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Vesicular glutamate transporter 2 and tyrosine hydroxylase are not co-localized in Syrian hamster nucleus accumbens afferents

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HIGHLIGHTS

- In Syrian hamsters, like in other species, VGLUT2 mRNA is present in the VTA.
- VGLUT2 does not co-localize with TH-positive fibers in the NAc or CPu in hamsters.
- The density of VGLUT-2- and TH-positive fibers/cells did not differ between sexes in hamsters.
- One interpretation is that DA and GLU are not co-released from the same terminals in NAc afferents.

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ABSTRACT

The nucleus accumbens (NAc) is an important brain region for motivation, reinforcement, and reward. Afferents to the NAc can be divided into two anatomically segregated neurochemical phenotypes: dopaminergic inputs, primarily from the midbrain ventral tegmental area (VTA) and glutamatergic inputs from several cortical and sub-cortical structures. A population of glutamatergic neurons exists within the VTA and evidence from rats and mice suggests that these VTA axons may co-release dopamine and glutamate into the NAc. Our laboratory has used sexual experience in Syrian hamsters as a model of experience-dependent plasticity within the NAc. Given that both dopamine and glutamate are involved in this plasticity, it is important to determine whether these neurotransmitters are co-expressed within the mesolimbic pathway of hamsters. We therefore used immunofluorescent staining to investigate the possible co-localization of tyrosine hydroxylase (TH), a dopaminergic marker, and vesicular glutamate transporter 2 (VGLUT2), a glutamatergic marker, within the mesolimbic pathway. PCR analyses identified VGLUT2 gene expression in the VTA. No co-localization of TH and VGLUT2 protein was detected in NAc fibers, nor was there a difference in immunolabeling between males and females. Further studies are needed to resolve this absence of anatomical co-localization of TH and VGLUT2 in hamster striatal afferents with reports of functional co-release in other rodents.

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1. Introduction

The nucleus accumbens (NAc) is a key brain region for motivated behaviors, such as copulation [24,27,34], aggression [11,28,36], and wheel running [18,42,44], as well as in substance abuse and addiction [9,14,45]. NAc afferents are primarily comprised of two neurochemical phenotypes: dopamine (DA) and glutamate (GLU). In particular, the NAc receives dense dopaminergic projections from the ventral tegmental area (VTA), forming the mesolimbic dopamine pathway [23,43]. The VTA also projects to cortical and sub-cortical structures including the prefrontal cortex (PFC), hippocampal ventral subiculum (VS), basolateral amygdala (BLA), and the central thalamic nucleus (CT), which are main sources of glutamatergic NAc afferents [15,35,39].

Historically, dopaminergic and glutamatergic projections to the NAc were identified as distinct populations of cells. More recently a discrete population of glutamatergic neurons has been identified within the VTA [38], whose axons may co-release dopamine and glutamate into the NAc. Furthermore, it has been demonstrated that this subset of dopaminergic neurons in the VTA contains vesicular glutamate transporter 2 (VGLUT2) [13], one of the 3 identified vesicular glutamate transporters and a biomarker for glutamatergic cells. Finally, *in vitro* studies have provided anatomical [13,25,38] and functional [10,29,40] support for the possibility of dopaminergic VTA neurons releasing GLU into the NAc.

We have used hamster sexual behavior to model the neurobiology of motivation. This model is valuable for elucidating the role of the mesolimbic DA pathway in naturally occurring, non-pathological motivated behaviors, and also to provide insight into

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how reproductive physiology and behavior can impact vulnerability to addiction [20,32]. As such, identifying whether DA and GLU are co-expressed within the mesolimbic pathway of hamsters would contribute to a mechanistic understanding of the neurobiology of motivation this model system. To investigate this, we used immunofluorescent staining of tyrosine hydroxylase (TH) and VGLUT2 and analyzed the degree of co-localization of axonal labeling within sub-regions of the NAc and caudate-putamen (CPu). As males and females differ in their reproductive physiology and behavior, as well as their response to drugs of abuse and vulnerability to addiction [2], we analyzed this possible co-expression in both male and female hamsters.

2. Methods

2.1. Animals

Adult male ($n = 8$) and female ($n = 8$) Syrian hamsters (Charles River Laboratories, Wilmington, MA, USA) were housed 2 per cage under a 14:10 light:dark cycle. Food and water were available *ad libitum*. Animal procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University of Minnesota IACUC.

