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Neuroscience. Author manuscript; available in PMC 2009 October 28.

Published in final edited form as:
Neuroscience. 2008 October 28; 156(4): 950–965. doi:10.1016/j.neuroscience.2008.07.070.

Transcriptome analysis identifies genes with enriched expression in the mouse central Extended Amygdala

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Abstract

The central Extended Amygdala (EAc) is an ensemble of highly interconnected limbic structures of the anterior brain, and forms a cellular continuum including the Bed Nucleus of the Stria Terminalis (BNST), the central nucleus of the Amygdala (CeA) and the Nucleus Accumbens shell (AcbSh). This neural network is a key site for interactions between brain reward and stress systems, and has been implicated in several aspects of drug abuse. In order to increase our understanding of EAc function at the molecular level, we undertook a genome-wide screen (Affymetrix) to identify genes whose expression is enriched in the EAc. We focused on the less-well known BNST-CeA areas of the EAc, and identified 121 genes that exhibit more than 2-fold higher expression level in the EAc compared to whole brain. Among these, forty-three genes have never been described to be expressed in the EAc. We mapped these genes throughout the brain, using non-radioactive in situ hybridization, and identified eight genes with a unique and distinct rostro-caudal expression pattern along AcbSh, BNST and CeA. Q-PCR analysis performed in brain and peripheral organ tissues indicated that, with the exception of one (Spata13), all these genes are predominantly expressed in brain. These genes encode signaling proteins (Adora2, GPR88, Arpp21 and Rem2), a transcription factor (Limh6) or proteins of unknown function (Rik130, Spata13 and Wfs1). The identification of genes with enriched

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expression expands our knowledge of EAc at a molecular level, and provides useful information to towards genetic manipulations within the EAc.

Keywords

adult mouse; Affymetrix microarray; in-situ hybridization; central Extended Amygdala; gene; marker

Introduction

The central Extended Amygdala (EAc) is a network formed by basal forebrain structures that include the Bed Nucleus of the Stria Terminalis (BNST) and the central Amygdala (CeA) (Alheid and Heimer, 1988, Cassell et al., 1999, de Olmos and Heimer, 1999, Swanson, 2003). However, it has been proposed that the shell of the Nucleus Accumbens (AcbSh) would be part of this network (Koob, 2003). This particular division of the Extended Amygdala interfaces reward circuitry with stress systems, and is involved in both the acute reinforcing effects of drugs of abuse and the negative reinforcing effects of drug dependence. As such, the EAc may represent a common anatomical substrate for drug reward and stress-induced drug seeking and reinstatement (Koob, 2003, Shaham et al., 2003, Koob and Kreek, 2007). The EAc receives inputs mainly from limbic cortices, and projects to the ventral tegmental area, the lateral hypothalamus, the tegmental pedunculopontine nucleus and other various brain stem nuclei (in (Koob, 2003)). A number of transmitter systems that operate within the EAc have been described. GABA immunoreactive neurons have been characterized within the 3 components of the EAc (Sun and Cassell, 1993). Also BNST and CeA neurons express a number of neuropeptides that include CRF, NPY, vasopressin and galanin, and modulate EAc function (see (Koob, 2003, Kash and Winder, 2006)). However, our knowledge of phenotypic characteristics of EAc neurons remains limited.

Understanding the function of specific brain areas or circuits requires detailed information on molecules expressed by neuronals. Cellular composition and neuron function varies greatly across the brain, and large-scale gene expression studies are growing to explore gene patterning in the adult mammalian brain on a genome-wide basis. A number of transcriptome studies have examined large areas of the brain, such as the cortex, hippocampus and striatum (Bonaventure et al., 2002, de Chaldee et al., 2003, Ghate et al., 2007, Stansberg et al., 2007). Comparison of central and peripheral nervous systems has led to identify genes whose expression is restricted to either spinal cord or dorsal root ganglia (LeDoux et al., 2006). Transcriptional imprint of 24 neural brain tissues helped to construct a gene expression-based brain map in the adult mouse (Zapala et al., 2005). Further the exploration of specialized brain networks has provided regional-specific gene profiles using fine-dissection procedures. Genes restricted to the CA1, CA3 and DG hippocampal territories (Lein et al., 2004), or even along the dorso-ventral axis of the CA1 field has been identified (Leonardo et al., 2006). The analysis of subregions of the hypothalamus highlighted genes restricted to the ventromedial hypothalamus in the adult (Segal et al., 2005) or developing mouse brain (Kurrasch et al., 2007). The study of Amygdala revealed gene subsets whose expression matched anatomical boundaries of amygdaloid nuclei (Zirlinger, 2003). Recently Olsen and coll. compared gene profiles of the BNST to those of ventral and dorsal striatum, and identified distinct signaling and plasticity genes in these areas that all respond to dopamine and are involved in disorders ranging from Parkinson’s disease to drug addiction (Olsen et al., 2008).

The present study aimed at identifying genes with enriched expression in the EAc, in order to broaden our knowledge of genes operating within this brain network. In this study we have focused on the BNST/CeA components of the EAc. Using a micropunch-dissection procedure,

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we prepared a tissue sample from mouse brain CeA and BNST and compared the transcriptome in this sample to that of the whole brain. We identified 129 probe sets with a 2-fold enrichment or more in the EAc and mapped the expression pattern of 49 genes by in situ hybridization in the mouse brain. Eight genes showed an enriched expression pattern in the EAc, that we analyzed in greater details. These genes potentially influence some aspects of addictive behaviors, and may be useful for further genetic manipulations within the EAc.

Experimental procedures

Tissue dissection

Tissues were dissected from male 3 to 6 month old C57Bl/6J wild-type mice using a microdissection procedure as follows. Briefly, mice were killed by cervical dislocation. Brain was removed, washed in PBS buffer and placed into a matrix cooled on ice (ASI Instruments Inc, Warren, MI, USA) to obtain slices of 1 mm thickness. Accurate localization of brain structures was based on the stereotaxic atlas of mouse brain (Paxinos and Franklin, 2001) and areas corresponding to the Bed Nucleus of the Stria Terminalis (BNST, +0.5 to −0.5), Central nucleus of Amygdala (CeA, −0.5 to −1.5) were taken by bilateral punches (1.2 mm diameter). BNST and CeA samples were pooled to obtain the central Extended Amygdala (EAc) sample (see Figure 1A). Additionally, samples of the whole brain (WB), lateral hypothalamus (LH), spinal cord, thymus, lung, spleen, heart, liver, intestine, lung, stomach, kidney, testis, and lung were collected. All samples were stored at −80°C until use. All animal use procedures were in strict accordance with standard ethical guidelines (European Community Guidelines on the Care and Use of Laboratory Animals 86/609/EEC) and approved by the local ethical committee (Comité régional d'éthique en matière d'expérimentation animale de Strasbourg, CREMEAS, 2003-10-08-[1]-58).

RNA preparation and microarray hybridization

Total RNA was extracted from the different tissues using TRIzol reagent (Invitrogen, Cergy Pontoise, France) and following the manufacturer's specifications. EAc samples were prepared with tissues pooled from 3 mice, WB samples were from 2 mice. The RNA quantity was measured using a spectrophotometer and quality was assessed by agarose gel electrophoresis. For the microarray experiments, cDNA synthesis, cRNA labeling, hybridization and scanning procedures were conducted according to standard Affymetrix'protocols (www.affymetrix.com) (Affymetrix Core Facility, IGBMC, Illkirch, France, http://www-microarrays.u-strasbg.fr). Three separate hybridizations were performed with independent pooled samples from both EAc and WB. In total, 6 Mouse Genome 430 2.0 oligonucleotide arrays representing 45101 transcripts or ESTs were used in this experiment.

Microarray data analysis

Microarrays were scanned using Affymetrix GeneChip Scanner 3000. Quantification and initial analysis of the microarrays were done using Gene Chip operating Software (GCOS v1, Affymetrix UK Ltd). The comparisons of gene expression profiles between EAc and WB samples were done using a standard significance analysis (MAS 5.0 software, Affymetrix UK Ltd). For selecting differentially expressed genes, we first searched for probes considered as detected. Probe sets were eliminated in the analysis when described as absent more than 4 times among 6, or when having all signal values bellow 25 (corresponding to the median value of signals for all samples). This step led to the selection of 25174 probe sets. We then used p-values from the MAS 5.0 comparative analysis to find probe sets, which led to acceptable False Discovery Rate (FDR). A statistical significance of 0.0025 corresponded to a FDR value of 2.5%. This threshold selected 2799 probe sets. To limit the number of genes for further analysis, we then applied more stringent criteria. We re-analyzed each hybridization set separately. We eliminated probe sets with signals under 100 in the EAc sample. We then selected probe sets

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with a “signal log2 ratio” of EAc/WB equal or superior to 1, corresponding to a difference in expression level of at least 2 fold. Finally, lists obtained from each hybridization set were combined and probe sets that were differentially expressed in at least 2 out of the 3 hybridizations were selected. Finally, this led to a group of 129 probe sets that we defined as enriched in the EAc. A student t-test was then performed on this subgroup to confirm that average signals from triplicate hybridizations from the EAc differed from those measured in the whole brain. Hierarchical clustering was performed on probe sets selected as enriched in the EAc using the Cluster 3.0 and Treeview software (Eisen et al., 1998, de Hoon et al., 2004). Genes enriched in the EAc were annotated for association with biological processes using an optimized Gene Ontology (GO) analysis (as described in (Chalmel et al., 2005, Abou-Sleymane et al., 2006, Befort et al., 2008b) and with biological functions using Ingenuity Pathway Analysis (IPA) network. For GO analysis, over-represented GO terms with a probability lower than 0.01 and including at least 4 proteins were selected. In IPA analysis, biological functions and/or diseases that are most significant to the dataset were identified. The p-value for a given annotation is calculated by considering the number of “focus genes” that participate in that function and the total number of genes that are known to be associated with that process in Ingenuity’s knowledge base (http://www.ingenuity.com). In our analysis, we show only the functions with the six highest p-values.

