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A Comprehensive Analysis of the Effect of DSP4 on the Locus Coeruleus Noradrenergic System in the Rat

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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. Alpha₁-adrenoreceptors binding site concentrations were unchanged in all brain regions at all three time points. However, an increase in α₂-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.

Keywords

norepinephrine; frontal cortex; hippocampus; 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 (P. 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 (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 α_2 -adrenoreceptor (α_2 -AR) (Wolfman et al., 1994; Harro et al., 1999b). The changes in these noradrenergic markers in 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, α_1 - (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: tyrosine hydroxylase (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) 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.

Administration of DSP4 for Catecholamine Levels, Binding and *In situ* Experiments

Saline (n=40) or DSP4 (50 mg/kg, ip; Sigma, St. Louis, MO) (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 brain regions. Whole brains were cut on a cryostat at 18 μ m onto Superfrost Plus slides divided into 3 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. Catecholamine levels were measured in the frontal cortex (FC), hippocampus (HP), cerebellum (CB), amygdala (Amy) and septum/bed nucleus stria terminalis (sep/BNST) by HPLC.

HPLC Measurement of Catecholamine

Each brain region was sonicated in 1 ml of 0.1 M perchloric acid. A 100 μ l aliquot of the sonicated material was stored at -80°C for protein determination using Pierce BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL). The supernate was collected from centrifugation of the sonicated material at 13,000g for 15 min and stored at -70°C until catecholamine extraction was performed. Catecholamine levels were measured in 6 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 μ l of the sonicated supernate as previously described (Eisenhofer et al., 1986). The eluted catechols were filtered through 0.22 Millex $\text{\textcircled{R}}$ GV syringe driven filter and detection was performed with the ESA Coulochem II electrochemical detector (conditioning cell set at +350mV, electrode 1 of analytical cell set at +90mV, electrode 2 of analytical cell set at -300mV)(ESA, Chelmsford, MA). Phenomonex reverse phase c18 Gemini column (150 $\times$ 4.6mm, 3 μ C, 110A) (Phenomonex, Torrance, CA) 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), 3,4-dihydroxyphenylalanine (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, values are expressed as the average % change from control $\pm$ SEM and analyzed using ANOVA, followed by a *post hoc* Tukey's test; statistical significance was taken at $p < 0.05$.

Receptor Binding

^3H -Prazosin (85.0 Ci/mmol; PerkinElmer, Boston, MA) was used to quantitate α_1 -AR binding sites, ^3H -RX821002 (55.0 Ci/mmol; PerkinElmer) was used to quantitate α_2 -AR binding sites and ^3H -nisoxetine (80.0 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) was used to quantitate NET binding sites. ^3H -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 μ l/slide of incubation buffer ($\sim$ 0.2 nM ^3H -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 μ M 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 ice-cold distilled water to remove salts and then rapidly dried under a stream of cool air. ^3H -RX821002 binding was performed as described previously (Szot et al., 2006). Briefly, slides were thawed as described above, and then 600 μ l/slide of incubation buffer ($\sim$ 2 nM ^3H -RX821002 in 50 mM NaPO_4 buffer, pH 7.4) was placed over the tissue. Non-specific binding was defined in the presence of 10 μ M rauwolscine. Slides were incubated for 45 min at room temperature and then washed for 2 min in ice-cold 50 mM NaPO_4 buffer, pH 7.4, dipped in ice-cold distilled water and dried as described above for ^3H -prazosin. ^3H -Nisoxetine binding was performed as described previously (Weinshenker et al., 2002; Szot et al., 2006). Briefly, slides were thawed as described above, and then 600 μ l/slide of incubation buffer ($\sim$ 3 nM ^3H -nisoxetine in 50 mM Tris buffer with 300 mM NaCl and 5 mM KCl, pH 7.7) was placed over the tissue. Non-specific binding was defined in the presence of 1 μ M mazindol. Slides were incubated for 2 h. at room temperature and then washed and dried as described above for ^3H -prazosin. Slides were apposed to Biomax MR film (Eastman Kodak Co, Rochester, NY) for 8 weeks. Films were developed by standard procedures (Szot et al., 1997) and the resulting images for α_1 -, α_2 -AR and NET were analyzed using the MicroComputer Imaging Device system (MCID) (InterFocus Imaging Ltd., Cambridge, England). α_1 -AR (^3H -prazosin) binding sites were quantitated (Optical Density; OD) in the following atlas matched regions of both saline and DSP4-treated animals at each time point: FC, BNST, HP, sep, thalamus, geniculate (gen), Amy and substantia nigra/ventral tegmental area (SN/VTa). Specific binding was obtained

