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Genetic alteration in the dopamine transporter differentially affects male and female nigrostriatal transporter systems

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ABSTRACT

Female mice with a heterozygous mutation of their dopamine transporter (+/− DAT) showed relatively robust reductions in striatal DAT specific binding (38–50%), while +/− DAT males showed modest reductions (24–32%). Significant decreases in substantia nigra DAT specific binding (42%) and mRNA (24%) were obtained in +/− DAT females, but not +/− DAT males (19% and 5%, respectively). The effects of this DAT perturbation upon vesicular monoamine transporter-2 (VMAT-2) function revealed significantly greater reserpine-evoked DA output from +/+ and +/− DAT female as compared to male mice and the DA output profile differed markedly between +/+ and +/− DAT females, but not males. No changes in VMAT-2 protein or mRNA levels were present among these conditions. On the basis of these data, we propose: (1) a genetic mutation of the DAT does not exert equivalent effects upon the DAT in female and male mice, with females being more affected; (2) an alteration in the DAT may also affect VMAT-2 function; (3) this interaction between DAT and VMAT-2 function is more prevalent in female mice; and (4) the +/− DAT mutation affects VMAT-2 function through an indirect mechanism, that does not involve an alteration in VMAT-2 protein or mRNA. Such DAT/VMAT-2 interactions can be of significance to the gender differences observed in drug addiction and Parkinson's disease.

1. Introduction

The dopamine transporter (DAT) plays an important role in calibrating the duration and intensity of DA neurotransmission in the central nervous system. The DAT accomplishes this goal by rapid reuptake of dopamine (DA) from the synaptic cleft into presynaptic terminals, and thereby controls the intensity and duration of dopaminergic neurotransmission by setting the concentration of DA in the extracellular space [1]. In addition, the DAT is the site through which amphetamine-like drugs result in reverse transport of DA [2,3] and the site of entry for neurotoxins that are relatively selective for dopaminergic neurons (e.g. MPP+ and 6-hydroxydopamine) [4–7]. Accordingly, the DAT can exert a significant influence upon dopaminergic function in both healthy and diseased conditions.

There exist significant differences in the DAT between females and males, which can contribute to gender differences in conditions ranging from drug addiction to Parkinson's disease [8,9]. The numbers/densities of DATs are greater in female versus male rats [10,11] and assays of DAT function indicate that a more active DAT is present within females versus males [12,13]. These findings of a sex difference in the DAT are not limited to animal studies, as clinical reports have shown greater numbers/densities of DATs within healthy adult women versus men [14–16].

One approach to study DAT function is with the use of mice possessing a mutation in the DAT [2]. However, given the predominant sex difference in DATs, an important issue to address is whether this mutation is processed equally in females and males. There are data which show that striatal DA concentrations of heterozygous mutant DAT (+/− DAT) female mice are significantly decreased as compared with +/− DAT males. No such difference is present between wild type (+/+ DAT) control female and male mice [17]. Accordingly, there exits some basis for the proposal that dopaminergic function is not affected equally in +/− DAT female and male mice, suggesting the existence of an important interaction between sex and a mutation of the DAT.

Abbreviations: ANOVA, analysis of variance; DA, dopamine; DAT, dopamine transporter; EDTA, ethylenediaminetetraacetic acid; KRP, kreb's ringer phosphate; MPP+, 1-methyl-4-phenylpyridinium; 6-OHDA, 6-hydroxydopamine; VMAT-2, vesicular monoamine transporter-2; WT, wild type.

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Additional considerations of significance regarding DAT mutations is that of the extent that other dopaminergic processes are affected, whether these represent direct or indirect effects of the mutation and, again, the potential for differential modulation within females and males. A precedent for this proposition can be traced back to data showing marked dissimilarities in dopaminergic function among different mouse strains. For example, the significant differences in striatal D2 receptors observed among different strains of mice can secondarily alter a variety of responses like neuroleptic-induced catalepsy [18] and amphetamine-induced locomotion [19]. Moreover, strain differences in striatal calmodulin can affect a number of striatal functions, and do so in an unpredictable manner, due to its generalized actions in regulating brain calcium concentrations [20]. Therefore, the importance of considering not only the primary, but also secondary, effects resulting from a targeted mutation can be appreciated. An example of a related dopaminergic function that could be affected by a DAT mutation is that of the vesicular monoamine transporter-2 (VMAT-2). The VMAT-2 sequesters cytoplasmic DA. This function prevents the oxidation of DA in the cytoplasm and thus contributes to the DA available for release. In this way, during normal dopaminergic neurotransmission, the DAT regulates extracellular concentrations of DA, while VMAT-2 regulates cytosolic concentrations of DA and indirectly regulates extracellular DA by affecting the amount of DA available for release from vesicular stores. Therefore, these two transporters play a critical role in dopaminergic function by regulating availability and access of intracellular and extracellular DA. It has been reported that the function of these two transporters may be linked [21,22]. To the best of our knowledge the potential for sex-dependent interactions between the DAT and VMAT-2 have not been investigated.

The information provided from this brief review highlight a key role for the DAT in dopaminergic functioning and the presence of sex-differences in this function. What is not clear is the extent to which a genetic DAT perturbation may be revealed in females and males and the potential for sex-dependent alterations in other dopaminergic functions. The purpose of this report is to evaluate the relationship among these variables. In specific, heterozygous mutant DAT (+/−DAT) and wild type control (+/+ DAT) male and female mice are compared in two series of experiments. First, sex differences in DAT specific binding within various regions of the striatum and substantia nigra and DAT mRNA within the substantia nigra are compared among +/+ and +/− DAT male and female mice. Since it has been observed that striatal DA concentrations and function are more severely affected within +/− DAT female versus male mice [17], in this initial series of experiments we determine whether the protein and message levels of DATs show a corresponding sex differences between +/+ and +/− DAT mice. In a second series of experiments we utilize these +/+ and +/− DAT mice to assess the possibility for a sex-dependent interaction between DAT and VMAT-2 function. To accomplish this goal responses of superfused striatal tissue to an infusion of the VMAT-2 blocker, reserpine, was evaluated within +/+ and +/− DAT male and female mice. As reserpine binds to the VMAT-2 to impair vesicular uptake and storage of DA within nerve terminals [23], it has been used as an agent to assess VMAT-2 function in various animal models [24–26]. To establish whether any potential differences in response to reserpine are attributable to a direct effect of DAT perturbation on the VMAT-2, VMAT-2 specific binding within the striatum and substantia nigra and VMAT-2 mRNA within the substantia nigra was measured among +/+ and +/− DAT male and female mice. With the performance of these experiments within male and female mice where a normal or perturbed DAT function is present, it will be possible to determine whether this DAT mutation differentially affects male and female mice as well as gain an understanding of the sex-dependent interaction that may exist between the DAT and VMAT-2.

