grooming was often performed out of sight, it may also suggest that mice prefer to perform grooming in a shelter, if a shelter is available.

Differences in engagement with enrichment items were also associated with improvements in animal welfare, albeit in a strain dependent way. C57 mice housed with a greater degree of enrichment, i.e., E and SE groups, were more exploratory, and therefore less anxious, in the elevated plus–maze. In the open field, C57 mice housed in the SE groups displayed a classic habituation response to the novelty of the arena across days of testing—on average lower levels of exploration and more time spent inactive in the corner of the arena. Thus, in general, C57 mice housed in SE cages displayed an anxiolytic profile in comparison to the other housing conditions.

In terms of our secondary outcome measures, some concordant differences were observed. For example, when comparing differences in stress physiology, a consistent difference was observed, but only in SWISS mice—animals housed in E and SE groups had lower levels of glucocorticoid metabolites in feces across all three time points of measurement compared to B and N groups. This result suggested that SWISS mice housed in E and SE conditions experienced lower levels of chronic stress. Our other measure of stress physiology, adrenal weight, varied differently with housing condition—SWISS mice housed in E groups had smaller adrenals compared to those housed in the N groups. Both adrenal weight and glucocorticoid metabolites in feces have been used previously as indicators of experienced chronic stress in mice (Tsai et al., 2002; Akre et al., 2011; Gurfein et al., 2014; Bailoo et al., 2018), albeit with different degrees of sensitivity relative to housing conditions. For example, one study has found that animals housed in enriched environments tended to have smaller adrenals; although this difference was not statistically significant (Tsai et al., 2002). In contrast, studies that measured corticosterone secretion in feces have consistently found that enriched housing conditions are associated with decreased levels of circulating glucocorticoids (Akre et al., 2011; Gurfein et al., 2014). Therefore, the discrepancy in the pattern of differences between fecal glucocorticoids and adrenal weight with respect to housing condition may simply reflect differences in measurement sensitivity.

Across time, food intake initially did not vary by housing condition—it did however vary by time. Food intake initially increased, peaking at around 7–8 weeks of age (puberty/early adulthood) and then either flattened or decreased with age. In contrast, water intake increased across time in the SE condition in both strains, but did not vary between the other housing conditions. In C57 mice only, animals housed in the SE condition weighed more than animals in all other groups. Notably, food intake and body weight (averaged at the cage level) were uncorrelated in C57 mice housed in E and SE groups. Taken together, these patterns of differences with respect to growth, most likely reflect variation in metabolic need. Importantly, as ambient temperatures in mouse facilities are kept below the thermoneutral zone of mice, increasing the risk of cold stress (Gordon, 1985, 1993; Johnson et al., 2017), increased opportunity for structural complexity, and in particular for the construction

of elaborate nests, may act as a buffer to cold stress—resulting in heavier animals. However, we were unable to examine this hypothesis further, as we did not measure temperatures inside the cages (micro-climate). Food and water intake are tightly linked to metabolism and are, in general, positively correlated (Gordon, 1993; Gordon et al., 1998). However, in relative terms, water intake in mice is an inelastic requirement—in the absence of water, mice quickly dehydrate (Harkness et al., 2013). Increased water intake in the SE housing condition is therefore most likely reflective of a difference in metabolic need prompted by increased activity and possibly reflecting increased engagement with the environment.

Animals housed in the different housing conditions did not vary in our measures of brain function, recurrent perseveration and patterned responding in a two-choice guessing task; at least not in the predicted way. Specifically, animals housed in barren environments have been reported to display a higher incidence of stereotypic behavior and, in turn, higher levels of perseverative behavior and patterned responding (Garner et al., 2011). In this experiment, no association was found between housing condition, stereotypy level and recurrent perseveration. This lack of association is, however, in line with more recent studies using this experimental paradigm (Latham and Mason, 2010; Gross et al., 2011a, 2012; Novak et al., 2016; Bailoo et al., 2018); the levels of stereotypy observed here were similar to those in the study reporting a relationship (Garner et al., 2011). These findings therefore indicate that either this test paradigm does not measure recurrent perseveration reliably or that cage stereotypies in these mice do not reflect behavioral disinhibition as measured by recurrent perseveration. A difference in patterned responding was observed with respect to housing condition, in C57 mice only—animals housed in the nesting condition made more repetitive choices, compared to all other groups; albeit to one side (LLLL). Previous studies have indicated that C57 mice tend to show more alternations (LRLR or RLRL) than repetitions (LLLL or RRRR; Bailoo et al., 2018) and that enriched housing reduces the number of repetitive choices compared to barren housing (Gross et al., 2011a). The series of paradoxical results across studies suggests that these may be study specific idiosyncrasies of unknown etiology.

Studies investigating the effect of transfer from standard housing conditions to enriched environments on judgment biases in rats have reported a shift in judgment biases from more "pessimistic" to more "optimistic" responses (Brydges et al., 2011; Richter et al., 2012). In the present experiment, the overall response pattern, a monotonic graded response, was observed in mice from both strains and across all four housing conditions—confirming that the internal consistency criteria of this test were met (for further details, see Hintze et al., 2018). However, the only variations depending on housing conditions were that C57 mice housed in the B group and SWISS mice kept in the SE group showed a lower number of go responses when confronted with near negative trials compared to the other housing conditions. These findings may indicate either that this test was not sensitive enough to detect variation in animal welfare or that the observed differences in animal welfare were not associated with differences in emotional valence. However, judgment bias was assessed at the end of a series of other tests and after extensive training, with daily removal from the home cage and handling for testing, which may have masked treatment effects. Without further study, this explanation remains essentially speculative. Therefore, these results—the first report of judgment biases in mice as a consequence of environmental supplementation—require replication and further study.

When comparing variation across our outcome measures in relation to housing conditions, no systematic patterns of differences were found. These results add to a growing body of evidence against the often heard, yet unsubstantiated, concerns that environmental enrichment would increase variation in outcome measures, thereby inducing a need for larger sample sizes to detect treatment effects (Augustsson et al., 2003; Wolfer et al., 2004; Baumans et al., 2010; van de Weerd et al., 2010).

Overall, we found that the mice readily used the enrichments when available, and that engagement with enrichment increased with increasing degree of enrichment—indicating that the enrichments offered the animals choices, which they integrated in the expression of their behavior. However, we also found that with the exception of extensive enrichment in the SE condition, variation in enrichment did not produce consistent variation in our measures of welfare. According to a large body of literature, there is no doubt that nesting material is beneficial for laboratory mice in many ways (Bult and Lynch, 1997; Sherwin, 1997; van de Weerd et al., 1997; Olsson and Dahlborn, 2002; Smith and Corrow, 2005; Gaskill et al., 2009, 2011, 2012; Gross et al., 2011b). Previous studies also found that nesting material alone attenuates stereotypic behavior (Gross et al., 2012). However, the lack of consistent differences between mice housed in B, N and E conditions together with the substantial differences between these mice and those housed in SE conditions suggested that considerably more extensive enrichment strategies may be needed to achieve substantial improvements in welfare. From the present results, we do not know whether it was the larger space, the frequency of bedding changes, the more complex environment, the types of enrichments, or the novelty that contributed most to the beneficial effect of the SE conditions as all of these factors were confounded. We therefore suggest that further studies under more extensive conditions are needed to inform decisions on minimal requirements for the housing of laboratory mice. Such an approach may allow for the identification of relevant resources or combinations of resources necessary for improving the welfare of laboratory housed rodents. It may also lend insight into relevant mental or cognitive capacities underlying species-typical behaviors (e.g., learning, memory, spatial navigation)—permitting for the development of innovative housing solutions that recapitulate the use of these capacities in the laboratory (for review, see Bennett et al., 2018; and for examples of evaluation and application, Dutton et al., 2018). In the 70s and 80s, such an approach was pursued in a range of farm animals (e.g., laying hens: Fölsch et al., 1983; rabbits: Stauffacher, 1992; pigs: Stolba and Wood-Gush, 1984), resulting in welfare-friendly prototypes

of housing conditions, from which practicable solutions were further developed for specific contexts of animal use (e.g., laboratory rabbits: Stauffacher, 1994).

The present results also highlight the difficulty in making general recommendations for improving the housing environment of laboratory mice. For example, even though the mice engaged more with the environment as the degree of supplementation increased, specific improvements in measures of welfare varied in a strain specific way. Furthermore, we only studied females and the needs of male mice may differ from those of female mice due to their higher propensity of escalating aggression (Kappel et al., 2017). Thus, we need to take into consideration that there may be no single solution to meet the needs of all strains of mice and both sexes, and enrichment strategies may always have to be adjusted to the specific strain and sex being used.

AUTHOR CONTRIBUTIONS

HW: funding acquisition and resources. JB, EM and HW: conceptualization. JDB and EM: methodology. JB: project administration, formal analysis and writing–original draft. JB, EM, MB-S, JV, SH, CB, KH, CG and RP: investigation. JB EM MB-S, JV, SH, KH, CG, RP, BV and HW: writing–review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00232/full#supplementary-material

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frontiers in Behavioral Neuroscience

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Enriched Environment Exposure Enhances Social Interactions and Oxytocin Responsiveness in Male Long-Evans Rats

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Both social and physical stimuli contribute to the complexity of an animal's environment, influencing biobehavioral responses to subsequent challenges. In the current study, male Long-Evans rats were randomly assigned to an isolate (ISO), social control (SC) or social enriched (SE) group (n = 8 per group). The SC and SE conditions were group housed with the SE group exposed to physical enrichment stimuli that were natural as opposed to manufactured (e.g., hollowed out log instead of plastic hiding place). On three occasions during their 40-day enriched environment exposure, night/dark phase videos were obtained for 1 h during the early part of the dark phase. During this time, the SE animals exhibited significantly more social grooming with no differences between the SE and SC in the frequency of play or self-grooming bouts. Subsequently, all animals were assessed in social interaction and problem-solving escape tasks during the last week of the enriched environment exposure. SE rats exhibited increased digging bouts toward the restrained conspecific in the social interaction task whereas the other groups exhibited more escape responses. In the problem-solving task, SE animals exhibited a decreased latency to cross the barrier to escape from the predator odor (i.e., cat urine and fur). Neural analyses indicated increased oxytocin-immunoreactive (OT-ir) tissue in the SE supraoptic and paraventricular nuclei of the hypothalamus compared to the other groups. Interestingly, blood samples indicated lower peripheral corticosterone (CORT) and higher OT levels in the ISO animals when compared to the SC and SE animals, an effect retrospectively attributed to separation anxiety in the SE and SC animals in preparation for histology procedures. When the behavioral, neural and endocrine data were visualized as a multifaceted dataset via a multidimensional scaling analysis, however, an association between social enrichment and higher OT involvement was observed in the SE animals, as well as heightened stress responsivity in the ISO and SC groups. In sum, the SE animals exhibited a facilitation of social responses, problem-solving ability and OT immunoreactive responsiveness. These findings provide new information about the influences of both physical and social stimuli in dynamic and enriched environments.

Keywords: enriched environment, natural habitat, use-dependent plasticity, oxytocin, corticosterone, acute stress, social interaction

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INTRODUCTION

In the late 18th century, Italian anatomist, Michele Vincenzo Malacarne, reported that when animals underwent extensive training, they developed more cerebellar folds than untrained animals (described in Rosenzweig et al., 1972). These provocative findings were controversial and, consequently, laid dormant until the mid-20th century when a prominent Canadian neuroscience researcher, Donald Hebb, reported that rats raised in an engaging environment in his own home exhibited enhanced learning compared to his standard laboratory raised animals (Hebb, 1949). Subsequently, a team of neuroscientists at UC Berkeley demonstrated that, contrary to prevalent assumptions about the fixed nature of the nervous system, rodents exposed to a complex environment developed heavier cortical areas and altered neurochemistry (Rosenzweig et al., 1962; Bennett et al., 1964). Instead of spending an inordinate amount of time training the animals, it was assumed that the animals would engage in a form of self-training if they were housed in engaging environments (Rosenzweig and Bennett, 1996). These complex environments became known as "enriched environments" to contrast with the typical cages used in the laboratory (Krech et al., 1960; Diamond et al., 1965). Rather than being enriched, however, these complex environments may be closer to an ecologically relevant environment—that is, a standard environment by nature's criteria (Juraska and Wise, 2015). Even so, the terms enriched and complex are used interchangeably throughout this manuscript to refer to engaging laboratory environments that include social and physical stimuli.

Early laboratory enriched environment studies utilized at least three groups: (1) an enriched social group with 8–10 animals housed in a large cage with objects that were rotated every few days; (2) a social control (SC) group of approximately three animals housed in a standard size cage; and (3) an isolate (ISO) control group with animals housed individually in a small cage (Rosenzweig et al., 1972). Compared to individual animals housed in standard laboratory cages, these enriched environments provided more diverse experiences for the animals with varied exposures to sensory, motor and social stimuli. Similar variations of these original groups are used in current studies; however, regardless of the size of the cage and social group, the physical stimuli are rotated every day or couple of days so that the animals experience novel conditions in their laboratory habitats (Nithianantharajah and Hannon, 2006).

The observed neurobiological effects of enriched environments have been both widespread and reliable. For example, the visual cortex increased approximately 6% in enriched animals (Bennett et al., 1964; Juraska et al., 1989) with more specific changes such as increased dendritic branching and spine density (Volkmar and Greenough, 1972; Globus et al., 1973). Although the volume changes are not as pronounced in the hippocampus (approximately 3%), increased dendritic spines have been observed in the absence of modifications in the dendritic tree in the dentate gyrus and CA3 area (Juraska et al., 1989; Moser et al., 1997). Enriched environments have also been shown to increase hippocampal neurogenesis rates (Kempermann et al., 1997), an effect that is enhanced when animals have the opportunity to engage in running behavior (Kobilo et al., 2011). Neurogenesis has been suggested as a mechanism for enhanced flexibility in the responses of animals housed in enriched environments, as varied responses are acquired in the dynamic, engaging environments (Garthe et al., 2016).

From the time that the earliest enriched environment studies were published, there has been an attempt to isolate the most influential underlying mechanisms of brain plasticity. Although adding environmental enrichment to single-housed animals has been shown to be beneficial to the animals' wellbeing over long periods of time (Abou-Ismail and Mahboub, 2011), the extent of brain changes observed in social enriched (SE) animals (that is environmental enrichment and social housing) has not been replicated in the single-housed enriched animals (Rosenzweig et al., 1978). Additionally, it is generally thought that ISO-housed animals represent impoverished conditions that render the animals susceptible to the subsequent emergence of anxiety-related responses such as motor stereotypies (Balcombe, 2006). Even though the importance of social housing has been established for the well-being of animals, the neuroanatomical enrichment effects have been suggested to be dependent on the animals' interactions with environmental stimuli (Rosenzweig et al., 1978). Considering that, since the original enriched environment studies were conducted, SC groups are often housed in the same size cage as the SE animals, the large cage size used in enriched environment studies has also been ruled out as the most important factor underlying the enriched environment effects (Lambert et al., 2016). Further, it has been acknowledged that many of the factors present in the enriched environment studies contribute to an *additive* effect on relevant neuroanatomical factors (Fabel et al., 2009).

Although the specific role of social responses in the enriched environment studies is still unknown, it has been suggested that playful social, physical and motor responses are critical for the neurobiological effects (Fagan, 1981, 1982). This play hypothesis was subsequently assessed when dark phase observations of the rats were evaluated to determine if the enriched, social animals engaged in more rough-and-tumble play than the standard SC animals. Interestingly, no differences in play behavior, or other measures of social interaction, were observed (Renner and Rosenzweig, 1986). Even so, the authors emphasized the importance of social housing since the social-housed control animals often exhibit brain effects that are intermediate between the SE environment and impoverished environment animals. A possible explanation for this putative additive effect may be related to local enhancement, or social facilitation, when an animal's activity attracts the attention of conspecifics which may facilitate orientation and subsequent physical and social interactions with the observed environmental interaction (Thorpe, 1963; Renner and Rosenzweig, 1986). The neuropeptide oxytocin (OT), involved in social responses ranging from maternal care to social trust, is a plausible mechanism for social facilitation in SE environments (Veenema, 2012). Because OT

has been associated with positive social interactions and reduced stress responsivity, it is a common target when investigating neurobiological mechanisms of affiliative social responses and accompanying enhancements in overall wellbeing (Uvnäs-Moberg, 1998).

In contrast to the previously mentioned study (Renner and Rosenzweig, 1986), when rats are exposed to an enriched environment with natural, as opposed to artificial, stimuli and observed during the dark phase, increased interactions with both physical and social stimuli have been observed. Further, compared to animals placed in an environment with artificial stimuli, the natural-enriched animals exhibited less anxiety-typical behavior in response to a predator odor (Lambert et al., 2016). In a similar study, natural enriched rats exhibited more evidence of emotional regulation in a challenging swim escape task, evidenced by shorter latencies and increased frequencies of diving responses as well as higher DHEA/corticosteroid ratios; however, no differences in hippocampal BDNF levels were observed between the natural- and artificial-enriched groups (Bardi et al., 2016). These findings confirmed earlier observations of the UC Berkeley team indicating more neuroanatomical modifications in enriched animals housed in a naturalistic outdoor habitat that provided opportunities for burrowing and exposed the animals to additional natural elements than encountered by the laboratory enriched animals (as described in Rosenzweig et al., 1972). If the natural environments stimulate more species-relevant responses (directed toward both physical and social stimuli), this habitat presents an optimal environment to investigate the influence of social interactions on neurobiological outcomes observed in various enriched environments (Thorpe, 1963; Renner and Rosenzweig, 1986; Bardi et al., 2016; Lambert et al., 2016).

Given the previous findings in our laboratory using natural-enriched environments, the purpose of the current study was to further explore the role of social interactions in laboratory enriched environments. Since increased social interactions have been observed in natural-enriched animals (group housed with natural physical enrichment), this type of enrichment was used in the current investigation. The natural-enriched group was compared to SC (group-housed with no physical enrichment) and ISO-housed (individual-housed with no physical enrichment) groups. Thus, the three groups were similar to the groups used in the classic enriched environment studies, with the addition of the natural elements in the enriched environment. The opportunity for social interactions was viewed as optimal in the natural-enriched animals, standard in the SC animals and absent in the ISO-control animals. Given the rich literature implicating OT in social behavior (Carter, 1998; Nelson and Panksepp, 1998), both central and peripheral OT activity were evaluated. Social responsiveness and problem-solving behaviors were also assessed, as well as peripheral corticosterone (CORT) levels. It was hypothesized that, as observed in past studies, the enriched animals would be most affected by their habitat exposure due to heightened social and physical interactions and would exhibit increased central and peripheral OT responsiveness, less stress responsiveness, heightened social attentiveness and more efficient problem-solving responses. It was anticipated that the focus on social interactions in the current study would provide meaningful information about the role of OT and social attentiveness in animals' neurobiological responses to enriched environments.

MATERIALS AND METHODS

Animals

Twenty-four male Long Evans rats were ordered from Envigo Laboratories (Indianapolis, Indiana) and arrived at 21–23 days of age. Rats were given 7 days to habituate to laboratory conditions before being assigned to one of three living environments: ISO, SC or SE groups. ISO rats were housed individually in cages (48 × 26 × 21 cm) with corncob bedding and food and water provided *ad libitum*. The SC group included eight males housed in a large cage (61 cm × 61 cm × 38 cm) containing a shallow floor pan for appropriate bedding substrate (corncob bedding), with food and water provided *ad libitum*. Provided as standard laboratory enrichment, 5 cm square nestlets (Ancare; Bellmore, NY, USA) were placed in the ISO and SC cages for the animals to manipulate. The SE group was housed in the same size cage as the SC animals, however, the bedding substrate consisted of shredded coconut husk substrate (Zoo Med Eco Earth; San Luis Obispo, CA, USA) that has the texture of dirt, as well as six patches of dried moss (Exo Terra; Mansfield, MA, USA). In addition to the natural bedding substrate, seven different objects (e.g., rocks, sticks, coconut shells) were placed throughout the SE cage (see **Figure 1**). The objects were either replaced or rotated within the cage every 4 days. Each object had an intended function (e.g., shelter, climbing, tunneling, or manipulating) which was maintained during changes. All three environments were kept on a 12-h light/dark schedule with lights on at 8 AM and lights off at 8 PM in a moderate temperature (approximately 22°C). This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee at Randolph-Macon College; further, the protocol was approved by the Institutional Animal Care and Use Committee at Randolph-Macon College.

Behavioral Assessments

Behavioral observations of social interactions among the group-housed animals were videotaped three times throughout

FIGURE 1 | Images depicting the natural-enriched environments utilized in the current study.

the duration of the study (at 1, 14 and 26 days after introduction to their respective environments). Each observation was videotaped for 1 h during the dark phase (8:30–9:30 PM). A red fluorescent light bulb was used to provide sufficient lighting to capture images of the animals during the dark phase while minimizing light-induced circadian disruptions.

Scan sampling was utilized to assess behavior during recordings. Briefly, every 30 s the social behavior of the animals was scored to determine the number of rats that were in contact with or within proximity of a conspecific. Each behavior was classified as active (physical activity) or passive (no observed movement). The number of animals in contact with each other, as well as, number of animals in close proximity (within one body-length) was also recorded. More detailed social behavior such as play and grooming were assessed by frequency and number of animals involved. A distinction was made in grooming behavior, noting bouts of self-grooming and social-grooming.

During the last week of assigned housing, animals were exposed to a social investigation task in which a novel male conspecific was placed in a plastic tube (22 cm × 0.9 cm × 6 cm), that allowed minimal movement of the animal. The tube had holes along the top to allow for the exchange of chemosensory (e.g., pheromonal) cues between the animal in the tube and the test animal placed in the aquarium (76.2 cm × 33 cm × 33 cm). The tube was placed on top of corncob bedding in one end of the aquarium with each of the 24 males placed at the opposite end of the tank during individual testing sessions (see **Figure 2A**). The stimulus males were rotated after every third session to avoid fatigue in the tube and to assure that each group was exposed to multiple stimulus males. The duration of the task was 7 min during which time a variety of behaviors were observed to assess social interest (i.e., latency to approach the tube, duration and frequency of tube investigation, frequency of tube manipulations (including digging around the tube, climbing on top of the tube and either touching and/or biting the tube), frequency of escape attempts, frequency of self-grooming bouts, frequency of sniffing bouts and duration of time spent in proximity (within 4 cm) of the tube).

A few days after the social investigation task, each of the 24 animals was also assessed in a problem-solving escape task involving the presence of multiple predator stimuli. For this assessment, rats were placed in an aquarium (76.2 cm × 33 cm × 33 cm) with corncob bedding and a partition in the middle of the tank that extended from the top of the tank to about 7 cm above the floor. During the habituation phase of training, animals had 5 min to explore the entire tank by easily walking under the partition to travel to the other side. During the test on the subsequent day, bedding was poured in the tank so that the space between the barrier and the floor was not apparent, requiring the rat to burrow through the bedding, under the partition, if they wanted to travel to the opposite side of the tank (see **Figure 2B**). During the problem-solving test, each animal was placed on one side of the partitioned aquarium that included cat urine and hair. Behaviors recorded during this 5-min test included: latency to burrow and dig under the partition and the frequency of digging bouts. All individual behavioral tasks were videotaped and coded so that each animal's behavior could be subsequently analyzed by an observer blind to group assignment.

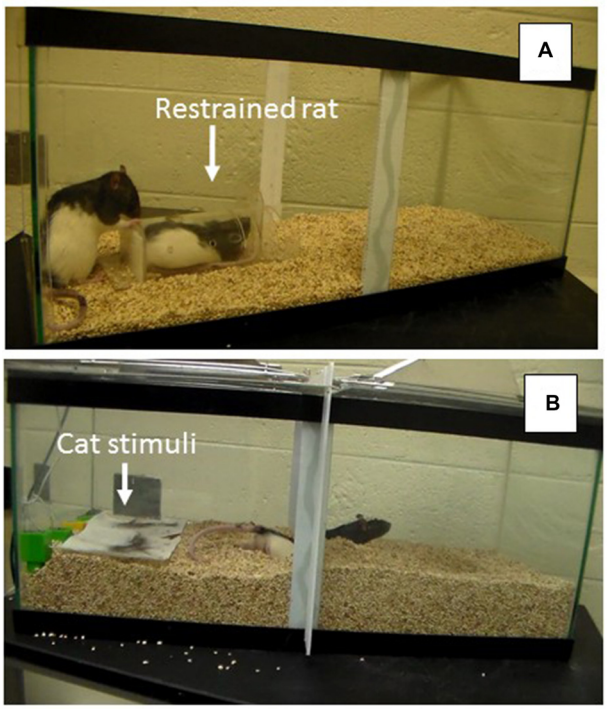

FIGURE 2 | (A) Social Interaction Task depicting restrained rat in container and experimental rat exploring the container. **(B)** Problem-Solving Task depicting experimental rat escaping from the predator odor compartment by burrowing under the partition to reach the other side of the arena.

Endocrine Assessments

To assess CORT and OT levels, blood samples were obtained prior to the perfusion of fixative solution through the cardiovascular system. Specifically, animals were anesthetized by exposure to 1 mL of Halothane liquid (Sigma-Aldrich; St. Louis, MO, USA) until respiratory rate slowed and were then given an intraperitoneal injection of 0.2 mL sodium pentobarbital at an overdose of 50 mg/Kg. Before the heart stopped beating, a needle was placed into the left ventricle and blood was slowly removed and stored in a −80°C freezer until the assays were conducted.

To assay for OT and CORT levels in blood samples, commercial kits were used (acquired from Enzo Life Sciences, Farmingdale, NY, USA). Before the assays, hormones were extracted by adding 1 mL of diethyl ether to each sample and then mixing the contents in glass tubes. Tubes were allowed to settle for about 30 min and then the aqueous phase was removed by freezing the samples at −80°C for a few minutes and then pouring the solution in a new tube. Subsequently, the ether was evaporated by placing the new tubes into a 42°C water bath inside a fume hood. The

samples were reconstituted with the addition of assay buffer at the correct dilution for each hormone. The selected dilution was 1:20 for OT and 1:50 for CORT. Samples, controls and standards were prepared in duplicates and added to the appropriate wells of the assay kits, following the instructions provided in the kits. Sample readings were completed using an automated microplate reader (BioTek, Winooski, VT, model Synergy) and Gen5 software (BioTek, Winooski, VT, version 2.04.11). Readings were assessed at a wavelength of 405 λ with correction at 490 λ. Data were calculated using log-logit transformations of the absorbance values recorded from the reader, and analyzed by least-squared regression analysis. Accuracy was demonstrated at each standard curve point: all readings off the 5% threshold value from the standard curve were discarded. Quality control pools were assayed in triplicate on each plate. The sensitivity of the assays, as reported by the manufacturer, was 26.5 pg/mL for OT and 171 pg/mL for CORT.

Histological Preparation

Following blood collection, animals were transcardially perfused at 40 mL/min using a MasterFlex L/S perfusion pump with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Following extraction, brains were post-fixed overnight with 4% paraformaldehyde at 4°C, then transferred to 10% sucrose solution for 24 h at 4°C followed by 20% sucrose at 4°C and finally into 30% sucrose at 4°C until time of sectioning. Brains were sectioned using a HM525 Microm cryostat in the anterior region (plate 46, Paxinos and Watcon, 2007) for assessment of the paraventricular and supraoptic nuclei (PVN and SON) of hypothalamus, as well as medial forebrain bundle (MFB) region. Six free-floating sections (40 μm) were collected, placed in PBS and prepared for immunohistochemistry.

For OT immunoreactivity assessment of the PVN, SON and the MFB, sections were washed in PBS to remove excess sucrose and paraformaldehyde. Sections were then incubated in 0.3% hydrogen peroxide for 10 min. Subsequently, the sections were blocked with 10% normal goat serum (Vector, Burlingame, CA, USA) in PBS-BT (BSA, Vector; Triton-X 100, Spectrum Chemical: Cardena, CA, USA) for 60 min before being incubated in the OT primary antibody (1:4,000 dilution, Immunostar, Inc., Hudson WI, USA) for 48 h at 4°C. Sections were subsequently washed in PBS-BT then exposed to the biotinylated secondary antibody for 90 min (goat anti-rabbit; 1:200 dilution, Vector). Following incubation, sections were processed with an Elite Vecastatin ABC kit (Vector). Finally, sections were visualized with DAB peroxidase substrate and then cleared through a series of 70, 95 and 100% ethanol followed by Citrasolv (Fisher Scientific, Fair Lawn, NJ, USA) and coverslipped with permount (Fisher Scientific).

Neural Quantification

Prior to being analyzed all slides were recoded to ensure experimenters would be blind to experimental conditions. A BA400 light microscope (Miotic, Richmond, BC, Canada) was used for neuroquantification. To assess the area of OT immunoreactivity, cell bodies and fibers were thresholded using a 135 × 135 μm area at 40× magnification. The percent area stained was determined using light-thresholding software (Bioquant Life Sciences, Nashville, TN, USA).

Statistical Analysis

To assess the combined effects of variables related to specific system outputs (oxytocin-immunoreactive (OT-ir) in different brain areas, and the peripheral levels of OT and CORT), MANOVA was used to test the overall effects of housing conditions (three levels: ISO, ES and CS groups). For the individual behavioral tasks, ANOVA was used to determine the effect of each of the three housing conditions on the behavioral output. The significance value for each analysis was set at $p = 0.05$. Following the analyses of variance, appropriate Tukey *post hoc* tests were conducted to identify the treatment group(s) responsible for the variation. During the dark phase observations in the control and enriched animals, the presence or absence of play behavior, self-grooming and social-grooming was recorded every 30 s for 1 h for three consecutive nights. Thus, a total of 360 data points were collected for each behavior. The marginal frequencies for binary output (presence/absence of focal behavior) was calculated for each of the three behavioral categories and compared among the three treatment groups. The likelihood ratio was used to determine if the frequencies in the two groups were significantly different.

To model the independent effects of housing conditions on each of the dependent variables, a non-parametric, ALSCAL, multi-dimensional scaling (MDS) model was used. It is important to remember that the main advantage of multivariate models is that the single contribution of each of the measures included in the analysis is independent of the other variable contributions. In other words, even if in itself a single measure appears to be higher in any given treatment condition, when the shared variance is partitioned out, the remaining individual contributions can provide a very different picture than provided by the initial mean values for a particular dependent measure. This is why multivariate models are essential in establishing associations in complex phenomena in which many different systems contribute to the final output. Generally speaking, MDS is a technique used to uncover the "hidden structure" to a set of data (Kruskal and Wish, 1978). To accomplish this, MDS generates graphical models that provide a spatial representation of the similarity structure of variables. Using the matrix of covariation among all the measures entered in the model, the relationships among variables can be displayed graphically. In order to map all of the variables into a desired space (two dimensional or greater), a certain lack of fit, referred to as the s-stress, is inevitable. The values of s-stress range from 0 (perfect fit) to 1 (worst possible fit). Thus, the aim of MDS is to find a map of the variables that minimizes the s-stress for a given number of dimensions. Kruskal's s-stress values <0.15 are typically deemed acceptable, and below 0.1 indicates an excellent fit. Additionally, a good model needs to explain most of the variance present in the original data, which is expressed by the R^2 value in the model. Typically, R^2 values of 0.8 or higher are desirable, with values above 0.9 considered to be excellent.

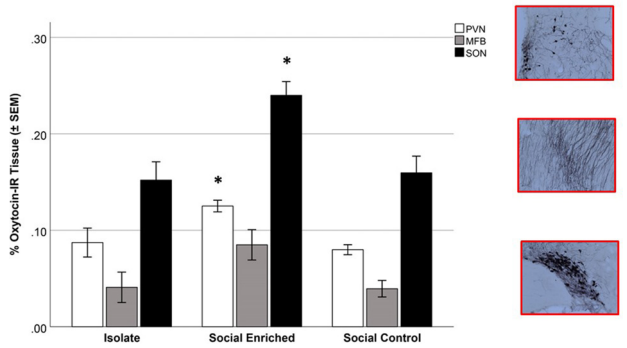

FIGURE 3 | Oxytocin-immunoreactive (OT-ir) tissue in isolate (ISO), social enriched (SE) and social control (SC) animals in the paraventricular nucleus of the hypothalamus (PVN), supraoptic nucleus (SON), and medial forebrain bundle (MFB). In the PVN and SON areas, the SE animals had higher OT-ir measures than the ISO and SC groups ($p < 0.05$ for each area). No significant differences were observed in the MFB area. *Indicates significant difference from ISO and SC animals in comparable brain areas.

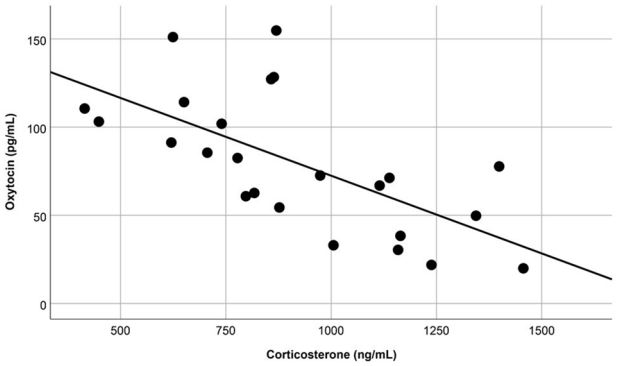

FIGURE 4 | Blood samples revealed that, regardless of treatment group, peripheral OT and corticosterone (CORT) levels were inversely related ($p = 0.01$).

RESULTS

Neuroendocrine Results

The overall OT-ir in all brain areas examined was significantly different among the three groups (Hotelling's Trace = 0.972; $F_{(6,36)} = 2.91$; $p = 0.020$). Specifically, SE animals had higher OT-ir than both SC and ISO groups in the PVN and SON (Tukey *post hoc* test *p*-values < 0.032), whereas no significant difference was found in the MFB (all p = values > 0.073; **Figure 3**). Additionally, peripheral OT and CORT levels were inversely related ($r = -0.65$, $n = 24$, $p = 0.01$ (**Figure 4**). The overall peripheral OT activity was significantly different among the three groups (Hotelling's Trace = 5.064; $F_{(4,38)} = 24.05$; $p < 0.001$). Specifically, ISO animals had the highest OT levels, whereas SC animals had the lowest (all Tukey *post hoc* test *p*-values < 0.001; **Figure 5A**). Conversely, ISO animals had significantly lower CORT levels than both SE and SC animals (all Tukey *post hoc* test *p*-values < 0.038). There was no significant difference between the SE and SC groups (Tukey *post hoc* test $p = 0.279$; **Figure 5B**).

Behavioral Tasks

During the Social Investigation Task, the latency to approach the tube and the frequency of total interactions with the tube (bite, paw, climb) were not significantly different by group (Latency: $F_{(2,21)} = 2.36$; $p = 0.119$; Interaction: $F_{(2,21)} = 0.85$; $p = 0.441$), but the frequency of sniffing the tube was higher in ISO animals ($F_{(2,21)} = 5.52$; $p = 0.006$; **Figure 6A**). Focusing on the additional behaviors, SE animals had the lowest number of escape attempts ($F_{(2,21)} = 7.95$; $p = 0.003$; **Figure 6B**) and the highest number of digging bouts directed toward the tube ($F_{(2,21)} = 9.00$; $p = 0.001$; **Figure 6C**).

During the Predator Stimuli Escape Task, the latency to escape from the side of the apparatus containing the predator stimuli to the other side was significantly different among the three groups ($F_{(2,21)} = 5.95$; $p = 0.009$; **Figure 6D**). Tukey *post hoc* tests indicated that the SE group was faster to escape than the SC group ($p = 0.008$), but there was no significant difference with ISO animals ($p = 0.069$). The number of digging bouts during the same task was not significantly different among the three groups ($F_{(2,21)} = 1.91$; $p = 0.173$).

Dark Phase Observations

The marginal frequencies for binary output (presence/absence) of play behavior, self-grooming and social grooming were recorded during the dark phase observations (**Table 1**). No significant differences were found between the SE and SC groups for play behavior (Likelihood ratio = 0.139, $p = 0.709$) and for self-grooming (Likelihood ratio = 1.639, $p = 0.201$), but there was a significant difference observed in the social

TABLE 1 | Marginal frequency cross tabulations.

		Play SC		
Count		**Absence**	**Presence**	**Total**
Play SE	Absence	197	58	255
	Presence	83	22	105
Total		280	80	360
	Value	**df**	***p*-value**	
Likelihood Ratio	0.139	1	0.709	

		SelfG SC		
Count		**Absence**	**Presence**	**Total**
SelfG SE	Absence	71	94	165
	Presence	71	124	195
Total		142	218	360
	Value	**df**	***p*-value**	
Likelihood Ratio	1.639	1	0.201	

		SG SC		
Count		**Absence**	**Presence**	**Total**
SG SE	Absence	272	19	291
	Presence	64	5	69
Total		336	24	360
	Value	**df**	***p*-value**	
Likelihood Ratio	3.851	1	0.048	

Numbers represent counts of behavior (absence / presence) in two groups: Social Control (SC) and Social Enriched (SE) during the night observation of spontaneous social behavior. Play, play behavior; SelfG, self-grooming; SG, social grooming.

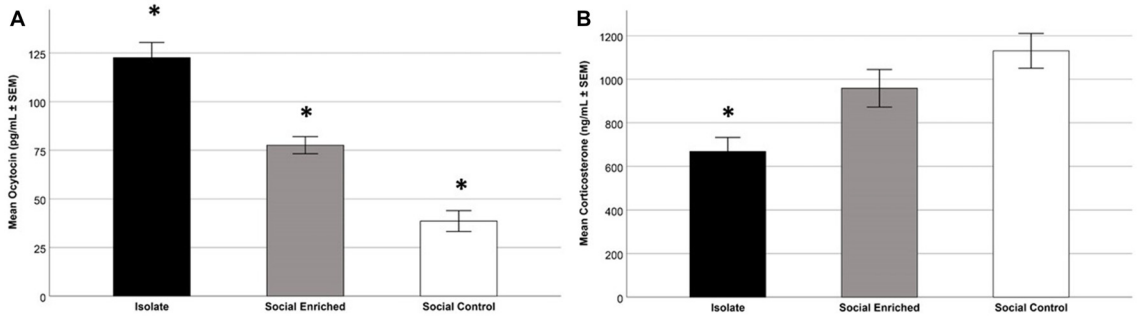

FIGURE 5 | Mean plasma OT levels in the three treatment groups; specifically, the ISO animals had higher levels than the other groups and the SE animals had higher levels than the SC (SE) animals ($p < 0.001$; **A**). Focusing on mean plasma CORT levels in the three treatment groups; as depicted, the ISO animals had lower levels than the other groups ($p < 0.05$; **B**). *Indicates significant difference from other groups.

grooming data; specifically, individuals in the SE condition were approximately four times more likely to engage in social grooming than individuals in the SC housing condition (Likelihood ratio = 3.851, $p = 0.048$).

Integrative Multivariate Model

We mapped the multivariate, independent association among all the significant measures assessing different system outputs (neural, endocrine and behavioral) using a MDS model. The map, provided in **Figure 7**, clearly indicated that housing conditions modified the system outputs to such an extent that we were able to discriminate efficiently among the individual subjects. Discrimination rate reached a perfect 100%, since no individuals with different housing conditions were clustered together. Moreover, both measures of accuracy for the MDS model indicated an excellent fit (Kruskal's stress index = 0.062; $R^2 = 0.97$). The two dimensions created by the MDS model, both linear combinations of the dependent variables entered in the model, were named *Social Activation* (dimension 1) and *Stress Response* (dimension 2). It is important to point out

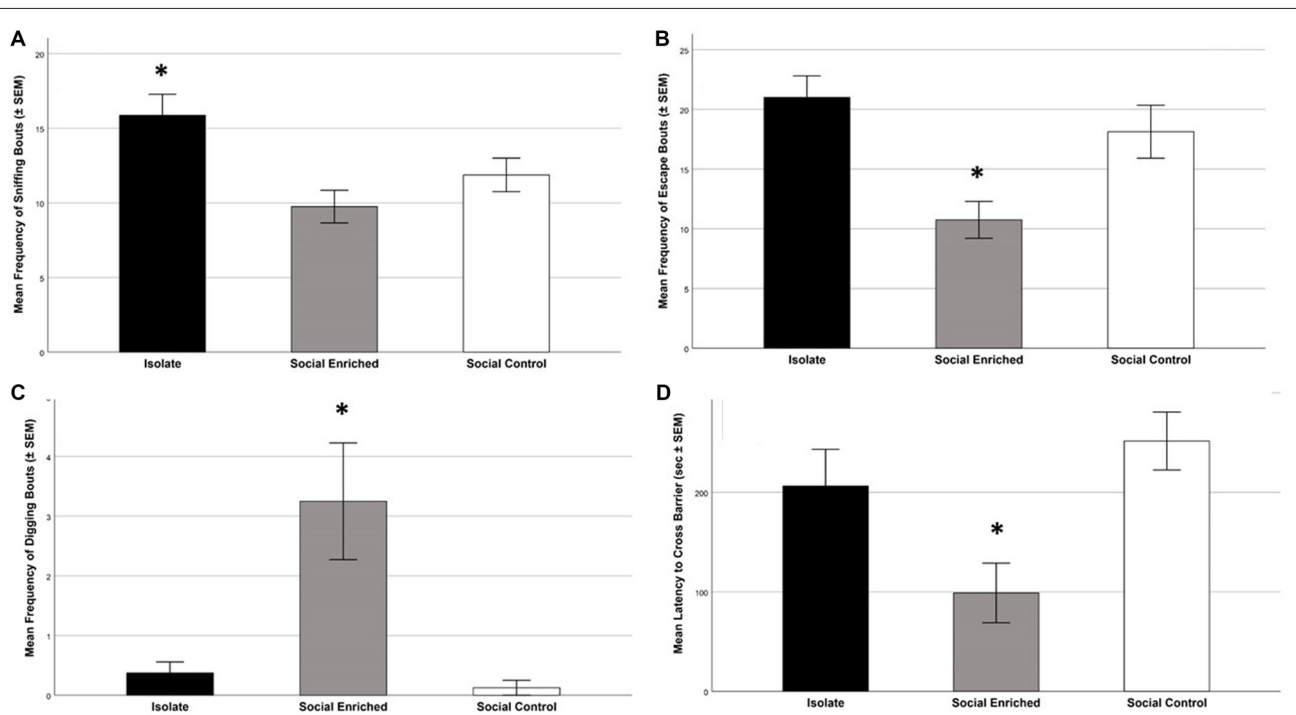

FIGURE 6 | The behavioral tasks revealed higher sniffing bouts in the ISO animals than the other groups (**A**; $p = 0.006$); further the SE animals had lower escape frequencies than the other groups ($p = 0.003$; **B**) and higher frequencies of digging bouts ($p = 0.001$; **C**). Focusing on the problem-solving digging task, the SE animals had a lower latency to cross the barrier than the SC group ($p = 008$; **D**). *Indicates significant difference from other groups in **(A–C)**; *indicates significantly different from SC group in **(D)**.

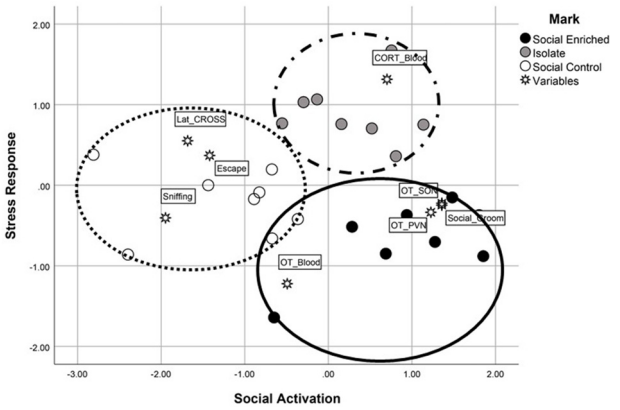

FIGURE 7 | The multidimensional scaling analysis, an additional statistical evaluation analysis, revealed high discrimination among the three groups. Using the two linear dimensions of *social activation* and *stress responsiveness*, the SE animals were characterized by higher social activation and lower stress responsiveness (e.g., higher OT-immunoreactivity, social grooming) whereas the SC animals were characterized by lower social activation responses (e.g., higher number of escape attempts in the social investigation task) and higher stress responsivity (e.g., longer latency to cross the barrier in the problem-solving task). The ISO animals were limited in behavioral responses due to their lack of cagemates during the dark observation phase; however, when the shared variance was subtracted from the scores, their plasma CORT levels placed them high along the stress responsiveness dimension.

that the ISO animals didn't have the full scale of behaviors to contribute to the analysis due to their restricted housing environment (i.e., behaviors in their home cages during the dark phase were not recorded since they were ISO housed, a condition that was necessary for the experimental design of the current study). Animals exposed to the SE environment were characterized by a higher OT-ir activity in both the PVN and SON areas, as well as a higher probability to engage in social grooming during the dark-phase observations. Alternatively, SC animals were characterized by higher levels of sniffing and escaping during both the Social Investigation task and Predator Stimuli task. Finally, although it was unexpected considering the MANOVA results, ISO animals were characterized by higher levels of stress arousal—after removing the partial effects shared with the other measurements (see **Figure 7**).

DISCUSSION

In the current study, animals were housed in habitats designed to yield varying amounts of social engagement (i.e., isolate-housed (ISO; no social contact); social-control (SC; moderate social contact) and contact) and social-enriched (SE; high social contact)) to explore the effects of environmental variables on social- and predator-stress responsiveness. In general, the results corroborate and extend previous research investigating social behavior in enriched environments by exploring interactions between physical and social stimuli used in the enriched environment investigations, as well as potential neurobiological correlates. As observed previously in our laboratory, animals housed in enriched environments containing natural elements demonstrated seemingly adaptive responses in the predator escape task (Lambert et al., 2016). The social interaction task used in the current study revealed that SE animals demonstrated more digging directed toward the stimulus animal, as well as fewer attempts to escape, whereas the ISO animals engaged in more exploratory sniffing. Collectively, these responses indicated that the SE animals directed more attention toward the stimulus animal in the restraint tube whereas the ISO animals directed their attention away from the distressed animal. Increased social/affiliative contact in the form of social grooming, but not social play, was observed in the SE animals during the dark phase. Neurobiological data revealed increased OT immunoreactivity in the SE animals; however, contrary to the hypothesized results, plasma levels of OT were highest in the ISO animals during the last phase of varied habitat exposures. Further, plasma CORT was lowest in the ISO animals at this time, an effect attributed to the unexpected role of separation anxiety during the procedure of separating animals for the histological/perfusion process. Beyond the MANOVA and ANOVA results, the MDS analysis suggests that the SE environment was also associated with a less responsive HPA axis, or CORT levels when shared variance among the dependent variables was accounted for in the data set. The relevance of these findings, along with potential contributing factors, are discussed below.

Focusing on social behavior, the results are in alignment with the initial hypothesis that the SE environment would generate the highest levels of affiliative social behavior. During the dark phase observations of spontaneous behavior, several responses, including social play, self-grooming and social-grooming, were observed. Interestingly, the SE animals exhibited an approximately 350% increase in social grooming when compared to SC animals. In agreement with Renner and Rosenzweig (1986), the SE habitat did not affect bouts of rough-and-tumble play behavior. In contrast to the current results, Renner and Rosenzweig failed to find enriched-induced increases in grooming interactions. Based on previous findings in our laboratory, it is likely that the heightened social interactions in the current study were due to the natural elements in the enriched habitat (Lambert et al., 2015, 2016; Bardi et al., 2016). Even so, further research with both natural and artificial enriched environments will further elucidate the role of natural elements in social facilitation in enriched environments.

Behaviors observed in the social interaction task corroborated the dark phase observations. When a conspecific was placed in a Plexiglass tube, ISO rats exhibited behavior directed away from the conspecific (e.g., sniffing, trying to escape) whereas the SE animals exhibited more digging bouts directed toward the conspecific in the tube. Thus, the SE animals exhibited more interest in the animal than the habitat, evidence of increased social attentiveness. The observation that the SC animals scored in an intermediate range in the sniffing and escape responses suggests a potential additive effect of the enrichment to the traditional social housing, as previously described in animals exposed to both running

exercise and enriched environments (Fabel et al., 2009). This heightened social interest may also be influenced by the previously described social facilitation effect proposed to explain the animals' engagement in enriched environments; specifically, that animals direct their attention to other animals in the SE environment, resulting in increased attentiveness to the behavioral responses of conspecifics (Thorpe, 1963).

The neuropeptide OT has been implicated in the formation of social bonds, in addition to other important physiological functions such as lactation and parturition (Carter, 1998; Nelson and Panksepp, 1998). OT is produced in the supraoptic and paraventricular hypothalamic nuclei with pervasive projections throughout the brain, including limbic, diencephalon, mesencephalon, brainstem and spinal cord areas of the rodent brain (Sofroniew, 1983). The extent of OT binding in the limbic system of humans appears to be less extensive than rodent observations; additionally, increased binding has been observed in the basal forebrain and substantia nigra in the human brain (Stevens et al., 2013). Because the roles of central and peripheral OT are still being evaluated, it is important to consider both sources when possible (Gordon et al., 2010), as was done in the current study. Interestingly, very different results were observed with these two measures. The central measures indicated, as hypothesized, more OT-immunoreactivity in the PVN and SON in the SE groups than the other groups. However, the highest peripheral levels were observed in the ISO group. Although not anticipated as an influence, it is likely that this effect is due to the protocol used during the perfusion/blood collection process. During this procedure, animals were maintained in isolation for a few minutes prior to anesthesia. Although being in a novel environment prior to the procedure was recognized as a potential stressor for all animals; in retrospect, the two social-housed groups likely experienced unintended stress in the form of separation anxiety once they were removed from their cagemates. Thus, during this brief time prior to the onset of the anesthesia, the ISO animals may have experienced less stress than their social-housed counterparts. The peripheral CORT data suggest that the ISO animals were less stressed at that time (i.e., had lower CORT levels). This inverse relationship between peripheral OT and plasma cortisol levels has been previously observed in humans (Heinrichs et al., 2003). If the social housed animals experienced separation anxiety, then OT levels were likely affected since separation and attachment anxiety have been associated with low OT levels (Eapen et al., 2014). Focusing on CORT, however, past research suggests that, following early life isolation, single-housed male rats have lower CORT levels during recovery from stress than group-housed conspecifics (Lukkes et al., 2009). Further, dynamic social hierarchies in group housed rodents have been associated with higher CORT levels than observed in ISO-housed animals (Bronson, 1973). Although no aggressive social interactions were observed in group-housed animals in the current study, it is likely that individual differences existed due to the animals' established social hierarchies (Beery and Kaufer, 2015). Regardless of the cause of the observed endocrine effects, these observations serve as a valuable reminder of the influence of social housing in laboratory animals at every stage of the experimental period.

Focusing on the central OT effects in the current study, the results indicated an interesting effect of enriched environments on central OT responsivity. A variation of this effect was observed in a previous study in which maternal rats housed in an enriched environment and exhibiting low-licking and grooming behavior resulted in their pups developing enhanced OT receptor binding than the pups raised by comparable mothers in a standard environment (Champagne and Meaney, 2007). Additionally, an effect of a limited enriched environment was observed in rats when exposure of single-housed animals to a nestlet (for nest building) accelerated wound healing in a pattern that was similar to the administration of OT (Vitalo et al., 2009). In the current study, the results are unique in that heightened measures of central OT and accompanying social behavior were observed in animals exposed to an enriched environment. Beyond the facilitation of social bonding, OT has also been implicated in the influence of experience-dependent, cross modal sensory experiences on the subsequent development of sensory cortical areas (Zheng et al., 2014). Accordingly, heightened engagement with both physical and social stimuli observed in the SE group may have been mediated through the oxytonergic system. Further, the increased OT-immunoreactivity observed in the SE animals may have had additional effects on the sensory cortical development of the animals. Expanding on the findings of increased OT immunoreactivity observed in the PVN of the hypothalamus in animals exposed to enriched environments, past research has confirmed that sensory deprivation leads to reduced OT-positive neurons in the same brain area (Zheng et al., 2014).

As the current study, and prior investigations in our lab, have confirmed, the addition of natural elements in the laboratory enriched environment habitats may provide valuable information about neural sensory-integration and healthy neural development (Bardi et al., 2016; Lambert et al., 2016). Because sensory integration therapies represent a common therapeutic approach for the treatment of neurodevelopment disorders (Green et al., 2006), a thorough analysis of cross-modality environmental interactions in preclinical models offers an opportunity to explore mechanisms in which complex environments facilitate the development of healthy brains. In contrast to enhanced sensory-integration, disruptions of sensory and motor development have been observed in several neurodevelopmental disorders such as Autism Spectrum Disorders (Reynolds et al., 2010). Thus, enriched environments, such as the natural enriched environments that heighten both physical and social interactions, provide a valuable opportunity to explore mechanisms leading to symptoms associated with neurodevelopmental disorders. Further, the timing and associated expectations of environmental changes may be relevant; for example, rats in a valproic acid model of enhanced vulnerability of autism disorders were less likely to develop hyper-emotionality symptoms if they were exposed to a predictable enriched environment as opposed to an unpredictable enriched environment (Favre et al., 2015). Because

the focus of the current study was the opportunity for social interactions, the natural-enriched environment was utilized based on previously described results indicating heightened social interactions in our laboratory (Bardi et al., 2016; Lambert et al., 2016); however, the continued use of comparable artificial environments are necessary to determine specific differences between the natural and artificial elements of the environments.

The rich accumulation of data accrued from the vast array of research investigating various aspects of enriched environments has persistently demonstrated neurobehavioral effects, especially related to cognitive effects and cortical neuroplasticity (Juraska et al., 1989). In the current study, the enriched environment, perhaps due to the utilization of natural stimuli, provided an environment that produced enhanced OT-related functions in comparison to the laboratory standard and control group-housed animals. In addition to enhancement of neural processes, research has also consistently indicated that enriched environments offer protection against the onset of disorders of the nervous system such as Alzheimer's Disease, Parkinson's Disease, amyotrophic lateral sclerosis (ALS), as well as enhanced recovery from brain trauma (Nithianantharajah and Hannon, 2006). Further, enriched environments have also ameliorated symptoms of psychiatric illnesses such as depression and addiction in rodent models (Puhl et al., 2012; Richter et al., 2013; Grippo et al., 2014). Increased social interactions observed in the SE group during the spontaneous dark phase observations and the social investigation task were associated with increased OT-positive cells in the hypothalamus, OT cells that may play a role in cross modal sensory integration important for healthy brain functions. One limitation of the current study, however, is the use of only male animals. Because sex-dependent effects of the enriched environments have been previously observed, future research should consider both males and females to obtain a more comprehensive evaluation of the effects of social enhanced environments (Kolb et al., 2003; Bakos et al., 2009). Looking to the future, urther research is necessary to characterize maximal engagement with the environment to more fully understand the impact of complex environments on adaptive neural functions as well as enhance the translational value of these studies.

AUTHOR CONTRIBUTIONS

KL designed the study, supervised data collection and prepared the manuscript. SN contributed to the study design, collected data and contributed to writing the manuscript. MK supervised data collection, managed the data files and contributed to writing the manuscript. MB contributed to endocrine data collection, statistical analysis and writing of the manuscript.

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frontiers in Behavioral Neuroscience

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Loss of Environmental Enrichment Elicits Behavioral and Physiological Dysregulation in Female Rats

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Chronic stress drives behavioral and physiological changes associated with numerous psychiatric disease states. In rodents, the vast majority of chronic stress models involve imposition of external stressors, whereas in humans stress is often driven by internal cues, commonly associated with a sense of loss. We previously exposed groups of rats to environmental enrichment (EE) for a protracted period (1 month), followed by removal of enrichment (ER), to induce an experience of loss in male rats. ER enhanced immobility in the forced swim test (FST), led to hypothalamic pituitary adrenal (HPA) axis hypoactivity, and caused hyperphagia relative to continuously enriched (EE), single-housed (Scon) and pair-housed (Pcon) groups, most of which were reversible by antidepressant treatment (Smith et al., 2017). Here, we have applied the same approach to study enrichment loss in female rats. Similar to the males, enrichment removal in females led to an increase in the time spent immobile in the FST and increased daytime food intake compared to the single and pair-housed controls. Unlike males, ER females showed decreased sucrose preference, and showed estrus cycle-dependent HPA axis hyperactivity to an acute restraint stress. The increase in passive coping (immobility), anhedonia-like behavior in the sucrose preference test and HPA axis dysregulation suggest that enrichment removal produces a loss phenotype in females that differs from that seen in males, which may be more pronounced in nature.

Keywords: anhedonia, coping behavior, corticosterone, estrus cycle, sex differences, stress

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INTRODUCTION

The experience of loss represents a significant risk factor for affective distress and disease. Life events connected with loss of financial resources, loved ones, relationships or liberties are among the most often reported experiences linked to negative mental health outcomes, prominently including depression and anxiety disorders (Rahe, 1968; Ganzini et al., 1990; Sikorski et al., 2014; Wang et al., 2015; Wiseman et al., 2015). However, there have been relatively few studies focusing on neurobiological mechanisms underlying the loss of positively rewarding life experiences.

Our lab has recently developed an enrichment removal model for induction of lasting behavioral and physiological responses to loss. In males, prolonged exposure to enrichment followed by removal to single housing results in reproducible increases in passive coping behavior, weight gain, and hypothalamic-pituitary-adrenocortical (HPA) axis hypoactivity that is not emulated by single housing alone, chronic restraint exposure or chronic variable stress, indicating that the behavioral and somatic phenotype is unique to experiencing cessation of enrichment. Moreover,

antidepressant treatment prevented the symptoms of enrichment removal, suggesting a linkage to biological/emotional processes regulated by this class of drug (Smith et al., 2017).

Work on the fundamental biology of stress indicates massive sex differences in behavioral and physiological endpoints. While the field is not in universal agreement on the nature and extent of sex differences, in general work suggests that chronic imposed stress (e.g., chronic mild stress, chronic variable stress, chronic social defeat) reliably affects coping behavior [e.g., increased immobility in the forced swim test (FST)], anhedonia and physiology (e.g., increased HPA axis reactivity) (Willner et al., 1987; Herman et al., 1995; Rygula et al., 2005, 2008; Kompagne et al., 2008). There is some evidence to suggest that females are more vulnerable to chronic mild stress, as demonstrated by decreased sucrose preference, increased immobility in the FST, decreased open field activity and more pronounced HPA axis responses to stress (Dalla et al., 2005; Xing et al., 2013; Lu et al., 2015; Rincon-Cortes and Grace, 2017). The enhanced stress phenotype seen in rats is in keeping with human reports indicating a nearly 2:1 incidence of stress-related diseases (depression, PTSD) in females relative to males (Kessler et al., 1993; Olff et al., 2007), suggesting that being female is a significant risk factor. Consequently, it is important to understand the impact of experience of loss on behavioral and physiological reactivity in the female. Therefore, the current study explored the impact of ER in females in light of what we know from males, focusing on behavioral endpoints showing the most consistent and pronounced male phenotypes. Our data suggest that while males and females share core features of enrichment removal, others (e.g., HPA axis reactivity and sucrose preference) differ substantially.

MATERIALS AND METHODS

Animals

Eighty female Sprague-Dawley rats, weighing between 180–220 g, were obtained from Harlan (Indianapolis, IN, United States) at approximately 9 weeks of age. Upon arrival, rats were randomly assigned to a housing manipulation and cohort. From the eighty rats, we created two cohorts, with each cohort containing forty rats. All rats were housed with corncob bedding and *ad libitum* water and chow (3.41 kcal/g, 0.51 kcal/g from fat; Harlan Teklad, Madison, WI, United States). The vivarium had a 12 h light:dark cycle (9am lights on, 9pm lights off) and was temperature (23°C) and humidity (50%) controlled. All procedures were conducted in compliance with the National Institutes of Health guidelines for the Care and Use of Animals, and were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Experimental Groups

Animals were divided into two cohorts to optimize behavioral testing (see **Supplementary Table S1**). Decreasing the number of animals tested per day allowed for the testing time to be done closer to the circadian corticosterone trough and thus minimizing the fluctuations in diurnal hormone levels at the time of testing (Atkinson and Waddell, 1997) Cohort 1 experienced 5 days of acclimation to the vivarium, and cohort 2 experienced 7 days. Each cohort underwent the same experimental timeline and protocols 2 days apart (**Figure 1**). Animals received housing manipulations during the 12 h dark (active) cycle only (Smith et al., 2017). Housing manipulations began for cohort 1 at lights out of day 5 and began 2 days later for cohort 2 at lights out of day 7. Each cohort consisted of four treatment groups, with treatment (housing manipulation) occurring during the dark (active) cycle only. Groups included single-housed animals (Scon n = 10 per cohort, n = 20 total); pair-housed control animals (Pcon n = 10 per cohort, n = 20 total); continuously enriched animals (EE n = 10 per cohort, n = 20 total); and enrichment-removed animals (ER n = 10 per cohort, n = 20 total). With the exception of the enrichment-removed group, all active cycle housing manipulations continued until the end of the experiment (**Figure 1**). Animals removed from enrichment were removed after 4 weeks. ER animals were given a week of single-housing during active cycle before behavioral testing began, to ensure a phenotype had time to develop (Smith et al., 2017).

For all experiments, the Pcon group serves as the control group for comparison of ER, EE and Scon manipulations. The Scon group evaluates the impact of isolation alone on the noted experimental endpoints.

Housing Manipulations

Standard housing for all rats during the inactive cycle was single-housing (home cage), which consisted of one rat per polycarbonate shoebox cage (20 cm H × 22 cm W × 43 cm L). Use of a home cage during the inactive cycle allowed for data collection from individuals. Animals that were single-housed during the active cycle remained in home cages but were handled briefly twice daily at the time when other animals were moving between housing environments to account for the stress of cage disruption (Sharp et al., 2003) and interactions with the researcher. Animals in the pair housed group were only pair housed during the active period (lights off), and the pair housing was done in a cage that was not the home cage of either animal in the pair. Cages used for pair housing were identical to the home cage. Cage mates remained constant throughout the experiment. Animals experiencing enrichment were moved to enrichment cages within a separate room during the dark cycle and received crinkle paper in the home cage when single-housed during the light cycle. 1 h before lights out and within 1 h of lights being on, all animals were handled in order to both transfer cages and to habituate the animals to interactions with the researcher.

Four enrichment cages (1 m H × 1 m W × 1 m L) were adorned with a large metal feeding bowl, three standard water bottles, and corncob bedding. One group (n = 10) was housed

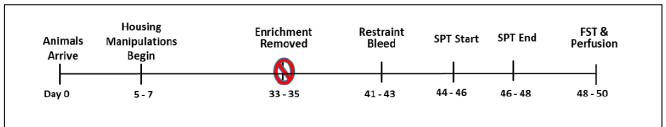

FIGURE 1 | Experimental time-line.

per enrichment cage. Each cage received 3–5 novel objects in addition to crinkle paper, metal ladders, and plastic huts. The interchanging enrichment objects consisted of: whiffle balls, Nyla bones, colored plastic rectangular tubes, colorful plastic key chains, plastic cones, cardboard huts of various shapes, nestlets, colored plastic bowls and cups, and plastic exercise balls. Objects in the enrichment cages were changed every 7 days when cages were cleaned. The enriched groups both received 28 days of enrichment. The enriched removal group was then removed from the enrichment cycle and single-housed 24 h/day with no crinkle paper in the home cage. The continuously enriched group continued enrichment for 14 more days, until sacrifice.

The issue of active cycle enrichment was addressed in our prior study, conducted in males (Smith et al., 2017). In this study we verified that active cycle enrichment replicated the stress profiles of EE and ER groups exposed to continuous enrichment. These data suggest that the physiological and behavioral effects of EE and ER can be replicated in this paradigm. In addition, the inactive cycle is the period of time where the animals are normally sleeping, and thus not likely to experience a negative impact of the lack of enrichment.

Food Intake and Body Weight

Immediately before housing manipulations began, body weight was recorded and used as a standard to calculate percent change from initial body weight for the duration of the study. Throughout experimentation, body weight was recorded every 7 days. Daily food and water intake, during the inactive period only, was measured for 1 week prior to and for 2 weeks following the enrichment removal.

Restraint and Blood Sampling

The acute restraint stress test began 1 h into the light cycle near the circadian trough of corticosterone secretion, 7 days post-removal. Rats were placed inside well-ventilated, clear Plexiglas® tubes for 30 min (6.35 cm inner diameter and 20.5 cm length). While in the restrainers, animals were video recorded for later analysis of struggling behavior as an index of helplessness (Atkinson and Waddell, 1997; Sharp et al., 2003). Parameters measured include active struggling; grooming; freezing (noticeable tensing); and still behavior (lack of movement, no noticeable tensing). For assessment of the HPA axis response to acute stress, blood collection was as follows: An animal was placed into the restraint tube and blood was collected by tail clip within 3 min from the initial disruption of the home cage for time 0. The initial tail clip is done by removing 1–2 mm of the distal portion of the tail with a razor blade. Blood samples (approximately 250 (μL) were collected from tail clips by milking blood from into a microcentrifuge tube containing 10 microliters of 100 mM EDTA, and were immediately placed on ice. This method of tail clipping provides for blood collection to occur at all time points without additional tail clipping to be required. At 15, 30, 60, and 120 min from the start of the restraint, blood was also collected. After blood was collected for the 30-min time point, the animal was removed from the restraint and placed back into the home cage. At time points 60 and 120 min, the animal was removed from the home cage and blood samples obtained by gently removing the clot while freely moving. All samples were collected within 3 min. Samples were centrifuged at 3000 × *g* for 15 min at 4°C, and plasma was stored at –20°C until radioimmunoassay. Females were swabbed to determine estrous cycle at 18:00, approximately 8 h after the start of the restraint.

