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Mitogen- and stress-activated kinases regulate progenitor cell proliferation and neuron development in the adult dentate gyrus

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Abstract

The neurogenic niche within the subgranular zone (SGZ) of the dentate gyrus is a source of new neurons throughout life. Interestingly, SGZ proliferative capacity is regulated by both physiological and pathophysiological conditions. One outstanding question involves the molecular mechanisms that regulate both basal and inducible adult neurogenesis. Here, we examined the role of the MAPK-regulated kinases MSK1 and MSK2 (mitogen and stress activated kinase 1 and 2) as regulators of dentate gyrus SGZ progenitor cell proliferation and neurogenesis. Under basal conditions, MSK1/2 null mice exhibited significantly reduced progenitor cell proliferation capacity and a corollary reduction in the number of DCX-positive immature neurons. Strikingly, seizure-induced progenitor proliferation was totally blocked in MSK1/2 null mice. This blunting of cell proliferation in MSK1/2 null mice was partially reversed by forskolin infusion, indicating that the inducible proliferative capacity of the progenitor cell population was intact. Further, in MSK1/2 null mice, DCX-positive immature neurons exhibited reduced neurite arborization. Together these data reveal a critical role for MSK1/2 as regulators of both basal and activity-dependent progenitor cell proliferation and morphological maturation in the SGZ.

Keywords

MAPK; MSK; progenitor cells; seizure; hippocampus; subgranular zone; neurogenesis

Introduction

A thin zone of self-renewing progenitor cells located within the subgranular zone (SGZ) continuously seeds the dentate gyrus with new cells (Aimone *et al.* 2007; Alvarez-Buylla and Lim 2004; Ming and Song 2011). A subset of these cells develop into adult granule cells

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that extend apical dendrites into the molecular layer, synapse on pyramidal cells of layer CA3 and contribute to hippocampal-dependent processes, such as learning and memory (Castilla-Ortega et al,. 2011; Deng *et al.* 2011; Koehl and Abrous 2011). Interestingly, neurogenesis is increased by diverse stimuli, such as environmental enrichment and motor activity (van Praag *et al.* 1999; Young *et al.* 1999). This varied rate of neurogenesis suggests that the SGZ progenitor cell population is primed to respond to changes in the level of neuronal activity, ostensibly adjusting the progenitor cell proliferation capacity to match the data processing demand of the dentate gyrus. Further, potentially pathophysiological stimuli, such as seizure activity and hypoxia also increase neurogenesis (a *et al.* 1997; Liu *et al.* 1998); with respect to dentate physiology, the ramifications of excitotoxic stimulus-evoked proliferation are not fully understood (Scharfman and Gray, 2009).

With regard to the SGZ, one key question relates to the intracellular signaling events that couple changes in neuronal activity to inducible neurogenesis. A potential clue comes from studies showing that seizure activity stimulates activation of the p42/44 mitogen-activated protein kinase (MAPK) cascade in neural progenitors of the dentate gyrus (Choi *et al.* 2008: Li *et al.* 2010). Further, proliferation of SGZ and subventricular zone neuronal precursors is attenuated by the disruption of MAPK signaling (Jiang *et al.* 2005; Howell *et al.* 2005; Choi 2008; Rosa *et al.* 2010; Learish *et al.* 2010).

As an activity-dependent kinase pathway, the MAPK cascade is responsive to an array of physiological and pathophysiological CNS stimuli. Interestingly, much of the transactivation potential of the MAPK cascade is regulated by downstream effector kinases. Along these lines, mitogen and stress activated kinase (MSK) 1 and 2 are key targets of the MAPK cascade (Pierrat *et al.* 1998). MSKs are nuclear-localized serine/threonine kinases composed of two distinct domains: an N-terminal kinase that phosphorylates MSK substrates, and a C-terminal kinase that functions in an autoregulatory role (Smith *et al.* 2004). MSKs exhibit a good degree of functional redundancy, however, some distinct differences in regulation of the kinase has been noted (Vermeulen *et al.* 2009). With respect to function, MSKs appear to principally serve as regulators of gene expression. Along these lines, MSKs have been shown to modulate chromatin structure (Vermeulen *et al.* 2009). Furthermore, MSKs are the dominant MAPK-regulated CREB kinases (Pierrat *et al.* 1998; Arthur et al, 2004). Interestingly, CREB-inducible gene expression has been implicated in the regulation of neuronal precursor proliferation and differentiation (Nakagawa et al 2002; Peltier *et al.* 2007; Jagasia *et al.* 2009; Dworkin *et al.* 2009; Grimm *et al.* 2009, Merz *et al.* 2011). These findings coupled with work showing that MAPK signaling influences progenitor proliferation and neuronal maturation (Samuels *et al.* 2008; Samuels *et al.* 2009) raises the possibility that MSKs function as essential intermediates that regulate SGZ neurogenesis. Here, we present data indicating that MSK1/2 play key roles in regulating progenitor proliferation capacity and in regulating adult-born neuron morphological maturation.

Methods

Animals

Mice were genotyped using the primer sets and cycling conditions described by Wiggin et al. (2002). $MSK1^{(-/-)}/2^{(-/-)}$ double-knockout and $MSK1^{(-/+)}/2^{(-/+)}$ heterozygous mice were generated by crossing $MSK1^{(-/+)}/2^{(-/+)}$ heterozygous mice: $MSK1^{(-/+)}/2^{(-/+)}$::$MSK1^{(-/+)}/2^{(-/+)}$. The MSK targeted strains were backcrossed into the C57/BL6 line over 8 generations. All animal procedures were in accordance with Ohio State University animal welfare guidelines and approved by the Institutional Animal Care and Use Committee. All experiments used male mice.

Pilocarpine-induced status epilepticus and cell proliferation

Initially, 8-9 week old $MSK1^{(-/-)}/2^{(-/-)}$ and $MSK1^{(-/+)}/2^{(-/+)}$ mice received an intraperitoneal injection (i.p.) with atropine methyl nitrate (1 mg/kg in saline, Sigma, St. Louis, MO), then 30 minutes later, status epilepticus (SE) was elicited via an i.p. injection of pilocarpine (325 mg/kg, Sigma, St. Louis, MO, USA) diluted in physiological saline. SE was defined as a continuous (6~7 hrs) motor seizure activity (stage 4 or greater, using the Racine grading scale: Racine, 1972). To label proliferating cells, 5-bromo-2′-deoxyuridine (100 mg/kg in saline, Sigma) was injected (i.p.) 6 hrs and 3 hrs before sacrifice.

