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Nonenzymatic Role of Acetylcholinesterase in Neuritic Sprouting: Regional Changes in Acetylcholinesterase and Choline Acetyltransferase After Neonatal 6-Hydroxydopamine Lesions

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Abstract

Acetylcholinesterase (AChE) is postulated to play a nonenzymatic role in the development of neuritic projections. We gave the specific neurotoxin, 6-OHDA to rats on postnatal day (PN) 1, a treatment that destroys noradrenergic nerve terminals in the forebrain while producing reactive sprouting in the brainstem. AChE showed profound decreases in the forebrain that persisted in males over the entire phase of major synaptogenesis, from PN4 through PN21; in the brainstem, AChE was increased. Parallel examinations of choline acetyltransferase, an enzymatic marker for cholinergic nerve terminals, showed a different pattern of 6-OHDA-induced alterations, with initial decreases in both forebrain and brainstem in males and regression toward normal by PN21; females were far less affected. The sex differences are in accord with the greater plasticity of the female brain and its more rapid recovery from neurotoxic injury; our findings indicate that these differences are present well before puberty. These results support the view that AChE is involved in neurite formation, unrelated to its enzymatic role in cholinergic neurotransmission. Further, the results for choline acetyltransferase indicate that early depletion of norepinephrine compromises development of acetylcholine systems, consistent with a trophic role for this neurotransmitter.

Keywords

6-hydroxydopamine; acetylcholine; acetylcholinesterase; neuritic sprouting; norepinephrine; trophic effects

Introduction

During brain development, neurotransmitters serve important trophic functions that control neuronal cell replication and differentiation, synaptic organization, and morphologic assembly of brain nuclei [19,54,55]. Recent work suggests that these unique developmental roles extend to proteins that are ordinarily associated with synaptic communication, notably acetylcholinesterase (AChE). Membrane-anchored AChE is a structural protein that is essential

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for axonal outgrowth [5], synaptogenesis [50], cell adhesion [4] and neuronal migration [9, 18], unrelated to its enzymatic role in hydrolyzing acetylcholine (ACh) [49]. Indeed, the functional moieties of the AChE molecule that participate in axonogenesis and other developmental processes appear to be remote from the catalytic core [7,26,29,44].

To date, most studies of the nonenzymatic functions of AChE have involved in vitro models [5,8,16,17,28,50]. The current study instead focuses on an in vivo model, 6-hydroxydopamine (6-OHDA) treatment in the neonatal rat, that elicits reproducible and well-characterized regional patterns of neuritic damage and reactive sprouting. 6-OHDA is a specific neurotoxin that destroys catecholaminergic nerve terminals, and when administered systemically to newborn rats, penetrates the immature blood-brain barrier [25,31]. Treatment on the first postnatal day (PN1) results in 75% depletion of norepinephrine in the forebrain within 24h of treatment, persisting into adulthood [31,53]. However, no depletion occurs in the brainstem, which instead shows reactive sprouting at the cell bodies [6,27,32,57]. Accordingly, if AChE does indeed play a role in neurite formation, we would expect to see loss of activity in the forebrain and subsequent increases in the brainstem; here, we conducted such evaluations spanning the immediate posttreatment period (PN2, PN4) and during the peak and completion of the formation of the majority of forebrain synapses (PN17, PN21) [12,13]. In addition, we contrasted the effects on AChE with parallel measurements of choline acetyltransferase (ChAT), a widely-used enzymatic marker for ACh nerve terminals. A postulated role for AChE in neurite formation does not entail effects that would be exclusive to ACh neurons, so we would therefore expect a dichotomy in the impact on the two markers. Further, effects of neonatal 6-OHDA lesioning on ChAT can provide corroborating evidence for a specific trophic role of norepinephrine itself in forebrain assembly, as postulated in earlier work [19,30,39, 41]. Finally, we examined sex differences in the response to neonatal 6-OHDA lesioning. Females generally recover from neurotoxic insults more readily because of greater synaptic plasticity as compared to males [1,21,36,38,46,52], and we determined if this was similarly reflected in the responses of AChE to neonatal lesioning.

