Alpha-2 Adrenergic Receptor Functional Coupling to G Proteins in Rat Brain During Postnatal Development

H. KEVIN HAPPE, DAVID B. BYLUND, and L. CHARLES MURRIN
Departments of Pharmacology (H.K.H., D.B.B., L.C.M.) and Internal Medicine Neurology Section (L.C.M.), University of Nebraska Medical Center, Omaha, Nebraska

Accepted for publication October 7, 1998 This paper is available online at http://www.jpet.org

ABSTRACT
During postnatal development, alpha-2 adrenergic receptors (A2AR) change in both density and distribution. In forebrain, receptor density increases about 4-fold over neonatal levels, reaching adult levels before postnatal day (P) 28, whereas in hindbrain, including cerebellum, there is a decrease in overall receptor density. We examined the coupling of A2AR to G proteins using agonist-stimulated [35S]GTPγS binding as a functional assay. In forebrain the A2AR agonist-stimulated [35S]GTPγS binding increases rapidly after P7, reaching its highest levels at P21 and then declining slightly to adult levels. This binding increases more slowly than receptor number, suggesting that the appearance of G proteins, rather than the A2AR, determines the developmental appearance of functional A2AR-G protein interactions in forebrain. Basal [35S]GTPγS binding and [35S]GTPγS binding stimulated by other neurotransmitter receptor systems (GABA-B, mu opiate, and muscarinic) increase with a time course similar to A2AR-stimulated [35S]GTPγS binding. In contrast, in hindbrain, A2AR-stimulated [35S]GTPγS binding decreases during postnatal development in parallel with the decrease in A2AR levels, whereas [35S]GTPγS binding stimulated by other neurotransmitter receptor systems increases in parallel with basal [35S]GTPγS binding. Functional receptor-G protein coupling in hindbrain appears to be dependent on the developmental appearance of G proteins for most neurotransmitter systems. However, for A2AR the decrease in receptor density is the overriding factor. These studies 1) demonstrate the functional measurement of A2AR-G protein coupling in native tissue for the first time, 2) demonstrate that A2AR are coupled to G proteins throughout postnatal development, and 3) describe developmental increases and decreases in functional A2AR in brain.

Alpha-2 adrenergic receptors (A2AR) are one of the three major classes of receptors for norepinephrine and epinephrine (Bylund, 1988; Bylund et al., 1994). They are widely distributed in the body and play important roles in a variety of physiological and pathological processes, including regulation of blood pressure, nociception, locomotion, and processing of stressful stimuli (Ruffolo et al., 1993, 1995). A2AR have been used to ameliorate withdrawal symptoms from opiates and alcohol, as anesthetic adjuvants in surgery, and may be of some benefit in treating cognitive deficits in the elderly (Ruffolo et al., 1995). These actions and uses point to the importance of understanding the role of A2AR in central nervous system (CNS) function.

A2AR are members of the G protein coupled receptor superfamily and appear to interact primarily with G_{i/o} proteins (Chabre et al., 1994; Limbird et al., 1995). When A2AR are stimulated, GDP is released from the G_{i/o} protein complex, allowing GTP to bind in its place. This leads to the dissociation of the α from the βγ subunits of the heterotrimeric G protein complex and the subsequent regulation of signal transduction systems within the cell. This activation of G proteins is one of the functional consequences of stimulation of A2AR and, as such, provides a measure of the functional activity of these receptors in tissue.

Relatively little is known about the development of central A2AR in general, and even less is known about their function during the developmental period. We examined A2AR functional activity during development using the [35S]GTPγS binding assay (Hilf et al., 1989; Sim et al., 1995). This approach has been used in the study of several receptors, including A2AR expressed in cultured cells (Tian et al., 1994; Gillison et al., 1997; Wise et al., 1997). We report here the functional linkage of A2AR to G proteins at birth and the subsequent alterations in the magnitude of this coupling during the first postnatal month. Increases in A2AR-agonist induced GTP-binding in forebrain generally parallel the increase in A2AR levels, but there are interesting discrepancies from this. In cerebellum and brainstem, the highest levels of

ABBRIRVEATIONS: A2AR, alpha-2 adrenergic receptors; CNS, central nervous system; carbachol, carbamyl choline; P, postnatal day.

1 Received for publication June 30, 1998.

1 This work was supported by National Institutes of Health Grant NS 38201. Part of this work has been reported previously in abstract form (Happe et al., 1998).
both receptors and functional receptor-G protein coupling are present at birth and decline in parallel to relatively low levels postnatally.