In situ hybridization

Plasmids containing candidate genes were obtained from the Deutsches Ressourcenzentrum für Genomforschung (RZPD, Berlin, Germany). Clone inserts were amplified by PCR (100 μl), using vector-specific primers and 0.25 μl of bacterial glycerol stock as template material. PCR reactions were purified using Millipore’s (Millipore Corporation, Bedford, USA) Montage 96 and amplicons used as template for in-vitro transcription of sense and anti-sense Dig-labeled riboprobes. To this aim 1μg linearized DNA was transcribed using T7, T3 or Sp6 polymerases and the 10x DIG RNA labeling mix (Roche Diagnostics, Meylan, France) according to the manufacturer’s instructions. Probes were quantified by spectrophotometry and quality assessed by agarose gel electrophoresis. Adult mice were killed by cervical dislocation and brains were rapidly extracted and fresh frozen in OCT. The OCT-embedded brain blocks were stored at −20°C until use. Brain sections 25 μm thick were processed for in situ hybridization using Genepaint robotic equipment and procedures (www.genepaint.org; (Carson et al., 2002) as previously described (Ghate et al., 2007). Briefly, 600 ng of probe at a concentration of 20 ng/μl was hybridized onto the sections at 64°C for 5.5 hours. The dig-label was detected using anti-Dig-POD antibody (Roche, 1:500 dilutions in Tris-NaCl pH 7.5 solution containing Perkin Elmer blocking reagent and 0.1% Tween). The signal was amplified and revealed using tyramide amplification process-kit (Perkin Elmer, Waltham, USA) and BCIP (0.15mg/ml)/NBT (0.4 mg/ml, Roche) color substrates. Images were recorded using a CCD camera (Leica Instruments, Rueil-Malmaison, France). In each in situ hybridization set, neuropeptide Y and preproenkephalin were used as positive controls and a blank hybridization (no probe) as the negative control. For ISH analysis, we used criteria of classification adapted from the GenePaint annotation procedures (http://www.genepaint.org/) as described in (Gofflot et al., 2007). Three different levels of expression were defined: 0: no color precipitate detected; 1: weak expression, a few particles of color precipitate per cell; 2: strong expression, color precipitate completely filling cells. For those, we distinguished two different types of expression patterns: ubiquitous distribution throughout the brain or restricted distribution to specific regions of the brain, including EAc.

Quantitative RT-PCR

Total RNA was extracted from the different tissues using TRIzol reagent (Invitrogen, Cergy Pontoise, France) with EAc and LH samples, prepared with tissues pooled from 3 mice, whole brain samples from 2 mice and all the other tissues from individual mouse. Total RNA (2.5

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μg) from each pool (n=2) was treated for 30 min at 37°C by DNase I RNase free (4 U, Invitrogen, Cergy Pontoise, France) in First strand Superscript buffer (Invitrogen, Cergy Pontoise, France) and reaction was stopped by incubating the mix 5 min at 75°C. RNA was then pre-incubated with oligodT primer (8 μM), random Hexamer (16 μM) and dNTPs (500 μM each) in a volume of 30 μl for 5 min at 65°C. Finally, First strand Superscript buffer, DTT (0,01M) and Superscript II (200U, Invitrogen, Cergy Pontoise, France) were added in final volume of 46 μl for 50 min at 42°C. Reaction was stopped by 15 min incubation at 70°C. Real-time PCR was performed in triplicate on a MyIQ BioRad instrument using iQ SYBR Green supermix, cDNA (0.5 μl) and gene-specific primers (200 nM) in a 25 μl reaction as recommended by the manufacturer (Bio-Rad, Marnes-la-Coquette, France). Gene-specific primers were designed using primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to obtain a 75–150 bp product (see supplemental Table S1). Relative quantification for a given gene in any area was normalized to its level in whole brain (ΔCt) and expressed as fold change (WB level being equal to 1).

Results

Identification of genes enriched in the central Extended Amygdala

To identify genes whose expression is enriched in the EAc, we searched for genes whose expression level is higher in EAc compared to WB. We used the Genechip Mouse Genome 430 2.0 oligonucleotide arrays (Affymetrix) and compared hybridization signals of RNA transcripts in the EAc to those obtained in WB. Our gene selection procedure involved a multi-step procedure based on the FDR, the gene expression level and the fold change EAc/WB (see methods). Using this approach, we identified 129 probe sets showing statistical differential expression with a fold-change of 2 at least, that we categorized as enriched in EAc (Table 1, Figure 1B). A statistical analysis further performed directly on mean signals of each probe set (see Methods) showed that 95.3 % of the EAc probe sets were significantly enriched over whole brain (p value<0.05). Gene annotation from Affymetrix identified distinct probe sets corresponding to identical genes, with 9 genes represented by two different probe sets. In the end we identified 121 genes enriched in the EAc. Figure 1B presents a hierarchical cluster analysis of the enriched EAc transcripts with the corresponding signals in all 6 arrays.

To characterize the enriched transcriptome in the Extended Amygdala, we used a GO database and annotated the 121 genes selected, as previously described (Chalmel et al., 2005, Befort et al., 2008b). We found 98 genes associated with proteins, among which 82 were associated with GO terms. These genes were then grouped into categories of biological processes, in which their encoded proteins are involved (GOBP) and these categories are illustrated in Figure 1C. This analysis showed that some GO categories are enriched, particularly in the response to stress (GO:0006950), behavior (GO:0007610), cell communication (GO:0007154), signal transduction (GO:0007165) and synaptic transmission (GO:0007268). Interestingly, a large group included neuropeptide signaling pathway (GO:0007218) and GPCR signaling pathway (GO:0007186) and some transcripts were also associated with neurogenesis (GO:0007399), organogenesis (GO: 00099887), central nervous system development (GO:0007417) and nerve maturation (GO:00042551). To interrogate potential functional interactions among EAc enriched genes, we also used the Ingenuity Pathways Analysis (Table 2). Our results showed that, among the top biological functions, potential interactions were revealed by putative protein networks involved in Cell Morphology, Gene Expression, Cell Signaling and Nervous System Development and Function. Interestingly, these two bioinformatics analysis were consistent and revealed that the genes we have identified in our study participate in functions described in the literature as involving the EAc and suggest new functions for this network.

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Altogether, this microarray screen identifies 23 genes that were already reported by others in EAc (see Table 1 column ISH label Litt.), some of which are widely studied (for example tachykinin 1 and 2, prodynorphin or dopamine D1 receptor). Importantly, the data also highlight a large number of genes whose expression in EAc is reported here for the first time.

Expression pattern by in situ hybridization

We further performed *in situ* hybridization (ISH) throughout the brain to examine expression patterns for a sub-selection of genes. We focused on 49 genes whose expression pattern in BNST and/or CeA was not studied in the mouse previously (Table 1 column ISH label S or SC). We first performed non isotopic ISH on sagittal sections of mouse brain. Our results show that a signal was detectable in the EAc for all tested probes confirming our microarray analysis (see Supplemental Figure S2). Expression patterns could be described as (i) low expression, (ii) strong and fairly ubiquitous expression or (iii) significant expression restricted to specific brain regions including the EAc (see Figure 2 and Methods). Altogether, 17 genes (see supplementary Figure S2A) showed a weak signal under our hybridization conditions and those were not further investigated. Figure 2A shows sagittal ISH for three of these genes, Limh8, Dock10 and Rasgrp2 whose expression was close to the detection limit. Twenty-four other genes showed a hybridization signal widely spread along the brain (see supplementary Figure S2B). Figure 2B shows an example for three of these genes, namely Mrg1, Rik263, PDLIM2 with staining throughout the brain. Lastly, 8 genes were clearly detected in EAc, with weak or restricted expression in other brain regions. Figure 2C shows the sagittal expression pattern for these genes namely Limh6, Adora2, Arpp21, GPR88, Wfs1, Spata13, Rem2 and Rik130.

