Neuroscience 166 (2010) 279–291

A COMPREHENSIVE ANALYSIS OF THE EFFECT OF DSP4 ON THE LOCUS COERULEUS NORADRENERGIC SYSTEM IN THE RAT

P. SZOT,[a,b]* C. MIGUELEZ,[c] S. S. WHITE,[a,b] A. FRANKLIN,[a] C. SIKKEMA,[a] C. W. WILKINSON,[b,d] L. UGEDO[c] AND M. A. RASKIND[a,b]

[a]*Northwest Network for Mental Illness Research, Education, and Clinical Center, Veterans Administration Puget Sound Health Care System, Seattle, WA 98108, USA*

[b]*Department of Psychiatry and Behavioral Science, University of Washington, Seattle, WA 98195, USA*

[c]*Department of Pharmacology, Faculty of Medicine and Dentistry, University of the Basque County, E-48940 Leioa, Vizcaya, Spain*

[d]*Geriatric Research, Education and Clinical Center, Veterans Administration Puget Sound Health Care System, Seattle, WA 98108, USA*

Abstract—Degeneration of the noradrenergic neurons in the locus coeruleus (LC) is a major component of Alzheimer's (AD) and Parkinson's disease (PD), but the consequence of noradrenergic neuronal loss has different effects on the surviving neurons in the two disorders. Therefore, understanding the consequence of noradrenergic neuronal loss is important in determining the role of this neurotransmitter in these neurodegenerative disorders. The goal of the study was to determine if the neurotoxin N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4) could be used as a model for either (or both) AD or PD. Rats were administered DSP4 and sacrificed 3 days 2 weeks and 3 months later. DSP4-treatment resulted in a rapid, though transient reduction in norepinephrine (NE) and NE transporter (NET) in many brain regions receiving variable innervation from the LC. Alpha$_1$-adrenoreceptors binding site concentrations were unchanged in all brain regions at all three time points. However, an increase in α_2-AR was observed in many different brain regions 2 weeks and 3 months after DSP4. These changes observed in forebrain regions occurred without a loss in LC noradrenergic neurons. Expression of synthesizing enzymes or NET did not change in amount of expression/neuron despite the reduction in NE tissue content and NET binding site concentrations at early time points, suggesting no compensatory response. In addition, DSP4 did not affect basal activity of LC at any time point in anesthetized animals, but 2 weeks after DSP4 there is a significant increase in irregular firing of noradrenergic neurons. These data indicate that DSP4 is not a selective LC noradrenergic neurotoxin, but does affect noradrenergic neuron terminals locally, as evident by the changes in transmitter and markers at terminal regions. However, since DSP4 did not result in a loss of noradrenergic neurons, it is not considered an adequate model for noradrenergic neuronal loss observed in AD and PD. Published by Elsevier Ltd on behalf of IBRO.

Key words: norepinephrine, α_2-AR, transporter, tyrosine hydroxylase, dopamine β-hydroxylase, single-unit extracellular recordings.

Degeneration of the locus coeruleus (LC) noradrenergic neurons is a major pathology of two neurodegenerative disorders, Alzheimer's disease (AD) (Mann et al., 1980; Bondareff et al., 1981; Tomlinson et al., 1981; Marcyniuk et al., 1986; Chan-Palay and Asan, 1989; German et al., 1992; Szot et al., 2000, 2006) and Parkinson's disease (PD) (Cash et al., 1987; Hornykiewicz and Kish, 1987; Chan-Palay and Asan, 1991; Patt and Gerhard, 1993; Bertrand et al., 1997; Marien et al., 2004). In AD, it appears that the surviving LC noradrenergic neurons undergo compensatory changes as evident by an increase in the expression of tyrosine hydroxylase (TH) mRNA (Szot et al., 2000, 2006), and sprouting of dendrites (Szot et al., 2006) and axons to forebrain regions (Szot et al., 2006, 2007). In PD, the surviving noradrenergic neurons in the LC do not appear to be compensating (Szot, unpublished observations). Despite the knowledge that these neurons are lost in these two disorders, it is unclear how the loss of LC noradrenergic neurons affects or is responsible for the progression of either of these neurodegenerative disorders. Therefore, studying the effect of LC noradrenergic neuronal loss in animals is an important step in determining the role of the noradrenergic nervous system in AD and PD. For this purpose, a well-characterized animal model of specific LC noradrenergic neuronal loss is important.

