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The pharmacological profile of L-glutamate transport in human NT2 neurones is consistent with excitatory amino acid transporter 2

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Abstract

The human teratocarcinoma cell line NTera2/D1 can be differentiated to produce post-mitotic neurones (NT2-N cells) by prolonged (> 3 week) exposure to retinoic acid. In this study, we describe the characterisation of high-affinity Na^+-dependent L-glutamate transport activity in post-mitotic differentiated NT2-N cells. NT2-N cells, but not the undifferentiated precursor cells, transported L-glutamate in a Na^+-dependent manner, as determined by equimolar replacement of Na^+ with choline. L-glutamate uptake was saturable and Eadie–Hofstee transformation of the saturation data revealed a K_m of 10.6 ± 0.8 μM, and a maximum transport capacity (V_{max}) of 100.3 ± 12.3 pmol min^{-1} mg^{-1} protein. Pharmacological characterisation of the transport activity in NT2-N cells produced a rank order of inhibitory activity which was identical to that determined for the human excitatory amino acid transporter 2 which we have analysed in a stable mammalian cell line (Madin Darby Canine Kidney (MDCK) cells). Of particular note, L-glutamate transport by NT2-N cells was sensitive to both dihydrokainate and kainate. The expression of human excitatory amino acid transporter mRNAs was studied using reverse transcriptase polymerase chain reaction. NT2-N cells expressed transcripts for excitatory amino acid transporters 2 and 3, but not for the subtypes 1, 4 and 5. We conclude that although the mRNA expression studies suggest the presence of transcripts for both excitatory amino acid transporter 2 and 3 in NT2-N cells, the sensitivity to dihydrokainate and kainate determined in the pharmacological analysis indicates that, of the known transporter subtypes, excitatory amino acid transporter 2 contributes to the bulk of the L-glutamate transport activity present in these cells.

Keywords: Excitatory amino acid transporter; L-glutamate; NT2-N cell

1. Introduction

Exposure of the human teratocarcinoma cell line NTera2/D1 to retinoic acid results in the differentiation of a population of these cells to produce post-mitotic neurones which have been designated as NT2-N cells (Andrews, 1984; Lee and Andrews, 1986). By combining retinoic acid treatment with a differential adhesion protocol and the use of mitotic inhibitors it has been shown that highly enriched (> 95%) cultures of NT2-N cells can be obtained (Pleasure et al., 1992). These NT2-N cells display a morphology which is very similar to that of primary neuronal cultures derived from rodents and it has been demonstrated that the neuronal phenotype is stable for up to two months in culture. Characterisation of NT2-N cells has revealed the expression of a wide range of neuronal markers including neurofilament proteins (Lee and Andrews, 1986; Pleasure et al., 1992), microtubule associated proteins (Pleasure et al., 1992), proteins associated with neurosecretion (Pleasure et al., 1992), tetrodotoxin-sensitive Na^+ channels (Rendt et al., 1989), tetanus toxin receptors (Andrews, 1984) and glutamate receptors of both the NMDA (Younkin et al., 1993; Munir et al., 1996) and non-NMDA receptor subtypes (Hardy et al., 1994; Kondoh et al., 1997). Although these neuronal markers have been identified in NT2-N cells there have been no reports describing the expression of neurotransmitter transporters by these neurones.

In this study, we describe the characterisation of high-affinity Na^+-dependent L-glutamate transport in NT2-N cells. L-glutamate is widely accepted as the major fast

