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Norepinephrine regulates locomotor hyperactivity in the mouse mutant coloboma

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Abstract

An imbalance between dopaminergic and noradrenergic systems is implicated in hyperactivity disorders such as attention deficit hyperactivity disorder (ADHD) and Tourette syndrome. We have identified the mouse mutant coloboma as an animal model for examining the neurological basis of hyperactivity. Coloboma mice exhibit spontaneous locomotor hyperactivity that is a result of a reduction in SNAP-25, a presynaptic protein that regulates exocytotic release. These mice exhibit an imbalance in catecholamine regulation whereby brain dopamine (DA) utilization is reduced while norepinephrine (NE) concentrations are significantly increased. Further, calcium-dependent NE release was also increased in these hyperactive mice, despite the reduction in SNAP-25. To determine the role of NE in the expression of hyperactivity, brain NE concentrations were reduced using the specific noradrenergic neurotoxin DSP-4 [*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride]. DSP-4 treatment specifically decreased NE concentrations, but had no effect on brain DA or serotonin. Depletion of NE by DSP-4 through either systemic or central administration significantly reduced the locomotor activity in coloboma mice. These results suggest that NE regulation in the CNS plays an important role in the expression of hyperactivity in this mouse model, consistent with results of human studies and current models of ADHD.

Keywords: Mouse mutant; Hyperactivity; Norepinephrine; Attention deficit hyperactivity disorder; SNAP-25; Dopamine; DSP-4; Coloboma

1. Introduction

Abnormalities in catecholaminergic regulation are implicated in the pathogenesis of hyperactivity syndromes such as attention deficit hyperactivity disorder (ADHD). The current hypotheses regarding the mechanisms underlying hyperactivity disorders focus on an imbalance between dopaminergic and noradrenergic regulation rather than an absolute functional increase or decrease in either system (Zametkin et al., 1985; McCracken, 1991; Malone and Swanson, 1993; Russell et al., 2000).

A dysregulation in catecholamine transmission may also contribute to the hyperactivity of the mouse mutant coloboma (gene symbol *Cm*). These mice display spontaneous hyperactivity that is at least threefold greater than the activity of control littermates. Coloboma mice have a semidominant mutation in which the heterozygous state (*Cm*/+) results in the mutant phenotype, while the homozygous mutation (*Cm*/*Cm*) is embryonic lethal (Theiler and Varnum, 1981). The genetic defect in coloboma mice is a deletion mutation that encompasses *Snap*, *PCLB*, *Jag1* and several unidentified genes (Hess et al., 1994; Xue et al., 1999). The *Snap* gene encodes *syn*aptosomal *a*ssociated *p*rotein-25 kD (SNAP-25), which plays an essential role in exocytotic neurotransmitter release. The synaptic membrane proteins, SNAP-25 and syntaxin, interact with integral vesicle-bound proteins, synaptotagmin and synaptobrevin to dock presynaptic vesicles at the active site in readiness for Ca^{2+}-triggered exocytosis (Söllner et al., 1993; Weber et al., 1998). The expression of SNAP-25 is reduced by 50% throughout the CNS in coloboma mice (Hess et al., 1992). This reduction in expression appears to be necessary for the hyperactivity because transgenic restoration of SNAP-25 expression in coloboma mice rescues the aberrant motor activity to near normal levels (Hess et al., 1996). In contrast, the JAG1 deletion accounts for the early lethality in homozygous mutants but not the hyperactivity (Xue et al., 1999). Although the *Snap* gene deletion appears necessary for the expression of hyperactivity, the loss of this gene is not sufficient because hemizygous *Snap* knockout mice are not hyperactive (Washbourne et al.,

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2002). Taken together, the results of the *Snap* transgene rescue and knockout suggest that the *Snap* gene plus one or more additional genes within the deletion contribute to the hyperactivity. Interestingly, the *Snap* gene has been linked to ADHD in humans (Barr et al., 2000; Mill et al., 2002), suggesting that SNAP-25 may regulate motor activity in both man and mouse.

