The presence of $\beta_2$-adrenoceptors sensitizes $\alpha_{2A}$-adrenoceptors to desensitization after chronic epinephrine treatment

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Abstract

Background: In addition to the regulation of blood pressure, $\alpha_2$- and $\beta$-adrenoceptor (AR) subtypes play an important role in the modulation of noradrenergic neurotransmission in the human CNS and PNS. Several studies suggest that the $\alpha_2$-AR responsiveness in cells and tissues after chronic epinephrine (EPI) or norepinephrine (NE) exposure may vary, depending on the $\beta$-AR activity present there. Recently, we reported that in BE(2)-C human neuroblastoma cells (endogenously expressing $\alpha_{2A}$- and $\beta_2$-AR), chronic EPI treatment (300 nM) produced a dramatic $\beta_2$-adrenoceptor-dependent desensitization of the $\alpha_{2A}$-AR response. The aim of this study is to determine if stable addition of a $\beta_2$-AR to a second neuroblastoma cell line (SH-SYSY), that normally expresses only $\alpha_{2A}$-ARs that are not sensitive to 300 nM EPI exposure, would suddenly render $\alpha_{2A}$-ARs in that cell line sensitive to treatment with the same EPI concentration.

Methods: These studies employed RT-PCR, receptor binding and inhibition of cAMP accumulation to confirm $\alpha_2$-AR subtype expression. Stable clones of SH-SYSY cells transfected to stably express functional $\beta_2$-ARs (SH$\beta_2$AR4) were selected to compare sensitivity of $\alpha_2$-AR to EPI in the presence or absence of $\beta_2$-ARs.

Results: A series of molecular, biochemical and pharmacological studies indicated that the difference between the cell lines could not be attributed to $\alpha_2$-AR heterogeneity. We now report that after transfection of functional $\beta_2$-AR into SH-SYSY cells (SH$\beta_2$AR4), chronic treatment with modest levels of EPI desensitizes the $\alpha_{2A}$-AR. This effect results from a $\beta_2$-AR dependent down-regulation of native $\alpha_{2A}$-ARs by EPI accompanied by enhanced translocation of GRK2 and GRK3 to the membrane (required for GRK-mediated phosphorylation of agonist-occupied receptors).

Conclusion: This study further supports the hypothesis that the presence of the $\beta$-AR renders the $\alpha_{2A}$-AR more susceptible to desensitization with physiological levels of EPI.
Background

Studying changes in α₂-adrenoceptor (AR) signaling is important for understanding the development and/or manifestation for several CNS (cerebral ischemia, pain, depression) and PNS disorders (hypertension and cardiac dysfunction). Under physiological conditions, norepinephrine and epinephrine (NE and EPI, respectively) activate the α₂-AR along with other members of the AR family, which also includes α₁- and β-ARs. The α₂- and β-ARs are often co-expressed on the same cell surface. Upon activation by NE and EPI, the independent signals initiated by the α₂- and β-ARs often converge to regulate specific physiological endpoints such as insulin release [1], maintenance of uterine smooth muscle tone [2], and noradrenergic transmission in the CNS and PNS [3,4]. The α₂- and β-ARs regulate many of these physiological mechanisms by mediating opposing actions on adenyl cyclase; α₂-AR inhibits while β-AR stimulates the adenyl cyclase pathway.

Continuous exposure to catecholamines leads to a declining receptor response, a phenomenon called desensitization. The process of desensitization generally includes receptor phosphorylation, internalization, and down-regulation. The process of desensitization generally includes receptor phosphorylation, internalization, and down-regulation. The waning α₂-AR signal is attributed primarily to down-regulation of the receptor and or phosphorylation of the agonist occupied receptor by G-protein coupled receptor kinases (GRK), specifically GRK2 and GRK3 [10,11]. Previous studies suggest that either of these two α₂A-AR desensitization mechanisms require supra-physiological (μM) concentrations of agonist [10,12-14].

However, our recent studies in the BE(2)-C human neuroblastoma cell line suggest that when β-ARs are present on the same cells lower, more physiologically relevant, concentrations of EPI (300 nM) are able to desensitize the α₂A-AR following chronic (24 hr) treatment [15]. In the absence of β-ARs, α₂A-AR desensitization occurs only with supra-physiological concentrations of EPI, if it occurs at all [15]. Concurrent activation of the β-AR and α₂A-AR also prompts down-regulation of cell surface α₂A-ARs while specifically up-regulating the expression of GRK3 within BE(2)-C cells [15]. Enhanced GRK3 expression plays a prominent role, as it is required for both β-AR-dependent α₂A-AR desensitization and down-regulation [15,16]. Recently we reported similar findings for the α₂B-AR subtype in mouse neuroblastoma cells [17-19]. Since both α₂A- and β-ARs are often co-localized and share the same endogenous ligands, it is reasonable to think that the α₂A-AR response is regulated differently in the presence and absence of the β-AR. Indeed, evidence suggests that the α₂A-AR responsiveness in cells and tissues after chronic EPI or NE vary, depending on the β-AR activity present there [2,15,20-23].

Results

Characterization of the model system and establishment of the SH/βAR4 cell line

Our first goal was to find a second model system that was similar to the BE(2)-C human neuroblastoma cell line (expressing modest levels of α₂A-AR), but that didn’t express β-ARs. Kazmi and Mishra previously identified the SH-SY5Y cell line as expressing two α₂AR binding sites [24], while Parsley et al. [25] reported that it expressed a single AR subtype, α₂C, based upon functional and molecular studies. Since receptor expression varies depending on differentiation state and passage number, it was necessary to determine which α₂A-AR subtypes were expressed in our population of SH-SY5Y cells, using a combination of binding, functional, and molecular approaches.

SH-SY5Y cells expressed α₂-AR levels slightly greater than the level detectable in BE(2)-C cells (Bₘₐₓ, SH-SY5Y, 67.6 ± 8.2; BE(2)-C, 40.8 ± 7.0 fmol/mg protein). According to nonlinear and linear regression analysis of saturation binding, the data best fit a single-site model in SH-SY5Y cells, as observed previously in BE(2)-C cells. Rauwolscine and yohimbine competed for specific [³H]rauwolscine binding to SH-SY5Y cell membranes with higher affinity than prazosin, the α₂B/C-selective antagonist (Table 1; [24]). Apparent Kᵢ values of agonists and antagonists against [³H]rauwolscine binding were determined for comparison with previously reported values in cells natively expressing α₂A-, α₂B, or α₂C-ARs (HT29 and BE(2)-C, NG108-15, OK, [15,26,27]) or cell lines expressing cloned α₁C10, α₁C2, and α₁C4 [28]. Values obtained from binding studies in SH-SY5Y cells correlated only to values from BE(2)-C cells and showed the greatest similarity with those derived from native and cloned α₂A-AR-containing cell membranes (Table 2). These results are
Consistent with binding of \[^{3}H\]rauwolscine to an \(\alpha_{2A}\)-AR in SH-SY5Y cells.

