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Phosphoinositide and Erk signaling pathways mediate activity-driven rodent olfactory sensory neuronal survival and stress mitigation

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Abstract

Olfactory sensory neurons (OSNs) are the initial site for olfactory signal transduction. Therefore, their survival is essential to olfactory function. In the current study, we demonstrated that while odorant stimulation promoted rodent OSN survival, it induced generation of reactive oxygen species in a dose- and time-dependent manner as well as loss of membrane potential and fragmentation of mitochondria. The MEK-Erk pathway played a critical role in mediating these events, as its inhibition decreased odorant stimulation-dependent OSN survival and exacerbated intracellular stress measured by reactive oxygen species generation and heat shock protein 70 (Hsp70) expression. The phosphoinositide pathway, rather than the cyclic AMP pathway, mediated the odorant-induced activation of the MEK-Erk pathway. These findings provide important insights into the mechanisms of activity-driven OSN survival, the role of the phosphoinositide pathway in odorant signaling, and demonstrate that odorant detection and odorant stimulation-mediated survival proceed *via* independent signaling pathways. This mechanism, which permits independent regulation of odorant detection from survival signaling, may be advantageous if not diminished by repeated or prolonged odor exposure.

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Keywords

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Introduction

The survival of sensory neurons is essential for sensory functions. Degeneration of sensory neurons contributes to the diminished sensory capacity seen with aging, diseases, and environmental stress (Semenchenko et al., 2004). Olfactory sensory neurons (OSNs) within the peripheral olfactory system are particularly at risk, given their peripheral location and the proximity of their receptive processes (sensory cilia) to the external environment (reviewed in ref. Ronnett and Moon, 2002). While our understanding of odorant signal transduction is significant, the mechanisms that mediate activity-driven OSN survival are less clear (reviewed in ref. Ronnett and Moon, 2002; Ache, 2010).

Sensory activity is vital to the survival of sensory neurons. In the auditory, olfactory, and visual systems, sensory deprivation causes structural changes of sensory neuronal tissues as well as cell death (Mostafapour et al., 2000; Madaïron et al., 2003; Tejedor and de la Villa, 2003). Sensory deprivation by naris occlusion causes neuronal loss in the olfactory epithelium (OE) (Farbman et al., 1988; Suh et al., 2006) and in the olfactory bulb (Corotto et al., 1994; Madaïron et al., 2003). Odorant stimulation in mammals induces long-term adaptive changes in OSNs (Moon et al., 1999; Watt and Storm, 2001), promotes neuronal survival in a competitive environment (Zhao and Reed, 2001), and rescues OSNs from apoptosis (Watt et al., 2004). However, the precise signal transduction mechanisms involved in odorant stimulation-dependent survival of OSNs are not well delineated.

Detection of an odorant is initiated when the odorant interacts with members of a large family of G protein-coupled receptors that activate adenylyl cyclase (AC) to produce cyclic AMP (cAMP), which gates a nonspecific cation channel to depolarize OSNs (Pace et al., 1985; Nakamura and Gold, 1987; Firestein and Werblin, 1989; Buck and Axel, 1991; Ronnett et al., 1993). Intriguingly, mice with a targeted gene-disruption in the cAMP cascade (e.g., deletion of G_{olf} or AC3) are anosmic; however, they display neither discernible loss of OSNs nor OE integrity (Belluscio et al., 1998; Wong et al., 2000), implying that one or more signaling pathways aside from the cAMP pathway may promote OSN survival in the OE. The other odorant-stimulated signaling pathways (i.e., phosphoinositide pathway) may have important roles in this context.

In addition to activating pathways for signal detection, neuronal activity causes cellular stress due to Ca^{2+} influx, reactive oxygen species (ROS) generation, and energy expenditure changes (Bredt, 1999; Paschen, 2000). Indeed, even habitual sensory stimulation can contribute to progressive cell damage and eventual neuronal death unless the concurrent stress conditions are alleviated (reviewed in refs. Strasser et al., 2000; Spierings et al., 2005). This may account for the neuronal loss associated with aging. Therefore, it is important to determine the relationship between activity-induced cellular stress and survival cues.

In this report, we investigated the role of odorant stimulation in generating cellular stress and the molecular mechanisms mitigating such stress and promoting neuronal survival upon odorant stimulation using rodent models. Our studies may provide a mechanism by which survival signaling is uncoupled from odorant transduction in order that independent regulation of these processes can be accomplished.

Materials & Methods

Animals

All experimental protocols were approved by Institutional Animal Care and Use Committees of both DGIST and The Johns Hopkins University, and all applicable guidelines for the care and use of laboratory animals from the National Institutes of Health Guide were followed. Adult male mice (C57/BL6, 6–7 week old) and rat pups (Sprague Dawley, days 0.5–1.5 postpartum) were obtained from Harlan (Indianapolis, IN) and KOATECH (Daegu, ROK).

