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Hyperactivity of the dopaminergic system in NTS1 and NTS2 null mice

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ABSTRACT

Neurotensin (NT) is a tridecapeptide that acts as a neuromodulator in the central nervous system mainly through two NT receptors, NTS1 and NTS2. The functional–anatomical interactions between NT, the mesotelencephalic dopamine system, and structures targeted by dopaminergic projections have been studied. The present study was conducted to determine the effects of NT receptor subtypes on dopaminergic function with the use of mice lacking either NTS1 ($NTS1^{-/-}$) or NTS2 ($NTS2^{-/-}$). Basal and amphetamine-stimulated locomotor activity was determined. *In vivo* microdialysis in freely moving mice, coupled with HPLC-ECD, was used to detect basal and D-amphetamine-stimulated striatal extracellular dopamine levels. *In vitro* radioligand binding and synaptosomal uptake assays for the dopamine transporters were conducted to test for the expression and function of the striatal pre-synaptic dopamine transporter. $NTS1^{-/-}$ and $NTS2^{-/-}$ mice had higher baseline locomotor activity and higher basal extracellular dopamine levels in striatum. $NTS1^{-/-}$ mice showed higher locomotor activity and exaggerated dopamine release in response to D-amphetamine. Both $NTS1^{-/-}$ and $NTS2^{-/-}$ mice exhibited lower dopamine D_1 receptor mRNA expression in the striatum relative to wild type mice. Dopamine transporter binding and dopamine reuptake in striatum were not altered. Therefore, lack of either NTS1 or NTS2 alters the dopaminergic system. The possibility that the dysregulation of dopamine transmission might stem from a deficiency in glutamate neurotransmission is discussed. The data strengthen the hypothesis that NT receptors are involved in the pathogenesis of schizophrenia and provide a potential model for the biochemical changes of the disease.

1. Introduction

The interaction between neurotensin (NT) and dopamine (DA) has been implicated in the pathogenesis and treatment of schizophrenia (Nemeroff et al., 1983b). The involvement of the dopaminergic system in the behavioral effects of schizophrenia has emerged from the observations that drugs used to treat schizophrenia act through blocking DA receptors (Carlsson and Lindqvist, 1963) and that DA agonists such as D-amphetamine, provoke certain psychotic reactions in normal humans and exacerbate some psychotic symptoms in schizophrenics (Gainetdinov et al., 2001).

NT is a neuropeptide that is co-localized with, and is expressed throughout the brain within the nigrostriatal and mesolimbic DA system (Jennes et al., 1982; Leeman and Carraway, 1982) modulating DA neurotransmission (Fuxe et al., 1992b; Kitabgi et al., 1989; Lambert et al., 1995). Additionally, NT and NT agonists exhibit neuroleptic-like properties in DA-mediated animal models of psychosis, such as amphetamine-induced locomotor activity, apomorphine-induced climbing, and drug-induced disruption of prepulse inhibition (PPI) (Boules et al., 2001; Cusack et al., 2000; Ervin et al., 1981; Feifel et al., 1999; Ford and Marsden, 1990; Jolicoeur et al., 1983, 1993; Kalivas et al., 1983, 1984; Nemeroff et al., 1983a; Sarhan et al., 1997; Shilling et al., 2003).Hence, NT has been labeled the endogenous neuroleptic (Nemeroff, 1980).

NT mediates its effects through its receptors that were first identified by radioligand binding techniques. The two, most widely studied receptors are: the high affinity, levocabastine-insensitive NT receptor subtype 1 (NTS1) (Tanaka et al., 1990; Vita et al., 1993) and the low affinity, levocabastine-sensitive subtype 2 receptor (NTS2) (Chalon et al., 1996; Mazella et al., 1996). NTS1 is localized both pre- and post-synaptically with DA D_2 receptors (Delle Donne et al., 2004) in the striatum, ventral midbrain, and nucleus accumbens. Its activation stimulates Ca^{++} release (Beauregard et al., 1992) and modulates DA transmission (Gully et al., 1993; Leonetti et al., 2004). Additionally, the lack of NT causes altered responses to antipsychotic drugs in mice (Dobner et al., 2001).

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NTS2 is widely distributed throughout the brain (Sarret et al., 2003) and has been implicated in spinal analgesia (Sarret et al., 2005).

Several studies have shown behavioral differences that are relevant to schizophrenia between wild type and NT or NT receptor knockout mice. Kinkead et al. (2005) reported that NT knockout mice had reduced PPI and were not sensitive to the PPI-disrupting effects of amphetamine as compared to wild type mice, a result suggesting the importance of endogenous NT in the effects of amphetamine on PPI. Additionally, administration of NT receptor agonists reversed apomorphine-induced climbing in wild type, but not in NTS1$^{-/-}$ mice (Mechanic et al., 2009), and administration of PD149163, an NT receptor agonist, significantly facilitated PPI and decreased the acoustic startle response in wild type but not in NTS1$^{-/-}$ mice (Feifel et al., 2010).