Functional studies were performed by measuring the ability of various \(\alpha_{2}\)-AR agonists to inhibit forskolin (10 \(\mu\)M)-stimulated cAMP accumulation in intact cells. All \(\alpha_{2}\)-AR agonists inhibited forskolin-stimulated cAMP accumulation in a concentration-dependent manner; no stimulation of cAMP accumulation was noted in the absence of forskolin. Inhibition of cAMP accumulation by the \(\alpha_{2A}\)-AR agonist UK14,304 (30 nM; Fig. 1) was completely reversed by 10 nM yohimbine, whereas the \(\alpha_{2B/C}\)-selective antagonist ARC-239, at a concentration over 30-fold higher than that of the agonist, failed to reverse the actions of UK14,304. Thus, both binding and functional studies support the classification of the \(\alpha_{2}\)-AR subtype in this neuroblastoma cell line as \(\alpha_{2A}\).

Since Parsley et al. [25] were unable to detect \(\alpha_{2A}\)-AR RNA by performing RT-PCR with total RNA extract, we optimized our chances for detecting \(\alpha_{2A}\)-AR RNA by generating RT-PCR products from SH-SY5Y mRNA using primer pairs selective for individual \(\alpha_{2}\)-AR subtypes (Table 3; [29,30]) or a primer pair that recognizes two \(\alpha_{2}\)-AR receptor subtypes distinguished by their restriction nuclease digestion products (Table 3; [30]). RT-PCR with \(\alpha_{2C}\)/C4 primers gave a 233 bp product specific for \(\alpha_{2}\)-\(\beta\)- and \(\alpha_{2C}\)-ARs; restriction digestion of this fragment with BglII, that would specifically cleave \(\alpha_{2A}\)-AR, resulted in two fragments of 117 bp and thereby established expression of \(\alpha_{2A}\)-AR mRNA in SH-SY5Y cells. RT-PCR with \(\alpha_{2C}\)/4 primers gave a 630 bp fragment, which was successfully digested by BstXI to produce three fragments of 271, 225, and 78 bp, consistent with the presence of an \(\alpha_{2C}\)-AR gene product (Fig. 2). RT-PCR products were neither noted in samples lacking reverse transcriptase (-), nor were they produced with primers selective for \(\alpha_{2C}\)/2 (\(\alpha_{2B}\)-AR; data not shown). While SH-SY5Y cells express mRNA for both \(\alpha_{2A}\) and \(\alpha_{2C}\)-ARs, it appears that the predominant functional \(\alpha_{2}\)-AR in our cell line is the \(\alpha_{2A}\)-AR.

Since these cells appear to express \(\alpha_{2A}\)-ARs with properties similar to those in BE(2)-C cells [15] but lack a \(\beta\)-AR, pcDNA 3.0 plasmid vector containing the human \(\beta\)-AR gene was transfected into SH-SY5Y cells. Colonies of stable transfectants were selected and maintained by their resistance to G418 (600 \(\mu\)g/mL) and subsequently clonal populations of \(\beta\)-AR-expressing SH-SY5Y cells (SH\(\beta\)/AR) were screened for \(\beta\)-AR expression using \[^{3}H\]CGP-12177 for binding studies as described in Methods. Since BE(2)-C cells express very low levels of \(\beta\)-AR (\(B_{max}: 18.5 \pm 6.2\) fmol/mg protein), the SH\(\beta\)/AR cell line that expressed 14.78 \pm 4.19 fmol/mg protein of the \(\beta\)-AR was selected for the subsequent studies. To ensure that the \(\beta\)-ARs were functional, the ability of isoproterenol (ISO) to stimulate Table 1: Pharmacological characteristics of adrenoceptors in SH-SY5Y and SH\(\beta\)/AR4 cells.

| Agonist | \(\log(K_{i})\) | \(\log(EC_{50})\) | \(\log(EC_{50})\) |
|---------|----------------|----------------|----------------|
| EPI     | -7.38 \(\pm\) 0.46 | -8.83 \(\pm\) 0.61 | -8.22 \(\pm\) 0.21 |
| UK 14,304 | -7.38 \(\pm\) 0.12 | -7.22 \(\pm\) 0.36 | -7.72 \(\pm\) 0.77 |
| Oxymetazoline (OXY) | -8.85 | -8.35 \(\pm\) 0.47 | N.D. |
| Isoproterenol | N.A. | N.A. | -7.02 \(\pm\) 0.28 |

Table 2: Correlation of SH-SY5Y cell \(\alpha_{2}\)-AR p\(K_{i}\) values with those of native and cloned \(\alpha_{2}\)-AR subtypes.

| Comparison | Reference | \# of Values Compared | Correlation Coefficient | Slope | p value |
|------------|-----------|----------------------|------------------------|-------|---------|
| v. HT29    | 21,22     | 6                    | 0.93                   | 1.48 \(\pm\) 0.41 | 0.07     |
| v. NG10B-15| 21        | 6                    | 0.13                   | 0.17 \(\pm\) 0.91 | 0.87     |
| v. OK      | 21,22     | 6                    | 0.62                   | 0.99 \(\pm\) 0.73 | 0.27     |
| v. \(\alpha_{2}\)/C10 | 23        | 6                    | 0.80                   | 1.04 \(\pm\) 0.45 | 0.10     |
| v. \(\alpha_{2}\)/C2 | 23        | 6                    | 0.40                   | 0.48 \(\pm\) 0.65 | 0.50     |
| v. \(\alpha_{2}\)/C4 | 23        | 6                    | 0.70                   | 0.94 \(\pm\) 0.52 | 0.16     |
| v. BE(2)-C | 6         | 6                    | 0.98*                  | 1.38 \(\pm\) 0.18 | 0.01     |

Correlation coefficient values \((r)\) were generated by comparing p\(K_{i}\) values from Table 1 with previously published values for one-site models using Pearson correlation analysis (GraphPad Prism). The slope of the linear regression line is also included. Correlations were considered significant \((*)\) if \(p \leq 0.05\).
cAMP accumulation was assessed (Table 1). The α_{2A}-AR responses were also tested in this new cell line to confirm that α_{2A}-AR function had not been altered by the expression of the β_{2}-AR (Table 1).

