CHAPTER 3

β2-ADRENOCEPTOR ACTIVATION RAPIDLY INDUCES THE PHOSPHORYLATION OF SYNAPTIC GLUCOCORTICOID RECEPTOR ON SERINE 154 IN THE RAT BASOLATERAL AMYGDALA

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Manuscript in preparation
ABSTRACT

Norepinephrine activity in the basolateral amygdala (BLA) interacts with glucocorticoid signaling to modulate the consolidation of memory of emotionally arousing experiences. There is growing evidence indicating that norepinephrine and glucocorticoids might synergistically and rapidly alter glutamate transmission within BLA synapses, suggesting that receptors for both hormones are located within the same synapses. In the present study, we investigated the ultrastructural distribution of the β2-adrenoceptor (β2-AR) and glucocorticoid receptor (GR) in the BLA by using immunofluorescence in combination with immuno-electron microscopy. Our findings indicate that both the GR and β2-AR colocalized preferentially with the vesicular glutamate transporter 2 (VGlut2), suggesting that the two receptors are located at glutamatergic synapses. Ultrastructural analysis of the distribution of each receptor confirmed that they were predominantly located in asymmetric excitatory synapses. Most importantly, we show the colocalization of the GR and β2-AR in spine heads, near postsynaptic densities in terminals and in dendritic shafts, suggesting that interactions between the two receptors might be involved in modulating different cellular and physiological processes. We further investigated a possible functional consequence of this colocalization and found that β2-AR activation by clenbuterol rapidly increases the phosphorylation of GR at serine 154, a ligand-independent phosphorylation site, possibly in a p38 mitogen-activated protein kinase (p38 MAPK)-dependent manner. We also showed that this clenbuterol activation provokes the formation of a molecular complex including β2-AR, p38 MAPK and GR within BLA synapses. These findings suggest that norepinephrine release and the activation of β2-ARs could rapidly set up the GR to prepare it for the coming surge of glucocorticoids in the cell. This preparation of the GR within BLA synapses may be required for an efficient alteration of neuronal physiology and its contribution to memory enhancement.
INTRODUCTION

The evidence that emotionally significant life events are remembered more vividly and with greater detail than mundane experiences (Bohannon, 1988; Neisser et al., 1996) has guided much research on the influence of stress-activated neuromodulatory systems, including central catecholamines and peripheral stress hormones, on memory consolidation (McGaugh and Roozendaal, 2002; McGaugh, 2004; Sandi and Pinelo-Nava, 2007; Schwabe et al., 2012). Norepinephrine release in the basolateral complex of the amygdala (BLA) induces a surge of vigilance and state of alertness and is essentially involved in enhancing the consolidation of memory of emotionally arousing experiences (McIntyre et al., 2002; McGaugh, 2004; Roozendaal and McGaugh, 2011). Adrenal glucocorticoid hormones (corticosterone in rodents, cortisol in humans) are also potent modulators of memory consolidation (Roozendaal et al., 2009; Schwabe et al., 2012). Although most studies have emphasized the importance of classical steroid actions of glucocorticoids via transcriptional regulation in modulating long-term plasticity and information storage processes (de Kloet, 2000), there is now ample evidence that glucocorticoids also rapidly interact with arousal-induced noradrenergic activity within the BLA to orchestrate the physiological response to stress and affect memory consolidation (Roozendaal, 2000; Joëls and Baram, 2009; Roozendaal et al., 2009; Joëls et al., 2011). Pharmacological suppression of noradrenergic transmission in the BLA with a β-adrenoceptor (β-AR) antagonist effectively blocks memory enhancement of inhibitory avoidance, object recognition or auditory-cue fear conditioning training induced by a simultaneously administered glucocorticoid (Quirarte et al., 1997; Roozendaal, 2002; 2006a & b). On the other hand, in the presence of a glucocorticoid receptor (GR) antagonist, a much higher dose of the β2-AR agonist clenbuterol is needed to induce memory consolidation enhancement (Roozendaal et al., 2002). These findings indicating that glucocorticoid signaling synergistically facilitates the effects of arousal-induced noradrenergic stimulation on BLA function could account for the observation from animal and human studies indicating that glucocorticoids appear to selectively modulate memory consolidation of emotionally arousing training experience or those acquired during arousing conditions (Roozendaal et al., 2006 Kuhlmann et al., 2006; de Quervain et al., 2009). However, the precise neural mechanism of how these two stress-response systems interact in promoting memory consolidation remains largely elusive.

Glucocorticoid hormones readily enter the brain and exert their modulatory action via binding to two types of steroid receptors: the high-affinity mineralocorticoid receptor (MR) and the low-affinity GR (de Kloet et al., 2005). The GR appears to be selectively involved in mediating the memory-enhancing effects of glucocorticoids (Oitzl and de Kloet, 1992; Roozendaal and McGaugh, 1997). Although anatomical studies have shown that most GRs are found in cytoplasmic and nuclear compartments (Cintra et al., 1994), there is now also structural evidence for the presence of GRs at presynaptic terminals and postsynaptic membrane densities in amygdala neurons (Johnson et al., 2005; Prager et al., 2010). A possible involvement of GRs residing at or near the cell surface in memory consolidation is supported by recent behavioral findings indicating
that the administration of a specific corticosterone ligand that is unable to penetrate the cell membrane because of covalent binding to a large albumin molecule (cort:BSA) is sufficient to enhance the consolidation of long-term memory, and that this cort:BSA effect depends on GR, and not MR, activation (Barsegyan et al., 2010; Roozendaal et al., 2010). On the other hand, β-ARs are G-protein-coupled receptors and expressed abundantly throughout the BLA, where they predominantly localize to postsynaptic densities and axon terminals (Abraham et al., 2008; Asan, 1998; Farb et al., 2010; Milner et al., 2000). At the cellular level, stimulation of β-ARs is known to rapidly change the activity of BLA neurons through an activation of the adenylyl cyclase/cAMP-dependent protein kinase (PKA) cascade as well as the calcium/calmodulin-dependent protein kinase II (CaMKII) pathway (Hu et al., 2007; Tenorio et al., 2010). Although norepinephrine most likely facilitates memory consolidation by having an impact on many different plasticity-related cellular processes (Marzo et al., 2009; Tully and Bolshakov, 2010), mechanistic studies have delineated that norepinephrine signaling can induce fast effects on neuronal activity via rapid changes in glutamatergic transmission. It was found that norepinephrine induces phosphorylation of the GluA1 subunit of AMPA receptors at sites critical for its synaptic delivery (Hu et al., 2007). Phosphorylation of these sites is necessary to lower the threshold for GluA1 synaptic incorporation during long-term potentiation. Accordingly, mice carrying mutations at these GluA1 phosphorylation sites failed to show norepinephrine-induced synaptic plasticity or memory enhancement (Hu et al., 2007). Importantly, recent findings indicate that a stress-level dose of corticosterone applied simultaneously with a β-AR agonist rapidly augments the surface expression of GluA1-containing AMPA receptors (Zhou et al., 2012) and facilitates AMPA receptor-mediated electrical responses in BLA neurons (Liebmann et al., 2009). These findings suggest that β-ARs and synaptic GRs might be colocalized within excitatory synapses to synergistically influence glutamatergic transmission and behavioral adaptation. Therefore, in the present study we used immunofluorescence in combination with immuno-electron microscopy to investigate the ultrastructural distribution of synaptic GRs and β-ARs within the BLA and whether they might be colocalized at the same synaptic compartments.

