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Activation of SRE and AP1 by olfactory receptors via the MAPK and Rho dependent pathways

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ABSTRACT

Whereas the activation of MAPKs (mitogen activated kinases) and Rho dependant pathways by GPCR (G protein coupled receptors) has been the subject of many studies, its implication in the signalling of olfactory receptors, which constitute the largest GPCR family, has been far less analysed.

Using an in vitro heterologous system, we showed that odorant activated ORs activate SRE containing promoters via the ERK pathway. We also demonstrated that RhoA and Rock kinases but not Rac were involved in ORs-induced SRE/SRF activation and that AP1 was activated, via JNK and p38 MAPKinase. Using real time PCR we found that mOR23, RnI7 and CfOR12A07 induced elevated levels of transcription factors ELK-4, srf, c-fos and c-jun mRNAs whereas mOREG induced an elevated transcription levels of c-fos and c-jun mRNA only. We showed also that odorant activated ORs stimulate the downstream MAPKs and Rho pathways in primary cultures of rat olfactory sensory neurons (OSNs). Similar results were also obtained with OE (olfactory epithelium) extracts prepared from rats exposed to odorants in vivo. Finally, we showed the important role of the AKT and MAPK signalling pathways in OSNs survival.

Taken together, these data provide direct evidence that the binding of odorants onto their ORs activates the MAPK and Rho signalling pathways that are involved in OSNs survival events. This suggests that these pathways could be implicated in the regulation of OSNs homeostasis.

1. Introduction

Olfactory receptors (ORs) constitute the largest G-protein-coupled receptor (GPCR) family described to date. ORs play a critical role in the recognition of many odorant molecules by olfactory sensory neurons (OSN) present in the nasal olfactory epithelium [1]. In response to odorants, OSNs transmit electric signals to the brain, resulting in odour perception. Mammals have between 500 and 1500 highly polymorphic odorant receptors [2–7]. It was shown by several studies that the binding of odorants to ORs triggers the production of cAMP and IP3, as second messengers via activation of adenylate cyclase and PLC respectively [8–14]. The effects of OR activation on other downstream pathways and cellular functions have been the subject of very few studies [15,16].

It is known that GPCRs, other than ORs activate mitogen activated protein kinases (MAPKs) [17–21]. MAPKs are essential components of many signalling pathways, linking extracellular signals to changes in transcription factor activity and expression of genes that are important for cell homeostasis [22,23]. Thus, the ternary complex factor (TCF) and the serum response factor (SRF), each of them controlled by a distinct mechanism [24,25], bind to the serum response element (SRE) included in the c-fos promoter.

Multiple signals induce the transcriptional activity of TCF via the activation of MAPKs, such as extracellular regulated kinases (ERK), Jun N-terminal kinases (JNK), and p38 [26,27], which phosphorylate the TCF trans-activation domain. In contrast, SRF is activated by RhoA, independently from the activity of MAPKs. The RhoA pathway is linked to the activation of SRF through the Rock kinase, which in turn phosphorylates the LIM kinase [28].

Among the MAPK pathways, the Jun N-terminal kinase (JNK) and p38 MAPK pathways, are activated by different types of stresses and have key roles in tissue homeostasis, as they control cell proliferation, differentiation, survival and cell migration. Depending on the type of stimuli, the cellular response can range from apoptosis to survival [29]. JNKs phosphorylate transcription factors, including c-Jun and ATF2 [30]. AP-1, which is formed by a dimer of Jun and Fos family members, regulates the expression of genes involved in cell proliferation and differentiation [31].

We thus wondered whether ORs would interfere with other downstream pathways and more specifically the MAPK and the RhoA pathways upon the binding of their cognate odorants. To this end we selected the rat OR gene RnI7, which has been the subject of

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many studies using octanal as ligand [8,32] as well as CfOR12A07, its canine orthologue [5,12,14]. We also included in this study two other ORs, which have been the subject of numerous reports in the literature, the mouse olfactory receptors mOR23 and mOREG, which bind lyral and eugenol respectively [4,33,34]. Using several luciferase reporter gene constructs and an in vitro heterologous system, we showed that ligand stimulated ORs are able to activate SRE transcriptional activity not only via MAPK but also via Rho dependent pathways. Also, we showed that ORs stimulation induced AP1 activation. Through in vivo analysis and OSN primary cultures, we confirmed that odorant stimulation does activate various MAPK pathways known to be implicated in cell survival and/or proliferation.

Altogether our results point to the additional role that ORs would have in inducing signalling pathways that may be involved in maintaining the homeostasis of the olfactory system, and more specifically the number of mature OSNs and the choice of OR expressed at their membrane surface.

2. Materials and methods

2.1. ORs cloning and plasmids

Olfactory receptors RnI7, CfOR12A07, mOR23 and mOREG were cloned in frame with a leader peptide sequence derived from Influenza haemagglutinin and a c-Myc epitope sequence in the pIRES plasmid vector (Clontech, Mountain View, CA) as previously described [12].

The plamids pSerum response element (SRE)-luciferase, pSerum response factor (SRF) binding site-luciferase, and pCRE-luciferase were from Stratagene (Agilent, France), pAP1 was from Clontech (France) and pRL-SV40 was from Promega (France).

2.2. Reagents

JNK, phospho JNK, phospho ERK1/2, phospho p38 and p38 antibodies were purchased from Cell Signalling Technology Inc (Ozyme, France). Erk2 antibody was purchased from Santa Cruz Biotechnology, Inc. (Tebu, France).

The following pharmacologic inhibitors and controls Y294002, UO126, UO124, SP600125, SB203580, Y-27632, CCG-1423, NSC23766 and SQ22536 were obtained from Merck–Calbiochem (VWR, France) and were used as indicated in the legend figures. The Rho inhibitor cell permeable exoenzyme C3 transferase was purchased from Cytoskeleton (Tebu, France). The AKT inhibitor IV was purchased from Santa Cruz Biotechnologies (Tebu).

All the odorants used were purchased from Sigma/Aldrich (Saint Quentin Fallavier, France). Stock solutions were prepared in DMSO immediately before each experiment. Solutions were then serially diluted in PBS (final DMSO concentration was <1/500). The composition of the cocktail of odorants for in vivo experiments was as described in the Fig. 7 legend.

2.3. 1E6 cell cultures

All culture reagents were from Invitrogen (France). We used a subclone (1E6) of a human embryonic kidney HEK293 expressing the Gα-olf as described previously [12]. Briefly, cells were maintained in complete medium (DMEM plus 10% FBS, nonessential amino acids, antibiotics) containing 800 μg/ml of G418. Cells were cultured at 37 °C, under an atmosphere of 5% CO_2. Cells were discarded after seven passages and new cultures were prepared from a frozen cell stock. The 1E6 cell clone was constructed with GMO authorization n° 12576 issued March 2003 by the genetic recombinant committee of the Ministry for Education and Research (France).

2.4. Dual-luciferase reporter assay

CRE, SRE or SRF-driven promoter activity was assayed using the PathDetect Signal Transduction Pathway cis-reporting Systems (Stratagene, France). AP1 activity was assayed using the Pathway profiling system of Clontech (France). Cells were co-transfected with 100 ng of plasmids encoding OR, 50 ng of pCRE-luc, pSRE-luc, p-SRF-luc or pAP1-Luc plasmid and 1 ng of pRL-SV40 control plasmid (Promega, France). At 24 h post-transfection, 1E6 cells were starved for an additional 15 h in DMEM-F12 without FBS and containing 0.1% bovine serum albumin (BSA). For the cAMP assay, cells were incubated with IBMX for 30 min and then exposed to various concentrations of odorants. Both firefly and *Renilla* luciferase were measured after 4–5 h incubation at 37 °C, using Dual Glo® luciferase reagent following the manufacturer's instructions (Promega, France) and a Fluostar Omega plate reader equipped with luminescence detectors (BMGLabtek). Data were normalised to *Renilla* activity levels by dividing the values obtained for firefly luciferase by the *Renilla* luciferase values. For some experiments, data were expressed as the ratio odorant/DMSO control. All experiments were carried out at least four times, with duplicate or triplicate technical measurements taken for each time-point.

2.5. Western blot analysis

For Western blot analysis, proteins from activated and non-activated 1E6 cells were separated on 12% SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were then incubated for 2 h with primary antibodies. Anti-rabbit IgG conjugated with HRP (Jackson Laboratories, Interchim), was used as a secondary antibody. Immunoreactivities were detected by ECL Reagents (Pierce, Thermofisher Scientific).