**Experimental Procedures**

**Materials.** \[^{35}\text{S}]\text{GTP}\gamma\text{S} (1000–1500 Ci/mmol) was obtained from New England Research Products (Boston, MA) and \[^{3}\text{H}]\text{RX 821002} (53 Ci/mmol) from Amersham Life Science (Arlington Heights, IL). Atropine sulfate, carbamyl choline (carbachol), epinephrine bitartrate, dithiothreitol, GTP\gamma\text{S}, and baclofen were purchased from Sigma Chemical Company (St. Louis, MO). UK 14,304 was a gift from Allergan Pharmaceuticals (Irvine, CA). Rauwolscine HCl and \[^{3}\text{H}]\text{RX 821002} \text{HCl were purchased from Research Biochemicals Inc. (Natick, MA). Methodone was purchased from Mallinckrodt (St. Louis, MO) and GDP was purchased from United States Biochemical Corp. (Cleveland, OH). All other chemicals were research grade.**

**Animals.** Adult female Sprague-Dawley rats, 185 to 250 g, (SASCO, Kingston, NY) were housed three to four per cage and fed ad libitum. Rat pups were bred in our colony. Litters were culled to nine pups and monitored for normal growth by body weight (Happe and Murrin, 1990). Brains were collected at P0 (day of birth), P7, P14, P21, and P28. All animal use procedures were in strict accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

**Membrane Preparation.** Adult female rats and rat pups at the designated ages were sacrificed by decapitation under halothane anesthesia, and brains were removed to ice and dissected into two regions (Fig. 1) designated forebrain and hindbrain (containing the cerebellum and brainstem). Tissue was homogenized in 20 volumes of ice-cold homogenization buffer (50 mM Tris-HCl, 3 mM MgCl\(_2\), and 1 mM EGTA, pH 7.4) and centrifuged at 48,000 g for 10 min. The pellet was resuspended in 20 volumes of buffer and centrifuged at 48,000 g for 10 min. The final pellet was resuspended in 20 volumes of incubation buffer (50 mM Tris-HCl, 3 mM MgCl\(_2\), 1 mM EGTA, and 100 mM NaCl, pH 7.4). For A2AR agonist-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding, we found that fresh membrane preparations were necessary to achieve detectable and consistent results and therefore all \[^{35}\text{S}]\text{GTP}\gamma\text{S}-binding assays reported here used fresh tissue.

Aliquots of resuspended membranes were stored at −80°C for receptor binding and protein assays. Protein levels were determined by the bicinchoninic acid method (Pierce Protein Detection System; Pierce, Rockford, IL; Smith et al., 1985).

**Agonist-Stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} Binding Assay.** The assay was performed according to Sim and colleagues (Sim et al., 1995) with minor modifications. Briefly, membrane preparations equivalent to 1 mg of wet weight (25–50 μg membrane protein) were incubated at 30°C for 1 h in incubation buffer (50 mM Tris-HCl, 3 mM MgCl\(_2\), 1 mM EGTA, 100 mM NaCl, 2 μM GDP, and 1 mM dithiothreitol, pH 7.4) containing 0.05 nM \[^{35}\text{S}]\text{GTP}\gamma\text{S}, in a volume of 1 ml. Nonspecific \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding was determined in the presence of 10 μM unlabeled GTP\gamma\text{S}. Agonists were used at a final concentration of 10 μM to determine receptor-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding. UK 14,304 was used as agonist for A2AR, baclofen for GABA-B receptors, methadone for mu opiate receptors and carbachol for muscarinic acetylcholine receptors. Basal binding was determined in the absence of agonist. In fresh tissue, agonist-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding increases linearly for at least 90 min (data not shown). The reaction was stopped by rapid filtration (Brandel Cell Harvester; Biomedical Research and Development, Gaithersburg, MD) through Whatman GF/B glass fiber filters (Whatman, Clifton, NJ), followed by three washes with 5 ml of ice-cold 50 mM Tris-HCl, pH 7.4. Filter disks were extracted overnight in Econo-Safe liquid scintillation cocktail (Research Products International Corp., Mount Prospect, IL), and bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency.