Anatomical evidence of colocalization of the β-AR and GR within BLA synapses would open up new possibilities for studying functional interactions between these two stress-response systems already at the level of the receptor. There is evidence that GR can potentiate the β-AR/cAMP/PKA pathway (Roozendaal et al., 2002). But as already mentioned, behavioral data clearly indicate that glucocorticoid effects on memory consolidation require noradrenergic activity, suggesting that norepinephrine or arousal level might also have a direct influence on GR signaling. Recently a novel phosphorylation site at the GR has been discovered on serine 134 in the human GR (serine 154 in rat). The phosphorylation status of this serine is independent of the presence of the hormone but importantly it is thought to integrate cellular stress via the action of p38 mitogen-activated protein kinase (p38 MAPK). In addition, the authors showed that phosphorylation on this specific site changes the gene expression profile by the altering GR capacity of interaction with signaling proteins on gene promoters (Galliher-Beckley et al., 2011). Interestingly, p38 MAPK has been shown
BETA-ADRENERGIC-INDUCED PHOSPHORYLATION OF GR IN THE AMYGDALA

to be activated by norepinephrine binding to the β2-AR (LaJevic et al., 2011; Zheng et al., 2000). Therefore, we hypothesize that stress-induced hyperphosphorylation of GR serine 154 (Ser154-GR) could be mediated by β2-AR activation and that this could constitute one of the mechanisms by which glucocorticoids and the noradrenergic system interact in the BLA. To test the hypothesis that β2-AR activation induces rapid phosphorylation of Ser154-GR at BLA synapses, we utilized biochemical methods to assess the phosphorylation status of membrane-associated GR after a brief incubation with the β2-AR agonist clenbuterol, and examined whether the two receptors were associated in a molecular complex.

MATERIALS AND METHODS

Animals
Male Wistar rats (Charles River, Kisslegg, Germany), weighing about 275-300 g at the time of arrival, were group-housed (2-3 to a cage) and maintained on a standard 12-h light: 12-h dark circle (7:00-19:00 h lights on) in a temperature- and humidity-controlled environment. Pellet food and water were available ad libitum. After their arrival, they were left undisturbed in their cages to acclimatize for at least one week prior to sacrifice. All efforts were made to minimize stress during housing of the animals or on the day of termination in order to avoid disturbances in basal arousal or corticosterone levels, which could influence the location and activity of the receptor types of interest. Sacrificing of rats was always performed in the morning, between 9:00 and 12:00 h, when endogenously circulating corticosterone levels are low. All experimental procedures were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

Primary antibodies and immunoblotting
The primary antibodies used in the study are listed in Table 1. These antibodies have all been successfully characterized and used in recent publications (Cox et al., 2008; Galliher-Beckley et al., 2011; Johnson et al., 2005; Lee et al., 2013; Marcellino et al., 2012; Montero-Pedrazuela et al., 2011; Nakadate et al., 2008; Prager et al., 2010; Zelano et al., 2009). Prior to use of these antibodies in microscopy, we performed immunoblotting to confirm the findings from previous Western-blotting studies and to demonstrate the presence of GRs and β-ARs in whole-cell and synaptic BLA fractions. For this, two rats were deeply anaesthetized with an overdose of sodium pentobarbital and decapitated 90 s later. Brains were rapidly removed and flash frozen by submersion for 2 min in a beaker filled with isopentane placed on dry ice. Frozen coronal sections containing the BLA (based on the atlas plates of Paxinos and Watson, 1998) were cut at a thickness of 350 μm on a cryostat, and BLA tissue punches (1.25 mm diameter) from both hemispheres were homogenized in 100 μl ice-cold buffer containing 124 mM NaCl, 5 mM KCl, 0.1 mM CaCl$_2$-2H$_2$O, 3.2 mM MgCl$_2$-6H$_2$O, 26 mM NaHCO$_3$ and 10
mM glucose (pH 7.4) with 20% protease inhibitor cocktail and 10% protease inhibitor cocktail II (Sigma-Aldrich). Fifty microliters was taken from each homogenate and stored as whole-cell BLA homogenate. Synaptoneurosome fractions were prepared with the rest of the homogenate as described in McReynolds et al. (2010). Briefly, cold homogenization buffer was added to each homogenate to a total volume of 500 μl. The preparations were then filtered through three layers of 100-μm nylon mesh (Small Parts), and then through a 5-μm Durapore® membrane (Millipore). The final filtered solutions were centrifuged at 10,000g for 10 min at 4°C. Subsequently, supernatants were removed and the pellets were suspended in 50 μl cold homogenization buffer. Total protein concentrations for both whole-cell homogenates and synaptoneurosome preparations were determined using Protein Assay Kit (BioRad).

Twenty microgram of total protein per sample was loaded on SDS-PAGE (7.5%), separated electrophoretically, and blotted onto a PVDF membrane (Millipore). After 1 h in blocking buffer (Odyssey blocker, LI-COR), diluted 1:2 in phosphate-buffered saline (PBS), the membranes were incubated with primary antibodies (cf Table 1) (dilution 1:200) at 4°C for 48 h. Subsequently the membranes were rinsed 3 times for 5 min in PBS and incubated with their secondary antibodies (goat anti-rabbit IRDye® 800CW, donkey anti-mouse IRDye® 680LT or donkey anti-guinea pig IRDye 680LT donkey anti-guinea pig; LI-COR) (dilution 1:5,000) for 2 h. Immunoreactivity was detected by fluorescence with the Odyssey® infrared imaging system.

