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2 # MITOGEN AND STRESS-ACTIVATED KINASES 1/2 REGULATE
3 ISCHEMIA-INDUCED HIPPOCAMPAL PROGENITOR CELL
4 PROLIFERATION AND NEUROGENESIS

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15 **Abstract—Pathophysiological conditions such as cerebral ischemia trigger the production of new neurons from the neurogenic niche within the subgranular zone (SGZ) of the dentate gyrus. The functional significance of ischemia-induced neurogenesis is believed to be the regeneration of lost cells, thus contributing to post-ischemia recovery. However, the cell signaling mechanisms by which this process is regulated are still under investigation. Here, we investigated the role of mitogen and stress-activated protein kinases (MSK1/2) in the regulation of progenitor cell proliferation and neurogenesis after cerebral ischemia. Using the endothelin-1 model of ischemia, wild-type (WT) and MSK1$^{-/-}$/MSK2$^{-/-}$ (MSK dKO) mice were injected with BrdU and sacrificed 2 days, 4 weeks, or 6 weeks later for the analysis of progenitor cell proliferation, neurogenesis, and neuronal morphology, respectively. We report a decrease in SGZ progenitor cell proliferation in MSK dKO mice compared to WT mice. Moreover, MSK dKO mice exhibited reduced neurogenesis and a delayed maturation of ischemia-induced newborn neurons. Further, structural analysis of neuronal arborization revealed reduced branching complexity in MSK dKO compared to WT mice. Taken together, this dataset suggests that MSK1/2 plays a significant role in the regulation of ischemia-induced progenitor cell proliferation and neurogenesis. Ultimately, revealing the cell signaling mechanisms that promote neuronal recovery will lead to novel pharmacological approaches for the treatment of neurodegenerative diseases such as cerebral ischemia.**
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Key words: MAPK, subgranular zone, ischemia, neuorogenesis, MSK, hippocampus. Q2 16

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Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; DCX, doublecortin; ET-1, endothelin-1; MAPK, mitogen activated protein kinase; MSK dKO, MSK1$^{-/-}$/MSK2$^{-/-}$; MSK1/2, mitogen and stress-activated protein kinases; NGS, normal goat serum; NSCs, neural stem cells; pMKS1, phospho-MSK1; SGZ, subgranular zone; WT, wild type.

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INTRODUCTION 17

Cerebral ischemia causes widespread neuronal degeneration, particularly in cortical and limbic regions, such as the hippocampus (Benveniste et al., 1984; Mattson et al., 1989). Given that hippocampal neuronal degeneration after ischemia leads to long-term functional and cognitive impairments (Rempel-Clower et al., 1996), there has been a longstanding interest in studying potential mechanisms of hippocampal neuronal repair. Importantly, some of the hippocampal vulnerability to cell death is mitigated by local neurogenesis: a process that is sustained throughout adulthood and is induced by ischemic injury (Jin et al., 2001; Yagita et al., 2001; Türeyen et al., 2004). Indeed, neural stem cells (NSCs) in the dentate gyrus subgranular zone (SGZ) are capable of regenerating a small, but significant, portion of the cell loss through ischemia-induced production of new neurons (Kee et al., 2001; Bendel et al., 2005). The majority of these immature neurons migrate to the granule cell layer, and integrate into the existing hippocampal network (Emsley et al., 2005). Importantly, interventions that increase post-ischemic neurogenesis promote greater functional and cognitive recovery (Chu et al., 2004; Matsumori et al., 2006b), while attenuation of neurogenesis has been shown to worsen post-ischemic recovery (Raber et al., 2004).

Given the inducible nature of SGZ neural progenitor proliferation, a good deal of effort has been focused on understanding the kinase signaling events that regulate this process. Consistent with this, an examination of the p42/44 mitogen-activated protein kinase (MAPK) cascade (Wu et al., 2000b) in the SGZ progenitor cell population has revealed that this pathway is activated within minutes following ischemic and other brain insults, and that it plays a key role in regulating inducible cell proliferation (Okuyama et al., 2004; Choi et al., 2008; Tian et al., 2009; Li et al., 2010).

The MAPK cascade is formed by a three kinase signaling cassettes, with the phospho-activation of extracellular signal-regulated kinases 1 and 2 (collectively referred to as ERK) (Chen et al., 1992) serving as its functional endpoint. Activation of MAPK occurs in response to extracellular stress signals produced by cerebral ischemia (Sugino et al., 2000; Lennmyr et al., 2002). Specifically, ERK phosphorylation occurs in response to growth factors, oxidative stress, glutamate receptor