Antibodies

Anti-Erk1/2 and phospho-specific antibodies were commercially available (Erk1/2, #9102; phosphorylated Erk1/2, #9101; Cell Signaling Technology, Beverly, MA, USA). Anti-Hsp70 antibody was available from StressGen Biotechnologies, Inc (#SPA810, San Diego, CA, USA). Anti-NST (β -tubulin III) antibody was purchased from Novus Biotechnology (#MMS-435p, Littleton, CO, USA). Anti-GFP antibody was available from Invitrogen, Inc (#6455, San Diego, CA, USA). Anti-GAPDH antibody was purchased from Chemicon (#MAB-374, Temecula, CA, USA).

Adenoviral infection

Administration of adenovirus was performed as previously reported (Ivic et al., 2000 ; Watt et al., 2004) with modifications. The epithelium of the right nostril was infected with 20 μ l of the viral stock solution (2.5×10^{10} pfu/ml). The viral solution was delivered 4 μ l at a time in intervals through loading tubes. At 48 hrs after infection, the animals were subjected to further studies.

Cell viability assay

Cell viability was determined by Calcein Red-Orange AM (Molecular Probes, Eugene, OR, USA), which determines intracellular esterase activity (Lichtenfels et al., 1994). OSNs were grown in 96 well tissue culture plates for 3 days and treated odorants with inhibitors or siRNA against specific pathway in odorant signaling. Cell viability was evaluated using a SpectraMax microplatereader (Molecular Devices Inc., Sunnyvale, CA, USA) with excitation and emission wavelengths of 567 and 590 nm, respectively.

Immunoblot analysis

Protein samples prepared from total lysates of primary OSN cultures or mouse OE tissues were subjected to SDS-PAGE, transferred to PVDF membrane, and probed with primary antibodies. For the secondary antibodies, horseradish peroxidase-conjugated IgGs were used. The immunoblots were visualized by using enhanced chemiluminescence kit (Pierce

Biotechnology, Inc. Rockford, IL, USA). For quantification analysis, Kodak Biomax film (PerkinElmer, Wellesley, MA) was scanned and quantified by using ImageJ software (NIH, USA).

Imaging and measurement of ROS and mitochondrial membrane potential in OSNs

We loaded CM-H₂DCFDA (10 μ M) and TMRM (10 nM) into OSNs for 30 minutes, and performed live cell imaging by confocal microscope (LSM7 live, Carl Zeiss, Germany) equipping incubating chamber maintaining 5% CO₂ and 37 °C. Images of CM-H₂DCFDA and TMRM were captured on 0 and 30 minutes after treatment of odorants. By using ImageJ software, we measured intensities of CM-H₂DCFDA and TMRM in OSNs and analyzed frequency of the intensity.

Immunohistochemistry

AdV-DN-Erk2 and AdV-GFP infection mice OE was post-fixed by paraformaldehyde and soaked in sucrose and embedded in Tissue-Tek OCT compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands). Cryo OE sections were permeated using Triton X-100 with 0.01 % hydrogen peroxide (H₂O₂) and incubated with 4% normal donkey serum (Jackson Laboratory, Bar Harbor, ME, USA). The sessions were incubated with primary antibody and Cy3-conjugated secondary antibody (Jackson Laboratory, Bar Harbor, ME, USA). For the case of Hsp70, tyramide signal amplification (TSA) was treated for signal amplification as previously described (Hansel et al., 2001). The image was visualizes and photographed under confocal fluorescence microscope (Carl Zeiss, Thornwood, NY, USA).

Intracellular fluorescence measurement of intracellular ROS

The membrane-permeable probe 5-(6)-chloromethyl-2',7'-dichloro-dihydro-fluorescein diacetate (CM-H₂DCFDA) (Molecular Probes, Eugene, OR, USA) enters the cells and produces a fluorescent signal after intracellular oxidation by ROS (Chernyak et al., 2006). We monitored intracellular oxidative stress by measurement of intracellular fluorescence intensity of CM-H₂DCFDA in a Wallac 1420 Victor multilabel counter (Wallac, Turku, Finland) using excitation and emission wavelengths of 485 and 535 nm, respectively. Fluorescence measurements were taken using Wallac 1420 Workstation software (version 2.00). H₂O₂ was used as a standard for intracellular ROS production. For fluorescence microscopic observation of intracellular ROS production upon odorant stimulation in OSN cultures, OSNs were loaded with 10 μ M CM-H₂DCFDA for 30 min at 37°C and washed with phosphate-buffered saline buffer. OSNs were sampled randomly using a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss MicroImaging Inc., Thomsonwood, NY, USA) equipped with a Hamamatsu digital camera (Hamamatsu, Bridgewater, NJ, USA) and Openlab image analysis software (Improvision Inc., Lexington MA, USA).

Measurement of mitochondrial length in OSNs

We transfected DsRed-mito plasmid, which can visualize mitochondrial matrix, into OSNs by using Calphos mammalian transfection kit (Clontech, USA) according to suggested protocol. Next day, we treated odorants during 1 hr, and fixed OSNs by 4% paraformaldehyde. To visualize OSNs, we performed immunocytochemistry by TuJ1 and

captured mitochondria on processes of TuJ1- labeled cells by confocal microscope (LSM700, Carl Zeiss, Germany). And then, lengths of individual mitochondria were measured by ImageJ.

