Published in final edited form as:

Neurochem Int. 2011 November ; 59(6): 787–803. doi:10.1016/j.neuint.2011.08.009.

Expression Profiling Reveals Differential Gene Induction Underlying Specific and Non-Specific Memory for Pheromones in Mice

Sudarshan C. Upadhy^a, Thuy K. Smith^a, Peter A. Brennan^b, Josyf C. Mychaleckyj^c, and Ashok N. Hegde^a

^aDepartment of Neurobiology and Anatomy, Wake Forest University Health Sciences, Winston-Salem, NC 27157 USA

^bDepartment of Physiology and Pharmacology, University of Bristol, Bristol, BS8 1TD, UK

^cCenter for Public Health Genomics, University of Virginia, Charlottesville, VA 22908-0717, USA

Abstract

Memory for the mating male's pheromones in female mice is thought to require synaptic changes in the accessory olfactory bulb (AOB). Induction of this memory depends on release of glutamate in response to pheromonal exposure coincident with release of norepinephrine (NE) in the AOB following mating. A similar memory for pheromones can also be induced artificially by local infusion of the GABA_A receptor antagonist bicuculline into the AOB. The natural memory formed by exposure to pheromones during mating is specific to the pheromones sensed by the female during mating. In contrast, the artificial memory induced by bicuculline is non-specific and results in the female mice recognizing all pheromones as if they were from the mating male. Although protein synthesis has been shown to be essential for development of pheromone memory, the gene expression cascades critical for memory formation are not known. We investigated changes in gene expression in the AOB using oligonucleotide microarrays during mating-induced pheromone memory (MIPM) as well as bicuculline-induced pheromone memory (BIPM). We found the set of genes induced during MIPM and BIPM are largely non-overlapping and Ingenuity Pathway Analysis revealed that the signaling pathways in MIPM and BIPM also differ. The products of genes induced during MIPM are associated with synaptic function, indicating the possibility of modification at specific synapses, while those induced during BIPM appear to possess neuron-wide functions, which would be consistent with global cellular changes. Thus, these results begin to provide a mechanistic explanation for specific and non-specific memories induced by pheromones and bicuculline infusion respectively.

Keywords

glutamate; norepinephrine; bicuculline; microarray; pathway analysis; signal transduction; plasticity

© 2011 Elsevier Ltd. All rights reserved.

Corresponding author: Ashok N. Hegde, Ph. D., Wake Forest University Health Sciences, Medical Center Boulevard, Winston-Salem, NC 27157-1010, USA, Phone: (336)716-1372, Fax: (336)716-4534, ahedge@wfubmc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Synaptic plasticity allows organism to learn and adapt to changes in the environment by storing the learned information. Research carried out over the last several decades in both invertebrates and vertebrates has yielded valuable information regarding molecular mechanisms underlying short-term and long-term memories (Kandel 2001; Matynia et al 2002; McGuire et al 2005; Reissner et al 2006; Yin and Tully 1996). It is generally believed that short-term memory requires posttranslational modifications such as phosphorylation of pre-existing proteins whereas long-term memory entails new gene expression and synthesis of new protein. Much of our knowledge on molecular mechanisms underlying long-term memory in vertebrates comes largely from studies on associative memory paradigms in which hippocampus or amygdala play a critical role. By contrast, molecular mechanisms underlying robust, single-trial learning that occurs in a sensory system are not clearly understood. Therefore we studied gene expression in the accessory olfactory bulb (AOB), the locus of a strong form of memory in female mice for the pheromones of the mating male.

Female mice acquire memory for the pheromones of the mating male with one-trial learning which lasts for up to 7 weeks, a significant period in the life span of mice (Brennan and Keverne 1997). The memory for male's pheromones is formed in females during a sensitive period around the time of mating and requires the association of mating and pheromonal exposure (Brennan and Keverne 1997). This memory is vital to reproductive success, as it prevents the pregnancy block effect that is elicited by exposure to pheromones from an unfamiliar (genetically different) male. The unfamiliar male's presence is not necessary to block pregnancy; exposure to the soiled bedding that contains his urinary pheromones is sufficient to produce the same result. In the absence of recognition memory, the pheromone signals detected by vomeronasal sensory neurons activate mitral cells in the AOB. Mitral cell output in turn activates a neuroendocrine pathway, via the amygdala, to the hypothalamus, ultimately leading to a decrease in prolactin secretion from the pituitary. Prolactin maintains progesterone secretion from the corpus luteum following mating until the implanting embryos become established. Exposure to strange male's pheromones during this vulnerable period causes progesterone levels to decline and the developing embryo fails to implant i.e. pregnancy is blocked (Brennan and Keverne 1997).

Formation of the pheromone memory depends on detection of pheromones by the vomeronasal sensory neurons and concomitant mating-induced release of norepinephrine (NE) from locus ceruleus projections to the AOB. This leads to the modification of reciprocal synapses between the mitral cells responding to the mating male's pheromones and inhibitory GABAergic granule cells (Brennan and Keverne 1997). This is thought to result in a long-lasting increase in gain of feedback inhibition from granule cells that can selectively inhibit transmission from mitral cells carrying the mate's pheromonal signal to the neuroendocrine pathway that mediates pregnancy block (*see* Dong et al 2009 Supplementary Data for depiction of the AOB circuitry relevant to pheromone memory). Thus the natural memory formed is specific to the mating male's pheromones. Experimentally, memory for pheromones can be induced by infusion of GABA receptor antagonist bicuculine into the AOB. This treatment, however, appears to modify all mitral to granule cell reciprocal synapses and induces a non-specific memory for the pheromones of all males (Kaba and Keverne 1988).

Our previous work has shown a role for Protein Kinase C α in early stages of memory formation (Dong et al 2009) and that PKC activation is linked to gene expression in cultured AOB neurons (Skinner et al 2008). The association of mating and pheromonal exposure has also been shown to increase expression of the immediate-early genes *Egr1* and *c-Fos* in the

AOB within 2 hours of mating (Brennan et al 1992). Also, pheromone memory formation is blocked by the protein synthesis inhibitor anisomycin only if the drug is applied at the late phase of the critical period of pheromone exposure coinciding with mating i.e. up to 4.5 h after mating (Kaba et al 1989). Therefore, it is highly likely that the second wave of genes (late genes) expressed after the immediate-early genes is critical for pheromone memory formation. As a first step towards elucidating gene expression underlying pheromone memory, we carried out studies using oligonucleotide microarrays at 4 h after induction of pheromone memory; at a time point following expression of immediate-early genes *Egr1* and *c-Fos*. We investigated gene expression in the AOB using natural pheromone memory induction protocol as well as memory induction using infusion of bicuculline. Our results indicate that different gene expression patterns are associated with specific and non-specific pheromone memories.

Materials and Methods

Animals

Adult, virgin female mice of Balb/c strain (6 weeks old) and male mice of C57/B16 (10 weeks old) were obtained from Charles River (Wilmington, MA). Males of DBA, CBA and SWR (10 weeks old) used in this study were obtained from Jackson Laboratory (Bar Harbor, Maine). Mice were housed under reverse light: dark cycle 12:12 h (lights on at 08:00 and lights off at 20:00). Food and water were available *ad libitum*. The estrous cycles of females were monitored daily by taking vaginal smears. All procedures were carried out using a protocol approved by the Institutional Animal Care and Use Committee of Wake Forest University Health Sciences.

Pheromone memory induction protocol

Most studies on the pregnancy block effect and mate recognition use inbred strains of mice. However, inbred strains have less diversity and complexity of their urinary pheromonal signals than their wild counterparts. For example, all inbred mice from the Castle (for example Balb/c) and Swiss (for example SWR) lineages express the same profile of major urinary proteins (Cheetham et al 2009). This profile differs from that of the C57/B16 line, and is reduced compared to the diversity of major urinary proteins found in urine from wild-derived males (Robertson et al 1997). To provide a diverse and maximal stimulation of the AOB, we used mixtures of urinary pheromones from males of different inbred strains. Soiled bedding from cages containing males of Balb/c, C57/B16, DBA, CBA and SWR (two males of the same strain in each cage) were mixed in equal proportions into a new cage and mating was set up in this cage. Experimental evidence shows that exposure of a mated female to a mixture of bedding of males of two strains during the post-mating sensitive period for memory formation results in memory formation to both components of the mixture. For example, when female Balb/c mice are mated with Balb/c males and are exposed to B10CB males, neither Balb/c males nor B10CB males block pregnancy. In other words, memory is formed in female mice for pheromones of males of both strains (Table 1) (see next section '*Pregnancy block experiments*' for experimental procedure). Thus our strategy is akin to inducing memory for pheromones of male mice of five strains.

The pheromone memory induction protocol with soiled bedding of males was adapted from a previously standardized procedure (Selway and Keverne 1990). Females with estrous cycle between proestrus and estrous were placed in the cage with a Balb/c male and mating was visually observed. For microarray studies, 4 h after mating the brains from females were dissected and the AOB was dissected out and frozen in liquid nitrogen. The AOB from control mice (non-mated mice matched for age and the exact stage of the estrous cycle) was similarly dissected and stored. For immunocytochemistry studies, the experiments were

terminated at 5 h after mating (to allow additional time for translation of mRNAs increased at 4 h and thus to detect protein expression) and the animals were perfused and brains were dissected.

Pregnancy block experiments

Balb/c females were placed in cages in which bedding from each inbred or hybrid male was mixed. The Balb/c female was mated with the first male and checked for a plug every 30 minutes. Immediately after finding a plug the male was removed and replaced with the second male. The female was removed and placed in a clean individual cage 5 hours following introduction to the male and mixed bedding. 24 hours later the blocking male was introduced to the female in her cage and removed 48 hours later. The female was killed and checked for uterine implantation sites 7-10 days after mating (Selway and Keverne 1990).

Infusion of bicuculline

Female mice received stereotaxic bilateral implantation with 23-gauge stainless steel guide cannulae immediately anterior to the AOB, under intraperitoneal ketamine (0.15mg/g)-xylazine (0.015mg/g) general anesthesia, with subcutaneous injection of the local anesthetic lignocaine (2%) below the scalp. The co-ordinates, relative to the intersection of the superficial sinuses and the surface of the olfactory bulb: longitudinal +0.5mm; lateral ± 0.8 mm; vertical -0.5mm. Previous dye infusion studies have shown that a 1 μ l infusion reaches all of the AOB and the dorsal part of the main olfactory bulb, without spreading to other areas of the brain. At least 3 days following surgery, bicuculline (0.1 nmol per AOB in 1 μ l of artificial CSF) was infused unilaterally, over a period of 10 minutes, through a 30-gauge infusion cannula, which fitted within the guide cannula (Experimental). Artificial CSF was similarly infused into the contralateral AOB (Control). The AOB samples were collected from four different animals. Four hours following the infusion, the mice were killed by cervical dislocation and the ipsilateral and contralateral AOBs were dissected out and frozen in liquid nitrogen. Surgery and drug infusions were performed in accordance with the Animals (Scientific Procedures) Act (UK).

Microarrays

Total RNA was isolated from AOBs using RNeasy kit (Qiagen). For preparation of cRNA targets, we used the 'Eukaryotic Small Sample Target Labeling' procedure from Affymetrix. We took 100 ng total RNA from AOB and linearly amplified it by converting it to double stranded cDNA and then transcribing the cDNA to cRNA using T7 RNA polymerase. The cRNA synthesized during the first cycle was reverse transcribed and double stranded cDNA was once again synthesized. Then biotin-labeled antisense cRNA was synthesized using *in vitro* transcription of the double stranded cDNA synthesized during the second cycle. Microarray hybridization on oligonucleotide microarrays (Murine Genome U74v2 Set) obtained from Affymetric, Inc. (Santa Clara, CA) was carried out at the Microarray Core Facility of Wake Forest University Health Sciences. The double-stranded cDNA synthesized as described above from AOB of each of the 5 control and 5 experimental animals was used for separate microarray hybridization. Similarly double-stranded cDNA synthesized from each of the 4 CSF-infused and the 4 bicuculline-infused AOB samples was used for separate microarray hybridization. In other words, in either of these experiments the samples were not pooled.