2. Materials and methods

2.1. Animals

The mice (3–7 months of age) were male and female littermate wild type controls (+/+ DAT) and heterozygous (+/− DAT) DAT mutant mice derived from the C57BL/6 strain [2] supplied through the generosity of Dr. Marc Caron of Duke University. In these experiments, comparisons between +/+ versus +/− DAT male and female mice were used to evaluate the impact of gender in a condition of a DAT deficit, as opposed to the complete absence of the DAT that would characterize the null mutant (−/− DAT) DAT mice. The use of +/− DAT for comparison with wild type mice was performed for three reasons. First, a DAT deficit, as opposed to a DAT absence (−/− DAT), provides for a more realistic condition that would be applicable and relevant to the clinical setting [27,28]. Second, −/− DAT mice show a marked hypopituitarism with glands being about half the size of that in normal animals [29]. Such secondary endocrine effects may confound some of the sex/hormonal parameters to be evaluated within the proposed experiments. Finally, the interpretations of results derived from −/− DAT mice can sometimes be problematic due to the extreme nature of the deficit and accompanying developmental adaptations that can result from the absence of the DAT [30]. While estrous cycle stage may influence the parameters to be measured, basic sex difference in nigrostriatal dopaminergic function remain present [31], and it is the evaluation of these basic sex differences that serve as the purpose of this report. Mice were genotyped by polymerase chain reaction and 1% agarose gel of electrophoresis of tail DNA. Primers for genotyping DAT mice were Neo2 5′ tga ccg ctt cct cgt gc 3′; JAH1 5′ ccc gtc tac cca tga gta aaa 3′; and JAH2 5′ ctc cac ctt cct agc act aac 3′ used in 1:1:1 ratio. The mutant fragment (900 kb) is larger than the wild type fragment (600 kb) and can be used to genotype mice as +/+ or +/−. All mice were housed in the vivarium under a 12 h light/dark cycle (lights on at 06:00 h) with food and water available *ad libitum*. The Animal Care and Use Committee at NEOUCOM in accordance with NIH guidelines approved all experimental procedures. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. General procedure

Two series of experiments were performed within this report. In the first series both protein (striatum and substantia nigra) and message levels (substantia nigra) of DAT were determined within +/+ and +/− DAT male and female mice. In the second series of experiments, the sex-dependent effects of this DAT perturbation upon the VMAT-2 were assessed. In these latter experiments two different approaches were used to evaluate the VMAT-2. These included measures of: (1) reserpine-evoked striatal DA output and (2) protein (striatum and substantia nigra) and message levels (substantia nigra) of VMAT-2. In this way, both a static and dynamic assessment of the VMAT-2 was achieved. To perform these assays, mice were euthanized by rapid decapitation, the brain removed and bisected. One half of the bisected brain was immediately frozen and stored at −80 °C until assay for DAT and VMAT-2 specific binding protein (striatum and substantia nigra) and mRNA (substantia nigra) levels. The striatum was dissected out from the contralateral half and prepared for measurement of reserpine-evoked striatal DA responses as determined using *in vitro* superfusion.

2.3. In vitro superfusion

Following a midline bisection, the ventricles on the medial side of the brain were pried open and the cortex cut away revealing

the striatum. The striatum was placed within a breaker containing cold (4 °C) Kreb's Ringer Phosphate (KRP) medium. The striatum was sliced into tissue fragments (approximately 0.5 mm × 0.5 mm × 0.5 mm) prior to placement into the superfusion chamber. The superfusion medium was a modified KRP medium: 120 mM NaCl, 4.8 mM KCl, 0.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10.2 mM Na_2HPO_4, and 0.18% glucose at a pH of 7.35–7.4. The KRP medium was filtered (0.45 μm, Millipore Filter) prior to use. Each of the superfusion chambers housing the striatal tissue fragments consisted of a tuberculin syringe cut off at the 0.3 ml level and was inserted into a 22 G lumbar puncture needle. The top of the chamber was sealed with a rubber stopper containing an entry port for filtered humidified air to oxygenate the tissue and an exit port for the perfusate. The tissue fragments (overall mean ± S.E.M. of tissue weight in chambers = 6.4 ± 0.4 mg) were suspended in the chamber on cellulose filter paper and were superfused with KRP at a flow rate of approximately 25 μl/min. The chambers were maintained in a temperature-regulated water bath at 37 °C. Following a 60-min equilibration period during which no samples were collected, effluent samples were collected at 10-min intervals for a total of 5 intervals. After determining the basal output from collection interval 1, KRP medium containing 1 μM of reserpine (Sigma Chemical Co., St. Louis, MO) was infused throughout the remainder of the superfusion period. At the end of the superfusion, tissue fragments were removed and weighed.

2.4. Neurochemical assay

Measurements of DA were determined through injection into a 20 μl loop on an ESA high-performance liquid chromatography system (ESA-Chelmsford, MA) with a coulochem II electrochemical detector set at E1: 400 mV, R1: 2 μA, E2: −350 mV, R2: −100 nA. Biogenic amines were separated on a Supelco column (Discovery C-18, 10 cm × 3 mm, 5 μm). The mobile phase consisted of 50 mM sodium acetate, 27.4 mM citric acid, 10 mM sodium hydroxide, 0.1 mM sodium octyl sulfate, 0.1 mM EDTA and 7% methanol in distilled water. The final pH of 4.5 was obtained with the addition of sodium hydroxide, and the mobile phase was filtered through a Millipore filter (0.45 μm) and degassed prior to use. The DA standard (Sigma Chemical Co., St. Louis, MO) was diluted in the KRP superfusion medium in doses of 6.25, 12.5, 25, 50, 100 and 200 pg/20 μl to construct a standard curve. DA from effluent superfusion samples was determined by comparing peak heights and retention times with that of standards using the software program provided by ESA. The sensitivity of this assay was <6.25 pg/20 μl as determined by the reliable determinations of peaks within standards.

