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Novel odors affect gene expression for cytokines and proteinases in the rat amygdala and hippocampus

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Abstract

Olfaction in rodents provides an excellent modality for the study of cellular mechanisms of information processing and storage, since a single occurrence of precisely timed stimuli has high survival value. We have followed up preliminary evidence of cytokine and proteinase involvement in normal (as opposed to pathologically-induced) brain plasticity by surveying for the presence of these factors in the olfactory circuitry of the rat. Genes for 25–30 common cytokines and their receptors, and over 30 cell matrix and adhesion molecules were found to be expressed across the olfactory bulb, insular cortex, amygdala, and dorsal hippocampus. We then measured by real-time PCR the transcriptional expression of seven of these genes following a one-time exposure to the novel odor of blueberry bars or cornnuts, in contrast to presentation of the familiar odor of lab chow. In the amygdala significant up-regulation of interleukin-1 receptor 1 (IL1r1), interleukin-4 receptor (IL4r), fibroblast growth factor 13 (FGF13), and cathepsin-H (CtsH) was observed in males in response to the odor of cornnuts only. Changes were less consistent and widespread in the hippocampus, but were again sex specific for three genes: cathepsin-L (CtsL), matrix metalloproteinase-14 (MMP-14) and MMP-16. Our results show that transcription for several specific cytokines, growth factors, and proteinases responds to a one-time exposure to a novel odor, in a manner that tends to be region- and sex-specific. This suggests considerable variation in the way that olfactory information is processed at the cellular level in different brain regions and by the two sexes.

Keywords

amygdala; hippocampus; interleukin; cathepsin; matrix metalloproteinase

1. Introduction

We have sought to identify events that can be associated with, and ultimately explain, the basis for lasting memory storage. Our strategy is to search for candidate genes and gene products at multiple relay points in the information processing circuit for robust and essentially one-trial learning experiences, where exposure to a novel stimulus can be tightly correlated temporally with changes at the cellular level.

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Olfaction in rodents is an excellent modality for testing cellular responses to novel stimuli. Odors are discriminated with high specificity by rodents. The information is processed sequentially through well-known neural pathways, and has high survival value, making one-trial learning robust and highly adaptive.

Elements of the immune response are of particular interest to us because of their involvement in the detection and storage of novel information, and their ability to interact in many combinations to account for context and consequence of specific chemical information (Dustin and Colman, 2002; Larson et al., 2002). Molecules that affect the functional association between neurons, including adhesion and the intercellular matrix, are frequently the signaling targets of cytokines, so they are of interest as well. If lasting memory depends on alteration of the functional association between brain cells, these molecules are likely to be involved (Milward et al., 2007).

The objectives of this study, therefore, were to (1) survey the expression of genes for a large number of (a) common cytokines and (b) cell matrix and adhesion molecules, in the amygdala, hippocampus, insular cortex and olfactory bulb, where critical processing of information for olfactory learning occurs; (2) determine whether a one-trial exposure to a novel odor affects the expression of any of those genes; and (3) discuss the implications of our finding for the plausibility that immune-like reactivity plays a role in the processing of new information by brain cells.

2. Results

Genes for 23–28 common cytokines and their receptors were expressed in the rat amygdala and hippocampus, with far fewer expressed in the insular cortex and olfactory bulb. Up to 34 cell matrix and adhesion molecules were expressed in the hippocampus, with slightly fewer across the other three brain regions studied (Fig. 1). A complete list of gene expression tested for and detected is given in Supplementary Tables 1 and 2.

In the amygdala, differential expression in response to novel odors occurred in 5 of the 7 genes whose expression was quantified by qPCR (Fig. 2). In 4 of the 5 differentially expressed genes, the change was restricted to males only, and was characterized by up-regulation in response to the odor of cornnuts but not blueberry bar. In the one case of a differentially expressed gene in females (FGF13, Fig. 2c), the change was up-regulation in response to both blueberry bar and cornnuts. Overall differences in expression attributable to sex were observed for two genes, *IL1r1* (Fig. 2a) and *MMP-14* (Fig. 2e).

Fewer changes in gene expression were seen in the hippocampus, and those followed no consistent pattern (Fig. 3). Systematic sex differences were observed in only one case, an overall increase in *IL4r* expression in females (Fig. 3b), though pair-wise differences were also seen for females in up-regulation of *CtsL* with blueberry bars (Fig. 3c) and *MMP-14* with cornnuts (Fig. 3e). Down-regulation of *MMP-16* with exposure to blueberry bars (Fig. 3f) was the only change seen in the hippocampus of males.