Microinfuion of forskolin

Mice were placed in a stereotaxic frame (David Kopf Instruments) under ketamine/xylazine anesthesia and guide cannulae (24G) were positioned in the lateral ventricle (stereotaxic coordinates: AP: −0.46 mm, ML: +1.10 mm, DV: −2.30mm). Following surgery, mice were allowed to recover for 10 days. Mice received two rounds of forskolin infusion and BrdU injection. Initially, mice were restrained by hand and infused (1 μL, 2 min) with forskolin (1 mM in DMSO, Sigma, St. Louis, MO) or vehicle (DMSO) through an injector needle (30G). To label proliferating cells, BrdU (100 mg/kg in saline) was injected (i.p.) 1 and 4 hrs after forskolin infusion. Twenty-four hrs later, mice received a second infusion of forskolin followed 1 hr later with injection of BrdU (100 mg/kg). Mice were perfused 3 hr after the last BrdU injection. To examine pCREB immunoreactivity, mice were perfused 1 hr after a single infusion of forskolin (1 μL, 1 mM) or DMSO vehicle.

Histochemistry and immunolabeling

Tissue was fixed via transcardial perfusion with 4% paraformaldehyde, under ketamine/xylazine anesthesia. Brains were then isolated and post-fixed (4% paraformaldehyde for 4 hr at 4 ° C) followed by cryoprotection with 30% sucrose. Coronal sections (20 or 40 μm) through the dorsal (stereotaxic coordinate AP: −1.40 ~ −2.20) hippocampus were prepared using a freezing microtome.

For immunohistochemistry, sections were washed with PBS and incubated in 0.3% hydrogen peroxide/PBS for 20 min to eliminate endogenous peroxidase activity. After several washes with PBS, sections were blocked with 10 % normal goat serum or 3% normal horse serum in PBS, then incubated overnight at 4°C with the following antibodies: rabbit anti-pCREB (1:1000, Cell Signaling, Danvers, MA, USA), rabbit anti-Ki-67 (1:2000, Vector labs, Burlingame, CA), Goat anti-doublecortin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-Sox-2 (1:1000, Santa Cruz Biotechnology), goat anti-MSK1 (1:1000, Santa Cruz Biotechnology: the specificity of this antibody was verified in Karelina et al. (2012). The ABC labeling method (Vector Labs) followed by nickel-intensified DAB development (Vector Labs) was used to visualize the signal. Images were acquired using a 16 bit digital camera (Micromax YHS 1300; Princeton Instruments, Trenton, NJ, USA) mounted on a Leica DM IRB microscope (Nussloch, Germany). For BrdU staining, sections were incubated in 2XSSC/50% formamide for 2 hr at 65 °C, followed by incubation in 2N HCl at 37 °C for 1 hr. After washing with 0.05 M borate buffer (pH 8.5) for 10 min and washing with PBS, sections were blocked with 10% normal goat serum in PBS and incubated at 4 °C with a rat anti-BrdU antibody (1:400, Accurate Chemical, Westbury, NY, USA). After washing with PBS, sections were incubated with HRP-conjugated anti-rat IgG (1:400, Jackson Immunoresearch, West Grove, PA) for 2 hr at room temperature and developed using nickel-intensified DAB.

For immunofluorescence labeling, sections were washed with PBS, DNA was denatured as noted above and blocked with 10% normal goat serum or 5% normal horse serum in PBS, followed by overnight incubation at 4 °C with rat anti-BrdU antibody (1:400), mouse anti-

NeuN antibody (1:1000, Millipore, Billerica, MA), goat anti-doublecortin antibody (1:500), rabbit anti-MSK1 antibody (1:1000) or goat anti-SOX2 antibody (1:500). After several washes, sections were incubated (2 hrs at room temperature) with secondary antibodies conjugated with Alexa 488 or Alexa 594 (1:1000, Invitrogen, Carlsbad, CA, USA), then mounted with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI, USA). Fluorescence images were captured using a Zeiss 510 Meta confocal microscope (2 μm-thick optical section).

Cresyl violet staining was performed as described in Choi et al., (2007).

Double labeling for BrdU and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Labeling was performed using the methods described by Kuhn et al. (2005) with minor modification. Briefly, sections were incubated in 0.1 M Tris–HCl (pH 7.4), and then in a graded (70%, 90%, 100%) isopropanol series for 10 min. Sections then descended through the isopropanol series and were finally placed in H_20. Tissue was then processed using the Apoptag TUNEL labeling kit (Chemicon) following the manufacturer's guidelines up to the antibody labeling step. At this point, sections were blocked (1 h) in 10% normal goat serum and incubated (overnight at 4 °C) with rhodamine-conjugated sheep anti-digoxigenin antibody (1:400) and anti-BrdU antibody (1:400). Next, the tissue was washed in PBS and incubated with Alexa-488-conjugated anti-rat antibody (1:1000) for 2 h in PBS. After washing, sections were coverslipped using Cytoseal.

Cell quantitation

Quantification of BrdU, Ki-67, doublecortin, and SOX-2 expression within the SGZ was performed by counting cells (bilaterally) in 3 dorsal hippocampal sections (AP coordinate of first, dorsal-most, section: −1.40 μm) separated by 160 μm intervals and averaged for each animal. The number of cells was expressed as the mean ± SEM from 5-7 mice for each group. Cell counts were analyzed statistically using Student's t-test, and significance was accepted for $P<0.05$.

Results

Generation of MSK1/2 double knockout mice

The disruption of MSK1 and MSK2 was confirmed by PCR (Fig. 1A and 1B**;** and see Wiggin et al. 2002). As noted by Wiggin et al. (2002), double knockout mice were viable and fertile and had no obvious health problems. As shown in Figure 1C, immunohistochemical labeling detected broad expression of MSK1 within the hippocampus of wild type mice; labeling was not detected in $MSK1^{(-/-)}/2^{(-/-)}$ mice. In the dentate gyrus, MSK1 expression was detected in the granule cell layer (GCL). Consistent with our recent work (Karelina *et al.*, 2012), we detected MSK1 expression in both SOX2-positive type1 and type2a stem/progenitor cells, and in doublecortin-positive type2b and type3 cells (Fig. 1D and 1E). Similar to MSK1, *in situ* data sets presented on the Allen Mouse Brain Atlas (http://mouse.brain-map.org/) show marked *MSK2* mRNA expression within the major hippocampus cell layers. Further, array based approaches have reported that both *MSK1* and *MSK2* mRNA are expressed in undifferentiated neural progenitor cell populations and in cultured neurospheres (Hartl *et al.* 2008; Ruau *et al.* 2008).

