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Amygdala nuclei critical for emotional learning exhibit unique gene expression patterns ☆

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Abstract

The amygdala is a heterogeneous, medial temporal lobe structure that has been implicated in the formation, expression and extinction of emotional memories. This structure is composed of numerous nuclei that vary in cytoarchitectonics and neural connections. In particular the Lateral nucleus of the Amygdala (LA), Central nucleus of the Amygdala (CeA), and the Basal (B) nucleus contribute an essential role to emotional learning. However, to date it is still unclear to what extent these nuclei differ at the molecular level. Therefore we have performed whole genome gene expression analysis on these nuclei to gain a better understanding of the molecular differences and similarities among these nuclei. Specifically the LA, CeA and B nuclei were laser microdissected from the rat brain, and total RNA was isolated from these nuclei and subjected to RNA amplification. Amplified RNA was analyzed by whole genome microarray analysis which revealed that 129 genes are differentially expressed among these nuclei. Notably gene expression patterns differed between the CeA nucleus and the LA and B nuclei. However gene expression differences were not considerably different between the LA and B nuclei. Secondary confirmation of numerous genes was performed by *in situ* hybridization to validate the microarray findings, which also revealed that for many genes, expression differences among these nuclei were consistent with the embryological origins of these nuclei. Knowing the stable gene expression differences among these nuclei will provide novel avenues of investigation into how these nuclei contribute to emotional arousal and emotional learning, and potentially offer new genetic targets to manipulate emotional learning and memory.

Keywords

Amygdala; Laser microdissection; Microarray; Gene expression

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.nlm.2013.06.015>.

1. Introduction

The amygdala is a complex structure, within the medial-temporal lobe, required for proper emotional learning. Extensive data collected over the last several decades widely support a critical role of the amygdala in acquisition, expression and extinction of appetitive and aversive emotional memories (Everitt, Cardinal, Parkinson, & Robbins, 2003; LeDoux, 2000; Pape & Pare, 2010). In addition, the amygdala is believed to modulate the formation of memories in other brain structures, such as the hippocampus and cortex, by regulating emotional arousal and emotional memory via a complex network of afferent and efferent connections with cortical and sub-cortical regions (McGaugh, 2004).

Given the complexity of the amygdala, underscored by its numerous nuclei that have differing cytoarchitectonics and neural connections, much effort has been devoted to elucidating the roles of the various amygdala nuclei (Pitkanen, Savander, & LeDoux, 1997). Most of the data discerning roles for particular amygdala nuclei in learning and memory have come from animal studies utilizing classical and instrumental associative fear learning paradigms (Goossens & Maren, 2001; McGaugh, McIntyre, & Power, 2002). In particular, Pavlovian fear conditioning has been widely used to study the roles of individual nuclei in acquisition, consolidation, expression and extinction of conditioned fear. In this learning paradigm an animal is presented with a benign stimulus, such as tone (conditioned stimulus; CS), followed by presentation of a noxious stimulus, such as a brief electrical shock (US; unconditioned stimulus). At a later time (typically 3 and 24 h later), the animal is exposed to the tone again without a foot shock to measure short term and long term memory, respectively. If the animal learns to associate the tone with the foot shock, it will exhibit defensive behavior (i.e. freezing and autonomic reactivity) where the degree of freezing is typically used as an indicator of memory strength (Rodrigues, Schafe, & LeDoux, 2004).

Studies of conditioned fear in rodents have elucidated an essential role in learning and memory for three amygdala nuclei. The Lateral nucleus of the Amygdala (LA) serves as the principal sensory input to the amygdala and it is believed to be an essential locus for plasticity during fear conditioning (Blair, Schafe, Bauer, Rodrigues, & LeDoux, 2001; Romanski, Clugnet, Bordi, & LeDoux, 1993). Accordingly, LA neurons alter their response properties during auditory fear conditioning (Maren, 2000; Quirk, Armony, & LeDoux, 1997; Quirk, Repa, & LeDoux, 1995; Repa et al., 2001). Associations formed in the LA and Basal (B) nuclei project to neurons in the lateral division of the Central nucleus of the Amygdala (CeA) where additional plasticity relevant to fear conditioning occurs (Ciocchi et al., 2010; Wilensky, Schafe, Kristensen, & LeDoux, 2006), and efferents from the CeA to the hypothalamus and brainstem trigger the autonomic expression of fear (Krettek & Price, 1978; LeDoux, 2000; LeDoux, Iwata, Cicchetti, & Reis, 1988; Petrovich & Swanson, 1997; Veening, Swanson, & Sawchenko, 1984). In addition, there are extensive inhibitory and excitatory neural connections within and between nuclei that further increase the complexity of information processing within the amygdala (Ehrlich et al., 2009; Pitkanen et al., 1995; Pitkanen et al., 1997). Notably these three nuclei have different embryological origins (CeA – striatal; LA and B – cortical) and they also differ morphologically, where the LA and B have proportionately more excitatory projection neurons compared to the CeA, which has proportionately more inhibitory projection neurons (Sah, Faber, Lopez De Armentia, &

Power, 2003). The LA and B nuclei are often collectively referred to as the Basolateral complex (BLA). This is in part due to the fact that it is often difficult to manipulate either the LA or the B nuclei specifically and therefore experimental manipulations typically target the BLA.