Agonist-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding data are expressed either as a percentage of basal binding ([stimulated − basal]/basal) \times 100, or as the total agonist-stimulated binding (stimulated − basal), expressed as femtomole bound per milligram of protein. For developmental studies, agonist-stimulated and basal \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding were determined for 7 to 11 animals for each age using triplicate samples. Total agonist-stimulated and basal \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding levels were transformed to \(B_{\text{max}}\) values using the formula \(B_{\text{max}} = (B × (K_d + [L])/[L]), \) where \(B\) is the measured binding, \(K_d\) is the

| Table 1: Membrane protein levels increase during postnatal development |
|-----------------------------|-----------------------------|
| Forebrain                  | Hindbrain                   |
| μg protein/mg wet wt.       |                             |
| P0                         | 27.6 ± 1.5                  | 25.8 ± 2.6               |
| P7                         | 37.1 ± 1.1                  | 39.6 ± 3.1               |
| P14                        | 45.4 ± 3.0                  | 40.5 ± 1.8               |
| P21                        | 46.6 ± 2.3                  | 44.1 ± 2.8               |
| P28                        | 47.9 ± 2.7                  | 45.8 ± 1.9               |
| Adult                      | 51.7 ± 2.3                  | 50.5 ± 3.4               |
affinity constant of [$^{35}$S]GTP$\gamma$S binding (see Table 2), and [L] is the [$^{35}$S]GTP$\gamma$S concentration (Bylund, 1980).

The affinity of [$^{35}$S]GTP$\gamma$S binding to G proteins was examined in hindbrain for P0 and in forebrain for P0, P21 and adult membrane preparations. Basal and 10 μM UK 14,304-stimulated binding of 50 pM [$^{35}$S]GTP$\gamma$S were determined with various concentrations of unlabelled GTP$\gamma$S (0–10 μM). Data were transformed and analyzed as saturation binding curves to estimate apparent $K_d$ values of agonist-stimulated GTP$\gamma$S binding (Prism; GraphPad, Inc., San Diego, CA). To compare $K_d$ values, the pK$_d$ values were subjected to one-way ANOVA with a Tukey-Kramer multiple comparison post test (InStat; GraphPad, Inc.). Differences were considered statistically significant when $p < 0.05$.

[$^{3}$H]RX 821002 Binding Assay. A2AR levels were measured in membrane preparations using [$^{3}$H]RX 821002 as ligand as described (O’Rourke et al., 1994) with minor modifications. Briefly, membrane preparations containing 100 μg of protein were incubated with 2.5 nM [$^{3}$H]RX 821002 in 0.3 mM MgCl$_2$, 0.1 mM EGTA, 5 mM Tris-HCl (residual from membrane preparation), and 50 mM sodium phosphate, pH 7.4, for 1 h at room temperature. The suspensions were then filtered through GF/B glass fiber filters and washed 3 times with 5 ml of ice-cold 50 mM Tris-HCl, pH 7.4. Radioactivity was determined by liquid scintillation spectrometry in Econo-Safe at 45% efficiency. Nonspecific binding was determined by addition of 10 μM rauwolscine. Binding levels are expressed as femtomoles of [$^{3}$H]RX 821002 (0.5 nM), and [L] is the [$^{3}$H]RX 821002 concentration.

For autoradiographic studies, 16-μm slide-mounted tissue sections were incubated with 2.5 nM [$^{3}$H]RX 821002 in 0.3 mM MgCl$_2$, 0.1 mM EGTA, and 50 mM sodium phosphate, pH 7.4, for 1 h at room temperature. Nonspecific binding was determined by addition of 10 μM rauwolscine. Sections were washed twice for 2 min in ice-cold 50 mM Tris-HCl, pH 7.4. Sections were apposed to tritium-sensitive film (HyperFilm-$^{3}$H; Amersham Corp) for 4 weeks. Films were developed in Kodak D19 for 5 min, Kodak indicator stop bath for 30 s, and Kodak Rapid Fixer for 4 min, all at 18°C.

**Results**

A2AR Development

Comparison of neonatal and adult CNS A2AR, using receptor autoradiography with the antagonist [$^{3}$H]RX 821002 (Fig. 1), demonstrates major changes in receptor distribution and density during postnatal development. In many brain regions, such as cortex, low levels of A2AR are present early in postnatal development and increase to much higher levels in the adult CNS. On the other hand, several brain regions, such as cerebellum and brainstem, have high levels of A2AR during the early postnatal period that decline to low levels in the adult. Forebrain receptor density undergoes a general increase, whereas hindbrain receptor density generally decreases during postnatal development. We therefore chose to study the forebrain and the hindbrain separately to examine the development of receptors and receptor-G protein coupling.

Membrane Receptor Binding. The developmental change in A2AR density was examined in forebrain and hindbrain membrane preparations throughout postnatal development. Consistent with the autoradiographic studies (Murrin et al., 1996), forebrain A2AR density increases and hindbrain A2AR density decreases with age (Fig. 2). The magnitude of change in forebrain receptor density from P0 to adult is dependent on expression of the data as either femtomole per milligram of protein (4-fold) or femtomole per milligram of wet weight (7-fold), as there is nearly a 2-fold increase from birth to adult in membrane protein content per milligram wet weight (Table 1). In forebrain there is a rapid increase in A2AR density from P0 to P14, when receptor density has essentially attained adult levels (Fig. 2A). In hindbrain there is a decrease in receptor density, falling to near adult levels by P14 (Fig. 2B).

