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AGED DOMINANT NEGATIVE p38 α MAPK MICE ARE RESISTANT TO AGE-DEPENDENT DECLINE IN ADULT-NEUROGENESIS AND CONTEXT DISCRIMINATION FEAR CONDITIONING

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Abstract

A major aspect of mammalian aging is the decline in functional competence of many self-renewing cell types, including adult-born neuronal precursors. Since age-related senescence of self-renewal occurs simultaneously with chronic up-regulation of the p38MAPK α (p38 α) signaling pathway, we used the dominant negative mouse model for attenuated p38 α activity (DN-p38 $\alpha^{AF/+}$) in which Thr180 and Tyr182 are mutated (T $\rightarrow$ A/Y $\rightarrow$ F) to prevent phosphorylation activation (DN-p38 $\alpha^{AF/+}$) and kinase activity. As a result, aged DN-p38 $\alpha^{AF/+}$ mice are resistant to age-dependent decline in proliferation and regeneration of several peripheral tissue progenitors when compared to wild-type littermates.

Aging is the major risk factor for non-inherited forms of Alzheimer's disease (AD); environmental and genetic risk factors that accelerate the senescence phenotype are thought to contribute to an individual's relative risk. In the present study, we evaluated aged DN-p38 $\alpha^{AF/+}$ and wildtype littermates in a series of behavioral paradigms to test if p38 α mutant mice exhibit altered baseline abnormalities in neurological reflexes, locomotion, anxiety-like behavior, and age-dependent

cognitive decline. While aged DN-p38 $\alpha^{AF/+}$ and wildtype littermates appear equal in all tested baseline neurological and behavioral parameters, DN-p38 $\alpha^{AF/+}$ exhibit superior context discrimination fear conditioning. Context discrimination is a cognitive task that is supported by proliferation and differentiation of adult-born neurons in the dentate gyrus of the hippocampus. Consistent with enhanced context discrimination in aged DN-p38 $\alpha^{AF/+}$, we discovered enhanced production of adult-born neurons in the dentate gyrus of DN-p38 $\alpha^{AF/+}$ mice compared to wildtype littermates. Our findings support the notion that p38 α inhibition has therapeutic utility in aging diseases that affect cognition, such as AD.

Keywords

Aging; behavior; context discrimination; adult neurogenesis; fear conditioning; hippocampus

1.0 INTRODUCTION

Aging is a complex organismal process that is controlled by genetic, environmental, and behavioral factors. A major aspect of mammalian aging is an age-associated decline in functional competence of many self-renewing cell types, including adult stem cells and other proliferative progenitors (Blau, Cosgrove, & Ho, 2015; Janzen et al., 2006; Molofsky et al., 2006). Further, age-related senescence of self-renewal occurs simultaneously with up-regulation of the p38MAPKalpha (p38 α) signaling pathway (Brien, Pugazhendhi, Woodhouse, Oxley, & Pell, 2013; Haq et al., 2002; Hsieh & Papaconstantinou, 2002; Hsieh, Rosenblatt, & Papaconstantinou, 2003; Iwasa, Han, & Ishikawa, 2003). One consequence of chronically elevated p38 α is a chronic pro-inflammatory state that promotes the expression of a physiologically complex aging phenotype both in the periphery and the CNS leading to susceptibility of age-related diseases including cancer and neurodegenerative diseases (Bachstetter & Van Eldik, 2010; Xu, Li, Xiang, & Sun, 2014). As such, it has been proposed that modulation of p38 MAPK activity may provide new avenues for treating certain age-related degenerative diseases.

Previous work by ourselves and others using mice that express a mutant form of p38 α in which Thr180 and Tyr182 are mutated (T→A/Y→F) to prevent phosphorylation activation results in dominant negative mitigation of p38 α (DN-p38 $\alpha^{AF/+}$) kinase activity. Consistent with the notion that p38 α is a significant regulator of cell senescence, aged DN-p38 $\alpha^{AF/+}$ mice are resistant to age-dependent decline in the proliferation and regeneration of several peripheral tissue progenitors when compared to wild-type littermates (Papaconstantinou et al., 2009, 2015; Wong et al., 2009). This has led to the hypothesis that in the periphery, p38 modulates the regenerative capacity of tissues that rely upon a progenitor population (e.g., muscle, liver, and pancreas).

Aging is the major risk factor for non-inherited forms of Alzheimer's disease (AD); environmental and genetic risk factors that enhance chronic inflammation and accelerate the senescence phenotype are thought to significantly contribute to an individual's relative risk for AD. It is not surprising then that p38 α MAPK represents an actively pursued therapeutic target for age-dependent cell senescence, neuroinflammation, and AD (Bachstetter & Van

Eldik, 2010; Munoz & Ammit, 2010; Pinsetta, Taft, & de Paula da Silva, 2014). In the present study, we evaluated aged DN-p38 $\alpha^{AF/+}$ and wildtype littermates in a series of behavioral paradigms to test if p38 α mutant mice exhibit altered baseline abnormalities in neurological reflexes, locomotion, anxiety-like behavior, and age-dependent cognitive decline. While DN-p38 $\alpha^{AF/+}$ and wildtype littermates appear equal in baseline neurological and behavioral parameters, DN-p38 $\alpha^{AF/+}$ exhibit superior context discrimination fear conditioning; a cognitive task that is supported by enhanced production of adult-born neurons in the dentate gyrus of the hippocampus, one of the major CNS progenitor sources (David J Creer, Carola Romberg, Lisa M Saksida, Henriette van Praag, & Timothy J Bussey, 2010; Sahay et al., 2011; Spalding et al., 2013). Quantification of new adult-born neurons revealed that aged DN-p38 $\alpha^{AF/+}$ have more BrdU/NeuN-positive neurons in the granule cell layer of the dentate gyrus compared to wildtype littermates. Our findings support the notion that p38 α inhibition has therapeutic utility in aging diseases that affect hippocampus-dependent cognitive function, such as AD.