Northern blot

Total RNA was isolated from primary cultures of OSNs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA samples were separated on 1 % agarose/2.2 M formaldehyde denaturing gel and transferred to nitrocellulose membranes (Amersham Biosciences Inc., Piscataway, NJ, USA). Hybridization was performed with labeled DNA probes as previously described (Sung et al., 2002). Membranes were exposed to autoradiography film and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Odorant stimulation to animals in vivo

Two groups of mice (n=3) were isolated in separate cages and exposed to the vapor phase of an odorant mixture (isobutylmethoxypyrazine (IBMP), citralva, and isovaleric acid; 1 mM each dissolved in 2% ethanol) or an equal volume of 2% ethanol solution for 90 min.

Pharmacological manipulation of signaling cascades

Chemicals and inhibitors were obtained from Calbiochem (La Jolla, CA, USA). The chemicals and inhibitors are cell-permeable, and each stock of inhibitor in DMSO was dissolved in medium. Cells were pre-incubated in medium containing each inhibitor for 30 min. Final concentration of chemicals and inhibitors in the medium was 200 μ M (U73343), 5 μ M (U73122), 10 μ M (NF449), 1 μ M (4 α -PDD), 50nM (4 β -PDD), 100 μ M (2-APB), 50 μ M (forskolin), 250 μ M (8-br-cAMP) and 20 μ M (U0126), respectively.

Primary culture of OSNs

Cultures were prepared as previously described with some modifications (Ronnelt et al., 1991). OSNs which were obtained from Sprague-Dawley rats were plated at a density of 2×10^6 cells/ml on tissue culture dishes (Falcon, Lincoln Park, NJ) coated with 25 μ g/ml laminin (BD Biosciences, USA) in modified Eagle's medium containing D-valine (MDV, Welgen Inc., Worcester, MA, USA). Cultures are placed in humidified 37°C incubator receiving 5 % CO₂. On 2 days in vitro (DIV) every day thereafter, cells are fed with MDV containing 15 % dialyzed fetal bovine serum (Gibco), gentamycin, kanamycin and 2.5 ng/ml NGF. Two days prior to use, the culture medium is changed to medium without NGF.

Recombinant adenoviruses

The recombinant adenovirus AdErk2DN that encodes the kinase-deficient K52R-Erk2, which can act as a dominant negative inhibitor of Erk1/2 activation (Frost et al., 1994), was constructed using the pAdTrackCMV shuttle vector and the pAdEasy adenoviral backbone, as previously described (He et al., 1998). High titer viral stocks were prepared from HEK293 cells. The control virus AdEGFP was described previously (Park et al., 2003). Virus was administered into the OE using hollow polyethylene tubes (PE50, 0.965 mm outer

diameter, Becton Dickinson, Sparks, Maryland, USA), as previously described with some modifications (Zhao et al., 1998; Ivic et al., 2000).

Transfection of OSNs with AC3 siRNA

OSNs were plated at 2×10^6 cells/ml on tissue culture dish for approximately 48 hrs before transfection. Just prior to transfection, the media were changed into serum free media. AC3 stealth siRNA oligonucleotides and negative control siRNA were purchased from Invitrogen (Darmstadt, Germany). OSNs were transfected with 50 nM siRNA using the magnetofection method from Chemicell (GmbH, Germany) as previously described (Krötz et al., 2003). Transfected cells were incubated for 48 hrs to suppress AC3 expression in OSNs in a siRNA-mediated manner.

Statistical analysis

For statistical analysis, one-way ANOVA followed by Turkey's post hoc test for unbalanced sample numbers were performed. In the case of experiments for measuring mitochondrial length in OSNs upon odorant stimulation, student *t-test* was performed. Statistical significances were indicated: * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.001$.

Results

Odorant stimulation induced ROS, and loss of membrane potential and fragmentation of mitochondria

We first determined whether odorant stimulation evokes a stress response in OSNs. As ROS generation has been implicated in stress-induced neuronal cell damage (Halliwell, 1992; Dugan et al., 1995), we therefore measured ROS generation in OSNs during odorant treatment. When primary cultures of OSNs pretreated with the cell permeable ROS-activated fluorophore CM-H₂DCFDA (Chernyak et al., 2006) were exposed to increasing doses of an odorant mixture [2-isobutyl-3-methoxypyrazine, citralva, and isovaleric acid] (Moon et al., 1999), fluorescence increased significantly in a dose-dependent manner. These data indicated the intracellular generation of ROS (Figure 1A). Although there was a dose-dependent increase in ROS generation using odorants up to 10 μ M, further increases in odorant stimulation did not increase ROS generation. Hydrogen peroxide treatment did increase ROS generation, indicating that the cells could produce, and the assay could detect, further increases in ROS levels.

It has been demonstrated that neuronal mitochondria are highly vulnerable to oxidative stress by ROS resulting in neurodegeneration (Lin and Beal, 2006). Therefore, we examined whether odorant-induced ROS insults mitochondria in OSNs. Interestingly, we found that mitochondrial membrane potential was significantly decreased by treatment of odorants in cells showing induction of ROS (Figure 1B). It implies that odorant-induced ROS impairs mitochondrial function. Next, we examined mitochondrial morphology in odorant-treated OSNs. We transfected DsRed-mito, which labels mitochondria by red fluorescence protein, and measured length of mitochondria on processes of TuJ1-labeled OSNs. We found that shorten mitochondria are increased in odorant-treated OSNs, thereby average length of

mitochondria is significantly decreased (Figure 1C). Therefore, we suggest that odorant-induced ROS mediates impairment on mitochondrial function and structure in OSNs.