[$^{35}$S]GTP$\gamma$S-Binding Assay

To determine whether the appearance of A2AR is indicative of the onset of receptor function, we used the A2AR agonist-stimulated binding of [$^{35}$S]GTP$\gamma$S to assess the functional coupling between receptors and G proteins. Agonist-stimulated binding of [$^{35}$S]GTP$\gamma$S has been used previously to demonstrate functional coupling of receptors to G proteins in both membrane preparations and in slide-mounted tissue sections (Sim et al., 1995; Traynor and Nahorski, 1995; Gillson et al., 1997). We examined this approach as a potential functional assay for A2AR in both adult and neonatal rat brain.

In our initial studies, we found that storage of tissue or membrane preparations at −80°C led to low and variable levels of A2AR agonist-stimulated [$^{35}$S]GTP$\gamma$S binding. It
has previously been reported that receptor coupling to G proteins, as determined by the exchange of bound GDP for \(^{35}\text{S}\)GTP\(_S\), can be altered by multiple freeze-thaw cycles of membrane preparations (Sim et al., 1995). It appears that the A2AR agonist-stimulated increase in \(^{35}\text{S}\)GTP\(_S\) binding is quite sensitive to freezing, as binding in preparations from previously frozen tissue was considerably reduced relative to fresh tissue. On the other hand, stimulation of binding by agonists for other receptors (GABA-B, muscarinic acetylcholine, and opiate receptors) was only slightly reduced in frozen tissue compared with fresh tissue (data not shown). Based on these results, fresh tissue was used throughout the present study.

The \(^{35}\text{S}\)GTP\(_S\) binding in membrane preparations from neonatal and adult rat forebrain is presented in Fig. 3. Basal \(^{35}\text{S}\)GTP\(_S\) binding is inhibited more than 90% by the addition of excess unlabeled GTP\(_S\) (1 \(\mu\)M). In all experiments agonists were used at 10 \(\mu\)M, which in preliminary studies gave maximum stimulation for each agonist. The A2AR agonist, UK 14,304, stimulates \(^{35}\text{S}\)GTP\(_S\) binding 24% over basal in P5 forebrain and 18% over basal in adults (Fig. 3). The UK 14,304-stimulated binding is blocked by the A2AR antagonist, RX 821002, which alone has no effect. We also found that the agonist epinephrine (10 \(\mu\)M) was as effective as UK 14,304 in stimulating \(^{35}\text{S}\)GTP\(_S\) binding (data not shown). Rauwolscine (10 \(\mu\)M), an A2AR antagonist without an imidazoline structure, was as effective as RX 821002 (10 \(\mu\)M) in blocking the stimulation produced by either agonist (data not shown). Binding characteristics in membrane preparations from P5 animals were qualitatively the same as found in adults, but the basal and agonist-stimulated \(^{35}\text{S}\)GTP\(_S\)-binding levels were lower. Stimulation of forebrain muscarinic cholinergic receptors with carbachol increases \(^{35}\text{S}\)GTP\(_S\) binding 26% in P5 animals and 22% in adults, an effect block by atropine (Fig. 3). As expected, both UK 14,304- and carbachol-stimulated \(^{35}\text{S}\)GTP\(_S\) binding are dependent on Mg\(^{2+}\) (Sim et al., 1995).

The affinity of GTP\(_S\)-binding to G proteins was determined in forebrain from P0, P21 and adult animals, and in hindbrain from P0 animals by isotopic dilution of \(^{35}\text{S}\)GTP\(_S\) with unlabeled GTP\(_S\) (10\(^{-10}\) to \(10^{-6}\) M; Fig 4). A one-site model was used throughout because a two-site model did not significantly improve the fit. No statistically significant differences in binding affinity are found among P0, P21, and adult forebrain and P0 hindbrain preparations for basal binding nor for A2AR agonist-stimulated GTP\(_S\) binding (Table 2). The A2AR agonist-stimulated GTP\(_S\) binding to G proteins has a higher affinity (\(K_d = 4.5\) nM) than basal GTP\(_S\)-binding (\(K_d = 21\) nM) at all ages and in both forebrain and hindbrain.

