Effects of short-term environmental enrichment on cognition and neurogenic potential: juvenile versus adult mice.

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Abstract

Early in telencephalic development, neural stem cells are born in the ventricular zone and migrate through the cortex before differentiating into neurons and glia. Adult neurogenesis, however, is limited to specific niches in the brain: the dentate gyrus of the hippocampus (DG) and the subventricular zone (SVZ). The proliferation potential of these neural stem cells is plastic and shows changes across states and in response to environmental manipulations. It has previously been shown that short-term environmental enrichment is sufficient to increase the proliferation of the astroglial stem cell pool in the DG of juvenile mice. Because longer-term enrichment protocols are typically used to induce behavioural and functional recovery in adult mice, it is expected that that short-term enrichment will be sufficient to induce an increase in neural stem cell potential only in juveniles. Using male C57 wild-type mice, we examined the potential of SVZ and DG neural stem cells (NSC's) in vitro following short-term enrichment using neurosphere assays in both juvenile (P35) and adult (P90) mice. The assays were examined for neurosphere (NS) size, a marker of proliferation. We also examined the effect of short-term environmental enrichment on cognitive abilities such as learning and memory as well as anxiety behaviour. As expected, we saw a trend that indicated that short-term enrichment increased NSC proliferation in the SVZ in juvenile but not adult mice. We also observed an effect of enrichment on the cognitive abilities of both age groups. Together, these data suggest that even short-term environmental enrichment can influence the proliferative potential of NSC's and play a role in increasing learning and memory in both juvenile and adult mice.

Acknowledgments

First and foremost, I would like to thank my supervisor Natalina Salmaso for taking a chance on me and allowing me to work in her lab. The experience has been difficult and wonderful. I would not have survived grad school without the constant encouragement to trust my instincts and endless opportunities to learn. I would also like to thank my parents who offered me just as much support and always listened to me ramble on about my mice and their brains.

I would also like to acknowledge the lab members and friends who kept me sane through this process. Special thanks to Gareth Rurak who was my partner in crime throughout this experience. Furthermore, I'd like to thank Hosnia Dosso who worked with me on the behaviour and kept a smile on my face.

Lastly, I'd like to thank my committee, Alfonso Abizaid, Jennifer Bruin, Michael Hildebrand and Natalina Salmaso for all discussions and input. I look forward to continuing on this path.

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Abbreviations

NSC- Neural stem cell

NPC- Neural progenitor cell

EGF- Epidermal growth factor

BDNF- Brain-derived neurotrophic factor

Enr- Environmental enrichment

GFAP- Glial acidic fibrillary protein

GFP- Green fluorescent protein

SVZ- Subventricular zone

SGZ- Subgranular zone

DG- Dentate gyrus

DMEM- Dulbecco's modified eagle's medium

FBS- Fetal bovine serum

FGF2- Fibroblast growth factor 2

Effects of short-term environmental enrichment on cognition and neurogenic potential: juvenile versus adult mice.

Introduction

Adult Neurogenesis

It was over a hundred years ago when Ramon Y Cajal first suggested that neurogenesis only occurred during early development. This resulted in years of research that upheld the assumption that there were no new neurons born in the adult brain and thus any injury resulting in neuronal loss could not be repaired. Evidence of adult neurogenesis was discovered almost a century later (Altman, 1962) and was strongly refuted by the scientific community. However, with the introduction of techniques such as radioactively-tagged thymidine, thymidine analogs such as bromodeoxyuridine, and fate mapping, it is now understood that although restricted to a few brain regions in vivo, there is ongoing neurogenesis even in the adult mammalian brain.

Early in telencephalic development, primary neural stem cells, also known as radial glial cells, are born in the ventricular and subventricular zones and migrate radially through the cortex before differentiating into neurons and glia (Rakic, 2009). These cells then extend dendrites and axons and form synaptic connections to create functional networks throughout the brain (Paton and Nottebohm, 1984). In postnatal mice, the potential of cortical neural stem cells (NSC's) diminishes over the first week after birth, but certain areas of the brain including the subventricular zone (SVZ) (Lois and Alvarez-Buylla, 1993) and dentate gyrus (subgranular zone) of the hippocampus (Gage et al, 1995) are considered postnatal neurogenic niches that store NSC's capable of producing neurons and glia. It has recently been proposed that the

hypothalamus may be a third neurogenic niche which can produce functional neurons in response to changes in feeding or energy balance (Kokoeva et al, 2005).

The SVZ is the secondary proliferative site in embryonic life and retains its neurogenic potential throughout adulthood (Noctor et al, 2004). The SVZ is a 4-layered region located adjacent to the walls of the lateral ventricles and contains both neural stem cells and progenitor cells that generate interneurons which travel to the olfactory bulb via the rostral migratory stream (Doetsch et al, 1999). Like stem cells, neural progenitor cells (NPC) differentiate asymmetrically into other cell types (Jhaveri et al, 2015). However, they show a more limited potential and only enter the cell cycle a few times before differentiating themselves (Gage, 2000). NSC's by definition must be 1) self-renewing and 2) multipotent, meaning they can enter the cell cycle an unlimited amount of times and differentiate into all neural cell types: astroglia, oligodendroglia and neurons (microglia arise in the yolk sac and are not of neural origin) (Barres, 2008). The balance between NSC's and NPC's is regulated by epidermal growth factor receptors which also stimulate the proliferation of the stem cell pool (Aguirre et al, 2010). This allows the brain to respond to stimuli and upregulate or downregulate neurogenesis when needed. Neurogenic niches typically maintain a pool of quiescent NSC's that can be stimulated and activated to increase the neuronal pool when needed (Doetsch et al, 1999). The SVZ-born cells that migrate to the olfactory bulb (OB) incorporate themselves into the OB network and contribute to olfactory associative learning, including odor discrimination (Sakamoto et al., 2014). Alonso et al., (2012) have also reported that by activating NSC's in the rostral migratory stream, mice have greater olfactory discrimination that correlated with increased learning and memory.

The dentate gyrus (DG) of the hippocampus is the second postnatal neurogenic niche in the adult mammalian brain. The DG is thought to play a primary role in learning and memory,

spatial navigation, and also serves as the primary regulator of the stress axis (Xavier and Costa, 2009., Nakashiba et al, 2012). This neurogenic niche was first identified by Altman in 1962 and has since been a target for research involving adult neurogenesis and its role in regulating various behaviours. The subgranular zone (SGZ) of the DG contains NSC's and NPC's that produce the neurons which form the granule cell layers. Granule cell projections send information to Cornu Ammonis area 3 (CA3) through the mossy fibre pathway; and together with the CA1, these form the trisynaptic loop (Hastings and Gould, 1999). The neurogenic niches have been shown to respond to various perturbations and environmental manipulations, including stress, exercise, injury and drugs such as anti-depressants. Finding ways in which we can either stimulate or restrict adult neurogenesis can help us elucidate novel therapies for neurodegenerative disease.

Changes in neurogenesis

Since the discovery and acceptance of postnatal neurogenesis, researchers have sought to understand its underlying mechanisms to determine how we can manipulate postnatal neurogenesis and stimulate neuronal repair and/or replacement in a multitude of disease states.

