Published in final edited form as:

Neuroscience. 2008 November 19; 157(2): 340–348. doi:10.1016/j.neuroscience.2008.09.016.

SIGNAL TRANSDUCTION AND GENE EXPRESSION IN CULTURED ACCESSORY OLFACTORY BULB NEURONS

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Abstract

Glutamate and norepinephrine (NE) are believed to mediate the long-lasting synaptic plasticity in the accessory olfactory bulb (AOB) that underlies pheromone recognition memory. The mechanisms by which these neurotransmitters bring about the synaptic changes are not clearly understood. In order to study signals that mediate synaptic plasticity in the AOB, we used AOB neurons in primary culture as a model system. Because induction of pheromone memory requires coincident glutamatergic and noradrenergic input to the AOB, and requires new protein synthesis, we reasoned that glutamate and NE must induce gene expression in the AOB. We used a combination of agonists that stimulate α -1 and α -2 adrenergic receptors in combination with NMDA and tested expression of the immediate-early gene c-Fos. We found that the glutamatergic and noradrenergic stimulation caused significant induction of c-Fos mRNA and protein. Induction of c-Fos was significantly reduced in the presence of inhibitors of protein kinase C, MAP kinase and phospholipase C. These results suggest that glutamate and NE induce gene expression in the AOB through a signaling pathway mediated by PKC and MAPK.

Keywords

glutamate; norepinephrine; protein kinase C; MAP kinase; immediate-early gene; c-Fos

The accessory olfactory bulb (AOB), for the past several years, has been the subject of investigations attempting to understand the role it plays in pheromone signal processing and pheromone memory formation. The AOB shares some similarities with the main olfactory bulb (MOB), such as having similar lamination patterns and similar neuronal types within the laminae. Much work has been carried out in order to better understand the signaling between neurons that underlies sensory processing in the AOB. The majority of this work has been restricted to behavioral and *in vivo* studies as well as some electrophysiological studies (Brennan and Keverne, 1997). Many of the important questions about the signal transduction within the AOB neurons, however, remain unanswered.

Previous studies have elucidated the neurotransmitter systems that are likely to play a role in activating the signaling mechanisms in the AOB. Several studies have infused pharmacological agents directly into the AOB in order to disrupt the normal signaling and thereby identify

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mechanisms important in, for example, pheromone memory formation (Kaba and Keverne, 1988; Kaba et al., 1989). These infusion studies have established a role for glutamate and norepinephrine (NE) in mediating signaling in the AOB. Also, the behavioral studies in mice have established that expression of immediate-early genes c-Fos and Egr1 occurs in the AOB by pheromone memory-inducing stimuli (Brennan et al., 1992). Details of the pathway connecting glutamate and adrenergic receptors to gene expression in the AOB are less well known. One set of experiments used infusion of anisomycin into the AOB demonstrating that protein synthesis is required for pheromone memory formation (Kaba et al., 1989).

Long-lasting changes in the AOB are likely to be mediated by gene expression. Understanding how glutamate and NE induce gene expression would be valuable for elucidating the AOB plasticity that is thought to underlie behavioral changes such as pheromone memory. Although some information regarding the signaling molecules that might be critical in AOB are available through prior behavioral studies, these studies used agonists or antagonists that were not highly selective. We used cultured AOB neurons with a view to develop a tractable model system that might allow us to mimic the glutamatergic and adrenergic signaling in the AOB.

We hypothesized that protein kinase C (PKC) plays a key role in linking glutamate and NE to gene expression. Previous experiments showed that infusion of a non-selective PKC inhibitor polymyxin B into the AOB of female mice immediately after mating prevented formation of pheromone memory (Kaba et al., 1989). Ongoing electrophysiology experiments in our laboratory indicated a role for PKC in mediating some of the immediate effects of glutamate and NE on ion channel activity (Hegde et al., 2005). Therefore, as a first step towards understanding AOB signaling, we stimulated cultured AOB neurons using glutamatergic and noradrenergic receptor agonists and tested the potential role of PKC in mediating gene expression. After stimulation, we examined the neurons for changes in expression of the immediate-early gene c-Fos. In addition, we used inhibitors of PKC, Erk1 and phospholipase C (PLC) to test the effect on agonist-induced c-Fos expression.

EXPERIMENTAL PROCEDURES

Animals

Mice were obtained from Charles River (Wilmington, MA) and all the experiments using animals were carried out under a protocol approved by the Institutional Animal Care and Use Committee of Wake Forest University Health Sciences.

Dissection of AOB from adult female mice

Adult, virgin, female Balb/c mice were deeply anesthetized using isoflurane. The top of the skull was removed and the frontal cortex with attached OB was pinned in a dissecting dish containing ice-cold Hank's balanced salt solution (HBSS, Invitrogen; Carlsbad, CA) and placed on an ice-cold block, the OB was viewed through a dissecting microscope and bisected revealing the laminations of the AOB. The AOB was removed using a fine-pulled pipette and kept in ice-cold Hibernate medium (Brain Bits; Springfield, IL) until all tissue was collected.