Table 1. Primary antibodies

<table>
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<th>Antigen</th>
<th>Host</th>
<th>Source</th>
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<tbody>
<tr>
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<td>rabbit</td>
<td>Santa Cruz Biotechnology, Inc</td>
<td>H300; sc-8992</td>
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<tr>
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<td>rabbit</td>
<td>Thermo Scientific</td>
<td>Clone BuGR2</td>
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<tr>
<td>β2-adrenergic receptor</td>
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<td>Santa Cruz Biotechnology, Inc</td>
<td>H20; sc-569</td>
</tr>
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<td>Clone 4F10.2</td>
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<td>rabbit</td>
<td>Cell Signaling</td>
<td>D13E1</td>
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<tr>
<td>Phospho-p38 MAPK (Thr180/Tyr182)</td>
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<td>Cell Signaling</td>
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</table>
**Immunohistochemistry**

To determine the cellular and sub-cellular distribution of the GR and β2-AR individually as well as in relation to each other, we used immunofluorescence followed by immunoelectron microscopy.

**Tissue fixation and preparation**

Ten rats were anaesthetized with sodium pentobarbital and perfused transcardially with 50 ml of heparinized 0.9% saline in phosphate buffer (PB, pH 7.4), followed by either 400 ml 4% paraformaldehyde in 0.1 M PB (n = 3; for confocal microscopy) or 400 ml 0.05% glutaraldehyde and 0.2% picric acid mixed into 4% paraformaldehyde in 0.1 M PB (n = 7; for electron microscopy). Brains were removed and post-fixed overnight in their respective perfusion solutions. Brains intended for immunofluorescence were cryoprotected in 25% sucrose and then frozen at -40°C. Coronal sections containing the BLA (between 2.0 and 3.2 mm posterior to Bregma) were cut at a thickness of 30 μm and collected in Tris-buffered saline (TBS) containing 0.1% sodium azide (storage buffer). For electron microscopy, coronal sections of the BLA were cut at 60 μm on a Vibratome and collected in the same storage buffer.

**Immunofluorescence**

Free-floating sections were preincubated with 5% normal donkey serum (nds) for 1 h and incubated overnight with either one primary antibody or a cocktail of appropriate primary antibodies (see Table 2). The next day, sections were rinsed in TBS for 90 min and subsequently incubated with the appropriate secondary antibodies for 2 h and rinsed again in TBS for 90 min. All primary and secondary antibody preparations were diluted in 0.3% Triton X-100 in TBS with 1% nds. Sections were mounted on gelatin-coated slides, air-dried, coverslipped with Mowiol and stored in the dark.

To control for possible non-specific staining, in some preparations the primary antibodies were omitted and replaced by normal serum. An extra control was performed for the GR and β2-AR antibodies: these were incubated with their respective blocking peptides (human glucocorticoid receptor G1542, Sigma-Aldrich; β2-AR blocking peptide sc-569 P, Santa Cruz Biotechnology) (five-fold the concentration of the primaries antibodies) overnight at 4°C. The antibody/peptide cocktails were then diluted into the appropriate medium and added to the tissue sections for incubation.

The final preparations were examined on a Leica TCS SP confocal microscope (Leica, Germany). Fluorescence of Alexa 488, TRITC and Alexa 633 dyes was excited with a 488-nm argon laser, a 543-nm HeNe laser and a 633-nm HeNe laser respectively.
Table 2. Combination and sequential processing of primary and secondary antibodies used for immunofluorescence labeling

<table>
<thead>
<tr>
<th>Series</th>
<th>Primary antibodies (overnight)</th>
<th>Secondary antibodies (2h)</th>
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<td></td>
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<td>Single-labeling</td>
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<td></td>
<td>GR</td>
<td>mouse</td>
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<td>β2-AR</td>
<td>β2-AR</td>
<td>rabbit</td>
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<tr>
<td>Double-labeling</td>
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<td>rabbit</td>
</tr>
<tr>
<td></td>
<td>VGluT2</td>
<td>guinea pig</td>
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<td>GR/GAD</td>
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<td>mouse</td>
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<td>GR/DBH</td>
<td>GR</td>
<td>rabbit</td>
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<td>DBH</td>
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</table>

Abbreviations: β2-AR, β2-adrenergic receptor; DβH, Dopamine β hydroxylase; GAD, Glutamic acid decarboxylase; GP, guinea pig; GR, glucocorticoid receptor; Ms, mouse; Rb, rabbit; TRITC, Tetramethyl Rhodamine Iso-Thiocyanate; VGluT2, vesicular glutamate transporter 2.

Immunocytochemistry for electron microscopy

**Pre-embedding immunohistochemistry**

We used pre-embedding immunohistochemistry to analyze the ultrastructural location of the GR and β2-AR. BLA sections were rinsed in PBS and cryoprotected overnight in 25% sucrose and 3.5% glycerol in PBS and freeze-thawed over liquid nitrogen in order to increase antibody penetration. Endogenous peroxidase activity was blocked with 1% H2O2 in 50% methanol/TBS for 30 min. Sections were preincubated with 5% nds in incubation medium (1% BSA, 0.1% glycine, 0.1% lysine and 0.1% cold...
water fish skin (CWFS) gelatin (Aurion, Wageningen, The Netherlands) in 0.05% Triton X-100 in TBS) for 2 h, and incubated with the GR antibody H300 or the β2-AR antibody H20 diluted 1:50 in incubation medium plus 5% nds at 4°C for 2 nights. After rinsing in TBS, sections were incubated with biotinylated donkey anti-rabbit (diluted 1:200 in incubation medium) for 2 h. After further rinsing in TBS, sections were incubated in ABC (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, USA). Immunoreactivity was enhanced according to the gold-substituted silver peroxidase method (van den Pol and Gorcs, 1986). Sections were then osmicated in 1% OsO₄ and 1.5% potassium hexacyanoferrate for 15 min, dehydrated in a graded series of ethanol and propylene oxide and flat embedded in epoxy resin. After polymerization, ultrathin sections were cut on a LKB Ultramicrotome, counterstained with 2% uranyl acetate and 2% lead citrate and examined with a Philips CM 100 transmission electron microscope (Eindhoven, The Netherlands).

Post-embedding immunohistochemistry
Post-embedding was used to confirm the findings obtained from the pre-embedding preparations and, most importantly, to determine the ultrastructural co-distribution of the two receptors. The post-embedding technique is most suited for this purpose due to the possibility of simultaneously using two secondary antibodies conjugated to gold particles of different size that can be easily distinguished (Oliver, 2010). BLA sections were processed for an osmium-free post-embedding labeling (Phend et al., 1995). Briefly, Vibratome sections were successively treated with 1% tannic acid, 1% uranyl acetate and 0.5% platinum chloride. They were then dehydrated in a graded series of ethanol and embedded in epoxy resin. For single labeling, ultrathin sections mounted on nickel grids were incubated with a primary antibody (for GR: H300 or BuGR2; for β2-AR: H20; diluted 1:40, 1:15 and 1:40, respectively) overnight at 4°C. After rinsing in PBS, sections were incubated with a secondary antibody, goat anti-rabbit or goat anti-mouse IgG conjugated to 10-nm gold particles (diluted 1:100, BioCell, Cardiff, UK). The two antibodies for GR gave a similar labeling pattern. For double labeling, sections were incubated with a cocktail of primary antibodies (the mouse-raised BuGR2 and the rabbit-raised H20, at the same dilution as indicated above) and after rinsing sequentially incubated with goat anti-rabbit conjugated to 5-nm gold particles and with goat anti-mouse IgG conjugated to 15-nm gold particles (Aurion, Wageningen, Netherlands). Secondary antibodies were diluted at 1:100 and the incubations lasted 2 h at room temperature.