From a molecular standpoint, progress has been made in identifying how fear learning transiently changes gene expression within the BLA during the consolidation phase of learning (Keeley et al., 2006; Ploski, Park, Ping, Monsey, & Schafe, 2010; Ressler, Paschall, Zhou, & Davis, 2002). However it is currently poorly understood how amygdala nuclei differ at the molecular level with respect to genome wide gene expression differences. Genes expressed uniquely to cells/brain structures likely contribute important functions that, in part, explain the cell's/structure's function. Therefore identifying gene expression differences among amygdala nuclei can provide insights to how these nuclei contribute to emotional arousal and emotional learning and open up novel avenues of investigation. For example Gastrin Releasing Peptide (GRP) has been previously identified to exhibit increased gene expression within the LA and was subsequently found to be a modulator of fear learning (Shumyatsky et al., 2002).

To further examine how amygdala nuclei contribute to emotional learning and arousal, we performed global gene expression profiling on the three amygdala nuclei critical for emotional learning (LA, B, and CeA). Because the amygdala is a heterogeneous structure containing numerous nuclei that change in size and shape through the anterior–posterior axis, we utilized laser micro-dissection to dissect the desired nuclei from the rat brain followed by gene expression analysis. The benefits of utilizing the precision of microdissection are enormous over conventional dissection approaches since dissection artifacts are virtually eliminated, allowing the detection of small changes to be remarkably enhanced. The current study provides a profile of genes, which are differentially expressed among individual amygdala nuclei, providing insights for the molecular basis of amygdala functioning.

2. Methods

2.1. Subjects

Adult male Sprague Dawley rats (Charles Rivers Laboratories) weighing 300–400 g were housed in pairs in plastic cages and maintained on a 12 h light/dark cycle. Food and water were provided *ad libitum* throughout the experiment. Animal use procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Texas at Dallas Animal Care and Use Committee.

2.2. Acetylcholinesterase staining, laser microdissection, RNA purification and labeling

Rats were lightly sedated by exposing them to CO₂ for ~1 min, followed by immediate decapitation; the brains were rapidly dissected and immediately frozen with powdered dry ice and stored at –80 °C until further processing. Brains were hemisected and 8–10 µm coronal sections containing the amygdala (–2.3 to –3.3 mm with respect to Bregma) were

mounted on MMI Laser Microdissection (LMD) slides (product #50102). Every fifth section was placed on Superfrost glass slides (Fisher Scientific) and stained for acetylcholinesterase activity by incubating the slides in a preheated (37 °C) acetylcholinesterase staining solution (1.73 mM acetyl thiocholine iodide, 0.1 M acetate buffer [pH 6.0], 0.1 M sodium citrate, 30 mM copper sulfate, 5 mM potassium ferricyanide) for 15–60 min, to differentiate the LA, CeA and B nuclei. Prior to LMD, the slides were dehydrated one-at-a-time using Histogene LCM Frozen Section Staining Kit (Invitrogen). Specifically, slides were transferred from –80 °C immediately to RNase-free 75% ethanol, 30 s; 95% ethanol, 30 s; 100% ethanol, 30 s; xylene, 30 s; and xylene, 5 min. Immediately following slide dehydration, the LA, CeA and B nuclei were laser microdissected using a SmartCut Laser Microdissection System configured on an Olympus CKX41 inverted microscope. Acetylcholinesterase stained sections no more than 3 sections apart from LMD slides were used as a reference to accurately identify the LA, CeA and B nuclei. Each microdissected fragment was detached from the slide using clean, RNase-free tweezers and deposited in 25 µL of cell lysis buffer (RNAqueous-Micro Kit; Ambion) at room temperature. Approximately 6–8 dissected nuclei were added to the 25 µL of lysis buffer before it was capped and frozen at –80 °C. This was repeated ~6–7 times per nucleus per animal and the resultant 25 µL aliquots per animal were thawed, pooled and the RNA was isolated according the manufacturer's instructions using the RNAqueous-Micro Kit. The resultant RNA was purified via precipitation using Pellet Paint NF (Novagen) to remove potential inhibitors of reverse transcription followed by RNA amplification and biotin labeling via a single round of amplification utilizing the Illumina TotalPrep RNA Amplification Kit (Ambion). The *in vitro* transcription reaction was performed for 14 h.

2.3. DNA microarray and analysis

DNA microarray hybridization was performed at the University of Texas at Southwestern Medical Center Genomics and Microarray Core Facility. Ten cRNA samples ($n = 3$ for B, $n = 3$ LA and $n = 4$ CeA) were hybridized to the Illumina RatRef-12 Expression BeadChip containing >22,000 probes for genome scale gene expression analysis. Data analysis was performed using Illumina GenomStudio using quantile normalization. Gene lists were created based on the relatively stringent criteria that the gene must exhibit an average fold difference of 3-fold or greater in pair wise comparisons between the LA and CeA, or B and CeA, or LA and B with a *t*-test *p*-value of $p < 0.05$. All genes in these lists exhibited a raw average signal value that was well above background. Importantly, the Microarray Quality Control (MAQC) Consortium has reported that this approach can be successful in identifying reproducible gene lists (Shi et al., 2006). However most of these genes also passed the multiple testing correction using Benjamini and Hochberg False Discovery Rate with a *p*-value of $p < 0.05$. Supplemental data including *p* values, gene bank accession numbers and full gene names, etc. are included in (S1, LA and B vs. CeA; S2, CeA vs. LA and B; S3, LA vs. B; and S4, B vs. LA).

