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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., *Cereb. Cortex* 18, 2789–2795), 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.

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Keywords

alpha_{2A} receptor; astrocytes; fluorescence-activated cell sorting; free cytosolic Ca²⁺ concentration; NG2-positive cells; oxidative metabolism

Introduction

Procedures activating locus coeruleus *in vivo* have astrocytes as a major target, increasing astrocytic free cytosolic Ca²⁺ concentration ([Ca²⁺]_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 α₁-adrenergic receptors are mainly postjunctional, although presynaptic α₁-adrenoceptors have also been reported (Herold et al., 2005). α₂-Adrenergic receptors were originally supposed to be presynaptic (Langer, 1980), but it is now recognized that α₂-adrenergic receptors in cerebral cortex are mainly postjunctional, reflected by adrenergic drug effects after locus administration of reserpine (depleting adrenergic nerves of noradrenaline) or of locus coeruleus destruction (U'Prichard et al., 1979; Reynoldson et al., 1979; Cai et al., 1993; Andrews and Lavin, 2006).

Astrocytic α₁-receptors have been demonstrated in vibrodissociated astrocytes showing a phenylephrine-induced increase in [Ca²⁺]_i, which was blocked by the α₁-specific antagonist prazosin (Thorlin et al., 1998). The presence of astrocytic α₂-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 α₂-adrenergic receptor binding, receptor expression, and signaling, including increase in [Ca²⁺]_i (reviewed by Hertz et al., 2004). However, neurons *in situ* also express postsynaptic α₂-adrenergic receptors (Modirrousta et al., 2004; Schambra et al., 2005; Wang et al., 2007), as well as α₁-adrenoceptors (Blume et al., 2002; Laorden et al., 2002), and cultured GABAergic cortical interneurons respond to the α₂-adrenergic agonist guanfacine (Hu et al., 2008).

The increase in astrocytic [Ca²⁺]_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 [Ca²⁺]_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 'Methods', to separate Thy1-positive neurons and astrocytes in order to determine gene expression of α₁-, α₂- 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 adrenoreceptor stimulation in astrocytes (ignoring potential astrocytic heterogeneity [Kimelberg, 2004; Maytash 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 $[\text{Ca}^{2+}]_i$ 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 -adrenoreceptor 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 'Methods' section.

Methods

Cell isolation

Dissociation of cortex—Brain tissue was from 10–12-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 $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PIPES/cysteine buffer, pH 7.4, for 1 h at $37^\circ\text{C}/5\%\text{CO}_2$. 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 $37^\circ\text{C}/5\%\text{CO}_2$. 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 $\mu\text{g ml}^{-1}$ propidium iodide (PI) (Sigma) and immediately sorted by fluorescence-activated cell sorting (FACS).

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 nm, 575/26 nm, and 675/20 nm discrimination filters, respectively. The signals were manually compensated, and cells were sorted into cold MEM with 1% BSA.

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.

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 3 were positive for the gene in question the result is indicated in Tables 1–3 as present, present, present, if 2 of the 3 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.

Primary cultures of mouse astrocytes

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 (neopallia) were carefully dissected out of the brains of newborn Swiss mice after removal of the meninges, cut into 1 mm cubes, vortexed and passed twice through Nitex nylon meshes (80 and 10 μ m pore sizes) to prepare a cell suspension in a slightly modified Dulbecco's medium (Hertz et al., 1982) with 20% horse serum. The cell suspension was introduced at low density (20 dishes per brain) directly into 35-mm Falcon Primaria tissue culture dishes for the metabolic studies or at twice this density onto very well rinsed (Chen and Hertz, 1999) glass coverslips (from Thomas Scientific Company) for studies of $[Ca^{2+}]_i$ and incubated in the slightly modified Dulbecco's medium with 20% serum at 37°C in a 95/5% (vol/vol) mixture of atmospheric air and CO₂. However, the cells grow equally well in conventional Dulbecco's medium. Due to the low seeding density oligodendrocytes do not develop, so that no subculturing is needed. The cultures were re-fed with fresh medium two times a week, and the serum concentration was decreased to 10% at the age of 7 days. The cells reached confluency after 2 weeks and were thereafter grown for another 1–2 weeks in the additional presence of 0.25 mM dibutyryl