Pharmacological treatment and assessment of p38 MAPK and Ser154-GR phosphorylation
Naïve rats were anaesthetized with pentobarbital and rapidly decapitated. Brains were immediately dissected and transferred to ice-cold dissection buffer containing 120 mM NaCl, 3.5 mM KCl, 1.25 mM NaH₂PO₄, 5 mM MgSO₄, 0.2 mM CaCl₂, 25 mM NaHCO₃, and 10 mM glucose (pH 7.4), saturated with carbogen (mixture of 95% O₂
and 5% CO₂). Brains were placed in a matrix and sections containing the BLA were cut and incubated at room temperature for 1 h in artificial cerebro-spinal fluid (ACSF) containing 120 mM NaCl, 3.5 mM KCl, 1.25 mM NaH₂PO₄, 1.3 mM MgSO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, and 10 mM glucose (pH 7.4) and continuously gassed with carbogen. BLA tissue was incubated with different doses of the β2-AR agonist clenbuterol hydrochloride (Sigma-Aldrich; 0, 50, 100 and 500 µM) for 10 min. After the treatment, the tissue was snap frozen and stored at -80 ºC until sample preparations, protein assays and immunoblotting were performed as described above to determine the phosphorylation of p38 MAPK and Ser154-GR.

The antibodies used for these experiments were the rabbit anti-p38 MAPK (1:1,000; Cell Signaling), rabbit anti-phospho-p38 MAPK (1:500; Cell Signaling), rabbit anti-phospho-Ser134-GR (1:1,000; gift from Dr. Cidlowski), mouse anti-GR (BuGR2) (1:200; Thermo Scientific), and mouse anti-β-actin (1:10,000; Abcam). Membranes were imaged and quantified using a LI-COR Odyssey scanner and the Odyssey 3.0 analytical software. Quantification was made on single channels. Boxes were manually placed around each band of interest, which returned near-infrared fluorescent values of raw intensity with intra-lane background subtracted. Ratio of phosphorylated proteins was calculated as the intensity values of those proteins divided by the intensity values of their respective total proteins. The results were calculated as percentage of the vehicle group values.

**Co-immunoprecipitation**

To determine a possible association of GR, β2-AR and p38 MAPK in a molecular complex, co-immunoprecipitation was performed on synaptoneurosomes prepared from BLA tissue treated with clenbuterol (50 µM) or vehicle as described in the previous section. Samples preparation and proteins assays were performed as mentioned above. Fifty µg of total protein was solubilized by addition of Nonidet P40 (Thermo Scientific) at 1% of total volume and by end-over-end agitation at 4°C for 1 h. Samples were centrifuged (10,000 g) at 4°C for 10 min and the supernatants were collected. Primary antibodies [anti β2-AR or anti GR (BuGR2), each at 1:200] and protein A/G Plus Agarose (Santa Cruz) (0.2-0.3 µl/nl of sample) were added to solubilizates and incubated overnight at 4°C. The next day, the samples were centrifuged (3000 g) at 4°C for 30 sec, the supernatants were removed, and the pellets were washed with homogenization buffer. The washing step was repeated 3 times. During this step, buffer was added to the samples followed by a 5 min-incubation at 4°C on a rotator. Subsequently, the samples were centrifuged (3000 g at 4°C for 30 sec) and supernatant was removed. Beads were eluted by incubation with electrophoresis sample buffer (1x) in water bath at 37°C for 30 min. The solubilizates were loaded on gel and Western blots were performed on SDS-PAGE gels as described above. The membranes were incubated overnight with rabbit anti-phospho-Ser134-GR (1:1000) and rabbit anti-p38 MAPK (1:1,000) for the immunoprecipitation of β2-AR, and with rabbit anti β2-AR (1:200) and rabbit anti-p38 MAPK (1:1,000) for the immunoprecipitation of GR.
Statistical analysis
Data are expressed as mean ± SEM. Non-parametric Kruskal-Wallis and two-tailed Mann-Whitney U tests were used to analyze the phosphorylation ratio of the proteins. For all comparisons, a probability level of p ≤ 0.05 was accepted as statistical significance.

RESULTS

Determination of the specificity of the antibodies
The specificity of the different antibodies used in this study (Table 1) had been established previously (Cox et al., 2008; Johnson et al., 2005; Lee et al., 2002; Marcellino et al., 2012; Montero-Pedrazuela et al., 2011; Nakadate et al., 2008; Prager et al., 2010; Zelano et al., 2009). Western blots on whole-cell preparations were performed for the antibodies that were reported suitable for this technique, and the findings were in general agreement with the literature as well as data sheets provided by the manufacturers (data not shown). Extra care was taken concerning the antibodies meant to detect GR and β2-AR. Immunoblotting was performed to confirm the expression of GR and β2-AR in the BLA. The H300 GR antibody did not appear very suitable for Western blotting. Although the manufacturer’s data sheet indicated a clear band around 90-100 kDa, this was not the case in our experimental set up (data not shown). As shown in Fig. 1, the GR antibody BuGR2 displayed a band at about 97 kDa representing the undigested GR. Three additional bands representing digested fragments of GR were observed at 25, 55 and 80 kDa. These bands have been described by the manufacturer as a product of GR cleavage. The β2-AR antibody H20 displayed a single band at around 68 kDa. Omission of the primary antibodies (H300, BuGR2 and H20) during the immunofluorescence staining procedure resulted in no labeling. Likewise, absence of staining was observed when the primary antibodies had been neutralized by their respective blocking peptides (data not shown). These test-experiments confirmed that the antibodies specifically recognized the GR and β2-AR.

