Aberrant amino acid transport in fibroblasts from children with autism

Elisabeth Fernell a, Aristea Karagiannakis b, Gunnar Edman c, Lars Bjerkenstedt d, Frits-Axel Wiesel e, Nikolaos Venizelos b, *

a Department of Neuropaediatrics, Astrid Lindgren Children’s Hospital, Karolinska University Hospital, SE 171 76 Stockholm, Sweden
b Department of Clinical Medicine, Biomedicine, Örebro University, SE-701 82 Örebro, Sweden
c Department of Psychiatry, R & D Section, Danderyd’s Hospital, SE-182 87 Danderyd, Sweden
d Department of Clinical Neuroscience, Karolinska University Hospital, SE-171 76 Stockholm, Sweden
e Department of Neuroscience, Psychiatry, Ulleråker, Uppsala University Hospital, SE-750 17 Uppsala, Sweden

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Abstract

Autism is a developmental, cognitive disorder clinically characterized by impaired social interaction, communication and restricted behaviours. The present study was designed to explore whether an abnormality in transport of tyrosine and/or alanine is present in children with autism. Skin biopsies were obtained from 11 children with autism (9 boys and 2 girls) fulfilling the DSM-IV diagnostic criteria for autistic disorder and 11 healthy male control children. Transport of amino acids tyrosine and alanine across the cell membrane of cultured fibroblasts was studied by the cluster tray method. The maximal transport capacity, \( V_{\text{max}} \) and the affinity constant of the amino acid binding sites, \( K_m \), were determined. Significantly increased \( V_{\text{max}} \) for alanine \((p = 0.014)\) and increased \( K_m \) for tyrosine \((p = 0.007)\) were found in children with autism. The increased transport capacity of alanine across the cell membrane and decreased affinity for transport sites of tyrosine indicates the involvement of two major amino acid transport systems (L- and A-system) in children with autism. This may influence the transport of several other amino acids across the blood–brain-barrier. The significance of the findings has to be further explored.

Keywords: Autism; Amino acid transport; Fibroblasts; Tyrosine; Alanine

Autism is a developmental disorder characterized by severely impaired social interaction, communication and restricted behaviours. Specific cognitive dysfunctions are linked to this triad of symptoms [18]. The vast majority of the patients have an overall low intellectual function with IQs below the normal variation. Attention deficits are usually present [2]. Hyperactivity and stereotypes are common. Other behavioural disturbances, such as self-injuries, tantrums and outbursts may occur, entailing a severe clinical picture. Treatment with post-synaptic dopamine-blockers, i.e. classical neuroleptic drugs, has been shown to reduce the symptom level in some patients [29]. The onset of clinical symptoms varies and a considerable number of children develop relatively normally until the age of 18–24 months. At that time deterioration with respect to communicative ability and behaviour becomes evident. This specific form of autism is referred to as the early regressive type [27].

In line with other developmental disorders several underlying etiologies have been suggested. Prenatal causes of various kinds dominate [14]. However, in most cases, available neurological assessments cannot reveal the underlying cause. Autism is a disorder with a strong genetic component [32]. Positive associations with autism were reported for more than 15 genes residing at different chromosomal loci [5].

Dopamine (DA) acts as a powerful regulator of different aspects of cognitive functions and is a key neurotransmitter in the brain. Several studies have shown its regulatory role for motor and cognitive/executive functions [25]. Already in 1987 Gillberg and Svennerholm [15] analyzed the major DA metabolite homovanillic acid (HVA) in the cerebrospinal fluid (CSF) in unmedicated children with autism, in children with other developmental disorders and in a healthy comparison group. Children with autism were found to have increased levels of HVA and the authors suggested that a hyperfunction of the dopaminergic system in the brain stem and the mesolimbic system could be
part of the pathogenesis in autism. However, in another study of HVA levels in the CSF in children with autism, no significant differences were found between children with autism and controls [24]. A link between autism, disintegrative disorder, i.e. autism with late onset (Heller’s syndrome; [27] and schizophrenia was proposed by Honjo [19] and also discussed by Schieveld [28]. In both conditions cognitive dysfunction is an important characteristic. Interestingly, in patients with schizophrenia, we have previously found a connection between tyrosine transport and cognitive functioning [33]. Tyrosine is the precursor of dopamine synthesis and a limitation of its access to the brain will influence dopamine content and functioning [6]. Amino acids play an important role in brain development and cognitive functioning, drastically demonstrated in children with phenylketonuria (PKU). In this context it should be of interest to investigate tyrosine transport in children with autism.

