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Developmental DSP4 Effects on Cortical *Arc* Expression

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Abstract

Activity Regulated Cytoskeleton Associated Protein (*Arc*) is an immediate early gene that is critical to brain plasticity. In this study, norepinephrine's regulation of *Arc* expression was examined during different stages of postnatal development. Rats were injected with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4), a selective noradrenergic neurotoxin, during preadolescence (PND 0 or 13), adolescence (PND 23 or 48) or adulthood (PND 60). After each DSP4 treatment, brains were harvested later in development and *Arc* mRNA levels analyzed with *in situ* hybridization. Rats lesioned with DSP4 during preadolescence showed no differences in *Arc* level compared to saline treated controls. In contrast, adolescence was a time of changing *Arc* mRNA response to DSP4. Rats lesioned during early adolescence showed *Arc* expression increases, while rats lesioned during late adolescence showed dramatic *Arc* expression decreases. Decreases in *Arc* level caused by late adolescent DSP4 were similar to those found in lesioned adults. These findings highlight a qualitatively different regulation of *Arc* expression by norepinephrine according to developmental stage, and indicate that mature regulation is not intact until late adolescence. These data point to important developmental differences in norepinephrine's regulation of brain plasticity. These differences may underlie contrasting psychotropic responses in children and adolescents compared to adults.

Keywords

norepinephrine; *Arc*; DSP4; development; cortex

Introduction

Norepinephrine is a neurotransmitter that plays influential roles within the developing brain through its stimulation of the α_1 , α_2 and β adrenergic receptor (AR) [1-4]. Seminal studies described important roles for neonatal norepinephrine in regulating synaptic density and

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dendritic length in cortical neurons [1, 3]. Other studies found that norepinephrine is important for modulating dendritic branching and the dendritic orientation of developing cortical neurons [3, 4]. Further research has indicated that norepinephrine may be critical for guiding the development of specific brain regions. Noradrenergic receptors and transporters, for instance, are enriched in many specific brain areas during times of early brain development and dramatically decrease in expression with maturity, suggesting important developmental roles [5, 6]. More recently, the noradrenergic system has been characterized by differences in its regulation of the developing versus mature brain [7-9]. The regulation of adrenergic receptors by norepinephrine, for example, differs according to developmental stage [7].

To further characterize norepinephrine's role in the developing brain, its postnatal modulation of Activity Regulated Cytoskeleton Associated Protein (*Arc*) was studied. *Arc* is an immediate early gene (IEG) that is of critical importance to synaptic plasticity and brain function [10, 11]. Neural activity results in *Arc*'s rapid enrichment in dendrites where it plays a role in synaptic strengthening [10-12]. *Arc* expression is increased during LTP and its disruption in the hippocampus interferes with learning and memory [13]. In the developing cerebral cortex *Arc* is essential for experience-dependent plasticity [14, 15]. These effects for *Arc* on neural plasticity may be clinically important to the pathophysiology and treatment of conditions such as depressive disorders. For example, *Arc* is decreased in the cortex of depressed humans and in rodent models of depression [16-18]. Conversely, antidepressant medication induces *Arc* expression in brain regions including the cerebral cortex [17, 19].

In adult rats, noradrenergic signaling plays a critical role in regulating *Arc* expression. For example, the α_2 -AR exerts a tonic inhibitory regulation of *Arc* level that is interdependent with α_1 and β -AR signaling [20]. The importance of norepinephrine to regulating *Arc* expression is further reflected in its maintenance of basal *Arc* levels. Lesioning adult rats with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP4), a selective noradrenergic neurotoxin, results in profound decreases in basal *Arc* expression [21]. The postnatal ontogeny of *Arc* regulation by norepinephrine, though, and how it manifests during crucial times of brain development, has not been well examined.

In this study, the developmental maintenance of basal *Arc* expression by norepinephrine was investigated. Rats were injected with DSP4 during preadolescence, adolescence or adulthood. Brains were harvested later in postnatal development and *Arc* levels analyzed with *in situ* hybridization. These data highlight a qualitatively different regulation of basal *Arc* expression by norepinephrine according to developmental stage.

Materials and Methods

Materials

N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP4) was purchased from Sigma (St. Louis, MO). [^3H]Nisoxetine (80 Ci/mmol) and [^{35}S]-dATP (1200 Ci/mmol) were obtained from Perkin Elmer Life Sciences (Boston, MA, USA). *In situ* hybridization

reagents were molecular biology grade and from Sigma Aldrich. All other chemicals were research grade.