Taken together, these results demonstrated that odorant stimulation generated ROS and thus mitochondrial-related cellular stress.

Odorant stimulation activates MEK/Erks to reduce ROS accumulation and support OSN viability

The Erk1/2 mitogen-activated protein kinase (MAPK) pathway has been implicated in neuronal survival in the olfactory system (Watt and Storm, 2001; Watt et al., 2004), leading us to investigate whether odorants activate this pathway in OSNs *in vivo*. Exposure to a 10 μ M odorant mixture activated Erk1/2 in the mouse OE as determined by immunostaining using antibodies against the dual-phosphorylation TEY site on the kinase (Figure 2A). Specifically, phosphorylated p42-MAPK (pErk2) increased nearly two-fold in odorant-stimulated animals as compared to vehicle-treated controls (vehicle-treated vs. odorant stimulated % $\pm$ SEM: pErk1, 100.0 $\pm$ 17.84 vs. 125.6 $\pm$ 31.58; pErk2, 100.0 $\pm$ 11.06 vs. 205.5 $\pm$ 25.00 [$p < 0.05$, one-way ANOVA]) (Figure 2B). We also determined the time course of activation of Erk1/2, as well as that of its upstream activator MEK1/2, in OSN primary cultures (Figure 2C). Upon odorant stimulation, MEK1 and Erk1/2 were activated within 30 minutes. Activations peaked by 60 minutes.

To further investigate these results, we determined whether MEK/Erk pathway activation in response to odorant stimulation affected odorant-induced ROS accumulation. This was accomplished through the use of U0126, an Erk1/2-specific inhibitor. When OSN cultures were pretreated with 20 μ M U0126 prior to exposure to a 10 μ M odorant mixture, ROS generation significantly increased compared to OSNs in either vehicle or treated with odorants alone (Figure 2D). In control experiments; the Erk1/2-specific inhibitor treatment alone did not affect basal ROS levels (Figure 2D) and odorant-induced Erk activation was completely blocked in OSNs pretreated with the inhibitor (data not shown). These data indicated that odorant-induced ROS accumulation was potentiated when the MEK/Erk pathway was inhibited, suggesting an important role for the MEK/Erk pathway in modulating odorant-induced ROS generation. We previously reported that deprivation of sensory stimulation increased apoptotic cell death in mouse OE *in vivo* (Suh et al., 2006). In current study, we tested our previous *in vivo* observation using *in vitro* techniques. When OSNs in primary culture were treated with a 10 μ M odorant mixture, cell viability was increased by 27 % as compared to controls (Figure 2E). As odorants induced Erk1/2 activation (Figure 2A, 2B and 2C), we subsequently determined whether the Erk1/2 pathway was involved in odorant stimulation-dependent neuronal survival. When OSN cultures were pretreated with 20 μ M U0126, odorant-dependent neuronal viability was significantly compromised by 6 hrs (Figure 2E). These data indicated that odorant stimulation-dependent activation of the Erk1/2 pathway was critical for reducing ROS accumulation and supporting OSN viability.

The cyclic AMP cascade does not mediate odorant-induced Erk activation and survival

A previous report suggested that cAMP may play a role in mediating odorant-induced Erk1/2 activation (Watt and Storm, 2001). However, we found that neither the adenylyl cyclase activator forskolin nor the cAMP analog 8-br-cAMP activated Erk1/2 in OSN cultures (Figure 3A). These results indicated that activation of the cAMP pathway was unrelated to- or insufficient for Erk1/2 activation and that one or more additional pathways must be involved. To investigate this directly, knockdown of AC3 expression using siRNA was performed and found to significantly block odorant-induced cAMP production (Figure 3B) without affecting odorant-dependent Erk activation (Figure 3C).

Next, we determined whether the Golf-AC3 cascade, which is known to be activated upon odorant stimulation, played a role in OSN survival. To study this, we used a cell permeable inhibitor of the stimulatory G protein (NF449) or AC3-directed siRNA for transcriptional inhibition (Figure 3D and Figure 3E, respectively). OSN cultures were pretreated with vehicle or 10 μ M NF449 prior to exposure to 10 μ M odorant mixture. OSN viability was not altered by pretreatment with NF449 as compared to vehicle pretreatment (Figure 3D). Similarly, NF449 did not affect the odorant-induced enhancement of cell viability (Figure 3D). Knockdown of AC3 expression also did not affect odorant-induced enhancement of OSN viability (Figure 3E). Collectively, these results demonstrated that MEK-Erk activation and odorant-dependent OSN survival were not mediated by the Golf-AC3-cAMP cascade.