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acting excitatory neurotransmitter in the mammalian CNS (Watkins and Evans, 1981). Recently, a family of human L-glutamate transporters have been identified by molecular cloning techniques and, to date, five members of this family have been described (Arriza et al., 1994, 1997; Fairman et al., 1995) and are designated excitatory amino acid transporter 1–5. The excitatory amino acid transporters 1–3 are the human homologs of the transporter clones isolated initially from non-human species which are designated as glutamate/aspartate transporter (GLAST), glutamate transporter (GLT-1) and excitatory amino acid carrier (EAAC1), respectively (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992). These transporters share the properties of high-affinity for the substrates L-glutamate, L-aspartate and D-aspartate and an absolute requirement for extracellular Na^+ for transport activity (see reviews by Kanner, 1993; Kanai et al., 1993; Gegelashvili and Schousboe, 1997). The excitatory amino acid transporters serve a dual function in the central nervous system (CNS) by terminating the neurotransmitter action of L-glutamate and by maintaining low extracellular glutamate concentrations under normal physiological conditions, thereby preventing glutamate neurotoxicity. This second property has been demonstrated most convincingly for the rat glutamate transporter GLT-1 and the glutamate/aspartate transporter subtypes by gene knockdown of these transporters using chronic antisense oligonucleotide administration which results in increased neurodegeneration in the treated animals (Rothstein et al., 1996). In addition, a GLT-1 knockout has been generated and the GLT-1 deficient mice exhibit spontaneous seizure activity and increased susceptibility to acute brain injury (Tanaka et al., 1997).

Following our observation of high-affinity L-glutamate uptake in NT2-N cells we performed a pharmacological characterisation of this transport activity and examined the expression of excitatory amino acid transporters in these neurones. Most significantly, the L-glutamate transport in NT2-N cells was sensitive to micromolar concentrations of dihydrokainate and kainate, compounds which are selective inhibitors of the excitatory amino acid transporter 2 (Arriza et al., 1994). Expression of the transcripts for excitatory amino acid transporters 1–5 in NT2-N cells was studied by reverse transcriptase polymerase chain reaction (RT-PCR). These studies revealed that NT2-N cells express transcripts for excitatory amino acid transporters 2 and 3 but not for the subtypes 1, 4 and 5.

The objective of this study was to characterise the L-glutamate transport activity which we observed in NT2-N cells. We present data which suggests that, of the known excitatory amino acid transporter subtypes, excitatory amino acid transporter 2 contributes to the bulk of the transport activity in these cells. This conclusion is supported both by the pharmacological analysis demonstrating sensitivity to dihydrokainate and kainate, and by the RT-PCR expression studies.

2. Materials and methods

2.1. Materials

The NTera2/D1 clone was obtained from Dr. Peter Andrews (University of Sheffield, UK) and the Madin Darby Canine Kidney (MDCK) cell line expressing excitatory amino acid transporter 2 was provided by Dr. S. Amara (Vollum Institute, Portland). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (D-PBS), foetal bovine serum, penicillin, streptomycin and trypsin were from Gibco BRL (Grand Island, NY). Retinoic acid, poly-D-lysine, cytosine arabinofuraside, 5-fluoro-2-deoxy-uridine and uridine were from Sigma (St. Louis, MO). L-[^{3}H]glutamate (40–80 Ci $mmol^{-1}$) was purchased from New England Nuclear (Boston, MA) and laminin from Collaborative Biomedical Products (Bedford, MA). Amino acids and glutamate uptake inhibitors were from Tocris Cookson (Ballwin, MO).

2.2. Cell differentiation and culture

The undifferentiated NTera2/D1 cells, cultured in T-175 flasks, were maintained in high glucose DMEM supplemented with 10% foetal bovine serum and penicillin/streptomycin. Cells were routinely grown to 80% confluency before passage (1:6 split). For differentiation, cells were plated at 1.3×10^4 cells cm^{-2} in T-175 flasks 24 h before changing the growth medium to one