Statistical analysis of microarray data

Raw data from the mouse RNA hybridization experiments was processed using the Bioconductor R package (<http://www.bioconductor.org>) to extract probe-level hybridization signals which were log₂ transformed. Since the A, B, and C arrays in the U74Av2 set

contain distinct probe sets and are assayed separately, we analyzed the 3 array classes separately also. The three arrays from each of the 10 groups were normalized using robust measures of distribution: median (central location, set to 0 for each array) and interquartile range (spread, for scaling expression values). Since the control and mating mouse pairs were carefully matched on age, day of mating, and stage of estrous cycle, and the bicuculline experiment AOB tissue samples were intracranially matched from left and right hemispheres, we analyzed the signal variance in each of the 10 sets of arrays using probe-level mixed effects models (Chu et al 2004a; Chu et al 2004b). Similarly A, B, and C arrays in the U74Av2 set were analyzed for each of the 4 control (CSF-infused) and 4 experimental (bicuculline-infused) samples. We treated the 5 mice pairs or 4 bilateral tissue sample pairs as a random sample with the variance partitioned into intra- and inter-cluster components, and the mating status (or bicuculline treatment) and probe terms treated as fixed effects. Before fitting the final model we tested for sample-specific probe outliers and dropped probe data points with a residual greater than the normal quantile after Bonferroni correction (probes per probeset $\times$ samples). We tested for a significant control versus mating (or bicuculline versus CSF infusion) effect for each probe set on each of the A, B, and C arrays, using a maximum p value threshold derived from False Discovery Rate (FDR) analysis (Storey and Tibshirani 2003). We set an identical maximum FDR = 0.001 (0.1%) for each array group, corresponding to p-value thresholds of 1.1×10^{-4} (A), 2.9×10^{-3} (B), and 2.0×10^{-5} (C). We applied a fold change (FC) criterion in addition to the FDR threshold (0.1 %, equivalent to 36 expected false positive probe set reports in approx 36,000 total) as suggested by a recent re-analysis of the MAQC data for maximizing reproducibility (Shi et al 2008). The FC criterion was applied to the mixed model adjusted FC, after adjustment for the fixed probe and random pair components of the model. The exact thresholds used are described in the section '*Ingenuity Pathway Analysis*'. Genes were mapped to probe sets and classified by molecular function (ontology classification) using EASE (<http://david.niaid.nih.gov/david>) (Hosack et al 2003) and Netaffyx (<http://www.affymetrix.com>). All statistical analyses were performed using R version 1.9 (<http://www.r-project.org>). The probe set mixed effects models were fitted using the R nlme package (Pinheiro and Bates 2000).

Quantitative Real-time PCR (Q-PCR)

Total RNA was isolated from the mouse AOB and cDNA was prepared with random hexamers and StrataScript reverse Transcriptase (Stratagene, La Jolla, CA) as per manufacturer's instructions. Expression of mRNA was measured by Q-PCR (ABI Prism 7700; Applied Biosystems, Foster City, CA) by using QuantiTect SYBR Green master mix (Qiagen Inc, Valencia, CA) and the primers specific for the dynein light chain 1 (dynein), kinesin family member 5C (kinesin) and cyclin-dependent kinase 5 regulatory subunit 1 (cdk5r1) coding region (Table 2). Genomic DNA contamination was ruled out by running a PCR with RNA alone and by running a PCR without cDNA template. Primer dimer formation was ruled out by checking the PCR product by running an agarose gel electrophoresis. Each independent sample was assayed in triplicate. The fluorescence signal from the SYBR Green dye was analyzed by using the ABI prism SDS software. Relative quantification of gene expression was performed using the comparative threshold (C_T) method as described by the manufacturer (Applied Biosystems; User Bulletin 2) (Giulietti et al 2001). Changes in dynein, kinesin and cdk5r1 mRNA expression levels were calculated after normalization to 18S rRNA (Table 2). All the primers had comparable PCR efficiency (Supplementary Table 3). The ratios obtained after normalization are expressed as fold induction over corresponding control AOB.

Immunohistochemistry and quantification of immunoreactivity

We anesthetized the Balb/c control and experimental females with isoflurane and perfused transcardially with cold phosphate buffered saline (PBS), pH 7.2, followed by cold 4% paraformaldehyde in PBS, pH 7.2. We then removed the brains and placed in fresh 4% paraformaldehyde for 2 h at 4°C. After transferring to a 30% sucrose solution in 0.2 M phosphate buffer the brains were left overnight at 4°C. We cut 100 µm frozen sections coronally from the AOBs. We rinsed the free-floating sections in PBS and then incubated separate sets of sections with polyclonal antisera against Cdk5r1, PKCα, and eEF1A (from Abcam, Cambridge, MA) overnight at room temperature. Antibody binding was visualized using the immunofluorescence with a secondary antibody conjugated to Alexa-488. No staining was observed in control sections incubated only with the secondary antibody conjugated to Alexa-488.

For quantification, we examined three sections through the central region of the AOB, separated by either 100 µm at a magnification of 20 X for all the antibodies. We analyzed the mitral and granule cell layers of the AOB for each section because these layers are easily identifiable. The granule cell layer is clearly demarcated from the adjoining main olfactory bulb and is separated from the rest of the AOB by the centrally projecting bundles of the lateral olfactory tract (LOT). The large cell bodies of the mitral cells form a wide band in the external plexiform layer, bounded by the LOT. Immunofluorescence for each antibody measured within each area using NIH image J software. For comparison between control and experimental sections the extent of immunofluorescence was normalized to area and expressed in arbitrary units (AU).

Ingenuity Pathway Analysis

We selected significant probe sets or genes with a cut off of $p < 0.0011$ and adjusted fold change > 1.1 . We used adjusted fold-change of expression value as cutoff value to reduce the noise within the dataset and to obtain comparable number of molecules eligible for generating networks in MIPM and BIPM. With 1.1 cutoff value, 156 genes from MIPM and 185 genes from BIPM were found to be network eligible genes (showing interaction with other molecules) which were further analyzed separately through the use of functional core analysis algorithm from Ingenuity Pathways Analysis (IPA: Ingenuity Systems®, www.ingenuity.com). The identified genes were mapped to functional networks in the IPA knowledge Base database and ranked by score. Each network is a graphical representation of the molecular relationships between genes. The score is a numerical value used to rank networks according to their degree of relevance to the network eligible molecules in the experimental dataset. The scores for all of the major mapped networks were higher than 22, indicating the networks selected were not due to random chance alone (Table 3). A score of 3 or greater was considered significant at $p < 0.001$ level. The network Score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's Exact Test. The score is the negative log of this p-value. This score was used as the cut-off for identifying significantly perturbed biological networks (Calvano et al 2005).

Core functions analysis of the genes induced during MIPM and BIPM was done separately and then compared to evaluate the set of genes induced by these two different memory mechanisms in the context of biological processes, canonical pathways and molecular networks. The relationships between the network generated in IPA and the known pathways that were associated with synaptic plasticity-related signaling were investigated further by canonical pathway analysis. Benjamini-Hochberg multiple-testing corrected p-values were used to calculate the significance value for determining the probability that each biological function analysis assigned to that data set was due to chance alone. Benjamini-Hochberg multiple-testing calculation returns adjusted or corrected p-values and controls the noise in

Functional Analysis (Benjamini and Hochberg 1995). At threshold 0.01 ($p \leq 0.05$), we can expect that the fraction of false positives among the significant functions is less than 5%. The significance of a canonical pathway is controlled by p-value, which was calculated using the right-tailed (over presented pathways) Fisher Exact Test. The significance threshold of pathways was set to 1.3 (derived by $-\log_{10} [p\text{-value}]$, whereas $p \leq 0.05$).

Hierarchical cluster analysis

To validate the observed effects of gene expression we performed cluster analysis on all of the significant genes assigned by our statistical analysis. Expression profiles of genes whose expression changed significantly ($p < 0.005$) between control animals and animals that underwent BIPM (186 genes) or MIPM (136 genes) were subjected to hierarchical clustering (Eisen et al 1998). We clustered the genes using agglomerative hierarchical average-linkage based on the Euclidian distance metric. The result is plotted using TreeView program, as a dendrogram that represents the clusters and relations between the clusters (Eisen, Spellman, Brown, and Botstein 1998).

Results and Discussion

Gene expression changes in the AOB during specific and non-specific pheromone memory-inducing protocols

Even though new protein synthesis has been implicated in induction of pheromone memory, no genome-wide gene expression studies have been carried out so far. A major constraint has been that only a small number of AOB synapses are modified during memory formation for pheromones of a specific strain of male such as Balb/c (Brennan and Keverne 1997). Thus it would be extremely difficult to detect the gene expression signal in a small number of neurons among thousands of unmodified neurons. Therefore, as a first step towards elucidation of gene expression in the AOB during pheromone memory formation, we used combined urinary pheromones from five different strains of males. Using this approach, we carried out microarray experiments on RNA isolated from AOB of females that underwent a pheromone memory inducing protocol by exposure to pheromones during mating (hereafter referred to as Mating Induced Pheromone Memory, MIPM). We also studied gene expression in the AOB of females that underwent chemical induction of pheromone memory using bicuculine (described in detail later in this section).

Our study design used specifically matched mice to control for confounders, and the mixed model analysis specifically incorporated this matching. In our microarray studies we performed detailed variance stabilization of all genes to ensure that we were not identifying genes that happened to have low variance, a factor that has been shown to be problematic in other cross-laboratory analysis (Etienne et al 2004).

We found significant differences in the levels of large number of transcripts between control and experimental samples. Cluster analysis of the data revealed non-matching hierarchical trees between genes induced during MIPM and bicuculine-induced pheromone memory (BIPM) (Fig. 1). In the AOB of females that underwent MIPM, 2076 transcripts were significantly increased and 65 transcripts were decreased (Supplementary Table 1). Although we employed rigorous statistical methods to determine the microarray results, as a way to validate the increase in these transcripts, we carried out Q-PCR experiments on AOB samples derived from an entirely different set of animals that were subjected to a pheromone memory inducing protocol. Since the number of genes chosen for Q-PCR validation tends to be arbitrary, as a quick way to corroborate the microarray results, we randomly selected 3 genes namely Cdk5r1, dynein and kinesin and carried out Q-PCR experiments on AOB samples derived from a different set of animals that were subjected to a pheromone memory

inducing protocol. We carried out Q-PCR on AOB samples from individual animals. We observed significant (about 3-5-fold; $P < 0.05$; $n = 4$; Student's t-test) induction of all three genes tested (Fig. 2).

As a complementary approach to microarray studies on the AOB from mice that underwent physiological induction of pheromone memory, we carried out expression profiling on mice that were subjected to an artificial memory induction protocol. Previous work has established that infusion of bicuculline into the AOB causes formation of memory which is similar in behavioral manifestation to that induced by pheromones except that it is generalized to all pheromones and thus constitutes a non-specific memory (Kaba and Keverne 1988). At the level of AOB, the memory induced by bicuculline which theoretically would result in modification of all the mitral-granule reciprocal synapses in the AOB. It was expected that such a modification would result in a higher signal-to-noise ratio of memory-related changes in gene expression. In the AOB of animals that underwent pheromone memory induction through bicuculline infusion (BIPM) 260 transcripts were significantly increased and 46 transcripts were decreased (Supplementary Table 2).