RNA Isolation

RNA isolation was carried out using the Ambion RNAqueous 4-PCR kit (Ambion, Austin, TX). Briefly, the culture medium was aspirated from a well and 100 μ L lysis buffer was added to the well to stop the reactions. A cell scraper was used to ensure that all cells were removed from the well floor. Lysis buffer was pipetted out into a clean, RNase free tube. RNA was isolated according to the manufacturer's instructions.

Primer design and RT-PCR of signaling molecules

Primer sequences (shown in Table 1) were designed by one of two strategies. Current literature was searched for potentially relevant primers which were used successfully for amplification of the target molecules. In some cases, those primers were used as a starting point for the selecting our primers (Yoshimura et al., 1997; Xiao et al., 1998; Pauken and Capco, 2000; Terao et al., 2003; Ren et al., 2004; Kitayama et al., 2004; Brand et al., 2005; Boon et al., 2005; Abe et al., 2005). When a suitable or useful primer was not found, primers were designed using the sequence in the NCBI database. In all cases, primers were designed according to the cDNA sequence for the specific mouse gene, Balb/C strain, when available. The design of all primers also had to fit the minimum requirements to avoid mispriming.

For comparison of expression of signaling molecules between adult and neonatal AOB, equal amount of cDNA (which directly corresponds to equal quantity of RNA) from the two samples was used for RT-PCR. Amplification of 18S rRNA was used as an internal control to ascertain equal quantity of cDNA used for RT-PCR.

Preparation of primary culture of AOB neurons

Cultures were prepared using standard procedures (Price and Brewer, 2001; Kivell et al., 2001). P3 female Balb/c mice were anesthetized using isoflurane and sacrificed by decapitation. The skin was removed and the skull pinned to sylgard base (Dow Corning; Midland MI) in a Petri dish filled with HBSS buffer at 4°C. Bone was removed from above the brain and the frontal cortex cut away with forceps. AOB tissue was dissected from the bulb and transferred to a tube containing 1 mL Hibernate medium (Brain Bits, Springfield, IL) at 4°C. After all tissue was collected, trypsin (0.05%) and DNase I (50 µg/ml) (Sigma-Aldrich, St. Louis, MO) were added. The tissue was then gently triturated using a fire-polished pasteur pipette and incubated at 37°C for 15 min. After incubation, the tissue was again triturated using a series of decreasing-bore fire-polished pipettes. The suspension was then centrifuged at 200 × g for 6 min and the supernatant removed. The pellet was then re-suspended in Neurobasal plating medium containing B27 supplement (1X), antimycotic antibiotic solution (1X) and GlutaMax-1 supplement for L-Glutamine (0.5mM) (Invitrogen, Carlsbad, CA). The cells were plated on Poly-D-lysine coated cover glass circles in 24-well plates at a concentration of 0.5 × 10⁶ per well.

Tissue staining

To ascertain the accuracy of dissection technique we used neonatal brains with or without dissection. In one set of samples AOB was removed using the technique described above, while in the other set AOB was left intact with only the meninges removed as necessary for AOB removal in the dissected bulb. Accessory olfactory bulbs used for histology were either untouched or dissected as usual then placed, *in situ* in the skull base, in 4% paraformaldehyde to fix. After fixation, the brains were dissected from the skull. This was carried out under a dissecting microscope with care taken to remove attached pieces of the cribriform plate. Brains were then cut in half and the anterior portion dehydrated in 20% sucrose. After dehydration, the brains were immersed in cryoprotectant, frozen in liquid nitrogen, and then stored in the freezer at -80°C. When preparing slides, the brains were cut into 10 µm sagittal sections in a cryostat, and adhered to glass slides. Sections were stained with hematoxylin and eosin, dried and mounted with glass coverslips.

Culture experiment conditions

For experiments, agonists were added to the fresh Neurobasal plating medium for a final concentration of 10 µM clonidine, 10 µM cirazoline and 20 µM NMDA (CNC cocktail) (Tocris; Ellisville, MO). For inhibitor conditions, chelerythrine and U0126 were dissolved in DMSO

and subsequently dissolved into Neurobasal plating medium for a final concentration of 10 μM and 20 μM , respectively. Chelerythrine and U0126 (Tocris) were added 30 min before the addition of agonists (CNC cocktail). In another set of experiments, cells were pre-incubated with the PLC inhibitor U73122 (Tocris; 1 μM) in Neurobasal plating medium. Agonist and inhibitor concentrations were adopted from concentrations found to be effective in electrophysiological experiments on female mouse AOB slices (Hegde et al., 2005). In all experiments, the volume of vehicle(s) mixed into control medium was the same as the volume of that solvent mixed into the medium from the experimental condition. Culture medium was exchanged with fresh Neurobasal plating medium 2 h prior to incubation with the agonists. The incubation with the CNC cocktail lasted 2 h, after which RNA from the cells was isolated.