We next performed ISH on coronal sections for the last 8 genes (Figure 3). We focused our analysis on sections corresponding to the EAc, at the levels of BNST (Figure 3B) and CeA (Figure 3C). As the AcbSh was proposed to be part of the Extended Amygdala (Koob, 2003), we also included analysis of the staining observed at the level of this structure (Figure 3A). The expressed sequence tag RIKEN E130309F12 probe (Rik130) presented a weak expression in the AcbSh that diminished gradually rostro-caudally and no signal could be detected in the CeA. The Rad and gem related GTP binding protein 2 transcript (Rem2) was strongly expressed in the Nucleus Accumbens including shell and core, and in the interstitial nucleus of the posterior limb of the anterior commissure (IPACL), which is part of BNST. A patchy distribution in the CeA was observed and Rem2 was also clearly expressed in the dorsal striatum. Concordant with our sagittal screen, the adenosine A2 receptor gene (Adora2) showed a strong and consistent expression in all areas of the EAc network. In the BNST, the lateral division was particularly labeled (Figure 3B). Adenosine A2 receptor mRNA expression was also present in several brain areas such as the striatum (Cpu and core of the Nucleus Accumbens), the cortex, the hippocampus and the piriform cortex. The cyclic AMP-regulated phosphoprotein 21 gene (Arpp21) presented an interesting expression pattern, with specific mRNA expression in the AcbSh, without signal in adjacent structures like the core of the Nucleus Accumbens and the caudate putamen (Figure 3B). At the level of the BNST, a specific signal was detected in the medial division. No detectable staining was obtained in the CeA while the Arpp21 gene was highly expressed in the basolateral and basomedial amygdaloid nucleus. Specific signal was also measured in the medial preoptic nucleus, the cortex and the hippocampus. GPR88 gene showed strong expression throughout the striatum, including the AcbSh (Figure 3A). GPR88 mRNA expression was undetectable in the BNST (Figure 3B). A strong staining was observed in the medial division of the CeA with no signal in the basolateral Amygdala. The GPR88 mRNA was also clearly present in the piriform cortex. For the LIM homeobox 6 (Limh6) mRNA, we obtained a strong and homogenous expression throughout areas of the Extended Amygdala including AcbSh, the lateral division of the BNST and the CeA. We also noticed expression of this gene on the nucleus of the vertical limb of the diagonal band (VDB) (Figure 3A). A strong signal was measured for the spermatogenesis associated 13

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(Spata 13) gene in the dorsal part of the lateral division of BNST compared to other BNST nuclei, as well as in the AcbSh and CeA. Additionally, a low Spata 13 staining was observed in the CA1 field of the hippocampus. Finally, the wolframin gene (Wfs1) showed strong expression throughout the Extended Amygdala. This gene was strongly expressed in the AcbSh and BNST, with a low staining in the caudate putamen. (Figure 3A and 3B). Interestingly, expression of this gene was strong in the CeA, and extremely low in the BLA clearly defining the boundaries between these two amygdaloid nuclei (Figure 3C). Wolframin mRNA was also strongly expressed specifically in the CA1 field of the hippocampus and the piriform cortex.

In conclusion, these 8 genes show a specific and distinct expression pattern within the EAc. They are expressed throughout the AcbSh and CeA, and show locally restricted expression in the subdivisions of the BNST structure.

Distribution of EAc-enriched genes in the central nervous system and peripheral tissues by qPCR

To examine the general expression pattern of these 8 genes throughout the central nervous system and in peripheral organs, we performed quantitative PCR on cDNA samples from WB, EAc and LH (a brain region that we have investigated in a separate study, see Befort et al (Befort et al., 2008a), as well as spinal cord, thymus, lung, spleen, heart, liver, intestine, stomach, kidney, testis, and muscle (Figure 4). The results confirmed high expression in the EAc for all the tested genes compared to whole brain. The Spata-13 gene showed the less restricted pattern of expression, with high expression levels in thymus, kidney and spleen (4.7-, 3.1- and 1.7-fold higher than in the EAc, respectively). In contrast, the GPR88 gene was detected only in the brain, with its highest level of expression in the EAc. Expression of the Rik130 transcript was restricted to the nervous system, including the spinal cord. Finally the Wfs1, Limh6, Rem2, Arpp21 and Adora2 genes showed detectable expression in several peripheral tissues, and none of the transcripts tested could be detected in muscle.

Discussion

Using Affymetrix microarrays, we have identified 129 probe-sets corresponding to 121 genes with prominent expression in the EAc. We used a strategy where gene expression in a sample from the region of interest (CeA and BNST) is compared with expression in a sample from the whole brain. Others have used a similar approach in the past, and successfully identified neuronal markers (Cahoy et al., 2008), subregional-specific genes within mouse Amygdala nuclei (Zirlinger et al., 2001, Zirlinger and Anderson, 2003), or regional gene markers within the several hippocampal fields (Lein et al., 2004, Leonardo et al., 2006).

Twenty-three genes enriched in EAc have been reported earlier as being expressed in the rodent EAc (Tables 1). For example, we detected enriched expression of neuropeptide precursor genes such as preprodynorphin (3.06-fold in EAc over WB), preproenkephalin (2.81-fold in EAc over WB) or preprotachykinin 2(6.35-fold in EAc over WB) genes, whose expression patterns were described previously in specific areas of the Extended Amygdala (Harlan et al., 1987,Iadarola et al., 1989,Song and Harlan, 1994). We also identified genes encoding myosin D (2.38-fold in EAc over WB), neuronatin (2.28-fold in EAc over WB) and myelin associated glycoprotein (2.22-fold in EAc over WB). These genes were described as specific markers of particular Amygdala nuclei in a microarray screen analysis from Zirlinger and coll (Zirlinger, 2003,Zirlinger and Anderson, 2003). Among the top enriched genes, we finally identified the wolframin gene (3.38-fold in EAc over WB). This transcript was previously described as specifically expressed in the CA1 region of the hippocampus, and central Amygdala (Takeda et al., 2001,Leonardo et al., 2006). Altogether, our identification of previously reported Amygdalar genes validates our gene selection strategy.

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Importantly, literature mining indicated that expression in the EAc was unknown for 49 other genes. Our further ISH mapping analysis revealed that 8 of these genes show an expression pattern of particular interest within the EAc. We compared the expression patterns of these 8 genes with the Allen Brain Atlas (Lein et al., 2007) and, with the exception of Arpp21 and Wfs1, our analysis showed a similar pattern in the three focused areas of interest. The two expression patterns for Arpp21 transcript presented in the Allen Brain Atlas showed a more widespread expression than our ISH data. However, our ISH results were consistent with Arpp21 protein distribution in rat brain (Ouimet et al., 1989). For the Wfs1 transcript, the pattern in the Allen Brain Atlas appeared ubiquitous, under our criteria (see methods), compared with the expression profile in the rat brain (Takeda et al., 2001) or shown here.

Altogether, with the exception of Rik130, all these genes encode a protein with either fully identified or potential function. For each of these genes, the unique and distinct expression pattern suggests a potential role in regulating the EAc network.

Spata13 showed moderate enrichment in EAc (ISH and qPCR), and was the only gene with strong expression in peripheral organs (Q-PCR) including thymus, lung or kidney. This gene (also called Asef2) was identified as a guanine-nucleotide exchange factor for Rac1 and Cdc42 (Hamann et al., 2007, Kawasaki et al., 2007). Rik130 was specifically expressed in the brain compared to peripheral tissues, but the expression in the EAc was quite low. No information is available on Rik130 distribution or function. A blast search in the Refprot database indicates that the Rik130 protein shows 74% homology with phosphatidic acid phosphatase type 2B (ppap2B), a gene ubiquitously and strongly expressed throughout the brain (http://brain-map.org/).

Limh6 encodes a protein belonging to the LIM homeodomain proteins, a family of transcription factors. Expression of this gene was high in brain, particularly in EA and barely detectable in the periphery. The LIM protein family is involved in many processes of CNS development, from cell fate specification to the establishment of neuronal connectivity. During embryogenesis Limh6 was found involved in the migration of cortical GABAergic interneurons (Cobos et al., 2006, Cuzon et al., 2008). Noticeably, several authors have proposed a role for Limh6 in the development of the Extended Amygdala in mice (Choi et al., 2005, Garcia-Lopez et al., 2008) or zebra fish (Mueller et al., 2008). Our Affymetrix analysis also identified Limh8, another member of the family, as a gene enriched in the EAc (Table 1), and the expression of Limh8 was also described in the adult mouse Amygdala in another report (Zirlinger and Anderson, 2003). These findings suggest that LIM genes, expressed in the adult brain, may contribute to remodeling of the EAc network (Grueter and Winder, 2005, Samson et al., 2005).

