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A simplified method to quantify dysregulated tyrosine transport in schizophrenia

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ABSTRACT

Background: Schizophrenia is associated with altered tyrosine transport across plasma membranes. This is typically demonstrated by measuring the uptake of radiolabeled tyrosine in cultured human fibroblasts. Our primary goal was to determine whether tyrosine uptake could be characterized using unlabeled tyrosine. A secondary goal was to assess the effect of antipsychotic drugs added during the incubation.
Method: Epithelium-derived fibroblast cultures were generated from patients with schizophrenia ($n = 6$) and age-matched controls ($n = 6$). Cells between cycles 8–12 were exposed to an amino acid free medium for 60 min and then for 1 min to media containing tyrosine (0.008–1.0 mM). Amino acid levels were measured and Michaelis–Menten parameters determined. Uptake of tyrosine (0.5 mM) was also measured in control cells after antipsychotic drugs were introduced during the depletion or uptake phases.
Results: Tyrosine uptake was sodium-independent. The maximal transport velocity (V_{max}) was significantly lower in patients with schizophrenia than in controls ($p < 0.01$). The transporter affinity (K_m) did not differ between the groups. Tyrosine uptake was differentially affected ($p < 0.001$) by inclusion of 10^{-4} M haloperidol, chlorpromazine or clozapine during different periods of incubation.
Conclusion: Dysregulated tyrosine kinetics in schizophrenia can be readily studied without the use of radiolabeled tracers. The data also indicate that tyrosine uptake may be subject to complex pharmacological effects.
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1. Introduction

Dysregulated tyrosine transport across plasma membranes is one of the most consistently demonstrated biomarkers in schizophrenia (Hagenfeldt et al., 1987; Wiesel et al., 1994; Ramchand et al., 1996; Flyckt et al., 2001). The abnormal tyrosine transport is maternally inherited (Flyckt et al., 2011), evident in antipsychotic drug naïve patients (Flyckt et al., 2001) and associated with greater cognitive dysfunction (Wiesel et al., 2005). Since common amino acid transporters are present throughout the body (Verrey et al., 2004), most data on aberrant tyrosine kinetics have been derived from cultures of human fibroblasts. Without exception, the maximal velocity (V_{max}) of tyrosine uptake has been found to be lower in schizophrenia (Hagenfeldt et al., 1987; Wiesel et al., 1994; Ramchand et al., 1996; Flyckt et al., 2001). Some groups have also reported a higher transporter affinity (Ramchand et al., 1996; Flyckt et al., 2001). Despite these highly consistent data, the link between abnormal tyrosine transport and the pathophysiology of schizophrenia remains to be fully defined. This process could be accelerated by simplifications in the methodology for measuring tyrosine transport.

In the standard approach, fibroblasts are cultured over several cycles, incubated for a short period in an amino acid free medium to deplete their internal stores and then briefly (1 min) exposed to various concentrations of radiolabeled tyrosine (Hagenfeldt et al., 1987). The intracellular content of radiolabeled tyrosine is then used to derive classical Michaelis–Menten parameters (Hagenfeldt et al., 1987; Wiesel et al., 1994; Ramchand et al., 1996; Flyckt et al., 2001). While the use of a radiolabel allows measurement of trace quantities of tyrosine, the cost and logistics of using radioactive materials can be limiting in some laboratories. Given improvements in analytic techniques for amino acids (Bongiovanni et al., 2001), we posited that unlabeled tyrosine could be used equally well to generate kinetic parameters. The testing of this hypothesis was the main purpose of the current study.

The pharmacological sensitivity of tyrosine transport in man is also incompletely characterized. One study showed that high concentrations of haloperidol or chlorpromazine present only during the 1 min uptake phase markedly inhibited tyrosine uptake (Wiesel et al., 1994). Since then, a longer antipsychotic drug exposure (≥3 h) has been reported to affect the transport of other classes of amino acids (Marchesi et al., 2006). Accordingly, our secondary aim was to determine whether tyrosine uptake parameters could be affected by a longer exposure to antipsychotic drugs. If dysregulated tyrosine transport mediates symptoms

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of schizophrenia, then pharmacologic agents that normalize tyrosine uptake in fibroblasts should prove to be therapeutic. In that case, fibroblast cultures could provide a valuable in vitro model for developing and evaluating new drug candidates.

2. Method

2.1. Subjects

Postmortem tissue was collected at autopsy following family consent under a study exempt from human subject approval. The Denver Schizophrenia Center Brain Bank was initiated in 1980. Human postmortem brain and matching skin fibroblasts were collected, following donation by the family. Subjects were identified by local coroners and the family contacted by Schizophrenia Center staff. In the current study, subjects with HIV or hepatitis B were excluded. The culturing of fibroblasts through several generations was used to attenuate medication and cause of death effects.

All subjects were Caucasian males and included controls ($n = 6$) with no history of mental illness and patients with schizophrenia ($n = 6$). Diagnosis (DSM-IV) was made by two board certified psychiatrists on the basis of medical records as well as family and physician interviews. The groups did not differ significantly in age (control 52.17 ± 10.40 yr; schizophrenia 62.33 ± 16.40 yr), or in postmortem interval (control 14.50 ± 5.90 h; schizophrenia, 17.00 ± 6.58 h). Medication information (Table 1) was collected from the chart. Two patients were on a single antipsychotic drug at the time of death (olanzapine or fluphenazine) and one was on multiple antipsychotic drugs (chlorpromazine, trifluoperazine, haloperidol). The medication status of the other three patients is not known.