It has been widely reported that stress and glucocorticoids cause a decrease in neurogenesis by inhibiting cell proliferation of neural stem cells in the dentate gyrus. Physiological and psychological stressors, both acute and chronic, decrease cell proliferation in the DG across various animal species and independent of age (Mirescu and Gould, 2006). In a study of stress and depression, Malberg et al (2000) report that anti-depressants may work by reversing the effects of stress by increasing cell proliferation and survival in the hippocampus. The loss of hippocampal volume associated with stress and depression (Duman et al, 1999) can be reversed with chronic anti-depressant treatment (ADT). The ADT is thought to increase brain-derived neurotrophic factor (BDNF) which increases cell proliferation (Nibuya et al, 1995) and in

turn can be linked to a decrease in psychiatric disorders such as depression and anxiety (Tamura and Kataoka, 2017). Increasing hippocampal volume via increased cell proliferation and neuronal replacement may play an important role in reversing the effects of stress and depression in the brain.

Alternately, some neuronal disorders such as epilepsy actually increase neurogenesis. Following prolonged induced seizures, it has been found via Ki67 and BrdU labelling that neurogenesis increases in the SVZ (Parent et al, 2002) and DG (Gray and Sundstrom, 1998). Huttmann et al (2003) found that following Kainate-induced seizures, there was a dramatic increase in astroglial cells but few of the new born cells were exhibiting neuronal markers. However, in a similar study, Parent et al (1997) found an increase in granule cells in the SGZ and suggested that the increase may help with network re-organization post-seizure. Other developmental models show that frequent seizures during the first post-natal week decreased neurogenesis in the DG (Holmes et al, 1998). Knowing the mechanisms that underlie these changes in critical periods for neurogenesis could help us better define how disease models affect the brain at every age.

Winner and Winkler (2015) suggest that dysregulation of the neurogenic system may even be a precursor to neurodegenerative disease such as Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD). Prior to disease onset, many patients report symptoms such as depression, anxiety and cognitive deficits across all 3 diseases (Winner et al 2015). Because the hippocampus is so largely involved in cellular plasticity and mediating these systems, a decline in neurogenesis may be a precursor to neurodegenerative disease. Previous studies into neurodegenerative diseases such as PD, have used transplanted embryonic cells to establish neuronal replacement (Hauser et al., 1999). In 2006, Shinya Yamanaka made a

breakthrough in the world of stem cell research that would win him the Nobel Prize and allow for an individualized stem cell treatment that does not require embryonic tissue. His group was able to induce Pluripotent Stem Cells (iPSCs) by activating 4 key genes- SOX2, Klf4, Oct3/4 and c-Myc in any somatic cell such as a fibroblast. These genes, when active, are responsible for maintaining states of cell proliferation and pluripotency, and can induce stem cell potential in a non-stem somatic cell (Takahashi and Yamanaka, 2006). These findings now permit scientists to de-differentiate any cell type, which can then be re-differentiated to replace specific cells lost through neurodegenerative disease.

Neurosphere assays

Increases in neurogenesis are commonly studied in vivo through thymidine analogue fate mapping. Thymidine analogs, such as BrdU, incorporate themselves into a cell during DNA replication (Russo et al., 1984). This allows us to track cell proliferation *in vivo*. While these increases may be due to increased stem cell activity, they may also be due to increased division of transit amplifying progenitor cells. To truly know the unlimited functional potential of neural stem cells, neurosphere assays need to be conducted. As aforementioned, neural stem cells must possess two qualities, to self-renew and to show multipotency, or the ability to differentiate into neurons, oligodendrocytes and astrocytes. Neurosphere assays allow us to distinguish stem cells from their progeny. The first neurosphere assays were developed by Reynolds and Weiss (1992) in which they dissected striatal tissue containing the SVZ and cultured the cells in media containing epidermal growth factor (EGF). The cells formed spheres of proliferating cells. When mechanically dissociated, the single cells formed secondary neurospheres. A differentiation assay includes plating these cells on an adherent surface without growth factors, where the cells

differentiate into both neurons and glia, representing the qualities of a neural stem cell: self-renewal and multipotency (Pastrana et al, 2011).

Environmental Enrichment

Environmental enrichment was first used in neuroscience research by Donald Hebb in 1947. Enrichment has been shown to increase cognitive abilities such as problem solving, place learning, and memory in rodents (Hebb, 1947). Since the initial studies conducted by Hebb, enrichment has been used as a manipulation to study neuroplasticity in many models of neuronal injury and disease.

Much of the work on environmental enrichment and its effects on the brain and behaviour have been conducted using long-term enrichment, typically a period of 6+ weeks. This has been shown to increase exploratory behaviour (Ferchmin and Bennet, 1975), learning (Kobayashi et al, 2002), synaptogenesis and angiogenesis (Black et al, 1990), and neurogenesis (Nilsson et al 1999) in healthy rodents. In disease models, environmental enrichment has been used to study its therapeutic potential in Autism, Alzheimer's disease, Huntington's disease, Parkinson's disease, stroke, and mood disorders. Rehabilitation and cognitive preservation have been observed in these models (Yamaguchi et al., 2017, Xu et al., 2016, Kreilaus et al., 2016, Jungling et al., 2017, Chen et al., 2017). Short-term enrichment consisting of 4 weeks or less is typically not used, presumably because longer enrichment is needed in order observe measurable effects (Diamond et al, 1966). While short term enrichment may not be sufficient in adults, it is possible that shorter durations of enrichment may benefit other neural changes and/or the effects may differ in younger rodents. In a recent study, juvenile mice that have undergone only 2 weeks of environmental enrichment showed an increase in the astroglial cell pool that produce new neurons in the DG. This increase, which was assessed *in vivo* using genetic fate-mapping and thymidine analogs, was

related to an increase in cognitive abilities and augmented recovery from hypoxia-induced deficits in both brain and behaviour (Salmaso, 2012). These data suggest that there may exist a different plasticity potential or responsiveness to environmental enrichment in juvenile rodents versus adults. However, this study did not examine whether the increase in stem cell pool was associated with an increase in functional stem cell potential as assessed through neurosphere assays *in vitro*. Both enrichment and physical exercise induce increases in neurogenesis: both in neuronal birth and survival. Multiple studies have shown that voluntary exercise in rodents increases adult hippocampal neurogenesis (Nokia et al 2016, van Praag et al, 2002, Ma et al 2017, Ehninger and Kempermann, 2003). Ma et al (2017) state that the increase in postnatal neurogenesis prevents decline in hippocampal related cognition and may play a role in preventing neurodegenerative diseases. Running not only increases neurogenesis, it also increases learning and cognition by way of increasing long term potentiation in the hippocampus (Praag et al 1999). Kemperman et al., (2010) suggests that voluntary exercise stimulates precursor cells and neurogenic proliferation, whereas the novel enrichment promotes cell survival. By providing a running wheel, we are incorporating exercise into environmental enrichment, we hypothesize that the predicted increase in cell proliferation will also be reflected in associated behavioural tests of cognition and show a correlated increase in learning and memory.