The coloboma mutation also results in region- and neurotransmitter-specific abnormalities in the CNS. The DA metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) are decreased in striatum and nucleus accumbens of coloboma mice (Jones et al., 2001a), suggesting a reduction in DA turnover. By contrast, NE is increased by ~40% in coloboma mice in both striatum and nucleus accumbens but is unchanged in other brain regions (Jones et al., 2001a). Furthermore, mRNA expression of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis, is significantly increased in cells of the noradrenergic nucleus locus ceruleus, but not the substantia nigra (Jones et al., 2001b).

To determine if the increase in NE contributes to the locomotor hyperactivity in coloboma mice, noradrenergic terminals were lesioned using the neurotoxin DSP-4. DSP-4 is transported into noradrenergic axons (Fritschy and Grzanna, 1992) and produces a marked and prolonged reduction in noradrenergic innervation due to the degeneration of locus ceruleus axons (Jonsson et al., 1981). The intent of these experiments was to reduce, not to eliminate, noradrenergic innervation to offset the increase in NE concentrations in coloboma mice without grossly perturbing catecholaminergic innervation.

2. Materials and methods

2.1. Mice

Coloboma mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and thereafter bred at Johns Hopkins University School of Medicine or Pennsylvania State University College of Medicine. In all experiments, coloboma mutant mice and wild type control littermates (8–10 weeks of age) were produced by breeding male *Cm*/+ mice with wild type C3H/HeSnJ mice. Mutant (*Cm*/+) mice were identified at weaning by their hyperactivity. Mice were maintained in group cages with ad lib food and water and a reverse 12-h light/dark cycle. All procedures in this study were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* as promulgated by the National Institutes of Health.

2.2. Neurotransmitter release

Calcium-mediated [^{3}H]DA and [^{3}H]NE release was examined in brain slices using a superfusion release assay. Brain slices (200 μm) from the striatum or nucleus accumbens were cut using a McIlwain tissue chopper and suspended in oxygenated Earles balanced salt solution (5.3 mM KCl, 0.8 mM $MgSO_4$, 117 mM NaCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5.6 mM glucose, 1.8 mM $CaCl_2$) plus 20 μM nialamide. Tissue slices were incubated in either 830 nM of [^{3}H]NE (Amersham Biosciences, Piscataway, NJ) or 125 nM of [^{3}H]DA (Amersham Biosciences) for 20 min at 37 °C and loaded into a 12-chambered Brandel superfusion apparatus maintained at 37 °C. Slices were rinsed with Earles buffer for 45 min at a flow rate of 0.6 ml/min. After the wash, the flow rate was reduced to 0.5 ml/min and 3-min fractions were collected throughout the experiment. Baseline fractions were collected and then the tissue was depolarized using 60 mM KCl in a 2-min pulse with and without calcium. The radioactivity released in each fraction and the total radioactivity remaining in each tissue sample was determined by liquid scintillation spectroscopy. Since the amount of loaded radioactive transmitter can vary between experimental day and even between slices, data were analyzed as a percentage of fractional release (Snyder et al., 1992) that is independent of the absolute DPM present in any one experiment or slice or genotype. Calcium-dependent release was calculated by adding the two fractions collected after stimulation minus the average of the three preceding baseline fractions for that tissue slice. All samples were assessed in duplicate with slices of striatum and nucleus accumbens from both genotypes run concurrently.

2.3. DSP-4 administration

Noradrenergic innervation was specifically depleted in wild type and coloboma mice using the neurotoxin DSP-4 (Sigma, St. Louis, MO) with either a subcutaneous or intracerebroventricular injection. For subcutaneous administration of DSP-4, locomotor activity of control and coloboma mice was first assessed after vehicle administration (subcutaneous) to provide baseline locomotor activity. Mice were then administered 50 mg/kg DSP-4 (10 ml/kg sc) freshly prepared in 0.9% NaCl. The effect of DSP-4 on locomotor activity was assessed 1 week after injection. Another group of mice was tested in parallel, but received saline instead of DSP-4 to provide controls for the high-performance liquid chromatography (HPLC) analysis.