2.0 MATERIALS AND METHODS

Materials

Chemicals and reagents were purchased from Sigma.

2.1 Animals

Dominant negative-p38 $\alpha^{AF/+}$ (DN-p38 $\alpha^{AF/+}$) mutant mice were created on a C57Bl/6j background strain in the laboratory of Dr. Dmitry V. Bulavin (David J Creer et al., 2010; Wong et al., 2009) and provided by Dr. John Papaconstantinou (Papaconstantinou et al., 2009). Wildtype and heterozygous DN-p38 $\alpha^{AF/+}$ were generated according to breeding strategies employed in (Papaconstantinou et al., 2009, 2015; Wong et al., 2009) and housed in the Animal Resource Center Facilities at the University of Texas Medical Branch (UTMB). UTMB Animal Resource Center Facilities operate in compliance with the USDA Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, under OLAW accreditation, and IACUC approved protocols. Male and female mice were housed separately, n = 5 per cage, with food and water *ad libitum* and aged (10–19 months of age). Median age of wild type and DN-p38 was 10MO, 13MO, respectively. Average age for wild type and DN-p38 $\alpha^{AF/+}$ was 12.5MO, 13.5MO, respectively. There was no significant difference in distribution of age as indicated by student t test [$t=0.87$, $df=22.02$] p value = 0.39. Genotyping was performed as described in (Papaconstantinou et al., 2009, 2015; Wong et al., 2009). Heterozygous DN-p38 $\alpha^{AF/+}$ were used as homozygous mutant mice die *in utero*. Experimenters were blinded to genotype during key data acquisition and analysis steps.

To optimize contextual-fear discrimination, 2.5-month old C57Bl/6J were purchased from Jackson Laboratory and aged to 3-months of age in animal care facilities here at UTMB, before testing. For this experiment, mice (n=10; 5 male, 5 female) were exposed to shock context on day 0 where they were allowed to explore for 178s before receiving a 0.75mA footshock for 2-s; the session was terminated 15 seconds after receiving the footshock (Figure 4A) and mice were placed back in their home cages. From day 1 and onward, %

freezing was recorded after mice were placed in the shock context A or the safe context B as indicated in Figure 4B.

General health, neurological, behavioral and cognitive tests were performed on 29 DN-p38 $\alpha^{AF/+}$ s and 13 wildtype mice (roughly equal for gender) in UTMB's *Rodent in Vivo Assessment (RIVA)* core facility (directed by Dr. Kelly Dineley). In spite of a disparate number of wildtype and DN-p38 $\alpha^{AF/+}$ mice, F tests for unequal variance indicated statistically equal variance between the two groups (*see statistics, below*). All mice were tested during 12hr light cycle (06:00 – 18:00hr) then returned to home cages prior to the 12hr dark cycle. Further, mice were acclimated to the testing room at least 1hr before behavioral and cognitive tests were performed. Furthermore our genotypes were analyzed as a single cohort in the order listed below and only one test battery, e.g., SHIRPA, grip strength, etc. was performed each day.

2.2 Behavioral Assessment

The *SHIRPA* (Rogers et al., 2001) general health and neurological screen was used to reveal discrete phenotypes in DN-p38 $\alpha^{AF/+}$ and wildtype mice. Briefly, weight, coat appearance, and spontaneous activity were recorded using the arbitrary scale 0–4; where 0=none, 1=low, 2=moderate, 3=fair, and 4=good. Next, observations related to cage activity included transfer to testing cage, arousal, gait, etc., were recorded on the same scale. Lastly, provoked activity i.e. trunk curl, wire maneuver and reflex tests were also recorded.

2.3 Forelimb Grip Strength

To assess forelimb strength, we utilized the DFIS-2 Digital Force Gauge (Chatillon®). Each mouse was gently but firmly held by the base of the tail and allowed to grab a metal bar with both forelimbs. While grasping the metal bar, grip strength was recorded by gently pulling the mouse away from the metal bar until the grip was lost. Average grip strength in (kg), was calculated for each mouse from three consecutive trials.

2.4 Open Field

The open field test measures exploratory behavior and locomotor activity in mice. Briefly, each mouse was placed in the center of a white 38 x 38cm Plexiglas box and allowed to explore for 10min. Digital video-based data capture and analysis was achieved with TopScan software (Clever Sys) using central (12.6 cm²) and peripheral (25.4 cm²) arenas to track exploratory behavior. Total distance travelled and velocity was calculated for the peripheral and central arenas.