2.4. Heat map

To generate the heat map, a comprehensive gene list was created, which included average fold difference values for the three possible pair-wise comparisons (LA, CeA; B, CeA; and LA, B) for any gene that passed the criterion (described above) for at least one of the three

pair-wise comparisons. The data in these tables were clustered based on average linkage using Gene Cluster 3.0 (de Hoon, Imoto, Nolan, & Miyano, 2004). The resultant clustered data file was analyzed in Java TreeView v1.1.6r2 (<http://jtree-view.sourceforge.net>) to create a dendrogram/heatmap.

2.5. In situ hybridization

In situ hybridization was performed as previously described (Newton, Dow, Terwilliger, & Duman, 2002; Ploski et al., 2008; Ploski et al., 2010). Briefly, rats were rapidly and deeply anesthetized with chloral hydrate (250 mg/kg, i.p.) and perfused through the heart with ice-cold 1X Phosphate buffered saline (PBS) (pH 7.4), followed by ice-cold 4% paraformaldehyde in 1X PBS. All solutions were made with water treated with Diethylpyrocarbonate (DEPC). Brains were removed, hemisected and 14 μ m coronal sections containing the amygdala (−2.3 to −3.14 mm with respect to Bregma) were mounted on Superfrost glass slides (Fisher Scientific) and immediately stored at −80 °C until further processing. Every fifth section collected was stained for acetylcholinesterase activity to differentiate the LA, CeA and B nuclei as described above. The remaining sections for *in situ* hybridization were placed 10 min in 4% paraformaldehyde; 1 min 1 × PBS; 1 min triethanolamine (TEA) buffer pH 8.0; 100 mM TEA pHed with NaOH ~60 mM); 15 min TEA buffer with 0.25% acetic anhydride; 2 min 2 × SSC; 2 min 2 × SSC; 2 min 30% ethanol; 2 min 70% ethanol; and 2 min 100% ethanol. Air dried slides were hybridized with ³⁵S-radioactive RNA probe mix containing 2 × 10⁶ dpm of RNA probe per 100 μ L of hybridization buffer (50% formamide, 0.6 M NaCl, 10 mM Tris–HCl (pH 7.4), 1 × Denhardt's solution, (10 mM DTT, 250 μ g/ml tRNA, 100 μ g/ml salmon sperm DNA, 10% dextran sulfate) overlaid on 2 sections per slide and coverslipped. Slides were incubated in a humidified chamber (50% formamide, 30% 20 × SSC, 20% H₂O) for 14–18 h at 55 °C. After incubation, coverslips and excess probe were removed in 2 × SSC, followed by a 30 min incubation in 20 μ g/ml RNase A in RNase buffer (0.5 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA). Slides were rinsed: 10 min 2 × SSC; 20 min 0.2 × SSC .1 mM DTT at 55 °C; 15 min 0.1 × SSC .1 mM DTT; 30 s 0.1 × SSC; 10 s Milli-Q water and air dried. Slide mounted sections were then exposed to autoradiographic film (BioMax MR, Kodak) for 2–14 days followed by film development. Gene specific RNA radio-active probes were generated by PCR amplification using gene-specific primers (see S5). The reverse primer includes a T7 template sequence. Rat brain cDNA was used as the template for PCR, which was performed using a Bio-Rad CFX96 Real-Time PCR Detection System. The PCR product was purified by ethanol precipitation and was resuspended in Tris–EDTA buffer. One microgram of the ~300 bp PCR product was used to produce radiolabeled probe using a T7-based *in vitro* transcription kit (Megashortscript; Ambion) using [³⁵S]CTP (1.5 ICi) (PerkinElmer). Removal of unincorporated nucleotides after the *in vitro* transcription reaction was performed using Sepharose spin columns (Roche). Gene expression intensity was measured using ImageJ (<http://rsb.info.nih.gov/ij/>) by measuring optical density of the LA, CeA, B nuclei from images captured from exposed autoradiographic films using a ZipScope USB Digital Microscope model #26700-300 (Aven Inc.). Obtained values were normalized to an autoradiographic film standard developed by exposing film to a Carbon-14 Standard Cat # (ARC 0146A(PL)) (American Radiolabeled Chemicals, Inc.). Relative gene expression differences between the LA, CeA and B nuclei were calculated by comparing

normalized expression values among each other. Data collected from 3 to 5 animals per group were analyzed using ANOVA and Scheffe's Post-hoc test. Differences were considered significant if $p < 0.05$ (uncorrected for multiple comparisons); however for most genes, the ANOVA reached significance correcting for multiple comparisons. Corrected comparisons were considered significant if $p < 0.003$. These statistical values are listed in S6. Relative gene expression differences between the hippocampal subfields; CA1, CA3, dentate gyrus and the hilus were calculated by comparing normalized expression values among each other essentially as described for the amygdala. Data collected from 3 to 5 animals per group were analyzed using ANOVA and Fisher's Post-hoc test. Differences were considered significant if $p < 0.05$ (uncorrected for multiple comparisons). These data are presented in S7.