For intracerebroventricular administration, animals were anesthetized with methoxyflurane (Metofane; Mallinckrodt Veterinary, Mundelein, IL), the scalp shaved and a 0.4-cm incision made. Subsequently, 1.5 μl of vehicle or 20 μg/μl of DSP-4 was slowly injected into the fourth ventricle using a 3 mm long needle on a Hamilton syringe. For intracerebroventricular injections, DSP-4 was prepared in equal parts of sterile Ringer solution and dimethylsulfoxide due to the high concentration of DSP-4. The incision was closed using Nexaband S/C topical skin closure (Veterinary Products Laboratories, Phoenix, AZ) and mice were placed on a warm heating pad. After waking, mice were observed for motor control and coordination. Mice exhibiting hemiparesis, com-

plete paralysis or other abnormal motor behaviors were excluded from the experiment. A week following DSP-4 or vehicle treatment, locomotor activity was assessed.

2.4. HPLC analysis

Immediately after behavioral testing, the forebrain (defined by the entire region anterior to the superior colliculus) was dissected and stored at −80 °C until HPLC analysis. The concentration of monoamines and metabolites was determined by HPLC with electrochemical detection as described previously (Jones et al., 2001a). Data were analyzed using ANOVA.

2.5. Behavioral testing

To quantify locomotor activity for the DSP-4 treatments, *Cm*/+ mice and control littermates were placed in individual, automated photocell activity cages (29.2 × 50.5 cm) with twelve 2-cm-high infrared beam detectors arranged in a 4 × 8 grid (San Diego Instruments, San Diego, CA). Mice were extensively habituated to the activity cages before testing with 6 h of exposure to the cages on the day before testing and 6 h on the experimental day. Recordings began 1 h into the dark cycle and lasted for 4 h. A computer recorded beam breaks, which were accumulated every 10 min for the duration of the test. Data were expressed as a percent of vehicle-treated locomotor activity or percent of baseline activity as appropriate.

There is considerable interanimal variability in the locomotor activity of coloboma mice, but intraanimal variability is very low. Therefore, coloboma mice were assessed after vehicle treatment and then again after drug treatment for the subcutaneous DSP-4 test. The invasive nature of the intracerebroventricular injections precluded testing mice after vehicle injection and then after DSP-4 injection. For the intracerebroventricular injections, mice were extensively habituated (12 h) to the locomotor activity cages and baseline locomotor activity recorded. After intracerebroventricular DSP-4 injection, mice were again habituated and then locomotor activity again assessed and compared to baseline. Another group of wild type and coloboma mice was tested in the same paradigm but received intracerebroventricular vehicle injections to exclude habituation or injections effects. After ruling out such extraneous effects, statistical analysis (repeated measures ANOVA) was performed comparing baseline (preinjection) locomotor activity to DSP-4-treated (postinjection) locomotor activity in a within-subjects design.

3. Results

NE and DA release in striatal and nucleus accumbens slices was induced by K^+ stimulation; exocytotic release was defined as K^+-induced calcium-dependent release. In calcium-free medium, little transmitter release was observed after K^+ stimulation (Fig. 1). However, in the presence of calcium, K^+ stimulation evoked DA and NE release in control and coloboma mice (Fig. 1). Calcium-dependent DA release in the striatum and nucleus accumbens was comparable in control and coloboma mice (Fig. 2). In contrast, there was a >35% increase in NE release in both the nucleus accumbens ($P<.01$) and striatum ($P<.05$) of coloboma mice compared to wild type mice (Fig. 2). The release of the radioactive transmitter was not influenced by differences in re-uptake because absolute amounts of DA and NE loaded in the tissue of both genotypes were comparable (data not shown).