Sucrose Preference Test (SPT)

Animals were habituated to two water bottles per home cage during the light cycle for 3 days before administration of sucrose. Habituation started on day 7 and testing was begun on day 10 after enrichment removal in the ER group. Once habituated, rats were given access to a water bottle with (1%) sucrose (Sigma-Aldrich) as well as a standard water bottle for 3 days in their home cages. Positions of the bottles were alternated each day to avoid place preference within the rats. Bottles were weighed prior to administration and after the end of the light cycle duration (11 h). Bottles containing sucrose solution were supplied 1 h after lights on and removed immediately prior to the start of the dark cycle. Data collection included grams of sucrose solution intake and grams of water intake. The percentage of sucrose solution intake per total fluid consumption was used to determine sucrose preference per day for each animal. Females were swabbed to determine estrous cycle at 18:00 following testing on each of the 3 days.

Forced Swim Test

Rats were exposed to the FST 14 days post-removal. Starting 1h into the lights on period, each rat was placed individually into a Plexiglas cylindrical tank (45 cm H × 20 cm diameter) containing 31 ± 3 cm of water (25 ± 2°C). The rats were monitored for ten minutes before removal from the tank. Behavior during the test was video monitored, with swimming, diving, immobility, and climbing behaviors assessed. The behaviors scored are defined as follows: (i) swimming — moving limbs in an active manner and making circular movements around the tank, (ii) diving-head fully immersed in the water, (iii) immobility — only necessary movements to keep head above water; and (iiii) climbing — rapid movement of limbs up the side of the tank. Animals were killed 85 min after FST initiation by overdose with Pentobarbital. Females were swabbed to determine estrous cycle at the time of kill, and organs removed and weighed.

Radioimmunoassay

Plasma corticosterone concentrations were measured with ^{125}I RIA kit (MP Biomedicals Inc, Orangeburg, NY, United States). Samples were run in duplicate when possible.

Estrus Cycle

Phase of estrus was assessed by vaginal cytology at time points noted above, using standard histological methods (Becker et al., 2005). Sampling time was late in the subjective day, optimized to detect proestrus (P) phase (1800 h) (Smith et al., 1975)) but less optimal for the estrus (E) phase (being late in E phase and nearing transition to diestrus. Due to these limitations, E and P groups were pooled (as per Egan et al. (2018)) to correspond to a time period where gonadal steroids would be particularly

elevated during the time of testing (8 h prior to vaginal swabbing). Similarly, diestrus 1 (D1) and diestrus 2 (D2) groups were pooled to correspond to a time period of reduced gonadal steroid secretion (Butcher et al., 1974; Smith et al., 1975).

Statistics

Cohort effects in these data were assessed by two-way ANOVA with housing manipulation and cohort as factors. If no cohort effect or interaction existed, data were pooled and analyzed by two-way ANOVA with cycle and housing manipulation as factors. This was the case for the behavioral data, organ weights, food intake and body weight. Due to a significant cohort effect, corticosterone responses to stress were assessed by three-way repeated measures ANOVA, using cohort, housing manipulation and cycle as between-subjects factors and time as the repeating factor (see **Supplementary Figure S1**). SigmaPlot (Systat Software, San Jose, CA, United States) was used for two-way ANOVAs and Statistica (Statsoft/Dell) was used for three-way ANOVAs. *Post hoc* testing, utilizing Fisher's Least Significant Difference (LSD), was performed following two-way and three-way ANOVAs to assess the effect of housing manipulation within given time and estrous cycle domains. For all data $p \leq 0.05$ denotes statistical significance. Due to specific hypotheses having been formed *a priori* on the effects of housing manipulation in females, planned comparisons between groups were performed (Maxwell and Delaney, 1990). The distribution of estrus cycle phase across groups was assessed by Chi-square tests. Outliers in the data sets were determined *a priori* by values that exist outside the mean ± 1.96 times the standard deviation and above the upper quartile + 1.5 times the interquartile range or below the lower quartile – 1.5 times the interquartile range. Data are graphed as mean ± the standard error of the mean (SEM).

RESULTS

HPA-Axis Response

At 7 days post removal of enrichment, the rats were subjected to a 30 min restraint stress in order to measure HPA axis responsiveness in the form of plasma corticosterone levels (CORT) (**Figure 2**). No differences were observed for amount of time that the animals in any group spent struggling. Analysis of the CORT response to acute restraint showed an increase in corticosterone secretion in all groups following the restraint [main effect of time; $F_{(4,284)} = 270.32$; $p < 0.001$], with levels returning to baseline at 120 min after stressor initiation.

There was a main effect of housing manipulation [$F_{(3,71)} = 3.0508$; $p < 0.05$], an interaction effect of housing manipulation × time [$F_{(12,284)} = 2.8065$; $p < 0.002$] and an interaction effect of housing manipulation × time × estrous cycle [$F_{(12,284)} = 2.8558$; $p < 0.002$]. For the females in the P and E phases, *post hoc* tests revealed that at time 15, the enriched-removed (ER) females had higher ($p < 0.05$) CORT levels compared to the continuously enriched (EE) and both the single (Scon) and pair (Pcon) housed controls. At time 30, the EE females showed higher ($p < 0.05$) CORT levels compared to the Pcon. At time 60, the Scon rats maintained higher ($p < 0.05$) CORT levels than all other animals (**Figure 2B**). For the females in the P and E phases, there was no effect of housing manipulation on CORT levels at times 0 and 120.

For females within the D1D2 phase of estrous cycle, there were no differences in CORT at any of the time points among the different housing manipulation groups (**Figure 2A**).

There was a main effect of cohort [$F_{(1,71)} = 23.228$; $p < 0.001$], with cohort 1 having a higher least square mean value than cohort 2, probably related to inter-assay variability in the corticosterone assay. As such, an individual analysis was also conducted for each cohort. The results of the individual analysis and corresponding graphs can be found in the **Supplementary Figure S1**.

The total corticosterone response to the acute restraint stress, in the form of time-integrated area under the curve (AUC) showed a main effect of housing manipulation [$F_{(3,71)} = 5.100$; $p = 0.003$], no main effect of cycle, and no interaction effect. *Post hoc* tests revealed a significant decrease ($p < 0.05$) in the total CORT response of the continuously enriched and pair housed control females when compared to the single-housed animals (**Figure 2C**). There was a main effect of cohort [$F_{(1,71)} = 6.300$; $p = 0.014$],with cohort 1 having a higher integrated corticosterone values than cohort 2. As such, an individual analysis was conducted for each cohort. The results of the individual analysis and corresponding graphs can be found in the **Supplementary Data**.

Sucrose Preference Test

Ten days after the enrichment removal, we assessed sucrose preference during the inactive cycle of the rats. Enrichment-removed (ER) animals had a decrease in sucrose preference [$F_{(3,70)} = 2.766$; $p = 0.048$] (**Figure 3A**) relative to both the single (Scon) and pair (Pcon) housed controls ($p < 0.05$). The continuously enriched (EE) animals showed a decrease in total sucrose intake [$F_{(3,73)} = 6.069$; $p < 0.001$] in comparison to the single (Scon) and pair (Pcon) housed controls ($p < 0.05$). However, the EE females also had a significant decrease in water intake [$F_{(3,73)} = 8.659$; $p < 0.001$] compared to the ER and Pcon females ($p < 0.05$). There was no cohort effect on sucrose preference [$F_{(1,72)} = 2.303$; $p = 0.133$], so the two cohorts were pooled for analysis. There was also no main effect of cycle [$F_{(1,71)} = 2.319$; $p = 0.132$] on the sucrose preference.

Forced Swim Test

Animals were tested for passive coping behavior in the FST 14 days after enrichment removal. Enrichment-removed (ER) females showed increased time spent immobile [$F_{(3,66)} = 3.310$; $p = 0.025$] (**Figure 3B**) in comparison to the single (Scon) and pair (Pcon) housed controls ($p < 0.05$). Pair housed control females spent more time swimming [$F_{(3,70)} = 7.114$; $p = 0.003$] than all other females ($p < 0.05$). There were no differences in any other behaviors in the FST. There were also no differences observed in any behaviors during the FST across the estrous cycle [$F_{(1,66)} = 1.461$; $p = 0.231$]. Two-way ANOVA comparison of time spent immobile in the females showed a main effect of housing manipulation [$F_{(3,68)} = 6.204$; $p < 0.001$] but no main effect of cohort [$F_{(1,68)} = 3.610$; $p = 0.062$] and no interaction effect. Based on this, we decided to pool both cohorts for analyses.

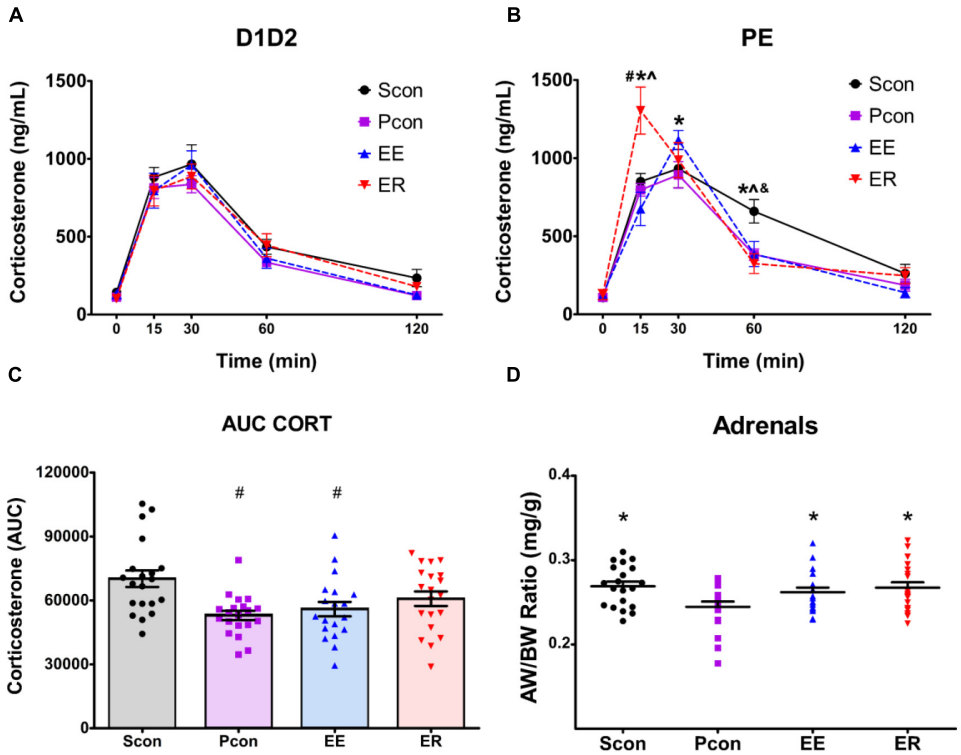

FIGURE 2 | Single-housing (SCon), enrichment (EE), and enrichment removal (ER) in females causes a dysregulation of the HPA axis response to acute restraint stress in a cycle dependent manner and increases adrenal size. Although hormonal time course data for all groups were analyzed by three-way repeated-measures ANOVA (see text), the time profiles of D1D2 and PE animals are shown separately for visual clarity of treatment effects. **(A)** No effects of housing manipulations on stress-induced corticosterone levels were observed for females in the D1D2 stage of the estrous cycle. **(B)** For females in the PE stage of estrous cycle, ER animals have increased corticosterone at 15 min after the start of restraint, EE animals have increased corticosterone at 30 min after the start of restraint, and Scon animals have increased corticosterone at 60 min after the start of restraint $^{\#}p < 0.05$ vs. Scon, $^{*}p < 0.05$ vs. Pcon, $^{\wedge}p < 0.05$ vs. EE, $^{\&}p < 0.05$ vs. ER. **(C)** Total corticosterone response to restraint is increased in the Scon group $^{*}p < 0.05$ vs. Pcon, $^{\wedge}p < 0.05$ vs. EE. **(D)** Single-housing, enrichment and enrichment removal in females causes adrenal hypertrophy $^{*}p < 0.05$ vs. Pcon.

Organ Weights

Somatic effects of enrichment removal were assessed in the form of specific organ weights. Following perfusion, the hearts, thymi, and adrenals of all animals were cleaned, weighed and normalized to animal body weight. There was a main effect of housing manipulation [$F_{(3,73)} = 3.518$; $p = 0.019$] on the adrenals. Single-housed, continuously enriched and enrichment-removed females had larger adrenals per bodyweight compared to the pair-housed controls ($p < 0.05$) (**Figure 2D**). No effects of housing manipulation were observed on the heart and thymus weights. There was a main effect of cohort [$F_{(1,72)} = 17.810$; $p < 0.001$],with cohort 1 having a higher least square mean than cohort 2. As such, an individual analysis was conducted for each cohort. The results of the individual analysis and corresponding graphs can be found in the **Supplementary Table S1**.

Food Intake and Body Weight

Daily food intake, during the inactive period only, was measured for 1 week prior to and for 2 weeks following the enrichment removal. Comparison of food intake prior to and following enrichment removal within each group showed a main effect of housing manipulation [$F_{(3,76)} = 12.747$; $p < 0.001$], a main effect of time [$F_{(1,76)} = 4.773$; $p = 0.032$], and an interaction effect of housing manipulation × time [$F_{(3,76)} = 10.390$; $p < 0.001$]. *Post hoc* tests revealed that only within the enrichment-removed (ER) group was there a significant increase in food intake following the enrichment removal ($p < 0.05$) (**Figure 4A**). Note that intake data measures food consumption during the inactive period only.

Body weight measurements were taken weekly and reported as the percentage of change from the original body weight of the animal prior to starting the housing manipulations. Analysis of body weight change during the first 4 weeks of the study showed a main effect of housing manipulation [$F_{(3,299)} = 22.877$; $p < 0.001$], a main effect of time [$F_{(4,299)} = 996.525$; $p < 0.001$], and an interaction effect of housing manipulation × time [$F_{(12,299)} = 13.050$; $p < 0.001$]. *Post hoc* tests revealed that starting 1 week into enrichment and until the time of enrichment removal, EE and ER animals had a significant attenuation of body weight gain compared to the Scon and Pcon animals ($p < 0.05$) (**Figure 4B**). Analysis of body weight at the time of enrichment removal, as well as once weekly for the next 2 weeks,

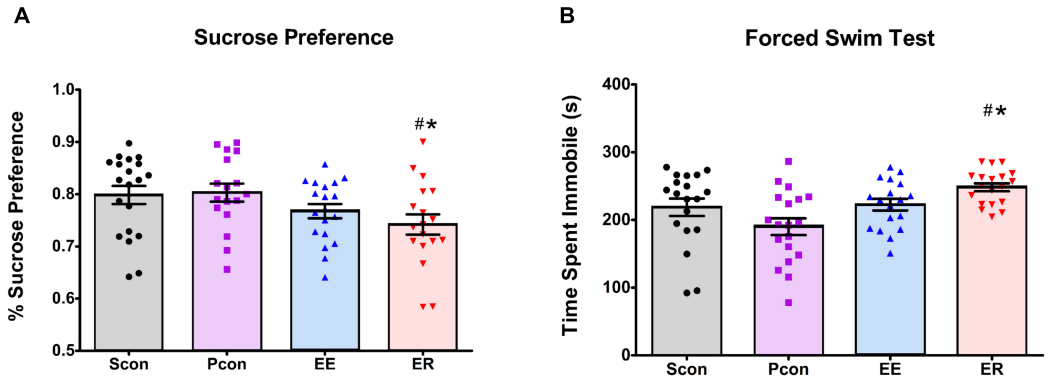

FIGURE 3 | Enrichment removal increases anhedonia in the sucrose preference test and passive coping behavior in the forced swim test. **(A)** ER animals have a decreased preference for sucrose on the first day of the sucrose preference test relative to PCon and Scon groups $^{\#}p < 0.05$ vs. Scon, $^{*}p < 0.05$ vs. Pcon. **(B)** ER females spent significantly more time immobile in the FST relative to pair housed controls (Pcon) and single housed animals (Scon) $^{\#}p < 0.05$ vs. Scon, $^{*}p < 0.05$ vs. Pcon.

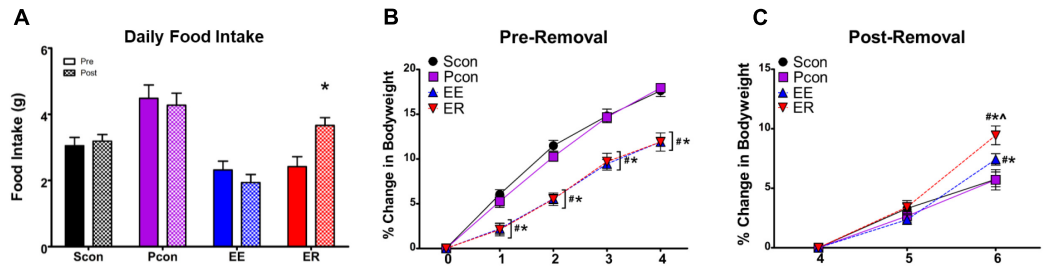

FIGURE 4 | Enrichment removal leads to hyperphagia and an increase in body weight gain. **(A)** Enrichment removal increases inactive cycle food intake $^{*}p < 0.05$ vs. pre for the ER group only. **(B)** Enrichment leads to an attenuation of body weight gain for both EE and ER groups relative to pair housed controls (Pcon) and single housed animals (Scon) for the first 4 weeks of enrichment $^{\#}p < 0.05$ vs. Scon, $^{*}p < 0.05$ vs. Pcon. **(C)** Rate of change in body weight is increased in the EE group and exaggerated in the ER group at 2 weeks post removal $^{\#}p < 0.05$ vs. Scon, $^{*}p < 0.05$ vs. Pcon, $^{\wedge}p < 0.05$ vs. EE.

showed a main effect of housing manipulation [$F_{(3,152)} = 14.612$; $p < 0.001$], a main effect of time [$F_{(2,152)} = 295.660$; $p < 0.001$], and an interaction effect of housing manipulation × time [$F_{(6,152)} = 6.026$; $p < 0.001$]. *Post hoc* tests revealed at the time of enrichment removal, as well as 1-week post, the continuously enriched (EE) and enrichment-removed (ER) females had an attenuation in body weight gain compared to the single (Scon) and pair (Pcon) housed controls ($p < 0.05$). At 2-weeks post removal, only the EE group was showing attenuated body weight gain compared to the single (Scon) and pair (Pcon) housed controls ($p < 0.05$) (**Figure 4C**). To understand the rate of change in body weight after enrichment removal in the same fashion as in the males (Smith et al., 2017), we calculated the percent change in body weight beginning at the point of enrichment removal in all groups. There was a main effect of housing manipulation on body weight change [$F_{(3,152)} = 3.643$; $p = 0.016$], a main effect of time [$F_{(2,152)} = 295.660$; $p < 0.001$], and an interaction effect of housing manipulation × time [$F_{(6,152)} = 6.026$; $p < 0.001$]. *Post hoc* tests revealed no differences in the rate of body weight gain 1 week after removal. At 2 weeks post removal, the ER group had a significant increase in weight gain compared to all other groups ($p < 0.05$) and the EE group had a significant increase in weight gain compared to the Scon and Pcon animals ($p < 0.05$) (**Figure 4C**).

Estrous Cycle

We monitored phase of estrus cycle at the conclusion of behavioral testing sessions to allow determination of the possible impact of housing conditions on cyclicity. As would be expected, the number of animals in diestrus was significantly greater than that of proestrus and estrus (**Table 1**), and this distribution did not differ with treatment condition (**Table 2**) (Chi-square).

DISCUSSION

Our studies indicate that loss driven by enrichment removal increases passive coping behavior and hyperphagia during the inactive cycle. These results recapitulate findings in males and suggest that loss engenders similar adaptations in both sexes. However, unlike males, enrichment removal decreases sucrose preference in females, suggesting that loss has sex-specific effects on reward circuits in the brain.

Enrichment removal increased immobility and decreased active behaviors in the FST relative to pair housed controls

TABLE 1 | Number of animals in either D1/D2 or P/E on days of testing.

Cycle:	FST (10AM)	Restraint bleed (6PM)	Sucrose preference (Day One) (6PM)	Sucrose preference (Day Two) (6PM)	Sucrose preference (Day Three) (6PM)
D1/D2	56	44	37	49	56
P/E	22	25	42	31	24
Undetermined	2	1	1	0	0

TABLE 2 | Number of animals in either D1/D2 or P/E across experimental groups.

Cycle:	Treatment:	FST (10AM)	Restraint bleed (6PM)	Sucrose preference (Day One) (6PM)	Sucrose preference (Day Two) (6PM)	Sucrose preference (Day Three) (6PM)
D1/D2	Single-Housed	13	10	9	16	12
	Pair-Housed	15	11	9	11	15
	EE	14	11	10	8	13
	ER	14	12	9	14	16
P/E	Single-Housed	7	10	11	4	8
	Pair-Housed	4	9	10	9	5
	EE	6	9	10	12	7
	ER	6	8	11	6	4

and single-housed animals, suggesting that the experience of loss selectively increased passive coping behavior (Commons et al., 2017; Smith et al., 2017). Immobility in the FST is interpreted by some as helplessness behavior, and can thereby linked to depressive symptomology [however, this is not the consensus interpretation (see de Kloet and Molendijk, 2016)]. This contention is made in the context of testing antidepressant drugs but is more difficult to apply as a "phenotyping tool" in the absence of antidepressant therapy here. However, it is important to note that in males, increased FST immobility following ER is reversed by antidepressant (imipramine) treatment (Smith et al., 2017), and thus the observed behavioral change may be consistent with alterations in mood.

Removal from enrichment differentially increased rate of body weight gain and food intake relative to other groups, an observation also seen in males. These data suggest that ER has a lasting impact on metabolic regulation, perhaps via modulation of central processes regulating food intake or energy expenditure. In ER males, increased body weight was accompanied by increases in non-homeostatic feeding (Smith et al., 2017)). Notably, stress is known to contribute to overeating, particularly of highly palatable calorically dense foods (Dallman et al., 2005; Ulrich-Lai et al., 2015; Packard et al., 2017), and these data suggest that ER may reflect stress facilitation of food intake. Continuously enriched females also show an increased rate of body weight gain compared to the pair housed controls and the single-housed animals. Although the data suggest no increase in daily food intake for these animals, it is important to note that due to the logistics of the study food intake was only measured during the inactive period for all animals and as such we may have missed any changes in food intake during the active period. Importantly, stress-induced eating has not been reproducibly observed in rodents exposed to most imposed stress regimens, such as social defeat, chronic variable stress, chronic restraint, etc. [e.g., see Harris, 2015; Herman et al., 1995; Tamashiro et al., 2004]. It is notable that chronic social stress regimens produce stress-related hyperphagia and weight gain in subordinate mice (Bartolomucci et al., 2004). Thus, the experience of loss may tap into physiological or psychological processes linking social stressors to ingestive behavior.

Despite the fact that enrichment-removed females show increased daily food intake, assessment of sucrose intake indicate reduced sucrose preference in ER females vs. both pair-housed controls and single-housed groups. These data are consistent with augmented anhedonic behavior following ER. Although full interpretation of these findings are limited by the inability to reliable assess food intake during enrichment periods, the data suggest that consequences of ER on hedonic processes may differ substantially in males and females, perhaps dictated by differential effects of ER on central reward pathways.

Female HPA axis responses to restraint revealed several effects of housing manipulations on plasma corticosterone (CORT) levels. Interestingly, all of the housing effects were observed only in the PE stages (and not D1D2 stages) of the estrous cycle at the time of the acute stressor. Continuously enriched females show a heightened peak CORT response compared to the pair-housed controls, which is reminiscent of the effect reported in males (Smith et al., 2017). However, enrichment removal in males leads to a blunted peak response (Smith et al., 2017), whereas in females, it drives an early and exaggerated peak response in the PE stage. Additionally, single housing delays CORT recovery, suggesting that social isolation is sufficient to alter HPA axis feedback in females. Given that estradiol levels are high in both P and E phases (Becker et al., 2005), these data suggest that housing conditions interact with HPA axis reactivity in an estrogen-dependent manner. The prolonged response observed in single-housed animals during P and E phase corresponds to the period of sexual receptivity, which perhaps intensifies the experience of individual housing (Becker et al., 2005).

Adrenal weights were increased in all experimental groups vs. pair-housed controls. Adrenal weight is determined in large part by history of ACTH release, and increases are generally thought to reflect a hyperactive HPA axis (Herman et al., 2016). Consequently, it appears that EE, ER, and single housing all produce some degree of prolonged HPA axis drive relative to the primary control group (pair housed). Enhanced HPA axis reactivity for all of these housing manipulations is supported by the observed increases in CORT mentioned above. These data add to a growing literature noting that females may be particularly susceptible to "stressful" effects of single housing or social isolation (Vieira et al., 2018). Increased adrenal weight in the continuously enriched group may also be related to increased need for glucocorticoid secretion during periods of high activity in the enrichment cages. Consistent with this possibility, it is known that voluntary exercise in male rats induces adrenal hypertrophy and increased corticosterone response to a forced swim stress (Droste et al., 2007). Thus, additional studies would need to be done to isolate which, if not both, enrichment or the subsequent social isolation is causing the adrenal hypertrophy in enrichment-removed females.

Estrus cycles were monitored at the conclusion of behavioral and HPA axis testing, to check for any stage effects on experimental endpoints. There was no effect of estrus stage on behavior in the FST or sucrose preference tests, suggesting that the behaviors tested were not sensitive to hormonal variations across the cycle. Moreover, none of the housing manipulations appear to affect estrus cycling, as there were no differences in the proportion of animals in D1D2 or PE at any point in the experiment (However, it is important to acknowledge that the design was not optimized to clearly differentiate individual stages of estrus, and thus our interpretation is limited to periods likely to have elevated (PE) or low (D1D2) estradiol and progesterone secretion during testing).

Continuous active-cycle enrichment does not recapitulate the effects of ER on passive coping and sucrose preference, indicating that the core findings are associated with the removal of enrichment, not the act of enrichment itself. Active cycle EE does not appear to deviate significantly from either single-housed animals or pair-housed controls in terms of passive coping, sucrose preference or daily food intake. The EE group does show an increased rate of body weight gain compared to the Scon and Pcon females but this effect is attenuated compared to the ER group. However, it is useful to note that the EE group drank less sucrose and water during the inactive cycle (with the same preference ratio). Rodents take in the majority of their daily calories and water during the active cycle, and it is possible that the lower raw intake level may be related to decreased activity or increased sleep during the inactive period (perhaps due to the high level of activity in EE period) (Abou-Ismail et al., 2010).

Overall, the data from the study support the usefulness of enrichment removal to induce behavioral symptoms associated with loss in humans, including passive coping, anhedonia and altered eating patterns. Importantly, ER causes symptoms consistent with negative mood in both males and females, indicating its utility for probing neurobiological underpinnings of loss-related dysphoria and dysfunction. Future studies are needed to explore the neural mechanisms and circuits that underlie loss phenotypes.

AUTHOR CONTRIBUTIONS

RM, OH, BS, and JH designed the experiments and wrote and edited the manuscript. RM and OH performed the experiments and analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00287/full#supplementary-material

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REVIEW
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Pre-reproductive Parental Enriching Experiences Influence Progeny's Developmental Trajectories

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While the positive effects of environmental enrichment (EE) applied after weaning, in adulthood, during aging, or even in the presence of brain damage have been widely described, the transgenerational effects of pre-reproductive EE have been less examined. And yet, this issue is remarkable given that parental environmental experience may imprint offspring's phenotype over generations through many epigenetic processes. Interactions between individual and environment take place lifelong even before conception. In fact, the environment pre-reproductively experienced by the mother and/or the father exerts a substantial impact on neural development and motor and cognitive performances of the offspring, even if not directly exposed to social, cognitive, physical and/or motor enrichment. Furthermore, pre-reproductive parental enrichment exerts a transgenerational impact on coping response to stress as well as on the social behavior of the offspring. Among the effects of pre-reproductive parental EE, a potentiation of the maternal care and a decrease in global methylation levels in the frontal cortex and hippocampus of the progeny have been described. Finally, pre-reproductive EE modifies different pathways of neuromodulation in the brain of the offspring (involving brain-derived neurotrophic factor, oxytocin and glucocorticoid receptors). The present review highlights the importance of pre-reproductive parental enrichment in altering the performances not only of animals directly experiencing it, but also of their progeny, thus opening the way to new hypotheses on the inheritance mechanisms of behavioral traits.

Keywords: environmental enrichment, maternal care, motor behavior, cognition, BDNF, oxytocin, stress response, rats

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INTRODUCTION

Organisms adapt their physiology and behavior in response to environmental modifications. The influence of environmental experiences during lifespan is confined not only to the neurobehavioral profiles of the directly exposed individual, but it can also be evident in the next generations (Arai and Feig, 2011; Thayer and Kuzawa, 2011; Lim and Brunet, 2013). Growing evidence demonstrates that both ancestral and parental histories are able to affect the offspring depending on the valence of the environmental circumstances and the period of exposure to them, and that environmental influences may be passed on to the next generations through epigenetic (i.e., non-DNA sequence-based rather than mutational) modifications (Jirtle and Skinner, 2007; Caldji et al., 2011; Bohacek and Mansuy, 2015;

Wang et al., 2017; Weaver et al., 2017). These studies shed new light on the Lamarckian theories of the inheritance of acquired traits that have been a matter of debate for over a century (Dröscher, 2015; Wang et al., 2017).

It is well-known that pre-natal/early post-natal as well as ancestral experience of environmental insults (e.g., undernutrition, stress) may result in modifications of the phenotype later in life (Shachar-Dadon et al., 2009; Franklin and Mansuy, 2010; Veenendaal et al., 2012; Xu et al., 2016; Ambeskovic et al., 2017). In the present review article we would address the effects of pre-reproductive enriching stimulations experienced by parents, such as the exposure to environmental enrichment (EE) or even exercise, on the behavioral and neurobiological phenotype of the offspring in rodents.

Environmental enrichment is an experimental paradigm apt to potentiate social, cognitive, and sensorimotor stimulations experienced by animals (Rosenzweig et al., 1964). By increasing environmental novelty and complexity, EE exerts potential therapeutic and neuroprotective effects as demonstrated by its efficacy in enhancing neural plasticity and delaying the progression and/or ameliorating the symptoms in the presence of brain injuries and diseases (Nithianantharajah and Hannan, 2006; Baroncelli et al., 2009; Nithianantharajah and Hannan, 2009; Petrosini et al., 2009; Cutuli et al., 2011; Simpson and Kelly, 2011; Sale et al., 2014; Mandolesi et al., 2017; Sampedro-Piquero and Begega, 2017; Gelfo et al., 2018). The broad beneficial effects of EE probably result from complex interactions among time window of exposure to EE, type of enrichment, and gender of enriched animals (Girbovan and Plamondon, 2013). On the other hand, scattered negative outcomes have also been reported, probably linked to the enhanced stress levels induced by the EE protocol (Schilling et al., 2004; Wood et al., 2011; Huzard et al., 2015; Mo et al., 2016).

Environmental enrichment as well as exercise may improve learning and memory, and enable neuroplasticity processes involving increased neurogenesis, possibly via neurotrophin-mediated mechanisms (Kempermann et al., 1997; van Praag et al., 1999; Olson et al., 2006; Bechara and Kelly, 2013; Livingston-Thomas et al., 2016). Notably, EE and exercise effects are difficult to disentangle, because most enrichment paradigms incorporate exercise elements. Anyway, many studies demonstrated that the motor component (i.e., voluntary running-wheel exercise) is the major neurogenic and neurotrophic stimulus in EE protocols in comparison to the cognitive and social components (i.e., complex environments comprised of inanimate objects with or without social interactions, but without running-wheel) (Kobilo et al., 2011; Mustroph et al., 2012; Bechara and Kelly, 2013; Grégoire et al., 2014).

In the last years, attention has been paid to the transfer of proactive effects of enriching experiences from parents to the progeny, and the number of researches on this issue is significantly increased over time (Arai and Feig, 2011; Girbovan and Plamondon, 2013; Sale et al., 2014; Taouk and Schulkin, 2016; Sale, 2018). By using different rat and mouse strains, schedules and protocols of physical, social and/or cognitive enrichment, and various behavioral tests, these studies demonstrated that parental positive manipulations are able to alter the neurodevelopmental trajectories of the progeny, likely to prepare the fetus to cope with a specific environment.

The effects of parental enrichment across generations have been mainly investigated by exposing mothers to different kinds of environmental stimulations during gestation and/or lactation (Sale et al., 2014).

A rising literature demonstrates the role of maternal (Dell and Rose, 1987; Arai et al., 2009; Leshem and Schulkin, 2012; Caporali et al., 2014, 2015; Cutuli et al., 2015, 2017, 2018) or paternal (Mashoodh et al., 2012; Mychasiuk et al., 2012; Dezsi et al., 2016; Short et al., 2017; Yeshurun et al., 2017) enrichment "before conception" (i.e., during the pre-reproductive period) in modifying the neurobiological and behavioral profile of the offspring. Since early nurturing experiences may influence brain plasticity and alter epigenome (Meaney, 2010; Weaver et al., 2017), part of these studies have also evaluated if the effects of pre-reproductive parental enrichment on offspring's phenotype were mediated by modifications of maternal behavior (Mashoodh et al., 2012; Caporali et al., 2015; Cutuli et al., 2015, 2017, 2018; Short et al., 2017; Yeshurun et al., 2017).

To our knowledge, the literature on the effects of early EE by parents in humans is still scarce, and principally deals with fetuses' and newborns' stimulation. In fact, besides an accumulating evidence on the beneficial consequences of tactile (i.e., body massage) and auditory stimulation (i.e., exposure to maternal voice) on the neurobehavioral development of preterm infants (Guzzetta et al., 2009; Picciolini et al., 2014; Webb et al., 2015) or the effects of antenatal auditory stimulations (i.e., training with music and maternal talk to the fetus during pregnancy) on the reduction of autistic-like behaviors (Ruan et al., 2018), to date only one article has demonstrated the proactive influence of pre-reproductive enrichment represented by maternal educational achievement on buffering offspring's stress sensitivity (Swartz et al., 2018).

In the following paragraphs, we will take into account the impact of pre-reproductive parental exposure to enriching experiences on maternal behavior and on physical, motor, cognitive and emotional features of the offspring in rodents. Furthermore, based on our studies and the few others present in literature, we will focus on the changes in a key neurotrophic factor, such as the brain-derived neurotrophic factor (BDNF), and in the oxytocinergic system following pre-reproductive parental housing in highly stimulating environments. Whenever possible, comparisons with the effects of parental enrichment during gestational and/or lactation periods (i.e., post-reproductive enrichment) will be also considered. Namely, in **Table 1** for each study we defined the period of enrichment exposure and if the enrichment was maternal and/or paternal, social (if the enriched cages contained more individuals than in the control groups), cognitive (if the objects, toys and other materials inside the enriched cages were systematically changed, rearranged or renewed), physical (if the enriched cages were bigger than the standard ones and

TABLE 1 | Parental enrichment protocols.

Parental enrichment protocols

A. Protocols of pre-reproductive parental enrichment

Authors	Year	Animals	Kind of enrichment	Enrichment period	Kind of parental enrichment
Arai et al. (2009)	2009	Ras-grf k/o mice	Moderate social, cognitive, physical and motor enrichment (with running-wheel)	Pre-reproductive period	Maternal or paternal
Benito et al. (2018)	2018	C57Bl/6J mice	Cognitive, physical and motor enrichment (with running-wheels)	Pre-reproductive period	Paternal
Caporali et al. (2014)	2014	Wistar rats	Social, cognitive, physical and motor enrichment (with running-wheel)	Pre-reproductive period	Maternal
Caporali et al. (2015)	2015	Wistar rats	Social, cognitive, physical and motor enrichment (with running-wheel)	Pre-reproductive period	Maternal
Champagne and Meaney (2007)	2007	Long–Evans rats	Social, cognitive and physical enrichment (without running-wheel)	Pre-reproductive period	Maternal
Cutuli et al. (2015)	2015	Wistar rats	Social, cognitive, physical and motor enrichment (with running-wheels)	Pre-reproductive period	Maternal
Cutuli et al. (2017)	2017	Wistar rats	Social, cognitive, physical and motor enrichment (with running-wheel)	Pre-reproductive period	Maternal
Cutuli et al. (2018)	2018	Wistar rats	Social, cognitive, physical and motor enrichment (with running-wheel)	Pre-reproductive period	Maternal
Dezsi et al. (2016)	2016	Genetic Absence Epilepsy rats	Cognitive, physical and motor enrichment (with running-wheel)	Pre-reproductive period	Paternal
Mashoodh et al. (2012)	2012	BALB/c mice	Social, physical and motor enrichment (with running-wheel)	Pre-reproductive period	Paternal
Short et al. (2017)	2017	C57Bl/6 mice	Motor enrichment (by running-wheel)	Pre-reproductive period	Paternal
Yeshurun et al. (2017)	2017	C57Bl/6J mice	Cognitive and physical enrichment (without running-wheel)	Pre-reproductive period	Paternal

B. Protocols of parental enrichment during pregnancy

Authors	Year	Animals	Kind of enrichment	Enrichment period	Kind of parental enrichment
Cymerblit-Sabba et al. (2013)	2013	Wistar rats	Social, cognitive, physical and motor enrichment with running-wheel	Pregnancy	Maternal
Gomes Da Silva et al. (2016)	2016	Wistar rats	Motor enrichment (by motor driven treadmill)	Pregnancy	Maternal
Herring et al. (2012)	2012	TgCRND8 mice	Motor enrichment (by running-wheel)	Pregnancy	Maternal
Kiyono et al. (1985)	1985	Fischer rats	Social, cognitive and physical enrichment (without running-wheel)	Pregnancy	Maternal
Koo et al. (2003)	2003	Sprague–Dawley rats	Social, cognitive, physical and motor enrichment (with running-wheel)	Pregnancy	Maternal
Lee et al. (2006)	2006	Sprague–Dawley rats	Motor enrichment (by swimming)	Pregnancy	Maternal
McKim and Thompson (1975)	1975	Sprague–Dawley rats	Physical and social enrichment	Pregnancy	Maternal
Park et al. (2013)	2013	C57Bl/6J mice	Motor enrichment (by motor driven treadmill)	Pregnancy	Maternal
Parnpiansil et al. (2003)	2003	Sprague–Dawley rats	Motor enrichment (by motor driven treadmill)	Pregnancy	Maternal
Rosenfeld and Weller (2012)	2012	WKY and Wistar rats	Cognitive, physical and motor enrichment (with running-wheel)	Pregnancy	Maternal

(Continued)

TABLE 1 | Continued

Parental enrichment protocols

C. Protocols of parental enrichment during pregnancy and lactation or only during lactation

Authors	Year	Animals	Kind of enrichment	Enrichment period	Kind of parental enrichment
Bick-Sander et al. (2006)	2006	C57Bl/6 mice	Motor enrichment (by running-wheel)	Pregnancy and lactation	Maternal
Branchi et al. (2010)	2010	CD-1 mice	Social and physical enrichment (without running-wheel)	Pregnancy and lactation	Maternal
Cancedda et al. (2004)	2004	C57Bl/6J mice, 148 CRE-LacZ transgenic mice	Social, cognitive and physical enrichment (without running-wheel)	Pregnancy and lactation	Maternal
Durán-Carabali et al. (2018)	2018	Wistar rats	Social, cognitive, physical and motor enrichment (with running-wheel)	Pregnancy and lactation	Maternal
Heiderstadt et al. (2014)	2014	C57BL/6J, DBA/2J and 129x1/SvJ mice	Social enrichment (without running-wheel)	Lactation	Maternal
Sale et al. (2004)	2004	C57Bl/6J mice	Social, cognitive, physical and motor enrichment (with running-wheel)	Pregnancy and lactation	Maternal
Sparling et al. (2010)	2010	Long–Evans rats	Physical and social enrichment (without running-wheel)	Pregnancy and lactation	Maternal
Welberg et al. (2006)	2006	Long–Evans rats	Moderate physical enrichment (without running-wheel)	Pregnancy and lactation	Maternal

D. Mixed protocols of parental enrichment

Authors	Year	Animals	Kind of enrichment	Enrichment period	Kind of parental enrichment
Bechard and Lewis (2016)	2016	Peromyscus maniculatus (deer mice)	Social, cognitive, physical and motor enrichment (with running-wheel)	Pre-reproductive and pregnancy periods	Maternal and paternal
Connors et al. (2015)	2015	Sprague–Dawley rats	Physical and cognitive enrichment (without running-wheel)	Pre-reproductive, pregnancy and lactation periods	Maternal
Curley et al. (2009)	2009	Balb/c mice	Social and physical enrichment (without running-wheel)	Lactation (F0), pre-reproductive period (F1)	Maternal
Leshem and Schulkin (2012)	2012	Sprague–Dawley rats	Social, cognitive, physical and motor enrichment (with running-wheels)	Pre-reproductive period (F0)/post-weaning (F0)	Maternal
Maruoka et al. (2009)	2009	C57Bl/6J mice	Cognitive, physical and motor enrichment (with running-wheel)	Pre-reproductive and pregnancy periods	Maternal
Mychasiuk et al. (2012)	2012	Long–Evans rats	Social, cognitive and physical enrichment (without running-wheel)	Pre-reproductive and pregnancy periods (mothers), pre-reproductive period (fathers)	Maternal or paternal
Zuena et al. (2016)	2016	Wistar rats	Social, cognitive, physical and motor enrichment (with running-wheel)	Pre-reproductive and pregnancy periods	Maternal

For each study the first author's name, year of publication, kind of animals used, kind of enrichment (social, cognitive, physical and/or motor), period of enrichment exposure (during pre-reproductive, pregnancy and/or lactation periods) and kind of parental enrichment (maternal and/or paternal) are reported. In each sub-section (A–D), the studies are reported in alphabetical order.

contained objects, toys, igloos, tunnels, nesting materials, etc.) and/or motor (with one or more running-wheels). It is evident that in literature different EE paradigms has been used, with little understanding of how differences in individual EE variables (such as social, cognitive, physical and/or motor ones) might impact on specific downstream biological mechanisms. Thus, given the importance of the motor component in EE (as above discussed), exercise only paradigms (by running-wheel, motor driven treadmill or swimming) were also taken into account as forms of parental enriching experiences. In addition, we described the effects of communal nesting, a condition in which parental responsibilities are shared by multiple individuals in a nest, as a source of social extra-stimulation in pups' early life.

IMPACT OF DIFFERENTLY TIMED PARENTAL ENRICHMENT ON MATERNAL BEHAVIOR

In mammals, the parental care ensures that the offspring effectively will survive until the reproductive age, thus transmitting the genetic information across generations. The maternal care in rodents is made up of a "*constellation*" of behaviors of preparation for the arrival of the newborns as well as their nurturing and protection (Kristal, 2009).

The literature especially reports maternal behavior alterations following various protocols of enrichment during gestational and/or lactation periods, while fewer studies are available on the effects of pre-reproductive parental enrichment.

Effects of Maternal Enrichment During Pregnancy and/or Lactation

Maternal exposure to EE during pregnancy and lactation increases maternal licking behavior, passive nursing and presence in the nest (Cancedda et al., 2004; Sale et al., 2004; Durán-Carabali et al., 2018).

Lactating rat dams enriched only during gestation display more pup-directed behaviors than control-reared rats during the first and third lactation weeks, but decrease the nighttime frequency of presence in the nest and licking/grooming (LG) and arched-back nursing (ABN) during the third lactation week (Rosenfeld and Weller, 2012).

Mother rats enriched before and during gestation show heightened LG behavior and reduced pup nursing (Zuena et al., 2016).

Maternal enrichment during pre-reproductive, gestational and lactation phases induces an increase in ABN, but a reduction in the time spent in the nest and nursing (Connors et al., 2015).

There are also studies in which parental enrichment does not affect (Bechard and Lewis, 2016) or even worsen the maternal behavior (Welberg et al., 2006). In fact, the study by Bechard and Lewis (2016) used a biparental EE protocol in deer mice during pre-reproductive and gestational phases, and found no differences in maternal behavior of enriched mothers. The study by Welberg et al. (2006) used a moderate EE protocol in rats during pregnancy and lactation, and found no differences in pup licking, but reduced nursing episodes and presence in the nest.

Taken together these data, even somewhat contrasting, seem to account for a modulatory effect of environmental conditions on maternal care that reflects the different EE protocols used or the timing of enrichment exposure.

The more effective EE protocols for increasing maternal care are the ones providing at least social, cognitive and physical stimulations during pregnancy and lactation (Cancedda et al., 2004; Sale et al., 2004; Durán-Carabali et al., 2018). Moreover, when EE protocol involves the transition from the enriched gestational environment to the standard environment, the increase in some pup-directed behaviors may be a substitute to object exploration activity, given the decreased space and stimuli (Rosenfeld and Weller, 2012). A similar explanation can be valid also if females enriched in groups before and during gestation are individually housed in enriched cages near parturition (Zuena et al., 2016).

A variable conserved across dams housed in EE is the reduction in the time/frequency in contact with the nest. It may be attributed to the increased physical space of the enriched cage, when the EE protocol is applied at least during gestation and lactation (Welberg et al., 2006; Connors et al., 2015), given the enriched housing conditions reproduce the natural situation in which the dam is given more space to explore and thus more chance to leave the nest. On the contrary, the reduced maternal contact with the nest can be a sign of distress induced by the transition from the enriched gestational environment to the standard environment; this transition may have an aversive meaning leading to a premature withdrawal of EE dams from their pups (i.e., spending less time in the nest and performing less LG and lactation in the third post-natal week) (Rosenfeld and Weller, 2012). This behavioral pattern seems analogous to the early weaning manipulation, where the pups are separated from their mothers in the third post-natal week and become more anxious and stressful (Ito et al., 2006; Kikusui et al., 2006).

Effects of Pre-reproductive Parental Enrichment

Few researches have investigated the effects of pre-conceptional parental enrichment on maternal care to date. In particular, when rearing their own offspring under standard conditions, communally reared females are reported to perform enhanced levels of *post-partum* care in comparison to standard-reared females, and their offspring show increased frequency of nursing (Curley et al., 2009).

Post-weaning exposure to EE of low LG offspring enhances LG behavior and oxytocin receptor binding across generations (Champagne and Meaney, 2007).

Recently, we have demonstrated that pre-reproductive maternal EE induces maternal care modifications consisting of higher levels of licking, ABN, and nest building activities, and a faster retrieving after maternal male intruder encounters (Cutuli et al., 2015, 2017, 2018). These effects of the pre-reproductive exposure of mothers to EE are accompanied by heightened levels of BDNF in the frontal cortex at pups' weaning (Caporali et al., 2015; Cutuli et al., 2015).

Paternal environmental experiences can modify offspring's phenotype even in the absence of paternal care. Unfortunately, only few studies addressed the role of mothers in the transmission of paternal effects by analyzing maternal care. Namely, standard-reared female mice mated with male mice enriched during the pre-reproductive period show increased frequency of pup nursing and licking during the first *post-partum* week. Such behavioral modifications are associated with gene expression modifications (i.e., higher levels of BDNF mRNA and lower levels of MeCP2 mRNA) in the hypothalamus of dams (Mashoodh et al., 2012). The effects of paternal enrichment via maternal investment could be the consequence of inherited paternal epigenetic variations that lead to variations in the level of maternal care requested by the offspring. In fact, pups provide

distal cues (e.g., sight, sound, tactile contact) for the mother, thus stimulating her contact with them. Interestingly, previous studies demonstrated that locomotion, ultrasonic vocalization and suckling ability are influenced by paternal genes (Curley et al., 2004; Plagge et al., 2004; Swaney, 2011). It can be speculated that the modulation of maternal behavior in standard-reared females mated with enriched males can be linked to a more demanding behavioral pattern of the pups.

Conversely, more recent studies did not find any effect of paternal enrichment on the maternal behavior (Short et al., 2017; Yeshurun et al., 2017).

Overall, the majority of the studies reported in this section seems to indicate that different pre-reproductive parental enriching experiences can potentiate the maternal care. Anyway, divergent data are emerged about the effects of pre-reproductive paternal enrichment on maternal behavior, possibly due to methodological reasons. In fact, in the study by Mashoodh et al. (2012) the fathers received increased social, physical and motor stimulations during their entire lifetime before breeding, and they were compared to male mice reared in isolated conditions. Differently, in the studies by Short and colleagues and Yeshurun and colleagues the fathers were exposed to only motor or only cognitive and physical enrichment and just in adulthood. Furthermore, in the study by Short et al. (2017) runner fathers and controls were both single-housed, while in the study by Yeshurun et al. (2017) enriched fathers and controls were both socially reared (four mice per cage).

It seems that the more is complete and long-lasting the set of stimulations provided to the fathers, more intense is the potentiating effects on the maternal behavior.

Furthermore, the use of socially isolated males (instead of standard-reared males) in the study by Mashoodh et al. (2012) could have contributed to shift the behavioral and neurobiological phenotypes of mates to extremes, thus influencing the maternal investment. Anyway, Mashoodh et al. (2012) showed that maternal investment is only partially dependent on the social and environmental experience of the mate (since environmentally induced anxiety levels of males did not fully predict the frequency of maternal nursing).

As evident, the implications of environmental factors on the complex relationship among paternal, maternal and offspring phenotypes still represent a crucial challenge in the study of the mechanisms driving paternal effects.

IMPACT OF DIFFERENTLY TIMED PARENTAL ENRICHMENT ON THE OFFSPRING'S PHENOTYPE

Effects of Pre-reproductive vs. Post-reproductive Parental Enrichment on Physical Development and Motor Behavior

A proper assessment of the transgenerational effects of parental experiences on offspring developmental trajectories can be achieved by examining the maturation of motor behaviors in rodents. This represents a useful tool to carefully assess early post-natal neurodevelopment, because the appearance of sensorimotor reflexes and motor skills typically follows a definite timing during the first 3 weeks after birth (De Souza et al., 2004).

Unfortunately, few studies paid specific attention to the transgenerational effects of EE on offspring's motor development and even fewer ones used the pre-reproductive EE paradigm, although it shows the possibility to distinguish pre- from post-natal effects of EE, thus allowing the study of Lamarckian inheritance.

To this aim, we performed a series of studies to examine the hypothesis that pre-reproductive maternal EE could affect progeny's phenotype, by rearing female Wistar rats in an enriched environment from weaning until mating (Caporali et al., 2014, 2015; Cutuli et al., 2015, 2017, 2018). To our knowledge, the study by Caporali and colleagues is the only available research analyzing the transgenerational effects of pre-reproductive maternal EE on offspring's development by using a battery of tests examining the acquisition of several developmental milestones in the physical and sensorimotor development (Caporali et al., 2014).

This pre-reproductive maternal EE did not affect litter characteristics, such as litter size and male/female ratio, thus suggesting that this experience does not influence the reproductive ability or the pregnancy of female rats (Caporali et al., 2014; Cutuli et al., 2015, 2017). Interestingly, similar results have been reported following differently timed maternal EE protocols (Welberg et al., 2006; Bechard and Lewis, 2016; Zuena et al., 2016) as well as following pre-reproductive paternal enrichment (Mashoodh et al., 2012; Mychasiuk et al., 2012; Yeshurun et al., 2017).

We found that pre-reproductive maternal enrichment affects offspring's body weight only at birth, with enriched mothers' offspring weight less than controls (Cutuli et al., 2015, 2017), similarly to their own enriched mother. As suggested by Sparling et al. (2010), this finding is consistent with researches demonstrating that enriched females are leaner (Pham et al., 1999; Larsson et al., 2002; Olsson and Dahlborn, 2002; Moncek et al., 2004; Brillaud et al., 2005) and maintain their weight stable over time (Brillaud et al., 2005). However, even opposing results have been collected. In fact, when female rats are exposed to a social colony designed to provide enhanced physical and social stimulations, during pregnancy and lactation, they give birth to heavier offspring (Sparling et al., 2010). Given higher weight of the offspring at birth is retained a good predictor of developmental success (Byrd and Weitzman, 1994; Prathanee et al., 2009), the authors conclude that heavier colony pups would have got a physiological advantage compared to their control mates (Sparling et al., 2010). No differences in the body weight have been reported in adulthood, even following different schedules of maternal enrichment (Welberg et al., 2006; Maruoka et al., 2009; Zuena et al., 2016). The pre-reproductive paternal exposure to enriched social, cognitive and physical stimulations (regardless running-wheel) does not affect body weight at birth, but it does predict male offspring's weight at adulthood, resulting in an increased weight gain (Mashoodh et al., 2012) that persists until the second generation (Yeshurun et al., 2017).

Overall, it seems that parental environmental experiences affect the body weight of the progeny. Namely, enriched mothers influence progeny's body weight only at birth, in a way ambiguously linked to different EE conditions, whereas enriched fathers influence offspring's body weight at adulthood. Thus, it is possible to speculate that both maternal and paternal influences are driven by pre-natal metabolic programming, even if we cannot exclude that a specific maternal behavior in the pre-weaning phase may account for the particular fitness outcome observed in the progeny of enriched fathers (Mashoodh et al., 2012).

The pre-reproductive maternal EE alters the motor development of the progeny, leading to an earlier acquisition of those abilities that require complex sequencing and coordination of the motor output (Caporali et al., 2014). Furthermore, maternal enrichment does not influence the postural development or affect the appearance of dynamic sensorimotor reflexes (negative geotaxis, cliff avoidance, and vestibular drop). Interestingly, similar results on negative geotaxis acquisition were obtained by Mychasiuk et al. (2012) evaluating the effects of pre-reproductive paternal enrichment. Nevertheless, the same authors found that the offspring of females enriched prior to and during pregnancy exhibit a reduction in time required to show negative geotaxis, but no EE effects were recorded on the day of the appearance. Thus, one possibility is that maternal EE, only if gestational, affects the time required to show negative geotaxis as days go by. A similar effect on negative geotaxis performance has been reported also by combining pre- and post-natal EE: the maternal exposure to social, cognitive, physical and motor enrichment occurring during pregnancy and lactation is able to promote a better reflex performance (i.e., reduced latency) counteracting the neurobehavioral delay induced by neonatal hypoxia ischemia, a common neurological complication occurring in preterm infants (Durán-Carabali et al., 2018). Given some researches demonstrated that post-natal EE exposure positively influences negative geotaxis development (Kiss et al., 2013; Schuch et al., 2016), it is possible that direct exposure to EE of fetus first and pup later leads to a better performance, without affecting the day of the appearance.

The open field test (OF) is one of the most frequently used method to assay locomotor behavior in rodents and is sensitive to EE-induced exploratory modifications. The analysis of the effects of pre-reproductive parental enrichment on explorative activity of the progeny provided conflicting results. In fact, increased OF activity levels have been reported in young male offspring of pre-reproductively enriched fathers (Mychasiuk et al., 2012), while adult male offspring of pre-reproductively enriched mothers exhibit activity levels similar to controls (Cutuli et al., 2015). Opposite findings were also obtained with differently timed maternal EE protocols. Mychasiuk et al. (2012) found increased OF activity levels in young male and female offspring of mothers enriched prior to and during pregnancy. Even cross-fostered pups of female rats exposed to EE during pregnancy show increased locomotion in the OF (McKim and Thompson, 1975). Anyway, reduced OF activity levels have been reported in young and adult female offspring in other experimental conditions (Maruoka et al., 2009; Rosenfeld and Weller, 2012). The conflicting findings now described, along with the well-known influence exerted by several factors on OF activity levels [for a review see (Simpson and Kelly, 2011)], make this issue worthy of further investigation.

In conclusion, the exposure of rodents to EE during the pre-reproductive phase significantly shapes the neurodevelopment of progeny, by accelerating the acquisition of motor abilities (Caporali et al., 2014). No significant effects have been described on litter features (Mashoodh et al., 2012; Mychasiuk et al., 2012; Caporali et al., 2014; Cutuli et al., 2015, 2017; Yeshurun et al., 2017), while contrasting findings, probably linked to different EE protocols and experimental parameters, have been reported on body weight and OF activity levels.

Effects of Pre-reproductive vs. Post-reproductive Parental Enrichment on Cognition and Anxiety

Effects of Maternal Enrichment During Pregnancy and/or Lactation

In rodents the effects of parental enrichment on the offspring's cognitive performances and anxiety levels have been primarily investigated by exposing mothers to complex stimulations during pregnancy and/or lactation, and by using different kinds of behavioral tasks and biochemical correlates. Namely, the exposure of mothers to different kinds of EE paradigms during pregnancy facilitates learning and memory abilities (Kiyono et al., 1985; Koo et al., 2003), and increases synaptic plasticity and hippocampal neurogenesis in the progeny (Koo et al., 2003). In addition, the maternal EE exposure during pregnancy reduces attentional performance (Cymerblit-Sabba et al., 2013), and controversially affects anxiety levels of the offspring (Maruoka et al., 2009; Rosenfeld and Weller, 2012; Cymerblit-Sabba et al., 2013).

Maternal exposure to EE during pregnancy and lactation improves spatial memory performances in the Morris water maze, reduces anxiety, and prevents the hippocampal tissue loss after neonatal hypoxia ischemia (Sparling et al., 2010; Durán-Carabali et al., 2018).

Also the exposure of mothers to exercise only paradigms during pregnancy (Parnpiansil et al., 2003; Lee et al., 2006; Herring et al., 2012) or during pregnancy and lactation (Bick-Sander et al., 2006) is able to improve learning and memory abilities, protect from neurodegeneration, improve brain plasticity and enhance hippocampal neurogenesis in the offspring.

The maternal exposure to EE before and during gestation affects offspring's development trajectories by modifying cognitive and emotional outcomes in a sex-specific manner with improved learning ability only in females and increased anxiety mainly in males (Connors et al., 2015; Zuena et al., 2016).

Lastly, data resulting from post-natal social enrichment obtained by communal nesting are variable and still

difficult to reconcile across studies. For example, a study by Heiderstadt et al. (2014) does not find any difference induced by communal nesting in learning or anxiety tests in the adult offspring. Conversely, in a study by Curley et al. (2009) communal nesting reduces anxiety levels and modifies oxytocin and vasopressin receptor densities in the adult offspring. These results were obtained by using different mice strains [i.e., C57BL/6J, DBA/2J and 129x1/SvJ mice (Heiderstadt et al., 2014) vs. Balb/c mice (Curley et al., 2009)], and suggest the importance of considering strain-specific effects in interpreting the long-term developmental effects of early social enrichment. In fact, communal nesting may be particularly effective and beneficial when using the Balb/c strain, since these mice display a more stress vulnerable phenotype [e.g., elevated stress responses and behavioral inhibition accompanied by reduced hippocampal glucocorticoid receptor expression and increased corticosterone (CORT) levels] (Brinks et al., 2007; Brodkin, 2007).

Effects of Pre-reproductive Parental Enrichment

The few studies available on the effects of pre-conceptional parental enrichment on the cognition and emotional response of the offspring generally indicate an amelioration of memory performances and a reduced anxiety. In fact, Arai et al. (2009) found rescued contextual fear conditioning memory and enhanced long-term potentiation not only in mice directly exposed to 2-weeks of EE when juveniles, but also in their offspring that never experienced EE. Benefits of EE across generations are associated with a signaling cascade in the CA1 hippocampal region and pass on through the mother (Arai et al., 2009). In rats, intergenerational effects of females' exposure to EE from weaning to reproductive age include improvements in cognitive performances as well as increased hippocampal BDNF levels in their male offspring, without changes in neurogenesis or reelin levels (Cutuli et al., 2015). A similar protocol of pre-reproductive maternal enrichment does not influence avoidance learning, and induces sex-dependent effects in female offspring by reducing anxiety and improving habituation to acoustic startle (Leshem and Schulkin, 2012).

Low LG female offspring housed under enriched conditions display increased LG behavior and higher levels of exploration in comparison to standard housed low LG females (Champagne and Meaney, 2007). These variations are also passed to the progeny.

In addition, communal nesting is able to increase maternal care and reduce anxiety across generations (Curley et al., 2009).

As for pre-reproductive paternal enrichment experiences, the existing studies mainly addressed the emotional responses of the offspring. Namely, a very recent study by Benito et al. (2018) demonstrated that pre-conceptional exposure of adult male mice to EE enhances LTP and induces a subtle memory improvement in the next generation, and that this phenotype is mediated by changes in the RNA composition in the sperm of the enriched fathers, especially through the upregulation of microRNA 212 and 132.

In a genetic rat model of absence epilepsy, early enrichment of fathers from weaning to breeding induces anti-epileptogenic and anxiolytic effects that were heritable across generations (Dezsi et al., 2016). These findings are in line with the anxiolytic effects of pre-reproductive paternal exercise. In fact, the male offspring of runner fathers show reduced anxiety levels and more robust fear extinction memory associated with alterations in the levels of small non-coding RNAs in sperm (Short et al., 2017). Conversely, it has been recently demonstrated that when the motor enrichment component is lacking, no differences in anxiety are found following pre-reproductive paternal enrichment (Yeshurun et al., 2017).

In conclusion, it seems that pre-reproductive paternal housing conditions which include an overt motor enrichment by running-wheel presence are able to induce anxiolytic effects.

With regard to indirect effects of parental enriching experiences on the pups' behavioral trajectories through the modulation of maternal care, the few data currently available are still little explicative, since maternal behavior has not been systematically investigated. However, it can be noted that when an enhancement in maternal care is evident following pre-reproductive maternal enriching experiences, the offspring's phenotype can be characterized by enhanced maternal care and/or reduced anxiety (Champagne and Meaney, 2007; Curley et al., 2009) or by improved cognitive performances (Cutuli et al., 2015).

As for paternal enrichment, it seems that a clear relationship between maternal care and offspring's behavior is still not well-definite. In fact, studies reporting behavioral modifications in the offspring of not-enriched females mated with males enriched during the pre-reproductive period fail to report maternal care modifications, thus suggesting that the progeny could be differently influenced by the pre-reproductive paternal experiences according to the different environmental manipulations used (motor enrichment vs. cognitive and physical enrichment), regardless maternal investment (Short et al., 2017; Yeshurun et al., 2017). Interestingly, enriching fathers preconceptionally significantly reduced global methylation levels in the frontal cortex and hippocampus of the developing offspring (Mychasiuk et al., 2012). And, other studies found modifications of RNA expression in the sperm (Short et al., 2017; Benito et al., 2018). As sperm development in rodents occurs continuously, as in humans, it can be speculated that pre-reproductive paternal enrichment experiences are able to alter gene expression in the sperm of sires, thus providing a means for the transmission of epigenetic change to the progeny.

Effects of Pre-reproductive vs. Post-reproductive Parental Enrichment on Social Behavior

Social interactions are essential for survival and proper neural and behavioral development. After weaning, playful interactions with peers allow the acquisition of social and cognitive competence. Social play behavior is a highly rewarding activity in humans and animals, as it can instill a sense of well-being and pleasure, motivates approach behaviors toward a specific social stimulus, and finally elicits associative learning in order to attribute salience to socially related cues (Trezza et al., 2011;

Vanderschuren et al., 2016). Interestingly, some studies indicate that EE may influence the social behavior in rodents. Namely, the exposure of pups to EE in the post-weaning period (Morley-Fletcher et al., 2003; Leshem and Schulkin, 2012) increases social interaction at adulthood and adolescence, respectively. Also, maternal exposure to EE before and during gestation enhances social behavior in the adolescent offspring with contrasting results depending on sex [i.e., higher social contact duration in the female offspring (Connors et al., 2015), and higher social play behavior in the male offspring (Zuena et al., 2016)].

As for pre-reproductive maternal EE, two studies demonstrated that it reduces social interaction in males, but not in females either in adult and adolescent offspring (Leshem and Schulkin, 2012; Cutuli et al., 2018). Similar effects are found in the adult offspring of pre-reproductively stressed females (Leshem and Schulkin, 2012), and are in line with the generally increased vulnerability of male rats to developmental disruption of social behavior by gestational ethanol (Mooney and Varlinskaya, 2011). The reduction in social play, and in particular in play solicitation (i.e., Pouncing) found in the enriched dams' male adolescent offspring by Cutuli et al. (2018), is probably linked to a less rewarding value of play initiation (Vanderschuren et al., 1997) or to not clarified neurohormonal modifications.

Nevertheless, pre-reproductive maternal EE does not induce any difference in sociability as assessed in the three-chamber sociability test (Cutuli et al., 2015), in which social interaction is prevented and the animal has to choose between a compartment containing a juvenile conspecific or an empty compartment.

Effects of Pre-reproductive vs. Post-reproductive Parental Enrichment on Stress Response

Environmental enrichment has been considered as prevention/intervention strategy to counteract the negative consequences of stress (McCreary and Metz, 2016).

The ability to cope with environmental challenges is essential to be successfully adapted. To face acute and chronic stressors, the sympathetic system and the hypothalamic-pituitary-adrenal (HPA) axis guarantee the recruitment of necessary resources, the inhibition of non-necessary ones, and fine feedback mechanisms for homeostasis. Glucocorticoids (GCs) secreted from adrenal glands act peripherally and in the brain through their binding with mineralcorticoid (MR) and glucocorticoid (GR) receptors. GR are recruited when GCs levels are elevated, as in a stressful condition, and are involved in negative feedback on HPA axis (De Kloet et al., 2005; Herman et al., 2016; Mifsud and Reul, 2018).

Genetic background (Kundakovic et al., 2013; Andolina et al., 2015; Di Segni et al., 2016) and parental environment (Meaney, 2001) shape individual differences in the ability to cope with stress and in the susceptibility of its negative consequences (Belsky and Pluess, 2009; Karatsoreos and McEwen, 2011; Daskalakis et al., 2013; Boersma and Tamashiro, 2015).

