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Adrenoceptors in brain: Cellular gene expression and effects on astrocytic metabolism and $[Ca^{2+}]_i$

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ABSTRACT

Recent *in vivo* studies have established astrocytes as a major target for locus coeruleus activation (Bekar et al., 2008), renewing interest in cell culture studies on noradrenergic effects on astrocytes in primary cultures and calling for additional information about the expression of adrenoceptor subtypes on different types of brain cells. In the present communication, mRNA expression of α_1-, α_2- and β-adrenergic receptors and their subtypes was determined in freshly isolated, cell marker-defined populations of astrocytes, NG2-positive cells, microglia, endothelial cells, and Thy1-positive neurons (mainly glutamatergic projection neurons) in murine cerebral cortex. Immediately after dissection of frontal, parietal and occipital cortex of 10–12-week-old transgenic mice, which combined each cell-type marker with a specific fluorescent signal, the tissue was digested, triturated and centrifuged, yielding a solution of dissociated cells of all types, which were separated by fluorescence-activated cell sorting (FACS). mRNA expression in each cell fraction was determined by microarray analysis. α_{1A}-Receptors were unequivocally expressed in astrocytes and NG2-positive cells, but absent in other cell types, and α_{1B}-receptors were not expressed in any cell population. Among α_2-receptors only α_{2A}-receptors were expressed, unequivocally in astrocytes and NG-positive cells, tentatively in microglia and questionably in Thy1-positive neurons and endothelial cells. β_1-Receptors were unequivocally expressed in astrocytes, tentatively in microglia, and questionably in neurons and endothelial cells, whereas β_2-adrenergic receptors showed tentative expression in neurons and astrocytes and unequivocal expression in other cell types. This distribution was supported by immunochemical data and its relevance established by previous studies in well-differentiated primary cultures of mouse astrocytes, showing that stimulation of α_2-adrenoceptors increases glycogen formation and oxidative metabolism, the latter by a mechanism depending on intramitochondrial Ca^{2+}, whereas α_1-adrenoceptor stimulation enhances glutamate uptake, and β-adrenoceptor activation causes glycogenolysis and increased Na^+, K^+-ATPase activity. The Ca^{2+}- and cAMP-mediated association between energy-consuming and energy-yielding processes is emphasized.

1. Introduction

Procedures activating locus coeruleus *in vivo* have astrocytes as a major target, increasing astrocytic free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in cerebral cortex. This is an α-adrenergic response, as evidenced by the ability of phentolamine, a non-specific α-adrenergic antagonist (Saeed et al., 1982) to block the response (Bekar et al., 2008). Among the two subtypes of α-adrenergic receptors α_1-adrenergic receptors are mainly postjunctional, although presynaptic α_1-adrenoceptors have also been reported (Herold et al., 2005). α_2-Adrenergic receptors were originally supposed to be presynaptic (Langer, 1980), but it is now recognized that α_2-adrenergic receptors in cerebral cortex are mainly postjunctional, reflected by adrenergic drug effects after locus administration of reserpine (depleting adrenergic nerves of noradrenaline) of or coeruleus destruction (U'Prichard et al., 1979; Reynoldson et al., 1979; Cai et al., 1993; Andrews and Lavin, 2006).

Astrocytic α_1-receptors have been demonstrated in vibrodissociated astrocytes showing a phenylephrine-induced increase in $[Ca^{2+}]_i$, which was blocked by the α_1-specific antagonist prazosin (Thorlin et al., 1998). The presence of astrocytic α_2-adrenergic receptors has also been shown immunochemically in intact brain (Aoki et al., 1998; Milner et al., 1998; Glass et al., 2001), and it is reflected in primary cultures of astrocytes by α_2-adrenergic

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receptor binding, receptor expression, and signaling, including increase in $[Ca^{2+}]_i$ (reviewed by Hertz et al., 2004). However, neurons *in situ* also express postsynaptic α_2-adrenergic receptors (Modirrousta et al., 2004; Schambra et al., 2005; Wang et al., 2007), as well as α_1-adrenoceptors (Blume et al., 2002; Laorden et al., 2002), and cultured GABAergic cortical interneurons respond to the α_2-adrenergic agonist guanfacine (Hu et al., 2008).