It has previously been shown *in vivo* that short-term enrichment is sufficient to increase the proliferation of the stem cell pool in the DG of juvenile mice, therefore, we hypothesize that astroglial cells will increase their self-renewing potential following enrichment in juveniles (P35) in both the SVZ and DG. In an additional experiment, we predict that this will be reflected in increased cognitive abilities and exploratory behaviour as well as a decrease in anxiety behaviour. Additionally, we hypothesize that adult mice (3 months) will show an attenuated response to

short-term enrichment as compared to juvenile mice on both behavioural and neurosphere measures.

Methods

Neurosphere assays- experimental animals

In this study, 32 male C57/BL6 wild type mice (Charles River) aged P21 or P90 were housed in groups of 2-3 in a standard environment or in groups of 5 in an enriched environment. Separated into four groups (Juvenile standard environment= P35SE, juvenile enriched environment= P35ENR, adult standard environment= P90SE, adult enriched environment= P90ENR) (see Table 1 for group membership and subject number breakdown), the animals were caged based on their respective group membership. Housing environments were similar to Salmaso et al, 2012 in order to remain consistent with their procedures/findings. Briefly, the standard environment consisted of a 27cm X 21cm X 14cm transparent polypropylene cage containing only a nestlet. Enrichment consisted of a larger cage (48cm X 25cm X 20cm) containing a running wheel, trapeze, plastic tubing, multiple houses and nestlets. The animals remained in these conditions for two weeks, during which the enrichment was reconfigured weekly to sustain novelty. A 12-hour light/dark cycle in a controlled (21 degrees) environment with food and water was maintained. All animal use procedures were approved by the Carleton University Committee for Animal Care, according to the guidelines set by the Canadian Council for the Use and Care of Animals in Research.

Isolation and culture of mouse NSC's

NSC cultures were conducted similar to previous studies (Salmaso, 2011). After a 2-week period of either standard or enriched environment, the mice were sacrificed by rapid decapitation. This was performed over two days, using mice from each group on each day to decrease any potential confound of time. In groups of 2 to 4, brains were removed and the subventricular zone and hippocampus were dissected into 1mL of cold DMEM/F-12 W/ HEPES and L-glutamine cell

culture media (Millipore, #DF-041-B). The SVZ and hippocampus were placed into separate petri dishes, each containing 1mL of 0.05% Trypsin EDTA (ThermoFisher, #25300062). Using the back of a 1mL syringe, sections were quickly mashed until the tissue was dissociated. Using a 1mL pipette, 1.5mL of trypsin was added to the dish and the mixture was mechanically dissociated until no large chunks remained. The tissue and trypsin solution was then transferred into a 15mL conical tube and incubated at 37˚C and 5%CO_2 for 15 minutes. In a Bio-safety cabinet, 25µL of DNAse I (Sigma-Aldrich, #4716728001) was added to each tube and incubated for another 2-5 minutes. The tissue was mechanically dissociated 20-30 times using a 1mL pipette. The entire solution was then passed through a 40 µm cell strainer (Fisher Scientific, #08-771-1) into a 50mL conical tube and rinsed with 10 mL of 10% FBS/ DMEM-F12 w/ HEPES and L-glutamine solution (FBS- ThermoFisher, #12484010). The new solution was then transferred into a 15mL conical tube and centrifuged for 5 minutes at 1200rpm. Using a 1mL pipette, the media was removed carefully and disposed of, being careful not to disturb the pellet at the bottom. One mL of fresh DMEM-F12 w/ HEPES and L-glutamine was added, mechanically dissociated 20-30 times and centrifuged for 5 minutes. The media was removed and disposed of and 1-2mLs of supplemented DMEM (containing fibroblast growth factor 2 (FGF2, ThermoFisher, #PHG0266, 200ng/ml), epidermal growth factor (EGF, ThermoFisher, #PHG0311, 100ng/ml), N2 (ThermoFisher, #17502001, 10µl/ml) and B27 (ThermoFisher, #17504044, 20µl/ml) supplements, penicillin/streptomycin (ThermoFisher, #15070063, 100U/ml) was added and the solution was mechanically dissociated.

Plating at clonal density

Using a 1:1 concentration, 20µL of cell solution was added to 20 µL of trypan blue (ThermoFisher, #T10282) in an Eppendorf tube. The solution was mixed by pipetting up and

down. Twenty µL of the solution was transferred to a hemacytometer and the cells were counted under an inverted microscope at 40X. The density of the cell solution was calculated and the cells were plated at 500, 000 per well in a 12-well plate containing 1mL of supplemented media. The remaining cells were frozen in 90%FBS-DMSO solution in liquid nitrogen for future experiments. The plates were incubated at 37˚C and 5% CO2, with a 60% media change every 48-72 hours.

Passaging cells

After 14-20 days, primary neurospheres formed in the wells. To passage the cells, 75% of the media was removed and replaced with 500 µL of 0.05% trypsin-EDTA. The wells were incubated for 10 minutes at 37˚C. The solution was transferred to a 15ml conical tube and centrifuged for 5 minutes at 1200rpm. The solution was removed carefully as not to disturb the pellet, washed with 1ml of DMEM-F12 w/ HEPES and L-glutamine and mechanically dissociated. This mixture was then centrifuged for 5 minutes, the media was removed and replaced with supplemented media and dissociated. The plating and counting procedure was performed as explained above.

Secondary Neurosphere analysis

After approximately 14 days, secondary neurospheres will have formed. Only wells that showed no contamination and healthy sphere formation were considered. The spheres were photographed and counted at 10X under an inverted microscope or an EVOS XL core (ThermoFisher). The spheres were measured using ImageJ software. The size of the five largest neurospheres from each well were measured in ImageJ and averaged. Data were calculated based on the average of 3.5 technical replicates for the SVZ and five for the DG. The mean of the technical replicates was calculated for each animal and contributed to the average neurosphere size per animal. The experimental timeline is outlined in Figure 1B.

Behaviour

In this experiment, 40 male C57/BL6 wild type mice (Charles River) aged P21 or P90 were housed in groups of 2-3 in a standard environment of in groups of 5 in an enriched environment. Separated into four groups, the animals were caged based on environment and age (Table 1). The housing and enrichment protocols are identical to those described above (see *experimental animals*). All animal use procedures were approved by the Carleton University Committee for Animal Care, according to the guidelines set by the Canadian Council for the Use and Care of Animals in Research.

After 13-14 days of enrichment, the animals were split into 2 groups at random and underwent 3 behavioural tests over 4 days including the open field, elevated plus maze and Morris water maze in order. The open field and elevated plus maze were conducted on days 1 and 2 while the Morris water maze was conducted on days 3 and 4, allowing one day of rest for each group of animals. The experimental timeline is outlined in Figure 1A.