2.6. Real time PCR

OR-transfected 1E6 cells were incubated with the corresponding ligand for the indicated time periods. Total RNA was extracted and purified with RNAII kit following the manufacturer's instructions (Macherey Nagel, France). Total RNA (1 μg) was used as a template for cDNA synthesis. cDNA was prepared using High capacity cDNA RT reagent kit (Applied Biosystems, France). Real-time PCR was performed using the following primer sets:

- Primers for the detection of human transcription factors in 1E6 cells were as follows:
  c-jun forward: CCAAAGGATAGTGCGATGTTT;
  c-jun reverse: CTGTCCCTCTCCACTGCAAC.
  c-fos forward: CTACCACTCACCCGCAGACT;
  c-fos reverse: AGGTCCGTGCAGAAGTCCT.
  srf forward: AGCACAGACCTCACGCAGA;
  srf reverse: GTTGTGGGCACGGATGAC.
  ELK-1 forward: GCTTCCTACGCATACATTGACC;
  ELK-1 reverse: GGTGCTCCAGAAGTGAATGC.
  ELK-4 forward: CTCGAGTTTCCAGCGTGAG;
  ELK-4 reverse: CAGGGTGATAGCACTGTCCAT.
  18S forward: TAGTTGGTGGAGCGATTTGTCTG;
  18S reverse: CTAAGCGGCATAGTCCCTCTAAG.
- Primers for the detection of rat transcription factors in OSNs or in vivo were as follows:
  c-jun forward: GCTGAACTGCATAGCCAGAA;
  c-jun reverse: GCCCCACTGACAGGTTGT.
  c-fos forward: CAGCCTTTCCTACTACCATTCC;
  c-fos reverse: ACAGATCTGCGCAAAAGTCC.
  srf forward: GCACAGACCTCACGCAGA;
  srf reverse: ATGTGGCCACCCACAGTT.
  ELK-1 forward: GCTCCCCACACATACCTTGA;

ELK-1 reverse: CGGTGCAATTGGACTCAGA.
ELK-4 forward: CCAGCACTCTTCTCACAGACA;
ELK-4 reverse: GTGCTCCAAAAGTGGATGCT.
β-actin forward: GCCGTCTTCCCCTCCAT;
β -actin reverse: AGGAGTCCTTCTGACCCATACC.

The SYBR Green used was from Applied Biosystems. The amplification reactions were performed in 10 μl of final volume containing 2× SYBR Green, 0.3 μM forward and reverse primers and cDNA dilution (2 μl). The thermal cycling conditions were 10 s at 95 °C and 40 cycles of 5 s, 95 °C and 30 s, 60 °C. Post-PCR melting curves confirmed the specificity of single-target amplification. Fold expressions of genes relative to 18S or ß actin was determined in triplicate. Data are expressed as ΔΔCT.

2.7. In vivo experiments and OSN primary cultures

Experiments were performed on 4–5 weeks old brown Norway rats (Elevage Janvier, France). All animal experiments were conducted in accordance with the local ethical committee. For in vivo experiments, rats were exposed to a cocktail of odorants for different time points. The rats were then killed by decapitation. The entire olfactory epithelium was dissected. The T-PER Tissue Protein Extraction Reagent (Thermo Scientific, France) supplemented with protease and phosphatase inhibitors (Sigma) was used for total protein extraction from olfactory epithelium (OE) samples. Total protein amount was determined with the standard protein assay Bradford (BioRad). Western blot analyses were then performed on these samples; 35 μg of proteins was loaded on each lane. mRNA preparations from OE samples were performed using the RNAII kit following the manufacturer's instructions (Macherey Nagel, France). The real time PCR was then performed as described above.

We also used primary cultures of OSNs. Briefly, for each experiment, 5–7 rats were killed and OE dissected. After digestion using collagenase (650 UI/ml) for 30 min at 37 °C and centrifugation, cells were washed in DMEM/Ham F12 (Invitrogen) and 10% foetal calf serum (FCS, Invitrogen). Cells were then suspended in Waymouth's medium containing N2 supplement, 10% FCS and antibiotics/antimycotic in the presence of human recombinant NGF (25 ng/ml). Cells were then seeded at approximately 7×10^3 cells/mm^2 on 100 mm Petri dishes previously coated with Poly-L-Lysine (Sigma) and cultured for 1–3 weeks. OSNs were then trypsinized and cultured in Poly-L-Lysine coated Petri dishes for 24 h, then cultures were starved for 18 h in DMEM/Ham F12 containing 0.1% BSA without FCS and NGF or other growth factors. Thus, OSNs were stimulated with the cocktail of odorants for the indicated time points. Protein extractions were performed using RIPA buffer (Thermo Scientific) supplemented with protease and phosphatase inhibitors. 4 μg of proteins was loaded on each lane for Western blots. mRNA was prepared from these stimulated cultures using the RNAII kit following the manufacturer's instructions.

2.8. Cell viability

OSN viability was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolism test (Sigma) following the manufacturer's protocol. OSNs were first cultured on Poly-L-Lysine coated 96-well plates (BioCoat, BD, France) at 7×10^3 cells/well in complete Waymouth's media containing N2 supplement, 10% FCS and antibiotics/antimycotic in the presence of human recombinant NGF (25 ng/ml). After 24 h incubation at 37 °C in a humidified atmosphere with 5% CO2, OSNs were starved for 18 h in DMEM/F12 containing 0.1% BSA in absence of N2 supplement, FCS, and NGF. After treatment with inhibitors for 2 h, the odorants were added for an additional 24 h and then MTT was added to the cultures at the final concentration 0.5 mg/ml. After 4 h incubation, formazan crystals were dissolved in isopropanol 0.1 N solution. Optical densities were measured at 570 nm using a scanning multi-well spectrophotometer (Omega star, BMGLabtek). The background absorbance was read at 690 nm and subtracted to the 570 nm values. All measurements were carried out in triplicate. Experiments were performed four times.

3. Results

3.1. SRE activation by mOR23, CfOR12A07 and RnI7 but not by mOREG

Previous studies have shown that ORs activated by their ligands transduced signals through the IP3 and cAMP pathways [8–14]. Here, we asked whether ORs, as members of the GPCR superfamily, have any effect on the MAP kinase and Rho pathways.

Olfactory receptors have no identified translocation sequence at their N-termini and are known to be difficult to express at the plasma membrane. We thus transfected 1E6 cells (HEK cells permanently expressing the Gαolf subunit) with an expression vector encoding an individual OR in frame with a peptide leader sequence added to the N-terminal end [12]. This leader peptide, derived from an influenza virus, was shown to facilitate OR expression at the cell surface [12,35]. We also added a c-Myc tag at the N-terminal of the recombinant protein to monitor its expression at the cell membrane by immunocytochemistry.

Phylogenetic and synteny comparison analyses indicated that the canine OR gene CfOR12A07 is the orthologue of the rat OR gene RnI7, which has been the subject of many studies using octanal as ligand [1,8,32]. For purpose of comparison, in addition to RnI7 and CfOR12A07, we included in this study the mouse olfactory receptors mOR23 and mOREG which bind lyral and eugenol respectively [4,33,34]. We first assessed the expression of these N-terminal Myc-tagged ORs by immunocytochemical

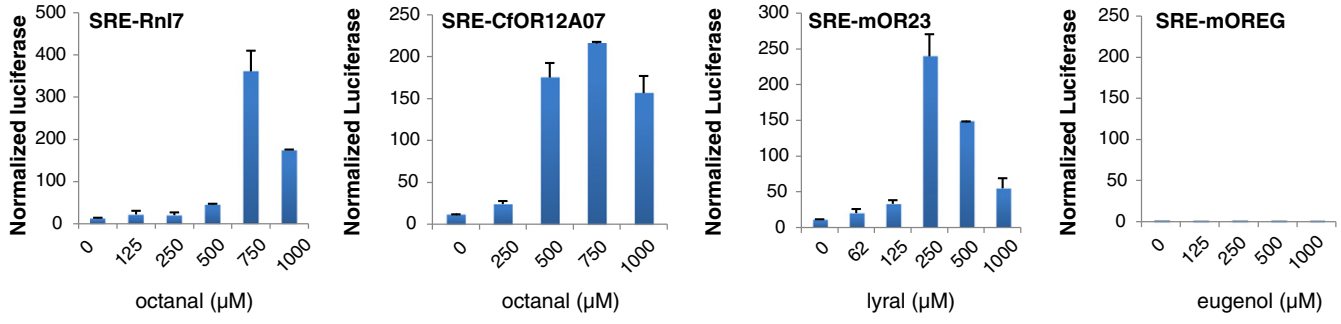

Fig. 1. SRE activation by ORs. Luciferase activity was measured to examine the activation of SRE. 1E6 cells were transiently transfected with an OR encoding plasmid together with the luciferase construct containing SRE and a third plasmid pRLSV40 which codes for *Renilla*. 24 h after transfection, cells were starved for an additional 15 h, and then were stimulated with the indicated odorants at different concentrations in PBS/DMSO for 4 h. Data are expressed as a ratio between luciferase firefly and *Renilla* emitted luminescence. Bars represent the standard deviation calculated with three technical replicates. A strong increase of SRE activity was observed for cells transfected with RnI7, its canine orthologue CfOR12A07 or mOR23 and stimulated with their respective ligands. In contrast, mOREG did not exhibit any SRE transcriptional activity following its stimulation, whereas the CRE activity is strongly induced with this same receptor (see Supplementary Fig. S2).