activation and cytokine release (Nozaki et al., 2001). These factors induce rapid activation (phosphorylation) of the MAPK/ERK pathway, measurable within minutes following cerebral ischemia (Wu et al., 2000a; Ferrer et al., 2003). Following phosphorylation, ERK translocates from the cytosol to the nucleus where it regulates transcription via the direct targeting of transcription factors, and via the actuation of effector kinase pathways. In line with this idea, our recent work has indicated that much of the proliferative capacity of MAPK signaling in the SGZ (Okuyama et al., 2004; Choi et al., 2008; Tian et al., 2009) is mediated by the ERK effector kinases mitogen and stress-activated kinases MSK1 and MSK2 (hereafter referred to as MSK1/2). MSK1/2 are a nuclear-localized family of serine/threonine kinases that tightly regulate the transactivational potential of numerous transcription factors (Deak et al., 1998; Wiggin et al., 2002; Hauge and Frödin, 2006). We recently reported that both basal and seizure-evoked progenitor cell proliferation, as well as adult neurogenesis, are disrupted in MSK1/2 null mice (Choi et al., 2012). In a separate line of work, the deletion of MSK1 was found to impair environmental enrichment-induced hippocampal neuron plasticity, as well as cognition (Karelina et al., 2012). Given the role that MSK1/2 activation plays in progenitor cell proliferation and neuronal plasticity, the experiments described here were designed to specifically assess the regulatory role of MSK1/2 in ischemia-induced neurogenesis. Using the endothelin-1 (ET-1) model of transient cerebral ischemia, we report a reduction in ischemia-induced progenitor cell proliferation in $MSK1^{-/-}/MSK2^{-/-}$ mice (hereafter referred to as "MSK dKO") compared to wild-type mice. Moreover, our analysis of immature newborn neurons indicates that MSK dKO mice exhibit impaired neuronal development after ischemia. Finally, the dendritic branching complexity of mature neurons was reduced in MSK dKO mice compared to WT. Taken together, our dataset reveals that MSK1/2 signaling is critical for the regulation of ischemia-induced progenitor cell proliferation, neuronal differentiation, and neurite outgrowth.

EXPERIMENTAL PROCEDURES

Generation of $MSK1^{-/-}/MSK2^{-/-}$ mice

Q3 Double knockout (MSK dKO) and wild-type (WT) mice were generated by crossing $MSK1^{-/+}/MSK2^{-/+}$ heterozygous mice (kindly provided by Dr. J. Simon Arthur; University of Dundee, Dundee, Scotland). Mice were backcrossed into a C57/bl6 line for over 10 generations. A subset of WT and MSK dKO mice were crossed with a line of transgenic mice expressing GFP under the *Thy1* promoter (Thy1-GFP mice were kindly provided by Dr. Gouping Feng; Duke University). Mouse genotype was confirmed using tail DNA samples subjected to PCR amplification as previously described (Karelina et al., 2012).

Stereotaxic surgery and endothelin-1 infusion

Adult WT and MSK dKO mice (6–9 week old/mixed sex) were anesthetized with an intraperitoneal (i.p.) injection of ketamine (95.2 mg/kg) and xylazine (30.8 mg/kg) approximately 15 min before surgery. The scalp was cleaned and sterilized and ointment was applied to the eyes. Mice were then placed in the stereotaxic apparatus (Cartesian Research, Inc.), a single hole was drilled into the skull (coordinates AP −2.06 mm; ML + 1.30 mm), and the tip of a 5-μL syringe was then lowered −2.00 mm to the position above the upper blade of the dentate gyrus. Mice were infused with 0.5 μL of ET-1 (1 μg/μL), a potent vasoconstrictor, or the vehicle saline, at the rate of 1 μL/min (Karelina et al., 2014). The needle was left in place for 5 min before retraction and suturing. All mice were returned to their home cages and monitored for the duration determined by experimental conditions.

BrdU administration

Following ischemia induction via ET-1 infusion, newly generated cells were labeled via i.p. injections of 5-bromo-2'-deoxyuridine (BrdU: 50 mg/kg in saline, Sigma Aldrich). BrdU-positive cell analysis was performed at 2 days, 4 weeks, or 6 weeks post injury. For the 2-day time point, BrdU was injected 4 and 2 h before sacrifice (Choi et al., 2008; Karelina et al., 2014). For the 4- and 6-week time points, BrdU was injected twice daily, 6 h apart, on days 6, 7, and 8 after injury. A parallel BrdU injection protocol was used for saline-infused control animals.

Tissue processing

Two days, 4 weeks, or 6 weeks after the induction of ischemia (or control), mice were anesthetized with an overdose of ketamine/xylazine and sacrificed via transcardial perfusion of cold saline, followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde overnight, then cryoprotected with 30% sucrose. Coronal sections (40 μm for 2-day and 4-week tissue, 80 μm for the 6-week tissue) through the hippocampus were prepared using a freezing microtome.

Immunolabeling

All immunohistochemical labeling was performed as previously described (Karelina et al., 2014). Briefly, sections were washed with phosphate-buffered saline with 0.1% Triton-X (PBST), and blocked with 10% normal goat serum before overnight incubation at 4 °C with rabbit anti-phosphorylated MSK1 (pMSK1, 1:250: Ser360, Cell Signaling, catalog # 9594). Sections were processed using the ABC staining method (Vector Labs) and visualized with nickel-intensified DAB (Vector Labs). Q4

For immunofluorescent labeling for BrdU, and doublecortin or GFP, sections were incubated overnight in rat anti-BrdU (1:200, Accurate Chemical & Scientific Corp., catalog code YSRTMCA2060GA), goat anti-doublecortin (1:1000, Santa Cruz Biotech, catalog code SC-8066) or chicken anti-green fluorescent protein (1:2500, Abcam, catalog code AB13970). Primary antibody labeling was visualized with AlexaFluor 488 and 594 antibodies (1:500; Invitrogen). DraQ5 (1:5000; Biostatus Limited) was used as a counterstain for the imaging of hippocampal cell layers. Images were

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