cyclic AMP (dBcAMP), an agent which induces differentiation of the cultures (Meier et al., 1991; Schubert et al., 2000) without leading to the formation of reactive astrocytes (Wandosell et al., 1993). The rationale behind this treatment is that innervation of cerebral cortex by noradrenergic fibers from locus coeruleus is just beginning at birth (Foote et al 1983), i.e., the age when the tissue for culture preparation was harvested. Therefore the cells have not received noradrenergic signals, which may be important for their functional development and can be replaced by the addition of dBcAMP. Besides a pronounced change in morphology (Fig. 1), there are major biochemical and physiological changes. In the present context it is of special importance that α_2 -adrenoceptor expression is deficient in cells that have not received dBcAMP (Enkvist et al., 1986). Moreover, L-channels for Ca^{2+} , a characteristic of astrocytes *in vivo* (Thorlin et al., 1998) develop only after dBcAMP treatment (Hertz et al., 1989b).

Functional characteristics—Astrocytes constitute >95% of the cell population in these cultures, neurons and oligodendrocytes are absent, and the contamination with macrophages is ~3% (Hertz et al., 1982). The information the cultures have provided about uptake of glutamate and GABA, glutamate formation and degradation, energy metabolism, and K^+ and Ca^{2+} homeostasis (Hertz et al., 1998) is consistent with subsequent observations and conclusions by other authors for astrocytic functions in the brain *in vivo*. The development of a key astrocytic enzyme, glutamine synthetase parallels that in the murine brain (Hertz et al., 1978) and its activity reaches under optimum conditions a value 2–3 times higher than in the brain *in vivo* (Juurlink et al., 1981).

Results

Expression of adrenergic receptors in different cell types

α_1 -Adrenergic receptors—Only one of the subtypes of the α_1 -adrenergic receptor, the α_{1A} -adrenergic receptor was expressed in any of the cell types, and it was found only in astrocytes and NG2-positive cells (Table 1). No significant expression of α_1 -adrenergic receptors was reported by Cahoy et al. (2008) in their FACS-separated cellular fractions, perhaps due to their use of relatively young animals (at most 17-day-old). The expression of α_1 -adrenoceptors in astrocytes is consistent with the demonstration that $[\text{Ca}^{2+}]_i$ in Bergmann glia is increased by (nor)adrenaline or the α_1 -adrenergic agonist phenylephrine and that the increase is inhibited by the α_1 -specific antagonist prazosin (Kirichuk et al., 1996), which also inhibits stimulus-induced $[\text{Ca}^{2+}]_i$ increase in these cells (Kulik et al., 1999) and phenylephrine-induced $[\text{Ca}^{2+}]_i$ increase in vibrodissociated astrocytes (Thorlin et al., 1998). In contrast to these observations as well as our own, Papay et al. (2006) found no expression of α_{1A} -adrenergic receptors in astrocytes. Also in contrast to our findings, they described widespread neuronal expression of α_{1A} -adrenergic receptors in cerebral cortex *in situ*. However, this expression was mainly in GABAergic interneurons, and Thy1-positive glutamatergic projection neurons were studied in the present communication.

We used adult Tg(Cspg4-DsRed.T1)1Akik/J to isolate NG2-positive cells. The majority of NG2-positive cells (>95%) in these transgenic mice are pericytes (own observation), cells apposed to CNS capillaries and containing contractile proteins (Hughes and Chan-Ling, 2004; Nishiyama et al., 2009). In cerebellar slices, superfused noradrenaline causes pericyte-mediated capillary constriction (Peppiat et al., 2006), and cultured pericytes from the mouse cerebral microvessels show isometric contractions and increase in $[\text{Ca}^{2+}]_i$ in response to noradrenaline (Noshio et al., 2007). Although receptor subtype was not determined in these studies, expression of α_1 -adrenoceptor on pericytes might explain their presence in the cellular fraction identified by the presence of NG2. Of note, our observations are not relevant for other types of NG-positive brain cells (glial precursors and/or specific glial cell types (polydendrocytes; synantocytes), interacting directly with neurons, astrocytes and pericytes

(Schools et al., 2003; Nishiyama et al., 2009; Bergles et al., 2009; Wigley and Butt, 2009). However, Palay et al. (2006) observed pronounced expression of α_{1A} -adrenergic receptors on NG2-positive cells, which they regarded as oligodendrocyte precursors.