Parents have a pivotal role in programming the offspring's HPA axis functioning as widely demonstrated in rodents (Meaney, 2001; Enthoven et al., 2010), and more controversially in non-human primates (Sanchez, 2006) and humans (Tollenaar et al., 2011).

Parental stress has been associated with offspring's greater risk of psychopathology (Glover, 2011), higher glucocorticoid sensitivity (Lehrner et al., 2014), immunological alterations (Laviola et al., 2004), enhanced neuronal activity (Bielas et al., 2014) and altered DNA methylation (Mulligan et al., 2012; Essex et al., 2013). Recently, epigenetic inheritance is assigned a captivating role in the intergenerational outcomes (Franklin et al., 2010), stressing the standing of "epigenetics prior to the birth" (Lo and Zhou, 2014).

Compelling evidence from animal models highlights the effectiveness of the EE in preventing, rescuing or normalizing the negative outcomes of stress (McCreary and Metz, 2016). In rodents post-weaning EE normalizes basal immune parameters altered by pre-natal stress (Laviola et al., 2004), reverses the negative effects of pre-natal stress on HPA axis reactivity and play behavior (Morley-Fletcher et al., 2003), attenuates CORT levels after restraint stress (Sztainberg et al., 2010) and exerts enduring effects on CORT daily pattern and levels in the response to a novel environment (Peña et al., 2009). Similar findings were also recently found in zebrafish (*Danio rerio*) (Marcon et al., 2018).

In rats long-lasting moderate maternal EE during pregnancy, and together with pups during lactation until weaning, modifies the response of the female offspring to a chronic stress (Welberg et al., 2006). Whereas chronically stressed females show increased basal CORT and reduced adrenocorticotropic (ACTH) levels in response to stress, no such effects neither on basal CORT nor in ACTH acute release are found in chronically stressed offspring exposed to early EE.

Moreover, the positive effects of EE on stress response are reported in different strains of mice. Branchi et al. (2010) research with outbred CD-1, Swiss-derived strain (ICR) of mice clearly demonstrates that social enrichment by communal nesting that provides maternal caregiving from three different mothers and higher peer interaction is able to modify the offspring's behaviors and neuroendocrine response to stress, by reducing anhedonia and decreasing CORT levels in response to social stress, enhancing time spent in immobility at the forced swim test (FST) and finally dampening the response to 3-weeks fluoxetine treatment. Also, in Balb/c mice, an inbred strain known for its high anxiety-like behavior, social enrichment by communal nesting reduces offspring's stress response when exposed to a novel environment (Curley et al., 2009).

Remarkably, the enhancement of stimulations provided by EE produces changes that propagate across generations influencing descendant's future responses to chronic and acute stress (Taouk and Schulkin, 2016) and promoting resilience even at the germline level (Gapp et al., 2016). The authors (Gapp et al., 2016) evidenced the role of both negative and positive environmental manipulations (i.e., maternal stress and unpredictable maternal separation, and exposure to EE, respectively) in influencing potential epigenome trajectories across generations. In particular, they proved that the detrimental effects of early stress on male offspring coping behaviors (F1) are related to a significant increase in GR expression in hippocampus and showed the propagation of the stress-related negative outcomes up until the

subsequent generation (F2). The intergenerational transmission of phenotype is associated with a decreased methylation in exons 1–7 of the GR gene both in the F2 offspring and in the F1 sperm. In addition, the authors disclosed that EE is effective in preventing the transgenerational effects of stress on F2. The GR gene hypomethylation found in the sperm of early-stressed males was in fact rescued by EE. A very recent study also emphasizes the role of specific sperm microRNAs in mediating the effects of paternal pre-conceptional EE (Benito et al., 2018).

Pre-reproductive EE can modify the effect of social isolation, in rats. The offspring born to standardly reared mothers and subjected to social isolation from weaning to adolescence show greater GR expression in amygdala compared to not-isolated controls, whereas socially isolated offspring born to pre-reproductively enriched mothers do not show any difference in GR expression when compared to the respective not-isolated controls. Furthermore, a blunted amygdaloid c-Fos immunoreactivity in response to the FST is evident in the offspring of pre-reproductively enriched mothers in comparison to the offspring of standardly reared dams (Cutuli et al., 2017).

Recently, the transgenerational effects of pre-conceptional paternal EE on despair behaviors and neuroendocrine phenotypes of the adult offspring in mice has been investigated (Yeshurun et al., 2017). EE effects skip the F1 generation and are sex-specific. While pre-conceptional paternal EE has no effect on despair behaviors of F1 male and female offspring neither on basal ACTH or CORT levels in response to FST, modifies F2 females' behavioral despair and CORT response in the FST. The transgenerational effect of paternal EE is not mediated by paternally induced changes in maternal care since no differences in maternal behaviors between EE and standardly reared male paired mothers.

Leshem and Schulkin (2012) showed that pre-reproductive maternal enrichment ameliorates the anxiogenic effect induced by pre-reproductive stress on offspring. The above-mentioned study also demonstrates stress-like effects of EE.

Environmental enrichment and stress indeed activate similar circuitries and involve HPA axis activity and changes in GR expression and functionality (Larsson et al., 2002; Moncek et al., 2004).

Several theoretical models have been proposed to account for the beneficial or contrasting effects of EE on stress response. Crofton et al. (2015) had addressed the issue in the inoculation stress hypothesis framework: the enhanced and complex stimulation provided by EE would represent a continuous mild form of stress that inoculates animals to subsequent challenges, as a controlled exposure to an harmless vaccine would protect against future encounter of the disease. Besides, the transition from eustress to detrimental stress follows the non-linear inverted U-shaped dose–response curve for which optimal levels of functionality are obtained at the moderate CORT expression while very high (e.g., overstimulation) or very low (e.g., understimulation) levels can exert similar deleterious effects on the individual (Sapolsky, 2015).

Considering conflicting transgenerational EE effects, the degree of fitness between the environmental challenges experienced by the offspring and the environmental demands for which parents and grandparents have equipped future generations to cope with has to be keep in mind (Marshall and Uller, 2007; Daskalakis et al., 2012; Nederhof and Schmidt, 2012; Prizak et al., 2014).

Finally, transgenerational inheritance could interact with ingrained individual differences to determine the phenotypic scenarios "for better or for worse" (Belsky et al., 2009).

IMPACT OF DIFFERENTLY TIMED PARENTAL ENRICHMENT ON BDNF

Neurotrophins constitute a protein family whose components show analogous structure and exert an essential action on the development and function of the neurons. They regulate cell proliferation and differentiation, growth and readjustment of axons and dendrites, and plastic changes involved in synaptic function (Park and Poo, 2013). In particular, BDNF is retained a key-player in the translation of the experience in neural structure and function modifications and a trigger and mediator of synaptic plasticity (Cowansage et al., 2010; Bekinschtein et al., 2011; Aarse et al., 2016). The direct effects of EE on brain BDNF levels have been demonstrated in a large number of animal studies (Angelucci et al., 2009; Gelfo et al., 2011; Mosaferi et al., 2015; Novkovic et al., 2015).

Recently, it has been evaluated if the known EE effects on BDNF-mediated brain plasticity could be transgenerationally transmitted. Namely, some research has been specifically devoted to study the effects of the pre-reproductive exposure of mothers to EE on the offspring's BDNF brain levels (Caporali et al., 2014; Caporali et al., 2015; Cutuli et al., 2015). Interestingly, while the pre-reproductive maternal EE does not modify pups' brain BDNF protein levels at birth, changes are evident at weaning and in adulthood (Caporali et al., 2014; Cutuli et al., 2015). It may be hypothesized that eventual latent genetic and epigenetic effects of pre-reproductive maternal EE on BDNF signaling need the interaction with the mother and the external ambient (that occurs only after the birth) to be manifest in term of BDNF protein differences. In fact, stable effects of pre-reproductive maternal EE are evident in pups' brain BDNF levels from weaning onward, and are different in association with the different experiences the pups are exposed to. When BDNF brain expression is evaluated at weaning in pups exposed to repeated motor challenging and exercise, BDNF increases in enriched mother's offspring at cerebellar and striatal levels, demonstrating an enhanced plasticity expression in the areas involved in motor performance (Caporali et al., 2014). On the other hand, when BDNF expression is evaluated in pups exposed only to maternal care and cage interaction with brotherhood (and not to behavioral testing), BDNF increases at weaning and in adulthood only in hippocampus, the brain area involved in memory formation, and thus solicited by every experience (Cutuli et al., 2015). The exposure after birth to the care of an enriched mother that shows in turn increased brain BDNF level (Caporali et al., 2015; Cutuli et al., 2015) is fundamental for any BDNF change. In fact, the overt increase in hippocampal BDNF levels is not anymore found

when the pups are born to an enriched mother but raised until weaning by a standard-reared mother (Caporali et al., 2015).

In line with our results, in a different model of maternal EE, pups born to mothers enriched during pregnancy and lactation do not show variations in cortical or hippocampal BDNF levels few days after birth (Durán-Carabali et al., 2018). In addition, it has been demonstrated that even a specific component of pre-reproductive EE, such as exercise, is able to change offspring's BDNF brain levels. Parnpiansil et al. (2003) reported that rat pups born to mother exposed to motor enrichment during pregnancy show BDNF mRNA hippocampal expression enhanced at birth, unchanged during lactation and reduced after weaning. More recently, it has been showed that motor enrichment during pregnancy induces enhanced BDNF protein expression in the hippocampus of pups when adult (Gomes Da Silva et al., 2016). Similarly, Park et al. (2013) showed that mice pups born to mother exposed to motor enrichment during pregnancy display enhanced hippocampal BDNF expression after weaning. Furthermore, maternal motor enrichment during pregnancy induces increased BDNF mRNA hippocampal expression in rat pups after weaning (Lee et al., 2006).

On the whole, the available evidence supports the influence of the maternal enrichment during pre-reproductive and post-reproductive periods on the pup brain BDNF-mediated neuroplasticity, regardless of the specific characteristics of the enrichment paradigm.

IMPACT OF DIFFERENTLY TIMED PARENTAL ENRICHMENT ON THE OXYTOCINERGIC SYSTEM

The neuropeptide oxytocin is widely implicated in the social behavior of mammalian by modulating maternal care, infant behavior, social bonding, agonistic behavior and social recognition (Lim and Young, 2006; Bosch, 2013; Crespi, 2016). It is synthesized in the magnocellular neurons of the hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus (SON), and is transported along their axons to the posterior pituitary and released from there into the blood stream to act on target organs in the periphery (Veenema, 2009). It is also released in the forebrain and hindbrain regions (Veenema, 2009). Oxytocin receptors are expressed in many brain regions, including cortical, limbic, hypothalamic and brain stem areas (Lee et al., 2009; Veenema, 2009).

Unfortunately, there is a scant literature on the effects of the enrichment on oxytocinergic system either in the dams and offspring. With regards to differently enriched dams, it seems that a potentiation of the oxytocinergic system is linked to increased maternal care. For example, communally reared dams exhibit elevated levels of *post-partum* care and of oxytocin receptor density in the lateral septum across generations (Curley et al., 2009). Similarly, post-weaning enrichment enhances LG behavior and oxytocin receptor binding of low LG offspring in the PVN, medial preoptic area and bed nucleus of the stria terminalis in comparison to low LG offspring housed in standard conditions (Champagne and Meaney, 2007).

A recent study has demonstrated the impact of pre-reproductive maternal EE on the hypothalamic oxytocinergic neurons on mothers and pups (Cutuli et al., 2018). Indeed, enriched dams show an increased number of oxytocinergic neurons either in PVN and SON associated with increased crouching levels and faster pups' retrieval. As for the adolescent offspring, while no differences have been found in the female pups, the male pups of pre-reproductively enriched dams exhibited higher levels of oxytocinergic neurons in SON and reduced play behavior. Interestingly, the anti-aggressive properties of the oxytocin are well-documented in humans and rodents (Todeschin et al., 2009; Crespi, 2016; Hathaway et al., 2016), and SON is the hypothalamic nucleus selectively activated after the display of offense (Kollack-Walker et al., 1997). Thus, being the social play a sort of preparation for the adult aggressive behavior (Aldis, 1975), the reduction in play behavior observed in male pups of enriched dams may be attributed to reduced aggressive tendencies linked to increased oxytocin levels in SON.

Due to the consistent interactions between oxytocinergic and dopaminergic systems in the establishment and maintenance of mother-pup relationship (Crespi, 2016), the differently enriched dams could be more attracted by pups because of a more rewarding effect of nurturing behaviors. And in turn, their pups could be more soliciting. To our knowledge, only one study has described an increased motivation of male pups born to pre-reproductively enriched females to contact the odor of their mothers (Cutuli et al., 2015). Furthermore, the increased maternal care found in enriched dams may potentiate the oxytocinergic system of their pups through epigenetic modifications, as demonstrated in previous studies (Champagne, 2008).

In conclusion, many questions remain to be answered and further studies are expected to clarify the role of oxytocinergic system in mediating the impact of parental enrichment in the subsequent generations.

DISCUSSION

An increasing body of evidence from animal studies shows the outcomes of maternal enrichment exposure during, before and/or after the gestation on development, behavior and physiological functioning of the progeny (Arai and Feig, 2011; Taouk and Schulkin, 2016).

In the present review article, we focused on the effects of pre-reproductive exposure of parents to highly stimulating environments on maternal behavior and offspring's phenotype.

A problem encountered in screening the current (still limited) literature is the diversity of paradigms as for timing of exposure and enhanced stimulations used. Nevertheless, a common pattern emerges in findings. As for dams, the pre-reproductive enrichment tendentially results in increased maternal care, brain BDNF and oxytocinergic levels. As for offspring, the pre-reproductive parental enrichment appears to accelerate the acquisition of complex motor

abilities, potentiate cognitive performances and coping skills, reduce anxiety, increase brain BDNF levels and modulate social behavior and oxytocinergic system in a sex-dependent manner.

CONCLUSION

On the whole, the available evidence supports an influence of pre-reproductive parental enrichment on mother's and pups' brain and behavior. However, given the diversity in the different parental enrichment paradigms, systematic analyses on the selective exposure to EE (and its social, cognitive, physical and motor components) in the different pre-reproductive and post-reproductive periods are required to provide exhaustive evidence on brain and behavior changes induced in pups by parental experiences.

It is interesting to speculate on the mechanisms of inheritance involved in the transmission of the environmental influence from parents to the offspring. The inheritance across generations may involve epigenetic modifications in the germline or can be passed to the offspring through maternal care during early post-natal life (Champagne and Curley, 2009; Bohacek and Mansuy, 2015; Kundakovic and Champagne, 2015). Non-genetic marks fluctuate throughout lifetime and carry important information about previous experiences and faced environments, and their outcomes on the organism (Campos et al., 2014). For these reasons, exposure to enriching experiences may be regarded as therapeutic intervention to support healthy aging (Ambeskovic et al., 2017) or reverse stress detrimental effects (Leshem and Schulkin, 2012).

Finally, the study of the inter- and transgenerational origin of age- or stress-related diseases offers the opportunity to identify predictive or diagnostic biomarkers crucial to develop new interventions according to a tailored clinical approach that claims healthy aging and mental health in today's population and future generations.

AUTHOR CONTRIBUTIONS

DC and LP coordinated the work, wrote, and edited the article. EB, FG, PC, and DL contributed in writing the article.

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frontiers in Behavioral Neuroscience

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Post-weaning Environmental Enrichment in Male CD-1 Mice: Impact on Social Behaviors, Corticosterone Levels and Prefrontal Cytokine Expression in Adulthood

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Environmental enrichment is typically associated with enhanced well-being, improved cognitive function and stress resilience. However, in some instances grouping adult male mice in enriched conditions promoted a stressful environment, which resulted in elevated endocrine, monoamine and inflammatory outcomes in response to subsequent stressor exposure. The current investigation examined whether raising male mice in an enriched environment (EE) would modulate social and anxiety-like behaviors in early adulthood and influence brain expression of pro-inflammatory cytokines and brain-derived neurotrophic factor (BDNF). Immediately after weaning (postnatal day [PD] 21), CD-1 male mice were housed with their siblings (3/cage) for 6 weeks in an EE or a standard (SE) environment. Body weights and aggressive interactions were monitored weekly. Social avoidance behaviors in the social interaction test and anxiety-like behaviors in the elevated-plus maze were examined in early adulthood. Ninety minutes following the behavioral tests, mice were sacrificed and a blood sample and the prefrontal cortex (PFC) were collected for the determination of plasma corticosterone levels as well as cytokine and BDNF mRNA expression. Mice raised in an EE exhibited more wounds and gained less weight than mice housed in a SE. Enriched mice also spent a greater amount of time in proximity of a social target in the social interaction test and made fewer transitions into the closed arms of the elevated-plus maze. Interestingly, the elevated plasma corticosterone and upregulated prefrontal interleukin (IL)-1β expression observed after the social interaction test among the SE mice were not apparent among those housed in an EE. Enrichment also increased prefrontal BDNF expression, especially among mice that experienced the social interaction test. These results suggest that although raising male mice in an EE may elicit aggressive interactions between sibling cage-mates (as indicated by a high number of wounds), this environment also enhances social behaviors and limits the corticosterone and cytokine impacts of mild social stressors encountered in early adulthood.

Keywords: aggression, enrichment, mice, PFC, cytokines, stress resilience

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INTRODUCTION

Environmental conditions may have a substantial influence over animal behaviors and well-being (Kentner, 2015). Enriched environments (EE) in laboratory rodents comprise housing in large cages equipped with toys and exercise items that stimulate playful behavior and physical activity. This manipulation may enhance cognitive abilities (He et al., 2017; Zeleznikow-Johnston et al., 2017) and diminish anxiety- and depressive-like behaviors (Galani et al., 2007; Nicolas et al., 2015; Reichmann et al., 2016; Aujnarain et al., 2018). Behavioral and cognitive effects of EE have been linked to increased neurogenesis and elevated expression of hippocampal brain-derived neutrophic factor (BDNF; Schloesser et al., 2010; Novkovic et al., 2015), and improved synaptic and transcriptomic capacity (Hüttenrauch et al., 2016; Zhang et al., 2018). In addition to improving psychological and physiological well-being in otherwise healthy animals, EE may limit behavioral, cognitive and biological disturbances provoked by stressful experiences. For instance, EE reduced anxiety-like behaviors and prevented cognitive impairments ordinarily elicited by acute and chronic stressors (Cordner and Tamashiro, 2016; Bahi, 2017; Marianno et al., 2017; Dandi et al., 2018). Mice housed in EE also exhibited limited corticosterone elevations and neuronal activation after stressor exposure (Branchi et al., 2013; Reichmann et al., 2013; Mesa-Gresa et al., 2016), indicating that housing conditions may modulate the impact of external stressors on hormonal and brain functions.

Although the stress buffering impacts of EE have frequently been confirmed, other reports, including from our laboratory, indicated that in some instances EE promoted territorial and aggressive behaviors that mitigated the positive effects that are often attributed to enrichment (McQuaid et al., 2012, 2013a). In this regard, male mice housed in EE in groups of 3–4 exhibited more anxiety-like behaviors and had increased corticosterone levels in response to stressors compared to their group-housed counterparts maintained in standard laboratory environments (Marashi et al., 2003; McQuaid et al., 2012, 2013a). They also displayed exaggerated monoamine and cytokine elevations ordinarily elicited by social and non-social stressors (McQuaid et al., 2012, 2013a,b). Precisely why EE elicits distress in group-housed male mice is uncertain, but it was suggested that the inherent territoriality of male mice could result in an unstable hierarchical structure and heightened aggression (Van Loo et al., 2003). In fact, although mice generally fare better in groups, laboratory confinement may inhibit natural territorial interactions that would be exhibited in the wild and thus accentuate territoriality and increase aggression (Howerton et al., 2008). Several reports have shown that the addition of stimulating objects (e.g., running wheels) to large environments among group-housed male mice promoted territorial and aggressive behaviors (Van Loo et al., 2002; Marashi et al., 2003; Howerton et al., 2008; McQuaid et al., 2012, 2013a).

Increased territoriality and aggression (and the resulting distress-related behavioral and biological outcomes) after the implementation of EE in laboratory mice have been primarily observed when the enrichment procedures were initiated in adult animals. Early environmental manipulations may dramatically influence behavioral, emotional and cognitive profiles in later life (Sánchez et al., 2001; Krugers et al., 2017). In relation to enrichment, post-weaning EE in male and female mice enhanced social preference, reduced anxiety-like behaviors, and improved spatial learning in adulthood (Hendershott et al., 2016; Aujnarain et al., 2018). As well, when housed in an EE with their siblings after weaning, male offspring born to dams stressed during pregnancy had reduced anxiety-like behaviors and limited Purkinje cell dendritic atrophy over the life course (Pascual et al., 2015). Thus, it is possible that the negative effects of social enrichment in male mice could be avoided by placing mice in EE immediately after weaning as opposed to early adulthood.

The current investigation examined the effects of a 6-week EE regimen on aggressive, social and anxiety-like behaviors, as well as on stress-induced corticosterone and brain inflammatory activation in CD-1 male mice. This outbred strain was selected as adult CD-1 male mice seem particularly prone to increased aggression in response to the addition of stimulating objects in their environment (Van Loo et al., 2002; Marashi et al., 2003; McQuaid et al., 2012). In contrast to earlier enrichment studies using this strain (Gross et al., 2011; McQuaid et al., 2012), mice were housed in sibling groups immediately after weaning in order to minimize social changes. We hypothesized that: (1) aggression levels and severity over the course of enriched housing would be comparable among SE and EE male CD-1 mice; (2) EE would increase social behaviors and reduce behaviors that reflect anxiety (Hendershott et al., 2016; Aujnarain et al., 2018); and (3) EE would limit plasma corticosterone elevations as well as brain cytokine and BDNF changes following exposure to a mild social (social interaction test) and non-social (elevated-plus maze) stressor (Rodgers et al., 1999; Koya et al., 2005; Skurlova et al., 2011).

MATERIALS AND METHODS

Animals and Housing Conditions

Mice were bred at Carleton University from CD-1 parent stock obtained from Charles River Canada (St. Constant, QC, Canada). Immediately after weaning at postnatal day (PD) 21, male mice were housed with their siblings (3/cage) for 6 weeks in a standard (SE; $n = 36$) or an enriched (EE; $n = 33$) environment. The SE consisted of a standard polypropylene cage (27 × 21 × 14 cm) containing one cotton nestlet. The EE comprised a large polypropylene rat maternity cage (50 × 40 × 20 cm) equipped with two running wheels, one orange polypropylene shelter attached to an angled running wheel, one red polypropylene shelter, three yellow polypropylene tunnels, and two cotton nestlets, as previously described (McQuaid et al., 2012, 2013a,b). Enrichment objects were replaced with clean duplicates once a week during cage cleaning but were otherwise not manipulated. Mice were left in their respective environments undisturbed, except during cage cleaning (once a week for EE mice and twice a week for SE mice due to the smaller dimensions of the housing environment) and tail marking (three times/week for both EE and SE mice; at the base of the tail, using a color-coding system with non-toxic markers). All mice were kept in the

same temperature (21°C) and humidity (63%) controlled room, given free access to food and tap water, and maintained on a 12-h light/dark cycle, with lights on from 08:00 h to 20:00 h. All experimental procedures were approved by the Carleton University Animal Care Committee and met the guidelines set out by the Canadian Council on Animal Care.

Weighing and Aggression Scoring

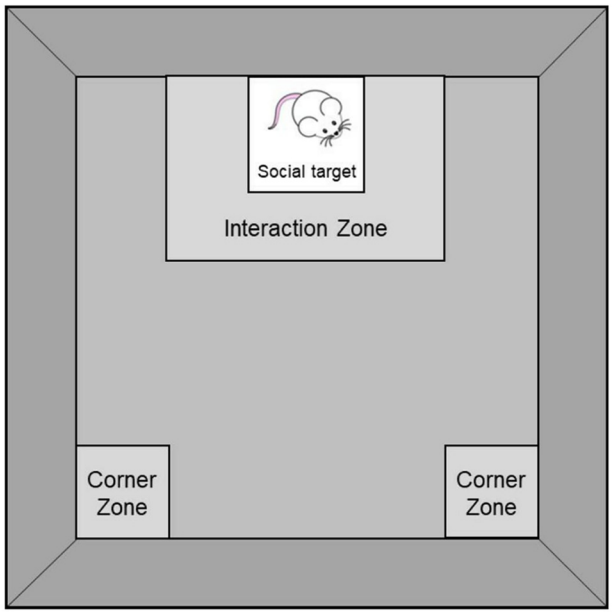
Mice were weighed immediately after weaning (before being placed into their respective environments) and then once a week during the 6-week SE and EE housing. The percentages of weight change from weaning were determined at the end of each week ([Weight at Week X − Weight at weaning]/Weight at weaning). Home-cage interactions were videotaped for 30 min at the same time of day (between 10:00 h and 11:00 h) every week. As hierarchical reorganization is highest after cage cleaning (Van Loo et al., 2000), videotaping was carried out immediately after mice were placed into their clean cages. Experimenters blind to the housing conditions subsequently scored, manually, aggressive behaviors from videotapes. These included Attacking (biting or attempting to bite, sometimes accompanied by tail rattling), Aggressive chasing (rushing or jumping at another mouse), Aggressive grooming (vigorous sniffing or licking), and Fighting (aggressive wrestling between two or more mice). The presence of wounds was also examined after cage cleaning and tail marking sessions as an indirect measure of aggression.

Behavioral Tests

After being housed in a SE or an EE for 6 weeks, mice were brought to their respective testing rooms 24 h prior to the behavioral test sessions to acclimate to the new laboratory environments. Behavioral testing took place between 08:00 h and 13:00 h to minimize effects related to diurnal factors. Mice within a same cage were randomly assigned to the social interaction test or the elevated-plus maze test (which also served as mild social or non-social stressors, respectively) or were not manipulated (no stressor condition). After the behavioral tests, mice were returned to their respective environments for 90 min, after which they were sacrificed and blood and brain tissue were collected.

Social Interaction Test

Social behaviors were assessed in the social interaction test (see **Scheme 1**). As previously described (Berton et al., 2006; Szyszkowicz et al., 2017), mice were placed, individually, into a black Plexiglas open field (42 × 42 × 60 cm) that contained a wire-mesh enclosure (10 × 10 × 30 cm) for two sessions of 150 s each, under red-light conditions. During the first session, the enclosure was empty whereas during the second session, a 6-month-old male CD-1 retired breeder (social target) was present in the enclosure. Between the two sessions, mice were placed into an empty cage with bedding for about 60 s during which open field surfaces were cleaned with a 70% ethanol solution and wire-mesh enclosures were switched. The two sessions were videotaped with an infra-red camera and movements were subsequently scored manually by an experimenter blind to the housing conditions. Time spent in an 8-cm corridor surrounding the enclosure (Interaction Zone) and in the 8 × 8 cm corner areas opposing the wire-mesh enclosure (Corner Zones) were used as measures of social avoidance. Because chronic social defeat, which is typically used to elicit social avoidance in this test (Berton et al., 2006; Szyszkowicz et al., 2017), was not used in the current experiment, a social interaction ratio (which classifies mice as susceptible or resilient to the effects of social stress) was not established.



SCHEME 1 | Social Interaction Test comprising an 8 × 8 cm Interaction Zone surrounding a wire-mesh enclosure (10 × 10 × 30 cm; where a social target is introduced during the second session of the test) and two 8 × 8 cm Corner Zones opposing the wire-mesh enclosure.

Elevated-Plus Maze

Anxiety-like behaviors were assessed in the elevated-plus maze, which was composed of two opposing open arms (50 × 10 cm) and two opposing closed arms (50 × 10 cm; with 21 cm-high walls) made of black Plexiglas. The apparatus was elevated 30 cm above a stable surface, in a dimly lit room (approximately 50 lux). Mice were individually placed at the extremity of a closed arm of the maze (facing away from the center) and their behaviors were recorded for 5 min by a ceiling-mounted video camera. Latency to enter an open arm as well as time spent in and number of entries to (defined by all four paws being in an arm) open and closed arms were subsequently scored manually by an experimenter blind to the housing conditions.

Blood and Brain Collection

Mice were euthanized by rapid decapitation 90 min after the social interaction test or the elevated-plus maze test (which served as mild social and non-social stressors, respectively), or at corresponding time for mice that were not manipulated (non-stressed mice). Trunk blood was immediately collected in tubes containing 10 μg of EDTA, centrifuged for 8 min

at 3600 RPM, and the plasma was aliquoted and stored at −80°C for subsequent determination of corticosterone levels. Brains were rapidly removed and placed on a stainless steel brain matrix (2.5 × 3.75 × 2.0 cm) positioned on a block of ice. The matrix had a series of slots spaced approximately 500 μm apart that guided razor blades to provide coronal brain sections. Once the brains were sliced, a tissue section from the prefrontal cortex (PFC) was collected based on the mouse atlas of Franklin and Paxinos (1997), placed immediately in nuclease-free tubes positioned on dry ice, and stored at −80°C for subsequent determination of the mRNA expression of the pro-inflammatory cytokines IL-6, IL-1β and tumor necrosis factor (TNF)-α and of the neurotrophin BDNF. The PFC as the brain region of interest and IL-1β, IL-6 and TNF-α as the pro-inflammatory cytokines of interest were selected based on our previous findings that male CD-1 mice had altered prefrontal mRNA expression of these cytokines after acute and chronic exposure to social stressors (see Audet et al., 2010, 2011).

Plasma Corticosterone Determination

A commercial radioimmunoassay kit (ICN Biomedicals Inc., Costa Mesa, CA, USA) was used to determine plasma corticosterone concentrations (in duplicate). The assay was conducted in a single run to prevent inter-assay variability. The intra-assay variability was less than 10%. The assay sensitivity was 1.7 ng/ml.

Reverse Transcription-Quantitative Polymerase Chain Reaction Analysis (RT-qPCR)

Brain sections were homogenized using Trizol and total brain RNA was isolated according to the manufacturer's instructions (Invitrogen, Burlington, ON, Canada). RNA yields and purity were tested using a NanoDrop 2000 (Thermo Fisher Scientific). Only RNA samples with purity ratios 260/280 and 260/230 between 1.90 and 2.10 were included and thus the N and df associated with prefrontal gene expression differed from behavioral and corticosterone outcomes. The total RNA was then reverse-transcribed using Superscript II reverse transcriptase (Invitrogen, Burlington, ON, Canada). The resulting cDNA aliquots were analyzed in simultaneous quantitative polymerase chain reactions (qPCR) using SYBR green detection and a MyiQ2 Real-Time PCR Detection System (Bio-Rad, Canada). All designed PCR primer pairs generated amplicons between 129 base pairs and 200 base pairs. Amplicon identity was verified by restriction analysis. Primer efficiency was measured from the slope relation between absolute copy number of RNA quantity and the cycle threshold using the Bio-Rad IQ5 version 2.0 software (Bio-Rad, Canada). All primer pairs had a minimum of 90% efficiency.

Primers that amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and synaptophysin were used as reference genes. The expression of each gene of interest within the PFC was normalized by subtracting the quantification cycle (C_q) of the two reference genes averaged from the gene of interest C_q (ΔC_q). The $2^{-\Delta\Delta C_q}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) was used to convert ΔC_q values to mRNA fold changes relative to the SE/No stressor group (calibrator). Primer sequences used were as follows: Mus GAPDH, F: 5′-AAA TGG TGA AGG TCG GTG TG-3′, R: 5′-GAA TTT GCC GTG AGT GGA GT-3′; Mus Synaptophysin, F: 5′-GGA CGT GGT GAA TCA GCT GG-3′, R: 5′-GGC GAA GAT GGC AAA GAC C-3′; Mus IL-1β, F: 5′-TGT CTG AAG CAG CTA TGG CAA C-3′, R: 5′-CTG CCT GAA GCT CTT GTT GAT G-3′; Mus IL-6, F: 5′-TTC TTG GGA CTG ATG CTG GTG-3′, R: 5′-CAG AAT TGC CAT TGC ACA ACT C-3′. Mus TNF-α, F: 5′-CTC AGC CTC TTC TCA TTC CTG C-3′, R: 5′-GGC CAT AGA ACT GAT GAG AGG G-3′. Mus BDNF, F: 5′-GTC TCC AGG ACA GCA AAG CCA C-3′, R: 5′-CCT TGT CCG TGG ACG TTT ACT TC-3′.

Statistical Analyses

Percentage of weight change from weaning to each of Weeks 1–6 as well as aggression scores and behaviors in the elevated-plus maze test were analyzed using a one-way analysis of variance (ANOVA) with Housing serving as the between-groups factor (SE vs. EE). The actual weight over the course the 6-week housing period was analyzed using a 2 (Housing) × 6 (Week: Weeks 1–6) mixed measures ANOVA with repeated measures on the within-groups factor comprising the 6 weeks. Behaviors in the social interaction test were analyzed using a 2 (Housing) × 2 (Session: Absence vs. Presence of CD-1 retired breeder) mixed measures ANOVA with repeated measures on the within-groups factor comprising the two test sessions. Plasma corticosterone concentrations and fold changes in prefrontal mRNA expression of pro-inflammatory cytokines and BDNF were analyzed using a 2 (Housing) × 3 (Stressor: No Stressor, Social Stressor [social interaction test] and Non-Social Stressor [elevated-plus maze]) between-groups ANOVA. Follow-up comparisons comprised *t*-tests with a Bonferroni correction to maintain the alpha level at 0.05.

RESULTS

Body Weights

Body weights increased during the 6-week housing period, $F_{(6,402)} = 2226.00$, $p < 0.0001$, and varied as a function of the interaction between Housing and Week, $F_{(6,402)} = 5.78$, $p < 0.0001$. As shown in **Figure 1A**, the analysis of the simple effects comprising the Housing × Week interaction confirmed that SE and EE mice had comparable weights during Weeks 1–4, but EE mice were lighter than SE mice during Weeks 5 and 6 (p's < 0.05). Percentages of weight change (i.e., weight lost or gained) were also affected by Housing during Weeks 5 and 6, F's$_{(1,67)} = 3.49$ and 5.43, p's < 0.05, with EE mice exhibiting slight, but significantly reduced weight gain compared to SE mice (**Figure 1B**).

Aggression

For ethical reasons, mice that were excessively aggressive (and thus could potentially have elicited serious injuries to their

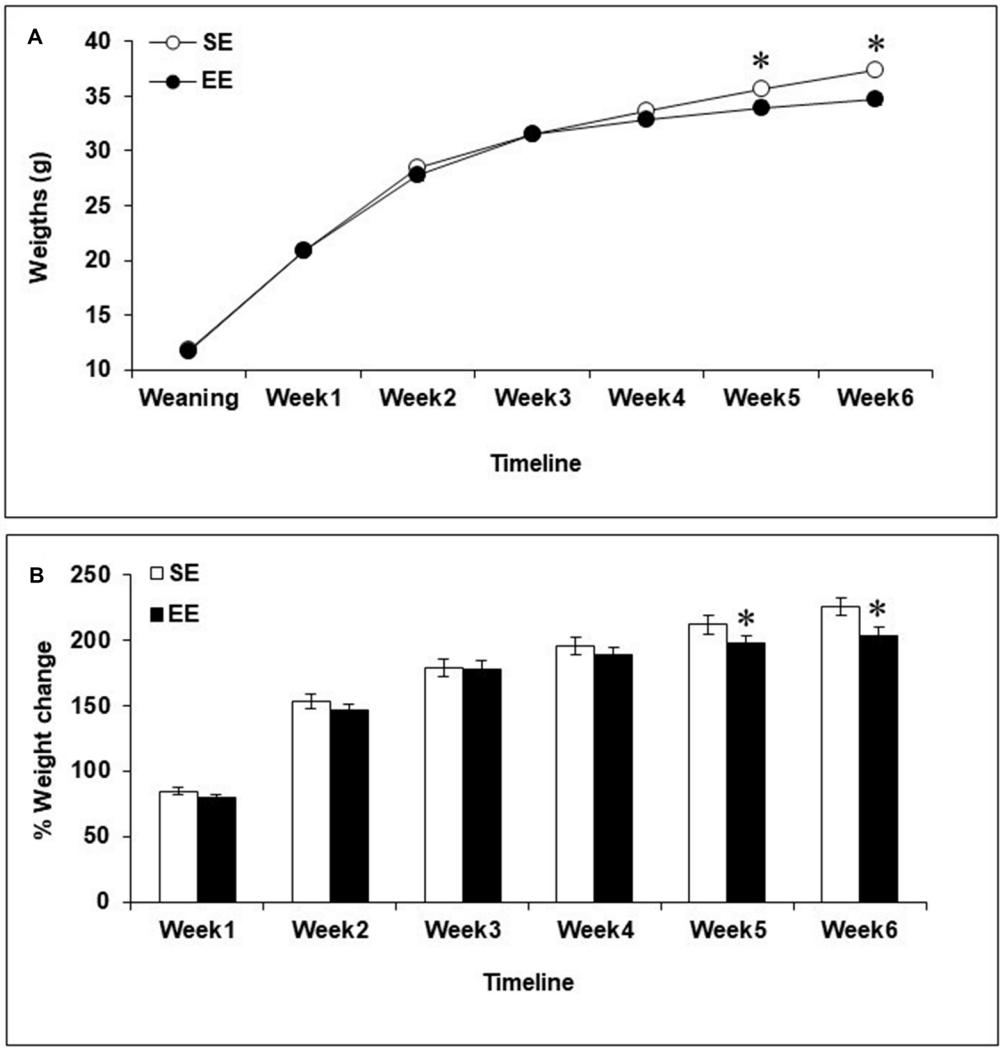


FIGURE 1 | Body weights among mice housed in a standard environment (SE) or an enriched environment (EE) during 6 weeks. **(A)** Actual weights increased over the course of the 6-week housing in both SE and EE mice, but EE mice weighed less than SE mice during Weeks 5 and 6. **(B)** Mice housed in EE gained as much weight as mice housed in SE at Weeks 1–4 but at Weeks 5 and 6, percentages of weight change from weaning were reduced in EE mice compared to SE mice. *$p < 0.05$ relative to mice housed in SE. Data represents means ± SEM (mice housed in SE, $n = 33$; mice housed in EE: $n = 36$).

cage-mates) were removed from their cages and from the study. By the end of Week 5, three EE mice had been removed, all from separate cages, but no SE mice had to be eliminated. **Table 1** displays the average number of aggressive encounters (total aggressions and specific types of aggressive behaviors), as well as the average number of wounds over the 6-week housing period in EE and SE mice. As depicted in **Table 1**, Fights, $F_{(1,10)} = 3.54$, $p = 0.089$, were marginally more frequent among SE mice than EE mice, but this difference did not reach significance. As well, aggressive chases appear to be more frequent among SE mice, but this was exceptionally variable over the 6-week housing and the difference observed did not approach significance, $F_{(1,10)} = 1.02$, $p = 0.40$. In contrast, EE mice had more wounds than SE mice, $F_{(1,10)} = 4.62$, $p < 0.05$.

TABLE 1 | Mean ± SEM of home-cage aggressive behaviors over the 6-week housing period among mice housed in a standard environment (SE) or an enriched environment (EE).

	Total aggressive encounters	Attacks	Aggressive chases	Aggressive grooming	Fights	Wounds
SE	49.50 ± 8.43	9.67 ± 1.93	23.83 ± 4.07	11.50 ± 1.95	4.83 ± 0.84	0.50 ± 0.20
EE	41.83 ± 8.22	11.83 ± 2.26	12.50 ± 2.11	15.67 ± 3.61	0.83 ± 0.22	6.67 ± 1.15*

$p < 0.05$ relative to mice housed in SE. Mice housed in SE: n = 33; mice housed in EE: n = 36. Scores were averaged per week.

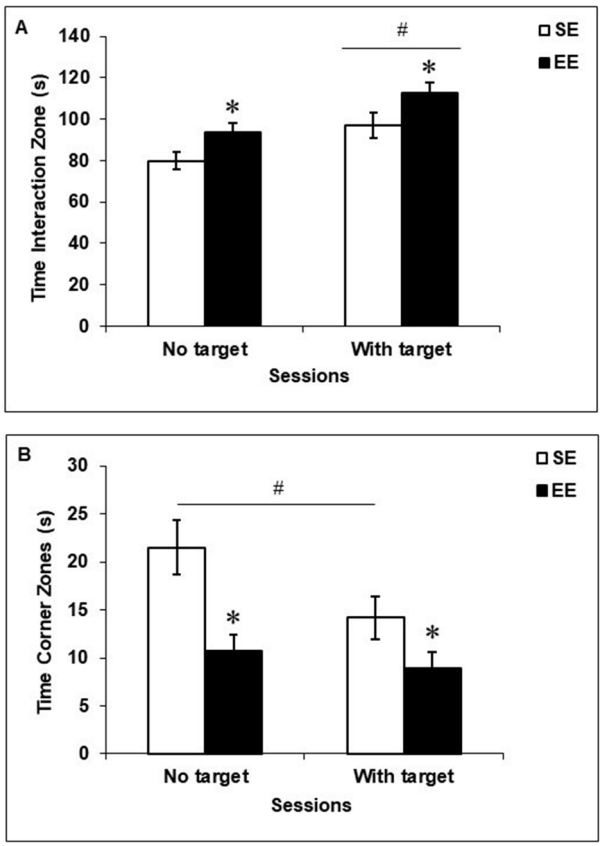


FIGURE 2 | Social behaviors in the social interaction test in mice housed for 6 weeks in a SE or an EE. **(A)** EE mice spent more time in the Interaction Zone than SE mice, irrespective of whether a mouse target was present in the enclosure (*$p < 0.05$ relative to mice housed in SE). Both SE and EE mice spent more time in the Interaction Zone when a mouse target was present (#$p < 0.001$ relative to the absence of a mouse target). **(B)** Mice housed in EE spent less time in the Corner Zones than SE mice, irrespective of whether a mouse target was present in the enclosure (*$p < 0.005$ relative to mice housed in SE). Only SE mice spent less time in the Corner Zones in the presence of a mouse target (#$p < 0.05$ relative to the absence of a mouse target). Data represents means ± SEM (mice housed in SE: $n = 12$; mice housed in EE: $n = 8$).

Finally, the total number of aggressive encounters as well as frequencies of attacks and of aggressive grooming bouts were comparable between EE and SE mice, F's$_{(1,10)} = 0.07$, 0.09 and 0.17, respectively.

Social Interaction Test

As shown in **Figure 2**, EE mice spent more time in the Interaction Zone, $F_{(1,19)} = 6.46$, $p < 0.05$ (**Figure 2A**) and less time in the Corner Zones, $F_{(1,19)} = 12.35$, $p < 0.005$ (**Figure 2B**), than did SE mice, regardless of whether a social target (CD-1 retired breeder) was present in the testing environment. When a social target was introduced, both SE and EE mice spent more time in the Interaction Zone, $F_{(1,19)} = 16.72$, $p < 0.001$, but only SE mice spent less time in the Corner Zones ($p < 0.05$) compared to when the social target was absent.

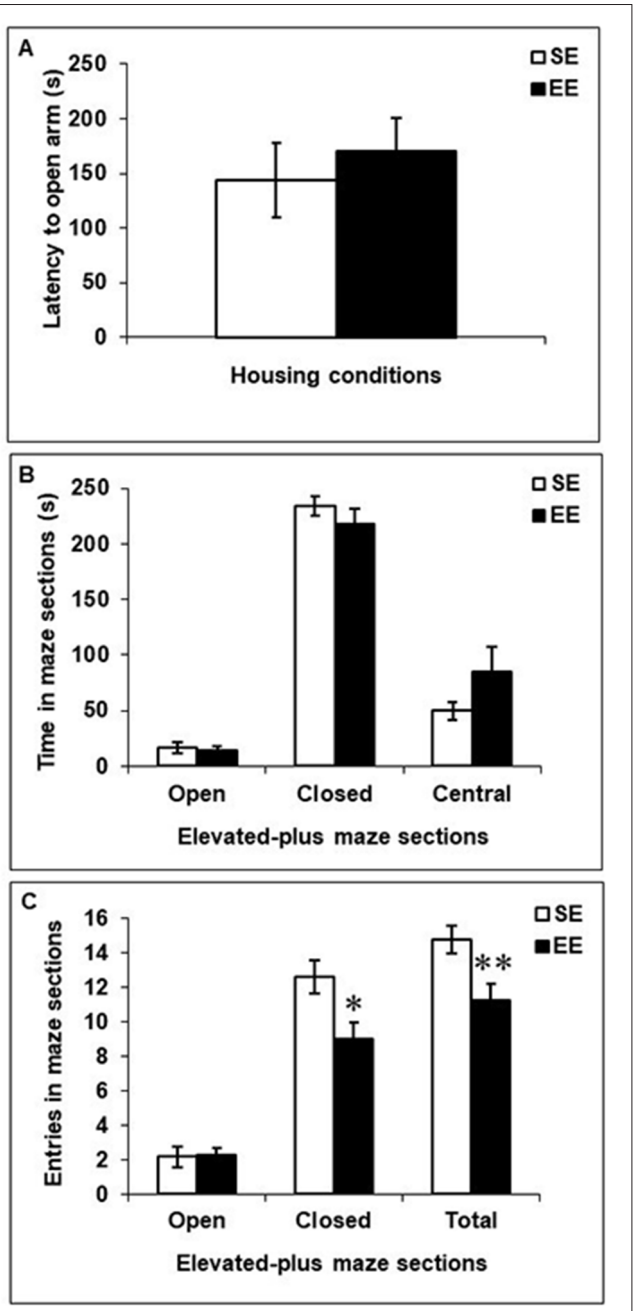


FIGURE 3 | Anxiety-like behaviors in the elevated-plus maze in mice that had been housed for 6 weeks in a SE or an EE. **(A,B)** Latency to enter an open arm as well as time spent in open arms, closed arms, and the central platform of the maze were comparable between EE and SE mice. **(C)** Mice housed in EE made fewer entries in the closed arms of the maze and fewer transitions between the different arms of the maze compared to SE mice. *$p < 0.05$ and **$p < 0.01$ relative to mice housed in SE. Data represents means ± SEM (mice house in SE: $n = 12$; mice housed in EE: $n = 8$).

Elevated-Plus Maze Test

Figure 3 depicts behaviors in the elevated-plus maze among SE and EE mice. EE and SE mice did not differ in their latencies to enter an open arm, $F_{(1,18)} = 0.29$ (**Figure 3A**), the time spent in the open arms, closed arms, or central

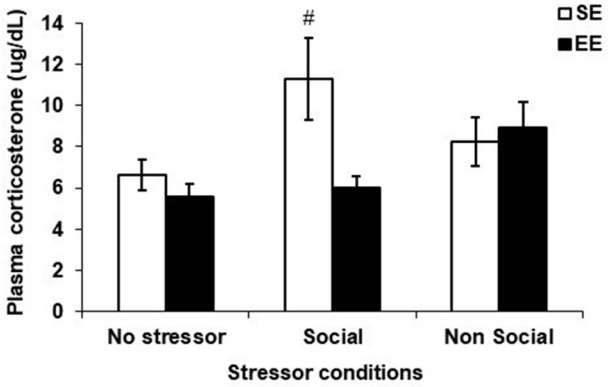


FIGURE 4 | Plasma corticosterone levels collected 90 min after the social interaction test (which acted as a Social Stressor), the elevated-plus maze (which acted as a Non-Social Stressor) or a control condition (No Stressor) among mice housed for 6 weeks in a SE or an EE. Mice housed in SE had increased plasma corticosterone levels after the Social Stressor but not the Non-Social Stressor. These corticosterone elevations were not apparent in mice housed in EE. $^{\#}p < 0.05$ relative to SE mice in the No Stressor condition. Data represents means ± SEM (SE mice/No stressor: $n = 12$; SE mice/Social: $n = 12$; SE mice/Non-Social: $n = 12$; EE mice/No Stressor: $n = 11$; EE mice/Social: $n = 8$; EE mice/Non-Social: $n = 8$).

platform, F's$_{(1,18)}$ = 0.10, 1.06 and 2.98, respectively (**Figure 3B**), or the number of entries into open arms, $F_{(1,18)} = 0.01$ (**Figure 3C**). However, mice housed in EE made fewer entries into closed arms and fewer transitions between arms (open and closed combined) than did SE mice, F's$_{(1,18)}$ = 6.42 and 7.47, p's < 0.05 and 0.01, respectively (**Figure 3C**).

Plasma Corticosterone Levels

Figure 4 shows the plasma corticosterone levels among SE and EE mice exposed to the social interaction test, the elevated-plus maze test or those that were left undisturbed (no stressor). Although the Housing, $F_{(1,58)} = 3.47$, $p = 0.068$, Stressor, $F_{(2,58)} = 2.85$, $p = 0.066$, and the interaction Housing × Stressor, $F_{(2,58)} = 2.91$, $p = 0.063$, did not reach significance, based on the *a priori* prediction that SE and EE mice would exhibit different corticosterone patterns after a mild social and non-social stressor, analyses of the simple effects comprising the Housing × Stressor interaction were conducted. These analyses confirmed that plasma corticosterone levels were increased 90 min after the social interaction test among SE mice ($p < 0.05$), which was not apparent in EE mice.

Prefrontal Expression of Pro-inflammatory Cytokines and BDNF

Fold changes in mRNA expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α as well as of the neurotrophin BDNF among SE and EE mice exposed to the social interaction test, the elevated-plus maze test, or left undisturbed are shown in **Figure 5**. Stressors affected prefrontal expression of IL-1β, $F_{(2,41)} = 4.39$, $p < 0.05$, whereas Housing did not, $F_{(1,41)} < 1$. Although the interaction between Housing and Stressor did not reach significance, $F_{(2,41)} = 2.68$, $p = 0.08$, based on the *a priori* prediction that SE and EE mice would exhibit different cytokine patterns after a mild social and non social stressor, analyses of the simple effects comprising the Housing × Stressor interaction were conducted. These analyses confirmed that, similar to corticosterone variations, PFC IL-1β expression was increased after the social interaction test in SE mice compared to non-stressed mice and mice exposed to the elevated-plus maze test (p's < 0.01 and 0.005), but this effect was not apparent among EE mice. Following stressor exposure, expression of IL-6 was elevated, $F_{(2,41)} = 3.75$, $p < 0.05$, irrespective of whether mice had been housed in SE or EE. Follow-up tests confirmed that IL-6 expression was increased after the social interaction test ($p < 0.05$), but not the elevated-plus maze test ($p = 0.25$) among both SE and EE mice. In contrast, prefrontal TNF-α expression was affected by Housing, $F_{(1,41)} = 4.53$, $p < 0.05$, but was not affected by the stressors, $F_{(2,41)} = 1.46$. Specifically, TNF-α expression was reduced in EE mice ($p < 0.05$), irrespective of whether they had been exposed to the mild stressors. Finally, prefrontal BDNF expression was modulated by both the Housing, $F_{(1,41)} = 11.27$, $p < 0.005$, and the Stressors, $F_{(2,41)} = 3.57$, $p < 0.05$. Follow-up tests confirmed that the neurotrophin was increased in EE mice compared to SE mice ($p < 0.005$) as well as in mice that had experienced the social interaction test compared to those that were not stressed ($p < 0.05$).

DISCUSSION

In the current investigation, male CD-1 mice housed in enrichment gained less weight, displayed increased social behaviors, had limited corticosterone and prefrontal IL-1β elevations in response to a mild social stressor, and exhibited reduced TNF-α and increased BDNF expression within the PFC in early adulthood. The current paradigm housed mice together with littermates immediately after weaning, and it appears that enrichment in this instance had overall beneficial actions. Mice housed in EE displayed more wounds than SE mice over the 6-week housing period and as indicated earlier, three mice in the EE condition were removed from the study because of their excessive aggressiveness (this was required for ethical reasons). Yet, a tendency was noted for SE mice to fight more often than EE mice. These findings contrast with our previous report that male CD-1 mice placed in EE as adults displayed very aggressive behaviors towards their cage mates and showed sensitized biological processes compared to SE counterparts when placed in a novel environment (McQuaid et al., 2012). Essentially enrichment in this case had been ineffective in limiting stress responses, but instead appeared to have increased vulnerability to stressor-related outcomes. These discrepant findings raise the possibility that the timing at which male CD-1 mice are placed in enrichment is an important factor to consider for territoriality, aggression and subsequent distress to be diminished. This said, although mice housed in EE did not exhibit particularly aggressive behaviors during the 30-min weekly videotaped sessions, the possibility

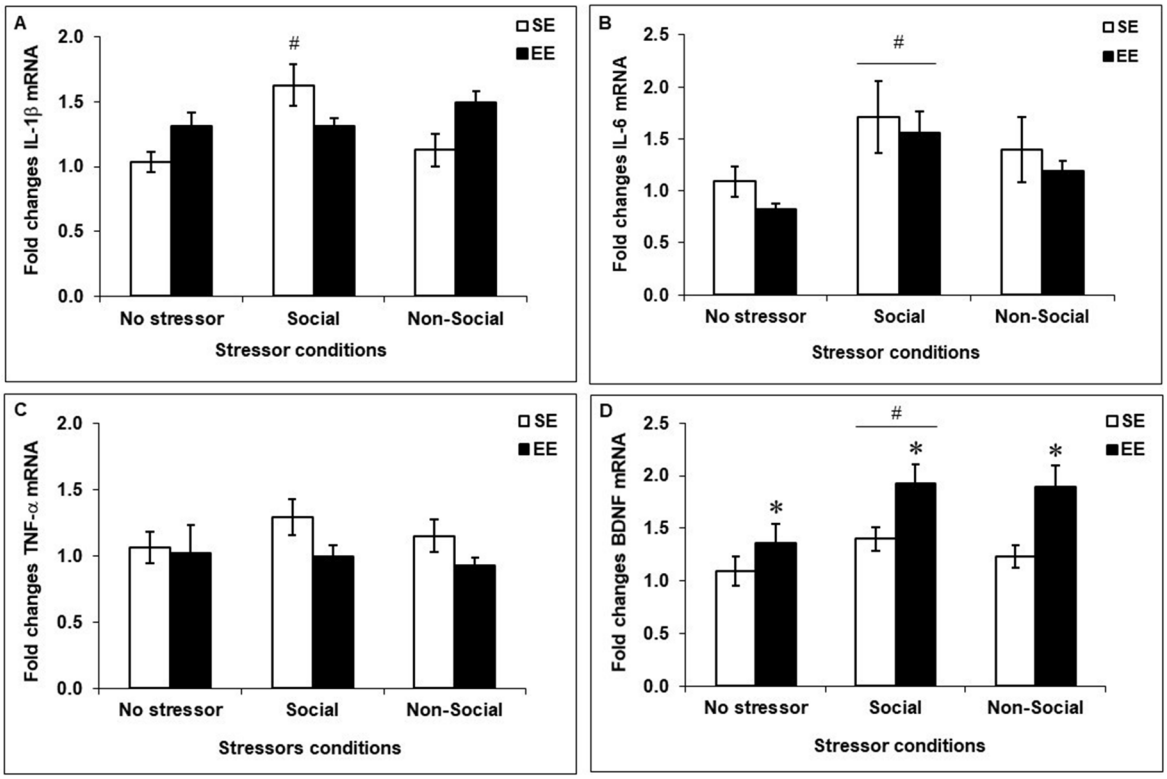


FIGURE 5 | Fold change in prefrontal mRNA expression of pro-inflammatory cytokines and BDNF assessed 90 min after the social interaction test (which acted as a Social Stressor), the elevated-plus maze (which acted as a Non-Social Stressor) or a control condition (No Stressor) among mice housed for 6 weeks in a SE or an EE. **(A,B)** Mice housed in SE had increased prefrontal IL-1β and IL-6 after the Social Stressor but not the Non-Social Stressor ($^{\#}p$'s < 0.01 and 0.05, relative to mice in the No Stressor condition). Whereas the IL-1β increases were prevented in mice housed in EE, the IL-6 expression remained elevated. **(C)** A main effect of Housing was observed with respect to prefrontal TNF-α expression, with the cytokine being reduced in EE mice ($p < 0.05$). **(D)** Mice housed in EE had increased prefrontal BDNF expression compared to SE mice ($^{*}p < 0.005$ relative to mice housed in SE). As well, BDNF expression was upregulated after the Social Stressor compared to the No Stressor condition ($^{\#}p < 0.05$ relative to mice in the No Stressor condition). Data represents means ± SEM (SE mice/No stressor: $n = 6$; SE mice/Social: $n = 10$; SE mice/Non-Social: $n = 11$; EE mice/No Stressor: $n = 6$; EE mice/Social: $n = 9$; EE mice/Non-Social: $n = 5$).

of agonistic encounters occurring in these mice cannot be entirely excluded, especially as they had more wounds than SE mice. Indeed, mice housed in EE were found to weigh less than SE mice during the last 2 weeks of housing, which might stem from increased distress among these mice (resulting from injurious aggression), as we previously observed (McQuaid et al., 2013a). However, the high levels of activity observed in the enrichment cages (due to access to running wheels) could also have contributed to the lower weights among EE mice, an effect found in other enrichment studies (Mesa-Gresa et al., 2016).

In the social interaction test, both SE and EE mice spent more time in proximity of a cage enclosure comprising a retired breeder (used as a social target), indicating that they were both attracted by social novelty, which is typically observed in non-stressed mice (Berton et al., 2006; Yu et al., 2011; Aujnarain et al., 2018). Importantly, mice housed in EE spent more time around the cage enclosure than SE mice, irrespective of whether a social target was present, suggesting that post-weaning enrichment may have reduced the fear of novelty (be it social or not) and/or increased the natural interest of mice for complex stimuli (e.g., a wire cage or another mouse). In line with this view, previous studies reported increased excitement and motivation to explore novel stimuli among EE-housed rodents (Roy et al., 2001; Larsson et al., 2002). Considering that mice chronically exposed to severe social stressors (e.g., social defeat) typically exhibit pronounced social avoidance in the social interaction test (Berton et al., 2006; Szyszkowicz et al., 2017), the current findings also confirm that when enrichment is provided immediately after weaning, the environment does not act as a social stressor as we have previously found when EE began during adulthood (McQuaid et al., 2012, 2013a,b), but instead enhanced sociability, as previously reported by others (Aujnarain et al., 2018).

The absence of an anxiolytic effect of enrichment in the elevated-plus maze contrasts with several reports showing a reduction of anxiety-like behaviors in the open-field test and the elevated-plus maze in male mice housed in EE from weaning onward (Chapillon et al., 1999; Aujnarain et al., 2018; Dandi et al., 2018). The reason for this discrepancy is not entirely clear, although it might be related to differences in the enrichment procedure used (e.g., dimensions of the enrichment cage, number

of mice per cage, different enrichment objects and different disposition) and/or the time at which anxiety-like behaviors were assessed (e.g., diurnal vs. nocturnal phase). The current findings also differ from our previous results showing increased anxiety-like behaviors in the elevated-plus maze among male CD-1 mice housed in EE in adulthood (McQuaid et al., 2012) and again, indicate that the developmental period in which male CD-1 mice are introduced to enrichment may be important in determining whether protective or detrimental effects would be apparent with respect to emotionality. Although open arm activity was not influenced by the housing conditions, EE mice made fewer total arm entries compared to SE mice. This may simply reflect reduced activity levels, as previously demonstrated (Aujnarain et al., 2018), although this effect may also be related to the smaller number of closed arm entries (and perhaps the tendency to spend more time in the center of the maze) among EE mice.

Plasma corticosterone levels in SE mice were increased 90 min after a social interaction test, but not after the elevated-plus maze. Importantly, these elevations were not apparent in EE mice, indicating that enrichment buffered the corticosterone response to the mild social stressor. This stress buffering effect of enrichment on corticosterone levels is consistent with previous reports (Ravenelle et al., 2013; Dandi et al., 2018), suggesting that the EE might have enhanced the ability to cope with the stress of a novel social situation. Alternatively, as corticosterone levels were measured 90 min after the social interaction test, it is also possible that EE mice displayed corticosterone elevations after the test, but that levels returned to baseline more quickly as a result of the protective effects of EE. We previously reported corticosterone increases following a novel cage stressor in EE mice but not in SE mice (McQuaid et al., 2012). In this study, unrelated CD-1 mice were placed in EE in adulthood, which appeared to have promoted aggressive interactions and created a stressful environment. It is possible that in the current study, relatedness and post-weaning introduction to EE among the male CD-1 mice prevented sensitization of the corticosterone response.

Prefrontal expression of the pro-inflammatory cytokines IL-1β and IL-6 was increased after the social interaction test but not after the elevated-plus maze, indicating that stressors of a social nature may be more potent in eliciting brain inflammatory activation, as previously reported (Audet et al., 2010, 2011), or that the social interaction test was simply more stressful than placement on an elevated-plus maze. Similar to plasma corticosterone patterns, enrichment prevented the IL-1β elevations, an effect that was not apparent with regard to IL-6, which remained elevated in EE mice. Why these two cytokines in the PFC were differentially modulated by enrichment is not certain. Similar to the current findings, prefrontal IL-6 increases elicited by repeated social defeat in adult male mice remained elevated in mice that had been housed in EE, but in the hippocampus, the cytokine increases were more pronounced (McQuaid et al., 2013b), suggesting that the brain cytokine effects of enrichment may be cytokine- and region-specific. In line with this view, hippocampal elevations of IL-1β in a mouse model of influenza were limited in mice that had been housed in an EE, whereas IL-6 increases in influenza-exposed mice were not influenced by enrichment (Jurgens and Johnson, 2012). As well, hippocampal IL-1β and TNF-α elevations in rats that had undergone surgery (Briones et al., 2013; Kawano et al., 2015) or that were treated with the bacterial endotoxin lipopolysaccharide (Williamson et al., 2012), were attenuated by enrichment. Interestingly, enrichment in the current investigation reduced TNF-α expression in the PFC, but this effect appeared to be most prominent among mice exposed to the social interaction test and the elevated-plus maze. Finally, enrichment increased prefrontal BDNF expression, irrespective of whether mice were tested in the social interaction test or the elevated-plus maze, although this effect was more pronounced in mice exposed to the social stressor (which may have acted as a social arousing stimulus). Enrichment-induced BDNF elevations are typically observed in the hippocampus (e.g., Jurgens and Johnson, 2012; Novkovic et al., 2015), and the current findings suggest that it might also be the case in other stress-related brain regions.

The current investigation provides insights concerning the effects of enrichment on stress reactivity, with a focus on the importance of this procedure being initiated relatively early in development. Although EE mice exhibited more wounds, and thus potentially were involved in more injurious fighting, it appears that the environmental conditions were not sufficiently distressing to alter social and anxiety-like behaviors or to sensitize the corticosterone and cytokine effects of a mild social stressor. In fact, enrichment provided immediately after weaning appears to be beneficial, as reflected by the enhanced sociability and attenuated corticosterone and IL-1β increases induced by a mild social stressor in EE mice. Early implementation of enrichment, long-term cohabitation, and familiarity between mice may thus be key factors to consider in enrichment studies in male mice in order to promote stress resilience. Despite these encouraging findings, it needs to be underscored that the present investigation involved a relatively small number of mice. Moreover, while fighting was reduced by the EE procedure used in the current study, some mice exhibited particularly pronounced aggressive behaviors. The individual factors responsible for this are uncertain, but determining what might produce such outcomes may be important in the understanding of aggressive behaviors.

AUTHOR CONTRIBUTIONS

M-CA, RJM and HA designed the experiment. M-CA, RJM, RD and SJ-P conducted the experiment. M-CA, RJM and SJ-P analyzed the data. M-CA, RJM, RD and HA wrote the manuscript.

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frontiers in Molecular Neuroscience

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Short-Term Environmental Stimulation Spatiotemporally Modulates Specific Serotonin Receptor Gene Expression and Behavioral Pharmacology in a Sexually Dimorphic Manner in Huntington's Disease Transgenic Mice

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Huntington's disease (HD) is a neurodegenerative disorder caused by a tandem repeat mutation encoding an expanded polyglutamine tract in the huntingtin protein, which leads to cognitive, psychiatric and motor dysfunction. Exposure to environmental enrichment (EE), which enhances levels of cognitive stimulation and physical activity, has therapeutic effects on cognitive, affective and motor function of transgenic HD mice. The present study investigated gene expression changes and behavioral pharmacology in male and female R6/1 transgenic HD mice at an early time-point in HD progression associated with onset of cognitive and affective abnormalities, following EE and exercise (wheel running) interventions. We have demonstrated changes in expression levels of the serotonin (5-HT) receptor Htr1a, Htr1b, Htr2a and Htr2c genes (encoding the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors, respectively) in HD brains at 8 weeks of age, using quantitative real-time PCR. In contrast, expression of the serotonin transporter (SerT, also known as 5-HTT or Slc6a4) was not altered in these brains. Furthermore, we identified region-specific, sex-specific and environmentally regulated (comparing EE, exercise and standard housing conditions) impacts on gene expression of particular 5-HT receptors, as well as SerT. For example, SerT gene expression was upregulated by exercise (wheel running from 6 to 8 weeks of age) in the hippocampus. Interestingly, when EE was introduced from 6 to 8 weeks of age, Htr2a gene expression was upregulated in the cortex, striatum and hippocampus of male mice. EE also rescued the functional activity of 5-HT_2 receptors as observed in the head-twitch test, reflecting sexually dimorphic effects of environmental stimulation. These findings demonstrate that

disruption of the serotonergic system occurs early in HD pathogenesis and, together with previous findings, show that the timing and duration of environmental interventions are critical in terms of their ability to modify gene expression. This study is the first to show that EE is able to selectively enhance both gene expression of a neurotransmitter receptor and the functional consequences on behavioral pharmacology, and links this molecular modulation to the therapeutic effects of environmental stimulation in this neurodegenerative disease.