2.5 Elevated Plus Maze

A standard elevated plus maze (30 cm long x 6 cm wide x 13 cm tall, closed arms) 50 cm off the ground) was used to measure anxiety-like behavior and exploration patterns. Mouse subjects were placed into the center arena of the maze and allowed to freely explore for 5min. Percent time spent in each arm, total distance travelled, and velocity was calculated using TopScan digital video-based data capture and analysis software (Clever Sys).

2.6 Fear Conditioning and Context Discrimination

The contextual fear-discrimination paradigm is a hippocampus-dependent cognitive task that measures an animal's ability to distinguish between two similar yet different environmental contexts (Frankland, Cestari, Filipkowski, McDonald, & Silva, 1998). On training day 0 (Figure 4), each subject was placed in a standard mouse fear conditioning chamber (Med Associates) which served as context A, the shock context. Context A training (Figure 4A) ensued for 195sec during which time a single 2sec 0.75mA shock was delivered through the electrified grid floor at the 178sec mark; mice then had an additional 15sec to explore the context then were returned to their home cages.

On testing days (day1 onward, Figure 4B), mice were randomly assigned to experience first either context A, or a similar yet non-identical "safe" context, context B. Context B resembled context A in terms of the grid floor being exposed. Context B contained cardboard inserts, vanilla extract (2 μ L pipetted onto a KimWipe®), and the chamber light and fan were turned off. Like context A, mice were placed in context B for 185s, however, no shock was delivered. A 2hr inter-trial interval between each context was utilized for each subject for each day of testing. Following each session, 70% ethanol was used to thoroughly wipe down Context A and 95% isopropyl alcohol was used to clean Context B.

Digital video-based data capture and analysis with FreezeFrame software (Actimetrics) was used to assess freezing behavior. Discrimination ratios (% freezing in Context B $\div$ % freezing in Context A+B) were calculated for each group on each test day.

As additional controls to test for memory of the shock context A and that a completely novel safe context can be recognized, we analyzed %freezing during the training session on day 0 and context discrimination in a completely unique context in which the electrified grid floor was concealed by Plexiglas on day 1.

2.7 Shock Threshold

Footshock perception was assessed using a protocol previously described (Dineley, Xia, Bui, Sweatt, & Zheng, 2002; Hernandez, Kaye, Zheng, Sweatt, & Dineley, 2010). Using Actimetrics FreezeFrame software, a protocol was created to deliver a 1-s foot shock every 30 seconds while the shock amplitude was manually increased by 1/10 mA until flinching, vocalization, and jumping behaviors were observed, then the protocol was terminated. Upon termination, mice were placed back in their home cages and the fear conditioning box was cleaned with 70% ethanol before the next subject was tested.

2.8 BrdU Incorporation

All animals tested received a total of five IP injections of 50 mg/kg BrdU over five days, 30 days prior to behavioral testing; animals were aged for an additional 30 days before harvesting tissue. At 60 days post-injection, animals were transcardially perfused first with 30 ml 0.1 M phosphate buffered saline (PBS) then with 4% paraformaldehyde (PFA) in PBS. Brains were harvested, post-fixed overnight in 4% PFA at 4°C then stored in 30% sucrose-PBS until sectioned. Left hemispheres were sagittal sectioned (30 μ m) on a Leica CM3050-S cryostat between interaural coordinates (0.36–2.4mm; Franklin and Paxinos) that

represent the entire dorsal dentate gyrus; right hemispheres were used for an alternative study. Serial sections were placed in cryoprotectant (30% ethylene glycol, 30% glycerol, 0.1 M PBS and distilled water) for storage at -20°C.

For BrdU staining, free floating sections were washed with 0.1 M PBS and then treated with 2N HCL for 20 minutes at 60°C for antigen exposure. Next, sections were blocked with 5% normal donkey serum-0.1MPBS-0.1%tween (Sigma-Aldrich 9663) for 1hr at room temperature and then probed in 3%BSA-0.1MPBS-0.1%tween (Sigma-Aldrich A9647) with rat anti-BrdU (1:1,00; Abcam 6326), mouse anti-NeuN (1:700; Millipore MAB377), goat anti-doublecortin (1:250; Santa Cruz sc-8066) and rabbit anti-Ki67 (1:400; Cellsignaling 9129s) overnight at 4°C. Sections were washed 5X with 0.1 M PBS then probed with secondary antibodies diluted in in 3% BSA-0.1MPBS-0.1%tween: anti-rat or anti-rabbit Alexa-Fluor® 488, anti-goat Alexa Fluor® 568, and anti-mouse Alexa Fluor® 647 (ThermoFisher A-21208, A-31571, A-11057, A-21206) for 1 hr at room temperature.

BrdU-positive cells were counted from five serial sections representing the entirety of the dorsal dentate gyrus. According to the coordinates interaural-Lateral 0.36–2.40mm based on Franklin and Paxinos 3rd edition (2007). The entire granule cell layer of each section was counted for cells co-labeled with BrdU and NeuN. Z-series were captured on a Nikon A1 confocal microscope system using the 20x objective magnification. Images were analyzed using ImageJ Software (NIH) and total cell counts were calculated for each animal. To remain unbiased, genotypes were revealed only after all BrdU, doublecortin, and Ki67 counts were totaled.