2.5. DAT and VMAT2 autoradiography

The striatum anterior (bregma 1.54–1.18), median (bregma 0.50–0.14) and posterior (bregma −0.34 to −0.70 mm) and the substantia nigra (bregma −2.80 to −3.88 mm) [32] of the contralateral hemisphere were cut on a cryostat in 12 μm slices. Slices were maintained at −80 °C until assay. DAT autoradiography in the striatum and the substantia nigra was performed as previously described [33]. DAT specific binding used 20 pmol of the ligand 3β-(4-[^{125}I]iodophenyl)tropane-2β-carboxylic acid isopropyl ester ([^{125}I]-RTI-121) (2200 Ci/mmol, PerkinElmer, Boston, MA, USA). Non-specific binding was evaluated using 100 nM of mazindol. Slices were apposed to Kodak films (Biomax), 16 h for the striatum and 78 h for the substantia nigra. VMAT2 autoradiography in the striatum and the substantia nigra was performed using the specific ligand [^{3}H]dihydrotetrabenazine ([^{3}H]-TBZ-OH, American Radiolabeled Chemicals, St. Louis, MO, USA) [34]. Specific binding was evaluated using 20 nM of [^{3}H]-TBZ-OH (20 Ci/mmol) and 1 μM of cold TBZ-OH for the non-specific binding. Slices were exposed to Kodak films (Biomax), 4 weeks for the striatum and 6 weeks for the substantia nigra. Films were analyzed using the software NIH Image 1.63.

2.6. DAT and VMAT2 in situ hybridization

DAT mRNA in the substantia nigra was measured by *in situ* hybridization using a cDNA probe labelled with [^{35}S]-UTP, as previously described [35]. VMAT2 mRNA was measured by *in situ* hybridization using a complementary oligonucleotide [^{35}S]-d-ATP labelled, as described [35]. Slices were exposed to Kodak films (Biomax), for 3 days (DAT mRNA) and 4 weeks (VMAT2 mRNA) and analyzed using the software NIH Image 1.63.

2.7. Statistical analysis

Statistical comparisons of autoradiography and *in situ* hybridization data were performed first with an overall analysis of variance (ANOVA) test using Stat View 4.51 for Macintosh Computer software. When this global test was significant ($p < 0.05$), two-way analyses of variance considering sex (female versus male) and genotype (+/+ versus +/− DAT) were performed.

The reserpine-evoked DA output was subjected to two different statistical analyses. First, to compare directly the effects of a DAT perturbation within each sex the reserpine-evoked DA output from collection intervals 2–5 was analyzed with use of a 2 (genotype: +/+ versus +/− DAT) × 4 (superfusion time: collection intervals 2, 3, 4 and 5) two-way ANOVA with one repeated measure. Second, to achieve an overall evaluation of sex differences a 2 (genotype: +/+ versus +/− DAT) × 2 (sex: female versus male) two-way independent groups ANOVA was performed. This analysis was performed separately on the data from collection interval 1, to establish whether any difference was present in basal DA output and on the total amount of DA output from collection intervals 2–5 to establish whether any differences were present in response to the reserpine infusion. For all analyses performed, subsequent post hoc pairwise comparisons were performed using the Fisher's protected LSD test to maintain the error rate at the 0.05 level. A $P < 0.05$ was required for results to be considered statistically significant.

3. Results

3.1. DAT specific binding in striatum

The results obtained from DAT specific binding determinations within the various areas of the striatum are presented in Fig. 1A and representative examples of this DAT binding are presented in Fig. 1B. Within the anterior region of the striatum, a significant interaction between sex and genotype was obtained in both medial and lateral sites. DAT binding of wild type (WT, +/+ DAT) females was significantly greater than WT males in both the medial and lateral sites. While +/− DAT females showed significantly decreased DAT binding as compared with their WT controls at both sites in the anterior striatum, for males a statistically significant decrease in +/− DAT mice was obtained only within the lateral site of the anterior striatum. No statistically significant differences were obtained between +/− DAT females and males in either the medial or lateral sites of the anterior striatum. In the medial site of the median striatum, a significant effect of genotype was obtained and DAT binding was significantly decreased in +/− DAT female, but not male, mice. Within all other striatal sites sampled, DAT binding of +/− DAT mice was decreased, but did not reach a statistically significant difference.