Keywords: tandem repeat disorder, polyglutamine disease, neurodegeneration, serotonin, dementia, depression, environmental enrichment, exercise

INTRODUCTION

Huntington's disease (HD) is a neurodegenerative disorder characterized by a triad of symptoms: cognitive deficits culminating in dementia, psychiatric symptoms such as depression and motor dysfunction including uncontrolled movements (chorea). Cognitive deficiencies and psychiatric symptoms begin prior to clinical diagnosis (motor onset) of HD (Zimbelman et al., 2007; Tyebji and Hannan, 2017). The brains of patients diagnosed with HD can display little or no detectable pathological change post-mortem (Vonsattel et al., 1985; Mo et al., 2015). Therefore, understanding subtle molecular changes that occur before clinical onset is very important, and may identify novel therapeutic targets. Transcriptional dysregulation is one of the characteristics of HD pathogenesis and is a possible pathological mechanism of action within the HD brain. Reversing abnormal gene expression may prevent downstream changes leading to neuronal dysfunction. Therefore, identifying early changes in gene expression is an important step in understanding HD pathogenesis and identifying potential therapeutic targets. It is these early changes that need to be targeted with therapies if HD progression is to be stopped or delayed.

It is known that environmental factors affect the time of onset, rate of progression and severity of HD (Sudarsky et al., 1983; Georgiou et al., 1999; Wexler et al., 2004; Friedman et al., 2005; Gomez-Esteban et al., 2007; Trembath et al., 2010). However, human studies have been unable to distinguish which facets of the environment influence disease onset. Therefore, in order to investigate environmental modifiers in HD, animal models must be used. Environmental enrichment (EE) provides increased cognitive, sensory and motor stimulation for experimental animals. EE and voluntary wheel running also affect transcription of specific genes in the cortex and hippocampus of wild-type (WT) rodents (Olsson et al., 1994, 1995; Torasdotter et al., 1996; Rasmuson et al., 1998; Rampon et al., 2000; Pinaud et al., 2001, 2002; Molteni et al., 2002; Mlynarik et al., 2004). Experimentally-enriched environments produce beneficial outcomes for HD mice (Chapillon et al., 1999; van Dellen et al., 2000; van Praag et al., 2000; Hockly et al., 2002; reviewed in Nithianantharajah and Hannan, 2006). Initially, EE was shown to delay onset of motor symptoms in R6/1 HD mice (van Dellen et al., 2000) and this has been extended to other models and phenotypic outcomes (Hockly et al., 2002; Spires et al., 2004; Nithianantharajah et al., 2008; Pang et al., 2009), as well as the beneficial effects of increased voluntary physical activity on running wheels (RWs) (Pang et al., 2006; van Dellen et al., 2008). However, these studies were predominantly performed in later stages of disease progression.

We therefore looked for changes in gene expression in the R6/1 transgenic mouse model of HD at early stages of pathogenesis (8 weeks of age) and also investigated the effects of EE and exercise (wheel running) on these HD mice and WT controls. Our prior findings (Renoir et al., 2011, 2012, 2013; Du et al., 2012). Suggested that serotonergic dysfunction was occurring in R6/1 HD mice. Therefore, in the present study, candidates within the serotonergic system were chosen for investigation, revealing dysregulation in early stages of HD pathogenesis, and differential modulation by environmental interventions.

MATERIALS AND METHODS

Animals and Housing

R6/1 hemizygote males (Mangiarini et al., 1996) were obtained from the Jackson Laboratory (Bar Harbor, ME, United States) and bred with CBB6 (CBAxC57/B6) F1 females to establish an R6/1 colony at the Florey Institute. These mice closely model HD and live for more than 6 months. Genotypes were determined by PCR (Mangiarini et al., 1996) with genomic DNA obtained from toe clips and mice were weaned at 3.5 weeks of age.

The WT and HD male and female mice were separately divided at 6 weeks of age into three groups—standard housed (SH), RW and EE. SH mice were housed in standard mouse boxes (10 cm × 16 cm × 38 cm) containing only bedding, with four mice per box. RW and EE mice were housed in larger sized (15 cm × 28 cm × 38 cm) rat boxes with elevated lids, containing bedding, four mice per box. RW mice were provided with two RWs per box. Housing boxes for EE mice contained a variety of novel objects (e.g., cardboard rolls, wire, mesh, various types of paper, wooden and plastic objects). The objects were changed twice weekly. Additionally, all mice in the EE housing groups were placed in larger (44 cm × 40 cm × 62 cm) activity boxes for 1 h three times weekly. Activity boxes were built up anew on each occasion with novel objects made of plastic, foam, rubber, wood, rope, wire, chains and paper. The differential EE, RW and SH

housing conditions were maintained from 6 to 8 weeks of age (i.e., the environmental intervention was performed over 2 weeks).

Mice were housed under a 12/12 light/dark cycle and food and water were provided *ad libitum*. Experiments were approved by the Howard Florey Institute Animal Ethics Committee and followed the guidelines of the National Health and Medical Research Council of Australia.

Tissue Collection, Dissections and Storage

Mice were killed by cervical dislocation between 9 and 11:30 am over 3 days. Differently housed groups were randomly allocated into balanced batches to eliminate batch effects. For quantitative real-time PCR analysis of gene expression, brains were bisected and hippocampus, striatum and whole cortex dissected out. Samples were immediately frozen on dry ice and transferred to a −80°C freezer for storage until required.

RNA Extraction, DNase Clean-Up and Analysis

Total RNA from the hippocampus and striatum was extracted using RNeasy Mini kits (Qiagen, Melbourne, VIC, Australia). Total RNA from whole cortex was extracted using RNeasy Midi kits (Qiagen, Melbourne, VIC, Australia). On-column DNase cleanup was performed on all samples using RNase-Free DNase set (Qiagen, Melbourne, VIC, Australia). The concentration and integrity of the extracted total RNA present in the final eluates were determined using an Agilent Bioanalyser 2100 (service provided by Australian Genomics Research Facility, Parkville, VIC, Australia).

Reverse Transcription

For each RNA sample, 1 μg of total RNA was reverse transcribed into cDNA using GeneAmp RT PCR kit (Applied Biosystems, Foster City, CA, United States) with random hexamers. The reverse transcription reactions were performed on a GeneAmp PCR system (Model 2700, Applied Biosystems, Foster City, CA, United States) at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. The cDNA products were stored at −20°C for subsequent use.

Quantitative Real-Time PCR

Expression levels of candidate genes were determined with quantitative real-time PCR performed on the PE-ABI Prism 7700 Sequence detection system version 1.9.1 (Applied Biosystems, Foster City, CA, United States) using SYBR Green JumpStart Taq ReadyMix (Sigma, Saint Louis, MI, United States). Primer 3 software (Rozen and Skaletsky, 2000) or Primer Express Software (Applied Biosystems, Foster City, CA, United States) were used to design reaction primers (Sigma Genosys, Castle Hill, NSW, Australia), which were designed across exon–exon boundaries. The primer sequences used were:

SerT_F:5′-CTTCAGCCCCGGATGGTT-3′;
SerT_R:5′-GTGGACTCATCAAAAAACTGCAAA-3′;
Ht1a_F:5′-CCCCAACGAGTGCACCAT-3′;
Ht1a_R: 5′-GCGCCGAAAGTGGAGTAGAT-3′;
Ht1b_F:5′-CACCAACCTCTCCCACAACT-3′;
Ht1b_R:5′-CCAGAGAGGCGATCAGGTAG-3′;
Ht2a_F:5′-CACTGTGAAGCGAGGCATAA-3′;
Ht2a_R:5′-AAGCCGGAAGTTGTAGCAGA-3′;
Ht2c_F:5′-TGCCATCGTTTGGGCAATA-3′;
Ht2c_R:5′-CGTCCCTCAGTCCAATCACA-3′;
Cyclophilin forward: 5′-CCCACCGTGTTCTTCGACA-3′;
Cyclophilin reverse: 5′-CCAGTGCTCAGAGCTCGAAA-3′.

To determine the optimal working volumes of forward and reverse primers, a set of primer dilutions was conducted in which different combinations of each primer ranging between 0.5 and 3 were used. The combination that yielded the lowest C*t*-value was used as the optimal working volume (data not shown). Optimization of primer efficiencies was carried out prior to commencement of quantification experiments. The real-time PCR cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was processed in triplicate and melt curve analysis was performed on all samples. As a validated endogenous control, cyclophilin A was amplified in separate triplicate reactions for normalization.

Determination of the relative gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Briefly, the C*t* values (threshold cycle at which the fluorescence intensity exceeds 10× the SD of background fluorescence) of the experimental genes and the control were determined for each sample. The difference in the C*t* values of the mean of the experimental gene triplicates and the mean of the control triplicates was determined (ΔC*t*) for each mouse, normalizing for amount of cDNA in each reaction. The mean ΔC*t* for the WT SH group was calculated, to use as a calibrator, and subtracted from the ΔC*t* of all the mice and giving the $\Delta\Delta$C*t*. Fold change was then determined by the formula $2^{-\Delta\Delta Ct}$.

DOI-Induced Head-Twitches

Eight-week-old mice were injected with the 5-HT_{2A} receptor agonist, 1-(2,5-dimethoxy-4-odophenyl)-2-aminopropane [(±)DOI] (1 mg/kg, i.p.) and immediately placed inside an observation area. The number of head-twitches the mice performed from 15 to 30 min post-injection was manually recorded (Renoir et al., 2011).

Statistical Analysis

Statistical analysis of quantitative real-time PCR and behavioral pharmacology (DOI) data was performed with the SPSS Package, version 16 (SPSS, Chicago, IL, United States). Fold changes were analyzed by three-way ANOVA (sex × genotype × housing condition). The critical value for significance was set at $p < 0.05$. In both cases, *post hoc* analyses were performed where appropriate using Tukey's test for housing condition and pairwise testing was conducted when significant interactions were present, using Bonferroni's adjustment for multiple comparisons. Fold changes were converted into percentage values for graphical representation.

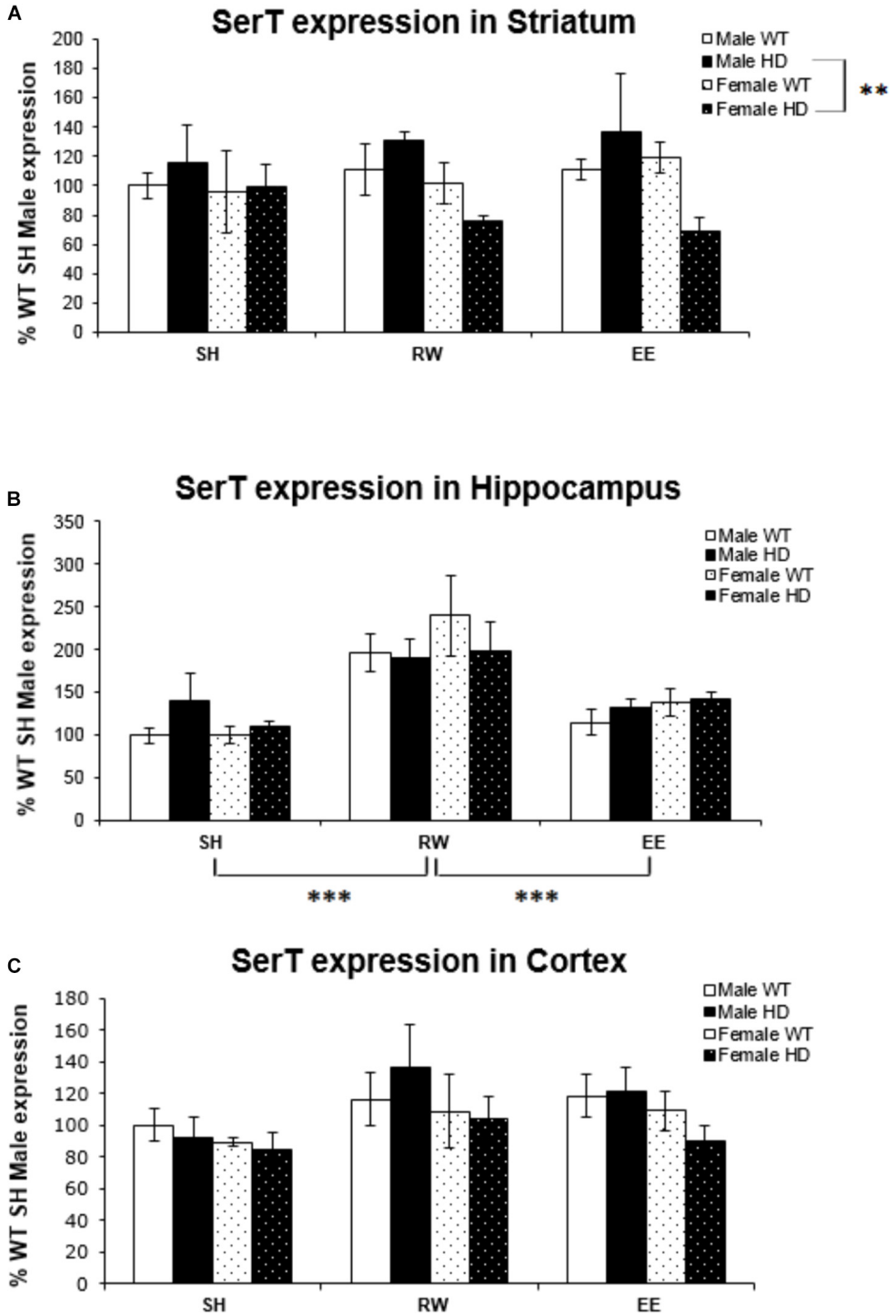

FIGURE 1 | Effects of environmental interventions and sex on SerT mRNA levels in 8-week-old WT and HD mice. **(A)** In Striatum, SerT expression was unaffected by genotype ($p = 0.862$) and housing environment ($p = 0.867$). Sex did significantly affect SerT mRNA levels ($p = 0.019$). There was a significant interaction between genotype × sex ($p = 0.030$); pairwise comparisons showed a significantly less SerT mRNA in female HD mice when compared to male HD mice ($p = 0.002$). **(B)** In Hippocampus, SerT expression was unaffected by genotype ($p = 0.774$) or sex ($p = 0.506$). Housing environment significantly affected SerT expression levels ($p < 0.001$). *Post hoc* testing showed that wheel running ($p < 0.001$), but not environmental enrichment ($p = 0.510$), significantly increased levels of SerT mRNA. **(C)** In Cortex, SerT expression was unaffected by genotype ($p = 0.816$). There were no effects of sex ($p = 0.065$) or housing environment ($p = 0.063$). $n = 4$–6 per group. Results are represented as mean ± SEM. Pairwise comparisons were conducted using Bonferonni's adjustment for multiple comparisons: $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

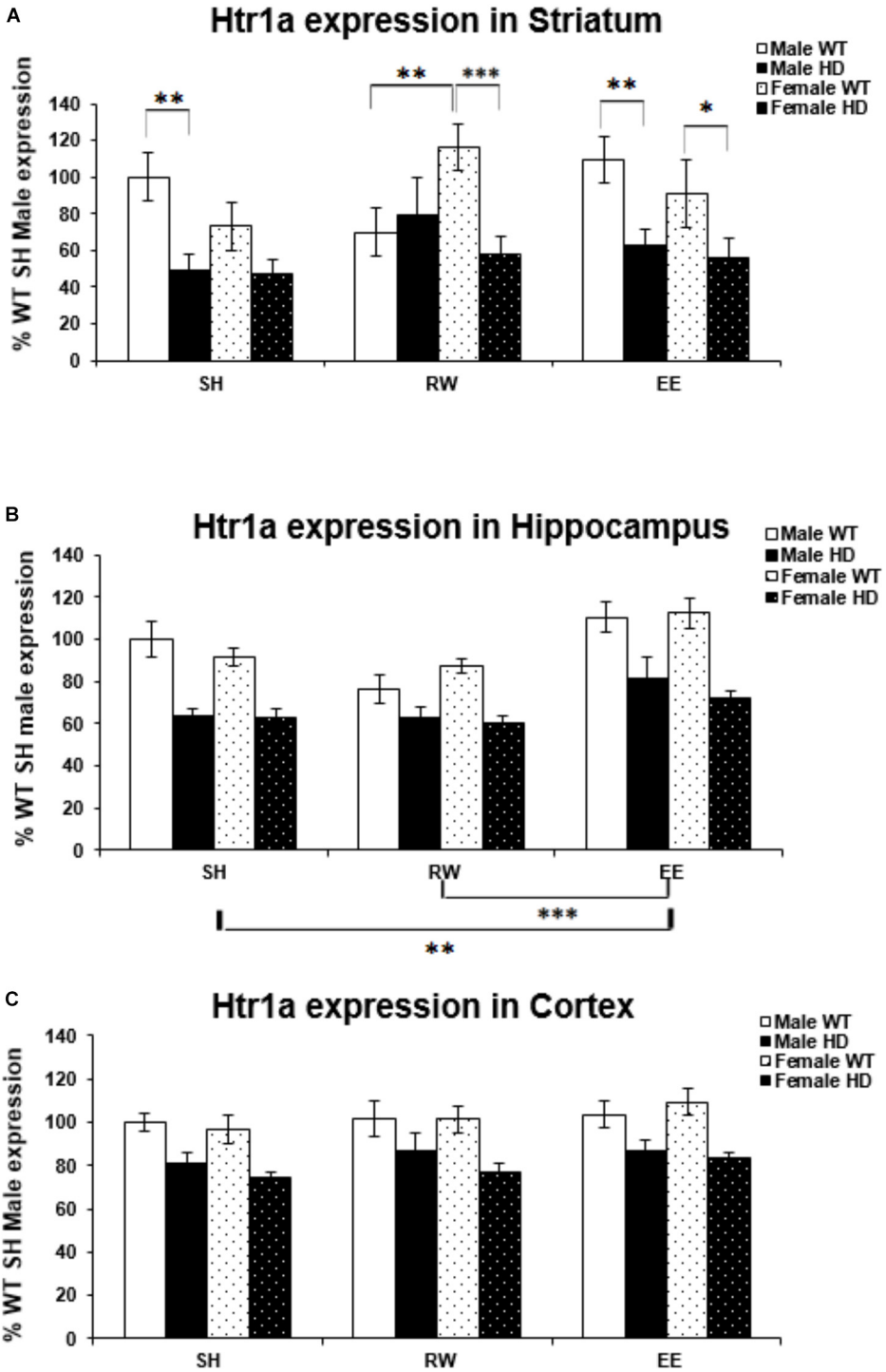

FIGURE 2 | Effects of genotype, environment and sex on Htr1a mRNA levels in 8-week-old WT and HD mice. **(A)** In Striatum, Htr1a expression was decreased significantly in 8-week-old HD mice compared to their WT counterparts ($p < 0.001$). There was a significant interaction between genotype × housing environment × sex ($p = 0.021$). Pairwise comparisons showed significant differences in Htr1a expression between male and female WT mice exposed to wheel running ($p = 0.009$), between female HD and WT exposed to EE ($p = 0.044$) and RW ($p = 0.001$) and between male HD and WT mice exposed to SH ($p = 0.004$) and EE ($p = 0.008$). **(B)** In Hippocampus, Htr1a expression was reduced significantly in 8-week-old HD mice ($p < 0.001$). Housing environment had a significant effect on Htr1a expression ($p < 0.001$). *Post hoc* testing showed that environmental enrichment ($p = 0.002$), but not wheel running ($p = 0.214$), significantly increased levels of Htr1a mRNA. **(C)** In Cortex, Htr1a expression was decreased significantly in 8-week-old HD mice compared to their WT counterparts ($p < 0.001$). Housing environment had no effect on Htr1a expression ($p = 0.150$). $n = 4$–6 per group. Results are represented as mean ± SEM. Pairwise comparisons were conducted using Bonferonni's adjustment for multiple comparisons: $^{*}p < 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

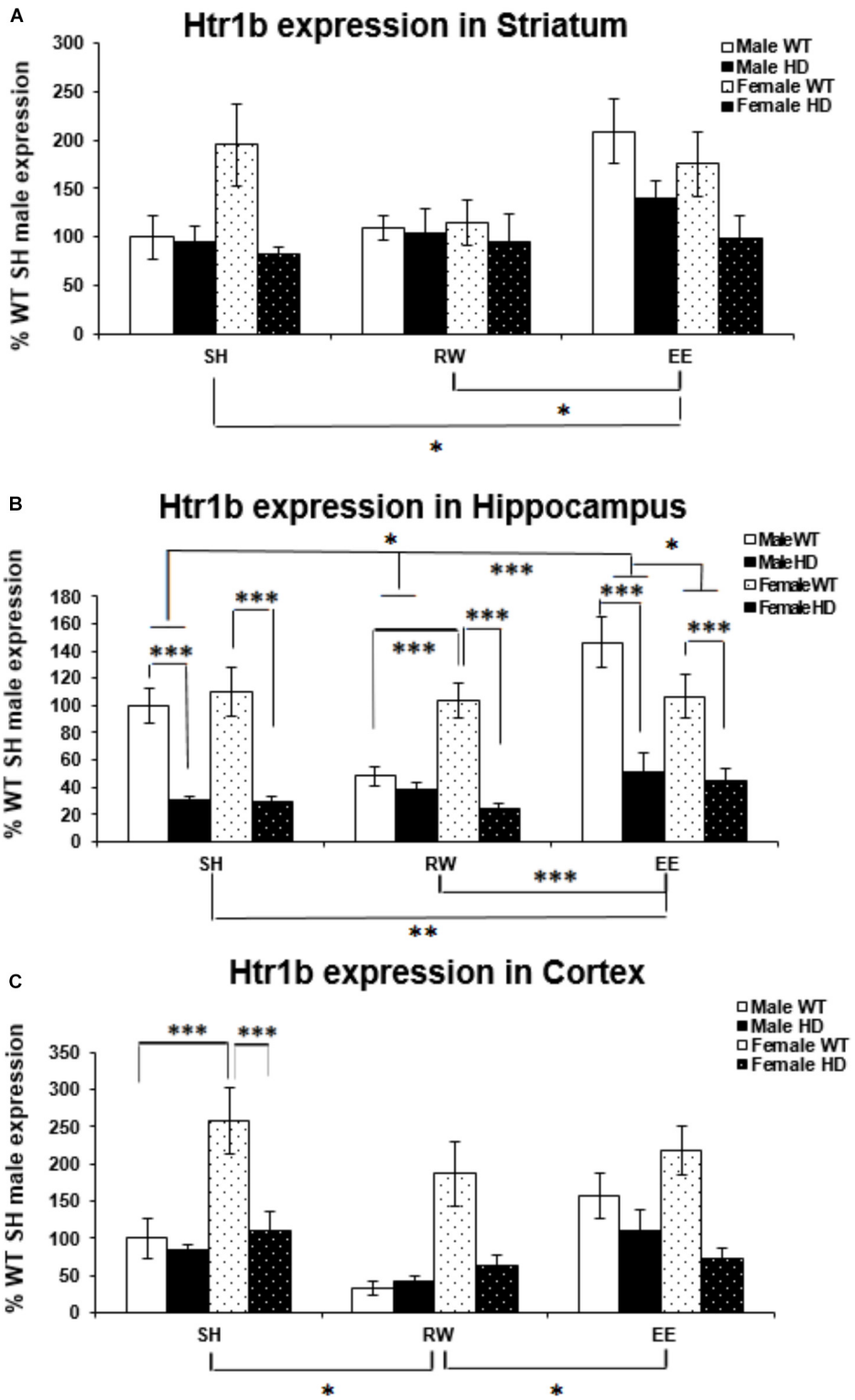

FIGURE 3 | Effects of genotype, environment and sex on Htr1b mRNA levels in 8-week-old WT and HD mice. **(A)** In Striatum, Htr1b expression was significantly affected by genotype in 8-week-old female mice ($p = 0.002$). Housing environment significantly affected Htr1b mRNA levels ($p = 0.016$). *Post hoc* testing showed that environmental enrichment ($p = 0.046$), but not wheel running ($p = 0.860$), significantly changed levels of Htr1b mRNA. There was no significant interaction between housing environment × sex ($p = 0.063$). **(B)** In Hippocampus, Htr1b expression was decreased significantly in 8-week-old HD mice compared to their

(Continued)

FIGURE 3 | Continued
WT counterparts ($p < 0.001$). Housing environment significantly affected Htr1b mRNA levels ($p < 0.001$). *Post hoc* testing showed that environmental enrichment ($p = 0.013$), but not wheel running ($p = 0.501$), significantly changed levels of Htr1b mRNA. There was a significant interaction between housing environment × sex ($p = 0.024$); pairwise comparisons showed a significant difference between male and female mice held in EE conditions ($p = 0.040$) and no difference between male and female mice held in RW conditions ($p = 0.069$). Male EE mice also showed significantly more Htr1a expression than male SH ($p = 0.012$) and RW ($p < 0.001$) mice. There was a significant interaction between genotype × housing environment × sex ($p = 0.008$); pairwise comparisons showed significant differences in Htr1a expression between male and female WT mice exposed to wheel running ($p = 0.001$) and environmental enrichment ($p = 0.014$). They also showed significant differences between female HD and WT mice raised under SH, RW and EE conditions ($p < 0.001$ for all) and between male HD and WT mice raised under SH and EE conditions ($p < 0.001$ for both). Additionally male WT mice raised in EE conditions had significantly increased levels of Htr1b mRNA when compared to SH ($p = 0.019$) and RW ($p < 0.001$) male WT mice and male WT mice raised in RW conditions had significantly less Htr1b expression when compared to SH ($p = 0.008$) WT male mice. **(C)** In Cortex, Htr1b expression was decreased significantly in 8-week-old female HD mice compared to their WT counterparts ($p < 0.001$). Housing environment significantly affected Htr1b mRNA levels ($p = 0.005$). *Post hoc* testing showed that wheel running ($p = 0.010$), but not environmental enrichment ($p = 0.921$), significantly changed levels of Htr1b mRNA. There was a significant interaction between genotype × sex ($p < 0.001$); pairwise comparisons showed a significant difference between Htr1b expression in male and female WT mice ($p < 0.001$) and a significant difference in Htr1b expression between HD and WT female mice ($p < 0.001$). $n = 4$–6 per group. Results are represented as mean ± SEM. Pairwise comparisons were conducted using Bonferonni's adjustment for multiple comparisons: $^{*}p < 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

RESULTS

We used quantitative real-time PCR to compare the effects of EE and RW, relative to standard housing (SH), on serotonergic gene expression in brains of male and female HD mice, and their WT control littermates. The use of these three housing conditions allowed us to compare the relative effects of both cognitive stimulation and physical activity (via EE), vs physical exercise alone (RW). We also investigated the effects of the HD genotype on gene expression, as well as the effects of sex.

Serotonin Transporter (Slc6a4/SerT) Gene Expression Is Affected by Sex and Environment

SerT gene expression was not affected by the HD genotype in the striatum [**Figure 1A**; $F(1,67) = 0.030$, $p = 0.862$], hippocampus [**Figure 1B**; $F(1,68) = 0.083$, $p = 0.774$], or cortex [**Figure 1C**; $F(1,71) = 0.54$, $p = 0.816$]. There was no effect of housing environment in the striatum [**Figure 1A**; $F(1,67) = 0.144$, $p = 0.867$]. However, there was a significant interaction between genotype × sex [**Figure 1A**; $F(1,67) = 4.956$, $p = 0.030$]; pairwise comparison showed a significant difference in SerT gene expression levels between male and female HD mice ($p = 0.002$).

Housing environment significantly affected SerT gene expression in the hippocampus [**Figure 1B**; $F(2,68) = 17.739$, $p < 0.001$]; *post hoc* testing revealed that exposure to wheel running significantly increased SerT mRNA levels when compared to SH mice ($p < 0.001$) and EE mice ($p < 0.001$). In the cortex, there was no effect of housing environment [**Figure 1B**; $F(1,71) = 2.863$, $p = 0.065$].

Deficits in Htr1a Gene Expression in HD Mice Are Differentially Modulated by Sex and Environment

Huntington's disease mice had significantly lower levels of Htr1a mRNA than WT mice in the striatum [**Figure 2A**; $F(1,70) = 23.938$, $p < 0.001$], hippocampus [**Figure 2B**; $F(1,72) = 73.098$, $p < 0.001$], and cortex [**Figure 2C**; $F(1,71) = 39.875$, $p < 0.001$]. In the striatum, analysis of Htr1a expression levels revealed a significant interaction between genotype × housing environment × sex [**Figure 2A**; $F(2,70) = 4.107$, $p = 0.021$]; pairwise comparisons showed that female WT mice exposed to wheel running had significantly higher levels of Htr1a mRNA than their WT male wheel running counterparts ($p = 0.009$). They also demonstrated that female HD mice exposed to EE had significantly less Htr1a mRNA than female WT EE mice ($p = 0.044$) and that female HD mice exposed to RW had significantly less Htr1a mRNA than female WT RW mice ($p = 0.001$). There were also significant differences between male HD and WT mice within the SH ($p = 0.004$) and EE ($p = 0.008$) groups. No change in Htr1a expression in female WT mice exposed to wheel running, in comparison to female WT mice exposed to standard-housing ($p = 0.061$), was evident.

Housing environment significantly affected Htr1a mRNA levels in the hippocampus [**Figure 2B**; $F(2,72) = 15.158$, $p < 0.001$]; *post hoc* testing demonstrated that EE significantly increased Htr1a expression levels when compared to SH ($p = 0.002$) and RW ($p < 0.001$). In the cortex, there was no effect of housing environment on Htr1a expression levels [**Figure 2C**; $F(1,71) = 1.960$, $p = 0.150$].

Region-Specific Changes in Htr1b Gene Expression HD Mice Are Differentially Modulated by Sex and Environment

Htr1b mRNA levels were significantly affected by genotype in the striatum [**Figure 3A**; $F(1,61) = 11.221$, $p = 0.002$]. Htr1b expression levels were also significantly affected by housing environment [**Figure 4A**; $F(2,61) = 4.511$, $p = 0.016$]; *post hoc* testing demonstrated that mice exposed to EE conditions had increased Htr1b expression in comparison to SH ($p = 0.046$) and RW ($p = 0.021$) mice.

Htr1b expression levels are reduced in HD hippocampus compared to WT expression levels [**Figure 3B**; $F(1,69) = 101.174$, $p < 0.001$]. There was also a significant effect of housing environment on Htr1b expression levels in the hippocampus [$F(2,69) = 9.321$, $p < 0.001$]; *post hoc* testing revealed that mice housed in EE conditions had significantly higher levels of Htr1b expression than mice housed in SH conditions ($p = 0.013$) and RW conditions ($p < 0.001$). A significant interaction between housing environment × sex was evident [**Figure 3B**;

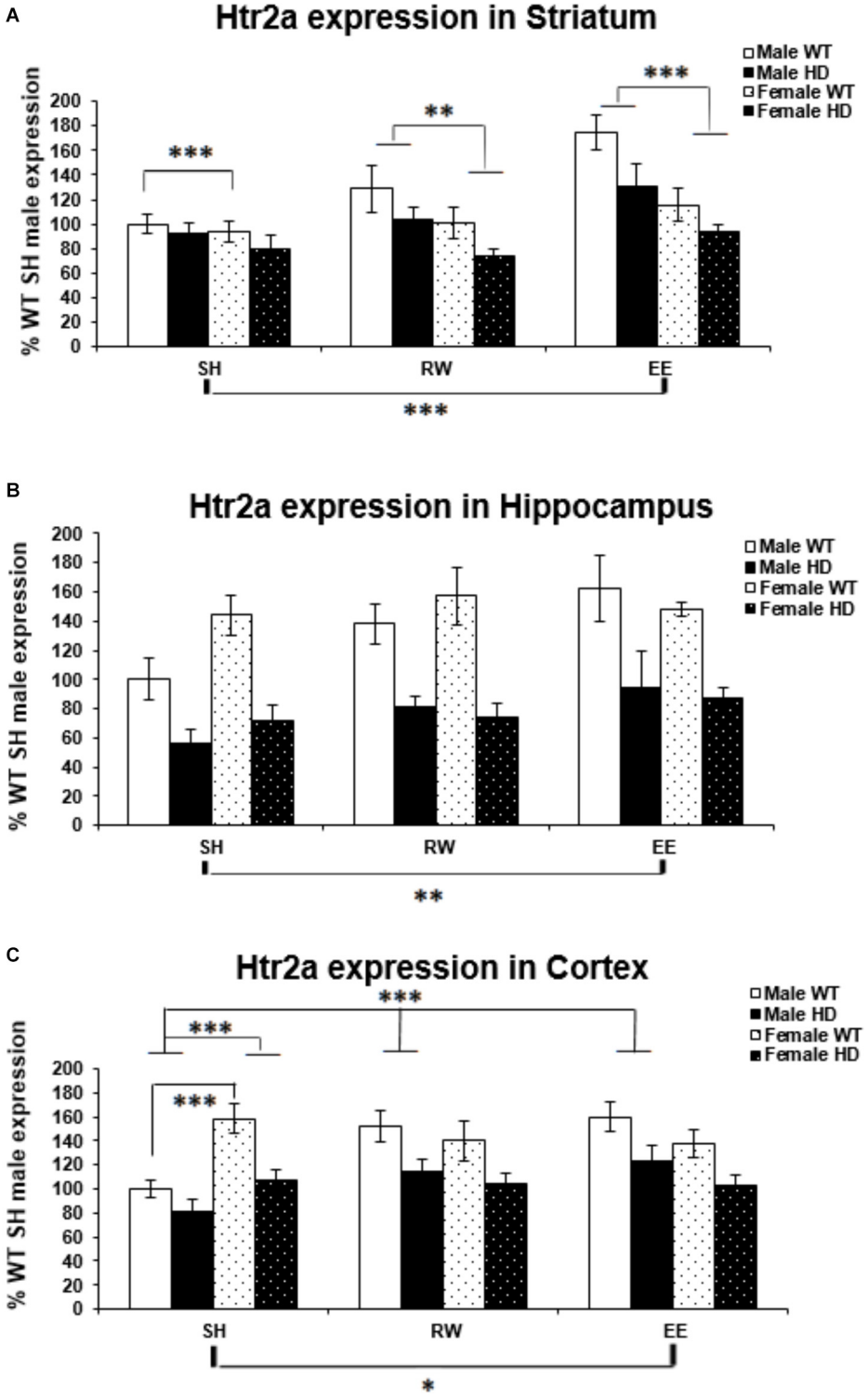

FIGURE 4 | Effects of genotype, environment and sex on Htr2a mRNA levels in 8-week-old WT and HD mice. **(A)** In Striatum, Htr2a expression was reduced significantly in 8-week-old HD mice ($p = 0.001$). Housing environment had a significant effect on Htr2a expression ($p < 0.001$). *Post hoc* testing showed that environmental enrichment ($p < 0.001$), but not wheel running ($p = 0.461$), significantly increased levels of Htr2a mRNA compared to SH. There was also a significant effect of sex on Htr2a expression ($p < 0.001$) and a trend toward an interaction between sex × housing environment ($p = 0.063$); pairwise comparison showed that male mice had significantly higher levels of Htr2a mRNA than their female counterparts when held under EE and RW conditions ($p < 0.001$ and $p = 0.018$, respectively) and that EE significantly increased Htr2a expression levels in male mice compared to SH and RW conditions ($p < 0.001$ and $p = 0.008$, respectively).

(Continued)

FIGURE 4 | Continued
(B) In Hippocampus, Htr2a expression was reduced significantly in 8-week-old HD mice ($p < 0.001$). Housing environment had a significant effect on Htr2a expression ($p = 0.014$). *Post hoc* testing showed that environmental enrichment ($p = 0.004$), but not wheel running ($p = 0.182$), significantly increased levels of Htr2a mRNA. **(C)** In Cortex, Htr2a expression was reduced significantly in 8-week-old HD mice ($p < 0.001$). Housing environment had a significant effect on Htr2a expression ($p = 0.038$). *Post hoc* testing showed that environmental enrichment ($p = 0.021$), but not wheel running ($p = 0.122$), significantly increased levels of Htr2a mRNA. There was a significant interaction between sex × housing environment ($p < 0.001$); pairwise comparison showed that female mice had significantly higher levels of Htr2a expression than their male counterparts when held under SH conditions ($p < 0.001$) and that EE and RW significantly increased Htr2a mRNA levels in male mice compared to SH ($p < 0.001$ and $p = 0.001$, respectively). $n = 4$–6 per group. Results are represented as mean ± SEM. Pairwise comparisons were conducted using Bonferonni's adjustment for multiple comparisons: $^{*}p < 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

$F(2,69) = 3.981$, $p = 0.024$]. Pairwise comparisons showed a significant difference between male and female mice within the EE group ($p = 0.040$), with a trend toward a difference between male and female mice in the RW group ($p = 0.069$); male EE mice also had significantly higher levels of Htr1b expression than male SH ($p = 0.012$) and male RW ($p < 0.001$) mice. A significant interaction between genotype × housing environment × sex was also apparent [**Figure 3B**; $F(2,69) = 5.303$, $p = 0.008$]; pairwise comparisons showed that female WT RW mice had significantly more Htr1b mRNA than male WT RW mice ($p = 0.001$), while male WT EE mice had significantly more Htr1b mRNA than female WT EE mice ($p = 0.014$). Male WT EE mice demonstrated increased levels of Htr1b expression when compared to male WT SH mice ($p = 0.019$) and male WT RW mice ($p < 0.001$), while male WT RW mice demonstrated significantly decreased levels of Htr1b expression when compared to male WT SH mice ($p = 0.008$). Pairwise comparisons also showed significant differences between HD and WT mice within the female SH ($p < 0.001$), RW ($p < 0.001$) and EE ($p < 0.001$) groups, as well as in the male SH ($p < 0.001$) and male EE ($p < 0.001$) groups, but not within the male RW group ($p = 0.533$).

Htr1b mRNA levels were significantly higher in female mice than in male mice [**Figure 3C**; $F(1,55) = 16.284$, $p < 0.001$]. They were significantly decreased in the HD cortex [**Figure 3C**; $F(1,55) = 24.079$, $p < 0.001$]. A significant interaction between genotype × sex was apparent [**Figure 3C**; $F(2,55) = 14.911$, $p < 0.001$]; pairwise comparisons showed significantly higher levels of Htr1b mRNA in female WT mice when compared to male WT mice ($p < 0.001$), but there was no difference between male and female HD mice ($p = 0.907$). A significant decrease in Htr1b expression was evident in female HD mice when compared to female WT mice ($p < 0.001$), whereas no difference was apparent between male WT and HD mice ($p = 0.489$).

Htr1b expression levels were also significantly affected by housing environment [**Figure 3C**; $F(2,55) = 5.901$, $p = 0.005$]; *post hoc* testing demonstrated that mice exposed to RW conditions showed decreased Htr1b expression in comparison to SH ($p = 0.010$) and EE ($p = 0.018$) mice.

Htr2a Gene Expression Is Decreased in HD Mice and Differentially Modulated by Sex and Environment

Expression of the Htr2a gene was decreased in HD mice at 8 weeks of age, when compared to their WT counterparts, in the striatum [**Figure 4A**; $F(1,65) = 11.700$, $p = 0.001$], hippocampus [**Figure 4B**; $F(1,66) = 60.316$, $p < 0.001$], and cortex [**Figure 4C**; $F(1,67) = 30.580$, $p < 0.001$].

In the striatum a significant effect of sex [**Figure 4A**; $F(1,65) = 18.304$, $p < 0.001$] indicated a significantly higher level of Htr2a mRNA in male mice then in female mice. There was a trend of interaction between sex and housing environment [**Figure 4A**; $F(2,65) = 2.915$, $p = 0.063$]; pairwise comparisons indicated that there was an increase in Htr2a expression in male mice, when compared to female mice, within the EE ($p < 0.001$) and wheel running ($p = 0.018$) groups, but not within the SH group ($p = 0.452$). And also that EE increased Htr2a expression compared to standard housing ($p < 0.001$).

Housing in EE condition increased Htr2a expression in the hippocampus [**Figure 4B**; $F(2,66) = 4.592$, $p = 0.014$, SH vs EE; $p = 0.004$, SH vs RW; $p = 0.182$]. In the cortex, a significant interaction between sex and housing environment [**Figure 4C**; $F(2,67) = 9.606$, $p < 0.001$], followed by pairwise comparison, indicated that female SH mice had significantly higher levels of Htr2a mRNA than their male SH littermates ($p < 0.001$); In male mice, EE ($p < 0.001$) and wheel running ($p = 0.001$) conditions increased Htr2a expression in comparison to SH conditions.

Region-Specific Htr2c Gene Expression Changes in HD Mice Are Differentially Modulated by Sex and Environment

There was a significant difference in Htr2c expression levels between WT and Huntington's disease mice in the striatum [**Figure 5A**; $F(1,71) = 15.306$, $p < 0.001$]. There was no difference in Htr2c expression between HD and WT mice in the hippocampus [**Figure 5B**; $F(1,70) = 2.134$, $p = 0.149$] and likewise in the cortex [**Figure 5C**; $F(1,71) = 3.712$, $p = 0.059$].

Female mice had significantly higher levels of Htr2c expression in the hippocampus than their male counterparts [**Figure 5B**; $F(1,70) = 11.543$, $p = 0.001$]. However, there was no difference in expression levels between the sexes in the striatum [**Figure 5A**; $F(1,71) = 0.000$, $p = 0.997$], or cortex [**Figure 5C**; $F(1,71) = 0.075$, $p = 0.785$].

Housing environment had no effect on Htr2c expression in the striatum [**Figure 5A**; $F(1,71) = 0.093$, $p = 0.912$]. In the hippocampus housing environment had a significant effect on Htr2c mRNA levels [**Figure 5B**; $F(2,70) = 6.166$, $p = 0.004$]; *post hoc* testing demonstrated that RW mice have significantly lower levels of Htr2c mRNA than EE mice ($p = 0.003$). There was also a trend toward lower levels of Htr2c mRNA in RW mice when compared to SH mice ($p = 0.054$). In the cortex there was again a significant effect of housing environment [**Figure 5C**;

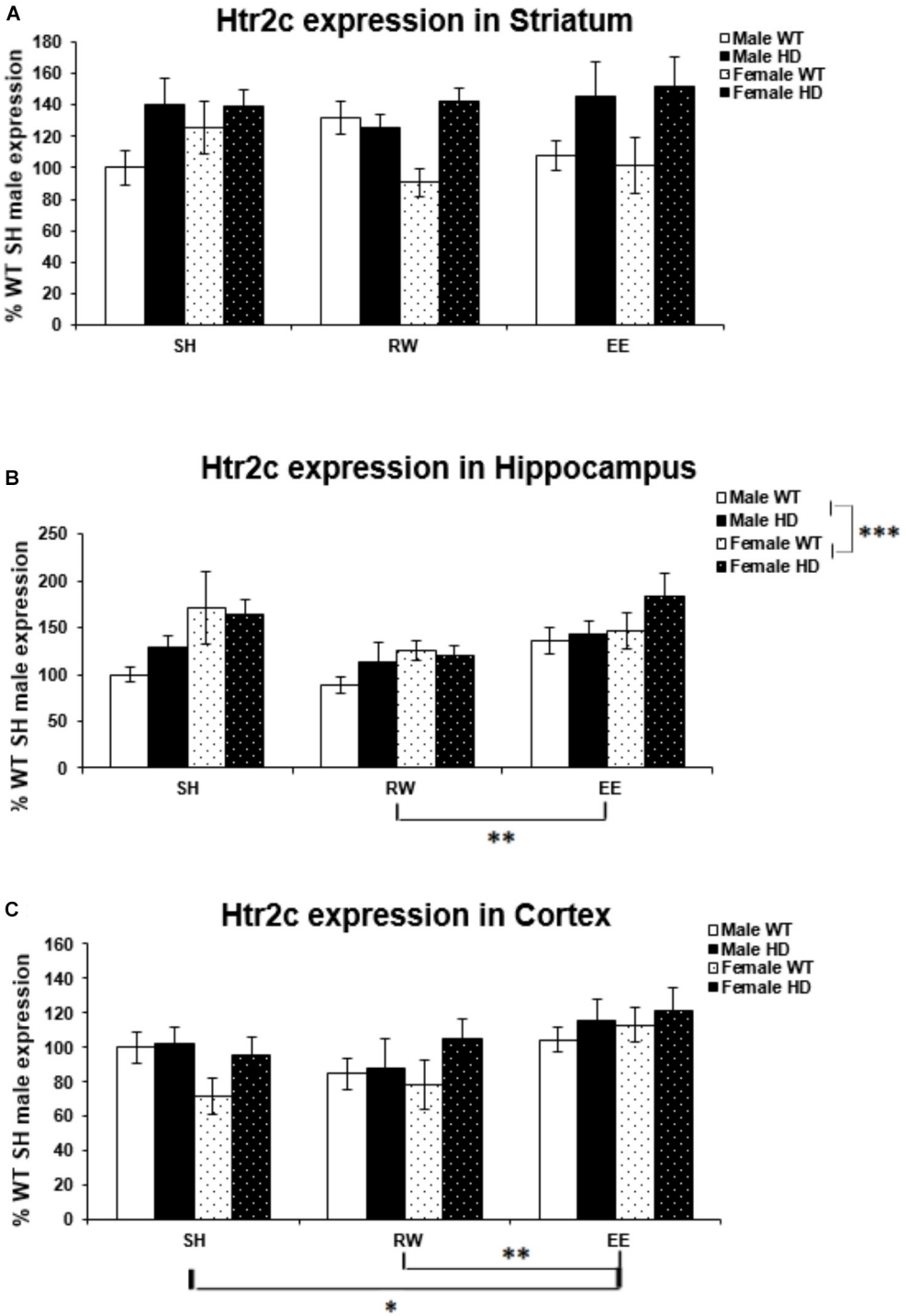

FIGURE 5 | Effects of genotype, environment and sex on Htr2c mRNA levels in 8-week-old WT and HD mice. **(A)** In Striatum, Htr2c expression was increased significantly in 8-week-old HD mice ($p < 0.001$). Housing environment had no effect on Htr2c expression ($p = 0.912$). **(B)** In Hippocampus, Genotype did not significantly affect Htr2c expression in 8-week-old HD mice ($p = 0.149$). Housing environment had a significant effect on Htr2c expression ($p = 0.004$). *Post hoc* testing showed that wheel running mice had significantly less Htr2c mRNA than mice exposed to EE ($p = 0.003$), and showed a trend toward less Htr2c mRNA than SH mice ($p = 0.054$). Male mice demonstrated significantly less Htr2c mRNA than female mice ($p = 0.001$). **(C)** In Cortex, HD mice demonstrated no change in levels of Htr2c mRNA compared to WT mice ($p = 0.059$). Housing environment had a significant effect on Htr2c expression ($p = 0.006$). *Post hoc* testing showed that environmental enrichment ($p = 0.040$), but not wheel running ($p = 0.846$), significantly increased levels of Htr2c mRNA. $n = 4$–6 per group. Results are represented as mean ± SEM. Pairwise comparisons were conducted using Bonferonni's adjustment for multiple comparisons: $^{*}p < 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

$F(1,71) = 5.541$, $p = 0.006$]; *post hoc* testing demonstrated that EE mice have significantly higher levels of Htr2c mRNA than SH mice ($p = 0.040$) and RW mice ($p = 0.009$).

Behavioral Pharmacological Analysis of 5-HT$_2$ Receptors Shows 2 Weeks of EE Boosts the Behavioral Response to Agonism of These Receptors in 8-Week-Old HD Mice

The administration of a 5-HT$_{2A/2C}$ receptor agonist, (±)DOI, induced head-twitches in WT and HD mice and this effect was reduced in both female and male HD mice [**Figure 6**, main effects of genotype, $F(1,70) = 7.6$, $p = 0.008$, and housing condition $F(1,70) = 7.0$, $p = 0.010$, and a significant interaction between sex and housing condition, $F(1,70) = 6.7$, $p = 0.012$]. *Post hoc* testing showed that EE significantly increased the number of head-twitches in both WT and HD male mice ($p < 0.001$) but not in female mice of either genotype (**Figure 6**).

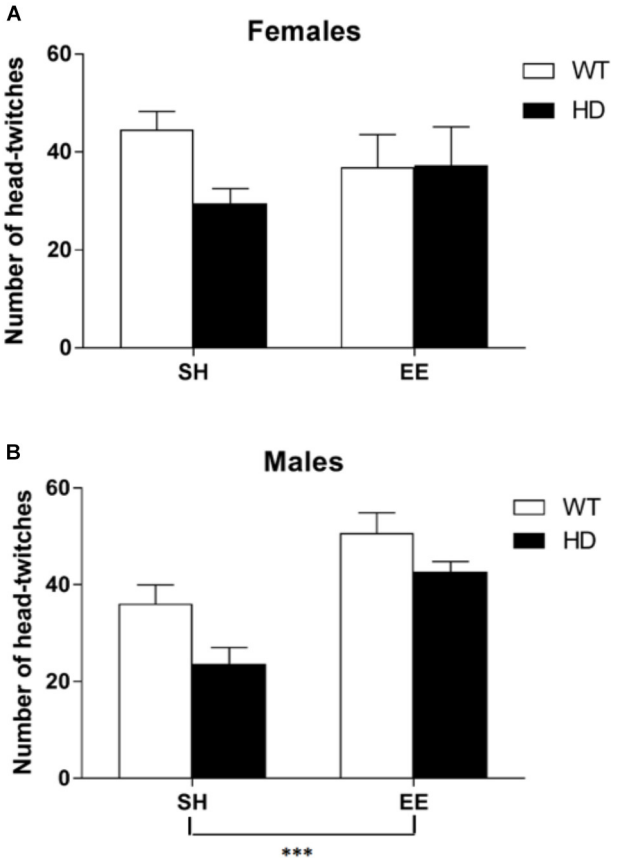

FIGURE 6 | Environmental enrichment induces a sexually dimorphic increase in the number of DOI-induced head-twitches in wild-type and HD mice at 8 weeks of age. The number of head-twitches induced by acute administration of the 5-HT$_2$ receptor agonist (DOI) was reduced in HD mice when compared to their wild-type counterparts ($p = 0.008$). There was no overall difference between male and female mice in number of head-twitches. **(A)** There was no significant effect of housing conditions in female mice; $n = 7$–10. **(B)** However, there was a significant effect of housing condition ($p = 0.010$) and a significant interaction between sex and housing condition ($p = 0.012$) with *post hoc* testing showing that environmental enrichment significantly increased the number of head-twitches in both wild-type and HD male mice; $n = 6$–11 ($p < 0.001$). Results are represented as mean ± SEM $^{***}p < 0.001$.

DISCUSSION

In this study, we have discovered various significant effects on gene expression due to genotype, environment, sex and brain region. This provides new insight into the pathogenesis of HD, and the therapeutic impacts of EE and exercise on the cognitive, psychiatric and motor symptoms of this currently incurable disease. Furthermore, the sexually dimorphic and region-specific effects we discovered may provide new insight into depression in HD (where sexual dimorphism occurs) and identify brain regions and molecular pathways that can be targeted with future therapeutic approaches.

The most intriguing change in gene expression after exposure to voluntary wheel running was an increase in SerT mRNA levels in the hippocampus in all four groups. An increase in serotonin transporter levels could indicate an increased ability to reuptake and recycle serotonin from the synaptic cleft in the hippocampus. SerT mRNA levels are reduced in the rat raphe nuclei after 3 weeks of running (Greenwood et al., 2005) and in a future experiment it would be interesting to investigate whether this is the case in HD mice.

There were significant decreases in Htr1a mRNA levels in 8-week-old male and female R6/1 HD mice in the hippocampus, cortex and striatum. Similar reduction in the hippocampus and cortex of 12-week-old male and female R6/1 HD mice have been demonstrated previously (Pang et al., 2009). This suggests that the transcriptional down-regulation of Htr1a occurs early in pathogenesis, prior to onset of behavioral deficits. Furthermore, our prior evidence of abnormal signaling through 5-HT1a autoreceptors in the raphe (Renoir et al., 2013) are consistent with transcriptional dysregulation of the Htr1a gene, and associated functional consequences.

The current study illustrates that EE and voluntary wheel-running paradigms do not have the same effect on gene expression patterns, far from it. In particular, Htr1b expression in the hippocampus and cortex of male WT mice is decreased by voluntary wheel running but increased by EE. In the cortex the same effect is apparent in the male R6/1 HD mice with a significant decrease in Htr1b mRNA levels due to voluntary wheel running and a significant increase caused by exposure to EE.

This study demonstrated dysregulation of the serotonergic system in the striatum, cortex and hippocampus of R6/1 HD mice, at early stages of the disease process. The R6/1 HD mouse model does not typically show any motor symptoms before 12 weeks of age (Pang et al., 2006; Nithianantharajah et al., 2008), however, both the male and female mice start to show cognitive behavioral changes (Mazarakis et al., 2005; Nithianantharajah et al., 2008; Mo et al., 2013, 2014a, 2015) including specific deficits in hippocampal-dependent, but not hippocampal-independent

memory (Nithianantharajah et al., 2008; Mo et al., 2014b). Therefore, any molecular and cellular changes seen in both male and female HD mice at 8 weeks of age in the present study are more likely to be involved in the onset of these cognitive symptoms.

Previous findings from our laboratory showed that female, but not male, R6/1 HD mice exhibited a depressive-like phenotype at 8–12 weeks of age (Pang et al., 2009; Renoir et al., 2011). In the present study, while Htr2c expression levels in 8-week-old female HD mice did not differ from those seen in WT in any of the regions examined, male HD mice had significantly increased Htr2c expression in the striatum. This specific increase in males may reflect a potential compensatory mechanism. With respect to potential mechanisms mediating this sexual dimorphism, sex hormones are the most obvious candidate. We have previously demonstrated abnormalities of the hypothalamic-pituitary-gonadal (HPG) axis in these HD mice (Du et al., 2015), and this may contribute to our observed findings.

Our findings in the present study provide new evidence that 2 weeks of EE can correct certain deficits in gene expression in R6/1 HD mice. The effect was restricted to male R6/1 HD mice, with no effect of EE on female R6/1 HD gene expression. The differential effect of EE on gene expression in male and female WT mice has not been previously demonstrated. We have found that EE can produce changes in gene expression (in a previous study focusing on BDNF) of a large magnitude in the brain of male mice where none occurs in females (Zajac et al., 2010).

Levels of Htr2a mRNA in 8-week-old male HD mice exposed to 2 weeks of EE were comparable to, or exceeded, levels in WT male SH mice, showing a rescue of the deficit caused by the HD transgene. These results differ from those in 12-week-old HD mice showing no change in Htr2a expression 4 weeks of EE (Pang et al., 2009). This agrees with the hypothesis that the time period of the EE paradigm is critical with regard to cellular responses associated with transcriptional regulation. This hypothesis is also relevant to exercise interventions and could also explain the difference between the present study and our previous study in which a wheel-running intervention occurred between 8 and 12 weeks of age (Renoir et al., 2012). Changes in gene expression in response to a novel environment are likely to be transient, with gene expression returning after a period of time to steady-state levels even if the environmental changes persist. Transient changes in the effects of EE are biologically relevant as they could affect long-lasting structural changes within cells.

We therefore used a test of 5-$HT_{2A/2C}$ receptor function to assess whether EE-mediated change in Htr2a receptor expression was translated into a functional increase in receptor activity. The study showed that exposure to 2 weeks of EE increased the number of head-twitches seen after an injection of the 5-HT2 receptor agonist, DOI, in both HD and WT male mice, suggesting that EE does indeed increase the functional activity of 5-HT_{2A} receptors in the mouse brain.

One limitation of the present study is that our gene expression measures were performed only at the mRNA level. It will be of interest in future studies to assess the impacts of these environmental interventions, as well as the associated effects of genotype, sex and brain region, at the protein level, using complementary approaches such as Western analysis and radioligand autoradiography, which would also provide more spatial resolution.

In this study, we have demonstrated a functional effect of early EE on behavioral pharmacology in WT mice, as well as a mouse model of a neurodegenerative disease. Our results provide new insight into the underlying mechanisms mediating the beneficial effects of EE, and will inform the development of this approach, and associated molecular targets, for future therapeutic interventions.

AUTHOR CONTRIBUTIONS

MZ and AH designed the experiments. MZ and TR performed the experiments. VP, SL, WA, and MvdB provided technical advice and support in preparing the manuscript and figures. MZ, TR, and AH wrote the manuscript. AH provided the funding for the experiments.

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Sex-Dependent Effects of Environmental Enrichment on Spatial Memory and Brain-Derived Neurotrophic Factor (BDNF) Signaling in a Developmental "Two-Hit" Mouse Model Combining BDNF Haploinsufficiency and Chronic Glucocorticoid Stimulation

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Neurodevelopmental disorders are thought to be caused by a combination of adverse genetic and environmental insults. The "two-hit" hypothesis suggests that an early first "hit" primes the developing brain to be vulnerable to a second "hit" during adolescence which triggers behavioral dysfunction. We have previously modeled this scenario in mice and found that the combined effect of a genetic hapolinsuffuciency in the brain-derived neurotrophic factor (BDNF) gene (1st hit) and chronic corticosterone (CORT) treatment during adolescence (2nd hit), caused spatial memory impairments in adulthood. Environmental enrichment (EE) protocols are designed to stimulate experience-dependent plasticity and have shown therapeutic actions. This study investigated whether EE can reverse these spatial memory impairments. Wild-type (WT) and BDNF heterozygous (HET) mice were treated with corticosterone (CORT) in their drinking water (50 mg/L) from weeks 6 to 8 and exposed to EE from 7 to 9 weeks. Enriched housing included open top cages with additional toys, tunnels, housing, and platforms. Y-maze novel preference testing, to assess short-term spatial memory, was performed at 10 weeks of age. At week 16 dorsal hippocampus tissue was obtained for Western blot analysis of expression levels of BDNF, the BDNF receptor TrkB, and NMDA receptor subunits, GluNR1, 2A and 2B. As in our previous studies, spatial memory was impaired in our two-hit (BDNF HET + CORT) mice. Simultaneous EE prevented these impairments. However, EE appeared to worsen spatial memory performance in WT mice, particularly those exposed to CORT. While BDNF levels were lower in BDNF HET mice as expected, there were no further effects of CORT or EE in males but a close to significant

female CORT × EE × genotype interaction which qualitatively corresponded with Y-maze performance. However, EE caused both sex- and genotype-specific effects on phosphorylated TrkB residues and GluNR expression within the dorsal hippocampus, with GluNR2B levels in males changing in parallel with spatial memory performance. In conclusion, beneficial effects of EE on spatial memory emerge only following two developmental disruptions. The mechanisms by which EE exerts its effects are likely via regulation of multiple activity-dependent pathways, including TrkB and NMDA receptor signaling.

Keywords: brain-derived neurotrophic factor, spatial memory, environmental enrichment, hippocampus, corticosterone, stress, neuroplasticity

INTRODUCTION

Cognitive impairment is a common symptom in a range of neurodevelopmental disorders, including schizophrenia, major depressive disorder (MDD) and anxiety. In schizophrenia, cognitive impairment occurs in ∼80% of cases and includes deficits in learning and memory, which have carry-on effects to social and intellectual functioning (Heinrichs and Zakzanis, 1998; Lewis, 2012). It has been reported that individuals with MDD and anxiety can have cognitive impairments in multiple domains including memory and attention (Gualtieri and Morgan, 2008; McIntyre et al., 2013).

One theory for the pathophysiology of neurodevelopmental disorders is the two-hit hypothesis. The "two-hit hypothesis" postulates that the combination of genetic predisposition and environmental insults during critical periods of development can culminate in significant behavioral disruption in adulthood (Klug et al., 2012). The "first hit" (genetic factor) during development creates a vulnerable brain, and when coupled with the "second hit" (environmental factor) triggers the onset of the disorder (Bayer et al., 1999).

Brain-derived neurotrophic factor (BDNF) is an essential neurotrophin responsible for a broad range of neuronal functions (Adachi et al., 2014) and is associated with neurodevelopmental disorders. Post-mortem studies have reported reduced levels of BDNF and its cognate receptor, Tropomysosin-related kinase B (TrkB) in the prefrontal cortex (PFC) and hippocampus of individuals with schizophrenia (Thompson Ray et al., 2011; Reinhart et al., 2015), suggesting a role of BDNF-TrkB signaling in the illness. There is support in the literature for altered BDNF in humans with depression (Lee and Kim, 2010; Zaletel et al., 2017) and anxiety (Soliman et al., 2010; Castrén, 2014). Stress has been recognized as a major environmental risk factor in the pathophysiology of schizophrenia models (van Os et al., 2010; Brown, 2011; Magariños et al., 2018), and depression and anxiety (Binder and Nemeroff, 2010; Zaletel et al., 2017). We therefore modeled the "two-hit" hypothesis by combining genetic haploinsufficiency in the BDNF gene (1st hit) with adolescent chronic corticosterone treatment (2nd hit). We previously found that these animals show short-term spatial memory deficits (Klug et al., 2012; Hill et al., 2014).

Prolonged corticosterone (CORT) administration in rodents is a well-established model to mimic the physiological parameters of chronic stress and disrupt the HPA axis (Buret and van den Buuse, 2014; Shahanoor et al., 2017). The hypothalamic pituitary adrenal (HPA) axis is the well-conserved control center for the body's stress response. While its role is to moderate the stress response, it can cause damage through prolonged release of glucocorticoids (GC) (Du and Pang, 2015). In humans this is cortisol and the rodent equivalent is corticosterone (Papadimitriou and Priftis, 2009), and these can act in a negative feedback loop to regulate the HPA axis in their respective mammalian systems (Du and Pang, 2015). Dysregulation of this loop can have a range of negative effects upon behavior and cognition. Indeed, in the hippocampus there is a dense expression of glucocorticoid receptors (GR), and it is thought that the excess activity of GC here could be contributing to the cognitive deficits associated with chronic stress (Mirescu and Gould, 2006; Jayatissa et al., 2008; Rainer et al., 2012; Du and Pang, 2015).

For humans, leading a healthy lifestyle or having a "positive environment," in both the physical and emotional sense, helps prevent and create resilience to neurodegenerative and mental health issues (Maass et al., 2014; Brown et al., 2017; Lee et al., 2018). A recent, comprehensive review by Arango et al. (2018) outlines that environmental risk factors such as poverty, stressful urban environments and negative social interactions such as bullying and abuse during childhood and adolescence can act synergistically to increase susceptibility to developing a neurodevelopmental disorder (Arango et al., 2018). It goes on to demonstrate that a range of interventions, including age-appropriate stimulation, proper nutrition and exercise can be important buffers against neurodevelopmental disorders. Another recent review by Devoe et al. suggested that cognitive behavioral therapy and family therapy are useful in the long-term reduction of attenuated psychotic symptoms (Devoe et al., 2018). This resilience is thought to be linked to a holistic health approach, which includes a "stimulating environment." A stimulating environment encapsulates many domains, including social, physical, and cognitive. Research in adulthood has found that focus on social groups and music therapy can prevent and alleviate depressive symptoms (Cruwys et al., 2013) and schizophrenia patient outcomes (Fachner et al., 2013; Geretsegger et al., 2017; Erkkilä et al., 2018). This is consistent with the

Arango et al. review that argued appropriate stimulation is necessary for a healthy mind (Arango et al., 2018). Positive environments in preclinical animal model research generally refer to environmental enrichment (EE), an experimental protocol that aims to provide the laboratory animals with a habitat with an enhanced sensory environment, in order to stimulate experience-dependent plasticity (Nithianantharajah and Hannan, 2006; Novkovic et al., 2015). Rodent EE studies vary in their protocols to create an enriched environment, and include larger living areas, giving the animals access to toys or other stimulating materials, living in larger social groups, and exercise (Clemenson et al., 2015). This has been found to have positive effects including improved cognitive functioning (Yuan et al., 2012), delay of disease progression (Garofalo et al., 2015) and recovering of disease symptoms, with learning and memory also modulated by EE (Burrows et al., 2015). However, some studies have shown that EE can also have a stressful and negative impact upon laboratory animals, including increased aggression (McQuaid et al., 2012).

It is well established in the literature that the hippocampus has a central role in cognition, is affected in human neurodevelopmental disorders (Lavenex et al., 2006; Barnea-Goraly et al., 2014; Ledoux et al., 2014; Blair et al., 2017), and in rodent studies has been particularly responsive to EE-induced effects (Teather et al., 2002). This is hypothesized to occur through the BDNF-TrkB signaling pathway (Novkovic et al., 2015). BDNF binding to TrkB induces receptor dimerization and subsequent phosphorylation of tyrosine residues (Minichiello, 2009), the most important being 705, 515, and 816. Y705 has been called the initiator of receptor autophosphorylation (Benmansour et al., 2016) and has an overall role in TrkB activation, with the extent of phosphorylation of this residue correlating with TrkB activity levels (Huang and McNamara, 2010). The tyrosine residue 515 (Y515) is the Shc adapter protein docking site (Ambjørn et al., 2013; Benmansour et al., 2016), which catalyzes multiple signaling cascades including pathways involved in learning and memory (Yang et al., 2011). Y816 is linked to the phospholipase (PLC)γ1 pathway, has a role in synaptic plasticity, cell survival and axon elongation (Ming et al., 1999; Atwal et al., 2000; Minichiello, 2009), and contributes to ERK activation (Ambjørn et al., 2013).