2.6. Ontology analysis

Comprehensive gene lists for BLA and CeA differentially expressed genes were organized into tab delineated files by Entrez gene ID and uploaded to WebGestalt (<http://bioinfo.vanderbilt.edu/webgestalt/>) for ontology analysis (Gene Ontology version 1.2) (Wang, Duncan, Shi, & Zhang, 2013; Zhang, Kirov, & Snoddy, 2005). Functional categories which contained at least one gene from the uploaded lists were identified and exported in Directed Acyclic Graph (DAG) files. Tables for each of these files were exported into Microsoft Excel for comparisons between lists and then pared to root category, sub-root, AmiGO ontology category ID, and number of differentially expressed genes within each sub-root category. The genes present in each of these categories are listed in S7.

3. Results

To determine the gene expression differences among the LA, CeA and B nuclei, these nuclei were first laser microdissected from coronal rat brain sections spanning -1.8 to -3.2 with respect to Bregma (Paxinos & Watson, 1998). Laser microdissection is necessary because the amygdala is composed of multiple nuclei that change in size and shape through the anterior-posterior axis of the rodent brain and these nuclei are too small to accurately dissect using traditional methods. To accurately identify the location of the various nuclei on fresh frozen cryocut coronal brain slices, sections adjacent to sections used for laser microdissection were stained for acetylcholinesterase activity. This staining procedure differentially stains the LA, CeA and B nuclei of the amygdala where the B nucleus stains intensely for acetylcholinesterase activity while the LA and CeA stain progressively less intensely, clearly differentiating these three nuclei (Fig 1). Unfortunately the tissue used for gene expression analysis cannot be directly stained for acetylcholinesterase activity, because exposing the tissue to aqueous solutions even briefly will result in RNA degradation and subpar gene expression analysis (data not shown).

Total RNA was purified from microdissected nuclei and subjected to RNA amplification followed by whole genome microarray analysis. The microarray data was filtered using a stringent 3-fold difference cutoff, which revealed 129 genes that are differentially expressed among the LA, CeA and the B nuclei. These genes include transcription factors, receptors, enzymes, ligand/peptides, channels/transporters and cytoskeletal associated proteins.

Notably gene expression patterns differ considerably between the CeA nucleus and the LA and B nuclei. For example there are 85 genes that exhibit increased gene expression in the LA and B nuclei compared to the CeA whereas 43 genes that exhibit increased gene expression in the CeA nucleus compared to the LA and B nuclei (Table 1 and 2 respectively). However, gene expression differences are not considerably different between the LA and B. Six genes exhibit increased gene expression in the B compared to the LA and 4 genes exhibit increased gene expression in the LA compared to the B nucleus (Table 3). Because the LA and B nuclei share such similar gene expression differences compared to the CeA, both the LA and B nuclei gene expression differences compared to the CeA are listed side by side (Tables 1 and 2). All genes presented in the lists meet the stringent criteria (see Section 2) for either the LA compared to the CeA or the B nucleus compared to the CeA; however, for the majority of the cases both the LA and B nuclei meet the criteria. The complete microarray data set is displayed graphically as a heat map (Fig 2). Table 4 and S8 lists the numbers of genes present in numerous gene ontology categories. Notably a large number of genes that were found to be expressed at a higher level in the BLA compared to the CeA are involved in neuron and brain development. In contrast a number of genes that were found to be expressed at a higher level in the CeA compared to the BLA are involved neurotransmitter and neuropeptide release.

To validate our microarray findings we performed *in situ* hybridizations to determine the mRNA expression pattern of selected genes we identified in our microarray screen. *In situ* hybridizations were performed on coronal cryocut rat brain sections for a total of 17 randomly chosen genes from our list that have not been extensively studied with respect to the amygdala previously (Fig. 3–6). All genes tested exhibit a gene expression pattern consistent with the microarray findings. Notably a number of the genes we identified have previously been determined to be differentially expressed among amygdala nuclei which provide additional validation of the microarray data. The *in situ* hybridization experiments also revealed that, for the majority of the genes tested, the gene expression patterns are consistent with the embryological origins of these nuclei. For example the LA and B nuclei are embryologically related to the cortex and thus have cortex-like features. Notably 7 of the 9 *in situ* hybridizations completed for LA/B genes that exhibit increased gene expression compared to the CeA also exhibit increased expression in the cortex. In contrast, the CeA is embryologically related to the striatum and thus has striatal-like features. Five of the 8 *in situ* hybridizations completed for CeA genes that exhibit increased gene expression compared to the LA/B also exhibit increased expression in the striatum.

A subset of genes that exhibit increased gene expression within the BLA compared to the CeA also exhibit differential gene expression within hippocampal subfields as analyzed by *in situ* hybridization. These data are presented in S7.

4. Discussion

One of the goals for learning and memory research is to identify the genes that are important for mnemonic processing. However since the genomes of mice, rats and humans contain approximately 25,000 genes, identifying plausible candidate genes that are important for learning and memory is not a trivial task. Reasonable approaches to identify potential

candidate genes for learning and memory include, but are not limited to, identifying genes whose expression changes during the protein synthesis dependent, consolidation phase of learning (Keeley et al., 2006; Ploski et al., 2010; Ressler et al., 2002), identifying genes whose gene products localize to synapses (Lyford et al., 1995; Steward, Wallace, Lyford, & Worley, 1998), and identifying genes that are expressed highly or uniquely within regions of the brain known to be critical for learning and memory such as the hippocampus or amygdala (Shumyatsky et al., 2002; Shumyatsky et al., 2005). Here we have examined the gene expression differences among three nuclei of the amygdala that are critical for emotional learning – the LA, CeA and the B nuclei. Whole genome microarray analysis has revealed numerous gene expression differences among these nuclei. The genes identified in this study represent, at least in part, a molecular basis for the differential function and roles of these nuclei and serve as potential candidates that could influence amygdala dependent learning and memory.