**Postnatal Development of \(^{35}\text{S}\)GTP\(_S\) Binding**

**Basal \(^{35}\text{S}\)GTP\(_S\) Binding.** Basal \(^{35}\text{S}\)GTP\(_S\) binding represents a large part of the total signal when examining agonist-stimulated binding at all ages. During postnatal development, basal \(^{35}\text{S}\)GTP\(_S\)-binding increases more than 3-fold in forebrain preparations and less than 1.5-fold in hindbrain preparations (Fig. 5). In the forebrain, basal \(^{35}\text{S}\)GTP\(_S\)-binding increases dramatically between P7 and P14, reaching adult levels at P14. On the other hand, hindbrain basal \(^{35}\text{S}\)GTP\(_S\)-binding remains low through P14 and then increases to adult levels by P21 (Fig. 5).

**A2AR-Stimulated \(^{35}\text{S}\)GTP\(_S\) Binding.** In forebrain membranes, A2AR-stimulated \(^{35}\text{S}\)GTP\(_S\)-binding increases nearly 4-fold from P0 to adult (Fig. 6A). The level of A2AR-stimulated \(^{35}\text{S}\)GTP\(_S\) binding is low but detectable at P0 and P7. Between P7 and P14, agonist-stimulated binding increases dramatically, reaching peak levels at P14 to P21, and then declines slightly to adult levels (Fig. 6A). The developmental pattern for A2AR-stimulated \(^{35}\text{S}\)GTP\(_S\)-binding is similar to the increase in A2AR determined by ligand binding and also parallels the increase in basal \(^{35}\text{S}\)GTP\(_S\)-binding. Comparison of Figs. 2A and 6A shows that the rise in receptor density precedes somewhat the increase in A2AR-stimulated \(^{35}\text{S}\)GTP\(_S\)-binding. This is particularly evident at P7 and indicates that receptor-G protein coupling lags receptor appearance early in postnatal development. The overall magnitude of developmental increases in A2AR density and levels of A2AR-stimulated \(^{35}\text{S}\)GTP\(_S\)-binding are similar, about 4-fold. The A2AR-stimulated \(^{35}\text{S}\)GTP\(_S\)-binding in adult membrane preparations is about 90% of the total signal when examining agonist-stimulated binding at all ages.
binding, expressed as percentage of basal binding, remains relatively constant throughout the postnatal period (Fig. 6B).

In hindbrain, A2AR-stimulated \([^{35}S]GTP\gamma S\) binding declines about 2-fold between P0 and P14 (Fig. 6C) and remains low into adulthood. The developmental pattern of A2AR-stimulated \([^{35}S]GTP\gamma S\) binding (Fig. 6C) parallels the decline in A2AR density (Fig. 2B), although the decline in agonist-stimulated \([^{35}S]GTP\gamma S\) binding levels (2-fold) is not as great as the decrease in receptor density (3-fold). When expressed as percentage of basal binding, A2AR-stimulated \([^{35}S]GTP\gamma S\) binding in hindbrain declines about 3-fold (Fig. 6B).

\([^{35}S]GTP\gamma S\) Binding Stimulated by Baclofen, Carbachol, and Methadone. For comparison with A2AR, we examined the development of functional receptor coupling to G proteins for the GABA-B, \(\mu\) opiate, and muscarinic cholinergic receptor systems, using the agonists baclofen, methadone, and carbachol, respectively. In forebrain, agonist-stimulated \([^{35}S]GTP\gamma S\) binding is similar at P0 for all three neurotransmitter systems (Fig. 7A). Baclofen-stimulated \([^{35}S]GTP\gamma S\) binding increases over 6-fold by P21, whereas methadone- and carbachol-stimulated binding each increase about 4-fold. In forebrain, the developmental pattern of agonist-stimulated binding of \([^{35}S]GTP\gamma S\) is remarkably similar for all three neurotransmitter systems (Fig. 7A). There is little or no change in agonist-stimulated \([^{35}S]GTP\gamma S\) binding between P0 and P7, but between P7 and P14 there is a dramatic increase for all three systems. After P14, binding levels increase slightly or stay the same through P21 and then decrease to adult levels. The developmental pattern of ba-
clofen-, methadone-, and carbachol-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding parallels both the increase in basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding (Fig. 5) and the increase in A2AR-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in the forebrain (Fig. 6A).

In hindbrain, baclofen-, methadone-, and carbachol-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding increase during postnatal development (Fig. 7B); the pattern of development is similar for each agonist. Between P0 and P7, agonist-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding remains at low levels and increases through P21. There are further increases in baclofen-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to adult levels after P28. The increase in methadone-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding is small after P21, whereas there is an apparent decrease in carbachol-stimulated binding (Fig. 7B). The increase in agonist-stimulated binding in hindbrain for these receptor systems differs from the A2AR-stimulated binding, which declines over the first 4 postnatal weeks. The major developmental increase in baclofen-, carbachol-, and methadone-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding occurs later in the hindbrain than in the forebrain (Fig 7). Basal $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding also increases later in the hindbrain relative to the forebrain (Fig. 5). The decline in A2AR-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding during this period of increased agonist-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding for other neurotransmitter systems underscores the unique nature of hindbrain A2AR development.

