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Uncovering the role of the nucleus accumbens in schizophrenia: A postmortem analysis of tyrosine hydroxylase and vesicular glutamate transporters

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ABSTRACT

The nucleus accumbens (NAcc) is often implicated in schizophrenia (SZ) pathology, but with little evidence to support its role. This study examined postmortem human tissue to determine if abnormalities are present in the dopaminergic or glutamatergic systems in the NAcc in SZ. We compared the protein levels of tyrosine hydroxylase (TH) and vesicular glutamate transporters vGLUT1 and vGLUT2 in control (n = 7) and schizophrenia (n = 13) subjects using Western blot analysis. The SZ subjects were further divided by treatment status: SZ on-drug (SZ-ON, n = 6) and SZ off-drug (SZ-OFF, n = 7), to assess the effects of antipsychotic treatment. TH protein levels were similar between control and SZ subjects, and there was no difference between SZ-ON and SZ-OFF subjects. Protein levels of vGLUT1 were similar in control and SZ subjects, and there was no difference in vGLUT1 protein levels between SZ-ON and SZ-OFF subjects. In contrast, vGLUT2 protein levels were significantly elevated in the SZ group (25% increase). Protein levels of vGLUT2 did not differ between SZ-ON and SZ-OFF subjects. Similar levels of TH suggest the presynaptic DA pathway may be normal in the NAcc in SZ. The elevated vGLUT2 protein levels, but not vGLUT1, suggest the NAcc receives increased glutamatergic input in SZ, possibly from thalamic or other subcortical origins. The similarity between SZ-ON and SZ-OFF subjects suggests that the results are not caused by APD treatment. These findings provide further insight into the role of the NAcc in SZ.

1. Introduction

The cause of schizophrenia (SZ) remains elusive, however decades of research have revealed common pathologies, some of which are now hallmarks of the disorder. One of these hallmarks is abnormalities in the dopamine (DA) system in the striatum of patients with SZ (Miyake et al., 2011). Of special interest is a subregion of the ventral striatum, the nucleus accumbens (NAcc), which has been assumed to be a prime location for the elevated DA levels in SZ, based on its functional properties and evidence of antipsychotic drug (APD) action here (Deutch and Cameron, 1992; Deutch et al., 1992; Robertson & Fibiger, 1992; Merchant and Dorsa, 1993). Until recently, imaging studies have had to analyze the striatum as a single region, without the ability to distinguish between key functional and anatomical areas. Thus, not only has the role of the NAcc has never been confirmed, but recent studies with improved imaging techniques suggest that this subregion may not be implicated as previously thought (Howes et al., 2009, 2011; Kegeles et al., 2010). Additionally, conclusions surrounding the DA abnormalities in the striatum lack solid support from postmortem studies, which offer the ability to study these subregions individually. Previous studies in postmortem NAcc report conflicting results, typically finding no change (Crow et al., 1979; Farley et al., 1977; Owen et al., 1978; Toru et al., 1982) or increases (Bird et al., 1979; Mackay et al., 1982) in DA measures.

Another, more recent hypothesis of SZ is a causal role of glutamate abnormalities. Within the NAcc, a hypothesis emerged that pathology of the glutamate system could drive DA dysfunction (Lodge and Grace, 2007, 2011). NMDA antagonists have been shown to enhance DA efflux in the striatum in both preclinical studies (Miller and Abercrombie, 1996) and imaging studies in healthy volunteers (Kegeles et al., 2000). Further, stimulation of glutamatergic input to the NAcc in rats causes elevated DA release in the region (Blaha et al., 1997; Legault and Wise, 1999). It is not known however, if glutamatergic abnormalities are actually present in the NAcc in SZ.

The purpose of this study was to determine if neurochemical abnormalities are present in the NAcc of postmortem SZ. To study the dopaminergic system, we analyzed tyrosine hydroxylase (TH), the rate-limiting synthesizing enzyme of DA. To study the glutamatergic system, we analyzed the vesicular glutamate transporters vGLUT1 and vGLUT2, which are essential for the uptake and storage of glutamate into synaptic vesicles (Bellocchio et al., 2000; Takamori et al., 2000). They have complementary localization patterns in cortical and

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subcortical structures, respectively (Hisano et al., 2000; Fremeau et al., 2001; Herzog et al., 2001) thus studying both markers provides a comprehensive analysis of glutamatergic input to the NAcc. The Western blot analyses were performed both in SZ subjects that were on medication at the time of death, and in SZ subjects that were off medication at the time of death to assess APD effects.

2. Methods

2.1. Postmortem tissue

Postmortem human brain tissue was obtained from the Maryland Brain Collection. DSM-IV diagnosis of SZ was confirmed by two psychiatrists based on patient medical records, family interviews, autopsy reports, and neuropathologic assessments. Schizophrenia subjects were considered off-drug if they had been untreated with APDs for at least 6 months prior to death. Control cases had no history of psychiatric or neurological disease. Cases were chosen based on the best match of age, race, sex, postmortem interval (PMI), and tissue pH level. Demographics are shown in Table 1.

Striatal tissue was fresh frozen on dry ice and stored at −80 °C until used for this study. The NAcc was dissected from tissue blocks of 7 control subjects, 6 SZ subjects on medication (SZ-ON), and 7 SZ subjects off medication (SZ-OFF). Thionin staining was used to assess the quality of the morphology for each case. Each group started with 10 cases but several were excluded due to poor tissue quality. The final numbers for each group listed above were those with good preservation that were included in the study. The tissue was sectioned on a cryostat at −16 °C in 4 series of 16 μm thick sections. It was not feasible to separate the core and shell of the NAcc since the boundary between the subregions is not visible in fresh tissue, so the NAcc was analyzed as a whole.