Open Field Test and locomotor activity

The open field test is used to measure exploratory and anxiety behaviours. Testing conditions had been established in previous protocols (Salmaso et al., 2012). Mice were brought into the room and allowed to acclimatize for 15 minutes prior to testing. The mice were placed in the corner of a brightly lit (650 lux) white Plexiglas box (50 X 50cm with 35cm high walls) and videotaped using AnyMaze Video Tracking System for 20 minutes. Time spent in the pre-determined zones (center and periphery) was recorded. Exploratory and anxiety behaviour was measured by calculating amount of time spent in each zone, as well as the frequency of each entry into the zones over the first 5 minutes of the test. Locomotor data was collected over the full 20 minutes. The testing zone was cleaned with Accel wipes between each trial. Once an animal had

completed the open field test, it was ear notched and replaced into its cage and allowed a two hour break before undergoing the elevated plus maze.

Elevated Plus Maze

The Elevated Plus Maze is used to measure anxiety behaviour. Testing conditions had been established in previous protocols (Salmaso et al., 2016). Mice were placed in the center of the maze and are allowed to freely explore both the closed arms which are encased in walls and the open arms with are devoid of walls. In general, mice tend to spend more time in the closed arms which is typically associated with anxiety behaviour. The exploration of the open arms therefore represents a lack of anxiety behaviour. The dimensions of the arms are 30 X 5cm, with the enclosed arms having walls 25cm high. The entire maze is 30cm above floor level. The activity of the mice was recorded using AnyMaze Video Tracking Software for 5 minutes. The amount of time spent in the open and closed arms, latency to enter the open arms and distance travelled in the open arms was recorded. This test was performed approximately 2 hours after the Open Field test on either Day 1 or 2. The testing area was cleaned with Accel wipes between trials.

Morris Water Maze

On the third and fourth days of testing, the mice underwent a shortened Morris Water Maze adapted from previous studies (Nunez, 2008; Salmaso et al, 2012) that assesses cognitive abilities such as learning and memory. The mice were brought into the room containing the maze to acclimatize to the environment for 15 minutes. The room was marked with visual cues on each wall surrounding the water basin. The mice were placed in quadrants in a random order and allowed to swim until they found the platform, or for 60 seconds at which point they were guided

to the platform and held there for 10 seconds. They were dried and replaced in their cages for at least 10 minutes before the next trial. Each mouse was given 3 sets of 4 trials separated by a 45-minute break. The mice were placed in 1 of 4 quadrants each trial and the latency to reach the platform (fixed location) was recorded. Following the 12th trial, the mice were placed into a probe trial in which the platform was removed and each mouse was placed in the same quadrant facing the tub and allowed to swim for 60 seconds. The time spent swimming in the target area (area surrounding location of platform from previous trials) was recorded.

Upon completion of behavioural testing, the animals were sacrificed using CO2 overdose and decapitation.

Statistics

All data were analyzed using IBM SPSS statistics data editor (version 20). Analysis of variance was conducted with age and environment as independent variables. When no statistically significant interactions were found, main effects were reported. When main effects were found to be significant, appropriate post-hoc tests were used to assess simple comparisons. Probability values were considered statistically significant when $p<0.05$.

Table 1: Number of Animals Used

	Standard Environment	Enriched Environment
P35	Behaviour n=10 Neurosphere Assay n=8	Behaviour n=10 Neurosphere Assay n=8
P90	Behaviour n=9 Neurosphere Assay n=8	Behaviour n=10 Neurosphere Assay n=8

