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LESIONING NORADRENERGIC NEURONS OF THE LOCUS COERULEUS IN C57BL/6 MICE WITH UNILATERAL 6-HYDROXYDOPAMINE INJECTION, TO ASSESS MOLECULAR, ELECTROPHYSIOLOGICAL AND BIOCHEMICAL CHANGES IN NORADRENERGIC SIGNALING

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Abstract—The locus coeruleus (LC) is the major loci of noradrenergic innervation to the forebrain. Due to the extensive central nervous system innervation of the LC noradrenergic system, a reduction in the number of LC neurons could result in significant changes in noradrenergic function in many forebrain regions. LC noradrenergic neurons were lesioned in adult male C57Bl/6 mice with the unilateral administration of 6-hydroxydopamine (6OHDA) (vehicle on the alternate side). Noradrenergic markers were measured 3 weeks later to determine the consequence of LC loss in the forebrain. Direct administration of 6OHDA into the LC results in the specific reduction of noradrenergic neurons in the LC (as measured by electrophysiology, immunoreactivity and *in situ* hybridization), the lateral tegmental neurons and dopaminergic neurons in the substantia nigra (SN) and ventral tegmental region were unaffected. The loss of LC noradrenergic neurons did not result in compensatory changes in the expression of mRNA for norepinephrine (NE)-synthesizing enzymes. The loss of LC noradrenergic neurons is associated with reduced NE tissue concentration and NE transporter (NET) binding sites in the frontal cortex and hippocampus, as well as other forebrain regions such as the amygdala and SN. Adrenoreceptor (AR) binding sites (α_1- and α_2-AR) were not significantly affected on the 6OHDA-treated side compared to the vehicle-treated side, although there is a reduction of AR binding sites on both the vehicle- and 6OHDA-treated side in specific forebrain regions. These studies indicate that unilateral stereotaxic injection of 6OHDA into mice reduces noradrenergic LC neurons and reduces noradrenergic innervation to many forebrain regions, including the contralateral side. Published by Elsevier Ltd. on behalf of IBRO.

Key words: frontal cortex, hippocampus, norepinephrine transporter, tyrosine hydroxylase, dopamine β-hydroxylase, single-unit extracellular electrophysiology.

INTRODUCTION

The noradrenergic nervous system is a major neurotransmitter system in the periphery and in the brain where it is involved in many physiological and behavioral processes; therefore, the central noradrenergic nervous system plays a role in many pathologies including depression, epilepsy, Parkinson's disease (PD), Alzheimer's disease (AD), and post traumatic stress disorder (Szot et al., 1999, 2006; Ressler and Nemeroff, 2000; Weinshenker and Szot, 2002; Zarow et al., 2003; Fitzgerald, 2010; McMillan et al., 2011; Olson et al., 2011). The locus coeruleus (LC) contains the vast majority of noradrenergic cell bodies in the brain, which sends projections throughout the forebrain. Therefore, a loss of LC noradrenergic neurons can have a profound effect on the function of the brain. A loss of LC noradrenergic neurons is a major neuropathological characteristic of two neurodegenerative disorders, AD and PD (Bondareff et al., 1981; Tomlinson et al., 1981; Marcyniuk et al., 1986; Cash et al., 1987; Chan-Palay and Asan, 1989, 1991; German et al., 1992; Patt and Gerhard, 1993; Bertrand et al., 1997; Szot et al., 2006; Zarow et al., 2003; McMillan et al., 2011). How this neuronal loss impacts the progression of these disorders remains unclear. To understand the role of LC neuronal loss in the pathogenesis of these neurodegenerative disorders and other disorders involving the noradrenergic nervous system, it is important to determine the consequence of LC noradrenergic neuronal