**Chronic 300 nM EPI exposure induces α_{2A}-AR desensitization only in SH-SY5Y cells transfected with functional β-AR**

To determine whether the presence of the β-AR influences α_{2A}-AR signaling, the ability UK14,304 to inhibit forskolin-stimulated cAMP accumulation was evaluated after wildtype (wt) and SHβ_{2}AR4 cells were exposed to vehicle or the indicated concentration of agonist for 16–24 hr. Wt SH-SY5Y cells (Fig 3A) require a 30-fold higher concentration of NE (30 μM) to desensitize the α_{2A}-AR signal than SHβ_{2}AR4 cells (1 μM; Fig 3B). Both the potency (-Log EC_{50} (M): 5.2 ± 0.1) and efficacy (I_{max} (%): 17.0 ± 1.6; P < 0.05 Fig. 3A) of UK14,304 were reduced by 30 μM NE compared to vehicle treatment in wt cells (-7.6 ± 0.2 M and 43.2 ± 6.8%); modest concentrations of NE (1 μM) and EPI (300 nM) are insufficient to alter the α_{2A}-AR signal in the wt SH-SY5Y cell line. In contrast, chronic treatment of the β-AR-expressing SHβ_{2}AR4 cells with 300 nM

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**Figure 1**
Reversal of the inhibitory effect of UK 14,304 on forskolin-stimulated cAMP accumulation. Yohimbine significantly antagonized the ability of UK14,304 (30 nM) to inhibit cAMP accumulation using an unpaired Student’s t-test (GraphPad Prism, San Diego, CA), while the α_{2B}-selective antagonist, ARC-239, had no effect. The results represent the mean ± S.E. of 2–9 experiments, performed in duplicate.

**Figure 2**
RT-PCR Products obtained from SH-SY5Y RNA using α_{2-AR} subtype selective primers. RT-PCR experiments were performed as described in “Methods” using primer pairs recognizing α_{2C10/C4} (corresponding to α_{2A} and α_{2C}) and α_{2C4} (corresponding to α_{2C}) gene products (Table 3). The reactions amplified fragments of the expected size from each set of primers. α_{2C10/C4} primers amplified 233 bp products from SH-SY5Y mRNA that were sensitive to digestion by BglII (specific for the α_{2A} product). Restriction digestion with BstXI of the 630 bp product of a C4 primer amplification gave three fragments of 271, 225 and 78 bp. All reactions were performed in the presence (+) or absence (-) of reverse transcriptase (RT) to rule out the possibility of DNA contamination. Lane M designates the 100 bp ladder; the 500 bp fragment is indicated by an arrow in each panel.
EPI desensitized the $\alpha_{2A}$-AR signal causing loss of UK14,304 potency ($-\log EC_{50}$ (M): Vehicle $6.9 \pm 0.2$; EPI $6.3 \pm 0.2$) and efficacy ($I_{\text{max}}$ (%): Vehicle $68.2 \pm 5.4$; EPI $49.3 \pm 10.4$; $p < 0.05$; Fig. 3B). Unlike EPI, which co-activates both ARs, NE, at the concentrations employed activates only $\alpha_{2A}$-ARs and does not alter $\alpha_{2A}$-AR signaling. We concluded that the difference in $\alpha_{2}$-AR signaling following EPI treatment between the transfected and wt SH-SY5Y was attributable to the presence of functional $\beta_{2}$-ARs, respectively. To ensure that the vector was not responsible for the observed difference between the wt and SH$\beta_{2}$AR4 cells, similar experiments were conducted in SH-SY5Y cells transfected with the vector alone (minus the $\beta_{2}$-AR gene). These vector only-expressing clones

| PCR Product | Primer: | Receptor | Expected size (bp) | Restriction Enzyme | Digestion Products (bp) |
|-------------|---------|----------|-------------------|-------------------|------------------------|
| $\alpha_{2A/C}$-AR | $\alpha_{2C10/C4}$ | $\alpha_{2A}$ | 233 | BglII | 117 (2) |
| $\alpha_{2C}$-AR | $\alpha_{2C}$ | $\alpha_{2C}$ | 233 | SacI | 153, 80 |
| $\alpha_{2C}$-AR | $\alpha_{2C}$ | $\alpha_{2C}$ | 630 | BstXI | 271,225,78 |

![Figure 3](http://www.biomedcentral.com/1471-2210/7/16)

**Figure 3**

Pretreatment with a modest concentration of EPI produces $\alpha_{2A}$-AR desensitization in SH-SY5Y cells only when the $\beta_{2}$-AR is present. Wild-type SH-SY5Y cells (A), cells expressing recombinant $\beta_{2}$-AR (SH$\beta_{2}$AR4, B and D), or SH-SY5Y cells expressing the vector alone (C) were pretreated 16–24 hr with any or all of the following: EPI (300 nM), NE (1 $\mu$M or 30 $\mu$M), EPI + Prop (30 nM), Prop (30 nM) alone or vehicle (0.1 mM ascorbate). Following pretreatment, the ability of UK14,304 to inhibit forskolin-stimulated cAMP accumulation was evaluated. A) Neither chronic EPI nor 1 $\mu$M NE pretreatments were sufficient to alter the $\alpha_{2A}$-AR signal ($n = 6$) in native SH-SY5Y cells. The $\alpha_{2A}$-AR signal in these cells desensitized only when exposed to higher agonist concentrations (30 $\mu$M NE, $n = 3$; 100 $\mu$M EPI, $n = 3$, data not shown). B) Unlike native SH-SY5Y cells, pretreatment with 300 nM EPI is sufficient to desensitize the $\alpha_{2A}$-AR signal in SH$\beta_{2}$AR4 cells ($n = 6$; $p < 0.05$). NE (1 $\mu$M), acting predominantly at $\alpha_{2A}$-AR with little affinity for the $\beta_{2}$-AR, does not produce $\alpha_{2A}$-AR desensitization. C) In SH-SY5Y cells transfected with the vector alone, neither EPI nor NE pretreatments altered $\alpha_{2A}$-AR signal ($n = 4$). D) Addition of propranolol (30 nM) prevents EPI-induced $\alpha_{2A}$-AR desensitization, suggesting a $\beta_{2}$-AR-dependent process (# $p < 0.05$ as compared to EPI treatment).
responded to EPI (300 nM) and NE (1 μM) pretreatments as the parent SH-SY5Y cells did (Fig. 3C).