Uncovering the roles NT receptors play in the brain has been approached with the use NT receptor agonists and antagonists, as well as mice lacking NT or its receptors (NTS1 or NTS2). In the present study, we used mice lacking either NTS1 or NTS2 to determine further the role of NT receptors on DA neurotransmission. Our results suggest that both NT receptor subtypes are necessary for normal dopaminergic function.

2. Materials and methods

All animal protocols were approved by the Mayo Foundation Institutional Animal Use and Care Committee in accordance with the NIH Guide for Care and Use of Laboratory Animals. All animals were kept in an environmentally controlled room with 12 h light/dark cycle and free access to water and food.

2.1. Generation of NTS null mice

NTS1$^{-/-}$ and NTS2$^{-/-}$ mice were established at Roche (Palo Alto, CA, USA) as previously described (Mechanic et al., 2009). The NTS null allele was originally created in Bruce-4 ES cells, which were derived from a C57BL/6 mouse strain. Cells were injected into BALB/c blastocysts to generate chimeras. Male chimera was mated with female C57BL/6J to give rise to the F1 or N1 heterozygotes (hets) (+/−). To ensure a pure genetic background, one additional backcross was performed (male F1 het × female C57BL/6J) to generate the N2 hets. Homozygotic knockout (−/−) and wild type (+/+) mice F2 were from N2 heterozygote × heterozygote intercrosses. These knockout and wild type mice were used to establish "in house" knockout and wild type colonies through heterozygous mating in each strain. The wild type mice were from the heterozygous progeny.

2.1.1. NT receptor (NTS) genotyping

Routine genotyping was conducted by PCR on purified tail DNA. Genomic DNA was extracted using a Qiagen DNEASY Tissue Kit (Qiagen, Valencia, CA, cat. # 69506) according to manufacturer's instructions. The PCR mixture consisted of 45 μL PCR Supermix (Invitrogen, Carlsbad, CA, cat. # 10572-014), 4 μL of each primer (20 μM), and 5 μL DNA (100 ng). Applied Biosystems (Foster City, CA) GeneAmp PCR system 9700 thermal cycling conditions were as follows: 94 °C, 3 min followed by 50 cycles of 94 °C, 45 s; 58 °C, 45 s; 72 °C, 45 s; followed by 72 °C, 7 min; and finally a 4 °C soak. For visualization purposes, 14 μL of the amplification product was subjected to electrophoresis on 3% agarose mini gel/1× TAE Buffer (BIO-RAD, Hercules, CA, cat. # 161-3024) and then stained with ethidium bromide. Three primers were used to amplify the DNA of interest for NTS1 – forward primer NT1-GT2 5′ CAGGAGTGCA GACCAACCACAG 3′, reverse primer NT1-GT3 5′ GTTCACGTCCAGGTTGCTGTT 3′ and a primer specific to the TK-neo construct in the KO mice Neo-GT1 5′ CCTTCTTGAC GAGTTCXTTCTGAG 3′. For NTS2 – forward primer NTS2-GT-C1 5′ GTCCATTCCCC ACCTCAGAAG 3′, reverse primer NTS2-GT1-C5 5′GCACCCTCCTGGTATCACACTG 3′ and a primer specific to the TK-neo construct in the KO mice NEO-GT4 5′CCTTCTATCGCCTTCTTGACGAG 3′. The expected sizes for the wild type alleles were 488 and 367 bp, for NTS1 351 bp, and for NTS2 563 bp.

2.1.2. NTS mRNA assay

mRNA levels of NTS were determined by real-time quantitative PCR. Real-time PCR was performed on the ABI 7900 TaqMan Sequence detector by using SYBR green as a dsDNA-specific binding dye. Brain total RNA was isolated using the Trizol method (Invitrogen, cat. #15596-018). RNA was purified using RNeasy cleanup/DNase set procedure (Qiagen, cat. #74103/79254). Total RNA quality was confirmed using a 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). mRNA was amplified using One-Step RT-PCR Master Mix kit (Applied Biosystems, cat. #4309169) according to the manufacturer's protocol. Levels of gene expression were quantitated using the comparative dd*CT* method (Perkin Elmer Life Sciences user bulletin number 2, 1997) with an 18s Ribosomal RNA Probe as an endogenous reference.

2.2. D-amphetamine-induced activity

Male knockout and wild type mice (25–32 g) were used for these experiments. Mice were divided into groups of 4–6 mice each. Activity was tested by placing each mouse in a Plexiglas Opto-Varimex Minor motility chamber (Columbus Instruments, Columbus, OH, USA) for 2 h for acclimation. Baseline activity was recorded for 60 min for each mouse. Mice were then injected with either saline or D-amphetamine (D-amphetamine sulfate salt, 4 mg/kg i.p.) (Sigma St. Louis MO, cat. # A-5880) and then placed in the chamber. Activity was recorded for 2 h. Results are expressed as average counts per 10 min ± standard error of the mean (S.E.M).

