Odorant-Induced Activation of Extracellular Signal-Regulated Kinase/Mitogen-Activated Protein Kinase in the Olfactory Bulb Promotes Survival of Newly Formed Granule Cells

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Extracellular signal-regulated kinase 1/2 (Erk1/2)/mitogen-activated protein (MAP) kinase (MAPK) plays a significant role in neuronal survival, including odorant-induced, activity-dependent survival of olfactory sensory neurons in the main olfactory epithelium. Here, we examined the role of MAPK for the survival of neurons in the olfactory bulb. To study odorant-induced activation of MAPK in the olfactory bulb, mice were exposed to odorants *in vivo*, and MAPK was assayed. Exposure of mice to some odorants *in vivo* activated MAPK in granule cells 10 min after exposure. Activation of MAPK was particularly evident in the nucleus and dendrites of granule cells. Because MAPK activation can augment neuronal survival, odorant enhancement of granule cell survival was monitored by bromodeoxyuridine (BrdU) incorporation. Long-term exposure to odorants increased the survival of newly formed granule cells as well as the number of granule cells that were both BrdU⁺ and phospho-Erk⁺. Inhibition of MAPK by administration of SL327 *in vivo* blocked the odorant-induced increase in newly formed granule cells, suggesting that activation of MAPK promotes the survival of granule cells in the olfactory bulb. Studies using cultured granule cells confirmed that activation of MAPK in granule cells protects them against strong apoptotic signals. These data suggest that stimulation of MAPK in olfactory bulb granule cells by some odorants may contribute to the survival of newly formed granule cells caused by odorant exposure.

Key words: Erk1/2; olfactory bulb; granule cell; BrdU; survival; odorants

Introduction

Mitogen-activated protein (MAP) kinase (MAPK) is a member of the MAPK family, enzymes that transduce changes in the environment into cellular responses. In neurons, neurotrophic factors and depolarization-induced Ca²⁺ influx activate signal transduction pathways that converge on MAPK kinase (MEK) (Xia et al., 1996; Hetman et al., 1999). Activated MEK, in turn, activates MAPK by phosphorylating threonine and tyrosine residues on residues 202 and 204 of human MAPK. Activated MAPK translocates into nuclei, leading to *de novo* gene induction. As a result of these molecular events, MAPK is believed to play an important role in neurotrophin-mediated survival and activity-dependent synaptic plasticity (for review, see Segal and Greengard, 1996; Grewal et al., 1999; Impey et al., 1999). Recently, we discovered that the MAPK pathway mediates odorant-induced survival of olfactory sensory neurons in the main olfactory epithelium (MOE) (Watt and Storm, 2001; Watt et al., 2004).

The olfactory bulb (OB) is a laminated structure that functions as a relay station in the olfactory pathway and for the inte-

gration of olfactory signals. Olfactory information generated in the MOE reaches glomeruli in the OB, where olfactory information is relayed to dendrites of mitral/tufted cells, the principal neurons in the OB. Activated mitral/tufted cells transduce olfactory information to the higher cortical regions of the brain through axons. In addition to mitral/tufted cells, there are several types of interneurons in the OB: granule cells, periglomerular cells, and short-axon cells. These interneurons modify the principal olfactory pathway by interacting with mitral/tufted cells laterally (for review, see Shepherd, 1972; Mori et al., 1999; Reed, 2003).

Several studies suggest that long-lasting, odorant-dependent activity in the OB may modulate the survival of granule cells and may improve olfactory learning (Najbauer and Leon, 1995; Petreanu and Alvarez-Buylla, 2002; Rochefort et al., 2002). Immunohistochemical studies have also revealed that the MAPK is expressed in granule cells (Flood et al., 1998), suggesting the possibility that MAPK may contribute to the survival of granule cells and/or activity-dependent plasticity. However, there are several important unanswered questions. Do odorants actually activate MAPK in the OB and, if they do, in which types of cells is MAPK activated? Does MAPK activation play a role in the survival of odorant-activated cells in the OB *in vivo*?

To address these questions, we examined the activation of MAPK in the OB after odorant stimulation *in vivo*. We discov-

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ered that some odorants activate MAPK in granule cells in the OB. Colocalization of phospho-extracellular signal-regulated kinase (pErk) and 5-bromo-2'-deoxyuridine (BrdU) labeling in granule cells suggests that activation of MAPK promotes the survival of granule cells during long-lasting odorant exposure. This was confirmed by demonstrating that the odorant-induced survival of newly formed granule cells in the OB is blocked by inhibition of MAPK activity *in vivo*.

Materials and Methods

Antibodies. Anti-pErk/MAPK polyclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-pan-Erk antibody and anti-Bcl-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MAP2 polyclonal antibody, anti-glia fibrillary acidic protein (GFAP) polyclonal antibody, and anti-S100 β monoclonal antibody were purchased from Sigma (St. Louis, MO). Anti-glutamate (Glu) decarboxylase 67 (GAD67) monoclonal antibody was purchased from Chemicon (Temecula, CA). Anti-bromodeoxyuridine monoclonal antibody was purchased from Roche Diagnostics (Indianapolis, IN).