The phosphoinositide cascade mediates odorant-induced Erk activation and supports cell viability

As other second messenger signaling pathways are also activated in response to odorants (Ache and Zhainazarov, 1995; Gold, 1999; Ache, 2010), including phosphoinositide hydrolysis by PLC to produce diacyl glycerol (DAG) and inositol-1,4,5-trisphosphate (IP₃) (Miyamoto et al., 1992; Ronnett et al., 1993; Bruch, 1996; Klasen et al., 2010), we investigated whether PLC was involved in odorant-induced Erk1/2 activation. Pretreatment of OSN cultures with the PLC inhibitor U73122 (5 μ M) blocked odorant-induced Erk1/2 activation, whereas U73343, the inactive form of the PLC inhibitor used as a negative control, did not block odorant-induced Erk1/2 activation (Figure 4A), even at higher concentration (i.e., 200 μ M). These data indicated that PLC was involved in odorant-induced Erk1/2 activation. In order to understand the role of downstream messengers generated from phosphoinositide hydrolysis, we next determined whether DAG was involved in odorant-induced Erk1/2 activation. Treatment with a DAG analog, phorbol ester (4 β -phorbol, 12,13-didecanoate, 4 β -PDD at 50 nM), strongly activated Erk1/2 in OSN culture (Figure 4B). Conversely, this activation was blocked by the MEK1/2-specific inhibitor U0126 (Figure 4B). This effect of phorbol ester was specific, as the inactive form (4 α -phorbol, 12,13-didecanoate, 4 α -PDD) had no effect, even at a higher concentration (1 μ M) (Figure 4B). Based on the involvement of DAG in odorant-induced Erk1/2 activation, we next examined whether IP₃ was also involved, as IP₃ signaling has been associated with changes in Ca²⁺ levels (Berridge, 1993). Indeed, in the presence of an IP₃R inhibitor, 2-aminoethoxydiphenyl borate (2-APB) at 100 μ M, odorant stimulation failed to activate Erk1/2 in OSN cultures (Figure 4C). Collectively, these data indicated that the PLC/phosphoinositide pathway played an important role in mediating the activation of the MEK/Erk pathway in response to

odorant stimulation. Moreover, in the presence of the PLC specific inhibitor U73122 (5 μ M), OSNs exhibited decreased viability (approximately 35% decrease at 18 hrs), whereas the inactive control compound U73343 did not affect cell survival, even at a much higher concentration (200 μ M) (Figure 4D). Odorant stimulation could not reverse the effect of U73122 (Figure 4D), indicating that the PLC pathway was important for both basal and odorant-dependent cell survival.

Odorant-induced MEK/Erks activation mitigates odorant-induced cellular stress *in vivo* and *in vitro*

Our *in vitro* data indicated that the MEK/Erks pathway played an important role in alleviating cellular stress caused by ROS generation and promoted viability during olfactory sensory signaling. We hypothesized that MEK/Erks pathway promotes cell viability by alleviating cellular stress (e.g., oxidative stress). To test this hypothesis, we determined whether the stress indicator Hsp70 was induced following odorant stimulation in OSN cultures. This seemed to be a fitting way to test our hypothesis, as OSNs are known to express heat shock proteins in response to various stresses including oxidative stress (Getchell et al., 1995; Mayer et al., 2008). As determined by both Northern and western blotting analyses, odorant stimulation increased the expression of Hsp70 *in vitro* (Figure 5A and 5B). The expression of Hsp70 increased significantly when the cells were pretreated with the MEK1/2 specific inhibitor PD98059 (50 μ M), although the inhibitor itself did not alter Hsp70 expression (Figure 5A and 5B). These data indicated that MEK/Erks pathway could play a protective role for OSNs, consistent with the role of MEK/Erks pathway in alleviating ROS generation during olfactory sensory signaling. Finally, we determined whether our *in vitro* findings could be demonstrated *in vivo*. When expression of Hsp70 was monitored in the OE of mice, odorant stimulation caused only a slight increase in the expression of Hsp70 (Figure 5C). However, when the epithelium was infected with a recombinant adenovirus, AdErk2DN, which encodes a kinase-deficient K52R-Erk2, odorant-induced Hsp70 expression significantly increased by inhibition of Erk1/2 signaling. The Erk2 mutant acts as a dominant negative inhibitor and has been used to block Erk1/2 signaling (Frost et al., 1994). Hsp70 expression was neither affected by the adenoviral infection nor augmented by the control, AdGFP (Figure 5D). When the mouse OE was immunohistochemically analyzed, odorant-mediated Hsp70 expression was increased in the OSN layer. Additionally, this expression was co-localized with GFP expression, indicating that the augmented Hsp70 expression was an Erk-specific effect in OSNs.

Discussion

In the current study, we demonstrated that sensory stimulation, specifically odorant stimulation, promoted OSN survival *via* PLC-mediated Erk activation in a Ca^{2+} -dependent manner. Odorant stimulation induced intracellular oxidative stress as demonstrated by ROS formation, which subsequently induced the expression of stress responsive proteins such as Hsp70. This odorant-induced oxidative stress was remediated by Erk activation. Thus, while the cAMP pathway mediated odorant detection in olfaction, the PLC pathway mediated stimulus-induced survival, permitting the independent regulation of survival cues from

sensory transduction. Furthermore, these data illuminated the role of the PLC transduction cascade in olfaction.