To determine the role of NE in the expression of locomotor hyperactivity in coloboma mice, noradrenergic innervation was depleted using the neurotoxin DSP-4. The NE concentration in saline-treated coloboma mice was significantly greater than wild type mice ($P<.05$), which corroborates our previous observations (Jones et al., 2001a). DSP-4 treatment (subcutaneous) resulted in ~50% reduction in forebrain NE concentration in both wild type and

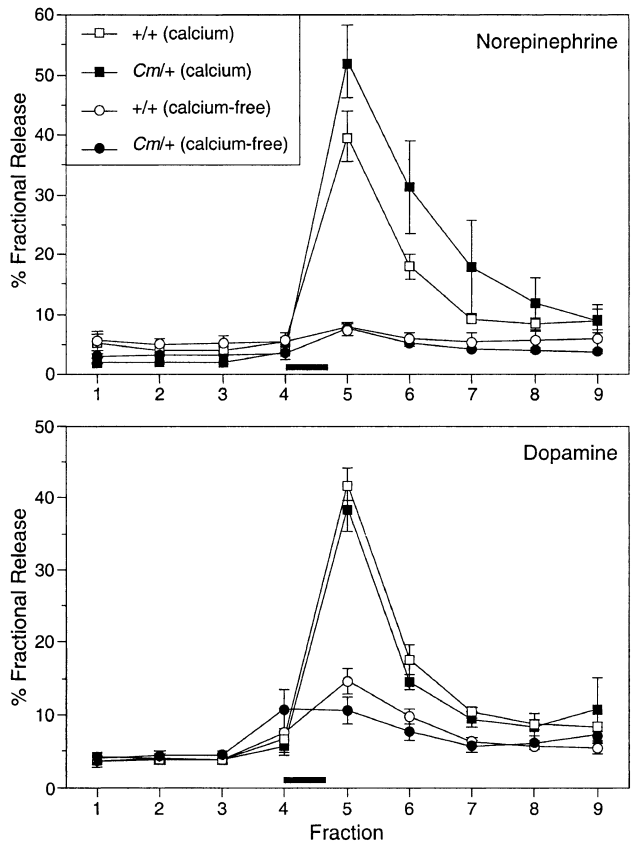

Fig. 1. K^+-stimulated release of [^{3}H]DA and [^{3}H]NE from slices of control and coloboma mouse nucleus accumbens in the presence or absence of calcium. Calcium-dependent release was observed in both control and mutant mice with an increase in NE release in the nucleus accumbens of coloboma mice compared to control mice. Black bar denotes the stimulation period. Data represent means ± S.E.M. (n = 7/genotype).