3. Discussion

The first objective of this study was to survey the presence of gene expression for cytokines and cell matrix and adhesion modulators in different components of the olfactory processing circuitry of the rat brain. We found that genes for up to 28 common cytokines are expressed in the rat amygdala and hippocampus, and from 25 to 34 cell matrix and adhesion molecules are expressed across all four brain regions studied. Interestingly, cytokines were minimally expressed in insular cortex and olfactory bulb (Fig. 1). This observation alone suggests an information processing role for cytokines in the amygdala and hippocampus.

Real-time PCR verified changes in the expression of a number of these genes following a one-time presentation of an odor that the rat had never previously experienced. Changes were seen more frequently in the amygdala, and more often in males than females. However, in all three cases of a systematic sex difference, females showed a greater magnitude of up-regulation in response to novel odors than did males (Fig. 2a, 2e, 3b).

Changes in gene expression also tended to be stimulus specific. When changes did occur in males, they more often were responsive to cornnuts only (Figs. 2a–d), while females were more likely to respond to both blueberry bar and cornnuts (Figs. 2c, 3c). While the prevalent theoretical assumption is that receptor specificity and precise circuitry provide the basis for conveying odorant identity to central processing areas of the brain (Belluscio et al., 2002; Mori et al., 1999), the influence of hormones on the maturation and function of those circuits in a sexually dimorphic manner is also well recognized (Wood, 1997; Bakker et al., 2002). The amygdala is a critical integration center for processing olfactory information related to sexual recognition and behavior (Been and Petrusis, 2011; DiBenedictis et al., 2012).

Our finding of sex- and stimulus-specific changes in gene expression, especially in the amygdala, likely reflects a complex interaction between hormones, sexually dimorphic neuronal circuits, and different subcellular signaling pathways unique to specific circuits. The genes demonstrated in this study to be differentially expressed in response to novel odors make strong candidates for future studies using hormonal manipulation to explore the influence of sex and hormones on the processing of olfactory information.

The influence of cytokines on behavior and affect is well established (Larson and Dunn, 2001). The robust response in this study of genes for both IL1r1 and IL4r in the amygdala provides additional evidence that these cytokines play a role in the normal processing of information by brain cells (Vitkovic et al., 2000).

Cathepsins H and L are endopeptidases that generate peptide neurotransmitters and neuromodulators (Brguljan et al., 2003; Hook et al., 2008; Yasothornsrikul et al., 2003). The possibility that cathepsins play a specific role in neuronal plasticity has gained recent traction (Stahl et al., 2007; Wendt et al., 2008; Zaidi and Kalbacher, 2008). This study shows that cathepsins H and L should be considered as candidates for involvement in the neuroplastic basis of olfactory learning.

Matrix metalloproteinases play a key role in normal development as well as pathological conditions (Candelario-Jalil et al., 2009; DeRosa et al., 2008; Bar-Or et al., 2003) Wright and co-workers ((Meighan et al., 2007; Meighan et al., 2006; Olson et al., 2008; Wright and Harding, 2004; Wright and Harding, 2009) have shown that matrix metalloproteinases contribute to synaptic remodeling that could underlie neural plasticity. Our results demonstrate that mere exposure to a novel odor alters gene expression for these enzymes, consistent with those suggestions.

Of particular relevance to this study is that gene expression for some metalloproteinases appears to be regulated by certain cytokines (Contasta et al., 1999; Gottschall and Deb, 1996). For instance, IL-1 plus oncostatin up-regulates the expression of MMP-14 but down-regulates MMP-16 (Milner et al., 2006). In this study, MMP-14 and MMP-16 likewise responded differently to novel odors, in both the amygdala and hippocampus. Thus these two enzymes may likewise play a role in modifying the neural circuitry underlying olfactory learning. Mechanistic studies aimed at unraveling the regulatory interactions among the cytokines would be a next logical step in this research.

Our results do not show conclusively that the genes we studied are involved in olfactory learning. Carefully controlled studies using learning paradigms, such as social transmission of olfactory information (Wrenn, 2004), or conditioned odor aversion (Grigson et al., 1997) should be carried out to reveal which aspects of the behavioral experience (e.g., short-term or long-term consolidation, retrieval, or modulation by affective state) are linked to specific cellular and molecular events. Our data provide a number of candidate molecular targets for focusing those investigations. In conjunction with good anatomical localization, such studies should lead to better insights into neural plasticity in the olfactory system at the cellular level

4. Experimental Procedures

4.1 Animals

This study reports the results of several independent experiments with different groups of young adult rats (*Rattus norvegicus*, Sprague-Dawley) of both sexes, ranging from 8 to 12 weeks of age. The research was approved by the Institutional Animal Care and Use Committee of the University of Texas at El Paso, in accordance with the United States Public Health Service *Guide to the Care and Use of Laboratory Animals*.