To validate the importance of the β2-AR in the desensitization of the α2-AR signal, we included the β-AR selective antagonist propranolol (30 nM) with the chronic 300 nM EPI treatment. Addition of propranolol blocks EPI-induced α2A-AR desensitization resulting in UK14,304 concentration-response curves indistinguishable from control (-Log EC50 (M) for EPI + Prop 6.7 ± 0.1; Iₜₐₓ (%) for EPI + Prop 67.9 ± 0.4; p < 0.05; Fig. 3D). Propranolol treatment alone did not alter UK14,304 potency or efficacy.

β2-AR signal is desensitized following exposure to 300 nM EPI

To ensure that the β2-AR is functioning properly following catecholamine treatment, we evaluated the ability of ISO to stimulate cAMP accumulation over basal in SHβ2AR4 cells. The β2-AR signal is desensitized following chronic EPI but not NE treatment, consistent with the fact that NE has a low affinity for the β2-AR. Inclusion of propranolol (30 nM) inhibited EPI-induced β2-AR desensitization (p < 0.05; Fig. 4), but had no effect in the absence of EPI.

Chronic EPI induces down-regulation of the α2A-AR in SHβ2AR4, but not wt SH-SY5Y cells

Our study in BE(2)-C cells suggests that β2-AR-induced α2A-AR desensitization following long-term EPI exposure is due in part to down-regulation of the α2-ARs. To determine if the same mechanism is responsible for the EPI-induced α2A-AR desensitization in SHβ2AR4 cells, changes in α2A-AR expression following catecholamine treatment were evaluated. Specific binding was measured with a single concentration of radioligand. We, and others, have shown that this is sufficient for accurate assessment of changes in receptor number for the α2A-AR [9,15]. Chronic exposure of SHβ2AR4 cells to 300 nM EPI down-regulates the α2A-ARs by 20% (p < 0.05; Fig. 5). The α2A-AR down-regulation in this cell line, as in BE(2)-C cells, requires β2-AR co-activation since loss of α2A-ARs is prevented when 30 nM propranolol is included with EPI. Down-regulation of the α2A-AR is not observed following chronic activation of α2A-AR alone by 1 μM NE. Further, 300 nM EPI does not alter the expression of α2A-AR in wt SH-SY5Y cells as compared to vehicle-treated cells (% of vehicle: 88.6 ± 25.9; n = 2) consistent with a lack of α2A-AR desensitization. Hence, it can be concluded that chronic EPI treatment induces a loss of α2A-AR response via β2-AR-dependent down-regulation of α2A-ARs in SHβ2AR4, but not in wt SH-SY5Y cells.
Chronic EPI exposure does not alter GRK2 or GRK3 levels in whole cells but instead enhances GRK2 and GRK3 expression at the membrane in SHβ2AR4 cells

We previously established that EPI-induced α2A-AR desensitization and down-regulation in BE(2)-C cells is mediated via β2-AR-dependent GRK3 up-regulation [15]. Therefore, GRK3 levels in whole cell SHβ2AR4 lysates were evaluated following 24 hr catecholamine treatments. Chronic EPI exposure altered neither GRK3 nor GRK2 levels in the transfected SH-SY5Y cell line (Table 4). Therefore, unlike results in BE(2)-C cells, increases in whole cell GRK3 levels do not contribute to the modest α2A-AR desensitization or down-regulation observed in the SHβ2AR4 cells.

Although GRK3 levels in whole cell lysates remain unaltered in SHβ2AR4 cells, it is not known whether GRK3 recruitment to the membrane is regulated via chronic EPI treatment in that cell line. Since GRK2 and GRK3 have been shown to regulate α2A-AR signaling [10], we wanted to determine whether the membrane recruitment of either GRK isoform was changed following chronic EPI exposure in SHβ2AR4 cells. GRK2 and GRK3 are cytosolic proteins that anchor to the membrane via interaction with free Gβγ subunits; thus both kinases translocate from the cytosol to the membrane to regulate receptor signaling upon activation. Taking this characteristic of GRK2 and GRK3 into account, the levels of both kinases in membrane fractions following chronic EPI exposure were evaluated. SHβ2AR4 cells exhibit an increase in membrane-associated GRK2 and GRK3 with 24 hr EPI treatment compared to vehicle (P < 0.05; Fig. 6). In SHβ2AR4 cells, the same propranolol concentration (30 nM) that inhibited EPI-induced α2A-AR desensitization and down-regulation also attenuated EPI-induced increase in GRK2 and GRK3 content in the membrane fraction (P < 0.05; Fig. 6). In contrast, no increased translocation of GRKs by EPI treatment was observed in wt SH-SY5Y cells that do not express β2-ARs. Therefore, this increased GRK2 and GRK3 translocation to the membrane following prolonged EPI treatment in SHβ2AR4 cells is β2-AR dependent.

Discussion

The major finding of the present study is the confirmation (using a different approach) that sensitivity of α2A-AR to desensitization following exposure to relatively low levels of epinephrine is significantly increased in cells expressing both α2A- and β2-AR. The first evidence for this was recently reported in a human neuronal cell line endog...
enously expressing \( \alpha_{2A} \) and \( \beta_2 \)-ARs. Alpha \( \alpha_{2A} \) and \( \beta_2 \)-ARs in BE(2)-C cells desensitized after chronic EPI (300 nM), but not NE (1 \( \mu \)M), treatment [15]. Interestingly, the \( \alpha_{2A} \)-AR responsiveness in SH-SY5Y cells (an alternative human neuroblastoma cell line that does not express \( \beta_2 \)-ARs) is not desensitized after chronic treatment with 300 nM EPI or 1 \( \mu \)M NE (Fig. 3).

Obviously, the difference in \( \alpha_2 \)-AR sensitivity to lower concentrations of EPI could be due to several factors, including differences in the \( \alpha_2 \)-AR subtypes expressed in each cell line. Since it is difficult to demonstrate with great certainty what \( \alpha_2 \)-AR subtypes are present in a given cell or tissue by biochemical or pharmacological means only, we took a molecular approach to ascertain which subtypes might potentially be expressed based on the presence of mRNA encoding each subtype. SH-SY5Y cells contained mRNA for \( \alpha_{2A} \) and \( \alpha_{2C} \)-ARs (Fig. 2). As we noted and as reported by others [25], no evidence for \( \alpha_{2B} \) mRNA was found. This was further confirmed by Northern blot analysis (data not shown). Initially, total RNA isolated from SH-SY5Y cells did not produce the \( \alpha_{2A} \)-AR PCR products using the antisense primer selective for \( \alpha_{2A} \)-AR previously described [30]. Instead \( \alpha_{2A} \)-AR RT-PCR product was obtained only with poly(A) mRNA. However, employing poly(A)-enriched mRNA in the RT-PCR did not yield an \( \alpha_{2B} \)-AR RT-PCR product. Parsley et al. [25]) identified only \( \alpha_{2C} \)-AR mRNA using total RNA isolated from SH-SY5Y cells; this observation may reflect the limitation associated with the \( \alpha_{2A} \)-AR primers used for RT-PCR of total RNA, similar to what we encountered.