Protein expression changes during MIPM

To test whether the gene expression changes that we observed are accompanied by corresponding changes in protein levels we carried out a set of experiments with a completely different group of animals. As before, we used a pheromone memory induction protocol that combined male pheromones from five different strains of mice. Five hours after mating we fixed the brains and subsequently subjected AOB sections to immunocytochemistry. We chose to test a candidate gene whose expression was increased during MIPM and the mRNA increase was validated by Q-PCR, namely Cdk5r1. In addition, we chose two other candidates identified by IPA analysis as important participants in signaling pathways relevant to synaptic plasticity, namely PKC α and eEF1A.

We tested the expression of Cdk5r1, PKC α and eEF1A using antibodies against these proteins and visualized the immunoreactivity using confocal microscopy. We found significant increase in expression of all three proteins. The increase in expression of the three proteins was not uniform across the whole AOB but rather cell-type specific. We found nuclear expression of Cdk5r1 as was expected for this protein in post-mitotic neurons (Zhang et al 2008). Cdk5r1 protein is expressed at higher basal levels in granule cells compared to mitral cells but with MIPM both cell types showed significant increase ($P < 0.001$; $n = 6$; Student's t-test). PKC α protein is found in the cytoplasm and the plasma membrane and is expressed uniformly in both mitral and granule cells. PKC α protein quantity was also significantly ($P < 0.01$; $n = 4$; Student's t-test) increased in both mitral and granule cells. eEF1A expression is cytoplasmic and the protein is expressed at slightly higher basal levels in mitral cells compared to granule cells but with MIPM there was a significant ($P < 0.01$; $n = 4$; Student's t-test) increase in expression in both cell types.

Biofunctional analysis of genes reveals different patterns of gene induction during MIPM compared to BIPM

We analyzed the genes whose expression is increased during MIPM and BIPM for 'Biofunctions' through Ingenuity Pathway Analysis (IPA). The IPA uses a manually-curated knowledge base to perform statistical tests on a given list of genes to identify functional associations supported by experimental results in the literature (Calvano, Xiao, Richards, Felciano, Baker, Cho, Chen, Brownstein, Cobb, Tschoeke, Miller-Graziano, Moldawer, Mindrinos, Davis, Tompkins, and Lowry 2005). The categories that exceeded the significance threshold were Neurological disease, Nervous system development and function, Cellular function and maintenance, Cell-to-cell signaling and interaction and

Cellular assembly and organization (Table 3). Overall, among the network eligible genes (showing interaction with other molecules) 92 genes were induced only by MIPM, 76 genes were induced only by BIPM and 16 genes were induced by both (Fig. 3). In each of the Biofunction categories some induced genes were common between MIPM and BIPM and several were not (Fig. 4, Supplementary Table 4). If one considers the sets of genes that do not overlap between BIPM and MIPM datasets, a pattern appears to emerge. That is, in BIPM the genes induced were the ones with cell-wide effects, whereas the genes induced in MIPM have more local functions at the synapse.

For example, under *Neurological disease*, histone deacetylase 5 (HDAC5) was increased in BIPM. In rodents, chronic stress or cocaine administration decreases HDAC function and allows histone acetylation and transcription (Renthal et al 2007). HDAC5 has been shown to be part of the molecular cascade that induces long-term depression in *Aplysia* neurons (Guan et al 2002). Since increased inhibitory transmission underlies pheromone memory (Brennan and Keverne 1997), an increase in HDAC5 might be suppressing the expression of genes that function in increasing the strength of excitatory transmission. In contrast to the cell-wide nature of the effect of genes induced in BIPM, the genes induced during MIPM are the ones that are known to have local role at the synapse. For example, the expression of eukaryotic translation elongation factor 1 alpha 1 (eEF1A) is increased following MIPM. In both invertebrates and vertebrates eEF1A plays an important role in synapse-specific plasticity by controlling local translation of proteins from pre-existing mRNAs at the synapse (Giustetto et al 2003; Tsokas et al 2005).

With respect to Nervous system development and function, during BIPM, transcription factors such as c-Fos, Egr1, Egr2, and Egr3 are induced. Egr1 is widely recognized as a plasticity-related gene and has been shown to be critical for certain types of memory formation (Jones et al 2001; Okuno and Miyashita 1996). Egr2 and Egr3, although less well studied compared to Egr1, are also known to have a role in plasticity (DeSteno and Schmauss 2008; Li et al 2007b). Although many types of external stimuli induce c-Fos in neurons, c-Fos also has a role in learning and memory (Fleischmann et al 2003) and c-Fos is induced in AOB *in vivo* when pheromonal stimulation is accompanied by mating but not by pheromonal stimulation alone (Brennan, Hancock, and Keverne 1992). Our studies on AOB neurons in culture have shown induction of c-Fos when $\alpha 1$ and $\alpha 2$ adrenergic receptors and NMDA receptors are stimulated together (Skinner, Upadhya, Smith, Turner, and Hegde 2008). During MIPM however, the quantity of synaptic proteins relevant to nervous system development and function is altered. For example mRNAs of neurexin 1 and neurexin 2 and that of synaptotagmin 1 are increased. Neurexins are presynaptic adhesion molecules that interact with postsynaptic neuroligins and regulate specific synapse formation during development. Neurexins induce clustering of PSD-95 (Graf et al 2004). Although a role for neurexins in synaptic plasticity has been suggested based on their role in synapse formation, our studies provide evidence of a role for neurexins in synaptic plasticity associated with pheromone memory. Conceivably, neurexins play a role in inducing the structural changes at AOB synapses that have been reported to occur during pheromone memory formation (Matsuoka et al 1997; Matsuoka et al 2004).

Synaptotagmins are one of the key proteins present on synaptic vesicles. Synaptotagmins act as Ca^{2+} sensors and regulate fast synchronous neurotransmitter release (Xu et al 2007). A model developed based on our recent study suggests that sustained neurotransmitter release at mitral-granule cell synapses in the AOB is a key step in the induction of pheromone memory (Dong, Godwin, Brennan, and Hegde 2009). It must also be noted that neurexins directly interact with synaptotagmins and neurexin-neuroligin interaction is thought to induce recruitment of proteins that are part of the molecular machinery regulating release of neurotransmitters from synaptic vesicles (Lise and El-Husseini 2006). Analysis of

Biofunction with respect to Cellular function and maintenance revealed a role for Egr1 and Egr3 in BIPM and that of cyclin-dependent kinase 5, regulatory subunit 1 (Cdk5r1; also known as p35) in MIPM. The role of Egr1 and Egr3 in plasticity is discussed above. The role of Cdk5r1 in synaptic plasticity and memory has been shown in other models of memory such as fear conditioning (Fischer et al 2003). In the other categories of biofunction such as Cell-to-cell signaling and interaction and Cellular assembly and organization, the pattern discussed above (genes with cell-wide effects in BIPM and genes with synapse-specific effects in MIPM) holds true.

Pathway analysis of transcription in the AOB suggests differential induction of signaling pathways in MIPM and BIPM

We used IPA to identify the known signaling pathways induced during MIPM or BIPM. Because the IPA knowledge base is hand-curated and relies on identification of genes that belong to a specific signaling pathway based on experimental evidence in the literature, IPA allows an assessment of signaling pathways represented in lists of genes such as the ones obtained by microarray studies. The IPA has been used to analyze microarray data on synaptic plasticity related to drug addiction (Renthall, Maze, Krishnan, Covington, III, Xiao, Kumar, Russo, Graham, Tsankova, Kippin, Kerstetter, Neve, Haggarty, McKinsey, Bassel-Duby, Olson, and Nestler 2007) and in studies of neuronal transcriptome in many other contexts (Cahoy et al 2008; Chan et al 2008; Osherooff and Hatten 2009).

As mentioned before, bicuculline induces fewer genes compared to mating. Therefore, we narrowed down the list of genes induced in MIPM and BIPM by considering only those genes that were induced 1.1 fold or higher. This approach brings the number of genes in MIPM and BIPM close enough for comparison.

IPA revealed a few canonical signaling pathways that were common to MIPM and BIPM (Fig 5A). We also found signaling pathways that were induced only in MIPM (Fig. 5B) or predominantly in BIPM (Fig. 5C).

Signaling pathways induced during MIPM and BIPM

The signaling pathway components induced during MIPM as well as BIPM were Huntington disease signaling, 14-3-3-mediated signaling, GABA receptor signaling, CXCR4 receptor signaling and axon guidance (Fig 5A, Table 4A, Supplementary Table 5A).

Huntington disease signaling

Under this category the key genes induced in MIPM were PKC α , PKC β , and cdk5r1 and in BIPM were BDNF, histone deacetylase 5 and histone deacetylase 9. The role of PKC α in synaptic plasticity is well documented. A cognition enhancing drug nefiracetam has been shown to increase LTP through activation of PKC α and CaM kinase II (Moriguchi et al 2008). In the cerebellum, PKC α modulates interaction between PDZ domain-containing protein PICK1 and GluR2/3 subunit, which is required for cerebellar long-term depression (LTD) (Xia et al 2000). Work in our laboratory has demonstrated a role for PKC α in the early signaling events in AOB plasticity (Dong, Godwin, Brennan, and Hegde 2009). PKC β and myristoylated alanine-rich C kinase substrate (MARCKS) substrate protein play an obligatory role in vesicular trafficking in neurons (Yang et al 2002). Also, mRNAs encoding some PKC isoforms have been shown to be present in dendrites (Matsumoto et al 2007).

Among the genes induced during BIPM, the role of BDNF as a plasticity-related molecule has been established by numerous studies (Bramham and Messaoudi 2005; Lu 2003). Our studies have revealed a role for HDAC5 and HDAC9 in AOB plasticity induced by bicuculline. HDAC5 has been shown to play a role in long-term plasticity in invertebrate

neurons (Guan, Giustetto, Lomvardas, Kim, Miniaci, Schwartz, Thanos, and Kandel 2002; Renthal, Maze, Krishnan, Covington, III, Xiao, Kumar, Russo, Graham, Tsankova, Kippin, Kerstetter, Neve, Haggarty, McKinsey, Bassel-Duby, Olson, and Nestler 2007) and in mammalian neuronal plasticity related to drug addiction (Renthal et al., 2007).

Proenkephalin gene, which encodes precursor of opioid peptides, is induced in both MIPM and BIPM. Previous studies showed that LTP in the perforant path/granule cell synapses in the hippocampus is associated with a post-synaptic induction of proenkephalin mRNA (Roberts et al 1997). Work by others has also demonstrated a role for opioid receptors in mossy fiber LTP (Williams and Johnston 1996) which is presynaptically induced. Presynaptic mechanisms on mitral-granule as well as granule-mitral cell synapses are likely to play a role in regulating plasticity in the AOB (Brennan and Keverne 1997). Thus it is possible that proenkephalin gene expression plays a role in the induction of presynaptic plasticity in the AOB.

14-3-3 signaling

The 14-3-3 proteins regulate diverse physiological processes. We found that the levels of several genes products with significant linkage to 14-3-3 signaling are increased during MIPM and BIPM. The brain-specific isoform 14-3-3 η has been shown to be required for a presynaptic form of LTP in the cerebellum (Simsek-Duran et al 2004). The level of 14-3-3 η was increased during MIPM (Fig 6, Table 4A, Supplementary Table 5A) suggesting that 14-3-3 η might have a similar role in presynaptic mechanisms of synaptic plasticity in the AOB. Some 14-3-3 isoforms are known to interact with PKC isoforms including PKC α (Oriente et al 2005) which is also increased during MIPM.

During BIPM, even though 14-3-3 η is not increased, signaling molecules such as MAP3K5 and cytoskeletal proteins such as tubulin α 1a and tubulin α 1c are increased (Fig. 6). The 14-3-3 proteins are known to bind MAP3K family of proteins and regulate their function (Fritz et al 2006). Also, based on research on *Drosophila* nervous system, it has been proposed that 14-3-3 proteins cause reorganization of the cytoskeleton which enables docking of synaptic vesicles and neurotransmitter release (Skoulakis and Davis 1998). A transcription factor induced during BIPM, c-Fos is known to induce some isoforms of 14-3-3 (Johnston et al 2000). In addition, 14-3-3 are recruited to c-Fos and c-Jun containing nucleosomes (Macdonald et al 2005).