Odorant treatments and subsequent Western blot analyses. Eight- to 12-week-old male C57BL/6 mice were individually housed and pre-handled for 4 d. On the test day, mice were exposed with a vapor from 100 μ M odorant [citralva, isoamyl acetate (IAA), heptanone (HEP), and ethyl vanillin] or odorless mineral oil through tubing to the cage. Mice were killed at the indicated times, and OBs as well as olfactory epithelia were dissected immediately, lysed, and sonicated in 4 $\times$ SDS sample buffers. A portion of the sonicated sample was electrophoresed and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore, Bedford, MA). After blocking with 10% skim milk in PBS, pH 7.4, the membrane was incubated with anti-pErk antibody or anti-Bcl-2 antibody at a dilution of 1:500 overnight at 4°C. After washing with PBS, the membrane was reacted with HRP-labeled secondary antibody. Immunoreactive proteins were visualized by a chemiluminescence reagent (ECL; Amersham Biosciences, Piscataway, NJ). For quantitative analyses of MAPK activation, pErk antibody on PVDF membrane was stripped off by soaking the membrane in a stripping buffer (100 mM glycine, pH 2.5, 1% SDS) for 30 min at 50°C. Blots were reprobed with anti-pan-Erk antibody at a dilution of 1:2 $\times$ 10⁴. Immunopositive bands were digitized and quantitated by Image Quant (Molecular Dynamics, Sunnyvale, CA), and the relative intensity of pErk-positive band to a pan-Erk-positive one was determined.

Odorant treatment and subsequent immunohistochemistry of olfactory bulb. After treatment with odorants (isoamyl acetate and heptanone) or mineral oil, OBs were dissected with a small portion of cortex to outline the rostrocaudal orientation when cutting. Bulbs were then immersed in 4% paraformaldehyde and then 0.1% glutaraldehyde in PBS for 24 h at 4°C. Then, OBs were immersed in 25% sucrose, embedded in OCT compound (Tissue-Tek, Miles, Elkhart, IN), cut into 8- μ m-thick section, and prepared as a series of three consecutive sections. One section from each series was used for immunostaining. Sections were rinsed in PBS and treated with 10% normal goat serum and rinsed with PBS and incubated with anti-pErk antibody, anti-GAD67 antibody, and anti-S100 β antibody at a dilution of 1:200, 1:200, and 1:5 $\times$ 10⁵, respectively. Then, sections were rinsed with PBS, treated with secondary antibody (Alexa 488 or 568; Molecular Probes, Eugene, OR). For labeling of nuclei, sections were incubated with 2.5 μ g/ml Hoechst 33342 at room temperature for 5 min. Images of sections were captured with a Bio-Rad (Hercules, CA) MRC600 confocal microscope (University of Washington Keck Imaging Center, Seattle, WA).

Long-lasting exposure to isoamyl acetate and BrdU labeling of granule cells in the olfactory bulb. Male mice housed individually were presented daily for 5 min with a cotton swab dipped in 100 μ M isoamyl acetate or odorless mineral oil (twice with a 5 min interval). In addition, mice were exposed daily for 15 h to microbaskets hanging from the edge of the cage that contained kimwipes (Kimberly-Clark, Neenah, WI) soaked either in isoamyl acetate or mineral oil. These treatments continued throughout the experiment (34 d). To inhibit MAPK activity *in vivo*, we used SL327, a selective MEK inhibitor (dissolved at 6 mg/ml in 20% DMSO) (Selcher

Figure 1. Some odorants stimulate MAPK activity *in vivo*. **A**, The vapor of odorants (CIT, citralva; EVA, ethyl vanillin) (100 μ M each) was delivered to mice as described in Materials and Methods. After 5 min, OBs and olfactory epithelia were dissected and subjected to Western blot analysis to quantitate the phosphorylation of MAPK. Erk was used as a loading control. Mineral oil (MIN) was used as a control. Lane B shows basal pErk in mice that were not exposed to MIN or odorants. The extent of MAPK activation was quantitated as the relative band intensity of pErk/Erk ($n = 5$). Error bars represent mean $\pm$ SD (* $p < 0.05$). **B**, The time course of MAPK activation was examined after exposure to IAA or MIN. OBs were taken at the indicated times after odorant exposure, and phosphorylation of MAPK (pErk) was quantitated as described above. Closed circles represent MAPK activity after exposure to IAA (mean $\pm$ SD; $n = 6$). Open circles represent MAPK activity after exposure to MIN (mean $\pm$ SD; $n = 3$). **C**, IAA exposure stimulates the expression of Bcl-2 in the OB and MOE. Eight hours after IAA or mineral oil (MIN) presentation, the MOE and OB were dissected and subjected to Western blot analysis to quantitate Bcl-2 expression. Erk was used as a loading control. The relative amounts of Bcl-2/Erk in the MOE and the OB were quantified (bottom). Error bars represent mean $\pm$ SEM ($n = 4$; * $p < 0.05$).

et al., 1999; Ohno et al., 2001). The inhibitory effect of SL327 persists for ≥ 3 h (Selcher et al., 1999). Therefore, we administered SL327 intraperitoneally (40 μ g/g) twice a week for 30 min before odorant presentation throughout the experiment. Seventeen days after the start of the exposure, mice were injected with BrdU (50 mg/kg, w/w) intraperitoneally (four times with a 2 h interval) to label newly generated precursors of granule cells. Seventeen days after injection, BrdU-injected mice were exposed to 100 μ M isoamyl acetate by the same method described above to examine colocalization of isoamyl acetate-dependent MAPK activation and BrdU incorporation. Then, OBs were dissected and fixed. After fixation of bulbs, coronal sections were prepared throughout the entire OB at 8 μ m thickness. Serial sections (every third; intervals of 24 μ m) were reacted with anti-BrdU antibody and pErk antibody. All sections were examined from the section at the rostral end to the caudal end, and all immunopositive cells in each section were counted. To prevent bias, this counting was done blind with the help of a second investigator who was unaware of the treatment of mice. The relative position of the coronal section in the whole tissue was determined by designating the coronal section that included the beginning of accessory olfactory bulb as 0.0 μ m.