**Discussion**

Stimulation of A2AR reduces adenylyl cyclase activity and alters ion channel activity in the adult CNS. A2AR signal transduction is pertussis toxin sensitive, indicating coupling primarily through $G_i/o$ proteins (Chabre et al., 1994; Limbird et al., 1995). In this report we use agonist-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding to study the coupling of native A2AR to G proteins in the CNS. Agonist-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding has been used to examine receptor-G protein coupling for several neurotransmitter receptor systems (Hilf et al., 1989; Gierschik et al., 1991; Lazareno et al., 1993; Lorenzen et al., 1993; Traynor and Nahorski, 1995; Newman-Tancredi et al., 1997; Waeber and Moskowitz, 1997), including the A2AR system in transfected cells (Tian et al., 1994; Wise et al., 1997), where receptor density is greater than in the CNS, and in RINm5f cells (Gillison et al., 1997). The characteristics of A2AR agonist-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in the current study are in general agreement with results in cell culture (Tian et al., 1994). This is the first study demonstrating the utility of this method for native A2AR in the CNS.

The present study provides evidence that A2ARs are functionally coupled to G proteins in the rat CNS throughout postnatal development based on the presence of A2AR agonist-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in membrane preparations. Our results show that norepinephrine and epinephrine can mediate cellular responses through A2AR as early as day of birth, and that developmental changes in A2AR agonist-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding generally parallel changes in A2AR density and distribution.

We used tissue separated into forebrain and hindbrain for functional receptor-G protein coupling studies to allow comparison of brain regions of increasing and decreasing A2AR
density during postnatal development. In forebrain, the predominant developmental pattern is a postnatal increase in A2AR density in regions expressing significant receptor levels in the adult. There are also a few regions in the forebrain that transiently express relatively high levels of A2AR at birth, but have few A2ARs in the adult. Functional coupling of A2AR to G proteins parallels the increase in receptor number determined by ligand-binding studies. In contrast to forebrain, transient expression of A2AR is a predominant developmental pattern in the hindbrain. The greatest receptor density is found at birth, followed by a rapid loss of receptors early in the postnatal period. Based on the parallel decline of A2AR agonist-stimulated [35S]GTPγS binding and A2AR binding in hindbrain, it appears that receptors expressed early in development, even in regions expressing few receptors in the adult, are nonetheless functionally coupled to G proteins. These results demonstrate that transiently expressed A2AR are capable of mediating norepinephrine- and epinephrine-stimulated effects during early postnatal development and suggest that these receptors may play a role during development distinct from that found in adults.

The postnatal increase in A2AR density in forebrain regions likely represents the maturation of the receptor system for noradrenergic function in the adult CNS. The coupling of A2AR to G proteins, as measured by [35S]GTPγS binding, generally increases in parallel with receptor number, although there is a delay in the increase of agonist-stimulated [35S]GTPγS binding compared with the increase in receptor number. This is most apparent at P7, when receptor density has doubled from P0 values (Fig. 2A) whereas agonist-stimulated [35S]GTPγS binding has increased only 25% (Fig. 6A). One interpretation of this pattern is that there is a delay in regulation of receptor-G protein coupling. The developmental changes in GTP-mediated shifts in the affinity state for A2AR agonist binding reported in rat cerebral cortex (Nomura et al., 1984) also suggest that regulation of A2AR-G protein-coupling changes during postnatal development.

The temporal development of A2AR-stimulated [35S]GTPγS binding in forebrain is remarkably similar to the pattern of basal [35S]GTPγS binding. Especially notable is the rapid increase after P7. In addition, the forebrain development of G protein coupling to three other neurotransmitter receptors, GABA-B, opiate, and muscarinic cholinergic receptors, respectively. Agonist-stimulated [35S]GTPγS binding was determined in forebrain (A) and hindbrain (B) membrane preparations from 7 to 11 animals at each age. Data are expressed as picomoles per milligram protein and are mean ± S.E.M. of Bmax values determined as described in Experimental Procedures.

The postnatal increase in A2AR density in forebrain regions likely represents the maturation of the receptor system for noradrenergic function in the adult CNS. The coupling of A2AR to G proteins, as measured by [35S]GTPγS binding, generally increases in parallel with receptor number, although there is a delay in the increase of agonist-stimulated [35S]GTPγS binding compared with the increase in receptor number. This is most apparent at P7, when receptor density has doubled from P0 values (Fig. 2A) whereas agonist-stimulated [35S]GTPγS binding has increased only 25% (Fig. 6A). One interpretation of this pattern is that there is a delay in regulation of receptor-G protein coupling. The developmental changes in GTP-mediated shifts in the affinity state for A2AR agonist binding reported in rat cerebral cortex (Nomura et al., 1984) also suggest that regulation of A2AR-G protein-coupling changes during postnatal development.

