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Interdependent Adrenergic Receptor Regulation of Arc and Zif268 mRNA in Cerebral Cortex

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Abstract

Norepinephrine is a neurotransmitter that signals by stimulating the α_1 , α_2 and β adrenergic receptor (AR). We determined the role of these receptors in regulating the immediate early genes, Activity Regulated Cytoskeleton Associated Protein (Arc) and Zif268 in the rat cerebral cortex. RX821002, an α_2 -AR antagonist, produced Arc and Zif268 elevations across cortical layers. Next we examined the effects of delivering RX821002 with an α_1 -AR antagonist, prazosin, and a β -AR antagonist, propranolol. RX821002 given with a prazosin and propranolol cocktail, or with each of these antagonists individually, decreased Arc and Zif268 to saline-treated control levels in most cortical layers. Arc and Zif268 levels were also similar to saline-treated control levels when rats were given a prazosin and propranolol cocktail alone, or when each of these antagonists were delivered individually. Taken together, these data reveal that α_2 -AR uniquely exert a tonic inhibitory regulation of both Arc and Zif268 compared to α_1 and β -AR. However, the ability of RX821002 to increase Arc and Zif268 is interdependent with α_1 and β -AR signaling.

Keywords

norepinephrine; Arc; zif268; adrenergic; cortex

Introduction

Norepinephrine is a neurotransmitter that works by stimulating the α_1 , α_2 and β adrenergic receptor (AR) [1]. Signaling through ARs serves critical modulatory roles for cortical plasticity [2–4]. For example, ocular dominance plasticity is facilitated by infusing

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norepinephrine into the developing occipital cortex [2]. Other investigations have shown that increasing norepinephrine levels assists in functional recovery from brain injury [3]. Conversely, destroying noradrenergic innervation impairs the ability of an animal to recover from cortical injury, relative to animals with noradrenergic systems intact [4].

Norepinephrine's influential roles in cortex may lie, in part, in its regulation of immediate early gene (IEG) expression [5–9]. Activity Regulated Cytoskeleton Associated Protein (Arc), for instance, is classified as an IEG due to its rapid activation by robust synaptic stimulation through long term potentiation (LTP) and memory-related behavioral paradigms [10]. Arc is unique since neuronal activity results its rapid enrichment within dendrites of the cortex and hippocampus [11, 12]. In the cerebral cortex Arc plays an important role in experience-dependent homeostatic plasticity of excitatory synaptic transmission [13], and in consolidating the activity of neural networks involved in motor learning [14].

Another critical IEG for brain function is Zif268, which is also critical for LTP and long-term memory formation [15]. Zif268 may also be important for endowing the brain with resiliency and repair capacity. For example, Zif268 is decreased in the cortex of depressed humans and in mouse models of depression [16]. Noradrenergic lesions deplete Zif268 [8, 9], and also cause the cortex to be vulnerable to neurotoxic insult [17]. Alternatively, optogenetic stimulation increases cortical Zif268 levels in mice in association with an antidepressant effect [16]. The overexpression of Zif268 also accompanies long-term functional recovery after cortical injury [18].

Given the potential importance of Arc and Zif268 to norepinephrine's regulation of cortical plasticity, we further characterized the noradrenergic regulation of these critical IEGs. For example, RX821002 is a α_2 -AR receptor antagonist that induces brain IEG expression [5, 19], demonstrating that α_2 -AR exert a tonic inhibitory regulation of IEG levels. In contrast to α_2 -AR, the role that α_1 and β -AR play in regulating basal Arc expression, however, has not been examined. Moreover, although ARs frequently work in coordinated manner to regulate plasticity [20–22], how α_1 , α_2 and β -AR integrate their signaling to modulate Arc and Zif268 has not been well studied.

In these experiments cortical Arc and Zif268 expression were therefore measured after delivering individual as well as combined AR antagonist treatments. Our data reveal distinctive roles for α_2 versus α_1 and β -AR in regulating Arc and Zif268. These data further characterize a coordination of these receptors to modulate levels of these key IEGs.