Genes with a noticeable expression pattern within the EAc include four genes involved in cell signaling, namely Adora2a, the orphan GPR88, Arpp21 and Rem2. Adora2a encodes the adenosine 2A receptor, a Gs-coupled G protein coupled receptor known to be expressed in the striatum and olfactory tubercules (Santicioli et al., 1993) and involved in many brain functions (for review see (Yaar et al., 2005)). Relevant to our finding of Adora2 expression in the EAc, previous evidence have suggested a role for Adora2 in Amygdala function. The Adora2 agonist CGS21680 was associated with an apoptosis regression in the Amygdala following myocardial infarction (Boucher et al., 2006) and a polymorphism in the human Adora2a gene was associated with panic disorders (Yamada et al., 2001, Hamilton et al., 2004, Lam et al., 2005). GPR88 is an orphan G-protein coupled receptor previously reported as a striatal transcript (Mizushima et al., 2000, Ghate et al., 2007). In accordance, our mapping data confirm prominent expression of this gene in both EAc and the caudate putamen. GPR88 function is unknown, but the alteration of GPR88 expression was reported under several experimental conditions. GPR88 mRNA was up-regulated in the rat arcuate ventromedial nucleus during

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lactation (Xiao et al., 2005), in the prefrontal cortex following metamphetamine or valpoate exposure (Ogden et al., 2004), and down-regulated in the striatum of human patients with Huntington's disease (Hodges et al., 2006). Arpp-21 is a cyclic AMP-regulated phosphoprotein of 21 kDa whose expression was reported in the caudate putamen and substantia nigra (Girault et al., 1990). Arpp-21 has been associated to several aspects of dopamine signaling (Tsou et al., 1993, Ivkovic and Ehrlich, 1999), and is down-regulated in substantia nigra of sporadic parkinsonian patients (Grunblatt et al., 2007). Finally, Rem2 encodes a Ras-like GTPAse (Finlin et al., 2000) initially described as a modulator of N-type current in primary neuron cultures (Chen et al., 2005). Recently, this protein was shown to play a key role in the development of glutamatergic and GABAergic synapses in rat hippocampus primary cultures (Paradis et al., 2007).

Most remarkable was the expression of Wfs1 gene, which showed best EAc enrichment among the final eight-gene subset. This gene appeared strongly expressed throughout the AcbSh, BNST and CeA, and weak -if no- expression was detected in most other brain regions. ISH indicated scarce expression in the caudate, olfactory tubercle, locus coeruleus, cerebellar cortex (Takeda et al., 2001). Our mapping study also highlighted the intriguing hippocampal expression of Wfs1, uniquely restricted to the CA1 field (see Figure 3C) as was previously described by two groups (Takeda et al., 2001, Leonardo et al., 2006). Expression of Wfs1 was also detectable in the spinal cord, heart and intestine. Mutations in the Wfs1 gene are responsible for the Wolfram syndrome, an autonomous recessive disorder which is highly variable in its clinical manifestations and includes diabetes mellitus, diabetes insipidus, deafness, optic atrophy and psychiatric abnormalities (Cryns et al., 2003). The Wfs1gene was originally characterized by positional cloning and encodes a transmembrane protein, wolframin, (Inoue et al., 1998) whose precise role in cell physiology remains to be determined. Knockout of the Wfs1 gene in mice leads to a loss of pancreatic beta-cell, suggesting a role in maintaining some populations of endocrine cells (Ishihara et al., 2004) (Riggs et al., 2005), however the potential neurological and behavioral phenotypes of Wfs1 null mutants have not been reported as yet. Interestingly the Wfs1 messenger was up-regulated in rat Amygdala (Koks et al., 2002) and mouse pre-frontal cortex (Raud et al., 2007) when animals were exposed to cat odor, suggesting a implication of wolframin in stress responses. Together, both anatomical and functional data suggest a prominent implication of Wfs1 in emotionally related behaviors.

In conclusion, we have identified a set of genes with enriched expression in the EAc, and examined the expression pattern for a number of these genes particularly in the EAc. This study extends our knowledge of genes expressed within a brain network, which is central in reward dysregulation and stress disorders. It will be interesting to further characterize neural populations expressing these genes, and particularly to examine whether these transcripts are present in GABAergic neurons that are best characterized within the EAc (Sun and Cassell, 1993). Additionally, BAC transgenic approaches has been used to label (Gong et al., 2003) or to isolate specific cells population in the brain (Nielsen et al., 2006, Cahoy et al., 2008) (Cahoy et al., 2008) (Lovatt et al., 2007). The generation of transgenic mice expressing eGFP under the control of EAc specific genes could be useful to label EAc neurons and study their function and plasticity under pathological stimulations.

Supplementary Material

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Acknowledgements

We thank Pascal Dollé, C. Thaller and G. Eichele for their advice about *in situ* hybridization and Julie Le Merrer for careful reading of the manuscript. We are thankful to IGBMC Core facilities. This work was supported by Centre National de la Recherche Scientifique, Institut de la Santé Et de la Recherche Médicale, Université Louis Pasteur. The authors wish to thank the National Institute of Health (NIH-NIAAA AA016658, NIDA 016768), and the European Union (GENADDICT/FP6 005166; EURExpress # LSHG-CT-2004-512003) for financial support. AG was supported by an IBRO PhD fellowship. JM was supported by grants from the National Research Fund and the Ministère de la Culture, de l'Enseignement Supérieur et de la Recherche of Luxembourg.

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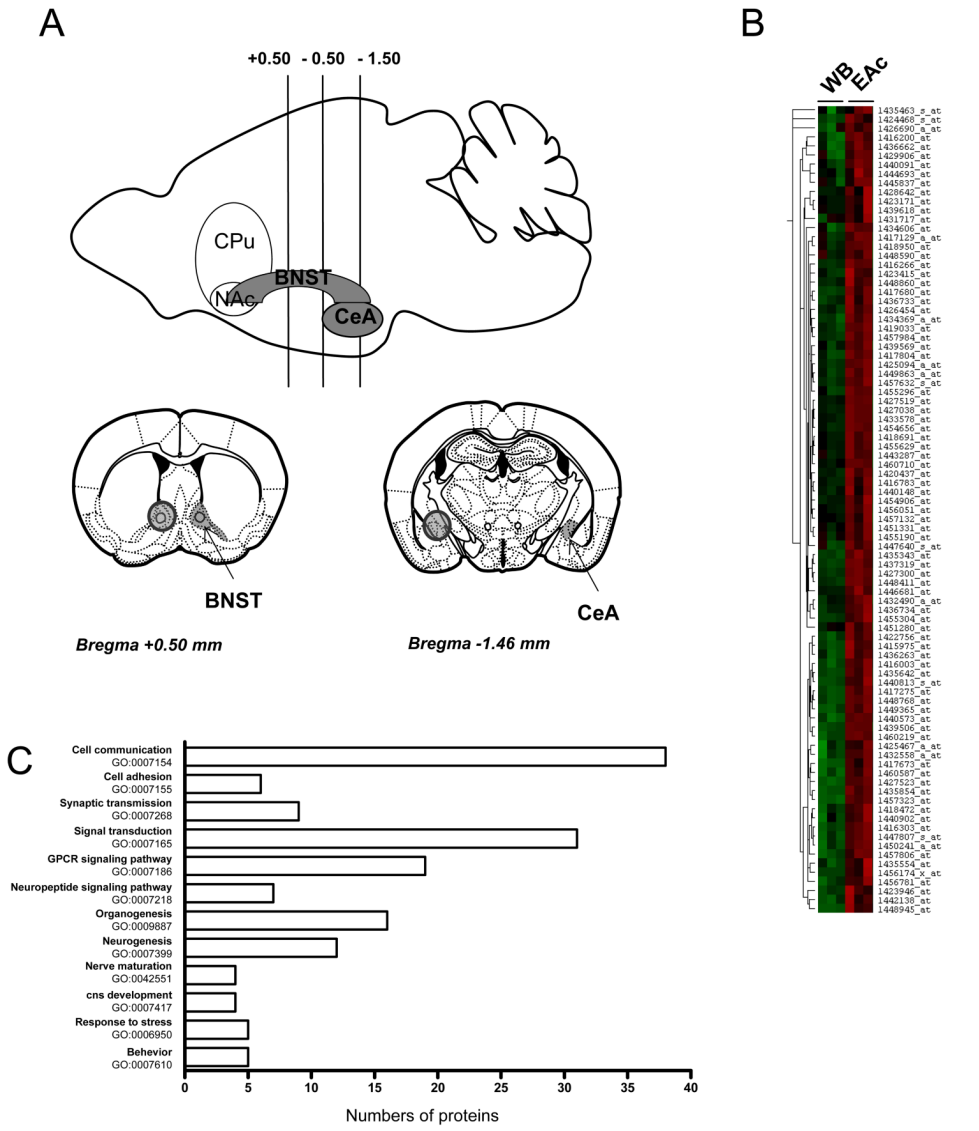

Figure 1. Affymetrix analysis for the identification of genes enriched in the EAc

(A) This scheme shows brain areas under study: bilateral punches (1.2 mm diameter) were taken from mouse brain coronal slices (1 mm thick) to collect the bed nucleus of stria terminalis (BNST, +0.5 to −0.5) and the central nucleus of the Amygdala (CeA, −0.5 to −1.5) (see Methods for details). BNST and CeA punches were pooled and corresponded to central Extended Amygdala (EAc) samples. (B) This hierarchical cluster illustrates raw microarray data from three independent hybridizations for the 129 selected probe sets, and shows high expression in EAc (right columns, red) compared to whole brain (WB, left columns, left). The probe set selection was based on a standard statistical analysis by MAS 5.0 and a threshold of 2-fold change in EAc over WB was used (see Methods for details). Hierarchical cluster analysis was performed using the Cluster 3.0 and Treeview softwares. (C) Gene ontology analysis of the EAc enriched genes. Genes were categorized with the Biological Process domain and significantly enriched GO terms with a probability lower than 0.01 and including at least four proteins are represented.