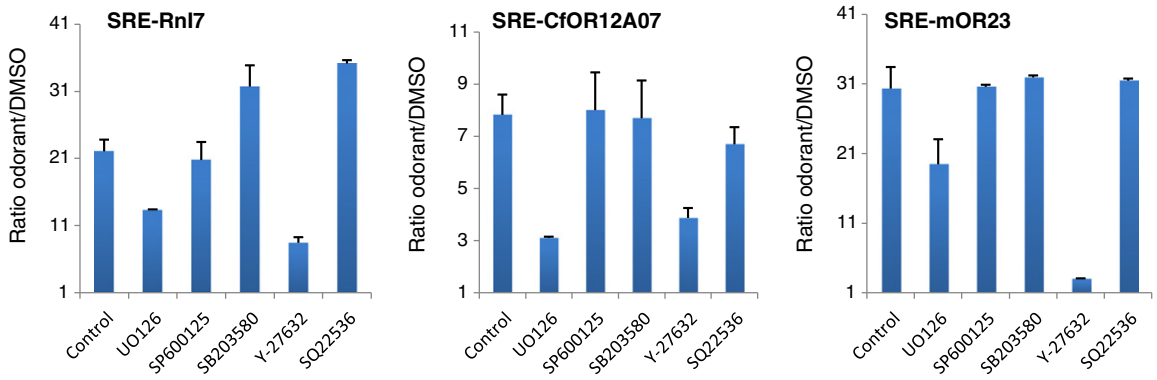

Fig. 2. SRE activation is mediated by both ERK and Rock kinases. Cells were transiently transfected with an OR encoding plasmid together with the luciferase construct containing SRE and the pRLSV40. 24 h after transfection, cells were starved for an additional 15 h, and then were incubated for 2 h with different inhibitors prior to stimulation: UO126 (10 μM, ERK inhibitor), SP600125 (10 μM, JNK inhibitor), SB203580 (20 μM, P38 inhibitor), Y27632 (20 μM, ROCK inhibitor) and SQ22536 (200 μM, AC inhibitor). Cells were stimulated with the odorants (octanal at 750 μM for RnI7 and CfOR12A07, and lyral at 250 μM for mOR23). Firefly values are normalised to *Renilla* values and data are expressed as a ratio between odorants versus DMSO control responses. Value 1 corresponds to an absence of response to odorant, value 10 to a 10 fold increase response of odorant compared to PBS/DMSO alone. The controls correspond to the response obtained by the odorant in absence of inhibitors. Data showed that SRE activation is dependent upon both ERK and Rock kinases as indicated by the responses obtained with UO126 and Y27632 whereas JNK and P38MAPK did not have any effect on SRE as shown by the absence of effect of SP600125.and SB203580. This inhibition did not result from loss of cell viability during the 4 h incubation required for luciferase synthesis (data not shown). SQ22536, which inhibits adenylate cyclase had no effect on the activation of the SRE cis element.

staining and showed their efficient translocation at the cell membrane of transfected 1E6 cells (Fig. S1).

Then we examined with a SRE-luciferase reporter construct whether SRE was activated following the exposure to odorants of these transfected 1E6 cells. As shown in Fig. 1, cells expressing RnI7, CfOR12A07 and mOR23 elicited a strong increase in SRE-luciferase activation following odorant exposure with a 21-fold, an 18-fold, and a 28-fold increase above background levels, respectively (Fig. 1). Although Fig. 1 does not show the usual linear dynamic curves, it clearly indicates a dose-dependent activation of SRE with a maximum activity at concentrations of 750 μM and 250 μM for octanal and lyral, respectively. In addition, we observed a relative decrease (compared to the maximum) in SRE activity at very high odorant concentration.

In contrast, cells expressing mOREG exhibited basal levels of SRE-luciferase activity following eugenol exposure. In order to check the functionality of mOREG, cells expressing the different ORs, were exposed to their respective ligands, and analysed for the CRE (Cre responsive element) responses. As detailed in Fig. S2, we obtained strong CRE responses showing clear linear dose dependant relationship with an EC50 of 500 μM for octanal, 60 μM for lyral and 30 μM for eugenol (Fig. S2) in agreement with previous studies [14,33,34,36]. This result indicated that the failure of mOREG to exhibit an SRE response was not due to a technical artefact (lack of functionality of the OR or the lack of efficacy of the ligand), but reflected actual differences between these ORs.

3.2. OR activation of SRE requires both the ERK and Rock pathways

The SRE element contains two distinct binding sites for TCF (ternary complex factor), a family of related transcription factors [37] and SRF (serum response factor). While the activity of TCF requires the sequential phosphorylation of Raf–Mek–Erk through the Ras signalling pathway [37] the SRF activity is Rho kinase dependent [24]. To evaluate the respective role of TCF and SRF we activated OR transfected 1E6 cells with their respective OR ligands as above but in the presence of a number of specific inhibitors. Results indicated that the addition of UO126, an ERK inhibitor, inhibited SRE activation induced with RnI7, CfOR12A07 and mOR23 by 45%, 62% and 36% respectively (Fig. 2). In contrast, inhibitors of JNK (SP600125) and p38 MAP kinase (SB203580) did not have any effect, as SQ22536, an adenylate cyclase inhibitor added in this experiment as a negative control.

These data show that the ERK pathway, but not JNK and p38 pathways, is involved in SRE activation.

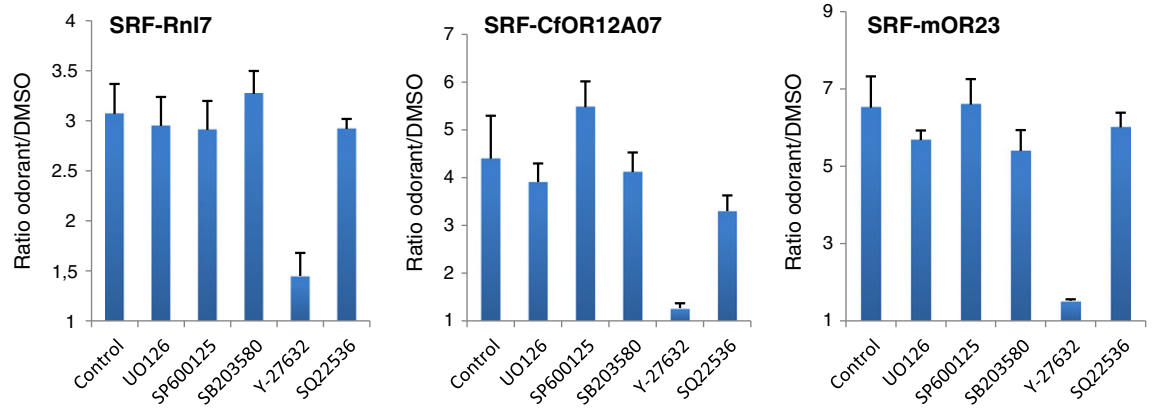

Fig. 3. SRF transcriptional activation following OR stimulation is mediated by Rock kinase. 1E6 cells were transiently transfected with OR and the luciferase construct containing the SRF binding site and with pRLSV40. Luciferase measurements in the presence of inhibitors are as described in Fig. 2. The profile of SRF transcriptional activity is the same as for SRE activation (see Supplementary Fig. S3); one observes an absence of SRF activation in mOREG-transfected cells while RnI7, CfOR12A07 and mOR23 are potent inducers of SRF. Data in Fig. 3 showed that SRF activation is inhibited by the Rock kinase inhibitor (Y27632). MAPkinases have no effect on SRF as demonstrated by the absence of response observed with the addition of UO126, SP600125 and SB203580.

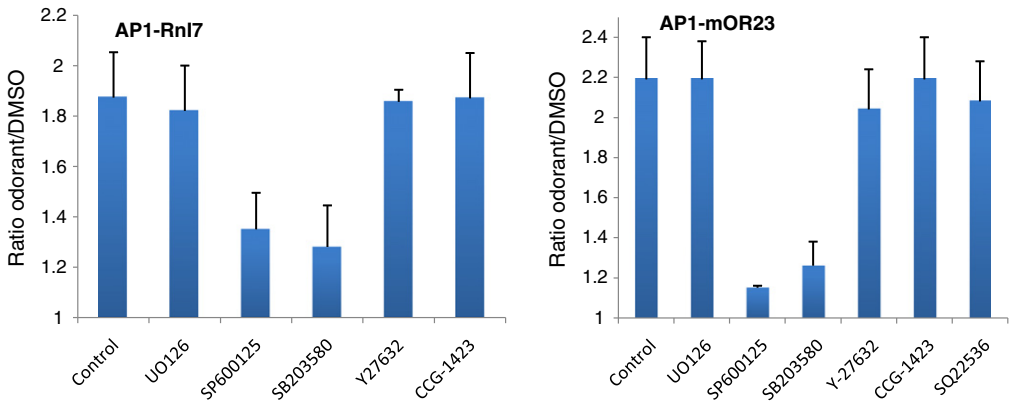

Fig. 4. AP1 activation following OR stimulation. AP1 transcriptional activity was measured in cells transfected with different OR containing plasmids together with pAP1-luc and pRL-SV40. Prior to stimulation, cells were incubated for 2 h in the presence of UO126 (10 μM, ERK inhibitor), SP600125 (10 μM, JNK inhibitor), SB203580 (20 μM, P38 inhibitor), Y27632 (20 μM, ROCK inhibitor), CCG-1423 (10 μM, a Rho/SRF pathway inhibitor) or SQ22536 (200 μM, AC inhibitor). Rnl7-transfected cells were stimulated with octanal (750 μM) and mOR23-transfected cells were stimulated with lyral (250 μM). Data were expressed as a ratio between odorant responses versus control responses as for Fig. 2. A significant increase of AP1 transcriptional activity was observed in cells transfected with Rnl7, CfOR12A07 or mOR23 following specific stimulation. Similarly to SRE profile, mOREG did not show any AP1 transcriptional activity, whereas CRE is strongly activated via this same OR (see Fig. S5). Results presented in Fig. 4 show that in contrast to SRE, AP1 transcriptional activity is strongly inhibited by JNK inhibitor (SP600125) and P38MAPkinase inhibitor (SB203580) indicating that AP1 activation is mediated through JNK and P38 MAPkinase pathways but not the ERK or Rock pathways.