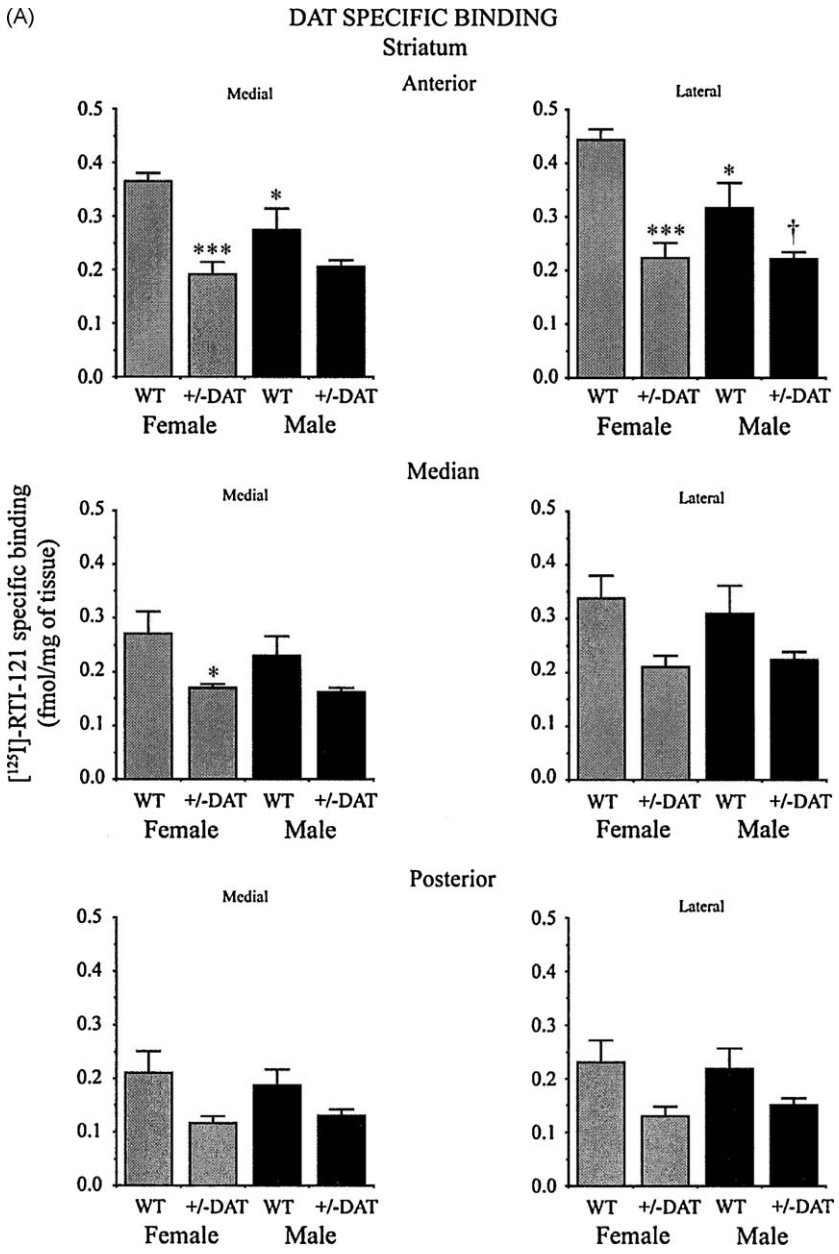

Fig. 1. (A) DAT specific binding in anterior, median and posterior striatum of +/+ DAT wild type (WT) and heterozygous (+/− DAT) female and male mice. $^{*}p < 0.05$ and $^{***}p < 0.005$ versus WT female; $^{\dagger}p < 0.05$ versus WT male. Values are the means (fmol/mg of tissue) ± S.E.M. of 4 individual mice per group. (B) Representative examples of [^{125}I]-RTI-121 DAT binding in anterior, median and posterior striatum of +/+ DAT wild type (WT) and heterozygous (+/− DAT) female and male mice.

3.2. DAT in substantia nigra

The results obtained for DAT specific binding and mRNA within the substantia nigra along with representative examples are presented in Fig. 2A and B, respectively. A statistically significant main effect for genotype was obtained for DAT binding and DAT mRNA levels within the substantia nigra with lower values for the +/− DAT compared to the +/+ DAT mice. DAT binding of WT females was significantly greater than that of WT males and +/− DAT females within the substantia nigra. In addition, substantia nigra DAT mRNA levels were significantly decreased in +/− DAT female and in WT males as compared with WT female mice. No statistically significant differences were present between +/− DAT females and males for DAT binding or mRNA.

3.3. Reserpine-evoked DA

The raw data reserpine-evoked DA output profiles are presented in Fig. 3A and a summary of the overall basal and reserpine-evoked DA responses are contained in Fig. 3B. In female mice a markedly different reserpine-evoked DA output profile was obtained between +/+ and +/− DAT mice. Whereas DA output of +/+ mice continued to rise over the reserpine-infusion period, producing statistically significant increases over basal DA levels (collection interval 1) at collection intervals 4 and 5 of the superfusion, that of +/− DAT females rose rapidly, producing statistically significant increases over basal DA at collection intervals 3 and 4, but decreased precipitously at collection interval 5. As a result of these differences in DA output profiles, a statistically significant interaction was obtained with an increase

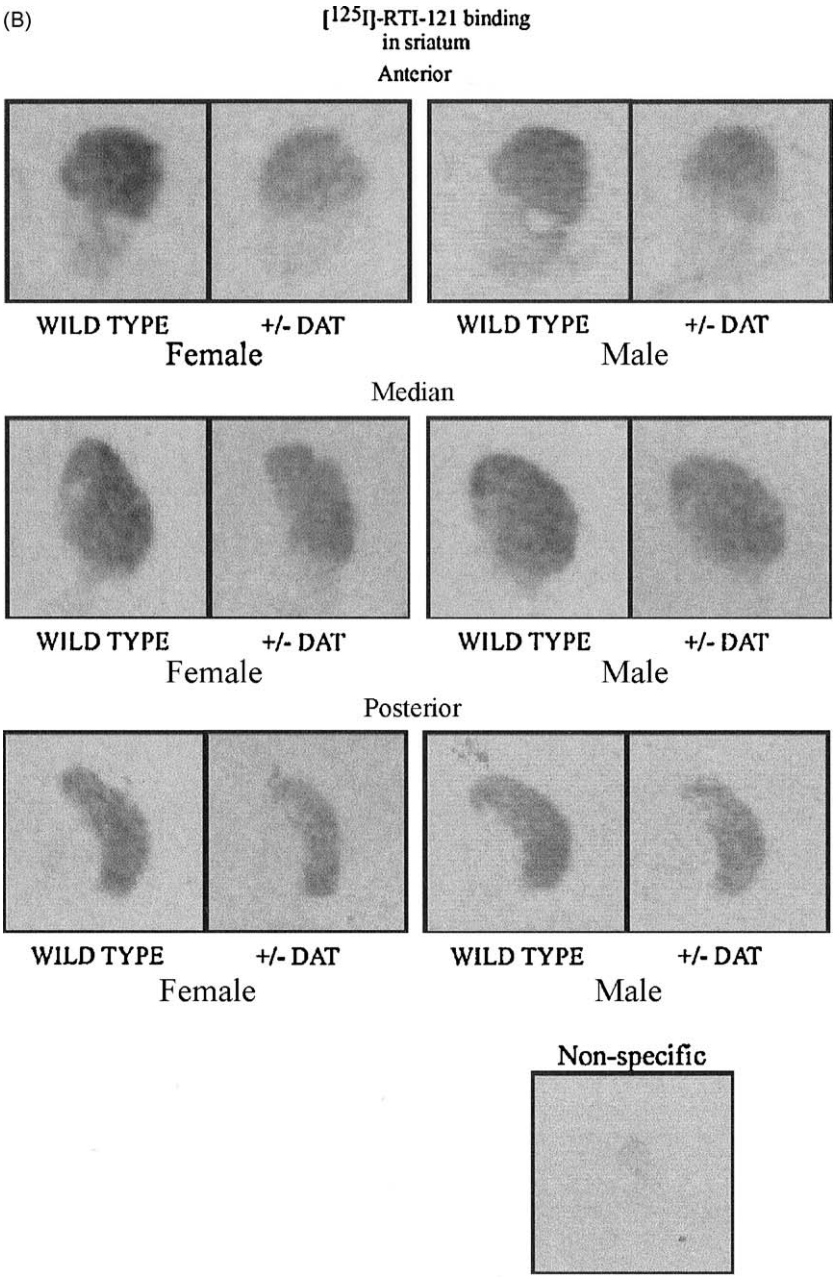

Fig. 1. (*Continued*).

of +/− versus +/+ DAT female mice at collection intervals 3 and 4 and a statistically significant decrease at collection interval 5. In males, the basic reserpine-evoked DA output profile was similar between +/+ and +/− DAT mice, with a statistically significant peak at collection interval 3 and a gradual decline thereafter. In +/− DAT male mice, levels at collection interval 4 also remained significantly increased over basal values. For +/− DAT males an overall statistically significant DA response over +/+ DAT males was achieved at collection interval 3.