Experimental timelines

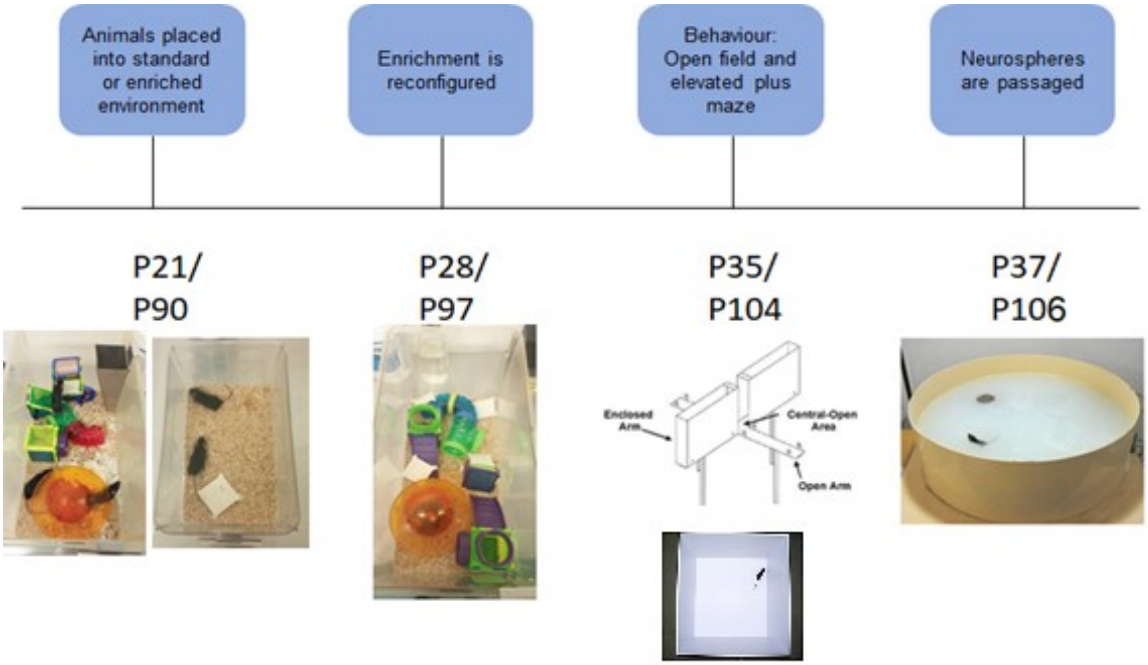

Figure 1A: Behavioural timeline

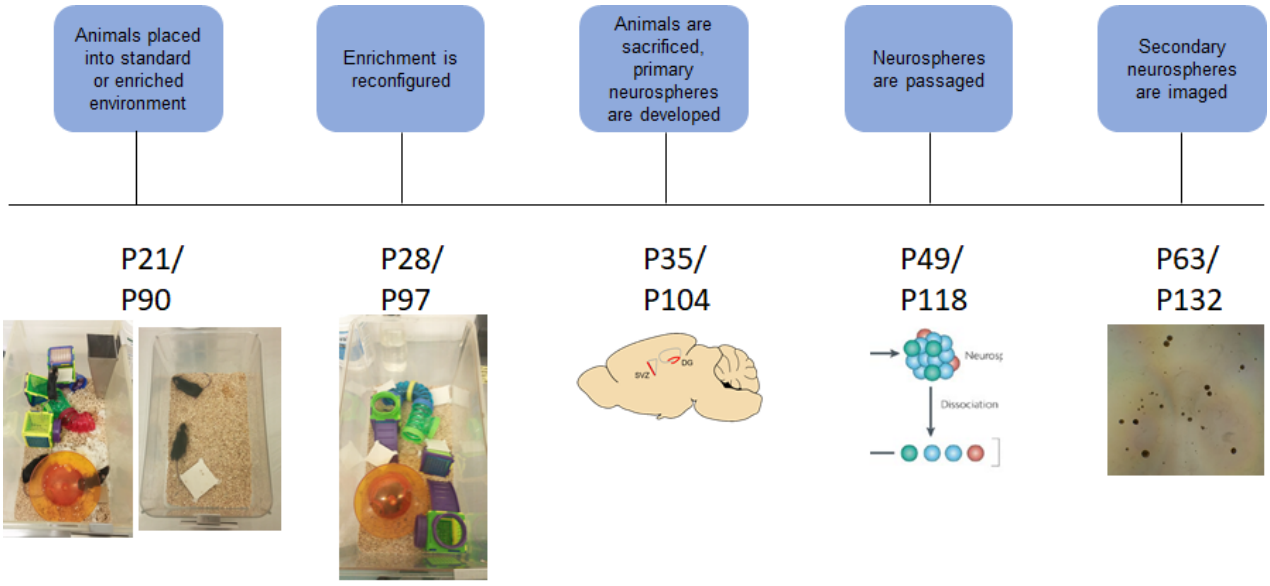

Figure 1B: Neurosphere assay timeline

Results

Behaviour

Locomotor activity- In order to assess if age or environmental enrichment have an effect on locomotor activity, mice were placed into the open field test for 20 minutes. Their distance travelled inside the box as well as average speed showed trends for a significant main effect of age (F, p=0.079; F, p=0.08, respectively) such that older mice were slower and travelled less than juveniles (Figure 2 A,B).

Anxiety Behaviours- To assess anxiety behaviours, both juvenile and adult mice from enriched and standard environments were tested in the open field and elevated plus maze. Juvenile and adult mice that were exposed to short-term environmental enrichment spent significantly more time in the center zone of the open field ($F_{1,38} = 4.674$, $p=0.038$) compared to controls in standard environment (Figure 3A). No freezing behaviour was observed during this test, indicated by the lack of difference between groups in distance travelled in the center of the test (Figure 3B). Latency to enter the center zone also showed no significant difference between groups (Figure 3C). To examine anxiety behaviours in the elevated plus maze, we assessed time spent in open arms, distance travelled in the open arms and latency to enter the open arms, all showed no significant differences between groups (Figure 4 A, B,C).

Cognitive behaviour- In order to assess cognitive behaviours often associated with environment enrichment, we used the modified one-day Morris Water Maze. Over twelve trials, enriched mice (both juvenile and adult) had a greater number of successes to find the platform compared to

standard controls ($F_{1,38}= 9.548$, $p=0.004$, Figure 5A) indicating that the enriched mice learned significantly faster, however post-hoc tests showed that this effect was only evident in P35 mice and not P90. Indeed, when we evaluated at which trial 70% of the mice found the platform, we found that P35SE reached this criterion at Trial 7; P35Enr at Trial 4; P90SE did not reach the criterion; and P90Enr at Trial 6; highlighting that environmental enrichment enhances the rate of acquisition, however this is augmented in juveniles. To assess memory abilities, we evaluated the time spent swimming in the target zone (location of platform after it was removed) and found that enriched mice also had a statistically significant higher rate of remembering the location of the platform ($F_{1,38}= 14.742$, $p=0.00$, Figure 5B). This suggests that enrichment improved memory for the platform regardless of age, however, juvenile mice showed a greater rate of acquisition (learning).

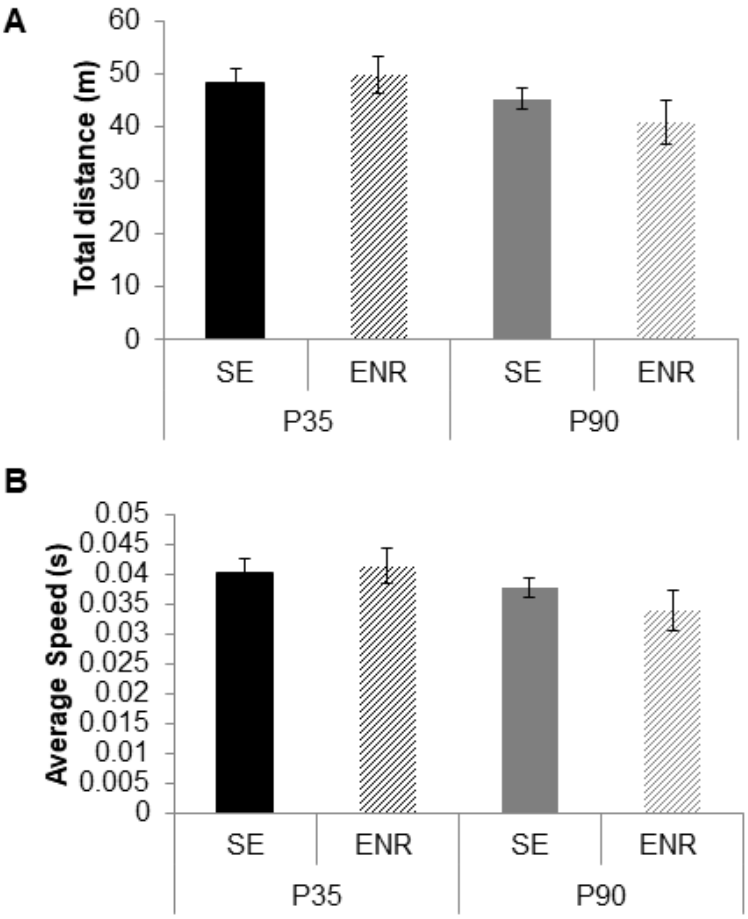

Figure 2. Locomotor Activity does not change with age or Enr. Panel A is a graphical representation of the total distance travelled over the entire open field in 20minutes. Panel B is a graphical representation of the average speed travelled over the entire open field in 20minutes. Bars depict group means and error bars represent ±SEM.