α_2 -Adrenergic receptors—Also among α_2 -adrenergic receptors only one subtype, the α_{2A} receptor was expressed, regardless of cell type. It was unequivocally expressed on astrocytes and NG2-positive cells (demonstrated in all 3 preparations), tentatively present on microglia (demonstrated in 2 out of 3 preparations), and absent or of questionable presence (demonstrated in 1 of the 3 preparations) on endothelial cells and Thy1-positive neurons (Table 2). The α_{2A} -adrenergic receptor was also the only α_2 -adrenergic receptor found by Cahoy et al. (2008) to be significantly expressed in astrocytes and neurons, and they described its virtual absence on oligodendrocytes. They found astrocytic expression to be 2–3 times higher than neuronal expression. It is important that neurons both in the studies by Cahoy et al. (2008) and by ourselves show less α_{2A} -adrenergic expression than astrocytes, since the criteria for the selection of neurons differed in the two studies (Thy1-positive cells in the present study *versus* cells remaining after other immunologically defined cell types had been removed in the study by Cahoy et al. (2008)). Our observations are consistent with the immunochemical demonstration of α_2 -adrenergic receptors on astrocytes in intact brain (Aoki et al., 1998; Glass et al., 2001). However, although little or no evidence was found for postsynaptic neuronal expression of α_2 -adrenoceptors, such receptors have not only been demonstrated on GABAergic interneurons (Modirrousta et al., 2004) but also been reported on glutamatergic pyramidal neurons in frontal cortex (Wang et al., 2007).

The possible α_{2A} expression in microglia is consistent with observations by Mori et al. (2002) of upregulation by clonidine, an α_{2A} -adrenergic agonist, of the gene encoding catecholamine-O-methyl transferase (COMT) in cultured microglial cells. The α_{2A} receptor expression on NG2-positive cells has to our knowledge not been described earlier, but these cells are targets for noradrenaline as indicated by their expression of both α_{1A} -adrenergic receptors and β_2 -adrenergic receptors. The same applies to cerebral endothelial cells, which also unequivocally express β_2 -adrenergic receptors (see below).

β -Adrenergic receptors—All cell types expressed β_2 -adrenergic receptors with relatively small differences, whereas β_1 -adrenergic receptors were unequivocally expressed only on astrocytes (where their expression was better established than that of β_2 -receptors) and microglia, of questionable presence or absence in endothelial cells and absent in Thy1-positive neurons (Table 3). These findings contrast those by Cahoy et al. (2008), who found almost equal expression of β_1 receptors on astrocytes and neurons and no significant expression of β_2 receptors. This discrepancy might on one hand be explained by our selection of Thy1-positive neurons (which might have reduced β_1 -adrenoceptor expression) and on the other hand by their use of not fully mature astrocytes (which might have reduced β_2 -adrenoceptor expression). The latter factor may be especially relevant for β -adrenergic receptors because of the 4–5-fold increase of β -receptor immunoreactive profiles on astrocytes in layer 1 of the rat visual cortex between days 14 and 21 (Aoki, 1997). The concept that a large fraction of β -adrenergic binding is astrocytic is consistent with binding experiments showing that β -adrenergic receptors on freshly isolated cells from cerebral cortex are more concentrated on astrocytes than on neurons (Morin et al., 1997).

On microglia β_2 -receptor expression was also better established than β_1 -receptor expression, consistent with the observation that cAMP levels were increased 10 times more strongly in cultured microglia by activation of a specific β_2 receptor agonist than by activation of a specific β_1 receptor (Mori et al., 2002). β -Adrenergic signaling has been demonstrated in a brain endothelial cell line (Smith and Drewes, 2006), and mRNA for β_2 -adrenoceptors in a pericytic cell line (Asashima et al., 2003).

β_3 -Adrenergic receptor expression has been demonstrated in brain but is more pronounced in hippocampus than in cerebral cortex, where its density only amounts to 3% of that in brown adipose tissue (Summers et al., 1995). Based on stimulatory effects of a β_3 -adrenergic agonist on glucose uptake in cultured cells from the chicken brain it appears to be confined to astrocytes (Hutchinson et al., 2009), and it has also been reported in mouse astrocytes (Catus et al., 2008).

Summary— α_{1A} , α_{2A} and β_1 receptors have a predominantly astrocytic localization, whereas β_2 receptors seem mainly to be expressed on neurons. Except for β -adrenoceptor expression NG2-positive cells are similar to astrocytes. Microglia express mainly α_{2A} -adrenergic receptors and both β_1 and β_2 receptors. The only adrenoceptor which with certainty was demonstrated on endothelial cells was the β_2 -receptor.