In support of the hypothesis that genes differentially expressed among amygdala nuclei may contribute to the unique functions of these nuclei and thus amygdala dependent learning and memory, numerous genes that we identified in our study have previously been associated with regulating emotional learning. For example Gastrin Releasing Peptide (GRP) was previously found to be a modulator of fear learning (Shumyatsky et al., 2002). Mouse knockouts of cholecystokinin (CCK) exhibit increased anxiety and reduced learning in the passive avoidance task and Morris water maze (Lo et al., 2008). Rats without a functional CCK-A receptor exhibit reduced learning of a radial arm task (Nomoto, Miyake, Ohta, Funakoshi, & Miyasaka, 1999). Behavioral pharmacology studies have found that agonists of CCK enhance learning while antagonists of CCK impair learning (Fekete, Bokor, Penke, & Telegdy, 1982a; Fekete, Penke, & Telegdy, 1982b; Fekete, Szabo, Balazs, Penke, & Telegdy, 1981; Kadar, Fekete, & Telegdy, 1981). Mice deficient in the receptor for adenylyl cyclase activating polypeptide 1 (Adcyap1) exhibit increased anxiety (Otto et al., 2001b) and reduced fear learning (Otto et al., 2001a). The receptor adenosine A2a receptor (Adora2A) is associated with modulating hippocampal dependent associative learning and LTP (Fontinha et al., 2009). Microinjection of neurotensin (NTS) into the CeA of rats resulted in a significantly increased latency time in a passive avoidance learning task (Laszlo et al., 2012).

Our screen has revealed that the transcript for the secreted peptide vasoactive intestinal polypeptide (VIP), is enriched in the LA and B nuclei while the transcript for VIP's receptor VIPR2 is enriched in the CeA. This pattern of ligand – receptor expression delineates a unique signaling network between the BLA and the CeA which contributes to emotional learning and expression of fear. For example VIP-deficient mice exhibit reduced fear conditioning (Chaudhury, Loh, Dragich, Hagopian, & Colwell, 2008) and VIP antagonists given during mouse embryological development induce anxiety-like behaviors (Hill et al., 2007).

Amygdala dependent emotional learning and memory has been intensely investigated in part because it may lead to insights into how pathological fear forms, or lead to new ways to reduce pathological fear. Interestingly a few of the genes we identified in our screen have been associated with disorders of fear. For example polymorphisms in Adcyap1 and its

receptor are associated with PTSD (Ressler et al., 2011). The receptor Adora2A is associated with panic disorder (Hamilton et al., 2004).

To our knowledge there is only one previous citation reporting microarray data from laser microdissected amygdala nuclei (Zirlinger & Anderson, 2003). In this previous study, the authors examined gene expression differences among the LA, CeA and the medial nuclei from mice. There is minimal overlap in our study vs. the Zirlinger study and we suspect that in part this may be due to the fact that the present study utilized rats; whereas the Zirlinger study used mice and that we amplified our RNA using one round of amplification, whereas the Zirlinger study used two rounds of RNA amplification. Multiple rounds of RNA amplification have been shown to reduce the quality of microarray data (Degrelle et al., 2008). Other sources of variability include differences in the DNA microarray platform used (Tan et al., 2003) and we have found that the thickness and degree of dehydration of cryocut sections required for laser microdissection is critical for successful gene expression analysis (data not shown). Differences in the quality of tissue could explain large differences in gene array data. Importantly the robustness of our data set is underscored by the extensive *in situ* hybridizations that were performed to validate our array data and the expression pattern indicated by the micro-array is also supported by the published literature for many of the genes we identified to be differentially expressed among the CeA, LA and B nuclei.

The current study provides a profile of genes that are differentially expressed among amygdala nuclei, providing insights for the molecular basis of amygdala functioning. This profile includes some genes that have previously been associated with emotional learning and expression of fear, and future studies will determine how other genes within this profile influence amygdala dependent learning and memory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Microdissection of amygdala nuclei from a 10 μ m coronal section. (i) Coronal amygdala anatomy as described by Paxinos and Watson (1998). (ii) Acetylcholinesterase-stained 10 μ m coronal brain slice differentially stains the LA, CeA and B nuclei within the amygdala region indicated in (i). (iii) 10 μ m coronal brain slice within the region indicated in (i) and (ii) before dissection. (iv) Tissue slice following dissection of the LA. (v) Tissue slice following dissection of the B nucleus. (vi) Tissue slice following dissection of the CeA. LA = lateral amygdala, B = basal amygdala, and CeA = central amygdala.

Fig. 2.

Heatmap of hierarchical clustering of the microarray data for the 129 genes differentially expressed among the LA, CeA and B nuclei. Comparisons of the gene expression profiles between the B and CeA, LA and CeA, and LA and B are depicted in columns left to right respectively. Red intensity indicates increasing levels of gene expression. Green intensity indicates decreasing levels of gene expression. Black indicates no difference in gene expression.

Fig. 3.

Representative autoradiograms from *in situ* hybridizations of selected genes identified to be expressed at a higher level in the LA and B nuclei compared to the CeA. (i) Images of *in situ* hybridizations of hemisected coronal brain sections for specified genes. (ii) Higher magnification of images presented in (i) of the region containing the amygdala. (iii) Same image presented in (ii), including labels for the lateral amygdala (LA), basal amygdala (B), and central amygdala (CeA). (iv) Acetylcholinesterase-stained reference tissue depicting boundaries of amygdala nuclei. Basal amygdala labeled with B.