Animals

Sprague-Dawley rats (Sasco, Kingston, NY) were bred in our colony. Rats of differing developmental ages received an i.p. injection of sterile saline alone or 50mg/kg of DSP4 (n=4-6). After injection, rats were returned to their home cage and brains harvested ~2-3 weeks later. This interval was chosen since this laboratory has confirmed a near complete loss of norepinephrine and noradrenergic innervation using a similar time frame [7]. Rats were taken to a separate room where they were killed by decapitation under isoflurane anesthesia and brains were removed, frozen on dry ice and stored at -80°C. 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. Studies were designed to minimize the number of animals used and their pain and suffering.

In situ hybridization

In situ hybridization to *Arc* mRNA was performed according to published methods [9, 20]. Sections 16 µm thick were thaw-mounted onto Superfrost Plus slides and stored at -80 °C (Fisher Scientific, Pittsburgh, PA). Sections were fixed in ice cold 4% paraformaldehyde and hybridized with oligonucleotide probe sequence to *Arc* mRNA. The oligonucleotide probe sequence was as follows; *Arc*: 5'-CTT-GGT-TGC-CCA-TCC-TCA-CCT-GGC-ACC-CAA-GACTGG-TAT-TGC-TGA-3'. Probes were 3' end labeled with [³⁵S]-dATP using terminal deoxyribonucleotidyl transferase (3' End Labeling System, Perkin Elmer). Hybridization buffer containing 1×10⁶ cpm of labeled probe was applied to each slide. Slides were coverslipped, sealed with D.P.X. (Aldrich Chemical Co., Milwaukee, WI) and placed overnight in a 1XSSC humidified sealed Tupperware container at 42 °C. The next day coverslips were removed in 55°C 1XSSC and slides were washed 4×15min in 1XSSC at 55 °C. Slides were apposed to Biomax film (Kodak, Rochester, NY) for 2–3 weeks. Nonspecific background was determined by inclusion of 10x unlabeled *Arc* probe. This resulted in a near complete loss of signal. The background was subtracted from quantification. At each developmental timepoint the brains from DSP4 and saline treated rats were analyzed for *Arc* in the same assay. Brains collected at different developmental timepoints, however, were processed in differing assays.

[³H]Nisoxetine autoradiography

Sections 16 µm thick were thaw-mounted onto subbed slides and stored at -80 °C. Sections were incubated in 10 mM Na₂HPO₄, 300 mM NaCl and 5 mM KCl, pH 7.4, containing 2 nM [³H]nisoxetine for 4 h at 4 °C. Following labeling, sections were washed in three separate 5 min washes with ice-cold incubation buffer and briefly dipped in ice-cold water to remove salts. Sections were then dried under a stream of cool air and slides were apposed to tritium-sensitive film for 8 weeks.

Image Analysis

Autoradiographic films were developed using standard techniques and analyzed using the MCID-M7 image analysis system (Interfocus Imaging, Ltd., Linton, England). After film processing, tissue slides were stained with cresyl violet. For *Arc* analysis, cresyl violet stained tissue sections were studied for cortical layers and the corresponding region was measured for the *Arc* signal on x-ray film. Cortical layers were defined as previously described [22]. Autoradiographic densities were quantified using commercial tritium standards (American Radiochemicals, St. Louis, MO) that were previously calibrated to ^{35}S [23].

Statistics

A student's t-test was used to compare *Arc* levels between saline versus DSP4 treated animals of comparable age. For graphical presentation these data are presented as percentage increase over the average saline expression level.

Results

The response of *Arc* was assessed after DSP4 was delivered on postnatal day (PND) 0, 13, 23, 48 or 60. These ages were chosen since they represent important stages of brain development. In rats, preadolescence lasts from PND0 until the time of weaning on PND 21. During this developmental period neuronal division and migration is complete and a robust increase in synaptogenesis begins near the end of the second postnatal week [24]. Adolescence lasts from approximately PND21 to PND59. It is a time of unique synaptic and behavioral plasticity [25, 26] and is characterized by peak cortical synaptogenesis [24]. Rats PND 60 and beyond are considered adults [27, 28].