N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine or DSP4 has been considered a LC-selective noradrenergic neurotoxin based on documented changes in terminal noradrenergic fibers in regions innervated mainly by the LC. Markers of terminal loss are observed as a reduction in norepinephrine (NE) tissue content (Ross, 1976; Jonsson et al., 1981; Grzanna et al., 1989; Theron et al., 1993; Wolfman et al., 1994; Hughes and Stanford, 1996, 1998; Kask et al., 1997; Harro et al., 1999a,b), NE transporter (NET) (Cheetham et al., 1996) and an increase in alpha$_2$-adrenoreceptor (α_2-AR) (Wolfman et al., 1994; Harro et al., 1999b). The changes in these noradrenergic markers in

*Corresponding author. Tel.: +1-206-277-5052; fax: +1-206-768-5456.
E-mail address: szot@u.washington.edu (P. Szot).

Abbreviations: AD, Alzheimer's disease; α, alpha; Amy, amygdala; ANOVA, analysis of variance; AR, adrenoreceptors; AVT, anteroventricular thalamic nucleus; BNST, bed nucleus of the stria terminalis; CB, cerebellum; CG, central grey; DA, dopamine; DBH, dopamine β-hydroxylase; DHPG, 3,4-dihydroxyphenylglycol; DOPAC, dihydroxyphenylacetic acid; DSP4, N-(2-chloroethyl)-N-ethyl-2-bromobenzyl amine; DTN, dorsal thalamic nucleus; FC, frontal cortex; Gen, geniculate; Hab, habenula; HP, hippocampus; HPLC, high performance liquid chromatography; Hypo, hypothalamus; LC, locus coeruleus; LP, lateral posterior thalamic nucleus; MCID, MicroComputer Imaging Device system; NE, norepinephrine; NET, norepinephrine transporter; NTS, nucleus tractus solitaris; PFC, prefrontal cortex; PVN, paraventricular hypothalamic nucleus; PVNT, paraventricular thalamic nucleus; PD, Parkinson's disease; TH, tyrosine hydroxylase; THL, anteroventricular thalamic nucleus; sep, septum; SN/VTA, substantia nigra/ventral tegmental area; Str, striatum.

0306-4522/10 $ - see front matter. Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2009.12.027

specific brain regions have suggested a selective loss of afferents from LC noradrenergic neurons (Olsen and Fuxe, 1971; Ungerstedt, 1971; Jones and Moore, 1977; Mason and Fibiger, 1979; Moore and Bloom, 1979; Waterhouse et al., 1983; Loughlin et al., 1986a,b). These changes appear to be rapid and transient (Wolfman et al., 1994), whereas in the LC, there appears to be a gradual loss of noradrenergic cell bodies that follows the loss of terminal noradrenergic markers. The hypothesis has been that the surviving LC neurons compensated for the loss of neurons and restored terminal innervation. However, there are also data to suggest that the neurons in the LC are not lost (Lyon et al., 1989; Robertson et al., 1993; Matsukawa et al., 2003), that noradrenergic terminals are not reduced (Booze et al., 1988), and that the amount of released NE into the synapse is not altered in animals with DSP4 (Kask et al., 1997; Hughes and Stanford, 1996, 1998). To validate DSP4 as a possible model of noradrenergic neuronal loss in either (or both) AD or PD, a comprehensive analysis of DSP4 effects on noradrenergic markers in forebrain regions was measured and correlated to LC noradrenergic neuronal loss in the same animals. Previous work suggesting the selectivity of DSP4 on LC noradrenergic neurons was performed in numerous laboratories employing a variety of techniques. To determine DSP4 induced changes in noradrenergic terminals the following studies were performed 3 days 2 weeks and 3 months after DSP4: NET, alpha1- (α_1) and α_2-adrenoreceptor (α_2-AR) binding. NE tissue content was determined in the frontal cortex (FC), hippocampus (HP), cerebellum (CB), LC and septum/bed nucleus of the stria terminalis (sep/BNST) 3 days 2 weeks and 3 months after DSP4. To assess whether noradrenergic neurons are lost in the LC following DSP4, the following measurements were performed at similar times in the same animals: TH, dopamine β-hydroxylase (DBH) and NET mRNA expression. To determine if DSP4 alters the function of LC noradrenergic neurons, single-unit extracellular recordings were performed to measure basal activity in anesthetized animals 3 days 2 weeks and 3 months after DSP4.