Determination of cell viability

Viability of AOB neurons in culture was determined using a Live/Dead Viability-cytotoxicity Kit (Molecular Probe Inc., OR). To determine live cells we treated AOB neurons with 2 μM calcein AM. Live cells convert a non-fluorescent calcein AM to fluorescent calcein. Dead neurons were detected by the treatment with 4 μM ethidium homodimer (EthD-1). EthD-1 enters only damaged neurons and undergoes enhancement of red fluorescence in dead cells (Jacobsen et al., 1996). The culture medium was removed from each well immediately after incubation with or without CNC-cocktail. After rinsing with phosphate-buffered saline (PBS), cultured neurons were incubated with 1 μM calcein AM, and 4 μM EthD-1 in PBS for 30 min at room temperature. Following incubation, cover slips containing AOB neurons were rinsed with PBS and mounted on microscopic slides and cells were counted as 'living' or 'dead' neurons under a fluorescence microscope. Neurons were counted across two perpendicular diameters of the coverslip using 20 X objective in fields of view that are continuous across the two diameters.

Quantification of living/surviving neurons based on morphology

Surviving neurons were defined by light microscopy as phase-bright neurons whose neurite length was at least twice the diameter of the cell body. Neurons were counted using 40 X objective in fields of view that are continuous across two perpendicular diameters of the culture dish (Robinson et al., 2005). Cells were counted before the CNC cocktail treatment and 24 h after the treatment.

Semi-quantitative RT-PCR

For quantification of c-Fos, linearity was determined as follows. After determining the optimal template concentration, we carried out PCR reactions with primers for c-Fos by varying the cycle number. We systematically increased the cycle number in 5 cycle increments starting with 20 cycles until the amount of PCR product reached saturation. Linearity of GAPDH amplification was determined similarly. The mid-point of the linear range of the PCR amplification was used for subsequent RT-PCR experiments (Pernas-Alonso et al., 1999; Spencer and Christensen, 1999). After running the PCR products on 2% agarose gels, we quantified the products using densitometry with Quantity One software (Bio-Rad Laboratories). Quantification of PCR products was then used to determine linearity.

The cells were plated in equivalent amounts and equal amounts of eluate from the RNA isolation column were subsequently used for RT-PCR. A 10 μL aliquot of RNA was used for reverse transcription. The RT reaction was carried out for 60 min. at 42°C using Stratascript reverse transcriptase (Stratagene, La Jolla, CA). Subsequently, 8 μL of the RT product was used to provide the template for PCR reaction.

The PCR cycling conditions for c-Fos were: 94°C for 15 sec, 58°C for 30 sec and 72°C for 2 min for 33 cycles. In order to normalize during analysis, a PCR of glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) was carried out for each sample of RT at the same time as the PCR for c-Fos. PCR products were analyzed by electrophoresis on a 2% agarose gel. After electrophoresis, images were taken and the PCR products were quantified using BioRad documentation system (GelDoc 2000; Bio-Rad Laboratories; Hercules, CA). Values for c-Fos were normalized using values obtained for GAPDH. The normalized value for experimental samples was then compared to the normalized value of control samples in order to calculate the percentage change.

Immunocytochemistry

In later experiments, immunocytochemistry was used to measure the expression of c-Fos. Experiments were terminated by a 10 min wash with 4% paraformaldehyde. After washing with PBS, cells were blocked in 8% bovine serum albumin and washed again with PBS. Cells were incubated for 1 hour with rabbit anti-Fos antibody (5 μ g/mL) (Upstate/Millipore; Charlottesville, VA), then, after washing out, cells were probed for 1 hour with 1:1000 AlexaFluor 488-conjugated goat anti-rabbit (Invitrogen; Carlsbad, CA) followed by PBS washes. This was then repeated using mouse anti-MAP2 (Calbiochem; San Diego, CA) and then donkey anti-mouse AlexaFluor594 (Invitrogen). In these experiments, confocal image files were converted to a standard image format (.jpeg), opened in Adobe Photoshop and processed according to a protocol modified from Lehr et al (Lehr et al., 1997). Briefly, the area of interest was selected by setting a pixel intensity threshold rather than circumscribing the cells by hand. Background intensity was subtracted from the intensity of the pixels in the cell body to obtain the luminance of the anti-rabbit fluorophore. The difference between the mean luminance of the control and experimental conditions was calculated and analyzed.