Since activation of SRF requires Rho function [24], we tested the ability of Rho small GTPase pathway to affect SRE reporter activity. As shown in Fig. 2, the use of Y-27632, a Rock inhibitor, induced a strong reduction of SRE-luciferase activation by 62%, 51% and 90% for Rnl7, CfOR12A07 and mOR23, respectively confirming the specific implication of the Rock pathway. Furthermore, with the simultaneous

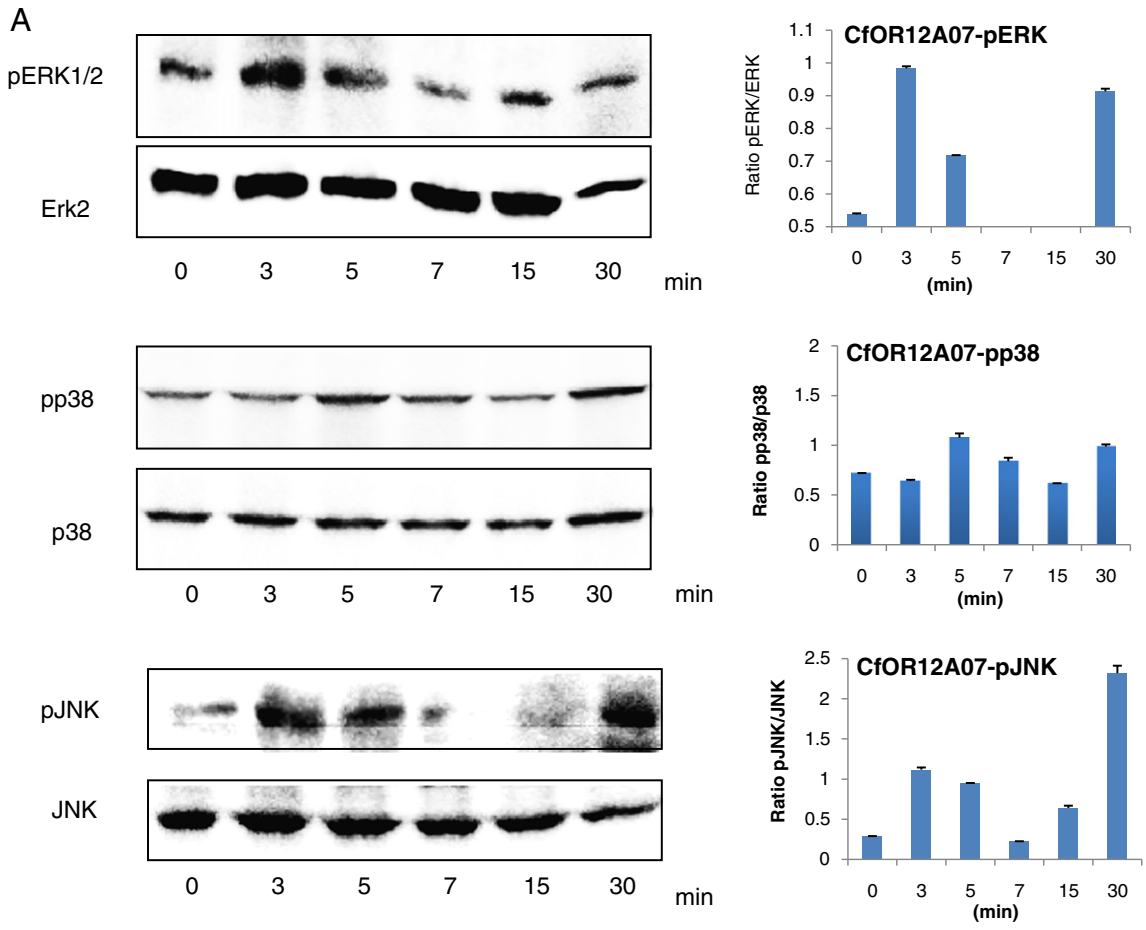

Fig. 5. Phosphorylation of MAPkinases following CfOR12A07 and mOR23 stimulation. Phosphorylation of MAPKs was measured by Western blot analysis. OR transfected cells were stimulated by lyral, octanal or eugenol for different time periods. MAPK phosphorylations were detected by Western blot analyses with specific antibodies to phospho ERK, phospho JNK and phosphop38MAPK. The same blots were stripped and probed again with specific antibodies directed against ERK2, JNK and p38 respectively (left panels). Densitometry analysis has been performed using ImageJ software (NIH) and results are represented as ratios phosphoMAPKs/total MAPKs (right panels). Results indicated that stimulation of CfOR12A07 (A) and mOR23 (B) induced a phosphorylation of ERK, p38 and JNK whereas eugenol stimulation of mOREG did not show any significant increase of ERK and p38 phosphorylation (see mOREG profiles in Supplementary Fig. S6). The phosphorylation profiles obtained with Rnl7 were equivalent to those obtained with CfOR12A7 (not shown).

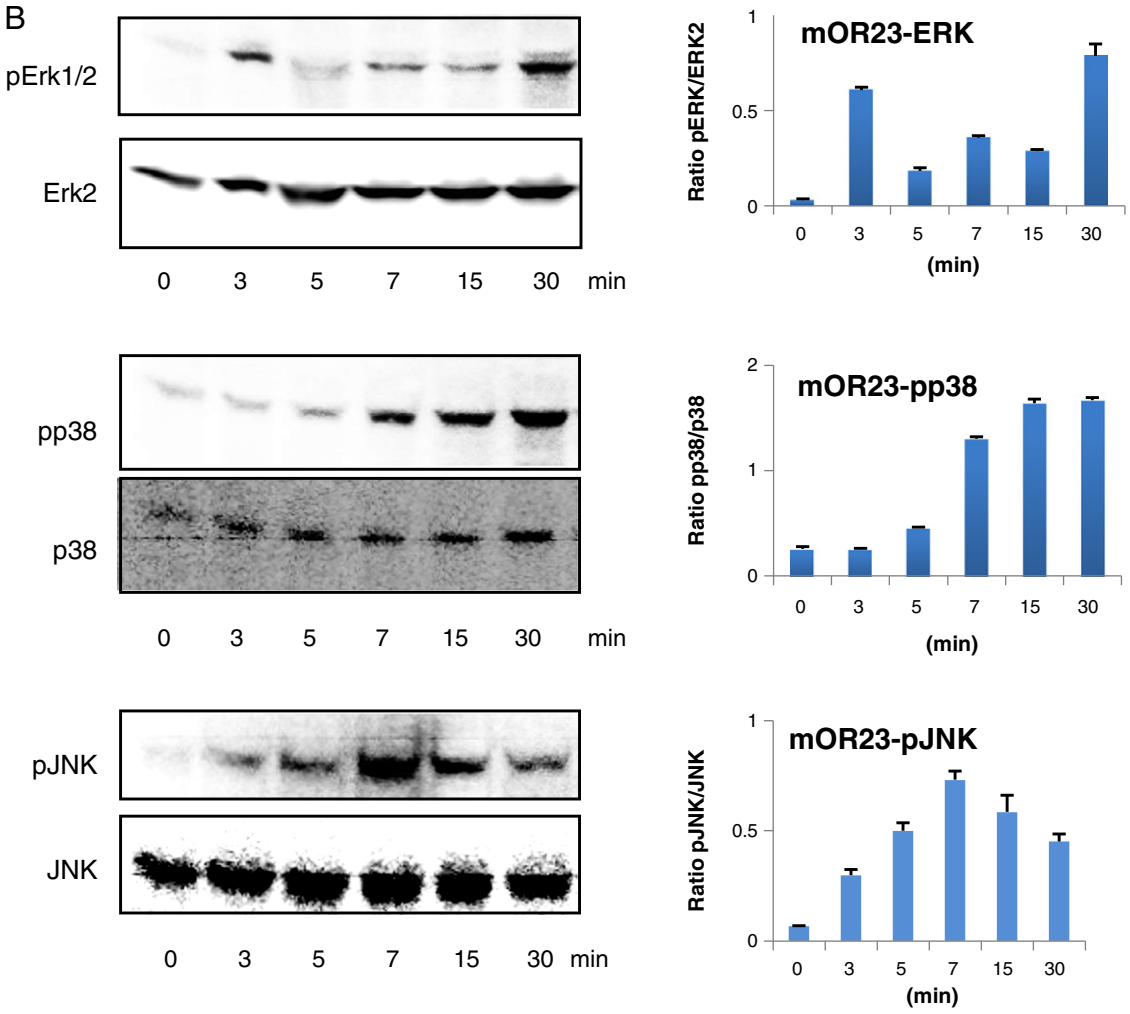

Fig. 5 (*continued*).

use of both ERK and Rock inhibitors, we observed a stronger inhibition of SRE-luciferase following stimulation of RnI7 and CfOR12A07 (respectively 74% and 79% instead of 45%, 62% and 62%, 51% when applied separately) (data not shown) indicating an additive or cooperative effect of the two pathways. These observations demonstrated that the activation of SRE observed upon the stimulation of some ORs by their ligands follows the usual pathways of activation of this element namely the ERK and the Rho pathways.