Several studies have demonstrated that EE increases BDNF levels in the hippocampus (Cao et al., 2014; Ramírez-Rodríguez et al., 2014; Novkovic et al., 2015) and, consequently exerts its positive effects upon cognition (Novkovic et al., 2015). Conversely, stress has been shown to negatively impact BDNF-TrkB signaling (Buckley et al., 2007). Chronic treatment with corticosterone (CORT) has been shown to decrease levels of BDNF mRNA and protein, as well as intracellular BDNF content (Nitta et al., 1999). Thus, we hypothesized that EE may recover the spatial memory deficit previously found in our two-hit model via regulation of the BDNF-TrkB signaling pathway. This hypothesis was tested by measuring protein expression of mature BDNF, TrkB, and multiple TrkB phosphorylation sites in the dorsal hippocampus.

Dysfunction of the inhibitory circuits and consequently the tilting of the excitatory/inhibitory balance toward over-excitation, is a major contributor to cognitive deficits present in neurodevelopmental disorders (Daskalakis et al., 2002; Heckers and Konradi, 2014; Fee et al., 2017; Selten et al., 2018). Excitotoxicity is characterized by increased extracellular concentrations of glutamate, which overactivate N-methyl-D-aspartate receptors (NMDAR) and allow an excess of Ca^{2+} influx. This activates a range of enzymatic effects that may cause cell damage or even cell death, resulting in a variety of detrimental neuronal and cognitive consequences. NMDAR are heteromeric tetramers consisting of different combinations of NMDAR subunits; usually including one NMDAR-1 (GluN1) subunit and at least one or more GluN2(A-D) or GluN3(A,B) subunits (Paoletti et al., 2013). NMDAR are located both at the pre- and post-synaptic sites, positioning them to play vital roles in long-term potentiation (LTP) and plasticity (Paoletti et al., 2013). Both of these processes are highly implicated in cognitive processes such as learning and memory (Nithianantharajah and Hannan, 2006; Vierk et al., 2014).

The first aim of this study was to investigate whether EE could reverse the spatial memory deficit in our "two-hit" model of BDNF haploinsufficiency and CORT treatment and how this would compare to EE effects in wildtype (WT) controls. The second aim of this study was to investigate any molecular changes to the BDNF-TrkB signaling pathway and NMDAR system in the dorsal hippocampus, and if these were modulated differentially by EE according to BDNF genotype, sex, and CORT treatment.

MATERIALS AND METHODS

Animals

Male and female BDNF heterozygous (HET) mice (Ernfors et al., 1994) and WT littermate controls were obtained from a breeding colony at the Florey Institute, Melbourne, Australia. All mice were on a C57Bl/6 background and breeders were originally obtained from The Jackson Laboratory (USA). 10 pairs of breeders were set up of WT female × HET male. Tail tissue samples were sent to Transnetyx (Cordova, TN, USA) for genotyping. Mice were weaned at 3 weeks and WT and HET mice were housed together. Males and females were housed separately, with an average of 3 mice per cage. Offspring were randomized into 8 experimental groups: (1) Group-housed males, water and standard housed (SH) (WT $n = 7$, HET $n = 7$), (2) Group-housed males, CORT, and SH (WT $n = 11$, HET $n = 10$), (3) Group-housed males, water, and EE (WT $n = 9$, HET $n = 9$), (4) Group-housed males, CORT, and EE (WT $n = 11$, HET $n = 12$), (5) Group-housed females, water, and SH (WT $n = 15$, HET $n = 11$), (6) Group-housed females, CORT, and SH (WT $n = 8$, HET $n = 10$), (7) Group-housed females, water, and EE (WT $n = 10$, HET $n = 10$), and (8) Group-housed females, CORT, and EE (WT $n = 10$, HET $n = 8$). No obvious competition or dominance ranking within the groups was observed. No overt aggressive behavior was observed for the EE groups. Six animals per group were used for molecular analysis. Mice had *ad libitum* access to food and water in a temperature controlled room maintained at ∼22°C and on a 12/12 h light/dark cycle. All procedures were

performed during the light phase. All procedures performed were done according to guidelines set by the National Health and Medical Research Council of Australia and approved by the Florey Institute for Neuroscience and Mental Health Animal Ethics Committee.

CORT Treatment

Adolescent/young adult mice were treated with corticosterone in the drinking water from 6 to 8 weeks of age (see **Figure 1**). These time points were based upon previous studies by our laboratory that show sexual maturation occurs during this period (Hill et al., 2012). Previous research has shown that in mice CORT can be administered between a dose of between 25 and 100 mg/L (Schroeder et al., 2015; Notaras et al., 2017). The CORT concentration of 50 mg/L was chosen with the assumption that the mice would increase water intake as they matured, and this CORT concentration would maintain CORT intake relative to body weight. A high CORT dose has been found in other models to create persistent stress phenotypes, which is important in a chronic model (Johnson et al., 2006; Gourley and Taylor, 2009). Corticosterone hemisuccinate (Q1662-000 Steraloids Inc, United States) was dissolved in water to a final concentration of 50 mg/L. CORT bottles were covered with aluminum foil to be protected from light degradation and were changed every 3–4 days. Bottles were weighed before they were replaced to measure CORT intake by the mice. CORT-treated mice tended to drink between 10 and 20 mg/kg/day. Once treatment stopped at the end of week 8, mice were left undisturbed for another 2 weeks. Control groups received water without CORT.

Environmental Enrichment

Mice received EE from 7 to 9 weeks of age (see **Figure 1**), during which they were kept in larger open top cages (44 × 30 × 15 cm) with various toys, tunnels, housing and platforms to provide novel cognitive challenges. These were changed once per week. Control mice were housed in open-top standard mouse cages (34 × 16 × 16 cm) with basic nesting materials and were designated "standard-housed" (SH). All mice were given 1 week to acclimatize to their environment when moved from open top to Individually-Ventilated Cages (IVC, 39.1 × 19.9 × 16 cm, Tecniplast, Italy) at the end of week 9.

Y-Maze Short-Term Spatial Memory Test

The Y-maze paradigm was performed as previously described (Hill et al., 2014) at week 11. The maze consisted of three arms (30 × 8 × 16 cm) at 120° angles to each other including geometric cues on the far end walls. Briefly, during the initial phase, the mouse was placed into the end of one arm (home arm) and was allowed to explore two arms for 10 min with one arm being closed (novel arm). After a 1 h retention time in the home cage, the mouse was placed into the same Y-maze with all arms open for 5 min. Behavior, including the time spent in each arm, was analyzed with video tracking software (TopScan, CleverSys Inc., Reston, VA, USA). A Discrimination Index (DI) was calculated, which was the amount of time spent in the novel arm divided by the average amount of time spent in the home arm and other familiar arm. Mice with intact spatial memory typically spend more time in the novel arm, reflective of intact memory of the original two familiar arms, and the DI tends to be around 1.5. A DI of around 1.0 represents equal times in all three arms (i.e., chance level) and is interpreted as no recollection of the two arms being familiar.

Western Blot Analysis

Mice were killed by cervical dislocation at 16 weeks of age and their brains were collected and stored at −80°C. The hippocampus was bilaterally dissected and separated into dorsal and ventral hippocampus (∼50/50). Protein extraction and Western blot analysis were performed as previously described (Hill et al., 2014). Primary antibodies were rabbit anti-BDNF (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA or Almone Labs, Israel), rabbit anti-NT-4 (1:200, Santa Cruz), rabbit anti-pTrkB Y705 (1:1,000, Signalway Antibody LLC, Maryland, USA), rabbit anti-pTrkB Y515 (1:1,000, Abcam, Cambridge, MA, USA), rabbit anti-pTrkB Y816 (1:500, Millipore, CA, USA), rabbit anti-TrkB (1:1,000, Santa Cruz), rabbit anti-NMDAR subunit 1 (GluNR1, 1:1,000, Cell Signaling Technology Inc, Danvers, MA, USA), rabbit NMDAR subunit 2A (GluN2A, 1:1,000, Cell Signaling Technology), rabbit NMDAR subunit 2B (GluN2B, 1:1,000, Cell Signaling Technology), or mouse anti-β-actin (1:10,000, Sigma-Aldrich). Secondary antibodies included anti-mouse or anti-rabbit IgG HRP-linked secondary antibodies (1: 2,000; Cell Signaling Technology; Danvers, MA, USA).

Statistical Analysis

All data are expressed as the mean ± the standard error of the mean (SEM). Groups were compared by ANOVA with the independent factors being sex (male or female), genotype (WT or HET), treatment (water or CORT), and environment (SH or EE), using the SYSTAT 13 (Systat Software Inc., San Jose, Ca, USA). Thus, there were 16 experimental groups. *Post-hoc* comparisons were done with Tukey's test. Group differences were considered significant when $P < 0.05$.

RESULTS

Y-Maze Behavior

Univariate ANOVA of the DI of time in the Y-Maze arms revealed that, while there were no main effects of either CORT or EE, there was a significant CORT × EE interaction [$F_{(1, 142)} = 10.62$, $P = 0.001$], suggesting that any effect of CORT depended on whether the animals also underwent EE. Furthermore, this interaction appeared to depend on the genotype of the animals [CORT × EE × Genotype interaction: $F_{(1, 142)} = 6.81$, $P = 0.010$; EE × Genotype interaction, $F_{(1, 42)} = 5.30$, $P = 0.023$]. Because there was also a CORT × Sex interaction [$F_{(1, 142)} = 5.69$, $P = 0.018$], further interrogation of the data was done in males and females separately (**Figure 2**).

In males, there was again a CORT × EE interaction [$F_{(1, 68)} = 7.37$, $P = 0.008$] although the CORT × EE × genotype interaction did not reach significance ($P = 0.079$). There was also a main effect of CORT treatment [$F_{(1, 68)} = 6.31$, $P = 0.014$]. Subsequent

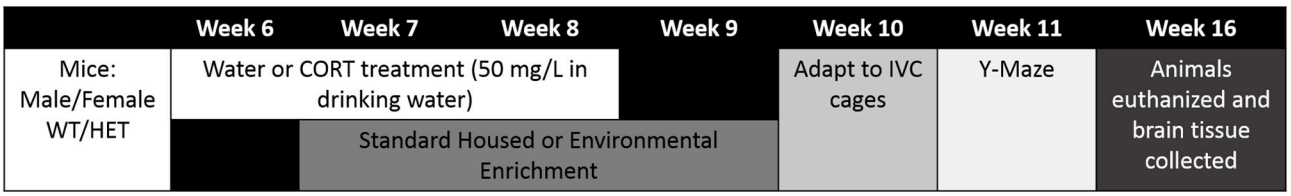

FIGURE 1 | Time line of the experiments. Male and female WT and BDNF heterozygote (HET) mice were used to investigate whether environmental enrichment could ameliorate an established spatial memory deficit in a neurodevelopmental "two hit" model. BDNF heterozygosity was used as the first hit with the second hit being chronic corticosterone (CORT) administered in the drinking water. Environmental enrichment (EE) was administered during and after chronic CORT treatment.

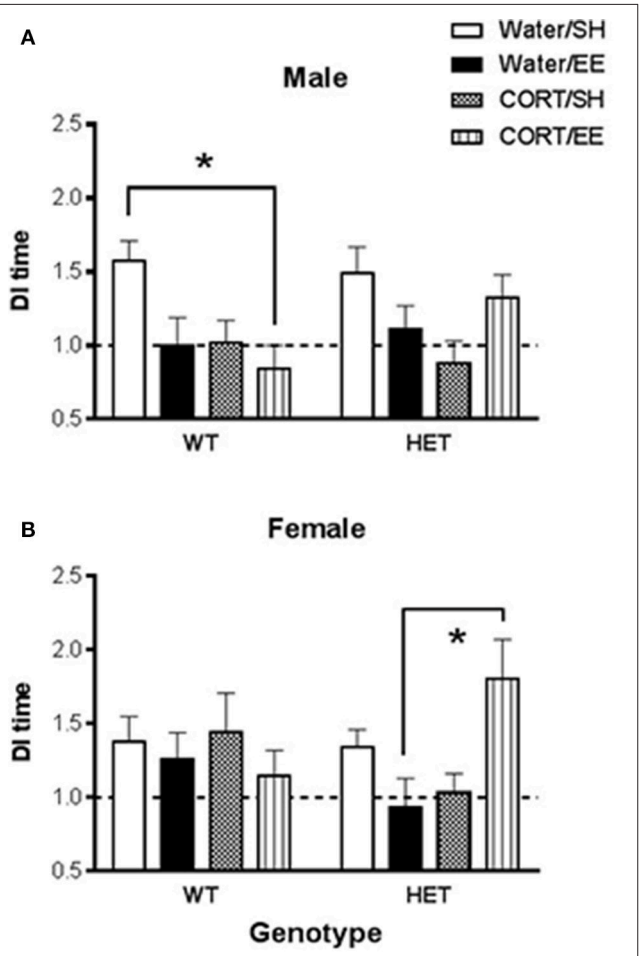

FIGURE 2 | Short-term spatial memory was measured using the Y-maze in wildtype (WT) and BDNF heterozygote (HET) mice which had been treated with chronic corticosterone (CORT), environmental enrichment (EE) or both. **(A)** Shows that male Y-maze behavior was impaired by both EE and CORT with the largest effect seen in male WT mice, but no such additive effect was observed in male BDNF HET mice. **(B)** Shows that female BDNF HET mice exposed to both CORT and EE had significantly higher Y-maze DI than controls. Data are mean ± SEM, $n = 7$–12. *Denotes a significant *post-hoc* interaction of $p < 0.05$.

pair-wise comparison with Tukey's test (**Figure 2A**) showed that DI values were significantly reduced in WT CORT/EE mice ($p = 0.045$) compared to WT water/SH.

In females, the CORT × EE × Genotype interaction was again significant [$F_{(1, 74)} = 7.16$, $P = 0.009$] suggesting differential effects of EE and CORT depending on the genotype. In HET mice, but not WT mice, pair-wise comparison with Tukey's test revealed that the combination of EE and CORT treatment resulted in significantly higher DI values compared to EE alone ($P = 0.009$) (**Figure 2B**).

Molecular Results

For the majority of investigated markers there was a main effect of sex when male and female data were combined, so it was decided to analyse the sexes separately. These main effects of sex were: mBDNF: $F_{(1, 67)} = 30.31$, $P < 0.001$; FL-TrkB: $F_{(1, 77)} = 227.13$, $P < 0.001$; Y705 ratio: $F_{(1, 72)} = 3105.0$, $P < 0.001$; Y816 ratio: $F_{(1, 68)} = 1061.98$, $P < 0.001$; GluN2A: $F_{(1, 51)} = 924.53$, $P < 0.001$; and GluN2B: $F_{(1, 67)} = 9.34$, $P = 0.003$.

mBDNF and NT4

Analysis of mBDNF levels revealed no effects of genotype, CORT, or EE on mBDNF expression in male dorsal hippocampus (**Figure 3A**). In females, there was similarly no main effect of genotype, CORT or EE, however there was a close to significant genotype × CORT × EE interaction [$F_{(1, 27)} = 4.13$, $P = 0.052$]. mBDNF expression levels qualitatively correspond with Y-maze performance with the female BDNF HET + CORT + EE group showing the highest expression (**Figure 3B**).

Analysis of NT-4 levels showed no effects of genotype, CORT, or EE either in males or females (**Figures 3C,D**).

TrkB and pTrkB

There were no effects of genotype, CORT or EE or interactions on FL-TrkB in the male and female DHP (**Figures 4A,B**).

Phosphorylation of TrkB was assessed at positions 705, 816, and 515. There was no main effect of genotype, CORT, or EE or interactions on pTrkB-Y705 in either males or females (**Figures 4C,D**). With respect to pTrkB-816, in the males there was a significant main effect of CORT [$F_{(1, 36)} = 16.45$, $P < 0.001$] and of EE [$F_{(1, 36)} = 8.22$, $P = 0.007$, **Figure 4E**], but no main effect of genotype or interactions. Inspection of the data (**Figure 4E**) shows that both CORT and EE increased the Y816 ratio and appear to have a cumulative effect when co-administered. This cumulative effect was also evident from a close to significant EE × CORT interaction [$F_{(1, 36)} = 3.82$, $P = 0.058$] for Y816.

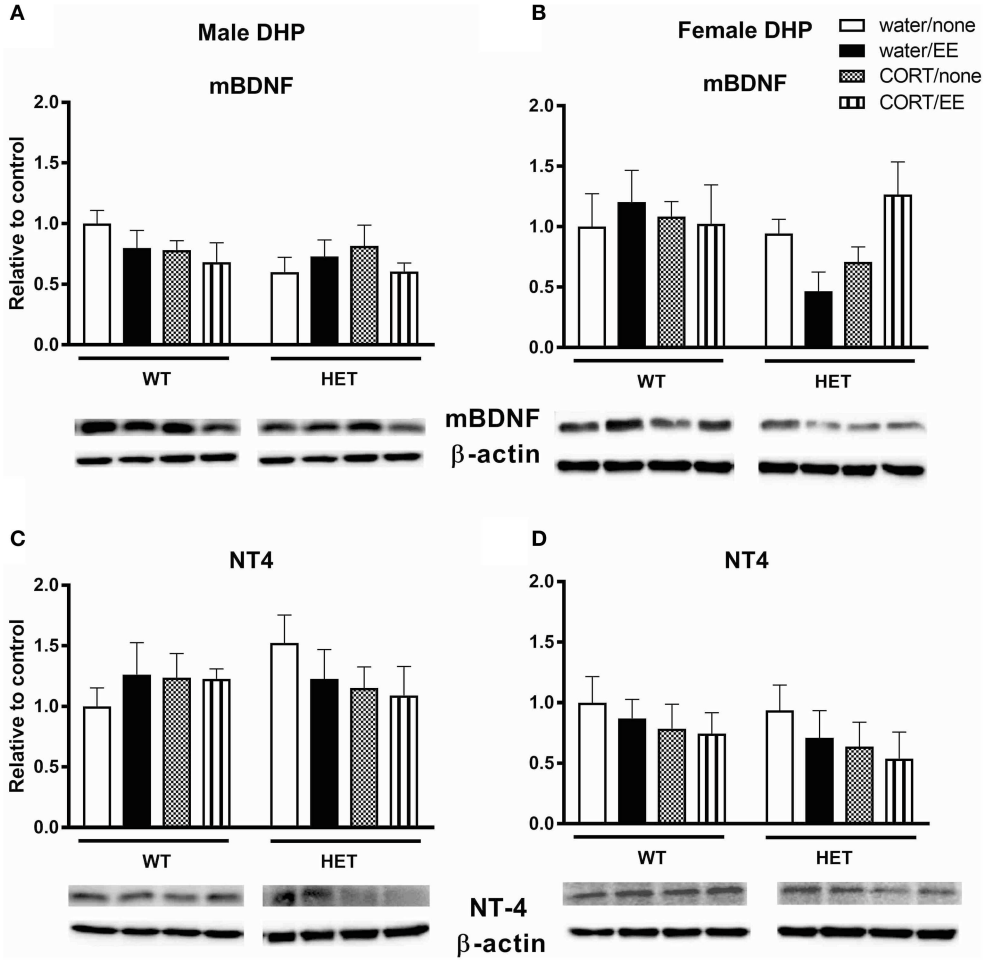

FIGURE 3 | Western blotting was used to measure protein expression of mature BDNF (mBDNF) and neurotrophin 4 (NT4) in the dorsal hippocampus (DHP) of wildtype (WT) and BDNF heterozygous (HET) mice after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both. Results are presented as standardized protein expression relative to control. There was a close to significant genotype × CORT × EE interaction on mBDNF in female DHP **(B)**. There were no significant changes to protein expression of BDNF in males **(A)** or to NT-4 in males and females **(C, D)**. Data are mean ± SEM, $n = 3$–6, no significant main effects.

In female mice, there was a significant main effect of genotype [$F_{(1, 36)} = 16.87$, $P < 0.001$] and CORT [$F_{(1, 36)} = 18.99$, $P < 0.001$], but not EE, on Y816 levels (**Figure 4F**). There was also a significant EE × CORT interaction [$F_{(1, 36)} = 5.21$, $P = 0.028$], reflecting that CORT tended to increase expression while EE tended to reduce expression of Y816, particularly in WT mice. This differential effect of EE in WT compared to BDNF HET mice was supported by a genotype × EE interaction [$F_{(1, 36)} = 4.94$, $P = 0.033$]. However, there was no genotype × CORT × EE interaction.

Analysis of pTrkB515 expression in male mice showed a significant genotype × CORT interaction [$F_{(1, 39)} = 4.24$, $P = 0.046$, **Figure 4G**] whereby CORT treatment increased expression in the WT mice, but reduced expression in BDNF HET mice. There was no main effect of genotype, CORT, EE on Y515 in male mice. No significant effects of genotype, CORT, EE or interactions were found in female mice (**Figure 4H**).

NMDAR Subunits

Analysis of GluN1 protein expression in the male dorsal hippocampus revealed a main effect of genotype [$F_{(1, 39)} = 5.73$, $P = 0.022$] but no effects of CORT or EE or interactions (**Figure 5A**). Male BDNF HET mice had higher protein expression of GluNR1 compared to male WT mice. Similarly, in females, there was a main effect of genotype [$F_{(1, 28)} = 4.54$, $P = 0.042$], but no effects of CORT or EE, with BDNF HET mice having a higher GluN1 protein expression levels compared to WT mice (**Figure 5B**).

In male mice, we found a significant genotype × CORT interaction for GluN2A protein expression [$F_{(1, 39)} = 5.40$, $P = 0.001$, **Figure 5C**) but no effect of genotype, CORT or EE. This significant interaction resulted from reduced GluNR2A protein expression in the male WT CORT/SH group but increased protein expression in male BDNF HET that underwent CORT/SH. *Post-hoc* Tukey's comparisons found while BDNF HET water/SH was significantly decreased ($p = 0.034$), BDNF

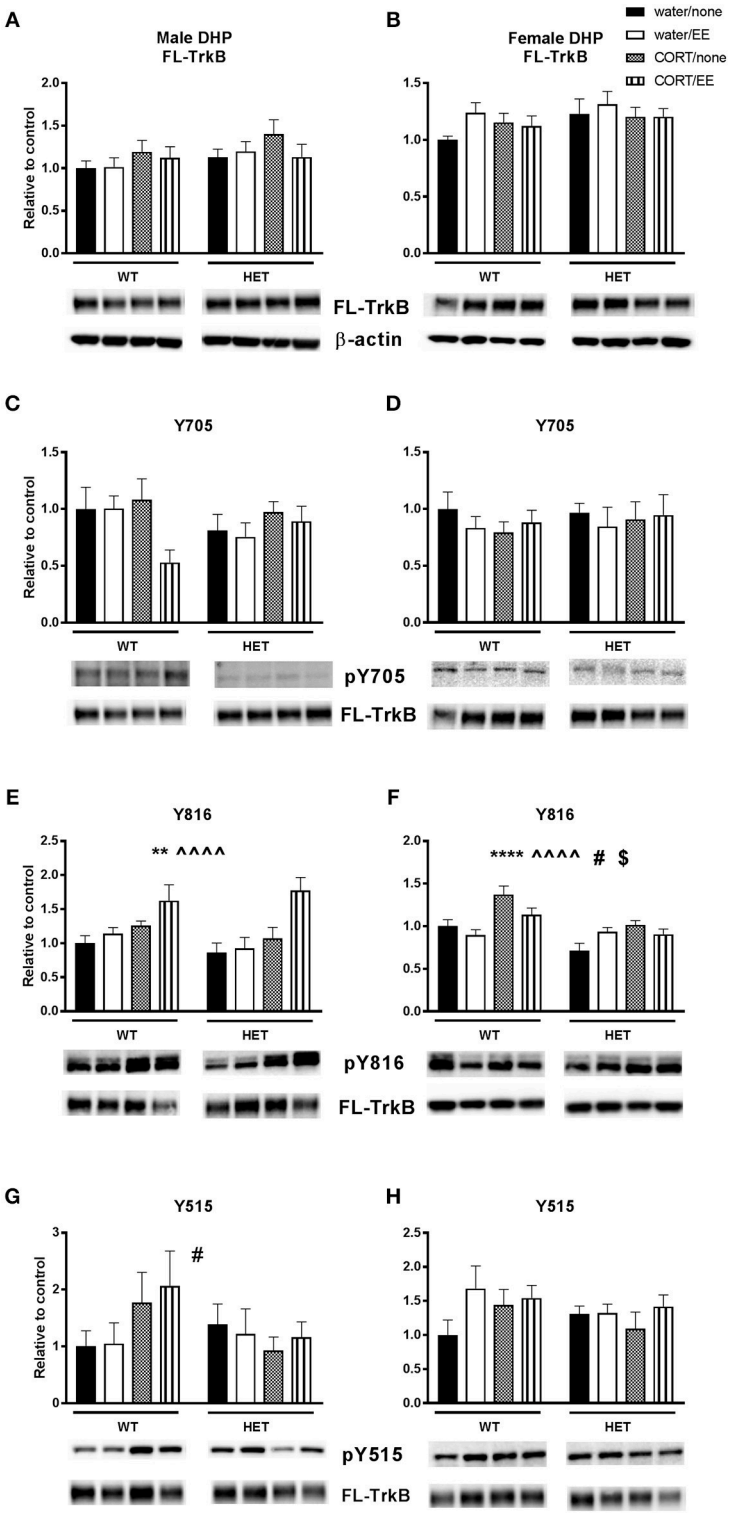

FIGURE 4 | Western blotting was used to measure protein expression of TrkB receptor and phosphorylated TrkB receptor residues after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the dorsal hippocampus of wildtype (WT) and BDNF heterozygous (HET) mice. Results are presented as standardized protein expression relative to control. Phosphorylated TrkB residues are always divided by full length TrkB. For male results refer to **(E)**, which shows a significant effect of CORT (^^^^) and of EE (**) and **(G)** which shows a significant genotype × CORT interaction ($^{\#}$). For female results refer to **(F)**, which shows a significant main effect of genotype (**), significant main effect of CORT (i, significant genotype × EE interaction ($^{\#}$) and significant EE × CORT interaction ($^{\$}$). There were no significant changes to protein expression of FL-TrkB in males and females **(A,B)**, Y705 in males and female **(C,D)** and Y515 in females **(H)**. Data are mean ± SEM, $n = 4$–6, main effect ** $P < 0.005$, ^^^^ or **** $P < 0.001$, significant interaction $^{\#}P < 0.05$ and $^{\$}P < 0.005$ for protein expression based on ANOVA. For full details of ANOVA results, see main text.

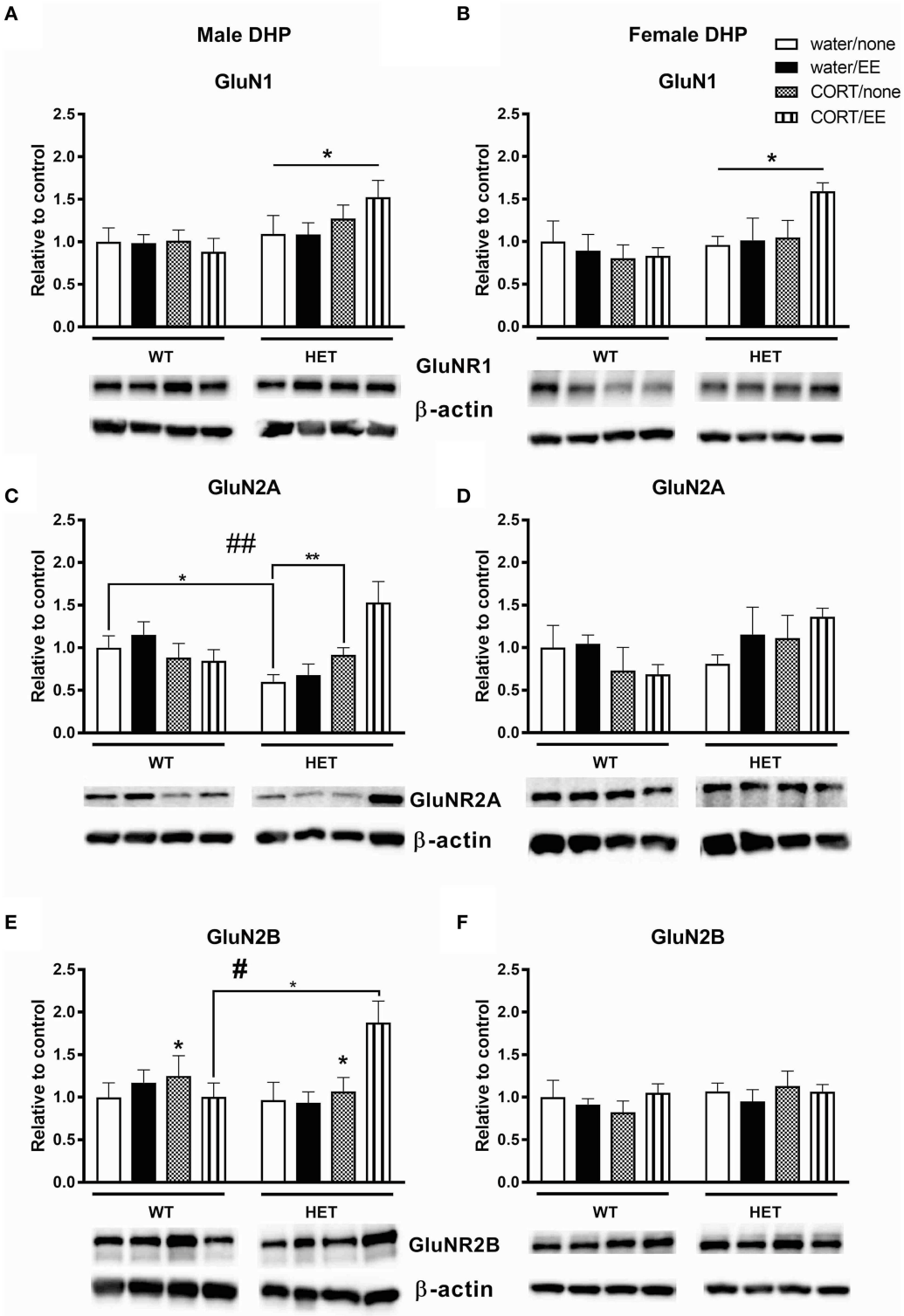

FIGURE 5 | Western blotting was used to measure protein of NMDAR subunits after exposure to chronic corticosterone (CORT), environmental enrichment (EE) or both in the dorsal hippocampus of wildtype (WT) and BDNF heterozygous (HET) mice. Results are presented as standardized protein expression relative to control. **(A,B)** Show protein expression of NMDAR subunit GluNR1, which had a significant main effect of genotype in males and females. **(C)** Shows male GluNR2A protein expression had a genotype × CORT interaction and **(E)** shows male GluNR2B protein expression had significant main effect of CORT and a significant genotype × EE × CORT interaction. There were no significant changes to GluN2A and GlunN2B protein expression in females **(D,F)**. Data are mean ± SEM, $n = 3$–6, main effect or *post hoc* comparison $^{*}p < 0.05$, significant interaction $^{\#}p < 0.05$ and $^{\#\#}p < 0.005$ for protein expression based on ANOVA and Tukey's *post-hoc*. For full details of ANOVA results, see main text. **Indicates a significant *post-hoc* interaction of $p < 0.005$.

HET CORT/SH was significantly increased ($p = 0.003$) compared to controls. In female mice, there were no main effects of genotype, CORT, EE or interactions for GluN2A (**Figure 5D**).

Analysis of GluN2B levels in male dorsal hippocampus revealed a main effect of CORT [$F_{(1, 39)} = 4.45$, $P = 0.041$] whereby CORT increased GluN2B protein expression (**Figure 5E**). We also found a significant genotype × CORT × EE interaction [$F_{(1, 39)} = 5.50$, $P = 0.024$], and *post-hoc* comparisons found that the BDNF HET CORT/EE group had higher GluN2B protein expression compared to WT CORT/EE ($p = 0.04$). There were no main effects of genotype or EE for GluN2B and no main effects of genotype, CORT, EE or interactions for GluN2B in the females (**Figure 5F**).

DISCUSSION

This study investigated the possible preventative benefits of environmental enrichment on memory impairments in a two-hit neurodevelopmental model. The current study is an extension of previous research by our group (Klug et al., 2012), showing in the two-hit model that chronic adolescent stress, modeled here by chronic CORT treatment, combined with BDNF haploinsufficiency leads to a spatial memory deficit in the Y-maze. The primary aim of this study was to investigate if this deficit can be prevented through environmental enrichment. In humans, negative environmental factors can act synergistically with other risk factors to trigger the onset of neurodevelopmental disorders. However, a range of environmental interventions including age-appropriate stimulation, proper nutrition, and exercise have been found to be important preventatives against the onset of neurodevelopmental disorders and mental health issues (Arango et al., 2018).

In the current study, while we anticipated positive modulation on cognition by EE alone, it appeared to have a detrimental effect on spatial memory performance, particularly in male WT mice. In this group, Y-maze DI was reduced in both the EE and CORT groups, with the WT CORT-EE reaching significance compared to the control WT. In contrast, albeit not at the level of a statistical genotype × CORT × EE interaction, in male BDNF HET mice, this additive effect was not observed, and EE/CORT-treated BDNF HET mice had Y-maze DI values not different from control BDNF HET mice. Analysis of data from both sexes combined showed a significant genotype × CORT × EE effect and this interaction remained statistically significant in female mice, where a significantly higher DI was found in the EE/CORT-treated BDNF HET group compared to EE only.

Previous studies examining the effects of EE on hippocampal-dependent memory tasks have shown beneficial effects of EE on long-term memory in the Morris Water maze (Leggio et al., 2005; Garthe et al., 2015) and spatial working memory in the radial arm maze (Leggio et al., 2005). In addition, EE has been shown to improve performance in the novel object recognition task, and this study showed that NR2B transgenic mice with enhanced NR2B function show much longer recognition memory when exposed to EE, and furthermore, they showed increased expression of NR1, NR2B, and NR2A subunits following EE exposure (Tang et al., 2001). This aligns with our study where we found significant effects of EE on NR2B subunits, however, we showed that this effect was specific to males, and these differential effects of EE on NMDA receptor subunits were contingent upon prior exposure to stress and BDNF genotype.

EE exposure for 4 weeks prior to behavioral testing has been shown to enhance spontaneous alternation in the Y-maze paradigm in mice (He et al., 2017) and rats (Jin et al., 2017). In addition, 6 weeks of EE treatment recovered Y-maze spatial memory preference for the novel arm in transient receptor potential channel (TRPC1)–/– mice (Xing et al., 2016). Mice exposed to chronic restraint stress for 4 weeks show impairments in spatial recognition memory in the Y-maze and here, simultaneously living in an enriched environment was able to ameliorate this deficit (Chen et al., 2010).

The above studies consistently show beneficial effects of EE on Y-maze performance, however, we found that EE alone appeared to negatively impact Y-maze novelty preference. This may be due to a number of important methodological considerations. Firstly, it has been shown that including a running wheel in the EE set up is critical particularly to the spatial memory-enhancing effects of EE (Lambert et al., 2005; Rogers et al., 2016). Our protocol did not include a running wheel. In addition, Zeleznikow-Johnston et al. (2017) also used an EE protocol that did not include a running wheel and while they found that EE enhanced visual discrimination and reversal learning, it had no effect on pattern separation or working memory in healthy mice. Thus, it appears that the beneficial effects of EE on hippocampal-dependent spatial memory tasks is heavily reliant upon physical activity as a component of EE. Secondly, the age at which EE is initiated is critical with most previous reports on the beneficial effects of EE initiating EE immediately post-weaning and continuing to maintain EE up to the time of behavioral testing (Simpson and Kelly, 2011). Our study began EE at 7 weeks of age and did not maintain EE until time of testing, thus the mice having experienced an enriched environment would have been in a state of deprivation prior to behavioral testing. Overall, in WT mice EE in our study appears to have functioned as another form of "stress," with the combined effect of EE and CORT being negatively additive. In contrast, a restorative effect of EE emerged only after CORT treatment in BDNF HET mice. This may represent a variation of the "inoculation hypothesis," which suggests that EE is a chronic mild stress, and as such creates a resilience to subsequent stressors (Crofton et al., 2015). It should be noted that in our study EE commenced after the introduction of CORT treatment, and mice were returned to standard housing prior to behavioral testing, thus EE followed by standard housing appeared to be another form of stress when compared to standard housing throughout the experimental timeline. Our results would suggest that this mechanism is particularly clear against a background of reduced BDNF levels. Two-hit animals (i.e., BDNF HET mice that received CORT treatment) may have been "inoculated" against EE, and as such EE was restorative to this group.

In the current study we found that Y-maze performance was impaired by chronic CORT treatment in both WT and BDNF HET male but not female mice, suggesting males are more

vulnerable to the effects of chronic corticosterone treatment. Female rats and mice are known to have higher levels of circulating corticosterone than males and a previous report in rats found that EE prevented a chronic stress-induced rise in corticosterone in females but not males, and showed a desensitization of the HPA axis to further exposure to an acute stress in female rats (Welberg et al., 2006). This may be due to interactions between female sex hormones such as estradiol and the HPA axis.

In our study, the close to significant genotype × CORT × EE interaction for mBDNF protein expression in the female dorsal hippocampus qualitatively follows the female pattern of Y-maze results, which had a significant genotype × CORT × EE interaction. This was sex-specific, as qualitatively the male Y-maze behavior did not align as closely to the measured mBDNF protein levels. Within the literature there is a broad range of results regarding EE and mBDNF levels. Most likely caused by the wide variety of protocols, studies have shown EE to both increase mBDNF (Cao et al., 2017) and to have no impact (Rogers et al., 2016). The diversity in approach is exemplified with two studies; Cao et al. exposed their mice to EE for 8 weeks, changing the cage every 4 days (Cao et al., 2017), while Rogers et al. (2016) had a similar set up to our study, with 4 weeks of EE and changing the cage weekly. It may be that the frequency of novelty and duration of exposure to EE are major considerations for effects on mBDNF protein expression. The current study found that the water/EE condition did not increase BDNF protein expression, which contrasts with other studies that show EE to increase BDNF (Chourbaji et al., 2012). BDNF is secreted in an activity-dependent manner (Hashimoto et al., 2000). However, in our study at the time-point the brains were collected, the mice were no longer exposed to EE. It could be that EE altered BDNF levels while it was on-going but after EE was stopped BDNF levels returned to pre-EE levels in response to the return to standard housing. In addition, sex is an obvious modifier of BDNF levels and, similar to our findings, a previous report in rats showed that female rats have higher levels of hippocampal BDNF and the effect of EE on increasing hippocampal BDNF levels was greater in females compared to males (Bakos et al., 2009).

In both sexes, both CORT and EE altered the expression of the phosphorylated Y816 TrkB residue. While CORT increased the Y816 ratio in both sexes, EE had a sexually divergent effect whereby it increased the Y816 ratio in males and decreased in females. A recent paper by Bengoetxea et al. (2018) found that only 1 week of EE in male rats increased the expression of TrkB, and improved performance in the Morris Water Maze. Comparatively in our study, when comparing both male genotypes' Y816 ratio to the respective Y-maze data, it would appear that the Y816 ratio shows the inverse trend compared to the Y-maze. This is indicating that while this residue has increased activation, this is not reflective of improved Y-maze performance. However, it should be noted that hippocampal lysates were analyzed 5 weeks following behavioral testing and thus do not reflect TrkB activation at the time of behavioral testing. A similar phenomenon is observed in the females, however it was more genotype specific to the female two-hit mice. Again, while the Y816 ratio is increased, the behavior did not reflect this, perhaps indicating that despite long-term increased activation of the residue this did not result in improved spatial memory performance.

An interesting divergence emerges in the male Y515 ratio results. The recorded genotype × CORT interaction demonstrated that CORT treatment increased Y515 ratio in WT but decreased it in the two hit animals. However, despite this divergence at the molecular level, possibly because of statistical power issues, the analysis did not show a CORT × genotype interaction for spatial memory performance, although this group clearly showed the lowest DI. Because both Y515 and Y816 contribute to the ERK1/2 signaling pathway, it is possible that both are needed to be upregulated to improve behavior.

It has been reported that chronic glucocorticoid treatment disrupts the interaction between glucocorticoid receptors and TrkB with subsequent dampening of the PLCγ signaling pathway (Numakawa et al., 2013), with implications for cognition. Yan et al. (2016) recently demonstrated that chronic CORT treatment decreased pTrkB, and Barfield and Gourley (2017) showed truncated TrkB, the inactive form, to be increased in relation to FL-TrkB after chronic adolescent CORT treatment. Our study adds to this literature by investigating the activation of the distinct residues, and the results discussed above contribute to creating a comprehensive molecular map of how glucocorticoids and the BDNF-TrkB signaling pathway dynamically interact.

Of the three NMDA receptor subunits investigated, the most interesting changes occurred on GluN2B in the males. CORT increased GluN2B protein expression but despite the role of NMDAR in cognition, Y-maze performance was impaired. It could be that this increase of GluN2B is excitotoxic and this is a possible mechanism through which CORT impairs spatial cognition in male mice. This is consistent with previous research by our lab (Klug et al., 2012), which found that CORT treatment increased NR2B protein levels in male BDNF HET mice and paralleled impaired spatial memory as measured by the Y-maze (Klug et al., 2012).

This study was specifically interested in EE as a preventative treatment during adolescence, however a study design limitation is the lack of ability to compare this treatment window with longer-term EE treatment that continues into behavioral testing and up until the point of brain collection. Another limitation of this study is that we present here findings from only one short-term spatial memory task. Future studies should include other spatial memory tasks as well as address other cognitive domains, such as working memory and recognition memory. Overall our study shows that the effects of EE on spatial memory are heavily dependent on BDNF genotype and prior exposure to stress, with our results showing benefit only in two-hit (BDNF HET × CORT) mice. In addition, we show an increased vulnerability of males to chronic CORT and EE and this coincides with male-specific alterations to phosphorylated TrkB residues and NMDA receptor subtypes following both CORT and EE exposure. Female spatial memory performance, however, aligned with mBDNF expression levels in the dorsal hippocampus. These results show that the timing and nature of EE, prior exposure to a model

of stress, BDNF genotype, and sex are all critical modifiers of EE-induced spatial memory and molecular outcomes.

AUTHOR CONTRIBUTIONS

AG performed all Western blot analysis and data analysis and wrote the first draft of the paper. UR performed the behavioral testing and its analysis. AH advised on the original project and on data analysis and interpretation. MvdB designed the project, oversaw behavioral testing, performed data analysis, and edited the manuscript. RH co-designed the project, oversaw Western blot analysis, performed data analysis, and edited the manuscript.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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Nesting Environment Provides Sex-Specific Neuroprotection in a Rat Model of Neonatal Hypoxic-Ischemic Injury

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Hypoxic-ischemic (HI) encephalopathy is a devastating injury that occurs when the fetal brain is deprived of oxygen and blood to a degree that may lead to neurological damage, seizing and cerebral palsy. In rodents, early environmental enrichment that promotes maternal care-taking behavior (mCTB) can improve neurobehavioral outcomes and protect against neurological decline. We hypothesized that an enhanced nesting environment would improve mCTB as measured by pup weight gain, and support greater HI recovery in developing rats. Pregnant dams (E15-16) were introduced to either control Standard Facility (SF) housing or closed nestbox (CN) conditions and maintained in larger cages through pup weaning. On postnatal day (PND) 7, male and female Long-Evans rat pups (*N* = 73) were randomly sorted into one of two surgical conditions: control and HI. HI pups received isoflurane anesthesia and right carotid artery ligation, a 2-h rest followed by 90 min exposure to a moist hypoxic (92% N, 8% O2) chamber. Pups (PND 8) were weighed daily, and tested on the Morris Water Maze (MWM) task (PND 35-50). Results demonstrate significant differences afforded to male and female pups based on weight measure, where CN-rearing modifies pre-weaning adolescent weights in females and increases post-weaning weights in males and females by an average of 10 g. Following successful MWM training and acquisition (PND 35-37), both male and female CN-raised animals demonstrated faster latency to find the hidden platform (HP) during HP trials (PND 38-42) and appeared to freely explore the MWM pool during an additional probe trial (PND 43). Moreover, after sacrifice (PND 60), CN rearing created sex-specific alterations in brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF) immunopositive cell staining of the dorsomedial striatum and CA1 of the hippocampus. CN-rearing afforded HI males higher BDNF levels in the striatum and produced greater GDNF levels in the hippocampus of HI-injured females. These results suggest that early life environmental enrichment positively modifies nesting environment, increases weight gain, as well as spatial learning and memory in a sex-specific directionality. Our findings also implicate correlative changes in corticolimbic neurotrophin protein levels in the CN-reared animals that may contribute to these benefits.

Keywords: Rice-Vanucci P7 HI model, Long Evans rats, environmental enrichment, Morris water maze, neurogenesis, neonatal hypoxic ischemic injury, hippocampus

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INTRODUCTION

Neonatal Hypoxic-ischemic encephalopathy (HIE) is a serious neurological injury resulting from oxygen and glucose deprivation during birth. Current estimates predict that 1–2 of every 1,000 full term neonates develop HIE (Volpe, 2009). Surviving infants may go on to develop long-term disabilities, including reoccurring seizures (Silverstein and Jensen, 2007; Björkman et al., 2010), motor impairments (Martinez-Biarge et al., 2011) and attentional and learning deficits (Lou, 1996; Perez et al., 2013).

Cognitive deficits are a hallmark of neonatal HIE, with some studies estimating 70% of adolescent HIE survivors demonstrate some degree of cognitive difficulty and 50% require special educational services (Lindström et al., 2006). Using the Rice-Vannucci method (Vannucci et al., 1999), the most prevalent rodent model of neonatal HIE, the injury is modeled in rats at postnatal day (PND) 7 by ligation of the right common carotid artery followed by oxygen deprivation. This model simulates both morphometric and functional sequalae of term neonatal hypoxic ischemic (HI) injury, including spatial and working memory deficits (Smith et al., 1991; Balduini et al., 2000; de Paula et al., 2009). The bilateral hippocampus and frontocorticolimbic system are highly vulnerable to HI injury, with studies suggesting that these areas are damaged in an estimated 90% of HI models and infarcted in another 56% of subjects (Rice et al., 1981). As a result of HI, early cell death occurs in the neocortex, and a second wave of cell death occurs from 12 h to 24 h after oxygen deprivation, targeting subcortical structures including the hippocampus, striatum and thalamus (Azzarelli et al., 1996; Northington et al., 2007).

Early environmental enrichment with large cages, social peers and sensorimotor stimulation has been shown to somewhat improve the functional memory outcomes of adolescent but not adult female rats with neonatal HI injury, without modifying the hippocampal or striatal damage (Pereira et al., 2008). Maternal care-taking behavior (mCTB) provided by rat dams in the first 2 weeks of life has also been shown to alter the stress response of offspring through direct modifications of the hypothalamic-pituitary-adrenal axis (Meaney, 2001) and neurotrophin levels in the central amygdala (Berman et al., 2014). During the pre-weaning period, greater licking and grooming, and arched back nursing result in the regulation of emotional as well as neural systems (Caldji et al., 1998; Roth and Sweatt, 2011). Pre-weaning environmental enrichment has been successfully applied to promote typical development in animal models (Koo et al., 2003; Szabadfi et al., 2009) and to improve learning and memory through alterations in hippocampal proteins and synapse formation (Venable et al., 1989; Bredy et al., 2003).

Early handling (Chou et al., 2001) and environmental enrichment during early life (Pereira et al., 2008) are neuroprotective in HI injured animals working via morphological changes to dendritic spine density and synaptic branching (Rojas et al., 2013; Zhao et al., 2013). In addition, this neuroprotection involves changes in the expression of neurotrophins like brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF; Lin et al., 1993; Skaper, 2012). Treatment with exogenous BDNF reduces HI-induced spatial deficits in rats trained on a Morris Water Maze (MWM) task (Almli et al., 2000). GDNF is a neurotrophin present in dopaminergic neurons that has been characterized as a marker of neuronal survival (Wang et al., 2004; Bakshi et al., 2006). Collectively, BDNF and GDNF are thought to act as endogenous neuroprotective agents (Kiprianova et al., 1999; Allen et al., 2013; Chen et al., 2013). The outcome of children who have experienced a brain injury has been correlated to levels of these two proteins, such that decreases in BDNF and GDNF indicate greater working memory deficits (Chiaretti et al., 2003). These findings indicate that environmental interventions may be a potential avenue for prevention or treatment of the dramatic cognitive deficits that often follow HI. Environmental enrichment has been shown to benefit individuals suffering with neurodegenerative diseases such as Huntington's, Parkinson's, or Alzheimer's diseases, through the utilization of the brain's plastic nature (for review, see Laviola et al., 2008). Although environmental enrichment has been well studied in terms of neural plasticity and recovery in many models of neural injury and disease there are few studies that investigate the effects of the early environment on developmental trajectory after HI, and fewer still that seek to understand the interplay between the quality of early environment and cognitive sequelae.

This study was designed to address the complexity of functional outcomes, while simultaneously examining potential proteomic changes as they reflect differences in recovery. We introduced rat dams to a closed nestbox (CN) condition as a protected environment as compared to the standard facility (SF) conditions, hypothesizing that the sheltered environment would provide positive enrichment for dams and pups during the pre-weaning period (Mychasiuk et al., 2012). We further hypothesized that the CN environment would improve phenotypic and proteomic features of HI in male and female pups in a sex-dependent manner. This potential benefit was theorized based on several sex-specific effects observed in environmental enrichment, pup weight gain and HI injury. The pattern of damage following HIE is thought to be different between males and females, with females displaying significantly less injury than males in physical and cognitive domains (Zhu et al., 2006; Hill and Fitch, 2012; Smith et al., 2014). Sex differences may also determine the effectiveness of treatment for HIE, to which end pre-weaning environmental modification may be useful (Fan et al., 2011; Nie et al., 2016).

MATERIALS AND METHODS

Animals

All procedures utilized in this experiment were approved by the University of Massachusetts Boston Institutional Animal Care and Use Committee and closely followed applicable portions of the Animal Welfare Act and the U.S. Department of Health and Human Services' "Guide for the Care and Use of Laboratory Animals." Pregnant Long Evans rats (N = 7; embryonic day 10)

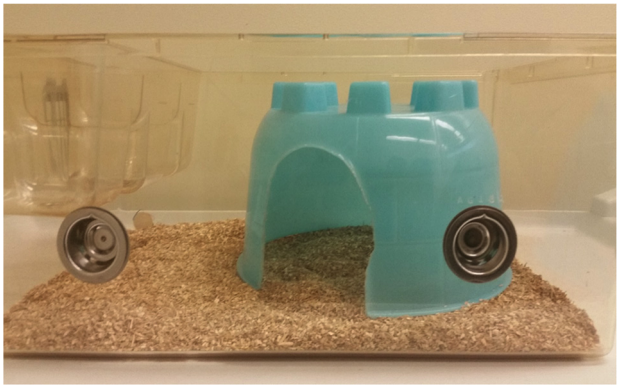

FIGURE 1 | Picture of Closed Nestbox (CN) inside standard cage with bedding. CN was added to the dam's cage at embryonic day (ED) 10 (E10-15) and remained until weaning on postnatal day (PND) 21.

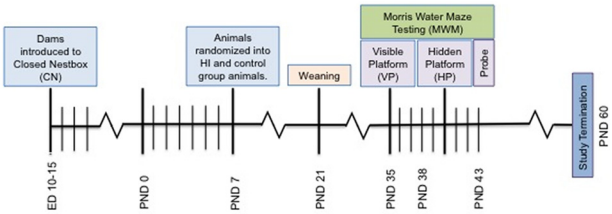

FIGURE 2 | Depiction of study timeline indicating the introduction of the CN enrichment at embryonic day 10–15, surgery on PND 7, Morris Water Maze (MWM) testing and study termination.

were purchased from Charles River (Wilmington, VA, USA) and upon arrival, were singly housed and randomly assigned to SF or CN (**Figure 1**) conditions in the animal vivarium in a light- and temperature-controlled environment at 22°C with lights on at 07:00 h and off at 19:00 h. Dams were kept in a Plexiglas cage with dimensions 31.75 cm × 41.7 cm × 17.8 cm. CN conditions were similar, but also included a small (7.75″L × 6″W × 4.5″H), opaque plastic shelter that contained one entrance placed in the center of the Plexiglas cage. Plastic shelters were inserted in the nesting environment of pregnant dams on embryonic day 10 and left undisturbed until time of delivery. After the dams gave birth, litters were sexed and humanely culled to 10–12 pups with even distribution of males and females in order to limit dramatic variations in maternal care (Champagne et al., 2003). Litters were also relatively stable in male to female ratio, never exceeding more than 1:3 females to males. Individual pups within litters were excluded from the research paradigm if they failed to reach a weight standard of 11 g at PND7 during weight check-in during randomization. Ten litters with a total of 73 pups were randomized within litters into one of two surgical conditions: control (n = 17 females, n = 18 males) and hypoxia ischemia (n = 19 females, n = 19 males; **Table 1**). The study timeline is depicted in **Figure 2**.

Surgery

On PND 7, male and female pups in each housing condition were randomly sorted within litters into one of two surgical conditions: HI and control groups. Animals in the HI condition underwent carotid artery ligation and hypoxia according to the Levine model of HIE, using the Rice-Vannucci modification (Rice et al., 1981). Pups were anesthetized with 3%–5% isofluorane and maintained on 1%–2% for the duration of the surgical procedure. We performed double ligation and severed the right common carotid artery followed by a 2 h rest period with the dam. HI pups were then removed from the litter and placed into a plastic container and supplied exclusively with 8% Oxygen and 92% Nitrogen for 90 min and then returned to their dams. Within this chamber, temperature was kept at a steady 36°C to mimic normative nesting conditions (Mortola and Dotta, 1992; Cameron et al., 2000). Animals in the control group remained in their home cage and were only removed from their dams to be marked for identification. In the current study, the mortality rate following right carotid artery ligation and post-operative 90-min hypoxia was 5% (two out of 40 total animals; one male and one female). This mortality rate is well within reported standards following HI induction in animals (Nakajima et al., 2000; Demers et al., 2005; Pereira et al., 2008). On PND 21, animals were weaned and housed by sex with littermates, with 2–3 animals per cage.

TABLE 1 | Total number of subjects, separated by sex and housing condition.

Subject Grouping	Standard Facility		Closed Nestbox (CN)	
	Male	Female	Male	Female
Control	n = 11	n = 11	n = 7	n = 6
Hypoxia-Ischemia	n = 7	n = 9	n = 12	n = 10
Total	18	20	19	16

Body Weight

Animals were weighed daily (grams) from PND 8–21 and every 3–4 days from PND 22–60. From PND 8–14, animals were weighed on a small plastic Sterilin weighboat (ThermoFischer, Cambridge, MA, USA), as appropriate for their size and physiological condition. Upon their maturation as denoted by the full appearance of fur at PND 15, animals were weighed in a closed-lid metal weight cage zeroed on an electronic scale (Basic Electronic Scale, Mettler Toledo International, Inc., Billerica, MA, USA).

Morris Water Maze—Visible Platform

All testing for the MWM trials took place within a pool (120.65 cm in diameter, 176.25 cm in height at its deepest point) filled with water heated to room temperature (±25–27°C) and colored with non-toxic milk powder. Around the pool, key shapes were pasted for internal orientation to the surrounding directions of North (N), West (W), East (E) and South (S) as reference cues for the rats.

PND 35 was the first day of testing, selected based on previous research suggesting that rats are able to perform with mature cognition by PND 30 (Ikeda et al., 2001; Arteni et al., 2003). On the initial test day, animals were placed on the

fixed, visible platform (VP) that was marked by bright green tape in the SE corner of the pool for 15 s, and were allowed to swim freely for up to 20 s Rats were tested in four trials each day from PND 35–42 and were given up to 60 s to find the VP on each trial. If rats did not find the platform within 60 s they were guided to the platform and were left on the platform for 10 s to familiarize them with its location. Each rat was dried and warmed between each trial and given a 10 min inter-trial interval throughout the four-trial testing period. The latency to reach the platform was recorded from trial to trial. Tests were conducted in a low light setting, with the experimenter standing at a distance out of view of the animals. Ethovision XT11 software and a digital camera (Microsoft Webcam, 12.0; Microsoft, Redmond, Washington, USA) were used to video record information for 78 of the 104 subjects.

For VP training, rats were tested from PND 35–38, and were dropped off from the South, North, East and West for each of the 3 days. The platform remained fixed in the SE corner. For the hidden platform (HP) trials, the platform was made translucent and remained fixed in the SW corner of the pool 0.5 inches under the surface of the opaque water. Each day, animals were placed in the pool in the directions of N, N, NW and NW for a total of four trials. Animals were tested in HP from PND38–42.

After completing VP and HP testing, we conducted probe trials (PND43) and captured video information for these trials with a subset of animals. Probe trials consisted of a 60 s swimming period for each animal, without a platform. For 60 s animals were allowed to freely swim around the pool and swimming behavior was coded for proximity to the most recent location of the platform.

Brain Morphology and Immunofluorescence

Animals were sacrificed at PND60 with the aid of a restraint cone (Harvard Apparatus, Holliston, MA, USA) and live decapitation. Brains were extracted and snap frozen with 2-methylbutane chilled on dry ice, and then stored at −80°C until the time of post-fixation and cryoprotection (increasing 5%–20% sucrose + 4% paraformaldehyde).

Following this, brain tissues were microsectioned at 20–25 μm (Leica CM 3050S; Leica Biosystems, Buffalo Grove, IL, USA) taken at Bregma 4.70–5.60 mm for the striatum (Paxinos and Watson, 2004) and Bregma −3.80 to −04.16 mm for the hippocampus thaw-mounted and placed directly on adhesive slides (SuperFrost Plus; ThermFischer, Cambridge, MA, USA). Mircosections were randomly divided into Nissl, control and positive immunofluorescent groups to include 5–6 animals per treatment and housing group. For Nissl stains, first we removed the fat (95% EtOH, 15 min) then rehydrated (70% and 50% EtOH, 5 min each). Next we ran the sections through dH_2O washes (1–2 min) followed by immersion in Cresyl Violet (Sigma, 0.1% (2–4 min); this was again followed by dH_2O wash (1 min). After, we returned to dehydration steps (50% EtOH (1 min), 75% acid EtOH, 95% and 100% EtOH (2 min) and the clearing agent, Histoclear (National Diagnostics, Atlanta, GA, USA). In models of HI injury, necrosis and apoptosis (Northington et al., 2007) are assessed close in time to the injury (see also Cai et al., 2008). Given that we sacrificed the animals ∼PND60, nearly 2 months after the HI injury, we instead think that information about the size of the infarct and relative damage to the hippocampus is important for the data presented in this manuscript (Ikeda et al., 2001).

After, sections were fixed with 4% paraformaldehyde for 30 min at a time and, repeatedly rinsed with phosphate buffered saline (PBS) for 10 min per wash. After five washes, slides were soaked in bovine serum albumin for 30 min, and then were covered with the primary antibodies BDNF (1:1,000; ThermoFischer, Cambridge, MA, USA) and GDNF (1:500, ThermoFischer, Cambridge, MA, USA) overnight. The next day, slides were recovered from the humidity chamber and washed with PBS again for 10 min per wash. Following this, slides were coated with two fluorophore conjugated secondary antibodies for approximately 1 h: goat anti-rabbit AlexaFluor 488 (IgG H&L) and goat anti-mouse AlexaFluor 647 (IgG H&L) at a concentration of 1:600 (Abcam, Cambridge, MA, USA and ThermoFischer, Cambridge, MA, USA). Excess antibody was washed with PBS three times, with 10 min per wash. Fluorescence was enhanced with anti-fade reagent (Prolong anti-fade reagent with 6-diamidino-2-phenylindole (DAPI); ThermoFischer, Cambridge, MA, USA), and slides were coverslipped. To preserve apparent fluorophores, slides were coated with clear nail polish around the edges to prevent oxidation. Slides were imaged within the week, with up to six images taken for each bilateral brain area of interest. Fluorescent images were converted from native high resolution .CZI to .JPEG format and dropped in ImageJ (National Institutes of Health, online). A 500 × 500 pixel yellow-colored region of interest (ROI; 2.23 μm per pixel) was manually drawn on the center of each image, and positional consistency was maintained through the use of an ImageJ placement macro (six images per subject, two merges in total overlap; 12 images if images were found to be ambiguous).

ImageJ was used to set color thresholds to maintain consistency for each fluorophore type and were subsequently processed using ImageJ to avoid experimenter bias and to increase objectivity. Initial GDNF counts proved inconsistent and variable across groups, measurement was altered to suit the diversity of the stain-type. Measurement analysis of GDNF fluorescent intensity level was restricted to a 500 × 500 pixel (2.23 μm per pixel) square. Circularity and overall size of GDNF-tagged cells were relatively unrestricted, as the main factor analyzed was mean brightness of areas contained within centered ROIs.

Data Analysis

All data were coded and interpreted with the use of SPSS 22.0 and 23.0 (Windows 10 and Mac 6.1 compatible versions). Body weight was analyzed using a Generalized Linear Mixed Model (GLMM) with repeated measures, with Housing and Surgical conditions. Each analysis was done separately for males and females. MWM trials were analyzed using GLMM with repeated measures. The latency to reach the platform was assessed according to Housing Condition (SF or CN) and Surgical

Condition (Control or HI) across the four trials of each testing day. Each analysis was done separately for males and females.

For the probe trial, collected data were analyzed with the use of the Ethovision XT11 (Leesburg, VA, United States). The MWM area was divided into four quadrants based on location of the symbols placed around the pool (NW, NE, SW, SE) and time spent in each quadrant was analyzed by the tracking system in sec. Videos were randomly coded for each subject and then run through data analysis for the variables of Movement (gauging the swimming style of the animal from its center) and Speed (how quickly an animal moved throughout the pool) as controls based on possible physical deficits between groups (Bona et al., 1997). Swimming speed was determined as also given by the independent variables of Housing Condition, Surgical Condition. The significance was set at probability of 0.05 or less. Confidence intervals were set at 95%.

A two-way between-subjects ANOVA was performed for the factors of Surgical condition (Control or Hypoxia-Ischemia) and housing condition (SF or CN) to compare means in BDNF and DAPI levels, as well as modifications to average GDNF levels based on fluorescent intensity following fluorescent microscopy in the dorsomedial striatum and CA1 of the hippocampus of subjects. All data was tested for normality. For two of the dependent measures, striatal counts of BDNF in males and striatal DAPI levels in females, homogeneity of collected data was not met under Levine's Test. While this was not complete unexpected due to the possible variability in targeted tissue, data were adjusted to reflect normal distributions through the exclusion of BDNF striatal counts in two male subjects and through the exclusion of DAPI striatal counts in one female subject.

RESULTS

Brain Morphology

Due to the severity of the injury sustained in the SH group, there was not enough intact tissue from this group to yield data from measurements of cortical or hippocampal area that could be statistically compared in a meaningful way. However, the lack of tissue is in itself an important finding that distinguishes animals from the two housing conditions. Therefore, the results from the measurements of hippocampal area will be reported in descriptive terms.

Observations of right and left hippocampal areas revealed that all CN animals retained tissue in both right and left hippocampal areas, whereas there were no SF animals which had a visible right hippocampus (ipsilateral to the injury). For SF animals, there were either large infarcts where the right hippocampus would be in un-injured animals, or the hippocampal area was not present in tissue that was intact. Females in the SF group had some visible left hippocampus tissue (contralateral to the injury), whereas SF males did not have any visible hippocampal tissue in the left hemisphere. In CN animals, all animals had both right and left hippocampi, although each displayed a disparity between right and left hippocampi, with the right measuring smaller than the left. Measurements of the cortical area of the right and left hemispheres showed some asymmetry between hemispheres, with the right (ipsilateral) hemisphere measuring smaller than the left (contralateral) hemisphere as expected. However, the degree of disparity appeared to differ greatly between groups. There was a dramatic disparity between right and left hemispheres for both males and females in the SF group, but comparatively little disparity in CN animals (**Figure 3**). This indicates that on average, regardless of sex, CN animals had less damage from infarct or atrophy in the ipsilateral hemisphere than SF animals.

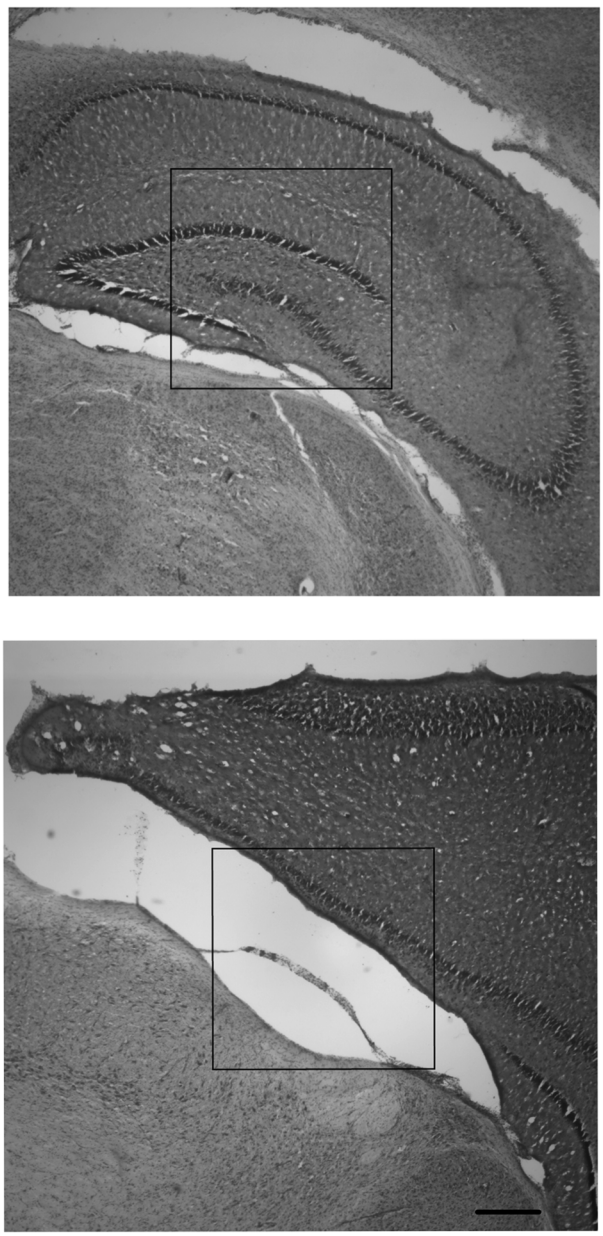

FIGURE 3 | Nissl-stained coronal hippocampal sections (ipsilateral to injury) from representative males exposed to Hypoxic-ischemic (HI) injury and reared in CN (top panel) and Standard Facility (SF; bottom). CN rearing provided enduring neuroprotection in late adolescence following neonatal HI insult. Scale bar = 200 μm for all images (bottom right); 20× magnification.

