Adult hippocampal neurogenesis, Rho kinase inhibition and enhancement of neuronal survival

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Running title: ROCK inhibition and hippocampal neurogenesis

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Number of words in Manuscript body: 3,110

Numbers of figures: 6

Abstract (300 words)

Adult neurogenesis occurs throughout life; however the majority of new neurons do not survive. Enhancing survival of these new neurons will increase the likelihood that these neurons could return function following injury. Inhibition of Rho kinase is known to increase neurite outgrowth and regeneration. Previous work in our lab has demonstrated a role for Rho kinase inhibition and survival of new born neurons from the sub-ventricular zone. In this study we examined the role of Rho kinase inhibition on hippocampal neurogenesis. Two concentrations of Rho kinase inhibitor Y27632 (20 μ M and 100 μ M) and the proliferative marker EdU were infused in the lateral ventricle for 7 days. Quantification of doublecortin+/EdU+ cells on the 7th day showed that cell numbers were not significantly different, suggesting no effect on neuroblast generation. Following infusion of 100 μ M Y27632, the number of newborn NeuN+/EdU+ neurons at 35 days in the granular cell layer of the dentate gyrus of the ipsilateral side of the infusion did not display a significant difference; however there was an increase on the contralateral side, suggesting a dose effect. Infusion of a lower dose (20 μ M) of Y27632 resulted in an increase in NeuN+/EdU+ cells in the granular cell layer of the ipsilateral side at 35 days. These mice also demonstrated enhanced spatial memory as tested by the Y maze with no significant changes in anxiety or novel object recognition. Rho kinase inhibition enhanced the survival of new born neurons in the dentate gyrus with a specific dosage effect. These results suggest that inhibition of Rho kinase following injury could be beneficial for increasing the survival of new neurons that may aid recovery.

Keywords: doublecortin, NeuN, Rho GTPases, dentate gyrus, granule cell layer

1.1 Introduction

Adult hippocampal neurogenesis is known to occur throughout life and influence both memory formation and emotional states (David et al., 2009, Deng et al., 2010). The birth of these adult-born neurons occurs in the sub-granular zone (SGZ) of the dentate gyrus, where progenitor cells generate doublecortin (DCX)+ neuroblasts that migrate into the granule cell layer (GCL). A fully mature NeuN+ neuron in the dentate GCL extends dendrites to the molecular layer and an axon to CA3, completing a circuit of information transfer in the hippocampus. Intriguingly, less than 50% of all newly generated granular neurons survive and this is largely due to an unknown mechanism (Cameron et al., 1993, Dayer et al., 2003). Leading hypotheses point to a lack of circuitry integration and a loss in the competition for target-derived factors, as potential mechanisms for neuronal loss (Bergami and Berninger, 2012). Within 2 weeks of their generation newborn neurons have generated an axon and commenced their extensive dendritic arborisation, followed by synaptogenesis beginning around 1 week later. Currently, it is unclear as to whether a new-born cell must compete with both other new-born neurons and mature GCL neurons or just one of the above. Either way, the formation of dendrites and synapses and the resultant competition for synaptic space appears to be a limiting step in the integration and subsequent survival of new-born neurons (Bergami and Berninger, 2012).

The Rho-GTPases are well-known for their roles in cytoskeletal rearrangement, leading to changes in neurite growth, regeneration, neurogenesis and cell migration (Kimura et al., 1996, Yang et al., 1998, Fukata et al., 1999, Arimura et al., 2000, Ng and Luo, 2004, Yoneda et al., 2005, Cheng et al., 2008, Ahmed et al., 2009, Keung et al., 2011, Vadodaria et al., 2013). Specifically for neurite outgrowth, Rac1 can induce neurite extension; while RhoA via its effector kinase Rho associated kinase (ROCK) leads to retraction and growth cone collapse (Kozma et al., 1997, Leeuwen et al., 1997, Luo, 2000). Inhibition of Rho kinase in

neurons by use of drugs such as Y27632 or HA1077 promotes neurite outgrowth and blocks effects of neurite outgrowth inhibitors (Borisoff et al., 2003, Bertrand et al., 2005, Lingor et al., 2007) and promotes axon regeneration and sprouting following injury (Dergham et al., 2002, Chan et al., 2005).