An overall summary of basal and reserpine-evoked DA responses among all experimental conditions is presented in Fig. 3B. No overall differences were observed for basal DA output as determined from the DA levels measured at collection interval 1. A statistically significant main effect for sex was obtained with regard to reserpine-evoked DA with females showing greater DA responses. No statistically significant effect of genotype was attained for these reserpine-evoked DA responses in either female or male mice.

3.4. VMAT-2 in striatum and substantia nigra

The results from striatal VMAT-2 binding are contained in Fig. 4A and representative examples are shown in Fig. 4B. No statistically significant differences in VMAT-2 binding were achieved for any of the sites sampled or as a function of the condition of the mice. Similarly, within the substantia nigra, VMAT-2 binding and mRNA levels were not significantly different among +/+ versus +/− DAT female or male mice (Fig. 5A and B).

4. Discussion

Results from this first series of experiments reveal that neither DAT protein nor mRNA levels are equally affected in +/− DAT female versus male mice. Specifically, while +/− DAT females show a more consistent and expected decrease in DAT binding and mRNA, this was substantially less evident in males where a

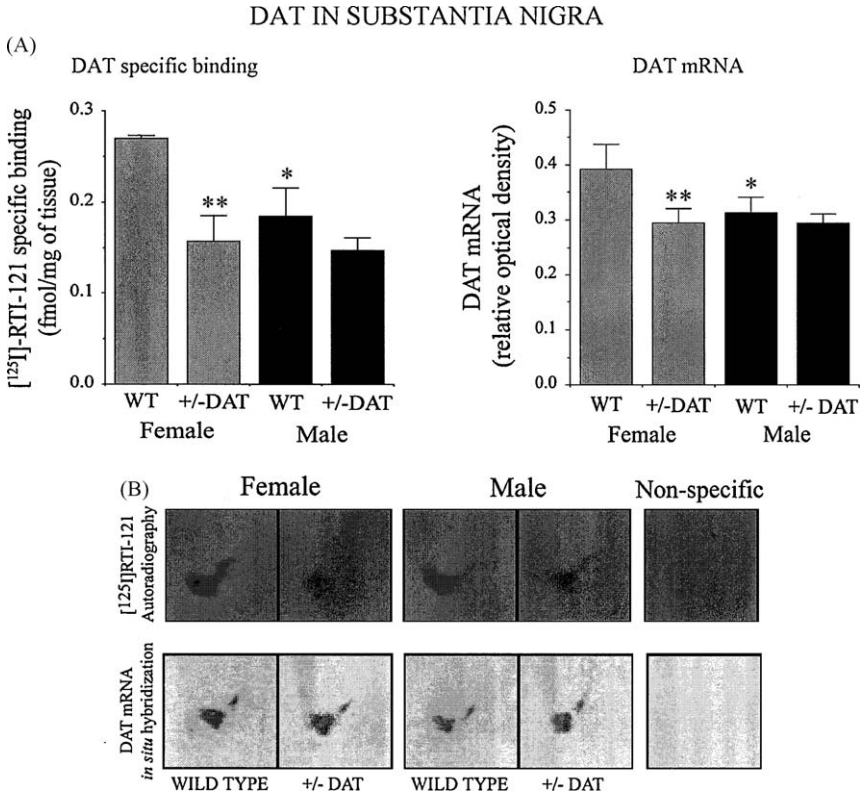

Fig. 2. (A) DAT specific binding and *in situ* hybridization of DAT mRNA in substantia nigra of +/+ DAT wild type (WT) and heterozygous (+/− DAT) female and male mice. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus WT female. Values are the means ± S.E.M. of 4 individual mice per group. (B) Representative examples of [^{125}I]-RTI-121 binding and DAT mRNA levels in substantia nigra are shown.

statistically significant decrease in DAT binding was seen only within one site of the striatum and not within the substantia nigra. Moreover, within female, but not male mice, the decrease in striatal DAT protein would appear to be related to DAT production as a corresponding decrease in substantia nigra DAT mRNA was present in these +/− DAT females. No such changes occurred in male mice. In this regard, females are clearly more affected by this genotypic alteration of their DAT. This sex differential was like that reported for DA concentrations and dopaminergic responses to the psychostimulant, methamphetamine, where more extreme changes were seen in +/− DAT females as compared with both their +/+ DAT wild type controls and +/− DAT males [17].

When combining the present results with previous findings [17], it seems quite apparent that females are more affected by this DAT perturbation. A clear explanation for this sex difference in response to a DAT mutation is not immediately apparent, but this observation has important implications. The findings that females show a more conventional response to a heterozygous DAT mutation (~50% decrease in DAT protein and mRNA) may be related to the initially augmented levels of DAT as compared to males [10,11]. When comparing DAT protein and mRNA levels between +/− DAT female and male mice, it is interesting that essentially equivalent levels are present. One interpretation of this phenomenon is that a heterozygous mutation may produce an *absolute* level of deficit that is sex-independent. As a result, females demonstrate responses (more consistent statistically significant decreases in DAT protein and mRNA) indicative of a more severe change to this DAT mutation that then may be due to their inherently higher basal levels of DAT. Such a result can have wide ranging implications. The first notable insinuation is that of considering the sex of the subject in these knock-out models; and, of greater significance, for considering the sex of the subject when performing genetic manipulations as part of future potential clinical applications. Second, age- and disease-related (e.g. Parkinson's disease) reductions in DATs can result in different consequences for females and males. Finally, is the prospective for a differential effect upon secondary dopaminergic functions within males and females. Given the key role of the DAT, a variety of ancillary dopaminergic functions can be disrupted, and disrupted in a sex-dependent manner. In fact, the results from the second series of experiments, as described below, provide data showing an interaction between sex and DAT mutation that affects secondary dopaminergic functions.