For each experimental group, brains were harvested ~2-3 weeks after DSP4 treatment. *Arc* levels were then analyzed with *in situ* hybridization and quantified with image analysis (Figure 1A). Animals were lesioned within a developmental window where DSP4 successfully removes noradrenergic innervation from the cerebral cortex [7]. Prior studies have confirmed this using autoradiographic analysis with [^3H]nisoxetine, a highly specific ligand for noradrenergic transporters (NET) and hence noradrenergic terminals. NET elimination in cortex may be used as an indicator of lesion completeness. This is because NET elimination follows a parallel and comparable loss of norepinephrine as determined by HPLC [7].

In the current study, a depletion of NET was confirmed with [^3H]nisoxetine autoradiography for rats lesioned with DSP4 on PND0 and PND60 (Figure 1B). A depletion of NET was also confirmed for additional timepoints, as reported in a previous publication [7]. These combined studies confirm that DSP4 results in a very reliable lesion of the developing and mature noradrenergic system.

DSP4 administered during preadolescence resulted in no difference in *Arc* expression. Animals treated with DSP4 on PND0 or on PND13, displayed minimal *Arc* mRNA response to noradrenergic de-innervation (Figure 2 A, B and Figure 4). In contrast to the minimal *Arc* mRNA response to preadolescent DSP4, this treatment during adolescence resulted in many

changes in cortical *Arc* level. Furthermore, the direction of these changes differed according to whether animals were lesioned in early versus late adolescence. DSP4 treatment in early adolescence (PND23) resulted in *Arc* expression that increased ~30-45% across cortical layers ($p < 0.05$ for each cortical layer, Figure 2C). Dissimilarly, animals treated with DSP4 in late adolescence (PND48) showed ~60% decreases in *Arc* level ($p < 0.001$ for each cortical layer, Figure 2D). These *Arc* expression decreases to late adolescent DSP4 were similar to ~60% decreases in *Arc* expression in DSP4 treated adults ($p < 0.001$ for each cortical layer, Figure 3 and Figure 4).

Discussion

In adult rats norepinephrine is essential to maintaining the basal expression of *Arc*. The postnatal ontogeny of this regulation, though, has not been charted. To probe the developmental regulation of *Arc* level by norepinephrine, rats were injected with DSP4, a selective noradrenergic neurotoxin, during brain development. Depleting brain norepinephrine had differing effects on *Arc* mRNA level according to whether it occurred in pre-, early, late or post-adolescence.

In pre-adolescence, DSP4 treated animals exhibited no difference in *Arc* expression compared to saline treated controls. This contrast from the adult response is consistent with many studies showing that the neonatal noradrenergic system is very different from the mature state [5, 7-9, 29]. In the neonatal brain, the α_2 -AR and IEGs are differentially expressed and regulated by norepinephrine compared to the adult brain [5, 6, 9]. The developmental response to specific adrenergic receptor agonists also differs in the neonate compared to the adult. Clonidine, a α_2 -AR agonist, has a well-documented role as an anti-epileptic in adult rats. In early preadolescent development, however, clonidine facilitates amygdaloid kindling [30]. Differences in the intracellular signaling of the α_2 -AR have also been shown. In the neonatal mouse hippocampus, α_2 -AR agonists will stimulate ERK phosphorylation, an effect that is not seen in the adult hippocampus [31]. These developmental differences are also seen for the β -AR. The β -AR exhibits developmental differences in its response to prolonged agonist stimulation [32]. Other studies of noradrenergic development have shown that neonatal lesions cause a decrease in phosphodiesterase, whereas adult lesions leave the enzyme unaffected [33].

Although preadolescent DSP4 did not alter *Arc* expression, it increased *Arc* level when given in early adolescence, and decreased *Arc* level when given in late adolescence. The decreases in *Arc* level caused by late adolescent DSP4 were similar to those found in lesioned adults. These data suggest that norepinephrine's regulation of cortical circuitry is undergoing important functional changes as the brain transitions towards maturity during adolescence. Developmental patterns of cortical synaptogenesis, for instance, may be critical to this maturation. The observed timeline for mature noradrenergic regulation of *Arc* correlates with the attainment of adult synaptic densities [24, 34]. During the first month of postnatal development the cortex undergoes robust increases in synaptic density. Around early adolescence, from PND 25-30, this density peaks. A decline in synaptic density occurs between PND 30 and 60, at which point the adult density of synapses is achieved [24]. In this paper the adult regulation of *Arc* expression by norepinephrine did not occur until late

adolescence, a time when peak synaptogenesis has occurred and synapses are being eliminated. Therefore, it may be that neural changes conferred by the adolescent processes of synapse formation and pruning, are necessary for attaining the adult regulation.