Body Weight

Pre-weaning weight measurements were collected daily from PND 8 to PND 21 and adolescent weights were collected every 3–4 days from PND 24–49. There were no significant differences between surgical groups or housing conditions for males during this pre-weaning period. However, pre-weaning female weights were significantly different between surgical conditions, with HI animals weighing significantly less than control animals ($F_{(1,34.02)} = 5.80, p < 0.05$). Analysis of adolescent weights yielded significant differences between housing conditions for males and females in the HI condition ($F_{(1,32.00)} = 6.25, p < 0.01$), with CN animals weighing an average of 10 grams more than SF animals. There were no other significant differences between conditions.

Morris Water Maze Performance

Visible Platform Training

VP training allows animals to learn the water maze testing procedure, and also measures spatial memory acquisition. The latency for animals to reach the platform decreased over the 3 days of VP testing indicating successful memory acquisition across study conditions for males ($F_{(2,462)} = 103.20, p < 0.05$), and females ($F_{(2,3478)} = 71.30, p < 0.05$). For females, there were significant differences between surgical conditions in latency to reach the platform regardless of housing condition ($F_{(1,29)} = 8.02$, $p < 0.05$), with control animals performing significantly better than HI animals. Unexpectedly, there was no significant effect of housing condition for males or females on latency to reach the platform independent of other study factors, and there were no significant interaction effects between study variables (**Figure 4**).

Hidden Platform Testing

HP testing with a stationary platform location, requires animals to develop memory strategies using spatial cues without the visual cue of the platform. A decrease in latency across days indicates functional long-term memory. There was a significant decrease in latency by day indicating functional long-term memory across treatment and surgical conditions for males ($F_{(4,800)} = 8.57, p < 0.05$), and females ($F_{(4,522)} = 6.08, p < 0.05$). For males, there was a significant effect of surgical condition on time to locate the platform, with HI animals performing significantly worse than control animals ($F_{(1,800)} = 11.35$ $p < 0.001$). There were no significant differences for males according to housing condition, or significant interaction effects between study factors. Females demonstrated a significant interaction effect between surgical condition and housing condition ($F_{(1,522)} = 9.37$ $p < 0.001$), with HI SF females performing significantly worse (12.04 s compared to HI CN (7.58 s), Control CN (6.06 s) and Control SF (6.00 s; **Figure 5**).

Probe Trial

Probe trials are conducted without a platform after VP training. These trials serve as control conditions to determine swimming speed and distance traveled as measures of locomotor ability, separate from memory function. Male animals displayed no significant differences between surgical condition or housing

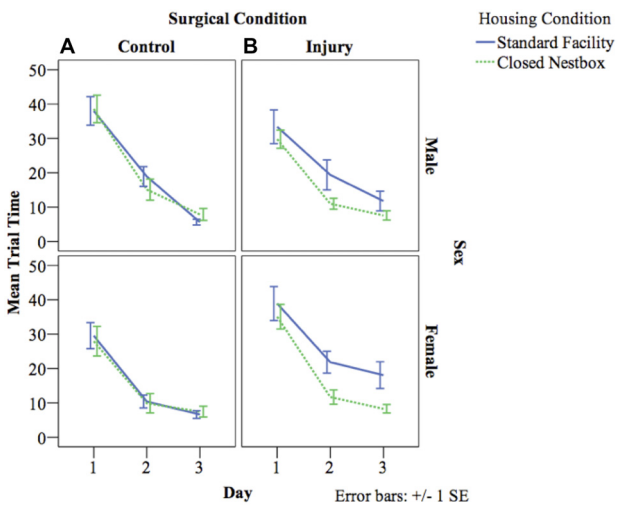

FIGURE 4 | (A,B) Mean latency by study condition in visible platform (VP) training. The following graphs mark the average latency of animals to reach the platform over the course of the four water maze trials each day for three consecutive days. The lines compare average latency for animals in the CN and SF housing conditions according to surgical condition: **(A)** Control, **(B)** HI Injury. Only main effects were observed for latency for males ($F_{(2)} = 103.20$, $p < 0.05$), and females ($F_{(2)} = 71.30$, $p < 0.05$) with both demonstrating decreased latency across the three training days indicating successful task acquisition.

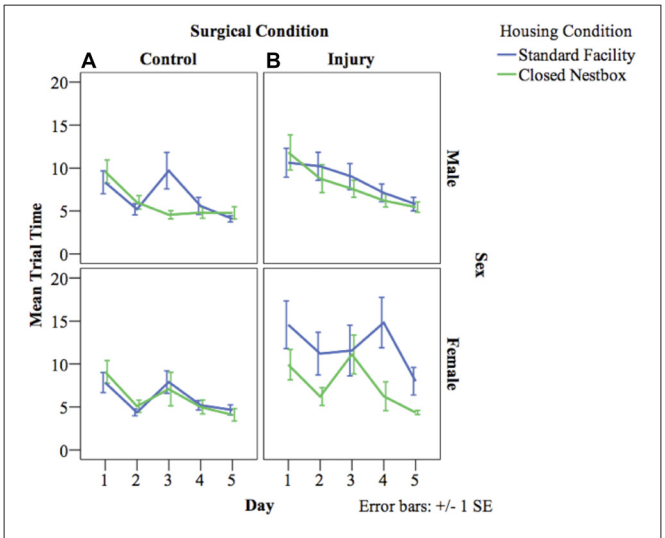

FIGURE 5 | (A,B) Mean latency by surgical and housing condition in hidden platform (HP) testing for males and females. The following graphs mark the average latency of animals to reach the HP over the course of the four trials each day for five consecutive days. The lines compare average latency for animals in the CN and SF housing conditions according to surgical condition: **(A)** Control, **(B)** HI Injury. There were significant differences between surgical conditions regardless of housing condition for males ($F_{(1)} = 11.35$ $p < 0.001$; HI males relative to Control males). Females demonstrated a significant interaction effect between surgical condition and housing condition ($F_{(1)} = 9.37$ $p < 0.001$; HI SF as compared to all other groups, same sex), with HI SF females performing significantly worse than all other groups.

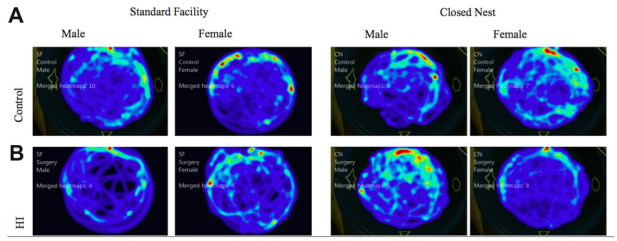

FIGURE 6 | (A,B) Probe trial: average swim path by surgery condition and housing for males and females. The following heat maps mark the average swim path during the probe trial. The markings represent aggregate data from CN and SF housing conditions for each sex according to surgical condition: **(A)** Control, **(B)** HI Injury. The maps illustrate that HI SF animals had significantly longer swim paths than either HI CN, Control SF, or Control CN animals ($F_{(1)} = 7.115$, $p < 0.01$). For swim speed velocity, there was no difference between surgical conditions, but there was a significant effect of housing condition, with SF females demonstrating a faster swim speed than CN females ($F_{(1)} = 7.05$, $p < 0.01$).

condition in either distance traveled or velocity of movement. Female animals however, displayed a significant interaction effect of housing condition and surgical condition, with HI SF animals demonstrating significantly longer swim paths than either HI CN, Control SF, or Control CN animals ($F_{(1)} = 7.115$, $p < 0.01$). For swim speed velocity, there was no difference between surgical conditions, but there was a significant effect of housing condition, with SF females demonstrating a faster swim speed than CN females ($F_{(1)} = 7.05$, $p < 0.01$; **Figure 6**). Male and female HI animals in both housing conditions demonstrated comparable swimming speed velocity and distance traveled in relation to intact animals, indicating that differences in latencies can be attributed to differential learning acquisition, search strategy, working memory and visuospatial memory ability; rather than differences in motor ability due to injury. We also have preliminary data that indicate HI injured pups show sex differences in a rope suspension task with HI-injured females reared in CN holding on to the rope longer (unpublished data). See **Figure 6** for representations of aggregate swim paths.

Expression of DAPI, and Neurotrophins BDNF and GDNF

In males, HI injury resulted in significantly lower average BDNF levels in both the hippocampus ($F_{(1,14)} = 14.672$, $p < 0.01$; **Figure 7**) and striatum ($F_{(1,12)} = 40.053$, $p < 0.001$; **Figure 7**) in comparison to control males, regardless of housing condition. We observed partial eta squared of 0.488 and 0.769, with 0.873 and 1.0 power, respectively. CN rearing increased average BDNF counts in the striatum for male subjects compared to SF males ($F_{(1,12)} = 7.232$, $p < 0.05$); here, we observed partial eta squared of 0.376, with 0.695 power. There was no interaction effect between housing condition and surgical condition on BDNF counts in males.

Females with HI did not display a lower hippocampal BDNF levels than control females ($F_{(1,13)} = 0.068$, $p > 0.05$, *NS*), however, there was a trend towards higher striatal BDNF counts for control females ($F_{(1,13)} = 4.315$, $p = 0.058$). Rearing in the CN

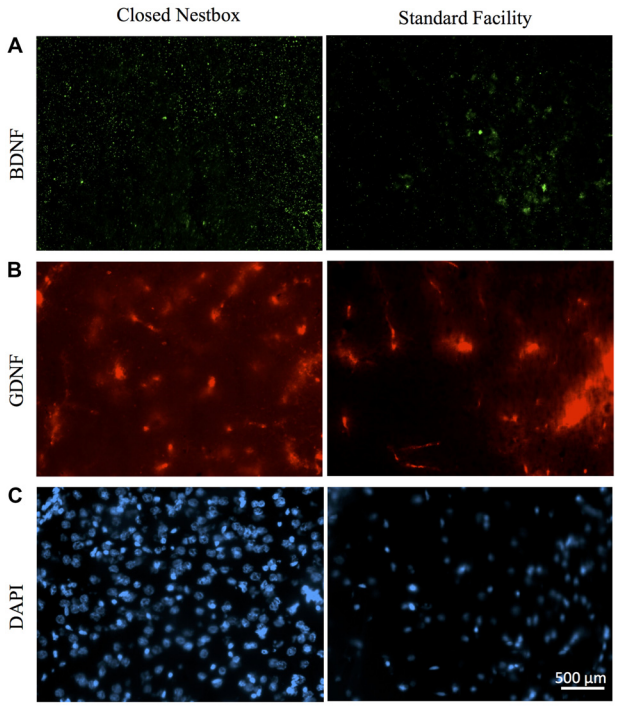

FIGURE 7 | Immunoflourescent staining for neurotrophic factors and DAPI vary by surgical and housing conditions in males. **(A)** Ipsilateral hippocampal CA3 staining for BDNF in HI-injured males housed in CN (left panel) and SF (right panel). **(B)** GDNF-positive cells in males that suffered HI insult and were reared in CN (left panel) and SF (right panel). **(C)** DAPI-stained cells of male rats following HI insult and CN (left panel) and SF (right panel) pre-weaning environments. Scale bar in bottom right image = 500 µm, 20× magnification for all images.

condition resulted in higher hippocampal BDNF compared to SF females ($F_{(1,13)} = 4.883$, $p < 0.05$). There was no significant interaction effect of surgical condition and housing condition ($F_{(1,13)} = 3.588$, $p > 0.05$, *NS*). However, there was an observable trend for HI females in the CN condition to exhibit hippocampal BDNF levels similar to that of control females. HI females in the SF conditions possessed lower BDNF hippocampal counts than all other groups (**Figure 8**).

The DNA-binding stain, DAPI (4′,6-diamidino-2-phenylindole) was used in order to both verify fluorescent staining but also to evaluate cell populations within each brain ROI. Interaction effects of surgical condition and housing condition indicated that the number of hippocampal cells present were significantly greater in control animals reared in SF conditions verses all other groups ($F_{(1,14)} = 18.938$, $p < 0.01$). Control males reared in CN conditions possessed lower levels of DAPI-stained cell bodies in the hippocampus as compared to all other groups, including all HI males. No significant differences were observed for males in striatal DAPI levels according to surgical condition ($F_{(1,14)} = 2.650$, $p > 0.05$, *NS*) or housing condition ($F_{(1,14)} = 0.327$, $p < 0.05$; **Figure 9**).

For female animals, two-way ANOVA revealed a significant difference between HI and control females ($F_{(1,12)} = 5.211$, $p < 0.05$), with control females exhibiting significantly greater

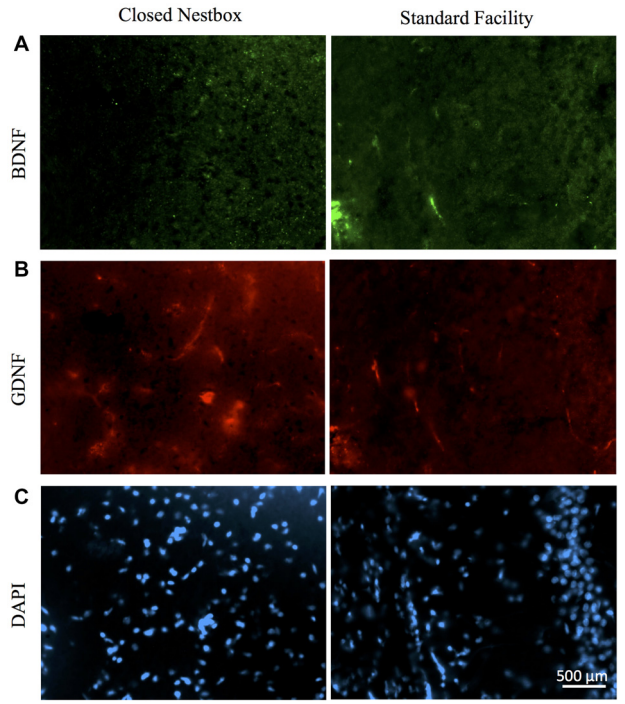

FIGURE 8 | HI injury differentially affects neurotrophic factors BDNF, GDNF and DAPI in the hippocampus of male rats reared in CN and SF conditions. **(A)** BDNF-positive staining in CA3 region of hippocampi of male rats after HI injury and CN (left panel) and SF (right panel) rearing. **(B)** Anti-GDNF positive cells in the hippocampus of HI-injured male rats from CN (left panel) and SF (right panel) housing. **(C)** Staining of DAPI cells in HI males from CN (left panel) and SF (right panel) environments. Scale bar = 500 µm, 20× magnification for all images.

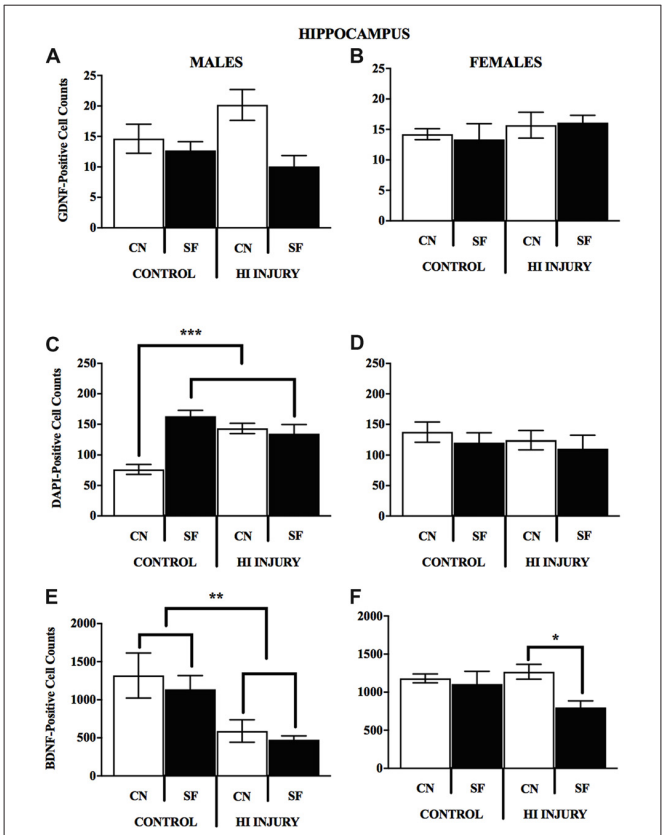

FIGURE 9 | (A–F) Immunopositive cell counts for GDNF, DAPI and BDNF in hippocampus by surgery and housing conditions for both sexes. Bar graphs show average ±SEM counts for **(A–B)** GDNF-positive cells, **(C–D)** DAPI-positive cells and **(E–F)** BDNF-positive cells in the hippocampus. No significant main effects or interactions were observed for GDNF. For DAPI, male control rats reared in CN had fewer immunopositve cells relative to all other groups, $^{***}p < 0.001$. HI injury males reared in SF had fewer cells relative to Control males reared in the same condition, $^{*}p < 0.05$, $^{**}p < 0.01$.

counts of DAPI-stained cells in the striatum than HI females (**Figure 10**). No differences in hippocampal DAPI-stained cells based on surgical condition ($F_{(1,13)} = 1.011$, $p > 0.05$, *NS*) or housing condition ($F_{(1,13)} = 0.291$, $p = 0.599$). There was also no effect of housing condition ($F_{(1,13)} = 3.345$, $p = 0.090$) on DAPI levels in the striatum of females.

Analysis of GDNF immunoreactive cells revealed a significant interaction effect between surgical condition and housing condition ($F_{(1,14)} = 5.743$, $p < 0.05$) indicating that CN improved the abundance of GDNF in the striatum of both control males and HI males (**Figure 10**). Although no significant differences were found between the factors of surgical and housing conditions on hippocampal GDNF, housing condition trended towards significance ($F_{(1,14)} = 4.337$, $p = 0.056$), with CN reared HI males showing the greatest levels of GDNF intensity compared to all other groups.

For females, there was a significant interaction effect between surgical condition and housing condition on GDNF counts in the striatum ($F_{(1,13)} = 5.777$, $p < 0.05$), with HI-CN females showing the highest counts of all groups. No significant differences were observed in regard to hippocampal measurement of GDNF for females or for males by housing or surgical condition, although a trend towards significance was observed in males by housing condition ($F_{(1,14)} = 4.337$, $p = 0.056$).

DISCUSSION

Overview of Findings

The present study was designed to assess the potential buffering effects of enrichment in the early pre-weaning environment on the negative neurological and cognitive effects of term neonatal HI injury. Dams were given a CN environment that we posited would provide early environmental enrichment for dams and pups and might promote mCTB, as measured by pup weight gain. Indeed, even though HI injury lowered pre-weaning weights for females, CN rearing protected against weight loss during adolescence following HI insult in males and females. The closed nest environment lead to significant changes in neurotrophin levels after HI injury in females and males suggestive of neuroprotection, namely higher levels of GDNF in the striatum in HI injured animals. The observed trend for HI-CN females to have near-normal levels of hippocampal BDNF may suggest even greater neuroprotection from early environmental enrichment for females.

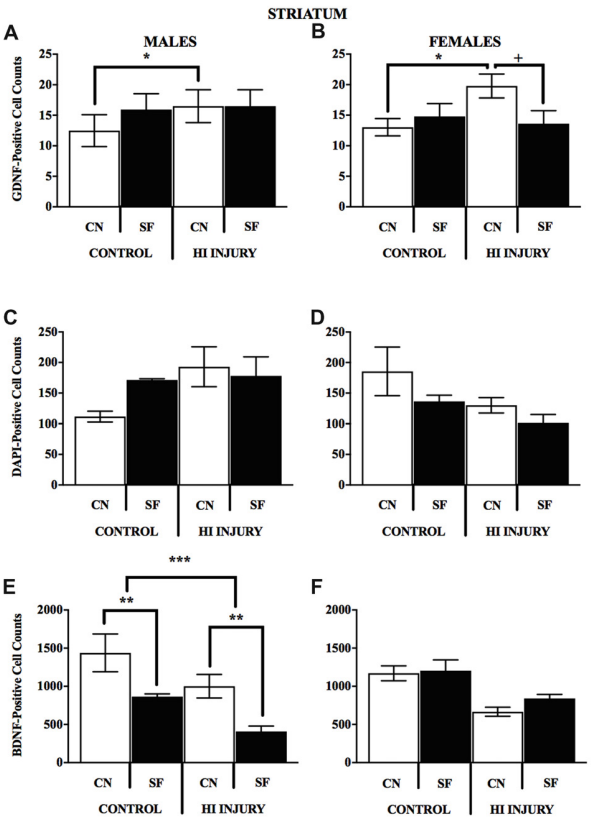

FIGURE 10 | (A–F) Positively stained cells for BDNF GDNF, DAPI in the striatum by surgery condition and rearing in males and females. **(A-B)** In both males and females, CN rearing increased GDNF counts following HI injury, $^{*}p < 0.05$, relative to Control CN; $^{+}p < 0.05$, compared to HI injury SF rearing. **(C-D)** No differences were observed for DAPI staining for males or females for any of the surgical or housing conditions. **(E)** CN rearing improved BDNF stained cells in the striatum of males, regardless of injury (control and HI); $^{**}p < 0.01$, CN vs. SF, same injury group. The overall number for BDNF-positive cells was greater in the control males relative to HI injured males, $^{***}p < 0.001$.

Learning and Memory

Neonatal HI injury is known to result in significant white matter damage (Jansen and Low, 1996) and cognitive impairments including learning and memory deficits (Huang et al., 2009). MWM is a standardized tool for assessing visuo-spatial learning and memory in rodents (Morris, 1984; Vorhees and Williams, 2006); we performed MWM using 3 days of VP training and acquisition followed by 5 days of HP testing and a probe trial (PND 35–43) in which the platform is removed. This developmental period maps onto school-age when many survivors of neonatal HIE demonstrate deficits in learning and memory (Lindström et al., 2006).

The results of the current study indicate that all animals were able to acquire new learning during the 3-day training period regardless of injury or housing condition, however, control female animals learned the location of the platform more quickly and demonstrated shorter latencies than HI females during training, regardless of housing condition. During the testing phase, when a HP was located in a fixed position for each testing day, all animals regardless of injury, and housing condition, were able to learn the location of the HP, as indicated by decreased latencies across testing days (Hill et al., 2011). This suggests that all animals demonstrated some degree of intact long-term memory and adaptive search patterns, however, there were significant differences between injury and housing groups in the speed with which they were able to remember and locate the HP. Only female animals with HI injury demonstrated a significant functional benefit from CN housing, showing comparable latencies to those of control females in both housing conditions, and SF HI females demonstrating significant impairment in comparison. Males with HI injury demonstrated significantly longer search times, indicating poorer long-term memory and search strategies than intact males, regardless of housing condition.

Previous studies have found that, in addition to being more vulnerable to neonatal HI (Hill and Fitch, 2012), males are less responsive to some treatments for neonatal HI injury than are females (Pereira et al., 2008; Fan et al., 2011; Nie et al., 2016). Research by Pereira et al. (2008) showed a selective benefit of environmental enrichment on visuospatial memory for HI injured female animals, without having an effect on males. We have unpublished data indicating improve rope suspension performance for HI injured females but not males reared in CN (unpublished data; Mason et al., 2016). There are several mechanisms for sex differences in HI injury and recovery that have been proposed, including sex-dependent cell-death pathways (Zhu et al., 2006), protective effects of estrogen (Gerstner et al., 2009), and structural neurological differences due to early testosterone exposure (Hill et al., 2011).

Other labs have demonstrated that adolescent and adult environmental enrichment leads to improved performance in MWM for mice and rats without injury (Hullinger et al., 2015; Garthe et al., 2016). Interestingly, there were no independent effects of housing condition on water maze performance for control animals. Some research has indicated that environmental interventions may be specifically beneficial for injured or neurologically compromised animals (Pereira et al., 2008; Ravenelle et al., 2014). It may be the case that interventions targeting early nesting environment are specifically helpful for functional repair after injury and less helpful for memory performance in healthy, normally developing animals. This may be particularly true for animals exposed to neonatal HI, due to the vulnerability of hippocampal tissue (Jansen and Low, 1996; McAuliffe et al., 2006).

Neurotrophic Factors

BDNF and GDNF likely contribute to neuroprotective effects following HI injury in neonatal rats (Abe, 2000; Jin et al., 2003) and novel treatments can be assessed through changes in these two factors (Miyazaki et al., 2001; Griesbach et al., 2004), as well as through overall number of cell bodies present (Levison et al., 2001). The neuroprotective effects of BDNF are mediated at least in part by ERK1/2 secondary signaling; this signaling blocks the activation of caspases necessary

for apoptotic mechanisms following HI damage and ensuing necrotic stress (Han and Holtzman, 2000; Jones and Bergeron, 2004). Furthermore, GDNF aids in the survival of brain tissue via inflammatory proteins activated during neonatal HI, such as interleukin-6 and caspase-3 (Miyazaki et al., 2001; Kilic et al., 2003).

Overall, average BDNF level was higher in intact animals, and lower in HI animals, as has been reported (Pereira et al., 2008; Chavez-Valdez et al., 2014). CN conditions *reversed* the overall reduction in hippocampal BDNF levels after HI injury for both males and females. HI-CN conditions resulted in non-significant differences BDNF levels in females in striatal tissue. By contrast, HI CN levels were significantly higher than HI SF and control levels were greater than HI injury levels. It is likely that endogenous hormones contribute to this dimorphism in some respect since BDNF levels in intact female rats fluctuate depending on estrous cycle (Franklin and Perrot-Sinal, 2006), and with higher levels of estradiol, female rats have increased recall, general memory and spatial navigation over males (Luine and Frankfurt, 2013).

We quantified the average number of cell bodies using DAPI immunofluorescence. It was surprising that HI insult did not have a significant effect on the number of DAPI-tagged cells in the striatum of study subjects. However, we posit that the lack of specificity in this fluorescent DAPI staining—which produced coloration of all activated, double-stranded DNA-containing nuclei rather than neurons alone—could be one possible explanation for this (Levison et al., 2001). Interestingly, we did find DAPI counts in CN control males were lower than all other groups, implying reduced brain structure following environmental enrichment (van Praag et al., 2000).

No notable distinctions were observed for hippocampal GDNF staining in either males or females. However, striatal GDNF counts were significantly affected by CN rearing conditions for both females and males with HI. In females, HI-CN animals had more striatal GDNF positive cells over HI-SF and control CN animals. In a rodent model of Parkinson's disease, viral vector transfer of BDNF and GDNF into nigrostriatal neurons was no more potent than GDNF alone (Sun et al., 2005), implying that current striatal changes in GDNF alone may underlie MWM sex differences.

Early environmental enrichment improved search patterns in the probe trial following MWM testing, regardless of injury. Despite observing similarities in swimming speed and latency across control and HI conditions, CN conditions appeared impactful for rodent navigation in the MWM. It is unlikely that induced differences between groups are due to physical movement and/or exercise related to the testing protocol. Past research has indicated that it may take 28–31 days to persistently upregulate endogenous BDNF through physical activity in the MWM (Adlard et al., 2004; Griesbach et al., 2004). While physical stimulation may indeed induce transient changes in neurotrophins from baseline (Huang et al., 2006; Ferris et al., 2007), animals in our current study were sacrificed more than 2 weeks following the conclusion of behavioral testing.

Conclusions From Cognitive and Neurotrophic Outcomes

The neuroprotective benefit of a CN environment following HI injury was partly sex-specific. For females exposed to HI, those reared in CN demonstrated cognitive abilities similar to those of non-injured females, in contrast to HI females reared in SF housing that demonstrated significant functional impairment and diminished weight gain. There was a trend for HI females to demonstrate a similar beneficial effect of CN on neurotrophic factors, which may depend on levels of estradiol (Luine and Frankfurt, 2013). In contrast, males with HI injury showed no significant cognitive benefit from CN. However, both males and females in the HI-CN condition did demonstrate higher levels of GDNF in the striatum, indicating that CN may have afforded some neuroprotection to HI males. HI injury most prominently affects cortical matter and hippocampal tissue (Busl and Greer, 2010), and males are more likely to incur severe immediate damage to these regions than females (Liu et al., 2007; Hill and Fitch, 2012; Smith et al., 2014). In terms of recovery period, females are thought to have "depressed" metabolic function in comparison to males (Morken et al., 2014). It is plausible that CN rearing, while theoretically protective from environmental stress in the laboratory setting, was not intensive enough to sufficiently rescue the damage induced by HI for males.

The difference between male and female levels of these neurotrophins could be directly related to the availability of the nest itself from birth. Reduced nesting environment leads to "fragmented," and stress-provoking maternal behavior in rat dams towards their offspring (Ivy et al., 2008). Adult laboratory rats do not spontaneously build nests (Van Loo and Baumans, 2004), and it has been suggested that researcher-provided materials for nesting may give rats a degree of control over their laboratory cage surroundings (as reviewed by Simpson and Kelly, 2011). Further, a small opaque enclosure similar to our CN can decrease environmental stress (Würbel, 2001). An early study by Manser et al. (1998) showed that rats will preferentially occupy an opaque nesting box with a roof and surrounding walls and a smaller entrance when offered multiple types of nesting, a design style similar to the CN provided here. Adult female rats have been hypothesized to have more complex interactions with their nesting environments as a result of hormones, and so it was expected the pregnant dams would adapt to the CN (Pietropaolo et al., 2004). Since rat dams demonstrate preferential licking and grooming for male pups, regardless of strain (Moore et al., 1997; McGowan et al., 2011), it is possible that a low-stress nursing environment provided for more equal distribution of maternal care for both sexes, thereby improving care specifically for female pups.

One future direction could include additional enrichment after weaning through the traditional social sensorimotor stimulation in housing animals with peers, toys and physical objects as it may promote further neuroplasticity through experience (Johnston et al., 2009). This may diminish apoptotic changes close to injury (Bondi et al., 2014) that we could investigate with earlier sacrifice days, and, therefore, create additive effects to the benefits presented here. In clinical

populations, most infants with HIE are provided with standard-of-care treatment, therapeutic hypothermia (Eicher et al., 2005), if they can be treated within 6 h of life. Environmental enrichment may provide adjunctive benefits in combination with hypothermia. In future studies, examining the combination of treatment approaches could provide further insight into clinical applications.

AUTHOR CONTRIBUTIONS

LR conceived the original pilot study and worked with SD to develop a full experiment. BM supported the expansion of the work and contributed novel insights in developing the experiment, particularly molecular targets. LR and BM completed surgeries and behavioral testing with support from EA, CC and NW. LR, BM, EA, CC, NW and SD completed the terminal procedures including animal sacrifice, brain extraction and tissue preparation for immunohistochemical analyses. BM completed all immunohistochemical work. SD, LR, BM, EA, CC and NW contributed to writing the Methods and entered data for analyses. BM and LR completed statistical analyses and wrote the Results. LR and BM both contributed equally to all aspects of the manuscript writing with support from SD.

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Enriched Environment Facilitates Anxiolytic Efficacy Driven by Deep-Brain Stimulation of Medial Prefrontal Cortex

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Deep brain stimulation (DBS) is a widely used treatment for neurodegenerative disorders like Parkinson's disease. Recently, several studies have used preclinical animal models to suggest that DBS has a potential to improve emotional symptoms in mental disorders such as treatment-resistant depression and post-traumatic stress disorder. An important difference between neurodegenerative and emotional disorders is the crucial role of environment in the ontogeny of the latter. Thus, it is important to understand the effects of DBS in the context of environmental variation. In this study, we show that DBS of ventromedial prefrontal cortex reduces anxiety in rats when it is coupled with simultaneous exposure to an enriched environment (EE). In contrast, effects of DBS on anxiety-like behaviors remained equivocal when animals were housed in standard laboratory conditions. These results suggest that the ability of DBS to treat anxiety and related phenotypes can be significantly enhanced by EE opportunities.

Keywords: anxiety, complex housing, plasticity, ventromedial prefrontal cortex, morphology, neurons

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INTRODUCTION

Deep brain stimulation (DBS) is a surgical therapeutic approach for treating disorders of the central nervous system. It uses electrodes implanted into anatomically defined brain targets to deliver current stimulation in a chronic manner (Kühn and Volkmann, 2017). It has been previously used as an effective treatment in movement disorders like Parkinson's disease (Hickey and Stacy, 2016) and several psychiatric disorders like Tourette syndrome and obsessive-compulsive disorder (Alonso et al., 2015; Fraint and Pal, 2015). This success has led to suggestions that this technique can also be used in treatment-recalcitrant cases of other brain-centric pathologies like post-traumatic stress disorder (Reznikov et al., 2016) or major clinical depression (Torres-Sanchez et al., 2017).

The DBS of the ventromedial prefrontal cortex in rats reduces learned helplessness in a forced swim task (Hamani et al., 2010). Stimulation of this brain region also rescues anhedonia caused by chronic unpredictable stress, as evidenced by the preference of sucrose over water (Hamani et al., 2012). Similarly, ventromedial prefrontal cortex stimulation rescues endophenotypes related to anhedonia and learned helplessness in a mouse strain selectively bred to show greater depression-like behaviors (Schmuckermair et al., 2013). Stimulation of the same brain region also rescues depression-like behaviors resulting from olfactory bulbectomy in rats (Jiménez-Sánchez et al., 2016). Human subcallosal cingulate gyrus is thought to be an analogous structure to rodent ventromedial prefrontal cortex. Rodent preclinical studies are, in fact, subsequent to several clinical case studies and open-label studies suggesting improvement of depressive symptoms after DBS in subcallosal

cingulate gyrus (reviewed in Dandekar et al., 2018). While some of the follow-up randomized controlled design studies confirm therapeutic benefits of DBS (Eitan et al., 2018), other studies do not show significant improvement (Holtzheimer et al., 2017; Merkl et al., 2018).

Other brain structures have also been examined as a target for DBS in treatment-refractory depression (Dandekar et al., 2018). Similarly, several brain regions have been examined as targets for lowering anxiety and depression-like behaviors. This includes the nucleus accumbens, ventral tegmental area, lateral habenula, subthalamic nucleus and medial forebrain bundle (Dandekar et al., 2018). Stimulation of ventromedial prefrontal cortex among these varied structures shows the most robust reversal of anxiety in stress-naïve animals; and reduction of stress-induced depressive and anxiety-like behaviors (Lim et al., 2015). Various brain structures have also been used as sites for DBS in animal models of fear and anxiety (Reznikov et al., 2016). This includes basolateral amygdala, prefrontal cortex, striatum and hippocampus. The choice of these brain structures in preclinical models arises from their role in forming brain circuits for fear learning and extinction. Among these structures, we have used DBS in ventromedial prefrontal cortex, showing that stimulation of this brain region also improves cognition in middle-aged rats (Liu et al., 2015). Thus, stimulation at the ventromedial cortex provides continuity with the previous work, while showing promise in preliminary clinical work. In this backdrop, we chose the ventromedial prefrontal cortex as the stimulation site in the current study.

In short, preclinical work shows promising results suggesting that DBS of the ventromedial prefrontal cortex can reduce anxiety and depression. Yet, the ontogeny of emotional disorders, for example, depression or consequences of prior trauma, is intricately linked to environmental factors. This contrasts with neurodegenerative disorders more commonly targeted by DBS. For example, a genetic predisposition to depression in both cohorts only manifests itself after exposure to childhood adversity (Caspi et al., 2003). Animal work congruently demonstrates the crucial role of environment in emotional behaviors. Stimuli that show robust anxiogenesis in a sparse housing environment fail to generate anxiety in complex housing regimes (Ashokan et al., 2016, 2018a; Koe et al., 2016) or when the ambient quality of the environment is changed (Abdulai-Saiku et al., 2017). Sensory enrichment of the housing environment induces robust structural changes in brain regions important for emotional behaviors including the ventromedial prefrontal cortex (Ashokan et al., 2018b), hippocampus (Darmopil et al., 2009), and basolateral amygdala (Ashokan et al., 2016; Koe et al., 2016). These studies collectively show that symptoms of emotional disorders are contingent on the environment of the individuals. Hence, the preclinical work for brain stimulation, when in the context of emotional plasticity, must also encompass interactions of the stimulation with the ambient environment of the animals. Housing conditions are known to influence anxiety and underlying plastic changes in the neuronal architecture. Thus, changes in the housing environment are a promising avenue to increase the efficacy of therapeutic changes in behavior brought about by DBS. This possibility has not yet been tested. In the present study, we attempt to bridge this gap by delineating if the efficacy of ventromedial prefrontal cortex DBS on anxiety-like behaviors depends on the housing environment of the rats. Specifically, we ask if ameliorating effects of DBS on anxiety can be enhanced by housing in an enriched environment (EE).

MATERIALS AND METHODS

Animals and Experimental Design

Adult Wistar male rats (average age: 8 weeks, average weight: 250 g) were used for this experiment. Animals were housed in reversed day–night cycle (lights on at 19:00 h) and *ad libitum* access to food and water. Animals were handled everyday to get them used to human handling to prevent stress during the behavior trials. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of NTU. All experiments were performed in accordance with IACUC guidelines and regulations.

Animals were randomly divided to receive either sham treatment or DBS to ventromedial prefrontal cortex. Both sham and stimulated animals were further subdivided and housed in either standard laboratory housing or enriched housing. Before assignment to experimental groups, all animals stayed in standard housing conditions postweaning (2/cage).

Housing Conditions

Standard laboratory housing consisted of two animals living in an animal facility cage (37 × 22 × 18 cm). Animals in the standard group were singly housed after surgery for electrode implantation. This was done to prevent damage to the electrode site due to physical interaction with cage mates. EE consisted of larger cages (72 × 51 × 110 cm), more animals per cage (four animals per cage), and presence of novel objects. Larger spaces within enriched housing prevented electrode damage during group housing. The novel objects included climbing walls made of wire-net, plastic tunnels, plastic and wooden objects of varied colors and textures, ample nesting material and gustatory variety in the form of fruit loops and sunflower seeds and layered tiers within the cage. Running wheel was not provided in the EE to minimize effects of exercise after recent surgery. The arrangement of the objects was changed every fourth day. Animals were placed in enriched housing from day 1 (surgery) to day 19 (sacrifice). Animals assigned to enriched housing were housed 2/cage in standard housing conditions before the surgery.

DBS

All animals were implanted with an electrode directed at the ventromedial prefrontal cortex under general anesthesia achieved by a cocktail of ketamine and xylazine. The plane of anesthesia was maintained using gaseous isoflurane during stereotaxic surgery (2.5% v/v). The rat was positioned and fixed in a standard stereotactic apparatus. A midline incision was made from the orbital level to the occipital lobe, which

allowed adequate exposure to the skull. A burr hole was made above the anatomical target followed by duratomy. Miniature screws (0.8 mm; 2 per hemisphere) were placed into the skull anteriorly and posteriorly to the burr holes to serve as anchors for cement. Electrodes were implanted into the ventromedial prefrontal cortex and fixed with dental cement that adhered to the electrode construction and miniature screws (AP: +2.70 mm; L: ±0.60 mm; V: 4.60 mm). Bipolar stimulating electrodes (Synergy, Singapore) with an inner platinum–iridium core wire with a gold-plated cannula were used (Technomed, Beek, Netherlands). Finally, the skin was carefully repositioned and stitched up. Experiments started after 10 days from surgery.

A digital stimulator DS8000 and stimulus isolators DLS100 (World Precision Instruments, Sarasota, FL, USA) were used to deliver the electrical stimuli in animals assigned to the DBS group. Pulse width of the stimulation was set at 100 μs and amplitude at 200 μA. Animals received stimulation for 1 h daily from day 11 to day 19 (100 Hz, surgery being day 1). Sham animals were brought to the stimulation room for the duration of stimulation protocol and connected to the stimulator, but no stimulation followed.

Behavioral Testing

All behavioral tests were conducted between 08:00 h and 12:00 h under dim red lights. Animals were allowed to habituate to the testing room for >30 min before the test. One day passed between successive behavioral tests in the sequence of home-cage emergence assay (day 11), object recognition task (days 13 through 15), and elevated plus-maze (day 16). Behavioral arenas were cleaned with 70% ethanol in-between trials.

Home Cage Emergence

A rat in its home cage was moved from the holding room to the test room. After habituation, the home-cage was left open, and the rat was offered the possibility of emerging via a grid. This was observed for 5 min. The latency to emerge from the home cage (i.e., the time until the rat was on the grid outside its home cage with all four legs) was scored. Trial duration was 300 s. A score of 300 was arbitrarily assigned to any animal which did not emerge for the duration of the trial.

Elevated Plus-Maze

Anxiety-like behavior was measured using an elevated plus-maze that consisted of a plus-shaped arena with two open (75 × 11 cm, 1 cm wall, 3–4 lux illumination) and two enclosed arms (75 × 11 cm, 26 cm wall, 0 lux illumination). The arena was elevated to 60 cm above the ground. The animal was placed at the center at the start of the trial (trial duration = 300 s). Exploration in open and enclosed arms was quantified. Open arm exploration (entries and occupancy time) relative to the sum of open and enclosed arm explorations was used as an index for anxiety. Mean of percentage open arms entries and percentage open arms time was subtracted from 100 to derive an index for anxiety. Entry in an arm was defined as the presence of the whole body including head, four paws, and at least the base of tail inside the open arm. Also, the number of head dips was quantified as a measure of risk assessment in the maze. Head dip was defined as downward movement of the head toward the floor, extending completely out of the open arm.

Object Recognition Task

The rats were introduced to a square arena (1 m × 1 m) with opaque walls. Two similar objects (1-liter laboratory glass bottles) were diagonally placed in the arena. Animals were allowed to explore the arena for 180 s. Short-term object recognition memory was tested 90 min afterward. One of the previously presented objects was replaced with a rectangular box during this phase. Exploration of novel and familiar objects was quantified over a period of 180 s. Object recognition was quantified as exploration of novel objects relative to the sum of exploration for novel and familiar objects.

Statistical Analysis

GraphPad Prism version 7 was used for statistical analysis. Figures represent mean and SEM of rank-transformed data, along with individual values for each animal. Numbers of animals used for analysis are depicted in each figure.

Normality for behavioral endpoints was tested using the Shapiro−Wilk test. Several endpoints exhibited significant departure from normality. Consequently, nonparametric statistics was used for intergroup comparisons (Kruskal–Wallis test). Data for each endpoint were further rank-transformed across four experimental groups.

This study was built to test an *a priori* premise that DBS shows greater clarity and more robust effects when applied in enriched housing rather than standard housing. Congruent with *a priori* assumptions, two planned comparisons were set before data collection started: sham and stimulated animals in standard housing and sham and stimulated animals in enriched housing (Ruxton and Beauchamp, 2008). Orthogonal comparisons were used, such that no experimental group was used in more than one comparison. For example, we did not test statistical significance for effects of enriched housing itself vis-à-vis standard housing in absence of DBS. Independent sample Student's *t*-test was used for parametric planned comparisons of rank-transformed data. Interpretation of these planned comparisons was buttressed by the calculation of effect size for rank-transformed data using Cohen's *d* (Lakens, 2013).

RESULTS

Animals housed in the standard laboratory housing or EE were subjected to either DBS directed at the ventromedial prefrontal cortex or sham stimulation, yielding four experimental groups.

The EE and DBS Decreased Latency to Emerge From the Home-Cage

A Kruskal–Wallis test revealed the presence of statically significant intergroup differences (H_4 = 17.4, p = 0.0006) in latency to emerge from home-cage. The data were rank-transformed and analyzed using planned comparisons.

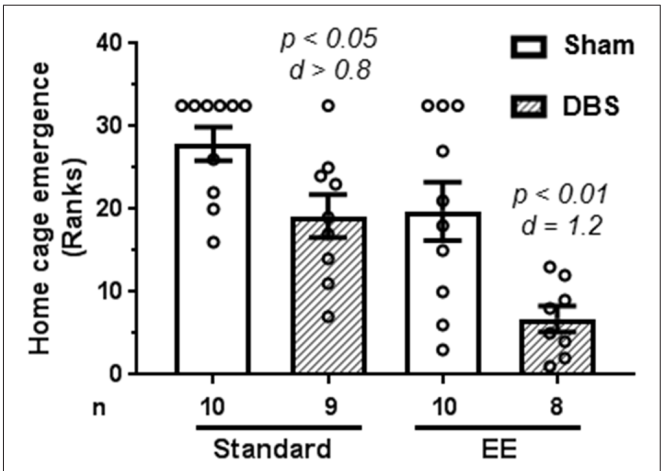

FIGURE 1 | Effects of deep brain stimulation (DBS) on latency to emerge from home-cage in presence or absence of enriched environment (EE). Emergence latency (in seconds) was rank-transformed for the illustration. Mean and SEM, along with individual rank of animals in each group, are depicted. Number of animals (*n*) is also presented. Both *p* and Cohen's *d* values are shown for statistically significant comparisons between DBS and corresponding sham groups.

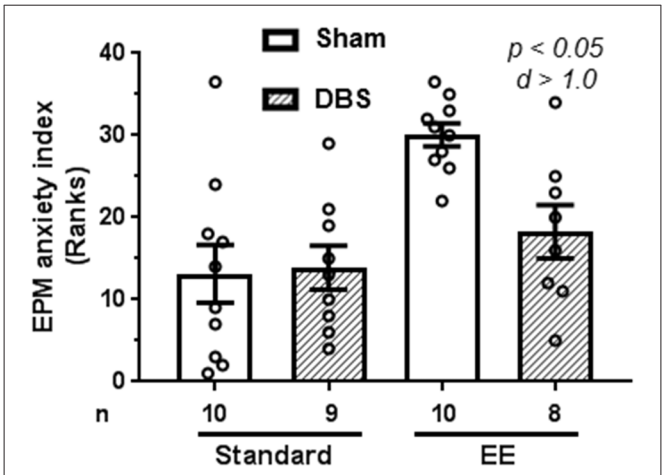

FIGURE 2 | Effects of DBS on anxiety in an elevated plus-maze in presence or absence of EE. Anxiety index was expressed as subtraction of open-arm exploration from 100. This endpoint was rank-transformed for the illustration. Mean and SEM, along with individual rank of animals in each group, are depicted. Number of animals (*n*) is also presented. Both *p* and Cohen's *d* values are shown for statistically significant comparisons between DBS and corresponding sham groups.

The DBS reduced emergence latency in animals housed in both standard housing (**Figure 1**; $t_{(17)} = 2.66$, $p = 0.016$) and EE (**Figure 1**; $t_{(16)} = 3.07$, $p = 0.007$). However, effects of DBS were more pronounced in presence of EE (Cohen's $d = 1.19$; rank difference = 13.0 ± 3.8) compared with absence of EE (Cohen's $d = 0.82$; rank difference = 8.7 ± 3.7).

DBS Reduced Anxiety-Like Behavior in Elevated Plus-Maze When Animals Were Placed in Enriched Housing

Anxiety-like behavior was quantified using reduction in anxiogenic open arms as a proxy. Open arm exploration was defined as the mean of the percentage of open arm entries and percentage of time spent in the open arm. Anxiety was defined as hundred minus percentage of open arm exploration.

A Kruskal–Wallis test revealed presence of statically significant intergroup differences ($H_4 = 15.4$, $p = 0.0015$) in anxiety-like behavior. Analysis of rank-transformed data revealed that DBS reduced anxiety-like behavior in animals housed in EE (**Figure 2**; $t_{(16)} = 3.60$, $p = 0.002$). In contrast, DBS did not cause significant effects on anxiety-like behavior in animals housed in standard laboratory cages (**Figure 2**; $t_{(17)} = 0.16$, $p = 0.872$). Effects of DBS on anxiety were more pronounced in presence of EE (Cohen's $d = 1.01$; rank difference = 11.8 ± 4.1) compared with absence of EE (Cohen's $d = 0.06$; rank difference = -0.7 ± 3.9).

EE and DBS Increased Risk Assessment in Elevated Plus-Maze

Risk assessment in elevated plus-maze was quantified as number of head dips made during the trial.

A Kruskal–Wallis test revealed presence of statically significant intergroup differences ($H_4 = 14.4$, $p = 0.0024$) in risk assessment behavior. Planned comparisons for rank-transformed data showed that DBS reduced risk assessment behavior in animals housed in EE (**Figure 3**; $t_{(16)} = 4.47$, $p < 0.001$). In contrast, DBS did not cause significant effects on risk assessment in animals housed in standard laboratory cages (**Figure 3**; $t_{(17)} = 1.87$, $p = 0.079$). Effects of DBS on anxiety were more pronounced in the presence of EE (Cohen's $d = 1.44$; rank difference = -17.3 ± 4.1) compared with absence of EE (Cohen's $d = 0.69$; rank difference = -7.9 ± 4.0).

EE and DBS Did Not Affect Memory Performance in Object Recognition Task

Animals were presented with two identical objects at time zero. Short-term object recognition memory was tested after 90 min by presenting animals a choice between a previously presented familiar object and a novel object. Memory was quantified as percentage time spent exploring novel object relative to sum of time spent exploring novel and familiar objects.

A Kruskal–Wallis test revealed absence of statistically significant intergroup differences in short-term memory ($H_4 = 5.2$, $p = 0.158$). The data were rank-transformed and analyzed using planned comparisons. Sidak's multiple comparisons test did not reveal statistically significant effects of DBS in presence (**Figure 4**; $t_{(14)} = 1.72$, $p = 0.108$) or absence (**Figure 4**; $t_{(16)} = 1.05$, $p = 0.307$) of EE.

DISCUSSION

Our results show that DBS of ventromedial prefrontal cortex consistently produces anxiolysis when animals are housed in EE, but not when animals are housed in standard laboratory housing. For example, DBS animals exhibited more robust exploration of anxiogenic parts of elevated plus-maze compared

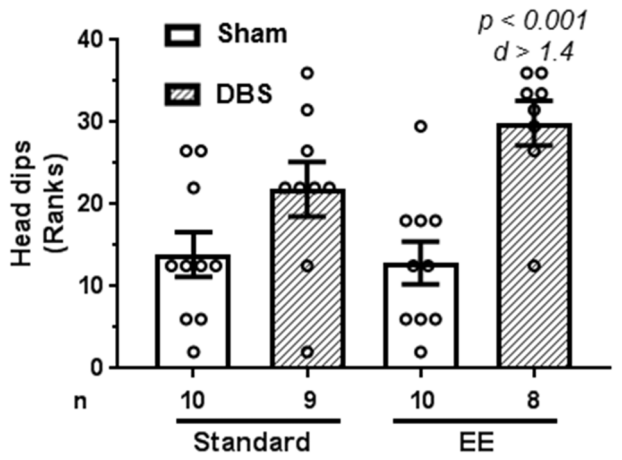

FIGURE 3 | Effects of DBS on risk assessment in an elevated plus-maze in presence or absence of EE. Risk assessment was measured as number of head dips made during the trial. This endpoint was rank-transformed for the illustration. Mean and SEM, along with individual rank of animals in each group, are depicted. Number of animals (*n*) is also presented. Both *p* and Cohen's *d* values are shown for statistically significant comparisons between DBS and corresponding sham groups.

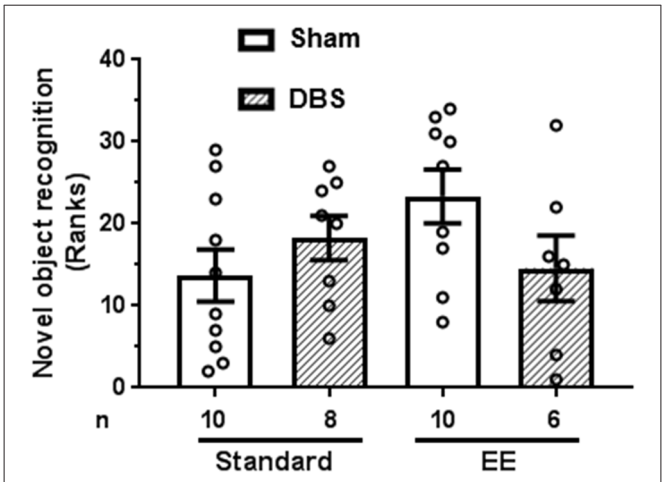

FIGURE 4 | Effects of DBS on short-term memory (90 min) in presence or absence of EE. Object recognition was measured as percentage time spent near novel object relative to total time spent with novel and familiar object (chance = 50%). This endpoint was rank-transformed for the illustration. Mean and SEM, along with individual rank of animals in each group, are depicted. Number of animals (*n*) is also presented.

with sham-stimulated animals. Yet this was true only if animals were housed in EE and not in standard housing environment. It is noteworthy that mean anxiety score of sham animals in standard housing was much lower than sham animals living in enriched housing. Thus, lack of DBS effect in sham animals might have been influenced by a floor effect of very low anxiety in control animals. Similarly, DBS-treated animals exhibited more active defense response in the plus-maze characterized by risk assessment rather than avoidance. This effect was also evident only when animals were housed in EE and not when animals were placed in standard non-EE conditions. These observations suggest that the complexity of housing environment is an important determinant of therapeutic outcome of DBS treatment in the context of emotional behaviors. This is further buttressed by comparisons of magnitude of DBS effects in the two housing environments. Effect size of DBS on anxiety-related endpoints was very robust with d values from 1.0 to 1.4 when housed in the EE. In contrast, DBS exhibited statistically significant effect in only one endpoint, and the observed effect size was weaker in that case (d = 0.8). To keep this in numerical perspective, a randomly chosen EE-housed DBS-treated subject had >75% probability of showing lower emergence latency from home-cage when compared with a randomly chosen EE-housed sham-treated animal. Similarly, a randomly chosen EE-housed DBS-treated animal had >83% probability of making more active risk assessment maneuvers compared with a randomly chosen EE-housed sham animal. In contrast, DBS induced anxiolysis did not reach statistical significance in elevated plus-maze and exhibited a mediocre effect size of 0.8 in case of home-cage emergence latency. This suggests that DBS might be more effective for emotional disorders if used in conjunction with environmental interventions. Effects of DBS and its higher efficacy with EE showed specificity to the anxiety-related endpoints, with nondiscernible effects on nonemotional object-recognition task.

The EE, in this study, comprises several social and nonsocial facets. For example, standard housing in our design entails housing animals singly after the surgery due to possibility of damage to the electrodes. Animals in the enriched housing, meanwhile, stay in a social setting of four animals per larger cage during the similar time window. This could have changed the social landscape of the housing environment including social interaction and dominance relationships. This is relevant because single housing is known to induce anxiety in rats (Balcombe, 2006). Our study design does not allow statistical comparisons between sham-treated animals living in standard housing vs. enriched housing, due to the orthogonal nature of the planned analysis. The enriched housing also consisted of changes in nonsocial aspects of the environment including greater availability of sensory stimuli and exposure to novelty. Our results cannot determine if greater efficacy of DBS in EE animals was due to social factors, nonsocial factors, or their emergent interaction.

Several studies suggest that DBS has the potential to manage fear and anxiety-related behaviors in preclinical animal models. For example, DBS of basolateral amygdala reduces anxiety when measured in a defensive burying task, but not when measured in elevated plus-maze (160 Hz for 4 h per day for 7 days; Langevin et al., 2010; Stidd et al., 2013). Similar stimulation paradigm also decreases the strength of fear conditioning to a discrete auditory tone, but not the contextual fear conditioning (200 Hz for 4 h per day for 7 days; Sui et al., 2014). The DBS effects in ventromedial prefrontal cortex show similar equivocality in experiments reported here. The DBS-treated animals in standard housing

regimes exhibit anxiolysis when measured in the home-cage emergence task, but not in elevated plus-maze. It is plausible that ambivalence in DBS effects in these studies is an artifact of the impoverished housing environment in standard laboratory practice.

Several EE paradigms have been previously used to show the beneficial effects of complex housing on emotional behaviors in animal models. For example, peripubertal EE reduces anxiety and depression (Francis et al., 2002; Cui et al., 2006; Ilin and Richter-Levin, 2009). Similarly, EE provided in adulthood reduces anxiogenesis brought about by historical stress exposure (Koe et al., 2016; Ashokan et al., 2018a). These studies have led to an emphasis on the critical role of living environment and potential to exhibit species-typical behaviors for emotional well-being. Our studies advance this by showing that EE can facilitate beneficial effects of a targeted and intensive surgical intervention such as DBS.

The DBS is a very appropriate exemplar of how animal studies can lead to clinical outcomes. Use of DBS is now mainstream for movement disorders like Parkinson's disease. The impetus for this adoption comes from carefully controlled animal studies, showing that stimulation of basal ganglia paradoxically had the same effect as lesions: which is to reduce motor symptoms in an animal model of the Parkinson's. This knowledge in animals directly led to experimental use of DBS in human patients. Observations in this report further that narrative by suggesting intimate interplay of DBS and ambient environment, whereby, environment enrichment enhances behavioral plasticity brought about DBS.

AUTHOR CONTRIBUTIONS

RM conceptualized, planned, analyzed and wrote the manuscript. YB and LL planned, executed and analyzed the data.

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frontiers in Behavioral Neuroscience

REVIEW
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Environmental Enrichment and Successful Aging

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The human brain sustains a slow but progressive decline in function as it ages and these changes are particularly profound in cognitive processing. A potential contributor to this deterioration is the gradual decline in the functioning of multiple sensory systems and the effects they have on areas of the brain that mediate cognitive function. In older adults, diminished capacity is typically observed in the visual, auditory, masticatory, olfactory, and motor systems, and these age-related declines are associated with both a decline in cognitive proficiency, and a loss of neurons in regions of the brain. We will review how the loss of hearing, vision, mastication skills, olfactory impairment, and motoric decline accompany cognitive loss, and how improved functioning of these systems may aid in the restoration of the cognitive abilities in older adults. The human brain appears to require a great deal of stimulation to maintain its cognitive efficacy as people age and environmental enrichment may aid in its maintenance and recovery.

Keywords: cognitive loss, cognitive impairment, cognitive enhancement, hearing impairment, mastication impairment, vision impairment, olfactory impairment, exercise

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INTRODUCTION

In more than a dozen animal models of human neurological pathology, environmental enrichment has been shown to ameliorate the human-like neurological symptoms present in these animals models, including the cognitive decline in aging (Segovia et al., 2006; Patel, 2012) and in Alzheimer's disease (Arendash et al., 2004; Jankowsky et al., 2005; Lazarov et al., 2005; Berardi et al., 2007; Costa et al., 2007; Basak et al., 2008; Arranz et al., 2011; Polito et al., 2014).

Humes et al. (2013) suggested that the multi-sensory decline that accompanies normal aging in humans might influence the decline in cognition. They systematically examined changes in hearing, vision, touch, and cognitive function, in young, middle, and older adults. By varying the difficulty and sensitivity of the tasks, as well as making allowances for deteriorating sensory skills, they not only were able to show that age, global sensory, and global cognitive abilities were correlated, but also they further suggested that the decline in sensory processing and acuity may affect the cognitive decline. We present here a brief summary of the deterioration of the sensory and cognitive systems that occurs in older adults and we will present evidence that suggests that enriching the environment of the aging brain by reversing the decline in sensory systems may aid cognitive functioning.

HEARING

Neural Deterioration With Hearing Loss

Age-related hearing loss is common in older adults, with almost two-thirds of those over 70 years old having compromised hearing, and about a third of older adults experiencing debilitating hearing loss (Lin et al., 2011b; WHO, 2018). The odds of hearing loss are 5.5-fold higher in men than in women (Agrawal et al., 2008) and hearing sensitivity declines two times faster in men than in women, with diminished sensitivity observed in males as early as age 30 years (Syka, 2002).

Changes in brain anatomy accompany the decrements in auditory functioning. A reduction in the volume of gray matter is observed bilaterally in auditory cortex that correlates with the loss of high-frequency age-related hearing loss (Peelle et al., 2011; Eckert et al., 2012). There also are neural losses in areas that do not process auditory information directly. Husain et al. (2011) found that older adults with hearing loss experienced decreases in the volume of gray matter in the superior and medial frontal gyri, both of which are involved in cognitive processes, along with losses in the superior temporal cortex, which includes the auditory cortex and multisensory association cortex, and right anterior cingulate cortex, which includes both emotional and cognitive areas. Wong et al. (2010) found that older adults who had limitations in their ability to perceive speech in a noisy environment had smaller frontal cortices than younger adults with normal auditory ability.

In a longitudinal study over 6 years, Lin et al. (2014) compared whole-brain volumes of older adults with hearing impairment to those without hearing loss and found that those with hearing loss had an accelerated loss of brain tissue. There were also volume losses in the right temporal lobe, which included the superior, middle, and inferior temporal gyri, and the parahippocampus, areas that also mediate cognitive processes.

As for functional changes in the brain in response to auditory signals in hearing-impaired individuals, differences in evoked responses correlated with hearing impairment in noisy environments (Campbell and Sharma, 2013). In noisy environments, older adults with hearing loss also respond to speech with both a reduction in the activity of auditory brain areas and an increase in the activity of parietofrontal brain centers (Wong et al., 2010). In general, additional cognitive resources are recruited to achieve auditory perception under challenging auditory conditions for older adults (Eckert et al., 2008; Harris et al., 2009; Erb and Obleser, 2013; Erb et al., 2013; Vaden et al., 2013, 2016).

Cognitive Deterioration With Hearing Loss

There is a consistent finding that has been revealed in five recent meta-analyses that hearing impairment in older adults increases the risk of cognitive deficiencies, including dementia (Thomson et al., 2017; Wei et al., 2017; Zheng et al., 2017; Loughrey et al., 2018; Yuan et al., 2018). Indeed, the risk of cognitive loss increased by 29% compared to normal-hearing older adults over the course of follow-ups of up to 6 years and that risk was increased by 57% when the patients were followed for a longer period, even when potential confounding factors were considered (Yuan et al., 2018). Moreover, increased peripheral damage of the auditory system predicted a greater risk of cognitive impairment in older adults (Yuan et al., 2018). Older adults with mild to severe hearing impairment have a two- to five-fold increase in their risk of developing dementia compared to older adults with normal hearing (Lin et al., 2011a). These cognitive impairments lead to a decreased ability to care for themselves, as confirmed by the impaired performance on the activities of daily living scales in older adults with hearing loss (Gopinath et al., 2013).

There are a number of ways that hearing impairment could affect cognition. The first is that there is a common mechanism that affects both neural systems independently, such as a gradual circulatory impairment, although the predictive value of hearing impairment appears to occur years before the cognitive decline (Albers et al., 2015). Another possibility is that the loss of hearing makes it difficult to have social interactions and increases the risk of depression, both of which could negatively impact cognitive ability (Mick et al., 2014; Dawes et al., 2015). Also possible is that as more effort is required for the listening process, fewer resources may be available for comprehension and memory, whereas the easing of the listening effort results in improvements in cognitive performance (Pichora-Fuller, 2006). Finally, it may be that the loss of auditory stimulation deprives the cognitive areas of their normal level of stimulation and thereby allows those areas to deteriorate.

Enhanced Auditory Stimulation and Cognitive Function

If the loss of auditory stimulation allows the brain's cognitive systems to deteriorate, would either the use of hearing aids or cochlear implants reverse the cognitive loss? Indeed, many studies have shown that the use of hearing aids is associated with improved cognitive abilities and decreased depression in older adults who have hearing loss (Mulrow et al., 1990; Tesch-Römer, 1997; Acar et al., 2011; Choi et al., 2011; Dawes et al., 2015; Castiglione et al., 2016; although see van Hooren et al., 2005).

Hearing aids, however, simply amplify and filter sounds and do not recreate the lost frequencies for their wearers. On the other hand, cochlear implants, even in older adults, restore a significant portion of the sound range that had been lost to the individual (Williamson et al., 2009; Carlson et al., 2010). Such implants restore not only auditory function, but also restore social interactions and quality of life for hearing impaired older adults (Olze et al., 2012). In addition, the cochlear implants induced improvements in cognitive function. Specifically, 12 months after cochlear implantation plus auditory training, 81% of the subjects with the lowest cognitive scores had improved their cognitive function (Mosnier et al., 2015). In another study of cochlear implantation in older adults with hearing impairment, the cochlear implants resulted in improvements in 70% of all cognitive tests, although the subjects did worse on 25% of the cognitive tests after surgery (Cosetti et al., 2016). Social isolation and depression also

improved in those with auditory rehabilitation (Castiglione et al., 2016).

Interestingly, enhanced auditory enrichment started early in life also appears to impart benefits to cognition in later life. Lifelong music training appears to ameliorate the cognitive decline observed in older adults (Hanna-Pladdy and MacKay, 2011; Parbery-Clark et al., 2012; Zendel and Alain, 2012). Moreover, older adults with musical experience have enhanced ability to process speech (Bidelman and Alain, 2015). White-Schwoch et al. (2013) further showed that even moderate amounts of early music training had a persistent effect on maintaining the cognitive ability of older adults, even with a lapse of decades since they had played their instrument.