Fig. 4.

Quantitation of *in situ* hybridization data collected for selected genes identified to be expressed at a higher level in the LA and B nuclei compared to the CeA (Fig. 4). $n = 3-6$.

*Indicates $p < 0.05$; error bars = standard error of the mean; LA = lateral amygdala, B = basal amygdala, and CeA = central amygdala.

Fig. 5.

Representative autoradiograms from *in situ* hybridizations of selected genes identified to be expressed at a higher level in the CeA compared to the LA and B nuclei. (i) Images of *in situ* hybridizations of hemisected coronal brain sections for specified genes. (ii) Higher magnification of images presented in (i) of the region containing the amygdala. (iii) Same image presented in (ii), including labels for the lateral amygdala (LA), basal amygdala (B), and central amygdala (CeA). (iv) Acetylcholinesterase-stained reference tissue depicting boundaries of amygdala nuclei. Basal amygdala labeled with B.

Fig. 6. Quantitation of *in situ* hybridization data collected for selected genes identified to be expressed at a higher level in the CeA compared to the LA and B nuclei (Fig. 6). *Indicates $p < 0.05$ compared to LA and B groups. Difference between B and LA not significant. Error bars = standard error of the mean; LA = lateral amygdala, B = basal amygdala, and CeA = central amygdala.

Table 1

Genes identified via DNA microarray analysis to be expressed at a higher level in the LA and B nuclei compared to the CeA. Data for the LA compared to the CeA and the B compared to the CeA were listed side by side since for most cases these data were very similar. These data were grouped based on gene classification. Gene names in bold indicate the amygdala gene expression pattern for this gene was previously published and consistent with these data with the associated reference located in the reference column. * Indicates that the fold difference value has a *t*-test *p*-value of *p* > 0.05. Bold fold difference value indicates gene expression pattern for respective comparison met criteria to be considered differentially expressed (see Section 2). Italics fold difference value indicates gene expression pattern for respective comparison had a *p* < 0.05 for the Benjamini and Hochberg False Discovery Rate multiple testing correction.

Gene symbol	LA/CeA AVG fold difference	B/CeA AVG fold difference	References	Gene symbol	LA/CeA AVG fold difference	B/CeA AVG fold difference	References
<i>Transcription factors</i>				<i>Peptides/ligands</i>			
Bhlhe22	34.0	<i>30.7</i>		Grp	<i>12.1</i>	2.9	Wada, Way, Lebacqz-Verheyden, and Battey (1990)
Neurod6	<i>13.9</i>	14.9		Cck	<i>11.1</i>	20.9	Giacobini and Wray (2008)
Zfpn2	6.3	<i>7.0</i>		Adcyap1	<i>10.8</i>	3.3	Skoglosa, Patrone, and Lindholm (1999)
Fezf2	4.8	<i>5.7</i>		Fam19a1	9.0	9.7	
Mef2c	4.6	3.0		Vip	<i>7.6</i>	6.4	Dussaillant, Sarrieau, Gozes, Berod, and Rostene (1992)
Neurod1	<i>3.9</i>	<i>1.9</i>		Nov	4.4	22.2	Su et al. (2001)
Satb1	<i>3.8</i>	<i>2.3</i>		Lingol	<i>3.7</i>	3.4	Carim-Todd, Escarceller, Estivill, and Sumoy (2003)
Nr4a3	3.6	5.4	Sun et al. (2007)	Bdnf	<i>3.7</i>	4.9	Karlen et al. (2009)
Tfap2d	<i>3.1</i>	3.8		Slit1	<i>3.5</i>	4.4	
Nr2f2	3.0	<i>2.7</i>		Ntng1	3.4	<i>2.2</i>	
Etv1	<i>1.5</i>	3.2		Npy	3.2	<i>2.8</i>	Smialowska et al. (2001)
<i>Channels/transporters</i>				Sytl2	3.0	<i>2.9</i>	
Mfsd4	7.4	7.0		Rasgrp1	<i>2.9</i>	4.4	
Slc17a6	<i>4.7</i>	3.3	Poulin, Castonguay-Lebel, Laforest, and Drolet (2008)	Crhbp	<i>2.8</i>	4.8	
Kcnj6	<i>4.6</i>	4.1		Cadps2	<i>2.7</i>	4.0	
Kcnv1	4.0	<i>2.5</i>		Cxcl12	<i>2.0</i>	6.5	Tham et al. (2001)
Slc17a7	<i>3.9</i>	4.6	Poulin et al. (2008)	Dkk3	<i>1.8</i>	3.3	