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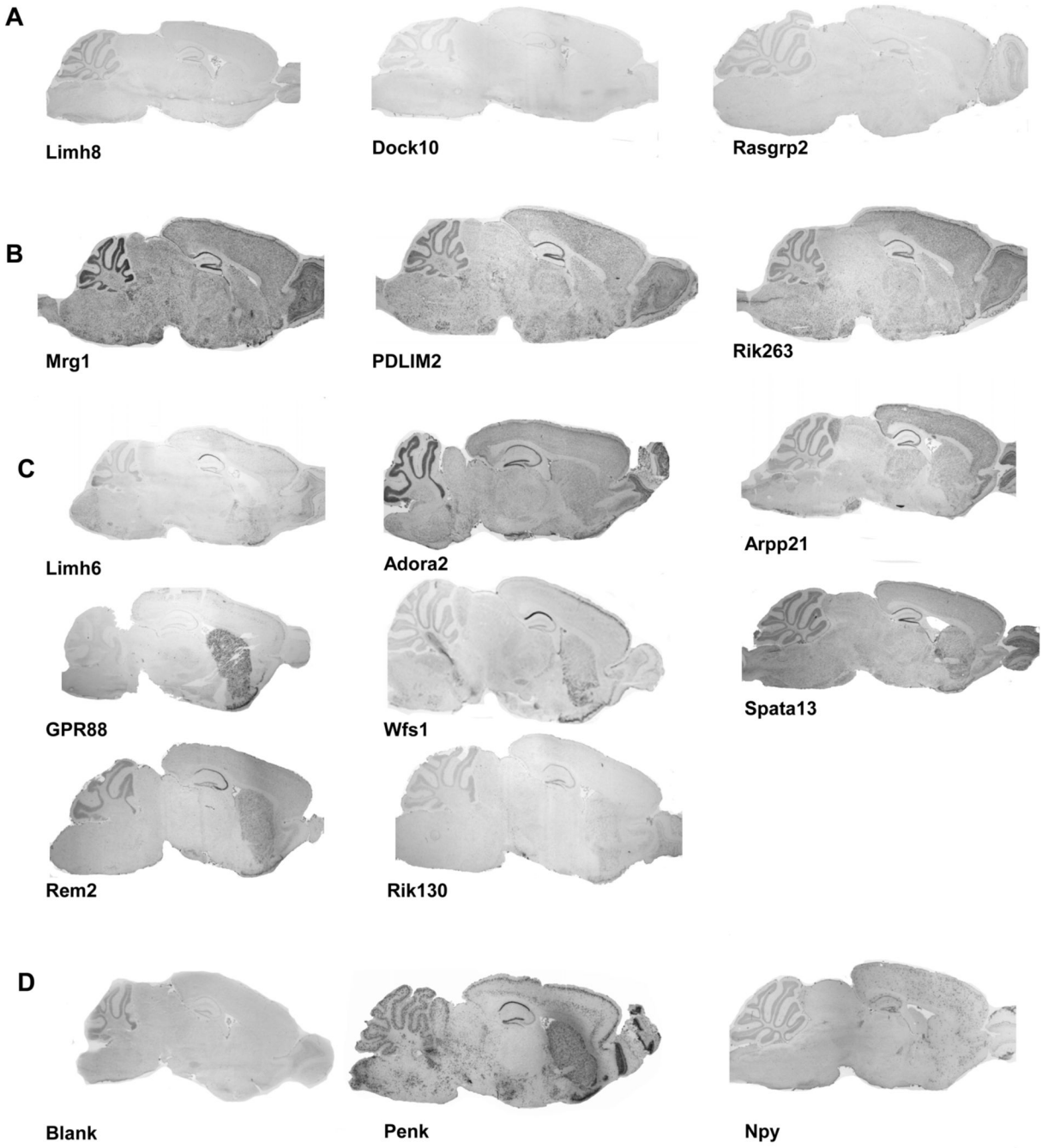

Figure 2. Expression pattern of EAc-enriched genes by in situ hybridization on sagittal brain sections

Dig-labeled RNA *in situ* hybridization (ISH) was performed on 25μm sagittal adult mouse brain sections 49 genes whose specific expression pattern in EAc network has not been reported earlier. In this figure, examples of low expressed genes (A), strong and ubiquitously expressed genes (B) and potential novel EAc markers (C) are shown. Neuropeptide Y (Npy) was used as a low intensity positive control, preproenkephalin (Penk) was used as a high intensity positive control and a blank hybridization was used as the negative control (D). Representative images are shown, and the complete ISH analysis on sagittal sections for all 49 genes is shown in

supplemental Figure S2. Expression patterns were classified as detailed in methods. Gene symbols are indicated as in Table 1.

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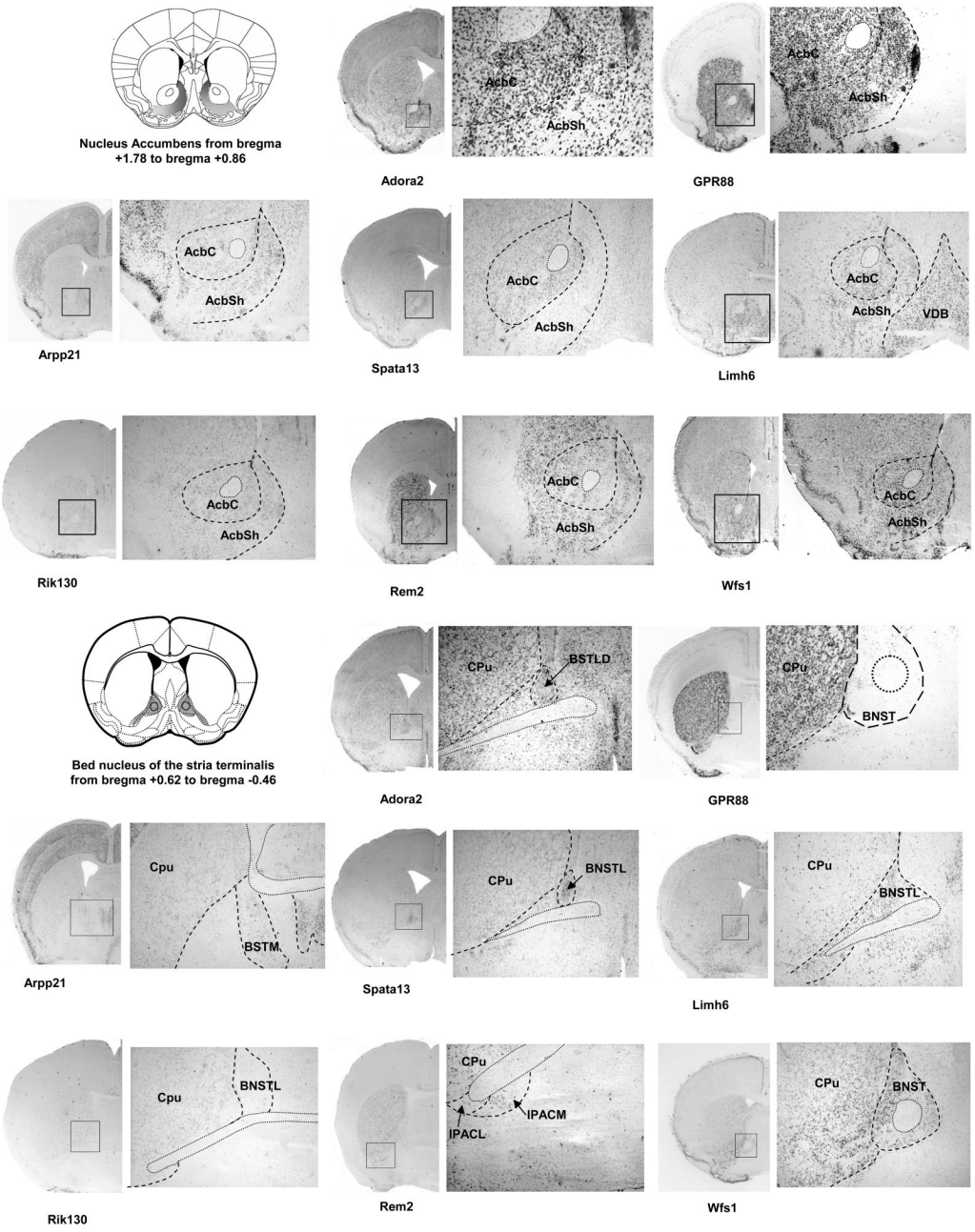