Materials and Methods

Animals and Adrenergic Receptor Antagonist Treatment

Sprague-Dawley rats (Sasco, Kingston, NY) were bred in our colony. Rats (250–300g) received i.p. injections of vehicle alone or drugs at the following dosages: RX821002 (α_2 -AR antagonist, 5mg/kg), prazosin (α_1 -AR antagonist, 15mg/kg), propranolol (β -AR antagonist, 10mg/kg) (n=4–6 animals per group). RX821002 and propranolol were dissolved in sterile saline immediately prior to use while prazosin was dissolved in 75% glycerol. After injection, animals were allowed to sit quietly in their home cage for 1hr and then taken

to a separate room where they were killed by decapitation under isoflourane anesthesia and brains were removed. Animals were handled for twice a day for 2 days prior to injection in order to minimize the effects of injection stress alone. All animal use procedures were in strict accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Nebraska Medical Center Animal Care and Use Committee.

***In Situ* Hybridization**

Sixteen micron tissue sections were cut in a cryostat and thaw-mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were fixed in ice cold 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4, for 5 min, washed in PBS for 1 min and in 75% ethanol for 2 min, and stored in 100% ethanol at 4 °C. Slides were allowed to dry at room temperature prior to *in situ* hybridization. Oligonucleotide probe sequences were as follows; Arc: 5'-CTT-GGT-TGC-CCA-TCC-TCA-CCT-GGC-ACC-CAA-GAC-TGG-TAT-TGC-TGA-3' (complementary to bases 789–833, NM_019361.1) and zif268: 5'-CCG-TTG-CTC-AGC-AGC-ATC-ATC-TCC-TCC-AGT-TTG-GGG-TAG-TTG-TCC-3' (complementary to bases 355–399, NM_012551.1). A Blast search of Genbank found that these sequences do not have significant homology with any other sequences. Probes were 3' end labeled with [³⁵S]-dATP (1200 Ci/mmol, Perkin Elmer, Boston, MA) using terminal deoxyribonucleotidyl transferase (3' End Labelling System, Perkin Elmer). Labeling reactions were purified of unincorporated radionucleotide with Biospin-6 chromatography columns (BioRad, Hercules, CA) by centrifuging at 2500 rpm for 5 min. Hybridization buffer (150 µl of 50% formamide, 4×SSC, 8% dextran sulfate, 1×Denhardt's, 500 µg/mL salmon sperm DNA, 270 µg/mL yeast tRNA, 0.1 mM DTT) containing 1×10⁶ cpm of labeled probe was applied to each slide. Non-specific labeling was determined by inclusion of 10× unlabeled probe. Slides were coverslipped, sealed with D.P.X. (Aldrich Chemical Co., Milwaukee, WI) and placed overnight in a 1×SSC humidified sealed Tupperware container at 42 °C. The next day coverslips were removed in 55°C 1×SSC and slides were washed 4×15min in 1×SSC at 55 °C. After washing, slides were briefly dipped in room temperature water to remove salts and in 70% ethanol to facilitate drying. Slides were apposed to Biomax film (Kodak, Rochester, NY) for 2–3 weeks. Films were developed using standard techniques and analyzed using the MCID-M7 image analysis system (Interfocus Imaging, Ltd., Linton, England).

Image Analysis

Autoradiographic densities were quantified using commercial tritium standards (American Radiochemicals, St. Louis, MO) that were previously calibrated to ³⁵S [23]. Cortical expression was measured at a coronal level 0.7 mm anterior to the bregma.

Statistics

Studies examining the effects of adrenergic antagonists on IEGs across cortical layers were analyzed by two-way ANOVA with the Bonferonni posthoc test. For graphical presentation these data are presented as percentage increase over the average basal expression level.

Results

RX821002 Produces Increases In Arc and Zif268 That are Blocked by a Prazosin and Propranolol Cocktail

In these experiments we first studied the response of Arc and Zif268 to RX821002 (α_2 -AR antagonist) delivered alone, or with prazosin (α_1 -AR antagonist) and propranolol (β -AR antagonist) as a cocktail. We also tested the response of Arc and Zif268 to a prazosin and propranolol cocktail alone.

We found that Arc was rich in layers 4 and 6 of cortex, and showed many changes to adrenergic receptor antagonist treatment. Statistical analysis showed an effect for both cortical layer ($F_{(3,152)}=13.74$, $p<0.001$) and drug treatment ($F_{(3,152)}=81.43$, $p<0.0001$). Injecting RX821002 caused a ~50% increase in Arc expression across cortical layers. In contrast, to increases in Arc caused by RX821002, delivering a prazosin and propranolol cocktail with RX821002 decreased expression to saline-treated control values (Figures 1 and 2A). A prazosin and propranolol cocktail, alone, did not cause statistically significant changes in Arc expression (Figure 2A).