Gene symbol	LA/CeA AVG fold difference	B/CeA AVG fold difference	References	Gene symbol	LA/CeA AVG fold difference	B/CeA AVG fold difference	References
Kcng1	2.8	3.3		Lxn	1.8	3.2	
Slc24a2	1.4*	5.1		Tnfrsf15	1.7	3.0	
<i>Enzymes</i>				Cort	1.4	4.1	de Lecea et al. (1997)
Ptgs2	9.1	4.6	Quan, Whiteside, and Herkenham (1998)	<i>Miscellaneous/unclassified</i>			
Trim54	8.2	2.0		Rasgef1c	6.1	2.6	
Rasl11b	5.0	4.0		Tmem178	5.6	5.7	
Hace1	4.4	3.3		Anxa11	4.1	5.1	
LOC365985	4.4	3.6		Olfm1	3.6	3.2	Nagano et al. (1998)
Cyp11b1	4.0	4.6		Mpdz	3.6	2.0	Becamel et al. (2001)
Ephx4	3.9	2.3		RGD1563065	3.3	2.2	
Prss23	3.2	2.4		Cpne4	3.3	2.0	
Mgst3	2.7	3.9	Fetissov et al. (2002)	Spon1	3.2	2.1	
Car4	2.7	3.2		Nptx1	3.2	3.9	
RGD1561381	2.5	3.4		Tspan8	3.1	1.1*	
Prss35	2.0	3.9		Arpp21	3.1	2.8	Becker et al. (2008)
Cyp26a1	1.6	5.5		Fam5b	3.1	2.4	
<i>Cytoskeletal associated</i>				Cntn4	3.1	2.4	
Nefl	2.4	3.3		Cabp7	3.1	8.5	
Pkp2	1.4	3.4		Khdrbs3	3.0	2.8	
Cdh9	1.2	5.0		RGD1561849	2.9	4.4	
<i>Receptors</i>				Yjefn3	2.8	5.2	
Mas1	5.5	5.9		Kctd6	2.2	3.2	
Rtn4r	4.4	4.5	Hasegawa et al. (2005)	Rgs12	2.1	3.5	
Olr59	3.8	1.2*		Fam188a	1.9	3.1	
Sstr1	3.7	4.0	Kong et al. (1994)	Serinc2	1.9	3.2	Inuzuka, Hayakawa, and Ingi (2005)
Nptxr	3.4	3.4		Anxa4	1.7	3.9	
Epha5	3.0	3.5	Cooper, Crockett, Nowakowski, Gale, and Zhou (2009)				
Grm2	2.8	4.5					
Sstr2	1.9	4.2	Kong et al. (1994)				
Rxfp1	1.3*	3.6	(Ma, Shen, Burazin, Tregear, and Gundlach (2006)				

Table 2

Genes identified via DNA microarray analysis to be expressed at a higher level in the CeA compared to the LA and B nuclei. Data for the CeA compared to the LA and the CeA compared to the B were listed side by side since for most cases these data were very similar. These data were grouped based on gene classification. Gene names in bold indicate the amygdala gene expression pattern for this gene was previously published and consistent with these data and includes the associated reference located in the reference column. * Indicates that the fold difference value has a *t*-test *p*-value of *p* > 0.05. Bold fold difference value indicates gene expression pattern for respective comparison met criteria to be considered differentially expressed (see Section 2). Italics fold difference value indicates gene expression pattern for respective comparison had a *p* < 0.05 for the Benjamini and Hochberg False Discovery Rate multiple testing correction.

Gene symbol	CeA/B AVG fold difference	CeA/LA AVG fold difference	References	Gene symbol	CeA/B AVG fold difference	CeA/LA AVG fold difference	References
<i>Transcription factors</i>				<i>Peptides/ligands</i>			
Zfhx3	6.6	7.4	Watanabe et al. (1996)	Nts	19.3	23.0	Day, Curran, Watson, and Akil (1999)
Dlx5	5.3	5.2	Wang, Lufkin, and Rubenstein (2011)	Nmb	<i>14.5</i>	<i>14.5</i>	Wada et al. (1990)
Meis2	4.3	3.7		Cartpt	11.8	15.5	Kang et al. (2010)
Zfhx4	3.3	3.1		Pdyn	<i>8.1</i>	7.1	Solecki et al. (2009)
Ebf1	3.0	3.0		Crh	6.9	5.3	Zambello et al. (2008)
<i>Channels/transporters</i>				Pnoc	6.6	7.6	Boom et al. (1999)
Crabpl	5.3	<i>3.1</i>		Penk	<i>4.7</i>	<i>6.1</i>	Poulin, Chevalier, Laforest, and Drolet (2006)
Rbpl	4.5	4.3	Zetterstrom, Simon, Giacobini, Eriksson, and Olson (1994)	Tac2	4.3	7.1	
Slc32a1	<i>3.4</i>	3.2	Peselmann, Schmitt, Gebicke-Haerter, and Zink (2012)	Tctex1d1	3.3	3.2	
<i>Enzymes</i>				Chga	3.2	3.6	
Ptpr	3.9	3.6		Sst	2.7	3.6	
Ptpro	3.3	4.2		Scg2	2.3	3.3	
Atp6v1c2	<i>3.1</i>	<i>3.0</i>		<i>Miscellaneous/unclassified</i>			
Gaa	<i>3.1</i>	3.4		Rgs9	6.6	5.0	Thomas, Danielson, and Sutcliffe (1998)
Camk1g	2.3	3.2	Takemoto-Kimura et al. (2003)	Coch	6.1	4.7	
Prkcd	<i>2.1</i>	3.5		Zfp503	<i>6.1</i>	6.7	

