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Increased expression of VMAT2 in dopaminergic neurons during nicotine withdrawal

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ABSTRACT

Evidence suggests that the vesicular monoamine transporter-2 (VMAT2) is regulated in striatum and dopamine (DA) may play a role in its regulation. DA is an important mediator of the behavioral actions of nicotine, and dopaminergic neurotransmission is altered following nicotine administration. We investigated the effect of nicotine withdrawal on the expression of VMAT2 in the midbrain DA neurons in animals dependent to nicotine. Mice were injected with nicotine free base 2 mg/kg, sc, four times daily for 14 days and killed 12–72 h after drug discontinuation. VMAT2 protein was increased in the striatum of nicotine-treated mice in a time-dependent fashion at all times studied. Furthermore, *in situ* hybridization studies demonstrated that VMAT2 mRNA was elevated in the substantia nigra pars compacta and ventral tegmental area, indicating enhanced gene expression and subsequent protein synthesis. Tissue DA content and synthesis were unaltered in the striatum of nicotine-treated mice at the times studied. However, basal DA release was decreased at 12 and 24 h after nicotine discontinuation which coincided with the elevated levels of VMAT2 protein. VMAT2 up-regulation might be a compensatory mechanism to restore and maintain synaptic transmission in dopaminergic midbrain neurons during nicotine withdrawal.

Synaptic vesicles play a major role in synaptic transmission by sequestering neurotransmitters for storage and subsequent release. The vesicular monoamine transporter-2 (VMAT2) is responsible for packaging newly synthesized or recaptured dopamine (DA) into the synaptic vesicles, and therefore controls the concentration and disposition of cytoplasmic DA within the nerve terminal. There is evidence suggesting that VMAT2 function is regulated following pharmacological manipulations of intracellular or extracellular DA concentrations, and a role for DA D2 receptors has been proposed ([11] for review). For example, in acute studies DA releasers, such as amphetamine analogs, attenuate DA uptake into synaptic vesicles prepared from striatum [3,17], while plasmalemmal DA transporter inhibitors, such as cocaine and buproprion, enhance it [4,30]. In addition, there are reports that activation of D2 receptors increases DA uptake into synaptic vesicles [5,36]. Notwithstanding, the question whether long-term changes of dopaminergic function affect VMAT2 regulation remains unsettled [23,24,37,40].

Nigrostriatal and mesoaccumbal dopaminergic neurons are important mediators of the behavioral pharmacology of nicotine ([1] for review). Diminished dopaminergic neurotransmission, as demonstrated by reduced extracellular DA concentrations in nucleus accumbens [6,16], is believed to contribute to the expression of the negative motivational and affective symptoms associated with the early nicotine withdrawal syndrome [20]. Since VMAT2 is an important component of synaptic dopaminergic function and is closely coupled to DA release [11], the question whether its regulation is altered during nicotine withdrawal is of importance and warrants examination. Using a mouse nicotine abstinence model [18], we investigated possible changes in VMAT2 mRNA in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA), as well as VMAT2 protein in the striatum during early nicotine withdrawal, 12–72 h. In addition, striatal tissue DA content and release and markers of DA synthesis were evaluated.

Male Swiss–Webster mice (Harlan), 30–35 g, were used for the studies, which were approved by the Institutional Laboratory Animal Care and Use Committee of the Ohio State University and conducted in accordance with the Guide for the Care and Use of laboratory Animals as adopted and promulgated by the National Institutes of Health. Mice were housed in our vivarium on a 12 h light:dark schedule with free access to food and water. They were treated with nicotine according to a protocol that induces a mild withdrawal syndrome over a 72 h period that is characterized by somatic symptoms and decreased locomotor activity [18]. Briefly, mice were injected four times daily (8AM, 12PM, 4PM, 8PM) with nicotine free base, 2.0 mg/kg, or saline, sc, for 14 consecutive days.