Similar to Arc, cortical Zif268 was enriched in layers 4 and 6 of cortex and increased with injections of RX821002. Statistical analysis showed an effect for drug treatment ($F_{(3,107)}=23.02$, $p<0.0001$) and cortical layer ($F_{(3,107)}=3.823$, $p<0.05$), RX821002 caused a ~50% increase in Zif268. A prazosin and propranolol cocktail delivered with RX821002, however, eliminated Zif268 increases. A prazosin and propranolol cocktail, delivered alone, resulted in no change in Zif268 expression (Figure 2B).

RX821002 Produces Increases In Arc and Zif268 That are Blocked by Prazosin and Propranolol Delivered Individually

Our next experiments studied the response of Arc and Zif268 to RX821002 delivered in combination with prazosin or with propranolol, individually. We also examined the response of basal Arc and Zif268 to prazosin or propranolol, each delivered individually.

Statistical analysis of Arc expression showed an effect for both cortical layer ($F_{(3,165)}=55.48$, $p<0.001$) and drug treatment ($F_{(5,165)}=126.81$, $p<0.0001$). Consistent with our initial experiment, injecting RX821002 caused a ~50% increase in Arc expression across cortical layers. RX821002 delivered with prazosin, though, decreased Arc levels to saline-treated control values. An exception was cortical layer 4 where Arc expression remained above control values (Figure 3A). RX821002 delivered with propranolol similarly decreased Arc levels that to saline-treated control values across cortical layers. Prazosin or propranolol, each delivered individually, did not cause any changes in basal Arc expression within any cortical layer (Figure 3A).

Statistical analysis of Zif268 expression showed an effect for cortical layer ($F_{(3,168)}=17.01$, $p<0.001$) and drug treatment ($F_{(5,168)}=77.38$, $p<0.0001$). RX821002 delivered with prazosin decreased zif268 to saline-treated control values in layers 1–3 and 5, while zif 268 levels in layer 4 and 6 remained elevated compared to saline-treated controls. RX821002 delivered with propranolol, however, decreased Zif268 to saline-treated control values across cortical

layers. Prazosin or propranolol, each delivered individually, did not cause any changes in Zif268 expression within any cortical layer (Figure 3B).

Discussion

In our studies the α_2 -AR antagonist, RX821002, produced significant increases in Arc and Zif268 mRNA in cerebral cortex of the rat. Injecting RX821002, with a prazosin and propranolol cocktail, abolished this response. When testing the effects of prazosin or propranolol, individually, propranolol was most effective at eliminating the Arc and Zif268 increases to RX821002. However, prazosin also blocked the response in many cortical layers. Collectively, these data show that modulation of Arc and Zif268 by RX821002 is dependent upon α_1 and β -ARs.

RX821002 is an α_2 -AR antagonist [24]. Because α_2 -AR function as important presynaptic inhibitory receptors of norepinephrine release, the interaction observed between α_1 , α_2 and β -AR indicates that RX821002 is acting on presynaptic α_2 -ARs to increase synaptic norepinephrine [25]. This synaptic norepinephrine would then act on postsynaptic α_1 and β to increase Arc and Zif268 levels. This interpretation is consistent with other findings from our lab showing that noradrenergic deinnervation abolishes IEG responses to RX821002 [5].

Prior studies have suggested that individual IEGs may be differentially regulated by adrenergic receptor type [26]. However, in our experiments we show that, in many cortical layers, giving RX821002 in the presence of either prazosin or propranolol alone blocks the Arc and Zif268 response as effectively as combining it with both of these drugs simultaneously. One possibility is that IEG increases caused by RX821002 are due to a level of post-synaptic excitation that relies upon a combination α_1 -AR and β -AR stimulation. This is suggested by experiments showing that Arc increases caused by RX821002 require excitatory neurotransmission [19], that may be downstream of α_1 -AR and β -ARs [27, 28]. Alternatively, the inability to attribute the IEG response exclusively to either α_1 or β -ARs may reflect a confluence of other α_1 -AR and β -AR signaling cascades that are important for downstream effects on IEG transcription. For instance, α_1 -ARs mediate biological processes due to their crosstalk with β -ARs [29]. By analogy, normal IEG regulation by adrenergic receptors may require an integration of α_1 and β -AR signaling cascades.