The coronal sections situated rostrally to the beginning of accessory olfactory bulb were assigned negative numbers, and the sections situated caudally were assigned positive numbers.

Cell culture. Because development of the OB has been investigated primarily in postnatal rats (Shepherd, 1972; Currie and Dutton, 1980; Frosch and Dichter, 1984), rat OB neurons were cultured from postnatal day 3 (P3) to P4 rat pups. OBs were dissected and digested in 15 ml of 10 U/ml papain in dissociation medium (DMEM; Invitrogen, San Diego, CA) at 37°C for 30 min. After rinsing, the tissue was triturated in the media (Neurobasal-A; Invitrogen) with a 10 ml plastic pipette, and, if necessary, the cell suspension was successively filtered through a series of nylon mesh filter (250, 50, and 10 μ m; Small Parts, Florida, MI). The cells were plated at a density of 0.5×10^6 cells/cm² onto a plastic culture plate that was precoated with laminin (10 μ g/ml) and poly-D-lysine (50 μ g/ml). Cells were maintained in the Neurobasal-A medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and $1 \times B27$ (Invitrogen). While the culture conditions were examined, the indicated concentration of either fetal bovine serum (FBS) or glutamine was added to this culture medium. On the next day [1 d *in vitro* (DIV1)], the indicated concentrations of cytosine arabinoside (Ara-C; Invitrogen) were added.

Immunocytochemistry of cultured cells. Cultured cells were plated on coverslips precoated with laminin (10 μ g/ml) and poly-D-lysine (50 μ g/ml). Cultured cells were fixed in 4% paraformaldehyde for 10 min at room temperature. After permeabilizing the cells and blocking with 5% normal goat serum, cells were incubated with antibody at 4°C overnight. After incubation with secondary antibody, detection and capturing of immunopositive cells were done similarly as described above. To visualize nuclei morphology, cells were stained with 2.5 μ g/ml Hoechst 33342.

Agonist treatment of cultured cells. At DIV5, 1 h before stimulation, culture medium was replaced with prewarmed fresh medium without any supplements. Cultured cells were then treated with Glu or isoproterenol (ISO) at indicated concentrations (0 nM, 10 nM, 1 μ M, and 100 μ M for Glu; 0 μ M, 1 μ M, 10 μ M, and 100 μ M for ISO) for varying times (0, 5, 10, and 15 min). For Western blot analyses, reactions were stopped by the addition of boiling $4 \times$ SDS sample buffers. For immunocytochemistry, paraformaldehyde solution was added, and subsequent incubation with antibody was performed.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for evaluation of cell viability. To evaluate cell viability after camptothecin (CPT) treatment, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done as described previously (Hetman et al., 1999). Briefly, MTT was converted from the yellow, water-soluble tetrazolium to the blue, water-insoluble formazan by cellular mitochondrial dehydrogenases. Because the rate of this reaction is proportional to the number of living cells, the amount of blue formazan reflects cell viability. In this assay, cultured cells in 96-well plates were treated with various concentrations (1, 2, 5, and 10 μ M) of CPT at DIV3. After 24 or 48 h, 100 μ M MTT was added. Twenty-four hours after the addition of MTT, DMSO was added to dissolve formazan, and OD₅₆₀ was measured.

Transient transfections of culture cells. To examine the effect of MAPK on survival, granule cells were transiently transfected at DIV3 with vehicle (pcDNA3; Invitrogen), dominant-negative MEK [K97M MEK, carrying M substitution at K97 (DNMEK)] or constitutively active MEK [Δ N3, S218E/S222D MEK (CAMEK)] genes. The expression plasmids were generous gifts from N. G. Ahn (University of Colorado, Boulder, CO) (Mansour et al.,

Figure 2. Activation of MAPK in granule cells of the olfactory bulb when mice are exposed to isoamyl acetate. Ten minutes after exposure to isoamyl acetate, the OB was dissected and fixed. Serial sections ($\sim 8 \mu$ m thick) were treated with anti-pErk antibody or normal serum and stained with toluidine blue. Scale bar, 200 μ m. **B**, Higher magnification of the granule cell layer and mitral cell layer. Sections were treated with anti-pErk (red) and anti-GAD (green) antibodies, and images were merged (merged). Representative granule cells that are pErk+ and GAD+ are indicated by arrowheads. A pErk+ and GAD+ cell in the mitral cell layer is also indicated by an arrow. Scale bar, 20 μ m. **C**, Representative pErk+ granule cells and S100 β + glial cells in the granule cell layer. Sections were treated with anti-pErk (red) and anti-S100 β (green) antibodies, and images were merged (merged). Glial cells that are S100 β + are indicated by arrowheads. Some pErk+ cells in the mitral cell layer are also indicated by arrows. Scale bar, 20 μ m. EPL, External plexiform layer; G, granule cell layer; GL, glomerular layer; M, mitral cell layer; IPL, internal plexiform layer.