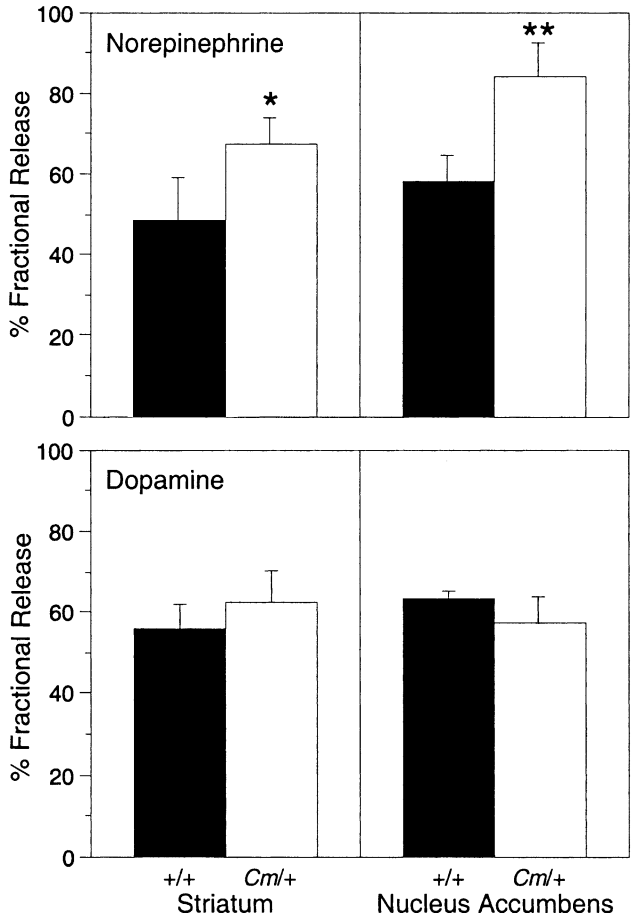

Fig. 2. K^+-stimulated release of [^{3}H]DA and [^{3}H]NE in striatal and nucleus accumbens slices. Release was determined by combining the two consecutive fractions following the stimulation period and normalizing to baselines. Data represent means ± S.E.M. (n = 7/genotype). Asterisks denote significant differences between coloboma and control mice (* P < .05 or ** P < .01 one-tailed t tests).

coloboma mice. There was no significant difference in the concentration of residual NE between genotypes after DSP-4 treatment (Table 1). Neither DA nor 5-HT forebrain concentrations were affected by DSP-4 administration (Table 1).

Table 1
Effect of DSP-4 treatment (subcutaneous) on forebrain monoamine concentrations

	DA	5-HT	NE (brain)
+/+ Vehicle	202 ± 33	49 ± 3	47 ± 2
+/+ DSP-4	193 ± 41	45 ± 4	20 ± 1 **
Cm/+ Vehicle	181 ± 31	43 ± 2	56 ± 3 *
Cm/+ DSP-4	223 ± 39	47 ± 5	26 ± 3 **

Values are expressed in ng/mg wet weight tissue (n = 6/group).
No significant differences were observed in DA or 5-HT concentrations after DSP-4 treatment.

* Significant difference (P < .05) from vehicle-treated wild type mice.

** Significant difference (P < .0001; ANOVA) from vehicle-treated mice.

Depletion of NE by subcutaneous DSP-4 administration produced a significant genotype × drug treatment interaction effect [repeated measures ANOVA; $F(1,16) = 7.129$; P < .05] whereby DSP-4 caused a greater reduction in the locomotor activity of coloboma mice than wild type mice (Fig. 3). DSP-4 treatment ameliorated but did not eliminate the hyperactivity in coloboma mice. As is typical for coloboma mice, interanimal variability in locomotor activity was high, likely due to partial penetrance of the semi-dominant gene defect. However, all coloboma mice were more active than wild type mice. DSP-4 consistently reduced the locomotor activity of all coloboma mice regardless of pretreatment level of motor activity (P < .01). DSP-4 also reduced the locomotor activity of wild type mice (P < .01) but this effect was not so consistent with some mice demonstrating little or no reduction in locomotor activity after DSP-4 treatment. The reduction in locomotor activity in wild type mice following DSP-4 treatment did not approach a floor effect with activity maintained at 70 ± 7%.

Because DSP-4 destroys both central and peripheral noradrenergic axons, it was possible that the reduction in activity was due to a decrease in blood pressure, adrenal activity or other peripheral processes. Therefore, DSP-4 was delivered into the fourth ventricle to eliminate possible confounds of peripheral effects. Intracerebroventricular injection of DSP-4 produced a significant decrease in NE forebrain concentration for control and coloboma mice after DSP-4 administration (Fig. 4), with no significant change in DA (data not shown). DSP-4 had no effect on spleen NE concentrations (Fig. 4) demonstrating that the effects of intracerebroventricular administration were limited to the CNS.