While Arc and Zif268 increased to α_2 -AR blockade, they were unaffected by combined or individual α_1 and β -AR blockade one hour after their administration. This lack of response to acute disruptions in α_1 and β -ARs is in contrast to IEG decreases caused by persistent depletion of brain norepinephrine when adult animals are treated with DSP4 [8, 9]. Taken together, these data suggest that a prolonged interruption in norepinephrine signaling through α_1 and β -ARs is necessary to decrease Arc and Zif268 levels in adult animals. This observation is consistent with other time-dependent adaptations that are secondary to interference in noradrenergic signaling. For example, adrenergic receptors show time-dependent patterns of reorganization secondary to norepinephrine depletion [30, 31].

This study has limitations. One caveat is that RX821002 has an affinity for serotonin receptors [24, 32, 33]. Furthermore, serotonergic manipulations have been shown to increase

Arc mRNA [34, 35]. Therefore, RX821002 could be increasing IEG levels through its action on serotonergic receptors. Additionally, the responses observed may, in part, reflect the systemic actions of the compounds as opposed to their direct effects on central neurotransmitter systems. We believe these are unlikely possibilities for two reasons. First, we have previously shown that central noradrenergic deinnervation abolishes IEG responses to RX821002 [5]. Second, we have found that the Arc and Zif268 response is mimicked in mice with a selective deletion in the α_{2A} -AR subtype [36]. Combined with our present data, these findings point to a central noradrenergic mechanism in mediating IEG responses to RX821002 as opposed to effects on other neurotransmitter systems or systemic effects.

Clinically, adrenergic receptors are commonly targeted by psychotropics. [37–40] and preclinical models suggest that they have cooperative roles in conferring therapeutic effects [20–22]. The α_2 -AR antagonist, mirtazapine, has anxiolytic and antidepressant effects in rodent models that require α_1 or β -AR signaling [21, 22]. Similarly, mirtazapine improves motor dysfunction in a mouse model of Parkinson's disease that depends upon concurrent α_1 -AR stimulation [20]. In light of our findings, these therapeutic effects may rely on an α_2 -AR regulation of IEG expression that depends on α_1 or β -AR signaling,

In summary, given the importance of norepinephrine to neuroplasticity, and the abundance of psychotropics with adrenergic properties [37–40], it is crucial to study the adrenergic receptor regulation of Arc and Zif268. Our experiments show that adrenergic receptors are interdependent in their regulation of these key IEGs. Clinically, these data may provide a foundation for understanding how medications with adrenergic receptor antagonist properties regulate cortical plasticity to impart neurotherapeutic effects.

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Abbreviations

Arc	activity regulated cytoskeleton associated protein
AR	adrenergic receptor
IEG	immediate early gene
LTP	long term potentiation
PBS	phosphate-buffered saline
RX821002	2-[2-(2-methoxy-1,4-benzodioxanyl)]imidazoline hydrochloride
DSP4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride

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Research Highlights

- α_2 -AR uniquely exert an inhibitory regulation of both Arc and Zif268 compared to α_1 and β -AR.
- The α_2 -AR antagonist, RX821002, increases Arc and Zif268 interdependent with α_1 and β -AR.
- A lack of adult IEG response to combined α_1 and β -AR blockade contrasts with decreases to N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP4).

Figure 1. *In situ* hybridization autoradiographs for Arc

These images correspond to 0.7 mm anterior to bregma in adult animals. The greatest enrichment for Arc mRNA is seen in layers 4 and 6. RX821002 causes increases in Arc mRNA that are blocked by co-administration of prazosin and propranolol. The scale bar indicates the density of mRNA calibrated for nCi/mg tissue.

Figure 2. The effects of RX821002 (5mg/kg) alone, or combined with prazosin (15mg/kg) and propranolol (10mg/kg), or the effects of prazosin and propranolol cocktail on Arc (A) and Zif268 (B) expression

Graph shows changes in Arc and Zif268 1hr after administration of adrenergic receptor antagonists. Each graph shows percent change in IEG levels relative to saline treated controls. Data are quantified according to cortical layer and analyzed by two-way anova with Bonferroni post-test. * $p < 0.05$, *** $p < 0.001$: significantly different from control.

Figure 3. The effects of RX821002 (5mg/kg) alone, or combined with prazosin (15mg/kg) or propranolol (10mg/kg) individually, or the effects of prazosin or propranolol individually on Arc (A) and Zif268 (B) expression

Graph shows changes in Arc and Zif268 1hr after administration of adrenergic receptor antagonists. Each graph shows percent change in IEG levels relative to saline treated controls. Data are quantified according to cortical layer and analyzed by two-way anova with Bonferroni post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significantly different from control.