Figure 1. Immunoblots of GR and β2-AR from whole BLA preparation. The first column represents the molecular weights. The mouse antibody against GR (BuGR2) recognizes the undigested form of GR at 97 kDa and three additional bands corresponding to digested fragments of GR as described by the manufacturer. The rabbit antibody against β2-AR (H20) detects a single band at around 68 kDa.
Cellular distribution of GR and β2-AR

To determine the specific cellular distribution of GR and β2-AR within the BLA, immunofluorescence labeling was applied on amygdala sections, and tissue was examined by confocal microscopy. In agreement with previous findings (Cintra et al., 1994; Farb et al., 2010; Johnson et al., 2005), we observed dense GR and β2-AR immunoreactivity in all subdivisions of the amygdala complex, with strongest labeling in the central nucleus (data not shown). Fig. 2A shows GR immunoreactivity (GR-ir) in the BLA. The lateral, basolateral and basomedial nuclei contained dense and homogenous immunoreactivity. High-magnification micrographs show that GR-ir was mainly present in nuclei (Fig. 2C), as expected based on its classical role as transcription factor. Most of the GR-positive nuclei were large, round or ovoid in shape, and are most likely nuclei of pyramidal neurons (Sah et al., 2003). Weaker immunoreactivity for GR was present in neuropil as puncta-like reaction product (Fig. 2B). Additionally, GR-ir was sometimes found in small nuclei and structures that are possibly inhibitory neurons and/or glial cells (McDonald et al., 2002). Fig. 2C shows that strong immunoreactivity for β2-AR was observed in the BLA. These β2-AR-ir cells, considering their triangular shape, are most likely pyramidal cells. The reaction product was observed in the cytoplasm as well as in nuclei, clearly delineating the nucleolus. Immunoreactivity was also observed in proximal dendrites, fibers and punctate profiles (Fig. 2D). The immunofluorescence data indicate that GR and β2-AR are abundant in pyramidal neurons in the BLA and in the neuropil.
Figure 2. Cellular distribution of GR and β2-AR in the BLA. (A-B) Numerous BLA cells contain GR-ir. This immunoreactivity is almost exclusively distributed in nuclei, with some punctate-like labeling in the cytoplasm and neuropil. (C-D) β2-AR-ir is also dense in the BLA, and at the cellular level, is wide spread in distinct compartments (somata, nuclei, proximal dendrites).

**GR-ir and β2-AR-ir are associated with BLA synapses**

Previous studies reported the association of GR with synapses of the lateral nucleus of the amygdala (Johnson et al., 2005; Prager et al., 2010). Moreover, it is well established that the β2-AR locates at synapses (Abraham et al., 2008; Asan, 1998; Farb et al., 2010; Milner et al., 2000). In order to further investigate the association of these two receptors with synapses in the BLA, we performed immunoblotting for GR and β2-AR antibodies on BLA synaptic fractions. BLA synaptic preparations showed immunoreactivity for both antibodies indicating that both GR and β2-AR are present at synaptic sites within the BLA. For GR, besides the main band at 97kDA, similar additional bands corresponding to digested fragments were found (Fig. 3).
To characterize the type of synapse at which each receptor is localized, double immunofluorescence for the receptor and a specific synaptic marker was performed. Dopamine β-hydroxylase (DβH), glutamic acid decarboxylase (GAD) and vesicular glutamate transporter 2 (VGLuT2) were used as markers for noradrenergic, GABAergic and glutamatergic synapses, respectively (Fremeau Jr et al., 2001; Hökfelt et al., 1977; Ribak et al., 1979). Qualitative examination of GR/DβH preparations showed that they were localized in separate profiles (Fig. 4A). Similarly, there was a distinct distribution of GR-ir and GAD-ir in the neuropil (Fig. 4B). In contrast, examination of sections stained for GR and VGLuT2 showed that GR-ir puncta were closely juxtaposed to some VGLuT2-ir puncta (Fig. 4C & 4C’), suggesting that GR might be preferentially localized at glutamatergic synapses. Similarly to GR, no evident juxtaposition of β2-AR-ir with DβH or GAD was observed (Fig. 4D & 4E). However, β2-AR-ir puncta were found in proximity to VGLuT2 puncta (Fig. 4F & 4F’).

To investigate the ultrastructural localization of the GR and β2-AR, single pre-embedding and post-embedding electron microscopy immunolabeling were performed. The analysis of those preparations was particularly focused on synaptic elements and gave details regarding the precise synaptic localization of each receptor. Apart from the classical localization of the GR in nuclei and cytoplasm, many GR-labeled synapses were observed. Most of these synapses was asymmetric in shape and therefore considered to be excitatory (Colonnier, 1981; Gray, 1959). GR immunogold labeling was found both in pre- and postsynaptic compartments such as in terminals (Fig. 5A), dendritic spines (Fig. 5A & B) and occasionally near postsynaptic densities or synaptic membranes (Fig. 5B). GR-ir terminals and boutons formed asymmetric synapses with dendritic spines (Fig. 5A), as well as on dendritic shafts. In those presynaptic elements, labeling was mainly associated with clear and spherical synaptic vesicles. Reaction product at the presynaptic membrane could be observed in the active zone and in the peri-synaptic area. Postsynaptically, GR labeling was found in spine heads and close to postsynaptic densities.

Similar to GR-ir, immunoreactivity for β2-AR was mostly present near asymmetric synapses (Fig. 5C & D). Labeling was detected in presynaptic terminals (Fig. 5C), associated with vesicles or close to the synaptic membrane. In postsynaptic compartments, spine heads and postsynaptic densities showed labeling (Fig. 5D). The ultrastructural distribution of GR-ir and β2-AR-ir in the BLA is thus in general concordance with our immunofluorescence data and suggests that both the GR and β2-AR are localized at excitatory glutamatergic synapses.

Figure 3. Presence of GR and β2-AR in BLA synaptic fractions. Immunoblots of GR (A) and β2-AR (B) show the association of GR and β2-AR with synaptic-enriched samples from the BLA. The two antibodies used, recognize similar bands as in the whole amygdala homogenates (including the digested fragment in the case of GR).
Figure 4. Merged confocal images in BLA after double labeling for GR, and synaptic markers DBH, GAD and VGluT2 (A-C'). There is no evident close apposition between GR and DBH (A) or GAD (B). Juxtaposition of GR and VGluT2 is indicated by white arrowheads (C'). Merged confocal images in BLA after double labeling for β2-AR and synaptic markers DBH, GAD and VGluT2 (D-F'). Neither DBH (D), nor GAD (E) were found in close proximity of β2-AR-leabeld punctate. Apposition between β2-AR and VGluT2 are indicated by white arrowheads (F'). Scale bars: 10 µm.
Figure 5. Electron micrographs showing GR (A & B) and β2-AR (C & D) at BLA synapses. GR labeled terminals forming asymmetric synapses onto spines. GR reaction product is present in terminals (A & B) associated within vesicles or near the synaptic membrane (B). Labeling is also located in spine heads, within the cytoplasm or near the post-synaptic density (B). (C) Pre-embedding labeling of β2-AR in terminals, reaction product is associated with vesicles (arrow) and the synaptic membrane (arrow head). (D) Post-embedding labeling of β2-AR (10 nm gold particles) in spine head. Scale bars: 200 nm.