2.2. Western blotting

2.2.1. Protein studies

Samples from human tissue were processed as described previously (Rice et al., 2014). Briefly, one series of tissue was sonicated in a lysis buffer (diluted 1:5) containing Tris–HCL (pH 8.0), EDTA, sodium dodecyl sulfate, and a protease inhibitor cocktail (Sigma; P8340). Tissue homogenate was centrifuged at 13,500 rpm for 15 min at 4 °C. Total protein concentration of the resulting supernatant was measured using a modified Lowry technique (Bio-Rad, Hercules, CA, USA; 500-0113, 500-0114).

2.2.2. Gel electrophoresis and Western blotting

Western blot analysis was used to measure protein levels of TH, vGLUT1, and vGLUT2 (Table 2). The gel electrophoresis and Western blot assays were performed as described previously (Rice et al., 2014) with one variation; samples used for vGLUT assays were not heated to 95 °C since this causes aggregation of the protein. Samples of 60 μg of total protein were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4–20% polyacrylamide gradient gels (Lonza, Basel, Switzerland; 58505), then transferred to polyvinylidene fluoride (PVDF) membranes for 21 h at 4 °C. One set of membranes was blotted for TH. A separate set of membranes was blotted for vGLUT2, stripped and reblotted for vGLUT1. Final reblots for actin were performed for all experiments as a control for tissue preservation and protein loading. All three groups were run together in the same experiment which required simultaneous incubation and development of two Western blot membranes. The experiments were performed in duplicate. Preincubation with the respective control peptide completely eliminates vGLUT1 (Barksdale et al., 2014) and vGLUT2 (Zeng et al., 2012) signal.

2.2.3. Analysis

Films were scanned using a flatbed scanner. Using NIH ImageJ, a box of consistent area was placed around each band at the expected molecular weight to measure optical density. An optical density standard curve was created using a step calibration tablet (Stouffer Industries Inc.; Mishawaka, IN, USA; T2120, series #130501), and each measurement was calibrated to the standard curve. A background subtraction in ImageJ was performed for each film. TH and vGLUT protein measures were normalized to actin. The normalized values for each duplicate were then averaged for the analysis.

All data sets were first assessed for normality with the Kolmogorov–Smirnov test. This was followed by an unpaired t-test or Wilcoxon test for CTRL vs SZ analyses. Welch's correction was used in cases of unequal variance. For CTRL vs SZ-ON vs SZ-OFF, a One-Way ANOVA or Kruskal–Wallis test was used. A Grubb's outlier analysis was used to identify potential outliers in each data set. Outliers were excluded only in situations where methodological considerations warranted exclusion. Presence of outliers, their inclusion/exclusion, and the impact on the results is noted in the Results section. All statistical tests were two-tailed with significance of $p < 0.05$.

3. Results

3.1. Actin protein levels in human NAcc

Protein levels of actin were measured in SZ and control subjects. Western blotting yielded an intense band at the correct molecular weight of ~42 kDa. Un-normalized actin levels did not differ between the NC and SZ groups. The mean un-normalized actin levels for the blots used in the TH analysis were 0.250 ± 0.007 (NC) and 0.247 ± 0.012 (SZ); $p = 0.563$. The mean un-normalized actin levels for the blots used in the vGLUT analysis were 0.251 ± 0.034 (NC) and 0.292 ± 0.046 (SZ); $p = 0.053$.

3.2. Tyrosine hydroxylase protein levels in human NAcc

Protein levels of TH were measured in SZ-ON, SZ-OFF, and control subjects. Western blotting yielded an intense band in the correct molecular weight range, 62–68 kDa and a less intense upper band present only in some samples (Fig. 1A). One control case was detected as an outlier. This case was noted to have poor protein concentration during protein extraction, so it was removed from the analysis. Removal of the data point resulted in normal distribution for the control group and had no effect on the significance of the results. TH protein levels were similar between control and SZ groups ($p = 0.35$, Fig. 1A). When the SZ cases were analyzed separately based on APD status, there was no difference in TH protein levels between the three groups ($p = 0.49$, Fig. 1A).

3.3. Vesicular glutamate transporter protein levels in human NAcc

Protein levels of vGLUT1 and vGLUT2 were measured in SZ-ON, SZ-OFF, and control subjects. Western blotting for vGLUT1 detected a light smear and one major broad band at the expected molecular weight, ~63 kDa (Fig. 1B). Protein levels of vGLUT1 were similar between control and SZ groups ($p = 0.81$, Fig. 1B). When the SZ cases were separated into SZ-ON and SZ-OFF groups, there were no significant differences in vGLUT1 protein levels between the three groups ($p = 0.36$, Fig. 1B). In the vGLUT1 data, one SZ-OFF case was detected as an outlier. There were no methodological issues that indicated the outlier should be excluded so it was left in the analysis. Inclusion of the outlier had no effect on the non-normal distribution of the data, or on the significance of the results.

Western blotting for vGLUT2 detected one major broad band at the expected molecular weight, 65 kDa, with multiple less intense bands (Fig. 1C). There was a significant increase in vGLUT2 protein levels in

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