VISION

Visual and Cognitive Deterioration in Older Adults

Vision is compromised with age (Klaver et al., 1998; Muñoz et al., 2000; Salvi et al., 2006) and there is an increased risk of cognitive loss, Alzheimer's disease and an increased clinical severity of Alzheimer's disease among those with visual impairment (Uhlmann et al., 1991; Lin et al., 2004; Reyes-Ortiz et al., 2005; Whitson et al., 2010, 2012; Ong et al., 2012; Chen et al., 2013, 2017; Mine et al., 2016). Indeed, older adults with good visual acuity have a 63% decreased risk for developing dementia over an 8.5-year period and older adults with diminished visual acuity are 5 times more likely to experience cognitive loss than are older adults with good vision (Rogers and Langa, 2010). In addition, older adults with age-related cataracts have a 1.4 times increased likelihood of developing Alzheimer's disease (Lai et al., 2014). It is also the case that older adults with poor vision engage in fewer activities that involve their cognitive ability than those with normal vision (Varin et al., 2017).

It is possible that there is a common mechanism that causes both vision and cognition to deteriorate. In fact, Drobny et al. (2005) found that older adults with poor vision did poorly on tasks that did not involve vision, suggesting that general cognition is deteriorating as vision deteriorates. When Dickinson and Rabbitt (1991) simulated visual impairment in normal subjects, the subjects were able to read the passage accurately, but their recall of material suffered, suggesting that the difficulty in storing the memory interfered with its retrieval. The cognitive loss that accompanies visual loss therefore may be due to such a situation.

Neural Deterioration With Compromised Vision

Older adults with macular degeneration causing their visual impairment have a smaller visual cortex, along with a thinner cortex, smaller surface area, and lower gray matter volume than older adults with normal vision (Hernowo et al., 2014; Prins et al., 2016). Chen et al. (2013) found deterioration of gray matter in the visual cortical areas in those individuals with open-angle glaucoma, along with finding increased gray matter in some cognitive areas, including the medial area of the superior parietal cortex. The size of the frontal lobe and other areas involved in cognition decreases in older adults with macular degeneration, along with deterioration of the connections between visual cortex and frontal cortex, suggesting a cognitive link in this situation (Chen et al., 2013; Hernowo et al., 2014).

Blind individuals also have a significantly larger anterior right hippocampus compared to that of normal sighted individuals, and the posterior right hippocampus is significantly smaller in blind individuals (Leporé et al., 2009), suggesting modifications to regions of the brain involved with cognitive processing in response to diminished visual information.

Compromised/Rectified Visual Stimulation, Cognitive Ability, and Visual Cortex

Wearing reading glasses is associated with improved cognitive outcomes for older adults (Spierer et al., 2016), although this condition could simply be the result of an increased ability to read. Indeed, the difference between groups was diminished when education level was considered. Unilateral cataract surgery in older adults can result in improved visual acuity, and that surgery improves cognitive functioning (Tamura et al., 2004; Ishii et al., 2008; Miyata et al., 2016), or maintains cognitive functioning (in comparison with other groups, which showed worsened cognitive functioning across the duration of the study; Elliott et al., 2009). It should be noted, however, that Anstey et al. (2006) did not find cognitive benefits after cataract surgery. Jefferis et al. (2015) found a small improvement in cognition after cataract surgery that was not correlated with improvement in visual acuity.

Older adults with visual impairments have a decrease in the volume of their visual cortex (Boucard et al., 2009) and unilateral cataract surgery can result in both an improvement in vision and an increase in visual cortex gray matter contralateral to the operated eye (Lou et al., 2013). Lin et al. (2018) found both improvements in visual acuity, cognitive functioning, and brain activity following bilateral cataract surgery, and these improvements were accompanied by an increase in gray matter volume in visual cortex, 6-months post-surgery.

Combined Hearing and Visual Impairment

Diminished functionality in both vision and hearing senses, or dual sensory impairment (DSI), is a common problem in the older adult population, with greater than 70% of individuals with major vision impairments also exhibiting hearing loss (Heine and Browning, 2002). Individuals with both visual and hearing impairment have an even higher risk of cognitive loss, a diminished capacity on the daily living scales measures, an increased incidence of depression, a greater likelihood of social isolation, an increased risk of falls, and an increased risk of mortality (Keller et al., 1999; Saunders and Echt, 2007; Gopinath et al., 2013, 2016; Mitoku et al., 2016). Indeed, when combined, visual and auditory acuity account for 49.2% of the total variance and 93.1% of the age-related

variance in intelligence in older adults (Lindenberger and Baltes, 1994). Both the high prevalence and the detrimental effects of DSI in the older adult population have resulted in recommendations for treatment that include mechanical aids to improve both hearing and vision deficits (Saunders and Echt, 2007; Zhou and Faure Walker, 2015; Gopinath et al., 2016).

OLFACTION

Neural Deterioration With Olfactory Loss

Normal human aging is accompanied by a deterioration of olfactory abilities (Hoffman et al., 2016; Dong et al., 2017; Seubert et al., 2017), with 18% of older adults having a significant olfactory impairment and 46% of those over 80 years old having very limited olfactory ability (Hoffman et al., 1998; Murphy et al., 2002; Doty et al., 2015; Toussaint et al., 2015; Liu et al., 2016).

Differing from other sensory systems, which have cortical projections that are gated by the thalamus, the olfactory system has direct projections to brain regions involved with cognition and the loss or compromise of the olfaction system results in volume losses to many of these cortical brain areas in humans at any age (Bitter et al., 2010a,b, 2011; Peng et al., 2013; Shen et al., 2013; Yao et al., 2014). Olfactory projection sites, which include primary and secondary sensory cortical areas, as well as cortical regions involved with cognitive processing, also deteriorate with age (Segura et al., 2013; Kollndorfer et al., 2015).

Olfactory System Deterioration and Cognitive Loss

Olfactory dysfunction also accompanies or precedes the early symptoms of cognitive disorders such as Alzheimer's disease, Parkinson's disease, Lewy body dementia, fronterotemporal dementia, Creutzfeldt-Jakob disease, mild cognitive impairment, and schizophrenia (Doty et al., 1988; Devanand et al., 2000; Ponsen et al., 2004; Tabaton et al., 2004; Ross et al., 2006; Luzzi et al., 2007; Wattendorf et al., 2009; Devanand et al., 2010; Li et al., 2010; Meusel et al., 2010; Nguyen et al., 2010; Parrao et al., 2012; Sohrabi et al., 2012; Conti et al., 2013). Given that these cognitive disorders have widely differing etiologies, it raises the possibility that the loss of olfactory stimulation contributes to the decline in cognitive ability in some of these disorders, particularly those in which olfactory loss precedes cognitive loss. Moreover, a degradation of olfactory ability predicts an elevated risk of mild cognitive impairment (MCI) and the degree of olfactory degradation may be used to predict which individuals with MCI will develop Alzheimer's disease (Devanand et al., 2000; Peters et al., 2003; Schubert et al., 2008, 2017; Roberts et al., 2016; Lafaille-Magnan et al., 2017; Adams et al., 2018).

Enhanced Olfactory Stimulation

Increasing olfactory stimulation in individuals who have experienced olfactory loss due to a variety of problems, such as post-infectious olfactory dysfunction, head trauma, Parkinson's disease, and aging, has been shown to improve olfactory identification, olfactory discrimination, and to a lesser extent, olfactory thresholds (Hummel et al., 2009; Haehner et al., 2013; Konstantinidis et al., 2013; Damm et al., 2014; Geißler et al., 2014; Patel et al., 2017). These results were achieved using twice daily fragrance exposures to four odorants taken from each of four odor groups: resinous (eucalyptus), flowery (rose), fruity (lemon), and aromatic (clove) and the individuals continued this regimen for varying durations, (i.e., 12 weeks to 6 months). Further improvements in olfactory ability were observed with increased duration of exposure, increased concentration of the odorants, or an increased number of odorants (Damm et al., 2014; Altundag et al., 2015; Konstantinidis et al., 2016). A recent review and meta-analysis by Pekala et al. (2016) concluded that olfactory training was effective in improving olfactory system function (e.g., odor discrimination and identification), and positive results were observed following the loss of olfactory function with different etiologies. In addition to improvements in sensory ability, older adults exposed to increased olfactory stimulation have an improvement in their cognitive function, as evidenced by increased verbal fluency, an improvement in their depressive symptoms, and an improved sense of wellbeing (Birte-Antina et al., 2018).

MASTICATION

Cognitive Deterioration With Dental Problems

There are hundreds of reports that have examined the relationship between mastication (chewing) and cognition. Researchers have found a strong correlation between cognitive decline and poor oral health (for reviews, see Chen et al., 2015; Azuma et al., 2017). For example, there are a number of studies showing that the fewer teeth an individual has, the worse their cognitive ability is (Bergdahl et al., 2007; Stein et al., 2007; Syrjälä et al., 2007; Grabe et al., 2009; Okamoto et al., 2010; Lexomboon et al., 2012; Del Brutto et al., 2014; Mummolo et al., 2014; Peres et al., 2014; Elsig et al., 2015; Luo et al., 2015; Stewart et al., 2015). Contributing factors that can lead to tooth loss are periodontal disease (Noble et al., 2009; Kamer et al., 2012; Gil-Montoya et al., 2015; Welmer et al., 2017), untreated tooth caries (Tonetti et al., 2017), and low socioeconomic scale (Cabrera et al., 2005; Matsuyama et al., 2017). The subsequent consequences of tooth loss include increased stress levels (Budtz-Jørgensen, 1980), decreased social interactions, lower quality of life (Griffin et al., 2012), and a limited diet (Walls et al., 2000; Spaccavento et al., 2009; Kimura et al., 2013). All of these factors may be involved in the degradation of cognition, making it difficult to conclude that chewing supports cognition directly.

On the other hand, when rats and mice have teeth removed, or are given only powdered or liquid food to eat, their cognitive ability also declines (Kato et al., 1997; Yamamoto and Hirayama, 2001; Fukushima-Nakayama et al., 2017), along with a decrease in brain-derived neurotrophic factor (BDNF), synaptic density,

and neuronal number in the hippocampus (Onozuka et al., 1999; Yamamoto and Hirayama, 2001; Okihara et al., 2014; Takeda et al., 2016). In addition, there is widespread volumetric loss of gray matter in the brain following tooth loss in mice (Avivi-Arber et al., 2016). At the same time, disruption of normal chewing leads to elevated levels of corticosterone (Kubo et al., 2007) and lower levels of hippocampal glucocorticoid receptors (Ichihashi et al., 2008) that are associated with chronic stress (Sapolsky et al., 1984). The advantage of examining chewing activity in non-human species is that the caloric intake and amount of food can be equalized across the hard and soft food eating conditions, thus maintaining similar nutritional values. The results of these animal studies support the idea that the active process of chewing plays a role in cognitive functioning and stress reduction. Indeed, in humans, a stronger correlation was observed between chewing ability (with or without the use of dental prostheses) and cognitive function than that observed between tooth loss and cognition (Lexomboon et al., 2012).

Enhanced Mastication/Corrected Dentition and Cognitive Restoration

While the loss of normal chewing ability is associated with cognitive decline, increased chewing activity has been shown to decrease stress and improve cognition in humans (Baker et al., 2004; Stephens and Tunney, 2004; Scholey et al., 2009; Ono et al., 2010; Yu et al., 2013; Azuma et al., 2017; for a review, see Weijenberg et al., 2011). Chewing gum alone can increase attention, decrease reaction times, and improve mood, even under stressful conditions (Smith, 2010; Kubo et al., 2015), as well as increasing cognitive processing speed (Hirano et al., 2013). A meta-analysis found positive effects of chewing on alertness or attention in 64% of the studies they examined (Hirano and Onozuka, 2015). In contrast, Tucha et al. (2004) observed chewing gum only changed aspects of attention without cognitive improvement. Consistent with the notion that chewing may aid cognition, the brain areas that are activated by chewing include cognitive centers such as the frontal cortex and the medial temporal lobe (Onozuka et al., 2002; Choi et al., 2017).

Rats and mice given wood dowels to chew have a reduced stress response and are able to maintain their hippocampal-dependent cognitive function (Ono et al., 2010; Miyake et al., 2012; Chen et al., 2015). In addition, the chewing action relieves the stress-induced suppression of cell proliferation in the hippocampal dentate gyrus that may underlie the impaired hippocampal functioning with tooth loss (Kubo et al., 2007). Also, switching mice from powdered food to hard pellet food reverses the suppression of neurogenesis in the forebrain subventricular zone that occurred while on the soft food diet (Utsugi et al., 2014).

Humans who have lost teeth and were subsequently given dentures or dental implants that restored dental function also had improved prefrontal cortex activity (Narita et al., 2009; Kimoto et al., 2011; Kamiya et al., 2016), along with improved cognitive performance (Cerutti-Kopplin et al., 2015; Banu et al., 2016; De Cicco et al., 2016).

SENSORIMOTOR/SOMATOSENSORY STIMULATION

Exercise Increases/Restores the Size of Cortical Structures

Decreases in the size of the brain, including the hippocampus, are observed in normally aging older adults, and this decrease accompanies the declines in cognitive functioning (Raz et al., 2005). Aerobic exercise can restore some of these decreases in brain volume. About 12 months of aerobic exercise resulted in an increase in the volume of the hippocampus (Erickson et al., 2011, 2014; Niemann et al., 2014) and 6 months of aerobic training increased the volume of gray and white matter, while stretching and toning for the same amount of time did not result in brain volume changes (Colcombe et al., 2006). In addition, both hippocampal volume and dorsolateral prefrontal cortex thickness were positively correlated with aerobic fitness (Jonasson et al., 2017).

Cognitive Benefits of Exercise

Multiple studies, reviews, and meta-analyses have concluded that in older adults, exercise training results in improvements in cognitive skills, along with improved health and mobility (Colcombe and Kramer, 2003; Hertzog et al., 2008; Kemoun et al., 2010; Bherer et al., 2013; Kirk-Sanchez and McGough, 2014; Barha et al., 2017; Gregory et al., 2017; Kennedy et al., 2017; Mavros et al., 2017; Saez de Asteasu et al., 2017; Northey et al., 2018; for review see Erickson et al., 2013). In addition, following exercise training, improvements in cognitive functioning are correlated with measurable increases in brain volume (Erickson et al., 2011; Mortimer et al., 2012) and increased BDNF levels (Erickson et al., 2011; Sungkarat et al., 2018), both of which may underlie the observed cognitive gains (Erickson et al., 2013). Exercise training also restores the decrease in interhemispheric inhibition typically observed in older adults, which could lead to improved motor control (McGregor et al., 2018). In addition, using fMRI to measure brain activity, exercise for 12 months improved functional connectivity among the cortices and the improved connectivity was associated with improved executive function (Voss et al., 2010). Moreover, adults who exercised showed altered activity in the right inferior frontal gyrus during a semantic verbal fluency task that was correlated with improvements in the task (Nocera et al., 2017). A recent meta-analysis that examined the influence of exercise, cognitive training, or the two combined on falls and cognition in older adults with MCI found that exercise or the combination of exercise and cognitive training resulted in gait speed and balance improvements, as well as cognitive function gains, all of which can contribute to decreased incidence of falls (Lipardo et al., 2017).

Mechanisms by Which Exercise Can Improve Cognition

Stimpson et al. (2018) have proposed a model for the mechanism underlying cognitive improvement following

exercise. Specifically, they first note that exercise increases cerebral angiogenesis and circulation in the brain. Pereira et al. (2007) reported that after 3 months of aerobic exercise, there was increased neurogenesis and increased cerebral blood volume in the human hippocampus, raising the possibility that increased angiogenesis mediated these improvements. There is also a transient elevation of serum BDNF after humans exercise (Ferris et al., 2007; Håkansson et al., 2017), and when the action of BDNF is blocked in rats after exercise, the cognitive benefits are also blocked (Vaynman et al., 2004). They next noted that exercise also decreases chronic inflammation in older adults (Cotman et al., 2007) and that restores elevated levels of insulin-like growth factor (IGF-1), which elevates BDNF levels and increases neurogenesis (Carro et al., 2000). The increased neurogenesis then allows the improved cognitive outcomes following exercise. It should be noted, however, that the presence of adult human neurogenesis has been recently questioned (Sorrells et al., 2018).

It is also the case that a relatively recent meta-analysis on studies showing aerobic gains with exercise came to the conclusion that there was no compelling evidence indicating that exercise improves cognitive abilities in older adults (Young et al., 2015). If increased aerobic capacity does not drive the effects of exercise, then it seems possible that the increase in somatosensory stimulation that is experienced during exercise may drive any improvements that are seen following exercise. This perspective would then bring any cognitive improvements with exercise in line with the effects of other types of sensory stimulation that we have discussed.

CONCLUSION

Deterioration of Sensory Systems Can Be Mitigated

It seems clear that the deterioration of sensorimotor systems contributes to the decline in cognition seen in older adults, either directly, by providing less stimulation to the cognitive areas of the brain, or indirectly, by depriving older adults of the nutrition, intellectual engagement, or social engagement that they need to thrive. Currently, there are methods that attempt to restore the declining systems, and although they are not always able to completely restore sensory system function, they do appear to aid individuals, with improvements to sensory acuity, cognition, and quality of life, thus improving chances of successful aging. Importantly, it is apparent that early detection of deficits to any of these systems is critical, and restoration or repair of any deficits should be prioritized to increase the likelihood of maintaining cognitive and full body health. Most certainly, new technologies are needed to continue to refine and improve the restoration of individual sensorimotor systems. Additional environmental enrichment using improvements to mastication or exercise training results in cognitive and health gains, which may potentially further aid in the successful aging of older adults.

Barriers to Maintenance or Restoration of Cognition Function by Restoring Deteriorating Sensory Systems in Older Adults

Although the aids described previously for use in the restoration of sensory and motor systems are promising, the benefits are not without a cost and may not be experienced by everyone. While it makes sense to do what one can to sustain or repair these systems, the cost of such repairs is not affordable by most people. Perhaps the greatest need is in hearing aids because a set can cost thousands of dollars and these devices are typically not covered by medical insurance in the United States. Consequently, only 3–4% of those with mild hearing loss who need hearing aids are wearing them (Chien and Lin, 2012). Cochlear implants, while even more expensive, can be covered by medical insurance, but are called for only in those with severe hearing impairments and in deaf individuals (Lin et al., 2012). The use of reading glasses seems to be a useful tool to allow older adults to engage with their environment effectively. While some investigators find a cognitive benefit for cataract surgery, others do not observe such an improvement, suggesting that the cognitive benefits may not be reliable. Dentures and dental implants are effective for maintaining or restoring cognitive ability for older adults with poor dentition, but again it comes at great expense, and it is something that Medicare does not cover. Enhanced olfactory stimulation has had limited tests for cognitive improvement and there are no studies showing improved neural responses after enhanced olfactory stimulation in older adults.

It is difficult to induce older adults to exercise regularly. Only about 16% of older adults engage in the recommended amount of physical activity on a daily basis (Centers for Disease Control and Prevention [CDC], 2018), which are at least 150 min of moderate-intensity aerobic activity or 75 min of vigorous-intensity aerobic activity and 2 or more days of muscle-strengthening activities per week (Centers for Disease Control and Prevention [CDC], 2018). Indeed, 33% of older adults reported no physical activity at all (Centers for Disease Control and Prevention [CDC], 2018). Barriers to exercise include lack of motivation, lack of knowledge, pain, poor health, physical limitations, peer pressure, and bad weather (Costello et al., 2011; Gothe and Kendall, 2016). Surprisingly, older adults do not have an increased risk of injury from exercise (Stathokostas et al., 2013). Although there is a concern that the oxidative stress associated with intense exercise would hasten aging in older adults, physically active older adults actually have reduced exercise-induced oxidative stress than older adults with a lower level of physical activity. Additionally, regular physical activity apparently improves the antioxidant defenses of older adults (Meijer et al., 2002).

The Possibility of a Common Factor

While each sensory system may have its impact on cognition in its own way, there is also the possibility that there is a

common factor that underlies their role in the maintenance of cognitive processes. For example, the loss or degradation of each sensory system may result in an emotional change that could impact cognitive performance. As discussed above, depression is associated with the loss of teeth (Shah et al., 2015), is associated with the loss of olfactory stimulation (Kohli et al., 2016), and often follows the degradation of the auditory and visual systems. Depression also often follows cognitive dysfunction in older adults (Yin et al., 2015). Alternatively, the common factor could be a physiological element that deteriorates both sensory and cognitive brain systems concurrently, but deficits are more easily detectable in the sensory systems. It also may be that continual exposure to environmental elements may wear on the sensory systems before they impact the cognitive brain areas.

Future Directions

One future direction may be to enhance stimulation of olfactory, visual, auditory, tactile, masticatory, and motor systems simultaneously or in conjunction. This type of sensorimotor stimulation had benefits for individuals with developmental neurological disorders (Woo and Leon, 2013; Woo et al., 2015; Aronoff et al., 2016). Environmental enrichment has also been shown to improve the symptoms of children with Rett syndrome (Downs et al., 2018). Similar forms of sensorimotor combination therapy may have health benefits for aging adults.

AUTHOR CONTRIBUTIONS

ML and CW conceived and wrote this review.

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frontiers in Behavioral Neuroscience

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Environmental Enrichment Elicits a Transient Rise of Bioactive C-Type Natriuretic Peptide in Young but Not Aged Rats

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Beneficial molecular and neuroplastic changes have been demonstrated in response to environmental enrichment (EE) in laboratory animals across the lifespan. Here, we investigated whether these effects extend to C-type Natriuretic Peptide (CNP), a widely expressed neuropeptide with putative involvement in neuroprotection, neuroplasticity, anxiety, and learning and memory. We determined the CNP response in 36 young (8–9 months) and 36 aged (22–23 months) male PVGc hooded rats that were rehoused with new cage mates in either standard laboratory cages or EE for periods of 14 or 28 days. Tissues were rapidly excised from four brain regions associated with memory formation (dorsal hippocampus, retrosplenial cortex, medial prefrontal cortex, and mammillary bodies) plus the occipital cortex and hypothalamus, and immediately frozen. Radioimmunoassay was used to measure bioactive CNP and the amino-terminal fragment of proCNP, NTproCNP. Because CNP but not NTproCNP is rapidly degraded at source, NTproCNP reflects CNP production whereas the ratio NTproCNP:CNP is a biomarker of CNP's local degradation rate. EE increased CNP at 14 days in all brain regions in young, but not old rats; this effect in young rats was lost at 28 days in all regions of interest. NTproCNP:CNP ratio, but not NTproCNP, was reduced in all regions by EE at 14 days in young rats, but not in old rats, which suggests a period of reduced degradation or receptor mediated clearance, rather than increased production of CNP in these young EE rats. Aged rats tended to show reduced NTproCNP:CNP ratios but this did not occur in dorsal hippocampus or mammillary bodies. This is the first study demonstrating modulation of CNP protein concentrations, and the effect of age, in response to environmental stimulation. Furthermore, it is the first to show that changes in degradation rate *in vivo* may be an important component in determining CNP bioactivity in neural tissues.

Keywords: C-type natriuretic peptide, environmental enrichment, aging, medial prefrontal cortex, hippocampus, retrosplenial cortex

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INTRODUCTION

Multiple beneficial effects of Environmental Enrichment (EE) have been established over 60 years of research using the paradigm. These variously occur at molecular, cellular and behavioral levels and relate to neurogenesis, synaptic plasticity, neuroprotection, and modifications to anxiety and learning and memory across the lifespan (see Rosenzweig and Bennett, 1996; van Praag et al., 2000; Will et al., 2004; Nithianantharajah and Hannan, 2006; Clemenson et al., 2015 for selected reviews). Less well-established is the neurological action of C-type Natriuretic Peptide (CNP), a proposed neuroendocrine regulator, with similar associations to those effects modified by EE.

CNP is the most recently discovered, but most primitive member of the Natriuretic Peptide family alongside Atrial and B-type Natriuretic Peptides. The family shares a ring structure consisting of 17 amino acid residues, flanked by two cysteine residues forming a disulfide bond critical to biological activity (Pandey, 2005; Potter et al., 2006). Three forms of the peptide occur: expression of the Nppc (Natriuretic Peptide Precursor-C) gene elicits the initial 126 residue preproCNP (Tawaragi et al., 1990) resulting in the 103 residue proCNP, which is cleaved intracellularly to produce CNP-53 and a biologically inactive amino terminal fragment, NTproCNP, both of which are secreted extracellularly in equimolar amounts (Wu et al., 2003; Prickett and Espiner, 2012). Further extracellular processing results in CNP-22, thought to be the fully active form of the peptide, with CNP-53 proposed as a storage form (Barr et al., 1996). CNP-22 is the most highly conserved of the Natriuretic Peptide family and is identical in all mammals studied to date (Pandey, 2005; Potter, 2011). CNP binds to a specific receptor, Natriuretic Peptide Receptor-B (NPR-B), through which it increases intracellular concentrations of cyclic Guanosine Monophosphate (cGMP; Yeung et al., 1996; Potter et al., 2006; Potter, 2011). Regulation of the peptide occurs via a membrane bound clearance receptor (NPR-C), which clears all Natriuretic Peptides from the extracellular space; and proteolytic degradation by neprilysin (primarily) and insulin degrading enzyme (Müller et al., 1992; Kenny et al., 1993; Watanabe et al., 1997; Potter, 2011).

It is important to note that the concentration of CNP at any time point is a function of both production and clearance or degradation rates. Since NTproCNP is not subject to rapid degradation or clearance (half-life of 30–40 min compared with 2–3 min for CNP), it provides a reliable measure of NPPC gene expression and peptide production (Schouten et al., 2011; Woodward et al., 2017). Calculation of NTproCNP:CNP ratio (by taking NTproCNP/CNP concentrations) provides an index of CNP's clearance or degradation in the tissue sampled, either via NPR-C mediated internalization or hydrolysis by neprilysin. Higher ratios reflect increases in the rate of loss of the bioactive form of the peptide. Combined measurement of CNP and NTproCNP, with calculation of the ratio, allows separate contributions of peptide production and metabolic clearance to be determined.

High concentrations of CNP are found in brain tissue, with the peptide constituting the major neuroactive member of the natriuretic peptide family (Komatsu et al., 1991; Kaneko et al., 1993; Totsune et al., 1994; Langub et al., 1995a; Pemberton et al., 2002; Jankowski et al., 2004; Wilson et al., 2017). In rodents, both CNP and NPR-B mRNA occur variably throughout olfactory bulb, basal forebrain, basal ganglia, limbic cortices, thalamus, amygdala, mammillary nuclei, hippocampus, and cerebellum (Langub et al., 1995a,b; Herman et al., 1996). As CNP is widely accepted to act in a paracrine/autocrine fashion, this regional distribution suggests a role for the peptide in the integration of sensation and emotion into memory.

This hypothesis receives initial support from the relatively sparse literature regarding CNP's effects within brain, and on behavior. Additionally, these findings indicate similarities between CNP's action and the beneficial effects of EE specifically in terms of neurogenesis, neuroplasticity, modification of anxiety behaviors, learning and memory. CNP induces a switch from proliferation to maturation in olfactory neuronal precursors (Simpson et al., 2002), is necessary for bifurcation of sensory axons (Schmidt et al., 2007, 2009; Zhao and Ma, 2009) and is associated with the onset of neurogenesis within the developing nervous system (DiCicco-Bloom et al., 2004). Within retinal ganglion cells, CNP is neuroprotective against cytotoxic injury both *in vivo* and *in vitro* (Ma et al., 2010). Within the hippocampus, CNP modifies several electrophysiological correlates of synaptic plasticity, and notably impedes long-term potentiation while facilitating long-term depression within CA1 (Decker et al., 2008, 2009, 2010). In relation to anxiety behavior, CNP may have a bidirectional effect as it is anxiolytic in low dose ranges, but anxiogenic when higher doses are administered (Bíró et al., 1996; Montkowski et al., 1998; Jahn et al., 2001). Such a bidirectional effect may also occur in relation to learning and memory behaviors: CNP improves learning in a passive avoidance task (Telegdy et al., 1999; Telegdy et al., 2000), but functional downregulation of NPR-B receptor signaling is associated with improved object-recognition memory (Barmashenko et al., 2014). Based on these emerging similarities between CNP's effects and effects demonstrated in EE, we posited that the paradigm would provide an excellent context for study of CNP within brain tissue.

In addition to a possible effect of EE on CNP, it has been recently suggested that Natriuretic Peptides may be involved in cognitive impairment and pathologies related to aging (Mahinrad et al., 2016). Although no relationship was previously found between age and CNP concentration in cerebrospinal fluid across a specific age-range in Parkinson's Disease patients (51–90 years; Schouten et al., 2011; Espiner et al., 2014), this does not exclude the possibility that regional variations may still be found within cerebral tissue. A primary question is whether CNP expression or degradation within brain tissue varies during normal aging. To this end, we analyzed brain tissue of both young and aged rats housed in either standard or enriched cages for two different periods of housing, selected to be representative of shorter- and longer-term exposure typical of many enrichment studies (Simpson and Kelly, 2011). Given

previously demonstrated reductions of neprilysin in fronto-temporal regions with brain aging (Iwata et al., 2002; Apelt et al., 2003; Fjell et al., 2014), general reductions to the NTproCNP:CNP ratio were expected in aged rats. As this is the first study of its kind, no predictions could be made about how enriched housing may affect concentrations of CNP, or synthesis of the peptide measured by NTproCNP concentrations in either age group.

METHODS

Subjects

Thirty-six male PVGc hooded rats aged 8–9 months old (weights between 306 and 413 g) and 36 male PVGc hooded rats aged 22–23 months (weights between 293 and 440 g) at the start of enrichment were treated equivalently in all respects. Prior to enrichment, all rats were housed in standard opaque plastic cages (45 × 27 × 22 cm high) in groups of three or four, from weaning until placement in enrichment at the stated ages. For each age group, on the first day of enrichment, 24 rats were re-housed in two enrichment cages (12 per cage) for either 14 or 28 days (1 cage per time-period; Enriched-14-day and Enriched-28-day). Twelve remaining rats were re-housed with new cage mates in standard cages (3 rats per cage) for the same time periods (2 cages per time-period; 6 rats total per time-period; Standard-14-day and Standard-28-day). All rats were rehoused with novel cage mates. Rats were maintained on a reversed light-dark cycle (lights off 08:00–20:00 h) and colony rooms were maintained at 22°C and 48% relative humidity. Food and water were available *ad libitum*. All procedures conformed to the NIH guide for the care and use of laboratory animals and were approved by the University of Canterbury Animal Ethics Committee.

Enrichment

A standardized enrichment protocol developed at the University of Canterbury was used (Harland et al., 2014; and see http://www.psyc.canterbury.ac.nz/Standardized%20Enrichment.shtml for details of, objects, arrangements and procedures). Enrichment cages were made of wire mesh with a sawdust covered metal floor and measured 85 cm x 60 cm x 30 cm high. Each day of enrichment consisted of a different combination of "junk" objects such as ceramic figurines, metal chains, PVC pipes and junctions and other small items, along with an ever-present wooden block to discourage chewing of enrichment objects. Enrichment configurations differed over 40 different days (though a maximum of 28 was used here) and ensured no object was repeated within 5 days of itself. On every seventh day, PVC pipes and junctions were presented in a "tubing only" day, and on every eighth day, all objects (except the wooden blocks) were removed from the cage. Additionally, food and water positions were changed daily, and cages were rotated through one of four possible positions in the colony room every fourth day. Objects were changed at the start of the dark period (between 09:00 and10:00h), during which rats from one enrichment cage were held together in a large opaque plastic cage (62 × 40 × 22 cm high).

Sacrifice and Tissue Extraction

After either 14 or 28 full days of enrichment, or the same period of standard housing with new cage mates, rats were placed in standard cages at the same time enrichment objects were usually changed and held in a novel, dimly lit room separate to both the colony room and tissue dissection room. Rats from Enriched and Standard Housing were euthanised throughout a single day, with two Enriched rats sacrificed per one Standard Housed, and order of sacrifice randomized within these groups of three. Rats were deeply anesthetised with an overdose of Sodium Pentobarbitone (1mL, 300 mg/mL, ip). Once unresponsive to both tail pinch and plantar reflex (∼5 min following injection), with no discernible heartbeat, rats were decapitated, and brains rapidly removed from the skull and placed in a brain matrix (Ted Pella). An initial coronal cut was made at the level of the optic tract. Additional coronal cuts were made 5 mm anterior and posterior to the initial cut, resulting in two "slabs" of fresh tissue (anterior extent approximately equivalent to Bregma +3.20 mm; posterior extent approximately equivalent to Bregma −6.30 mm; from the atlas of Paxinos and Watson, 1998). These tissue slabs were placed anterior-face-upwards on a glass petri dish, previously sterilized with 70% ethanol and rinsed with saline. Sterilization and rinsing was repeated between rats. Microdissection scissors were used to acquire tissue samples from seven regions of interest, starting in the posterior slab, in the order: occipital cortex, retrosplenial cortex (containing both dysgranular and granular B tissue), dorsal hippocampus (left and right hemispheres separately), mammillary bodies, hypothalamus and medial prefrontal cortex. Tissue samples were placed in pre-weighed Eppendorf tubes, weighed, and snap frozen with liquid nitrogen, within 15 min post-sacrifice, before long-term storage at −80°C.

Tissue Preparation and Peptide Measurements

CNP and NTproCNP were measured by radioimmunoassay (RIA), described in detail in (Yandle et al. (1993) CNP) and (Yandle et al. (1993) NTproCNP). Prior to assay, frozen tissue samples were transferred to dry ice chilled scintillation vials, then 10 mL of boiling distilled water containing 0.01% Triton X-100 was added and the vials were held at 98°C in a water bath for 5 min. Samples were acidified with 610 μL glacial acetic acid and homogenized prior to extraction on Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA). Following extraction, samples were dried under an air stream and frozen for later re-suspension in assay buffer for RIA. All tissues from an individual rat were processed in the same extraction, with tissues from housing groups counterbalanced across extractions.

CNP Assay

CNP-22 is identical in all mammals studied thus far. CNP-22 and the amino terminal extended form CNP-53, both contain the 17-amino acid ring essential for bioactivity and show 100% cross reactivity in the assay used to measure CNP. Antiserum to CNP-22 (G-012-03, Phoenix Pharmaceuticals, Belmont, CA) was diluted 1:2,000 with assay buffer. Labeled CNP was prepared by chloramine-T iodination of [Tyr0] CNP-22 (Peninsula Labs) and purified by reverse-phase HPLC. Fifty microliters each of

antiserum and CNP standard (0.7–235 pmol/L) or sample extract (all in duplicate) were mixed and incubated for 22–24 h at 4°C, followed by addition of 50 μL labeled CNP containing 2,000 cpm for 22–24 h at 4°C. Bound and free labeled CNP were separated by a solid-phase secondary antibody method (Sac-cell, Rabbit-Anti Goat, IDS Ltd., England). CNP assays had a detection limit of 0.6 pmol/L and ED50 of 7.3 pmol/L; intra- and inter-assay coefficients of variation were 5.9 and 7.4%, respectively, at 17 pmol/L.

NTproCNP Assay

For NTproCNP an in-house antiserum was used that recognizes the C-terminal epitope in the region of proCNP (38–50), which is identical in human, mouse, and rat (Prickett et al., 2012). Fifty microliters of sample extract or 0.5–372 pmol/L proCNP(36–50) standards (again in duplicate) were incubated with 50 μL ovine antiserum (Sheep43) for 22–24 h, followed by addition of 50 μL tracer solution (proCNP(38-50)-[125I]Tyr37) containing 2,000 cpm for 22–24 h at 4°C. Bound and free labeled proCNP were separated by solid-phase second antibody method (Sac-cell, Donkey-Anti Sheep, IDS Ltd., England). NTproCNP assays had a detection limit of 0.4 pmol/L and ED50 9.9 pmol/L; intra- and inter-assay coefficients of variation were 6.8 and 7.5%, respectively, at 45 pmol/L.

Statistical Analyses

CNP and NTproCNP concentrations were expressed as femtomoles per gram of wet tissue (fmol/g). NTproCNP:CNP ratio was calculated by dividing NTproCNP concentration by CNP concentration. In young rats, CNP assay failed in one sample from each of retrosplenial (Standard-14-day group) and occipital (Enriched-14-day group) cortices and data was excluded, reducing sample Ns in these regions. In aged rats, a portion of one sample from the hypothalamus (Enriched-28-day group) was lost, and data subsequently excluded. Two tissue samples had NTproCNP concentrations beyond the detection limit of the assay: one from mammillary bodies (Enriched-14-day group) and one from hypothalamus (Enriched-28-day group). Thus, sample Ns for aged rats are reduced for each of these regions (mammillary bodies and hypothalamus). In both age groups, final Ns for all other regions were: Enriched-14-day, $N = 12$; Enriched-28-day, $N = 12$; Standard-14-day, $N = 6$; Standard-28-day, $N = 6$. Initial analysis indicated there were no differences in any measure (CNP, NTproCNP or ratio) between left and right hemispheres of the dorsal hippocampus. Data was averaged across hemispheres for each rat and analyzed as a single value. Data acquisition and analysis for Young rats was conducted prior to acquisition of Aged rat data. As the Null Hypothesis was rejected in most cases for Young rats (see results), data from Aged rats were compared directly using cohen's *d* effect sizes. Analysis of data for Young rats was by Robust 2-way ANOVA with 20% trimmed means (after Mair and Wilcox, 2016; using WRS2 package for "R"). The statistical outcome measure of this test—Q—is interpreted in the same fashion as the traditional F statistic. *Post-hoc* testing of Young rats and Aged rat comparisons by effect size (cohen's *d* [± 95% CI]) calculations were all based on trimmed measures (using compute.es package for "R"). Figures provide boxplots displaying trimmed mean, standard error and 95% confidence intervals, overlaid with all data points (constructed using ggplot2 for "R").

RESULTS

NTproCNP Concentrations

For Young rats, NTproCNP concentrations did not vary in occipital cortex (**Figure 1**; overall M [95% CI] = 749.38 [691.36, 807.40]), medial prefrontal cortex (**Figure 2**; overall M [95% CI] = 1847.26 [1739.99, 1954.53]), or mammillary bodies (**Figure 4**; overall M [95% CI] = 4938.76 [4519.69, 5357.83]). NTproCNP concentrations also did not vary for Aged rats in occipital cortex (**Figure 1**; overall M [95% CI] = 854.61 [801.54, 907.68]), medial prefrontal cortex (**Figure 2**; overall M [95% CI] = 1599.42 [1448.72, 1750.12]), or mammillary bodies (**Figure 4**; overall M [95% CI] = 4245.68 [3547.89, 4943.48]). Concentrations of NTproCNP for Aged rats were higher than for Young rats in occipital cortex (**Figure 1**; $d = 0.62$ [0.14, 1.11], $p = 0.01$), lower than Young rats in medial prefrontal cortex (**Figure 2**; $d = 0.62$ [0.14, 1.10], $p = 0.01$), and equivalent in mammillary bodies (**Figure 4**). In retrosplenial cortex of Young rats (**Figure 3**), NTproCNP concentrations were lower at 14 days after rehousing than 28 days after rehousing, in Standard cages only (Housing x Time Interaction: $Q_{(1,31)} = 6.40$, $p = 0.026$; Young-Standard-14-day vs. Young-Standard-28-day ($d = 2.12$ [0.41, 3.83], $p = 0.02$). No significant between group differences were identified for NTproCNP for Aged rats in retrosplenial cortex (**Figure 3**). In dorsal hippocampus, NTproCNP concentrations were lower in Enriched housing than Standard housing, regardless of Time since rehousing (**Figure 5**; Main effect of Housing: $Q_{(1,32)} = 7.55$, $p = 0.017$; Young-Enriched vs. Young-Standard $d = 0.87$ [0.12, 1.62], $p = 0.02$). Again, no effects were identified in Aged rats (**Figure 5**). In hypothalamus, NTproCNP concentrations were higher following 14 days of rehousing than 28 days, regardless of Housing condition (**Figure 6**; Main effect of Time $Q_{(1,32)} = 11.05$, $p = 0.01$; Young-14-day vs. Young-28-day $d = 1.13$ [0.4, 1.86], $p < 0.001$). This effect was also not apparent in Aged rats (**Figure 6**).

CNP Concentrations

For Young rats, significant two-way interactions (Housing × Time) were identified in occipital cortex [$Q_{(1,31)} = 16.77$, $p = 0.001$], medial prefrontal cortex [$Q_{(1,32)} = 12.56$, $p = 0.003$], retrosplenial cortex [$Q_{(1,31)} = 4.45$, $p = 0.055$] and mammillary bodies [$Q_{(1,32)} = 7.41$, $p = 0.015$]. In each region (**Figures 1–4**), CNP concentrations were elevated in Young-Enriched-14-day rats compared to all other groups of rats (between group comparisons, **Table 1**). Additionally, in retrosplenial cortex CNP concentrations were reduced across time since rehousing for Standard Housed rats (Young-Standard-14-days vs. Young-Standard-28-days $d = 1.37$ [0.21, 2.54], $p = 0.02$). In dorsal hippocampus, higher concentrations of CNP were seen 14 days after rehousing with new cage mates, regardless of type of Housing [**Figure 5**; Main Effect of Time: $Q_{(1,32)} = 5.74$, $p = 0.03$; Young-14-day vs. Young-28-day $d = 0.7$ [0, 1.4], $p = 0.05$]. Although there was no significant effect of

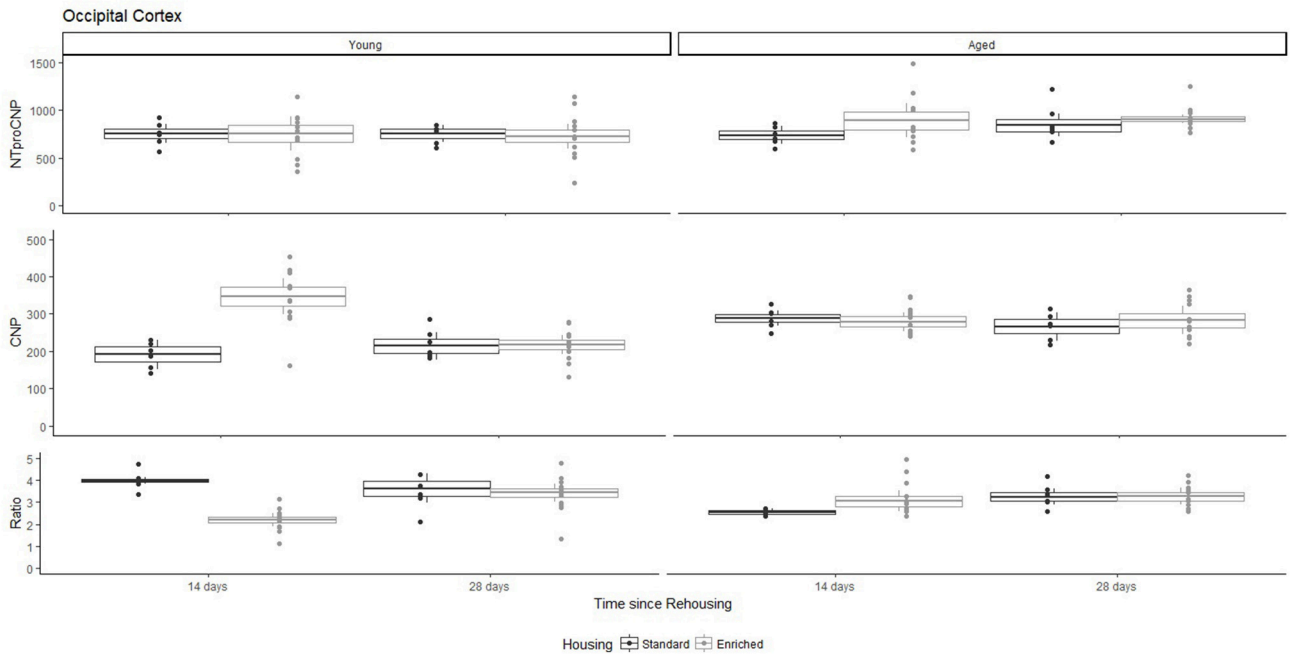

FIGURE 1 | Concentration (femtomoles/g wet tissue) of NTproCNP **(Top)**, CNP **(Middle)**, and NTproCNP:CNP ratio **(Bottom)** within occipital cortex. Summary data is 20% trimmed means (resulting in a reduction of $n = 2$ for all groups), standard error (box) and 95% confidence interval (whisker), overlaid by individual data points. For NTproCNP: Aged rats > Young rats. For CNP: Young-Enriched-14-day > Aged overall > Other Young rats. For Ratio: Young-Enriched-14-day < Aged overall < Other Young rats.

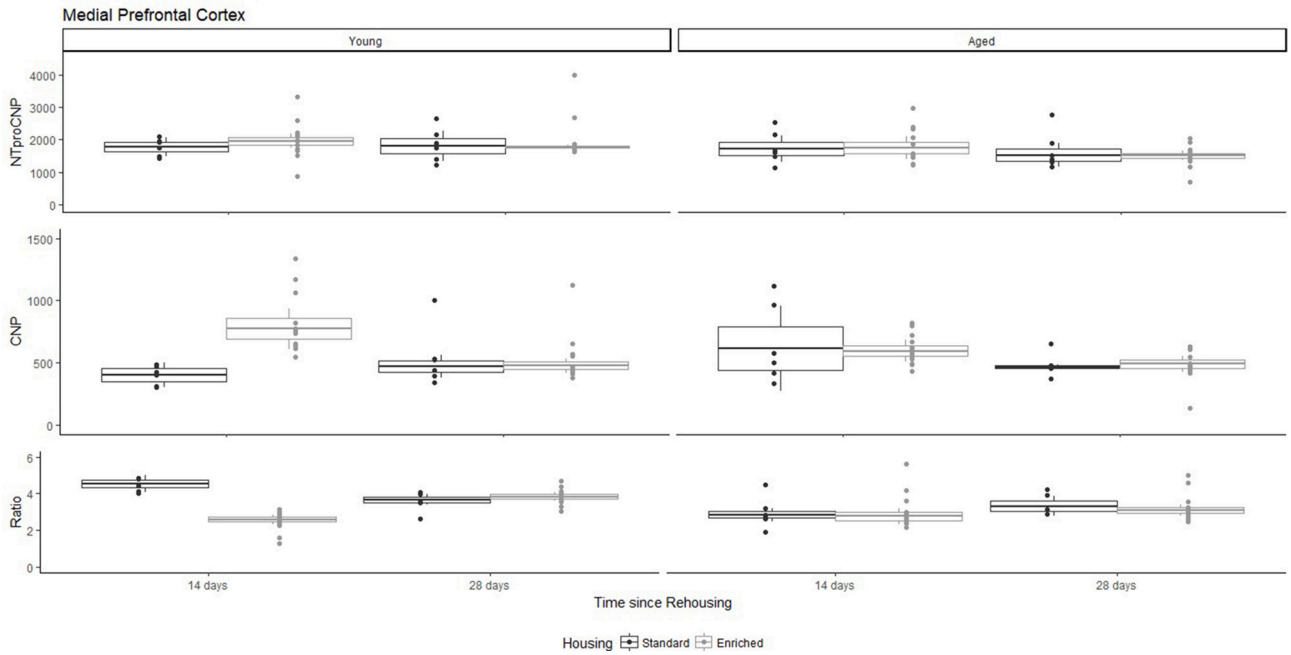

FIGURE 2 | Concentration (femtomoles/g wet tissue) of NTproCNP **(Top)**, CNP **(Middle)**, and NTproCNP:CNP ratio **(Bottom)** within medial prefrontal cortex. Summary data is 20% trimmed means (resulting in a reduction of $n = 2$ for all groups), standard error (box) and 95% confidence interval (whisker), overlaid by individual data points. For NTproCNP: Aged rats < Young rats. For CNP: Young-Enriched-14-day > Other Young rats; Aged-14-day > Aged-28-day. For Ratio: Young-Enriched-14-day = Aged overall < Other Young rats.

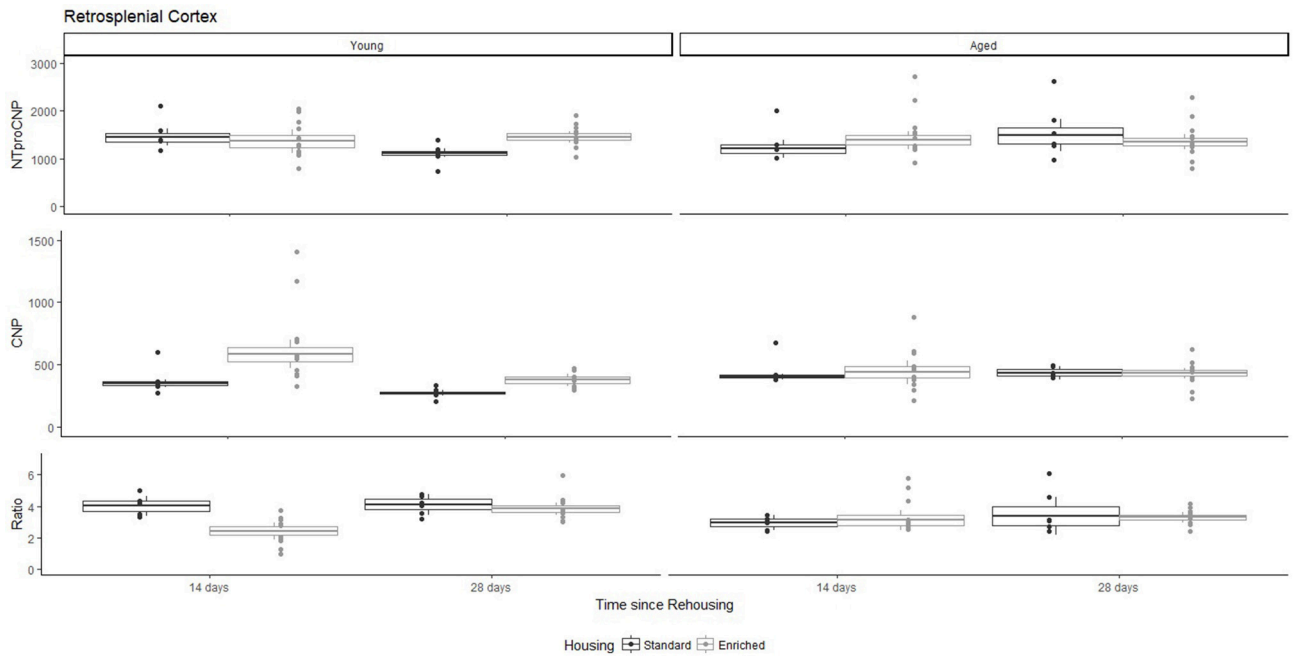

FIGURE 3 | Concentration (femtomoles/g wet tissue) of NTproCNP **(Top)**, CNP **(Middle)**, and NTproCNP:CNP ratio **(Bottom)** within retrosplenial cortex. Summary data is 20% trimmed means (resulting in a reduction of $n = 2$ for all groups), standard error (box) and 95% confidence interval (whisker), overlaid by individual data points. For NTproCNP: Young-Standard-14-day > Young-Standard-28-day. For CNP: Young-Enriched-14-day > Aged overall > Other Young rats. For Ratio: Young-Enriched-14-day < Aged overall < Other Young rats.

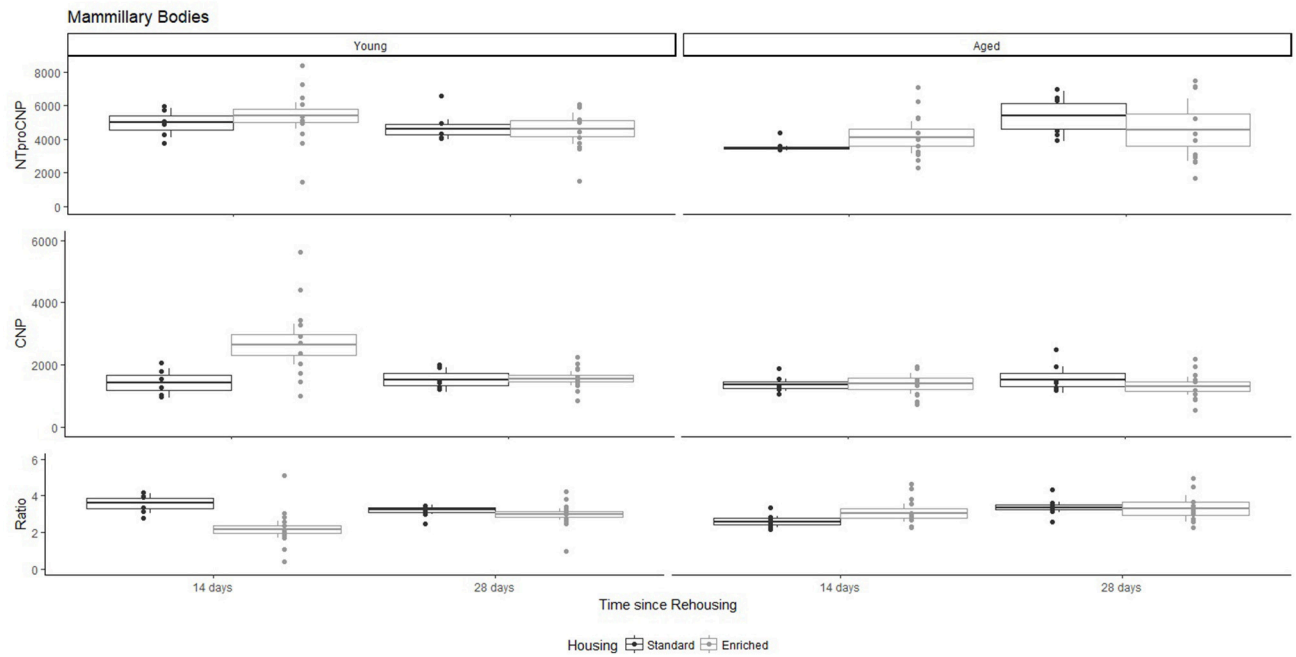

FIGURE 4 | Concentration (femtomoles/g wet tissue) of NTproCNP **(Top)**, CNP **(Middle)**, and NTproCNP:CNP ratio **(Bottom)** within mammillary bodies. Summary data is 20% trimmed means (resulting in a reduction of $n = 2$ for all groups), standard error (box) and 95% confidence interval (whisker), overlaid by individual data points. For NTproCNP: Aged rats = Young rats. For CNP: Young-Enriched-14-day > Aged overall = Other Young rats. For Ratio: Young-Enriched-14-day < Aged overall = Other Young rats.

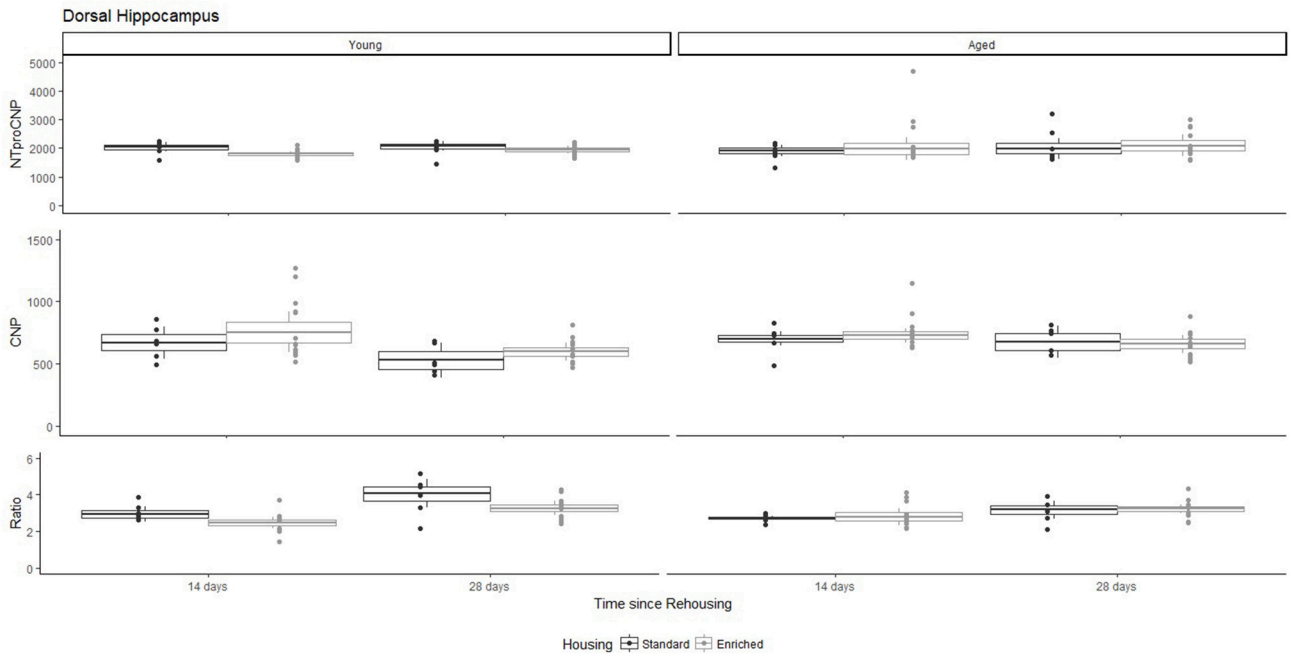

FIGURE 5 | Concentration (femtomoles/g wet tissue) of NTproCNP **(Top)**, CNP **(Middle)**, and NTproCNP:CNP ratio **(Bottom)** within dorsal hippocampus. Summary data is 20% trimmed means (resulting in a reduction of n = 2 for all groups), standard error (box) and 95% confidence interval (whisker), overlaid by individual data points. For NTproCNP: Young-Enriched < Young-Standard. For CNP: Young-14-day = Aged overall > Young-28-day. For Ratio: Young-Enriched-14-day < Aged overall = Other Young rats; Young-14-day < Young-28-day.

Housing, highest concentrations of CNP occurred within Young-Enriched-14-day rats (**Figure 5**). In hypothalamus, significant Main Effects of Housing [$Q_{(1,32)} = 12.66$, $p = 0.002$] and Time [$Q_{(1,32)} = 21.00$, $p = 0.001$] were such that CNP concentrations were higher in Enriched rats than Standard Housed rats, and higher 14 days following rehousing with new cage mates (**Figure 6**). The additive nature of these effects was reflected in significantly higher concentrations of CNP in Young-Enriched-14-day rats vs. all other groups (between group comparisons, **Table 1**).

For Aged rats, CNP concentrations did not vary significantly in any region, in individual group comparisons (**Figures 1**–**6**). The sole effect on CNP concentrations identified within Aged rats was in medial prefrontal cortex (**Figure 2**), wherein CNP concentrations were higher 14 days after rehousing with new cage mates than at 28 days of rehousing ($d = 0.68$ [−0.01, 1.38], $p = 0.005$). CNP concentrations for Aged rats overall were lower than the peak seen in Young-Enriched-14-day rats in occipital cortex, retrosplenial cortex, mammillary bodies and hypothalamus (between group comparisons, **Table 1**). In occipital and retrosplenial cortices (**Figures 1**, **3**), CNP concentrations were higher in Aged rats than for other Young rats (i.e., excluding Young-Enriched-14-day rats; between group comparisons, **Table 1**). In the Aged hippocampus, concentrations of CNP were equivalent to Young rats rehoused for 14 days ($d = 0.12$ [−0.46, 0.47], ns), but higher than Young rats rehoused for 28 days ($d = -1.01$ [−1.62, −0.4], $p < 0.001$).

NTproCNP:CNP Ratio

For Young rats, significant two-way interactions were identified in occipital cortex [$Q_{(1,31)} = 14.76$, $p = 0.004$], medial prefrontal cortex [$Q_{(1,32)} = 49.00$, $p = 0.001$], retrosplenial cortex [$Q_{(1,31)} = 5.55$, $p = 0.039$] and mammillary bodies [$Q_{(1,32)} = 9.20$, $p = 0.009$]. In each region (**Figures 1**–**4**) NTproCNP:CNP ratio was reduced in Young-Enriched-14-day rats compared with all other Young rats (between group comparisons, **Table 2**). In dorsal hippocampus (**Figure 5**), NTproCNP:CNP ratio was lower 14 days following rehousing than at 28 days of rehousing [Main Effect of Time: $Q_{(1,32)} = 15.87$, $p = 0.003$] and lower in rats housed in Enrichment than in Standard cages [Main Effect of Housing: $Q_{(1,32)} = 7.03$, $p = 0.026$]. Lowest ratio values therefore occurred in Young-Enriched-14-day rats, but this difference was not significant compared with Young-Standard-14-day rats (between group comparisons, **Table 2**). In hypothalamus (**Figure 6**), NTproCNP:CNP ratio was lower in Enriched rats than Standard Housed rats [Main Effect of Housing: $Q_{(1,32)} = 12.08$, $p = 0.005$]. However, an overall mean comparison was not statistically significant (Young-Standard vs. Young-Enriched $d = 0.55$ [−0.18, 1.28], $p = 0.14$). Only Young-Enriched-14-day rats had lower ratio values than Young Standard Housed rats (between group comparisons, **Table 2**).

NTproCNP:CNP ratio did not generally vary across groups for Aged rats (**Figures 1**–**6**). Overall, NTproCNP:CNP ratio for Aged rats was lower than Young rats (excluding Young-Enriched-14-day rats) in occipital, medial prefrontal and retrosplenial cortices,

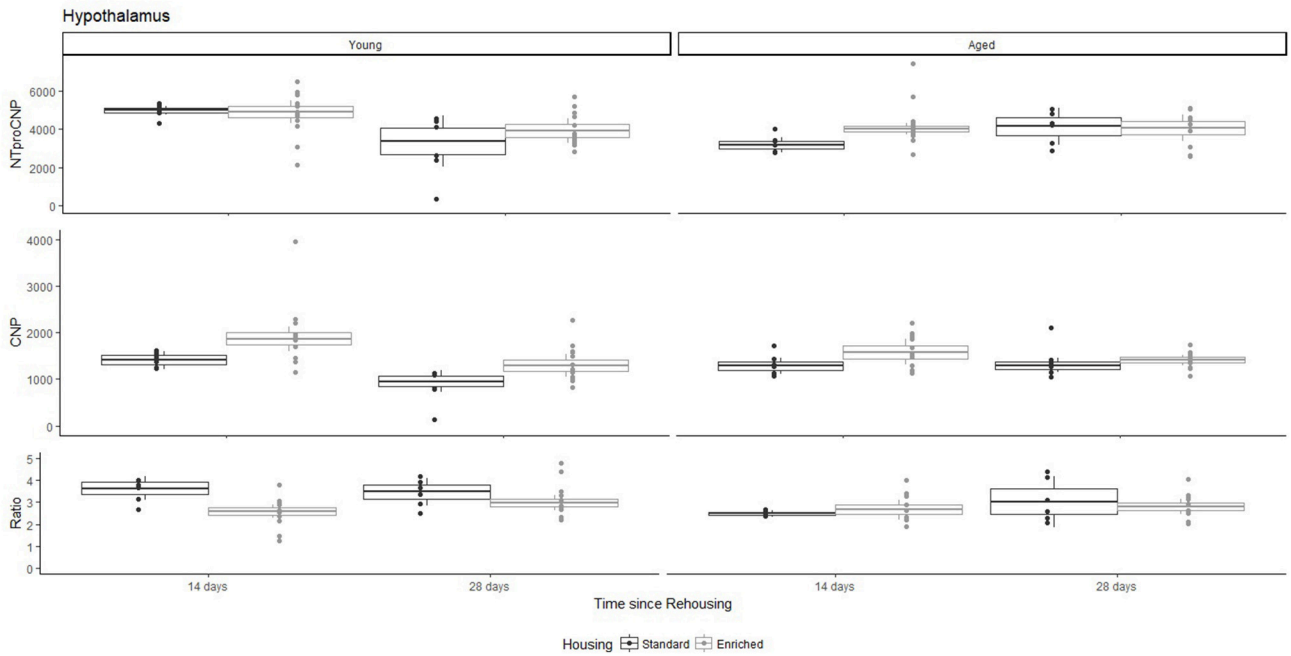

FIGURE 6 | Concentration (femtomoles/g wet tissue) of NTproCNP **(Top)**, CNP **(Middle)**, and NTproCNP:CNP ratio **(Bottom)** within hypothalamus. Summary data is 20% trimmed means (resulting in a reduction of $n = 2$ for all groups), standard error (box) and 95% confidence interval (whisker), overlaid by individual data points. For NTproCNP: Young-14-day > Young-28-day. For CNP: Young-Enriched-14-day > Aged overall = Other Young rats. For Ratio: Young-Enriched-14-day < Young-Standard. Aged overall < Other Young rats.

TABLE 1 | Between group comparisons (cohen's *d* [±95% CI]) for CNP concentrations within five regions of interest (see text for hippocampus and Aged medial prefrontal cortex).

Region		Young SH 14	Young SH 28	Young EE 28	Aged overall
Occipital Cortex	Young EE 14 vs.	$d = 2.14$ [0.8, 3.47] ***	$d = 1.85$ [0.57, 3.13]**	$d = 1.98$ [0.92, 3.04]***	$d = 1.22$ [0.48, 1.96]***
	Other Young vs.				$d = -1.57$ [−2.17, −0.97]***
Medial Prefrontal Cortex	Young EE 14 vs.	$d = 1.50$ [0.31, 2.68]*	$d = 1.22$ [0.08, 2.36]*	$d = 1.37$ [0.43, 2.31]**	
Retrosplenial Cortex	Young EE 14 vs.	$d = 1.35$ [0.11, 2.58]*	$d = 1.84$ [0.6, 3.09]**	$d = 1.32$ [0.38, 2.25]**	$d = 1.31$ [0.59, 2.03]***
	Other Young vs.				$d = -1.13$ [−1.71, −0.56]***
Mammillary Bodies	Young EE 14 vs.	$d = 1.22$ [0.08, 2.36]*	$d = 1.13$ [0.0, 2.26]*	$d = 1.27$ [0.34, 2.19]**	$d = 1.75$ [0.99, 2.52]***
	Other Young vs.				$d = 0.30$ [−0.24, 0.83] ns
Hypothalamus	Young EE 14 vs.	$d = 1.11$ [0.02, 2.24]*	$d = 2.17$ [0.86, 3.48]***	$d = 1.26$ [0.33, 2.18]**	$d = 1.21$ [0.49, 1.94]***
	Other Young vs.				$d = -0.45$ [−0.99, 0.09] ns

*EE, Enriched Environment; SH, Standard Housed; Other Young indicates pooled Young data, excluding Young-Enriched-14-day rats. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.*

and hypothalamus (between group comparisons, **Table 2**), but not in mammillary bodies or dorsal hippocampus.