1994). An expression construct for β -galactosidase was used to normalize transfections. Transfections were performed using a calcium-phosphate coprecipitation protocol as described previously (Hetman et al., 1999). Briefly, cells were plated onto a 12-well plate and cultured as described above. At DIV3, the medium was replaced with 600 μ l/well Neurobasal-A containing 60 μ l of varying amounts (3 and 5 μ g) of DNA/calcium phosphate. After incubation for 1 h, cells were rinsed and incubated again with presaved-conditioned medium, initiating expression of transgenes. On the next day, 10 μ M CPT was added. Twenty-four hours after CPT treatment, CPT-dependent apoptosis of cultured cells was examined by nuclear morphological change (see below).

Quantification of apoptosis by nuclear morphological change. For analyses of effects of transfected genes on CPT-induced cell death, changes of nuclear morphology in cultured cells were examined. In this experiment, at DIV3, cells plated onto a coverslip in 12-well plates were transiently transfected with expression vectors described above. Twenty-four hours after transfection, cells were treated with 10 μ M CPT. After 24 h, cells were fixed and stained with Hoechst 33342. Uniformly stained nuclei were considered healthy, whereas condensed or fragmented nuclei were scored as apoptotic. To evaluate the extent of apoptosis, the number of apoptotic cells was counted and scored as a percentage of apoptotic cells to total transfected cells. To prevent bias counting, the slides were coded to enable blind counting.

Results

Exposure of mice to some odorants *in vivo* stimulates MAPK activity in olfactory bulbs

To examine odorant-dependent activation of MAPK in OBs and olfactory epithelia, several odorants (citralva, isoamyl acetate, heptanone, and ethyl vanillin) were delivered *in vivo*, and MAPK activation was examined by Western blot analysis. After a 5 min

Figure 3. Odorant-induced MAPK activation in granule cells. **A**, Ten minutes after odorants were presented (IAA and HEP), OBs were dissected, and sections were prepared. Odorless mineral oil (MIN) was used as a control. Scale bar, 20 μ m. Insets, Toluidine blue staining of serial sections. Scale bar, 200 μ m. **B**, Segregation of pErk+ granule cells was seen in one area (activated) and not in the adjacent area (silent). Scale bar, 200 μ m. **C**, Spatial pattern of pErk+ cells throughout the OB. The position of the coronal section in the whole tissue was determined by designating the coronal section that included the beginning of accessory olfactory bulb as 0 μ m. The spatial patterns of pErk+ granule cells throughout the OB were illustrated from six sections situated approximately at the indicated levels (–520, –430, –310, –210, –120, and 0 μ m). The contour in each section represents mitral cell layers, and the dots represent pErk+ granule cells. The hatched area at 0 μ m represents the beginning of AOB. Scale bar, 800 μ m. **D**, Higher magnification of representative granule and mitral cell layers. Some pErk+ dendrites of granule cells extended into the mitral cell layer (arrowheads). A pErk+ cell in the mitral cell layer is also indicated (arrow). Scale bar, 20 μ m. **E**, Higher magnification of representative granule cell layers. Sections were treated with anti-pErk antibody (red), and nuclei staining was done with Hoechst 33342 (blue). The subcellular localization of pErk was seen in nuclei and in the dendrites of some granule cells (arrowhead). Scale bar, 20 μ m. EPL, External plexiform layer; G, granule cell layer; Gl, glomerular layer; M, mitral cell layer.

exposure, MAPK activity was increased by citralva (~300% of basal level) and isoamyl acetate (~200%) but was unchanged by heptanone and ethyl vanillin (Fig. 1A). IAA was used in subsequent studies for comparison with other studies (Guthrie et al., 1993) and because the purity of IAA was greater than citralva. Furthermore, the SDs for IAA activation of MAPK activity were superior to citralva. This indicates that exposure of mice to some, but not all, odorants activates MAPK in the OB. Either heptanone and ethyl vanillin evoke signaling pathways in the OB distinct from isoamyl acetate and citralva (Lin et al., 2004), or activation of MAPK is below the limits of detection of the assay.

Odorant activation of MAPK in the OB was similar to the profile seen in the MOE, suggesting that activation of MAPK in the OB may be related to signal intensity in the MOE (Fig. 1A). The peak of MAPK activation in the OB was 10–15 min after exposure of animals to the odorant and declined to baseline thereafter (Fig. 1B). Interestingly, maximal activation of MAPK in the hippocampus after contextual fear conditioning also occurs 5–10 min after treatment (J. Athos and D. R. Storm, unpublished observations). Furthermore, glutamate stimulation of MAPK in cultured cortical neurons reaches a maximum at ~10 min (Hetman et al., 1999).

MAPK is thought to contribute to the survival of neurons (Xia et al., 1995) through activation of cAMP response element (CRE)-binding protein (CREB)-mediated transcription and increased expression of Bcl-2, a CRE-regulated gene product (Bonni et al., 1999; Riccio et al., 1999; Mabuchi et al., 2001; Watt et al., 2004). To test whether exposure of odorant increases Bcl-2 expression in the OB, we exposed mice to IAA *in vivo*. Eight hours after odorant presentation, expression of Bcl-2 in the OB as well as MOE was increased, suggesting that odorant-induced increases in Bcl-2 expression may contribute to the survival of OB neurons.