MSK1/2 regulate progenitor cell proliferation

The expression of MSK1 in the SGZ raised the possibility that MSKs contribute to cell proliferation and/or post-mitotic cellular survival/differentiation. To examine progenitor cell

proliferation in the SGZ, mice were injected (two times, at a 3 hr interval) with BrdU (100 mg/kg in saline. i.p.) and perfused 3 hrs after the last injection. Compared to $MSK1^{(+/+)}/2^{(+/+)}$ and $MSK1^{(-/+)}/2^{(-/+)}$ mice, the number of BrdU-labeled cells was markedly lower in $MSK1^{(-/-)}/2^{(-/-)}$ mice (Fig. 2A and 2B). BrdU analysis was complemented by immunolabeling for Ki-67, a marker of actively proliferating progenitor cells (Fig. 2C and 2D). Consistent with the results using BrdU-labeling, quantitative analysis of Ki-67 revealed a significant reduction in the number of proliferating progenitor cells in the SGZ of $MSK1^{(-/-)}/2^{(-/-)}$ compared to $MSK1^{(+/+)}/2^{(+/+)}$ and MSK1(−/+)/2(−/+) mice. Of note, the number of BrdU- and Ki-67-positive cells was not significantly different between $MSK1^{(+/+)}/2^{(+/+)}$ and $MSK1^{(-/+)}/2^{(-/+)}$ heterozygous mice. Together, these results indicate that MSKs function as regulators of progenitor cell proliferation in the SGZ.

To determine whether reduced cell proliferation results from reduced progenitor cell density, sections were labeled for SOX-2. The density of SOX-2-labeled cells in the SGZ was not significantly different between $MSK1^{(-/+)}/2^{(-/+)}$ and $MSK1^{(-/-)}/2^{(-/-)}$ littermates (Fig. 2E and 2F), indicating that MSKs influence either the rate of progenitor cell proliferation or the number of proliferating cells, rather than the total number of progenitor cells. Finally, given that the proliferation in $MSK1^{(+/+)}/2^{(+/+)}$ and $MSK1^{(-/+)}/2^{(-/+)}$ mice was not significant different, in the remainder of the paper, we focus our comparative analysis on $MSK1^{(-/+)}/2^{(-/+)}$ and $MSK1^{(-/-)}/2^{(-/-)}$ littermates.

MSK1/2, neurogenesis and cell survival

To determine whether this reduction in proliferation in MSK1/2 null mice manifests as a reduction in the number of immature neurons, we quantitated doublecortin (DCX)-expressing cells. DCX is transiently expressed in proliferating progenitor cells and newly generated neuroblasts (Brown et al, 2003), and thus serves as a useful marker of maturing neurons. Relative to $MSK1^{(-/+)}/2^{(-/+)}$ heterozygous mice, the density of DCX-positive cells was significantly lower in $MSK1^{(-/-)}/2^{(-/-)}$ mice (51% reduction; Fig. 3A and 3B).

To examine the potential effects of MSKs on neuronal lineage commitment, $MSK1^{(-/-)}/2^{(-/-)}$ and $MSK1^{(-/+)}/2^{(-/+)}$ mice were killed 4 weeks post-BrdU injection, and the tissue was processed for incorporation of the mitotic marker and for NeuN, a marker of mature neurons. As shown in Figure 3C and 3D, there was no significant effect of MSKs on the relative percentage of adult-born neurons.

We then examined whether MSK1/2 signaling regulates the survival of newborn cells. To this end, tissue was processed using TUNEL, a marker of apoptotic cell death. Relative to $MSK1^{(-/+)}/2^{(-/+)}$ heterozygous mice, the average number of TUNEL-positive cells in the SGZ was significantly lower in the $MSK1^{(-/-)}/2^{(-/-)}$ mice (Fig. 3E and 3G). Since this may simply reflect a reduction in cell generation in the $MSK1^{(-/-)}/2^{(-/-)}$ mice, the percentage of BrdU- that were also TUNEL-positive cells was determined. Animals were sacrificed 10 days after BrdU injection (100 mg/kg, 2 times: 3 hr interval). We chose the 10-day post-injection time point for analysis, since a significant decrease in cell viability is observed from 1 to 2 weeks after cell birth (Gould *et al.* 1999). The percentage of dead or dying BrdU-labeled cells was not significantly different between $MSK1^{(-/+)}/2^{(-/+)}$ and $MSK1^{(-/-)}/2^{(-/-)}$ littermates (Fig. 3F and 3H), indicating that MSKs are not critical for the survival of newborn cells. Together, these data suggest that MSK1/2 signaling plays a key role in progenitor cell proliferation, but not in survival or neuronal lineage commitment.

MSK1/2 and cell cluster size

To further address the role of MSK1/2 in cell proliferation, we examined the size of BrdU-positive cell clusters in the SGZ. For this study, mice injected twice with BrdU (100 mg/kg,

2 times: 3 hr interval) and killed 3 hrs after the second injection. In both MSK1$^{(-/+)}$/2$^{(-/+)}$ and MSK1$^{(-/-)}$/2$^{(-/-)}$ mice, clusters of BrdU labeled cells were found throughout the SGZ (Fig. 4A). However, MSK1$^{(-/-)}$/2$^{(-/-)}$ mice had a higher relative level of lone BrdU-positive cells, and two-cell clusters. Conversely, MSK1$^{(-/+)}$/2$^{(-/+)}$ mice exhibited a markedly higher level of clusters consisting of 3 or more cells (Fig. 4B). These data provide support for the idea that MSKs regulate progenitor proliferative capacity.