Tyrosine transport is mediated from plasma to brain through membranes mainly via the L-system, named from its function to transport large, neutral, branched and aromatic amino acids (LNAA), i.e. phenylalanine, tyrosine, tryptophan, methionine, leucine, isoleucine, valine and histidine. The L-system is sodium-independent and consists of four isoforms; LAT1, LAT2, LAT3 and LAT4 [7]. The L-system is widely expressed in the body and occurs in the blood–brain-barrier, kidney and small intestine [20]. In fibroblasts only the total tyrosine transport has been studied, without discriminating between the isoforms of the L-system [16,20]. To a smaller extent, tyrosine is also transported from plasma to brain by the sodium-dependent A-system (“A” is an abbreviation for alanine) [20]. Three variants of system A, designed ATA1, ATA2 and ATA3, have been identified [1]. Transport of short-chain neutral amino acids such as alanine is mainly mediated by the transport A-system [30] but also by the L-system [20]. ATA2 is widely expressed in mammalian tissues [17] and occurs like the L-system in the blood–brain-barrier [23] and fibroblasts [13,26].

The aim of this study was to test our working hypothesis that children with autism have changes in amino acid transport mechanisms, based on the profound importance of amino acids in brain development and for cognitive functioning. A 2 mm skin punch biopsy was taken after anaesthetizing the mid-forearm under aseptic conditions. The tissue was placed immediately in tubes containing complete culture medium.

The study group consisted of 11 children with autism, according to the DSM-IV criteria [3], 9 boys and 2 girls, aged 5–11 years (mean 8 years). All were all patients at the Neuropaediatric Outpatient Clinic at the Department of Neuropaediatrics, Karolinska University Hospital, Stockholm and had been assessed and diagnosed by experienced neuropaediatric-neuropsychiatric teams. Ten had mental retardation (U.K. learning disabilities) and one had an IQ in the lower normal area. Four of the 11 children also had epilepsy, and in at least 2 children without clinical epilepsy, the EEG had revealed epileptiform discharges. All boys and one of the girls had an uneventful neonatal period and first year of life and clinically they were classified as being of the early regressive type. No specific disease had earlier been demonstrated. The second girl had a different clinical picture, starting with muscular hypotonia, however, her cognitive problems later became evident, first as a mental retardation, and eventually at the age of 6 years autism was diagnosed. (Etiologically a specific syndrome was suspected but had not been proven).

Eight of the 11 children had undergone a magnetic resonance tomography or a computerized tomography with normal results. The four children with epilepsy were treated with antiepileptics; and one of these children had also received a low dose of risperidone. Two other children were treated with a low dose of risperidone and serotonin reuptake inhibitor, respectively. Routine plasma amino grams had been performed on all the children with normal results.

The Ethics Committee at Karolinska Hospital approved the study. The parents were introduced to the study procedure and gave informed consent to let their child participate in the study. Skin biopsies were taken in connection with surgery for hypospadias in 11 boys between the ages of 1 and 13 years old (mean 4 years). All were healthy and there was no suspicion of developmental disorders in any of these children.