Odorant-dependent activation of MAPK in granule cells of the olfactory bulb

To determine in which cells MAPK is activated, OBs were dissected 10 min after exposure of IAA to the mice, and the distribution of pErk, the activated form of MAPK, was examined immunohistochemically. Although the pErk signal was evident in several areas of the OB, including the mitral cell layer and internal plexiform layer, the majority of pErk+ cells were within the granule cell layer (Fig. 2A). Within the granule cell layer, pErk+ cells were also GAD+ and S100 β –, indicating that it was expressed primarily in granule cells and not glial cells (Fig. 2B, C). On the basis of morphology, the pErk signal in the mitral cell layer may be displaced granule cells (Figs. 2B, 3C, arrow). However, pErk+ cells were absent from the glomerular layer. This distribution of pErk is in good agreement with a previous report showing that Erk protein is abundant in granule cells and less in mitral cells and the glomerular layer (Flood et al., 1998).

To characterize odorant-induced MAPK activation in granule cells, IAA and HEP were delivered *in vivo*, and activation of MAPK was examined immunohistochemically 10 min after odorant exposure. The number of pErk+ granule cells was increased by exposure of the animals to IAA but was unchanged by heptanone (Fig. 3A), consistent with the Western blot analysis as shown in Figure 1. Interestingly, the pErk signal was not uniformly distributed throughout the granule cell layer, and there were zones of activation and neighboring “silent zones,” suggesting that exposure of mice to IAA activates subsets of cells (Fig. 3B). To explore the spatial pattern of IAA-activated pErk+ granule cells in OB, the location of pErk+ granule cells was reconstructed using six sections from adjacent layers of the OB (Fig. 3C). Immunopositive granule cells were localized mostly in the dorsal area of the entire OB and the ventral area in the caudal part of the OB. This spatial pattern is similar to that reported for IAA stimulation of *c-fos* expression in the OB (Guthrie et al., 1993). These data suggest that IAA activates a subset of granule cells located at stereotypical positions in the OB.

Higher magnification revealed that most pErk-immunopositive cells overlapped with nuclei staining, and some staining for

pErk was apparently localized in the dendrites of granule cells (Fig. 3D). This suggests that MAPK is activated not only in nuclei but also in dendrites. Some dendritic staining for pErk was present in the mitral cell and external plexiform layers (Fig. 3D, arrowheads). To estimate the proportion of MAPK-activated granule cells, the ratio of pErk-positive cells to all Hoechst+ granule cells was calculated (Fig. 3E). Because the cell body of granule cells is $\sim 10 \mu\text{m}$ and the sections prepared were $8 \mu\text{m}$ thick, overlapping staining of pErk and Hoechst reflects staining in the same cells. In the “activated zone” of the granule cell layer, the number of pErk+ granule cells was $\sim 12\%$ of the total, whereas in the silent zone, it was $<1\%$. This indicates that IAA induces MAPK activation mostly in subzones of the granule cell layer, although the percentage of activated granule cells is relatively small.

Long-lasting odorant exposure increases the survival of newly formed granule cells

MAPK is believed to be important for neuronal survival (Meyer-Franke et al., 1995; Xia et al., 1995; Friedman and Greene, 1999; Hetman et al., 1999) and protection against injury-dependent neuronal cell death (Han and Holtzman, 2000; Kuroki et al., 2001; Wang et al., 2003). Therefore, odorant-induced activation of MAPK in the OB raised the interesting possibility that this enzyme may play a role in survival of granule cells in the OB. Granule cells are known to originate continuously in the subventricular zone of the lateral ventricle as immature neurons and to migrate through a rostral migratory stream for 1 week to reach the OB (Altman, 1969; Kaplan and Hinds, 1977; Kishi, 1987; Luskin, 1993). Recent studies indicate that long-lasting odorant exposure increases the number of granule cells, not by increasing cell proliferation but by supporting survival of newly generated cells (Rocheffort et al., 2002). Consequently, we tested whether long-lasting exposure of mice to IAA increases the survival of newly formed granule cells and, if so, whether this survival correlates with MAPK activation.

After a preconditioning period (17 d of exposure to IAA), BrdU was injected intraperitoneally to label newly generated cells, and mice were exposed daily to IAA for another 17 d. Before dissection of OBs on the last day, both pre-exposed and control mice were exposed to IAA for 10 min to stimulate MAPK in granule cells. In this experiment, the number of BrdU-incorporated granule cells (BrdU+) was proportional to the extent of granule cell survival. We examined the OB 17 d after administration of BrdU, because the maximum number of newly formed granule cells falls within this time scale (Petreanu and Alvarez-Buylla, 2002). If activation of MAPK contributes to the survival of granule cells, odorant exposure should increase the number of granule cells that are both BrdU+ and pErk+. The number of BrdU+ granule cells was significantly greater in mice pre-exposed to IAA compared with control mice (Fig. 4A, arrowheads). In contrast, exposure of mice to heptanone did not in-