Previous work in our lab examined the role of Rho kinase on neural precursor cell migration. We demonstrated that inhibition of Rho kinase promoted migration of cultured neurosphere-derived neural precursor cells and in explants of subventricular zone promoted single cell rather than chain migration. Further, infusion of 100 μ M Y27632 Rho kinase inhibitor into the lateral ventricles of adult mice promoted ectopic migration of subventricular zone-derived neuroblasts away from the rostral migratory stream, resulting in decreased numbers of newborn neurons in the olfactory bulb, but increased numbers in the olfactory accessory nucleus and anterior cortex after 1 week of infusion. Surprisingly, in these mice ROCK inhibition also led to increased survival of the ectopic newborn neurons in the accessory olfactory nucleus at 1 month after infusion (Leong et al., 2011). Based on this we sought to elucidate the effect of ROCK inhibition on the survival of newborn granule cell neurons in the dentate gyrus in the current study.

Granule cell neurogenesis is also linked to functional behaviours such as learning, spatial memory, anxiety, and depression (Gould et al., 1999, Shors et al., 2001, David et al., 2009, Revest et al., 2009, Snyder et al., 2011); we wished to determine whether alteration of neurogenesis by ROCK inhibition would affect these behaviours. Here, we demonstrate that inhibition of ROCK leads to an increase in the survival of new born neurons that is not the result of increased neuroblast generation. Further, this increase in NeuN⁺ neurons leads to enhanced spatial memory.

2.1 Experimental Procedures

2.2 Mice

8-9 week-old male C57BL/6 mice (Animal Resource Centre, Western Australia) were used for all experiments (n=75; 6-8 per experimental condition and timepoint). All use of experimental animals was approved by the Animal Experimentation Ethics Committee of the Florey Neuroscience Institutes at the University of Melbourne. All procedures were conducted in strict accordance with the National Health and Medical Research Council of Australia guidelines.

2.3 Lateral Ventricle Infusion of Y27632 and/or EdU

Brain infusion cannulae and Alzet osmotic pumps (Model 1007D, 0.5 µl/hr, 7-day duration; BioScientific, NSW, Australia, www.alzet.com) were used. Control mice were infused with 5-ethynyl-2'-deoxyuridine (EdU, 5 mg/ml, Invitrogen) and treated mice were infused with EdU plus the ROCK inhibitor Y27632 (100 µM or 20 µM, Sigma, Sydney, Australia). Adult mice were anesthetized with isofluorane. Using aseptic techniques, cannulae were inserted into the left lateral ventricle (0.5 mm anterior to Bregma, 0.7mm lateral, 2.5-mm deep). Animals were given analgesia (Metacam, 3mg/kg, Boehringer Ingelheim, North Ryde, Australia, www.boehringer-ingelheim.com) post-surgery. Animals were single-housed following infusion. The infusion assembly was left in place for 7 days or 35 days and the brains were removed for analysis at 7 days or 35 days after cannula insertion (only infused for 7 days in both cases).

2.4 Tissue Preparation

Mice were anesthetized with 100 mg/kg of sodium pentobarbitone (Lethabarb, Virbac, Milperra, Australia, www.virbac.com.au) and perfused transcardially. Brains were removed

and postfixed in 4% paraformaldehyde overnight then cyroprotected in 30% sucrose. Whole brains were frozen in Tissue-Tek optimal cutting temperature compound (OCT; Sakura-Finetek, The Netherlands, www.sakuraeu.com), and 30- μ m coronal sections were collected at the level of the dentate gyrus (-1.2- 2.0 mm Bregma) for analysis. Coordinates according to (Paxinos and Franklin, 2001).

2.5 Immunohistochemistry

For fluorescence immunohistochemistry tissue was blocked with 5% v/v goat serum with 0.1% v/v Triton-X 100 (Sigma) in phosphate-buffered saline (PBS). Tissue was incubated with mouse anti-NeuN (Millipore), or goat anti-Doublecortin (Santa Cruz Biotechnology) overnight and then goat anti-mouse Alexa 488 (Molecular Probes, Invitrogen) or donkey anti-goat Alexa 488 for 1 hour (Molecular Probes, Invitrogen). Tissue was then processed for fluorescent EdU labelling using the Click IT kit for Alexa 555 (Molecular Probes, Invitrogen). All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI), then coverslipped with mounting media (Dako). Sections were viewed on an IX81 Olympus fluorescent microscope (x40 lens, 0.60 NA) and an Olympus FV 1000 Confocal microscope (x60 lens, Z-plane step of 1 μ m).