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At various times during nicotine withdrawal (12, 24, 48, and 72 h) mice were decapitated, brains quickly removed, and either frozen whole or striatum dissected and used immediately. For the VMAT2 *in situ* hybridization studies, fixed frozen brain coronal sections, 12 μm, through the midbrain (bregma −4.5 to −6.7 mm) were used. They were hybridized with a [^{35}S]-labeled DNA oligonucleotide probe complementary to bases 100-153 of the rat cDNA sequence (Integrated DNA Technologies), as we have previously described [14]. Quantitative analysis of the autoradiographs was performed by image analysis (Metamorph), using [^{14}C] standards. The boundaries of SNc and VTA were manually traced, and average grey values on the X-ray film were estimated and converted to units of radioactivity tissue equivalents. The average hybridization signal density for each nucleus/animal was calculated and transformed to percent of respective control.

VMAT2 protein was estimated in whole striatum postnuclear lysates by Western blot. One striatum was homogenized briefly with a cell disruptor in 200 μl of ice-cold lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 1 mM EGTA, 2 mM EDTA, 1 mM DTT, 1 mM PMSF and 1 μg/μl aprotinin), and samples incubated on ice for 30 min. Insoluble material was removed by centrifugation at 15,000 × *g* for 10 min. Equal amounts of protein, 20 μg, were separated by 12% SDS-PAGE electrophoresis in parallel with molecular weight markers (Invitrogen). Proteins were electro-transferred to nitrocellulose membrane (Amersham), and incubated in TBS-Tween (10 mM Tris–HCl, pH 7.8, 150 mM NaCl, 0.1% Tween 20) containing 5% dry milk overnight at 4 °C. The blots were then incubated with a VMAT2 antibody (Chemicon AB 1598, 1:2000) for 1 h at room temperature followed by horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology, 1:1000). Following stripping, membranes were subsequently immunoblotted with tyrosine hydroxylase (TH; Chemicon AB 152, 1:5000) or aromatic L-amino acid decarboxylase (AAAD; Chemicon AB 1569, 1:1000) antibodies [25]. Bound antibody was detected with Enhanced Chemiluminescence (ECL, Amersham). VMAT2 antibody AB 1598 is made to the C-terminal of the rat VMAT2, and reportedly detects a major band at ~55 kDa in postnuclear supernatants of CHO cells transfected with VMAT2. Higher molecular weight bands detected by AB 1598 are due to glycosylation; while, the source of lower immunoreactive bands is unknown ([27] Manufacturer's product information sheet). Multiple exposures of blots were obtained to ensure linearity of the signal on the X-ray film, and the density of the bands of interest were estimated by image analysis (Metamorph) using grey scale standards. All samples of a study were immunoblotted and analyzed in one run under the same experimental conditions, and there was no significant variance in the estimated control density among the various gels [$F(4,14) = 1.668$; $P = 0.2372$]. In addition, in each gel a nicotine-treated sample and its corresponding time-matched saline-treated control were loaded next to each other and control and treated samples from all time points, 12–72 h, were included in each gel.

The release of endogenous DA was estimated in striatal slices, as we have previously described [14]. Slices prepared from the striata of two mice were pooled and aliquots, about 150–200 μg of protein, were used for the estimation of basal or stimulated DA release in duplicate. Following several washes and DA release equilibration, slices were incubated in oxygenated Kreb's buffer, pH 7.4 (120 mM NaCl, 4.5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1 mM EGTA, and 11 mM glucose) in the presence of 10 μM of pargyline and 170 μM of ascorbic acid for 5 min at 37 °C, and release terminated by centrifugation. When high potassium was added to the samples the buffer was changed to 50 mM KCl and 70 mM NaCl. Dopamine in both supernatant and pellet was estimated by HPLC-ED, and DA release was expressed as the percentage of DA released in the supernatant compared to total DA estimated in the supernatant and pellet. Data were analyzed by parametric