GABA receptor signaling

The α 2 subunit of GABA_A receptor was increased in animals that underwent BIPM. This would be consistent with an increased GABAergic inhibitory feedback from granule to mitral cells which is thought to underlie the maintenance of the pheromonal memory. The increased expression of GAD1, the enzyme critical for biosynthesis of GABA, in MIPM would similarly be expected to enhance this inhibitory feedback from granule cells (Fig. 7). This finding is supported by previous work showing that GABA release was increased during and following formation of pheromone memory (Brennan et al 1995). Dynamin 1, which is a GTPase (Clayton and Cousin 2009), and ubiquitin C have been implicated in endocytosis and both were increased in MIPM, (Fig. 7, Table 4A, Supplementary Table 5A). Attachment of a single ubiquitin serves as a tag for neurotransmitter receptor endocytosis (Robinson et al 1994) and endocytosed neurotransmitters are either recycled back to the plasma membrane or are degraded by the lysosome or the proteasome (Hegde 2004). Since GABA_A receptors are known to be degraded by the proteasome (Bedford et al 2001) and there were no significant increases in proteasome subunits, the increase in dynamin 1 might cause enhanced endocytosis and recycling of GABA_A receptors for fresh use, which should in turn help sustain high levels of inhibition of mitral cells by granule cells.

Axonal guidance signaling

Under this category, IPA recognizes signaling molecules that affect the axons as well as dendrites (Fig. 5A, Table 4A; Supplementary Table 5A). Among the molecules identified by IPA are PKC α , PKC β and neurotrophic tyrosine kinase receptor NTRK2 (TrkB). IPA also revealed increases in expression of IRSp53 (Insulin receptor substrate p53; also called BAIAP2), which is a component of the postsynaptic density (Soltau et al 2004).

Mice lacking IRSp53 exhibit enhanced NMDA receptor transmission, increased LTP and impaired learning (Kim et al 2009). Thus adequate amounts of IRSp53 might be required for normal plasticity that enables learning. IRSp53 mediates the effects of Rac1 and Cdc42, small GTPases that are known to control spine morphology. Experiments in cultured neurons showed that IRSp53 overexpression increases the density of spines (Choi et al 2005). Structural changes of AOB synapses have been shown to occur during pheromone memory formation (Matsuoka, Kaba, Mori, and Ichikawa 1997; Matsuoka, Kaba, Moriya, Yoshida-Matsuoka, Costanzo, Norita, and Ichikawa 2004). Thus increase in IRSp53 might have a role in causing structural changes in AOB synapses. IRSp53 is also known to be translocated to the synapses in an NMDA receptor-dependent fashion. Synaptic translocation of IRSp53 depends on PKC signaling (Hori et al 2005). The isoforms of PKC that mediate IRSp53 translocation have not been identified, however. It is possible that either PKC α or PKC β mediates translocation of IRSp53. During MIPM and BIPM, subunits of Arp2/3 complex (actin related protein 2/3) ARPC2 and ARPC1B are increased respectively (Table 4A). Arp2/3 complex is critical for development of dendritic spines and synapses (Wegner et al 2008). Arp2/3 function seems to be critical for synaptic plasticity and learning and memory. Disruption of signaling molecules upstream of Arp2/3, such as WAVE-1 (Wiskott-Aldrich Syndrome Protein family Verpolin homologous protein-1) alters LTP and LTD and impairs memory retention (Soderling et al 2003; Soderling et al 2007). Thus an increase in Arp2/3 subunits might have a positive effect on pheromonal memory formation.

CXCR4 signaling

Another pathway that is above the threshold only in MIPM is *CXCR4 signaling* (Fig. 5A, Table 4A; Supplementary Table 5A). CXCR4 is a G-protein coupled chemokine receptor that has been shown to activate a signaling cascade leading transcription of a specific set of genes in non-neuronal cells (Locati et al 2002). In NT2-N human neurons, CXCR4 activation causes transcription of a kinase MAP3K2, a G-protein α subunit and RGS4 (Valerio et al 2004). A previous study has shown that CXCR4 signaling modulates the frequency of Ca²⁺ spikes in cultured hippocampal neurons (Liu et al 2003).

Signaling pathways induced only during MIPM

Among the pathways induced only during MIPM were, α -adrenergic signaling, Neurotrophin/TRK signaling (Fig 5B, Table 4B, Supplementary Table 5B).

Neuregulin signaling and Synaptic LTP

The signaling pathways identified under *Neuregulin signaling* mainly comprise PKC α , PKC β and cdk5r1 (Fig. 5B; Table 4B; Supplementary Table 5B). Neuregulin has a role in synaptic plasticity and influences the expression of glutamate as well as GABA receptors (Rieff et al 1999). In cerebellar neurons for example, neuregulin-1 induces the GABA_A receptor β 2 subunit and increases the amplitude of GABA_A-mediated currents (Rieff, Raetzman, Sapp, Yeh, Siegel, and Corfas 1999). Neuronal activity causes cleavage of NRG1 cytoplasmic domain (Bao et al 2003) and the cleavage requires PKC (Ozaki et al 2004). It is

interesting to note that expression of neuroligins, the presynaptic binding partners of neuroligins, is increased only during MIPM.

PKC α and PKC β are also linked to signaling for *Synaptic LTP* along with adenylylase and calmodulin 1. The roles of these molecules in synaptic plasticity are discussed elsewhere.

cAMP-mediated signaling and G-protein-coupled signaling

The molecules linked to these signaling pathways are mainly adenylylase and calmodulin 1 (Fig. 5B; Table 4B; Supplementary Table 5B). Work carried out many years ago showed that a *Drosophila* learning mutant rutabaga had a defect in adenylylase (Livingstone et al 1984). In *Aplysia* Ca²⁺/calmodulin-sensitive adenylylase plays a role in associative synaptic plasticity that underlies classical conditioning (Abrams et al 1991; Onyike et al 1998). In mice, expression of adenylylase (type-1) increases LTP and memory (Wang et al 2004). Prior to our work, a role for adenylylase in pheromone memory was not indicated, and it remains to be seen how the adenylylase-cAMP pathway contributes to plasticity in the AOB.

Alpha adrenergic signaling

During MIPM molecules linked to α -adrenergic receptor signaling are induced. One such molecule is Gna2 (guanine nucleotide binding protein [G protein], α inhibiting activity polypeptide 2) which is known to be coupled to α -2 adrenergic receptors (Milligan et al 1990). Other key molecules identified by IPA under adrenergic receptor signaling are PKC α and PKC β . Electrophysiological studies carried out in our laboratory have identified PKC α as a coincidence detector that integrates glutamatergic and α -adrenergic signaling in the AOB. These studies also have identified roles for α -1 and α -2 adrenergic receptors (Dong, Godwin, Brennan, and Hegde 2009).

Oxidative phosphorylation

During MIPM several genes related to *Oxidative Phosphorylation*, a key function of mitochondria, such as cytochrome c oxidase are increased (Fig. 5B; Table 4B; Supplementary Table 5B). A role for mitochondria in synaptic plasticity has begun to be appreciated only recently (Mattson et al 2008). Mitochondria are present in axons, presynaptic terminals and dendritic spines, and movement of mitochondria into dendritic spines correlates with changes in spine morphology. Manipulations of dynamin-related protein 1 (a GTPase that regulates mitochondrial morphology and distribution) that reduce the amount of dendritic mitochondria cause loss of synapses and dendritic spines (Li et al 2004). In this context, it is interesting to note that dynamin 1 mRNA is increased in MIPM. Mitochondrial alterations have also been linked to neurological disorders that have synaptic plasticity defects, such as Alzheimer's disease (AD) (Chen and Yan 2007). In the brains of AD patients, activity of cytochrome c oxidase is decreased (Maurer et al 2000).

Signaling pathways induced only during BIPM

The signaling pathways significantly induced only in BIPM were: Neurotrophin/TRK signaling, Protein ubiquitination pathway, Regulation of actin-based motility by Rho and Phosphatidylinositol 3 kinase (PI3K)/AKT signaling (Fig 5C, Table 4C, Supplementary Table 5C).

Neurotrophin/TRK signaling

During BIPM, several components of the Neurotrophin/TRK signaling are elevated. Among the molecules directly connected to this pathway are BDNF, NTRK2 (TrkB) neurotrophin

receptor and Fos (Fig. 5C; Table 4C; Supplementary Table 5C). BDNF is rapidly induced in the hippocampus during contextual learning (Hall et al 2000). BDNF mRNA is also induced during LTP (Lee et al 2005). It is thought that the post-synaptically produced BDNF mRNA gives rise to increased BDNF protein that has both post- and pre-synaptic actions in strengthening the synapse. Many plasticity-related actions of BDNF are mediated by the NTRK2 (TrkB) receptor (Bramham and Messaoudi 2005). Application of BDNF causes an increase in long-term synaptic plasticity by promoting local protein synthesis in dendrites. Mice lacking either the BDNF gene or the TrkB gene show decreased synaptic plasticity (Korte et al 1995; Patterson et al 1996). Therefore, increased expression of NTRK2 (TrkB) during BIPM suggests a possible mechanism for amplification of the BDNF signal.

Protein ubiquitination pathway

Components of the ubiquitin-proteasome pathway such as the von Hippel Landau protein (VHL), Ube2J1, and Ubiquitin C are elevated in the AOB during induction of BIPM (Fig. 5C; Table 4C; Supplementary Table 5C). VHL protein is now recognized to be multifunctional adaptor protein that links different enzymes to their targets: for example, ubiquitin ligases and protein kinases to their substrates, co-activators to transcription factors and cargoes to molecular motors (Nyhan et al 2008). When VHL interacts with elongin B and elongin C, the complex has ubiquitin ligase activity. Ube2J1 (also known as Ubc6) is a ubiquitin conjugating enzyme which has a role in nuclear degradation of certain transcription repressors (Lenk and Sommer 2000). Ubiquitin C is a gene that encodes tandemly-linked polyubiquitin. In eukaryotic cells the polyubiquitin protein is processed to monoubiquitin which is used to mark protein substrates for ubiquitin-proteasome-mediated degradation. The role of ubiquitin-proteasome-mediated degradation in synaptic plasticity is well established. For example, proteolysis regulates protein kinases, transcription factors and synaptic molecules critical for synaptic plasticity (Hegde 2004; Hegde and Upadhyaya 2007).

Regulation of actin-based motility by Rho

Components of a signaling pathway that governs actin-based motility were up-regulated during BIPM. The actin-cytoskeleton is critical for formation of synapses as well as for regulating synaptic transmission and plasticity. *In vivo* LTP in the hippocampal dentate gyrus is accompanied by enhancement in F-actin and interfering with actin polymerization hinders late phase LTP (Fukazawa et al 2003). Furthermore, interfering with the actin cytoskeleton prevents conversion of early phase LTP to late phase LTP (Ramachandran and Frey 2009). Presynaptic actin is believed to regulate the availability of synaptic vesicles, and actin anchors postsynaptic receptors such as NMDA and AMPA receptors in the postsynaptic compartment (Cingolani and Goda 2008). The dendritic spines, the sites where bulk of the synapses are made in the central nervous system, show remarkable actin-based plasticity. Dendritic spine plasticity is highly relevant to pheromone memory because structural changes at the dendritic synapses between mitral and granule cells of the AOB accompany formation of pheromone memory (Matsuoka, Kaba, Mori, and Ichikawa 1997; Matsuoka, Kaba, Moriya, Yoshida-Matsuoka, Costanzo, Norita, and Ichikawa 2004). Even though multiple signaling pathways can influence actin-based plasticity of dendritic spines, the 'common denominator' appears to be a family of small proteins called Rho, which are GTPases (Carlisle and Kennedy 2005). Proteins that interact directly or indirectly with Rho such as subunit 1B of the Arp2/3 complex, IRSp53 and the catalytic subunit of phosphatidylinositol 4-kinase are increased during bicuculline-induced pheromonal memory. These molecules might help reshape dendritic spines during the heightened neuronal activity induced by inhibition of GABA_A receptors by bicuculline. In support of this idea, it has been shown that excitation of neurons by NMDA and AMPA receptor stimulation helps spine formation and stabilization respectively (Fischer et al 2000).