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Abbreviations: 6OHDA, 6-hydroxydopamine; AD, Alzheimer's disease; Alpha, α; aCSF, artificial cerebrospinal fluid; Amy, amygdala; AR, adrenoreceptors; BNST, bed nucleus of the stria terminalis; CRF, cerebrospinal fluid; DA, dopamine; DBH, dopamine β-hydroxylase; DHPG, 3,4-dihydroxyphenylglycol; DOPA, 3,4-dihydroxyphenylalanine; DOPAC, dihydroxyphenylacetic acid; DSP4, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine; DTN, dorsal thalamic nucleus; FC, frontal cortex; Gen, geniculate; Hypo, hypothalamus; HP, hippocampus; ICV, intracerebroventricular; ISH, *in situ* hybridization; IR, immunoreactivity; LC, locus coeruleus; MCID, MicroComputer Imaging Device; NE, norepinephrine; NET, norepinephrine transporter; NTS, nucleus tractus solitaris; OD, optical density; PVTN, paraventricular thalamic nucleus; PBS, phosphate buffer; PD, Parkinson's disease; Sep, septum; SN, substantia nigra; Str, striatum; TH, tyrosine hydroxylase; Thal, thalamus; VTA, ventral tegmental area.

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loss on noradrenergic markers in the LC and at terminal forebrain regions. The consequences of LC neuronal loss alone have not been adequately defined.

The vast majority of research on LC neuronal loss has focused on the effects of the noradrenergic neurotoxin *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP4). DSP4 consistently results in a temporary reduction in NE tissue concentration in terminal forebrain regions (Ross, 1976; Jonsson et al., 1981; Grzanna et al., 1989; Theron et al., 1993; Wolfman et al., 1994; Kask et al., 1997; Hughes and Stanford, 1998; Szot et al., 2010), a temporary reduction in NE transporter (NET) binding sites in specific forebrain regions (Cheetham et al., 1996; Szot et al., 2010) and an increase in α_2-adrenoreceptor (AR) binding sites in specific forebrain regions (Wolfman et al., 1994; Szot et al., 2010). However, there is data indicating that DSP4 does not result in a loss of LC noradrenergic neurons but does cause these changes in noradrenergic forebrain markers (Booze et al., 1988; Lyons et al., 1989; Robertson et al., 1993; Matsukawa et al., 2003; Szot et al., 2010).

Another neurotoxin, 6-hydroxydopamine (6OHDA), has been shown to reduce LC noradrenergic neurons when administered directly into the lateral ventricles, but dopaminergic neurons in the substantia nigra (SN) and ventral tegmental area (VTA) are reduced as well (Descarries and Saucier, 1972; Coradazzi et al., 2010). Administration of 6OHDA directly into the LC also results in a significant loss of noradrenergic neurons (Harik, 1984; Biancardi et al., 2008), although a comprehensive analysis of the consequence of this loss on noradrenergic terminals has not been performed. The objective of this study is to determine the effect of unilateral 6OHDA in C57Bl/6 mice on LC neuronal loss and changes in forebrain noradrenergic markers 3 weeks after administration. Alterations in noradrenergic markers 3 weeks after 6OHDA administration would indicate long-term changes. Changes in noradrenergic terminals were assessed by: NET, α_1- and α_2-adrenoreceptor (AR) binding, and NE tissue concentration in specific forebrain regions. To assess the degree of LC neuron loss tyrosine hydroxylase-immunoreactivity (TH-IR), TH and dopamine β-hydroxylase (DBH) mRNA expression were measured in the same animals where catecholamine or binding assays were performed. Electrophysiological characteristics of viable LC neurons were measured in a separate set of animals.

EXPERIMENTAL PROCEDURES

Animals

Adult male C57Bl/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in standard enriched environment cages in a temperature-controlled room with a 12-h light/dark cycle. Food and water were provided *ad libitum*. The animals were given at least 2 weeks acclimating period to the facility before administration of 6OHDA. All animal procedures were in accordance with the Animal Care Committee at the VA Puget Sound Health Care System, Seattle, WA, Whitman College, Walla Walla, WA and National Institute of Health guidelines. The minimum number of animals was used for these studies and care was taken to minimize any suffering.