Materials and Methods

All studies were performed humanely and were approved by the Duke University Institutional Animal Care and Use Committee. Twenty timed-pregnant Sprague-Dawley rats (Zivic Laboratories, Pittsburgh, PA) were shipped by climate-controlled truck (transit time, 12 h) and were housed individually, with a 12h light-dark cycle and free access to food and water. On the day after birth, all pups were randomized and redistributed to the nursing dams with a litter size of 10 pups. Animals in 10 litters were then given a subcutaneous injection of 6-OHDA HBr (150 mg/kg body weight; Sigma Chemical Co., St. Louis, MO), whereas the remaining 10 litters received equivalent volumes (1 ml/kg) of control injection vehicle, consisting of 0.9% saline plus 0.1% ascorbic acid. To minimize maternal effects on litter development, animals within each treatment group were randomized and redistributed to the nursing dams at intervals of several days. No more than one male and one female were selected from each litter at each age point. After selection, the randomization procedure was conducted so as to maintain litter sizes, reducing the total number of litters within each treatment group to 7 litters by the end of the study on PN21.

Animals were decapitated and the brains were dissected by removing the cerebellum (including peduncles) and dividing the forebrain from the brainstem with a cut rostral to the thalamus. Tissues were frozen in liquid nitrogen and stored at -45 °C. Tissues were homogenized and assayed for AChE and ChAT by standard techniques described in detail previously [20,42, 43,48]. For AChE, the substrate was 0.5 mM acetylthiocholine iodide (Sigma) and enzyme activity was assessed kinetically by spectrophotometry, using mercaptoethanol as a reference standard. For ChAT, each tube contained 50 µM [¹⁴C]acetyl-coenzyme A (PerkinElmer Life

Sciences, Boston, MA) as a substrate. Enzyme activities were then calculated relative to tissue protein [47].

Data were compiled as means and standard errors, and, to enable visual comparisons across different regions and measures, are presented as the percent change from the corresponding control group; however, statistical analyses were conducted on the original data. Although not shown here, the control values were similar to those reported previously [14,15,37,48,56]. To avoid an increased probability of type 1 errors from repeated testing of the data set, the results were first subjected to a global ANOVA, incorporating all variables in a single test: treatment, region, sex, age and enzyme measure, with the latter considered as a repeated measure, since both ChAT and AChE were measured in the same tissue sample. The data were log-transformed because of heterogeneous variance across the different tissues, ages and measures. Lower-order tests were then conducted as permitted by the interactions of treatment with the other variables; Fisher's Protected Least Significant Difference was used post-hoc to determine individual values that differed from the corresponding control. Significance was assumed at $p < 0.05$.

Results

The global statistical test (excluding PN2, where sex was not determined) indicated a significant main treatment effect ($F_{1,109}=19.5$, $p < 0.0001$) and interactions of treatment $\times$ age ($F_{2,109}=3.1$, $p < 0.05$), treatment $\times$ sex ($F_{1,109}=7.9$, $p < 0.006$), treatment $\times$ region ($F_{1,109}=19.7$, $p < 0.0001$), treatment $\times$ measure ($F_{1,109}=13$, $p < 0.0005$) and treatment $\times$ sex $\times$ measure ($F_{1,109}=5.4$, $p < 0.03$). After separating the two enzyme measures, interactions of treatment with the other variables were still present: AChE, treatment $\times$ age ($F_{2,110}=3.1$, $p < 0.05$), treatment $\times$ region ($F_{1,110}=23$, $p < 0.0001$), treatment $\times$ sex $\times$ region ($F_{1,110}=3.9$, $p < 0.05$); ChAT, treatment main effect ($F_{1,114}=18$, $p < 0.0001$), treatment $\times$ age ($F_{2,114}=3.2$, $p < 0.05$), treatment $\times$ sex ($F_{1,114}=8.9$, $p < 0.004$), treatment $\times$ region ($F_{1,114}=5.3$, $p < 0.03$). Accordingly, we subdivided the data into the individual values for each age, region and sex for presentation.

Neonatal 6-OHDA treatment evoked deficits in forebrain AChE that emerged by PN4 in males, continuing through the end of the third postnatal week (Figure 1A). Females were initially spared but then showed a later-emerging deficit that by PN21 was equivalent to that seen in males. Forebrain ChAT likewise showed substantially reduced values by PN4 but in this case involving both sexes (Figure 1B). Values showed partial recovery in males by PN17 and PN21, but total recovery in females. In the brainstem, AChE was not significantly reduced by 6-OHDA treatment and instead showed significant later-emerging *increases* (Figure 1C). For brainstem ChAT, there was an initial deficit of about half the magnitude seen in the forebrain, with eventual, complete recovery for both sexes (Figure 1D). Females then showed a rebound elevation by PN21.