There appears to be a site specificity within the striatum as not all regions sampled showed a statistically significant difference in DAT binding. The anterior striatum is clearly more sensitive as significant differences in DAT binding were obtained for both medial and lateral sites of the anterior striatum in females and even within males for the lateral site. The anterior lateral region of the striatum also shows a relatively more severe deficit in DAT binding following treatment with a neurotoxic regimen of the psychostimulant, methamphetamine [36]. In this way, the maximal debit in DATs as can result from a DAT mutation or from an agent capable of producing neurotoxicity within the nigrostriatal dopaminergic system occurs at the same striatal region. It is interesting to note that the lateral striatum is also more responsive to estrogen-dependent effects upon dopamine D2 receptors, with significant increases over control levels being reported in the lateral, but not medial region of the striatum [37]. The significance of this finding is twofold. Not only does it support further the site specificity of striatal effects, but they also reveal that the predominant female gonadal steroid hormone, estrogen, may be critically associated with this site specificity. It has been reported that peak densities of DAT sites are present at proestrus, when estrogen is increasing, are decreased in ovariectomized rats [38] and are increased in ovariectomized rats treated with estrogen

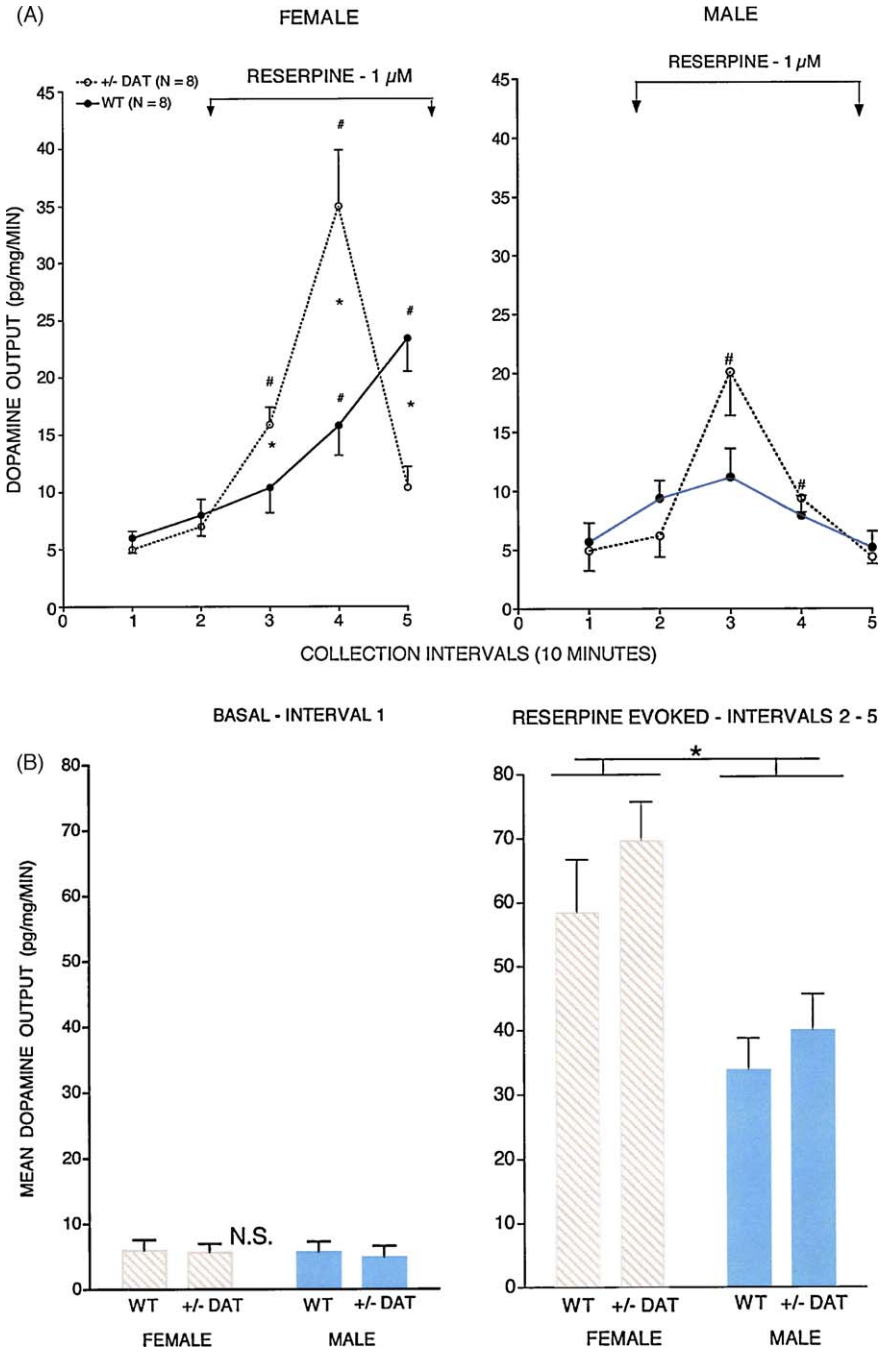

Fig. 3. (A) Dopamine output values (mean ± S.E.M. in pg/mg/min-line graphs, 8 mice per group) obtained during superfusion of +/+ DAT wild type (WT) and heterozygous (+/− DAT) female and male mice in response to an infusion of 1 μM reserpine stimulation. $^{*}p < 0.05$ WT versus +/− DAT; $^{\#}p < 0.05$ versus collection interval 1. (B) Summary of basal (collection interval 1) and reserpine-evoked (total from intervals 2–5) DA output among +/+ DAT wild type (WT) and heterozygous (+/− DAT) male and female mice. $^{*}p < 0.05$ females versus males.

[39–41]. Therefore, the salient sex differences seen in +/− DAT females, might be related to estrogen-dependent dissimilarities between females and males; and, these estrogen-dependent dissimilarities might be exerted at specific sites within the striatum. There are also data that show that estrogen can decrease DAT densities [42,43]. Whether, these reports indicating either positive or negative effects of estrogen upon DATs can be related to the potential for regional effects of this gonadal steroid hormone and/or other conditions of the animal model await further investigation.

In the second series of experiments an evaluation of reserpine-evoked DA was performed as a means to assess whether an associated sex-dependent effect upon the VMAT-2 would occur with an alteration in the DAT. The data from these experiments also reveal that female striatal tissue is more affected by infusion of this VMAT-2 blocker and show an interaction with their genotypic condition. In support of the conclusion that females are more affected are the data from Fig. 3B, which show that following reserpine infusion the overall amount of DA evoked is significantly greater in females. These results support previous findings from both clinical [44] and laboratory [24] reports that suggest the presence of a sex difference in VMAT-2 function. In support of the conclusion that females show a genotypic interaction to the reserpine infusion are the marked differences in DA output profiles