The increase in astrocytic $[Ca^{2+}]_i$ observed by Bekar et al. (2008) was unaffected by the β-adrenergic antagonist propranolol, which does not indicate the absence of β-adrenergic signaling but reflects that β-adrenergic signaling generally occurs *via* cyclic AMP (cAMP) and protein kinase A (PKA), and thus is not linked to an increase in $[Ca^{2+}]_i$. It has previously been shown that β-adrenergic receptors on freshly isolated cells from cerebral cortex are more concentrated on astrocytes than on neurons (Morin et al., 1997), and that there is a huge increase of β-receptor immunoreactive profiles on astrocytes in layer 1 of the rat visual cortex between days 14 and 21 (Aoki, 1997). However, Milner et al. (2000) reported that in the rat dentate gyrus β-adrenergic receptors are primarily located on dendrites of granule cells and interneurons, although they were also found on astrocytes and a few presynaptic profiles. On the other hand Szot et al. (2007) concluded that 'even though adrenoceptors are found on astrocytes, the level of these binding sites appears to be much lower than that of neurons.' In the light of these differences of opinion more general and averaged semi-quantitative information would be desirable about expression of adrenergic receptors and their subtypes on neurons compared to astrocytes, not to mention other cell types.

Recently, dissociation procedures of freshly excised brain cortex (Lovatt et al., 2007) or forebrain (Cahoy et al., 2008) and subsequent fluorescence-activated cell sorting (FACS) of specifically labeled cell types have successfully been used to assess mRNA expression in astrocytes, neurons and oligodendrocytes. In the present study we have used the procedure of Lovatt et al. (2007), as described under 'Section 2', to separate Thy1-positive neurons and astrocytes in order to determine gene expression of α_1-, α_2- and β-adrenergic receptors and their subtypes. Using additional fluorescent labeling, receptor expression on microglia, endothelial cells and NG-positive cells was also determined. Since the method shows mRNA expression, not protein expression, and since microarray analysis not always provides identical results in replicates from different animals, the observed gene expression is interpreted in the light of morphological, mainly immunohistochemical literature data.

An observed preferential expression of several subtypes of adrenoreceptors to astrocytes raises the question of the functional implications of adrenoceptor stimulation in astrocytes (ignoring potential astrocytic heterogeneity [Kimelberg, 2004; Matyash and Kettenmann, 2009]). Very limited information is available *in vivo*, but previous experiments in cultured mouse astrocytes have indicated a profound influence on astrocytic energy metabolism, including glycogen synthesis, glycogenolysis and oxidative metabolism, as well as on several energy-requiring processes, including glutamate uptake and Na^+, K^+-ATPase activity. This information will be reviewed, but sufficient metabolic information is not available for the other cell types. The simultaneous stimulation of energy-requiring and energy-yielding reactions is important, because it supplements the classical concept of regulation of energy metabolism by the availability of ADP (Berg et al., 2002) with a mechanism independent of previous ATP degradation (McCormack and Denton, 1990; Rutter et al., 1996; Robb-Gaspers et al., 1998; Griffiths and Rutter, 2009). In the case of oxidative metabolism the stimulation is effectuated by an increase in $[Ca^{2+}]_i$ followed by an increase in intramitochondrial Ca^{2+}, and in the case of glycogenolysis by cAMP/protein kinase A. Since especially the expression of the α_2-adrenoceptor is critically dependent on the culturing method (Enkvist et al., 1996), a brief description of the culturing technique used and the characteristics of the cultures will be included in the 'Section 2'.