In preliminary experiments to assess the influence of social transmission, only males were used, to eliminate variation due to sex (Irwin and Byers, 2005). It became apparent, however, that odor by itself could affect gene expression, so new experiments were designed to focus only on exposure to novel odors, and to include both females and males. Sample sizes varied somewhat due to the availability of animals bred in house within a consistent age range. In all experiments rats were housed in group cages in a constant-temperature vivarium on a 12L:12D photoperiod, and fed Purina Lab Chow® with water available ad lib until the last four days of the experiment. The stage of estrus for each female was assessed by cytological examination of vaginal lavage samples obtained immediately after decapitation.

4.2 Olfactory stimulation

On each of the final four days of the experiment, food was removed at about 0900 each morning. At about 1700, rats were individually moved to a single cage where they were exposed for 10 min to one of three odors from food just beyond the reach of their nose through a hole at the back of the cage. Control rats (Group C, n = 6 males and 11 females) were exposed to the familiar odor of lab chow. Group B rats (n = 6 males and 4 females) were exposed to the novel odor of blueberry snack bars (Kellogg's NutriGrain®). Group N rats (n = 6 males and 10 females) were exposed to the novel odor of cornnuts (Kraft Foods, original flavor). Test foods were selected based on preliminary experiments to determine the rats' preference for a sweet reward and an alternative that was non-sweet and of different texture. The lights went off at 1800. Rats were left in their individual cages for two hours following olfactory stimulation, then weighed and returned to their group home cages on the first three nights, or taken to a dark room and decapitated under red light on the final night.

4.3 Gene Array Analysis

To survey overall gene expression for cytokines and proteinase in the circuitry for olfactory information processing, brains were quickly removed, placed immediately in chilled phosphate-buffered saline, and taken to the dissection lab. Transverse slabs ~ 3 mm thick centered on the infundibulum were made from each brain. Bilateral samples of the amygdala, hippocampus, insular cortex, and olfactory bulb were later dissected from the frozen sections. Total RNA was isolated using the RNAqueous kit (Ambion®) following the manufacturer's protocol. RNA was reverse transcribed to generate 16-UDP-labeled cRNA

probes using TrueLabeling-AMP™ linear RNA Amplification Kit (SABioscience). Probes were hybridized to Oligo GEArrays (SABioscience) ORN-011 for Rat Inflammatory Cytokines and Receptors, and to ORN-013 for Rat Extracellular Matrix and Adhesion Molecules, containing 96 and 111 gene sequences, respectively. After hybridization, arrays were washed and prepared for detection using the GEArray™ Chemiluminescent Detection Kit D-01 (SABioscience). Chemiluminescent images were visualized and photographed with the FX Imager (Bio-Rad), and scanning densitometry software (Molecular One™, Bio-Rad) was used to quantify the intensity of individual spots. The results shown in Fig. 1 were obtained from male brain samples only, but subsequent analyses from females yielded images qualitatively indistinguishable from those for males. A complete list of genes available for hybridization on the arrays, and the ones for which hybridization was visibly detected, are provided in Supplementary Tables 1 and 2.

4.4 Gene selection

Seven genes were selected for quantification of mRNA levels as a measure of transcriptional gene expression on the basis of preliminary studies indicating the possibility that they could be involved in the process of olfactory learning (Byers and Irwin, 2008; Irwin and Byers, 2005). Originally, 13 genes were selected for further study based on an apparent change in level of expression due to social transmission at a confidence level of $p < 0.05$. This was subsequently reduced to eight genes, based on insufficient levels of expression in some samples, or poorly performing primers. One of the eight -- FGF receptor activating protein 1 (Frag1) -- was not affected by novel odors in either brain region for either sex. The remaining seven genes reported here include the interleukin-1 receptor 1 (IL1r1), interleukin-4 receptor (IL4r), fibroblast growth factor 13 (FGF13), cathepsins H and L (CtsH and CtsL), and matrix metalloproteinases -14 and -16 (MMP-14 and MMP-16)

4.5 Tissue collection and RNA extraction

Follow-up experiments were performed on additional cohorts of animals to analyze changes in the expression of individual candidate genes. Brains were quickly removed, cut into coronal sections 2–3 mm thick, then frozen quickly on dry ice. Micropunched samples of the amygdala and dorsal hippocampus were collected bilaterally with a blunted 16 gauge needle and transferred to an enzyme digestion solution. Samples were not taken from the insular cortex or olfactory bulb in this phase of the study because of the much lower level of expression of the target genes in these two regions (Fig. 1). Total RNA was then isolated from tissue samples using the MELT™ Total RNA Isolation kit (Ambion), following the manufacturer's protocol for very small tissue sections.