The rank order binding affinity of the various agonists and antagonists tested is in agreement with that previously reported in cells expressing recombinant [3,28,31] or native \( \alpha_{2A} \)-ARs [26,32]. When we compared apparent pKi values for various \( \alpha_2 \)-AR agonists and antagonists against binding to \([^3]H\)rauwolscine in SH-SY5Y membrane homogenates with previously reported values, we saw a correlation only with those cells that expressed \( \alpha_{2A} \)-ARs (Table 2). Another means of distinguishing between various \( \alpha_2 \)-AR subtypes involves comparing the prazosin/oxytocin and oxyhobine affinity ratios (Table 1; [4]). Prazosin/OXY (74.4) and OXY/yohimbine (1.95) ratios were within the range reported for native and recombinant \( \alpha_{2A} \)-ARs, and differ by at least 10-fold from values reported for \( \alpha_{2C} \)-AR (from [4]). The agonist potency series in SH-SY5Y cells also most closely parallels that reported for the \( \alpha_{2A} \) [26-28,32]. The inhibitory effect of \( \alpha_2 \)-AR agonists on cAMP production in SH-SY5Y cells is readily reversed in a concentration-dependent fashion by the antagonist yohimbine (Fig. 1); the failure of the selective \( \alpha_{2B} \) antagonist ARC-239 to antagonize UK 14,304 is consistent with activation of \( \alpha_{2A} \)-ARs in SH-SY5Y cells.

Therefore, our results strongly support the designation of the functional \( \alpha_2 \)-AR in SH-SY5Y cells as \( \alpha_{2A} \).

The present study supports our previous findings that pre-treatment with a modest EPI concentration readily desensitizes the \( \alpha_{2A} \)-AR signal in the presence, but not in the absence, of the \( \beta_2 \)-AR. This conclusion is based on several results. First, in wt SH-SY5Y cells (no \( \beta_2 \)-AR), the \( \alpha_{2A} \)-AR signal is not desensitized following 24 hr treatment with modest concentrations of EPI or NE (300 nM and 1 \( \mu \)M, respectively). Instead wt cells required chronic exposure to supra-physiological concentrations of catecholamines (30 \( \mu \)M NE and 100 \( \mu \)M EPI; data not shown) for desensitization of the \( \alpha_{2A} \)-AR signal; supporting the fact that \( \alpha_{2A} \)-ARs do not desensitize and/or down-regulate readily in response to low to moderate levels of EPI. Second, 300 nM EPI induces \( \alpha_{2A} \)-AR desensitization only in SH\( \beta_2 \)AR4 cells which express functional \( \beta_2 \)-AR. Finally, EPI-generated waning of the \( \alpha_{2A} \)-AR response is not observed in transfected cells expressing the pcDNA plasmid vector minus the \( \beta_2 \)-AR gene. This observation suggests that introduction of the \( \beta_2 \)-AR, and not the vector, is responsible for the difference in the \( \alpha_{2A} \)-AR signal between wt and SH\( \beta_2 \)AR4 cells exposed chronically to modest EPI concentrations.

As previously observed in BE(2)-C cells [15], desensitization of \( \alpha_{2A} \)-AR signal with 24 hr EPI exposure is due, in part, to down-regulation of the receptor in SH\( \beta_2 \)AR4 cells. Chronic co-activation of both \( \alpha_{2A} \) and \( \beta_2 \)-AR is required for desensitization and down-regulation of the \( \alpha_{2A} \)-AR in SH\( \beta_2 \)AR4 cells as indicated by the following results. First, 300 nM EPI, but not 1 \( \mu \)M NE, produces \( \alpha_{2A} \)-AR desensitization and down-regulation in the recombinant cell line. Lands et al. [33] established that EPI has equal affinity for \( \alpha_{2A} \) and \( \beta_2 \)-AR while NE has a higher affinity for the \( \alpha_{2} \)-AR than \( \beta_2 \)-AR; therefore, EPI activates both \( \alpha_{2A} \) and \( \beta_2 \)-ARs simultaneously while NE activates the \( \alpha_{2A} \)-AR alone. It is evident that the modest EPI concentration readily activates the \( \beta_2 \)-AR since chronic pretreatment with 300 nM EPI, but not 1 \( \mu \)M NE, desensitized the \( \beta_2 \)-AR response. Second, the inclusion of the \( \beta_2 \)-AR blocker propranolol prevented EPI-induced \( \alpha_{2A} \)-AR desensitization and down-regulation in \( \beta_2 \)-AR-transfected SH-SY5Y cells. This propranolol concentration (30 nM) is sufficient to prevent EPI activation of \( \beta_2 \)-AR as indicated by the inhibition of EPI-induced \( \beta_2 \)-AR desensitization.

Although chronic EPI treatment desensitized and down-regulated \( \alpha_{2A} \)-AR in both BE(2)-C and SH\( \beta_2 \)AR4 cells, several differences were observed. First, a more profound loss of efficacy is observed following 24 hr EPI exposure in BE(2)-C cells as compared to SH\( \beta_2 \)AR4 cells. The maximal inhibition of forskolin-stimulated cAMP accumulation by UK14,340 was reduced 54% in BE(2)-C, but only 27% in...
SHβ2-AR4, cells following EPI treatment (Fig. 3). The greater down-regulation of α2A-ARs observed in BE(2)-C versus SHβ2-AR4 cells most likely accounts for the greater change in efficacy: in SHβ2-AR4, chronic EPI treatment produces a 20% loss of α2A-ARs while in BE(2)-C cells, there is a 60% α2A-AR down-regulation (Fig. 5). This more profound α2A-AR desensitization and down-regulation observed in BE(2)-C is mediated via the up-regulation of GRK3 [15]. The lack of GRK3 up-regulation in the SHβ2-AR4 cells is the second major difference between the two cell lines. At present, it is unknown what prompts GRK3 up-regulation in BE(2)-C but not in the SHβ2-AR4 cells. However, we have observed that ERK1/2 activation is required for this induction of GRK3 following chronic exposure of BE(2)-C cells to EPI [16]. Moreover, while α2A-ARs do not readily activate this pathway in neuronal cells, and β-AR activation by ISO can activate ERK1/2 at high concentrations, we have observed that ERK1/2 activation by EPI at concentrations that up-regulate GRK3 appears to require the simultaneous activation of both α2A-ARs and β-ARs. Conversely, the inability of transfected β-ARs to prompt ERK1/2 activation in SH-SY5Y cells could explain the lack of GRK3 up-regulation in SHβ2-AR4 cells.