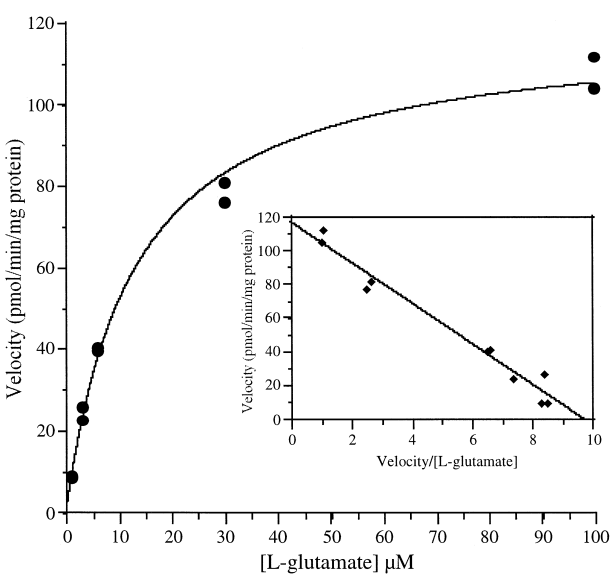

Fig. 1. The uptake of L-glutamate by NT2-N cells is a high-affinity saturable process. Cells were incubated in the presence of increasing concentrations of L-glutamate and uptake was calculated and expressed as pmol min^{-1} mg^{-1} protein after correction for the uptake observed in the absence of extracellular Na^+. A representative experiment is presented with duplicate determinations for each L-glutamate concentration. Inset: Eadie–Hofstee transformation of the saturation data was used to estimate kinetic parameters which for the representative experiment shown were; K_m 12.1 μM and V_{max} 116.3 pmol min^{-1} mg^{-1} protein.

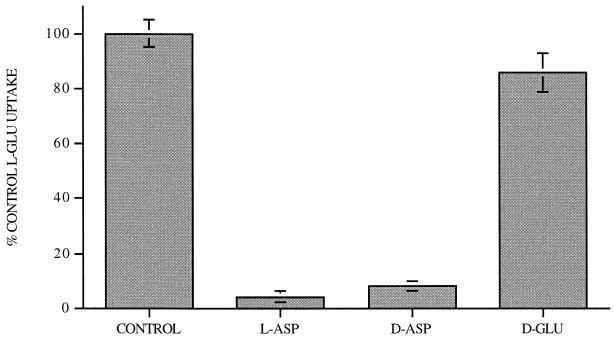

Fig. 2. The uptake of L-glutamate by NT2-N cells is inhibited by both L- and D-aspartate, but is insensitive to D-glutamate. Cells were incubated in the absence (control) or presence of 100 μM L-aspartate, D-aspartate or D-glutamate and the uptake of L-glutamate was measured. Results are expressed as a percentage of the control uptake in the absence of added compound and data are mean values ± S.E.M. from three independent experiments.

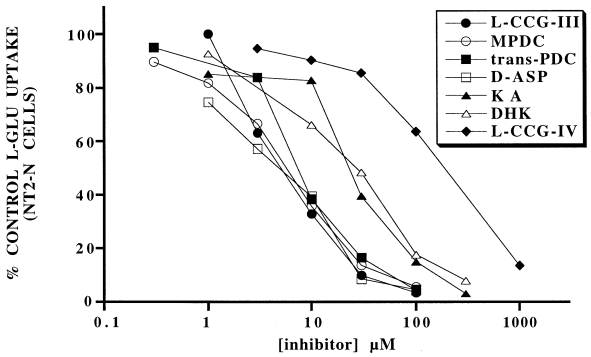

Fig. 3. Pharmacological characterisation of L-glutamate transport in NT2-N cells. Results are expressed as a percentage of the control uptake measured in the absence of inhibitor. Data are mean values from three independent experiments.

supplemented with 10 μM retinoic acid. Cells were exposed to retinoic acid for a minimum of 3 weeks and the growth medium was changed three times a week during this period. Following retinoic acid removal cells were split 1:4 and cultured for 24 h prior to a mechanical dislodging procedure (flasks were struck a minimum of 10 times). Dislodged cells were cultured overnight and the mechanical dislodge procedure was repeated, dislodged cells were counted and the cells were cultured in T-175 flasks pre-coated with poly-D-lysine and laminin at a density of 2×10^5 cells cm^{-2} in medium supplemented with mitotic inhibitors (0.25 μg ml^{-1} cytosine arabinofuraside, 2.5 μg ml^{-1} 5-fluoro-2-deoxy-uridine and 2.5 μg ml^{-1} uridine). These cells were maintained for one week before treatment with a low concentration of trypsin (0.0025%) to remove the top layer of neurones. At this stage the neurones are counted and plated in poly-D-lysine/laminin coated 24-well culture plates at a density of 2×10^5 cells cm^{-2} in medium supplemented with mitotic inhibitors. L-[^{3}H]glutamate uptake was assayed 48 h after this final replate.