2.9 Statistics

All statistics were performed using GraphPad Prism and SPSS. Analyses for general health, neurological, behavioral, and cognitive performance were reported as mean $\pm$ SEM with 95% confidence intervals, where appropriate. Genotype differences were tested using an unpaired two-tailed t test. The F-test was used to test for equal variance between groups. In all cases but one (Context C testing, Figure 5B), the F test did not fail. Context discrimination ratios were calculated and tested using a one sample t test against chance, a theoretical mean of 0.5. A repeated measures two-way ANOVA using discrimination ratios was used to determine an effect via genotype vs day design. The Levene's test was used to determine equal variances for within subject's design (days). Further, a within-subject repeated measures two-way ANOVA between subjects using raw freezing data was used (total freezing Context A vs total freezing Context B). BrdU counts were totaled from five mice of each genotype from five sagittal sections (every sixth) that represented the lateral extent of the hippocampus. Statistical significance was determined using a p value <0.05 .

3.0 RESULTS

The SHIRPA primary behavioral screen scores a variety of general health measures as well as baseline behaviors and reflexes (Rogers et al., 2001). Across 34 metrics evaluated (Table 1), we found no significant differences between aged DN-p38 $\alpha^{AF/+}$ and wildtype littermates.

Previous studies found significant delay of age-dependent decline of skeletal muscle myofibers and progenitor cells (Papaconstantinou et al., 2009). Therefore, we gauged muscular performance in aged DN-p38 $\alpha^{AF/+}$ and wildtype mice using forelimb grip strength testing. Average grip strength was not statistically significantly different between groups as indicated by the unpaired t test (Figure 1).

Baseline locomotion and exploratory behavior was comparable between DN-p38 $\alpha^{AF/+}$ and wildtype littermates. Neither distance travelled (Fig. 2A) in peripheral and central arenas nor velocity (Fig. 2B) was significantly different. This result suggests that suppression of p38 activity has no influence on baseline exploratory or locomotor behaviors. Open field is also a preliminary screen for anxiety-like behavior (Crawley, 1985) and our results indicate that DN-p38 $\alpha^{AF/+}$ mice do not differ from wildtype mice in this parameter. However, to investigate this possibility, we performed additional testing using the elevated plus maze.

To further ensure that motivation to explore and anxiety levels were no different between genotypes, each mouse was evaluated in the elevated plus maze. Percent time in the open, closed, and center arenas of the maze as well as total distance and velocity were compared between wildtype and DN-p38 $\alpha^{AF/+}$ groups. The time spent in the open, closed, and center arenas of the EPM were no different between groups (Figure 3A). Similarly, there was no significant difference in total distance traveled (Figure 3B) and speed while mice explored the maze (Figure 3C). In summary, DN-p38 $\alpha^{AF/+}$ mice displayed no notable differences in general health measures as well as baseline behaviors and reflexes as determined with SHIRPA and they perform indistinguishably from their wildtype littermates in tests for locomotion, exploration, anxiety-like behavior, and grip strength. Thus, attenuation of p38 MAPK activity does not impinge upon the overall health or behavioral phenotype of these mice compared to wildtype littermates.

Based on the notion that DN-p38 $\alpha^{AF/+}$ mice are a model for delayed senescence, we wanted to test our cohort in a cognitive task that has been shown to be highly dependent upon adult neurogenesis. We therefore adopted the context discrimination fear conditioning paradigm (Frankland et al., 1998; Sahay et al., 2011). To validate the protocol, we subjected young (3 months old) wildtype C57Bl/6j mice to context discrimination (Figure 4B). Young wildtype mice were able to significantly discriminate between contexts on days 3–5 as determined by analyzing %freezing (Figure 4C) and discrimination indices (Figure 4D). The discrimination ratio was calculated as the ratio of total freezing in context A over the sum of total freezing in both context A + B and statistically tested against chance (0.5 or 50%). Young C57Bl/6j mice learn to context discriminate by day 3 onward but not on the first two days of testing.

We next tested our cohort of aged wildtype and DN-p38 $\alpha^{AF/+}$ mice in the context discrimination fear conditioning paradigm, a hippocampus-dependent memory task that relies upon intact neurogenesis (Kempermann, 2015). During training in context A on day 0, both wildtype and DN-p38 $\alpha^{AF/+}$ displayed equivalent freezing (Figure 5A). On testing day 1 (Figure 4B), mice were tested to discriminate a grossly different context, context C, after testing in both contexts A and B. Both groups of mice exhibited similar %freezing in context C (Figure 5B), and statistically significant discrimination ratios compared to context A on day 1 ($p < 0.05$, one sample t test against a theoretical mean = 0.5), data not shown. Repeated