As assessed by multivariate ANOVA (sex, region, age), control values for AChE and ChAT did not display any significant sex differences (data not shown), although the lack of significant differences may reflect the small number of animals used in this study.

Discussion

Neonatal lesioning with 6-OHDA elicited decreases in AChE in the forebrain and later-emerging increases in the brainstem. This pattern parallels the loss of noradrenergic projections in the terminal zones and reactive sprouting at the cell bodies, and is entirely consistent with a postulated role for AChE in neurite formation. Our findings thus provide one of the first confirmations of a nonenzymatic function of AChE in an *in vivo* model. Nevertheless, there were a number of unusual features that indicate an involvement of additional factors in the response to lesioning. First, the initial loss of AChE in the forebrain of males was 15%, far

more than the proportion of noradrenergic nerve terminals in the forebrain [11], which points to a more pervasive involvement than just the impact on this one neurotransmitter. Norepinephrine is itself an important trophic factor for forebrain assembly [19,30,39,41], and the lesioning treatment was given during the phase in which neurotransmitter inputs control the development of ACh systems [3,22,23]. In turn, this suggested that the noradrenergic lesion compromises the development of neuritic connections for other types ACh synapses, an hypothesis that we evaluated through measuring ChAT, a marker for ACh presynaptic terminals. We found large decrements in forebrain ChAT and lesser, but significant deficits in the brainstem; indeed, the magnitude of the forebrain effect exceeded that for AChE (note different scales in Fig. 1A vs. 1B). Our results thus afford support for a specific trophic role of norepinephrine in the development of forebrain ACh connections. This likely also explains why females showed a late-emerging deficit in forebrain AChE even though they were spared the initial effects of 6-OHDA: an indirect, but more widespread disruption of synaptic connections would be expected to emerge only during the spurt of synaptogenesis that occurs in the second to third postnatal weeks [12,13].

A second issue that needs to be addressed is the pronounced difference between males and females. Although the brainstem AChE sprouting response was similar, the forebrain effects in females were far less robust and recovered more quickly than in males. Many aspects of neuronal plasticity are promoted by estrogen receptor activation [51,52], and since sexual differentiation of the brain is initiated prenatally [33,35], our results are compatible with a superior recovery capacity in females. Nevertheless, as already noted for forebrain AChE, there are likely to be later-emerging deficits that reflect longer-term disruption of synaptic connections as a result of the neonatal lesion. Indeed, the overshoot for brainstem ChAT in females likely represents a consequence of such actions.

In conclusion, our results for neonatal 6-OHDA lesioning of catecholamine inputs, supply some of the first in vivo evidence to support a nonenzymatic function of AChE in neuritic outgrowth in the developing brain. Further, our observations of regional differences in the response of AChE and ChAT indicate a coordinated trophic role of norepinephrine acting together with AChE in forebrain assembly. However, there are more extensive ramifications of these findings. In studies of the developmental neurotoxicity of organophosphate and carbamate insecticides, AChE activity is commonly used as an indicator of persistent inhibition resulting from the direct effects of these agents [10,34,40]. Our findings indicate that long-term reductions or increases in AChE activity can instead reflect their actions on neurite formation [2,24,45], effects that are obviously of key importance in the neurobehavioral teratology of these agents.

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Abbreviations

6-OHDA	6-hydroxydopamine
ACh	acetylcholine
AChE	acetylcholinesterase
ANOVA	analysis of variance
ChAT	choline acetyltransferase
PN	postnatal day

Figure 1.

Effects of neonatal 6-OHDA treatment on ACh-related enzyme activities: (A) AChE in the forebrain, (B) ChAT in the forebrain, (C) AChE in the brainstem, (D) ChAT in the brainstem. Values represent means and standard errors obtained from 5-7 animals in each group, shown as the percentage change from control values. Asterisks below the panels denote significant main treatment effects at each age that do not show sex-selectivity; daggers within the panels denote significant treatment $\times$ sex interactions and indicate which sex is affected. Sex was not determined for the PN2 point.