The temporal development of A2AR-stimulated [35S]GTPγS binding in forebrain is remarkably similar to the pattern of basal [35S]GTPγS binding. Especially notable is the rapid increase after P7. In addition, the forebrain development of G protein coupling to three other neurotransmitter receptors, GABA-B, opiate, and muscarinic cholinergic, also increases after P7. As opposed to basal and agonist-stimulated [35S]GTPγS binding, mu opiate, and muscarinic cholinergic receptor density increases extensively between P0 and P7 (Clendeninn et al., 1976; Coyle and Pert, 1976; Kuhar et al., 1980; Miyoshi et al., 1987). To our knowledge, GABA-B receptor binding has not been examined during development.

In the hindbrain the decrease in A2AR-G protein coupling contrasts with the G protein coupling to the three other neurotransmitter systems examined, all of which increase during postnatal development. The decline in A2AR density in hindbrain membranes, more than 3-fold, is greater than the 2-fold loss of agonist-stimulated [35S]GTPγS binding; this is consistent with the presence of spare receptors in the neonatal hindbrain. The difference could also be due to developmental changes in the expression of different A2AR subtypes and/or G protein subtypes in hindbrain. It remains to be determined whether A2AR loss is due to a loss of cells or whether receptor expression is turned off in cells that remain into adulthood.

The relationship of basal [35S]GTPγS binding to G protein levels is not clear. Basal binding likely represents [35S]GTPγS binding to a large number of different GTP-binding proteins. Nonetheless, pertussis toxin-sensitive Gi/o proteins comprise as much as 1% of total membrane protein in the CNS and represent the predominant subtype of GTP-binding proteins, at least in the adult brain (Sternweis and Robishaw, 1984; Harmouch et al., 1997). Given the quantity of Gi/o and the known favorable binding of [35S]GTPγS to Gi/o over Gs, it is likely that Gi/o is the major site of basal [35S]GTPγS binding. Basal [35S]GTPγS binding increases later in hindbrain than forebrain, as does binding stimulated by GABA-B, opiate, and muscarinic cholinergic agonists. The correspondence between basal [35S]GTPγS binding and agonist-stimulated [35S]GTPγS binding in both forebrain and in hindbrain suggests that the expression of G proteins may be rate-limiting for development of functional receptor-G protein coupling.

Although little is known about A2AR development or their role in regulating development, a transient perinatal expression of A2AR in some brain regions and a rapid postnatal increase in A2AR in other brain regions has been demon-
strated by receptor autoradiography (Murrin et al., 1996). Similar developmental patterns in mRNA are seen using in situ hybridization (Wang and Limbird, 1997; Winzer-Serhan and Leslie, 1997; Winzer-Serhan et al., 1997a,b). Our results are in general agreement with previous studies (Murrin et al., 1996; Winzer-Serhan and Leslie, 1997; Winzer-Serhan et al., 1997a,b) indicating both postnatal increases and perinatal transient expression of A2AR in different brain regions. Our studies, however, do not distinguish different A2AR subtypes or small brain regions because of technical limitations of the assay. The transient expression of receptors in cerebellum and brainstem may indicate a specific role in development. Recently it has been suggested that embryonic A2AR expression is associated with apoptotic events, based on the anatomic and temporal correlation between A2AR mRNA expression and markers of apoptosis (Wang and Limbird, 1997). Likewise, based on the temporal and regional pattern of A2AR mRNA expression, it has been suggested that the perinatal increase in receptor density may serve specific roles in development, including neuronal migration, maturation of neurons, and mediation of sensory functions (Winzer-Serhan and Leslie, 1997; Winzer-Serhan et al., 1997a,b). All of these hypotheses require further study.