To compare effect of Rho kinase inhibition versus controls, for each mouse five hippocampal sections 150 μ m apart from -1.2- 2.0 mm Bregma coordinates were counted and area of dentate was recorded with DAPI labelling in ImageJ, with the experimenter blinded to condition. All counts were performed in real time using an epifluorescent microscope with so the experimenter could focus on all planes through the 30 μ m tissue slice and assess antigen colocalisation. Quantification of cell number was normalized to area of the dentate gyrus for each section and represented as number of EdU + cells per area (mm²). The average was obtained for each mouse which was used to determine the average for all mice in the group

(n=6-8 mice/group). Confocal images were used to verify the accuracy of the antibody colocalisation.

2.6 Behavioural Testing

2.6.1 Elevated Plus Maze On day 33 post-infusion animals (n=8 per group for all tests) were given 5 minutes to explore the maze. Their movements were tracked with TopScan software. Time spent in open and closed arms was quantified.

2.6.2 Object Recognition On day 34 post-infusion animals were habituated in boxes for 3 ten minute trials without objects. The animals were then put back into their box with two identical objects for 5 minutes and tracked with TopScan software. A bout of exploration was set at 1 second with nose on the object. Following an inter-trial interval of 1.5 hours the animal was put back into the box with one familiar and one novel object for another 5 minutes. Latency to novel object, total time on novel object, percent time exploring objects, and number of bouts on objects was quantified.

2.6.3 Y maze On day 35 post-infusion animals were placed in a plastic Y maze for 5 minutes with one arm blocked and tracked with TopScan software. Visual cues were placed at the end of each arm of the maze. Animals were placed into their home cage for 1.5 hours and then returned to the Y maze with all arms accessible. Preference for initial arm, latency to novel arm, entries to novel arm, and duration in novel arm in seconds and percent of total time was quantified.

2.7 Statistics

All data were presented as mean $\pm$ SEM and statistical analyses were performed using GraphPad Prism v4.03 (GraphPad Software, Inc., San Diego, CA, www.graphpad.com) with $p < 0.05$ considered statistically significant. Comparisons between groups were analyzed

using analysis of variance (ANOVA), with Tukey's test. Otherwise data was analyzed using the unpaired student's t-test.

3.1 Results

To determine the effect of ROCK inhibition on hippocampal neurogenesis, the ROCK inhibitor Y27632 was infused into the lateral ventricle, along with the proliferative marker EdU, for 7 days and brains taken for analysis at 7 or 35 days from commencement of infusion. Hippocampal sections were then examined for EdU incorporation and either the neuroblast marker DCX (7 days) or the mature neuron marker NeuN (35 days). The lateral ventricle was chosen as the infusion site, as factors infused into the lateral ventricle diffuse widely to different brain regions, including hippocampus. We also wanted to avoid the confounder of local hippocampal injury if infusion was directly into hippocampus.

We initially infused 100 μ M Y27632, as per our previous study (Leong et al., 2011) but as we found an unexpected effect on newborn neurons on the contralateral side of the hippocampus, as described below, we then repeated our analyses using a lower concentration (20 μ M).

3.2 ROCK inhibition did not increase neuroblast generation in the SGZ

Evaluation of neuroblast generation was carried out in animals infused with both concentrations of Y27632 (100 and 20 μ M) or saline (Figure 1a). Following 7 days of infusion, there was no difference in the density of EdU+ cells in either side of the SGZ (Figure 1 b-c, f-g). The average area of the dentate gyrus was not significantly different between groups or concentrations of inhibitor (Figure 1 d-e). Furthermore, ROCK inhibition did not change the percentage of the EdU+ cells that co-label led with DCX (Figure 1 b-c, h-i).

3.3 ROCK inhibition increased the survival of new-born neurons in the granule cell layer (GCL)

Infusion of Y27632 increased the density of EdU+ cells remaining in the GCL of the dentate gyrus (DG) at 35 days (Figure 2). Interestingly, 100 μ M resulted in a significant increase in the non-infused side of the DG (Figure 2f), while 20 μ M infusion produced an increased density of EdU+ cells in the infused DG (Figure 2b, g). The average area of the dentate gyrus was not significantly different between groups or concentrations of inhibitor (Figure 2 d-e). Under normal conditions the majority of newborn cells in the adult hippocampus are neuronal and this did not change following ROCK inhibition. Similar percentages of NeuN+/EdU+ neurons were counted in the GCL under control and treated conditions (Figure 2 h-i) and verified by confocal microscopy (Figure 2c), indicating that ROCK inhibition did not change the differentiation fate of the EdU+ cells. If it is assumed that all DCX+ neuroblasts would either mature to NeuN+ neurons or die by the 35d timepoint and that the numbers of DCX cells we obtained for the 7d timepoint are reflective of the numbers that would have been present at 7d in the brains assessed at 35d, then ROCK inhibition increased the percentage of neuroblasts that survived to become mature neurons. Comparing numbers of EdU+/NeuN+ cells present at 35d to EdU+/DCX+ cells at 7d (Figure 3) then Y27632 infusion resulted in enhanced survival of DCX+ cells that became NeuN+ 70% (100 μ M non-infused side) and 66% (20 μ M infused side) compared to survival in controls of 46% (100 μ M non-infused side) and 44% (20 μ M infused side) (Figure 3).

3.4 ROCK inhibition did not alter anxiety

An elevated plus maze was run to assess anxiety levels of mice infused with 20 μ M Y27632 and controls on day 33 post surgery (Figure 4a). There was no significant difference between Y27632 and control mice in total time spent in either open or closed arms (Figure 4 b,c).

3.5 ROCK inhibition did not alter object recognition

Mice were taken through a novel object recognition paradigm on day 34 post surgery (Figure 5a). An inter trial interval of 1.5 hours was used to assess memory of the objects. Y27632 (20 μ M) and control animals did not show any differences in total time exploring objects, latency to the novel object, number of times exploring the novel object, or the total time spent exploring the novel object (Figure 5 b-e).

3.6 ROCK inhibition enhanced spatial discrimination and place recognition

Spatial discrimination and place recognition was assessed with a Y maze, complete with visual cues on the ends of each arm (Figure 6a). Mice explored the maze for 5 minutes with the barrier occluding one arm. An inter trial interval of 1.5 hours separated the second trial where all arms were open. One hundred percent of the Y27632 (20 μ M) mice explored the novel arm first as opposed to 62.5% of controls. Y27632 mice also reached the novel arm quicker (Figure 6b) and had more entries into the novel arm over the 5 minutes (Figure 6c). Further, Y27632 mice spent more time exploring the novel arm in both duration and percent of total time in the maze (Figure 6 d,e).

4.1 Discussion

Inhibition of ROCK, from lateral ventricle infusion, lead to an increase in the numbers of mature new born neurons in the dentate gyrus that correlated with enhanced spatial memory. Importantly we showed that the increase in EdU+/NeuN+ cells is not the result of increased neuroblast generation suggesting an increase in survival of the new-born neurons in the GCL. While the mechanism that promotes this ROCK-mediated survival is yet to be elucidated, we propose it may be due to enhanced integration of new neurons, which may be amplified as

synaptic connections are made with existing circuitry within the molecular layer and CA3.

This could be achieved by an increased growth of dendrites and/or increased plasticity of the axon and dendrites. Synaptic connections rely on spine formation and plasticity. Importantly, dendritic morphology is shaped by the Rho-GTPases; specifically RhoA and Cdc42 activity is required for plasticity of spine structures in the hippocampus (Murakoshi et al., 2011).

Inhibition of ROCK suppresses spine volume changes; however this occurs with short term application of the inhibitor (30 min) and dendritic growth and spine maturation of new neurons in the dentate rely heavily on Rac1 (Vadodaria et al., 2013). In this study, we infused Y27632 for 7 days and tracked only the EdU labelled cells from this time point. This timeline of inhibition could accelerate migration of neuroblasts to the GCL and enhance initial dendritic and axonal outgrowth. We would not expect the ROCK inhibitor to still be available at later time points to inhibit spine volume changes following the end of the infusion (7 days). ROCK inhibition induces increased neurite outgrowth, including length and branching, as demonstrated in multiple cell types (Lehmann et al., 1999, Borisoff et al., 2003, Bertrand et al., 2005, Chan et al., 2005, Cheng et al., 2008). It also increases spine formation and synaptic integration as shown in mature hippocampal pyramidal neurons (Nakayama et al., 2000), while complete knock out of ROCK in ROCK2^{-/-} mice led to deficits in spine formations and synaptic transmission (Zhou et al., 2009).

The concentration of ROCK inhibitor must be optimized for each outcome; in spinal cord injuries a high dose of Y27632 was needed to stimulate sprouting where a low dose was detrimental (Chan et al., 2005). Conversely, in DRG neuronal cultures a curve of optimal concentration of ROCK inhibition to enhance neurite outgrowth was shown, increasing doses above the optimal concentration decreased neurite outgrowth (Cheng et al., 2008). Here, we demonstrate that high concentration of ROCK inhibition had no effect on survival of new

neurons on the infused side. However, a lower concentration increased unilateral survival in the infused dentate gyrus.

Surprisingly, we showed that this unilateral increase in neuron survival could produce an enhancement of spatial memory. However, others have also demonstrated that a unilateral change in neurogenesis can generate a behavioural outcome; recently it was shown that unilateral optogenetic stimulation of one dentate gyrus was capable of producing a learned fear conditioned freeze response (Liu et al., 2012). Many other studies have highlighted the role of adult neurogenesis in memory formation, primarily via ablation models (reviewed in (Deng et al., 2010). Although the field is full of inconsistent results with regards to neurogenesis and some hippocampal dependent tasks, it is noteworthy that novel object recognition, which was unaltered in our paradigm, is not affected by ablation (Madsen et al., 2003, Rola et al., 2004, Clelland et al., 2009) or enhancement of neurogenesis (Sahay et al., 2011). Potentially, novel object recognition tasks are dentate gyrus independent and direct inputs from the entorhinal cortex to the CA3 and CA1 may be used instead (Deng et al., 2010). A similar outcome with an effect on spatial memory but no effect on novel object recognition has been reported in another study. When examining role of Fmrp in neurogenesis, Guo and colleagues specifically note the lack of change in the object recognition task as further evidence to the role Fmrp has in hippocampus-dependent learning (Guo et al., 2011). Importantly, the Y maze paradigm used in this current study utilized visual cues that allowed the mouse to spatially orientate and discriminate. This paradigm is similar to the T maze used to show deficits in exploring the novel arm when adult neurogenesis was irradiated (Madsen et al., 2003). Overall, the increase in new neurons shown here was specific to spatial memory only, as we did not see any changes in anxiety even though adult neurogenesis is known to play a role in anxious and depressive behaviours (Revest et al., 2009). This may suggest that behaviours such as anxiety require changes in neurogenesis on

1 both sides of the hippocampus. Alternatively, changes in hippocampal neurogenesis may
2 produce more profound effects on depressive behaviours, as opposed to anxiety-like
3 behaviours (Snyder et al., 2011).
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8 The use of ROCK inhibitors for therapeutic benefit is increasing for both neural and non-
9 neural conditions (Masumoto et al., 2002, Shibuya et al., 2005, Zhao et al., 2006, Suzuki et
10 al., 2008, Iwabuchi et al., 2011). The current findings suggest that ROCK inhibition can lead
11 to changes in adult hippocampal neurogenesis. Increased survival of new born neurons could
12 aid in recovery of function following dementia or injury to the brain. Further investigation is
13 needed to explore the benefits of increased survival of new neurons following injury.
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30 **Acknowledgments**

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32 We acknowledge support of this project from Stem Cells Australia and the National Health
33 and Medical Research Council of Australia (Project grant; ID: 1045125 and Fellowship to
34 AMT; ID: 628344).
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Figure Legends

Figure 1. ROCK inhibition does not alter neuroblast generation. A) Schematic of overall experimental design. Groups of animals had osmotic pumps infusing either EdU alone or with Y27632 at either 20 or 100 μ M into the left lateral ventricle. For neuroblast generation analysis animals were perfused at 7 days and labelled for DCX. For neuronal survival analysis animals were perfused at 35 days and labelled for NeuN. B) Coronal section of dentate gyrus from control (saline) and C) Y27632 (20 μ M) treated mice at 7 days showing DCX+ (green) cells and EdU+ cells (red). D) Average areas of the dentate gyrus in control and 100 μ M Y27632 animals. E) Average areas of the dentate gyrus in control and 20 μ M Y27632 animals. F) Quantification of EdU+ cells per area of dentate gyrus in both infused and non-infused sides (100 μ M). G) Quantification of EdU+ cells per area of dentate gyrus in both infused and non-infused sides (20 μ M). H) Percentage of EdU+ cells that co-labelled with DCX (100 μ M). I) Percentage of EdU+ cells that co-labelled with DCX (20 μ M). Error bars represent $\pm$ SEM (n=8/group). Scale bar is 100 μ m. ML: molecular layer, H: hilus.

Figure 2. ROCK inhibition increased the survival of new-born neurons in the granule cell layer. A) Coronal section of dentate gyrus in control (saline) and B) Y27632 (20 μ M) at 35 days showing NeuN+ (green) cells and proliferating EdU+ cells (red). Arrows point to double labelled cells. Scale bar is 100 μ m. C) Confocal images of NeuN+ and EdU+ cells. Inset highlights 3D co-localization of EdU and NeuN. Scale bar is 20 μ m. D) Average areas of the dentate gyrus in control and 100 μ M Y27632 animals. E) Average areas of the dentate gyrus in controls and 20 μ M Y27632 animals. F) Quantification of EdU+ cells per area of dentate gyrus in both infused and non-infused sides (100 μ M). G) Quantification of EdU+ cells per area of dentate gyrus in both infused and non-infused sides (20 μ M). H) Percentage of EdU+ cells that co-labelled with NeuN (100 μ M). I) Percentage of EdU+ cells that co-

labelled with NeuN (20 μ M). Error bars represent $\pm$ SEM (* p <0.05, n =6/group). GCL:
granular cell layer.

Figure 3. ROCK inhibition increased the survival neuroblasts that become mature

neurons. Comparison of the total number of DCX+ cells at 7 days and the NeuN+ cells at 35 days for (A) non-infused side 100 μ M Y27632 and (B) infused side 20 μ M Y27632. Error bars represent $\pm$ SEM (* p <0.05, n =6-8/group).

Figure 4. ROCK inhibition did not alter anxiety. A) Schematic drawing of the elevated plus maze. B) Graph of total time spent in closed arms. C) Graph of total time spent in open arms. Error bars represent $\pm$ SEM (n =8/group).

Figure 5. ROCK inhibition did not alter object recognition. A) Schematic drawing of the novel object recognition paradigm. B) Graph of total time spent exploring objects. C) Graph of latency to novel object. D) Graph of the number of bouts exploring the novel object. E) Graph of the total time spent exploring the novel object. Error bars represent $\pm$ SEM (n =8/group). ITI: inter trial interval.

Figure 6. ROCK inhibition enhanced spatial discrimination and place recognition. A) Schematic drawing of the Y maze, note the visual cues at the end of each arm. B) Graph of latency to novel arm. C) Graph of the number of entries exploring the novel arm. D) Graph of the total time spent exploring the novel arm. E) Graph of the percent of time spent exploring the novel arm. Error bars represent $\pm$ SEM (* p <0.05, ** p <0.01, n =8/group).

Figure 1

Figure 2

Figure 3

Figure 4

A

B

Total time in Closed Arms

C

Total time in Open Arms

Figure 5

Figure 6

Highlights

- Increased survival of adult new-born hippocampal neurons with Rho-kinase inhibition.
- Rho-kinase inhibition did not change neuroblast proliferation.
- Inhibition of Rho-kinase enhanced spatial memory.
- Inhibition of Rho-kinase did not change anxiety levels.

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Title:

ADULT HIPPOCAMPAL NEUROGENESIS, RHO KINASE INHIBITION AND ENHANCEMENT OF NEURONAL SURVIVAL

Date:

2013-09-05

Citation:

Christie, K. J., Turbic, A. & Turnley, A. M. (2013). ADULT HIPPOCAMPAL NEUROGENESIS, RHO KINASE INHIBITION AND ENHANCEMENT OF NEURONAL SURVIVAL. NEUROSCIENCE, 247, pp.75-83. <https://doi.org/10.1016/j.neuroscience.2013.05.019>.

Publication Status:

Accepted manuscript

Persistent Link:

<http://hdl.handle.net/11343/41818>