measures ANOVA of discrimination ratios did not show a significant effect for day or genotype [$F(5,200)=0.83$; $p=0.52$]. However, repeated measures ANOVA of discrimination ratios in young wild type mice shows significance compared to aged C57Bl6 mice [$F(1,20)=11.6$] $p=0.002$; Sidak multiple comparisons revealed significant discrimination on day 5. Further, a within subjects two-way repeated measures using raw freezing data for each aged group showed a significant discrimination on day 3 for DN-p38 $\alpha^{AF/+}$, [$F(5,140)=4.0$; $p=0.001$]; Sidak multiple comparisons reveal significant discrimination on day 3 but no significant discrimination was observed for aged wild type mice [$F(5,60)=0.94$; $p=0.45$]. Upon testing for discrimination between context A and context B on days 1–6, aged wildtype mice were only able to discriminate between context A and B on the last day of testing (Figure 5E). In contrast, DN-p38 $\alpha^{AF/+}$ exhibited significant context discrimination on days 3, 5 and 6 (Figure 5F), suggesting that attenuated p38 kinase activity is protective against age-dependent decline in context discrimination. At the completion of context discrimination testing, all mice were subjected to shock threshold testing for flinching, vocalization, and jumping with no significant differences noted (Table 2).

Since it has been established that hippocampal adult born neurons aid performance in context discrimination (Besnard & Sahay, 2016; Frankland, Kohler, & Josselyn, 2013; Lacar, Parylak, Vadodaria, Sarkar, & Gage, 2014; Wojtowicz, 2012), we evaluated the production of adult born neurons in the dentate gyrus of aged wildtype and DN-p38 $\alpha^{AF/+}$. By counting BrdU-positive neurons within the granule cell layer in aged wildtype and DN-p38 $\alpha^{AF/+}$ 60 days post injection of BrdU we were able to compare how many dentate gyrus neurons were produced and survived within this timeframe. Aged DN-p38 $\alpha^{AF/+}$ exhibited significantly higher number of cells dually positive for BrdU and NeuN compared to wildtype littermates (Figure 6) suggesting that hippocampal neurogenesis is more pronounced in aged DN-p38 $\alpha^{AF/+}$. Moreover, DN-p38 $\alpha^{AF/+}$ exhibited numbers of immature (DCX-positive) neurons compared to aged wild type mice as indicated by two-tailed unpaired t test [$t=5.31$ $df=8.0$; $p<0.05$] (Figure 7). However, proliferation in hippocampus between young DN-p38 $\alpha^{AF/+}$ and wild type mice was not different for BrdU and Ki67 markers, [$t=0.68$ $df=4$; $p=0.53$], and [$t=1.17$ $df=4$; $p=0.34$], respectfully (Figure 8).

In summary, C57bl/6j mice exhibit an age-dependent decline in pattern separation as measured by the context discrimination fear conditioning paradigm in 3 months old versus aged C57bl/6j; DN-p38 $\alpha^{AF/+}$ mice are resistant to this age-dependent phenotype. DN-p38 $\alpha^{AF/+}$ and wildtype mice froze to the same extent during fear conditioning training, have similar shock thresholds, and are able to discriminate a wholly unique context (context C), suggesting that fear conditioning deficits in aged wildtype mice are specific to context discrimination fear conditioning rather than an inability to perceive the shock or associate it with a particular context. Enhanced context discrimination in DN-p38 $\alpha^{AF/+}$ mice was accompanied by enhanced BrdU-positive neurons and immature doublecortin neurons in the dentate gyrus suggesting that enhanced neurogenesis underlies this behavioral phenotype. Furthermore, DN-p38 $\alpha^{AF/+}$ do not present with any alterations in baseline general health, neurological function, locomotor exploration, or anxiety-like behavior. Thus, as has been demonstrated with a variety of p38 MAPK manipulations in a range of model systems as well as reported for the DN-p38 $\alpha^{AF/+}$ mouse model, attenuation of p38 MAPK activity

delays tissue senescence; in this case, decline of adult hippocampal neurogenesis that underlies context discrimination.

4.0 DISCUSSION

Aging is the primary risk factor for all neurodegenerative diseases and other leading causes of death. Based on the early observation of aberrant MAPK signaling (e.g., ERK, p38) in human AD brains and AD mouse models (Dineley et al., 2001; Dineley et al., 2002; Hensley et al., 1999; Hyman, Elvhage, & Reiter, 1994; Savage, Lin, Ciallella, Flood, & Scott, 2002; Zhu et al., 2000), aberrant MAPK activity has long been thought to be a significant contributor to AD. Furthermore, the age-dependent induction of CNS p38 α activity that drives stress signals to promote neuroinflammation and cognitive impairment in AD and the converse outcome of acute inhibition of p38 α activity that improved spatial memory in AD mouse models (Ashabi, Alamdary, Ramin, & Khodagholi, 2013; Ashabi et al., 2012) has driven recent pursuits of p38 α inhibitors as a therapeutic strategy (Bachstetter & Van Eldik, 2010; Munoz & Ammit, 2010; Pinsetta et al., 2014).

DN-p38 $\alpha^{AF/+}$ mice express a mutant form of p38 α in which Thr180 and Tyr182 are mutated (T $\rightarrow$ A/Y $\rightarrow$ F) to prevent phosphorylation activation of p38 α and exhibit resistance to age-dependent decline in the regenerative potential of liver, pancreatic islet, and skeletal muscle tissues (Papaconstantinou et al., 2009, 2015; Wong et al., 2009). To determine if reduced p38 α MAPK activity affected a cognitive metric associated with adult neurogenesis, we aged heterozygous DNp38 $\alpha^{AF/+}$ mutant and wildtype mice then tested our cohorts in the context discrimination fear conditioning paradigm. Control studies were included to determine whether general health, neurological function, locomotor exploration, or anxiety-like behavior were affected. Finally, BrdU injections and immunofluorescence imaging was employed to quantify adult-born neuronal maturation and survival.