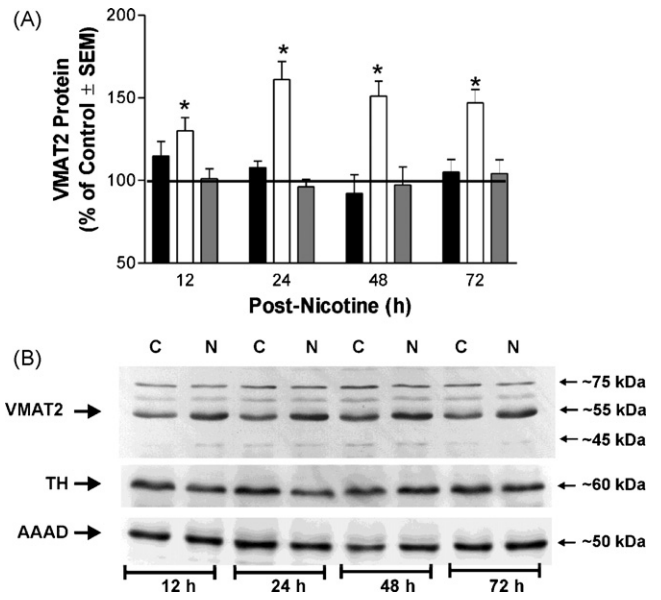

Fig. 1. Increased VMAT2 protein levels in striatum following chronic nicotine treatment and withdrawal. Animals were treated with nicotine (N) free base, 2 mg/kg, or saline (control; C), sc, four times daily for 14 days and killed at the indicated times. VMAT2, TH and AAAD proteins were estimated in lysates prepared from whole striatum as described. (A) Quantitative presentation of the VMAT2 protein change. For presentation purposes, data were expressed as percent of respective saline-treated controls ± SEM: black bars, VMAT2 ~45 kDa; open bars, VMAT2 ~55 kDa; grey bars, VMAT2 ~75 kDa. *$P < 0.05$ compared with respective control. (B) Representative immunoblot images of VMAT2, TH and AAAD at 12, 24, 48 and 72 h post-nicotine in the same blot. $N = 4$–5 animals/treatment group.

(ANOVA) or nonparametric (Kruskal–Wallis test) analysis of variance followed by a Tukey–Kramer or Dunn post hoc test for multiple group comparisons as indicated. When appropriate, a *t*-test or a Mann–Whitney test was used for two group comparison. GraphPad InStat was used for the analysis, and $P \leq 0.05$ was accepted for statistical significance.

Western blot of whole striatum postnuclear supernatants with AB 1598 showed two major bands of ~55 and ~75 kDa (Fig. 1B), of which the ~55 kDa band displayed the strongest immunoreactivity. Although, a band was identified at ~45 kDa the signal was relatively weak (Fig. 1B). Immunoreactive bands of similar molecular weight have been detected in synaptosomal preparations from mice striata as well, using an antibody to human VMAT2C terminus [13,38]. Reported discrepancies in VMAT2 band immunoreactivity density and molecular weight forms [8,13,19,26,30,36,38] might reflect differences in antibody specificity, animal species, tissue preparation, cellular fractions, Western blot conditions, etc. Image analysis of the three identified VMAT2 bands showed no change in the density of the ~75 and ~45 kDa bands (Fig. 1A and B). However, the density of the ~55 kDa band was increased in the striatum by 12 h after nicotine discontinuation and remained elevated for over 72 h [$F(7,30) = 8.534$, $P < 0.001$] (Fig. 1A and B). The ~55 kDa protein was increased about 30% over control at 12 h, it reached maximal levels, about 60% over control, by 24 h, and it was still elevated 72 later. The ~55 kDa protein is presumed to be the predicted full-length rodent VMAT2 protein [13,27]. Although, there is consensus that the ~75 kDa protein is the glycosylated form of the transporter [8,13,19,27], the molecular identity of the ~45 kDa protein remains elusive, being explained as a truncated form, proteolytic product, core protein, as well as artifact of gel mobility or experimental conditions ([19] and discussion there in).

Although, VMAT2 is present in norepinephrine and serotonin neurons as well, most of vesicular transporter in the DA-rich striatum is located in small synaptic vesicles in dopaminergic ter-