Statistical analysis

For analyses of data from culture experiments, cultures were prepared from separate groups of P3 animals and each culture prepared from a group of animals was considered one sample in an experiment (i.e. $n=1$). Typically AOBs from four P3 animals were processed as one sample and all the procedures up to plating the cells in culture wells were carried out separately for each sample. In each culture well, a minimum of two fields were analyzed. Values are expressed as Mean $\pm$ Standard error. Student's t-test (unpaired) was used for comparison between two groups. Multiple groups were compared by using one-way ANOVA followed by a post-hoc Tukey test (pairwise multiple comparison).

RESULTS

Expression of key signaling molecules in the neonatal and adult AOB

We wished to examine the signaling pathways initiated by glutamate and NE leading to gene expression in the AOB using cultured AOB neurons. The cultured neurons offer a good model system to efficiently ascertain the intracellular signaling pathways and also allow for future manipulation of signaling molecules. The ultimate goal of these experiments would be generation of testable hypotheses for behavioral and other studies in the adult animals. Therefore, it was important to test whether key signaling molecules were expressed in the neonatal AOB.

Prior to proceeding with our studies, we histologically confirmed that our dissection procedure removed only the neonatal AOB (Fig. 1). For our PCR study, we took a sampling approach and tested major isoforms of neurotransmitter receptors, cytoplasmic signaling molecules connected to the PKC pathway and transcription factors. We carried out RT-PCR on RNA isolated from neonatal and adult AOB. Previous behavioral studies using infusions of phentolamine into AOB, have demonstrated effects of α -adrenoceptors. Because phentolamine blocks both $\alpha 1$ & $\alpha 2$ adrenergic receptors, we examined expression of these receptors in the

AOB of female mice. Both receptor types are expressed in the AOB of adult and neonatal mice (Fig. 2A). When we tested for the presence of NMDA receptor, mRNA for the obligatory NR1 subunit was observed in the AOB of both the neonate and the adult female. In addition, mRNA for isoforms NR2A and NR2D of the subunit were present in the AOB at both ages (Fig. 2B). Earlier behavioral studies have implicated GABA_A receptors in negatively regulating the AOB output. Our RT-PCR demonstrated the expression of this GABA_A $\gamma 2$ subunit in both the adult and the P3 female AOB (Fig. 2C).

We tested the expression of the major isoform of PKC expressed in the brain PKC γ and another classical isoform PKC $\beta 1$ (The classical isoforms of PKC require Ca²⁺ and DAG for activation). Both of these PKC isoforms are expressed in adult and neonatal AOB in comparable amounts (Fig. 2D). PLC activity is required for activation of classical PKC isoforms. The RT-PCR experiments showed expression of two PLC isoforms PLC $\beta 1$ and PLC $\gamma 1$ in the AOB of P3 and adult animals (Fig. 2E). PKC is known to activate Erk1. We found that Erk1 mRNA is expressed in the adult as well as neonatal AOB (Fig. 2F). In addition, we have found the expression of two immediate-early genes (IEGs) of interest, c-Fos and Egr1, in the AOB at both ages (Fig. 2G, H). As a negative control, to demonstrate the accuracy of our RT-PCR results, cDNA from adult and P3 mice was probed with primers for mGluR6, a protein known to be expressed exclusively in the neurons of the retina (Nakajima et al., 1993). Expression of mGluR6 was observed in the adult eye, however no expression was found in the AOB of either the adult or neonate (Fig. 2I).

Glutamate receptor and NE receptor agonists induce gene expression in the AOB

Glutamate and norepinephrine have both been implicated as the neurotransmitters underlying the formation of pheromone memory in the AOB (Brennan and Keverne, 1997). Glutamate is released by axons from the vomeronasal organ and can mediate pheromonal information (Kaba and Keverne, 1992). Norepinephrine levels in the AOB rise after mating, signaling that mating has taken place (Brennan et al., 1995). Furthermore, NMDA receptors as well as α -1 and α -2 adrenergic receptors are capable of affecting pheromone memory formation (Brennan and Keverne, 1997). Thus, these receptors were chosen as targets for modeling the input that AOB neurons receive during pheromone memory formation. For testing the effects of glutamate and adrenergic receptor agonists we used a cocktail of 10 μ M clonidine ($\alpha 2$ -adrenergic agonists), 10 μ M NMDA (NMDA-type glutamatergic agonist) and 20 μ M cirazoline ($\alpha 1$ -adrenergic agonist) (CNC cocktail). We ascertained that the CNC cocktail did not affect the viability of AOB neurons (because of possible apoptosis) by counting 'live' and 'dead' cells by labeling the live cells with calcein-AM and dead cells with ethidium homodimer (EthD-1) (Jacobsen et al., 1996). Non-fluorescent calcein-AM is converted to fluorescent calcein-AM by cells with an intact plasma membrane ('live' cells). EthD-1 labels nuclei of 'dead' cells or cells with damaged plasma membrane. When we counted the live and dead AOB neurons with or without CNC cocktail treatment, there was no significant difference in the number of live cells (Fig. 3A; $P=0.310$; $n=6$; Wilcoxon Rank Sum test) or dead cells (Fig. 3B; $P=0.805$; $n=6$; unpaired t-test) between untreated and CNC cocktail-treated cultures. As an additional measure of cell viability, we counted surviving neurons based on morphological criteria (Robinson et al., 2005). We found no significant difference in the number of surviving neurons between untreated and CNC cocktail-treated cultures when counted 24 h after CNC treatment suggesting that the treatment did not have any adverse effect on viability of AOB neurons (Fig. 3C; $P=0.890$; $n=12$; Wilcoxon Rank Sum test).