3.3. Induction of SRF reporter activation following stimulation of RnI7, CfOR12A7 and mOR23

We then transfected the 1E6 cells as above but with a different reporter plasmid named SRF-luciferase which contains only the CCATATTAGG sequence instead of the whole SRE sequence. This sequence binds SRF but not TCF [38]. As shown in Fig. S3, the binding of octanal or lyral to their specific receptors, RnI7, CfOR12A07 and mOR23, induced a SRF luciferase reporter activity, which was dose dependent. As above, stimulation of mOREG by eugenol had no effect (Fig. S3). As shown in Fig. 3, the Rock inhibitor Y27632 induced a strong decrease of SRF activity, whereas the MAPK inhibitors for ERK, JNK and p38 did not have any effect on SRF induction. Altogether these experiments showed that following OR activation, SRE activity is under the control of both SRF via the Rho/Rock kinases and TCF via the Raf–Mek–Erk cascade.

3.4. ORs stimulate SRE-luciferase via Rho and Rock but not Rac

To further elucidate the role of the Rho pathway, we used two different specific inhibitors. As shown in Fig. S4A and B, the permeable form of C3 transferase or CCG-1423, a newly described inhibitor of Rho pathway [39], blocked RnI7-, CfOR12A07- and mOR23-induced SRE activation, demonstrating that the response of this cis-reporter element is Rho-dependent. As it has been shown that some GPCR agonists activated simultaneously Rac and RhoA [18], we used a specific inhibitor to determine whether Rac was also involved in SRE activation. Data indicated that unlike the RhoA inhibitor, the Rac inhibitor NSC23766 had no effect indicating that RhoA but not Rac regulated the downstream signalling pathway leading to SRE activation following odorant OR interactions (Fig. S4A.).

3.5. Regulation of AP1 reporter activation by ORs through JNK and P38 MAPK pathways

Next, we investigated the capacity of ORs to activate other transcription factors, including AP1, NFAT and NF-KB. Of the cis elements tested here, only AP1 was activated by ORs upon the presence of odorants (Fig. S5). Conversely, the cis elements corresponding to NF-KB and NFAT were not activated by odorant stimulated ORs (data not shown). Unlike RnI7, CfOR12A07 and mOR23, activated mOREG failed to significantly stimulate the AP1-luciferase activity. As shown in Fig. 4, 28% decrease and 31% decrease in AP1 activation were observed in the

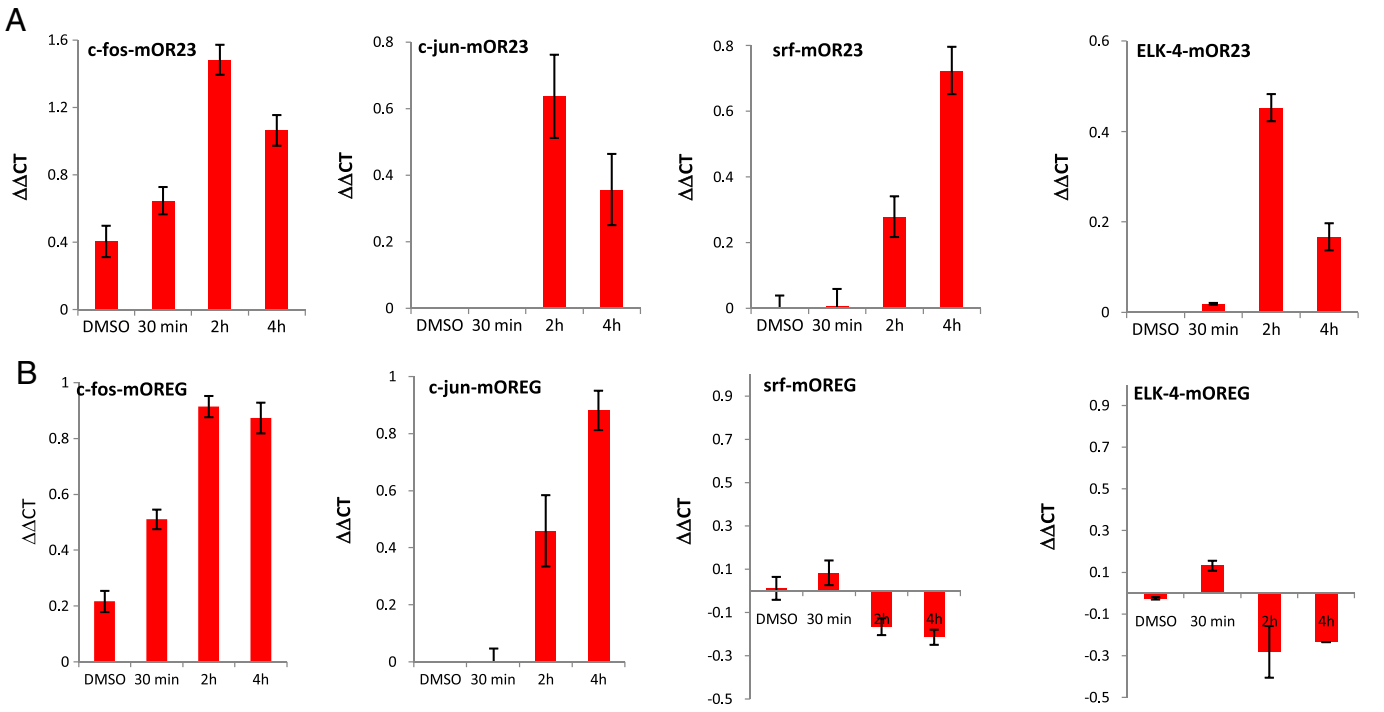

Fig. 6. c-fos, c-jun, SRF and ELK-4 mRNA expression following OR stimulation. Transcription of c-fos, c-jun, SRF, ELK-1 and ELK-4 was measured by RT-PCR. Time stimulation is indicated for each OR. Optimal odorant concentrations were 250 μM for lyral, 250 μM for eugenol (or 750 μM for octanal — see Supplementary Fig. S7). ΔΔCt values were expressed with respect to 18S mRNA as internal reference as specified in Materials and methods. Data showed that c-fos and c-jun mRNA concentrations were increased with all ORs, whereas SRF and ELK-4 mRNA concentrations were not increased in mOREG-transfected cells. These results suggested that the lack of SRE activation in mOREG-transfected cells accounted at least partly for an absence of both SRF and ELK-4 transcription. Bars correspond to the standard deviation calculated with four technical replicates.

presence of SP600125 (JNK inhibitor) and SB203580 (p38MAPK inhibitor), respectively under RnI7 activation. A decrease in cis AP1 activation of about 48% and 43% was observed using the same MAPK inhibitors on mOR23-transfected cells (Fig. 4). Of note, identical results were observed with CfOR12A07 (data not shown). Altogether these experiments indicated that the profile of AP1 cis element activation following specific ligand exposure was the same as observed with SRE and SRF reporter elements. However unlike SRE, which is under the control of ERK, AP1 cis element is under the control of both JNK and p38MAPK pathways.

3.6. Analysis of MAPK phosphorylation

Using inhibitors we have observed that MAPKs were involved in SRE and AP1 reporter activities following OR activations. We then wondered whether an early phosphorylation of MAPKs could be observed. Phosphorylations of ERK, JNK and P38 MAPK were analysed by Western blot, with specific antibodies directed against the MAPK phosphorylated form and normalised with respect to the amount of their respective total protein determined by specific antibodies against the nonphosphorylated protein. Data indicated that stimulation of CfOR12A07 and mOR23 induces a phosphorylation of ERK, JNK and p38AMPK at early time points (Fig. 5A and B). Of note, similar results were obtained with RnI7 (data not shown), whereas eugenol stimulation of mOREG did not show any significant increase of ERK and p38 phosphorylation in agreement with our results obtained with reporter genes and inhibitors. However a slight but significative phosphorylation of JNK was observed following eugenol stimulation of mOREG (Fig. S6).

3.7. Cjun, cfos, srf and ELK-4 mRNA expression following OR stimulation

To analyse the expression levels of several MAPK and Rho target genes, we prepared mRNA at different time points following 1E6 cell odorant exposure and performed real time PCR. We found c-fos, c-jun, srf and ELK-4 markedly up-regulated by exposure of CfOR12A07-RnI7 and mOR23 to their cognate ligand (Figs. 6 and S7). In contrast, we observed no change in the transcriptional level of ELK-1. Interestingly cells expressing mOREG and exposed to eugenol displayed a down regulation of Srf and ELK-4 mRNAs and an up regulation of c-fos and c-jun (Fig. 6). This result along with the slight phosphorylation of JNK observed above suggested the implication of a different downstream pathway for mOREG compared to the three other ORs.

3.8. MAPK phosphorylation in OSNs in culture and in vivo

The in vitro experiments reported above, indicated that several MAPKs were activated upon the binding of odorants onto their cognate ORs. We thus wondered whether this activation could correspond to some artefacts due to the heterologous nature of the system used or whether they actually mimicked OSN behaviour. To approach this question we performed two types of experiments. In one of them, OE was dissected from unexposed rats and OSN subcultures were made as described in the Materials and methods section. Following a limited number of cell passages (<3), rat OSNs were exposed to a cocktail of odorants. In another set of experiments, rats exposed to the same cocktail of odorants were sacrificed few minutes later and their olfactory epithelium (OE) promptly removed for further analyses.