Co-distribution of GR and β2-AR at BLA synaptic sites

The findings described above suggest that the GR and β2-AR might share a similar localization in their synaptic distribution pattern in the BLA. Therefore, we next investigated whether the two receptors could be colocalized within the same pre- and post-synaptic compartments. Post-embedding double immunolabeling was performed, examined and quantified.

Analysis of the preparations revealed the existence of GR/β2-AR-labeled synapses in the BLA. Synapses were considered to be double labeled if GR and β2-AR immunoreactivity was present within the same either pre- or postsynaptic compartment. Co-distribution of the two receptors was observed in postsynaptic compartments,
principally spine heads (Fig. 6A), sometimes close to the postsynaptic density (Fig. 6B) and in dendrites (Fig. 6C). Presynaptically, colocalization was detected in terminals associated with synaptic vesicles (Fig. 6D) and extrasynaptic membranes (Fig. 6E). In all types of pre- and postsynaptic distributions, GR and β2-AR were found in clusters (Fig. 6A, B & F) or segregated (Fig. 6B, C, D & E). Sometimes, double immunolabeling was found in both pre- and postsynaptic compartments of the same terminal.

To obtain an estimation of the quantitative distribution of colocalized GR/β2-AR in synaptic sites, 50 double-labeled asymmetric synaptic profiles were randomly selected and we analyzed the distribution among terminals, dendritic profiles and spines. Fig. 7 showed this distribution: in 26 cases, double immunolabeling was found in postsynaptic compartments (16 spines and 10 dendrites) whereas at 23 synapses double labeling was found in their terminals. In one synapse among the 50 analyzed, double labeling was found in both the pre- and postsynaptic compartments.

These findings demonstrate that GR and β2-AR are colocalized within the BLA in specific subcellular compartments close to the synapse (Fig. 8).
Figure 6. Electron micrographs of post-embedding labeling showing GR (15 nm gold, arrow heads) and β2-AR (5 nm gold particles, arrows) located at the same synaptic sites in BLA. Co-distribution of GR and β2-AR is found in spine heads (A & B), dendrites (C & D) and terminals and postsynaptic dendrites (E & F). Scale bars: 200 nm.
Figure 7. Quantification of GR/β2-AR co-distribution occurrence in a sample of 50 synaptic sites.

Figure 8. Schematic drawing representing GR/β2-AR co-distribution in the synapse. GR and β2-AR were co-localized in the postsynaptic compartment at the postsynaptic density (1), in dendritic spine cytoplasm (2), and in dendritic shaft (3). In terminals, GR and β2-AR were co-localized near the synaptic membrane in the active zone (4), near neurotransmitter vesicles (5) and in the cytoplasm (6).

**Activation of β2-AR induces phosphorylation of synaptic GR on the serine 154 via activity of p38 MAPK**

The GR contains a ligand-independent phosphorylation site on serine 134 (serine 154 in rat) that is hyperphosphorylated in response to cellular stress (such as glucose starvation, UV irradiation, osmotic shock or oxidative stress), via activation of p38 MAPK (Galliher-Beckley et al., 2011). Besides, stimulation of β2-AR is also known to activate p38 MAPK via PKA (Zheng et al., 2000). To determine whether GR serine...
154 (Ser154-GR) is hyperphosphorylated following the activation of β2-AR, BLA slices were treated for 10 min with increasing concentrations of clenbuterol (0 to 500 µM). Subsequently, the phosphorylation status of Ser154-GR, and p38 MAPK relative to their respective total protein were assessed in synaptoneurosomes. Fig. 9A shows that activation of β2-AR by clenbuterol increases the phosphorylation of Ser154-GR and of p38 MAPK. Kruskal-Wallis test for Ser154-GR phosphorylation ratio revealed a significant group effect \[H(3) = 12.7, p = 0.05\]. Mann-Whitney U tests revealed that the phosphorylation ratio of the 50 µM clenbuterol group (\(Z = -2.9; p = 0.01; n = 4\)), the 100 µM group (\(Z = -2.9; p = 0.04; n = 4\)) and the 500 µM group (\(Z = -2.9; p = 0.01; n = 4\)) were significantly higher than that of the vehicle control group (\(n = 6\)). The three different doses of clenbuterol did not result in significantly different phosphorylation of Ser-154-GR [50 µM vs 100 µM (\(Z = 0; p = 1.00\)); 50 µM vs 500 M (\(Z = -1.2; p = 0.34\)); 100 µM vs 500 M (\(Z = -1.2; p = 0.34\))].

Similarly, Kruskal-Wallis test revealed a significant group effect on p38 MAPK phosphorylation ratio [H(3) = 12.2, p = 0.007]. Mann-Whitney U test comparison of groups revealed that the phosphorylation ratio of the 50 µM clenbuterol group (\(Z = -2.9; p = 0.01; n= 4\)), the 100 µM group (\(Z = -3.0; p = 0.04; n = 5\)), and the 500 µM group (\(Z = -2.8; p = 0.02; n = 3\)) were significantly higher than that of the vehicle control group. The three different doses of clenbuterol used did not result in significantly different phosphorylation ration [50 µM vs 100 µM (\(Z = -0.6; p = 0.56\)); 50 µM vs 500 M (\(Z = -0.7; p = 0.63\)); 100 µM vs 500 M (\(Z = -0.6; p = 0.57\)]

These results indicate that β2-AR stimulation induces a rapid hyperphosphorylation of the hormone-independent phosphorylation site Ser154-GR and of p38 MAPK in the BLA synapses.

![Figure 9](image.png)

**Figure 9.** Clenbuterol increases p38 MAPK and GR phosphorylation in synaptoneurosomes preparation from BLA tissue.

**β2-AR, p38 MAPK and GR are associated in a molecular complex**

Previous studies have shown that the β2-AR co-precipitates with p38 MAPK which leads to the phosphorylation of the latter (LaJevic et al., 2011; Zheng et al., 2000). Similarly the association of p38 MAPK and GR in a molecular complex may be necessary for the phosphorylation of Ser154-GR (Galliher-Beckley et al., 2011). We
have shown that β2-AR stimulation induces phosphorylation of the GR at serine 154 and of p38 MAPK. To determine whether β2-AR, p38 MAPK and GR are involved in a molecular assembly upon stimulation of β2-AR, co-immunoprecipitation was performed with synaptoneurosomes prepared from the BLA tissue treated clenbuterol (50 µM) for 10 min as described earlier.

Fig. 10A shows that after treatment with clenbuterol, the β2-AR co-precipitates with the phosphorylated GR and the phosphorylated p38 MAPK. In the same condition, GR (total GR) also co-precipitates with the β2-AR and p38 MAPK (Fig. 10B). In tissue treated with vehicle, bands are barely detectable, indicating that in baseline conditions, β2-AR, p38 MAPK and GR do not form a molecular assembly. These data indicate that after its activation, β2-AR is involved in a molecular assembly including p38 MAPK and GR, in order to phosphorylate the serine 154 of GR.