All growth media, antibiotics, fetal calf serum and Amnio-Max™ were purchased from Gibco Invitrogen cell culture, Sweden. Phosphate buffered saline (PBS) from Statens Veterinärmedicinska Anstalt (SVA), Uppsala, Sweden. [U-14C]-labelled L-tyrosine (specific activity 434 mCi/mmol) and [U-14C]-labelled L-alanine (specific activity 128 mCi/mmol) were purchased from Moravek Biochemical Inc., California, U.S.A. Unlabelled L-tyrosine and L-alanine were purchased from Sigma–Aldrich, Sweden.

Fibroblasts were cultured from skin biopsies using Eagle’s Minimum Essential Medium (MEM) supplemented with fetal calf serum (10%, vol/vol), penicillin V (125 U/ml), streptomycin (125 μg/ml), L-glutamine (2 mmol/l) and Amnio-Max™. Stock cultures of individual fibroblast strains were cultured in 75 cm² Costar plastic tissue culture flasks in a humidified atmosphere of 5% CO₂ at 37 °C. Fibroblast lines were used experimentally between the 5th and 9th passage.

Amino acids transport assays were performed according to the fibroblast model [10,12,16] for rapid measurement of amino acid flux in adherent intact fibroblast cells. Approximately 5 × 10⁴ cells were seeded in a 24 multiwell tray (2 cm diameter; Costar Europe Ltd., Costar, NY) and grown to confluence in 1.5 ml of MEM for 5 days. The cells were washed twice with 1ml phosphate buffered saline (PBS) and directly pre-incubated with 1 ml PBS containing 1% glucose for 1 h at 37 °C, 5% CO₂ to deplete endogenous amino acids pools. After removal of the pre-incubation medium, the initial rate of a particular amino acid uptake was measured during incubation for 60 s at 37 °C in 0.2 ml PBS, containing a constant amount of 14C-labelled amino acid (0.2 μCurie) and 12 different concentrations (varying between 0.01 and 1.5 mmol/l for tyrosine and between 0.02 and 6 mmol/l for alanine) of unlabelled amino acids. Amino acid transport assay was terminated by rapidly washing the cells twice with 2 ml of ice-cold PBS. After washing, the wells were drained and 230 μl of trichloroacetic acid (10%, v/v) was added to each well for 20 min at room temperature. Two hundred microliters of the radioactive soluble amino acid extract was removed for liquid scintillation counting. The
trays were drained and the precipitated proteins were dissolved in 1 mol/l NaOH and assayed by the modified Lowry method using bovine serum albumin as standard.

The amino acid kinetic parameters were calculated from the Lineweaver–Burk plot equation \[ \frac{1}{V_0} = \left( \frac{K_m}{V_{\text{max}}} [S] \right) + \left( \frac{1}{V_{\text{max}}} \right), \]
by using computerized software as described previously by Flyckt et al. in 2001. \( V_0 \) is the initial transport velocity and \([S]\) is the transport substrate concentration, \( V_{\text{max}} \) is the maximal uptake rate for the carrier-mediated process (nmol/min × mg protein) and \( K_m \) is the affinity constant (the concentration at half-saturation; μmol/l).

Each experiment was performed at 12 different concentrations in duplicate in the same incubation for both tyrosine and alanine.

The tyrosine transport was studied on two occasions at different time points, to diminish cell artefacts that can be caused by the condition of the cells, and could influence the low \( V_{\text{max}} \) and \( K_m \) tyrosine values. Alanine was analyzed only once since the experiment precision is higher than tyrosine (controls are presented in Table 1).

Brain tyrosine is dependent on the influx of this amino acid across the blood–brain-barrier. Tyrosine is critical for maintaining adequate levels of dopamine and therefore of vital importance for adequate cognitive functions. A major reason for studying tyrosine transport is the association between PKU and the other hand the \( K_m \) \( t (13.9) = 1.05, p = 0.311 \) for alanine did not differ significantly between the two groups.

\( V_{\text{max}} \) and \( K_m \) for tyrosine and alanine for the four children with autism and epilepsy were compared with those of the autistic children without epilepsy. There were no significant differences between the groups (data not shown).