Next, we tested the effect of CNC cocktail on the levels of c-Fos mRNA. We observed that a two hour exposure to the cocktail induced a significant increase in c-Fos mRNA ($216 \pm 17\%$; $P < 0.01$; $n=4$; unpaired t-test) compared to controls as observed using semi-quantitative RT-PCR (Fig. 4).

Subsequent tests of c-Fos expression changes measured with immunocytochemistry have yielded similar results. The expression of c-Fos in cultured neurons exposed to CNC cocktail was significantly increased ($221 \pm 24\%$; $P < 0.01$; $n=9$; unpaired t-test) compared to control levels (Fig. 5). We ascertained that induction of c-Fos occurred in neurons by co-localization of c-Fos staining cells with MAP2 staining. This increase in protein expression corroborates the mRNA finding reported above, and demonstrates that noradrenergic and glutamatergic stimuli induce both c-Fos mRNA and protein.

Effect of kinase inhibitors on c-Fos expression

Because of the implication of protein kinase activity in pheromone memory formation (Kaba et al., 1989) we explored the role of kinases in AOB neuron response to the CNC cocktail. AOB neurons were treated with CNC cocktail after pre-incubation with chelerythrine and U0126 (PKC and MEK inhibitors, respectively) for 20 min. As before, we observed an increase in c-Fos protein with the CNC cocktail compared to controls ($207 \pm 45\%$; $n=5$; $P < 0.05$; ANOVA). When pre-incubated with a cocktail of inhibitors for PKC and MEK inhibitors, however, CNC cocktail effect on c-Fos expression was inhibited by 74% (Fig. 6A; $P < 0.01$; $n=8$; ANOVA).

In order to test for differential roles for the two kinases, we carried out a separate set of experiments in which we induced c-Fos expression with the CNC cocktail and used each inhibitor separately. As before, we observed significant induction with the CNC cocktail ($189 \pm 27\%$, $n=5$; $P < 0.05$; ANOVA). Both U0126 and chelerythrine separately inhibited the effects of the CNC cocktail on c-Fos expression in cultured AOB neurons. c-Fos expression in U0126-treated CNC cultures was inhibited by 77% (Fig. 6B; $P < 0.001$; $n=4$; ANOVA) while chelerythrine inhibited c-Fos expression by 82% relative to CNC treatment alone (Fig. 6B; $P < 0.001$; $n=4$; ANOVA).

A role for PLC in c-Fos expression

We were also interested in the role PLC plays since PLC is known to affect signal transduction leading to kinase activity, and could possibly be activated as a result of coincident adrenergic and glutamatergic receptor stimulation that is implicated in pheromone memory. PLC activity produces diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP3). The former reduces the threshold required for PKC activity, while the latter induces the release of calcium from internal stores thereby increasing the activation of PKC. We tested the potential role of PLC in c-Fos expression in AOB neurons. We exposed neurons to the PLC inhibitor U73122 (1 μ M). The result was an inhibition of c-Fos expression by 81% relative to the CNC cocktail alone (Fig. 6B; $P < 0.01$; $n=3$; ANOVA). This result supports the hypothesis that PLC plays a role in signal transduction resulting from receptor activation to gene expression.

DISCUSSION

Studies of signal transduction and other molecular mechanisms that govern plasticity in the AOB are likely to aid the elucidation of pathways that underlie long-lasting behavioral changes such as pheromone memory formation. Such an undertaking has been hampered by lack of availability of a model system. Although acute brain slices are highly useful for electrophysiological studies, slicing has been shown to induce significant induction of IEGs (Taubenfeld et al., 2002) which would be a major confound in any experiment on gene induction. Therefore, we chose to test mouse AOB neurons in primary culture as a model system in which to study gene expression.

Prior to embarking on cultural studies it was important to test the presence of basic elements of the signaling pathways that we intended to examine. Presence of signaling molecules in the

mouse AOB was mainly inferred based on infusion of agonists and antagonists before the availability of highly selective reagents against neurotransmitter receptor subtypes and protein kinases. Furthermore, prior studies of the AOB have been carried out in widely different species such as mouse, rat, hamster, and turtle. Thus there are large gaps in knowledge of the AOB in mice. Furthermore, an even greater paucity of information prevails over expression of signaling molecules in the neonatal AOB. Our studies of gene expression in neonatal and adult AOB, although not exhaustive, demonstrated equivalent expression of neurotransmitter receptors, protein kinases and other molecules relevant for the hypothesized PKC-mediated signaling pathway.