by taking average value minus non-specific OD from the same region. Data for each time point were adjusted to percent control and control values from all 3-time points were combined. Specific binding values (saline, 3 days, 2 weeks and 3 months) were expressed as the average % change from saline $\pm$ SEM and analyzed using ANOVA, followed by a *post hoc* Tukey's test; statistical significance was taken at $p < 0.05$. α_2 -AR (^3H -RX82002) binding sites were quantitated in the following atlas matched regions of both saline and DSP4-treated animals at each time point: FC, BNST, HP, sep, striatum (str), dorsal thalamic (DTN), paraventricular thalamic nucleus (PVTN), Amy, hypothalamus (hypo), SN/VTa, lateral posterior thalamic nucleus (LPT) and central grey (CG). Specific values were quantitated and analyzed as described above for α_1 -AR. NET (^3H -nisoxetine) binding sites were quantitated in the following atlas matched regions of both saline and DSP4-treated animals at each time point: FC, BNST, HP, sep, habenula (Hab), paraventricular hypothalamic nucleus (PVN), hypo, Amy, anteroventricular thalamic nucleus (AVT) and SN/VTa. Specific binding was analyzed as described above for α_1 -AR.

***In situ* Hybridization**

Tissue preparation and labeling of the TH, DBH and NET oligonucleotides was performed as described previously (Szot et al., 1997, 2006). The TH oligonucleotide probe was a 48 base probe complementary to nucleotides 1351-1398 of the rat TH mRNA (Grima et al., 1985). The DBH oligonucleotide probe consisted of three oligonucleotides complementary to nucleotides 454-505, 994-1045, and 1414-1465 of the rat sequence (McMahon et al., 1990). The NET oligonucleotide probe consisted of three oligonucleotides complementary to nucleotides 601-652, 1123-1174, and 1726-1777 of the rat sequence (Gen Bank NM_Y13223). The oligonucleotide probes were 3' end-labelled with ^{33}P -dATP (PerkinElmer) using terminal deoxyribonucleotidyl transferase (Invitrogen, Piscataway, NJ). The TH probe contained 0.45×10^6 cpm/50 μl and was washed as described in detail in previously published work with the oligonucleotide (Szot et al., 1997). The DBH probe contained 2.8×10^6 cpm/50 μl and was washed at 50°C . The NET probe contained 0.60×10^6 cpm/50 μl and was washed at 55°C . Slides were coated with NTB2 Nuclear Track Emulsion (undiluted) (Eastman Kodak) and stored at -20°C for 3 days for TH, 4 days for NET and 10 days for DBH. Slides were developed by standard procedures (Szot et al., 1997).

Quantitation of TH, DBH and NET mRNA expression was similar to that performed by Szot et al., (2006) using the MCID system. The number of cells that achieved labeling threefold higher than background was counted bilaterally on 7 atlas matched consecutive LC sections. The 7 consecutive sections comprise approximately 80% of the labeled LC neurons. Data for each oligonucleotide probe were adjusted to percent control, and control values from all 3- time points were combined. Data for number of positively labeled neurons (saline, 3 days, 2 weeks and 3 months) were expressed as the average % change from saline $\pm$ SEM and analyzed using ANOVA, followed by a *post hoc* Tukey's test; statistical significance was taken at $p < 0.05$. The density of TH, DBH and NET mRNA expression/neuron was performed by measuring the amount of silver grains over cell bodies of labeled neurons that were threefold higher than background under 20X dark-field illumination using MCID (InterFocus Imaging Ltd) and analyzed as described above. Therefore, all labeled neurons

that were counted as positively labeled for each oligonucleotide probe were also quantitated for the amount of TH, DBH and NET per neuron.

Animal Preparation and Surgery for Electrophysiology

Thirty-three adult Sprague Dawley rats were treated as described above with either saline or DSP4 (50 mg/kg). All animal procedures were in accordance with the European Community Council Directive on “The Protection of Animals Used for Experimental and Other Scientific Purposes” (86/609/EEC Spanish Law (RD 1201/2005). Three days (5 control and 6 DSP4), 2 weeks (5 control and 6 DSP4) and 3 months (4 control and 7 DSP4) after the injection of DSP4 or saline, animals were anesthetized with chloral hydrate (400 mg/kg, i.p.). After cannulating the trachea, a catheter was inserted in the jugular vein for additional administrations of anesthetic. The rat was placed in the stereotaxic frame and the body temperature was maintained at $\sim 37^{\circ}\text{C}$ for the entire experiment by means of a heating pad connected to a rectal probe.

The head was oriented at 15° to the horizontal plane (nose down). A bur hole was drilled and an electrode was placed in the following coordinates (relative to *lambda*): AP: -3.7 mm, ML: +1.1 mm, DV: -5.5 to -6.5 mm (Paxinos, 1997) by means of a hydraulic microdrive.

Recording and neuronal identification—Single-unit extracellular recordings of LC neurons were performed as described previously (Migueluez *et al*, 2009). The recording electrode, consisting of an Omegadot single-barrel glass micropipette, was filled with a 2 % solution of Pontamine Sky Blue in 0.5 % sodium acetate and broken back to a tip diameter of 1-2 μm . The electrode was lowered into the brain by means of a hydraulic microdrive. LC neurons were identified by standard criteria (Cedarbaum and Aghajanian, 1976) which included: spontaneous activity displaying a regular rhythm and a firing rate between 0.5-5 Hz, characteristic spikes with a long-lasting positive-negative waveform and biphasic excitation-inhibition response to pressure applied to the contralateral hind paw (paw pinch).