2.2. qPCR

An additional 4 males were anesthetized with Sleepaway (0.2 ml, i.p., Fort Dodge Animal Health, Fort Dodge, IA, USA), rapidly decapitated, and 1-mm thick coronal sections containing either the VTA (Bregma −4.6 mm) or the medial dorsal thalamus (Bregma −1.5 mm) were taken. Bilateral tissue punches (diameter: 0.67 mm) from each area were collected into RNAlater (Qiagen, Germantown, MD, USA) and stored overnight at room temperature. mRNA was extracted using a RNAeasy Mini Kit (Qiagen) and reverse transcribed using a Transcriptor First-Strand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, Indiana, USA). Because the sequence for VGLUT2 is unknown in Syrian hamsters, primers were designed using known sequences from the Chinese hamster (*Cricetulus griseus*; Forward: 5′-GAAACCGTGGGGATGATTC-3′; Reverse: 5′-CCGGAATCTGGGTGATGAT-3′).

qPCR amplifications were performed using LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) on a LightCycler 480 II PCR (Roche Diagnostics). PCR for individual cDNA samples was performed in triplicate. Threshold values were calculated using the second derivative max method and standardized to the housekeeping gene *GAPDH* (LightCycler 480 software version 1.5). The thermal cycling program used a preincubation step at 95 °C for 5 min, followed by 45 cycles consisting of a 10-s denaturing step at 95 °C, annealing step for 10 s at 60 °C, an extension step for 10 s at 72 °C, and a measurement of fluorescent intensity. At the end of each cycling program, a melting curve was run.

2.3. Vesicular glutamate transporter 2 and tyrosine hydroxylase staining

Subjects were anesthetized with an, i.p. injection of 0.2 ml of Sleepaway (Fort Dodge Animal Health) and transcardially perfused with 25 mM phosphate buffered saline (PBS, pH 7.2) for 3 min at a flow rate of 25 ml/min, followed by 4% paraformaldehyde in 25 mM PBS for 20 min. After perfusion, brains were removed, post-fixed for 1 h in 4% paraformaldehyde in PBS, and then placed in a 10% sucrose solution in PBS overnight at 4 °C.

Serial sections (40 μm) cut from frozen tissue coronally through the rostral–caudal dimension of the nucleus accumbens or sagittally through the lateral–medial dimension were placed into 25 mM PBS with 0.1% bovine serum albumin (BSA; wash buffer). Sections then were incubated in primary antibodies for guinea pig anti-vesicular glutamate transporter 2 (1:2000, AB2251, Millipore, Temecula, CA, USA) and mouse anti-tyrosine hydroxylase (1:2000, 22941, ImmunoStar, Hudson, WI, USA) in wash buffer plus 0.3% Triton-X 100 for 24 h at room temperature, followed by 24 h at 4 °C. Sections were then rinsed three times for 10 min in wash buffer, and incubated in anti-guinea pig Alexa 488 (1:500, A11001, Invitrogen Life Technologies, Grand Island, NY, USA) and anti-mouse Alexa 633 (1:500, A21105, Invitrogen Life Technologies) in wash buffer for 1 h at room temperature. Following this incubation, sections were washed three times for 10 min in wash buffer, and then mounted on slides and coverslipped while still wet with 5% n-propyl galate in glycerin.

2.4. Confocal imaging

A Leica TCS SPE confocal microscope (Leica, Mannheim, Germany) was used to image tissue sections labeled with both TH and VGLUT2, tagged with the Alexa 488 or Alexa 633 fluorophore, respectively. The complete 40-μm thickness of the slice was captured with a 63× oil immersion lens using a 5.61 optical zoom and XY pixel distribution of 512 × 512 at a frequency of 400 Hz. The tissue section was scanned every 0.29 μm along the *Z*-axis until the entire thickness of the slice was imaged. Three images, one at each anatomically matched plane of section (rostral, middle, and caudal), were collected for each subject. A systematic approach was taken to ensure that sections from each animal were taken from the same anatomical plane. Because the medial–lateral distance between the anterior commissure and ventral tip of the lateral ventricle changes monotonically in the rostro-caudal direction, a template cresyl violet stained section was selected from the rostral, middle and caudal levels of the nucleus accumbens and the distance between the medial edge of the anterior commissure and tip of the lateral ventricle measured. Fluorescent labeled sections were selected for analysis based on those measured distances. Within each section, the dorsal NAc core, dorsal NAc shell and dorsomedial CPu were analyzed. Accordingly, a total of 24 sections (3 sections × 8 animals) and 72 images (3 brain areas × 24 sections) were analyzed in both female and male subjects.

2.5. Data analysis

Unprocessed Z-stack images were reconstructed using the Surpass module of the Imaris software package (Bitplane Inc., South Windsor, CT, USA). The Spot function was used to define a region of interest that was 35 μm × 35 μm × 40 μm thick. The Spot function automated Wizard was used to threshold intensity and size of fluorescent labeling obtained from immunofluorescent staining in tissue sections. A total spot count was collected for both TH and VGLUT2 channels in each region of interest. A threshold value of 0.2, requiring minimal overlap of the pixel maps for TH and VGLUT2 identified spots, was used within the Colocalize Spots Wizard to identify presumptive terminals stained for both TH and VGLUT2. Total spot counts were used in a Bonferroni *t*-test to evaluate whether statistical differences existed between rostral–caudal dimensions or sex (male *vs.* female) and a χ^2 test was used to detect significant differences in the total density of TH or VGLUT2 immunoreactivity between males and females, and to determine whether the number of colocalized spots identified significantly differed from zero.

3. Results

qPCR demonstrated that in Syrian hamsters, as in other species, the VTA contains mRNA for VGLUT2 (Cp: 27.02 ± 0.62; ΔCt: