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

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Abstract—Adult neurogenesis occurs throughout life; however the majority of new neurons do not survive. Enhancing the 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 and 100 μM) 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 μM 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 μM) 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.

Key words: doublecortin, NeuN, Rho GTPases, dentate gyrus, granule cell layer.

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 the use of drugs such as Y27632 or HA1077 promotes neurite outgrowth and blocks the effect 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 the 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 μM Y27632 Rho kinase inhibitor into the lateral ventricles of adult mice promoted ectopic migration of subventricular zone-derived neuroblasts

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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.

Experimental procedures

Male. C57BL/6 mice at 8–9 weeks of age (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.

Lateral ventricle infusion of Y27632 and/or EdU. Brain infusion cannulae and Alzet osmotic pumps (Model 1007D, 0.5 μl/h, 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 Life Technologies, Mulgrave, Victoria, Australia) 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, 0.7 mm lateral, 2.5-mm deep). Animals were given analgesia (Metacam, 3 mg/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 or 35 days and the brains were removed for analysis at 7 or 35 days after cannula insertion (only infused for 7 days in both cases).

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-μm coronal sections were collected at the level of the dentate gyrus (−1.2 to 2.0 mm Bregma) for analysis. Coordinates according to Paxinos and Franklin (2001).

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, Dallas, TX, USA) overnight and then goat anti-mouse Alexa 488 (Molecular Probes, Invitrogen) or donkey anti-goat Alexa 488 for 1 h (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 (×40 lens, 0.60 NA) and an Olympus FV 1000 Confocal microscope (×60 lens, Z-plane step of 1 μm).

To compare the effect of Rho kinase inhibition versus controls, for each mouse five hippocampal sections 150 μm apart from −1.2 to 2.0 mm Bregma coordinates were counted and the area of dentate was recorded with DAPI labelling in ImageJ, with the experimenter blinded to the condition. All counts were performed in real time using an epifluorescent microscope so that the experimenter could focus on all planes through the 30-μm tissue slice and assess antigen colocalisation. Quantification of cell number was normalized to the area of the dentate gyrus for each section and represented as number of EdU+ cells per area (mm^2). 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.

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

Object recognition. On day 34 post-infusion animals were habituated in boxes for three 10-min trials without objects. The animals were then put back into their box with two identical objects for 5 min and tracked with TopScan software. A bout of exploration was set at 1 s with nose on the object. Following an inter-trial interval of 1.5 h the animal was put back into the box with one familiar and one novel object for another 5 min. Latency to novel object, total time on novel object, percent time exploring objects, and number of bouts on objects was quantified.

Y maze. On day 35 post-infusion animals were placed in a plastic Y maze for 5 min with one arm blocked and tracked with TopScan software. Visual cues were