Effects of adrenoceptor stimulation on astrocyte metabolism

Glucose metabolism in astrocytes: energy and glutamate production—In order to understand the importance of adrenoceptor effects on glucose metabolism metabolic pathways for glucose must be briefly described. Glucose is normally the predominant substrate used by the brain for energy metabolism and, in addition, it is the sole source of transmitter glutamate. Astrocytes have rates of oxidative metabolism and activities of the enzymes involved that on a per volume basis are at least equal to those in neurons (Hertz et al., 2007; Lovatt et al., 2007; Zielke *et al.* 2007). Moreover, in contrast to neurons, they are able to use glucose to form glutamate, which can then be transferred to glutamatergic neurons and used as transmitter glutamate or to GABAergic neurons and used as a GABA precursor. As illustrated in Fig. 2, glucose, containing six carbon atoms (C), is initially converted to 2 molecules of the 3C compound pyruvate. In both astrocytes and neurons this occurs in a multi-step glycolytic process. In astrocytes, but not in neurons, this process can be diverted by initial formation of glycogen, which is then degraded by glycogenolysis, catalyzed by glycogen phosphorylase, bypassing the initial step in glucose degradation, and thereafter followed by similar downstream processes towards pyruvate as glycolysis. Such a 'glucose-glycogen shunt' is slightly less advantageous energetically than direct degradation of glucose. Anyhow glycolysis *per se* contributes only little to energy formation, but it does generate pyruvate, which yields large amounts of ATP upon oxidative metabolism, and pyruvate formation from glycogen has the advantage that it can occur very rapidly. This shunt is operating during brain activation (Swanson et al., 1992; Hertz et al., 2007; Dienel et al., 2007), including learning in day-old chicken, where glycogen is used for production of glutamate, following the pathway described below (Gibbs et al., 2008).

In both neurons and astrocytes, oxidative metabolism of pyruvate in the tricarboxylic acid (TCA) cycle is initiated by an oxidative decarboxylation by pyruvate dehydrogenase and condensation with coenzyme A to form acetyl coenzyme A (Acetyl CoA). The acetyl moiety (2C) is transferred to oxaloacetate from the TCA cycle (4C) to form citrate (6C). Through an intermediate step, citrate is decarboxylated to α -ketoglutarate (5C), which is further decarboxylated to succinate (4C). Through several steps oxaloacetate is re-generated from succinate and ready to condense with another molecule of acetyl CoA for continued production of energy, *but without net synthesis of any TCA cycle constituent* (Fig. 2).

Pyruvate can also be carboxylated by pyruvate carboxylase to give rise to a *new* molecule of oxaloacetate (Fig. 2), a process which occurs in astrocytes but not in neurons, which lack pyruvate carboxylase activity (Yu et al., 1983; Shank et al., 1985; Hertz and Zielke, 2004; Hertz et al., 2007). After condensation of oxaloacetate with acetyl CoA, the resulting citrate molecule can be converted to α -ketoglutarate. α -Ketoglutarate can leave the cycle to form glutamate, which is transferred to neurons in the glutamate-glutamine cycle (which is also used for the

return of astrocytically accumulated glutamate). An increase in *de novo* glutamate synthesis has been demonstrated in the chicken brain during the initial phase of learning (Gibbs et al., 2008). This is consistent with other evidence that increased brain activity is associated with enhanced pyruvate carboxylation (Öz et al. 2004, Serres et al. 2008). A high glutamate content in neurons is useful at times of increased glutamatergic activity, because vesicular filling with glutamate depends upon the cytosolic glutamate concentration (Wilson et al. 2005). During learning the increased brain glutamate content soon returns to normal, following glutamate exit from the TCA cycle and its oxidation predominantly in astrocytes (Yu et al. 1982, McKenna et al. 1996). These are major metabolic processes, as indicated by the conclusion that on the average about one third of all released transmitter glutamate has been recently synthesized from glucose, with the other two thirds representing recycled transmitter glutamate, to a large extent returned to neurons in the glutamate-glutamine cycle after initial accumulation in astrocytes (Hertz et al., 2007).