The quality of the OSNs primary cultures in our hands was checked by in situ hybridization experiments (ISH). As OSN apparition and maturation occurred after day 7 [40], we performed all experiments after day 10 and after less than 3 passages. For ISH experiments, non-stimulated and octanal-stimulated cultures were fixed and labelled with a mixture of three probes specific to RnI7 as described in the Supplementary Fig. S8 legend. Images were obtained with a Leica microscope, and show confluent neurons. OSNs expressing RnI7 mRNA can be detected in 2 h-odorant treated cultures (Fig. S8), indicating that

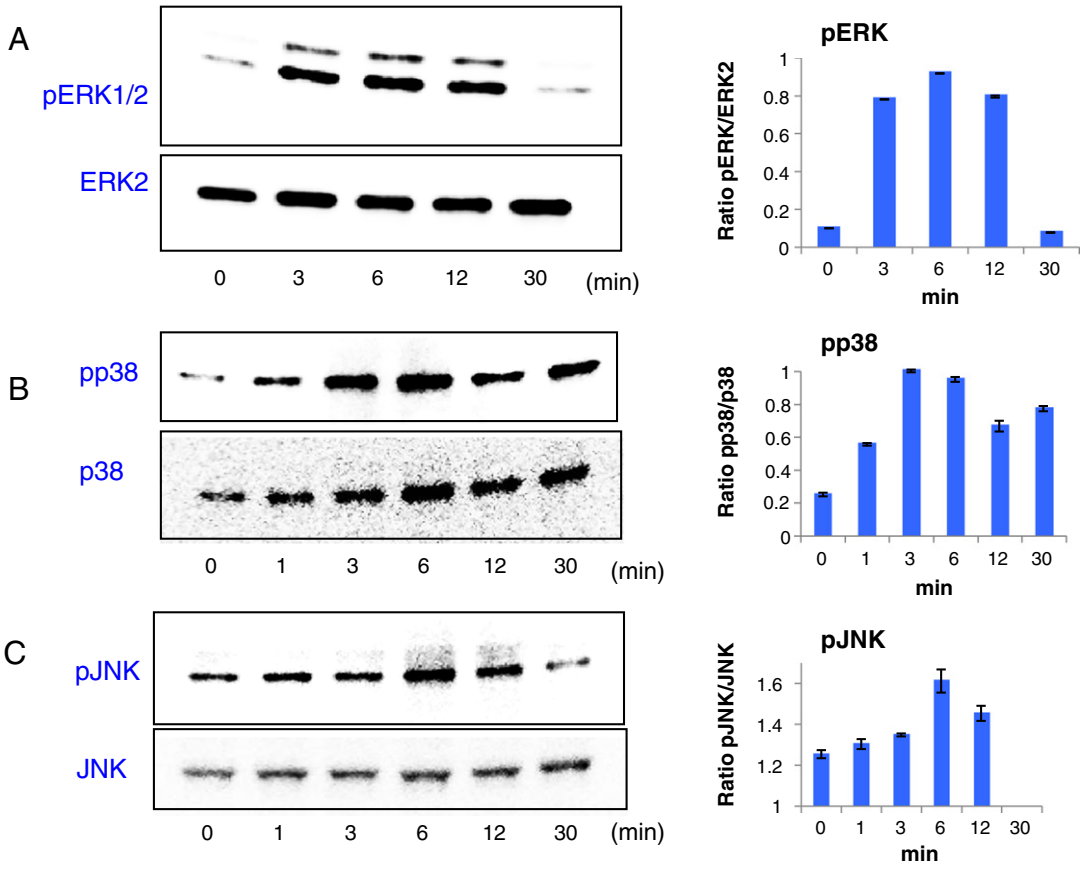

Fig. 7. Activation of MAPkinases in OSNs following odorant stimulation. Activation of MAPKs by odorants was determined in OSN primary cultures by Western blot analysis. Cells were stimulated by a cocktail of odorants for different time periods. The cocktail of odorants used for in vivo and OSN experiments was composed of a mixture of the following odorants (equal volumes): Aldehydes (hexanal, 1-heptanal, octanal, nonanal, decanal, undecal, dodecanal), alcohols (1-hexanol, 1-heptanol, 1-octanol, 1-nonanol, 1-decanol, 1-undecanol), acids (hexanoic acid, heptanoic acid, octanoic acid, nonaoic acid), ester (ethyl caproate, ethyl heptanoate, ethyl caprylate, ethyl nonaoate, ethyl caprate, ethyl dodecanoate), ketones (hexanone, 4-heptanone, 3-octanone, 3-nonanone, 3-decanone, 2-undecanone), limonene, lyral, eugenol, vanillin, methyl benzoate, acetophenone, cyclohexanone, benzaldehyde, butyric acid. Phosphorylated MAPKs were detected by Western blots with specific antibodies to phospho ERK, phospho JNK and phosphop38MAPK. The same blots were stripped and probed again with specific antibodies directed against ERK2, JNK and p38 respectively (lower left panels). Densitometry analysis results are presented as ratios phosphoMAPKs/total MAPKs. Data indicated a clear phosphorylation of ERK, p38 and JNK.

the neuronal cultures corresponding to OSNs are able to express endogenous ORs.

As illustrated in Fig. 7, exposure of OSN primary cultures to a cocktail of odorants stimulated the activity of ERK, p38 and JNK. Using olfactory epithelium prepared from rats exposed to odorants prior to be sacrificed we also observed an increase in the phosphorylation of JNK and p38. A stimulating effect of JNK was detected after as early as 7 min of incubation and peaked at around 15 min before to decline to basal level at 30 min (Fig. 8) and phosphorylation of P38 MAPK was significantly increased at later time points. However, ERK phosphorylation was not increased in these experimental conditions (Fig. 8). The lack of observation of ERK phosphorylation could be due to the delay between the time of odorant exposure and the time of analysis (7 min.).

3.9. C-jun, c-fos, srf and ELK mRNA prepared from rat OE and OSN cultures

To further determine the behaviour of transcription factors involved in odorant stimulation, real time PCR analyses were performed with RNA extracted from OSN cultures and OE dissected from rats exposed to odorants prior to be sacrificed. As shown in Fig. 9, we found a strong up-regulation of c-fos in OE prepared from odorant exposed rats. Expression of c-jun, srf, ELK-1 and ELK-4 was also up regulated. Similar results were obtained with OSNs in culture (Fig. 10), which gave the same profiles as odorant stimulated 1E6 cells (Fig. 6). These results demonstrated and reinforced the implication of the downstream MAPK pathways following OR-ligand interactions.

3.10. Both MAPK and AKT pathways are essential for odorant-mediated OSN survival

As shown in Fig. 11A, we found that activation of OSNs by odorants promoted cell survival in a dose dependent manner as indicated by the MTT assay. It has been previously shown that MAPK, Rho and AKT signalling pathways were involved in neuronal survival [41,42]. Therefore, to examine whether the activation of these signalling pathways by odorants was implicated in the neuroprotective effect that we observed, we treated OSN cultures for 2 h with several specific inhibitors of the MAPK members and AKT prior to odorant activation. Fig. 11B shows that AKT, ERK and JNK, and to a lesser extent P38 and Rho are required for OSN survival in the presence of odorants. Inhibition of AKT or ERK1/2 reduced cell viability by nearly 95% and 85% respectively in odorant-treated cells and by 90% and 22% respectively in odorant untreated OSNs (Fig. 11B). Without odorant, data showed that only AKT and JNK seem to be significantly important for OSNs survival.

4. Discussion

It is well known that GPCRs interact with multiple signalling molecules including MAP kinases to participate to cellular homeostasis [17–21]. GPCR family members are able to enhance SRE transcriptional activity by stimulating the activity of SRF in NIH3T3 cells [43]. Among different transcription factors phosphorylated and activated by the