Fibroblast cell cultures offer a model for experimental studies on the transport of amino acids across cell membranes. The kinetic parameters \( V_{\text{max}} \) and \( K_m \) were studied, reflecting the maximal transport capacity of tyrosine/alanine and the affinity of tyrosine/alanine for the binding sites of the transporters, respectively.

The main finding of this study was that fibroblasts from children with autism had an elevated \( V_{\text{max}} \) for alanine and a higher \( K_m \) for tyrosine. The higher \( K_m \) corresponds with a decreased affinity between the transport protein and the substrate tyrosine. The lower affinity for tyrosine indicates that a higher concentration of extracellular tyrosine is required to reach the maximal transport capacity, resulting in a decreased competition between tyrosine and other amino acids. Alanine on the other hand showed a higher transport capacity in this group of children with autism, but the \( K_m \) did not differ significantly between the two groups. This indicates that fibroblasts from the children with autism in this study group had an elevated expression of alanine transporting protein. Hence, our main result shows that there is a change in the kinetics of tyrosine and alanine transport in this study group of children with autism.

Recently, we have investigated the two major transporters of tyrosine and alanine (L- and A-systems) in fibroblast from patients with schizophrenia and controls [16,26]. This study provided evidence that 90% of the total uptake of tyrosine and 65% of alanine were transported through the sodium-independent L-system, whereas 10% of tyrosine and 75% of alanine was transported by the A-system. Consequently, alanine inhibited tyrosine uptake by 65% in fibroblasts in both patients with schizophrenia and controls. Therefore, there seems to be a competition between alanine and tyrosine for transport across membranes [16,26].

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Table 1

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Children with autism (n = 11)</th>
<th>Controls (n = 11)</th>
<th>( p )-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>S.D.</td>
<td>M</td>
</tr>
<tr>
<td>Tyrosine*, ( V_{\text{max}} )</td>
<td>12.4</td>
<td>1.80</td>
<td>10.9</td>
</tr>
<tr>
<td>Tyrosine*, ( K_m )</td>
<td>23.8</td>
<td>3.42</td>
<td>19.2</td>
</tr>
<tr>
<td>Alanine, ( V_{\text{max}} )</td>
<td>27.0</td>
<td>5.10</td>
<td>21.6</td>
</tr>
<tr>
<td>Alanine, ( K_m )</td>
<td>114.0</td>
<td>15.5</td>
<td>126.0</td>
</tr>
</tbody>
</table>

The results are presented as mean (M) and standard deviation (S.D.).

\( V_{\text{max}} \) indicates maximal transport capacity (nmol/min × mg protein).

\( K_m \), affinity of binding sites for a specific amino acid (μmol/l).

\( ^* \) M ± S.D. for tyrosine are based on the average of the two repeated uptake measurements.
The number of children with epilepsy in our study is in agreement with the reported frequency of epilepsy in autism, being about one-third [8,31]. According to Tuchman and Rapin [31], epilepsy and autism are both heterogeneous clinical disorders associated with an array of etiologies and pathologies and the present evidence suggests that there are common pathophysiological mechanisms that account for both the autism and the epilepsy. We have no idea whether the proportion of children with both autism and epilepsy in our study group influences our results since the relation of clinical and subclinical epilepsy to autistic behaviour and early regression is unsettled.

In conclusion, our findings may indicate an elevated access of alanine and less availability of tyrosine in the brain, resulting in a lower dopaminergic activity. On the other hand, in vitro data is hard to translate to in vivo situations with reciprocal interactions involving many complex systems. Our findings demonstrate that there may be disturbances in transport mechanisms for amino acids, at least at the membrane level. A further exploration of this research field concerning disturbances of amino acid transport in children with autism includes molecular investigations, looking for polymorphisms in gene loci, and further transport studies, which will hopefully provide more valid answers.

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