Here, we demonstrated that aged DN-p38 $\alpha^{AF/+}$ mice appear to have no behavioral or gross neurological abnormalities as they perform comparably to aged wildtype mice in the SHIRPA primary screen for general health, baseline behaviors and reflexes. Although, aged DN-p38 $\alpha^{AF/+}$ showed higher hind limb gastrocnemius density (Papaconstantinou et al., 2009), we found no significant difference in forelimb grip strength between aged DN-p38 $\alpha^{AF/+}$ and wildtype mice. This is somewhat surprising given that previous studies found delayed loss of age-dependent gastrocnemius muscle mass (Papaconstantinou et al. 2009). Perhaps, forelimb muscle mass is not affected to the same extent as hind limb gastrocnemius.

Further, DN-p38 $\alpha^{AF/+}$ mice appeared to have similar motivation to explore open field and elevated plus maze while showing no difference in control metrics like speed and distance traveled. Since aged DN-p38 $\alpha^{AF/+}$ mice appear to have no baseline behavioral or neurological deficits, we tested cognition in a context fear-discrimination task. Context discrimination is a hippocampus-dependent task that challenges an animal's ability to distinguish contextual cues in similar contexts. Several studies have demonstrated that increasing production of adult born neurons in the hippocampus; that is increasing adult neurogenesis levels improves performance in context discrimination while decreasing

neurogenesis has the opposite effect (Clemenson et al., 2015; D. J. Creer, C. Romberg, L. M. Saksida, H. van Praag, & T. J. Bussey, 2010; Danielson et al., 2016; Frankland et al., 1998; Merritt & Rhodes, 2015; Sahay et al., 2011). Young neurons are more sensitive to perforant pathway input necessary for driving the encoding of new contexts (Frankland et al., 2013; Lacar et al., 2014; Wojtowicz, 2012). Previous work showed that nearly complete inhibition of p38 α activity in CA1 abolished context discrimination in C57Bl6 mice (Jin, Arai, Tian, Kumar-Singh, & Feig, 2013), whereas our data showed that partial reduction of p38 activity enhanced context discrimination. While this seems contradictory, we would argue that extensive inhibition of CA1 p38 is interfering with the consolidation of memory trace required for context discrimination and that the partial activity remaining in DN-p38 $\alpha^{AF/+}$ mice is sufficient to complete the hippocampal tri-synaptic circuit and preserve context discrimination. Analyzing hippocampal proliferation markers, BrdU and Ki67 in young DN-p38 $\alpha^{AF/+}$ and wild type mice revealed no significant difference between either group. However, DN-p38 $\alpha^{AF/+}$ maintained immature neuronal pools while also showing higher retention of new born neurons compared to aged wild type mice. Together, this data reveals attenuation of p38 activity preserves neurogenesis mechanisms with age. Similarly, interventions slowing age dependent decline of adult neurogenesis show benefit in a spatial navigation task ((van Praag, Shubert, Zhao, & Gage, 2005).

Thus, our finding that aged DN-p38 $\alpha^{AF/+}$ mice produce more adult-born neurons than wildtype littermates suggests that p38 α negatively contributes to age-dependent decline in neurogenesis that underlies the age-dependent decline in context discrimination.

Our study is the first to demonstrate that *in vivo* attenuation of p38 α activity slows age-associated decline in dentate gyrus neurogenesis and associated cognitive function. Overall, our findings suggest that the p38 α MAPK pathway plays a role in the decline of cognition with age, and potentially contributing to age-dependent neurodegenerative disease.

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Figure 1. Equivalent grip strength between aged WT and DN-p38α^{AF/+} mice
Two-tailed unpaired t test (p=0.7). ns, not significant.

Figure 2. Equivalent performance in the open field between aged WT and DN-p38 $\alpha^{AF/+}$ mice
 A, Comparison of distance in the periphery and center arenas of the open field are similar between WT and DN-p38 $\alpha^{AF/+}$ mice. Two-tailed unpaired t test ($p=0.3$, 0.2 for the peripheral and center arenas, respectively). Velocity, B, was also comparable. Two-tailed unpaired t test ($p=0.2$, 0.3 for velocity in the peripheral and center arenas, respectively) ns, not significant.

Figure 3. Equivalent performance in the elevated plus maze between aged WT and DN-p38^{AF/+} mice

A, Comparison of time spent in the open, closed, and center areas of the elevated plus maze are comparable between WT and DN-p38^{AF/+} mice. Two-tailed unpaired t test ($p=0.6$, 0.4 , 0.2 for open, closed and center arms, respectively). Total distance travelled, B, and velocity, C, are also similar. Two-tailed unpaired t test ($p=0.6$, 0.07 for distance travelled and velocity, respectively) ns, not significant.