MDCK cells expressing excitatory amino acid transporter 2 were maintained in high glucose DMEM supplemented with 10% foetal bovine serum, penicillin/streptomycin, 2 mM glutamine and 0.5 mg ml^{-1} G418. These cells exhibit Na^+-dependent, high-affinity (K_m 74 ± 8 μM) and saturable (V_{max} 1.68 ± 0.19 nmol min^{-1} mg^{-1} protein) uptake of L-glutamate (Dunlop et al., 1998). The expression of excitatory amino acid transporter 2 in MDCK cells is stable at least up to 30 passages (unpublished observations).

2.3. Glutamate uptake studies

Uptake of L-[^{3}H]glutamate by NT2-N cells was assayed at room temperature directly in the 24-well plates. The growth medium was removed by aspiration and the cells were washed (3×) with a HEPES buffered saline (HBS) with the following composition; (mM), HEPES (10), Tris Base (5), NaCl (140), KCl (2.5), $CaCl_2$ (1.2), $MgCl_2$ (1.2), K_2HPO_4 (1.2), glucose (10), pH 7.4. L-[^{3}H]glutamate substrate solution was added for 20 min and removed rapidly by aspiration followed by two washes with ice-cold HBS. In preliminary experiments it was determined that the uptake of L-[^{3}H]glutamate by NT2-N cells was linear at least up to 30 min (not shown). Saturation analysis was undertaken with concentrations of 1, 3, 6, 30 and 100 μM glutamate and each of these solutions contained 1 μCi L-[^{3}H]glutamate/assay as tracer. Inhibition studies were undertaken using 1 μM glutamate in the presence of 1 μCi tracer/assay and inhibitors were evaluated at 100 μM for the stereospecificity studies, or over the concentration range 0.1–1000 μM for the pharmacological characterisation. Accumulation of L-[^{3}H]glutamate was measured by adding 500 μl 0.5 N NaOH to each well to solubilise the cells. Aliquots (2 × 100 μl) were removed for liquid scin-

Table 1
Comparison of K_i values for L-glutamate uptake inhibitors in the NT2-N cell preparation and in excitatory amino acid transporter 2 expressing MDCK cells

Compound	NT2-N cells (μM)	MDCK/EAAT2 cells (μM)
L-CCG-III	4.1 ± 0.7	6.3 ± 2.3
MPDC	6.4 ± 1.2	6.4 ± 0.8
trans-PDC	7.6 ± 0.9	7.1 ± 0.9
D-ASP	17.4 ± 7.1	37.6 ± 8.4
Kainate	34.5 ± 2.0	39.9 ± 6.4
Dihydrokainate	26.8 ± 8.1	14.7 ± 3.0
L-CCG-IV	214.2 ± 12.4	455.7 ± 117.0
L-α-AA	≫ 1000	≫ 1000

IC_{50} values for the inhibition of L-glutamate uptake in NT2-N cells and excitatory amino acid transporter 2 expressing MDCK cells were generated from log–concentration response curves by non-linear regression analysis using Kaleidagraph™.
Data were converted to K_i values according to the equation described by Cheng and Prusoff (1973) and the mean values ± S.E.M. from three independent experiments are presented.

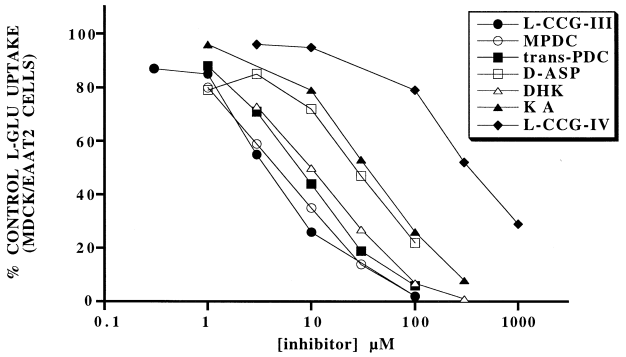

Fig. 4. Pharmacological characterisation of L-glutamate transport by excitatory amino acid transporter 2 expressed in the mammalian MDCK cell line. Results are expressed as a percentage of the control uptake measured in the absence of inhibitor. Data are mean values from three independent experiments.

tillation counting and protein was determined using the commercially available BioRad assay kit.