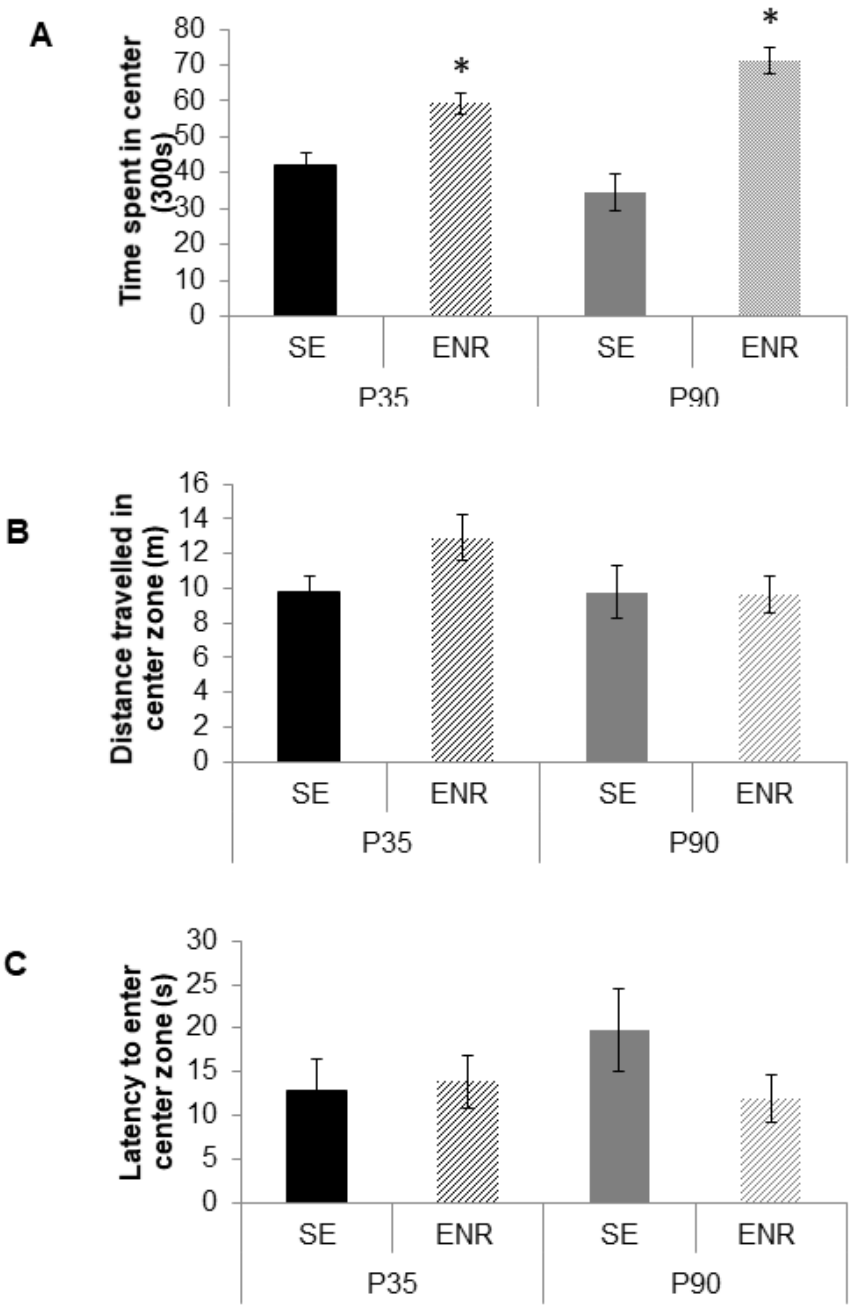

Figure 3. Enrichment decreases anxiety of both juvenile and adult mice. Panel A is a graphical representation of time spent in the center of the Open Field Test over 5 minutes. Panel B is a graphical representation of distance travelled in the center zone (m). Panel C is a graphical representation of latency to enter the center zone (s). Bars depict group means and error bars represent ±SEM. * depicts statistical significance from standard controls.

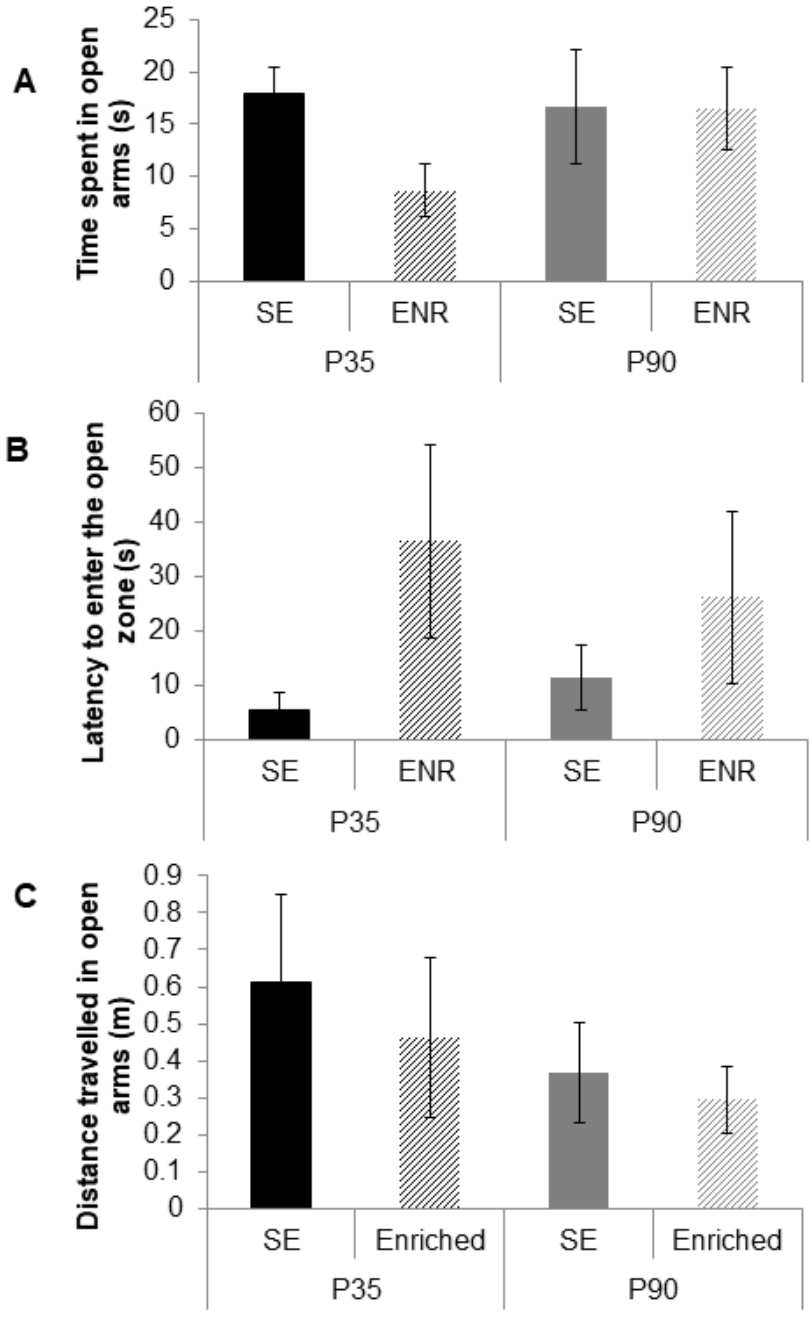

Figure 4. Elevated plus maze shows no difference in anxiety behaviours with respect to age or environment. Panel A is a graphical representation of time spent in open arms (s). Panel B is a graphical representation of latency to enter the open arms (s). Panel C is a graphical representation of distance travelled in the open arms (m). Bars depict group means and error bars represent ±SEM.