PI3K/AKT signaling

In non-neuronal cells PI3K is linked to cell division and growth control. A protein regulated by PI3K, cyclin-dependent kinase inhibitor 1 a (cdkn1a; also called p21 or Cip1), is induced during BIPM. Cdkn1a inhibits cyclin-Cdk2 or -Cdk4 complexes, and treatment of slices with Cdk4 inhibitor reduces mGluR-dependent LTD in the hippocampal CA1 region (Li et al 2007a). PI3K is required for expression but not induction or maintenance of LTP (Sanna et al 2002) and it has been suggested that PI3K might influence AMPA receptor insertion into the plasma membrane during LTP. In cultured hippocampal neurons, PI3K inhibition blocks NMDA receptor dependent exocytosis of AMPA receptor subunit GluR1 (Passafaro et al 2001). AMPA receptors contribute to excitatory transmission from mitral to granule cells in the AOB (Brennan and Keverne 1997). Therefore, it is possible that during BIPM, increased activity of the PI3K pathway enhances AMPA receptor insertion on the postsynaptic granule cell membrane, which would in turn strengthen granule-to-mitral cell inhibitory synaptic transmission and thereby contribute to BIPM.

Conclusion

The IPA analyses on the microarray data indicated that several pathways are induced only during MIPM or BIPM indicating induction of a large number of non-overlapping sets of genes in these two memory paradigms. There was induction of a small set of common genes between the two paradigms as well. Our analyses suggest that signaling molecules that function at the synaptic level were induced during MIPM. Therefore it is possible that the protein products induced during MIPM (such as translation factors and synaptic structural proteins) contribute to synapse-specific local alterations that underlie the specificity of the female's memory for the mating male's pheromones. In contrast, during BIPM, it appears that the induced genes encode proteins likely to contribute to global changes (such as transcription factors), which could explain the non-specific nature of the memory. This would result from widespread alteration of most, if not all, of the mitral-granule cell synapses in the AOB. This is reminiscent of local and global effects on synaptic plasticity found in invertebrates as well as in the vertebrate hippocampus (Casadio et al 1999; Frey and Morris 1997; Sossin 1996; Yin 1999). Nonetheless, several molecular players discovered by the current analysis are novel. For example, our studies revealed a role for PKC α and PKC β in long-term synaptic plasticity (Dong, Godwin, Brennan, and Hegde 2009). Although it is likely that the candidate genes we have observed have a role on AOB plasticity underlying pheromone memory, with the present studies one cannot link gene and protein expression to causation of AOB plasticity. Future studies will be required to address the possible causative roles of the key molecules found in this study and the mechanisms by which these molecules contribute to plasticity of the dendrodendritic synapses in the AOB underlying pheromone memory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from Tab Williams Endowment Fund and Whitehall Foundation grant to ANH. SCU was supported by a training grant from NIH (DC 000057).

References

- Abrams TW, Karl KA, Kandel ER. Biochemical studies of stimulus convergence during classical conditioning in *Aplysia*: dual regulation of adenylate cyclase by Ca^{2+} /calmodulin and transmitter. *J. Neurosci.* 1991; 11:2655–2665. [PubMed: 1679120]
- Bao J, Wolpowitz D, Role LW, Talmage DA. Back signaling by the Nrg-1 intracellular domain. *J. Cell Biol.* 2003; 161:1133–1141. [PubMed: 12821646]
- Bedford FK, Kittler JT, Muller E, Thomas P, Uren JM, Merlo D, Wisden W, Triller A, Smart TG, Moss SJ. GABA(A) receptor cell surface number and subunit stability are regulated by the ubiquitin-like protein Plic-1. *Nat. Neurosci.* 2001; 4:908–916. [PubMed: 11528422]
- Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)*. 1995; 57:289–300.
- Bramham CR, Messaoudi E. BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. *Prog. Neurobiol.* 2005; 76:99–125. [PubMed: 16099088]
- Brennan PA, Hancock D, Keverne EB. The expression of the immediate-early genes *c-fos*, *egr-1* and *c-jun* in the accessory olfactory bulb during the formation of an olfactory memory in mice. *Neuroscience*. 1992; 49:277–284. [PubMed: 1279452]
- Brennan PA, Kendrick KM, Keverne EB. Neurotransmitter release in the accessory olfactory bulb during and after the formation of an olfactory memory in mice. *Neuroscience*. 1995; 69:1075–1086. [PubMed: 8848096]
- Brennan PA, Keverne EB. Neural mechanisms of mammalian olfactory learning. *Prog. Neurobiol.* 1997; 51:457–481. [PubMed: 9106902]
- Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, Xing Y, Lubischer JL, Krieg PA, Krupenko SA, Thompson WJ, Barres BA. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J. Neurosci.* 2008; 28:264–278. [PubMed: 18171944]
- Calvano SE, Xiao W, Richards DR, Felciano RM, Baker HV, Cho RJ, Chen RO, Brownstein BH, Cobb JP, Tschoeke SK, Miller-Graziano C, Moldawer LL, Mindrinos MN, Davis RW, Tompkins RG, Lowry SF. A network-based analysis of systemic inflammation in humans. *Nature*. 2005; 437:1032–1037. [PubMed: 16136080]
- Carlisle HJ, Kennedy MB. Spine architecture and synaptic plasticity. *Trends Neurosci.* 2005; 28:182–187. [PubMed: 15808352]
- Casadio A, Martin KC, Giustetto M, Zhu H, Chen M, Bartsch D, Bailey CH, Kandel ER. A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. *Cell*. 1999; 99:221–237. [PubMed: 10535740]
- Chan JP, Cordeira J, Calderon GA, Iyer LK, Rios M. Depletion of central BDNF in mice impedes terminal differentiation of new granule neurons in the adult hippocampus. *Mol. Cell Neurosci.* 2008; 39:372–383. [PubMed: 18718867]
- Cheetham SA, Smith AL, Armstrong SD, Beynon RJ, Hurst JL. Limited variation in the major urinary proteins of laboratory mice. *Physiol Behav.* 2009; 96:253–261. [PubMed: 18973768]
- Chen JX, Yan SD. Amyloid-beta-induced mitochondrial dysfunction. *J. Alzheimers. Dis.* 2007; 12:177–184. [PubMed: 17917162]
- Choi J, Ko J, Racz B, Burette A, Lee JR, Kim S, Na M, Lee HW, Kim K, Weinberg RJ, Kim E. Regulation of dendritic spine morphogenesis by insulin receptor substrate 53, a downstream effector of Rac1 and Cdc42 small GTPases. *J. Neurosci.* 2005; 25:869–879. [PubMed: 15673667]
- Chu TM, Deng S, Wolfinger R, Paules RS, Hamadeh HK. Cross-site comparison of gene expression data reveals high similarity. *Environ. Health Perspect.* 2004a; 112:449–455. [PubMed: 15033594]
- Chu TM, Weir BS, Wolfinger RD. Comparison of Li-Wong and loglinear mixed models for the statistical analysis of oligonucleotide arrays. *Bioinformatics*. 2004b; 20:500–506. [PubMed: 14990445]
- Cingolani LA, Goda Y. Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. *Nat. Rev. Neurosci.* 2008; 9:344–356. [PubMed: 18425089]

- Clayton EL, Cousin MA. The molecular physiology of activity-dependent bulk endocytosis of synaptic vesicles. *J. Neurochem.* 2009; 111:901–914. [PubMed: 19765184]
- DeSteno DA, Schmauss C. Induction of early growth response gene 2 expression in the forebrain of mice performing an attention-set-shifting task. *Neuroscience.* 2008; 152:417–428. [PubMed: 18280047]
- Dong C, Godwin DW, Brennan PA, Hegde AN. Protein kinase Calpha mediates a novel form of plasticity in the accessory olfactory bulb. *Neuroscience.* 2009; 163:811–824. [PubMed: 19580852]
- Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. U. S. A.* 1998; 95:14863–14868. [PubMed: 9843981]
- Etienne W, Meyer MH, Peppers J, Meyer RA Jr. Comparison of mRNA gene expression by RT-PCR and DNA microarray. *Biotechniques.* 2004; 36:618–6. [PubMed: 15088380]
- Fischer A, Sananbenesi F, Schrick C, Spiess J, Radulovic J. Regulation of contextual fear conditioning by baseline and inducible septo-hippocampal cyclin-dependent kinase 5. *Neuropharmacology.* 2003; 44:1089–1099. [PubMed: 12763101]
- Fischer M, Kaech S, Wagner U, Brinkhaus H, Matus A. Glutamate receptors regulate actin-based plasticity in dendritic spines. *Nat. Neurosci.* 2000; 3:887–894. [PubMed: 10966619]
- Fleischmann A, Hvalby O, Jensen V, Strekalova T, Zacher C, Layer LE, Kvello A, Reschke M, Spanagel R, Sprengel R, Wagner EF, Gass P. Impaired long-term memory and NR2A-type NMDA receptor-dependent synaptic plasticity in mice lacking c-Fos in the CNS. *J. Neurosci.* 2003; 23:9116–9122. [PubMed: 14534245]
- Frey U, Morris RG. Synaptic tagging and long-term potentiation. *Nature.* 1997; 385:533–536. [PubMed: 9020359]
- Fritz A, Brayer KJ, McCormick N, Adams DG, Wadzinski BE, Vaillancourt RR. Phosphorylation of serine 526 is required for MEKK3 activity, and association with 14-3-3 blocks dephosphorylation. *J. Biol. Chem.* 2006; 281:6236–6245. [PubMed: 16407301]
- Fukazawa Y, Saitoh Y, Ozawa F, Ohta Y, Mizuno K, Inokuchi K. Hippocampal LTP is accompanied by enhanced F-actin content within the dendritic spine that is essential for late LTP maintenance in vivo. *Neuron.* 2003; 38:447–460. [PubMed: 12741991]
- Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods.* 2001; 25:386–401. [PubMed: 11846608]
- Giustetto M, Hegde AN, Si K, Casadio A, Inokuchi K, Pei W, Kandel ER, Schwartz JH. Axonal transport of eukaryotic translation elongation factor 1alpha mRNA couples transcription in the nucleus to long-term facilitation at the synapse. *Proc. Natl. Acad. Sci. U. S. A.* 2003; 100:13680–13685. [PubMed: 14578450]
- Graf ER, Zhang X, Jin SX, Linhoff MW, Craig AM. Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell.* 2004; 119:1013–1026. [PubMed: 15620359]
- Guan Z, Giustetto M, Lomvardas S, Kim JH, Miniaci MC, Schwartz JH, Thanos D, Kandel ER. Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. *Cell.* 2002; 111:483–493. [PubMed: 12437922]
- Hall J, Thomas KL, Everitt BJ. Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. *Nat. Neurosci.* 2000; 3:533–535. [PubMed: 10816306]
- Hegde AN. Ubiquitin-proteasome-mediated local protein degradation and synaptic plasticity. *Prog. Neurobiol.* 2004; 73:311–357. [PubMed: 15312912]
- Hegde AN, Upadhyia SC. The ubiquitin-proteasome pathway in health and disease of the nervous system. *Trends Neurosci.* 2007; 30:587–595. [PubMed: 17950927]
- Hori K, Yasuda H, Konno D, Maruoka H, Tsumoto T, Sobue K. NMDA receptor-dependent synaptic translocation of insulin receptor substrate p53 via protein kinase C signaling. *J. Neurosci.* 2005; 25:2670–2681. [PubMed: 15758177]
- Hosack DA, Dennis G Jr, Sherman BT, Lane HC, Lempicki RA. Identifying biological themes within lists of genes with EASE. *Genome Biol.* 2003; 4:R70. [PubMed: 14519205]
- Johnston IM, Spence HJ, Winnie JN, McGarry L, Vass JK, Meagher L, Stapleton G, Ozanne BW. Regulation of a multigenic invasion programme by the transcription factor, AP-1: re-expression of