2. Methods

2.1. Cell isolation

2.1.1. Dissociation of cortex

Brain tissue was from 10 to 12-week-old FVB/NTg(GFAP GFP)14Mes/J, Tg(Cspg4-DsRed.T1)1Akik/J, B6.129P-*Cx3cr1*^{tm1Litt}/J, Tg(TIE2GFP)287Sato/J, or B6.Cg-Tg(Thy1-YFPH)2Jrs/J mice (The Jackson Laboratory, Bar Harbor, ME). These transgenic mice combine each cell-specific marker with a specific fluorescent signal, allowing fluorescence-activated sorting of specified cell fractions, although it should be emphasized that Thy1 is mainly a marker of large projection neurons rather than a general neuronal marker (Feng et al., 2000; Seki et al., 2002). The mice were anesthetized with pentobarbital (50 mg kg^{-1}, i.p.), perfused with cold Hanks buffer (Invitrogen, Carlsbad, CA), and decapitated. The brain was immediately removed to cold Hanks buffer containing glutamate receptor antagonists, 3 μM DNQX and 100 μM APV (Tocris, Ellisville, MO). The frontal, parietal and occipital cortex was dissected free of white matter, cut into small pieces, and digested with 8 U/ml papain (Worthington, Lakewood, NJ) in Ca^{2+}/Mg^{2+}-free PIPES/cysteine buffer, pH 7.4, for 1 h at 37 °C/5% CO_2. After one wash, the tissue was further digested with 40 U/ml DNase I (Sigma, St. Louis, MO) in Mg^{2+}-containing minimum essential medium (MEM) (Invitrogen) with 1% bovine serum albumin (BSA) (Invitrogen) for 15 min at 37 °C/5% CO_2. The tissue was then carefully triturated in cold MEM with 1% BSA, centrifuged over a 90% Percoll gradient (GE Healthcare, Piscataway, NJ) to collect all cells below and including the lipid layer, which then was further diluted five times (MEM with 1% BSA) and centrifuged to collect the pellet, which normally included five million viable cells per brain used. The cells were then re-suspended in cold MEM with 1% BSA and 4 μg ml^{-1} propidium iodide (PI) (Sigma) and immediately sorted by fluorescence-activated cell sorting (FACS).

2.1.2. FACS

Cells were sorted using either the BDFACSVantage Cell Sorting System (13 psi sheath pressure, Cell Quest software) or the BD FACSAria Cell Sorting System (35 psi sheath pressure, FACSDiva software; BD Biosciences, San José, CA). GFP/YFP, R-PE, and PI were all excited by a 488 nm laser, and emissions were collected by 530/30, 575/26, and 675/20 nm discrimination filters, respectively. The signals were manually compensated, and cells were sorted into cold MEM with 1% BSA.

2.1.3. RNA processing and microarray

After FACS, cells were immediately extracted for total RNA and DNase treatment using the RNAqueous Micro kit (Ambion, Austin, TX). RNA quantity was assessed using the NanoDrop-1000 (NanoDrop Technologies, Wilmington, DE), and RNA integrity was assessed using the 2100-Bioanalyzer (Agilent Technologies). For microarray, 20 ng of total RNA was amplified and labeled with biotin using the Ovation kit (NuGEN, San Carlos, CA) according to the manufacturer's instruction and hybridized to the Affymetrix (Santa Clara, CA) GeneChip Mouse Genome 430 2.0 Array.

2.1.4. Microarray data mining and statistical analysis

Microarray data were analyzed using the Arrayassist 5.0.0 software package (Stratagene, La Jolla, CA). We normalized the data using the MAS5 algorithm, followed by log2 transformation and filtering out any probe set that had only one present call across all samples or a maximum intensity value <100 across all samples, which reduced the initial data set of 45,101 probe sets to 19,369 probe sets. To assure reproducibility among independent biological replicates in the genomic data set, we compared the correlation coefficient within groups of sorted cells (19,369 probe sets). The degree of similarity across all samples was assessed by hierarchical clustering using Euclidean average distances. All statistical comparisons between groups were performed by the nonpaired parametric Student's *t* test using the Benjamini–Hochberg false discovery rate (FDR) correction algorithm of 5%.

Results for each cell-type was obtained in three biological replicates, i.e., in cells isolated from three different animals. If all three were positive for the gene in question the result is indicated in Tables 1–3 as present, present, present, if two of the three were positive by two times present and one time absent, if only one was positive by one time present and two times absent, and if all three were negative as absent, absent, absent. Three positive calls were interpreted as unequivocal presence, two positive calls as tentative presence, one positive call as questionable presence or absence, and three negative calls as absence. However, classification as absent does not necessarily exclude a minor expression, as in the case of β_3-adrenergic receptors, giving three absent calls in all cell fractions but shown by Summers et al. (1995) to be weakly expressed in rodent brain.

2.2. Primary cultures of mouse astrocytes

2.2.1. Preparation

Cultures of astrocytes were prepared as previously described (Hertz et al., 1982, 1998). The areas superficial to the lateral ventricles of the cerebral hemispheres