Figure 4. Context discrimination protocol validation in young C57Bl/6j mice. Context discrimination protocol validation in young C57Bl/6j mice

A, Behavioral timeline for aged DN-p38 $\alpha^{AF/+}$ and wild type mice. B, For testing, mice are randomly assigned to either context A or context B to measure freezing. On day 1, an additional test is performed in a completely novel context C. C, By day 3, young C57Bl/6j mice freeze significantly less in context B (two-tailed unpaired t test $p=0.006$, 0.009 , <0.0001 for days 3, 4, and 5, respectively) indicative of context discrimination. D, Analysis of discrimination ratios by one sample t test against a theoretical mean of 0.5 revealed significant context discrimination on days 3, 4, and 5 ($p=0.01$, 0.02 , 0.0004 , for days 3, 4, and 5, respectively).

Figure 5. Enhanced context discrimination in aged DN-p38 $\alpha^{AF/+}$ mice

A, Wildtype and DN-p38 $\alpha^{AF/+}$ freeze equivalently during the training session on day 0. Unpaired two-sided t test $p=0.3$. **B**, Wildtype and DN-p38 $\alpha^{AF/+}$ freeze equivalently in a novel context C on day 1 following exposure to contexts A and B. Unpaired two-sided t test with Welch's correction for unequal variance [$F(28,12)=3.18$], $p=0.9$. **C** and **D**, two-way RM ANOVA for raw freezing. **C**, no significant context discrimination in (Context vs. Day) wildtype [$F(5,60)=0.9$] $p=0.4$. **D**, significant discrimination (Context vs Day) Dn-p38 $\alpha^{AF/+}$ [$F(5,140)=4.0$] $p=0.001$; sidak multiple comparisons reveal significant discrimination on Day3. **E**, One sample t test against a theoretical mean of 0.5 revealed significant context discrimination in aged wildtype between context A and B only on day 6 ($p=0.03$). **F**, DN-p38 $\alpha^{AF/+}$ exhibit significant context discrimination on days 3, 5, and 6 ($p=0.02$, 0.04, and 0.02, respectively). ns, not significant.

Figure 6. Dominant negative p38 α mutation has no effect on hippocampal adult neuron production in 6MO mice

6MO mice received a single IP BrdU injection 24hrs before brain harvest. **A**, DN-p38 $\alpha^{AF/+}$. **B**, WT. sagittal sections were co-labeled with antibodies against BrdU (**green**) and Ki67 (red) then double-labelled cells were counted as described in Methods. **C**, No significant difference for either BrdU or Ki67 markers between DN-p38 $\alpha^{AF/+}$ and wild type mice as indicated by two-tailed unpaired t test [$t=0.68$, $df=4$, $p=0.53$] and [$t=1.17$ $df=4$, $p=0.3$], respectively.

Figure 7. Enhanced production of BrdU-positive neurons in dentate gyrus of DN-p38 $\alpha^{AF/+}$ compared to wildtype mice

Sixty days after BrdU injection wildtype, **A**, and DN-p38 $\alpha^{AF/+}$, **B**, sagittal sections were co-labeled with antibodies against BrdU (green) and NeuN (purple) then double-labelled cells were counted. DN-p38 $\alpha^{AF/+}$ had a greater number of adult-born neurons in the granule cell layer of the dentate gyrus than wild type mice, **C**. Two-tailed unpaired t test * $p < 0.0001$).

Figure 8. Increased immature neuron pool in aged DN-p38 $\alpha^{AF/+}$ mice compared to aged wt mice 10-19MO mice received a single IP BrdU injection for five consecutive days, 60 days before brain harvest. Brain sections were co-labeled with antibodies against BrdU (**green**), doublecortin (red), and NeuN (**purple**) then counted. **A**, DN-p38 $\alpha^{AF/+}$, and **B**, WT, triple labelling of the dentate gyrus of the hippocampus. **C**, DN-p38 $\alpha^{AF/+}$ had a greater number of immature neurons in the dentate gyrus than wild type mice as indicated by a Two-tailed unpaired t test (*** $p < 0.001$).

Table 1

SHIRPA protocol reveals no phenotypic differences between DN-p38 α ^{AF/+} and wildtype mice