The extracellular signal from the electrode was pre-amplified and amplified later with a high-input impedance amplifier, and then monitored on an oscilloscope and on an audio monitor. This activity was processed using computer software (Spike2 software, Cambridge Electronic Design, UK). Firing patterns were determined by analyzing the interspike interval histogram, firing rate, coefficient of variation (percentage ratio of standard deviation to the mean interval value of an interspike time-interval histogram), percentage of spikes in burst, mean spikes/burst, percentage of cells exhibiting burst firing and response to paw-compression intensity. The number of spontaneously active noradrenergic neurons was determined in 9 additional tracks separated 50 μm around the first track through the LC before the drug administration (10 tracks/rat).

At the end of each experiment, a Pontamine Sky Blue mark was deposited in the recording site, passing a 5 μA cathodic current for 10 minutes through the recording electrode. Subsequently, the animals were perfused and the brain removed. Brain sections containing the LC were processed for Neutral Red Staining and the location of the recording site was examined microscopically. Only measurements from cells within the LC were included in the study.

Experimental data were analyzed by using the computer program GraphPad Prism (v. 5.01, GraphPad Software Inc.). Statistical significance was assessed by means of two- or one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test. Burst firing activity and percentage of cells exhibiting burst were analyzed by nonparametric tests (Mann Whitney) and χ^2 , respectively. The level of significance was considered as $p < 0.05$. Data are reported as mean $\pm$ S.E.M.

Results

Norepinephrine tissue content is transiently reduced in specific brain regions

Administration of DSP4 to rats did not result in any obvious behavioral changes. Animals that received DSP4 initially (3 days later) gained weight at a slower rate than control animals, however, they were equivalent to controls 2 weeks and 3 months later (Figure 1).

NE, DA, DOPA, DOPAC and DHPG tissue content were measured in the PFC, HP, LC, Amy, CB and sep/BNST 3 days, 2 weeks and 3 months after the administration of DSP4. NE tissue content varied in the 6 different brain regions across all time points; however, as time progressed from the day of DSP4 administration, the number of animals exhibiting a reduction in NE tissue content (specifically in the PFC, HP and CB) (Figure 2). There is no correlation of NE tissue content to the order of DSP4 administration after the solution was prepared, addressing the stability of DSP4. NE tissue content was significantly reduced 3 days after DSP4 in the PFC and HP, regions that receive sole innervation from the LC (Olsen and Fuxe, 1971; Ungerstedt, 1971; Jones and Moore, 1979; Mason and Fibiger, 1979; Moore and Bloom, 1979; Waterhouse et al., 1993; Loughlin et al., 1986a); however, NE tissue content was also reduced in sep/BNST, a region that receives only partial innervation from the LC (Segal and Landis, 1974; Mason and Fibiger, 1979; Loughlin et al., 1986a, b) (Figure 2). In fact the loss of NE 3 days after DSP4 was greatest in the sep/BNST. There was no correlation between NE tissue content and the weight of the animal 3 days after DSP4. Two weeks after DSP4 administration there was still a significant reduction of NE tissue content in the HP and a reduction in the CB, but in the sep/BNST there was a rebound significant increase in NE tissue content (Figure 2). Three months after DSP4 all brain regions had normal NE tissue content (Figure 2). This pattern of changes calls into question the LC selectivity of DSP4 to affect specifically LC terminals. DA tissue content was also variable in the 6 different brain regions at all time points, with a significant reduction in DA content only in the HP 2 weeks after DSP4 administration (Figure 2). DOPA, DHPG and DOPAC were not statistically different in any brain region at any time point (data not shown).

NET binding site concentrations are reduced in several forebrain regions; the loss is not reflective of LC innervation

NET binding sites, which are exclusively localized to adrenergic terminals, were significantly reduced 3 days after DSP4 in the FC and HP (Figure 3), i.e., the same regions that exhibited a significant loss of NE tissue content. However, NET binding sites are back to normal 2 weeks later. Two weeks after DSP4 administration NET binding site concentrations were significantly reduced in the sep, AVT and Hab (Figure 3 and 4

autoradiograms), regions that receive partial innervation from the LC. Three months after DSP4 all brain regions showed normal NET binding site concentrations (Figure 3). NET binding site concentrations were unchanged in the rest of the regions at all time point (Figure 3).