The uptake of L-[^{3}H]glutamate by MDCK cells expressing the excitatory amino acid transporter 2 was assayed in 24-well plates with cells plated at 1×10^5 cells/well the day prior to the experiment. Cells were washed ($3\times$) with Dulbecco's phosphate buffered saline (D-PBS) followed by the addition of L-[^{3}H]glutamate substrate (1 μM glutamate, 2 μCi L-[^{3}H]glutamate tracer/assay) for 10 min. Reactions were stopped at 10 min by aspiration and two washes with ice-cold D-PBS. Samples were further processed as described above for the NT2-N cells. Inhibitors were assayed over the concentration range 0.1–1000 μM.

2.4. Measurement of transcripts

Expression of the human excitatory amino acid transporters 1–5 by NT2-N cells was examined by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated using the RNAqueous™ (Ambion) phenol-free isolation kit as described in the manufacturers protocol. The specificity of the primers used for the RT-PCR reactions was confirmed by BLAST search of the GenBank database and their ability to amplify from the appropriate template was confirmed using plasmids containing excitatory amino acid transporters 1–4 (obtained from Dr. S. Amara) and human retinal cDNA for excitatory amino acid transporter 5 (not shown). The synthesis of cDNA and the PCR amplification were performed in one step using the SUPERSCRIPT ONE-STEP™ RT-PCR system from Life Technologies. 0.1 μg of total RNA was added to 50 μl (final volume) buffered reaction mix which contained 1.2 mM $MgCl_2$, 200 μM dNTPs, SUPERSCRIPT II RT/Taq mix and 1 μM forward and reverse primers for one of the excitatory amino acid transporters. The synthesis of cDNA and pre-denaturation were performed in one cycle at 50°C for 30 min for the reverse transcription of mRNA and at 94°C for 2 min for the pre-denaturation. This was followed immediately by the PCR amplification of cDNA with 40 cycles at 94°C (30 s) for denaturation, 55°C (30 s) for primer annealing and 70°C (1.5 min) for DNA elongation. A final extension of one cycle at 72°C for 10 min was used. The PCR products were separated on a 1% agarose gel stained with ethidium bromide and were visualised under UV illumination.

3. Results

The Na^+-dependency of L-glutamate uptake by NT2-N cells was established by replacing Na^+ in the extracellular medium with choline. NT2-N cells exposed to 1 μM L-glutamate accumulated 10.2 ± 1.2 pmol min^{-1} mg^{-1} protein ($n = 5$) in the presence of extracellular Na^+. Equimolar replacement of Na^+ with choline in the incubation buffer abolished 95% of this transport activity. In all subsequent experiments on the L-glutamate transport activity in the NT2-N cells parallel determinations were performed in the absence of extracellular Na^+ and all data has been corrected to represent the Na^+-dependent transport activity. L-glutamate transport activity in the NTera2/D1 precursors was evaluated using the same protocol described for the NT2-N cells, and the activity was found to be too small to accurately quantify (data not shown).

Transport experiments performed over a range (1–100 μM) of L-glutamate concentrations demonstrated that the accumulation of L-glutamate by NT2-N cells is a saturable process (Fig. 1). Eadie–Hofstee transformation of the saturation data presented in Fig. 1 was used to generate estimates of apparent affinity (K_m) and maximum transport capacity (V_{max}). The kinetic constants estimated for the representative experiment shown were; $K_m = 12.1$ μM and $V_{max} = 116.3$ pmol min^{-1} mg^{-1} protein, and those obtained from three independent determinations were;

Table 2
Primers used for RT-PCR analysis (5′–3′)