**Figure 10.** β2-AR, p38 MAPK and GR are involved in a molecular complex after activation of β2-AR by clenbuterol. (A) β2-AR co-precipitates with P-GR-Ser154, and P-p38 MAPK after treatment with clenbuterol. (B) GR co-precipitates with β2-AR and p38 MAPK after clenbuterol treatment.

**DISCUSSION**

In the present study, we used immunofluorescence in combination with immuno-electron microscopy to investigate the ultrastructural distribution of synaptic GRs and β2-ARs within BLA neurons. The rationale for this investigation stems from previous work, in both animals and humans, indicating that glucocorticoid administration, via GR activation, selectively enhances the consolidation of memory of emotionally salient training experiences (Buchanan and Lovallo, 2001; Okuda et al., 2004; Abercrombie et al., 2006; Kuhlmann and Wolf, 2006) because of a critical dependence on arousal-induced noradrenergic activity within the BLA (Roozendaal et al., 2006; Van Stegeren et al., 2007). Although some interactions between glucocorticoids and noradrenergic activity might have a slow onset and are likely exerted through their ability to modulate gene transcription in the nucleus, physiological and cellular studies have provided critical evidence indicating that this crosstalk is able to induce rapid changes in glutamatergic transmission and synaptic plasticity (Zhou et al., 2012). However, there is still no anatomical and molecular evidence supporting the interaction of the GR and β2-AR at synaptic compartments.
After confirming the presence of both GR and β2-AR immunoreactivity at BLA synapses with Western blotting, we used double immunofluorescence to determine the type of synapse at which each receptor is located. Our findings indicating that the GR and β2-AR colocalized preferentially with VGluT2, a marker commonly used to identify glutamatergic synapses (Fremeau Jr et al., 2001), suggest that these two receptors are located at glutamatergic synapses. This observation was supported by our immuno-electron microscopy experiments indicating that both receptor types were predominantly found at asymmetric excitatory synapses on dendritic spines. At the ultrastructural level, we found β2-AR immunoreactivity in postsynaptic compartments as would be expected in view of previous neuroanatomical reports and electrophysiological studies showing its action on postsynaptic electrical responses (Farb et al., 2010; Marzo et al., 2009). We also found β2-AR immunoreactivity in presynaptic compartments associated with vesicles or concentrated at the synaptic membrane (Farb et al., 2010; Milner et al., 2000). Our single electron microscopy immunolabeling for GR revealed that this receptor was also localized in both pre and postsynaptic elements of asymmetric terminals. This expression profile of synaptic GR in the BLA is generally consistent with that of prior ultrastructural studies in the amygdala and hippocampus (Johnson et al., 2005; Ooishi et al., 2012; Prager et al., 2010). The existence of GR associated with the synapse supports the findings from cellular and behavioral studies indicating that glucocorticoids can have fast and presumably non-genomic effects on synaptic transmission and memory. A major finding of our electron microscopic experiments is that the GR and β2-AR were found in close proximity to each other at both pre and postsynaptic compartments of BLA neurons. We showed colocalization of GR and β2-AR in spine heads, near postsynaptic densities in terminals and in dendritic shafts. The colocalization of GR and β2-AR at multiple sites on BLA principal neurons suggests that interactions between the two receptors might be involved in modulating different cellular and physiological processes, including glutamatergic receptor current and neurotransmitter release. In support of a functional interaction between both receptors, we demonstrated that β2-AR activation with clenbuterol rapidly increased phosphorylation of the only hormone-independent phosphorylation site of the GR, serine 154. Furthermore, upon its activation a molecular complex consisting of the β2-AR, p38 MAPK and GR was formed as illustrated by the co-precipitation of the three molecules.

The crosstalk between glucocorticoids and norepinephrine in regulating BLA activity in the context of memory consolidation has been the focus of many studies in the past decade (Joels et al., 2011; Krugers et al., 2012; Roozendaal et al., 2000 and 2009). This interaction was first reported by Quirarte et al. in 1997. These authors showed that specific blockade of β-AR activity within the BLA prevented the enhancing effects of a systemically administered GR agonist on memory consolidation. Later studies confirmed that glucocorticoid effects on memory depend on the integrity of the amygdala noradrenergic system (Roozendaal et al., 2006; Okuda et al., 2004). An important conclusion was that GR activation could induce rapid changes in the efficacy of arousal-induced noradrenergic activity (Roozendaal et al., 2002). At the
behavioral level, the effect of glucocorticoids on amplifying noradrenergic activity is now well established. For instance, a 100-times higher dose of the β2-AR agonist clenbuterol is needed to enhance memory consolidation when a GR antagonist is infused into the BLA (Roozendaal et al., 2002). A non-genomic action of GR activity on the β-AR/cAMP/PKA pathway appears to be involved in the potentiation of norepinephrine effects on memory consolidation. This interference is thought to result in an overall increase in BLA excitability (Duvarci and Pare, 2007). In addition, in vivo microdialysis studies showed that systemic glucocorticoid administration after training on an emotionally arousing task augments norepinephrine levels in the BLA and that norepinephrine levels positively correlate with later retention performance (McReynolds et al., 2010). Evidence from memory studies in healthy human subjects also indicated that the combination of cortisol and a noradrenergic stimulant correlates with improved memory for emotionally arousing information (Smeets et al., 2009; van Stegeren et al., 2010).

At the cellular level, glucocorticoids have been shown to enhance β-adrenergic influences on the facilitation of long-term potentiation (Pu et al., 2007). A recent study indicated that the combination of corticosterone and the β-AR agonist isoproterenol rapidly increased AMPA receptor GluA1 subunit phosphorylation and surface expression at the postsynaptic membrane as well as facilitated miniature excitatory postsynaptic currents, whereas the application of each compound alone was less or not effective (Zhou et al., 2012). Such a rapid synergetic action of two transmitters requires their signaling machinery to be located within the same sub-cellular compartment, preferentially close to the synapse. This is in agreement with our finding that the GR and β2-AR are colocalized in spine heads, near the postsynaptic density. At this site, the two receptors are likely to functionally interact and as a result positively modulate glutamatergic transmission in a rapid and nongenomic manner. However the nature of this functional interaction is not known. Activation of the β-AR rapidly increases the phosphorylation of the GluA1 subunit of the AMPA receptor as well as induces insertion of AMPA receptors into the synaptic membrane (Hu et al., 2007). Another recent study demonstrated that upon activation, β2-AR forms a molecular complex that includes PKA and the subunit GluA1, resulting in the phosphorylation of GluA1 and its increased expression at the cell surface (Joiner et al., 2010). It is not known whether GR is also part of this molecular complex. If that were the case, its activation could potentiate the β-AR/cAMP/PKA pathway, as suggested by the behavioral studies, and thereby accelerate the β-AR-induced phosphorylation and increased surface expression of GluA1. However, Zhou and colleagues did not investigate whether the corticosterone effects were mediated by GR activation. Recent reports have shown that glucocorticoids via MR rapidly enhance GluA2 containing AMPA receptors surface trafficking (Groc et al., 2008; Martin et al., 2009). However, these studies have not investigated whether this corticosterone effect requires concomitant noradrenergic activity.