α_2 -AR binding site concentrations are elevated in specific brain regions 2 weeks and 3 months after DSP4

α_2 -AR binding sites are localized presynaptically on noradrenergic terminals as autoreceptors and postsynaptically on dendrites and axon terminals of other transmitter systems. Therefore, α_2 -AR binding sites are observed in many brain regions including the FC, str, sep/BNST, DTN, PVTN, Amy, hypo, SN/VTA and hindbrain regions. Figure 5 illustrates the changes in α_2 -AR binding site concentrations in DSP4 animals. α_2 -AR binding site concentrations were unchanged in all brain regions 3 days after DSP4, but significantly elevated 2 weeks later in the DTN, PVNT and Hypo; 3 months later α_2 -AR binding site concentrations in these regions were not significantly different from those in saline controls (Figure 5 and 6 autoradiograms). However, 3 months after DSP4 α_2 -AR binding site concentrations were significantly elevated in the anterior portion of the sep and the Amy (Figure 5). It is unclear if the DSP4-induced increase in α_2 -AR binding site concentrations in specific brain regions was in pre- or post- (or both) synaptic receptors. α_1 -AR binding sites, which are postsynaptic receptors, were measured in DSP4-treated animals. α_1 -AR binding site concentrations did not differ from those of saline-treated animals in all regions, at all time points (data not shown).

LC noradrenergic neurons are not altered at any time point following DSP4

To determine if DSP4 affects the number of LC noradrenergic neurons, three different genes (TH, DBH and NET mRNA) that are expressed in LC noradrenergic neurons were measured. TH and DBH mRNA are synthesizing enzymes for NE, with TH being the rate-limiting enzyme; DBH and NET mRNA are found only in adrenergic neurons. The number of TH, DBH and NET positively labeled neurons and the amount/neuron were counted over 7 consecutive levels of the LC. The 7 consecutive sections constituted the majority of labeling in the LC. Table 1 shows the average value over the 7 LC sections of cell number and grains/neuron for TH, DBH and NET mRNA in saline and DSP4-treated animals at all three time points. The number of neurons expressing TH, DBH and NET was not significantly different in DSP4 animals at any time point including 3 months later, a time point where significant neuronal loss has been documented with DBH immunohistochemistry (Grzanna et al., 1989; Fritschy and Grzanna, 1991, 1992; Zhang et al., 1995). This indicates that there was no neuronal loss of noradrenergic neurons in the LC up to 3 months after DSP4. A loss of NE tissue content 3 days and 2 weeks after DSP4 did not result in a compensatory change in either synthesizing enzymes as to expression per neuron. NET mRNA expression per neuron was significantly different (ANOVA $p=0.03$) between 3 days and 2 weeks post DSP4 (*post hoc* Tukey test). Between these two time points there was a decrease in NET mRNA expression per neuron that corresponded to a time when there was a reduction in NET binding site concentrations in several forebrain regions, but the regions that demonstrated reduced NET binding sites 2 weeks after DSP4 are regions that receive only partial LC innervation (Figure 3). The significant reduction in

NET binding site concentrations in the FC and HP, regions solely innervated from LC, at 3 days after DSP4 was not associated with a change in NET mRNA expression (Figure 3). Therefore, there does not appear to be a correlation of NET binding and NET mRNA expression in the LC, suggesting that the alteration in NET binding sites in forebrain regions was a localized effect of DSP4.

DSP4 reduces the number of spontaneously active neurons, but does not affect basal firing rate of LC noradrenergic neurons

A total of 298 neurons were recorded in the LC: 3 days (n= 54 control; n=52 DSP4), 2 weeks (n= 53 control; n=52 DSP4) and 3 months (n=41 control; n=46 DSP4). All neurons possessed previously described electrophysiological characteristics of LC neurons (see Material and Methods) and were located within the LC. Mean number of neurons encountered per track was significantly reduced in DSP4 relative to control group (for treatment $p<0.001$, $F_{1,27}=45.71$, two-way ANOVA) time variable ($p<0.001$, $F_{2,27}=22.51$, two-way ANOVA) and interaction ($p<0.05$, $F_{(2,27)}=5.81$, two-way ANOVA). Basal firing rate of LC neurons was not affected by the administration of DSP4 at any time point, nor was the percentage of spikes fired in bursts (Table 2). Significant differences were observed in the coefficient of variation between the studied groups ($p<0.001$, $F_{(1,288)}=15.31$ for treatment variable). Subsequent *post hoc* analysis showed that only at 2 weeks after DSP4 injection did neurons discharge with significantly more irregular firing pattern compared with the corresponding control group ($p<0.001$, Bonferroni *post hoc* test) (Table 2). Significant differences were also observed only at 2 weeks after administration for the percentage of neurons firing in bursts ($\chi^2=21.55$, $df=5$, $p<0.001$).