Even though total GRK3 levels are unaltered, GRKs play a role in β2-AR-regulated α2A-AR signaling in SHβ2-AR4 cells as indicated by several results. First chronic EPI treatment enhances localization of GRK2 and GRK3 to the membrane. As indicated previously, translocation of these two cytosolic kinases to the membrane is required for phosphorylation and subsequent desensitization of its receptor substrate, which in this study is the α2A-AR. Second, addition of propranolol attenuated EPI-mediated translocation of both GRK isoforms. This same propranolol concentration also inhibited α2A-AR desensitization and down-regulation as discussed above. Therefore, β2-AR co-activation with α2A-AR is required for enhanced GRK2 and GRK3 translocation to the membrane and subsequent α2A-AR desensitization and down-regulation.

Translocation of both GRK2 and GRK3 to the membrane following chronic EPI treatment in SHβ2-AR4 cells differs from the selective translocation of GRK3 (but not GRK2) observed in BE(2)-C cells following the same treatment (unpublished observations). The selective GRK3 up-regulation in BE(2)-C cells could account for the enhanced GRK3 levels at the membrane in these cells since in a previous study increase in total GRK2 levels promoted increased GRK2 expression at the membrane [34]. It is unknown at present why chronic EPI treatment translocates both GRK2 and GRK3 in SHβ2-AR4 cells, and not in BE(2)-C cells. A possible explanation for the difference in the GRK isoform translocation between the two cell lines is differences in the β subunit expressed and/or released upon EPI exposure. GRK2 and GRK3 require the βγ subunit of the G proteins to anchor to the membrane but GRK2 and GRK3 exhibit distinct binding preferences for individual β subunits [35]. The β3 isofrom preferentially binds GRK3 but not GRK2, whereas β1 and β2 bind equally to both GRK3 and GRK2 [35,36].

Conclusion
Based on results obtained in this series of experiments, we conclude that exposure to modest EPI concentrations readily desensitizes and down-regulates α2A-ARs in the presence, but not in the absence, of a functional β-AR. The β-AR-dependent down-regulation of α2A-ARs is modulated via GRKs. In BE(2)-C cells, chronic co-activation of β- and α2A-AR prompts enhanced expression of GRK3, but not GRK2, in whole cells [15] and membrane fractions. In contrast, EPI pretreatment of SH-SY5Y cells transfected with functional β2-ARs does not increase either GRK3 or GRK2 expression per se, but does increase translocation of GRK2 and GRK3 to the plasma membrane. Like α2A-AR desensitization and down-regulation, this translocation of GRK2 and GRK3 in SHβ2-AR4 cells is β-AR-dependent and thus presents an alternate mechanism for the regulation of the α2A-ARs by β-ARs.

Methods

Materials
The following drugs were purchased or obtained from the indicated sources: (-) epinephrine (EPI), (±)norepinephrine (NE), sodium ascorbate, UK14,304 (Sigma-Aldrich, St. Louis, MO); cell culture media (Gibco, Grand Island, NY); fetal bovine serum (Atlanta Biologicals, Norcross, GA); and antibiotics (Mediatech, Inc., Herndon, VA). GRK2 (C-15) and GRK3 (C-14) primary antibodies and horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Research Diagnostics, Inc., Flanders, NJ).

Cell culture
SH-SY5Y (passages 37–55) human neuroblastoma cells (Dr. Robert A. Ross, Fordham University, Bronx, NY) were maintained in a humidified atmosphere (6% CO2:94% air) in a 1:1 mixture of Eagle’s minimum essential medium with non-essential amino acids and Ham’s F-12 that contains 10% fetal bovine serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin sulfate. Plates of cells greater than 60% confluence were used throughout the study.

Transfection
Plasmid cDNA with the human β2-AR gene (provided by Dr. Brian Knoll; University of Houston, Houston, TX) or vector alone was stably transfected into SH-SY5Y cells with the fuGENE 6 Transfecting Reagent (Roche). Ten
positive clones were isolated by their resistance to 800 μg/ml of G418 and maintained in media containing 600 μg/ml of G418. SHβ2AR4 was selected for use in all experiments because it expressed similar levels of β2-ARs as that expressed natively in BE(2)-C cells; SHβ2AR4 expressed 14.78 ± 4.19 fmol/mg protein while BE(2)-C express 18.5 ± 6.2 fmol/mg protein [15]. This β2-AR level remained consistent to passage 12 in SHβ2AR4 cells. After passage 12, SHβ2AR4s neither expressed β2-ARs nor maintained resistance to G418, suggesting that the cells no longer expressed the transplanted plasmid.

**RNA isolation and RT-PCR**

Total RNA was isolated from several different passages of freshly harvested SH-SY5Y cells by the guanidinium isothiocyanate/phenol-chloroform extraction method [37]. Total RNA concentrations were determined by UV spectrophotometry; integrity of each isolate was determined by electrophoresis through a 1% agarose gel in the presence of 0.01 M sodium phosphate buffer. Poly(A) mRNA was isolated using a Dynabead oligo(dT)25 Kit (Dynal, Oslo, Norway) and was used for RT-PCR reactions. Each RT reaction (20 μL) contained 5–10 μg total or poly(A) RNA preincubated with 5 ng/μL oligo(dT)12-18, for 10 min at 70°C. The reaction mixture contained 80 μM each of deoxynucleotides (dATP, dCTP, dGTP and dTTP), RT buffer (50 mM KCl [containing 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and dTTP, 8 μM each RT reaction tube were diluted to a final volume of 50 μL with 10% DMSO, 80 μM each of dATP, dCTP, dGTP and dTTP, 8 μM each of the appropriate sense/antisense primer pair, 1.5 mM MgCl2, and magnesium free buffer [containing 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 50 mM KCl] in sterile distilled water. Reaction mixtures were overlaid with mineral oil and subjected to a hot start for 5 min at 95°C. DNA polymerase (2.5 U/μL, Promega, Madison, WI) was added to each reaction tube after the hot start, and the tubes were subjected to a PCR reaction of 30 cycles in a thermal cycler (MJ Research Inc., Watertown, MA) for 1 min at 94°C, 1.5 min at 55°C, and 2 min at 72°C with a final elongation step at 72°C for 7 min. Reaction products were separated by electrophoresis through 2% agarose gels and visualized by ethidium bromide staining. PCR products were isolated from the gel using a DNA extraction kit (Amicon Inc., Bedford, MA). Identity of the purified PCR products was confirmed by their susceptibility to digestion with restriction enzymes specific for each reaction product (see Table 1; [30]).