Numerous lines of evidence have indicated that odorant detection proceeds *via* a G-protein-coupled receptor mechanism involving AC, Golf, and cAMP that regulates a cyclic nucleotide-gated channel (Ronnelt and Moon, 2002). However, odorants have also been shown to induce phosphoinositide hydrolysis, which has led to the hypothesis that odorants activate either cAMP or inositol phosphates to signal odorant detection (Boekhoff et al., 1994). While IP₃ receptors have been identified in the mammalian olfactory system (Cunningham et al., 1993; Smutzer et al., 1997), the results of studies using mice with targeted disruptions of genes for proteins involved in the cAMP signal transduction pathways argued against a role for PLC in odorant detection (Brunet et al., 1996; Belluscio et al., 1998; Wong et al., 2000). Data from the current study support a role for PLC, not in odor detection, but in sensory stimulation-mediated neuronal survival. In fact, PLC pathway might be involved in cell survival in distinct manners including growth factors, cellular stimulation etc. Thus, we performed our *in vitro* examination in a serum-free condition to monitor the sole effect of odorant stimulation on cell survival, showing that PLC signaling pathway may play a crucial role in sustaining cell viability in our experimental condition. Recently, both cAMP and inositol phosphates signal transduction pathways have been reported to be activated *via* a single type of odorant receptor (Ko and Park, 2006), suggesting that an odorant(s) may activate both signal transduction pathways to mediate distinct functions in the olfactory sensory system (Ache, 2010).

Mice with targeted disruptions of genes encoding Golf and AC3 (cAMP cascade components) were anosmic, but, intriguingly, retained OE integrity (Belluscio et al., 1998; Wong et al., 2000). These reports suggested that cAMP was critical to odorant detection, but may not have been essential to sensory stimulation-dependent OSN survival. In the current study, we showed that sensory stimulation-dependent OSN survival was promoted by MAPK activation, and that MAPK activation was not mediated by cAMP production, but rather by phosphoinositide hydrolysis. In addition, our results showed that inhibition of PLC decreased OSN viability even in the absence of odorants, while MEK inhibition did not alter basal viability. These findings suggested that PLC may have regulated one or more additional pathways aside from the MAPK pathway to promote OSN survival. Spontaneous activity has been implicated in OSN survival (Yu et al., 2004), and therefore, PLC may also play a role in spontaneous activity-dependent OSN survival. Collectively, the results of the current study provided insight into the role of phosphoinositide hydrolysis in OSNs.

Sensory stimulation has long been implicated in sensory neuronal survival; however, whether sensory stimulation can also increase intracellular stress by Ca²⁺ influx, changes in energy expenditure, and ROS production has not been studied in the olfactory system. In particular, even during normal physiological processes, ROS production occurs, and may lead to compromising levels of intracellular stress unless alleviated (Halliwell, 1992; Adams et al., 1996; Paschen, 2000). In the current study, we demonstrated that odorant stimulation indeed produced ROS in OSNs. Interestingly, the intracellular ROS amount induced by odorants at 100 μ M was not significantly different from that at 10 μ M, implying that regulatory mechanisms are activated once intracellular oxidative stress reaches a certain

level. The regulation of intracellular oxidative stress during odorant stimulation may be crucial to supporting neuronal viability, as oxidative stress has been implicated as a key trigger of neuronal death (Adams et al., 1996). It has been demonstrated that neuronal activation induces ROS and this process is involved in neuronal pathology (Sengpiel et al., 1998; Suberbielle et al., 2013) as well as physiology including synaptic plasticity (Bindokas et al., 1996). Especially, sensory neurons may be exposed to oxidative stress by ROS over-produced by continuous external sensory cues. In addition, neuronal pathology by ROS has been known to be mainly mediated by mitochondrial dysfunction (Lin and Beal, 2006). However, there is no report about mitochondrial dysfunction evoked by activity of sensory neurons. In this study, we found that physiological activation of OSNs induces loss of membrane potential and fragmentation of mitochondria. Considering that loss of mitochondrial membrane potential leads to failure of oxidative phosphorylation and intracellular calcium buffering with mitochondrial fragmentation resulting in acceleration of neuronal death (Knott et al., 2008), we suggest that physiological activation of OSNs induces mitochondrial stress. However, this mitochondrial stress not only results in neurodegeneration, but also turns on defensive mechanism. Together with mitochondrial stress, we also found that Erk1/2 is activated by odorants and inhibition of Erk1/2 augments ROS generation. It has been demonstrated that mild levels of ROS activate Erk1/2 pathway and this process contributes to resistance and survival against cellular stress (Anderson and Tolkovsky, 1999; Rosseland et al., 2005; Bhagatte et al., 2012). Therefore, we concluded that neuronal activity by odorants induces Erk1/2 activation via defensive mechanism evoked by mitochondrial stress in OSNs.

MAPK pathways play critical roles in many neuronal processes including survival (Bonni et al., 1999) and development in the olfactory system (Hirotsu et al., 2000). More recently, it was reported that Erk1/2 was involved in rodent OSN survival (Watt and Storm, 2001; Watt et al., 2004). Among Erk1 and Erk2, results from the current study indicated that Erk2 appeared to be a more critical component in odorant stimulation-dependent OSN survival. Moreover, the sensory stimulation-induced Erk2 activation mediated a protective response to intracellular oxidative stress.