Discussion

DSP4 resulted in rapid and transient changes in NE tissue content, NET binding sites and α_2 -AR binding sites in many forebrain regions, but these changes in forebrain regions occurred without a loss of LC noradrenergic neurons. The changes in noradrenergic markers observed in this study do not support the LC selectivity of DSP4-induced lesion of noradrenergic neurons or terminals. DSP4, although reduced the number of spontaneously active neurons, did not affect basal activity of LC noradrenergic neurons at any time point, but did increase the irregularity and the burst firing of noradrenergic neurons only at 2 weeks after DSP4. Our data clearly indicates that DSP4 affects some noradrenergic terminals, which could alter the local function of these terminals early after DSP4 administration. The regions affected in the forebrain indicate it is not just LC innervation that is affected by DSP4. Since the number of LC noradrenergic neurons is not reduced with DSP4, the findings also indicate that DSP4 administration does not yield a good model in rats for the loss of noradrenergic neurons in the LC in association with either (or both) AD or PD.

The loss of NE tissue content in the FC, HP and CB has been well documented, though the degree of loss induced by DSP4 has varied greatly in the literature depending on the dose of DSP4 and the method for catecholamine analysis (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). Because these regions receive sole innervation from the LC (Olsen and Fuxe, 1971; Ungerstedt, 1971; Jones and Moore, 1979; Mason and Fibiger, 1979; Moore and Bloom, 1979; Waterhouse et al., 1983; Loughlin et al., 1986a, b), the original hypothesis was that DSP4 affected only LC terminals. However, as other brain regions that receive the majority of innervation from either the lateral tegmental area or the nucleus solitary tract (NTS) demonstrated a significant reduction in NE tissue content (Grzanna et al., 1989; Kask et al., 1997; Theron et al., 1993; Wolfman et al., 1994), the selectivity of DSP4 for LC innervation is called into question. In this study the greatest loss of NE tissue content was observed in the sep/BNST, and the loss in this region is greater than that observed in the FC, HP and CB (Figure 2)(regions that receive sole LC innervation), indicating that DSP4 is not selective for LC terminals. The reduction of NE tissue content is transient and not associated with cell loss or a change in the expression of two enzymes involved in the synthesis of NE. This indicates that DSP4 has a localized effect on NE tissue content in specific forebrain noradrenergic terminals. A localized reduction in NE content with no neuronal loss has likewise been observed in the vesicular monoamine transporter 2 (VMAT2) deficient mouse (Colebrooke et al., 2006).

The loss of NET binding site concentration in the cortex 3 days after DSP4 (Cheetham et al., 1996) is another piece of evidence that has been used to support the selectivity of DSP4 on LC terminals. However, in that study no other region other than the cortex was examined to justify this conclusion. Our study confirms the reduced NET binding site concentrations in the cortex but extends the number of regions that also exhibit reduced NET binding site concentrations to include regions that receive full LC innervation (HP) and a mixture of LC and other noradrenergic innervation (sep, AVT and Hab). The reduction in these noradrenergic terminal markers does not necessarily indicate a loss of terminals per se, but suggests that existing terminals have altered transporter. Booze et al., (1988) examined noradrenergic terminals in the HP with TH immunohistochemistry after DSP4 and determined there was no terminal loss. The loss of NET binding site concentrations induced by DSP4 was transient (returned to normal 3 months after DSP4) and not associated with a loss of NET mRNA expression neurons or expression of NET per neuron in the LC. This would suggest again that DSP4 is having a localized effect on noradrenergic terminals. Zhao et al., (2009) also demonstrated that changes in NET binding sites can occur with chronic antidepressant treatment, without an accompanying change in NET mRNA, supporting the possibility of a local effect on NET binding sites at terminal regions.

The loss of NET binding site concentrations in forebrain regions does not correspond to the regions that exhibited reduced NE tissue content. Therefore, the reduction in NE tissue content probably is not the reason for the reduction in NET binding sites. The hypothesis that NE tissue content does not reflect NET binding sites, is supported by the normal NET binding site concentration observed in the CNS of DBH knockout mice (DBH $-/-$), a transgenic mouse that lacks the capacity to synthesize NE (Weinshenker et al., 2002). However, the converse may be true, that reduced NET binding site concentrations may contribute to reduced NE tissue content in the cortex and HP. If less NE is being taken back into terminals and there is no compensation for synthesis, tissue content may be reduced. It is unclear if the reduced NET binding sites in other brain regions (other than cortex and HP) are associated with a reduction in tissue NE content since tissue content was not measured in

those specific regions. The reduction of NET binding site concentrations in the cortex after DSP4 could also account for the normal synaptic NE levels in the cortex that is observed 5-7 days after DSP4, even though there is reduced NE tissue content at the time (Hughes and Stanford, 1996; Kask et al., 1997).