Adrenoceptor effects on glycogen synthesis and glycogenolysis—Stimulation of α_2 receptors leads to a rapid increase of glucose incorporation into glycogen, as required for the operation of the glucose-glycogen shunt during enhanced brain activity, whereas α_2 receptor inhibition leads to a decreased glycogen synthesis (Hertz et al., 2007; D. Hutchinson and M.E. Gibbs, personal communication). Noradrenaline also increases glycogenolysis in cultured astrocytes (Magistretti et al., 1983; Cummins et al., 1983), mainly by a β -adrenergic effect, although an α_2 -adrenergic effect also contributes to the enhanced glycogenolysis, and complete inhibition of noradrenaline-induced glycogenolysis requires administration of both β - and α_2 -adrenergic antagonists (Subbarao and Hertz, 1990). Brain slice experiments have identified the β -adrenergic subtype involved in the mouse as the β_1 -adrenoceptor (Quach et al., 1988), but in the chicken brain it is the β_2 -adrenoceptor (Gibbs et al., 2008). Since glycogenolysis in brain parenchyma is virtually restricted to astrocytes these observations confirm β -adrenoceptor-mediated glycogenolysis in non-cultured brain tissue. Thus, both glycogen synthesis and glycogenolysis are activated by adrenoceptor subtypes shown to be unequivocally expressed in astrocytes, with less evidence of neuronal expression (Fig. 2).

Adrenoceptor effects on pyruvate dehydrogenation—Stimulation by noradrenaline of pyruvate dehydrogenase activity, measured as rate of production of labeled CO_2 from $[1-^{14}\text{C}]$ pyruvate (Fig. 2), is an α_2 -adrenergic effect. This has been shown by the ability of noradrenaline and either clonidine (Chen and Hertz, 1999) or the highly α_2 -specific and potent drug dexmedetomidine (Chen et al., 2000), but not of phenylephrine or isoproterenol, a β_1 - and β_2 -adrenergic agonist, to increase $^{14}\text{CO}_2$ production (Fig. 3). The concentration dependence of the stimulation by dexmedetomidine is mimicked by a similar effect of dexmedetomidine on $[\text{Ca}^{2+}]_i$ (Fig. 4). The left, high-affinity portion of this Figure indicates an α_2 -adrenergic effect, which was almost completely inhibited by the α_2 -adrenergic antagonist yohimbine, whereas the right, low-affinity portion is mainly due to a stimulation of an imidazoline-preferring site (Chen et al., 2000). An increased $[\text{Ca}^{2+}]_i$ is not sufficient for the metabolic response, since phenylephrine also increases $[\text{Ca}^{2+}]_i$ in a subtype-specific manner (Fig. 5). However, removal of extracellular Ca^{2+} combined with the addition of 10 mM MgCl_2 , which prevents a concomitant increase in intramitochondrial Ca^{2+} , abolishes the response (Table 4). A maximum $[\text{Ca}^{2+}]_i$ response is not required for the stimulation, since full metabolic stimulation is reached at a noradrenaline concentration yielding a $[\text{Ca}^{2+}]_i$ of only twice the resting concentration of 100 nM (Fig. 6).

Adrenoceptor effects on α -ketoglutarate dehydrogenation— α -Ketoglutarate dehydrogenation (*via* succinyl coenzyme A [succinyl CoA] to succinate [Fig. 2]) was examined in a similar fashion as pyruvate dehydrogenation, albeit with the difference that α -ketoglutarate does not easily enter mitochondria, so that instead production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ glutamate,

an α -ketoglutarate precursor, was studied. Again, both noradrenaline and clonidine stimulated metabolism (Subbarao and Hertz, 1991). However, in addition phenylephrine had a stimulatory effect on $^{14}\text{CO}_2$ production, but this can probably be explained by stimulation of glutamate uptake by an α_1 -adrenergic effect (see below), which will increase the specific activity of the cellular pool of glutamate and thus also of its product, α -ketoglutarate.

Thus α_2 -adrenoceptors, shown to be unequivocally present in astrocytes, were of key importance for stimulation of both TCA cycle enzymes studied. However, there was the difference that while maximum effect on pyruvate dehydrogenation was reached at $<0.2 \mu\text{M}$ noradrenaline (Fig. 6), a >10 -fold higher concentration was needed for stimulation of α -ketoglutarate dehydrogenation (Subbarao and Hertz, 1991). This difference could suggest that lower concentrations of noradrenaline have little effect on α -ketoglutarate dehydrogenation, but facilitate its formation and thus might enhance glutamate formation from glucose. This may be of importance for the supply of glutamatergic neurons with transmitter glutamate during brain activation, including learning (Gibbs et al., 2008). Accordingly the α_{2A} -adrenergic agonist guanfacine, which alleviates attention deficit hyperactivity disorder (ADHD) by acting on frontal cortex to facilitate working memory (Levy, 2008) might owe part of its therapeutic effect to an action on astrocytes. This concept is supported by the recent demonstration that pharmacological inhibition of glial cells by the toxin fluorocitrate affects working memory (Wang et al., 2009). At low doses, working memory is improved by fluorocitrate, which could be due to reduction of astrocytic uptake of released transmitter glutamate (see below). At higher doses memory is impaired, perhaps reflecting interference with astrocytic glutamate production by an inhibition of the enzyme aconitase, needed for citrate metabolism. This inhibition is more potent in astrocytes than in neurons (Zielke et al., 2009), and therefore fluorocitrate at appropriate dosage prevents α -ketoglutarate production specifically in the astrocytic TCA cycle (Fig. 2).