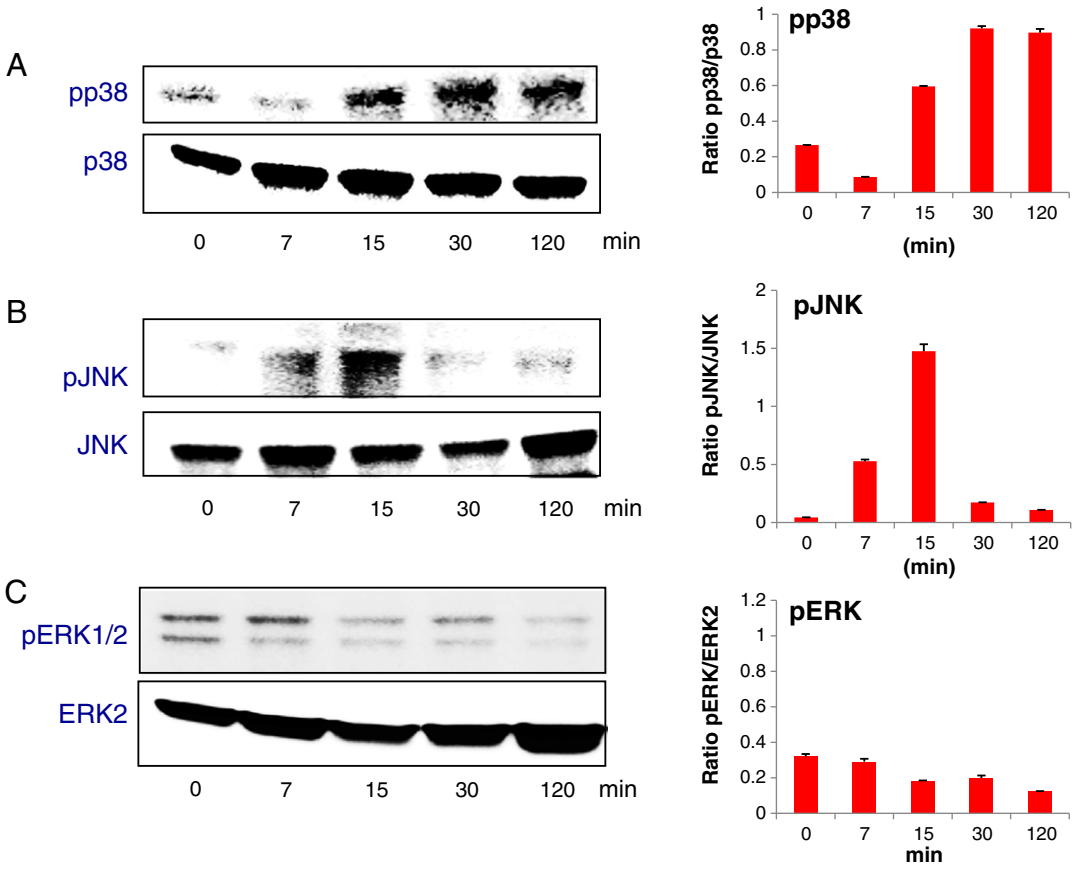

Fig. 8. Activation of MAPkinases in vivo. Activation of MAPKs by odorants was determined in dissected OE by western blot analysis with specific antibodies to phosphop38MAPK, phospho JNK and phospho ERK. Rats were stimulated by the cocktail of odorants for different time periods. Results are expressed as in Fig. 7. Densitometry analysis has been performed using ImageJ software and results (right panels) indicated a clear phosphorylation of JNK and p38MAP kinase but not of phospho ERK2.

MAPKs are TCFs, which include ELK-1, ELK-4, and ELK-3 [44,45]. Each of these proteins forms a ternary complex on target promoters made of one molecule of TCF with two molecules of SRF [44]. In addition, Rho GTPases including RhoA, Rac1, and Cdc42, and their downstream targets can also be activated by a variety of GPCRs [46], thus regulating a wide variety of signalling processes and much work has been attempted to elucidate the role of Rho pathways [47,48]. We thus wondered whether ORs, known to constitute so far the largest group of GPCRs, could also interfere with these pathways and be implicated in the differentiation and survival of olfactory sensory neurons (OSNs).

Our results showed a crucial relationship between OR/odorant stimulation and phosphorylation of MAPKs in a heterologous system using 1E6 cells (expressing the Gαolf). Furthermore, we showed that stimulations of mOR23, RnI7 and CfOR12A07 induced the activation of both SRE and SRF luciferase reporter elements. In contrast, mOREG activated neither of them. Physiological activation of SRE occurs following a sequential phosphorylation of the Raf–Mek–Erk signalling cascade [37]. In conjunction with the use of chemical inhibitors of the phosphorylation of MEK1/2 (UO126), JNK (SP600125) and p38MAPKs (SB203580), we showed that stimulated ORs use the ERK pathway to activate SRE but not SRF, whereas JNK and P38 remained without effect on SRE. We also showed that the use of Y-27632 (a ROCK inhibitor) as well as C3toxin (RhoA inhibitor) and CCG1423 (RhoA/SRF inhibitor) strongly inhibited the activation of SRE demonstrating a link between RhoA

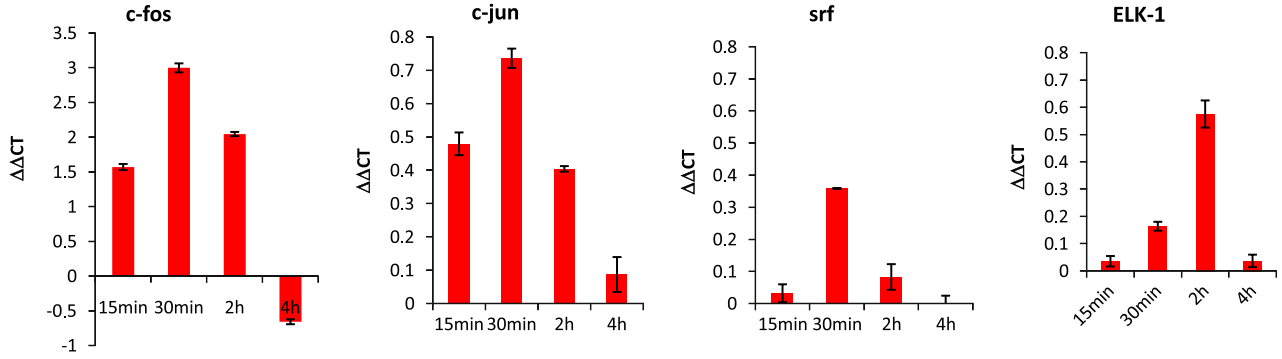

Fig. 9. cfos, cjun, srf and ELK-1 mRNA expression in vivo. Transcription of c-fos, c-jun, srf, ELK-1 and ELK-4 was measured by real time RT-PCR in rats exposed to odorants in vivo. Time of stimulation start is indicated on the x axis. ΔΔCt values were expressed with respect to *beta-actin* mRNA used as internal reference and normalised to control rats. Data showed a strong c-fos expression, and a lower but significant c-jun, SRF and ELK-1 mRNA.

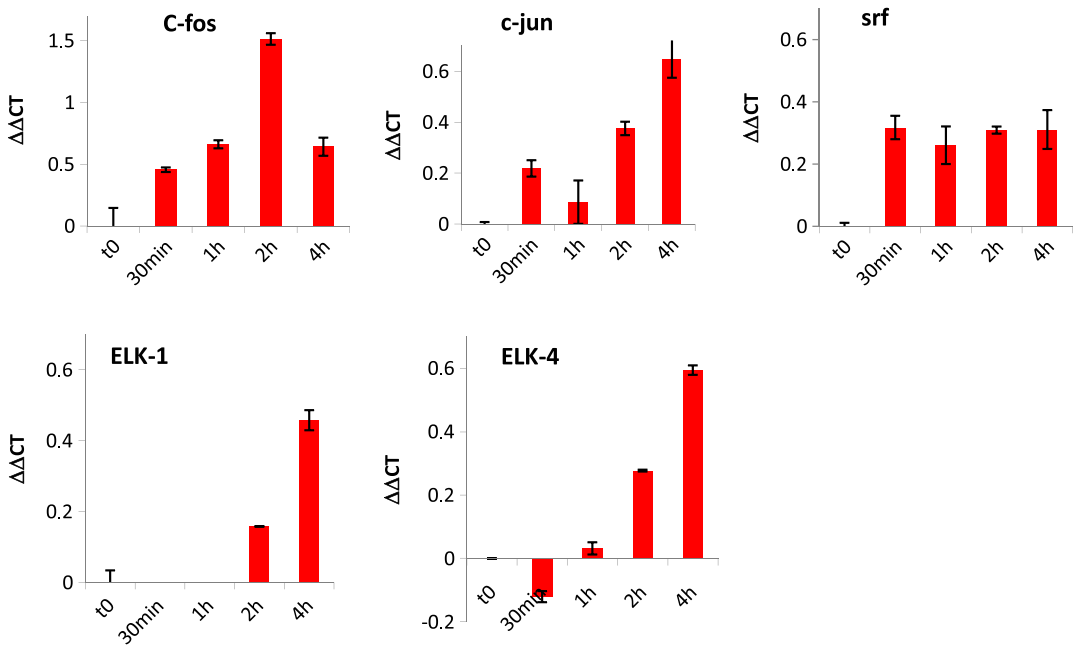

Fig. 10. c-fos, c-jun, SRF and ELK-4 mRNA expression in primary OSNs. Transcription of target genes was calculated by real time quantitative RT-PCR in primary OSNs exposed to odorants. ΔΔCt values were expressed with respect to *β-actin* mRNA as internal reference as in Fig. 9. Data showed an increase of c-fos, c-jun, SRF, ELK-1, and ELK-4 mRNA expression.

and SRF pathways following OR stimulation. In agreement with our data, it has been shown that RhoA regulated the SRF-dependent transcription of a number of neuronal specific genes [24,49]. Altogether these results showed that Rho/Rock and ERK did function in a cooperative manner to activate SRE. Our observations showing that ERK was an activator of SRE in 1E6 cells confirmed previous observations on the direct phosphorylation of ELK-1 by ERK [44]. Furthermore, the possibility of a cross-talk between the Rho and ERK pathways may still exist, as several investigations have linked these two signalling pathways to Rho/Rock/LIMK inhibition in a MEK-dependent manner [50,51].