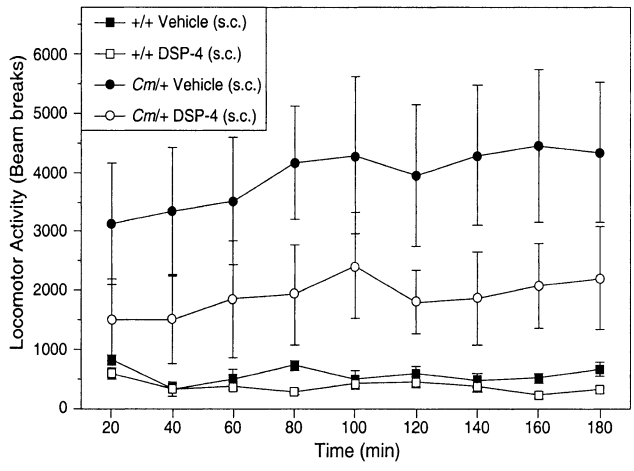

Fig. 3. Locomotor activity of adult control and coloboma mice was assessed after saline treatment and then a week after specific depletion of noradrenergic innervation using the neurotoxin DSP-4 (50 mg/kg sc). Data represent means ± S.E.M. (n = 9/genotype). There was a significant genotype × drug treatment interaction effect [repeated measures two-way ANOVA; $F(1,16) = 7.129$; P < .05].

Similar to depletion of NE by subcutaneous DSP-4 administration, intracerebroventricular injection of DSP-4 produced a significant genotype × drug treatment interaction effect [repeated measures ANOVA; $F(1,16)=7.129$; $P<.05$] whereby the reduction in locomotor activity caused by DSP-4 in coloboma mice was exaggerated compared to the effect on wild type mice activity (Fig. 5). Intracerebroventricular delivery of DSP-4 dramatically reduced coloboma mouse locomotor activity to less than 30% of baseline whereas wild type mouse activity after DSP-4 treatment was ~80% of baseline. Wild type mouse activity was not significantly reduced with intracerebroventricular DSP-4 administration. Similar to subcutaneous treatment, DSP4 reduced but did not abolish the coloboma mouse hyperactivity.

It is possible that the experimental design comparing pre- and postinjection locomotor activity affected locomotor activity independent of the effects of DSP-4. Therefore, locomotor activity was assessed in coloboma and normal mice before and after intracerebroventricular vehicle injection. Locomotor activity of both coloboma and wild type mice after intracerebroventricular vehicle injection actually slightly exceeded preinjection levels (data not shown), suggesting that effects of habituation or route of drug administration could not account for the decrement in locomotor activity observed after DSP-4 treatment.

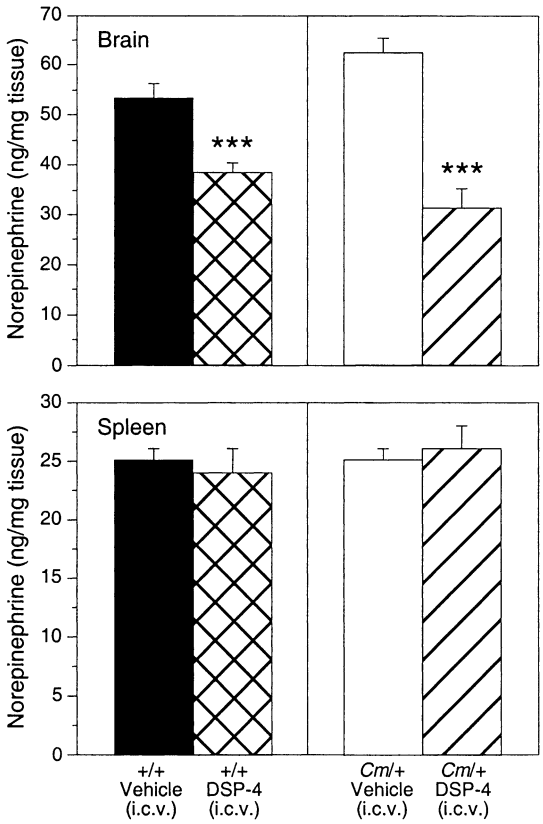

Fig. 4. NE concentrations in the forebrain and spleen after central administration of DSP-4. Data represent means ± S.E.M. ($n=4-5$). DSP-4 significantly reduced NE concentrations in wild type and coloboma mouse brain (ANOVA; $F(3,16)=61.011$; *** $P<.001$) but not in spleen after intracerebroventricular administration.