In the current study, we evaluated intracellular stress in OSNs *in vivo* by monitoring Hsp70 expression. Our results demonstrated that Hsp70 was induced even during normal sensory stimulation and that its expression increased even further when Erk2 activation was inhibited. Similarly, it has been reported that Hsp70 induction in the hippocampus was critical for adaptation to stress and that its expression was dramatically increased under intensive stress conditions (Shao et al., 2007). Moreover, oxidative damage has been shown to result in age-specific Hsp70 expression (Wheeler et al., 1995). The induction of Hsp70 expression by odorant stimulation and its exacerbation *via* Erk2 inhibition further supports the importance of these pathways in OSN survival.

In summation, the results of our current study demonstrated that odorant stimulation promoted OSN survival and also induced intracellular oxidative stress, which was exacerbated when Erk2 was inhibited. Sensory stimulation simultaneously activated at least two parallel pathways, the AC/cAMP cascade responsible for odorant detection, and phosphoinositide hydrolysis to promote odorant stimulation-dependent neuronal survival

(Figure 6). Odorants activated the Erk2 *via* PLC activation to respond to elevated intracellular oxidative stress. Therefore, sensory stimulation mediated not only rapid signal detection, but also long-term cellular responses that included sensory stimulation-dependent neuronal survival. The survival of OSNs may be continually compromised by the elevated intracellular oxidative stress that accompanies habitual sensory stimulation. In turn, sensory stimulation appears to activate signaling pathways parallel to sensory detection to control sensory stimulation-related intracellular oxidative stress. This may permit activation of survival pathways independent of stimulus detection, which can be crucial even in circumstances where signaling of odorant detection has been desensitized physiologically. The response to stress may be exacerbated in pathological conditions. For example, in aging and neurodegenerative diseases, neurons may no longer be able to tolerate oxidative stress induced in the course of habitual cellular events. The results from our study elucidated the roles of phosphoinositide hydrolysis and MAPK activation in OSN survival and may provide information that could lead to the development of therapeutic strategies to treat such aging and neurodegenerative conditions.

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Abbreviation footnote

OSN	olfactory sensory neurons
CNG2	olfactory cyclic nucleotide-gated channels
AC	adenylyl cyclase
GC	guanylylcyclase
ROS	reactive oxygen species
CM-H₂DCFDA	5-(6)-chloromethyl-2',7'-dichloro-dihydro-fluoresceindiacetate
IBMP	2-isobutyl-3-methoxypyrazine
MAPK	mitogen-activated protein kinase
IP₃	inositol-1,4,5-trisphosphate
4β-PDD	4β-phorbol, 12,13-didecanoate
Hsp70	heat shock protein 70
2-APB	2-aminoetoxydiphenyl borate

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FIGURE 1.

A, Time course of intracellular ROS generation upon odorant stimulation in OSN cultures. Measurements were taken at 0, 0.75, 2, 3, 4, 5, and 6 h after exposure to odorants at increasing concentrations. H₂O₂ (10 μM) was used as positive control for intracellular ROS generation. Odorant stimulation increased ROS generation in a dose-dependent manner. B, Time-lapse images of TMRM and CM-H₂DCFDA in culture OSNs during odorants treatment (10 μM). Images of TMRM before and after treatment of odorants for 30 minutes. Arrows indicate cells exhibiting decreased TMRM. Right, cumulative frequency of TMRM in OSNs (N=289 cells) treated by odorants were summarized. Images of CM-H₂DCFDA before and after treatment of odorants for 30 minutes. Arrow heads indicate cells exhibiting increased CM-H₂DCFDA. Right, cumulative frequency of CM-H₂DCFDA in OSNs (N=289 cells) treated by odorants were summarized. C. Images of mitochondria labelled by DsRed-mito in culture OSNs untreated and treated by odorants for 1 hr. Lower panels are straightened images of mitochondria in boxes of vehicle and odorants. Tuj1 was count-

stained as neuronal marker. Cumulative frequency and average of mitochondrial length in OSNs untreated (N=448 from 13 neurons) or treated by odorants (N=471 from 14 neurons) were summarized (Student *t*-test, *** $p < 0.001$).

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FIGURE 2.

A, Odorants increased Erk2 phosphorylation *in vivo*. Mice (n=3) were exposed to a vehicle (1 ml of 2% EtOH) or odorant mixture (1 ml of 1 mM odorant mixtures in 2% EtOH: IBMP, citralva, and isovaleric acid) for 90 min in separate cages. Activation of Erk1/2 was measured using antibodies against phospho-Erk1/2 (pErk1/2) and total Erk1/2 (Erk1/2). Equal number of neurons were confirmed by monitoring the *neuron-specific tubulin* (NST) expression levels. Equal loading of samples was confirmed by monitoring the GAPDH. B, Quantification of phosphorylated Erk1/2. Quantification of protein expression levels was determined by stereological analysis using the ImageJ analysis program. Equal loading of samples was confirmed by monitoring the total Erk 1/2 protein level, respectively. Odorants significantly increased Erk2 phosphorylation, but not Erk1 phosphorylation. C, Time course of odorant-induced MAPK activation in OSN cultures. Odorant stimulation induced activation of MEK and Erk1/2, which peaked at 60 min after stimulation. D, Erk1/2 inhibition increased the odorant stimulation-induced intracellular ROS generation *in vitro*.