MSK1/2 signaling regulates activity-dependent progenitor cell proliferation

Next, we examined the role of MSK1/2 in progenitor cell proliferation following pilocarpine-evoked repetitive seizure activity (i.e. status epilepticus: SE). As expected, progenitor cell proliferation, as assessed by BrdU incorporation, was significantly increased in MSK1$^{(-/+)}$/2$^{(-/+)}$ heterozygous mice examined at 2 days post-SE (Fig. 5A and 5B). In marked contrast, SE-induced cell proliferation was completely blocked in MSK1$^{(-/-)}$/2$^{(-/-)}$ mice. These data suggest that MSK1/2 is a critical signaling intermediate for activity-dependent progenitor cell proliferation.

Signaling via the MAPK pathway and the downstream target CREB has been suggested to play a role in neurogenesis and differentiation (Nakagawa *et al.* 2002; Giachino *et al.* 2005; Fujioka *et al.* 2004, Samuels *et al.* 2009, Merz *et al.* 2011). Given this, we tested whether deletion of MSK1 and MSK2 altered levels of the Ser-133 phosphorylated form of CREB (pCREB). Under basal conditions, pCREB was detected in both the GCL and SGZ of MSK1$^{(-/+)}$/2$^{(-/+)}$ heterozygous mice (Fig. 5C). This expression pattern is consistent with the pCREB expression pattern reported by Nakagawa et al. (2002). In contrast with MSK1$^{(-/+)}$/2$^{(-/+)}$ mice, MSK1/2 nulls exhibited a near total loss of pCREB immunoreactivity within both the GCL and SGZ (Fig. 5C). Next, we tested whether MSKs contribute to SE-evoked CREB activation. To this end, mice were injected with pilocarpine, and killed 2 days post-SE. Relative to saline-injected mice (Fig. 5C, Sal), SE triggered a marked upregulation of pCREB in both the GCL and SGZ of MSK1$^{(-/+)}$/2$^{(-/+)}$ heterozygous mice. Conversely, SE-induced CREB activation was not detected in MSK1$^{(-/-)}$/2$^{(-/-)}$ mice. These data indicate that MSKs are principal regulators of CREB activation in the SGZ. Of note, in MSK1/2 null mice, a decrease in pCREB labeling was also observed in all of the major cell layers (e.g., CA3, CA1 and GCL) of the hippocampus (Supplemental figure 1).

The effect of forskolin on progenitor cell proliferation

MSKs are activity-dependent proline-directed Ser-Thr kinases which exhibits homology with other activity-dependent kinases, such as PKA and CaMKIV (Manning et al, 2002; McCoy et al. 2005). Given this, we examined whether the proliferation and pCREB phenotype in MSK1/2 null mice could be rescued via the stimulation of other kinase pathways. This experiment also addresses potential developmental deficits associated with MSK1/2 ablation. As expected, forskolin microinfusion (1 mM in DMSO, 1 μL) into the lateral ventricle significantly increased the progenitor cell proliferation in the SGZ of MSK1$^{(-/+)}$/2$^{(-/+)}$ heterozygous mice. Importantly, in MSK1$^{(-/-)}$/2$^{(-/-)}$ mice, forskolin infusion triggered a significant increase in the progenitor cell proliferation (Fig. 6A and 6B). The forskolin-induced fold-increase in proliferation was similar between the MSK1$^{(-/-)}$/2$^{(-/-)}$ and MSK1$^{(-/+)}$/2$^{(-/+)}$ mice. Further, microinfusion of forskolin also triggered robust CREB phosphorylation in MSK1$^{(-/+)}$/2$^{(-/+)}$ mice, and, importantly, triggered a remarkable recovery of CREB phosphorylation in the SGZ of MSK1$^{(-/-)}$/2$^{(-/-)}$ mice (Fig. 6C). Interestingly, forskolin-induced progenitor cell proliferation as well as CREB phosphorylation was more prominent in MSK1$^{(-/+)}$/2$^{(-/+)}$ mice than in the MSK1$^{(-/-)}$/2$^{(-/-)}$ mice. One potential reason is that the forskolin-evoked cAMP increase may, in addition to working through PKA, couple to both CREB and cell proliferation via a MAPK cascade-

dependent process (Frödin *et al.* 1994). Thus, in MSK1$^{(-/-)}$/2$^{(-/-)}$ mice, this cAMP actuated-MAPK-dependent route to both CREB and cell proliferation would not be functional.

The effect of MSK1/2 on neuronal morphological maturation and dentate gyrus volume

Given that MSKs regulate CREB and that CREB is a key regulator of neuronal morphology, we examined the effects of MSK1/2 deletion on morphological maturation of newborn cells. To this end, sections were labeled for DCX, and, using the criteria employed by Plümpe et al. (2006: with minor modifications) immature neurons were organized into morphological groups based on process length and degree of branching (Fig. 7A). Interestingly, the percentage of type I-II DCX-positive cells, which are defined by short plump processes or no processes (type I) or processes that do not reach the molecular layer (type II), was significantly higher in MSK1$^{(-/-)}$/2$^{(-/-)}$ mice than in MSK1$^{(-/+)}$/2$^{(-/+)}$ heterozygous mice. On the other hand, a higher percentage of type III-IV DCX-expressing cells, which are defined as having processes that reach the molecular layer and either do (type IV) or do not have well-developed branches (type III), were detected in MSK1$^{(-/+)}$/2$^{(-/+)}$ heterozygous mice compared to MSK1$^{(-/-)}$/2$^{(-/-)}$ mice (Fig. 7B and 7C). Together, these data indicate that MSK1/2 signaling regulates neuronal maturation and/or the degree of neurite outgrowth and process arborization.

Since MSK1/2 signaling reduces progenitor cell proliferation, we examined the total volume of the granule cell layer. In 5 months old mice the volume of the granule cell layer in MSK1/2 KO mice was significantly lower than in MSK1/2 heterozygous mice (Supplemental figure 2**)**, suggesting that the reduced progenitor cell proliferation results in a decrease in granule cell number. Importantly, body weight was not significantly different between two groups, suggesting that the difference of GCL volume is not because of developmental deficits (29.17 ± 0.54 and 28.05 ± 0.42 grams for MSK1/2 heterozygous and KO mice, respectively. n = 6 for each group).

Discussion

A key goal of this study was to examine potential signaling effectors by which the MAPK pathway influences SGZ neurogenesis. Here, we present data supporting the idea that MSKs are essential regulators of both basal and inducible progenitor cell proliferation and neuronal development.