Effects of adrenoceptor stimulation on energy-requiring processes

Glutamate uptake—Glutamate is avidly taken up by astrocytes both in primary cultures (Hertz et al., 1978) and in the brain *in vivo*, where most of the re-uptake of released transmitter glutamate occurs in astrocytes (Danbolt, 2001). Hansson and Rönnbäck (1991) showed that phenylephrine greatly increases the V_{max} for the uptake into cultured astrocytes, although with relatively low affinity. An α_1 -mediated uptake stimulation in cultured rat astrocytes was confirmed by Fahrigh (1993), who provided the additional information that the subtype stimulated was the α_{1B} receptor. This observation is not consistent with the present demonstration of gene expression of the α_{1A} but not the α_{1B} adrenoceptor in astrocytes from the mouse brain, but α_1 adrenoreceptors are known to show subtype variability between species (García-Sáinz et al., 1992). Since astrocytes account for most of the re-uptake of glutamate in the brain *in vivo* the observation that clearance of extracellular glutamate in the brain *in vivo* is stimulated by the α_1 -adrenergic agonist phenylephrine (Alexander et al., 1997) confirms the results obtained in tissue culture.

Na^+, K^+ -ATPase activity—The astrocytic Na^+, K^+ -ATPase has sufficiently low affinity for K^+ that an elevated extracellular K^+ concentration stimulates its activity (Grisar et al., 1979; Hajek et al., 1996). It is now accepted that the astrocytic Na^+, K^+ -ATPase plays a major role in clearance of activity-induced increase in extracellular K^+ concentration in the brain (Somjen et al., 2008). Na^+, K^+ -ATPase activity in astrocytes, and thus probably also K^+ uptake (and Na^+ extrusion), is enhanced by isoproterenol and perhaps also phenylephrine, but not by the α_2 -adrenergic agonist clonidine, indicating β - and possibly α_1 -adrenergic effects (Hajek et al., 1996). Both of these effects would be consistent with astrocytic receptor localization if the α_1 -adrenergic effect is exerted on α_{1A} -adrenoceptors.

Discussion

Noradrenaline co-ordinates energy-yield and energy-requirement

The stimulatory effects of noradrenergic receptor activation on both energy-requiring and energy-yielding processes (albeit often by activation of different subtypes of adrenoceptors) enable the energy-requiring processes to simultaneously stimulate oxidative metabolism and/or glycogenolysis (Fig. 7). Simultaneous effects on both energy use and energy production supplement the classical linkage between energy supply and demand, i.e., regulation of energy production by products of work such as ATP hydrolysis to ADP (Berg et al., 2002). In contrast to the classical mechanism they allow energy metabolism to be stimulated without preceding decreases in ATP. Stimulation of glycogenolysis matters, because brain glycogenolysis no longer is regarded as primarily an emergency function, but as an integral part of glucose breakdown *via* a substantial 'glucose-glycogen shunt' (Swanson et al., 1992; Dienel et al., 2007; Hertz et al., 2007). A noradrenaline-induced increase in mitochondrial Ca^{2+} has been observed in astrocytes (Simpson et al., 1998), but stimulation of oxidative metabolism by increased $[\text{Ca}^{2+}]_i$, followed by increased intramitochondrial Ca^{2+} , has mainly been studied in muscle and liver, where a direct stimulation was demonstrated of the mitochondrial dehydrogenases pyruvate dehydrogenase, isocitrate dehydrogenase (which stimulates degradation of isocitrate, an intermediate between citrate and α -ketoglutarate in the TCA cycle) and α -ketoglutarate dehydrogenase (McCormack and Denton, 1990; Rutter et al., 1996; Gaspers and Thomas, 2008; Griffith and Rutter, 2009). Fluxes through the metabolic steps catalyzed by two of these enzymes were shown above to be stimulated by noradrenaline, whereas flux catalyzed by the isocitrate dehydrogenase has not been studied in astrocytes, because no convenient substrate entering the mitochondria exists for this reaction. In addition to the stimulation of the 3 dehydrogenases a direct stimulation of oxidative phosphorylation has also been demonstrated (Robb-Gaspers et al., 1998; Gaspers and Thomas, 2008).