EAAT	Upper	Lower
1	GAAACGCTTGTGGGTGCTG	ATCTTGGTTTCACTGTCGATGGGT
2	ACAGTCATCTTGGCTCAGAGGAAC	CAGGCCCTTCTTGATAACCATCTT
3	TTGGCATGTATTCAGATGGCATAA	CAAGGCAAAGGGATTCACAATGT
4	GAAACGCCTTCATTCTGCTGACG	TGTGCCATGAGGGACTTGTAGGGT
5	CCCGAGGTCGTTTACAAGTCAGAG	CTCGCAGGGCAGCAGTTTCT

$K_m = 10.6 \pm 0.8$ μM and V_{max} 100.3 ± 12.3 pmol min^{-1} mg^{-1} protein.

The stereospecificity of the L-glutamate transport activity in NT2-N cells was examined by evaluating the effects of L- and D-aspartate and D-glutamate. As shown in Fig. 2, both L-aspartate and D-aspartate were effective inhibitors of L-glutamate uptake producing $96 \pm 1\%$ and $92 \pm 1\%$ inhibition, respectively, at 100 μM. In contrast, 100 μM D-glutamate failed to produce an inhibition of NT2-N L-glutamate transport.

Pharmacological characterisation of the NT2-N L-glutamate transport activity was carried out using a number of L-glutamate transport inhibitors. Of particular note both dihydrokainate (K_i 26.8 μM) and kainate (K_i 34.5 μM) were potent inhibitors of the L-glutamate transport activity in NT2-N cells (Fig. 3 and Table 1). The rank order of inhibitory potency determined for the compounds tested was (2S,1′S,2′R)-2-(carboxycyclopropyl)glycine-(L-CCG-III) (4.1 μM, K_i) = L-*trans*-pyrrolidine-2,4-dicarboxylic acid (*trans*-PDC) (7.6 μM) = L-*anti-endo*-3,4-methanopyrrolidine dicarboxylic acid (MPDC) (6.4 μM) = D-aspartate (17.4 μM) > dihydrokainate (26.8 μM) = kainate (34.5 μM) > (2*S*,1′*R*,2′*S*)-2-(carboxycyclopropyl)glycine (L-CCG-IV) (214.2 μM) ≫ α-aminoadipic acid (> 1 mM).

The pharmacology of the human excitatory amino acid transporter 2, expressed in an MDCK cell line which itself has no endogenous glutamate transport activity (data not shown), was found to be identical to that described above for the NT2-N cells (Fig. 4 and Table 1).

RT-PCR of total RNA prepared from NT2-N cells using primers (Table 2) specific for each of the excitatory amino acid transporters demonstrated that NT2-N cells express transcripts for excitatory amino acid transporters 2 and 3 (Fig. 5). Parallel reactions performed in the absence of reverse transcriptase failed to produce amplification products confirming that the products were derived from RNA and not contaminating genomic DNA (data not shown).

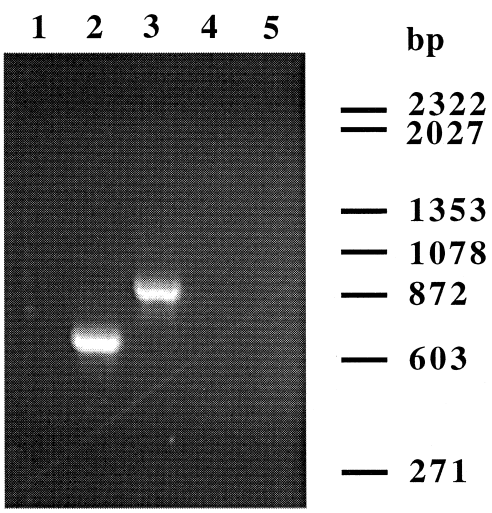

Fig. 5. RT-PCR analysis of excitatory amino acid transporter subtype transcripts in NT2-N cells. Amplification products were separated on a 1% agarose gel stained with ethidium bromide and were visualised under UV illumination. Lanes 1–5 correspond to primer sets, indicated in Table 2, for excitatory amino acid transporters 1–5, respectively.