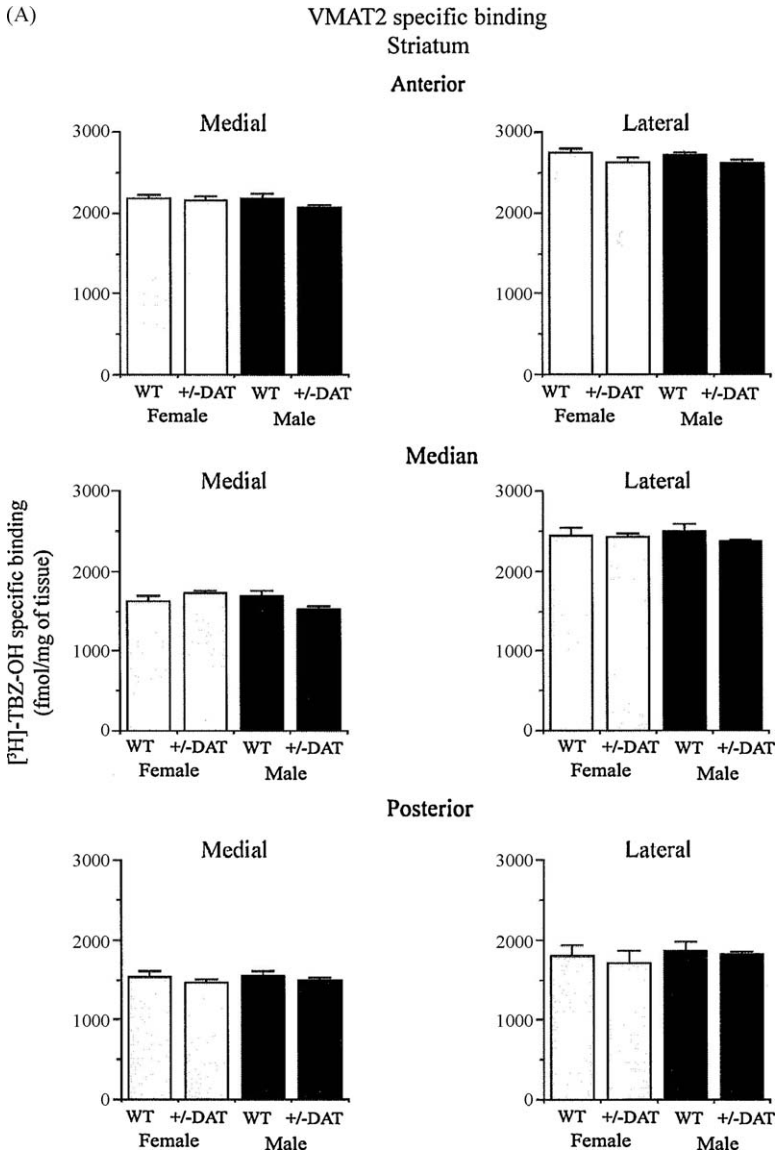

Fig. 4. (A) VMAT2 specific binding in anterior, median and posterior striatum of +/+ DAT wild type (WT) and heterozygous (+/− DAT) female and male mice. Values are the means (fmol/mg of tissue) ± S.E.M. of 4 individual mice per group. (B) Representative examples of [^{3}H]-TBZ-OH VMAT2 binding in anterior, median and posterior striatum of +/+ DAT wild type and heterozygous (+/− DAT) female and male mice.

between +/+ and +/− DAT female mice (Fig. 3A) that generate a statistically significant interaction in DA output. This can be contrasted with the similarity in DA output profiles that characterize reserpine-evoked DA responses in +/+ and +/− DAT male mice. Interestingly, in spite of this sex-dependent differential and genotypic interaction in females in response to reserpine, no differences were observed for VMAT-2 protein or mRNA levels (Figs. 4 and 5). These findings would suggest that the alteration in VMAT-2 function, as indicated by DA output responses to reserpine infusion, would be the result of an indirect effect of the DAT mutation upon VMAT-2. In this way, reserpine-evoked DA responses of females more likely represent deficits in DAT function which secondarily affect the amount of reserpine-evoked DA collected from superfused striatal tissue. Accordingly, the interaction between these two transporters as shown in the present results and as proposed previously [21,22], might involve interactions that result from indirect/secondary effects exerted upon the VMAT-2 due to alterations in extracellular DA uptake from diminished DAT activity.

Female mice are clearly more responsive to reserpine, as seen in the present results, and as demonstrated previously under *in vivo* and *in vitro* conditions of reserpine treatment [45]. When these findings are combined with data showing that potassium-stimulated DA output, which releases DA from vesicular stores, is greater in females [46], they suggest that females possess a greater vesicular storage capacity. Such an enhanced VMAT-2 function may be related to the relatively lower degree of striatal neurotoxicity to methamphetamine seen in females [47–49] as mutant mice with diminished VMAT-2 expression show an increased degree of striatal neurotoxicity to methamphetamine [50]. The significantly greater DA concentrations of +/+ versus +/− DAT female, but not male, mice [17] imply that this vesicular pool is selectively diminished within +/− DAT female mice. Similarly, reserpine-evoked DA responses of +/+ versus +/− DAT females show unique profiles that are not observed within +/+ versus +/− male mice, providing further support for a sex-dependent difference in VMAT-2 function, that is affected by their DAT genotype (Fig. 3A). With the depletion of their vesicular stores as achieved with reserpine in +/+ DAT females, a gradual, temporally dependent increase in extracellular DA is obtained, like that reported previously [45]. Such a DA output profile could result from the accumulating effects of increasing numbers of VMAT-2