- a down-regulated gene, TSC-36, inhibits invasion. *Oncogene*. 2000; 19:5348–5358. [PubMed: 11103936]
- Jones MW, Errington ML, French PJ, Fine A, Bliss TV, Garel S, Charnay P, Bozon B, Laroche S, Davis S. A requirement for the immediate early gene *Zif268* in the expression of late LTP and long-term memories. *Nat. Neurosci.* 2001; 4:289–296. [PubMed: 11224546]
- Kaba H, Keverne EB. The effect of microinfusions of drugs into the accessory olfactory bulb on the olfactory block to pregnancy. *Neuroscience*. 1988; 25:1007–1011. [PubMed: 2841623]
- Kaba H, Rosser A, Keverne B. Neural basis of olfactory memory in the context of pregnancy block. *Neuroscience*. 1989; 32:657–662. [PubMed: 2601837]
- Kandel ER. The molecular biology of memory storage: a dialogue between genes and synapses. *Science*. 2001; 294:1030–1038. [PubMed: 11691980]
- Kim MH, Choi J, Yang J, Chung W, Kim JH, Paik SK, Kim K, Han S, Won H, Bae YS, Cho SH, Seo J, Bae YC, Choi SY, Kim E. Enhanced NMDA receptor-mediated synaptic transmission, enhanced long-term potentiation, and impaired learning and memory in mice lacking *IRSp53*. *J. Neurosci.* 2009; 29:1586–1595. [PubMed: 19193906]
- Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T. Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc. Natl. Acad. Sci. U. S. A.* 1995; 92:8856–8860. [PubMed: 7568031]
- Lee PR, Cohen JE, Becker KG, Fields RD. Gene expression in the conversion of early-phase to late-phase long-term potentiation. *Ann. N. Y. Acad. Sci.* 2005; 1048:259–271. [PubMed: 16154938]
- Lenk U, Sommer T. Ubiquitin-mediated proteolysis of a short-lived regulatory protein depends on its cellular localization. *J. Biol. Chem.* 2000; 275:39403–39410. [PubMed: 10991948]
- Li C, Li X, Chen W, Yu S, Chen J, Wang H, Ruan D. The different roles of cyclinD1-CDK4 in STP and mGluR-LTD during the postnatal development in mice hippocampus area CA1. *BMC. Dev. Biol.* 2007a; 7:57. [PubMed: 17535444]
- Li L, Yun SH, Keblesh J, Trommer BL, Xiong H, Radulovic J, Tourtellotte WG. *Egr3*, a synaptic activity regulated transcription factor that is essential for learning and memory. *Mol. Cell Neurosci.* 2007b; 35:76–88. [PubMed: 17350282]
- Li Z, Okamoto K, Hayashi Y, Sheng M. The importance of dendritic mitochondria in the morphogenesis and plasticity of spines and synapses. *Cell*. 2004; 119:873–887. [PubMed: 15607982]
- Lise MF, El-Husseini A. The neuroligin and neuroligin families: from structure to function at the synapse. *Cell Mol. Life Sci.* 2006; 63:1833–1849. [PubMed: 16794786]
- Liu Z, Geng L, Li R, He X, Zheng JQ, Xie Z. Frequency modulation of synchronized Ca^{2+} spikes in cultured hippocampal networks through G-protein-coupled receptors. *J. Neurosci.* 2003; 23:4156–4163. [PubMed: 12764103]
- Livingstone MS, Sziber PP, Quinn WG. Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a *Drosophila* learning mutant. *Cell*. 1984; 37:205–215. [PubMed: 6327051]
- Locati M, Deuschle U, Massardi ML, Martinez FO, Sironi M, Sozzani S, Bartfai T, Mantovani A. Analysis of the gene expression profile activated by the CC chemokine ligand 5/RANTES and by lipopolysaccharide in human monocytes. *J. Immunol.* 2002; 168:3557–3562. [PubMed: 11907119]
- Lu B. BDNF and activity-dependent synaptic modulation. *Learn. Mem.* 2003; 10:86–98. [PubMed: 12663747]
- Macdonald N, Welburn JP, Noble ME, Nguyen A, Yaffe MB, Clynes D, Moggs JG, Orphanides G, Thomson S, Edmunds JW, Clayton AL, Endicott JA, Mahadevan LC. Molecular basis for the recognition of phosphorylated and phosphoacetylated histone h3 by 14-3-3. *Mol. Cell.* 2005; 20:199–211. [PubMed: 16246723]
- Matsumoto M, Setou M, Inokuchi K. Transcriptome analysis reveals the population of dendritic RNAs and their redistribution by neural activity. *Neurosci. Res.* 2007; 57:411–423. [PubMed: 17207874]
- Matsuoka M, Kaba H, Mori Y, Ichikawa M. Synaptic plasticity in olfactory memory formation in female mice. *Neuroreport*. 1997; 8:2501–2504. [PubMed: 9261816]
- Matsuoka M, Kaba H, Moriya K, Yoshida-Matsuoka J, Costanzo RM, Norita M, Ichikawa M. Remodeling of reciprocal synapses associated with persistence of long-term memory. *Eur. J. Neurosci.* 2004; 19:1668–1672. [PubMed: 15066163]

- Mattson MP, Gleichmann M, Cheng A. Mitochondria in neuroplasticity and neurological disorders. *Neuron*. 2008; 60:748–766. [PubMed: 19081372]
- Matynia A, Kushner SA, Silva AJ. Genetic approaches to molecular and cellular cognition: a focus on LTP and learning and memory. *Annu. Rev. Genet.* 2002; 36:687–720. [PubMed: 12429705]
- Maurer I, Zierz S, Moller HJ. A selective defect of cytochrome c oxidase is present in brain of Alzheimer disease patients. *Neurobiol. Aging*. 2000; 21:455–462. [PubMed: 10858595]
- McGuire SE, Deshazer M, Davis RL. Thirty years of olfactory learning and memory research in *Drosophila melanogaster*. *Prog. Neurobiol.* 2005; 76:328–347. [PubMed: 16266778]
- Milligan G, Mullaney I, McKenzie FR. Specificity of interactions of receptors and effectors with GTP-binding proteins in native membranes. *Biochem. Soc. Symp.* 1990; 56:21–34. [PubMed: 2175190]
- Moriguchi S, Shioda N, Han F, Narahashi T, Fukunaga K. CaM kinase II and protein kinase C activations mediate enhancement of long-term potentiation by nefiracetam in the rat hippocampal CA1 region. *J. Neurochem.* 2008; 106:1092–1103. [PubMed: 18445137]
- Nyhan MJ, O'Sullivan GC, McKenna SL. Role of the VHL (von Hippel-Lindau) gene in renal cancer: a multifunctional tumour suppressor. *Biochem. Soc. Trans.* 2008; 36:472–478. [PubMed: 18481984]
- Okuno H, Miyashita Y. Expression of the transcription factor Zif268 in the temporal cortex of monkeys during visual paired associate learning. *Eur. J. Neurosci.* 1996; 8:2118–2128. [PubMed: 8921303]
- Onyike CU, Lin AH, Abrams TW. Persistence of the interaction of calmodulin with adenylyl cyclase: implications for integration of transient calcium stimuli. *J. Neurochem.* 1998; 71:1298–1306. [PubMed: 9721756]
- Oriente F, Andreozzi F, Romano C, Perruolo G, Perfetti A, Fiory F, Miele C, Beguinot F, Formisano P. Protein kinase C- α regulates insulin action and degradation by interacting with insulin receptor substrate-1 and 14-3-3 epsilon. *J. Biol. Chem.* 2005; 280:40642–40649. [PubMed: 16216880]
- Osheroff H, Hatten ME. Gene expression profiling of preplate neurons destined for the subplate: genes involved in transcription, axon extension, neurotransmitter regulation, steroid hormone signaling, and neuronal survival. *Cereb. Cortex*. 2009; 19(Suppl 1):i126–i134. [PubMed: 19398467]
- Ozaki M, Itoh K, Miyakawa Y, Kishida H, Hashikawa T. Protein processing and releases of neuregulin-1 are regulated in an activity-dependent manner. *J. Neurochem.* 2004; 91:176–188. [PubMed: 15379898]
- Passafaro M, Piech V, Sheng M. Subunit-specific temporal and spatial patterns of AMPA receptor exocytosis in hippocampal neurons. *Nat. Neurosci.* 2001; 4:917–926. [PubMed: 11528423]
- Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER. Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron*. 1996; 16:1137–1145. [PubMed: 8663990]
- Pinheiro, JC.; Bates, DM. Mixed-effects models in S and S-PLUS. Springer; New York: 2000.
- Ramachandran B, Frey JU. Interfering with the actin network and its effect on long-term potentiation and synaptic tagging in hippocampal CA1 neurons in slices in vitro. *J. Neurosci.* 2009; 29:12167–12173. [PubMed: 19793974]
- Reissner KJ, Shobe JL, Carew TJ. Molecular nodes in memory processing: insights from Aplysia. *Cell Mol. Life Sci.* 2006; 63:963–974. [PubMed: 16596335]
- Renthal W, Maze I, Krishnan V, Covington HE III, Xiao G, Kumar A, Russo SJ, Graham A, Tsankova N, Kippin TE, Kerstetter KA, Neve RL, Haggarty SJ, McKinsey TA, Bassel-Duby R, Olson EN, Nestler EJ. Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. *Neuron*. 2007; 56:517–529. [PubMed: 17988634]
- Rieff HI, Raetzman LT, Sapp DW, Yeh HH, Siegel RE, Corfas G. Neuregulin induces GABA(A) receptor subunit expression and neurite outgrowth in cerebellar granule cells. *J. Neurosci.* 1999; 19:10757–10766. [PubMed: 10594059]
- Roberts LA, Large CH, O'Shaughnessy CT, Morris BJ. Long-term potentiation in perforant path/granule cell synapses is associated with a post-synaptic induction of proenkephalin gene expression. *Neurosci. Lett.* 1997; 227:205–208. [PubMed: 9185686]