4. Discussion

4.1. Properties of L-glutamate transport in NT2-N cells

This study describes the characterisation of high-affinity Na^+-dependent L-glutamate transport activity in differentiated human NT2 neurones (NT2-N cells). Significantly, the ability to transport L-glutamate was restricted to the NT2-N cells and was absent in the NTera2/D1 precursors indicating a requirement for terminal differentiation to the neuronal phenotype. The properties of the L-glutamate transport activity in NT2-N cells described here are consistent with those observed using a number of preparations derived from rodent brain including synaptosomes and primary cultures of neurones and astrocytes (Kanner and Schuldiner, 1987; Nicholls and Attwell, 1990; Schousboe, 1981). These are a high-affinity (2–50 μM) for the substrate and an absolute requirement for the presence of extracellular Na^+. Additionally, both L-aspartate and D-aspartate are transportable substrates for high-affinity L-glutamate transporters, however these transporters are stereospecific with respect to glutamate and the D-enantiomer is neither a substrate or inhibitor. Consistent with these properties, both L- and D-aspartate were effective inhibitors of L-glutamate uptake in NT2-N cells and D-glutamate was ineffective.

The recent cloning of five human excitatory amino acid transporter subtypes and their rodent homologs has facilitated the pharmacological characterisation of these transporter subtypes by the use of expression systems to study each transporter in isolation. In this regard, the two main approaches have been the use of *Xenopus* oocytes for expression of transporter subtypes with electrophysiological assays used to measure glutamate elicited transporter currents, and the transient or stable expression of transporters in mammalian cell lines to allow studies of L-[^{3}H]glutamate transport. One of the most important observations from these expression studies has been the demonstration of the selective inhibitory activity of dihydrokainate and kainate on the excitatory amino acid transporter 2. In this study, we clearly demonstrate that the L-[^{3}H]glutamate uptake in NT2-N cells is sensitive to both dihydrokainate and kainate, thus providing the first indication that the transport activity in these cells may be mediated predominantly by excitatory amino acid transporter 2. In order to provide a direct comparison between the pharmacology of the L-glutamate transport activity in the NT2-N cells with that of the human excitatory amino acid transporter 2 we characterised the pharmacology of

this subtype when expressed in the mammalian MDCK cell line. Our results demonstrate that the pharmacological profile of L-glutamate transport in NT2-N cells correlates extremely well with that determined for the human excitatory amino acid transporter 2. In addition, the pharmacological profile presented here for the L-glutamate transport activity in NT2-N cells is consistent with that described for forebrain synaptosome preparations (Ferkany and Coyle, 1986; Robinson et al., 1991, 1993) in which excitatory amino acid transporter 2 is believed to predominate, and in other expression studies where this subtype has been characterised (Arriza et al., 1994; Tan et al., 1997).

The excitatory amino acid transporter 2 subtype is reported to be localised exclusively to astroglial cells in the CNS (Rothstein et al., 1994), however the main conclusion of this study is that a human neuronal cell line exhibits L-glutamate transport activity with pharmacological properties consistent with that of the cloned excitatory amino acid transporter 2. There are other examples in the literature where the in vitro expression of transporter subtypes fails to recapitulate the localisation studies performed in whole brain. The pharmacology of L-glutamate transport in astrocyte-enriched primary cultures is not consistent with that of excitatory amino acid transporter 2 (Garlin et al., 1995), although this subtype is believed to represent the predominant astroglial transporter (Rothstein et al., 1994). In fact, expression studies indicate that the glutamate/aspartate transporter subtype is predominantly expressed by astrocytes in culture (Gegelashvili et al., 1996; Swanson et al., 1997) and the expression of excitatory amino acid transporter 2 by these cells requires neuronal factors which can be introduced with neuronal conditioned medium or by co-culture with neurones (Gegelashvili et al., 1997; Swanson et al., 1997). In another CNS derived preparation, the widely utilised rat C6 glioma cell line, the neuronal excitatory amino acid carrier 1 is expressed but not the astroglial transporters (Dowd et al., 1996; Palos et al., 1996). Primary cultures of rat cortical neurones which do express the neuronal excitatory amino acid carrier 1, also exhibit L-glutamate transport activity which is sensitive to both kainate and dihydrokainate suggesting the presence of a neuronal excitatory amino acid transporter 2-like transporter (Wang et al., 1998). This observation is consistent with the results of our study suggesting the presence of an excitatory amino acid transporter 2-like subtype in human neurones. In relation to this there has been one study supporting the expression of excitatory amino acid transporter 2 in neurones (Chaudhry et al., 1995) although the levels detected were at best 10% of that observed in astrocytes. More recently, significant neuronal expression of the rat GLT-1 subtype in hippocampal microcultures has been described (Mennerick et al., 1998) indicating that the expression of GLT-1 is not always restricted to astroglial cells.