**cAMP accumulation**

To determine the effects of α2-AR agonists on forskolin-induced cAMP accumulation, intact cells were incubated for 5 minutes at 37°C in HBSS buffer (in mM): NaCl (137), KCl (5), Na2HPO4 (0.6), KH2PO4 (0.4), NaHCO3 (4), D-glucose (6), MgCl2 (0.5), MgSO4 (0.4) and CaCl2 (1), containing the phosphodiesterase inhibitor IBMX (0.5 mM). In some experiments, antagonists also were included in this step. To inhibit oxidation, sodium ascorbate (0.11 mM) was included when assaying catecholamines. Upon addition of forskolin (10 μM) and agonist, assay tubes were incubated for an additional 10 min at 37°C. Removing the tubes to a boiling water bath for 5 min terminated the assay. All assays were performed in duplicate in a total volume of 0.5 ml. After boiling, samples were centrifuged for 5 min at 14000 × g, and cAMP levels from the supernatant fractions were determined in a [3H]cAMP (0.8 pmol) binding assay as previously described [38]. β-AR-mediated stimulation of cAMP accumulation was performed in the same manner except that forskolin was not included in the assay mixture. Forskolin (10 μM) stimulated cAMP accumulation to 587 ± 88 pmol/mg protein (n = 46), 15-fold over basal levels (40.5 ± 2 pmol/mg protein).

**Receptor binding**

**Preparation of cell membranes**

Cells were homogenized in 20 volumes of Tris-HCl buffer (50 mM, pH 7.4) containing NaCl (100 mM), Na2 EDTA (1 mM) and PMSF (0.1 mM), and the membranes sedimented by centrifugation for 30 minutes at 34000 × g at 4°C. Pellets were resuspended in 0.32 M sucrose, and aliquots of the membrane fractions were stored frozen (-80°C) until use.

**Saturation experiments**

The level of α2A-ARs in SH-SY5Y cell membranes (0.5 mg/ml) was determined with various concentrations of [3H]rauwolscine (60–80 Ci/mmol, 0.3 – 12 nM) in a total volume of 1–2 ml in potassium phosphate buffer (50 mM, pH 7.4) containing MgSO4 (5 mM) at 37°C for 45 min. Thereafter, 2 ml Tris-HCl (5 mM, pH 7.4, 4°C) was added to the homogenate to terminate the binding reaction and the contents of the tubes was filtered over #32 glass fiber filter strips (Schleicher & Schuell, Keene, NH) using a PHD cell harvester (Cambridge Technology, Cambridge, MA). The reaction tubes and the filter strips were rinsed twice with a further 2–3 ml of buffer. Levels of radioactivity were determined by scintillation spectroscopy in
a Beckman LS6000 liquid scintillation counter. All assays were performed in triplicate, and specific binding was determined by subtracting the binding in the presence of yohimbine or phentolamine (10 μM; nonspecific) from the binding in its absence.

Previously we have shown that agonist treatments do not alter the Kd of the ligand for the β2-AR [15]. Therefore, levels of β2-ARs in SHβ2AR4 cell membranes (0.1 – 0.2 mg/mL) were determined using a single concentration (2 nM) of either [3H]rauwolscine or [3H]RX821002 following catecholamine treatment.

β2-AR binding was performed with [3H]CGP-12177. For saturation studies, cell membranes (0.5 mg/mL) were incubated with [3H]CGP-12177 (0.2 to 40 nM) in Tris-HCl buffer (50 mM, pH 7.5) containing MgCl2 (0.5 mM) at 37°C for 30 min. Specific binding was determined by subtracting the binding in its absence.

Competition experiments
Cell membrane fractions were incubated as described above except that the concentration of [3H]rauwolscine was fixed (1 nM), and various (4–9) concentrations of unlabeled drugs were included.

Immunoblotting
Membrane proteins were separated from cytosolic proteins by centrifugation, were resolved by SDS-PAGE through 10% gels and relative levels of GRK2 and GRK3 determined by immunoblotting as described previously [15]. Briefly, proteins were transferred to PVDF membrane, blocked with 5% nonfat dried milk in PBS containing 0.1% Tween (PBS/T) and incubated overnight at 4°C with dilutions of a rabbit polyclonal antibody directed against GRK2 (1:1000), GRK3 (1:1000), or both GRK2 and GRK3 (GRK2/3; 1:1000; wt SH-SY5Y). Blots were subjected to 4 washes before incubating for 60 min at room temperature with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000) in PBS/T. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL or Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The intensity of each immunoreactive band was determined using a NucleoImaging Workstation (Nucleotech Corp., San Carlos, CA), and normalized to the GAPDH loading control (1:5000).

Protein determination
Bovine serum albumin was used as a standard in the determination of protein levels in intact cells and cell membranes as described [39].

Data analysis
Kd, Bmax, IC50 and LogEC50 values were determined by nonlinear regression analysis using GraphPad Prism (GraphPad Software http://www.graphpad.com). The Kd values were calculated according to the Cheng-Prusoff equation [40] in which Kd = (IC50)/(1+S), where S = [concentration of radioligand]/[Kd of radioligand]. Comparisons between groups were made by two-way Student’s t-tests or ANOVA and Tukey’s or Dunnett’s post hoc test (where appropriate; GraphPad Software, San Diego, CA), and groups were considered significantly different if p ≤ 0.05.

Abbreviations
IBMX, 3-isobutyl-1-methylxanthine; HBSS, Hank’s balanced salt solution; UK 14,304, 5-Bromo-N-(4,5-dihydro-1H-imidazole-2-yl)-6-quinoxalamin; ARC-239, 2-(2,4-(O-methoxyphenyl)-piperazin-1-yl)-ethyl-4,4dimethyl-1,3-(2H,4H)-isoquinolindione, AR, adrenergic receptor; ISO, isoproterenol; EPI, epinephrine; NE, norepinephrine; wt, wild-type.