Spatial and temporal restriction of cellular Ca^{2+} signals

Both α_{2A} and α_1 -adrenergic stimulation raised $[\text{Ca}^{2+}]_i$, but only the former increased pyruvate dehydrogenation. These different patterns of Ca^{2+} signaling may result from a limited range of cytoplasmic Ca^{2+} diffusion from different locations towards mitochondria and temporal regulation of mitochondrial Ca^{2+} uptake (Thomas et al., 1996; Putney and Thomas, 2006). Both IP_3 - and ryanodine/cafeine-sensitive Ca^{2+} stores have been found in cultured astrocytes (Reyes and Parpura, 2009), and they might be differently located and differently affected by α_1 - and α_2 -adrenergic stimulation. Moreover, due to different kinetics for opening and inhibition of the mitochondrial uniporter for Ca^{2+} , the possibilities for mitochondrial Ca^{2+} uptake may vary with the kinetics of the Ca^{2+} signal (Putney and Thomas, 2006). Additional information about the effect of locus coeruleus stimulation on intracellular distribution of increases in $[\text{Ca}^{2+}]_i$ and its kinetics may in the future help clarifying different functions of elevated intracellular Ca^{2+} concentrations.

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Fig. 1. Phase contrast micrographs of 4-week-old primary cultures of astrocytes from neonatal mouse brain grown in the absence (upper row) or presence (lower row) of dBcAMP (0.25 mM) from the age of two weeks. Two different magnifications are shown, with bars in right lower corner indicating 200 μ m. From Hertz et al., 1982.

Fig. 2.

Glucose metabolism in astrocytes. Substrates and metabolites are shown in blue, reactions by black arrows, stimulation by noradrenergic agonists by stippled green arrows, and inhibition by fluorocitrate in red. For details of the metabolic pathways involved in energy metabolism in cytosol (glycolysis with or without participation of a glucose-glycogen shunt) and mitochondria (complete oxidative metabolism of glucose by acetyl CoA entry and uninterrupted circling in the TCA cycle, decarboxylating 2 carbon atoms per turn), and glutamate formation (by *de novo* formation of oxaloacetate and its condensation with acetyl CoA to eventually form the glutamate precursor α-ketoglutarate) and its transfer to neurons, see text.

Fig. 3. Effects of adrenergic agonists (10 μ M) on $^{14}\text{CO}_2$ production from [1- ^{14}C]pyruvate during a 30-min incubation in tissue culture medium, an indication of pyruvate dehydrogenase activity. Values are expressed as means $\pm$ SEM values relative to those under control conditions (no agonist) in the same experiment; the SEM value of the control indicates the variability between experiments; $n=6-15$ individual experiments. *Significantly different from control ($P<0.05$). From Chen and Hertz, 1999.

Fig. 4.

Effects of the α_2 -specific agonist dexmedetomidine on free cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (open squares) and pyruvate dehydrogenase (PDH) activity (filled squares), measured as in Fig. 3. Concentrations of $[\text{Ca}^{2+}]_i$ are absolute values (nM), measured by aid of Indo-1 fluorescence, and the PDH activity was measured as $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]$ pyruvate, as in Fig. 3. Results are means $\pm$ SEM values for 3–18 individual experiments. Only the high-affinity part of the Figure shows a pure α_2 -adrenergic effect. The 70 and 100 nM values are significantly different from control for both $[\text{Ca}^{2+}]_i$ and PDH activity ($P < 0.05$). From Chen et al., 2000.

Fluorescence ratio

Fig. 5.

Both the α_1 -adrenergic agonist phenylephrine and the α_2 -adrenergic agonist clonidine ($10 \mu\text{M}$) increase $[\text{Ca}^{2+}]_i$ as indicated by the increased 405/485 nm emission ratio for INDO-1.

Horizontal lines indicate drug administration. The responses are inhibited by, respectively, the α_1 -adrenergic antagonist phentolamine and the α_2 -adrenergic antagonist yohimbine ($10 \mu\text{M}$).

From Chen and Hertz, 1999.

Fig. 6.