4.6 Verification of differential gene expression

To obtain a realistic measure of inter-animal variation, total RNA was extracted from each regional sample from individual rats. mRNA was then reverse transcribed into unlabeled cDNA templates using the Verso™ cDNA Kit (ABgene, Thermo Scientific). Specific forward and reverse primers for candidate genes (Table 1) were designed using Primer Designer™, Version 2.0 (Scientific & Educational Software), and synthesized by IDT, Inc. (Coralville, IA). The FastStart SYBR Green Master® (Roche Diagnostics) fluorescent labeling kit and custom primers (0.4 mM) were used to perform qPCR using the iCycler™ (Bio-Rad) and the following protocol: 40 cycles, each consisting of 30 sec for melting, 30 sec for annealing, and 20 sec for elongation. Melt curve analysis confirmed a single amplification product for each target gene assayed.

All samples were run in duplicate along with a standard curve to calculate copy numbers of mRNA for each gene, and normalized to ribosomal 18S RNA as an internal standard.

4.7 Statistical Analysis

To determine if stage of estrus affected gene expression in female rats, results from those in proestrus or estrus were first separated out from results for all females for each gene. Expression profiles were virtually identical for all rats compared to those in proestrus or estrus only in all but two cases, and in those, a two-way analysis of variance (ANOVA) confirmed that the proestrus/estrus females showed no significant differences in the expression of any gene from that shown by all females. Therefore, in all following analyses, stage of estrus was disregarded.

We next determined by two-way ANOVA if results differed systematically according to sex or to the odorants presented. Statistical interactions were frequent, indicating that odorants affected gene expression differently in the two sexes. A Mann-Whitney two-tailed U-test was used to test for individual pair-wise differences. This is a non-parametric test, used because of the substantial differences in variance among many of the samples. All statistical tests were carried out with software embedded in GraphPad® Prism 4 for Windows.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

IL1r1	interleukin-1 receptor 1
IL4r	interleukin-4 receptor
FGF13	fibroblast growth factor 13
CtsH	cathepsin-H
CtsL	cathepsin-L
MMP-14	matrix metalloproteinase-14
MMP-16	matrix metalloproteinase-16

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FGF13

- Genes for numerous cytokines, cell matrix proteinases, and adhesion molecules are expressed in the olfactory pathway
- Specific interleukins, cathepsins, and matrix metalloproteinases were up- or down-regulated in response to 1-time exposure to odor of cornnuts or blueberries
- Sex-specific changes were observed in transcription of interleukin-1 and -4 receptors, FGF13, and in matrix metalloproteinase-14 in the amygdala
- Sex-specific changes occurred in gene expression for the interleukin-1 receptor, cathepsin-L, and for matrix metalloproteinases-14 and -16 in the hippocampus
- These results demonstrate cytokine and proteinase involvement in normal information processing functions in the brain, often in a sex-specific manner

Many cytokines and cell matrix proteinases are expressed in the olfactory pathway

- Gene expression was altered in response to a single exposure to novel odors
- Olfactory stimulation up-regulated IL1r1, IL4r, FGF13, and MMP-14 in the amygdala
- Sex affected changes in IL1r1 and MMP-14 in amygdala, and IL4r in hippocampus
- Cytokines and proteinases play a role in normal olfactory information processing