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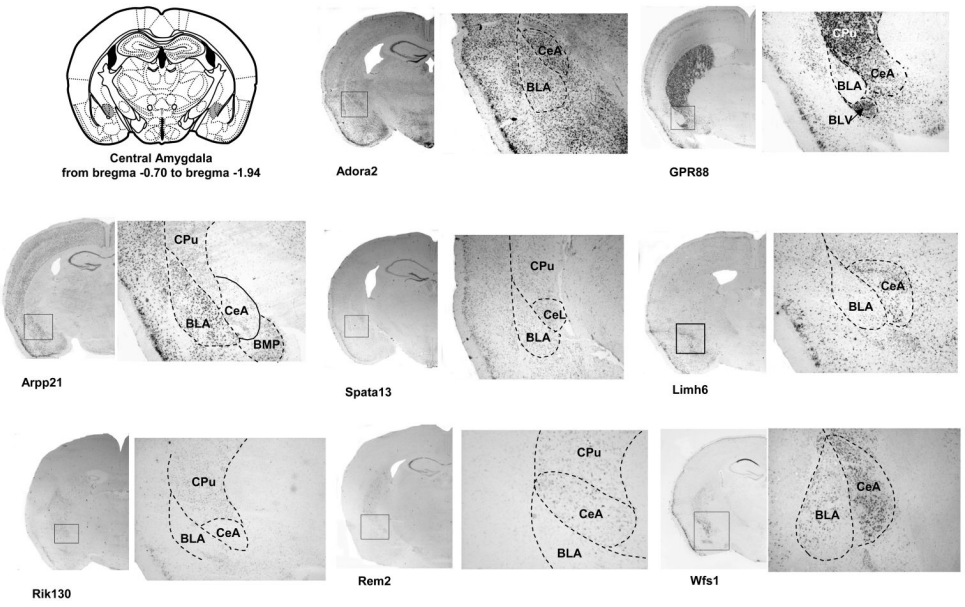

Figure 3. Expression analysis of EAc-enriched genes in AcbSh, BNST and CeA on coronal brain sections

Dig-labeled RNA *in situ* hybridization (ISH) was performed for a selection of 8 potential EAc markers using 25μm coronal sections of mouse brain. A scheme from the mouse brain atlas with the coordinates (Paxinos and Franklin, 2001) shows location of (A) the shell of the Nucleus Accumbens (AcbSh), (B) the bed nucleus of stria terminalis (BNST) and (C) the central nucleus of the Amygdala. Representative ISH images are shown for each candidate gene with an enlargement (zoom) for specific areas of interest. Abbreviations: AcbSh, accumbens nucleus, shell; AcbC, accumbens nucleus, core ; VDB, nucleus of the vertical limb of the diagonal band; CPu, caudate putamen; IPACL, interstitial nucleus of the posterior limb of the anterior commissure, lateral part; IPACM, interstitial nucleus of the posterior limb of the anterior commissure, medial part BSTLD, Bed Nucleus of the Stria Terminalis, lateral division, dorsal part; BSTL, Bed Nucleus of the Stria Terminalis, lateral division; BSTM, Bed Nucleus of the Stria Terminalis, medial division; BLA, basolateral amygdaloid nucleus, anterior part ; BLV, basolateral amygdaloid nucleus, ventral part CeA central amygdaloid nucleus; CeL, central amygdaloid nucleus, lateral division; BMP, basomedial amygdaloid nucleus, posterior part (Paxinos and Franklin, 2001)

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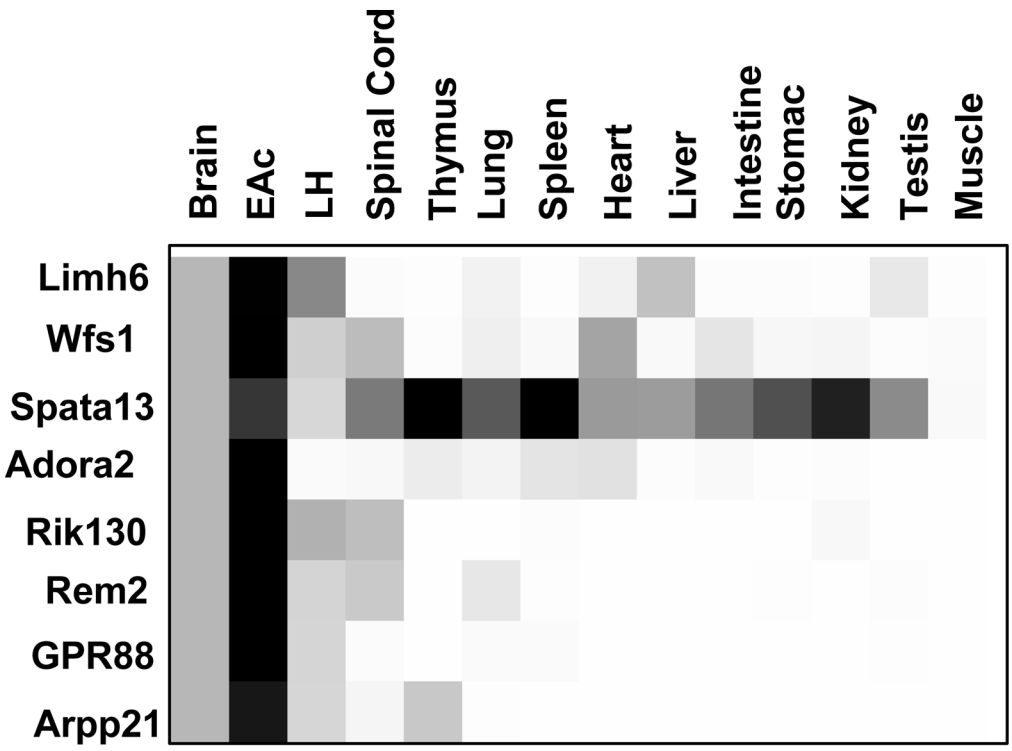

Figure 4. Expression of EAc-enriched genes in the central nervous system and peripheral tissues by qPCR

Quantitative PCR reactions were performed in triplicate on 2 independent samples (3 mice pooled for EAc, 2 mice pooled for WB and individual n=2 mice all other tissues, see Methods for details) and data are expressed as a fold-change over WB considered as the reference sample. Each gene is represented by a single box row, with expression levels illustrated using a grey scale from white (low level) to black (high level). All the genes are mainly expressed in the central nervous system (CNS), with the exception of Spata-13 expressed in most tested tissues at levels similar or higher to CNS.

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Table 1

EAc-enriched genes

This table shows the list of 129 probe sets selected from the Affymetrix analysis, with high expression in EAc compared to whole brain (WB). Data are expressed as fold-change of EAc over WB, with corresponding p values from the MAS5.0 analysis (Affy p-value). A FDR of 2.5% was used for the initial selection of probe sets (see details in Methods). Student t-test was further performed to compare mean signals for EAc versus WB data and validate the selection. Probe sets whose expression was investigated in this study by in situ hybridization (ISH) on sagittal (S) or coronal (C) sections are indicated (see Figures 2, 3 and Supplemental Figure 1). Gene encoding a glial protein (Gli) or previously described in EAc using ISH analysis (Litt) have been identified from literature mining and are also indicated in the table. Twins indicate probe sets corresponding to the same gene.