GPCRs have been shown to stimulate a variety of other downstream signalling pathways such as STAT3 [52,53], NFKB [52], AP1 [54], and NFAT [55]. Using reporter genes, we found that in the 1E6 cell line neither NFAT nor NFKappaB was involved in response to odorant stimulation (data not shown). In contrast, mOR23, RnI7 and CfOR12A07 activated directly AP1, in correlation with the induction of JNK phosphorylation and p38MAPK as demonstrated by Western blot analysis. Using chemical inhibitors we demonstrated that the MAPK pathway was involved in the activation of both SRE and AP1 pathways but differently. Activation of AP1 cis element involves the direct phosphorylation of AP1 components (c-fos and c-jun) as well as the phosphorylation and activation of transcription factors that induce elevated expression of c-jun and c-fos [56]. The DNA-binding activity of AP1/c-jun and its role in OR signalling remains unknown.

We also showed by real time PCR an elevated expression of c-fos, c-jun, srf, and ELK-4, but not of ELK-1 in response to RnI7, CfOR12A07 and mOR23 odorant stimulation, in line with the activation of both SRE and AP1 cis elements. The transcriptional activation of ELK-4 instead of ELK-1 suggests that ELK-4 is involved in the MAPK pathway leading to SRE activation in 1E6 cells. Whereas eugenol stimulation of mOREG failed to induce phosphorylation of ERK as well as SRE/SRF transcriptional activation, it induced a strong c-fos and c-jun mRNA synthesis. One explanation for this latter observation would be the induction of c-fos/c-jun transcription via CREB activation [57] since we showed that mOREG is a potent activator of CRE following its ligand stimulation.

It is rather intriguing that mOREG behaved differently from the three other tested ORs and we suggest that mOREG induces the activation of other independent signalling pathways such as PI3K/AKT pathway that has been shown to promote mature neuronal survival [41,58]. It would be of interest to determine whether other ORs behave as mOREG.

We then questioned whether the findings we obtained using an heterologous in vitro system, in which kidney cells of human origin were transfected to express a human Gα subunit and canine/rat or murine ORs, were relevant in primary OSNs cultures or in vivo. To this end, OSNs were prepared from 4 to 5 week old rats and were subjected to odorant stimulation in vitro. We also exposed rats to odorants prior to sacrifice them to prepare at various times thereafter OE for further analysis. In both cases, odorant stimulations were performed using a cocktail of odorants, in order to increase the number of target OSNs. First, in line with the profile obtained using the in vitro heterologous system, we showed that odorant exposure of OSNs enhanced ERK1/2, JNK, and p38 pathways as shown by the increased phosphorylation of these kinases. Similarly, odorant exposure of rats also resulted to a significant increase of phosphorylation of JNK and p38 MAPK in microdissected nasal epithelia. We also demonstrated that odorant stimulations induce an increase in OSNs survival as judged by the increase response in the MTT assay. MAPK and Akt pathways are predominant mediators of trophic signalling for many neuronal systems [41,59]. To examine the contribution of PI3K/AKT signalling and MEK/ERK1/2 signalling to odorants-mediated OSN survival, MTT assays were performed in the presence of various specific inhibitors. Our data suggest that PI3K-AKT is important for OSN survival but that ERK1/2 signalling contributes also to odorant-mediated cell survival. Furthermore, we demonstrated for the first time that the JNK and to a less extent p38 are involved in odorant-induced OSN survival. Along this line, various reports also suggest the involvement of AKT in promoting neuron survival. AKT is known to phosphorylate the proapoptotic protein BAD or caspase-9, inhibiting their proapoptotic function [60,61] and AKT pathway is also involved in the regulation of expression of the anti-apoptotic Bcl2 [41]. Interestingly, mRNA level of survival genes such as Bcl2 was found higher in OSNs treated with odorants as compared to control cells (data not shown). The roles of particular MAPK in cell death and survival are more complex and depend on cell type, magnitude and timing of stimulation. ERK pathways are mainly linked to cell survival and proliferation [62], whereas JNK and p38 pathways are characterized as stress-activated protein kinases and implicated in apoptosis [63,64].

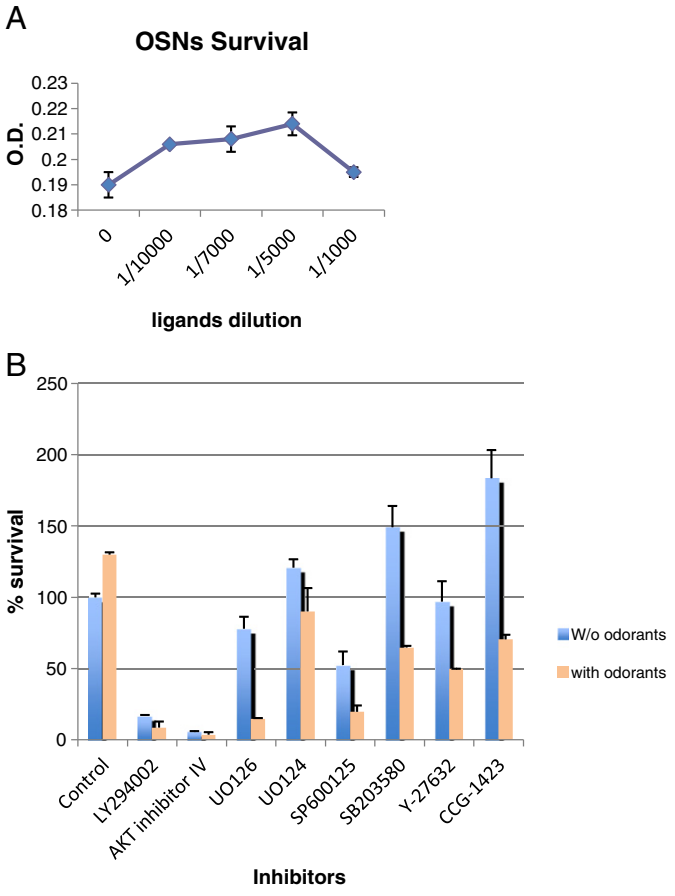

Fig. 11. Survival of OSNs in culture. OSN viability was determined using the MTT test as described in Materials and methods. A/ OSNs were cultured in the presence of different dilutions of the cocktail of odorants. Cultures were performed in triplicate and O.D. are shown. Vertical bars show the standard deviation of OD measured on the replicates. B/ OSNs were treated for 2 h in the presence of LY294002 (20 μM, AKT inhibitor), AKT inhibitor IV (2 μM), UO126 (10 μM, ERK inhibitor), UO124 (10 μM, an analogue negative control of UO126), SP600125 (10 μM, JNK inhibitor), SB203580 (20 μM, P38 inhibitor), Y27632 (20 μM, ROCK inhibitor) and CCG-1423 (10 μM, a Rho/SRF pathway inhibitor). The cocktail of odorants was then added at dilution 1/7000 (in PBS) for 18 h. MTT was then added for a further 4 h, and results were expressed as means of % survival as compared to control OSNs (without odorant). Data demonstrated that OR activation enhanced OSN survival through the activation of PI3K/AKT as well as of the MAPK and Rho pathways.

However, in other cellular contexts such as malignancies or in neuronal progenitor cells as well, JNK and p38 MAPK activity may conversely promote cell survival and differentiation, in line with our results [65–67]. Moreover, we showed that Rho (and Rho/SRF) inhibitors induced cell death in the presence of odorants. It is known that Rho family GTPases are also key regulators of neuronal survival. Interestingly, individual Rho family members have been shown to play either a pro-death or pro-survival role in the nervous system depending on both the type of neurons and the particular neurodegenerative insult involved [42,68]. In particular, it has been shown that RhoA functions to prevent death by inducing the AKT pathway [69].

The signalling effectors aforementioned play a critical role in neuronal regeneration and survival in the central nervous system [41,65,67,68,70]. Particularly, c-fos affects the regulation of many genes involved in cell growth, differentiation and transformation under strictly regulated conditions [71]. In addition to its role in cell survival, the MAPK pathway is well known for influencing proliferation and has been reported to promote differentiation as well [41].

We are aware that primary cultures of olfactory sensory neurons (OSNs) are difficult to establish. However the concordant results we obtained with cultured OSN and OE micro-dissected from rats following their exposure to odorants leave no doubt on the quality of the OSN per se and on the faithfulness of the results we obtained with the three experimental systems.

These results raise an intriguing possibility that odorant activated ORs would utilize more than one mechanism to protect OSNs and could act as a survival factor. Given the role of these signalling pathways, first we hypothesize that the binding of an odorant onto its OR not only elicits the IP3 and cAMP pathways that transform a chemical signal into an electrical signal that conveys information to different brain structures for odorant recognition, but also induced the AKT, MAPK and Rho and possibly other signalling cascades to prolong OSN survival and induce the differentiation programmes of neighbouring immature cells. Indeed, in vivo, olfactory OSNs undergo continuous regeneration, differentiation and maturation to replace damaged OSNs [72,73] and it has been shown that odorant exposure support OSNs survival and neo-neurogenesis [15,16] in agreement with our data. Second, we hypothesize that the direct induction of these programmes through the binding of an odorant onto its receptor does concern specific OSN populations. In other words we postulate that it is the very set of OSNs, of which the ORs are activated by the binding of their ligands that would be the target of the survival programme and would induce the differentiation of neighbouring OSN progenitors.

Authors' contributions

NB conceived of the study, performed experiments and drafted the manuscript. SE participated in the design of experiments and helped to draft the manuscript. FG participated in the design of the study and its coordination, performed in vivo experiments and helped to draft the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2013.02.019.

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