The morphologies of neurons observed in our mouse AOB culture system closely resembles the cells observed by the Ichikawa laboratory in cultures of rat AOB (Kato-Negishi et al., 2003). In primary culture of the rat AOB, two primary groups of neurons (MAP2-positive cells) were observed, medium-sized multipolar neurons with thick dendrites and smaller unipolar and bipolar neurons with thinner dendrites (Kato-Negishi et al., 2003). Of the neurons in our cultures, nearly all resembled either the multipolar neurons or the uni/bipolar morphology observed in rat AOB culture. Generally multipolar neurons tend to be mitral/tufted cells and uni/bipolar neurons tend to be granule cells although there is no straightforward way to distinguish granule cells from periglomerular cells. Detailed investigations would be necessary to tease apart the differences in signaling mechanisms in different types of AOB neurons.

A series of studies conducted by Keverne, Brennan and colleagues has established that coincident action of glutamate and NE is required for pheromone memory formation, the trace for which is thought reside in the AOB. Based on their behavioral studies these authors have established that protein synthesis is required for pheromone memory formation. Brennan and Keverne have also shown induction of c-Fos and Egr1 by pheromone memory inducing stimuli (Brennan et al., 1992) i.e. concomitant glutamate and NE release onto the AOB. Although gene expression in AOB plasticity has been implicated by earlier studies, there have been no investigations on how glutamate and NE regulate gene expression in the AOB. We designed experiments to elucidate some of the initial steps. We used neurotransmitter receptor agonists to approximate glutamatergic and noradrenergic stimuli that induce pheromone memory *in vivo*. The results of these experiments show that c-Fos expression increases after agonist exposure. Furthermore, our experiments showed that when PLC, PKC and MAPK activity was blocked, c-Fos increase was prevented. The results suggest that PKC and MAPK are likely to operate in series rather than in parallel because the inhibitory effect on c-Fos induction produced by the combination of PKC and MEK inhibitors is not additive. Thus our results successfully model c-Fos induction in AOB during pheromone memory formation (Brennan et al., 1992; Kaba et al., 1989) and identify key signaling cytoplasmic signaling molecules critical for c-Fos induction in the AOB.

Glutamatergic signaling is the principal component of neuronal activity within the AOB (Brennan and Keverne, 1997). Thus, it follows that glutamate receptors will play an important role in AOB signal transduction. Both NMDA and AMPA receptors mediate ionotropic glutamate neurotransmission in the AOB. The NMDA receptor, however, appears to play a greater role in pheromone memory formation. (Jia et al., 1999; Kaba and Keverne, 1992; Saito-Ito et al., 2001; Taniguchi and Kaba, 2001). Of these reports, no thorough study was made of expression changes resulting from glutamate signaling. Rather, the aforementioned studies focused on behavioral, electrophysiological and neurotransmitter changes resulting from pharmacological manipulation often without directly monitoring changes in gene expression. The cocktail used in our experiments which induced c-Fos expression included NMDA thus implicating NMDA receptors in controlling gene expression in the AOB.

Previous studies of pheromone memory demonstrated an important role for the adrenergic input from the locus ceruleus (Brennan and Keverne, 1997). An early study described a rich innervation of the AOB by the noradrenergic locus ceruleus (Shipley et al., 1985; McLean et al., 1989). Another study indirectly observed an increase in noradrenergic levels after vaginocervical stimulation (Rosser and Keverne, 1985), while a later, more direct approach demonstrated an increase in noradrenergic levels in the AOB of female mice after mating (Brennan et al., 1995). In addition to these, several other studies reported the effects of noradrenergic agonists and inhibitors in the AOB. These studies established that adrenergic receptors are important mediators of neuronal plasticity in the AOB (Brennan and Keverne, 1997). Our experiments showed the CNC cocktail containing $\alpha 1$ & $\alpha 2$ adrenergic receptor agonists induced c-Fos expression thus suggesting a role for both of these receptor subtypes in induction of gene expression in the AOB.

In our experiments we used neurotransmitter receptor agonists rather than neurotransmitters themselves. The combination and concentration of agonists were based on those successfully employed in our laboratory for *in vitro* electrophysiology experiments on AOB slices (Hegde et al., 2005). While the utilization of agonist cocktail is an artificial way of stimulation, it has been useful in establishing the AOB culture model for studying gene expression. Thorough future studies would be required to address contribution of glutamate and NE and individual neurotransmitter receptors towards activation of gene expression. Also, because the agonists applied to the culture medium would bathe all types of neurons of the AOB, our experiments may not replicate the local release of neurotransmitters in the AOB. Nonetheless, the types of experiments described here have a utility in identifying the intracellular signaling pathways in an efficient manner.