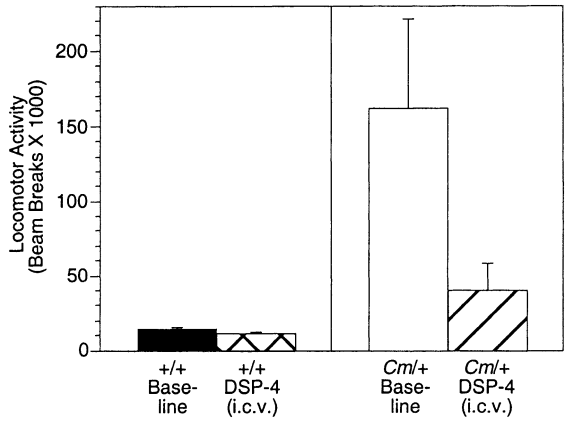

Fig. 5. Locomotor activity of control and coloboma mice was assessed before and 1 week after treatment with the neurotoxin DSP-4 (intracerebroventricular). Data represent means ± S.E.M. ($n=4-5$) of total locomotor activity recorded over 4 h. There was a significant genotype x drug treatment interaction effect (repeated measures two-way ANOVA; $F(1,7)=7.978$; $P<.05$).

4. Discussion

The results suggest that in a pathological state, NE plays a significant role in the regulation of locomotor activity. Consistent with the observed increase in NE concentrations in striatum and nucleus accumbens (Jones et al., 2001a), calcium-dependent NE release was significantly increased in these regions of the hyperactive mouse mutant coloboma. Reducing brain NE concentrations with DSP-4 disproportionately reduced the locomotor activity of the hyperactive mouse mutant coloboma compared to wild type mice. Administration of DSP-4 through either a peripheral or central route consistently reduced NE content in forebrain with no effect on DA or 5-HT suggesting that the reduction in locomotor activity was specific to the loss of central NE. Although NE concentrations were reduced to below wild type levels, the hyperactivity was not completely eliminated suggesting that additional, as yet undefined, factors contribute to the mutant phenotype.

Despite the reduction in SNAP-25 expression, calcium-dependent release was apparently not perturbed in coloboma mice. Similarly, in amperometric recordings from chromaffin cells, no differences in quantal size, frequency of release or kinetics of catecholamine release were detected between control and coloboma mice (Colliver et al., 2000). A previous report demonstrated near complete loss of exocytotic DA release in coloboma mice while calcium-dependent release of other transmitters was near normal (Raber et al., 1997). The discrepancy between these results is difficult to reconcile although different methods were used to measure release. That neurotransmitter release is apparently spared despite the reduction in SNAP-25 suggests that excess

SNAP-25 may be present in presynaptic terminals. Indeed, without excess SNAP-25, small fluctuations in demand for neurotransmitter release could not be met. It is possible that stressing the release machinery in coloboma mice by repetitive K^+ or electrical stimulation would reveal abnormalities in release. Currently, the relationship between SNAP-25 deficiency and hyperactivity is not understood, but it is clear that presynaptic proteins are highly interactive. SNAP-25 and syntaxin interact directly with ion channels, G-proteins and G-protein-coupled autoreceptors (Wiser et al., 1996; Rettig et al., 1996; Stanley and Mirotznik, 1997; Charvin et al., 1997; Kim and Catterall, 1997; Linial et al., 1997). A change in the balance among these presynaptic elements may result in dysregulation of specific transmitter systems depending on the protein interactions and molecular dynamics of each system.