Gene symbol	CeA/B AVG fold difference	CeA/LA AVG fold difference	References	Gene symbol	CeA/B AVG fold difference	CeA/LA AVG fold difference	References
<i>Cytoskeletal associated</i>				Rap1gap	4.9	5.5	
Cdhl	4.8	4.4		Arhgap36	3.7	3.6	
Actn2	3.2	3.6		RGD1565489	3.7	3.1	
<i>Receptors</i>				LOC100910460	3.5	3.3	
Adora2a	8.4	5.4	Becker et al. (2008)	Mt1a	3.4	2.0	
Gpr88	7.8	2.7	Becker et al. (2008)	RGD1564664	3.2	3.0	
P2ry1	3.0	2.7*		Hap1	3.0	3.1	Fujinaga et al. (2004)
Fgfr1	2.9	3.0	Gonzalez, Berry, Maher, Logan, and Baird (1995)				
Vipr2	2.0	3.0					

Table 3

Genes identified via DNA microarray analysis to be differentially expressed between the LA and B nuclei. These data were grouped based on gene classification. Gene names in bold indicate the amygdala gene expression pattern for this gene was previously published and consistent with these data and includes the associated reference located in the reference column.

Gene symbol	B/LA AVG fold difference	References	Gene symbol	LA/B AVG fold difference	References
<i>Channels/transporters</i>			<i>Enzymes</i>		
Slc24a2	3.6		Trim54	4.1	
<i>Enzymes</i>			<i>Receptors</i>		
Cyp26a1	3.4		Olr59	3.1	
Cyp26b1	3.0		<i>Peptides/ligands</i>		
<i>Cytoskeletal associated</i>			Grp	4.2	Wada et al. (1990)
Cdh9	4.2		Adcyap1	3.3	Skoglosa et al. (1999)
<i>Peptides/ligands</i>					
Nov	5.1	Su et al. (2001)			
Cxcl12	3.3	Tham et al. (2001)			

Table 4

Genes differentially expressed between the CeA and the B or LA (BLA) were placed in functional categories based on ontology analysis. The following table indicates the number of differentially expressed genes that were identified to be part of each ontology category. The table is separated into three root categories, Biological Processes, Cellular Component and Molecular Function respectively. Sub-root, AmiGO ontology category ID, and number of differentially expressed genes within each sub-root category are listed. The genes present in each of these categories are listed in S7.

Root category	Sub-root category	AmiGO category ID	CeA	BLA
<i>Biological process</i>				
	Amine transport	GO:0015837	5	0
	Axonogenesis	GO:0007409	0	13
	Blood circulation	GO:0008015	9	0
	Catecholamine secretion	GO:0050432	4	0
	Cell morphogenesis involved in neuron differentiation	GO:0048667	0	13
	Central nervous system development	GO:0007417	0	17
	Circulatory system process	GO:0003013	9	0
	Feeding behavior	GO:0007631	5	0
	Forebrain development	GO:0030900	0	14
	Nervous system development	GO:0007399	0	26
	Neuron development	GO:0048666	0	17
	Neuron projection development	GO:0031175	0	17
	Neuron projection morphogenesis	GO:0048812	0	13
	Neuropeptide signaling pathway	GO:0007218	6	0
	Positive regulation of cAMP metabolic process	GO:0030816	4	0
	Regulation of catecholamine secretion	GO:0050433	4	0
	Regulation of norepinephrine secretion	GO:0014061	3	0
	Regulation of system process	GO:0044057	9	0
	Single-organism behavior	GO:0044708	0	13
	Telencephalon development	GO:0021537	0	10
<i>Cellular component</i>				
	Anchored to membrane	GO:0031225	0	4
	Axon part	GO:0033267	4	0
	Axon	GO:0030424	0	8
	Cell projection part	GO:0044463	7	0
	Cell projection	GO:0042995	10	14
	Cone cell pedicle	GO:0044316	1	0
	Cytoplasmic membrane-bounded vesicle	GO:0016023	7	0
	Cytoplasmic vesicle	GO:0031410	7	0
	Endoplasmic reticulum	GO:0005783	0	14
	Extracellular region	GO:0005576	10	20
	Extracellular space	GO:0005615	0	11
	Membrane-bounded vesicle	GO:0031988	7	0

Root category	Sub-root category	AmiGO category ID	CeA	BLA
	Neuron projection	GO:0043005	8	13
	Plasma membrane	GO:0005886	0	25
	Synapse part	GO:0044456	0	8
	Synaptic vesicle	GO:0008021	0	4
	Vesicle	GO:0031982	7	0
	<i>Molecular function</i>			
	ADP-activated nucleotide receptor activity	GO:0045032	1	0
	G-protein coupled adenosine receptor activity	GO:0001609	2	0
	Gamma-aminobutyric acid:hydrogen symporter activity	GO:0015495	1	0
	Hormone activity	GO:0005179	0	6
	Isoprenoid binding	GO:0019840	0	3
	Neuromedin B receptor binding	GO:0031710	1	0
	Neuropeptide hormone activity	GO:0005184	0	3
	Neuropeptide receptor activity	GO:0008188	0	3
	Opioid peptide activity	GO:0001515	3	0
	Oxidoreductase activity, acting on peroxide as acceptor	GO:0016684	0	3
	Peroxidase activity	GO:0004601	0	3
	Protein binding	GO:0005515	23	0
	Receptor binding	GO:0005102	11	0
	Retinal binding	GO:0016918	2	0
	Retinoic acid 4-hydroxylase activity	GO:0008401	0	2
	Retinoic acid binding	GO:0001972	0	3
	Retinoid binding	GO:0005501	2	3
	Retinol binding	GO:0019841	2	0
	Somatostatin receptor activity	GO:0004994	0	2