The experiments described above support a signal transduction pathway in AOB neurons which could underlie pheromone memory formation. NE and glutamatergic inputs activate their receptors, which, through a combination of ionotropic and metabotropic effects, increase the concentration of intracellular Ca^{2+} . NE input also activates PLC which generates IP3 and DAG. IP3 contributes to the rise in Ca^{2+} through release from intracellular stores. IP3 and DAG together activate PKC which can then activate the Erk1 pathway. The result of this activation is IEG expression such as that of c-Fos (Fig. 7). While the precise connections between separate portions of this signal transduction pathway need to be more clearly understood and ultimately tested through behavioral studies, the present study has identified the basic elements of signal transduction that may comprise the molecular response producing the robust pheromone memory acquired through single-trial learning.

In terms of the components of signal transduction pathways downstream of glutamate and adrenergic receptors, there appears to be notable similarity between adult and neonatal AOB neurons. Even though the sensory stimulation and behavioral manifestation of pheromone memory only occur in adult female mice, the basic signal transduction machinery seems to be already in place in neonatal AOB. The main difference between the two ages is likely to be in the maturation of neuronal circuitry of which AOB is a part. Cultured neurons from other parts of the brain such as the hippocampus have been used to model neuronal responses that occur in the adult; for example, estradiol-induced synaptic plasticity (Murphy and Andrews, 2000). Our studies suggest that cultured AOB neurons can be used to model at least some aspects of signaling relevant to pheromone memory. Extensive additional studies would be necessary to determine the utilities and limitations of the AOB culture model. If utilized in conjunction with the behavioral studies, the culture system might be fruitful for screening pharmacological compounds and for identifying molecules that might play a role in pheromone memory.

Acknowledgements

This work was supported in part by a grant from the Whitehall Foundation to ANH. SCU was supported by a training grant from NIH (DC 000057).

Abbreviations

AOB, Accessory Olfactory Bulb
 AR, Adrenergic
 CNC, Agonist Cocktail (Clonidine, NMDA, Cirazoline)
 DAG, Diacylglycerol
 Erk, Extracellular signal Regulated Kinase
 Glu, Glutamate
 GAPDH, glyceraldehyde-3-phosphate dehydrogenase
 IEG, immediate-early gene
 IP₃, Inositol 1,4,5 trisphosphate
 MAPK, Mitogen-activated protein kinase
 MEK, MAPK/ERK kinase
 NE, Norepinephrine
 NMDA, N-methyl-D-aspartic acid
 PKC, Protein Kinase C
 PLC, Phospholipase C
 RT-PCR, reverse transcription followed by polymerase chain reaction

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Fig. 1.

Histological confirmation of neonatal AOB dissection.

(A) The figure shows hematoxylin and eosin stained sections of neonatal olfactory bulb before removal of AOB. (B) The figure shows a comparable AOB after removal of AOB. Some portion of the AOB granule cell layer is still left in this section. The top right hand portion of this section shows edges of the tissue surrounding AOB curling up. The arrow points to the area from which the AOB was removed. VNNL, vomeronasal nerve layer; MC, mitral cell/external plexiform layer; IPL, internal plexiform layer; LOT, lateral olfactory tract, GC, granule cell layer; MOB, main olfactory bulb. Scale bar = 200 μ m.

Fig.2.

Expression of key signaling molecules in the neonatal and adult AOB.

RT-PCR analysis of RNA from adult and neonatal AOB is shown. 18S rRNA was used as internal control. Representative images of agarose gel electrophoresis from one of the three independent experiments are shown. (A) Adrenergic receptors (ARs) α 1A and α 2A. (B) NMDA receptor subunits, NR2A, NR2D and NR1. (C) GABA $_A$ γ 2 receptor. (D) PKC isoforms β 1 and γ . (E) PLC isoforms β 1 and γ 1. (F) Erk1. (G) c-Fos. (H) Egr1. (I) mGlu6 was used as negative control. mGluR6, which is known to be expressed only in the retina, shows expression in the eye tissue of the adult but not in adult or neonatal AOB.

Fig. 3.

CNC-cocktail treatment does not affect the survival of AOB neurons in culture.

(A) Neuron viability assay showed that CNC treatment did not alter the number of living AOB neurons ($P=0.310$). (B) Number of dead cells in viability test after CNC treatment did not differ from that of control ($P=0.805$). (C) Number of surviving AOB neurons was determined based on their neurite length 24 h after of CNC treatment. CNC treatment did not change the number of surviving AOB neurons ($P=0.890$).

Fig. 4. Upregulation of c-Fos mRNA in AOB neurons. (A) c-Fos RT-PCR product from AOB culture without any treatment (Control) or with CNC treatment; GAPDH was used as control. (B) Quantification of c-Fos mRNA showed a significantly higher level (** $P < 0.01$) after CNC treatment. AU=arbitrary units of RT-PCR product intensity.