MSK1/2 and SGZ progenitor proliferation

Progenitor cell proliferation was determined using a combination of markers for actively proliferating cells (e.g. BrdU and Ki-67), and a marker of both proliferating and quiescent progenitor cells, SOX-2 (Nyberg *et al.* 2005). This analysis revealed that the total number of progenitor cells was not altered in MSK1$^{(-/-)}$/2$^{(-/-)}$ mice, but rather, that there was a reduction in proliferation. Mechanistically, a reduction in proliferation could result from either a decrease in the number of actively proliferating cells or a decrease in the rate of proliferation. Although we did detect a decrease in the size of cell clusters (via BrdU labeling), which is suggestive of a decrease in proliferative capacity, neither mechanism was conclusively tested. Clearly, additional experiments using specific markers of cell proliferation and/or acute mitotic labeling approaches will be required to clarify precisely how MSKs influence the proliferative process.

The pilocarpine model of SE revealed a marked deficit in induced progenitor proliferation in the MSK1/2 null mice. To our knowledge this is the first report examining the role of MSKs in cell proliferation, although a significant body of work has implicated the MAPK pathway as a regulator of cell division outside of the nervous system (Zhang and Liu, 2002; Yoon and

Seger, 2006; Sebolt-Leopold, 2000). With respect to potential effectors of MSK1/2 activity in the SGZ, a number of neurotransmitters, including dopamine (Höglinger *et al.* 2004), and trophic factors, including IGF-1 and VEGF (Aberg *et al.* 2000; Jin *et al.* 2002) have been shown to influence progenitor proliferation. Importantly, these transmitters and trophic factors are capable of stimulating MAPK activity and, one would assume, MSK1/2 signaling. Further, with respect to the SGZ, several studies have reported that inducible cell proliferation is dependent on the MAPK pathway. For example, neuropeptide Y-mediated proliferation is dependent on MAPK signaling (Howell *et al.* 2005). Similarly, cannabinoid- and valproate-induced proliferation is mediated by a MAPK-dependent mechanism (Jiang *et al.* 2005; Hao *et al.* 2004). These findings, coupled with our work raise the possibility that MSKs are a principal downstream effector that couple stimulus-induced MAPK cascade activation to cell proliferation. However, it is important to note that our examination focused on a single time point: 2 days-post seizure onset. Thus, additional studies will be required to determine whether acute (i.e., hrs post-seizure onset) induction of cell proliferation is also dependent on MSKs. Further, as briefly described above, it is unclear whether MSK1/2 couple SE to increased proliferation via an increase in the mitotic activity of proliferating progenitors, and/or whether MSKs couple SE to the activation of quiescent progenitors. With respect to the latter idea, it is interesting to note that Parent et al. (1999) found that enhanced proliferative capacity actuated by SE results from an increase in the proliferative activity of dividing cells rather than from the recruitment of quiescent SGZ progenitors. Finally, the cell-autonomous nature of the effects described here will need to be tested. Hence, given that we used a germ-line MSK1/2 deletion line, which results in a loss of MSK1/2 expression in all cells, it is conceivable that the SGZ proliferation phenotype could result in part from alterations in the extracellular trophic environment, which in turn, could affect proliferative capacity within the SGZ neurogenic niche.

MSK1/2 and SGZ neuron morphological maturation

The MAPK pathway and its downstream effector CREB have been shown to regulate neuronal morphological development (Giachino *et al.* 2005; Fujioka *et al.* 2004; Samuels *et al.* 2009, Merz *et al.* 2011). Our data support the idea that MSK1/2 serve as intermediates within this MAPK/CREB-regulated developmental signaling pathway within the SGZ. Along these lines, MSK1/2 null mice exhibit reduced pCREB expression within the SGZ, and adult born neurons displayed impaired morphological development (e.g. neurite outgrowth and process arborization). There are numerous potential mechanisms by which the disruption of the MAPK/MSK/CREB signaling cassette could impact neuronal morphological development. Along these lines, a number of CREB target genes, including Wnt-2 and miR-132 have been shown to affect neuronal morphological maturation (Redmond *et al.* 2002; Wayman *et al.* 2006; Wayman *et al.* 2008; Magill *et al.* 2011).

It is worth noting that a number of studies have shown that pCREB levels are relatively low in the progenitor pool, and only upon exiting the cell cycle and commitment to the neuronal lineage is there a marked increase in pCREB expression (Jagasia *et al.* 2009; Merz *et al.* 2011). Given this, the SGZ progenitor cell proliferation deficit in MSK1/2 mice would likely not be related to the pCREB deficiency. Rather, with respect to the proliferation phenotype, MSKs would likely be functioning through a number of alternate signaling pathways. Along these lines, MSKs have been shown to affect gene expression through a number of epigenetic mechanisms, including the phosphorylation of histone H3 and the phosphorylation non-histone chromosomal protein HMG-14 (HMG-14: also referred to as HMG1 and IPO38), (Soloaga *et al.* 2003; Thomson *et al.* 1999). In addition, MSK phosphorylation of H3S28 was recently found to be a key event that couples both mitogenic and differentiation signals to the displacement of polycomb group proteins from trimethylated H3K27 (Gehani *et al.* 2011). This MSK-actuated set of events allows for the

de-repression of gene expression during development, and was found to affect cell fate. Clearly, further work on the precise mechanisms by which MSKs influence SGZ progenitor proliferation is merited.

Finally, beyond the SGZ, we noted a marked decrease in pCREB levels throughout the hippocampus in MSK1/2 null mice. This observation is consistent with work performed using a MSK1 null mouse line, which detected a deficit in pCREB levels within the hippocampus (Chwang et al. 2007). Interestingly, they also reported MSK1 deficient mice exhibit significant deficits in learning and memory. Of note, we recently completed a study which revealed an important role for MSK1 in environmental enrichment-induced cognitive enhancement (Karelina et al., 2012). These data, coupled with the deficit in pCREB expression reported here, lead us to predict that the knockout of both MSK1 and MSK2 would result in a profound cognitive impairment.