2.2. Fibroblast cultures

Skin fibroblasts were cultured from cadavers as described (Canastar et al., 2012). Briefly, the skin was swabbed with betadine and alcohol before harvesting. Skin (approximately 1 cm^2) was dissected from the upper and inner arm of the deceased and immediately placed in media with antibiotics (DMEM/F12 1:1, 15% fetal calf serum, 2 mM L-glutamine, penicillin/streptomycin) and brought to the laboratory. The tissue was dissected into small pieces with a sterile scalpel and forceps and placed into a Corning T-25 flask. Medium, 2.0 ml was slowly dripped onto the tissue in the flask. Cultures were placed in a 37 °C, 5% CO_2 incubator overnight. After 24 h, 4.0 ml of additional media was added slowly. Medium was changed twice weekly. Fibroblast growth is usually seen in 7–14 days. Cells were passaged once and then frozen in media with 20% fetal calf serum and 10% DMSO at −80 °C. After thawing, individual cell lines were cultured in 75 cm^2 plastic (Costar®) flasks in a humidified atmosphere of 10% CO2 at 37 °C. The cells were frequently checked for mycoplasma and other bacterial contamination. Primary cultures between the 8th and 12th passages were then frozen in 10% dimethyl sulfoxide in Dulbecco minimal essential medium with 10% fetal calf serum and stored in liquid nitrogen for later use.

Culture reagents were purchased from Sigma-Aldrich: Dulbecco's Modified Eagle Medium (DMEM), insulin/transferrin/selenium supplement (ITS) (1 mg/ml/0.55 mg/ml/0.5 μg/ml respectively), fibroblast growth factor (25 μg/ml), fetal bovine serum (FBS), penicillin/streptomycin (10,000 U/ml/10,000 μg/ml respectively), phosphate buffer saline (PBS) with 0.1% glucose, L-glutamine 200 mM. The culture medium was made by adding the following to 500 ml DMEM: penicillin/streptomycin 5 ml, L-glutamine 5 ml, ITS 5 ml, fibroblast growth factor, FBS 100 ml. Thus the culture medium contained FBS (17%, vol/vol), penicillin (85 U/ml), streptomycin (85 μg/ml), glutamine (1.7 mM), fibroblast growth factor (45 ng/ml), and ITS (insulin 8.5 μg/ml, transferrin 5 μg/ml, selenium 4.5 ng/ml).

2.3. Amino acid depletion and tyrosine uptake

Following the cluster-tray method (Gazzola et al., 1981; Hagenfeldt et al., 1987) approximately 4×10^4 cells were seeded into each well (4.8 cm^2, 12/tray) of a multiwell tray (Corning® CellBIND® Surface). Two equivalent trays were set up for each cell line and cultured to confluence in 5 days. A partial depletion of intracellular endogenous amino acid stores was achieved by incubating the fibroblasts in an amino acid-free medium (Gazzola et al., 1980). The cells grown to confluence were washed with 2 ml of phosphate buffered saline (PBS) containing 1 g/l glucose and incubated with an additional 2 ml PBS for one hour at 37 °C.

The PBS incubation medium was then removed and tyrosine uptake measured during a 1 min incubation at 37 °C in 0.5 ml of PBS containing a known concentration of tyrosine. Uptake was terminated rapidly by placing the tray on ice and washing twice with ice-cold PBS. The residual PBS was removed from the wells, 250 μl of 5% trichloroacetic acid TCA was added and the trays incubated for an additional 30 min at room temperature. The acid soluble extract from each well was transferred to a 1 ml tube for measurement of free amino acids. The cells in each well were then digested by the addition of 250 μL of 1 N NaOH and then incubation for 30 min. Protein quantities were assayed directly in the tray utilizing a modified Bio-Rad method (Bradford, 1976).

Since the concentrations of glucose and TCA used differ across published studies (Gazzola et al., 1980; Hagenfeldt et al., 1987), we conducted a preliminary experiment. Glucose and TCA concentrations were varied and large neutral amino acid levels measured in the PBS removed at the end of the 60 min incubation as well as in the acid soluble fraction subsequently extracted with TCA. In a second experiment, only glucose levels were varied and the 1 min uptake of 0.5 mM tyrosine measured. Insofar as tyrosine uptake in fibroblasts is sodium-independent (Hagenfeldt et al., 1987), we conducted a confirmatory study, comparing tyrosine uptake (0.063–1.5 mM) in regular PBS and in a medium in which sodium

Table 1
Subject Characteristics.

Age (yr)	Dx	COD	PMI (h)	Drugs	Smoker
53	CON	Acute disease	17.0	Thyroxine, antilipidemic	Y
72	CON	Heart failure	18.5	Digoxin, vancomycin, hydrocortisone, famotidine cyclosporin, famotidine	N
45	CON	Acute disease	15.0	Furosemide, sertraline, codeine, prochlorperazine, dextromethorphan, vancomycin, lorazepam, diphenhydramine	N
51	CON	Renal	6.0	Sevelamer, phenytoin, warfarin, amlodipine, insulin	N
43	CON	Trauma	21.5	None	Y
49	CON	Cardiac	9.0	Alcohol	Y
63	SZ	Cardiac	18.0	Digoxin, procainamide, isosorbide, captopril, ketorolac	N
38	SZ	Suicide	11.0	Cannabis, alcohol	N
59	SZ	Acute disease	19.5	Chlorpromazine, trifluoperazine, haloperidol	Y
57	SZ	Heart failure	18.0	Fluphenazine, insulin	Y
68	SZ	Heart failure	8.5	Amitriptyline, risperidone, venlafaxine	N
89	SZ	Heart failure	27.0	Olanzapine, sertraline, trazodone	Y

CON (control), SZ (schizophrenia), Diagnosis (Dx), cause of death (COD), postmortem interval (PMI).

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