- Robertson DH, Hurst JL, Bolgar MS, Gaskell SJ, Beynon RJ. Molecular heterogeneity of urinary proteins in wild house mouse populations. *Rapid Commun. Mass Spectrom.* 1997; 11:786–790. [PubMed: 9161047]
- Robinson PJ, Liu JP, Powell KA, Fykse EM, Sudhof TC. Phosphorylation of dynamin I and synaptic-vesicle recycling. *Trends Neurosci.* 1994; 17:348–353. [PubMed: 7526507]
- Sanna PP, Cammalleri M, Berton F, Simpson C, Lutjens R, Bloom FE, Francesconi W. Phosphatidylinositol 3-kinase is required for the expression but not for the induction or the maintenance of long-term potentiation in the hippocampal CA1 region. *J. Neurosci.* 2002; 22:3359–3365. [PubMed: 11978812]
- Selway R, Keverne EB. Hippocampal lesions are without effect on olfactory memory formation in the context of pregnancy block. *Physiol Behav.* 1990; 47:249–252. [PubMed: 2333338]
- Shi L, Jones WD, Jensen RV, Harris SC, Perkins RG, Goodsaid FM, Guo L, Croner LJ, Boysen C, Fang H, Qian F, Amur S, Bao W, Barbacioru CC, Bertholet V, Cao XM, Chu TM, Collins PJ, Fan XH, Frueh FW, Fuscoe JC, Guo X, Han J, Herman D, Hong H, Kawasaki ES, Li QZ, Luo Y, Ma Y, Mei N, Peterson RL, Puri RK, Shippy R, Su Z, Sun YA, Sun H, Thorn B, Turpaz Y, Wang C, Wang SJ, Warrington JA, Willey JC, Wu J, Xie Q, Zhang L, Zhang L, Zhong S, Wolfinger RD, Tong W. The balance of reproducibility, sensitivity, and specificity of lists of differentially expressed genes in microarray studies. *BMC. Bioinformatics.* 2008; 9(Suppl 9):S10. [PubMed: 18793455]
- Simsek-Duran F, Linden DJ, Lonart G. Adapter protein 14-3-3 is required for a presynaptic form of LTP in the cerebellum. *Nat. Neurosci.* 2004; 7:1296–1298. [PubMed: 15543142]
- Skinner CB, Upadhyia SC, Smith TK, Turner CP, Hegde AN. Signal transduction and gene expression in cultured accessory olfactory bulb neurons. *Neuroscience.* 2008; 157:340–348. [PubMed: 18848604]
- Skoulakis EM, Davis RL. 14-3-3 proteins in neuronal development and function. *Mol. Neurobiol.* 1998; 16:269–284. [PubMed: 9626666]
- Soderling SH, Guire ES, Kaech S, White J, Zhang F, Schutz K, Langeberg LK, Banker G, Raber J, Scott JD. A WAVE-1 and WRP signaling complex regulates spine density, synaptic plasticity, and memory. *J. Neurosci.* 2007; 27:355–365. [PubMed: 17215396]
- Soderling SH, Langeberg LK, Soderling JA, Davee SM, Simerly R, Raber J, Scott JD. Loss of WAVE-1 causes sensorimotor retardation and reduced learning and memory in mice. *Proc. Natl. Acad. Sci. U. S. A.* 2003; 100:1723–1728. [PubMed: 12578964]
- Soltau M, Berhorster K, Kindler S, Buck F, Richter D, Kreienkamp HJ. Insulin receptor substrate of 53 kDa links postsynaptic shank to PSD-95. *J. Neurochem.* 2004; 90:659–665. [PubMed: 15255944]
- Sossin WS. Mechanisms for the generation of synapse specificity in long-term memory: the implications of a requirement for transcription. *Trends Neurosci.* 1996; 19:215–218. [PubMed: 8761954]
- Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. U. S. A.* 2003; 100:9440–9445. [PubMed: 12883005]
- Tsokas P, Grace EA, Chan P, Ma T, Sealton SC, Iyengar R, Landau EM, Blitzer RD. Local protein synthesis mediates a rapid increase in dendritic elongation factor 1A after induction of late long-term potentiation. *J. Neurosci.* 2005; 25:5833–5843. [PubMed: 15958750]
- Valerio A, Ferrario M, Martinez FO, Locati M, Ghisi V, Bresciani LG, Mantovani A, Spano P. Gene expression profile activated by the chemokine CCL5/RANTES in human neuronal cells. *J. Neurosci. Res.* 2004; 78:371–382. [PubMed: 15389840]
- Wang H, Ferguson GD, Pineda VV, Cundiff PE, Storm DR. Overexpression of type-1 adenylyl cyclase in mouse forebrain enhances recognition memory and LTP. *Nat. Neurosci.* 2004; 7:635–642. [PubMed: 15133516]
- Wegner AM, Nebhan CA, Hu L, Majumdar D, Meier KM, Weaver AM, Webb DJ. N-wasp and the arp2/3 complex are critical regulators of actin in the development of dendritic spines and synapses. *J. Biol. Chem.* 2008; 283:15912–15920. [PubMed: 18430734]
- Williams SH, Johnston D. Actions of endogenous opioids on NMDA receptor-independent long-term potentiation in area CA3 of the hippocampus. *J. Neurosci.* 1996; 16:3652–3660. [PubMed: 8642409]

- Xia J, Chung HJ, Wihler C, Huganir RL, Linden DJ. Cerebellar long-term depression requires PKC-regulated interactions between GluR2/3 and PDZ domain-containing proteins. *Neuron*. 2000; 28:499–510. [PubMed: 11144359]
- Xu J, Mashimo T, Sudhof TC. Synaptotagmin-1, -2, and -9: Ca(2+) sensors for fast release that specify distinct presynaptic properties in subsets of neurons. *Neuron*. 2007; 54:567–581. [PubMed: 17521570]
- Yang H, Wang X, Sumners C, Raizada MK. Obligatory role of protein kinase C β and MARCKS in vesicular trafficking in living neurons. *Hypertension*. 2002; 39:567–572. [PubMed: 11882609]
- Yin JC. Location, location, location: the many addresses of memory formation. *Proc. Natl. Acad. Sci. U. S. A.* 1999; 96:9985–9986. [PubMed: 10468544]
- Yin JC, Tully T. CREB and the formation of long-term memory. *Curr. Opin. Neurobiol.* 1996; 6:264–268. [PubMed: 8725970]
- Zhang J, Cicero SA, Wang L, Romito-Digiacomo RR, Yang Y, Herrup K. Nuclear localization of Cdk5 is a key determinant in the postmitotic state of neurons. *Proc. Natl. Acad. Sci. U. S. A.* 2008; 105:8772–8777. [PubMed: 18550843]

Highlights

- > We analyzed gene expression underlying two types of memory for pheromones.
- > We studied mating-induced and chemically-induced memories.
- > We used oligonucleotide microarrays for our study.
- > Using Ingenuity Pathway Analysis (IPA) we determined the signaling pathways.
- > IPA indicated that mating induces molecules that locally alter synapses.
- > Chemical induction of memory brings about neuron-wide changes.

Fig. 1.

Hierarchical cluster plot showing patterns of gene expression in the AOB induced during bicuculline-induced pheromone memory (BIPM) and mating-induced pheromone memory (MIPM).

Fluorescent log ratios of significant probe sets for BIPM (Panel A) and MIPM (Panel B) assigned by the statistical analysis with adjusted fold induction cut off value of 1.1 were subjected to hierarchical clustering to identify clustering within groups. Fluorescent log ratio is the logarithm of the ratio of a given gene's expression level in AOB of animals that underwent MIPM or BIPM to that particular gene's expression level in the reference state (untreated AOB). Horizontal rows of pixels represent different genes. The columns represent comparison between controls vs. experimental conditions. For example, in Panel A, C4-BICU4 is the gene expression in a bicuculline (BICU4) treated (BIPM) AOB compared to the gene expression in the control AOB treated with CSF (C4). In Panel B, gene expression in the AOB of an experimental animal (E1) that underwent MIPM is compared to gene expression in the AOB of a control animal (C13) ($n=4$ for BIPM & $n=5$ for MIPM). Each gene is represented by a single column or cell. Gene with fluorescent log ratios of 0 are colored black, increasingly positive log ratios with reds of increasing intensity, and increasingly negative log ratios with greens of increasing intensity. A dendrogram is shown at left of cluster plot to indicate the nature of the computed relationship among genes in the table. We observed two non-matching hierarchical trees between genes induced during BIPM and MIPM suggesting that different set of genes are induced in the two different memory pathways.

Fig. 2.

Validation of microarray data by real-time PCR. Quantification of levels of mRNA expression of dynein, kinesin and Cdk5r1 by real-time PCR showing significant (* $P < 0.05$; $n = 4$; student t-test) induction of these mRNAs with mating-induced pheromone memory. 18s rRNA was used as control. The expression levels of all three genes were calculated after normalization to expression of 18S rRNA.

Fig. 3.

Immunocytochemistry showing increased expression of Cdk5r1 in the AOB during MIPM. Control (Top panels): The figure shows confocal images of anti-Cdk5r1 immunoreactivity in the AOB (top middle). Nuclei were counterstained with TOPRO-3 iodide which stains double-stranded nucleic acids (top left). The first two panels are 'merged' in the top third panel. Experimental (Bottom panels): Confocal images of anti-Cdk5r1 (bottom middle) TOPRO-3 (bottom left) and merged (bottom right) of AOB from animals that underwent MIPM. GC: granule cell layer; MC/EPL: mitral cell/external plexiform layer; LOT/IPL: lateral olfactory tract/internal plexiform layer; Scale bar: 20 μ m.

Fig. 4. Immunocytochemistry showing increased expression of PKC α in the AOB during MIPM. Control (Top panels): The figure shows confocal images of anti-PKC α immunoreactivity in the AOB (top middle). Nuclei were counterstained with TOPRO-3 iodide which stains double-stranded nucleic acids (top left). The longish red TOPRO-3 stains probably resulted from squishing of cells together in a slightly distorted section. The first two panels are 'merged' in the top third panel. Experimental (Bottom panels): Confocal images of anti-PKC α (bottom middle) TOPRO-3 (bottom left) and merged (bottom right) of AOB from animals that underwent MIPM. GC: granule cell layer; MC/EPL: mitral cell/external plexiform layer; LOT/IPL: lateral olfactory tract/internal plexiform layer; Scale bar: 20 μ m.

Fig. 5. Immunocytochemistry showing increased expression of eEF1A in the AOB during MIPM. Control (Top panels): The figure shows confocal images of anti-eEF1A immunoreactivity in the AOB (top middle). Nuclei were counterstained with TOPRO-3 iodide which stains double-stranded nucleic acids (top left). The first two panels are 'merged' in the top third panel. Experimental (Bottom panels): Confocal images of anti-eEF1A (bottom middle) TOPRO-3 (bottom left) and merged (bottom right) of AOB from animals that underwent MIPM. MC/EPL: mitral cell/external plexiform layer; LOT/IPL: lateral olfactory tract/internal plexiform layer; GC: granule cell layer. Scale bar: 20 μ m.

Fig. 6.

Quantification of increase in expression of Cdk5r1, PKC α , and eEF1A during MIPM.

(A) Cdk5r1 immunoreactivity was significantly ($P < 0.001$; $n = 6$; Student's t-test) higher in both mitral cell (MC) and granule cell (GC) layers of the AOB during MIPM compared to controls.

(B) eEF1A immunoreactivity was significantly ($P < 0.01$; $n = 4$; Student's t-test) higher in both MC and GC layers of the AOB during MIPM compared to controls.

(C) PKC α immunoreactivity was significantly ($P < 0.01$; $n = 4$; Student's t-test) higher in both MC and GC layers of the AOB during MIPM compared to controls. AU: Arbitrary units.

Notation on Y-axis: $2e+5 = 2 \times 10^5$; $1e+6 = 1 \times 10^6$ etc.

Fig. 7.

Analysis of Biological Functional Pathways underlying BIPM and MIPM.

Biofunctional analysis was carried out using IPA knowledge Base database. Bar chart representing the significant changes in the seven functional categories among genes induced in the AOB during BIPM and MIPM. The functional categories are displayed along the x-axis, and the y-axis indicates the significance score (negative log of p-value calculated using Benjamini-Hochberg multiple-score test). The horizontal yellow line indicates the significance threshold. The significance threshold of pathways was set to 1.3 (derived by $-\log_{10} [p\text{-value}]$, whereas $p \geq 0.05$). Functional categories below the threshold line are not significant. Our result shows the statistically significant association ($p < 0.05$) of genes induced in the AOB during BIMP and MIPM among top seven biofunction categories (neurological disease etc as indicated along the x-axis). The bar chart also shows the higher significance value (lower Benjamini-Hochberg p-value) and higher number of focus molecules for genes induced during MIPM relative to the genes induced during BIPM in all seven functional categories

Fig. 8.