α_2 -AR binding sites are localized both as prejunctional autoreceptors, and postjunctional receptors on dendrites or axon terminals. Determining the location (pre- versus post-synaptic) of a change in α_2 -AR receptors by binding assays is not feasible. The effect of DSP4 on α_2 -AR binding site concentrations in the literature has not been clear. One study observed a decrease in α_2 -AR binding sites 3 days after DSP4 in the cortex, HP and hypothalamus, but normalized 15 days later (Heal et al., 1993), while another study observed an increase in α_2 -AR binding sites in the cortex 7 days after DSP4 with normal levels a year later (Wolfman et al., 1994). The initial reduction in α_2 -AR binding sites documented 3 days after DSP4 in the cortex, HP and hypothalamus was proposed to represent the loss of autoreceptors on noradrenergic terminals (Heal et al., 1993), a hypothesis based on the assumption that terminals were lost. The normalization/increase in binding sites (Heal et al., 1993; Wolfman et al., 1994) was then proposed to be upregulation of postsynaptic sites. The data generated in our study do not demonstrate an early decrease in α_2 -AR sites, but do demonstrate an increase in specific regions at later time points. However, since there does not appear to be a loss of noradrenergic terminals (Booze et al., 1988), the increase observed in our and other studies can not be attributed to postsynaptic sites. The differences in results between the three studies examining α_2 -ARs may be attributed to differences in the binding method (membrane binding versus autoradiography) and the ligand used to measure α_2 -AR binding sites. It is unclear why α_2 -AR binding sites would be elevated in the brain after DSP4. The regions where an increase was observed are regions that receive partial innervation from the LC, and which might or might not have shown a loss of NE tissue content. The HP and FC, typical regions studied following DSP4, did not demonstrate significant differences in α_2 -AR binding sites, but did demonstrate reduced NET binding sites and NE tissue content. It is also unclear if the changes in the α_2 -AR are localized at pre-versus post-synaptic sites. To clarify where the α_2 -AR are altered (pre- versus post-synaptic) either gene expression or protein analysis of the different α_2 -AR subtypes in these regions are required. Interestingly, measurement of α_1 -AR, a postsynaptic receptor, was unchanged following DSP4 (data not shown). Wolfman et al, (1994) is the only other group to measure α_1 -AR after DSP4 and they observed a modest increase 7 days later. Again the discrepancies in the results may be attributed to the different time points and binding methods used.

All the previous data published concerning changes in noradrenergic markers at terminal regions following DSP4 were performed without assessing concomitant changes in LC noradrenergic neuronal cell bodies. Unlike the previous work, our study can link the changes measured in the terminal forebrain regions to LC noradrenergic neurons. This is the first comprehensive study of the effects of DSP4 on the noradrenergic nervous system at terminals in the forebrain and in the LC of rats.

Our study did not observe a change in the number of noradrenergic neurons in the LC, as measured by three different noradrenergic markers (TH, DBH and NET mRNA) up to 3

months after DSP4 administration. Two of these markers (DBH and NET) are exclusive to adrenergic neurons. This result differs from previously published work with DSP4, which observed a significant loss of LC noradrenergic neurons starting 2 weeks after DSP4 (Grzanna et al., 1989; Fritschy and Grzanna, 1991; Zhang et al., 1995) using DBH immunohistochemistry. However, reduced DBH immunohistochemistry does not necessarily indicate a loss of LC noradrenergic neurons with DSP4 (Lyons et al., 1989). As already shown with NET, protein levels can be reduced without a change in mRNA levels.

Electrophysiological data indicate that DSP4 does not affect the functioning of the LC neurons; LC neuronal basal activity and percentage of spikes fired in a burst are not affected by the administration of DSP4. Only 2 weeks after DSP4, not 3 days or 3 months after DSP4, there is an increase in irregular activity of the noradrenergic neurons and an increase in the percent of neurons with burst activity. An increase in the number of neurons with burst activity measured 2 weeks after DSP4 could be due to a change in the threshold of the noradrenergic neurons or an alteration in the excitatory/inhibitory input to cell bodies. Interesting, the number of active neurons per track is reduced in anesthetized DSP4-treated animals at all three time points. The cause of this reduced number of active neurons per track is unclear. The decrease in active neurons cannot be attributed to a loss of noradrenergic neurons, as determined by TH and DBH mRNA expression, but changes in NET or α_2 -AR binding sites in the LC could result in a change in the number of active neurons in the LC. These proteins are changed in forebrain regions, but were not measured in the LC. Also, it is unclear if innervation to the LC in DSP4 animals is altered that could result in a change in afferent connection. A loss of dopaminergic innervation results in a significant loss of active neurons per track in the LC, but the noradrenergic neurons have increased spontaneous firing (Guiard et al., 2008).