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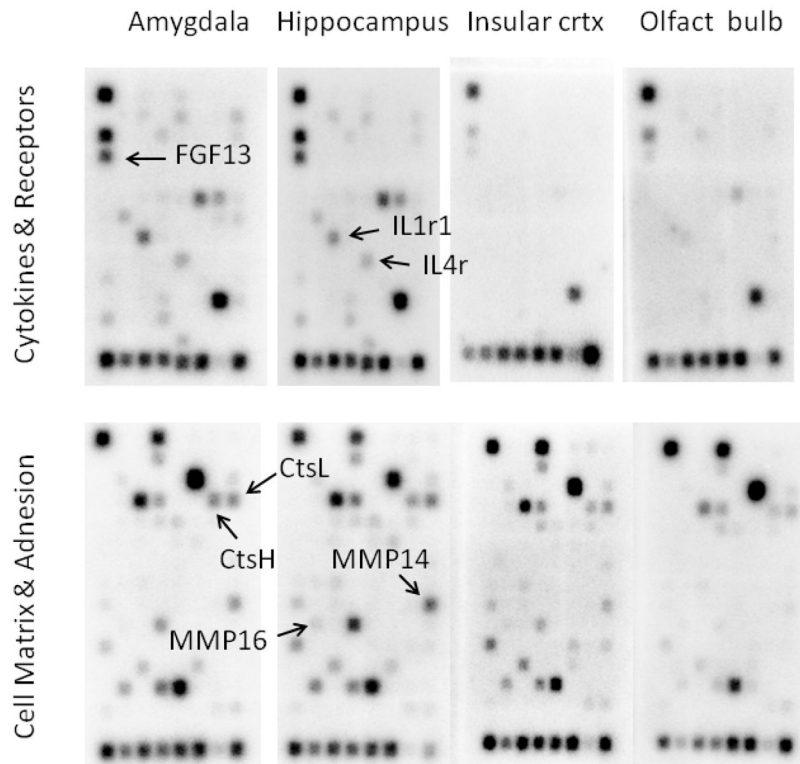


Fig. 1. Neuroanatomical distribution of genes for cytokines, growth factors, and proteases in the rat brain. The density of each chemiluminescent spot is proportional to the amount of mRNA produced for that gene. The position of seven genes analyzed by qPCR in this study is indicated by arrows.

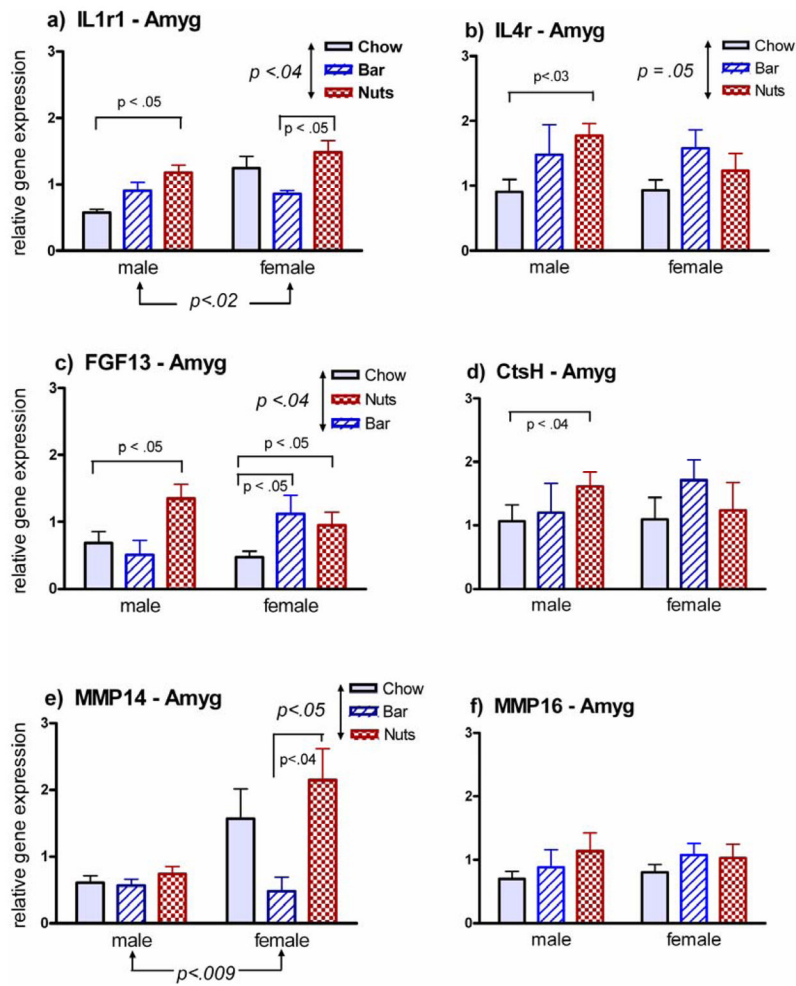


Fig. 2. Gene expression in the amygdala of male and female rats 2 h following exposure to the familiar odor of lab chow, or the novel odor of a blueberry bar (bar) or cornnuts (nuts). Arrows show differences attributable to either sex or odorant by 2-way ANOVA, at the indicated level of significance. Pair-wise comparisons are shown for 2-tailed U-tests at the indicated level of significance. Results for six of the seven genes tested are shown; no changes were seen in this brain region for CtsL.