The immaturity of the adolescent noradrenergic system, as characterized in this study and in others [35], may contribute to its vulnerability to environmental stressors or pharmacological interventions. Adolescent social stress, for example, leads to persistent activation of the locus coeruleus [36], and altered coherence with the prefrontal cortex [37]. Methylphenidate treatment during adolescence alters cortical norepinephrine transporter function in adults [38]. Adolescent exposure to the norepinephrine reuptake inhibitor, atomoxetine, sensitizes adult responses to amphetamine [39].

Collectively, these data highlight important differences in the noradrenergic system according to stage of postnatal development. Continued research to elaborate such developmental differences is clinically important to understanding and treating psychopathology within developing populations. Anxiety and depressive disorders, for example, frequently emerge during adolescence [40, 41] with limited pharmacological treatment options during this stage of development [42]. Indeed, many antidepressants used in childhood and adolescence have FDA black-box warnings regarding their potential for increasing suicidal thoughts [43]. The challenges associated with treating these conditions may be due to qualitative differences in developing versus mature neurotransmitter systems as highlighted in this study.

Norepinephrine, in particular, is implicated in the pathogenesis of anxiety and major depressive disorder [44, 45]. Tricyclic antidepressants targeting the noradrenergic are effective in the treatment of adult depression but may not be effective in children [8, 35]. In light of the data in this paper, differential responses to antidepressants in children versus adults may be due to fundamental differences in noradrenergic regulation of brain plasticity across postnatal development. An appreciation of qualitative differences in the developing versus mature brain is important for clinicians and researchers whose aim is to treat and understand psychopathology across the lifespan.

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Abbreviations

Arc	activity regulated cytoskeleton associated protein
IEG	immediate early gene
LTP	long term potentiation
PBS	phosphate-buffered saline
DSP4	N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride
PND	postnatal day

NET norepinephrine transporter

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Research Highlights

- *Arc* mRNA is differentially regulated by norepinephrine at different ages.
- There is an absence of *Arc* mRNA response to DSP4 in preadolescence.
- There is a changing *Arc* mRNA response to DSP4 in adolescence.
- Mature noradrenergic regulation of *Arc* mRNA is not intact until late adolescence.
- *Arc* mRNA decreases in DSP4 lesioned adult rats.

Figure 1. Diagram of experimental design and images of NET depletion by DSP4

A) Rats were treated with DSP4 (50mg/kg) or sterile saline during preadolescence (PND 0 or 13), early adolescence (PND 23), late adolescence (PND 48) or adulthood (PND 60). Brains harvested ~2-3 weeks later and processed with in situ hybridization to measure *Arc* mRNA. **B)** DSP4 successfully destroyed noradrenergic terminals in development and in adulthood. Representative images are of NET in rats treated with saline versus DSP4 on PND0 or PND60. PND=postnatal day, NET=norepinephrine transporter. The scale bar for NET indicates its density calibrated for fmole/mg tissue.

Figure 2. Developmental DSP4 effects on *Arc* expression

A) DSP4 treatment on PND0 did not change *Arc* level on PND25. **B)** DSP4 treatment on PND13 did not change *Arc* level on PND35. **C)** DSP4 treatment on PND23 increased *Arc* expression on PND45 **D)** DSP4 on PND48 decreased *Arc* expression on PND62. * $p < 0.01$, *** $p < 0.001$. PND=postnatal day.

Figure 3. Adult DSP4 effects on *Arc* expression

DSP4 treatment on PND60 resulted in profound decreases in *Arc* level on PND 74.

*** $p < 0.001$.

Figure 4. Images of *Arc* mRNA for rats treated with DSP4 at PND0 versus PND60

Rats were treated with sterile saline or 50mg/kg of DSP4 at PND0 or PND60 and sacrificed ~2-3 weeks later. *Arc* expression was not affected when DSP4 was delivered at PND0, but was decreased when delivered at PND60. The scale bar for *Arc* indicates the mRNA density calibrated for nCi/mg tissue.