Receptor-coupling efficiency reflects the ability of a receptor to stimulate $[^{35}S]GTP\gamma S$ binding to multiple G proteins upon agonist stimulation (Tian et al., 1994), and is estimated from the $B_{\text{max}}$ of agonist-stimulated $[^{35}S]GTP\gamma S$ binding and the $B_{\text{max}}$ of ligand-binding sites. Based on our data, A2AR coupling efficiency is 6 to 10 mol of $[^{35}S]GTP\gamma S$ bound/mol A2AR/h and does not change significantly with developmental age or between forebrain and hindbrain. The coupling efficiency of A2AR in our study is similar to results in PC-12 cells (Tian et al., 1994, 1996) but lower than studies in COS-7 cells (Wise et al., 1997) and RINm5F cells (Gillison et al., 1997). The discrepancies may be due, in part, to the expression of different levels and ratios of A2AR and G proteins in cultured and/or transfected cells as opposed to native tissue or to differences in G proteins expressed. Our coupling efficiency is also similar to that reported for several neurotransmitters systems (Hilf et al., 1989; Lazarenko et al., 1993; Lorenzen et al., 1993; Traynor and Nahorski, 1995; Newman-Tancredi et al., 1997; Sim et al., 1997a) and lower than reported for some more robust systems (Gierschik et al., 1991; Tian et al., 1996; Gillison et al., 1997; Sim et al., 1997a).

Recently, agonist-stimulated $[^{35}S]GTP\gamma S$ binding has been adapted for autoradiographic use on slide-mounted tissue sections (Sim et al., 1995, 1997a) and applied to several neurotransmitter receptor systems (Sim et al., 1996a,b, 1997b; Sim and Childers, 1997; Waeder and Moskowitz, 1997). Autoradiography provides more detailed regional analysis of agonist-stimulated $[^{35}S]GTP\gamma S$ binding than is possible with membrane preparations; however, we were unable to detect significant A2AR agonist stimulation of $[^{35}S]GTP\gamma S$ binding over basal levels in adult brain sections (data not shown). In contrast, with the agonists bacofox, methadone, and carbamol we obtained autoradiographic results (data not shown) similar to previously published studies for GABA-B, mu opioid, and muscarinic receptors (Sim et al., 1995; Waeder and Moskowitz, 1997). Receptor density and coupling efficiency can limit the detection of $[^{35}S]GTP\gamma S$ binding by autoradiography (Sim et al., 1997a). In this regard, A2ARs have similar densities to mu opioid (Clendeninn et al., 1976; Coyle and Pert, 1976) and muscarinic (Kuhar et al., 1980; Miyoshi et al., 1987) receptors in the CNS, and A2AR and muscarinic receptors have similar catalytic efficiencies. Therefore, neither of these factors provides a clear explanation for the inability to detect A2AR-stimulated $[^{35}S]GTP\gamma S$ binding autoradiographically.

The specific heterotrimeric G protein coupled to a receptor may affect autoradiographic determination of $[^{35}S]GTP\gamma S$ binding. For example, receptor coupling to $G_\alpha$ has not been detected by this method, probably due to the very slow dissociation of GDP from $G_\alpha$ (Sim et al., 1997a; Waeder and Moskowitz, 1997). Among receptors coupled to $G_{\alpha\alpha}$ proteins, serotonin 5-hydroxytryptamine1A and 5-hydroxytryptamine1B receptor-stimulated $[^{35}S]GTP\gamma S$ binding can be demonstrated, but 5-hydroxytryptamine1F receptor-stimulated $[^{35}S]GTP\gamma S$ binding can not (Waeder and Moskowitz, 1997). The difference may be due to receptor subtype coupling to particular $G_{\alpha\alpha}$ subtypes, with some pairings more amenable to autoradiographic analysis than others (Waeder and Moskowitz, 1997). This requires further study. In membrane preparations we found that A2AR-stimulated $[^{35}S]GTP\gamma S$ binding was very sensitive to freezing, to a much greater extent than found with other neurotransmitter receptor systems (data not shown). The use of frozen tissue sections for autoradiography also may contribute to the lack of success in obtaining A2AR-stimulated autoradiographic signal in the $[^{35}S]GTP\gamma S$-binding assay.

In summary, our data suggest that there may be important differences in the role of A2AR between the early postnatal period and adult rat brain. We have used agonist-stimulated $[^{35}S]GTP\gamma S$ binding to examine A2AR function in rat brain during postnatal development. Our data show that A2AR are functionally coupled to G proteins throughout postnatal development and, therefore, are able to mediate signal transduction upon stimulation by norpirenephrine and epinephrine. Functional A2AR in some brain regions are transiently expressed, suggesting a specific role in brain development. In many brain regions A2ARs are at low levels at birth, acquire adult receptor density by P14, and are functionally coupled to G proteins as soon as expression can be detected. Coupling of GABA-B, mu opiate, and muscarinic receptors to G proteins has also been demonstrated and these functions develop with a time course similar to A2AR coupling to G proteins and to the increase in basal $[^{35}S]GTP\gamma S$ binding. These data suggest that a similar mechanism is involved in the development of functional receptor-G protein interactions for several different neurotransmitters and that G protein expression may be a limiting factor in this process.

Acknowledgments

We thank Dr. Steven Childers for helpful discussions and for providing preprints of several articles.

References