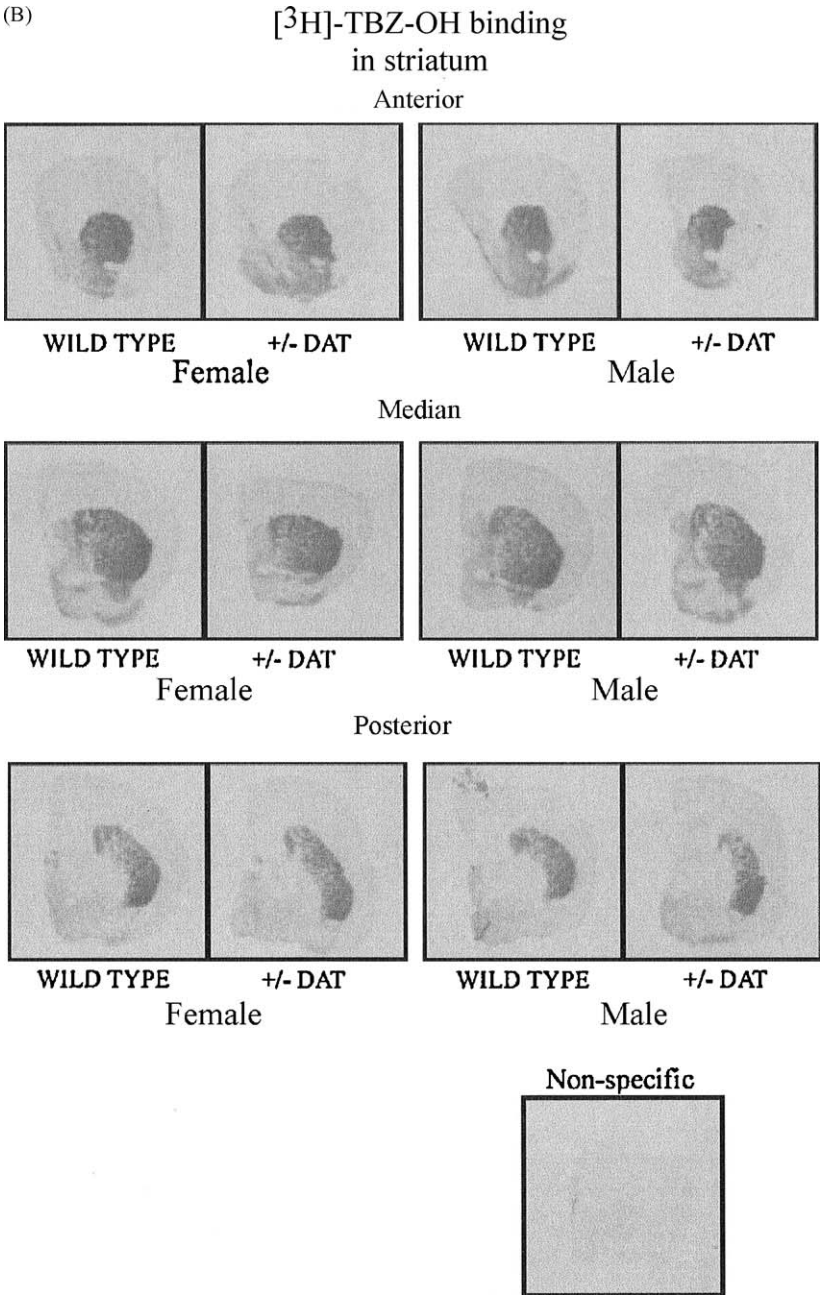

Fig. 4. (*Continued*).

inhibition and vesicular depletions combined with the prevention of sequestering DA recovered through the DAT. As a result, the DA output profile of these +/+ DAT females continues to increase over the entire superfusion period, and presumably would yield significantly greater amounts of DA had the superfusion collection been extended. This DA profile can be contrasted with that of the +/− DAT female. In these mice, the limited amount of vesicular DA combined with a diminished DA recovery capacity would generate an acute, surge-like DA output followed by a rapid decrease due to depletion of the vesicular pool. Again, based upon data from the VMAT-2 protein and mRNA, these results would not seem attributable to a direct change in the VMAT-2. While caution must be exercised in interpreting results derived with reserpine, this agent is effective in revealing the extent of qualitative as well as quantitative differences in striatal dopaminergic function present in these mice. Accordingly, when these reserpine data are collated with the related findings, as described above, they provide a composite of the interactions among sex, DAT genotype and VMAT-2 function.

In summary, the present results reveal a number of important and novel relationships of the nigrostriatal dopaminergic transporter systems. A genetic mutation of the DAT does not exert equivalent effects upon the DAT in female and male mice. We had reported previously that striatal DA concentrations differed between +/− female versus male mice but not between WT females and males [17] and now confirm that striatal DAT and substantia nigra DAT protein and mRNA are clearly different in these heterozygous mutant female and male mice. With the use of these +/− DAT mice we were also able to demonstrate that an alteration in the DAT affects VMAT-2 function, and does so in a sex-dependent manner. However, this modulation of VMAT-2 function in DAT mutant mice appears to involve an indirect or secondary

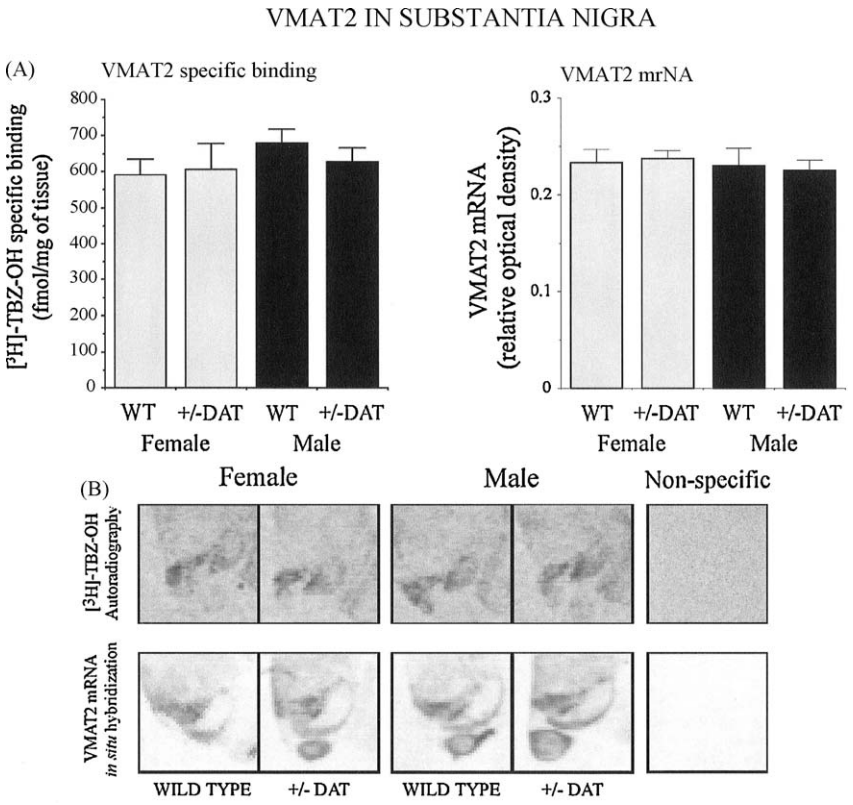

Fig. 5. (A) VMAT2 specific binding and *in situ* hybridization of VMAT2 mRNA in substantia nigra of +/+ DAT wild type (WT) and heterozygous (+/− DAT) female and male mice. Values are the means ± S.E.M. of 4 individual mice per group. (B) Representative examples of [^{3}H]-TBZ-OH binding and VMAT2 mRNA levels in substantia nigra are shown.

effect, since no change in VMAT-2 protein or mRNA was observed as a function of the sex or genotype of the mice. These findings may provide critical information of significance to the gender differences observed in conditions ranging from drug addiction to Parkinson's disease.

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