Expression studies using RT-PCR can be employed to provide information on which genes are being transcribed by a given cell. For example this approach has been used previously with the NT2-N cells to demonstrate the expression of non-NMDA glutamate receptor transcripts (Hardy et al., 1994). By using primers specific for each of the excitatory amino acid transporters we were able to demonstrate that NT2-N cells transcribe both excitatory amino acid transporter 2 and 3. Since the excitatory amino acid transporter 3 subtype is known to be insensitive to both dihydrokainate and kainate it is unlikely that this subtype contributes to the L-glutamate uptake in NT2-N cells. Thus, from the pharmacological characterisation and expression analysis we conclude that excitatory amino acid transporter 2 is the L-glutamate transporter in NT2-N cells. However, the possibility that another excitatory amino acid transporter subtype exhibiting sensitivity to dihydrokainate and kainate exists, but remains to be cloned, cannot be excluded.

4.2. Significance of L-glutamate uptake in NT2-N cells

It has been clearly established that the over-activation of both NMDA and non-NMDA glutamate receptors results in neuronal cell death. This excitotoxic response to glutamate has been studied extensively using in vitro preparations (Garthwaite et al., 1986; Olney et al., 1986; Choi, 1987; Garthwaite and Garthwaite, 1989; Meldrum and Garthwaite, 1990) and is believed to represent a fundamental mechanism of neurodegeneration in vivo (Choi, 1988). The excitotoxic response of NT2-N cells to glutamate has been characterised in some detail (Munir et al., 1995) and is triggered by exposure to both NMDA or non-NMDA receptor agonists. Interestingly, the appearance of sensitivity to glutamate toxicity, assessed by measuring release of lactate dehydrogenase, and the expression of NMDA receptors by ^{125}I-MK-801 ((5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine) binding are well correlated. Glutamate toxicity appears in 3 week old NT2-N cultures and the extent of the excitotoxic response increases up to 8 weeks. Correspondingly, there is an increase in NMDA receptor expression between weeks 2 and 3 and there is a further age-dependent increase in NMDA receptor expression up to 8 weeks.

Our studies with relatively immature neurones (9 days after purification) indicate that the expression of L-glutamate transport activity occurs at a time before the expression of NMDA receptors has been reported to increase. As described earlier, one of the main functions of the high-affinity glutamate transporters is to maintain low extracellular concentrations of glutamate, thus preventing glutamate neurotoxicity. It is possible that the expression of glutamate transport activity by differentiated NT2-N cells represents an important step in the maturation of these neurones. Specifically, the expression of glutamate transport activity prior to the increased expression of NMDA recep-

tors may represent a neuroprotective mechanism. We are currently attempting to optimise conditions for the long term maintenance of NT2-N cells in order to study the expression of the excitatory amino acid transporters as the cells mature in culture, in particular with respect to transport capacity and the developmental expression of transporter subtypes. Preliminary data obtained from neurones maintained for 3 weeks after the final replate indicates that the kinetic parameters are unchanged and the transport activity is completely abolished by dihydrokainate (unpublished observations).

In summary, differentiation of NTera2/D1 precursors in the presence of retinoic acid to produce NT2-N cells results in the expression of glutamate transport activity. Analysis of the pharmacological profile of this transport activity indicates that, of the known transporter subtypes, excitatory amino acid transporter 2 accounts for the transport activity in NT2-N cells.

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