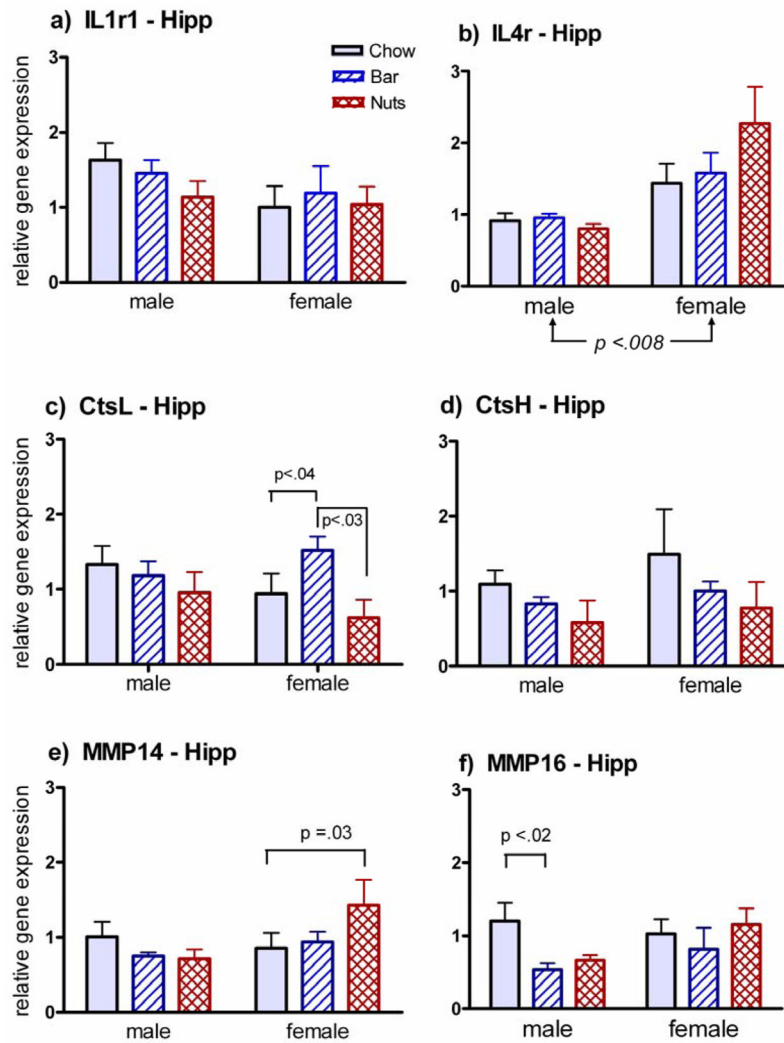


Fig. 3. Gene expression in the hippocampus of male and female rats 2 h following exposure to the familiar odor of lab chow, or the novel odor of a blueberry bar (bar) or cornnuts (nuts). Arrows show differences attributable to either sex or odorant by 2-way ANOVA, at the indicated level of significance. Pair-wise comparisons are shown for 2-tailed U-tests at the indicated level of significance. Results for six of the seven genes tested are shown; no changes were seen in this brain region for FGF13.

Table 1

Primers used for q-PCR, in the forward (F) and reverse (R) directions

Gene	Sequence (5' → 3')
IL1r1 - F	gcc agt cat ctg aag agc
IL1r1 - R	gcc aag tgg taa gtg tgt c
IL4r - F	gct gag gtc tgt gct aag g
IL4r - R	cta tgc cag gac tgc tgt g
FGF13 - F	cct ctc ctt cct act gtc c
FGF13 - R	caa tgc cac tgt tcc ac
CtsH - F	ggc tat gga gaa cag aat gg
CtsH - R	gta tgg ctg gtg atg tc
CtsL - F	ggg ggt tgg cta tgg tta tg
CtsL - R	gaa ctc ctt cgg atg tag tg
MMP-14 - F	ctg aag gta gag cca gg
MMP-14 - R	ctt gtc cag cag tga acg
MMP-16 - F	gat gga cca aca gac cga g
MMP-16 - R	gga ctg aag aac ggc aga tac