Differential expression of canonical pathway molecules during BIPM and MIPM. Significant canonical pathways as determined by using Ingenuity Pathway Knowledge Base functional analysis tools are displayed along the x-axis. The y-axis displays the significance score (negative log of p-value calculated using right-tailed Fisher Exact Test). The yellow threshold line that appears in the bar chart represents a p-value of 0.05. The significance threshold of pathways was set to 1.3 (derived by $-\log_{10} [P\text{-value}]$, whereas $P \leq 0.05$). Canonical pathways below the threshold line are not statistically significant. Beyond the threshold, the taller bars represent participation of higher number of genes in the pathway compared to shorter bars. (A) Genes induced during MIPM displaying higher statistical significance value (low p-value) or stronger association to the canonical pathways (Huntington's disease signaling, 14-3-3 mediated signaling, GABA receptor signaling, CXCR4 signaling and axon guidance signaling) compared to genes induced during BIPM. (B) Bar chart showing the significant association of mating induced genes to the canonical pathways like neuregulin signaling, synaptic long-term potentiation, cAMP-mediated signaling, G-protein coupled receptor signaling, α -adrenergic signaling, and oxidative phosphorylation. The genes in these pathways are expressed only in during MIPM. (C) Graph showing the significant association of genes induced during BIPM with the canonical pathways like neurotrophin.Trk signaling, protein ubiquitination pathway, regulation of actin-based motility by Rho and PI3K/AKT signaling. Association with these pathways among genes induced during MIPM is either absent or statistically not significant.

Fig. 9. Differential association of 14-3-3 mediated signaling among genes induced during BIPM compared to the genes induced during MIPM. Canonical pathway diagram representing 14-3-3 mediated signaling pathways matched to the network eligible genes induced during BIPM (A) and network eligible genes induced during MIPM (B). Mitogen-activated protein kinase 5 (ASK1), glial fibrillary acidic protein (GFAP), tubulin, microtubule-associated protein tau (Tau) and c-Fos show significant association with this pathway in BIPM dataset. Glial fibrillary acidic protein (GFAP), tubulin, glycogen synthase kinase 3 (GSK3A), protein kinase C (PKC) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3, YWHA1) show significant association with this pathway in the MIPM dataset. Red color nodes represent genes that appear in the bicuculline or mating dataset. Greater intensity of red represents a higher degree of upregulation.

Neurochem Int. Author manuscript; available in PMC 2012 November 1.

Table 1

Pregnancy block experiments showing formation of memory for pheromones of two different strains of inbred male mice

Group	Strain of mating male	Exposed to male of strain	Strain of blocking male	Percent showing pregnancy block	Sample size (n)
1	Balb/c	B10CB	Balb/c	21%	n=14
2	Balb/c	B10CB	B10CB	10%	n = 10
3	Balb/c	B10CB	DBA	80%	n = 10
4	Balb/c	SWR	SWR	0%	n = 10
5	Balb/c	SWR	Balb/c	20%	n = 10
6	Balb/c	SWR	B10CB	70%	n = 10

Groups 1 ($p = 0.007$) and 2 ($p = 0.003$) significantly differ from group 3. Groups 4 ($p = 0.001$) and 5 ($p = 0.035$) significantly differ from group 6 (Fisher exact probability test, 1-tailed).

Table 2

Real-time quantitative PCR (Q-PCR) Primers

Name	Protein ID	Primer length	Primer sequence
Dynein, light chain 1	NP_062656	11-36bp. 150-175bp	Sense: 5' GGAAGCGGTGATCAAAAATGCAGAC 3' Anti: 5' CCACAATGCAGTGCCAGGTAGGGTTG 3'
Kinesin family member 5C	NP_032475	130-156bp 251-285bp	Sense: 5' GGGAAGCCGTATGTCTTGACCGAGTG 3' Anti: 5' GGTATGAGTTTTTCCTGATGAAGTCTGCCCATATG 3'
Cyclin-dependent kinase 5, regulatory subunit (p35) 1 (CDk5r1)	NP_034001	207-231bp 356-383bp	Sense 5' GCCCAACAGCAGCTACCAGAGCAAC 3' Anti 5' GCCTTCTTGACAGAAGAGGAGACCCAG 3'

Table 3

List of Ingenuity networks generated by mapping the focus molecules that were upregulated during bicuculline and mating induced memory.

Analysis	Molecules in Network	Score *	Focus Molecules	Top Functions
Bicuculline-induced	Ap1, ATF3, CCL13, CEBPD, DIO2, EGR3, ERK, FOSB, FOSL2, FTH1, GFAP, HMGCS1, IL1, INHBA, JAK, JUND, KLF6, LMO4, NFIL3, NMDA Receptor, OSMR, Pdgf, PDGF BB, PDGF-AA, PENK, PLK2, SFRS10, SOCS2, SOCS3, SPRED2, STAT, STAT5a/b, TAC1, TPT1, UB	46	25	Gene Expression, Organismal Development, Nervous System Development and Function
Bicuculline-induced	AHCYL1, ALP, AP3B2, BAIAP2, Caspase, CDKN1A, CLTC, CSDC2, CSTB, Cytochrome c, EIF2C2, GAPDH, Hsp90, HSPA1A, HSPH1, IL12, Interferon alpha, Jnk, LDL, LMNA, MAP4K5, MCL1, NFKBIA, PI3K, Proteasome, PSMA3, PTGDS, Ras homolog, RNA polymerase	34	20	Cardiovascular Disease, Hematological Disease, Neurological Disease
Bicuculline-induced	ACTN1, ADCY, ADCYAP1, Alpha Actinin, BASP1, Calmodulin, Calpain, CCK, CHGB, CNN3, CUGBP2, DCLK1, EGR1, EGR2, ERK1/2, FOS, hCG, Insulin, JDP2, MAP2K1/2, MAPT, Mek, MRPS6, NEFM, P38 MAPK, Pka, Pkc(s), PLC, PP2A, PPM1L, PTPRN, Ras, RPS24	31	20	Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Cellular Assembly and Organization
Mating-induced	Actin, APBA1, Arp2/3, ARPC2, BAIAP2, Calpain, CAMK2N1, CaMKII, CDK5R1, CNR1, CX3CL1, DCLK1, DIO2, ENC1, ERK, F Actin, GAD1, GFAP, GPRASP1, GSK3A, KND1, MAP2, MBP, MYO5A, NCALD, NCKAP1, NRXN1, PHACTR1, Rac, SV2A, SYT1, TUBA1C, TUBB4, TUBB2A, Tubulin	53	27	Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Cellular Assembly and Organization
Mating-induced	ADCY, ADCY1, Ap1, ARPP-21, BMPR2, Calcineurin A, Calcineurin protein(s), CALM1, Calmodulin, Ck2, DLGAP1, DNM1, DYNLL1, EEF1A1, IFN Beta, Insulin, MT3, NFkB, NRG, NTRK2, P38 MAPK, Pdgf, PENK, Pkc(s), PPP3CB, PRKCA, PRKCB, PTMA, PTPN11, RAP1GDS1, RGS4, SL	40	22	Cellular Function and Maintenance, Hair and Skin Development and Function, Cancer
Mating-induced	ACO2, BBS2, CCNG2, CNBP, EPM2AIP1, FEM1B, GAS1, HNF4A, MRPL18, NDFIP1, NDUFA1, NDUFA4, NDUFA5, NDUFB1, NDUFB3, NDUFB5, NDUFS1, NDUFS3, NDUFS4, NDUFV1, NR2F2, OLIG1, PAPOLA, PJA2, PSME3, R3HDM1, RAD51, RFC3, RPL41, SFRS2, SHH, SNRPA, TYMS, USMG5, ZRANB2	22	14	Genetic Disorder, Neurological Disease, Connective Tissue Development and Function

* A score of 3 or greater was considered significant ($p < 0.001$).

Table 4A

Differential gene regulation in canonical pathways

Ingenuity Canonical Pathways	Analysis Name	−Log(P-value)	Ratio	Molecules
Huntington's Disease Signaling	Bicuculline-induced	2.63E00	3.88E-02	HSPA8, BDNF, HSPA1A, PENK, CLTC, HDAC9, UBC, HSPA5, HDAC5
Huntington's Disease Signaling	Mating-induced	3.36E00	3.88E-02	DNM1, DNAJC5, CPLX2, GLS, PENK, UBC, CDK5R1, PRKCA, PRKCB
14-3-3-mediated Signaling	Bicuculline-induced	2.36E00	5.36E-02	FOS, TUBA1A, MAPT, TUBA1C, GFAP, MAP3K5
14-3-3-mediated Signaling	Mating-induced	5.49E00	8.04E-02	YWHAH, TUBB2A, TUBA1C, GFAP, GSK3A, TUBB4, TUBB, PRKCA, PRKCB
GABA Receptor Signaling	Bicuculline-induced	8.68E-01	3.64E-02	UBC, GABRA2
GABA Receptor Signaling	Mating-induced	1.85E00	5.45E-02	DNM1, GAD1, UBC
CXCR4 Signaling	Bicuculline-induced	4.68E-01	1.83E-02	FOS, MLC1, EGR1
CXCR4 Signaling	Mating-induced	1.63E00	3.05E-02	GNAI2, MRLC2, ADCY1, PRKCA, PRKCB
Axonal Guidance Signaling	Bicuculline-induced	2.58E-01	1.27E-02	NTRK2, ARPC1B, BDNF, BAIAP2, LINGO1
Axonal Guidance Signaling	Mating-induced	1.38E00	2.03E-02	GNAI2, NTRK2, PPP3CB, PTPN11, ARPC2, BAIAP2, PRKCA, PRKCB

Table 4B

Differential gene regulation in canonical pathways

Ingenuity Canonical Pathways	Analysis Name	−Log(P-value)	Ratio	Molecules
Neuregulin Signaling	Mating-induced	1.76E00	4.08E-02	PTPN11, CDK5R1, PRKCA, PRKCB
Synaptic Long Term Potentiation	Mating-induced	2.21E00	4.35E-02	PPP3CB, ADCY1, PRKCA, CALM1, PRKCB
G-Protein Coupled Receptor Signaling	Mating-induced	1.62E00	2.79E-02	GNAI2, PDE2A, ADCY1, RGS4, PRKCA, PRKCB
α-Adrenergic Signaling	Mating-induced	2.48E00	4.72E-02	GNAI2, ADCY1, PRKCA, CALM1, PRKCB
cAMP-mediated Signaling	Mating-induced	2.15E00	3.7E-02	GNAI2, PDE2A, PPP3CB, ADCY1, RGS4, CALM1
Oxidative Phosphorylation	Mating-induced	2.52E00	4.46E-02	HCG 25371, COX6A1, NDUFS7, COX6C, NDUFB5, ATP6V1A, ATP6V1G2

Table 4C

Differential gene regulation in canonical pathways

Ingenuity Canonical Pathways	AnalysisName	−Log(P-value)	Ratio	Molecules
Neurotrophin/TRK Signaling	Bicuculline-induced	2.63E00	6.58E-02	FOS, NTRK2, BDNF, SPRY2, MAP3K5
Neurotrophin/TRK Signaling	Mating-induced	7.76E-01	2.63E-02	NTRK2, PTPN11
Protein Ubiquitination Pathway	Bicuculline-induced	2.45E00	3.47E-02	HSPA8, PSMA3, HSP90AA1, UBC, HSPA5, UBE2J1, VHL
Regulation of Actin-based Motility by Rho	Bicuculline-induced	2.36E00	5.43E-02	MLC1, ARPC1B, ACTB, BAIAP2, PI4KA
Regulation of Actin-based Motility by Rho	Mating-induced	1.28E00	3.26E-02	MRLC2, ARPC2, BAIAP2
PI3K/AKT Signaling	Bicuculline-induced	1.62E00	3.7E-02	NFKBIA, PPM1L, CDKN1A, HSP90AA1, MAP3K5
PI3K/AKT Signaling	Mating-induced	4.31E-01	1.48E-02	YWHAH, GSK3A