Our study indicates that DSP4 does not selectively lesion LC noradrenergic cell bodies or affect the function of LC noradrenergic neurons, though DSP4 does affect noradrenergic markers at terminals in a variety of brain regions that vary in their degree of LC innervation. The reasons why these terminals are affected by DSP4 are unclear. It is also apparent that these noradrenergic markers are transiently altered and all these terminal changes in noradrenergic markers occur without a loss of LC noradrenergic neurons, indicating that DSP4 is not a neurotoxin in the rat. DSP4 could be characterized as a compound that has localized effects on proteins in noradrenergic terminals in rats. It is unknown if DSP4 has similar effects in mice. The loss of tissue NE content in forebrain regions like the cortex and HP appears to be more pronounced and persistent in mice than in rats (Haberle et al., 2001; Archer and Fredriksson, 2006; Dailly et al., 2006; Thomas et al., 2007). A loss of noradrenergic LC neurons has been documented following DSP4 in mice but the treatment involved two 50 mg/kg doses (Heneka et al., 2006). This would suggest that rats have a different sensitivity to DSP4 than mice, which has been suggested before (Fornai et al., 1996). Mice and rats also have a difference in response to the neurotoxic effects of MPTP, in that mice exhibit a loss of dopaminergic neurons to MPTP but rats do not (Sedelis et al., 2000).

LC noradrenergic neurons are lost in AD and PD, but the response of the surviving noradrenergic neurons is different. The consequence of this loss as to the progression of

these disorders is unknown. The goal of this study was to determine if DSP4 (a noradrenergic neurotoxin) could serve as an animal model of noradrenergic loss in either AD or PD (or both). The results of this study indicate that DSP4 does not reduce LC noradrenergic number or function, suggesting that DSP4 is not an appropriate rat model to study the functional consequence of noradrenergic neuronal loss in the degenerative disorders of AD and PD. There is a mouse model of AD, the amyloid precursor protein/presenilin 1 (APP/PS1) double transgenic mice, in which plaques are expressed and a 24-50% reduction in the total number of TH immunohistochemical labeling of LC noradrenergic neurons occurs (O'Neil et al., 2007; Liu et al., 2008). At the present time it is unclear if the surviving LC noradrenergic neurons demonstrate a compensatory response. However, only the double transgenic mouse has shown a modest reduction in the LC noradrenergic neurons, the APP transgenic mouse has normal LC noradrenergic neurons and expression (Szot et al., 2009). Because these animal models of AD have limitations, our laboratory is continuing to determine a pharmacological means of lesioning LC noradrenergic neurons and the consequence of this loss on terminals in the forebrain to determine the role of the noradrenergic nervous system in AD and PD.

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Abbreviations

AD	Alzheimer's disease
Alpha	α
Amy	amygdala

AR	adrenoreceptors
CB	cerebellum
CG	central grey
DA	dopamine
DBH	dopamine β -hydroxylase
BNST	bed nucleus of the stria terminalis
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
Hab	habenula
HP	hippocampus
Hypo	hypothalamus
Gen	geniculate
LC	locus coeruleus
LP	lateral posterior thalamic nucleus
PFC	prefrontal cortex
PVN	paraventricular hypothalamic nucleus
PVNT	paraventricular thalamic nucleus
NE	norepinephrine
NET	norepinephrine transporter
NTS	nucleus tractus solitarius
PD	Parkinson's disease
TH	tyrosine hydroxylase
THL	anteroventricular thalamic nucleus
Sep	septum
SN/VTA	substantia nigra/ventral tegmental area
Str	striatum

Figure 1.

Weight of animals 3 days, 2 weeks and 3 months after DSP4 (50 mg/kg, ip) or saline. Animals that received DSP4 initially (3 days later) gained weight at a slower rate than control animals but quickly caught up to control at 2 weeks. * Indicates significant difference from saline-treated animals.

Figure 2. Norepinephrine (NE) and dopamine (DA) tissue content in prefrontal cortex (PFC), hippocampus (HP), locus coeruleus (LC), cerebellum (CB), amygdala (Amy) and septum/bed nucleus of the stria terminalis (sep/BNST) 3 days, 2 weeks and 3 months after administration of DSP4 and saline. Values are converted to percent control for each time point. NE is significantly reduced 3 days after DSP4 in the PFC, HP and sep/BNST and 2 weeks after DSP4 in HP and CB. NE tissue content is significantly elevated in the sep/BNST 2 weeks after DSP4. NE levels are normal 3 months after DSP4 in all regions. DA

levels are only reduced in the HP 2 weeks after DSP4. * Indicates significant difference from saline-treated animals.

Figure 3.

Norepinephrine transporter (NET) (³H-nisoxetine) binding site concentrations in forebrain regions 3 days, 2 weeks and 3 months after administration of saline or DSP4. NET binding site concentrations were measured in the frontal cortex (FC), septum (sep), bed nucleus of the stria terminalis (BNST), anterior ventricular thalamus (AVT), paraventricular hypothalamic nucleus (PVN), hippocampus (HP), habenula (Hab), hypothalamus (hypo), amygdala (Amy) and substantia nigra/ventral tegmental area (SN/VTA). Values are converted to percent saline for each time point. Three days after DSP4 NET binding site concentrations are significantly reduced in the FC and HP. Two weeks after DSP4 NET binding site concentrations are significantly reduced in the sep, AVT and Hab with normal levels in FC and HP. Three months after DSP4 NET binding site concentrations are similar to saline in all regions studied. * Indicates significant difference from saline treated animals.

Figure 4.