We also observed co-localization of GR and β2-AR in terminals indicating that the
two receptors could be involved in neurotransmitter release. In several brain regions, including the BLA, presynaptic β-AR/cAMP/PKA signaling enhances synaptic transmission by increasing glutamate release from terminals through the increase of calcium influx via P/Q-type (also known as CaV2.1) calcium channels (Gereau and Conn, 1994; Herrero et al., 1996; Huang et al., 1996 and 1998). In that case also, GR could be involved in the potentiation of β-AR/cAMP/PKA cascade or act directly on neurotransmitter release. Stress and glucocorticoids rapidly increase extra-cellular glutamate levels, as measured by microdialysis (Hascup et al., 2010; Reznikov et al., 2007; Venero and Borrell, 1999). Electrophysiological studies suggested that corticosterone could rapidly increase glutamate release probability and that this effect was mediated by a membrane-associated MR and not GR (Karst et al., 2005; Olijslagers et al., 2008). This is similar to what was found with AMPA mechanisms. Another study showed that acute corticosterone treatment of hippocampal synaptoneurosomes facilitated glutamate release and that this effect was mediated by GR and not MR (Wang and Wang, 2009). The mEPSCs measured by Karst and colleagues are thought to reflect spontaneous fusion of glutamatergic vesicles to the presynaptic membrane, whereas Wang and colleague measured an evoked glutamate release. Both studies claim that the doses of corticosterone they used reflected post-stress elevation of the hormone. As for the findings on postsynaptic effects of corticosteroids, the lack the noradrenergic activity makes it difficult to draw a conclusion about the exact role of GR and MR.

In our study, not all the BLA asymmetric synapses were double-labeled. Some synapses contained only one of the receptors at either compartment, whereas in others each receptor was in a different compartment. Therefore besides the possible direct interaction between GR and β2-AR, there might be indirect interactions between glucocorticoid and noradrenergic systems. One example is the action of glucocorticoids on central norepinephrine levels. Several animal studies have shown that systemic corticosterone treatment causes a rapid and transient increase of norepinephrine levels in the amygdala as in others brains regions via a membrane receptor (McReynolds et al., 2010; Thomas et al., 1994). Glucocorticoids may alter norepinephrine re-uptake by acting on catecholamine transporter (Grundemann et al., 1998; Iversen, 1965). There is also growing evidence showing that glucocorticoids effects on norepinephrine release involve the endocannabinoid system. Glucocorticoids induce a rapid release of endocannabinoids in the brain (Hill et al., 2010; Malcher-Lopes et al., 2006). In the BLA, the presence endocannabinoids is necessary for the effects of glucocorticoids on memory consolidation (Campolongo et al., 2009). A new model of interaction between stress mediators has been proposed in which the rapid glucocorticoids-induced release of endocannabinoids increases noradrenergic activity by reducing GABAergic transmission, the resulting enhanced noradrenergic activity will ultimately enhance memory consolidation (Freund et al., 2003; Hatfield et al., 1999; Hill et al., 2009). In this model, GR and β-AR do not need to be in the same synaptic compartment, and can even be located on different synapses as we also observed in this study. However the rapid action on the endocannabinoid system also requires the presence
of noradrenaline (Atsak et al., 2012; Campolongo et al., 2009). Additionally, a recent report proposed that the rapid and transient elevation of endocannabinoid following systemic corticosterone administration could be due to the interaction between corticosterone itself and the arousal induced by the injection (Hill et al., 2010). This indicates that similarly to what is observed at the behavioral levels, glucocorticoids signaling is regulated by norepinephrine.

Our findings that β2-AR activation induces a change in the GR phosphorylation status confirm this hypothesis. This is the first evidence at the molecular level to support this view, which is logical from a physiological perspective, since stress and arousal first activate the autonomic nervous system which rapidly leads to an important release of noradrenaline in the brain and particularly in the BLA, meaning that neurons are first exposed to high level of norepinephrine. Before glucocorticoids reach these neurons, norepinephrine, through activation of its receptors has time to exert different effects, among which causing and increase phosphorylation of the ligand-independent serine (serine 154 in rat, serine 134 in human) of the GR at the synapse. This phosphorylation site has been discovered recently, and is thought to integrate cellular stress influence on GR functions (Galliher-Beckley et al., 2011). The authors found that the presence of the ligand did not modify the phosphorylation of serine 134 of human GR, whereas different types of cellular stress induced a hyperphosphorylation at this specific site. Our study indicates that β2-AR activation is one of the mediators of stress on cells. Further, we showed that the two receptors co-precipitated with each other and with the protein kinase p38 MAPK in synaptoneurosomes, indicating that this phenomenon takes place near membrane and synapse and therefore implicates synaptic GR. In the study of Galliher-Beckley and colleagues, serine 134 is phosphorylated by p38 MAPK. Another study demonstrated that β2-AR activation induces the phosphorylation of p38 MAPK via activation of PKA. This evidence suggests that the p38MAPK pathway mediates the effect of β2-AR on GR phosphorylation on serine 154. The role of this serine on GR functions needs to be further examined. So far, it has been shown to change gene expression pattern (Galliher-Beckley et al., 2011). The fact that we found that synaptic-associated GR undergoes this specific phosphorylation could mean that it plays a role in the rapid effects of glucocorticoids. Further study will determine this possible role.

In summary, the present findings show that the GR and β2-AR are colocalized in BLA excitatory synapses, and that β2-AR activation causes a modification of GR phosphorylation status via the formation of a molecular complex. In addition, our finding that this colocalization of GR and β2-AR was present at different subcellular compartments suggests that the interaction between norepinephrine and glucocorticoids affects several synaptic mechanisms. These findings are important because they suggest that norepinephrine release and the activation of β2-ARs could set up the GR to prepare it for the coming surge of glucocorticoids in the cell. This preparation of the GR may be necessary for an efficient alteration of neuronal physiology and its contribution to memory enhancement. These findings might help explaining why arousal or norepinephrine is necessary for the genomic and nongenomic effects of GR.
REFERENCES


BETA-ADRENERGIC-INDUCED PHOSPHORYLATION OF GR IN THE AMYGDALA


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