Surgery and LC microinjections

Mice were anesthetized with Isoflurane (Baxter Healthcare, Deerfield, IL, USA) and mounted in a Kopf Model 1900 stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA) with the skull balanced in the medial/lateral and anterior/posterior planes. Carprofen (5 mg/kg) and ceftriaxone (50 mg/kg) were administered to animals before incision to prevent pain and infection. 6OHDA was administered into the right LC, while vehicle (0.2% ascorbic acid/saline) was administered into the left LC at the following coordinates: A/P: −5.4 mm from bregma; M/L: ±0.9 mm; D/V: −3.25 mm from the surface of the skull, at a rate of 0.13 μl/min over ∼8 min. The needle was left in place for an additional 4 min after injection and then the needle was slowly withdrawn. Drilled holes were filled with wax and the skin sutured. Animals were allowed to recover in a cage placed halfway on a heating pad for the next 3 days. During this time animals were given 2 additional doses of carprofen for pain relief.

To assess the dose–response effect of 6OHDA in the LC, 6OHDA (7 (n = 7), 10 (n = 5) and 14 (n = 5) μg/μl) was administered unilaterally to mice as described above. Animals were sacrificed 3 weeks later. In addition to these animals, 5 mice that did not receive LC injection (non-surgery) were sacrificed on the same day as surgery animals to examine the noradrenergic markers in naïve animals. All animals had TH mRNA measured in LC noradrenergic neurons to assess the degree of LC neuronal loss; in addition, TH mRNA was measured in dopaminergic neurons of the SN and VTA in mice that demonstrated a loss of LC neurons.

Functional assessment of neuron loss in the LC following unilateral 6OHDA (10 μg/μl) injection

To examine the loss of noradrenergic neurons following 6OHDA and to assess if any of the surviving LC neurons were functional, we implemented intracellular, sharp electrode recordings to assay the number of electrophysiologically viable cells. Twelve C57Bl/6 mice had 6OHDA (10 μg/μl) injected unilaterally into the LC; vehicle was administered in the alternate LC. Of the 12 animals 1 died following surgery and 2 were lost during acute slice preparation. Three additional animals were administered 1,1′-dilinoleyl-3,3,3′,3′-tetramethylindocarbocynanine and 3,3′-dioctadecyloxacarbocyanine (Biotium, Hayward, CA, USA) bilaterally for verification of stereotaxic placement. For *in vitro* slice electrophysiology, brain slices containing LC were prepared following a modified protocol as previously published (Henderson et al., 1982; Williams et al., 1984). Four to 7 weeks (n = 9) after stereotaxic injection, mice were deeply anesthetized with halothane, rapidly decapitated, and the brains were carefully removed and transferred to cold, oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM) 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaCO_3$, 2 $CaCl_2$ and 10 dextrose. Brains were blocked to contain the LC by making two coronal cuts at approximately the caudal and rostral limits of the pons. Serial sections were cut at 300 μm thickness using a vibroslicer (Electron Microscopy Sciences, Hatfield, PA, USA) and transferred to a holding chamber at room temperature with oxygenated, circulating aCSF. Individual slices were then transferred to a slice interface recording chamber (Scientific Systems Design Inc, Mississauga, Ontario, Canada) perfused with warmed (32–35 °C), oxygenated aCSF; the slice surface was exposed to a warmed, humidified 95% O_2/5% CO_2 air. Left and right hemispheres were noted and slice orientation was carefully monitored to track the 6OHDA-injected right hemisphere and the vehicle control-injected left hemisphere.

Microelectrodes were made from borosilicate glass pulled on a horizontal puller (Sutter Instruments, Novato, CA, USA) and filled with 4 M potassium acetate (30–60 MΩ). These microelectrodes were used to estimate the number of viable neurons in the vehicle- versus 6OHDA-treated LC. Viable LC neurons