In coloboma mice, the reduction in locomotor activity after DSP-4 treatment was exaggerated compared to normoactive mice suggesting that an increase in NE may have a disproportionate effect on locomotor activity. It is interesting to note that the activity of control animals is reduced after subcutaneous, but not intracerebroventricular DSP-4 administration, suggesting that the peripheral depletion of NE contributed to the reduction observed with subcutaneous DSP-4 treatment. In fact, previous reports have demonstrated similar subtle reductions in locomotor activity after DSP-4 treatment (Ogren et al., 1983; Harro et al., 2000), although this effect is not entirely consistent among all investigations (Lapiz et al., 2000; Harkin et al., 2001).

Because the primary treatment for ADHD is stimulant medication, including amphetamine and methylphenidate, research on hyperactivity disorders has focused on the neurotransmitter DA. Genetic linkage studies have demonstrate an association between the DA transporter and ADHD (Cook et al., 1995; Gill et al., 1997; Waldman et al., 1998) and the D4 DA receptor has also been associated with novelty seeking and with ADHD in some population studies (LaHoste et al., 1996; Smalley et al., 1998; Faraone et al., 1999). However, others were unable to demonstrate such an association (Castellanos et al., 1998; Rowe et al., 1998). SPECT and PET studies in ADHD patients have demonstrated decreased metabolic activity in the basal ganglia (Lou et al., 1989, 1990), a region that contains high concentrations of DA and DA receptors. Assessments of catecholamine metabolites in cerebral spinal fluid of ADHD children support the imaging studies, demonstrating a positive correlation between the DA metabolite HVA, the degree of hyperactivity (Castellanos et al., 1994), and behavioral response to stimulant medication (Castellanos et al., 1996).

Although defects in dopaminergic function are often associated with disorders such as ADHD, evidence is mounting for a role of NE in hyperactivity disorders in humans. Clinical trials in children comparing selective dopaminergic and noradrenergic agents have shown that the most effective drugs are those that have effects on both catecholamines (Zametkin et al., 1985). Genetic analysis has demonstrated an association between ADHD and noradrenergic genes, including the α_{2A}-adrenergic receptor, α_{2C}-adrenergic receptor and DA β-hydroxylase (Feng et al., 1998; Comings et al., 1999); indeed, linkage analysis demonstrates that these genes are more important factors in ADHD than either the dopaminergic or serotonergic genes (Comings et al., 2000). The role of NE in hyperactivity disorders is also supported by pharmacological evidence. Clinical drug trials have demonstrated that the symptoms of some patients improve following treatment with clonidine, a mixed α_1/α_2 adrenergic agonist or guanfacine, a more selective α_2 adrenergic agonist (Hunt et al., 1985, 1995). Indeed, most drugs used to treat ADHD, including amphetamine and methylphenidate, exert effects at α_2-adrenergic receptors (Shenker, 1992). In children with ADHD whose symptoms are improved by amphetamine, the NE metabolite MHPG is significantly reduced by the drug treatment (Shekim et al., 1979, 1983). Collectively, these data suggest that abnormalities in noradrenergic regulation may be central to the pathophysiology of hyperactivity disorders.

Because ADHD is a heterogeneous disorder (Todd et al., 2002), it is not likely that noradrenergic dysregulation underlies the pathophysiology in all patients. For example, there are no consistent changes in urinary MHPG excretion in ADHD patients. Some studies report no differences (Rapoport et al., 1978; Zametkin et al., 1985); others report increases (Khan and Dekirmenjian, 1981); and still others show decreases (Shekim et al., 1979, 1983). Such heterogeneity could account for individual differences in drug responses whereby subsets of patients respond preferentially to amphetamine, methylphenidate or clonidine (Arnold et al., 1978; Elia et al., 1991). Several current models of hyperactivity disorders include an increase in noradrenergic tone (Mefford and Potter, 1989; Malone et al., 1993; Pliszka et al., 1996), but it is unlikely that any single model can account for the pathophysiology in all patients. Indeed, it is likely that coloboma mice reflect the neurochemical imbalance of a subset of patients, as the *Snap* gene is only one of many genes that has been linked with hyperactivity disorders in humans (Barr et al., 2000; Mill et al., 2002).

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