EXPERIMENTAL PROCEDURES

Animals

Eighty adult male (60 days) Sprague–Dawley rats were purchased from Charles River (Wilmington, MA, USA) and housed in standard cages in a controlled environment with a 12-h light/dark cycle. Food and water were provided *ad libitum*. The animals were given a 7-day acclimating period to the facility before treatments were started. All animal procedures were in accordance with the Animal Care Committee at the VA Puget Sound Health Care System, Seattle, WA, NIH guidelines. The minimum number of animals were used for these studies and care was taken to minimize any suffering.

Administration of DSP4 for catecholamine levels, binding and *in situ* experiments

Saline (n=40) or DSP4 (50 mg/kg ip; Sigma, St. Louis, MO, USA) (n=40) was administered to animals. Due to the instability and light sensitive nature of DSP4, DSP4 was made fresh twice and placed into a light tight container. After half of the animals were injected with DSP4, any remaining solution was thrown away and a new batch of DSP4 was made for the remaining animals. Animals were sacrificed 3 days 2 weeks and 3 months after DSP4. An equal number of animals were obtained from the two different batches of DSP4 for each time point. At each time point animals were sacrificed, brains removed and frozen on dry ice, whole or dissected into specific brains regions. Whole brains were cut on a cryostat at 18 μm onto Superfrost Plus slides divided into three sets of slides with alternating sections and stored at −80 °C. Slides containing forebrain regions had NET, α_1- and α_2-AR binding performed, while slides containing the LC had TH, DBH and NET mRNA *in situ* hybridization performed. At the 3 months time point after DSP4, the lateral tegmental nuclei (nucleus tractus solitarius (NTS) and A1) were also cut in addition to the LC and TH and NET mRNA *in situ* hybridization was performed. Catecholamine levels were measured in the FC, HP, CB, amygdala (Amy) and septum/bed nucleus stria terminalis (sep/BNST) by high performance liquid chromatography (HPLC).

HPLC measurement of catecholamine

Each brain region was sonicated in 1 ml of 0.1 M perchloric acid. A 100 μl aliquot of the sonicated material was stored at −80 °C for protein determination using Pierce BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). The supernate was collected from centrifugation of the sonicated material at 13,000 g for 15 min and stored at −70 °C until catecholamine extraction was performed. Catecholamine levels were measured in six different assays, an assay for each brain region at every time point, to reduce variable effects of the assay on catecholamine levels. Catecholamines were extracted by alumina extraction from 100 μl of the sonicated supernate as previously described (Eisenhofer et al., 1986). The eluted catechols were filtered through 0.22 Millex® GV syringe driven filter and detection was performed with the ESA Coulochem II electrochemical detector (conditioning cell set at +350 mV, electrode 1 of analytical cell set at +90 mV, electrode 2 of analytical cell set at −300 mV) (ESA, Chelmsford, MA, USA). Phenomonex reverse phase c18 Gemini column (150×4.6 mm, 3 μC, 110 A) (Phenomonex, Torrance, CA, USA) and Scientific Software Inc. was used for data collection and analysis. The following catecholamines were measured for each brain region at each time point: NE, dopamine (DA), 3,4-dihydroxyphenylglycol (DHPG), DOPA, dihydroxyphenylacetic acid (DOPAC). DOPA is a precursor for both NE and DA, while DHPG is a metabolite of NE and DOPAC is a metabolite of DA.

Catecholamine values were expressed as ng catecholamine/mg protein. Data for each time point were adjusted to percent control and values are expressed as the average percent change from control±SEM. Control values from all three-time points were combined. Experimental data were analyzed by using the computer program GraphPad Prism (v. 5.0, GraphPad Software Inc.). Statistical significance was assessed by means of two-way analysis of variance (ANOVA) followed a post hoc Tukey test; statistical significance was taken at $P<0.05$.

Receptor binding

^{3}H-Prazosin (85.0 Ci/mmol; PerkinElmer, Boston, MA, USA) was used to quantitate α_1-AR binding sites, ^{3}H-RX821002 (55.0 Ci/mmol; PerkinElmer) was used to quantitate α_2-AR binding sites and ^{3}H-nisoxetine (80.0 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO, USA) was used to quantitate NET binding sites. ^{3}H-Prazosin binding was performed as described previously (Sanders et al., 2006; Szot et al., 2006, 2007). Briefly, slides were thawed at room temperature for 10 min and then 600 μl/slide of incubation buffer (~0.2 nM ^{3}H-prazosin in 50 mM Tris buffer, 1 mM EDTA, pH 7.4) was placed over the tissue. Non-specific binding was defined in the presence of 10 μM phentolamine. Slides were incubated for 40 min at room temperature, washed twice for 2 min in ice-cold 50 mM Tris buffer, pH 7.4, dipped in