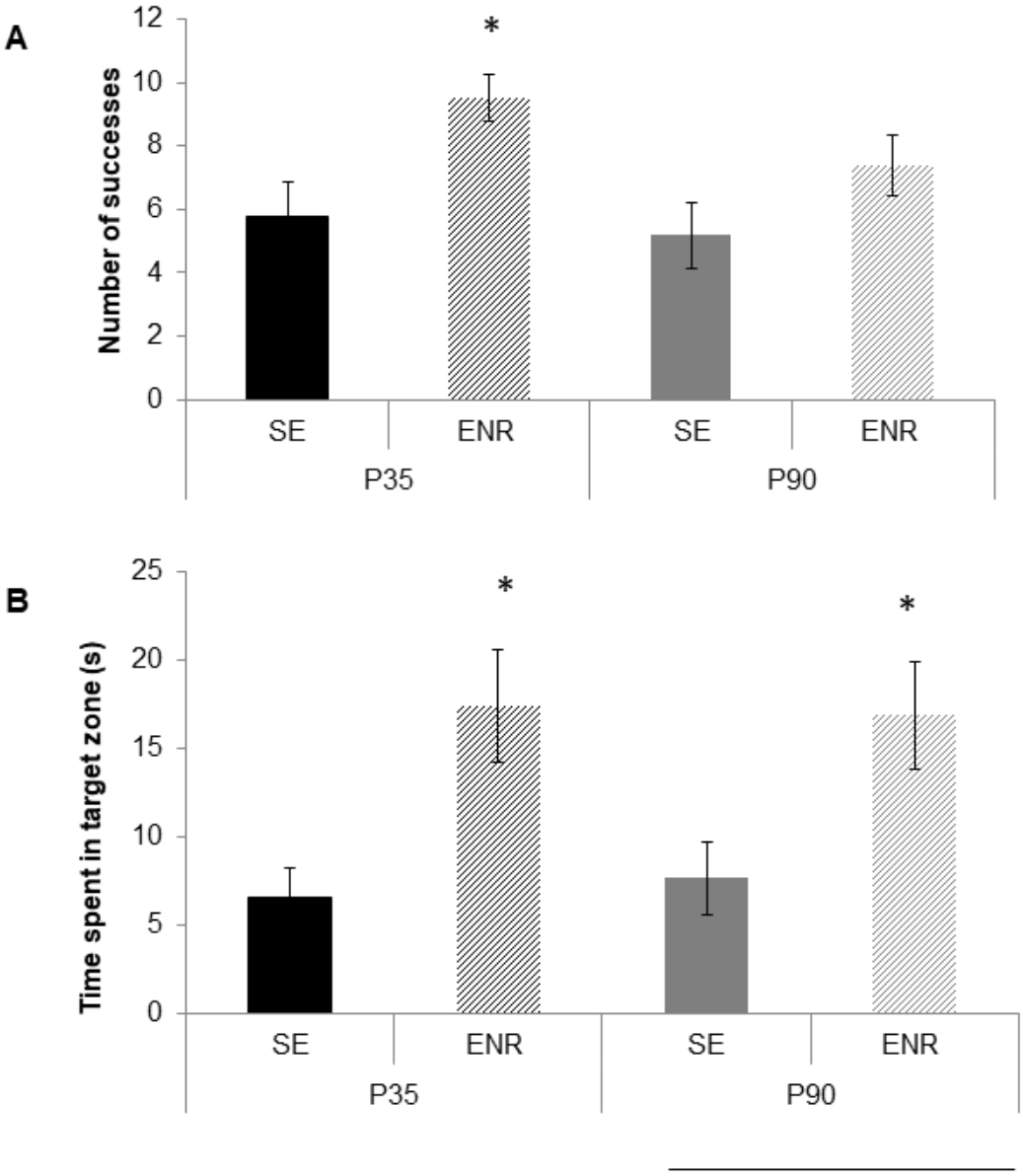

Figure 5.Enrichment improves cognitive abilities of both juvenile and adult mice. Panel A is a graphical representation of the number of successes to reach the platform over 12 trials in the Morris water maze. Panel B is a graphical representation of the time spent swimming in the target zone during the probe trial.. Bars depict group means and error bars represent ±SEM. * depicts statistical significance from to standard controls.

Neurosphere Assays

To assess the differences in proliferative potential of NSC's from the SVZ and DG, neurosphere assays were conducted. Size of secondary neurospheres were measured as a marker for proliferation. In the SVZ, there was a trend for an interaction ($p=0.082$) such that enriched juvenile mice tended to have larger neurosphere size and thus greater proliferation in comparison to their adult and standard counterparts (Figure 6A). Formation of healthy neurospheres from the DG were observed only in a subset of cultures from one cohort. Therefore, results cannot be adequately analysed due to lack of power (1 or 2 subjects per group, 6 technical replicates), however, the pattern of results obtained in the first cohort are similar to the SVZ (Figure 6B) suggesting a potentially generalised effect across niches.

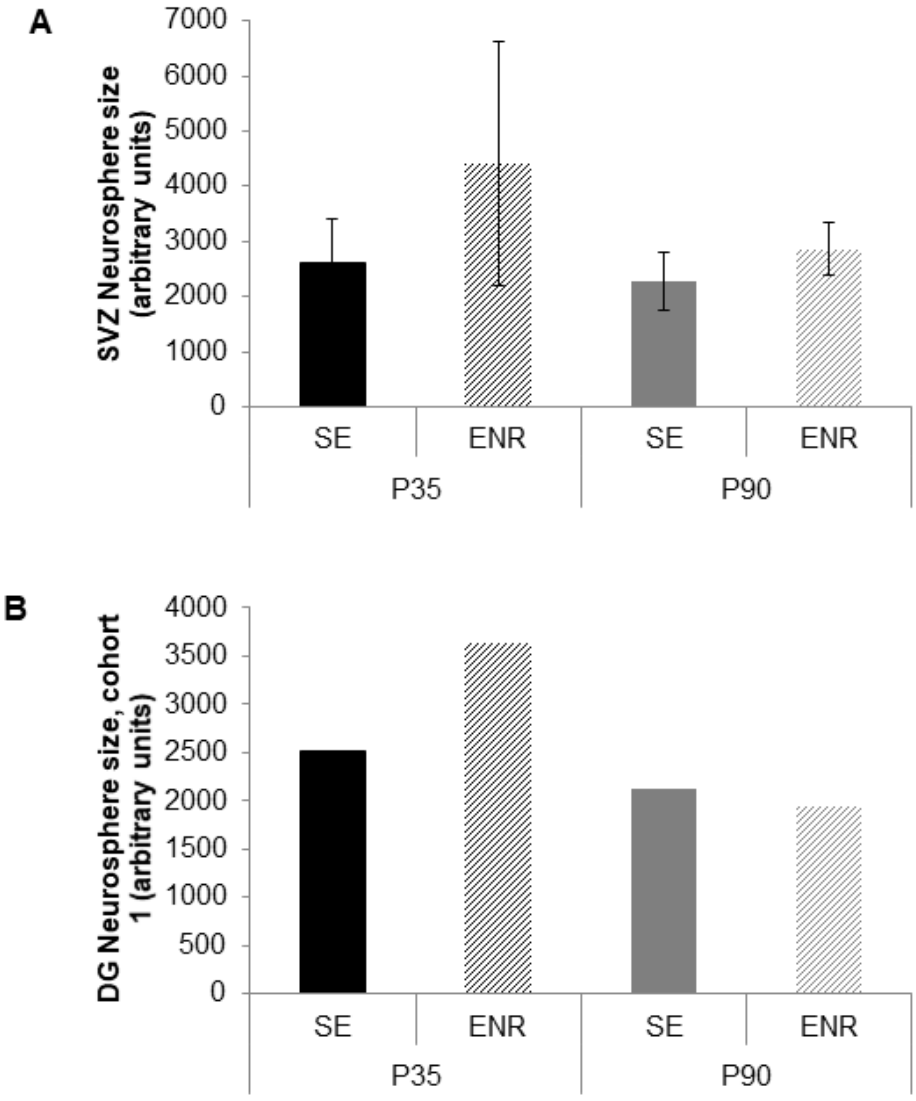

Figure 6.Enrichment shows no statistical significant difference in neurosphere size. Panel A is a graphical representation of neurosphere size in the Subventricular zone. Panel B is a graphical representation of neurosphere size in the Dentate gyrus, no error bars are used because of low power. Bars depict group means. Error bars represent ±SEM in Panel A.