Enriched EA markers

ProbeSet	RefSeq ID	Gene Name	Symbol	ISH	Fold Change EAc	Affy p-values	t-test EA vs WB	twins
1427300_at	NM_010713	LIM homeobox protein 8	Limh8	S	11.05	2E-05	0.006	
1450723_at	NM_021459	ISL1 transcription factor, LIM/ homeodomain (islet 1)	Isl1		6.73	2.7E-05	0.014	
1419411_at	NM_009312	tachykinin 2	Tac2	Litt	6.35	2E-05	0.084	
1432558_a_at	NM_013779	melanoma antigen, family L, 2	Magel2	S	4.84	2E-05	0.003	1417217_at
1431717_at	AK014386	RIKEN full-length enriched library, clone:3526401B18	Rik352		4.44	2E-05	0.221	
1441382_at	NM_001033360	Mus musculus G protein-coupled receptor 101 (Gpr101),	Gpr101		4.21	5.2E-05	0.041	
1422586_at	NM_021306	endothelin converting enzyme-like 1	Xce	Litt	4.21	2E-05	0.034	
1421978_at	NM_008078	glutamic acid decarboxylase 2	GAD65	Litt	3.94	4E-05	0.001	
1456781_at	AK043872	RIKEN full-length enriched library, clone:A830044L07	RikA83		3.70	4.6E-05	0.014	
1444693_at	NM_023116	Mus musculus calcium channel, voltage-dependent, beta 2 subunit (Cacnb2),	Cacnb2		3.64	6.8E-05	0.031	
1417997_at	NM_022414	neuroglobin	Ngb		3.59	2.7E-05	0.011	1417996_at
1439569_at	NM_010287	G protein-coupled receptor 83	GPR83	S	3.52	2E-05	0.026	1423415_at
1427509_at	AK122358	Mus musculus similar to mKIAA0734 protein (LOC381075), mRNA	KIAA073		3.50	2.3E-05	0.023	
1460668_at	NM_010253	galanin	galn	Litt	3.47	2E-05	0.042	
1420437_at	NM_008324	indoleamine-pyrrole 2,3 dioxygenase	Indo	Gli	3.45	1.3E-04	0.037	
1441429_at	NM_010572	insulin receptor substrate 4	Irs4	S	3.41	2E-05	0.025	
1427523_at	NM_011381	sine oculis-related homeobox 3 homolog (Drosophila)	Six3		3.39	1.1E-04	0.003	
1448411_at	NM_011716	Wolfram syndrome 1 homolog (human)	Wfs1	SC - Litt	3.38	2E-05	0.002	
1422860_at	NM_024435	RIKEN cDNA 5033428E16 gene	Nts	Litt	3.31	2E-05	0.068	
1425094_a_at	NM_008500	LIM homeobox protein 6	Limh6	SC	3.27	5.2E-05	0.012	
1446681_at	Mm.184283	Mus musculus transcribed sequences			3.24	3E-05	0.037	
1451280_at	NM_028755	Mus musculus cyclic AMP-regulated phosphoprotein, 21 (Arpp21)	Arpp21	SC	3.15	2E-05	0.013	
1457806_at	NM_001033420	dedicator of cyto-kinesis 1	Dock1		3.12	2E-05	0.007	
1419033_at	NM_001037750	Mus musculus transcribed sequence with weak similarity to protein pir:I58401	PirI584		3.12	2E-05	0.028	
1416266_at	NM_018863	prodynorphin	Pdyn	Litt	3.06	7.8E-05	0.006	
1440148_at	NM_199058	G protein-coupled receptor 6	GPR6		3.06	3E-05	0.070	
1417680_at	NM_145983	potassium voltage-gated channel, shaker-related subfamily, member 5	Kcna5	Gli	3.05	5.2E-05	0.018	
1425467_a_at	NM_011123	proteolipid protein (myelin)	Myelin	Gli	2.97	2E-05	0.059	
1433652_at	NM_183336	immunoglobulin superfamily, member 1	IGSF1	Litt	2.96	2E-05	0.007	
1454906_at	NM_011243	retinoic acid receptor, beta	Rarb	Litt	2.96	2E-05	0.067	
1440091_at	NP_034955	myeloid ecotropic viral integration site-related gene 1	Mrg1	S	2.96	2.1E-04	0.057	

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Enriched EA markers

ProbeSet	RefSeq ID	Gene Name	Symbol	ISH	Fold Change EAc	Affy p-values	t-test EA vs WB	twins
1455304_at	XM_146948	Mus musculus similar to Munc13-3 (LOC235480), mRNA	Munc13-3		2.94	2E-05	0.003	1437319_at
1435854_at	NM_153520	transmembrane protein 10	Tmem10		2.88	2E-05	0.014	
1433578_at	NM_173403	hypothetical protein E130304D01	E130	S	2.83	2E-05	0.002	
1427038_at	NM_001002927	preproenkephalin 1	PENK1	S - Litt	2.81	2E-05	0.003	
1455296_at	NM_001012765	adenylate cyclase 5	Adcy5	S	2.80	2E-05	0.009	
1423415_at	NM_010287	G protein-coupled receptor 83	GPR83	S	2.78	2E-05	0.019	1439569_at
1440570_at	AK045773	RIKEN full-length enriched library, clone:B230310I20	Rik230		2.77	2.3E-05	0.022	
1460587_at	AK131933	Gabrg1	Sox2		2.77	1.7E-04	0.039	
1433434_at	NM_178737	expressed sequence AW551984	AW55		2.77	2E-05	0.035	
1442561_at	NM_207010	MAM domain containing 1	MAM1		2.76	6.8E-05	0.026	
1427227_at	NM_010252	gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 1	GABA-A	Litt	2.75	4.6E-05	0.066	1460408_at
1443287_at	XM_917427	RIKEN full-length enriched library, clone:E330020H17	Rik3300		2.73	2E-05	0.001	
1436733_at	NM_178756	RIKEN cDNA E130309F12 gene	Rik130	SC	2.72	2E-05	0.063	1436734_at
1439618_at	NM_011866	phosphodiesterase 10A	Pde10A		2.72	5.2E-05	0.031	1432490_a_at
1423946_at	NM_145978	PDZ and LIM domain 2	PDLIM2	S	2.71	7.8E-05	0.051	
1445837_at	NM_023872	Mus musculus potassium voltage-gated channel, subfamily Q, member 5 (Kcnq5),	Kcnq5		2.70	2E-05	0.021	
1417217_at	NM_013779	melanoma antigen, family L, 2	Magel2	S	2.69	4.6E-05	0.019	1432558_a_at
1447640_s_at	NM_016768	Mus musculus pre B-cell leukemia transcription factor 3 (Pbx3),	Pbx3	S	2.69	2E-05	0.038	
1434394_at	NM_001024917	FLJ10680 protein	FLJ106	S	2.69	1.7E-04	0.019	
1454656_at	XM_147847	spermatogenesis associated 13	Spata13	SC	2.68	1E-04	0.003	
1441629_at	NM_028736	Mus musculus glutamate receptor interacting protein 1 (Grip1)	Grip1		2.65	3E-05	0.070	
1416783_at	NM_009311	tachykinin 1	Tac1	Litt	2.65	2E-05	0.050	
1460408_at	NM_010252	gamma-aminobutyric acid (GABA-A) receptor, subunit gamma 1	GABA-A		2.63	2E-05	0.043	1427227_at
1450975_at	NM_019431	calcium channel, voltage-dependent, gamma subunit 4	Cacng4		2.62	3E-05	0.595	
1436263_at	NM_008614	myelin-associated oligodendrocytic basic protein	Mobp	Gli	2.62	3E-05	0.001	
1434594_at	NM_177336	RIKEN cDNA B230373P09 gene	Dho6	S	2.61	3.5E-4	0.008	
1448590_at	NM_009933	procollagen, type VI, alpha 1	Col6a1	Gli	2.61	2E-05	0.038	
1416997_a_at	NM_010404	huntingtin-associated protein 1	Hap1	Litt	2.61	1.3E-04	0.091	
1448860_at	NM_080726	rad and gem related GTP binding protein 2	Rem2	SC	2.60	1.3E-04	0.051	
1417996_at	NM_022414	neuroglobin	Ngb	Litt	2.60	7.8E-05	0.050	1417997_at
1429906_at	AK032623	RIKEN full-length enriched library, clone:6430710A17	Rik643		2.59	4.6E-05	0.169	
1435343_at	XM_976471	dedicator of cytokinesis 10	Dock10	S	2.58	2E-05	0.005	
1436734_at	NM_178756	RIKEN cDNA E130309F12 gene	Rik130	SC	2.54	1.9E-04	0.002	1436733_at
1455629_at	NM_010076	dopamine receptor D1A	DRD1a	Litt	2.54	2.3E-05	0.004	1456051_at
1456051_at	NM_010076	dopamine receptor D1A	DRD1a	Litt	2.54	2.7E-05	0.042	1455629_at
1423171_at	NM_022427	G-protein coupled receptor 88	GPR88	SC	2.54	2E-05	0.157	
1450241_a_at	NM_010161	ecotropic viral integration site 2a	Evi2a	S	2.54	2E-05	0.006	
1455190_at	NM_010319	guanine nucleotide binding protein (G protein), gamma 7 subunit	Gng7	Litt	2.53	2E-05	0.023	
1428642_at	NM_029529	frc, fringe-like 1 (Drosophila)	Slc35d3	S	2.53	4.6E-05	0.049	
1440573_at	NM_021563	Mus musculus Erbb2 interacting protein (Erbb2ip), transcript variant 2, mRNA	Erbb2ip	S	2.53	3.5E-05	0.012	