In summary, the data provided here reveal a key role for MSK1/2 as regulators of basal and inducible cell proliferation and adult-born neuron development. Given the regenerative potential of adult progenitor cells, targeted regulation of MSK activity may prove to be a useful therapeutic intervention against hippocampal neurodegenerative disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

SGZ	subgranular zone
MSK	mitogen and stress activated kinase
MAPK	mitogen activated protein kinase
CREB	cAMP response element-binding protein
GCL	granule cell layer
TUNEL	terminal transferase dUTP nick end labeling
BrdU	bromodeoxyuridine
PKA	protein kinase A

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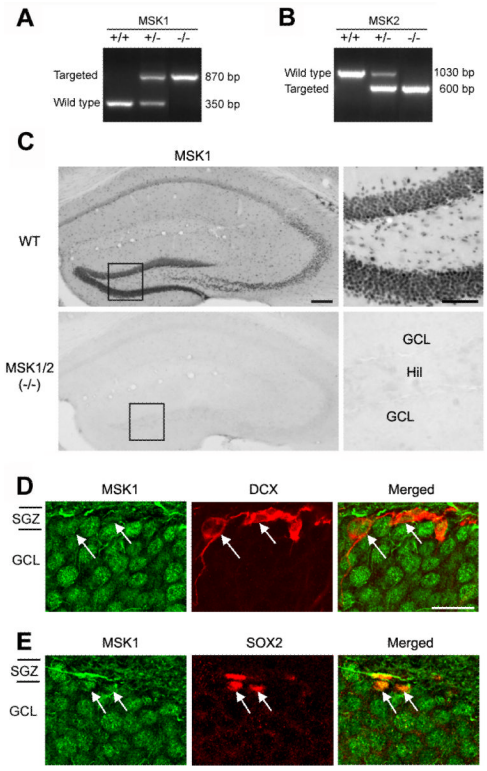

Figure 1. Generation of MSK1/2 double knockout mice

A-B) Genotyping of MSK1 (A) and MSK2 (B) knockout (−/−), heterozygous (−/+) or wild type (WT) (+/+) mice from tail biopsy samples. DNA samples were subjected to PCR amplification as described in Materials and Methods. DNA was electrophoresed on 1% agarose gels and examined by ethidium bromide staining. (C) Upper left panel: immunohistochemical labeling revealed marked MSK1 expression throughout the hippocampus. Lower left panel: MSK1 expression was not detected in MSK 1/2 null mice. The granule cell layer (GCL), and hilar regions of the dentate gyrus are magnified (right panels). Scale bar = 200 μm (low magnification images), 100 μm (high magnification images). D-E) Double labeling revealed MSK1 expression in doublecortin- (DCX) positive cells (D) and in SOX-2 positive stem cells, (E). Arrows denote relevant MSK1-positive cells. Scale bar = 20 μm. SGZ: subgranular zone, GCL: granule cell layer.

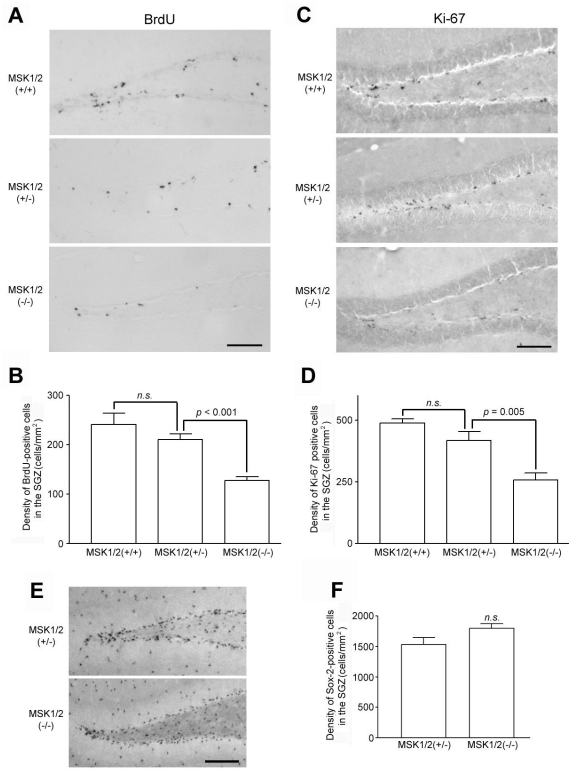

Figure 2. Decreased progenitor cell proliferation in MSK1/2 knockout mice

(A) Representative images of BrdU incorporation in MSK1/2 WT (MSK1/2 (+/+)), $MSK1^{(-/+)}/2^{(-/+)}$ (MSK1/2 (−/+)) and $MSK1^{(-/-)}/2^{(-/-)}$ (MSK1/2 (−/−)) mice. Mice were killed 3 hrs after the second of two BrdU (100 mg/kg) injections. Representative images reveal a reduced number of BrdU-labeled cells in $MSK1^{(-/-)}/2^{(-/-)}$ mice, relative to WT and $MSK1^{(-/+)}/2^{(-/+)}$ littermates. Scale bar = 100 μm. (B) Quantitative analysis of SGZ BrdU-labeling in the three genotypes shows that $MSK1^{(-/-)}/2^{(-/-)}$ mice have a reduced number of proliferating progenitor cells. There was no significant difference between WT and $MSK1^{(-/+)}/2^{(-/+)}$ mice. (C) Representative images of Ki-67 expression, a marker of proliferating progenitor cells. Similar to BrdU, the number of Ki-67-labeled cell was reduced in $MSK1^{(-/-)}/2^{(-/-)}$ mice. Scale bar = 100 μm. (D) Quantitative analysis of Ki-67-labeled cells in the SGZ. (E) Representative images of SOX-2 (a marker of both proliferating and quiescent progenitor cells) expression are shown. Scale bar = 100 μm. (F) Quantitative analysis reveals of the SGZ revealed that the number of SOX-2-labeled cells was not significantly different across the genotypes. For this study, only SOX-2-positive cells in the subgranular zone were counted. For all three markers, 6 mice from each genotype were used and data are presented as mean ± SEM.