Normal behavior was observed in all mice tested as indicated by the following arbitrary scales for each SHIRPA metric: **Body Position**, 0=Completely flat, 1=Lying on side, 2=Lying prone, 3=Sitting or Standing, 4=Rearing on hind legs, 5=Repeated vertical leaping; **Spontaneous Activity** 0=None, resting, 1=Casual scratch, groom, slow movement, 2=Vigorous scratch, groom, moderate movement, 3=Vigorous, rapid/dart movement, 4=Extremely vigorous, rapid/dart movement; **Respiration Rate** 0=Gaspings, irregular, 1=Slow, shallow, 2=Normal, 3=Hyperventilation; Tremor 0=Important, 1=Mild, 2=None; Transfer Arousal 0=Coma, 1=Prolonged freeze, then slight movement, 2=Extended freeze, then slight movement, 3=Brief freeze (few seconds), then active movement, 4=Momentary freeze, then swift movement, 5=No freeze, immediate, 6=Extremely excited; **Palpebral Closure** 0=Eyes closed, 1=Eyes half closed, 2=Eyes wide open; **Piloerection** 0=Coat stood on end, 1=None; **Gait** 0=Incapacity, 1=Limited movement only, 2=Fluid but abnormal, 3=Normal; **Pelvic Elevation** 0=Markedly flattened, 1=Barely touches, 2=Normal, 3=Elevated; **Tail Elevation** 0=Dragging, 1=Horizontally extended, 2=Elevated/straub tail; **Touch Escape** 0=No response, 1=Mild, 2=Moderate, 3=Vigorous; **Positional Passivity** 4=Struggles when held by tail, 3=Struggles when held by neck, 2=Struggles when laid supine, 1=Struggles when held by hind legs, 0=No struggle; **Trunk Curl** 0=Absent, 1=Present; **Limb Grasping** 0=Absent, 1=Present; **Visual Placing** 0=None, 1=Upon nose contact, 2=Upon vibrassey contact, 3=Before vibrassey contact, 4=Early vigorous extension; **Body Tone** 0=Flaccid, no return of cavity to normal, 1=Slight resistance, 2=Extreme resistance, board like; **Pinna Reflex** 0=None, 1=Active retraction, moderately brisk flick, 2=Hyperactive, repetitive flick; **Corneal Reflex** 0=None, 1=Active single eye blink, 2=Multiple eye blink; **Toe Pinch** 0=None, 1=Slight withdrawal, 2=Moderate withdrawal, 3=Brisk, rapid withdrawal, 4=Very brisk repeated extension and flexion; **Wire Maneuver** 0= Falls immediately, 1=Unable to lift hindlegs, falls within seconds, 2=Unable to grasp with hindlegs, 3=Difficulty to grasp with hindlegs 4=Active grip with hindlegs; **Skin Color** 0=Blanched, 1=Pink, 2=Bright, deep red flush; **Heart Rate** 0=Slow, bradycardia, 1=Normal, 2=Fast, tachycardia; **Limb Tone** 0=No resistance, 1=Slight resistance 2=Moderate resistance, 3=Marked resistance, 4=Extreme resistance; **Abdominal Tone** 0=Flaccid, no return of cavity to normal, 1=Slight resistance, 2=Extreme resistance, board like; **Lacrimation** 0=Present, 1=None; **Salivation** 0=Wet zone entire sub-maxillary area, 1=Slight margin of sub-maxillary area, 2=None; **Provoked Biting** 0=Present, 1=Absent; **Righting Reflex** 0=Fails to right when placed on back, 1=Lands on back, 2=Lands on side, 3=No impairment; **Contact Righting Reflex** 0=Absent, 1=Present; **Negative Geotaxis** 0= Falls off, 1=Does not move within 3- seconds, 2=Moves, but fails to turn, 3=Turns but then freezes, 4=Turns and climbs the grid; **Fear** 0=Freezes during transfer arousal, 1=None; **Irritability** 0=Freezes during transfer, 1=Struggle during supine restraint; **Aggression** 0=None, 1=Provoked biting or attack, **Vocalization** 0=None, 1=Provoked during handling.

SHIRPA	Wildtype	DN-p38 α ^{AF/+}	Two- tailed
Metric	n=13	n=29	t Test
	Average		p Value
Weight(g)	30.7	32.8	0.07
Body Position	4	4	0.53
Spontaneous Activity	2	1.92	0.5

SHIRPA	Wildtype	DN-p38 α ^{AF/+}	Two- tailed
Metric	n=13	n=29	t Test
	Average		p Value
Respiration Rate	2	2	>0.99
Tremor	2	2	>0.99
Transfer Arousal	5	5	0.21
Palpebral Closure	2	2	>0.99
Piloerection	1	1	>0.99
Gait	3	3	>0.99
Pelvic Elevation	2	2	>0.99
Tail Elevation	1	1	>0.99
Touch Escape	2	2	0.91
Postional Passivity	4	4	>0.99
Trunk Curl	1	1	>0.99
Limb Grasping	1	1	>0.99
Visual Placing	3	3	>0.99
Body Tone	1	1	0.6
Pinna Reflex	1	1	>0.99
Corneal Reflex	1	1	>0.99
Toe Pinch	0	0.96	0.23
Wire Maneuver	4	4	>0.99
Skin Color	2	2	>0.99
Hear Rate	1	1	>0.99
Limb Tone	3	3	>0.99
Abdominal Tone	1	1	>0.99
Lacrimation	1	1	>0.99
Salivation	2	2	>0.99
Provoked Biting	1	1	>0.99
Righting Reflex	3	3	>0.99
Negative Geotaxis	4	4	>0.99
Fear	1	1	>0.99
Irritability	1	1	>0.99
Aggression	1	1	>0.99
Vocalization	1	1	>0.99

Table 2**Footshock perception equivalent in aged DN-p38 α ^{AF/+} and wildtype mice**

Footshock threshold was employed to determine shock intensity that provoked jumping, vocalization and flinching behaviors. Average amplitude for all behavior was not statistically different between groups as indicated by Student's t test.

Response	DN- p38 α ^{AF/+}	Wildtype	Student's t Test
to Footshock	Average Amplitude(mA)		p Value
Jump	0.49	0.47	0.84
Vocalization	0.34	0.33	0.69
Flinch	0.18	0.15	0.15