DISCUSSION

Here, we provide the first data indicating a markedly different response of CNP and its signaling system in aged compared to young rats. The major finding in Young rats, is an increased availability of CNP in all regions of interest following 14 days of enriched housing, although this effect was weak within dorsal hippocampus. This increase in CNP can be accounted for by reduced proteolytic degradation or NPR-C mediated clearance of the peptide (indicated by NTproCNP:CNP ratio reductions) as opposed to increased production. Relatively minimal modifications to peptide production (NTproCNP) were restricted to tissues from hypothalamus, retrosplenial cortex and dorsal hippocampus. In contrast, within aged rats changes to CNP concentrations were restricted to medial prefrontal cortex and related to rehousing with new cage mates with higher concentrations of CNP at 14 vs. 28 days of housing regardless of housing condition. NTproCNP:CNP ratios were reduced in comparison with young rats generally in occipital cortex, medial prefrontal cortex, retrosplenial cortex, and hypothalamus as hypothesized, but this age-related reduction was not

TABLE 2 | Between group comparisons (cohen's *d* [±95% CI]) for NTproCNP:CNP ratio within six regions of interest.

Region		Young SH 14	Young SH 28	Young EE 28	Aged overall
Occipital Cortex	Young EE 14 vs.	$d = -4.34$ [−6.25, −2.42]***	$d = -2.28$ [−3.65, −0.92]***	$d = -2.0$ [−3.07, −0.94]***	$d = -1.33$ [−2.07, −0.58]***
	Other Young vs.				$d = 0.9$ [0.34, 1.45]***
Medial Prefrontal Cortex	Young EE 14 vs.	$d = -4.10$ [−5.89, −2.30]***	$d = -2.64$ [−4.05, −1.23]***	$d = -3.0$ [−4.23, −1.76]***	$d = -0.63$ [−1.32, 0.05] ns
	Other Young vs.				$d = 1.52$ [0.92, 2.11]***
Retrosplenial Cortex	Young EE 14 vs.	$d = -1.80$ [−3.11, −0.49]**	$d = -1.85$ [−3.10, −0.61]**	$d = -1.69$ [−2.67, −0.7]***	$d = -1.0$ [−1.70, −0.30]**
	Other Young vs.				$d = 1.24$ [0.66, 1.82]***
Mammillary Bodies	Young EE 14 vs.	$d = -1.85$ [−1.99, −0.60]**	$d = -1.59$ [−2.78, −0.39]**	$d = -1.20$ [−2.12, −0.28]**	$d = -1.21$ [−1.93, −0.49]***
	Other Young vs.				$d = 0.15$ [−0.39, 0.68] ns
Hippocampus	Young EE 14 vs.	$d = -0.88$ [−1.99, 0.22] ns	$d = -2.26$ [−3.58, −0.93]***	$d = -1.31$ [−2.25, −0.38]**	$d = -0.84$ [−0.53, 0.15]*
	Other Young vs.				$d = 0.45$ [−0.08, 0.99] ns
Hypothalamus	Young EE 14 vs.	$d = -1.82$ [−3.06, −0.58]**	$d = -1.43$ [−2.60, −0.25]*	$d = -0.68$ [−1.55, 0.19] ns	$d = -0.13$ [−0.81, 0.55] ns
	Other Young vs.				$d = 0.77$ [0.21, 1.32]**

*EE, Enriched Environment; SH, Standard Housed; Other Young indicates pooled Young data, excluding Young-Enriched-14-day rats. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.*

evident in mammillary bodies or hippocampus. Compared with young rats, NTproCNP was increased in occipital cortex, and decreased in medial prefrontal cortex. Moreover, modifications to NTproCNP seen in young rats within retrosplenial cortex and dorsal hippocampus were not evident in aged rats. Varying combinations of changes to degradation and production in aged rats also resulted in increased concentrations of CNP in the aging occipital and retrosplenial cortices. Overall, these results indicate age-related modification to both the CNP signaling system broadly, and its response to environmental conditions. In addition, they indicate degradation or clearance rate to be an important factor in determining bioactivity within neural tissue.

Previous work has shown that 2 weeks of enrichment is sufficient to stimulate neural progenitor cell mobilization (Magalon et al., 2007), upregulate multiple genes associated with neuroplasticity in the hippocampus (Keyvani et al., 2004), improve cognitive performance in intact animals (e.g., Tang et al., 2001; Frick and Fernandez, 2003; Bruel-Jungerman et al., 2005) and ameliorate cognitive deficits in animals with neurological insults (e.g., Passineau et al., 2001; Hicks et al., 2002; Wagner et al., 2002). Although here we have not explicitly linked increases in CNP concentrations at this time point to other neurological or behavioral sequelae, this finding encourages the view that the peptide may contribute to such outcomes as part of the early neurological response to enrichment in young rats. Interestingly, the hippocampus is often a focus of structural and molecular responses to enrichment (see Simpson and Kelly, 2011; Hirase and Shinohara, 2014 for reviews), but here CNP exhibited the smallest effect in terms of young rats' response to short-term enriched housing. Plausibly, the size of this effect may vary throughout the hippocampal complex. Within hippocampus, CNP mRNA predominates in CA1 through CA3, whereas NPR-B mRNA is largely expressed within DG (Langub et al., 1995b; Herman et al., 1996). Substantive changes to bioactive CNP in any specific region similar to those in other regions of interest analyzed here may be masked by the inclusion of all three subregions used for hippocampal tissue analysis. This highlights a need within future studies of CNP to consider these sub-regions independently. Interestingly, an effect of enrichment on NTproCNP concentrations was seen in dorsal hippocampus for NTproCNP concentrations, suggesting a modification to gene regulation as opposed to the effect seen on bioactive CNP in other regions of interest. An explanation for this cannot be generated from this data. Future work may consider the use of PCR to analyse gene expression of Nppc directly, alongside NPR-B/NPR-C and degradative enzyme (neprilysin and insulin-degrading enzyme) gene expression to elucidate on the findings.

The relatively minimal response of aged rats to environmental manipulations used here should now encourage further study of the CNP signaling system in the aging brain. In a recent review, Mahinrad et al. (2016) outline evidence suggesting Natriuretic Peptides contribute to cognitive decline and pathologies associated with aging, which we believe this experimental work supports. Primarily, the lack of response to enrichment of bioactive CNP in aged rats compared to young is suggestive of age-related loss of sensitivity to external stimulus. Additionally, evidence of loss or change in the NTproCNP response to rehousing or enrichment in the aged retrosplenial cortex, hippocampus and hypothalamus indicates age-related modification to peptide production in these cortical nodes. Apparent accumulation of CNP with aging in occipital and retrosplenial cortices may align with the facilitation of long-term depression with aging (Kelly et al., 2006). Notably, while expected reductions to degradative activity were identified in most regions, this effect was absent in hippocampus and mammillary bodies, contrary to the hypothesis. Region specific, age-related reductions in neprilysin (the proteolytic enzyme regulating CNP) have been demonstrated previously within hippocampus (Iwata et al., 2001, 2002; Yasojima et al., 2001a,b; Higuchi et al., 2005; Hellström-Lindahl et al., 2008). Thus, the absence of hypothesized reductions to degradative activity within this region specifically raises interesting questions. Because NTproCNP:CNP ratio is influenced by both proteolytic enzyme activity and NPR-C clearance activity, one plausible line of inquiry is to examine age-related modifications to this receptor within hippocampus. Additionally, these age-related changes to production, bioactive availability and degradation or clearance of CNP should now be examined in association

with other neurological factors wherein CNP may contribute to cognitive decline such as modifications to synaptic regulation, neurovascular function, anxiety, and memory (Mahinrad et al., 2016). In this context it is relevant to note that in contrast to other neuroprotective factors, the diverse molecular events potentially regulated by CNP signaling—including ion channel activity and cyclic AMP concentrations (Kuhn, 2016)—need to be considered.

The major modification to CNP in medial prefrontal cortex of aged rats seemingly related to the period of rehousing with new cage mates rather than the rehousing environment. Other minor modifications to measures from aged rats (not directly reported here, but see **Figures 1**, **4** Aged-Standard ratios; **Figure 6** Aged-Standard NTproCNP for examples), also generally related to rehousing period. Because CNP has been previously related to anxiety regulation and related behaviors (Bíró et al., 1996; Montkowski et al., 1998; Telegdy et al., 1999, 2000; Jahn et al., 2001), this now encourages further investigation of CNP in the context of anxiety and aging, because this response in aged rats seems to relate to an anxious event (rehousing with new cage mates). CNP's effects on anxiety are dependent on corticotropin releasing hormone (Jahn et al., 2001) and medial prefrontal cortex, hippocampus and other limbic system structures participate in regulation of the hypothalamo-pituitary adrenal axis (Herman et al., 2005). Moreover, changes to hypothalamo-pituitary adrenal axis signaling are thought to contribute to neurological and behavioral changes during aging (Mizoguchi et al., 2009; Swaab and Bao, 2011) as are molecular and physiological changes throughout these same frontal and temporal cortical networks (Nestor et al., 2003). Although anxious and mnemonic behaviors were not studied here, age-related modifications to this peptide system identified within fronto-temporal regions provides initial experimental support for an association with cognitive decline (Mahinrad et al., 2016). However, a lack of reported impairment in cognition in the homozygous human loss-of-function mutation of the NPR-B receptor (Wang et al., 2016) suggests at least two additional caveats. First, is that this conclusion may be limited to rodents. Cognitive and neurological effects have been reported in rodents with a loss of NPR-B function (Tamura et al., 2004; Barmashenko et al., 2014; Buttgereit et al., 2016). Alternatively, it may be that the NPR-C receptor is of greater importance in mediating central functions of CNP than is currently believed, but suggested by others (Trachte, 2000, 2005; Gong et al., 2017; Moghtadaei et al., 2017). Clearly further work is required to clarify the importance of CNP and its receptors in regulating neural function in both rodents and humans. As the first report of CNP and related molecules within rodent medial prefrontal cortex and retrosplenial cortex, and changes to bioactive CNP during environmental stimulation, the current findings now call for more focused studies of CNP activity, function and receptor expression throughout these important neural circuits.

DATA AVAILABILITY STATEMENT

Data generated and analyzed in this experiment is available from corresponding author on request.

AUTHOR CONTRIBUTIONS

JD-A and EE developed initial concept for young rats, which was expanded to aged rats in consultation with SR and TP. SR conducted animal trials, tissue acquisition and homogenization, peptide assays for aged rats, data analysis, and visualization. TP conducted assays for young rats and supervised aged rat assays. SR prepared the manuscript in consultation with all other co-authors.

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REVIEW
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Is Environmental Enrichment Ready for Clinical Application in Human Post-stroke Rehabilitation?

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Environmental enrichment (EE) has been widely used as a means to enhance brain plasticity mechanisms (e.g., increased dendritic branching, synaptogenesis, etc.) and improve behavioral function in both normal and brain-damaged animals. In spite of the demonstrated efficacy of EE for enhancing brain plasticity, it has largely remained a laboratory phenomenon with little translation to the clinical setting. Impediments to the implementation of enrichment as an intervention for human stroke rehabilitation and a lack of clinical translation can be attributed to a number of factors not limited to: (i) concerns that EE is actually the "normal state" for animals, whereas standard housing is a form of impoverishment; (ii) difficulty in standardizing EE conditions across clinical sites; (iii) the exact mechanisms underlying the beneficial actions of enrichment are largely correlative in nature; (iv) a lack of knowledge concerning what aspects of enrichment (e.g., exercise, socialization, cognitive stimulation) represent the critical or active ingredients for enhancing brain plasticity; and (v) the required "dose" of enrichment is unknown, since most laboratory studies employ continuous periods of enrichment, a condition that most clinicians view as impractical. In this review article, we summarize preclinical stroke recovery studies that have successfully utilized EE to promote functional recovery and highlight the potential underlying mechanisms. Subsequently, we discuss how EE is being applied in a clinical setting and address differences in preclinical and clinical EE work to date. It is argued that the best way forward is through the careful alignment of preclinical and clinical rehabilitation research. A combination of both approaches will allow research to fully address gaps in knowledge and facilitate the implementation of EE to the clinical setting.

Keywords: environmental enrichment, stroke, rehabilitation, neuroplasticity, recovery, clinical translation

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EARLY BEGINNINGS

History of Environmental Enrichment

Environmental enrichment (EE) was first studied by Canadian scientist Donald Hebb, who raised rats in his home and later showed they were superior to laboratory raised animals in tests of problem solving ability (Hebb, 1947). His influential book, the Organization of Behavior: A Neuropsychological Theory (Hebb, 1949), emphasized the importance of experience in shaping behavior and provided the stimulus for research examining how EE changes the brain and subsequently behavior. Much of the work in the 1960's focused on the effects of EE on the undamaged brain. Seminal studies by Rozenzweig and others showed that brain plasticity (e.g., dendritic branching) was dramatically altered by varying experience (Rosenzweig et al., 1962; Bennett et al., 1964; Diamond et al., 1964; Greenough et al., 1973). These use-dependent neuroplastic changes can be induced across the life span and are associated with improved performance on various learning and memory tasks. Later efforts investigated how EE affected the damaged brain (Will et al., 2004). For example, studies showed that EE attenuated the effects of frontal cortex injury (Kolb and Gibb, 1991), as well as both global (Farrell et al., 2001) and focal ischemia (Ohlsson and Johansson, 1995; Johansson, 1996; Puurunen et al., 2001; Risedal et al., 2002).

Based on relatively little preclinical evidence many "so-called" neuroprotective drugs were advanced into clinical stroke trials where they met universal failure (O'Collins et al., 2006). In contrast, an overwhelming amount of preclinical evidence, accumulated over several decades, shows that EE enhances learning and memory, promotes various forms of neuroplasticity and consistently improves recovery from brain injury, including stroke. In spite of this evidence there has been limited translation of this promising intervention into the clinical setting (Livingston-Thomas et al., 2016). The purpose of this review article, is to summarize the widespread preclinical evidence for utilizing EE as a therapeutic intervention for stroke recovery and examine why EE has largely remained a laboratory phenomenon. Additionally, how preclinical and clinical investigators can facilitate the transition of EE into the clinical setting is discussed.

Defining Environmental Enrichment

A major impediment to clinical translation has been inconsistency in how EE is defined experimentally. This has created confusion in the clinical community because it's unclear which EE paradigm or what critical elements of EE should be adapted for patients. As originally conceived, EE was designed to provide a more enriching, stimulating environment for animals to more closely mimic conditions encountered in the wild. There is no standardized form of EE; for some, enrichment means little more than housing several animals together in a standard sized cage containing a tube and a running wheel. Other configurations are much more elaborate and engaging, consisting of a very large, multi-level cage, that includes toys, ramps, ladders and ropes, which are replaced or moved at intervals (e.g., daily, or weekly) throughout an experiment. The elements of the enrichment cage (**Figure 1**) provide opportunities for social interaction, to stimulate exploration (e.g., multi-level floors connected by tubes) and engage in activities (e.g., nesting, crossing beams and hanging platforms) that tax balance, strength and provide somatosensory stimulation. The replacement of objects and changing their location within the cage provides

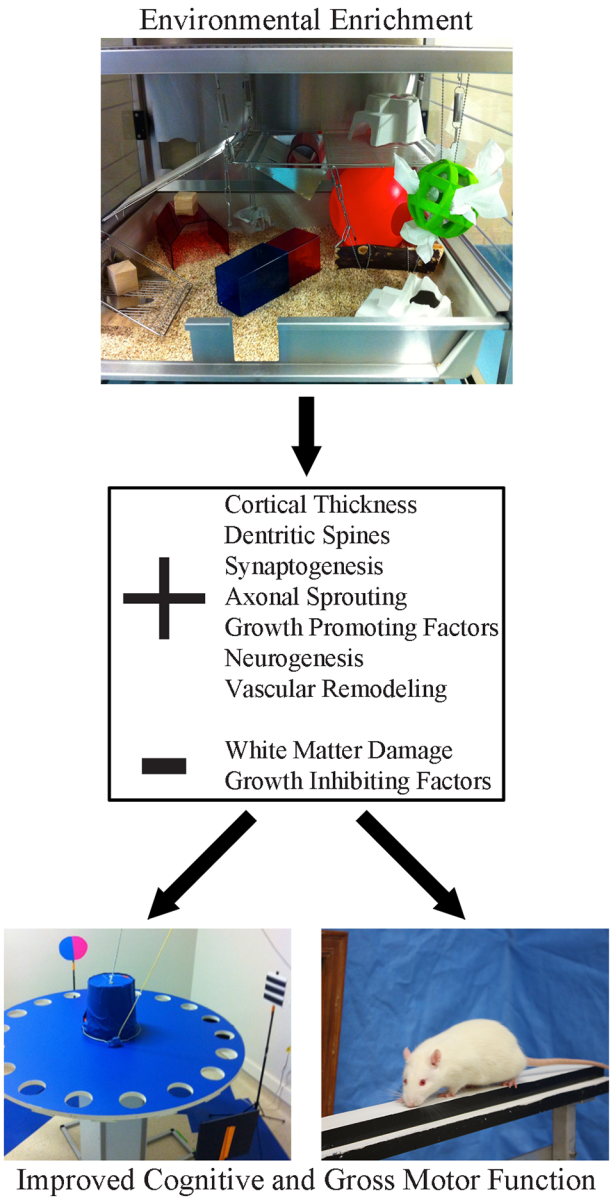

FIGURE 1 | Environmental enrichment (EE) is a multi-faceted form of housing that provides enhanced motor, cognitive, sensory and social stimulation, relative to the standard conditions of rodent housing. This form of housing has been shown to create widespread changes in the neuroplastic milieu of the brain. Following stroke, these beneficial changes create a neural environment that is permissive to recovery, resulting in robust improvements in both cognitive and gross motor function.

cognitive stimulation, additional olfactory and visual stimulation and further encourages exploration and physical activity. Introduction of new materials into the cage can be used to provide added sensory stimulation (Zubedat et al., 2015). In the context of stroke recovery, it is important to recognize that EE needs to include a task specific component that targets the animals' primary deficits. For example, upper limb impairment is very common clinically (Duncan et al., 1992; Kwakkel et al., 2003) and consequently, most preclinical investigators target the forelimb motor cortex in their stroke studies (Murphy and Corbett, 2009; Corbett et al., 2017). EE alone is not effective in promoting recovery of skilled forelimb movements (e.g., reaching; Grabowski et al., 1993), since there is no opportunity to engage in this activity in standard EE configurations. As such, to fill this void our group adds a daily reaching task component to EE which dramatically improves the level of recovery provided by EE (Biernaskie and Corbett, 2001; Biernaskie et al., 2004; Jeffers and Corbett, 2018). Thus, the ideal definition of EE, unlike typical stroke rehabilitation in the clinic, encompasses a changing environment that encourages socialization, exercise, sensory and cognitive stimulation, and task-specific therapy targeting the primary impairment.

Most animal studies provide unlimited access to enrichment 24 h a day, 7 days a week with relatively few studies using shorter enrichment exposures (Leger et al., 2015). This feature of EE raises immediate concerns with clinicians when attempting to extrapolate results from animal studies where not only the configuration of EE, but also practical concerns, limit the duration or amount of therapy time that can be allocated to EE vs. other forms of patient care. Another important consideration related to the duration of EE is that most of the demonstrated benefits in fostering stroke recovery, and the postulated mechanisms underlying these benefits, may not hold if shorter durations of EE are employed. This is an important consideration in view of translational limitations inherent in most preclinical exercise studies. For example, running wheel exercise has long been known to enhance neurogenesis (van Praag et al., 2000; Voss et al., 2013) which in turn is suggested to contribute to improvements in learning, memory, and recovery from brain injury, including stroke (Voss et al., 2013). However, access to this form of exercise, like EE, is typically provided to rodents 24 h per day. It is unclear how such prolonged exercise regimens could be possible for stroke patients who typically are older, experience fatigue, have sensorimotor impairments and are much more sedentary than age-matched controls (Bernhardt et al., 2004; Duncan et al., 2012). In animal studies, the effects on neurogenesis are much more modest when running wheel access has been limited to several hours per day on alternate days (Nguemeni et al., 2018).

A concern with the implementation of EE in the clinic is that rodents experience a relatively impoverished environment in standard animal facilities, and EE may simply normalize typical living conditions (Würbel, 2001). If this is indeed the case, then EE may not be effective in humans who are viewed as already living in an enriched, stimulating environment. However, Bernhardt et al. (2004) have shown that after stroke patients spend a large proportion of time in isolation and physically inactive (Fini et al., 2017). Further, patients frequently report the rehabilitation setting as being unstimulating and boring (Kenah et al., 2017). Thus, the early post-stroke environment for humans and impoverished animals may actually be relatively similar.

Environmental Enrichment as a Combination Therapy

A question, often encountered when discussing the beneficial effects and potential mechanisms underlying the neuroplasticity enhancing actions of EE, is what element of the EE is most important? Is it socialization, exercise, sensorimotor activation or cognitive stimulation? There have been a number of attempts to dissect EE into the relative importance of its individual components. Prior to bilateral cortical injury, rats given 2 h per day of EE for 25 days performed better on a motor task than those given the same amount of running wheel exercise (Gentile et al., 1987). Similarly, improved motor outcomes of EE compared to running wheel exercise-alone have also been observed after middle cerebral artery occlusion (MCAo) in rats, indicating the important influence of socialization on recovery (Johansson and Ohlsson, 1996; Risedal et al., 2002). Using a modified EE paradigm in which EE was combined with daily reach training (i.e., enriched rehabilitation, ER), it was found that EE, running exercise and reach training all produce a uniform pattern of activation throughout all layers of the sensorimotor cortex after stroke, however ER causes a more specific pattern of activation, targeting layer II and layer III motor neurons (Clarke et al., 2014). Recently, we showed that ER is more effective than either EE alone or reach training alone at restoring skilled forelimb function after stroke (Jeffers and Corbett, 2018). Similarly, others have shown a synergistic benefit when EE is paired with either resistance exercise or increased social interaction (Brenes et al., 2016; Prado Lima et al., 2018).

The pattern emerging from studies using EE to promote post-stroke recovery is that the whole is greater than the sum of the parts (Jeffers and Corbett, 2018). In this regard, EE shares similarity with other pleiotropic treatments such as exercise, hypothermia and ischemic tolerance, that have proven to be effective in reducing ischemic damage to the brain (Iadecola and Anrather, 2011). Cell death, like stroke recovery, is not dependent on a single mechanism. Indeed, attempts to rescue cells from ischemic injury or restore lost function after stroke with single target interventions have been met with little success (Murphy and Corbett, 2009; Iadecola and Anrather, 2011; Corbett et al., 2014; Hayward et al., 2014; Carmichael, 2016). The advantage of using EE or ER is that these synergistic approaches engage multiple, potentially beneficial mechanisms (described below and listed in **Table 1**) whereas the single target approach has failed completely in stroke neuroprotection and other conditions, including Alzheimer's disease (Iadecola and Anrather, 2011; Corbett et al., 2014). As such, EE and ER should be viewed as combination therapies that create a permissive, regenerative state in the brain that is receptive to use-dependent, task-specific forms of rehabilitation and other recovery promoting treatments.

TABLE 1 | Potential underlying mechanisms of environmental enrichment (EE) beneficial for promoting stroke recovery.

EE-induced plasticity	References
↓ Lesion volume	Buchhold et al. (2007) and Zhang et al. (2017)
↑ Dendritic remodeling	Biernaskie and Corbett (2001) and Johansson and Belichenko (2002)
↑ Synaptogenesis	Jones et al. (1999), Xu et al. (2009) and Hirata et al. (2011)
↑ Axonal remodeling	Papadopoulos et al. (2009) and Li et al. (2015)
↓ White matter damage	Hase et al. (2017, 2018)
↑ Antioxidant activity	Cechetti et al. (2012)
↑ Angiogenesis	Hu et al. (2010), Matsuda et al. (2011), Zheng et al. (2011), Yang et al. (2012), Ma et al. (2013), Seo et al. (2013) and Zhang et al. (2017)
↓ BBB leakage	Hase et al. (2017) and Zhang et al. (2017)
↑ Neurogenesis	Komitova et al. (2005a,b, 2006); Buchhold et al. (2007), Wurm et al. (2007) and Venna et al. (2014)
↑ Growth-promoting factors (BDNF, Gap43, FGF-2)	Gobbo and O'Mara (2004), Ploughman et al. (2007), Mizutani et al. (2011), Seo et al. (2013) and Venna et al. (2014)
↓ Growth-inhibiting factors (aggrecan-containing perineuronal nets, NOGO-A)	Madinier et al. (2014) and Li et al. (2015)

Up and down arrows indicate an increase or decrease in the corresponding factor in response to EE, respectively.

HOW DOES ENRICHMENT ENHANCE PLASTICITY AND RECOVERY FROM STROKE?

Underlying Mechanisms

Until the work of Mark Rosenzweig and Marian Diamond in the 1960s it was generally thought that the adult brain was fixed and unable to undergo any degree of neuroplasticity. Their work was the first to show that the brains of rats that lived in an EE weighed more, had increased cortical thickness, and demonstrated increased cortical acetylcholinesterase activity compared to their restricted littermates (Rosenzweig et al., 1962; Bennett et al., 1964; Diamond et al., 1964). In response to stroke, synaptogenesis, axonal sprouting, gliogenesis and neurogenesis are significantly upregulated, creating an environment that is highly permissive to behavior-driven plasticity (Murphy and Corbett, 2009; Zeiler and Krakauer, 2013; Carmichael, 2016). It is now recognized that an EE stimulates a number of neuroplastic processes, such as structural changes (dendritic arborization, synaptogenesis, and axonal sprouting), enhanced brain activity, angiogenesis, neurogenesis, and the release of growth factors (brain-derived neurotrophic factor (BDNF), growth-associated protein 43 (GAP43)). Importantly, the upregulation of the aforementioned processes and growth factors play a significant role in facilitating motor and cognitive recovery following ischemic stroke. As discussed above, EE is multi-faceted, incorporating a number of behavioral experiences. The mechanisms upregulated in response to EE alone, or in combination with other components of ER paradigm (exercise, task-specific training), are discussed in relation to their role in promoting recovery following stroke (**Table 1**).

Structural Changes (Dendritic Arborization, Synaptogenesis, Axonal Sprouting, White Matter, Lesion Volume)

While some have demonstrated reduced lesion volume following EE (Buchhold et al., 2007; Zhang et al., 2017), the vast majority of studies do not show a difference in the size of the infarct in standard housed animals compared to EE (Johansson and Ohlsson, 1996; Biernaskie and Corbett, 2001; Risedal et al., 2002; Hirata et al., 2011; Clarke et al., 2014; Madinier et al., 2014). In fact, if EE is introduced within the first few days after stroke it can increase infarct volume and cell loss (Risedal et al., 1999; Farrell et al., 2001). These findings indicate that the beneficial effects of EE for stroke recovery go beyond simple neuroprotection.

A prevailing view of how stroke rehabilitation reduces neurological impairments is by enhancing use-dependent activation of intact tissue adjacent to the infarct and contralesional cortical regions, thereby shaping neural reorganization (Nudo et al., 1996a,b; Dijkhuizen et al., 2001; Binkofski and Seitz, 2004). Experience-induced plasticity following stroke results in remodeling of dendrites in perilesional tissue, and possibly protects vulnerable neurons from further damage (Johansson and Belichenko, 2002; Brown et al., 2008). In healthy rats, EE alone also increases dendritic spines in all cortical layers (Johansson and Belichenko, 2002), while social isolation has been reported to have the opposite effect (Bryan and Riesen, 1989). In hypertensive rats, EE following MCAo increases dendritic spines in pyramidal neurons in layers II/III compared to standard housing conditions (Johansson and Belichenko, 2002). Further, pairing a task-specific reaching paradigm with EE 15 days after MCAo results in increased basilar dendritic growth in layer V pyramidal neurons within the uninjured motor cortex, and corresponding improved functional recovery (Biernaskie and Corbett, 2001). Similarly, EE promotes synaptogenesis in perilesional and contralesional cortex and enhances use-dependent activity in perilesional cortex compared to standard housing (Jones et al., 1999; Hirata et al., 2011; Clarke et al., 2014). Following MCAo the change in synaptic density and structure following 2 weeks of EE has also been associated with improved functional recovery on a spatial memory task (Xu et al., 2009). Further, both exercise and EE enhance axonal sprouting and reduce white matter damage (Papadopoulos et al., 2009; Li et al., 2015; Hase et al., 2017, 2018). Running wheel exercise, often included in EE paradigms and associated with improved functional recovery, enhances axonal remodeling following focal cortical stroke (Li et al., 2015). In models of chronic hypoperfusion, glial

damage in white matter, and neuroinflammation, is also attenuated in mice exposed to EE (Hase et al., 2017, 2018). Similarly, chronic cerebral hypoperfusion and oxidative stress in the hippocampus are prevented following 12 weeks of EE in rats, likely due to heightened antioxidant enzyme activity (Cechetti et al., 2012).

Vasculature

The cerebrovasculature plays a potentially important role in promoting post-stroke recovery (Ergul et al., 2012). Following stroke, angiogenesis is upregulated in order to increase blood flow to damaged tissue and thereby engage endogenous recovery mechanisms such as synaptogenesis, synaptic plasticity and neurogenesis. Similar to the proangiogenic effects of exercise alone (Hu et al., 2010; Matsuda et al., 2011; Zheng et al., 2011; Yang et al., 2012; Ma et al., 2013), EE delivered in the recovery period following ischemic stroke can stimulate angiogenesis throughout the brain and perilesional tissue through vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), and astrocytic high-mobility group box-1/interleukin-6 (HMGB1/IL-6) signaling (Seo et al., 2013; Yu et al., 2014; Chen et al., 2017; Zhang et al., 2017). Importantly, these changes in the cerebrovasculature occur in parallel with varying degrees of functional recovery post-stroke such as grip strength, motor coordination and function (Seo et al., 2013; Yu et al., 2014), decreased depression and anxiety (Chen et al., 2017), and enhanced learning and memory (Yu et al., 2014). Additionally, EE also attenuates blood brain barrier leakage following focal cerebral ischemia and in models of vascular cognitive impairment (Hase et al., 2017; Zhang et al., 2017).

Neurogenesis

Migration of new immature neurons to the site of stroke damage has been shown to occur following ischemic cell death, and in close association with newly formed vasculature (Ohab et al., 2006). Significant literature has demonstrated the benefit of EE on neurogenesis concurrent with enhanced spatial learning and memory (van Praag et al., 2000; Simpson and Kelly, 2011; Leger et al., 2015). Likewise, enhanced neurogenesis is recognized to be upregulated following EE in different models of stroke (Komitova et al., 2005b, 2006; Buchhold et al., 2007; Wurm et al., 2007; Venna et al., 2014). For example, after MCAo in rats, both early (24 h post-stroke) and late (7 days post-stroke) administration of EE for 5 weeks results in significantly more newly born cells in both ipsi- and contra-lateral cortical regions than standard housing (Komitova et al., 2006). This increase in neurogenesis is often accompanied by improved cognitive and sensorimotor function (Komitova et al., 2005a; Wurm et al., 2007). Furthermore, the exercise component of EE may be largely responsible for these neurogenic effects (Grégoire et al., 2014), which is confounded by findings that exercise also results in upregulation of many neuroplasticity-promoting factors such as BDNF (Bechara and Kelly, 2013). This suggests that although neurogenesis and post-stroke recovery may occur in tandem, this may be coincidental, with recovery being more directly related to the upregulation of a variety of growth-promoting factors such as BDNF and GAP43 (Rossi et al., 2006; Ploughman et al., 2009; Clarkson et al., 2011; Mizutani et al., 2011; Cook et al., 2017).

Growth Promoting and Inhibitory Factors

Both the early phase following stroke and initiation of EE are associated with an increase in growth promoting factors (glial-derived synaptogenic thrombospondin 1 and 2, GAP43, MARKS, CAP23, BDNF, etc.) that have varying effects on the aforementioned changes in neuronal structure (Murphy and Corbett, 2009). Thus, the timing of when rehabilitation is delivered is important, with the goal to actively engage in this early time period post-stroke (Corbett et al., 2015). BDNF has a major role in spontaneous and rehabilitation-induced recovery following stroke (Ploughman et al., 2009; Clarkson et al., 2011; Cook et al., 2017). For example, administration of BDNF intravenously or via a hydrogel significantly improves tissue repair and motor recovery in two different rodent models of stroke (Schäbitz et al., 2004; Cook et al., 2017). While EE increases BDNF in some studies of ischemic brain injury (Gobbo and O'Mara, 2004; Venna et al., 2014), others have reported negative findings (Risedal et al., 2002; Hirata et al., 2011). However, it is important to note that rehabilitation and exercise intensity are significant determinants as to whether rehabilitation is accompanied by increases in BDNF and whether significant functional recovery occurs (Ploughman et al., 2007; MacLellan et al., 2011a). Likewise, in the perilesional cortex of rats with cortical injury, running wheel exercise has been associated with increased GAP43, as well as its phosphorylated form (pSer41-GAP43), a key protein involved in neuronal plasticity (Mizutani et al., 2011). Other neurotrophic factors such as insulin-like growth factor-1 (IGF-1), FGF-2, nerve growth factor (NGF) and neurotrophin-3 (NT-3) are also increased by varying amounts of EE (Hu et al., 2013; Seo et al., 2013; Yu et al., 2016).

A critical window for stroke recovery has been linked to post-stroke upregulation of growth promoting factors (described above), with closing of this window related to the upregulation of growth inhibiting genes, such as NOGO and chondroitin sulfate proteoglycans (CSPGs; Murphy and Corbett, 2009). In order for recovery to occur beyond this finite period, interventions should attempt to promote a more permissive environment for neuroplasticity and recovery. For example, administering chondroitinase ABC, which degrades inhibitory CSPGs in the extracellular matrix, or blocking neurite inhibitory protein Nogo-A, enhances sensorimotor recovery following focal stroke due to new axonal connections and increased dendritic arborization in contralesional cortex (Papadopoulos et al., 2002, 2006; Soleman et al., 2012). Similarly, providing EE for 9 weeks after photothrombotic stroke results in a reduction of aggrecan-containing perineuronal nets surrounding parvalbumin containing GABAergic neurons in the peri-infarct area (Madinier et al., 2014). Additionally exercise results in a downregulation of Nogo-A signaling in perilesional tissue, promoting axonal remodeling (Li et al., 2015).

Establishing which EE-induced mechanisms are critical for stroke recovery is difficult to investigate experimentally, with the vast majority of studies being correlative in nature.

A substantial body of preclinical work has focused on the potential role of neurogenesis, yet the precise role of neurogenesis or the degree to which it occurs in adult humans has recently been questioned (Sorrells et al., 2018). Nonetheless, the aforementioned mechanisms and processes discussed above likely have a collective role in promoting recovery following stroke rather than any single one. Indeed, the post-stroke time course of these neuroplasticity processes strongly relate to the functional recovery observed across different domains (cognitive, sensorimotor, etc.).

BENEFITS OF ENVIRONMENTAL ENRICHMENT ON FUNCTIONAL RECOVERY IN ANIMALS

Sensitive Periods Following Stroke: The Importance of Maximizing Therapy Dose in the Early Post-stroke Phase

Corresponding with the aforementioned changes in growth factors, recovery of post-stroke motor impairment is thought to plateau within the first 4–5 weeks in rodents (Biernaskie et al., 2004; Murphy and Corbett, 2009) and the first 3–4 months in humans (Jørgensen et al., 1995; Kwakkel et al., 2006; Langhorne et al., 2011), with a large degree of improvement during this time being attributable to spontaneous recovery in both species (Prabhakaran et al., 2008; Krakauer et al., 2012; Winters et al., 2015; Jeffers et al., 2018a,b). Although recovery can still occur outside of this period, these changes may be mediated by compensatory strategies, rather than restitution of neurological impairments (Zeiler and Krakauer, 2013). This highlights the need for preclinical work to consider more sensitive measures of sensorimotor recovery, such as kinematics (Corbett et al., 2017). Furthermore, although some degree of recovery may occur at any time following stroke, the rate of change becomes more limited as time post-stroke increases (Lohse et al., 2016). Evidence from both preclinical and clinical studies suggest that rehabilitation therapies should be maximized in the early weeks and months following stroke, with caution being taken to not intervene too early (i.e., <3 days), when intensive therapy may have contradictory, or even detrimental effects (Humm et al., 1998; Risedal et al., 1999; Farrell et al., 2001; Dromerick et al., 2009; Lang et al., 2015; Langhorne et al., 2017).

Despite some experiments not finding a relationship between therapy dose and recovery (Winstein et al., 2016), overall meta-analysis across clinical trials have indicated that increased therapy dose augments recovery across a range of post-stroke impairments, using a variety of intervention strategies and outcome measures (Lohse et al., 2014; Schneider et al., 2016). Additionally, the benefits of post-stroke task-specific training have been shown to be transferrable to non-trained tasks (Schaefer et al., 2013). As rehabilitation resources are often limited, alternative methods for increasing therapy dose are highly desirable. EE may provide one such adjunctive intervention for increasing non-specific therapy dose, as this treatment paradigm provides a stimulating environment that enhances stroke recovery in rodents across a variety of impairment domains without requiring provision of specific training (Ohlsson and Johansson, 1995; Risedal et al., 2002; Livingston-Thomas et al., 2016). Furthermore, this stimulating environment has a potentiating effect on task-specific therapy, resulting in recovery beyond what would have occurred with either EE, or task-specific therapy alone (Jeffers and Corbett, 2018).

Efficacy of Environmental Enrichment in Non-motor and Motor Recovery Domains

As previously mentioned, early work with EE focused on how stimulating early life experience promotes enhanced cognitive development (Hebb, 1947). Later, cortical injury models in rodents were used to probe the various functions and network connectivity of the brain, while investigating how early-life EE could ameliorate impairments in learning and memory associated with these injuries (Kolb and Elliott, 1987; Kolb and Gibb, 1991). EE's efficacy in improving cognitive function in these studies led to utilization of this treatment for adult focal ischemia in rodents, with a continued focus on cognitive performance. Following stroke, EE has been shown to significantly enhance spatial learning of the Morris Water Maze (Risedal et al., 1999; Dahlqvist et al., 2004; Rönnbäck et al., 2005; Sonninen et al., 2006) and spatial memory in Radial Arm Maze tasks (Buchhold et al., 2007). These benefits appear to be robust across injury types, as similar benefits of EE have been observed in Morris Water Maze acquisition (Puurunen et al., 1997) and switching between relevant reward-cues in the Win/Shift-Win/Stay version of the T-maze task (Farrell et al., 2001) in models of global ischemia. EE also alleviates depression-like behaviors in mice (Jha et al., 2011), which is an important consideration, as depression in humans after stroke is common (Arwert et al., 2018). Overall, these studies (see **Table 2**) demonstrate the robust cognitive benefits of EE, and the potential for this treatment to be applied to other domains of impairment in preclinical models of stroke.

The preclinical stroke field has primarily used EE to promote motor recovery and study its underlying neuroplastic mechanisms. Many studies have demonstrated benefits of EE on post-stroke recovery of a variety of sensorimotor tasks (see **Table 2**), including: rotarod (Ohlsson and Johansson, 1995; Johansson and Ohlsson, 1996; Johansson, 1996; Nygren and Wieloch, 2005; Nygren et al., 2006; Buchhold et al., 2007), ladder crossing (Biernaskie et al., 2004; Windle et al., 2007; Wurm et al., 2007), limb placement (Puurunen et al., 2001), and adhesive strip removal (Kuptsova et al., 2015). While some studies have shown neutral, or slightly negative effects of EE on similar sensorimotor tasks (Hicks et al., 2008), meta-analysis of these results indicates that EE has a significant benefit on general sensorimotor function (Janssen et al., 2010). Furthermore, these benefits also extend to models of intracerebral hemorrhage (Auriat and Colbourne, 2008), which receives relatively little attention compared to focal ischemia in the preclinical literature.

TABLE 2 | Benefits of EE on functional recovery in animals following stroke.

Benefits	Task	References
↑ Spatial learning	Morris Water Maze	Puurunen et al. (1997), Risedal et al. (1999), Dahlqvist et al. (2004), Rönnbäck et al. (2005) and Sonninen et al. (2006)
↑ Spatial memory	Radial Arm Maze	Buchhold et al. (2007)
↑ Working memory	T-maze	Farrell et al. (2001)
↓ Depression-like behaviors	Tail suspension test, open-field and sucrose preference test	Jha et al. (2011)
↑ Motor recovery	Rotarod	Ohlsson and Johansson (1995), Johansson (1996), Johansson and Ohlsson (1996), Nygren and Wieloch (2005), Nygren et al. (2006) and Buchhold et al. (2007)
	Ladder crossing	Biernaskie et al. (2004), Windle et al. (2007) and Wurm et al. (2007)
	Limb placement	Puurunen et al. (2001)
	Adhesive strip removal	Kuptsova et al. (2015)
	Montoya staircase	Biernaskie and Corbett (2001) and Jeffers et al. (2014)
	Single pellet reaching	Jeffers and Corbett (2018)

Up and down arrows indicate an increase or decrease in the corresponding factor in response to EE, respectively.

One caveat to this positive outlook on EE for enhancing motor recovery is that tasks of fine motor dexterity, such as pellet retrieval, do not demonstrate the same benefits as less-skilled motor outcomes (Grabowski et al., 1993; Ohlsson and Johansson, 1995; Auriat and Colbourne, 2008; Kuptsova et al., 2015). As such, EE may not substitute for task-specific (e.g., upper limb) therapy; however, it could potentially serve as an adjunct to conventional care that would enable greater recovery than possible with task-specific training alone (Livingston-Thomas et al., 2016). This adjunctive approach to EE and task-specific training is supported by evidence that such combination therapies augment recovery of fine-motor skills that normally do not benefit from EE alone, in both models of focal ischemia (Biernaskie and Corbett, 2001) and intracerebral hemorrhage (MacLellan et al., 2011b; Caliaperumal and Colbourne, 2014). Additional combinations of EE with various pharmacological agents has also yielded promising synergistic results; however, this work is still in its infancy (Corbett et al., 2014; Mering and Jolkkonen, 2015; Malá and Rasmussen, 2017). Our previous work has demonstrated that the combination of EE, task-specific reaching and growth factor administration accelerates the rate of recovery of fine motor dexterity (Jeffers et al., 2014). Studies such as these further emphasize that the naturalistic behaviors and heightened activity encouraged by EE has the potential to produce a powerful synergistic interaction to promote recovery of even very specific skilled functions post-stroke (Zeiler and Krakauer, 2013; Corbett et al., 2015).

Generalization of the Benefits of Environmental Enrichment

An important consideration in attempting to translate a potential preclinical stroke treatment, such as EE, to human clinical practice is the robustness of the benefits observed in the preclinical environment. Stroke is a heterogeneous disorder, affecting both sexes at all points throughout the lifespan, causing damage in diverse brain regions and an array of functional impairments (Ramsey et al., 2017). In contrast, preclinical rodent studies of stroke typically utilize young adult, male rats, with cortical lesions that do not represent those most commonly observed in clinical studies (Edwardson et al., 2017). These factors hamper the translation of preclinical stroke treatments to clinical practice, and have led to concerted international efforts to better align preclinical and clinical experimental methodologies in stroke (Bernhardt et al., 2017a; Corbett et al., 2017). As a general principle, before considering translation to the clinic, a potential preclinical therapy should demonstrate robust benefits across a range of experimental conditions.

Undoubtedly EE has been studied under an array of conditions and preclinical demographics (Simpson and Kelly, 2011). In addition to the diverse benefits outlined above, EE has also been shown to exhibit significant effects throughout the lifespan, from neonatal (Kolb and Gibb, 1991; Rojas et al., 2013) to aged animals (Buchhold et al., 2007). However, with aging, animals may need to be subjected to more intense stimulation than younger animals in order to obtain the same benefits of EE (Bennett et al., 2006). The literature regarding sex-differences in the efficacy of EE is much less clear. Studies have shown greater benefits of EE for females (Pereira et al., 2008), males (Langdon et al., 2014), or similar effects between sexes (Frick et al., 2003; Saucier et al., 2010; Schuch et al., 2016). As only ∼17% of EE studies have included both male and female animals, and of this subset only a minority of studies has been concerned with the effects of stroke, or stroke recovery, it is unlikely that enough data currently exists in the literature to definitively answer the conditions under which sex-specific effects of EE may occur (Simpson and Kelly, 2011). As previously outlined, EE has shown beneficial effects for both cognitive and motor recovery using a variety of models of neurological damage including: global ischemia (Farrell et al., 2001), neonatal hypoxia-ischemia (Pereira et al., 2007; Rojas et al., 2013), intracerebral hemorrhage (Auriat and Colbourne, 2008), and cortical injury in a variety of regions using different lesion induction methods (Kolb and Gibb, 1991; Johansson, 2004; Buchhold et al., 2007; Windle et al., 2007; Jeffers et al., 2014; Kuptsova et al., 2015). Another important consideration is whether the beneficial effects of EE are lasting, since the vast majority of preclinical EE studies maintain enrichment until the time of sacrifice. One study provided ER for 9 weeks, at which time animals post-stroke recovery had plateaued. Thereafter, animals were given two cycles ("tune-ups") of 5 weeks of no treatment followed by 2 weeks of additional ER. However, these

tune-ups provided no additional benefits to recovery. Re-testing throughout this period revealed that the initial functional gains from the first 9-week exposure to ER were maintained, suggesting the benefits of ER are long lasting (Clarke et al., 2009). The demonstrated efficacy of EE across a wide variety of stroke models and conditions, together with the overall positive effects on stroke recovery in meta-analysis, suggests that EE may be an ideal intervention for clinical trial assessment (Janssen et al., 2010).

ENVIRONMENTAL ENRICHMENT AS AN ADJUNCTIVE THERAPEUTIC IN HUMANS

Current State of Post-stroke Activity Levels

Despite the above-mentioned literature highlighting the importance of experience to shape behavior and recovery, people with stroke who are inpatients in hospital have limited exposure to a range of experiences, activities and therapy opportunities. A large body of evidence has demonstrated that stroke patients in hospital (up to 3 months post-stroke) consistently exhibit an activity profile of "inactive and alone". In 2004, Bernhardt et al. reported that stroke patients spend 50% of their time resting in bed, 88.5% in their bedroom and 60% of time alone (Bernhardt et al., 2004) and little has changed in the ensuing years. Patients remain inactive, alone and in their bed/bedroom for large proportions of the day (**Table 3**, Fini et al., 2017). While evidence is limited, it also appears that stroke patients demonstrate low levels of social and cognitive activity: in acute care, social activity represented ∼29.3% of time observed, while cognitive activity represented ∼44.7% of time (Rosbergen et al., 2016) and in subacute rehabilitation, social activity occurred in 32% of observations and cognitive activity in only 4% of observations (Janssen et al., 2014).

These low activity levels of stroke patients raise concerns regarding the rehabilitation environment and demonstrates that little patient-initiated therapeutic activity (i.e., without a therapist) occurs during acute and subacute stroke rehabilitation. Synthesizing perspectives and preferences of stroke patients in acute and subacute inpatient rehabilitation shows that patients highly value physical activity and believe that physical activity levels are highly related to enhanced recovery (Luker et al., 2017). Stroke survivors indicate that they want to practice meaningful activities and have more opportunities to engage in recreational activities (Luker et al., 2017). Indeed, a recent review showed that boredom was a very common experience during inpatient rehabilitation for patients with acquired brain injuries (Kenah et al., 2017). Patients highlight that communal areas and outdoor spaces, which provide opportunities for engagement in activities, reduce boredom (Kenah et al., 2017). Importantly, patients recognize that current inpatient rehabilitation is not meeting their activity needs and remain insufficiently engaging.

Animal studies of ER have provided opportunities for very intensive therapy, whereas human stroke patients are typically limited in this regard. From observational studies, direct therapist time focused on active upper limb therapy has been found to be <5 min per day in the acute setting and <17 min per day in the subacute setting (Hayward and Brauer, 2015), and consistent with ∼32 repetitions (Lang et al., 2009). With regards to lower limb activities, Fini et al. (2017) reported across acute and subacute settings, 9.2% of therapy time was directed to standing and walking. Mean time spent walking was 31 min per day in subacute rehabilitation, with likely even less time spent on walking in acute stroke units as patients are more dependent early after stroke.

As outlined above, the present clinical setting contrasts dramatically with preclinical EE and ER where animals are exposed to a high level of social interaction, cognitive stimulation, opportunities for physical activity and intensive rehabilitation to achieve sensorimotor stimulation (Biernaskie et al., 2004). Therefore, optimization of how stroke patients spend their day in acute or subacute inpatient rehabilitation after stroke may be an avenue for improving stroke outcome by emulating preclinical EE in patient care.

Optimizing the Post-stroke Environment

It is essential to explore alternative opportunities to promote greater social, cognitive, and physical activity post-stroke. EE and ER may be a critical aspect that has been long overlooked in rehabilitation units. Similar to animal models, a

TABLE 3 | % of observed time in bed, in bedroom and alone.

Study	Location	% Observations in bed	% Observations in bedroom	% Observations alone
Bernhardt et al. (2004)	Acute	50	88.5	60
Askim et al. (2012)	Acute and subacute	30.3	–	–
Åstrand et al. (2016)	Acute group	33	82	54
	Subacute group	21	53	52
English et al. (2014)	Subacute	0	55	47
Hokstad et al. (2015)	Acute and subacute	44	74	56
Janssen et al. (2014)	Acute and subacute		Inactive and alone 40	
King et al. (2011)	Subacute	52	76	47
Prakash et al. (2016)	Acute and subacute	52	15	78
Rosbergen et al. (2017)	Acute	68	94.5	58.9
Skarin et al. (2013)	Subacute	38	–	52
van de Port et al. (2012)	Acute and subacute	62	87	61
West and Bernhardt (2013)	Acute and subacute	60	76.1	51.9

natural environment for a human is quite enriched; however, hospital environments have been generally considered to be impoverished. An EE is a non-direct therapy approach that can help to equip stroke survivors with the skills to drive their own activity levels and recovery (Barker and Brauer, 2005). Creating an EE that stimulates activity beyond direct therapy time is an important line to explore in the clinical setting and could address the needs of therapists and stroke survivors. While translation is in its infancy, there are global efforts to learn from animal models of enrichment and translate the EE and ER approach to human stroke rehabilitation settings. This line of research will be discussed in order of stroke progression (i.e., acute to subacute), but will not include enrichment strategies that target a specific activity domain alone such as physical activity through group therapy (English et al., 2015), personalized out of therapy protocols (Harris et al., 2009); or social activity using groups (Higgins et al., 2005).

Translation to Acute Stroke Unit

The acute stroke unit is a unique rehabilitation environment, as the majority of stroke patients are more dependent and require frequent assistance from staff to undertake activities. The EE adaptation tested by Rosbergen et al. (2017) in the acute stroke unit included access to communal areas with a variety of equipment to enhance activities away from the bedside including iPads, books, puzzles, newspapers, games, music and magazines available 24 h a day. Daily group sessions (1-h duration) were provided with a focus on different aspects of stroke recovery such as stroke education, emotional support, communication and upper limb, balance, mobilization activities. An opportunity for communal breakfast and lunch was included to stimulate frequency of mobilization and social interaction, as well as encourage sitting upright for mealtimes (Rosbergen et al., 2016). In addition to environmental changes, stroke patients and families received information that explained the importance of activity after stroke, outlined organizational structure of the unit and how stroke patients and families could contribute to encourage activity out of therapy hours (Rosbergen et al., 2016). Under this protocol, the EE group (n = 30) spent a significantly higher proportion of their day engaged in "any" activity (71% vs. 58%) compared to the usual care group (n = 30) and were significantly more active in physical (33% vs. 22%), social (40% vs. 29%) and cognitive domains (59% vs. 45%). Furthermore, the enriched group experienced significantly fewer adverse events (e.g., falls), with no differences found in serious adverse events (e.g., death). The increased activity levels remained evident in the acute stroke unit environment 6-months post-implementation of the EE paradigm.

Translation to Inpatient Rehabilitation

Janssen et al. (2014) focused on access to communal and personal enrichment spaces with the view to increase activity that was driven by the environment. Patients were recruited during the first 4 weeks post-stroke and communal enrichment strategies included computers with internet connection, reading material, jigsaw puzzles, board games and tablets. Strategies targeting personal enrichment were also used and included access to music, audio books, books, puzzles and board games; family members were encouraged to bring in hobbies and activities that patients enjoyed pre-stroke; staff were advised to encourage stroke patients to access communal areas or use personal enrichment resources when patients were observed inactive. Per this 2-week protocol, Janssen et al. (2014) demonstrated that stroke survivors engaged in an EE were: (a) 1.2 times more likely to do "any activity" compared to individuals with stroke in the control group with no EE (activity change from timepoint 1 to timepoint 2 (ΔT1-T2): 13% EE vs. 2% control observations); (b) 1.1 times more physical (ΔT1-T2: 8% EE vs. 5% control); (c) 1.2 times more social (ΔT1-T2: 3% EE vs. −5% control); and (d) 1.7 times more cognitively active (ΔT1-T2: 7% EE vs. 1% control). This pilot study was small (n = 15 intervention group) but was a critical piece of translation work showing how the field is beginning to approach the post-stroke environment.

An alternative approach to enrichment was explored by Khan et al. (2016) in a larger sample using a randomized controlled trial (n = 103, 51% stroke survivors). Individual and communal EE was offered, including an activity stimulating area, the "activity arcade." In contrast to Janssen, where access to activities was available throughout the entire day, in Khan et al. (2016), access to the activity arcade was for 2-h per day only. Activities provided in the arcade were consistent with Janssen et al. (2014) including computers with internet access; workstations with gaming technology; books; music; life-size mirrors for visuo-perceptual deficits; as well as novel training tasks including simulated shopping corner with groceries, electronic payment machines, and bank teller machines; wood workshop, and other activities. This multifaceted approach is more comparable to preclinical EE, where rodents are exposed to a variety of activities in enrichment chambers (Hannan, 2014). Findings (for stroke patients only) demonstrated significant improvements in depression (Depression Anxiety Stress Scale, DASS mean difference from baseline −24.1 (95%CI −40.1, −7.2) and general function (Functional Independence Measure motor, FIM-motor mean difference from baseline 6.7 (95%CI 0.2, 13.1) at discharge compared to the control group, who received standard therapy on the ward at the same time as enrichment patients. However, no differences in Cognition (Montreal Cognitive Assessment and FIM-cognition) and overall health (EQ-5D) were found between groups and improvements were not maintained within patients at 3-months follow-up. As observation of activity levels was not an outcome measure, the impact of enrichment on activity levels remains unknown.

Collectively the studies completed to date demonstrate important outcomes in activity and function, as well as the ability to embed adjunctive indirect therapy through enrichment of the environment within acute and subacute rehabilitation settings.

Contrasts Between Preclinical and Clinical Enriched Environments

To date, it is clear that the approaches used in preclinical and clinical stroke rehabilitation settings have differed.

TABLE 4 | Differences between preclinical and clinical housing conditions, delivered care and therapy routines.

Housing conditions	
Preclinical EE	**Clinical EE**
Animal cages can be built to have standardized physical environments	Stroke and rehabilitation units physical build varies widely from hospital to hospital
Easy to change housing environment	Difficult to change housing environment (e.g., built floor plan, walls and communal space locations)
Animals unlimited access to all areas	Patient with contact precautions and higher stroke severity (e.g., unable to mobilize independently) have limited access
Controlled number of animals with uniform stroke severity in the environment	Controlled number of patients, but large number of staff, visitors, and non-stroke patients also interacting in environment
Length of stay is based on biology of recovery	Length of stay is pragmatic and limited by funding
Species, care and therapy	
Predominantly young, male rodents	Stroke patients are largely older, mixed sex populations
Controlled daily routine	Daily routine frequently interrupted (e.g., medical investigations, visitors, medical emergencies on acute ward)
Rodents activities are spontaneous, rather than directed by a therapist	Humans activities based on learned behaviors and influenced by therapists, carers and other medical staff
Rodents can engage in any activities as soon as they desire, at any level of intensity (not restricted by investigator)	Human activities may be restricted by care givers (e.g., number of people to assist to mobilize) and/or hospital procedures (e.g., safety measures to prevent falls)
Rodents access only the cage	Humans have access to areas beyond the unit e.g., therapy spaces, outdoor areas, hospital grounds and beyond
Rodent EE encourages more physical, social, and cognitive activity and often contains a variety of self-initiated opportunities for exercise, and in ER, includes intensive reaching practice	Human EE also encourages more physical, social, and cognitive activity, but has fewer opportunities for strenuous exercise or task-specific reaching practice

Key distinctions between animal and human stroke studies are presented in **Table 4**. A significant barrier to clinical implementation is configuration of the EE environment. In animal studies cages are not difficult to standardize, it is easy to increase the novelty of objects and tasks while allowing unlimited access to all areas of the cage. In human stroke rehabilitation it is much more difficult to standardize EE conditions across sites, since stroke rehabilitation units vary, some patients have limited access due to impairment levels, length of stay can vary, and due to cost restrictions, the EE cannot be physically rearranged very easily. Although no sex-specific differences in EE have been identified with regards to stroke rehabilitation, a limitation in preclinical work to date is that most studies have utilized young male rodents. While clinical EE has attempted to mirror the physical, social and cognitive focus of preclinical EE, the opportunity for more strenuous exercise, similar to rodent running wheels, is lacking. Further, few clinical studies to date have attempted to include more task-specific rehabilitation into their EE paradigm similar to ER, which preclinical work has shown to be even more advantageous than EE alone (Jeffers and Corbett, 2018). Nonetheless, taking these differences into account, there are considerable research opportunities to better align preclinical and clinical EE and ER research.

Implementation of EE in Clinical Practice: Are We Ready?

Before wide-spread implementation of EE in a clinical setting, stronger evidence for its benefits in post-stroke patients is required. So far, no large scale clinical trials of effectiveness and cost efficacy have been undertaken (e.g., Phase III). To date, the few small to medium sized studies (n = 14 to n = 52 stroke patients) have demonstrated that activity levels can be increased (Janssen et al., 2014) and appear to remain sustained over time within units (Rosbergen et al., 2017), but not within individuals (Khan et al., 2016). However, we have limited evidence of improved stroke recovery in terms of disability (e.g., modified Rankin Scale), function (e.g., Fugl Meyer Assessment, Action Research Arm Test, walking ability) or participation (e.g., return to meaningful activities); nor evidence of biological changes (e.g., altered functional connectivity, growth factors, etc.) like that found in animal models. It is likely that enrichment is one piece of a complex rehabilitation intervention and thus, trial design is challenging.

There is considerable cause for optimism that EE can increase stroke patient activity indirectly, but potential translational roadblocks need to be addressed prior to wide-spread implementation of EE in a clinical setting. There is a need to consider how we best design an effectiveness trial (e.g., cluster trial), but to progress translation of EE to the clinical setting we need early phased studies as well. Such studies need to focus on building an understanding of how EE works, focusing on the neurobiology and individual differences. While human research cannot always probe the same biological mechanisms available to preclinical research, human studies can use data collected preclinically to guide key biomarkers of interest for the clinic (Boyd et al., 2017). This includes using functional imaging such as resting and functional MRI, EEG and MEG to understand the influence of EE on cortical and subcortical networks, as well as TMS to investigate cortical excitability and inhibitory patterns. Further, structural changes at the macrolevel can be probed, for example using diffusion weighted imaging to explore whole brain white matter fiber integrity, as well as various MRI scans to model microlesion load. Inclusion of blood (to model potential growth-promoting and inflammatory biomarkers) and genetic (to explore BDNF polymorphisms) assays could also

be included to help understand who might benefit most from EE. Exploring biomarker candidates that have been identified in parallel preclinical research may also inform stratification of patients in future trials (Jeffers et al., 2018b).

A better understanding of the optimal dose of EE is required. Trials that attempt to understand the dose characteristics of EE could use novel 3 + 3 designs that progressively increase exposure across physical, social, and cognitive activities that may shape behavior. This can allow sophisticated and detailed analysis of the effect of EE on activity levels, well-being, functional outcomes and fatigue levels. As well, any models of EE must consider the impact of ER evidence in animals. We cannot assume that EE alone will be the recovery breakthrough without considering the need to substantially increase the dose of complex and challenging therapy opportunities. While human studies use behavioral mapping to profile individual patient activities, technological advancements have also enabled rodent tracking on the individual level, using methods such as video shape recognition, or RFID tagging. This alignment of preclinical and clinical research methodologies will enable parallel, and complementary, research to be conducted across species in order to determine the optimal EE environment for promoting neuroplasticity and stroke recovery.

Finally, EE requires the environment to be novel and complex. At present there are limited opportunities for stroke patients to engage in physical, social and cognitive activities within the inpatient rehabilitation environments. To enable access to meaningful activity for stroke patients there is a need to create activities that are accessible outside of therapy. Self-directed upper limb and mobility activities, including smart use of technology such as gaming, robotics and virtual reality may contribute to enhance EE translation.

FUTURE DIRECTIONS

As discussed by the international Stroke Recovery Roundtable group, for stroke recovery research to progress forward there is a need for closer alignment of preclinical and clinical research (Bernhardt et al., 2017a,b; Boyd et al., 2017; Corbett et al., 2017). Despite a significant amount of preclinical research being conducted on the ability of EE and ER to enhance stroke recovery, questions still remain to translate this adjunctive model of therapy to the clinic. For example, while rehabilitation strategies that promote neuroplasticity are important for functional recovery following stroke it is also recognized some forms of neuroplasticity may actually be maladaptive (Jones, 2017). Training the unaffected limb on a reaching task following focal stroke actually worsens behavioral recovery in the affected limb (Allred and Jones, 2008). This maladaptive plasticity is mediated by transcallosal projections (Allred et al., 2010), and has also been linked to abnormal synaptogenesis and decreased neural activation of perilesional cortex (Allred and Jones, 2008; Kim et al., 2015). To lessen the potential for aberrant neuroplasticity when engaging in rehabilitation, such as EE, it is important to try and limit compensatory strategies using the unaffected limb. However, the way in which EE may promote or negate compensatory strategies and learned-nonuse of the stroke-affected limb has not been widely studied in preclinical and clinical studies.

To date, studies that have investigated different EE paradigms in the clinical setting have incorporated a number of cognitive and social components that have been shown to promote greater activity. While increasing any aspect of physical, cognitive, or social activity is important, preclinical EE also has motor components that provide the ability to engage in intense physical activity, more akin to exercise (running wheel, climbing, beam walking, etc.). Since preclinical work has shown that the effects of EE are multi-factorial in nature, to demonstrate clinical efficacy future clinical translation should attempt to better mirror animal EE environments. Integrating more opportunities for patient-initiated goal directed exercise into clinical EE would likely be quite valuable, tapping into both cognitive and motor domains. Indeed, evidence from animal work demonstrates that exercise and cognitively stimulating environments alone do not provide the same magnitude of benefits as when they are provided together (Langdon and Corbett, 2012).

On the other hand, preclinical experiments should attempt to mirror the clinical setting more closely. As previously mentioned, the majority of animal studies have used young male adult rodents (Simpson and Kelly, 2011) while within the clinical setting stroke patients' characteristics vary widely in age, stroke features, comorbidities, and prior living situations. Further, most preclinical EE studies have also administered EE 24 h a day, something that is not achievable in the clinical setting. Experiments that mimic variables encountered in the human stroke population can further contribute to the translation of EE.

Lastly, future design of acute stroke and inpatient rehabilitation units should facilitate early rehabilitation and indirect therapeutic activity. Hospitals are currently moving away from co-location of multiple patients in a bedroom to single patient bedrooms to minimize risk of infection, which results in reduced social stimulation (Anåker et al., 2017). However, to facilitate brain repair and recovery processes after stroke the architectural layout needs to promote early rehabilitation and safe indirect therapeutic activity. In this modern era for clinical practice, there is a need to break down the barriers between the disciplines that can support optimal translation and work collaboratively across the translation pipeline (Bernhardt et al., 2017a,b). This means increasing communication between preclinical and clinical researchers, as well as architecture and technology experts, and health care consumers (i.e., patients and caregivers) to create optimal health environments for stroke survivors that promote activity and recovery. Co-design is a novel methodology that could be integral to unravelling the translational hurdles of EE.

Decades of preclinical research have established that EE is a robust intervention for fostering brain plasticity and recovery from various types of brain injury, including stroke. A number of important questions remain regarding the optimal delivery of EE for promoting recovery from stroke. However, aligning the preclinical and clinical approaches to these questions may greatly accelerate our ability to undertake these challenges, and to work towards implementation of EE into the clinical domain on a large scale.

AUTHOR CONTRIBUTIONS

MM, KH, IR, MJ and DC contributed to the conception, literature search, drafting and revising of the manuscript. Furthermore, all authors approve the publication of this content and agree to be accountable for all aspects of the work.

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