Autoradiographic norepinephrine transporter (NET) binding in specific forebrain regions of saline and DSP4-treated animals 2 weeks after DSP4 administration. Arrows indicate NET specific binding in the frontal cortex (FC), septum (sep), anterior ventral thalamic nucleus (AVT), paraventricular nucleus (PVN), hippocampus (HP) and habenula (Hab).

Figure 5.

α_2 -Adrenoreceptor (α_2 -AR) (³H-RX821002) binding sites concentrations in forebrain regions 3 days, 2 weeks and 3 months after administration of saline or DSP4. α_2 -AR binding site concentrations were measured in the frontal cortex (FC), striatum, (str), anterior septum (Ant sep), ventral septum (Vent sep), bed nucleus of the stria terminalis (BNST), dorsal thalamic nucleus (DTN), paraventricular thalamic nucleus (PVTN), hippocampus (HP), hypothalamus (hypo), amygdala (Amy), substantia nigra/ventral tegmental area (SN/VTA), lateral posterior thalamic nucleus (LPT), central grey (CG). Values are converted to percent control for each time point. α_2 -AR binding site concentrations in DSP4-treated animals are not different from saline-treated animals 3 days later, but 2 weeks after DSP4 α_2 -AR binding site concentrations are significantly elevated in the DTN, PVNT and hypo. Three months after DSP4 α_2 -AR binding site concentrations are significantly elevated in the Ant sep and Amy. * Indicates significant difference from saline-treated animals.

Figure 6.

Autoradiographic α_2 -adrenergic receptor (α_2 -AR) binding in specific forebrain regions of saline and DSP4-treated animals 2 weeks after DSP4 administration. Arrows indicate specific α_2 -AR binding in the frontal cortex (FC), septum (sep), paraventricular thalamic nucleus (PVNT), dorsal thalamic nucleus (DTN), amygdala (Amy) and hypothalamus (Hypo).

Table 1

TH, DBH and NET mRNA expression in the LC of saline and DSP4 treated rats.

	Cell number (% saline)	Grains/cell (% saline)
TH mRNA	(p=0.82)	(p=0.76)
control	100.0 ± 4.5	100.0 ± 3.9
3 days	100.4 ± 3.8	96.6 ± 4.9
2 weeks	95.4 ± 3.3	107.4 ± 11.1
3 months	95.2 ± 6.5	101.2 ± 8.2
DBH mRNA	(p=0.63)	(p=0.58)
control	100.0 ± 3.0	100.0 ± 3.2
3 days	94.0 ± 3.3	107.9 ± 8.8
2 weeks	94.3 ± 4.0	107.9 ± 8.8
3 months	96.9 ± 5.9	100.3 ± 6.5
NET mRNA	(p=0.54)	(p=0.03)
control	100.0 ± 2.8	100.0 ± 6.5
3 days	98.1 ± 6.0	106.0 ± 5.8*
2 weeks	92.5 ± 3.4	89.0 ± 3.4*
3 months	95.4 ± 4.0	91.0 ± 4.9

* Indicates significant difference, p<0.05.

Table 2

Electrophysiological characteristics of LC cells recorded under basal conditions from DSP4 pretreated rats (3 days, 2 weeks and 3 months after 50mg/kg i.p. administration) and the corresponding control group.

	3 days		2 weeks		3 months	
	Control	DSP4	Control	DSP4	Control	DSP4
Active neurons per track (% saline)	100 ± 5 (n=5)	45 ± 3 (n=6)***	100 ± 9 (n=5)	71 ± 9 (n=6)*	100 ± 8 (n=4)##	51 ± 4 (n=7)**
Basal Firing Rate (Hz)	2.17 ± 0.13 (n=54)	2.40 ± 0.16 (n=52)	2.24 ± 0.16 (n=53)	2.28 ± 0.19 (n=52)	2.09 ± 0.18 (n=41)	2.06 ± 0.20 (n=46)
Coefficient of variation (%)	41 ± 2 (n=54)	44 ± 1 (n=51)	37 ± 1 (n=52)	45 ± 2 (n=51)#	38 ± 2 (n=40)	41 ± 2 (n=4)
% spikes in burst	2.20 ± 0.38 (n=36)	3.64 ± 1.06 (n=40)	2.96 ± 0.76 (n=24)	4.86 ± 0.98 (n=39)	3.11 ± 0.79 (n=18)	3.58 ± 1.06 (n=26)
Neurons with burst firing (%)	67	77	45	75 ^a	44	57

Each cell was recorded for 300 seconds. The parameter "Active neurons per track" was carried out in 10 consecutive tracks.

Indicates p<0.001

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indicates p<0.01

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indicates p<0.05 vs respective control group (Bonferroni *post hoc* test).

Indicates p<0.01 vs control 3 days (Bonferroni *post hoc* test).

Indicates p<0.001 vs control 2 weeks (Bonferroni *post hoc* test).

^a
Indicates p<0.01 vs control 2 weeks