Authors’ contributions
TB-K participated in the design of the study, generated and selected stable SHβ2AR4-expressing clones, carried out all chronic treatment experiments, performed the statistical analyses and immunoblotting experiments, and drafted the manuscript. GFA carried out the binding and functional assays characterizing the α2-AR subtype. CDM conducted the molecular analysis studies. DCE participated in the conception and design of the study and helped draft the manuscript. LAS participated in the design and coordination of all experiments and helped draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements
This work was supported by grants from the Department of Health and Human Services (DA10738 and DA01738) and Texas Advanced Research Program (003652-014-1999 and 003652-0182-2001) to KMS and the American Heart Association, Texas Affiliate, Inc (Grant 0555032Y) to DCE. We gratefully acknowledge Dr. Brian Knoll (University of Houston, Houston, TX) for the gift of the plasmid cDNA with the human β2-AR gene and Ms. Vanessa Ramirez and Dr. Hibah Awwad for their excellent technical expertise.

References
1. John GW, Doxey JC, Walter DS, Reid JL: The role of alpha- and beta-adrenergic receptor subtypes in mediating the effects of catecholamines on fasting glucose and insulin concentrations in the rat. Br J Pharmacol 1990, 100(4):699-704.
2. Lecrivain JL, Mhaoût-Kodja S, Cohen-Tannoudji J, Maltier JP, Legrand C. In vivo stimulation of the beta(2)-adrenergic pathway increases expression of the Gi proteins and the alpha(2)A-adrenergic receptor genes in the pregnant rat myometrium. J Endocrinol 1998, 156(2):379-387.
13. Jewell-Motz EA, Donnelly ET, Eason MG, Liggett SB: An overview in alpha 2-adrenoceptor-subtype function. Trends Pharmacol Sci 1997, 18(3):121-129.

14. Hieble JP, Ruffolo, R.R. Jr, Starke, K.: An overview of alpha2-adrenergic receptors: structure, function and therapeutic implications. In Identification, characterization and subclassification of alpha2-adrenoceptors Edited by: Lanier SMILLE. Amsterdam , Harwood Academic Publishers; 1997:1-18.

15. MacMillan LB, Hein L, Smith MS, Piascik MT, Limbird LE: Central hypotensive effects of the alpha2a-adrenergic receptor subtype. Science 1996, 273(5276):801-803.

16. Kable JW, Murrin LC, Bylund DB: In vivo gene modification elucidates subtype-specific functions of alpha2-adrenergic receptors. J Pharmacol Exp Ther 2000, 293(1):1-7.

17. Jones SB, Halenda SP, Bylund DB: Alpha 2-adrenergic receptor stimulation of phospholipase A2 and of adenylate cyclase in transfected Chinese hamster ovary cells is mediated by different beta-adrenoceptor subtypes. J Pharmacol Exp Ther 1995, 278(2):239-245.

18. Jones SB, Leone SL, Bylund DB: Desensitization of the alpha-2-adrenergic receptor in HT29 and opossum kidney cell lines. J Pharmacol Exp Ther 1990, 254(1):294-300.

19. Eason MG, Liggett SB: Subtype-selective desensitization of alpha2-adrenoceptors. Different mechanisms control short and long term agonist-promoted desensitization of alpha2C10, alpha2C4, and alpha2C2. J Biol Chem 1992, 267(35):25473-25479.

20. Jewell-Motz EA, Liggett SB: G protein-coupled receptor kinase specificity for phosphorylation and desensitization of alpha2-adrenergic receptor subtypes. J Biol Chem 1996, 271(30):18082-18087.

21. Benovic JL, Regan JW, Matsui H, Mayor F Jr., Coteccchia S, Leeb-Lundberg LM, Caron MG, Lefkowitz RJ: Agonist-dependent phosphorylation of the alpha2-adrenergic receptor by the beta-adrenergic receptor kinase. J Biol Chem 1987, 262(36):17251-17253.

22. Heck DA, Bylund DB: Mechanism of down-regulation of alpha2-adrenergic receptor subtypes. J Pharmacol Exp Ther 1997, 282(3):1219-1227.

23. Jewell-Motz EA, Donnelly ET, Eason MG, Liggett SB: Role of the amino terminus of the third intracellular loop in agonist-promoted downregulation of the alpha2A-adrenergic receptor. Biochemistry 1997, 36(28):8058-8063.

24. Eason MG, Moreira SP, Liggett SB: Four consecutive serines in the third intracellular loop are the sites for beta-adrenergic receptor kinase-mediated phosphorylation and desensitization of the alpha2A-adrenergic receptor. J Biol Chem 1995, 270(19):4681-4686.

25. Bawa T, Atteneimi GF, Eikenburg DC, Standifer KM: Desensitization of alpha2A-adrenergic receptor signalling by modest levels of adrenaline is facilitated by beta 2-adrenergic receptor-dependent GRK3 up-regulation. Br J Pharmacol 2003, 138(5):921-931.

26. Salim S, Standifer KM, Eikenburg DC: Extracellular signal-regulated kinase 1 and 2-mediated transcriptional regulation of G-protein-coupled receptor kinase 3 expression in neuronal cells. J Pharmacol Exp Ther 2007, 321(1):51-59.

27. Desai AN, Standifer KM, Eikenburg DC: Cellular G protein-coupled receptor kinase levels regulate sensitivity of the (alpha)2b-adrenergic receptor to agonist-induced down-regulation. J Pharmacol Exp Ther 2005, 312(2):767-773.

28. Desai AN, Salim S, Standifer KM, Eikenburg DC: Involvement of G protein-coupled receptor kinase (GRK) 3 and GRK2 in down-regulation of the alpha2b-adrenergic receptor. J Pharmacol Exp Ther 2006, 317(3):1027-1035.

29. Desai AN, Standifer KM, Eikenburg DC: Simultaneous alpha2b- and beta2-adrenergic receptor activation sensitizes the alpha2b-adrenergic receptor for agonist-induced down-regulation. J Pharmacol Exp Ther 2004, 311(2):794-802.

30. Apparsundaram S, Eikenburg DC: Prejunctional alpha adrenoceptor desensitization in rat heart after chronic epinephrine treatment. J Pharmacol Exp Ther 1996, 278(2):862-870.

31. Schwartz DD, Eikenburg DC: Enhanced endogenous neurotransmitter overflow in the isolated perfused rat kidney after chronic epinephrine administration: lack of a prejunctional beta adrenoceptor influence. J Pharmacol Exp Ther 1988, 244(1):11-18.