Figure 4. Long-term exposure to isoamyl acetate increases the number of BrdU+ granule cells and the number of cells that are BrdU+ and pErk+. **A**, After 17 d of pre-exposure to IAA, coronal sections were double labeled with anti-BrdU (BrdU) and pErk (pErk) antibodies, and a serial section was stained with toluidine blue. Representatives of BrdU+ cells are marked (arrowhead). Scale bar, $200 \mu\text{m}$. Control mice of mice were exposed to a cotton swab without odorant. **B**, The number of BrdU+ and pErk+ cells in each section was counted throughout the OB (left panel in each row). The section in which the accessory olfactory bulb starts was used for a landmark of each bulb and designated $0 \mu\text{m}$. The rostral regions are shown as negative numbers, and the caudal regions are shown as positive numbers. The mean relative number of BrdU+ cells was calculated in pre-exposed (Pre-exp) and control mice (Cont) (right). Each bar represents mean $\pm$ SEM (pre-exposed, $n = 4$; control, $n = 3$; $*p < 0.05$). **C**, Higher magnification of two representative regions in the granule cell layer stained with anti-BrdU (BrdU; green) and anti-pErk (pErk; red) antibodies and then merged (merged). Top, A representative BrdU+, pErk+ granule cell is indicated by an arrowhead. Bottom, A representative BrdU+ granule cell that is located just next to pErk+ granule cell is indicated by an arrow. Scale bar, $20 \mu\text{m}$. **D**, The number of granule cells that were double stained by anti-BrdU and anti-pErk antibodies in each section was counted throughout the OB (overlap; left). The mean relative number of BrdU+ cells in each case was calculated in pre-exposed (Pre-exp) and control (Cont) mice (right). Each bar represents mean $\pm$ SEM (pre-exposed, $n = 4$; control, $n = 3$; $*p < 0.05$).

crease survival of granule cells. Despite considerable fluctuation throughout sections, the number of BrdU+ cells was greater in the OB of mice that had been pre-exposed to IAA than that of controls (Fig. 4B, left BrdU). The mean number of BrdU+ cells from mice pre-exposed to IAA was twice that of control mice ($n = 4$) (Fig. 4B, right BrdU). The number of pErk+ granule cells was similar in pre-exposed and control mice, because we activated pErk right before the tissue was collected (Fig. 4B, left and right pErk). Higher magnification revealed the presence of granule cells that were both BrdU+ and pErk+ (Fig. 4C, top column, arrowhead). The number of these double-labeled cells in mice that had been pre-exposed to IAA for 17 d was approximately fourfold greater than that for control mice, which were not exposed to IAA (Fig. 4D).

To determine whether MAPK activation is required for odorant-induced survival of newly formed granule cells *in vivo*, we administered a selective MEK inhibitor (SL327) by intraperitoneal injection (Selcher et al., 1999; Ohno et al., 2001). Administration of SL327 before daily odorant presentation reduced the number of BrdU+ granule cells compared with DMSO-injected mice, both sets of which were pretreated with IAA ($n = 4$) (Fig. 5). These data suggest that activation of MAPK may contribute to the odorant-induced increase in the survival of newly generated granule cells.

Figure 5. Inhibition of MAPK *in vivo* blocked odorant-induced increases in the survival of newly formed granule cells. Mice were pre-exposed to IAA for 17 d as described in Material and Methods, except that experimental mice received intraperitoneal administration of SL327 (40 μ g/g; dissolved in 20% DMSO; twice each week) or 20% DMSO as a vehicle control. Control mice were not exposed to an odorant but, rather, were exposed to a cotton swab without odorant. After odorant exposure, the OBs were dissected, sectioned, and stained with anti-BrdU antibody. **A**, The number of BrdU+ granule cells in each section was calculated in nonexposed mice (control), mice that been exposed to IAA for 17 d (pre-exposed), pre-exposed mice injected either with 20% DMSO (DMSO) or SL327 (SL327), an MEK inhibitor. **B**, The mean relative number of BrdU+ cells in control (cont), pre-exposed (pre-exp), pre-exposed mice with DMSO injection (DMSO), or pre-exposed mice that received SL327. Error bars represent mean $\pm$ SEM (cont, $n = 3$; pre-exp, $n = 4$; DMSO, $n = 4$; SL327, $n = 4$; * $p < 0.05$).

MAPK activity in cultured granule cells

To understand the effect of MAPK on the survival of granule cells more directly, we cultured granule cells *in vitro* and examined the effect of MAPK activation on cell survival. There are several published methods for culturing OB neurons (Currie and Dutton, 1980; Trombley and Westbrook, 1990; Carlson et al., 1997; Puche and Shipley, 1999; Osako et al., 2000; Muramoto et al., 2001), which lead to differences in cellular organization and morphology of cultured cells. Furthermore, because these cultured cells were generally used for single-cell activity in electrophysiological and/or optical recordings, the biochemical responses of granule cells in culture remain unknown. Therefore, it was necessary to establish a culture system in which granule cells are enriched.

To increase the population of neurons relative to glia, we used serum-free Neurobasal-A medium with $1 \times$ B27 (Brewer et al., 1993), glutamine (0.5 mM), and Ara-C (2 μ M) added on the day after dissociation. To identify cell types in culture at DIV4, MAP2 and GFAP were used as neuronal and glial markers, respectively. Addition of FBS increased the number of GFAP+ cells, with little or no effect on the number of MAP2+ cells (Fig. 6A). Increasing the concentration of Ara-C inhibited the survival of MAP2+ cells with little effect on GFAP+ cells (Fig. 6A). Removal of 0.5 mM glutamine decreased MAP2+ cells and did not affect GFAP+ cells (Fig. 6A). From these results, the supplemental component of the culture medium was serum-free, $1 \times$ B27, 2 μ M Ara-C, and 0.5 mM glutamine.

To enrich granule cells among cultured neurons, we tried to separate granule cells from other components by size. The mean soma size of granule cells is relatively small ($\sim 10 \mu$ m) compared with mitral/tufted cells (15–50 μ m). Because almost all granule cells and some periglomerular cells contain GABA (Ribak et al., 1981), we used GAD67, a synthesizing enzyme of GABA, as a marker for granule cells. Consecutive filtration increased the percentage of cells that were both GAD+ and MAP2+ to 75% (Fig. 6A). Among the GAD– cells, the majority of the cell bodies were $>20 \mu$ m. Although there may be some periglomerular cells in

these cultures, we estimated that $>70\%$ of the neurons are granule cells.