2.3. Real-time PCR analysis

Total RNA was extracted from brain tissue with the use of TRIzol reagent (Life Technologies, Frederick, MD) and purified by RNeasy cleanup/DNase set procedure as above. Total RNA content was determined by OD_{260} with the use of a 2100 Agilent Bioanalyzer. Total RNA was converted to single stranded cDNA with the use of the high capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. Real-time RT-PCR was then performed to determine relative mRNA levels of dopamine D_1 (DA D_1) and dopamine D_2 receptor (DA D_2), striatum. The real-time RT-PCR reactions were performed with the use of predesigned primers and probes from Applied Biosystems, which use TaqMan DNA minor groove binding probes that are fluorescently labeled with FAM™ dye for detection. Murine 18s ribosomal RNA (Mm02601776_g1) was used for the internal control. The total reaction volume was 20 μL containing TaqMan universal PCR master mix, TaqMan primers and probes, and cDNA. Reactions were run in triplicate in optical grade 384-well plates on an ABI Prism 7900HT PCR machine with the following thermal cycling conditions: 2 min @ 50 °C, 10 min @ 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Expression levels of each gene were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with 18s RNA as an internal control. To perform this calculation, the difference in cycle threshold (ΔCt) between the gene of interest and the corresponding 18s threshold for each sample was calculated. There was no significant difference in the expression of 18s RNA between wild type, NTS1$^{-/-}$, and NTS2$^{-/-}$ strains (14.6 ± 0.3, 14.5 ± 0.2 and 14.8 ± 0.5 for wild type, NTS1$^{-/-}$, and NTS2$^{-/-}$, respectively). Mean ΔCt's were calculated for each group (wild type, NTS1$^{-/-}$, and NTS2$^{-/-}$). The ΔΔCt was calculated by subtracting the mean ΔCt for wild type animals (control group) from the mean ΔCt for each knockout group. Numbers were then normalized to the wild type group by calculating the fold change in expression ($2^{-\Delta\Delta Ct}$).

2.4. In vivo microdialysis

2.4.1. Surgery

All procedures were approved by the Mayo Foundation Institutional Animal Use and Care Committee. Wild type, NTS1$^{-/-}$, and NTS2$^{-/-}$ mice ($n = 5$) were housed in a temperature controlled room with 12 h light–dark cycle and free access to food and water. On the day of surgery, the adult mouse was anesthetized with gasiform isoflurane (1% isoflurane in a mixture of 20% oxygen and 80% nitrogen gas), and immobilized in a stereotaxic frame (KOPF Ins., Tujunga, CA). The anesthesia was maintained during the entire experiment. Each mouse was cannulated in the right striatum (A 0.5, L 1.6, V 2.5) according to the bregma and skull (Paxinos and Franklin, 2001). Following surgery, each mouse was housed individually. The mice were kept for 3–5 d to recover from the cannulation surgery before conducting microdialysis.

2.4.2. Microdialysis procedure

Microdialysis experiments were carried out on conscious, freely moving mice. On the day of the experiment, the stylet in the guide cannula was replaced with the microdialysis probe (CMA/7 with 2 mm membrane CMA Microdialysis Inc., Acton, MA). The probe was perfused at 2 μl/min with artificial cerebrospinal fluid (146 mM NaCl, 1.2 mM $CaCl_2$, 3 mM KCl, 1.0 mM $MgCl_2$, 1.9 mM Na_2HPO_4, 0.1 mM NaH_2PO_4, pH 7.4). After at least 2 h equilibration, dialysate samples were automatically collected every 20 min into vials containing 2 μL perchloric acid (0.5 M) to retard oxidation of monoamines. Four baseline fractions were collected before saline i.p. injection. D-Amphetamine (D-amphetamine sulfate salt, 4 mg/kg, i.p.) was injected 1 h after saline injection. Monoamines and their metabolites were measured in the dialysate with the use of HPLC and electrochemical detector (ED) as described below. Results are reported as % increase over baseline for each strain (mean ± SEM). The position of the probe was verified by visual inspection at the end of each experiment.

2.4.3. Monoamine assay

Monoamines and their metabolites were measured on an ESA HPLC coupled with Coulochem II electrochemical detector system (ESA Inc., Chelmsford, MA) with a 20-μL sample loop. They were separated on an MD-150 analytical column (3 × 150 mm, 3 μm C18, ESA Inc.) with MDTM mobile phase (ESA Inc.) at 0.6 ml/min. Potential settings for detection were E1 at −175 mV, E2 at 250 mV, GC at 350 mV. Peaks were displayed, integrated, and stored with ESA 501 Chromatography data system (ESA Inc.). The detection limit of DA was 23 pmol.