Maximum PDH activity is reached at a noradrenaline concentration giving a relatively small increase in $[\text{Ca}^{2+}]_i$. Measurements as in previous Figures. $n=5-20$ individual experiments.

*Significantly different from control ($P < 0.05$). From Chen and Hertz, 1999.

Fig. 7.

Noradrenaline stimulates both energy-requiring and energy-yielding processes (indicated by red arrows), allowing a *direct* stimulation of energy-yielding processes simultaneously with increased use of energy. cAMP increase in astrocytes by β -adrenergic stimulation increases K⁺ uptake (and Na⁺ extrusion) as well as the activity of glycogen phosphorylase and thus glycogenolysis. Increase in [Ca²⁺]_i by α_1 -adrenergic stimulation increases glutamate uptake and a corresponding effect by α_2 -adrenergic stimulation leads to an increase in mitochondrial Ca²⁺ and thus stimulation of mitochondrial enzymes.

mRNA expression of α_1 -adrenoceptor subtypes in FACS-isolated cell fractions from 3 separate dissociates obtained from the adult murine brain, determined by microarray analysis of 20 ng of total RNA.

Table 1

Gene symbol	Receptor	Astrocyte	NG2 ⁺ cell	Microglia	Endothelial	Thy-1 ⁺ Neurons
Adra1a	$\alpha 1A$	Present Present Present	Present Present Present	Consistently absent	Consistently absent	Consistently absent
Adra1b	$\alpha 1B$	Consistently absent	Consistently absent	Consistently absent	Consistently absent	Consistently absent

The Table indicates Present calls and Absent calls. Three Present calls normally indicate that a gene is unequivocally present.

mRNA expression of α_2 -adrenoceptor subtypes in FACS-isolated cell fractions from 3 separate dissociates obtained from the adult murine brain, determined by microarray analysis of 20 ng of total RNA.

Table 2

Gene symbol	Receptor	Astrocyte	NG2 ⁺ cell	Microglia	Endothelial	Thy1 ⁺ Neuron
Adra2a	$\alpha 2A$	Present Present Present	Present Present Present	Present Present Absent	Present Maybe Absent	Absent Present Absent
Adra2b	$\alpha 2B$	Consistently absent	Consistently absent	Consistently absent	Consistently absent	Consistently absent
Adra2c	$\alpha 2C$	Consistently absent	Consistently absent	Consistently absent	Consistently absent	Consistently absent

The Table indicates Present call, Absent call and Maybe call. For a gene to be unequivocally present 3 Present calls are normally expected. Two Present calls have been interpreted as tentatively present, partly based on comparison with literature data, whereas a single Present call at most indicates questionable expression.

mRNA expression of β -adrenoceptor subtypes in FACS-isolated cell fractions from 3 separate dissociates obtained from the adult murine brain, determined by microarray analysis of 20 ng of total RNA.

Table 3

Gene symbol	Receptor	Astrocyte	NG2 ⁺ cell	Microglia	Endothelial	Thy1 ⁺ Neuron
Adra1	β 1	Present Present Present	Consistently absent	Present Present Maybe	Absent Present Absent	Consistently absent
Adra2	β 2	Absent Present Present	Present Present Present	Present Present Present	Present Present Present	Present Present Present
Adra3	β 3	Consistently absent	Consistently absent	Consistently absent	Consistently absent	Consistently absent

The Table indicates Present call, Absent call and Maybe call. For a gene to be unequivocally present 3 Present calls are normally expected. Two Present calls have been interpreted as tentatively present, partly based on comparison with literature data, whereas a single Present call at most indicates questionable expression.

Table 4

Effects of noradrenaline on rates of $^{14}\text{CO}_2$ production from 5 mM $[1-^{14}\text{C}]$ pyruvate during incubation in normal tissue culture medium and in an otherwise similar Ca^{2+} -free, Mg^{2+} -enriched medium.

Noradrenaline	Control	0 Ca^{2+} , 10 Mg^{2+}
0 μM	100 $\pm$ 6.8 (n=16) *	95.9 $\pm$ 8.1 (n=9)
0.3 μM	162 $\pm$ 12.8 (n=16)	91.2 $\pm$ 9.1 (n=9)
Stimulation, %	61.5 (P<0.0001)	-4.7 (NS)

* Rates are expressed as means $\pm$ SEM values relative to those under control conditions in the same experiment (incubation in conventional tissue culture medium in the absence of noradrenaline); the SEM value of the control indicates the variability between experiments.

From Chen and Hertz, 1999.