Olfactory signaling is relayed to mitral/tufted cells in the OB, which release glutamate on the dendrites of granule cells at the dendrodendritic synapse. In addition to this afferent input, β -adrenergic efferent input from the pontine nucleus has been reported previously (Shipley et al., 1985; McLean et al., 1989; McLean and Shipley, 1991). To examine the effect of these inputs on activation of Erk/MAPK in granule cells, cultured granule cells were incubated with glutamate or isoproterenol, a β -adrenergic agonist. Both ligands activated Erk/MAPK in nuclei and dendrites (Fig. 6C). Maximal activation of Erk/MAPK was 10 min after addition of agonists, a kinetic response similar to that seen for odorant activation of Erk/MAPK in the OB *in vivo* (Fig. 6D).

Activation of MAPK in cultured granule cells is neuroprotective

To directly determine whether activation of MAPK in granule cells is neuroprotective, we induced apoptosis in cultured cells

using CPT. CPT is an inhibitor of DNA topoisomerase 1, which induces DNA strand breaks, inducing neuronal apoptosis via cyclin-dependent kinase and p53 pathways (Morris et al., 2001). CPT-induced apoptosis has been used as a model system to study neuronal apoptosis (Morris and Geller, 1996). CPT treatment induced nuclei fragmentation, a morphological characteristic of apoptotic cells (Fig. 7A). To examine cell viability, cultured cells were subjected to the MTT assay after CPT treatment. CPT reduced MTT metabolism in a dose- and time-dependent manner (Fig. 7B), indicating that, like cortical neurons (Hetman et al., 1999), CPT causes cell death in granule cells.

To directly examine the effect of MAPK activation on the survival of cultured cells, the cells were transiently transfected with plasmids expressing DNMEK or CAMEK, an upstream activator of MAPK. Approximately 75% of granule cells exhibited an apoptotic phenotype (condensed or fragmented nuclei) 48 h after treatment with CPT (Fig. 7C). Inhibition of MAPK by DNMEK expression increased the percentage of apoptotic cells, even without CPT treatment (Fig. 7C). Moreover, constitutive activation of MAPK by CAMEK expression decreased apoptosis caused by CPT cells ($\sim 50\%$, 3 μ g of CAMEK transfection; $\sim 30\%$, 5 μ g) and without CPT treatment ($\sim 25\%$, 3 μ g; $\sim 20\%$, 5 μ g). These data indicate that activation of MAPK in cultured granule cells is neuroprotective against a strong apoptotic signal, but they do not imply that odorant-induced MAPK activation may alter DNA strand breaks. Most importantly, MAPK activity is required for odorant-induced increases in the survival of granule cells *in vivo* (Fig. 5).

Discussion

Although there is active neurogenesis in the OB of adult mice, only a small percentage of newly formed granule cells survive. Consequently, it is of interest to identify regulatory mechanisms that promote the survival of newly formed granule cells. Previously, we demonstrated that odorants activate MAPK in sensory neurons of the main olfactory epithelium, a process that leads to

Figure 6. Characterization of cultured granule cells from the olfactory bulb. **A**, OBs from pups (P3–P4) were dissociated and cultured using several different types of culture media, including filtered cultured as well as Ara-C, glutamine, FBS, and containing media. At DIV4, MAP2+ cells, GFAP+ cells, or GAD+ cells were counted. The number of counted cells was an average of three individually dissociated coverslips. In each coverslip, the count was an average of different areas per coverslip, in each area of which the score was the summation from nine neighboring fields. The bar graphs represent the mean percentage of MAP2+, GFAP+, or GAD+ cells relative to MAP+ cells under standard conditions (black bars, MAP2+; hatched bars, GFAP+ or GAD+). **B**, At DIV4, cultured cells were double stained with anti-MAP2 (red) and anti-GFAP antibody (green), and images were merged (left) to discriminate neurons from glia cells. In addition, cells were double treated with anti-MAP2 (red) and anti-GAD antibody (green), and images were merged (right). **C**, At DIV4, cells were incubated with medium alone (no add), glutamate (100 μ M), or ISO (100 μ M). After 10 min of incubation, cells were fixed and double treated with anti-pErk (red) and anti-GAD antibody (green). For examination of time dependency and dose dependency of Erk activity, cells were treated with varying concentrations of glutamate (0 nM, 10 nM, 1 μ M, and 100 μ M) or ISO (0, 1, 10, and 100 μ M), and samples were subjected to Western blot analyses. The relative MAPK activity was plotted as a function of time (mean $\pm$ SEM; $n = 6–9$).