Fig. 5.

Upregulation of c-Fos protein in AOB neurons. (A). Control cells (top middle panel) showed less immunoreactivity to c-Fos than those treated with a CNC cocktail (bottom middle panel). MAP2 was used to label neurons and overlay was utilized to help identify cells and quality of neurons before quantification. [Note: c-Fos immunoreactivity is largely concentrated in the nuclei. There is no significant c-Fos immunoreactivity in the dendrites and other processes.] (B). Quantification showed that c-Fos immunoreactivity was significantly (** $P < 0.01$) higher after CNC cocktail treatment compared to controls. AU=arbitrary units of intensity of immunofluorescence.

Fig. 6.

Blockade of c-Fos induction by inhibitors of PKC, MAPK and PLC.

(A) The c-Fos immunoreactivity with CNC treatment alone was increased significantly ($***P<0.05$). When the AOB neurons were pre-treated with a cocktail of MEK inhibitor U0126 and PKC inhibitor chelerythrine, the CNC treatment-mediated increase in c-Fos immunoreactivity was abolished ($***P<0.01$ compared to CNC). AU=arbitrary units of intensity of immunofluorescence.

(B) Single inhibitors block c-Fos induction. The AOB cultures were subjected to treatment with CNC cocktail alone, or pre-treated separately with PKC inhibitor chelerythrine, MEK inhibitor U0126, or PLC inhibitor U73122. As before, we observed significant increase in c-

Fos immunoreactivity with CNC treatment (* $P < 0.05$ compared to control). Significant reduction in c-Fos immunoreactivity was observed with chelerythrine (*** $P < 0.001$), U0126 (*** $P < 0.001$), and U73122 (** $P < 0.01$) compared to CNC alone.

Fig. 7.

Schematic diagram of signaling mechanisms in the AOB. Glutamate binds to NMDA receptors causing Ca^{2+} influx into AOB neurons. NE binding to α 1-ARs stimulates PLC leading to generation of inositol 1,4,5 tris phosphate (IP3) and diacyl glycerol (DAG). IP3 releases intracellular Ca^{2+} . NE binding to α 2-ARs might contribute to an increase in Ca^{2+} as well. Ca^{2+} and DAG together activate PKC. Activated PKC in turn causes stimulation of ERK1 which then leads to induction of c-Fos in the nucleus.

Table 1

Primers for receptors, signaling components and other molecules

Name	Sequence 5' → 3'
$\alpha 1A$ AR-sense	GTAGCCAAGAGAGAAAGCCG
$\alpha 1A$ AR-anti	CTAGACTTCCTCCCGTTTT
$\alpha 2A$ AR-sense	CCTGCAGGTGACACTGACGCTGGTTTGC
$\alpha 2A$ AR-anti	CAAGGCGCGAAGAAGGAACCGATGGAC
NR1-sense	CAGGAGCGGGTAAACAACAGCAAC
NR1-anti	GACAGCCCCACCAGCAGCCACAGT
NR2-A-sense	AGCCCCCTTCGTCATCGTAGA
NR2-A-anti	CAGAAGGGGAAACAGTGCCATTA
NR2-D-sense	CGATGGCGTCTGGAATGG
NR2-D-anti	CTGGCAAGAAAGATGACGGC
GABA _A γ II-sense	GGTGGAGTATGGCACCCCTGCATT
GABA _A γ II-anti	AGGCGGTAGGGAAGAAGATCCGA
PKC- β 1-sense	ATCTGGGATGGGGTGACAAC
PKC- β 1-anti	TAGGACTGGTGGATGGCGGG
PKC- γ -sense	GCTGTATGAGATGTTGGCAGG
PKC- γ -anti	GAGATTACATGACAGGCACGG
PLC- β 1-sense	GTTCTCAGCAGACCGGAAGCGC
PLC- β 1-anti	GCTGCTGTTGGGCTCATATTTC
PLC- γ 1-sense	GGATACACTGCAGGCAGCCACAC
PLC- γ 1-anti	CTCCTCAATCTCTCGCAAGGGG
Erk-1-sense	TCCAAGGGGTACACCAAATC
Erk-1-anti	GCTCCATGTCGAAGGTGAAT
c-Fos-sense	GAATGGTGAAGACCGTGTGAGG
c-Fos-anti	CGTTGCTGATGCTCTTGACTGG
Egr1-sense	GGAGATGATGCTGCTGAGCAACG
Egr1-anti	GGATGAAGAGGTGCGGAGGATTGG
18s RNA-sense	CAAGAACGAAAGTCGGAGGTTTGAAGACGATC
18s RNA-anti	CCTGTTATTGCTCAATCTCGGGTGGCTGAAC
GAPDH-sense	GGCTGCCCAGAACATCATCC
GAPDH-anti	CGGCATCGAAGGTGGAAGAGTGG