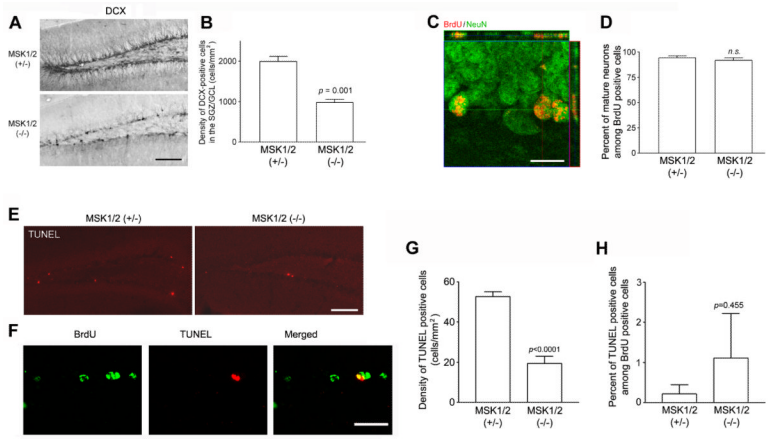

Figure 3. MSK1/2: neuronal survival and maturation

(A) Representative image of doublecortin (DCX) expression from MSK1$^{(-/+)}$/2$^{(-/+)}$ and MSK1$^{(-/-)}$/2$^{(-/-)}$ mice. Scale bar = 100 μm. (B) Quantitative analysis shows that the number of doublecortin-labeled cells was significantly reduced in MSK1$^{(-/-)}$/2$^{(-/-)}$ mice compared to MSK1$^{(-/+)}$/2$^{(-/+)}$ mice. (C) Representative image of double-labeling for BrdU (red) and NeuN (green) at 4 weeks after BrdU injection. Scale bar = 20 μm. (D) The percentage of BrdU/NeuN double-labeled cells was not significantly different between the two genotypes. A total 165 BrdU-labeled cells from MSK1$^{(-/+)}$/2$^{(-/+)}$ (n=6) and 177 cells from MSK1$^{(-/-)}$/2$^{(-/-)}$ (n=6) mice were examined. (E) To examine newborn cell survival, hippocampal sections were processed using TUNEL labeling. Representative images from MSK1$^{(-/+)}$/2$^{(-/+)}$ and MSK1$^{(-/-)}$/2$^{(-/-)}$ mice are shown. Scale bar = 100 μm. (F) Mice were perfused 10 days after BrdU injection and double-labeled for BrdU and TUNEL. Scale bar = 50 μm. GCL: granule cell layer, SGZ: subgranular zone, Hil: hilus. (G) Quantitative analysis of cell density revealed that the number of TUNEL-labeled cells was significantly increased in MSK1$^{(-/+)}$/2$^{(-/+)}$ mice compared to MSK1$^{(-/-)}$/2$^{(-/-)}$ mice. (H) However, when analyzed as a percentage of the BrdU positive cells there was no significant difference in cell death (TUNEL-positive) between the two genotypes. For each histogram shown here, data were collected from 6 MSK1$^{(-/+)}$/2$^{(-/+)}$ and 6 MSK1$^{(-/-)}$/2$^{(-/-)}$ mice and the results are reported as the mean ± SEM.

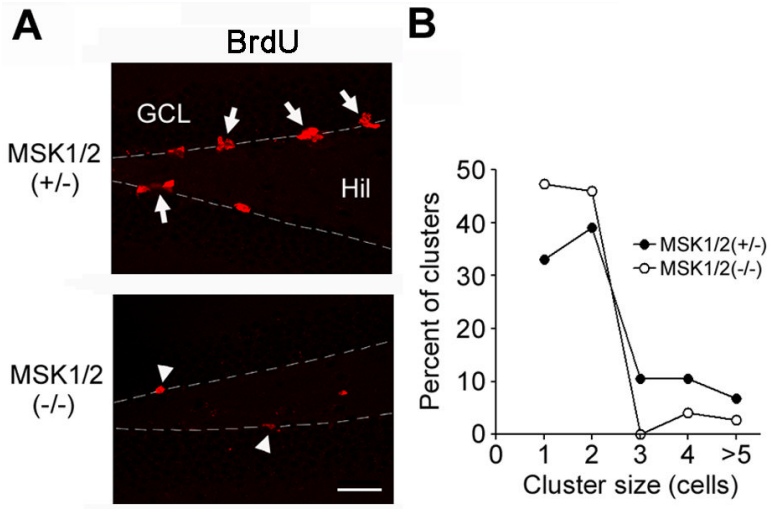

Figure 4. MSK1/2 and proliferating cell cluster size

(A) To monitor progenitor cell proliferation, mice received two injections of BrdU (100 mg/kg, i.p., 3 hr interval) and were perfused 2 hrs after the last BrdU injection. Representative BrdU labeling is shown for MSK1$^{(-/+)}$/2$^{(-/+)}$ and MSK1$^{(-/-)}$/2$^{(-/-)}$ mice. Arrows denote cell clusters; arrowheads denote single BrdU-positive cells. Hil: hilus, GCL: granule cell layer. Dashed lines approximate the SGZ. Scale bar = 50 μm. (B) Quantitation of BrdU-positive cell clusters. Relative to MSK1$^{(-/+)}$/2$^{(-/+)}$ mice, MSK1$^{(-/-)}$/2$^{(-/-)}$ mice exhibited a reduced percentage of multi-cell (≥ 3) clusters. N = 6 for each genotype.

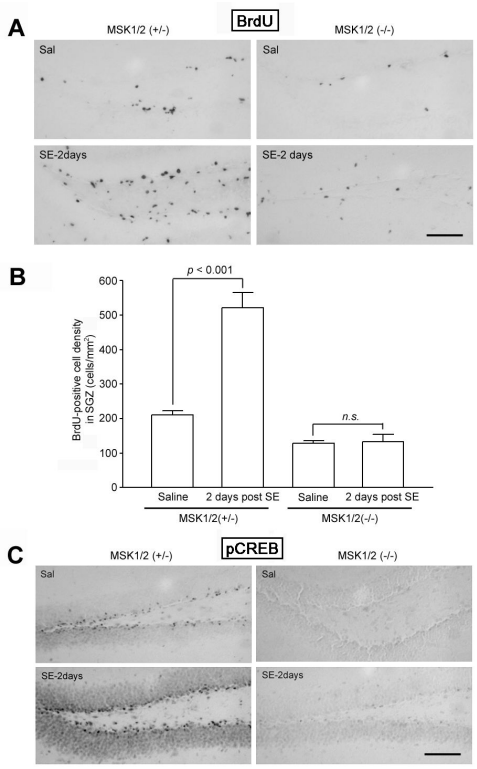

Figure 5. MSK1/2 couples seizure activity to progenitor cell proliferation and CREB activation