Figure 7. MAPK protects cultured granule cells from the OB against CPT-induced apoptosis. **A**, Cells were transiently transfected with β -galactosidase genes at DIV3. On the next day, cells were treated in the absence (–CPT) or presence (+CPT) of 10 μ M CPT. After 48 h of treatment, cells were fixed and reacted with anti- β -galactosidase antibody (red) and stained with Hoechst 33342 (blue). Scale bar, 10 μ m. **B**, At DIV3, cultured granule cells were treated with varying concentration of CPT, and 24 or 48 h after incubation, cells were assayed by MTT metabolism. The relative cell viability was scored as a percentage of viability on DIV3 and plotted as a function of time. **C**, At DIV3, cells were transiently transfected with β -galactosidase expression vectors and vehicle (vehicle), DNMEK (3 or 5 μ g), or CAMEK (3 or 5 μ g). On the next day, cells were treated in the absence (black bars) or presence (hatched bars) of 10 μ M CPT. The percentage of cells that underwent apoptotic morphological changes relative to total transfected cells was counted as described above (* $p < 0.05$). Error bars represent mean $\pm$ SEM.

activity-dependent survival of neurons expressing receptors for the conditioning odorant (Watt et al., 2004). The general objectives of this study were to determine whether odorants stimulate MAPK in the OB and to ascertain whether MAPK contributes to odorant-induced survival of newly formed granule cells in the OB. To accomplish this goal, we examined activation of MAPK in OB neurons after odorant stimulation *in vivo*.

Several, but not all, odorants stimulated MAPK in the OB when mice were exposed to odorants *in vivo*. Immunohistochemical analysis of mice exposed to odorants indicated that odorant activation of MAPK is mainly in granule cells, in restricted zones of the granule cell layer. Long-term exposure to odorants *in vivo* increased the number of BrdU+ granule cells in the OB as well as the number of cells that were both BrdU+ and pErk+. MAPK inhibition by administration of SL327 antagonized this increase in the number of BrdU+ cells. In studies using cultured granule neurons from the OB, we demonstrated that activation of MAPK protects cultured granule cells from apoptosis. Collectively, these data support the notion that odorant activation of MAPK may protect newly formed granule cells in the OB.

Several studies have reported that activation of CREB-mediated transcription through stimulation of MAPK in the OB may be necessary for short-term (<60 min) olfactory-based learning (Yuan et al., 2003; Zhang et al., 2003). The cellular localization of MAPK activation and how this activation might contribute to olfactory learning has not been defined. Our data indicate that odorants activate MAPK in the nucleus and dendrites of granule cells, which is consistent with a previous report showing preferential localization of MAPK protein in granule cells (Flood

et al., 1998). Olfactory-based learning may depend, at least in part, on this MAPK activation in a selected population of granule cells in the OB.

Granule cells receive at least two inputs: glutamatergic input from the dendrites of mitral cells after odorant stimulation (Fuller and Price, 1988; Trombley and Shepherd, 1992) and noradrenergic input from the pontine locus ceruleus (LC) (Shipley et al., 1985; McLean et al., 1989; McLean and Shipley, 1991). In this respect, it is interesting that glutamate and isoproterenol both activated MAPK in cultured granule cells. This indicates that input from mitral cells or the LC has the potential to stimulate MAPK in granule cells of the OB. Although odorant information is relayed through the OB, granule cells are thought to contribute to lateral inhibition through reciprocal dendrodendritic synapses between mitral/tufted cell and granule cells (Mori and Takagi, 1978; Yokoi et al., 1995). This lateral inhibition plays a role in enhancing contrast in activities of neighboring mitral/tufted cells, contributing to odorant discrimination (Mori and Takagi, 1978; Yokoi et al., 1995). Therefore, the dendrites of granule cells may be an important site for odorant discrimination. MAPK activation in dendrites plays a role in changing the efficacy of the synapse in CA1 hippocampal neurons (Winder et al., 1999). Therefore, it is possible that MAPK activation in the dendrites of granule cells modulates the efficacy of reciprocal dendrodendritic synapse, participating in lateral inhibition and odorant discrimination.

Potential role of MAPK activation in newly generated granule cells

MAPK mediates trophic factor-supported neuronal survival (Meyer-Franke et al., 1995; Xia et al., 1995; Friedman and Greene, 1999) and protects against injury-dependent neuronal cell death (Han and Holtzman, 2000; Kuroki et al., 2001; Wang et al., 2003). Our data suggest that activation of MAPK correlates with the survival of newly generated granule cells, and its activation protects against apoptosis in cultured granule cells. Therefore, we hypothesize that odorant stimulation of MAPK in the OB during long-lasting odorant exposure may contribute to the survival of newly generated granule cells. Previous studies suggest that survival of newly generated granule cells are associated with improved olfactory memory (Rochefort et al., 2002). How could the increased survival of newly generated granule cells participate in olfactory function? This could be attributable to persistent enhancement of lateral inhibition and odorant discrimination, resulting from enhanced survival of newly generated granule cells.

Interestingly, physical activity and environmental enrichment increase the survival of newly generated granule cells in the dentate gyrus without affecting the total number of neurons and/or size of the tissue (Barnea and Nottebohm, 1994; Gould et al., 1999). Similarly, our data indicate that the numbers of pErk+ cells in pre-exposed and control mice are not different. New granule cells may be formed to replace dying cells without a net increase in granule cells. Instead of an increase in the number of granule cells, the change in strength of neural circuits derived from newly formed granule cells and mitral/tufted cells may increase during persistent enhancement of odorant discrimination. Several previous studies using naris-closed or anosmic mice showed that loss of olfactory input decreased the number of granule cells (Najbauer and Leon, 1995; Petreanu and Alvarez-Buylla, 2002). However, this does mean that odorant exposure should increase the number of granule cells. Indeed, our experiments indicate that odorants increase the survival of newly formed granule cells without increasing the total number of granule cells.

In conclusion, we discovered that exposure to some odorants activates MAPK in granule cells of the OB and stimulates increased expression of Bcl-2. This suggests that stimulation of MAPK by odorant exposure may increase the survival of subpopulations of newly formed granule cells in the OB.

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