OSNs were loaded with 10 μ M CM-H₂DCFDA for 45 min at 37°C. Upon odorant stimulation (10 μ M) for 6 hrs, preincubation with U0126 (20 μ M) did not affect the basal level of ROS generation, but significantly increased the odorant-induced ROS generation. The data were acquired from three independent experiments. Statistical significances are indicated (one-way ANOVA, *** $p < 0.001$). E, Effects of Erk1/2 inhibition on OSN viability. OSN cultures were exposed to odorants for 6 hrs in the presence or absence of U0126. OSN viability was increased upon odorant stimulation. Inhibition of Erk1/2 significantly reduced the odorant stimulation-induced OSN survival. The data were acquired from three independent experiments. Statistical significances are indicated (one-way ANOVA, ** $p < 0.01$, *** $p < 0.001$).

FIGURE 3.

A, Odorant-induced Erk1/2 activation. Cultures were treated with vehicle, forskolin (FSK), 8-br-cAMP or odorants in the presence or absence of U0126. Odorants increased pErk1/2 levels, whereas forskolin and 8-br-cAMP did not. Preincubation with U0126 completely blocked the odorant-induced Erk1/2 activation. Quantitative analysis from two independent experiments was performed. B, Functional knock-down of AC3. Functional knock-down of AC3 was confirmed by cAMP assay upon odorant stimulation. 48 hr after magnetofection of AC3 stealth siRNA oligonucleotides, OSNs failed to produce cAMP production whereas OSNs transfected with negative siRNA oligonucleotides successfully produced cAMP. Statistical analysis was performed with one-way ANOVA (** $P < 0.01$, *** $P < 0.001$). C. Effect of knock-down of AC3 using siRNA on odorant-induced Erk1/2 activation. OSNs were cultured after magnetofection with control or AC3 siRNA. The western blot was performed 48 hrs after magnetofection. AC3 knocked-down with siRNA had not changed Erk1/2 signaling in OSNs. D, Effect of inhibition of Golf on OSNs viability *in vitro*. OSN viability was increased upon odorant stimulation. When incubated with NF449 had no effect on viability at baseline or upon odorant stimulation. E, Effect of inhibition of AC3 using siRNA on OSN viability. OSN cultures were exposed to odorants for 6 hrs in the presence or vehicle. OSN viability was not changed upon odorant stimulation. The data were acquired from three independent experiments. Statistical significances are indicated (one-way ANOVA, *** $p < 0.001$).

FIGURE 4.

A, Odorant-induced activation of Erk1/2 was mediated by PLC. Cultures were exposed to a vehicle or odorants in the presence or absence of U73122 (5 μ M), or U73343 (200 μ M). Preincubation with PLC inhibitor, U73122 inhibited the odorant-induced Erk1/2 activation, whereas U73343, the inactive form, failed to block the odorant-induced Erk1/2 activation. B, Phorbol ester activation of Erk1/2 in OSNs. When OSNs were stimulated with a DAG analog, 4- β phorbol 12,13-didecanoate (PDD; 50 nM) *in vitro*, Erk1/2 were strongly activated. The effect of 4- β PDD was not mimicked by the inactive form, 4- α PDD, at 1 μ M. C, Ca²⁺ is critical in the odorant-induced Erk1/2 activation. Treatment of cultures with 2-APB (100 μ M) blocked the odorant-induced Erk1/2 activation. D, Effect of inhibition of PLC on OSN viability *in vitro*. OSN viability was increased upon odorant stimulation. When

incubated with U73122 (5 μ M), odorant-dependent OSN viability was significantly decreased. The data were acquired from three independent experiments. Statistical significances are indicated (one-way ANOVA, *** $p < 0.001$).

FIGURE 5.

A, Northern blot analysis of *hsp70* expression. OSN cultures were exposed to odorants for 6 h. Inhibition of MEK increased *hsp70* expression upon odorant stimulation. B, Western blot analysis of HSP70 expression. OSN cultures were exposed to odorants up to 18 h. Inhibition of MEK using PD98059 increased Hsp70 expression upon odorant stimulation. Infection rates were compared by NST (β -tubulin III) expression. C, Western blot analysis of Hsp70 expression in adenoviral vector infected animals. Hsp70 expression is another indicator of intracellular stress, and its expression was monitored following odorant treatment in animals treated with AdV-DN-Erk2. Equal rates of infection were confirmed by GFP expression. Odorant stimulation induced Hsp70 expression, which was increased following infection of

the OE with AdErk2DN compared to cultures infected with AdEGFP. D. Immunohistochemistry of Hsp70 expression in AdEGFP and AdErk2DN infected animals. OE was infected with AdEGFP or AdErk2DN and immunostained for Hsp70. Inhibition of Erk2 increased Hsp70 expression upon odorant stimulation. Infected cells in the OE were visualized by GFP expression.

FIGURE 6.

Working Hypothesis. Odorants may activate parallel signaling cascades to mediate sensory detection and sensory stimulation-dependent survival. See text for details.