(A) To elicit seizure activity, mice were injected with pilocarpine (325 mg/kg). Two days later, mice were injected with BrdU (100 mg/kg) 3 and 6 hr before sacrifice. Representative image shows BrdU labeling in the SGZ. Control animals were injected with saline (sal) instead of pilocarpine. Representative images reveal that seizure activity increased cell proliferation in $MSK1^{(-/+)}/2^{(-/+)}$, but not in $MSK1^{(-/-)}/2^{(-/-)}$ mice. Scale bar = 100 μm. (B) Quantitative analysis of basal and seizure-induced BrdU density in the SGZ. Data are represented as mean ± SEM for six mice for each group. **(**C**)** Immunolabeling for the Ser-133 phosphorylation of CREB (pCREB) 2 days post-pilocarpine injection. Relative to control mice (Sal), seizure activity triggered a robust increase in pCREB in $MSK1^{(-/+)}/2^{(-/+)}$ mice. However under both basal conditions and following seizure activity, marked pCREB expression was not detected in $MSK1^{(-/-)}/2^{(-/-)}$ mice. Scale bar = 100 μm.

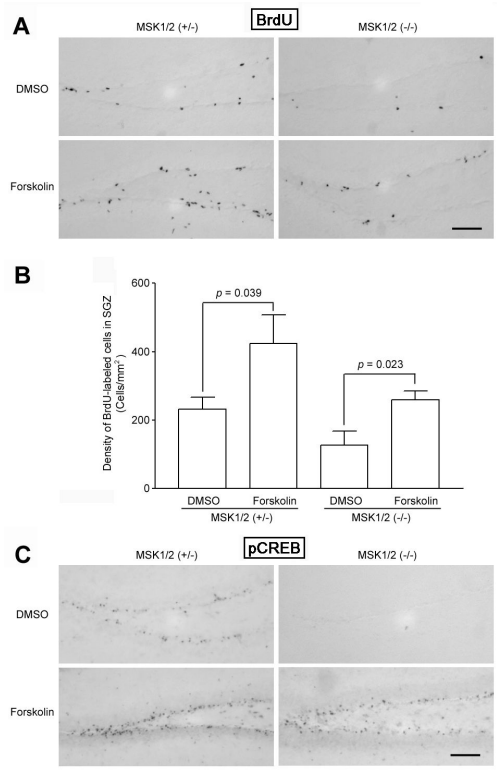

Figure 6. Elevating cAMP ameliorates the cell proliferation and pCREB phenotypes in MSK1/2 null mice

(A) Mice were microinfused with either drug vehicle (DMSO) or forskolin (1 μL, 1 mM) and mitotic activity was assayed via BrdU injection. Please refer to the Methods section for a detailed description of the infusion/injection protocol. Representative images of BrdU labeling in the SGZ. Note that forskolin increased cell proliferation in both $MSK1^{(-/+)}/2^{(-/+)}$ and $MSK1^{(-/-)}/2^{(-/-)}$ mice. Scale bar = 100 μm. (B) Quantitative analysis of BrdU labeling. Although forskolin-induced progenitor cell proliferation was higher in $MSK1^{(-/+)}/2^{(-/+)}$ mice, $MSK1^{(-/-)}/2^{(-/-)}$ mice also showed a significant increase in progenitor cell proliferation relative to vehicle-infused $MSK1^{(-/-)}/2^{(-/-)}$ mice. For this study, 5-7 mice were used for each group and data are represented as mean ± SEM. (**C**) pCREB immunoreactivity was examined in the dentate gyrus 1 hr after microinfusion of forskolin. Relative to vehicle-infused $MSK1^{(-/-)}/2^{(-/-)}$ mice, forskolin induced a marked increase in pCREB expression within the SGZ. Scale bar = 100 μm.

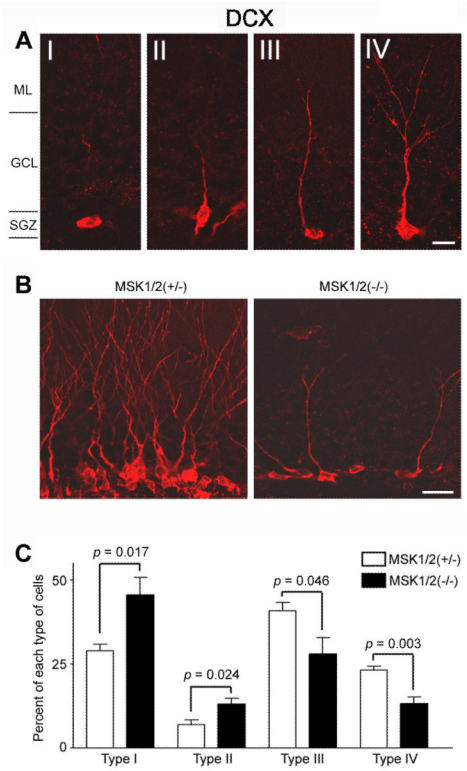

Figure 7. MSK1/2 and morphological maturation

(A) Representative images of DCX-positive cells for each morphological group. Cells which have short (less than the width of the cell body), plump processes or no processes are defined as type I; cells that have longer process which do not reach molecular layer are defined as type II; cells that have processes reaching the molecular layer, but do not have well developed branches are defined as type III and cells that have well developed branches within the molecular layer are defined as type IV. Scale bar = 20 μm. ML: molecular layer, GCL: granule cell layer, SGZ: subgranular zone. (B) Representative images of DCX-positive cells from $MSK1^{(-/+)}/2^{(-/+)}$ and $MSK1^{(-/-)}/2^{(-/-)}$ mice. Note the limited number of cells and poorly developed DCX-positive processes in the $MSK1^{(-/-)}/2^{(-/-)}$ line. Scale bar = 50 μm. (C) The percentage of each type of DCX-positive cells is shown. $MSK1^{(-/-)}/2^{(-/-)}$ mice have a higher population of type III-IV cells compared to $MSK1^{(-/-)}/2^{(-/-)}$ mice. Over 100 DCX-expressing cells were examined from each animal and 5 mice were used for each group. Data are represented as mean ± SEM.