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Enriched EA markers

ProbeSet	RefSeq ID	Gene Name	Symbol	ISH	Fold Change EAc	Affy p-values	t-test EA vs WB	twins
1445148_at	AK049014	RIKEN full-length enriched library, clone:C230091K16	RikC23		2.52	2E-05	0.078	
1447807_s_at	XM_126961	pleckstrin homology domain containing, family H (with MyTH4domain) member 1	Max-1	S	2.49	2E-05	0.021	
1448945_at	NM_026385	transmembrane 4 superfamily member 11	Tm4sf11	Gli	2.47	2E-05	0.039	
1429589_at	AK018118	RIKEN full-length enriched library, clone:6330404F12	Rik633		2.46	2E-05	0.035	
1427519_at	NM_009630	adenosine A2a receptor	Adora2	SC	2.45	2E-05	0.001	1460710_at
1433551_at	NM_173016	hypothetical protein 9430073I07	Vat-1	S	2.45	2E-05	0.023	
1418950_at	NM_010077	dopamine receptor 2	DRD2	Litt	2.44	5.2E-05	0.050	
1417804_at	NM_011242	RAS, guanyl releasing protein 2	CDC25L	SC	2.42	2E-05	0.006	
1442138_at	XM_488192	Mus musculus G protein-coupled receptor 62 (Gpr62)	Gpr62		2.42	2.3E-05	0.058	
1449863_a_at	NM_010056	distal-less homeobox 5	Dlx5	S	2.42	1.3E-04	0.004	
1437319_at	XM_146948	Mus musculus similar to Munc13-3 (LOC235480), mRNA	Munc13-3		2.41	2E-05	0.005	1455304_at
1449682_s_at	NM_023716	TUBULIN, BETA 5 homolog	Tubbh	S	2.39	2E-05	0.045	
1418472_at	NM_023113	aspartoacylase (aminoacylase) 2	Aspa	Gli	2.39	2E-05	0.029	
1436662_at	NM_021377	Mus musculus VPS10 domain receptor protein SORCS 1 (Sorcs1),	Sorcs1	S	2.38	3.5E-04	0.067	
1435463_s_at	NM_177390	Mus musculus myosin ID (Myo1d), mRNA	MyosinID		2.38	2.1E-04	0.050	
1457323_at	XM_905537	hypothetical protein LOC632191	LOC632191		2.37	1.3E-04	0.006	
1457984_at	NM_205769	Mus musculus transcribed sequence with strong similarity to protein sp:P00722	CRF	Litt	2.36	1E-04	0.014	
1451331_at	NM_144828	protein phosphatase 1, regulatory (inhibitor) subunit 1B	DARPP32	Litt	2.35	2E-05	0.013	
1425263_a_at	NM_001025245	myelin basic protein	Mbp	Gli	2.35	6.1E-04	0.166	
1435642_at	NM_178774	RIKEN cDNA 9630019K15 gene	Rik963	S	2.33	2E-05	0.017	
1428434_at	NM_028325	RIKEN cDNA 2810028A01 gene	Sizn	S	2.32	2E-05	0.006	
1418691_at	NM_011268	regulator of G-protein signaling 9	RGS9	Litt	2.31	2E-05	0.009	
1440803_x_at	NM_021382	tachykinin receptor 3	Tacr3	Litt	2.31	6.8E-05	0.005	
1434098_at	NM_183427	glycine receptor, alpha 2 subunit	Glra2	S	2.31	3E-05	0.064	
1417129_a_at	NM_010825	myeloid ecotropic viral integration site-related gene 1	Mrg1	S	2.30	2E-05	0.007	1457632_s_at
1436449_at	AK142834	Mus musculus transcribed sequences	Pcdhx		2.28	0.000147	0.010	
1417090_at	NM_009037	reticulocalbin	Rcn1	S	2.28	4.6E-05	0.002	
1423506_a_at	NM_010923	neuronatin	Nnat	S	2.28	2E-05	0.078	
1432490_a_at	NM_011866	phosphodiesterase 10A	Pde10A	S	2.27	1.8E-04	0.011	1439618_at
1417275_at	NM_010762	myelin and lymphocyte protein, T-cell differentiation protein	Mal	Gli	2.27	2E-05	0.013	
1460710_at	NM_009630	adenosine A2a receptor	Adora2	SC	2.24	2E-05	0.002	1427519_at
1448768_at	NM_010814	myelin oligodendrocyte glycoprotein	Mog	Gli	2.24	2E-05	0.005	
1437418_at	Mm.11171	Mus musculus transcribed sequence with weak similarity to protein ref:NP_061720.1	NP061720		2.24	2.4E-04	0.124	
1417673_at	NM_016719	growth factor receptor bound protein 14	Grb14	ppGANTase	S	2.23	8.9E-05	0.029
1440902_at	NM_029972	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5			2.23	2E-05	0.071	
1460219_at	NM_010758	myelin-associated glycoprotein	Mag	S	2.22	2E-05	0.008	
1422756_at	NM_009508	vesicular inhibitory amino acid transporter	Slc32a1	Litt	2.22	2E-05	0.006	
1449365_at	NM_053190	endothelial differentiation, sphingolipid G-protein-coupled receptor, 8	Edg8	Gli	2.21	4E-05	0.002	
1456174_x_at	NM_010884	N-myc downstream regulated 1	Ndrg1	S	2.20	2E-05	0.081	

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Enriched EA markers

ProbeSet	RefSeq ID	Gene Name	Symbol	ISH	Fold Change EAc	Affy p-values	t-test EA vs WB	twins
1457132_at	Mm.26805	Mus musculus transcribed sequences			2.20	2.7E-05	0.140	
1439506_at	XM_983842	Mus musculus gene model 98, (NCBI) (Gm98)	C11orf9		2.19	7.8E-05	0.004	
1457632_s_at	NM_010825	myeloid ecotropic viral integration site-related gene 1	Mrg1	S	2.19	2E-05	0.001	1417129_a_at
1425892_a_at	NM_010932	Prepronociceptin	Pnoc	Litt	2.18	2.3E-05	0.012	
1444345_at	AW123227	Mus musculus transcribed sequences			2.16	3E-04	0.359	
1440813_s_at	XM_621025	plexin B3	PLNX6	Gli	2.16	2E-05	0.020	
1416200_at	NM_133775	RIKEN cDNA 9230117N10 gene	Rik923	S	2.16	2.3E-05	0.023	
1434369_a_at	NM_009964	crystallin, alpha B	Crya2	Gli	2.15	2E-05	0.033	
1424468_s_at	NM_153537	RIKEN cDNA D330037A14 gene	Rik330	S	2.15	1.7E-04	0.042	
1416003_at	NM_008770	claudin 11	Cldn11	S	2.14	3E-05	0.008	
1435554_at	NM_172051	Transmembrane and coiled coil domains 3 (Tmcc3), mRNA	Tmcc3		2.13	2E-05	0.073	
1415975_at	NM_025821	calcium regulated heat stable protein 1	Chrsp-24		2.11	7.8E-05	0.012	
1434606_at	NM_010153	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	Erbb3	Gli	2.09	1E-04	0.021	
1449106_at	NM_008161	glutathione peroxidase 3	Gpx3		2.09	8.6E-04	0.142	
1426690_a_at	NM_011480	sterol regulatory element binding factor 1	Sebf1	Gli	2.06	1.1E-04	0.073	
1424843_a_at	NR_002840	growth arrest specific 5	Gas5	S	2.06	4.6E-05	0.008	
1426454_at	NM_007486	Rho, GDP dissociation inhibitor (GDI) beta	Arhgdib	S	2.05	6.2E-04	0.045	
1416303_at	NM_019980	LPS-induced TN factor	LITAF	S	2.04	2E-05	0.009	
1426545_at	NM_144812	trinucleotide repeat containing 6b	Tnrc6b	S	2.04	3.5E-04	0.832	

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Table 2

Top gene networks identified in Ingenuity Pathway analysis. Number of genes and corresponding top functions for which expression is higher in the Extended Amygdala as compared to the whole brain.

Top biological functions	Score	Focus genes	genes
Behavior, Digestive System Development and Function, Nervous System Development and Function	52	24	Adcy5, Adora2, Rik352, CRF, Dlx5, Drd1a, Drd2, Galn, Gpx3, Grb14 Grp1, Rik923, Kcnq5, Mrg1, Nts, Pdyn, Penk, Pnoc, PirI584, Sebf1,, Tac1, Tac2, Tacr3
Neurological Disease, Cell Morphology, Nervous System Development and Function	28	15	Akt, Cacnb2, Col6a1, Erbb3, Gad2, Gas5, Indo, Irs4, Isl1, Litaf, Mag, Mbp, Mog, Slc12A7, SlcA1
Cancer, Neurological Disease, Cell Death	17	10	Arpp21, Chrsp-24, Dock10, Glra2, MyosinID, Nnat, Rik330, Plnx6, Tubbh, Wfs1
Reproductive System Development and Function, Gene Expression, Amino Acid Metabolism	16	10	Cacng4, Dock1, Edg8, Gaba-A, Igsf1, Kcna5A, Mal, Pbx3, Pdlim2, Rgs9
Cell Death, Hepatic System Disease, Liver Necrosis/Cell Death	15	9	Cldn11, Gng7, Hap1, Limh6, Limh8, Ngb, Pde10A, Cdc25L, Six3
Cell Signaling, Molecular Transport, Vitamin and Mineral Metabolism	14	9	Arhgdib, Aspa, ppGantase5, GPR6, GPR83, Flj106, Ndrg1, Rcn1, Sorcs1