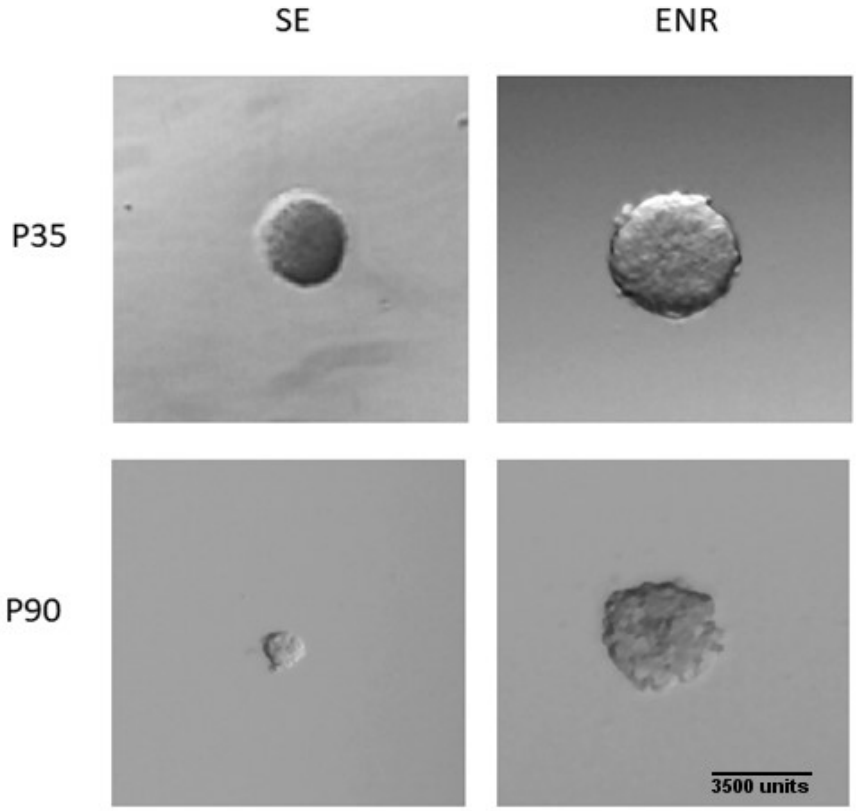

Figure 7. Representative photomicrographs of neurospheres from each condition taken on EVOS XL core at 10X magnification. Photos depict secondary neurospheres from the SVZ.

Discussion

In the current study, we examined how short-term environmental enrichment affects the proliferative potential of NSC's in the SVZ and DG of juvenile and adult mice. We also examined whether age and enrichment would affect behaviours associated with hippocampal function and neurogenesis- specifically, cognitive abilities and anxiety behaviour. We used neurosphere assays to culture NSC's from both the SVZ and DG. As expected, NSC's dissected from the SVZ of young (juvenile) mice that had experienced an enriched environment showed increased proliferation when compared to both older (adult) mice and those raised in a standard environment, suggesting a decrease in neurogenic potential with age. The SVZ as a neurogenic niche is susceptible to many environmental cues that can influence stem cell activity and directly influence behaviour of the animal (Urban and Guillemot, 2014). It is well documented that long term enrichment may increase neurogenesis in the DG (Huttenrauch et al., 2016), however we have shown that even short-term enrichment can have an effect on the stem cell pool from the SVZ in vitro. A similar pattern of results was seen in neurospheres generated from the DG, however, our number of subjects is too low to make conclusive interpretations.

The difficulties in generating neurospheres from the DG are not necessarily surprising. The DG contains only a fraction of the NSC potential that the SVZ possesses meaning that the DG stem cells produce fewer progenitor cells because it is restricted spatially more than the SVZ and rostral migratory stream (Riddle and Lichtenwalner, 2007). Technically, isolating stem cells from the SVZ is also simpler then dissecting the DG, which is a more difficult anatomical structure to delineate and contains many more cell types that may contaminate the culture. Therefore, together with the additive impact of aging, culturing DG born NSC's represents a larger challenge than the standard SVZ neurosphere assay. Nevertheless, the similarity in the pattern of results between the

two niches may suggest a universal response to enrichment and aging on neurogenic potential in the brain. However, given the preliminary nature of the current study, further replications would be needed to ascertain the reliability of this finding.

In future studies, it will be vital to further optimise our protocol to examine how the DG responds to short-term enrichment in both juvenile and adults, although it is possible that generation of neurospheres from the DG is simply not viable in adults. Future studies will also examine the second characteristic of stemness: multipotency. Differentiating stem cells allows us assess the multipotency of these cells and how this potential may change in response to aging and environmental enrichment.

In addition to examining the proliferative potential of NSC's, this study also wanted to examine the functional outcome of age and enrichment on behaviours previously associated with hippocampal function and neurogenesis. It was previously demonstrated (Salmaso et al., 2012) that juvenile mice show increased cognitive abilities in the Morris water maze. The Morris water maze is a measure of learning and memory typically associated with the function of the hippocampus. In the current study, we found that enriched juvenile mice learned the task more quickly than other groups, however both juvenile and adult mice showed similar increases in memory retention in response to enrichment. This suggests that while enrichment is useful in increasing cognitive abilities in both age groups, the younger mice benefit slightly more and have a faster rate of acquisition.

In our current study, we also examined the effects of age and enrichment on anxiety behaviour, as assessed in the open field test and elevated plus maze. Our data show that enriched mice spent more time in the center zone of the open field test as compared to their standard counterparts, suggesting that short-term enrichment decreased anxiety irrespective of age. These

results suggest a dissociation between cognitive and anxiety behavior and the interaction of age and enrichment. Indeed, although both behaviours are hippocampal dependant, substantial research has shown that the dorsal hippocampus is primarily responsible for cognitive behaviours versus the ventral hippocampus, which is believed to be more responsible for emotive behaviours such as those seen in depressive and anxiety phenotypes (Bannerman et al., 2003). Further studies would be required to test whether the effects of enrichment are differentially affecting the dorsal versus the ventral hippocampus.

Although preliminary, altogether these data suggest that short-term enrichment can have beneficial effects on memory and anxiety behaviour regardless of age, enriched adult mice show an attenuated increase in rate of learning as compared to the effect of enrichment at P35 as seen in the Morris water maze. Neurogenic potential showed a similar pattern of results to cognitive abilities such that the effects of short-term enrichment were greater at P35 then in adults. Whether the changes in neurogenic potential are related to the observed changes in cognitive behaviour remains to be determined.

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